

Rif1 Controls DNA Replication Timing in Yeast through the PP1 Phosphatase Glc7

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SUMMARY

The Rif1 protein, originally identified as a telomere-binding factor in yeast, has recently been implicated in DNA replication control from yeast to metazoans. Here, we show that budding yeast Rif1 protein inhibits activation of prereplication complexes (pre-RCs). This inhibitory function requires two N-terminal motifs, RVxF and SILK, associated with recruitment of PP1 phosphatase (Glc7). In G1 phase, we show both that Glc7 interacts with Rif1 in an RVxF/SILK-dependent manner and that two proteins implicated in pre-RC activation, Mcm4 and Sld3, display increased Dbf4-dependent kinase (DDK) phosphorylation in *rif1* mutants. Rif1 also interacts with Dbf4 in yeast two-hybrid assays, further implicating this protein in direct modulation of pre-RC activation through the DDK. Finally, we demonstrate Rif1 RVxF/SILK motif-dependent recruitment of Glc7 to telomeres and earlier replication of these regions in cells where the motifs are mutated. Our data thus link Rif1 to negative regulation of replication origin firing through recruitment of the Glc7 phosphatase.

INTRODUCTION

DNA replication in eukaryotes is initiated from specific chromosomal sites (origins), which fire in a temporal pattern during S phase that depends on cell type and developmental stage. The unfolding of this replication program is controlled through mechanisms that remain poorly understood to date. Recent studies show that premature firing of normally late or dormant origins in yeast can lead to activation of a DNA-damage response, most likely as a consequence of deoxynucleotide triphosphate depletion (Mantiero et al., 2011). This finding suggests that control of origin usage may be connected in some way to replication fork progression.

The control of DNA replication initiation is best understood in the budding yeast *Saccharomyces cerevisiae*, where, unlike in all other eukaryotes studied to date, potential replication origins, or autonomously replicating sequences (ARS), are well defined by a conserved sequence bound constitutively by the origin recognition complex (ORC) (Siddiqui et al., 2013). Origins are first prepared for replication through the loading of the replicative helicase (MCM2–MCM7 hexamer) to form the prereplication complex (pre-RC). Activation of the pre-RC requires the combined action of two kinase complexes, the cyclin-dependent kinase (CDK) and the Dbf4-dependent kinase (DDK), the latter consisting of the Cdc7 kinase and its activator Dbf4 (Labib, 2010), and is associated with the recruitment of additional proteins, including Cdc45, implicated in MCM2–MCM7 release during initiation; a set of adaptor proteins, Sld2/Sld3 and Dpb11; the GINS complex, containing four proteins implicated in polymerase assembly at the origin; and the leading strand DNA polymerase itself, Pole (Araki, 2011).

The temporal pattern of DNA replication during S phase in yeast has been extensively studied (Aparicio, 2013). In this organism, only a small fraction of potential origins actually fire early during S phase. Other origins fire during middle or late S phase or not at all and are thus passively replicated. Significantly, overexpression of several factors, particularly Sld2/Sld3, Dbf4, and Dpb11, accelerates initiation of normally late-firing origins, suggesting that the temporal pattern of initiation is entrained by a competition for limiting factors (Mantiero et al., 2011). Telomeres tend to replicate late in S phase (Donaldson, 2005), despite their proximity to nearby ARS elements, due to the action of two proteins involved in gene silencing at telomeres, Sir3 and the Ku heterodimer (Yku70/Yku80) (Cosgrove et al., 2002; Stevenson and Gottschling, 1999). More recent studies have shown that a third telomere-binding protein, Rif1, also determines late telomere replication (Lian et al., 2011). Mutation of *RIF1*, in addition to causing earlier telomere replication, also leads to elongation of TG_(1–3) tract length at telomeres, yet paradoxically, in otherwise wild-type cells, short telomeric TG-repeat tracts entrain the linked telomere to replicate earlier (Bianchi and Shore, 2007). Taken together, these data implicate the telomere length

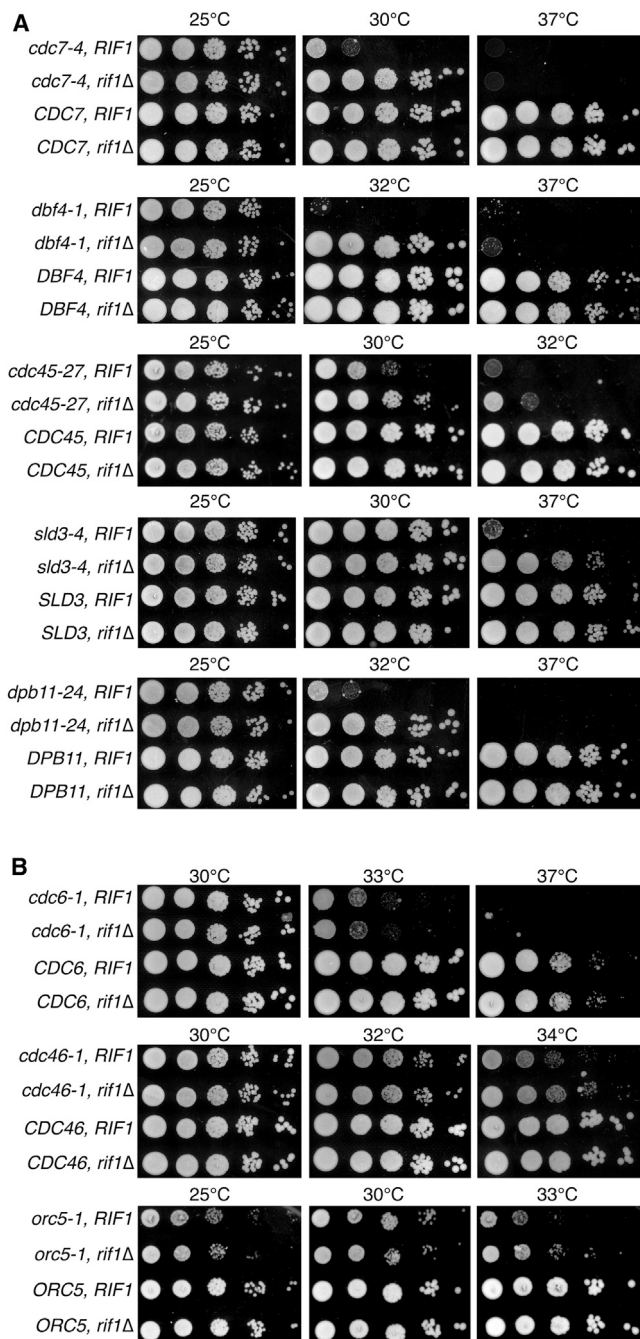


Figure 1. RIF1 Deletion Suppresses Mutations Affecting DNA Replication Initiation

Temperature-sensitive (ts) replication mutants, together with either *RIF1* or *rif1Δ*, were grown overnight in rich medium (YPAD) at 25°C. Serial 10-fold dilutions were spotted onto YPAD plates and incubated at the indicated temperatures for 48 hr. In each panel, isogenic wild-type controls for the different ts mutants are shown. See also Figure S1.

regulatory mechanism and telomeric gene silencing in control of telomere-proximal origin firing, though the mechanisms involved remain obscure.

Here, we explore in molecular detail the role of Rif1 in telomere-replication timing and more generally in the regulation of genome-wide origin activation. Our findings suggest a model in which Rif1 affects replication initiation through recruitment of the PP1 phosphatase (Glc7), most likely at replication origins themselves, where it counteracts the effect of the DDK.

RESULTS

Rif1 Has a Global Effect on pre-RC Activation in Budding Yeast

The previous finding that deletion or knockdown of *RIF1* in yeast or mammalian cells leads to earlier DNA replication at some chromosomal sites suggests a role for Rif1 in directly regulating replication origin activation (Cornacchia et al., 2012; Hayano et al., 2012; Lian et al., 2011; Yamazaki et al., 2012). In support of this notion, studies in the fission yeast *Schizosaccharomyces pombe* showed that deletion of *RIF1* can rescue the lethality of an *hsk1⁺* deletion, which removes the essential DDK required for replication initiation (Hayano et al., 2012). We found a similar effect of *RIF1* deletion in a *Saccharomyces cerevisiae* strain carrying a *cdc7-4* temperature-sensitive (ts) mutation (*CDC7* is the budding yeast ortholog of *hsk1⁺*) but noted that *rif1Δ* does not completely bypass the requirement for Cdc7, because *rif1Δ cdc7-4* cells still do not grow at 37°C (Figure 1A), and *rif1Δ* does not permit growth of cells deleted for *CDC7* (data not shown).

To ask whether the action of *RIF1* is specific to *CDC7*, we examined the effect of *rif1Δ* on other factors required for pre-RC activation, again using temperature-sensitive alleles of the corresponding (essential) genes. Notably, we found that *rif1Δ* strongly suppressed *dbf4-1*, *sld3-4*, and *dpb11-24* mutations, whereas it displays a more modest effect on *cdc45-27*, encoding a third activation factor (Figure 1A). In contrast, the influence of *rif1Δ* on alleles affecting the helicase or its loading onto origins (*cdc46-1*, *orc5-1*, and *cdc6-1*) was either minimal or undetectable (Figure 1B). This analysis suggests that Rif1 has an inhibitory effect related to the DDK and three pre-RC activator proteins (Cdc45, Sld3, and Dpb11), whereas relatively little or no effect on pre-RC assembly (e.g., Cdc6 and Cdc46/Mcm5).

The fact that late-firing origins are controlled by limiting amounts of some pre-RC activation factors (Mantiero et al., 2011; Tanaka et al., 2011) raises the possibility that the suppression effect we observe for *rif1Δ* might result from an increase in the amount of one or more of these proteins. However, we found no evidence for this (Figure S1). Although we cannot rule out a small abundance change that could have phenotypic consequences, we present data below showing that at least two initiation factors, including Sld3, display increased phosphorylation in *rif1Δ* cells.

Conserved RVxF- and SILK-like Motifs in Rif1 Mediate Its Effect on Pre-RC Activation

To address the mechanism of Rif1 inhibition of pre-RC activity, we initiated a structure-function analysis. Rif1 is a large protein with a long, centrally located array of helical repeats and a C-terminal domain required for telomere binding through interactions with Rap1 (Shi et al., 2013). Deletion of the C-terminal domain

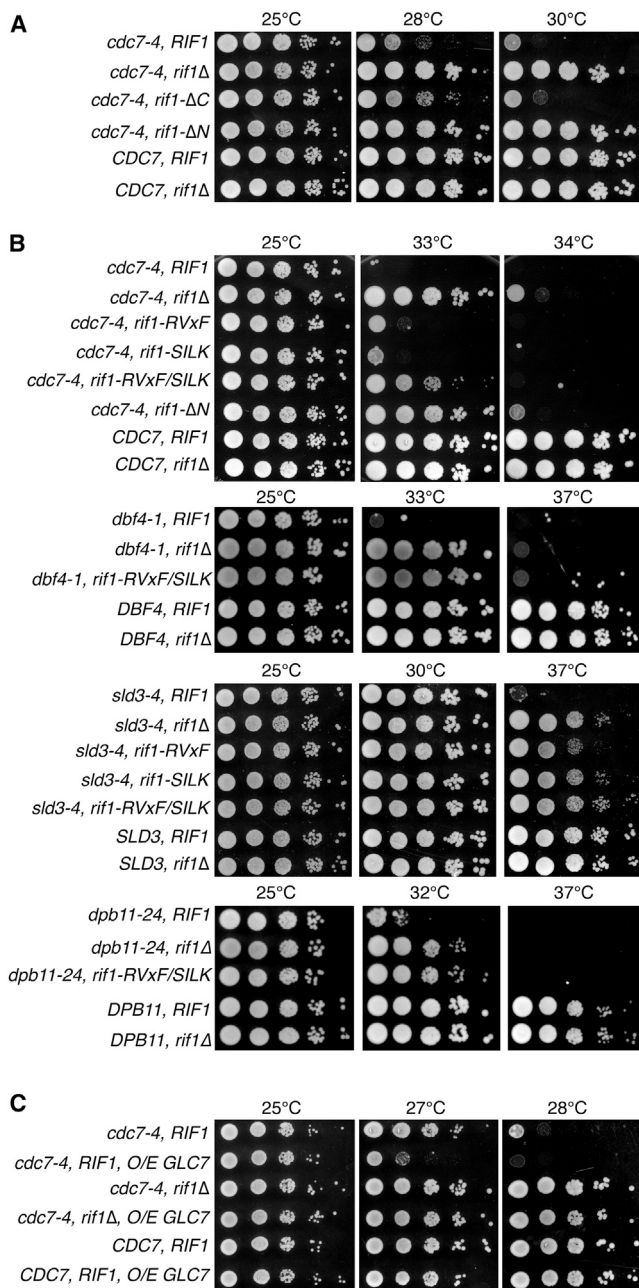


Figure 2. Glc7 Interaction Motifs in Rif1 and *GLC7* Dosage Modulate DNA Replication Initiation

(A and B) Cells with the indicated genotypes (*rif1-RVxF* and *rif1-SILK* cells carry point mutations in, respectively, RVxF and SILK motifs) were assayed as described in Figure 1.

(C) Cells with the indicated genotypes carrying either an empty plasmid or a plasmid containing the *GLC7* gene (*pGLC7*) were grown overnight in liquid medium lacking uracil (SC-Ura) at 25°C. Serial 10-fold dilutions were spotted onto SC-Ura plates and incubated at indicated temperatures for 48 hr. See also Figure S2.

(from residue 1323 to the end at 1916) had no effect on growth of a *cdc7-4* mutant (Figure 2A). In contrast, an N-terminal deletion (residues 2–176; *rif1-ΔN*) resulted in strong suppression of

cdc7-4, similar to that observed for *rif1Δ* (Figures 2A and 2B). The yeast Rif1 N terminus was shown recently to contain two short peptide motifs (commonly abbreviated as RVxF/SILK and referred to as such here, but actually KSVAF/SILR in *S. cerevisiae*; Sreesankar et al., 2012) that have been associated with recruitment of the PP1 phosphatase (Hendrickx et al., 2009), encoded by *GLC7* in yeast. PP1 (Glc7) is an abundant, highly conserved serine/threonine phosphatase that controls many different processes in all eukaryotic cells (Cannon, 2010). Interestingly, these motifs are conserved in fly and mammalian Rif1 homologs, though their position within the protein and their orientation are not. We generated point mutations in these two motifs and introduced them individually or in combination at the endogenous *RIF1* locus.

Strikingly, the combined RVxF/SILK domain mutations suppressed the *cdc7-4*, *dbf4-1*, *sld3-4*, and *dpb11-24* growth defects as well (or nearly, in the case of *cdc7-4*) as *rif1Δ* (Figure 2B). Where we examined either RVxF or SILK mutations alone (in *cdc7-4* and *sld3-4* strains), suppression was evident but weaker, indicating that the two motifs have an additive effect. We observed a similar effect of RVxF/SILK mutation on growth of *cdc45-27* (Figure S2A) and showed that the ΔN, RVxF, and SILK mutations do not act by reducing Rif1 protein levels (Figure S2B). Despite the fact that *rif1-ΔN* cells show a clear increase in Rif1 protein level compared to wild-type (Figure S2B), this mutation, lacking the two Glc7 interaction motifs, suppresses the *cdc7-4* growth defect as well as *rif1Δ* (Figure 2B).

If the RVxF/SILK mutations improved growth of *cdc7-4* by decreasing Glc7 recruitment at origins, overexpression of Glc7 might be expected to have the opposite effect. Indeed, we found that modest overexpression of Glc7 is sufficient to reduce the permissive temperature of a *cdc7-4* mutant (Figure 2C) but that this effect is abolished by *rif1Δ*. Taken together, these data strongly suggest that Glc7 acts downstream of Rif1 in the inhibition of origin firing.

Rif1 Interacts with Both Glc7 and Dbf4 and Regulates the Phosphorylation of Mcm4 and Sld3

The results described above suggest that Rif1 affects pre-RC activation through recruitment of the Glc7 phosphatase, which in turn directly counteracts the effect of DDK (and perhaps CDK) at replication origins. To test this idea, we first asked whether Rif1 and Glc7 physically interact in cells. We immunoprecipitated FLAG epitope-tagged Glc7 protein (expressed from the native *GLC7* locus) and probed the precipitate for Rif1-Myc (also expressed from the endogenous locus). As shown in Figure 3A, Rif1-Myc is enriched in precipitates from Glc7-FLAG-expressing G1-arrested cells, and this binding is strongly decreased in cells expressing the *rif1-RVxF/SILK*-Myc mutant protein (see also Figure S3A for two independent experiments and a coimmunoprecipitation [co-IP] in which the epitope tags were reversed).

We next asked whether Rif1 has an effect on the phosphorylation state of the pre-RC or its activators by focusing on a well-established DDK target, the MCM helicase component Mcm4 (Sheu and Stillman, 2006). We first examined Mcm4-Myc protein mobility by SDS-PAGE in extracts from *RIF1*, *rif1Δ*, and *rif1-RVxF/SILK* cells arrested either in G1 (following treatment with

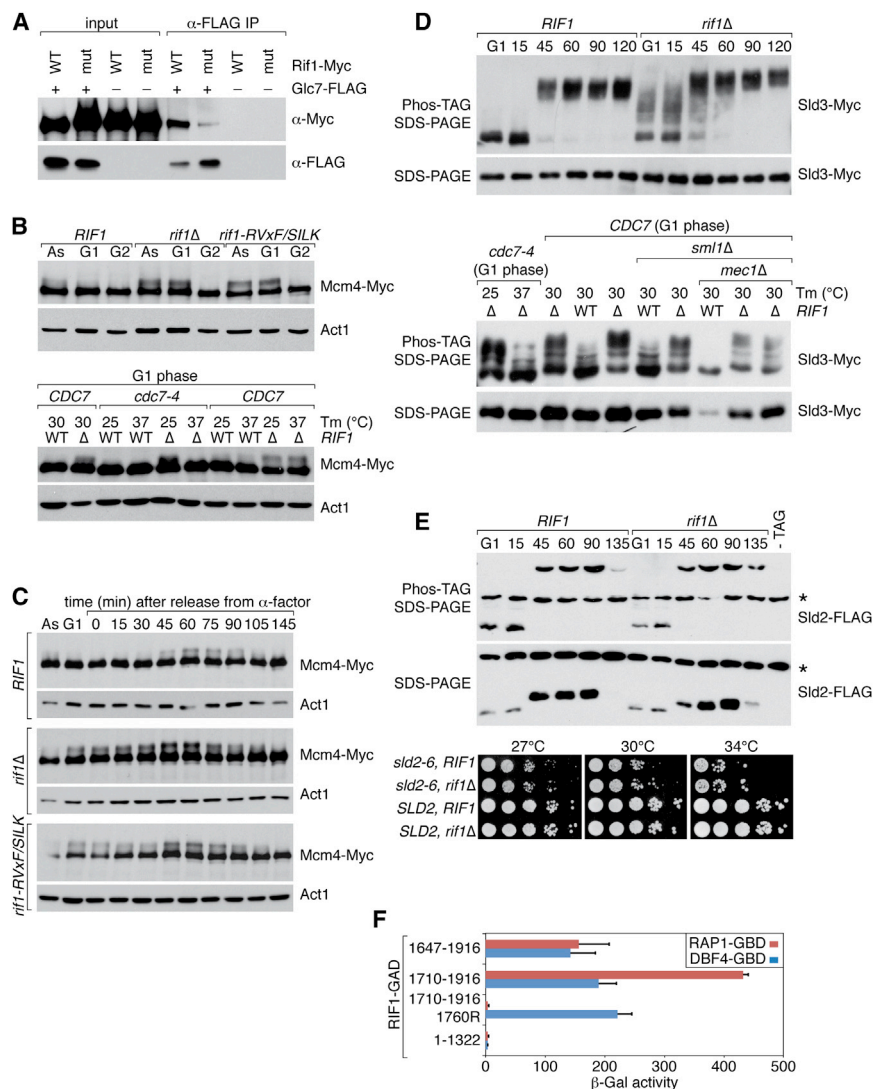


Figure 3. Rif1 Interacts with Both Glc7 and Dbf4 and Regulates the DDK-Dependent Phosphorylation of the Replicative Helicase Protein Mcm4 and the Pre-RC Activator Sld3

(A) Western blot using a Myc monoclonal antibody (mAb) following immunoprecipitation with anti-FLAG M2 beads from cells expressing Glc7-FLAG and either Rif1-Myc (WT) or *rif1-RVxF/SILK*-Myc (mut) or from control cells expressing only Rif1-Myc or *rif1-RVxF/SILK*-Myc. Extracts were prepared from G1-arrested cells. The same samples were analyzed by western blot using FLAG antibody.

(B) *RIF1*, *rif1-RVxF/SILK*, and *rif1 Δ* cells, all of which express a Mcm4-Myc protein from the endogenous *MCM4* locus, were arrested either in G1 or G2 phase by α factor or nocodazole (10 μ g/ml) treatments, respectively. Protein extracts were prepared and analyzed by western blot with a Myc mAb and with actin antibody as a loading control. As, asynchronous culture (top panel). *CDC7* or *cdc7-4* cells expressing Mcm4-Myc were grown at 25°C and then shifted to the indicated temperatures in the presence of α factor for 2 hr. Western blots were carried out as above (bottom panel).

(C) The strains described in (B, top panel) were arrested in G1 phase and then released into a synchronous S phase at 18°C. Protein extracts were prepared at the indicated times following release from the G1 block and analyzed by western blot using Myc and actin antibodies.

(D) Phos-tag SDS-PAGE western blots of extracts from either *RIF1* or *rif1 Δ* cells probed for Sld3-Myc (expressed from the endogenous *SLD3* locus). Cells were G1-arrested and released into a synchronous S phase at 18°C. Aliquots were taken at the indicated time points. The same samples were analyzed by standard SDS-PAGE western blot as a measure of total Sld3 protein (top panel). Cells of the indicated genotypes, all expressing Sld3-Myc, were grown at 25°C and then shifted to the indicated temperatures in the presence of α factor for 2 hr. Western blots were carried out as above (bottom panel).

(E) Phos-tag and normal SDS-PAGE western blot using FLAG mAb of extracts from either *RIF1* or *rif1 Δ* cells expressing Sld2-FLAG from the endogenous *SLD2* locus. Cells were G1-arrested and released as in (D, top panel; top panel; *, nonspecific band). Cells with the indicated genotypes were grown and spotted in YPAD plates as indicated in Figure 1 (bottom panel).

(F) Two-hybrid assays in a *rif1 Δ* derivative of the PJ69-4a reporter strain were performed using GBD-Dbf4 or GBD-Rap1 fusions as “bait” with indicated GAD-Rif1 fragments “prey.” The β -galactosidase activity levels reported are averages (with SD) of at least two independent biological replicates. See also Figure S3.

mating pheromone) or G2/M (by nocodazole treatment). This preliminary analysis revealed a prominent slower-mobility species specific to both G1-arrested *rif1 Δ* and *rif1-RVxF/SILK* mutant cells (Figure 3B, top panel) that is abolished by phosphatase treatment (data not shown) and absent when *cdc7-4* protein is inactivated (Figure 3B, bottom panel; 37°C). To examine Mcm4 phosphorylation in more detail, we monitored cells released from G1 arrest into a synchronous S phase. As shown in Figure 3C, and consistent with the previous experiment, we detected a modified form of Mcm4 only in G1-arrested *rif1 Δ* and *rif1-RVxF/SILK* mutant cells, which increased in intensity and reached a maximum at the 60 min time point but was unde-

tectable at 90 min, when S phase was completed (as shown by fluorescence-activated cell sorting [FACS] analysis and Clb2 accumulation; see Figure S3B, left panels). Interestingly, wild-type (*RIF1*) cells showed no evidence for Mcm4 phosphorylation at early times (0–30 min following release from G1) but displayed a slower-mobility species starting at 45 min (when early ARSs are fired, as measured by RPA recruitment; see Figure S3B, right panel) and increasing at 60 and 75 min, similar to that observed in the *rif1 Δ* cells (Figure 3C; for a separate experiment in which samples were loaded on one gel, see Figure S3C).

We also examined the phosphorylation state of two well-known CDK targets, Sld2 and Sld3 (Tanaka et al., 2007;

Zegerman and Diffley, 2007), using a Phos-tag gel to enhance the mobility shift of phosphorylated protein (see [Experimental Procedures](#)). We observed a prominent mobility shift in Sld3-Myc from G1 phase cells, and until 30 min following G1 release, that was specific for the *rif1* Δ mutant (Figure 3D, top panel; Figure S3D, left panel). As both *RIF1* and *rif1* Δ cells enter S phase at 45 min, Sld3 becomes highly phosphorylated (Figure 3D, top panel; Figure S3D, left panel), which previous studies have shown to result from S phase CDK action (Tanaka et al., 2007; Zegerman and Diffley, 2007). Total Sld3 levels are similar in these strains, indicating that the early-stage appearance of the shifted Sld3 band is not due to increased Sld3 expression in the *rif1* Δ mutant. Because the S phase CDK is not active in G1 cells, we asked whether Sld3 phosphorylation might require Cdc7 activity. We also examined the Mec1 kinase, which had previously been implicated in Sld3 phosphorylation (Lopez-Mosqueda et al., 2010; Zegerman and Diffley, 2010). Interestingly, we found that the G1-specific phosphorylation of Sld3 in *rif1* Δ cells is abolished when *cdc7-4* protein is inactivated (37°C sample) but unaffected by the absence of Mec1 (Figure 3D, bottom panel; Figure S3D, right panel). These data suggest that, as for the case of Mcm4, Sld3 is targeted by the DDK in G1 but that this phosphorylation is strongly counteracted by Rif1-Glc7. Interestingly, identical experiments in cells expressing Sld2-FLAG show no difference between *RIF1* and *rif1* Δ cells in the mobility of this protein either in G1 or as cells enter and proceed through S phase, where the protein becomes highly modified (Figure 3E, top panel). Consistent with this absence of Rif1-dependent phosphorylation of Sld2, we found that an *sld2-6* mutant is not suppressed by *rif1* Δ (Figure 3E, bottom panel). Another target of CDK, Orc6 (Chen and Bell, 2011), shows no change in its phosphorylation status between *RIF1* and *rif1* Δ cells (Figure S3E). Taken together, these data suggest that the action of Rif1-Glc7 might be limited to G1 phase and to DDK targets (Mcm4 and Sld3, though perhaps others).

In the course of investigating the role of Dbf4 in DNA replication initiation, we performed a yeast two-hybrid screen using full-length Dbf4 as bait and identified a C-terminal portion of Rif1 (amino acids 1647–1916) as an interacting polypeptide (Figure 3F). Interestingly, a nearly identical clone of *RIF1* was originally identified through its two-hybrid interaction with a C-terminal domain of Rap1 (Hardy et al., 1992). More recently, we demonstrated a direct interaction between a short α -helical region within the Rif1 C terminus (Rif1_{RBM}, amino acids 1752–1772) and a conserved groove in the Rap1 C terminus (Shi et al., 2013). Mutation of a critical residue within this peptide abolishes the interaction with Rap1, as shown before (Shi et al., 2013), but has no effect on the interaction with Dbf4 (Figure 3F). The experiments shown in Figure 3F were all performed in a *rif1* Δ reporter strain to eliminate the possibility of bridging effects with endogenous Rif1, which we showed can form tetramers through a C-terminal domain (Shi et al., 2013). The Dbf4 interaction we detect is thus not mediated through other parts of Rif1. We next asked if Rif1-Glc7 affects phosphorylation of Dbf4 by monitoring cells released from G1 arrest into a synchronous S phase. We observed a prominent mobility shift of Dbf4-FLAG throughout S phase but no effect of Rif1 on this modification (Figure S3F).

Rif1 RVxF and SILK Mutations Affect Glc7 Recruitment and Replication Timing at Telomeres

Because Rif1 is concentrated at telomeres in yeast (Mishra and Shore, 1999) and known to play a role in determining their late replication (Lian et al., 2011), we next asked whether Rif1 recruits Glc7 to telomeres. Indeed, we detected modest but reproducible association of Glc7-Myc to both the chromosome VI-R and XV-L telomeres, at sequences very close to their linked ARS sites (Figure 4A). Significantly, this binding was reduced or eliminated by mutations in the RVxF and SILK motifs, to an extent similar to that observed in *rif1- Δ N* or *rif1* Δ mutants (Figure 4A). The effect of the RVxF and SILK mutations is not due to decreased recruitment of Rif1 to the telomeres (Figure S4). These data thus indicate that Glc7 interacts specifically with two telomeres in a manner that depends upon the Rif1 RVxF/SILK motifs.

We then asked whether reduced Glc7 binding caused by *rif1* mutations is associated with altered replication profiles at chromosome VI-R and XV-L telomeres. We measured replication kinetics in synchronized cells by performing chromatin immunoprecipitation (ChIP) on a Myc epitope-tagged Pol2 (DNA polymerase ϵ) protein (Bianchi and Shore, 2007). This analysis showed that, although the firing of the early *ARS607* occurs at the same time in wild-type and all *rif1* mutant cells, the appearance of Pol2 immediately adjacent to the VI-R and XV-L telomeres occurs approximately 15 min earlier in the *rif1* mutants, consistent with the activation of the telomere-proximal *ARS610* and *ARS1503* origins (Figures 4B, right panels, and S4B). We confirmed the earlier telomere replication in *rif1* mutants by measuring DNA amounts for the input samples of the Pol2 ChIP experiment (Figure 4B, left panels), normalized to the *ARS607* region, where replication timing is unaffected by Rif1 (Figure 4B, top right panel). The two telomeric regions that replicate after *ARS607* display a decrease in DNA copy number at the time of *ARS607* replication that is reversed when they themselves replicate (Figure 4B, left panels; compare 60 and 105 min time points). Consistent with the Pol2-Myc ChIP results, we observed an earlier copy-number increase at both telomeres in all the *rif1* mutants compared to wild-type (Figure 4B, left panels; see 75 and 90 min time points). Our results thus suggest that the Rif1-Glc7 interaction controls origin firing adjacent to two different telomeres.

DISCUSSION

The genetic analysis described here provides several insights into the role of Rif1 protein in the control of DNA replication in budding yeast by both genetically and physically linking its action to the PP1 phosphatase, Glc7, and by pinpointing its effect to specific events involved in pre-RC activation.

In considering how Rif1 might exert a negative effect on replication initiation, we focused on two short motifs (similar to RVxF/SILK) that were recently observed in fungal Rif1 N termini (Sreesankar et al., 2012) and that have previously been shown to be docking elements allowing regulatory proteins to bind to the conserved PP1 phosphatase (Cannon, 2010; Hendrickx et al., 2009). Our genetic data strongly suggest that these two motifs are together absolutely required for the ability of Rif1 to negatively regulate origin firing, because their mutation suppresses

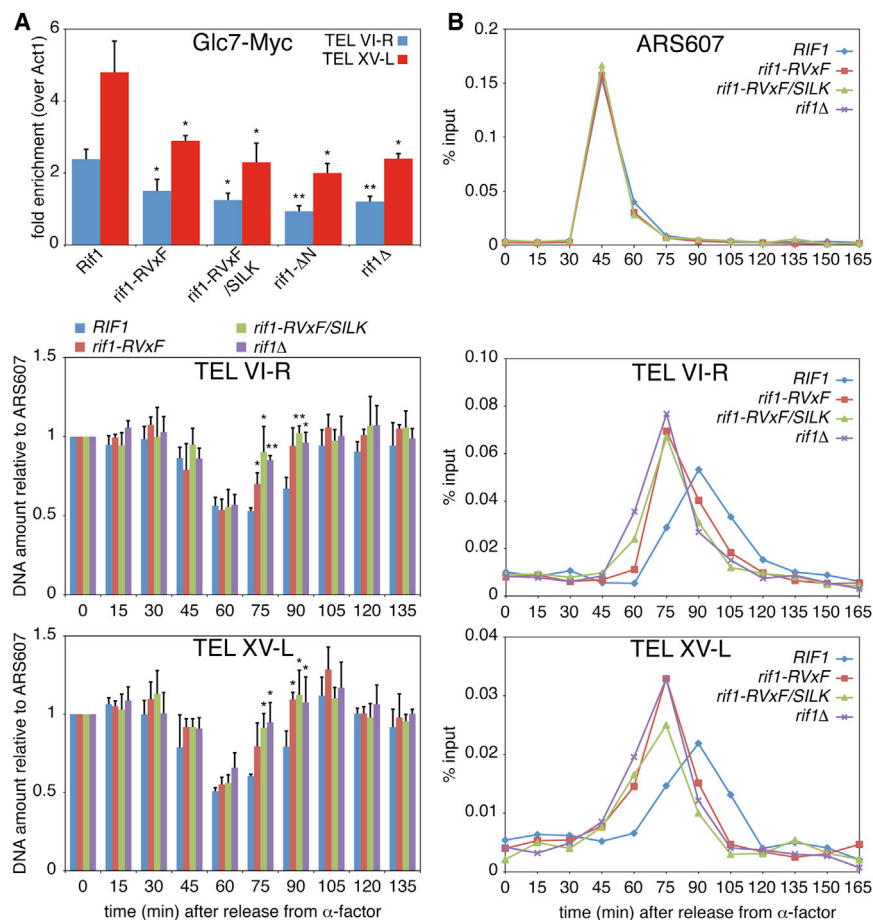


Figure 4. Telomeric Recruitment of Glc7 and Replication Timing Are Dependent upon Rif1 RVxV/SILK Motifs

(A) ChIP analysis of Glc7-Myc protein at the native chromosome VI-R and XV-L telomeres in *RIF1*, *rif1Δ*, and the indicated *rif1* deletion and point mutants cells. Results are reported as average fold enrichment and SD relative to the *ACT1* gene. Reduced recruitment of Glc7 is statistically significant in all of the *rif1* mutants tested (for TEL VI-R and TEL XV-L, respectively: *rif1-RVxV*, $p = 0.049$ and $p = 0.048$; *rif1-RVxV/SILK*, $p = 0.007$ and $p = 0.038$; *rif1-ΔN*, $p = 0.004$ and $p = 0.037$; *rif1Δ*, $p = 0.0013$ and $p = 0.019$).

(B) *RIF1*, *rif1-RVxV*, and *rif1-RVxV/SILK* cells were blocked in G1 phase and then released at 18°C. Pol2-Myc protein recruitment was assayed by quantitative PCR-ChIP analysis on aliquots taken at the indicated times following release from the G1 block. Results are reported as average and SD of three or four independent experiments (TEL VI-R: *rif1-RVxV* 75 min $p = 0.03772$; *rif1-RVxV/SILK* 75 min $p = 0.01628$, 90 min $p = 0.01684$; *rif1Δ* 75 min $p = 0.00049$, 90 min $p = 0.04867$. TEL XV-L: *rif1-RVxV* 75 min $p = 0.00951$; *rif1-RVxV/SILK* 75 min $p = 0.01684$, 90 min $p = 0.04186$; *rif1Δ* 75 min $p = 0.02524$, 90 min $p = 0.04905$; right panels). See also Figure S4.

various ts-lethal mutations affecting pre-RC activation to nearly the same extent as deletion of the whole *Rif1* open reading frame without having any obvious effect on *Rif1* protein levels. Our observation that *Rif1* coimmunoprecipitated with *Glc7* in an RVxV/SILK-dependent manner further supports the idea that *Rif1* and *Glc7* indeed interact in G1 phase to affect replication. We note that a high-throughput proteomic screen of yeast kinases and phosphatases has also revealed a *Rif1*-*Glc7* interaction (Breitkreutz et al., 2010).

Our detailed suppressor analysis of several different mutants involved in either pre-RC assembly or activation gives insights into the mode of *Rif1*-*Glc7* action. We found no evidence for *Rif1* action during pre-RC assembly but instead observed strong suppression by *rif1Δ* or *rif1-RVxV/SILK* mutations of specific alleles affecting pre-RC activation. Examination of the phosphorylation state of four different proteins involved in pre-RC activation provided further insights. Thus, we were able to show that *Rif1* affects the phosphorylation state of both *Mcm4* and *Sld3* in a *Cdc7* (DDK)-dependent manner in G1 cells, but not that of either *Dbf4* or *Sld2*. Our phosphorylation data are thus strikingly consistent with the genetic suppression data, both of which point to the DDK axis of pre-RC activation and two DDK targets, *Mcm4* and *Sld3*. Significantly, the S phase CDK target *Sld2* is unaffected by *Rif1* in either genetic or biochemical assays. Taking

all of the above into account, we propose that *Rif1*-*Glc7* inhibits replication initiation by counteracting the DDK in G1-phase cells. Our data are consistent with recent findings that *Cdc45* and *Sld3* (but not *Sld2*) are recruited to the pre-RC in G1 in a *Cdc7*-dependent manner and specifically at early-firing origins (Heller et al., 2011; Tanaka et al., 2011).

Although the suppression effect we observe for the *rif1-RVxV-SILK* mutation suggests that it might provoke a global increase in the phosphorylation state of at least two pre-RC activators, thus partially compensating for reduced DDK activity (*cdc7-4* or *dbf4-1*) or reduced activity of structural components (*sld3-4* and *dpb11-24*), it is still unclear if or how *Rif1*-*Glc7* activity is targeted at a genome-wide level. *Rif1* localizes to telomeres in budding yeast through an interaction with the telomere-binding protein *Rap1* (Mishra and Shore, 1999; Shi et al., 2013) but has not been shown to bind elsewhere in the genome. Consistent with this, we do indeed find that telomeres are a direct site of *Rif1*-*Glc7* action. One possibility is that the relief of *Rif1*-*Glc7* inhibition at telomere-proximal origins in *rif1-RVxV-SILK* mutants is sufficient to cause the ts mutant rescue that we observe. However, we know that *Rif1* mutants incapable of telomere localization, such as *rif1-ΔC* and *rif1-RBM* (Shi et al., 2013; data not shown), still strongly inhibit growth of *cdc7-4*, indicating that *Rif1* does not have to be telomere-bound to counteract DDK function

globally. So, can Rif1-Glc7 exert their inhibitory effect at other specific genomic sites? Although we suspect that this is the case, we have so far failed to detect either Rif1 or Glc7 at nontelomeric origins by ChIP, so have no evidence that their effect at these sites is direct. Further work will be required to resolve this issue.

Our identification of Rif1 as a Dbf4-interacting protein in a yeast two-hybrid screen provides evidence for a physical link between Rif1 and the DDK. Interestingly, the C-terminal domain of Rif1 that suffices for this interaction is not required for Rif1 to exert a negative effect on pre-RC activity, and we detect no effect of Rif1 on Dbf4 phosphorylation. This argues that the DDK itself is not a direct target of Rif1-Glc7, nor is it required for Rif1-Glc7 to operate on its actual targets. Instead, the Dbf4-Rif1 interaction may be best understood as part of a feedback mechanism by which the DDK limits the inhibitory action of Rif1-Glc7 on the pre-RC during G1, up to the point at which early origins fire. Indeed, we note the presence of five phospho-serine residues flanking the RVxF/SILK motifs in Rif1 that have been identified in proteomic studies (<http://www.phosphopep.org/>) and which are potential DDK targets. We thus speculate that the DDK targets the Rif1 N terminus in order to limit Glc7 recruitment and action at origins to the G1 phase. This model is consistent with the proposal that DDK activity increases as cells move into and through S phase (Rhind et al., 2010) and is also supported by our biochemical data.

We note that the findings described here, and in two recent related reports (Davé et al., 2014; Hiraga et al., 2014), are likely to have important implications for Rif1 function in other eukaryotes, including mammals. Recent reports show that fission yeast (Hayano et al., 2012), mouse (Cornacchia et al., 2012), and human Rif1 (Yamazaki et al., 2012) homologs are also involved in regulation of the temporal program of DNA replication. Furthermore, mammalian RIF1 has been shown to interact with PP1 (Moorhead et al., 2008). We thus predict that a RIF1-PP1 interaction in mammalian cells (as well as in fission yeast) will play a role in controlling the replication timing program in these systems, presumably through similar targets in the pre-RC activation pathway, many of which are also highly conserved between yeast and human.

EXPERIMENTAL PROCEDURES

Strains and Plasmids

All yeast strains and plasmids used in this study are listed in Tables S1 and S2, respectively. General yeast manipulations were done according to standard methods (Rose et al., 1990).

Viability Assays

Yeast strains were grown in the appropriate media to a concentration of 1×10^7 cells/ml. Serial 10-fold dilutions were spotted on plates containing yeast extract, peptone, adenine, and dextrose medium (YPAD) or selective medium at the indicated temperature. Plates were photographed after 2 days of incubation.

Western Blotting and CoIP Assays

Protein extracts (trichloroacetic acid-urea method) and SDS-PAGE western blot analyses were performed as described (Lempiäinen et al., 2009). See Supplemental Experimental Procedures for further details. For coimmunoprecipitation experiments, 500 ml of cells in exponential growth were treated with

50 ng/ml of α factor (Bachem) for 120 min in YPAD at 30°C. Cells were harvested and lysed by bead beating (0.5 mm zirconia-silica beads) in a buffer containing 20 mM Tris (pH 8.0), 150 mM KCl, 1 mM EDTA, 1% NP-40, 20% glycerol, 1 mM dithiothreitol, and 1 mM phenylmethanesulfonyl fluoride, supplemented with a protease and phosphatase inhibitor mix (Roche Molecular Biochemicals). Lysates precleared by centrifugation were subjected to anti-FLAG M2 or anti-Myc (9E10) immunoprecipitation by following the manufacturer's instructions (Sigma Aldrich). The beads were washed three times with the lysis buffer, and the immunoprecipitates were eluted in SDS sample buffer.

Yeast Two-Hybrid Screens and Assays

Yeast two-hybrid screens and quantitative beta-galactosidase assays were carried out as described previously (James et al., 1996).

ChIP and DNA Quantification Assays

ChIP assays were performed as described previously (Bianchi et al., 2004), with minor modifications (see Supplemental Experimental Procedures for details and for description of the DNA quantification assay).

Cell Synchrony Experiments

Overnight cultures of *MATa bar1 Δ* strains were diluted 10-fold into 450 ml of YPAD. After 3.5 hr growth at 30°C, cultures were blocked by treatment with 50 ng/ml of α factor (Bachem) for 130 min in YPAD at 30°C. Cells were released from the arrest by washing and by Pronase Nuclease-free treatment (Calbiochem; 50 mg total) and then placed in YPAD medium at 18°C. Samples were taken at 15 min intervals for ChIP and FACS analysis. Cell-cycle synchrony and release were checked by microscope and FACS analysis as described previously (Bianchi and Shore, 2007).

Statistical Analysis

The differences in values obtained in either ChIP or DNA quantification assays were assessed for significance with Student's *t* test. Average values and SD are reported.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and two tables and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2014.03.010>.

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