Rif1 controls DNA replication by directing Protein Phosphatase 1 to reverse Cdc7-mediated phosphorylation of the MCM complex

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Initiation of eukaryotic DNA replication requires phosphorylation of the MCM complex by Dbf4-dependent kinase (DDK), composed of Cdc7 kinase and its activator, Dbf4. We report here that budding yeast Rif1 (Rap1-interacting factor 1) controls DNA replication genome-wide and describe how Rif1 opposes DDK function by directing Protein Phosphatase 1 (PP1)-mediated dephosphorylation of the MCM complex. Deleting RIF1 partially compensates for the limited DDK activity in a cdc7-1 mutant strain by allowing increased, premature phosphorylation of Mcm4. PP1 interaction motifs within the Rif1 N-terminal domain are critical for its repressive effect on replication. We confirm that Rif1 interacts with PP1 and that PP1 prevents premature Mcm4 phosphorylation. Remarkably, our results suggest that replication repression by Rif1 is itself also DDK-regulated through phosphorylation near the PP1-interacting motifs. Based on our findings, we propose that Rif1 is a novel PP1 substrate targeting subunit that counteracts DDK-mediated phosphorylation during replication. Fission yeast and mammalian Rif1 proteins have also been implicated in regulating DNA replication. Since PP1 interaction sites are evolutionarily conserved within the Rif1 sequence, it is likely that replication control by Rif1 through PP1 is a conserved mechanism.

[Keywords: DNA replication; protein phosphorylation; protein kinase; protein phosphatase; PP1]

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step in initiating replication, as it triggers recruitment of Cdc45 and GINS, a four-subunit protein complex, to create the CMG (Cdc45–MCM–GINS) complex that forms the active replicative helicase (Fu et al. 2011). The N-terminal domain of Mcm4 plays an inhibitory role in CMG assembly that is alleviated by DDK phosphorylation [Sheu and Stillman 2010]. Either removal of this Mcm4 N-terminal domain or the introduction of phosphomimetic mutations is sufficient to bypass the requirement for DDK in CMG formation and DNA replication, identifying Mcm4 as the only DDK target whose phosphorylation is essential for DNA replication [Sheu and Stillman 2010].

Rif1 (Rap1-interacting factor 1) was originally identified as a telomeric chromatin component required for telomere length regulation in budding yeast through its physical interaction with Rap1 [Hardy et al. 1992; Shi et al. 2013]. It has since been demonstrated that S. cerevisiae Rif1 suppresses activation of the DNA damage checkpoint near telomeres (Xue et al. 2011; Ribeyre and Shore 2012) and affects telomere replication time [Lian et al. 2011]. The presence of Rif1 is evolutionarily conserved, but in other eukaryotes, Rif1 has been shown to play nontelomeric roles, such as directing the pathways used in DNA double-strand break repair and DNA recombination (Chapman et al. 2013; Di Virgilio et al. 2013; Escribano-Diaz et al. 2013). Recent studies have implicated the fission yeast (Schizosaccharomyces pombe) and mammalian Rif1 proteins in the regulation of DNA replication genome-wide. Deleting the S. pombe rif1+ gene suppresses the block to replication caused by mutations in DDK subunits [Hayano et al. 2012]. Removal of S. pombe or mammalian Rif1 also derails the replication temporal program, affecting the order in which origins initiate replication (Carnachia et al. 2012, Hayano et al. 2012, Yamazaki et al. 2012). Rif1 binds most strongly to telomeric regions within the fission yeast genome but can also be detected at hundreds of internal sites. The relationship between Rif1-binding sites and origins affected by the rif1Δ mutation is ill-defined, however, and the mechanism through which Rif1 affects origin replication initiation time and imposes the requirement for DDK has been unclear. Mammalian Rif1 moreover seems to be involved in large-scale chromatin organization, but again the mechanism is unclear (Carnachia et al. 2012, Yamazaki et al. 2012). In budding yeast, a role for Rif1 in global control of DNA replication has not previously been reported.

Eukaryotic Rif1 proteins are evolutionarily divergent but do share some features, in particular HEAT [Huntingtin, elongation factor 3, Protein Phosphatase 2A, and yeast kinase TOR1] repeats as well as SILK and RVXF motifs (Xue et al. 2011; Sreesankar et al. 2012). HEAT is a tandem repeat of ~50 amino acids found in a wide variety of eukaryotic proteins. HEAT repeats are proposed to form a “solenoid” domain functioning in protein–protein interactions [Andrade et al. 2001]. SILK and RVXF motifs are conserved sequence signatures of Protein Phosphatase 1 (PP1)-interacting proteins that mediate binding to PP1 (Bollen et al. 2010). Consistent with the presence of PP1 interaction motifs, Rif1 was reported to interact physically with PP1 in budding yeast [Breitkreutz et al. 2010], but the physiological significance of PP1–Rif1 interaction has been unclear.

PP1 is a conserved Ser/Thr protein phosphatase with multiple functions in eukaryotes [Cohen 2002]. Most eukaryotes contain several PP1 isoforms, but S. cerevisiae has a single PP1 encoded by the essential gene GLC7. PP1 proteins have indiscriminate phosphatase activity in vitro, and in vivo are directed by PP1 targeting proteins to physiologically relevant substrates or cellular locations. PP1 targeting proteins are diverse, generally showing no sequence similarity beyond the presence of at least one PP1 interaction [e.g., RVXF or SILK] motif [Wakula et al. 2003]. These PP1 interaction motifs are also found in other PP1-interacting proteins, including PP1 substrates and inhibitory regulators [Bollen et al. 2010]. For all such PP1-interacting proteins, physical interaction via PP1 interaction motifs is important for them to direct PP1 function in vivo.

As the sole S. cerevisiae PP1, Glc7 is essential for cell viability. Glc7 has been implicated in many cellular pathways, including glycogen metabolism [Feng et al. 1991], control of budding [Black et al. 1995], premeiotic DNA synthesis [Ramaswamy et al. 1998], mitotic control [Hisamoto et al. 1994; Black et al. 1995], and recovery fromcheckpoint arrest [Bazzi et al. 2010]. Compared with protein kinases, little is known about the involvement of protein phosphatases in the regulation of DNA replication.

In this study, we show that the S. cerevisiae Rif1 protein controls DNA replication genome-wide and that Rif1 exerts this control through PP1-mediated dephosphorylation of the MCM complex early in the cell cycle. We confirm that Rif1 and Glc7 interact, implicating Rif1 as a previously unidentified PP1 substrate targeting subunit. Targeting of PP1 by Rif1 appears itself to be controlled by DDK through phosphorylation to inactivate the Rif1 PP1 interaction motifs. By both controlling and being controlled by DDK phosphorylation, Rif1 contributes to the sharp rise in DDK-mediated phosphorylation of Mcm4 that allows cells to begin replication.

Results

A rif1Δ mutant escapes from a cdc7 block and partially suppresses cdc7-1 temperature sensitivity

In the course of an attempt to synchronize rif1Δ cells using the cdc7-1 temperature-sensitive allele, we noticed that the cdc7-1 rif1Δ strain escapes a cdc7+ [37°C] block. To test the effect of RIF1 deletion on cdc7-1 arrest systematically, wild-type, cdc7-1, and cdc7-1 rif1Δ strains were arrested in G1 phase at 23°C using α-factor and then released at 37°C, and their DNA content was followed by flow cytometric analysis (Fig. 1A). Wild-type cells quickly transit S phase and enter a second cell cycle 2–3 h after removal of α-factor. DNA content did not change on release of the cdc7-1 mutant strain, confirming that these cells fail to begin DNA replication. In contrast, the majority of cdc7-1 rif1Δ cells began DNA replication [as demonstrated by their exit from the 1C peak] and, after 2–3 h, had accumulated as a near-2C population; however, the majority of cdc7-1 rif1Δ cells did not complete mitosis, suggest-
ing that DNA replication was incomplete after 4 h at 37°C.

Figure 1. Deletion of RIF1 partially suppresses temperature-sensitive defects of cdc7-1. (A) Wild-type [AW31], cdc7-1 [RM14-3a], and cdc7-1 rif1Δ [HYLS1] strains were arrested with α-factor and released at 37°C. Cells were sampled, and DNA content was analyzed by flow cytometry. (B) As in A but released at 30°C. (C) Growth of cdc7-1 [RM14-3a] and cdc7-1 rif1Δ [HYLS1] strains at 23°C and 30°C. See also Supplemental Figure S1. (D, top panel) DNA replication profile of chromosome IV in the cdc7-1 rif1Δ strain [HYLS1] after release from α-factor for 1 h (orange), 2 h [green], and 3 h [blue] at 37°C. The black curve shows the extent of replication in G1 control. The Y-axis shows the percent replication. (Bottom panel) The gray curve shows the DNA replication profile of chromosome IV in wild-type cells in early S phase 40 min after release from α-factor [redrawn from McCune et al. 2008]. Early initiating replication origins ARS418, ARS428, and ARS432.5 are indicated. Yellow circles indicate CEN4. See also Supplemental Figure S2.
caused by deleting CDC7 or DBF4 [Supplemental Fig. S1]. In summary, deletion of *S. cerevisiae* RIF1 can partially compensate for compromised DDK function but does not remove the requirement for DDK altogether.

Replication initiation within telomeric and nontelomeric chromosome regions enables the rif1Δ mutant to escape a cdc7ts block

*S. cerevisiae* Rif1 regulates telomere function [Hardy et al. 1992], and we previously showed that replication origins near telomeres are prematurely activated in a rif1Δ mutant [Lian et al. 2011]. We therefore tested whether, in the *cdc7-1 rif1*Δ strain, replication depends on deregulated telomere-proximal origins or genome-wide initiation events. To distinguish between these possibilities, we used an isotopic labeling method [Alvino et al. 2007] to monitor the appearance of replicated DNA in the *cdc7-1 rif1*Δ strain released from α-factor at 37°C ([Fig. 1D, top panel; Supplemental Fig. S2]). Consistent with our flow cytometry analysis ([Fig. 1A]), chromosomal DNA replicated slowly and neared completion after 3 h. Importantly, 1 h after release, we observed zones of replicated DNA not only close to telomeres but also within internal chromosomal regions [e.g., chromosome IV 350–630 kb], implying that at 37°C in the *cdc7-1 rif1*Δ strain, replication begins from internal as well as telomere-proximal origins.

The chromosome regions that are first to be replicated in the *cdc7-1 rif1*Δ strain at 37°C generally correspond to chromosome domains that are early replicating in the normal S phase of a wild-type strain ([Fig. 1D, bottom panel; McCune et al. 2008]). This replication pattern indicates that the sites most prone to replication initiation at 37°C in *cdc7-1 rif1*Δ are likely to correspond to normally early initiating origins, for example, origins ARS418, ARS428, and ARS432.5 (arrowheads in [Fig. 1D]; Nieduszynski et al. 2007; Siow et al. 2012).

Rif1 suppresses Mcm4 phosphorylation

The data above suggest that Rif1 regulates DNA replication by counteracting DDK activity. Mcm4 is the major target of DDK in DNA replication control, so we investigated whether Rif1 affects the phosphorylation status of Mcm4. In whole-cell extracts from wild-type yeast, phosphorylated forms of a Flag-tagged Mcm4 protein are barely detectable because only a small fraction of Mcm4 protein is phosphorylated ([Fig. 2A, top panel; lanes 1–3; Sheu and Stillman 2006]). Deleting *RIF1* increased the fraction of Mcm4 protein that displayed a slower mobility in SDS-PAGE analysis ([Fig. 2A, top panel, lanes 4–6]. This slower mobility form corresponds to hyperphosphorylated Mcm4, as it is lost upon phosphatase treatment [Supplemental Fig. S3A]. Therefore, loss of Rif1 function leads to hyperphosphorylation of Mcm4 protein. Importantly, this hyperphosphorylation depends on DDK function, since the amount of Mcm4 showing lowest mobility is reduced if Cdc7 activity is compromised [in a *cdc7-1 rif1*Δ strain at 30°C] ([Fig. 2A, bottom panel]). Lack of Rif1 therefore does not substitute for the DDK requirement but rather compensates for reduced DDK activity—a result consistent with the observation that *RIF1* deletion does not suppress lethality of *cdc7*Δ or *dbf4*Δ [Supplemental Fig. S1]. In the *rif1*Δ strain, the majority of chromatin-associated Mcm4 appears to be hyperphosphorylated [Supplemental Fig. S3B], suggesting that Rif1 preferentially affects chromatin-bound Mcm4.

Rif1 therefore restricts DDK-mediated phosphorylation of Mcm4 even during G1 phase in cells blocked with α-factor ([Fig. 2A]). DDK activity is generally low during G1 due to low Dbf4 levels [Oshiro et al. 1999; Weinreich and Stillman 1999; Ferreira et al. 2000], although the presence of some functional DDK during G1 phase has been demonstrated [Katou et al. 2006; Tanaka et al. 2011]. The increase in Mcm4 phosphorylation caused by deleting Rif1 was not due to increased or precocious Dbf4 expression, as Western analysis revealed no change in Dbf4 levels in the *rif1*Δ mutant in either S phase [Supplemental Fig. S3C] or G1 phase (data not shown).

![Figure 2](image-url)
Next, we examined whether increased Mcm4 phosphorylation is productive for the DNA replication process. One consequence of DDK-mediated Mcm4 phosphorylation is Cdc45 recruitment by the MCM complex to form CMG, the active replicative DNA helicase [Gambus et al. 2006; Moyer et al. 2006]. We tested whether elevated Mcm4 phosphorylation in the absence of Rif1 leads to increased CMG complex formation. In the rif1Δ strain, we found a significant increase in the amount of Cdc45 protein interacting with Mcm4 protein during S phase in both CDC7+ and cdc7-1 contexts (as assessed by analyzing Cdc45 coimmunoprecipitating with Mcm4) (Fig. 2B, cf. lanes 4,8 and 3,7, respectively). Deregulated Mcm4 phosphorylation in the absence of Rif1 therefore appears to allow increased CMG formation, potentially stimulating additional origin initiation events that could be related to S-phase deregulation in the absence of Rif1 [Lian et al. 2011; Xue et al. 2011; Cornacchia et al. 2012; Yamazaki et al. 2012]. Mcm4–Cdc45 interaction was not detected in G1 phase in rif1Δ cells despite the fact that Mcm4 phosphorylation is increased. Presumably other cell cycle controls prevent premature Mcm4 phosphorylation from triggering aberrant DNA replication during G1 phase in the rif1Δ strain. In summary, Rif1 seems to regulate DNA replication by repressing Mcm4 phosphorylation.

Rif1 C terminus physically interacts with Dbf4

In investigating how Rif1 counteracts DDK function, we first considered the possibility that Rif1 binds to DDK directly and negatively regulates its kinase activity. A two-hybrid-based investigation of Dbf4 had revealed that it interacts with the C-terminal domain of Rif1. Further mapping identified Rif1 residues 1790–1916 as the minimal region required for interaction with a BRCT domain in the N-terminal region of the Dbf4 protein [Fig. 3A, left plate; Supplemental Fig. S4B]. This 1900–1916 region overlaps the C-terminal Rif1 domain that interacts with Rap1 protein at telomeres. However, the minimal Rif1 domain required for Dbf4 interaction (residues 1790–1916) is shorter than that required for Rap1 interaction (residues 1761–1916) [Fig. 3A, right plate], suggesting that Rif1 interacts with Dbf4 and Rap1 through distinct molecular mechanisms.

To confirm this interaction biochemically, full-length HA-tagged Cdc7 protein, a series of truncated Dbf4 proteins, and the C-terminal fragment of Rif1 [residues 1735–1916; referred to as Rif1-CT] were coexpressed in insect cells. Cdc7 was immunoprecipitated from the cell extracts and tested for recovery of Dbf4 and Rif1-CT. We found that the Rif1-CT was recovered with Cdc7 and that this coprecipitation depended on the presence of Dbf4 with an intact BRCT domain [BRCT domain is residues 118–221 in Dbf4] [Fig. 3B, Supplemental Fig. S4A]. This result was therefore consistent with the two-hybrid analysis, indicating that the C-terminal region of Rif1 can bind to DDK through an interaction with Dbf4.

A physical interaction between Rif1 and Dbf4 appeared to favor the suggestion that Rif1 restrains replication by binding DDK and repressing its activity. We found, however, that addition of purified Rif1-CT [fused to GST] does not affect in vitro kinase activity of DDK toward Mcm4 (Supplemental Fig. S4C). Moreover, overexpressing the Rif1 C-terminal domain in yeast did not affect growth of a cdc7-1 strain, the binding of Dbf4 to an origin, or Dbf4 binding to chromatin [data not shown], as tested using established assays [Dowell et al. 1994; Sheu and Stillman 2006]. These results indicate that the Rif1 C terminus is not detrimental to DDK function, although, as we were unable to express or test GST-fused full-length Rif1, we cannot exclude the possibility that full-length Rif1 might be capable of repressing DDK activity.
Mapping of Rif1 domains required to counteract DDK

To identify domains of the Rif1 protein important for regulating DNA replication and DDK function, we constructed a series of RIF1 alleles with internal deletions or terminal truncations [Fig. 4]. These constructs were designed to remove putative functional elements, including the C-terminal Dbf4-interacting region identified above, the HEAT repeats [Xu et al. 2010; Sreesankar et al. 2012], potential PP1 interaction motifs in the N-terminal domain [Sreesankar et al. 2012], and a region with weak homology with a putative Holliday junction (HJ)-binding domain in vertebrate Rif1 [Xu et al. 2010].

We tested the effect of these constructs on growth of a cdc7-1 strain by introducing centromeric plasmids with the corresponding alleles into the cdc7-1 rif1Δ strain. If a mutated RIF1 allele retains the replication-repressive function of wild-type RIF1, it will prevent the cdc7-1 strain from growing at the restrictive temperature of 30°C [Fig. 4, full-length]. If, on the other hand, the RIF1 allele cannot repress replication, it will permit cdc7-1 cells to grow at 30°C [Fig. 4, empty vector]. We found that any deletion within its N-terminal half made Rif1 nonfunctional for replication repression [Fig. 4], including relatively small deletions covering the PP1 interaction motifs Δ[114–225] or the HEAT repeats Δ[321–455], suggesting that these features are important for the function of Rif1 in regulating DNA replication. The abundance of Rif1-Δ[114–225] was not reduced compared with wild-type Rif1 [Supplemental Fig. S5], so loss of repressive activity is not due to instability of this mutant protein.

To our surprise, the entire C-terminal half of Rif1, including the region mediating Dbf4 interaction, was found to be dispensable for replication repression [Fig. 4, 30°C panel, bottom three strains]. In fact, rather than alleviating its repressive effect, removal of the C-terminal Dbf4-interacting domain appeared to make Rif1 even more repressive toward replication. Specifically, these deletions exacerbated the temperature sensitivity of the cdc7-1 mutant so that it grew very poorly even at 26.5°C, a temperature at which a cdc7-1 Rif1 strain can grow moderately well [Fig. 4, 26.5°C panel, cf. Δ[1790–1916] and Δ[997–1916] with full-length]. This enhanced repressive effect suggests that the C-terminal region of Rif1 may down-regulate its repressive function in replication.

PP1 mediates DNA replication control by Rif1

Our deletion analysis highlighted a potential PP1-interacting region of Rif1 as important for replication repression. Sequences capable of PP1 interaction are called SILK and RVXF motifs, their consensus sequences more precisely expressed as [S/G][I/L][K/R] and [K/R][x1][V/I][x2][F/W], where x1 may or may not be present. Two potential PP1 interaction motifs have been described in the N-terminal region of Rif1: KSVAF [at position 114] and SILR [at 146] [Sreesankar et al. 2012]. We noticed two further potential PP1 interaction motifs in the N-terminal region: GLIR [at 222] and KIVKW [at 316] [Supplemental Fig. S5]. To investigate the significance of these PP1 interaction motifs, we constructed a Rif1 allele in which all of the four putative PP1 interaction motifs were mutated [rif1-pp1bs] [Fig. 5A]. These mutations completely reproduced the effect of Rif1 deletion with respect to replication repression [Fig. 5B], that is, a cdc7-1 strain carrying rif1-pp1bs was able to grow at 30°C, like cdc7-1 rif1Δ or cdc7-1 rif1-Δ[114–225] [Fig. 5B]. The rif1-pp1bs gene product was expressed at levels similar to wild-type Rif1 [Supplemental Fig. S5]. Its phenotype demonstrates that the N-terminal PP1 interaction motifs are essential for Rif1 function in regulating DNA replication and strongly suggests that repression of replication by Rif1 is mediated through PP1.

Rif1 has previously been reported to interact physically with Glc7, the sole PP1 in budding yeast [Breitkreutz et al. 2010]. We sought to confirm this interaction using a Rif1–Glc7 two-hybrid assay. Growth on selective—adenine—histidine medium [Fig. 5C, top middle panel] demonstrated interaction between a slightly truncated Glc7 prey fragment (amino acids 1–287) and a Rif1 bait fragment containing the PP1 interaction motifs (amino acids 1–339). A weaker interaction was observed between Glc7 and a longer Rif1 fragment (amino acids 1–846), as demonstrated interaction between a slightly truncated Glc7 prey fragment (amino acids 1–287) and a Rif1 bait fragment containing the PP1 interaction motifs (amino acids 1–339). A weaker interaction was observed between Glc7 and a longer Rif1 fragment (amino acids 1–846), as indicated by growth under less stringent selection conditions [low levels of adenine and histidine] [Fig. 5C, right panel, bottom three strains]. In fact, rather than alleviating its repressive effect, removal of the C-terminal Dbf4-interacting domain appeared to make Rif1 even more repressive toward replication. Specifically, these deletions exacerbated the temperature sensitivity of the cdc7-1 mutant so that it grew very poorly even at 26.5°C, a temperature at which a cdc7-1 Rif1 strain can grow moderately well [Fig. 4, 26.5°C panel, cf. Δ[1790–1916] and Δ[997–1916] with full-length]. This enhanced repressive effect suggests that the C-terminal region of Rif1 may down-regulate its repressive function in replication.

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mutating the Rif1 PP1 interaction motifs prevented the two-hybrid interaction, as expected if Rif1 directly recruits Glc7 protein (Fig. 5C, bottom middle panel; Supplemental Fig. S6A). Our two-hybrid analysis therefore confirmed interaction between Rif1 and Glc7 protein domains, dependent on the Rif1 PP1 interaction motifs.

On the basis of our results, we hypothesized that Rif1 represses DNA replication through PP1, counteracting DDK by directing Glc7-mediated dephosphorylation of the MCM complex. If this model is correct, Glc7 will be required to restrict phosphorylation of Mcm4 protein, in particular during G1 phase, when the effect of the rif1Δ mutation is especially marked. Whole-cell extracts were prepared from α-factor-arrested glc7-10 cells (Andrews and Stark 2000), and phosphorylation of Mcm4 protein was compared with wild-type cells. Since glc7-10 is temperature-sensitive for growth, we prepared extracts from cells grown at the permissive temperature (26° C) and after shift for 1 h to the restrictive temperature (37° C). Mcm4 phosphorylation is increased in glc7-10 cells (Fig. 5D, lanes 2, 4), demonstrating that PP1 plays a role in suppressing Mcm4 phosphorylation during G1 phase. Increased Mcm4 phosphorylation was seen at both 26° C and 37° C, consistent with the observation that, even at permissive temperature, the glc7-10 allele compromises PP1 function and results in slow growth (data not shown).

If Glc7 acts to reverse DDK-mediated phosphorylation of the MCM complex, then reducing levels of active Glc7 might alleviate the growth defect of a cdc7-1 strain, similar to the rif1Δ mutation. To test this possibility, we examined the effect of removing one copy of the GLC7 gene in a cdc7-1/cdc7-1 diploid background. The cdc7-1/cdc7-1 GLC7+/glc7Δ heterozygote grew better than cdc7-1/cdc7-1 GLC7+/GLC7+ at 27° C (Fig. 5E), indicating that reduced Glc7 levels can partially compensate for compromised DDK activity. Conversely, we would predict that increased Glc7 phosphatase activity might interfere with replication when DDK activity is limited. We found that even a mild increase in Glc7 levels, caused by the introduction of a centromeric plasmid bearing the GLC7 gene, compromised growth of a cdc7-1 rif1Δ mutant at temperatures where its growth is marginal (30° C–32° C) [Supplemental Fig. S6C]. Overall, these tests confirmed that the cdc7-1 mutant, whose growth is limited by available DDK, is extremely sensitive to Glc7 phosphatase levels but only if Rif1 is present. These results therefore support the suggestion that Rif1 binds Glc7 and directs it to dephosphorylate Mcm4.

**Rif1–PP1 interaction may be regulated by DDK phosphorylation**

The Rif1 C-terminal domain can interact with Dbf4 [Fig. 3], but the Rif1 C terminus is dispensable for replication repression and apparently plays a negative role that restricts Rif1 function [Fig. 4]. We considered whether the
The sequence surrounding the first two Rif1 RVXF and SILK motifs (shaded gray), with DDK target consensus sequences indicated by black bars, and CDK target consensus sequences indicated by broken bars. Serine residues identified as phosphorylated in vivo are shown in pink. (B) The top Rif1 cartoon illustrates the locations of the nine potential DDK target sites mutated. Vertical black lines indicate the nine potential DDK phosphorylation sites between amino acids 100 and 250 or all 14 CDK and DDK sites (Fig. 6B) were substituted with nonphosphorylatable alanine or phosphomimetic glutamate residues, creating rif1-9A, rif1-9E, rif1-14A, and rif1-14E alleles (sequences shown in Supplemental Fig. S7A). If phosphorylation is required to down-regulate the Rif1–Glc7 interaction, then we would predict that the rif1-9A and rif1-14A gene products will constitutively target PP1 activity to counteract DDK and will therefore be repressive toward replication. As predicted, the rif1-9A and rif1-14A alleles were hyper-repressive toward growth of the cdc7-1 strain (Fig. 6C), showing a phenotype resembling the rif1Δ(1790–1916) truncation mutant (Fig. 4). This hyperrepressive effect is not caused by increased protein levels (Supplemental Fig. S7B). Conversely, rif1-9E and rif1-14E alleles alleviated the cdc7-1 growth defect, partially mimicking the effect of rif1Δ by allowing robust growth at 27°C, consistent with compromised interaction between PP1 and the phosphorylated form of Rif1. Neither rif1-9E nor rif1-14E permitted strong growth of the cdc7-1 strain at 30°C (unlike rif1Δ), perhaps because glutamate substitution does not fully mimic phosphorylation or because some other aspect of control is missing. For both nonphosphorylatable and phosphomimetic alleles, mutating the nine DDK consensus sites resulted in a clear phenotype, which was mildly enhanced by additionally mutating the CDK sites. Phosphorylation of Rif1 by DDK therefore appears to be largely sufficient for regulating interaction with PP1, with phosphorylation by CDK additionally making a contribution. In summary, the results of our investigation suggest a model in which Rif1 represses DNA replication by directing PP1 to dephosphorylate the MCM complex (illustrated in Fig. 7A), with the Rif1–PP1 interaction itself likely to be regulated by DDK phosphorylation.

Discussion

Here we demonstrated that budding yeast Rif1 is involved in regulating DNA replication genome-wide, consistent with findings in fission yeast and mammalian cells and suggesting that DNA replication control by Rif1 is conserved through eukaryotes (Cornacchia et al. 2012; Hayano et al. 2012; Yamazaki et al. 2012). Rif1 restrains replication by counteracting the action of DDK, and loss of Rif1 partially compensates for impaired DDK function in a cdc7-1 mutant strain. In particular, we found that deleting Rif1 increases Mcm4 phosphorylation levels and concomitantly alleviates the temperature sensitivity in growth of cdc7-1 cells. Depletion of Rif1 leads to increased Cdc7-mediated phosphorylation of Mcm4 protein even in

Figure 6. Phosphorylation at DDK consensus sequences regulates replication repression by Rif1. (A) The sequence surrounding the first two Rif1 RVXF and SILK motifs (shaded gray), with DDK target consensus sequences indicated by black bars, and CDK target consensus sequences indicated by broken bars. Serine residues identified as phosphorylated in vivo are shown in pink. (B) The top Rif1 cartoon illustrates the locations of the nine potential DDK target sites mutated. Vertical black lines indicate the nine potential DDK phosphorylation sites between amino acids 100 and 250 in the Rif1 sequence. Vertical gray bars indicate PP1 interaction motifs 1–4. The bottom Rif1 cartoon illustrates the locations of the 14 potential DDK and CDK target sites mutated. Vertical dashed lines indicate consensus CDK phosphorylation sites, with other annotations as above. See also Supplemental Figure S7. (C) Plasmids containing rif1-9A, rif1-9E, rif1-14A, or rif1-14E alleles mutated at the sites shown in B were transformed into cdc7-1 rif1Δ strain (SHYS55). Serial dilutions of cultures were spotted onto SC-Ura plates and incubated at the indicated temperatures.
G1 phase, when DDK activity is limited. PP1-binding motifs in the Rif1 N-terminal region are essential for replication repression, implicating PP1 in the mechanism. We confirmed an earlier report that Rif1 can interact physically with Glc7, the S. cerevisiae PP1 (Breitkreutz et al. 2010). Reduced PP1 levels alleviate the cdc7-1 growth defect, and PP1 appears to act in the same pathway as Rif1 [Fig. 3], recruiting Cdc7 and causing phosphorylation of the cluster of DDK target sites in the Rif1 N terminus, leading to inactivation of the PP1-binding motifs [Rif1-P in Fig. 7A, bottom panel]. As a result, Glc7 is released from Rif1 and no longer directed to dephosphorylate Mcm4, favoring the buildup of DDK-mediated Mcm4 phosphorylation and consequent origin activation. We identified multiple phosphorylated serine residues within DDK target consensus sequences in the PP1-interacting region of Rif1 (Sasanuma et al. 2008; Wan et al. 2008). One such phosphorylation site (Ser119) is immediately adjacent to an RVXF PP1 interaction motif (KSVAF; 114–118) and corresponds to a priming-independent DDK consensus. Phosphorylation adjacent to an RVXF motif has been reported to disable PP1 binding (Kuntziger et al. 2006), and the effects of nonphosphorylatable and phosphomimetic Rif1 mutants support the notion that Rif1–Glc7 interaction is down-regulated by DDK phosphorylation. Such a dual mechanism—in which DDK both phosphorylates Mcm4 and down-regulates an Mcm4 phosphatase—would reinforce the switch from low to high DDK activity as cells pass from G1 into S phase, ensuring that DDK targets undergo a quick and robust shift from “hypophosphorylated” to “hyperphosphorylated” status.

Effects of mutating Rif1 domains

The effects of the various mutant constructs support our model. We propose that, when RIF1 is deleted, Glc7 can no longer dephosphorylate Mcm4. As a consequence, any premature phosphorylation that occurs cannot be reversed and therefore accumulates, predisposing cells and origins to replication initiation [Fig. 7B]. Our model is supported by the observation that deleting RIF1 alleviates the defects of cdc7-1 in particular, since Rif1 exacerbates effects of low DDK activity by promoting the reversal of DDK phosphorylation. It is therefore as expected that removing Rif1 is beneficial in the cdc7-1 context. In fact, a cdc7-1 strain is
probably especially sensitive to the presence of Rif1, since limited DDK activity may also cause Rif1 itself to remain unphosphorylated so that Glc7 is constitutively associated with Rif1 and targeted to dephosphorylate Mcm4. Our model therefore explains why deleting Rif1 is particularly effective in ameliorating *cdcl* temperature sensitivity.

Removal of the N-terminal domain of Rif1 or mutation of the PP1 interaction motifs causes effects similar to the deletion mutant *rif1Δ*. Such a mutated Rif1 is unable to direct Glc7 to dephosphorylate DDK substrates, relieving *cdcl* temperature sensitivity [Figs. 4, 5A,B, 7C] in a similar way to *rif1Δ*.

Removal of the Rif1 C-terminal domain, in contrast, had a mild but reproducible negative impact on *cdcl* growth at semipermissive temperature [Fig. 4]. This phenotype also supports our model, which predicts that the *rif1ΔC* mutant is unable to recruit DDK, as it lacks the Dbf4-interacting domain [Fig. 7D]. As a result, the Rif1 N-terminal domain may remain unphosphorylated so that Glc7 is targeted to dephosphorylate DDK substrates throughout the cell cycle. Nonphosphorylatable *rif1-9A* and *rif1-14A* mutations caused an effect similar to *rif1ΔC*, consistent with their constitutive targeting of PP1 to dephosphorylate Mcm4. Cells with wild-type Cdc7 can presumably accumulate sufficient DDK to overwhelm such constitutive dephosphorylation activity, but defects caused by limiting DDK will be accentuated [Fig. 7D], consistent with our observation that *rif1ΔC* exacerbates *cdcl* temperature sensitivity.

Our deletion analysis also indicated that the conserved HEAT repeats in the N-terminal half of Rif1 are required for replication repression [Fig. 4]. HEAT repeats form a "solenoid" domain that mediates protein–protein interactions [Andrade et al. 2001]. We suspect that the HEAT repeats may be involved in directing Glc7 to the appropriate targets, including the MCM complex. Mcm4 itself contains two RVXF motifs [positions 148 and 343], which may also contribute to Glc7 recruitment.

**Backup S-phase controls and regulation of replication timing**

A *rif1Δ* mutant does not begin replication during α-factor arrest despite the fact that Mcm4 is already hyperphosphorylated at this cell cycle stage [Fig. 2A]. This control presumably reflects the fact that multiple events are required for replication initiation so that the precocious buildup of DDK-mediated MCM phosphorylation alone cannot trigger replication. In particular, Cdk-mediated phosphorylation of Sld2 and Sld3 is required for origin initiation [Tanaka et al. 2007; Zegerman and Diffley 2007]. It will be of interest to test whether removing Rif1 causes replication to initiate in α-factor in the context of mutated Sld2 and Sld3 variants that bypass the requirement for CDK to initiate replication [Tanaka et al. 2007; Zegerman and Diffley 2007].

Previous investigations showed that deleting *S. pombe rif1* suppresses mutations in DDK and that *S. pombe* and mammalian Rif1 affect genome-wide replication timing [Cornacchia et al. 2012; Hayano et al. 2012; Yamazaki et al. 2012]. A previous study of the *S. cerevisiae rif1Δ* replication program showed a shortened interval between replication of early and late sequences, with telomeric origins in particular replicating aberrantly early [Lian et al. 2011]. Loss of Rif1-mediated targeting of PP1 to dephosphorylate Mcm4 could conceivably cause a derailed replication program, since the level of DDK-mediated MCM phosphorylation is one of the factors likely to affect the initiation time of specific origins [Mantiero et al. 2011; Tanaka et al. 2011].

**Spatial localization of Rif1**

The effect of *S. pombe* Rif1 on origins is related to the proximity of chromosomal binding sites [Hayano et al. 2012], but intranuclear spatial localization may also be important [Yamazaki et al. 2012]. *S. cerevisiae* Rif1 is reported to be localized at the nuclear periphery through association with peripherally localized telomere clusters [Gotta et al. 1996; Hiraga et al. 2008] and by palmitoylation-mediated nuclear envelope anchoring [Park et al. 2011]. It will be interesting to study whether localization of Glc7 is affected by Rif1 and whether the spatial localization of Rif1 is related to its impact on replication origin initiation. During mitosis, Glc7 is localized by regulatory subunits to counteract the activity of Ipl1 kinase [Pinsky et al. 2006].

**Evolutionary conservation of PP1 interaction motifs in Rif1**

PP1 interaction motifs exist in Rif1 proteins from yeasts through to higher eukaryotes [Supplemental Fig. 5C] despite the generally limited conservation of Rif1 protein sequence. *S. pombe* Rif1 contains RVXF and SILK motifs in its N-terminal domain, like *S. cerevisiae* Rif1 [Supplemental Fig. 5C], even though the overall similarity of this region is low. The conservation raises the possibility that *S. pombe* Rif1 also represses DNA replication through PP1. Notably, the RVXF and SILK motifs of fission yeast Rif1 are both flanked by potential DDK phosphorylation sequences [Supplemental Fig. 5C,D]. Both mammalian and Drosophila Rif1 proteins also have an N-terminal RVXF motif [Supplemental Fig. 5C] in addition to previously reported SILK and RVXF motifs close to their C termini [Sreesankar et al. 2012]. We suggest that the role of mammalian Rif1 in repressing DNA replication also involves targeting of PP1 to counteract DDK phosphorylation. Consistent with this possibility, MCM phosphorylation is increased in human cells depleted for Rif1 [Yamazaki et al. 2012]. Interestingly, PP1 was recently shown to be responsible for rapid reversal of DDK-dependent phosphorylation of Mcm4 in *Xenopus* egg extract [Poh et al. 2014].

Originally discovered for its role at yeast telomeres and later identified as important for controlling DNA replication, Rif1 is now known to mediate various additional chromosome stability functions, including DNA break repair pathway choice [Chapman et al. 2013; Di Virgilio et al. 2013; Escribano-Diaz et al. 2013] and down-regulation of checkpoint activity close to telomeres [Xue et al. 2011; Ribeyre and Shore 2012]. All of these control mech-
anisms could potentially involve regulated dephosphorylation of specific proteins. It will be intriguing to discover whether phosphatase targeting is a common theme among the manifold cellular roles of Rif1.

Materials and methods

Yeast strains and plasmids

Yeast strains and plasmids are listed in Supplemental Material along with construction and two-hybrid assay procedures.

Microarray analysis of genomic DNA replication profiles

Isotopic density transfer experiments and microarray hybridizations were performed as described [Pohl et al. 2012]. Genome-wide percent replication values were normalized, and the data were smoothed as described [Alvino et al. 2007; Feng et al. 2009].

Analysis of Mcm4 phosphorylation

In vivo Mcm4 phosphorylation was analyzed in either whole-cell extracts or chromatin fractions. Whole-cell extracts were prepared essentially as described [Kushnirov 2000]; details are in the Supplemental Material. Chromatin fractions were prepared as described [Sheu and Stillman 1999]. See the Supplemental Material for additional details.

Coommunoprecipitation experiments and DDK assays

A series of Dbf4 proteins, HA-Cdc7, and Rif1-CT were expressed in insect Sf9 cells, and the whole-cell extracts were immunoprecipitated with anti-HA monoclonal 12CA5. Kinase assays were performed as described [Weinreich and Stillman 1999]. See the Supplemental Material for additional details.

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