Palmitoylation controls the dynamics of budding-yeast heterochromatin via the telomere-binding protein Rif1

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The posttranslational addition of palmitate to cysteines occurs ubiquitously in eukaryotic cells, where it functions in anchoring target proteins to membranes and in vesicular trafficking. Here we show that the *Saccharomyces cerevisiae* palmitoyltransferase Pfa4 enhanced heterochromatin formation at the cryptic mating-type loci *HMR* and *HML* via Rif1, a telomere regulatory protein. Acylated Rif1 was detected in extracts from wild-type but not *pfa4*Δ mutant cells. In a *pfa4*Δ mutant, Rif1-GFP dispersed away from foci positioned at the nuclear periphery into the nucleoplasm. Sir3-GFP distribution was also perturbed, indicating a change in the nuclear dynamics of heterochromatin proteins. Genetic analyses indicated that *PFA4* functioned upstream of *RIF1*. Surprisingly, the *pfa4*Δ mutation had only mild effects on telomeric regulation, suggesting Rif1's roles at *HM* loci and telomeres were more complexly related than previously thought. These data supported a model in which Pfa4-dependent palmitoylation of Rif1 anchored it to the inner nuclear membrane, influencing its role in heterochromatin dynamics.

transcriptional silencing | chromosome architecture

Transcriptional silencing in *Saccharomyces cerevisiae*, a form of gene repression that occurs at the *HM* loci and telomeres, requires formation of distinctive chromatin structures that span extended chromosomal domains forming the budding-yeast version of heterochromatin (1–3). Silencing is established by direct recruitment of Sir2/3/4 proteins (Sir complex) by sequence-specific DNA binding proteins bound to DNA elements called "silencers." The Sir2 NAD-dependent deacetylase positioned at silencers removes acetyl groups from an adjacent nucleosome, creating another binding site for an additional Sir complex. Iterative cycles of histone deacetylation and Sir complex binding expand heterochromatin ("silent" chromatin) over several kilobases of DNA. Yeast heterochromatin is dispensable for cell viability but critical for controlling gene expression at the *HM* loci and telomeres, both easily measured with phenotypic assays. Thus, yeast silencing has served as a powerful tool for defining mechanisms of heterochromatin formation.

The *SIR* genes were isolated in forward genetic screens focused on *HM* silencing and encode nonessential proteins that are structural components of yeast heterochromatin (4). However, several modified genetic screens have identified proteins with impacts in chromosome biology that extend beyond heterochromatin. For example, mutant versions of the origin recognition complex (ORC), a silencer binding protein that also functions as the eukaryotic initiator of DNA replication, were identified in silencing screens (5–7). A common feature of these modified screens is that silencing is genetically compromised by mutation to sensitize it to further exacerbating defects; alleles of ORC were identified in screens focused on *HMR* silencing in which the *HMR* silencer had been compromised by *cis*-mutation (6, 7). Variations of sensitized silencing screens have uncovered roles in chromosome biology for other conserved factors that are essential for viability, highly redundant, or otherwise difficult to define (8–10).

We exploited a genetically sensitized form of *HMR* silencing and identified *PFA4*, a gene that encodes a DHHC (asp-his-his-cys) domain-containing, integral membrane S-palmitoyltransferase of the endoplasmic reticulum (ER) (11, 12). The data supported a model in which Pfa4 modulated *HM* silencing, Sir-complex dynamics, and macromolecular aspects of telomere architecture by palmitoylating the telomere binding protein Rif1.

Results and Discussion

***PFA4* Contributed to *HMR* Silencing.** To uncover new regulators of chromatin, several genetic modifications of *HMR* silencing were exploited. First, *SIR1* was deleted. Sir1, one of four Sir proteins, contributes to the initial recruitment of the Sir complex to the *HM* loci (13). In its absence, silencing is weakened but not abolished, indicating that Sir1-independent mechanisms for Sir complex recruitment exist (14). Second, the parent strain was further sensitized by use of a synthetic silencer (*HMR*-SS), making *HMR* more sensitive to *SIR1* loss (15). Third, modest overexpression of the cell-cycle regulator *FKH1* (*FKH1*^{hc}) was used to partially restore silencing crippled by these modifications. *FKH1*^{hc} recreates bona fide Sir complex silencing at *HMR* in *sir1Δ HMR*-SS cells (16). The strain was subjected to transposon-based mutagenesis and screened for mutants that reduced silencing (17). *LEU2* insertion mutations in *YKU80* and *PFA4* were identified and cosegregated with the silencing defect. *YKU80* was discussed previously (17).

To confirm a role for *PFA4* in *HMR* silencing, the effect of a complete deletion of the gene on transcription of the a1-gene was determined by RT-PCR (Fig. 1*A*). Transcription of a1 is the direct consequence of reduced silencing at *HMR*. In *SIR1* cells, a1 mRNA was not detected regardless of *PFA4* genotype. In contrast, *FKH1*-assisted silencing was sensitive to *PFA4* as a1 mRNA levels were twofold greater in the *pfa4*Δ mutant compared with wild-type cells. These data solidified the conclusion that *PFA4* contributed to *FKH1*-assisted silencing and that *SIR1* masked *PFA4*'s contribution.

FKH1-assisted silencing was used as a tool to sensitize silencing to *SIR1*-independent mechanisms that are likely to be more conserved in eukaryotes (17). However, if *PFA4* had a fundamental role in chromosome biology that affected *HMR*, its contribution should not be limited to this specialized condition. Therefore, quantitative mating experiments with *sir1*Δ cells containing a native *HMR* locus and chromosomal *FKH1* were performed (Fig. 1*B*). Loss of *PFA4* caused a threefold reduction in *HMR* silencing in these cells. Silent chromatin in these cells was also monitored by ChIP with monoclonal antibodies against Sir3 (16). Sir3 levels at *HMR* were reduced more than twofold in the *pfa4*Δ mutant cells (Fig. 1*C*). Thus, *PFA4* could modulate heterochromatin formation at the native *HMR* locus.

Because *PFA4* encodes a palmitoyltransferase, we tested whether its catalytic activity was required for *HMR* silencing. A *pfa4C108A* allele produces a catalytically inactive version of Pfa4 that fails to direct the trafficking of the chiten synthase, a Pfa4

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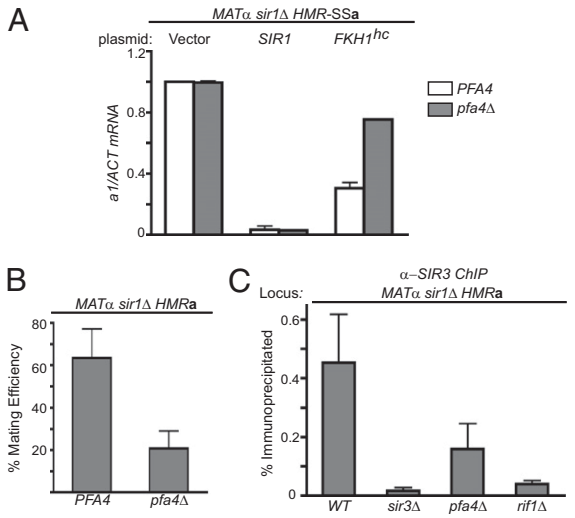

Fig. 1. *PFA4* in *HMR* silencing. (*A*) A *pfa4*Δ mutation reduced *FKH1*-assisted silencing but not *SIR1*-mediated silencing. *MATα sir1*Δ *HMR-SSa* cells that were either *PFA4* or *pfa4*Δ were transformed with a 2-μ *URA3*-plasmid that was empty (Vector) or contained either *SIR1* or *FKH1 (FKH1* "high copy"; *FKH1^{hc})* and a1 mRNA levels were measured by RT-PCR (17). (*B*) *PFA4*'s role in silencing natural *HMR* was examined by quantitative mating assays in which the precise number of cells in a population that can mate is determined by serial dilutions into an excess of *MAT*a cells, as in *A*, except all of the cells are counted (17). Mating efficiency is indicated as the percentage of cells that could form diploid colonies on selective agar plates. The significance of the difference between *PFA4* and *pfa4*Δ cells was assessed with a *t* test ($P = 0.0006$). (*C*) Sir3 binding to *HMR* was examined by ChIP using monoclonal Sir3 antibodies (16). The significance of the differences between *PFA4* and the mutant cells indicated in the figure were assessed with *t* tests and the *P* values were: *sir3*Δ, $P = 0.010$; *pfa4*Δ, $P = 0.052$; *rif1*Δ, $P = 0.012$.

substrate (12). Importantly, in contrast to a wild-type *PFA4-HA* allele, a *pfa4C108A-HA* allele expressed on either a CEN or 2-μm plasmid failed to complement the *HMR* silencing defect of a *pfa4*Δ mutant (Fig. S1). Thus, the catalytic activity of Pfa4 was required for *HMR* silencing in *sir1*Δ cells.

***PFA4* Was Required for S-Acylation of Rif1.** Palmitate is a 16-carbon fatty acid, and as a protein modification functions as a membrane anchor, tethering a protein domain to cellular membranes. Although palmitoylation is most recognized for its role in vesicular trafficking, it seemed significant that Pfa4 resides within the ER membrane, as this organelle's membrane is contiguous with the nuclear membranes (11, 18). The catalytic site of S-palmitoyltranferases face the cytoplasm. Thus, Pfa4 could palmitoylate a nuclear protein that would anchor it to the ER membrane, allowing it to enter the nucleus via translocation through nuclear pores. Thus, an attractive hypothesis was that Pfa4 directly palmitoylated a nuclear protein relevant to silencing. Rif1, a protein with roles in telomere structure and silencing, was recovered in a proteomic survey of palmitoylated proteins in yeast (19). Thus, the modification status of Rif1 was examined more closely with an acyl-biotin exchange (ABE) protocol (20) (Fig. S2*A*) in which free thiols are first protected by treatment with *N*-ethylmaleimide (NEM). Hydroxylamine is then used to cleave thiol ester bonds of any existing protein-acyl linkages. Finally a thiol-specific biotinylation reagent (EZ-Link; Thermo Scientific) covalently links biotin to the newly liberated thiols, and biotinylated proteins are purified by neutravidin-affinity chromatography. GNP1-3xHA, a positive control, was acylated in a *PFA4*-dependent manner, whereas Orc1 and Fkh1 were not (19) (Fig. 2*A* and Fig. S2*B*). Rif1-9xMyc was also enriched in the acylated fraction, and this enrichment was reduced by omission of the hydroxylamine step. Most significantly, this enrichment was reduced to background levels by deletion of *PFA4* (Fig. 2*A* and Fig. S2*B*). NEM protection was performed under highly denaturing conditions (6 M Urea), making it unlikely that Rif1 was purified on the basis of its interaction with a secondary modified protein. Therefore, one or more cysteines of Rif1 were acylated. Given the dependence on *PFA4*, these data provided evidence that Rif1 was palmitoylated.

Palmitoylation of Rif1 would be expected to modulate Rif1's association with membranes and thus its solubility in cellular extracts. Therefore, the solubility of Rif1-9xMyc in *PFA4* and *pfa4*Δ mutant cell extracts was compared by differential centrifugation (21) (Fig. 2*B*). Immunoblotting revealed two bands that corresponded to full-length Rif1 with epitope (Rif1-9xMyc) and an N-terminal truncated form that lacked about 400 amino acids but retained the C-terminal Myc epitope (Rif1*-9xMyc). Rif1-9xMyc and Rif1*-9xMyc were enriched in the insoluble pellet of *PFA4* relative to *pfa4*Δ extracts. Correspondingly, these proteins were diminished in the soluble fraction of *PFA4* relative to *pfa4*Δ extracts. In addition, several smaller Rif1-9xMyc protein fragments were detected (**) that associated primarily with the soluble fraction of both the *PFA4* and *pfa4*Δ extracts. Finally, Fkh1, which is not palmitoylated (Fig. S1*B*), distributed similarly between the pellet and soluble fractions regardless of *PFA4* genotype (Fig. 2*B*). Thus, *PFA4* altered the solubility of Rif1 in yeast extracts, consistent with palmitoylation of Rif1 helping anchor it to the nuclear membrane.

***PFA4* Was Required for Rif1-GFP Foci at the Nuclear Periphery.** Rif1 associates with telomere clusters that are themselves anchored to the inner nuclear membrane, forming distinct foci (22–25). To test whether Rif1's localization to these foci was affected by *PFA4*, a Rif1-GFP fusion protein was coexpressed in a strain expressing an HDEL-DsRed fusion protein, which is retained in the ER to allow visualization of the contiguous nuclear and ER membranes (26). Confocal sectioning of wild-type cells revealed 10 to 12 discrete Rif1-GFP foci per nucleus, most of which abutted the nuclear membrane (Fig. 2*C*). This pattern was similar to that observed by immunoflorecense of Rif1 and is reminiscent of other telomere-binding proteins (22, 25). In contrast, in *pfa4*Δ mutant cells, Rif1-GFP fluorescence was more broadly distributed across the nucleus, and only two to four larger, more diffuse foci per nucleus could be identified (Fig. 2*D*). Importantly, a *pfa4*Δ mutant did not affect levels of Rif1-GFP as measured by protein immunoblotting, and a catalytically inactive *pfa4(C108A)* mutant produced similar dispersion of Rif1-GFP foci to a *pfa4*Δ mutant (Fig. S3). Therefore, enrichment of Rif1-GFP in distinct foci at the nuclear periphery required *PFA4*-dependent palmitoylation.

***PFA4* and *RIF1* Functioned in a Common Pathway in *HM* Silencing.** The data presented above indicated that Rif1's nuclear distribution was affected by *PFA4*, suggesting that this regulation was relevant to *PFA4*'s role in silencing. In the current view, Rif1 acts as a direct negative regulator of telomeric silencing and an indirect positive regulator of *HM* silencing (22). Specifically, Rif1 and the Sirs interact exclusively with Rap1; multiple Rap1 sites exist at telomeres serving as the telomeric version of a silencer. Thus, Rif1 attenuates telomeric silencing by competing with Sir complexes for binding to Rap1. Because Sir complexes are limiting, increases in Sir complexes at telomeres are balanced by reductions in Sir complexes at the *HM* loci (27). Therefore, deletion of *RIF1* enhances telomeric silencing and simultaneously reduces *HM* silencing. This model might account for *HMR* silencing defects caused by deletion of *PFA4*. However, *HM* silencers contain single Rap1 binding sites important for silencing (28, 29), and genome-wide analysis of Rif1 binding provides evidence that it binds the *HM* loci (25). Thus, it is possible that Rif1 also has more direct negative roles in *HM* silencing. Regardless, if palmitoylation regulates Rif1's role in *HM* silencing, then defects caused by a *rif1*Δ mutation should not be further exacerbated by loss of *PFA4*. Therefore, we asked whether *RIF1* and *PFA4* functioned in a common pathway in *HM* silencing (Fig. 3 *A* and *B*).

Data for *HML* were straightforward. Quantitative mating showed that *HML* silencing was reduced similarly when either *PFA4* or *RIF1* was deleted, and that the effect was no greater when the two deletions were combined (Fig. 3*A*). The data for *HMR* were more complex, but nevertheless consistent (Fig. 3*B*). Quantitative mating showed that *HMR* silencing was reduced when either *PFA4* or *RIF1* was deleted, but the defect caused by *RIF1*

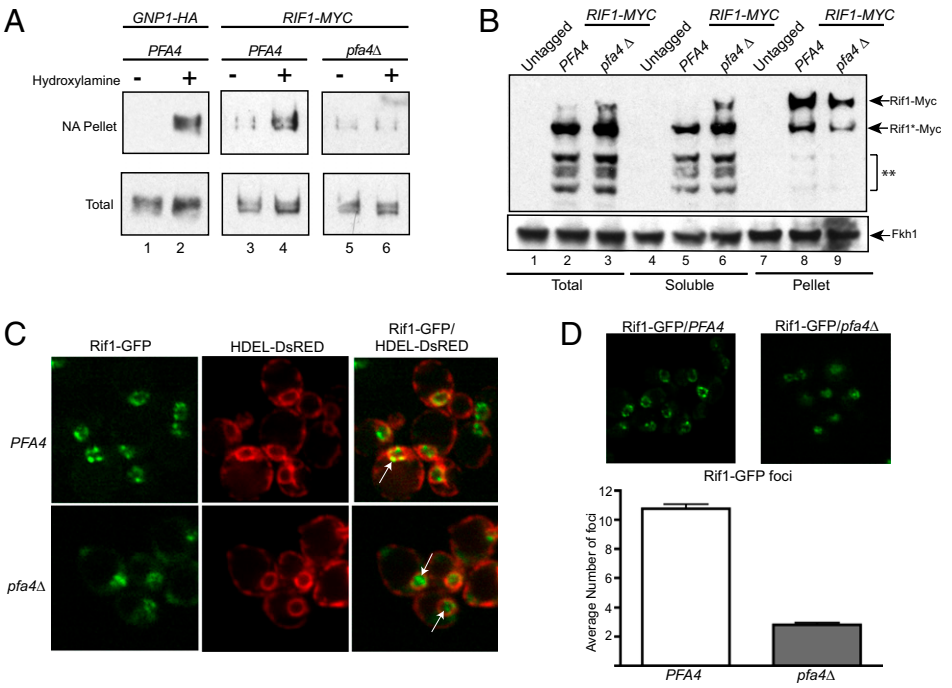

Fig. 2. *PFA4* was required for S-acylation of Rif1 and formation of Rif1-GFP foci. (*A*) ABE chemistry (20) was used to test whether Gnp1, a protein known to be palmitoylated by *PFA4* (19), and Rif1 were acylated (also see Fig. S1). Biotinylated proteins should be present in the neutravidin-agarose (NA) pellet when the extracts are treated with hydroxylamine (+). Protein immunoblotting was used to detect Gnp1-3xHA and Rif1-9xMyc (22). (*B*) Wild-type yeast cells (Untagged) or cells containing *RIF1-9xMYC* that were either *PFA4* or *pfa4*Δ were fractionated by centrifugation into membrane-containing and soluble fractions (21). Rif1-9xMyc was detected by protein immunoblotting. Rif1-9xMyc contains the Myc epitopes at its C terminus. Full-length Rif1-9xMyc is indicated (Rif1-Myc). Rif1-Myc* is a truncated form of the protein that lacks about 400 amino acids of the N terminus but retains the C-terminal Myc epitopes. The shorter fragments (**) are further N-terminally truncated Rif1-9xMyc fragments. Fkh1 was detected with an anti-Fkh1 antibody (16). (*C*) Serial sectioning and confocal microscopy was used to visualize Rif1-GFP. The ER was detected with HDEL-DsRED (26) that stains the ER, which is contiguous with the nucleus (small red circles), as well as the cortical ER that leads to signal intensity near the plasma membrane. White arrows in the merged data panel indicate selected Rif1-GFP foci. (*D*) The number of Rif1-GFP foci in wild type (*PFA4*) and *pfa4*Δ mutant cells was determined by counting distinct foci from 97 *PFA4* cells and 98 *pfa4*Δ mutant cells. Confocal sections were acquired at 300-nm intervals through the nucleus. The significance of the difference was determined by a *t* test ($P = 1.76 \times 10^{-48}$). Images were acquired on a swept field confocal microscope (Nikon Ti-E) equipped with a Roper CoolSnap HQ2 CCD camera using a Nikon 60×, 1.4NA Planapo oil objective lens, and a 1.5× Optivar auxillary magnifier.

deletion was more severe. This different quantitative contribution was also revealed by Sir3-ChIP experiments (Fig. 1*C*). Significantly, however, cells lacking both *PFA4* and *RIF1* exhibited an *HMR*-silencing defect that was the same as that of cells lacking only *RIF1* (Fig. 3*B*, *Inset*). The absence of an additive defect was not a result of assay limitations because cells lacking both *RIF1* and *RIF2* [*RIF1* and *RIF2* have overlapping functions (30)] showed greater silencing defects. Thus, *RIF1* and *PFA4* acted in a common genetic pathway to silence *HML* and *HMR*.

***PFA4* Had Mild Effects on Telomeric Silencing and Length Control.** As discussed above, Rif1 acts as a negative regulator of telomeric silencing by binding to telomere-bound Rap1 and blocking recruitment of the Sir complex. Thus, in a *rif1*Δ mutant, telomeric silencing is enhanced. This effect is also thought to be the basis of *RIF1*'s positive role in *HM* silencing. Thus, based on the *HM* silencing data as well as delocalization of Rif1-GFP, we expected that a *pfa4*Δ mutation would enhance telomeric silencing. Silencing of the left telomere of chromosome VII was assessed using a *TRP1* reporter gene (Fig. 3*C*). In wild-type cells, *TRP1* at telomere VII-L is silenced, hence cells grow poorly on medium lacking tryptophan (31). The Sir complex is essential for telomeric silencing, hence cells lacking *SIR2* express *TRP1* and grow on medium lacking tryptophan (32). As expected, deletion of *RIF1* enhanced telomeric silencing (33). However, a *pfa4*Δ mutation had only a slight effect on silencing of telomere VII-L.

Rif1 also negatively regulates telomere length (34). We therefore tested whether *PFA4* regulated telomere length by measuring the average length of bulk telomeres (Fig. 3*D*). DNA was cleaved with XhoI at a location near yeast chromosomal ends, and telomeres were detected by DNA blot hybridization with a probe complementary to yeast telomeres. Telomeres appear as a smear in these experiments (Fig. 3*D*, telomeres are bracketed). As expected, a deletion of *RIF1* led to an increase in telomere length. A deletion of *PFA4*, however, had no effect on telomere length. Telomeres in cells lacking both *PFA4* and *RIF1* were similar in length to those in cells lacking only *RIF1*. As in silencing (Fig. 3*B*), *RIF1* and *RIF2* have additive roles in telomere length, such that deletion of both genes leads to substantially longer telomeres (30). Thus, a *rif2*Δ mutation might sensitize telomere length to defects in *RIF1* caused by a *pfa4*Δ mutation. A *pfa4*Δ mutation did lead to slightly longer telomeres in *rif2*Δ mutant cells (compare *rif2*Δ to *rif2*Δ *pfa4*Δ) but the effect was small. Thus, *PFA4* had only a very modest role in controlling telomere length. Therefore, Rif1's molecular roles at telomeres remained largely intact in *pfa4*Δ mutant cells.

To reconcile dispersion of Rif1-GFP with retention of telomere functions in a *pfa4*Δ mutant, ChIPs in cells expressing Rif1-9xMyc were performed (Fig. 3*E*). Deletion of *PFA4* reduced Rif1 binding to the right telomere of chromosome III (*TEL* III-R) by ~30%. A similar reduction occurred at *TEL VII-L*. Rif1-9xMyc association with a third telomere *TEL VI-R* was not reduced. Thus, Rif1's average occupancy at telomeres did not change substantially in a *pfa4*Δ mutant, providing an explanation for the mild effects that the *pfa4*Δ mutant had on telomere silencing and length control. Considering the substantial dispersion of Rif1-GFP in *pfa4*Δ mutants, these data suggest that Rif1 is normally present in excess concentration at telomeres for its roles in telomeric silencing and length regulation.

Because a genome-wide analysis detected Rif1 binding to *HMR* (25), Rif1-9xMyc binding to *HMR* was examined (Fig. 3*E*). At *HMR*, Rif1-9xMyc occupancy was actually enhanced by ~30% by deletion of *PFA4*. One explanation was that in a *pfa4*Δ mutant, Rif1, now released from pools normally tightly sequestered into foci at the nuclear periphery, was free to sample Rap1 proteins bound to more internal sites, such as *HM* silencers, more efficiently. Thus, perhaps reduced *HMR* silencing in a *pfa4*Δ mutant was caused in part by direct negative regulation of this locus by Rif1, and in part by some sequestration of Sir proteins at telomeres, as in the current model. These two mechanisms might act additively to cause reductions in HM silencing, and could explain how *PFA4*-dependent regulation of Rif1 could reduce *HM* silencing measurably without simultaneously affecting telomeric silencing or length control substantially.

***PFA4* and *RIF1* Acted in a Common Pathway to Control Sir3 Dynamics.** The *HM* data supported the idea that *PFA4* regulated *RIF1*, but the modest effects of *PFA4* on telomeres precluded epistasis analysis. Therefore, more macromolecular aspects of telomere

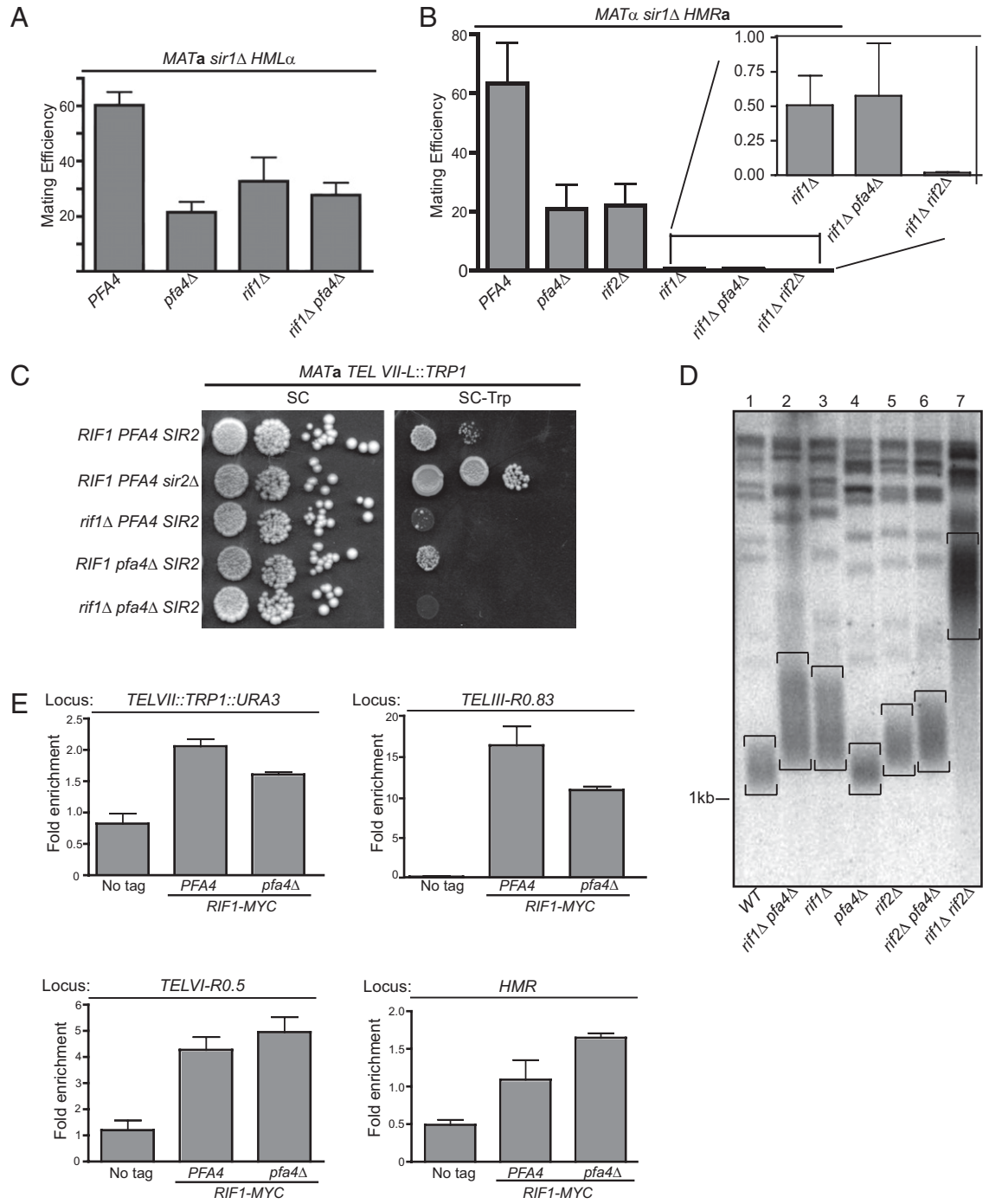

Fig. 3. *RIF1* and *PFA4* in silencing, telomere length and Rif1 binding. Quantitative mating-assays were used to measure the epistatic relationship between *RIF1* and *PFA4* at (*A*) *HML*, and (*B*) *HMR*. Quantitative mating assays were performed as previously described (17). (*C*) Telomeric silencing was assessed by measuring expression of the *TRP1* reporter gene engineered at the left telomere on chromosome VII (*TEL VII-L::TRP1*) by comparing growth on rich medium to that on synthetic medium lacking tryptophan. (*D*) Average length of bulk telomeres for a set of congenic strains was determined by DNA blot hybridization (45). (*E*) The effect of *PFA4* on Rif1-9xMyc binding to several loci was measured by ChIP.

behavior were used to examine the relationship between *PFA4* and *RIF1*. Because most telomeres contain neighboring domains of heterochromatin, a Sir3-GFP fusion protein was used as a marker for telomere clusters. Serial sectioning by fluorescence microscopy revealed an average of 8.8 Sir3-GFP foci in nuclei of wild-type cells, and this number dropped to 7.0 and 7.4 in *pfa4*Δ and *rif1*Δ mutants, respectively (Fig. 4 *A* and *B*). Cells with both mutations (*rif1*Δ *pfa4*Δ) produced a similar number of Sir3-GFP foci as cells with either mutation alone (average = 7 per nucleus). These data suggest that both *RIF1* and *PFA4* acted in a common pathway to modulate telomere clustering. However, *RIF1* and *PFA4* had relatively small effects on telomere clustering; although the number of Sir3-GFP clusters was reduced, most appeared to remain intact. Importantly, Sir3-GFP levels were similar in wild-type and *pfa4*Δ mutant cells (Fig. S3*D*). Thus, the striking loss of Rif1-GFP foci in *pfa4*Δ mutants was likely not caused by a large-scale breakdown of telomere clusters but rather a dispersion of Rif1 away from these, and possibly other structures, at the nuclear periphery.

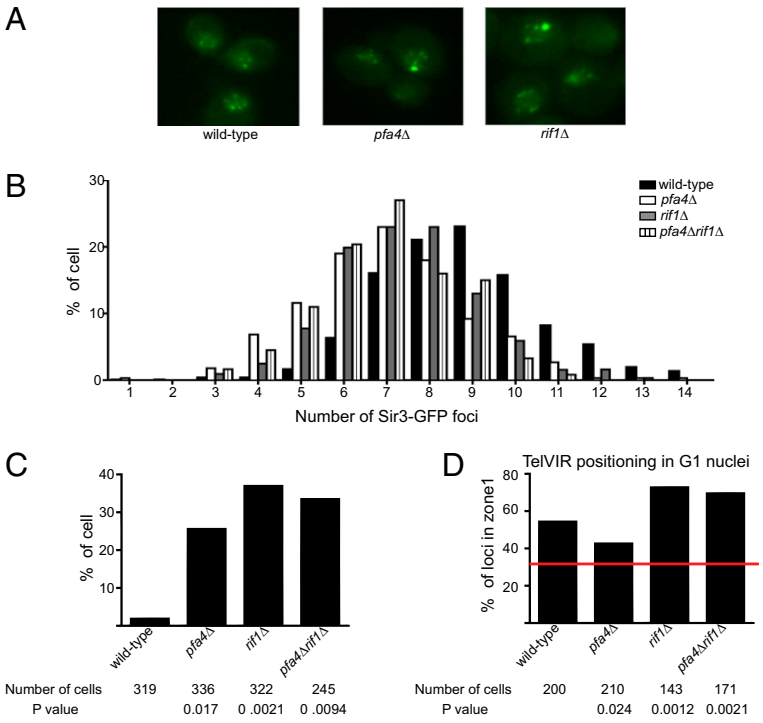

Fig. 4. *PFA4* modulated Sir3-GFP distribution and the perinuclear association of telomere VIR. (*A*) Examples of Sir3-GFP foci observed in wild-type, *pfa4*Δ, and *rif1*Δ cells. (*B*) Sir3-GFP foci were counted and the percentage of cells (*y* axis) containing a given number of foci (*x* axis) was determined. (*C*) The percentage of cells containing SBF formed by Sir3-GFP was determined. (*D*) The percentage of cells containing TelVIR in zone I of the nucleus (region closest to the nuclear periphery) was determined. Statistical data included in the figure.

An additional phenomenon was noted. Although the intensity of individual Sir3-GFP foci in wild-type cells did not vary dramatically, one or two of the foci within *pfa4*Δ or *rif1*Δ mutant cells were more intense than other foci. We therefore counted the number of exceptionally intense foci by setting an arbitrary cutoff to define the most intense spots as single bright foci (SBFs). Only 1.9% of wild-type cells contained a SBF (Fig. 4*C*). In contrast, the mutants contained 15- to 20-fold more (*pfa4*Δ = 25.6%; *rif1*Δ = 37%). Deletions of both genes produced a similar number of SBFs as either deletion alone (*rif1*Δ*pfa4*Δ = 33%). These data suggest that *RIF1* and *PFA4* acted in a common pathway to prevent formation of foci that contained abnormally high quantities of Sir3-GFP.

Because telomeres form clusters that associate with the nuclear periphery, we also examined whether *PFA4* and *RIF1* influenced the anchoring of a telomere to the nuclear membrane by measuring distances between a GFP-tagged lac operator array at the right end of telomere VI and the rim of fluorescence created by a GFP-tagged nuclear pore protein, Nup49-GFP (24). Measurements were normalized to the nuclear diameter and binned into three concentric zones of equal surface area. This analysis was restricted to G1 cells. In wild-type cells, TelVIR was found in zone I, the zone closest to the nuclear periphery, in 58% of the cells examined (Fig. 4*D*). A *pfa4*Δ mutation reduced the fraction in zone I to 43%, suggesting that palmitoylation helped promote telomere anchoring to the nuclear membrane. However, in contrast to *HM* silencing and Sir3-GFP behavior, deletion of *RIF1* caused a phenotype opposite to that of *pfa4*Δ and actually increased the fraction of cells in zone I to 71%. Deletion of both *RIF1* and *PFA4* gave rise to 70% of the cells with TelVIR in zone I, similar to *RIF1*deletion alone. A possible explanation for these data were that palmitoylation of Rif1 by Pfa4 favored telomere anchoring by one mechanism and revealed Rif1's contribution to this anchoring, but the presence of Rif1 restricted perinuclear anchoring by another mechanism that was able to dominate only when *RIF1* was completely absent. For example, absence of Rif1 would be expected to enhance the presence of Sir complexes at telomeres substantially, promoting Sir-mediated mechanisms of telomere anchoring.

In summary this study provided evidence that palmitoylation controlled the behavior of Rif1, a protein that regulates both telomere dynamics and silencing. *PFA4* was important for Rif1-GFP's localization to discrete foci at the nuclear periphery. Several studies provide evidence that these foci correspond to telomeric clusters (22, 25, 35). Rif1 binds telomeres via direct interactions with the telomere-binding protein Rap1, and this interaction is relevant to Rif1's molecular roles at telomeres (25, 33). However, our data demonstrat that Rap1-Rif1 interactions were insufficient to explain the discrete Rif1-GFP foci that form at the nuclear periphery, and instead suggest that palmitoylation of Rif1 promoted formation of these foci. Perhaps concentrating Rif1 at the inner nuclear membrane via palmitoylation favors its association with telomere clusters that are anchored to this membrane by several other mechanisms, thus forming concentrated pockets of Rif1 revealed by the Rif1-GFP foci (36). Simultaneously, this mechanism could sequester Rif1 from many internal Rap1-associated loci, including *HMR* and *HML*, where it might be detrimental. Regardless of the precise mechanisms that explain *PFA4*'s role in *HM* silencing, the genetic data suggest that *PFA4* and *RIF1* acted in a common pathway, as would be predicted if palmitoylation of Rif1 by Pfa4 regulated Rif1. Although several studies establish functional links between the inner nuclear membrane and yeast silencing (37–39), palmitoylation of Rif1 is unique as an example in which a known silencing protein's association with the inner nuclear membrane is controlled by a fatty acid modification. A recent study shows that carbohydrate modification of Sir2 can regulate silencing (40). Perhaps these unexpected modifications represent an emerging theme for how cells use nonhistone proteins to make chromatin structures more responsive to a cell physiology. S-acylation catalyzed by palmitoyltransfereases is particularly interesting in that it is reversible, providing a way to make Rif1 nuclear distribution highly responsive and dynamic. Further studies will be needed to test this idea, and reveal whether the conservation of *PFA4* and *RIF1* results in the use of this unique pathway in metazoans. Moreover, it is likely that other nuclear proteins are controlled by palmitoylation because it provides a dynamic means to control protein distribution and chromosome architecture.

Materials and Methods

Yeast Genetics and Molecular Biology. Yeast strains (Table S1) were constructed by standard procedures. Mating assays were performed as previously described (17). Primers are listed in Table S2.

Quantitative PCR to determine a1 mRNA levels was performed as previously described (17).

ABE assay used the modified version of ABE chemistry (41) that used the three steps previously described (42) (Fig. S2*A*), except that proteins were denatured by 6 M urea and free cysteines blocked by NEM (Fluka) under these conditions. Extracts were prepared from cells growing in log-phase (A_{600} ~1.0).

Rif1-9xMyc Solubility. Extracts made from cells grown in YPD and harvested at A_{600}~1.0 were fractionated as previously described (21) and examined for Rif1-9xMyc (25) by protein immunoblotting.

ChIP was performed as described (16, 43, 44) with anti-Sir3 to monitor Sir3 binding or anti-Myc to monitor Rif1-9xMyc binding. *HMR* and telomeres were detected with specific primers (Table S2). Rif1-Myc ChIPs were performed in *MATa SIR1* cells that contained natural versions of the *HM* loci. Signals were normalized to the *ADH4* locus (17).

Telomere length measurements were performed as previously described (45).

Rif1-GFP Imaging. Cells were grown in liquid synthetic media, diluted to an A_{600} = ~0.1 to 0.2, and grown to an additional 5 h to A_{600} = ~0.7. One milliliter of culture was pelleted, washed with ~1 mL water, and resuspended in 100 μL water. Rif1-GFP and HDEL-DsRED were visualized by serial sectioning and confocal microscopy (Nikon Ti-E, equipped with a Roper CoolSnap HQ2 CCD camera). Confocal sections were acquired at 300-nm intervals through the nucleus. Foci were counted manually in images of ~100 live cells.

Sir3-GFP Imaging and Telomere VIR Positioning. Cells were grown in liquid YPD, diluted, and regrown to A_{600} = 0.2 to 0.3, pelleted, and washed with water before mounting on slides with 1.4% agarose plugs. Fields of 15 to 50 cells were imaged with a Zeiss Axioplan II fluorescence microscope (100× Plan-Apochromat objective, NA = 1.4) and Axiocam HR camera. Additional *z*-stacks were composed of 17 elevations, each separated by 250 nm and an acquisition time of 250 ms. Foci were counted manually in images of live cells. Datasets for each strain consisted of three trials of 50 to 150 cells and were pooled after the average values of spots per nucleus were found to lie within the same 95% confidence interval. Pooled data were evaluated relative to wild-type by *t* test. For foci intensity, Zeiss Axiovision software was used to obtain a unitless intensity value I = (Sf – Bf)/Bf, where Sf equals signal intensity of an area encompassing the bright spot and Bf equals intensity of a comparable nuclear area lacking a fluorescent spot. An arbitrary cutoff of I > 1.1 was chosen to define an SBF. To measure perinuclear position of TelVIR, GFP positions were determined as previously described (24).

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