Reversal of DDK-Mediated MCM Phosphorylation by Rif1-PP1 Regulates Replication Initiation and Replisome Stability Independently of ATR/Chk1

Highlights

- DDK-dependent phosphorylation of Mcm4, but not Mcm2, tracks with DNA replication
- DDK-inhibitors, PHA-767491 and XL-413, differentially affect MCM phosphorylations
- Vertebrate Rif1 targets PP1 to replication origins that reverse MCM phosphorylation
- Replisomes are destabilized by Rif1/PP1-mediated dephosphorylation

Authors

Robert C. Alver, Gaganmeet Singh Chadha, Peter J. Gillespie, J. Julian Blow

Correspondence

j.j.blow@dundee.ac.uk

In Brief

Alver et al. find that DDK-dependent MCM2-7 phosphorylation is opposed by Rif1-targeted PP1. Lack of Rif1 increases MCM phosphorylation and abolishes the ability to block origin firing, even when undergoing replication stress. In the absence of CHK1, the stability of stalled replisomes is severely compromised upon Rif-PP1-mediated dephosphorylation.
Reversal of DDK-Mediated MCM Phosphorylation by Rif1-PP1 Regulates Replication Initiation and Replisome Stability Independently of ATR/Chk1

Robert C. Alver,1,2,3 Gaganmeet Singh Chadha,1,2 Peter J. Gillespie,1 and J. Julian Blow1,4,*
1Centre for Gene Regulation & Expression, School of Life Sciences, University of Dundee, Dundee DD1 5EH, UK
2Co-first author
3Present address: Division of Oncology Research, Mayo Clinic, Rochester, MN 55905-0002, USA
4Lead Contact
*Correspondence: j.j.blow@dundee.ac.uk
http://dx.doi.org/10.1016/j.celrep.2017.02.042

SUMMARY

Dbf4-dependent kinases (DDKs) are required for the initiation of DNA replication, their essential targets being the MCM2-7 proteins. We show that, in Xenopus laevis egg extracts and human cells, hyper-phosphorylation of DNA-bound Mcm4, but not phosphorylation of Mcm2, correlates with DNA replication. These phosphorylations are differentially affected by the DDK inhibitors PHA-767491 and XL413. We show that DDK-dependent MCM phosphorylation is reversed by protein phosphatase 1 (PP1) targeted to chromatin by Rif1. Loss of Rif1 increased MCM phosphorylation and the rate of replication initiation and also compromised the ability of cells to block initiation when challenged with replication inhibitors. We also provide evidence that Rif1 can mediate MCM dephosphorylation at replication forks and that the stability of dephosphorylated replisomes strongly depends on Chk1 activity. We propose that both replication initiation and replisome stability depend on MCM phosphorylation, which is maintained by a balance of DDK-dependent phosphorylation and Rif1-mediated dephosphorylation.

INTRODUCTION

The ordered assembly of replication proteins onto chromatin is highly regulated to ensure that DNA is precisely copied only once each cell cycle. Beginning in late M/early G1 phase, double hexamers of Mcm2–7 are loaded in excess onto replication origins. In S phase, Dbf4-dependent Cdc7-kinase (DDK) and cyclin-dependent kinase (CDK) promote the conversion of Mcm2–7 into active Cdc45-MCMs-GINS (CMG) replicative helicases.

DDK is an essential and conserved serine/threonine kinase that is formed of a Cdc7 catalytic subunit associated with its regulatory partner, Dbf4. In vertebrates, a second regulator of Cdc7, Drf1, has been identified (Montagnoli et al., 2002; Yanow et al., 2003; Takahashi and Walter, 2005; Yoshizawa-Sugata et al., 2005). The key target of DDK for the initiation of DNA replication is the Mcm2–7 complex. DDK phosphorylates the N terminus of several Mcm2–7 subunits (minichromosome maintenance proteins, or MCMs), specifically Mcm2, Mcm4, and Mcm6, (Lei et al., 1997; Sato et al., 1997; Jiang et al., 1999; Masai et al., 2000; Jares and Blow, 2000; Sheu and Stillman, 2010). Although the contribution of each of these phosphorylation events to initiation is not well understood, a key consequence of the hyper-phosphorylation of Mcm4 is the relief of an inhibitory signal that prevents replication (Sheu and Stillman, 2006, 2010). Significantly, cells lacking this Mcm4 N-terminal tail no longer require DDK activity to complete replication. There is evidence that Mcm2 phosphorylation by DDK is also required for DNA replication (Hardy et al., 1997; Bruck and Kaplan, 2015).

In Xenopus laevis egg extracts, Mcm4 hyper-phosphorylation by DDK occurs primarily on chromatin (Poh et al., 2014), though experiments in mammalian cells have suggested that DDK phosphorylation of Mcm2 occurs primarily when it is not bound to DNA (Montagnoli et al., 2006).

Recent studies have revealed that protein phosphatase 1 (PP1) restricts DDK activity by reversing the Cdc7-dependent phosphorylation of Mcm4 (Poh et al., 2014; Hiraga et al., 2014; Davè et al., 2014; Mattarocci et al., 2014). In Xenopus egg extracts, DDK executes its essential function early in S phase by phosphorylating Mcm4 at all replication origins (Poh et al., 2014). However, PP1 continually removes these Mcm4 phosphorylations so that when DDK activity is inhibited in the middle of S phase, PP1 dephosphorylates MCMs at origins that have not yet fired, and replication fails to finish. In addition, induction of cell-cycle checkpoints resulted in increased PP1 recruitment and subsequent Mcm4 dephosphorylation without altering DDK activity (Poh et al., 2014).

In Saccharomyces cerevisiae and Schizosaccharomyces pombe, PP1 is targeted to MCMs by Rap1-interacting factor (Rif1) (Hiraga et al., 2014; Davè et al., 2014; Mattarocci et al., 2014). Loss of this interaction leads to premature Mcm4 phosphorylation and misregulation of origin firing and allows cells to compensate for reduced DDK activity. Loss of S. pombe or mammalian Rif1 deregulates the replication timing program (Corpacchia et al., 2012; Hayano et al., 2012; Yamazaki et al., 2012). These findings are consistent with the hypothesis that Rif1 negatively regulates DNA replication by recruiting PP1 to replication origins to reverse DDK-dependent MCM phosphorylation.
In this paper, we show that DDK-dependent hyper-phosphorylation of Mcm4, but not phosphorylation of Mcm2, correlates well with replication initiation in both Xenopus extracts and human cells. We show that Rif1-directed PP1 opposes DDK-dependent phosphorylation on MCMs, acting in parallel with the ATR-dependent checkpoint pathway. In human cells, loss of Rif1 results in accelerated progression through S phase due to increased initiation, disrupting the normal replication timing program. Loss of Rif1 can also alleviate the slow progression through S phase that results when DDK is inhibited. Finally, we provide evidence that Rif1-PP1 destabilizes replication forks that have stalled in the absence of Chk1 activity.

**RESULTS**

**DDK Differentially Phosphorylates MCM2–7 Subunits**

The phosphorylation of Mcm2 on S40 and S53 has been exploited as a readout for Ddc7 kinase activity and has been used in screens for the development of small-molecule inhibitors of Cdc7 (Montagnoli et al., 2008; Koltun et al., 2012). We used Xenopus egg extracts to evaluate the hyper-phosphorylation of Mcm4 and the phosphorylation of Mcm2 at the Xenopus orthologous S40 and S53 residues (S25 and S38, respectively) as readouts for the DDK activity required for DNA replication (Figure 1A). As reported previously, DNA-bound Mcm4 was hyper-phosphorylated by DDK (Poh et al., 2014). Treatment with the DDK inhibitor PHA-767491 resulted in completely dephosphorylated Mcm4. Surprisingly, the more specific and potent DDK inhibitor XL413 (Hughes et al., 2012) caused only inefficient inhibition (Figure 1A). Phosphorylation of Mcm2 S53 behaved similarly to Mcm4, appearing only on chromatin and being reduced by DDK inhibition. In contrast, phosphorylation of S40 was strong in the chromatin fraction, as well as in the supernatant where it was insensitive to DDK inhibition.

To further investigate the effects of PHA-767491 and XL413 on different MCM phosphorylation sites, we isolated chromatin at short time intervals (Figure 1B) while monitoring DNA replication (Figure 1C). PHA-767491 efficiently inhibited phosphorylation of all assayed MCMs, including chromatin-bound Mcm2 at S40. As detailed previously (Poh et al., 2014), PHA-767491 also abolished DNA replication (Figure 1C). In contrast, XL413 only delayed the hyper-phosphorylation of Mcm4, although it efficiently inhibited Mcm2 phosphorylation on S40 and S53 (Figure 1B). XL413 delayed progression through S phase but did not prevent most of the DNA from being replicated. This shows that, among the different DDK-dependent MCM phosphorylations, Mcm4 phosphorylation provides a better functional correlation with DNA replication than phosphorylation of Mcm2 on S40 or S53.

After DDK phosphorylates the Mcm2–7 complex, CDK activity promotes the initiation of replication (Jares and Blow, 2000; Walter, 2000). We used a CDK inhibitor, p27Kip1, to prevent replication and, hence, stabilize phosphorylated MCMs on DNA. We previously showed that, in these conditions, inhibition of DDK activity leads to rapid dephosphorylation of Mcm4 by PP1 (Poh et al., 2014). Inhibition of DDK with either PHA-767491 or XL413 resulted in the loss of Mcm2 S40 and S53 phosphorylation, but only PHA-767491 caused efficient dephosphorylation of Mcm4. Notably, CHL2, which is a Chk1 activator, is insensitive to DDK inhibition. This suggests that Chk1 activity leads to rapid dephosphorylation of Mcm4 by PP1 (Poh et al., 2014).

**Figure 1. Phosphorylation of Different MCM Subunits by Xenopus DDK Is Reversed by PP1**

(A–C) Xenopus egg extracts were supplemented with demembranated sperm nuclei and optionally supplemented with 50 μM PHA-767491 (PHA) or 100 μM XL413 (XL). (A) After incubation for 40 min, isolated chromatin along with 0.5% (v/v) of the supernatant was immunoblotted for Mcm4, Mcm2-P-S40, Mcm2-P-S53, and total Mcm2. (B) At the indicated times, chromatin was isolated and immunoblotted for Mcm4, Mcm2-P-S40, Mcm2-P-S53, total Mcm2, PCNA, and histone H3. (C) Extract was supplemented with [α-32P]dATP. Total DNA synthesis was determined at indicated times. (D and E) Sperm nuclei were incubated for 60 min in Xenopus egg extracts treated with p27kip1 to allow Mcm4 hyperphosphorylation. 50 μM PHA-767491 or 100 μM XL413 plus or minus 1 μM tautomycetin (Tauto) was then added, and chromatin was isolated either immediately after inhibitor addition (60 min) or later at every 5 for next 20 min (65–80 min). Chromatin samples were immunoblotted for Mcm4, Mcm2-P-S40, Mcm2-P-S53, total Mcm2, and histone H3.
of Mcm4 (Figures 1D and 1E). When tautomycetin, a specific inhibitor of PP1 (IC50 [half maximal inhibitory concentration] = 1.6 nM and 62 nM for PP1 and PP2, respectively), was added along with PHA-767491 or XL413, virtually all MCM dephosphorylation was abolished (Figure 1E). This suggests that PP1 activity reverses DDK-dependent phosphorylation on all its MCM substrates. This result also suggests that the inability of PHA-767491 or XL413 to inhibit Mcm2 S40 phosphorylation in solution (Figure 1A) is due to the lack of PP1 activity, which, as we have previously shown, is recruited to chromatin to promote MCM dephosphorylation (Poh et al., 2014).

We next investigated the kinetics of DDK-dependent MCM phosphorylation in HeLa cells by using mitotic shakeoff to monitor cells as they move from G1 to S and onto G2 (Figure 2A). As has been previously reported (Masai et al., 2006), phosphorylation of chromatin-bound Mcm4 (evidenced by slower migrating bands on SDS-PAGE) occurred as cells entered into and progressed through S phase (Figure 2B). Consistent with results from Xenopus, Mcm4 in the HeLa soluble fraction behaved differently and was typically seen as one strong, fast migrating band and one slower migrating weak band that did not vary over the cell cycle. Phosphorylation of Mcm2 S40 was most evident in the soluble fraction rather than on chromatin. Moreover, the phosphorylation of soluble Mcm2 S40 was strongest in G1, it nearly disappeared during S phase, and it returned as cells moved into G2 phase.

The efficacy of both PHA-767491 and XL413 has been assessed by their ability to inhibit Cdc7-dependent phosphorylation of soluble Mcm2 in human tissue culture cells (Montagnoli et al., 2008; Koltun et al., 2012). To our knowledge, neither was assessed for Mcm4 phosphorylation status. To test this, HeLa cells were replated after a mitotic shakeoff and then challenged with PHA-767491 or XL413. Each compound inhibited DNA replication and S-phase entry, with PHA-767491 displaying stronger activity (Figure 2C). The stronger effect of PHA-767491 is likely explained by its promiscuous activity against other kinases, including Cdk9, which is required for S-phase entry (Montagnoli et al., 2008; Hughes et al., 2012). PHA-767491 induced apoptosis in HeLa cells as cells enter S phase after mitotic shakeoff (Figure S1A). However, when the inhibitors were added just after the start of S phase, PHA-767491 no longer had a strong apoptotic effect, and both PHA-767491 and XL413 appeared to inhibit S-phase entry to a similar degree (Figures S1B and S1C). PHA-767491 or XL413 also suppressed phosphorylation of chromatin-bound Mcm4, as well as S40 and S53 of Mcm2, consistent with these being direct DDK targets (Figure 2D). Congruent with results in Xenopus egg extracts, inhibition of PP1 with tautomycetin resulted in the accumulation of phosphorylated, chromatin-bound Mcm2 and Mcm4. Tautomycetin also reduced the dephosphorylation of chromatin-bound MCMs induced by XL413 or PHA-767491. In contrast to the experiments in egg extracts, both inhibitors reduced phosphorylation of soluble Mcm2, suggesting more active dephosphorylation of soluble Mcm2 in human cells. Both XL413 and PHA-767491 consistently increased the proportion of cells that remained in the near-2N population (Figure S1C) when added to cells synchronized in S phase; co-addition of tautomycetin partially reversed this effect. These results show that, in both Xenopus egg extracts and human cells, phosphorylation of Mcm4, but not Mcm2, provides the best indicator of DDK activity required for DNA replication initiation and that phosphorylation of chromatin-bound MCMs by DDK is counteracted by phosphatase activity of PP1.

**PP1 Requires Rif1 to Dephosphorylate the Mcm2–7 Complex**

Previous data in yeast has established that Rif1 recruits PP1 to replication origins to dephosphorylate MCMs (Davé et al., 2014; Hiraga et al., 2014; Mattarocci et al., 2014). The motifs on Rif1 required for interaction with PP1 are conserved in Xenopus and mammalian Rif1 homologs but are located within the C terminus of the protein (Sreesankar et al., 2012). In order to test whether Rif1 directs PP1 to replication origins in vertebrates, we raised an antibody to Xenopus Rif1 (Figure S2A). Immunoprecipitation of Rif1 from Xenopus egg extracts co-precipitated PP1γ and PP1β (Figure 3A). Moreover, PP1β was detected by mass spectrometry in immunoprecipitates of Rif1 from chromatin (Figure S3A). Inhibition of Xenopus Cdc7 did not obviously reduce the Rif1–PP1 interaction (Figure S3B) as observed in yeast (Hiraga et al., 2014). In human tissue culture cells both PP1α and PP1γ, Mcm2 and Cdc7 were co-precipitated with chromatin-bound Rif1 (Figure 3B).

If Rif1 targets PP1 to DNA-bound Mcm2–7, then depleting Rif1 from Xenopus egg extracts should mimic the effect of inhibiting PP1. Depletion of >90% of Rif1 from Xenopus egg extracts (Figure S3C) substantially decreased the amount of PP1 on chromatin and reduced the rate of dephosphorylation of Mcm4 and Mcm2 that occurred after DDK inhibition (Figures 3C and S3D). A more robust Rif1 depletion (>95%) caused even greater loss of chromatin bound Rif1 and PP1γ with a corresponding increase in Mcm4 phosphorylation (Figures S3E and S3F). The process of immunodepletion slightly compromises Cdc7 nuclear import (Poh et al., 2014), reducing MCM4 hyper-phosphorylation (Figures S3C, S3D, and S3F) and sensitizing DNA replication to further DDK inhibition (Figure S4A).

We performed parallel experiments in asynchronous HeLa cells using small interfering (si)RNA against Rif1. Hyper-phosphorylated Mcm4 accumulated in proportion to the amount of Rif1 knocked down by RNAi treatment (Figure 3D). The accumulation of hyper-phosphorylated Mcm4 was more pronounced in cells synchronized in S phase following a mitotic shakeoff (Figure 3E). As we observed in Xenopus (Figure 3C), knockdown of Rif1 in human cells rendered phosphorylation of Mcm4 relatively insensitive to DDK inhibition (Figure 3E). These data clearly show that, in both Xenopus and human systems, loss of Rif1 increases DDK-dependent phosphorylation of MCMs in a manner that is similar to inhibition of PP1 and implices Rif1 in directing PP1 to origins of replication.

**Rif1 Is Essential for Regulating S-phase Progression**

We next investigated the potential role of Rif1 in regulating progression into and through S phase. When Xenopus extracts were immunodepleted of Rif1, the rate of replication increased compared to control immunoglobulin-G (IgG)-depleted extract (Figures 4A and S4B). Furthermore, Rif1 depletion reduced the inhibitory effect of PHA-767491 on DNA replication (Figure 4B).
Figure 2. Human Mcm4 Phosphorylation Predicts DNA Replication

(A and B) HeLa cells were synchronized in early M phase by mitotic shakeoff and then replated for different times. (A) DNA content of cells as determined by flow cytometry. (B) Immunoblot of Mcm4, total Mcm2, and Mcm2-P-S40 on and off chromatin. Lamin B and tubulin serve as loading and fractionation controls.

(C) Cells were synchronized as in (A) and were replated with 10 mM PHA-767491 or 10 mM XL413 in the media. At 7.5 hr, cells were pulsed for 30 min with EdU and then analyzed by flow cytometry for DNA content and EdU incorporation. An example plot and gate are shown plus the mean and SD of EdU+ cells from three experiments.

(D) Cells were synchronized as in (A) and replated for 8 hr (t-2); media was then optionally supplemented with 10 mM PHA-767491 (PHA), 10 mM XL413 or 225 nM tautomycetin (Tauto) as indicated for 2 hr. Cells were then immunoblotted for Mcm4, Mcm2-P-S40, Mcm2-P-S53, or total Mcm2 on and off chromatin. Lamin B and tubulin served as loading and fractionation controls.

See also Figure S1.
These results show that, in Xenopus egg extracts, Rif1 counteracts the function of DDK in driving S-phase progression.

A similar effect was seen when Rif1 levels were reduced in human cells using RNAi. On release from a double thymidine block, siRif1 HeLa cells moved more quickly through S phase and man cells using RNAi. On release from a double thymidine block, acts the function of DDK in driving S-phase progression.

To test if deoxyuridine (EdU) at a higher rate

S-phase progression observed with XL413 treatment was highly disrupted chromatin structure (Foti et al., 2016). Therefore, in showing less discrete labeling patterns. Incorporation of EdU 90 min after release from a thymidine block was greater in siRif1-treated cells (Figure 5B), implying that aberrant EdU incorporation due to the absence of Rif1 occurs primarily in early S phase.

DNA fiber analysis showed that the replication fork speed was similar in siCtrl and siRif1-treated cells (Figure 5E). Previous work in mammalian cells has shown that loss of Rif1 results in limited change in inter-origin distances (Cornacchia et al., 2012) and a highly disrupted chromatin structure (Foti et al., 2016). Therefore, the increased levels of EdU incorporation we observed after Rif1 depletion likely reflects initiation occurring across larger timing domains at an otherwise unchanged origin density, consistent with an underlying change in chromatin structure.

Rif1 Regulates S-phase Progression in Parallel with ATR/Chk1

Previous work has implicated Rif1 in the regulation of replication initiation in response to activation of cell-cycle checkpoints (Kumar and Cheok, 2014; Renard-Guillet et al., 2014). Therefore, we sought to understand whether Rif1 was regulated by the S-phase checkpoint kinases ATM and ATR. We observed S-phase progression of synchronized HeLa cells after treatment with an ATM inhibitor (KU55933), a Chk1 inhibitor (CHIR-124), or a generic inhibitor of ATM/ATR/DNA-PK (caffeine) in the presence and absence of Rif1 (Figure 6). Both caffeine and CHIR-124, but not KU55933, inhibited S-phase progression in siCtrl cells, consistent with the known role of ATR and Chk1 in promoting fork stability (Smits and Gillespie, 2015). siRif1 cells treated with caffeine or CHIR-124 showed an increase in EdU incorporation and S-phase progression (Figure 6A). This implies that Rif1 has a negative
regulatory effect on S-phase progression that is independent of ATR and Chk1. To determine whether this Rif1 function occurs by opposing DDK activity, we observed S-phase progression in synchronized cells treated with a DDK inhibitor, a Chk1 inhibitor, or both in the presence and absence of Rif1. In control cells, both CHIR-124 and XL413 individually caused a slight reduction in EdU incorporation, which was enhanced when combined with each other (Figure 6B). In contrast, XL413 had no significant effect on EdU incorporation or S-phase progression in siRif1 cells, either alone or when combined with CHIR-124. This strongly implies that, in the absence of exogenously applied replicative stresses, Rif1 restricts S-phase progression by opposing DDK function in a manner that is independent of ATR and Chk1.

We next investigated Rif1 function under conditions of replication stress. It has previously been shown in Xenopus egg extracts that the topoisomerase II inhibitor etoposide inhibits DDK activity via both checkpoint-dependent and checkpoint-independent pathways (Costanzo et al., 2003; Poh et al., 2014). Therefore, we added etoposide to control and Rif1-depleted HeLa cells synchronized in early S phase. As expected, this caused a reduction in EdU incorporation and S phase progression (Figure 6C). When treated with both etoposide and CHIR-124, control cells had great difficulty proceeding with S phase, and incorporation of EdU was significantly decreased. This is consistent with the known role of Chk1 in stabilizing stalled replication forks, as confirmed by DNA fiber analysis, which showed a decrease in the average labeled track length of ~50% (Figure 6D). Remarkably, in Rif1-deficient cells, co-treatment of etoposide and CHIR-124 resulted in near-normal replication profiles as judged by fluorescence-activated cell sorting (FACS). DNA fiber analysis showed that, in Rif1-deficient cells, the length of labeled tracks was largely unaffected by etoposide or CHIR-124. These data are consistent with the idea that, in addition to its role in regulating initiation, Rif1 destabilizes replication forks that have stalled in the absence of Chk1 activity.

**PP1-Dependent Dephosphorylation of CMG Destabilizes Replication Forks**

The results presented earlier show that Rif1-PP1 reverses DDK-dependent phosphorylation of chromatin-bound MCMs. However, at any given moment, only a fraction of the total chromatin-bound MCMs are active as replicative CMG complexes. Therefore, we analyzed the effect of Rif1-PP1 on CMG complexes using Xenopus egg extracts. DNA was incubated for 60 min in extract containing 100 μM aphidicolin (to inhibit...
replication fork progression) and 5 mM caffeine (to allow dormant origins to fire, thereby increasing the number of active CMGs) (Woodward et al., 2006). PHA-767491 and/or tautomycin were then added, after which CMG complexes were isolated by immunoprecipitation of Cdc45 (Figure 7A). DDK inhibition caused significant dephosphorylation of total chromatin-bound MCM4 but only partial dephosphorylation of MCM4 in the CMGs. In both cases, dephosphorylation was inhibited by tautomycin. However, there was strong PP1-dependent dephosphorylation of MCM2 S40, both on total chromatin-bound MCM2 and in the CMGs. MCM2 S53 phosphorylation was not significantly affected by DDK inhibition. These results suggest that PP1-dependent MCM dephosphorylation occurs on the CMG, but with different kinetics at different MCM sites compared to MCM complexes at licensed origins.

Previous work has shown that, unlike the case in mammalian cells, replisomes in Xenopus extracts are not destabilized when Chk1 is inhibited (Luciani et al., 2004). Since our results in human cells indicate that replisome stability involves both MCM phosphorylation and Chk1 activity (Figure 6D), we considered whether the very high degree of DDK-mediated MCM phosphorylation in Xenopus egg extracts (Figure 1) helps stabilize replisomes, even when Chk1 is inhibited. To test this, replication forks in Xenopus egg extracts were stalled with aphidicolin, new initiation events were blocked with p27kip1, and the incubation was continued in the presence of PHA-767491 plus or minus caffeine (which, by inhibiting ATR, prevents Chk1 activation). Chromatin was then isolated, and replication fork elongation was measured in a run-on assay (Figure 7B). When caffeine was added in addition to PHA-767491, the overall rate of replication was reduced to 76% ± 5% (mean ± SEM, six independent experiments; p < 0.05, an unpaired, two-tailed t test). The results of the experiments shown in Figures 7A and 7B are consistent with the data from HeLa cells in Figure 6D, and they suggest that, in the absence of ATR/Chk1 activity, DDK-dependent phosphorylation of CMG complexes becomes important to maintain replisome stability.

Finally, we investigated Rif1’s ability to inhibit S-phase progression in response to a range of different replication stresses. To this end, we observed EdU incorporation in the presence and
Figure 6. Rif1 Regulates S-phase Progression and Replisome Stability in Parallel with ATR/Chk1

(A–C) HeLa cells were treated with control or Rif1 siRNA, synchronized and released from a double thymidine block for 2 hr, and then optionally treated for 2 hr with caffeine (5 mM), CHIR-124, KU55933, XL413, or etoposide as indicated. EdU was added in the last 30 min of treatment and the cells were analyzed by flow cytometry.

(D) HeLa cells were treated with control or Rif1 siRNA, synchronized and released from a double thymidine (Thy) block for 70 min, labeled with CldU for 20 min, washed, and subsequently labeled with IdU while being incubated with CHIR-124 (CHIR), etoposide (ETO), or both for 1 hr. DNA fibers were prepared, and the IdU length of 100 tracks were counted per experimental condition. Data were evaluated for significance using an unpaired, two-tailed t test, with significant differences in fiber length between siCtrl untreated and siCtrl ETO + CHIR (p < 0.0001), and between siCtrl untreated and siRif1 untreated (p = 0.0321). There was no significant difference found between any siRif1 treatments.
Figure 7. Rif1-PP1 Regulates Replisome Stability and S-Phase Progression

(A) Sperm nuclei were incubated for 60 min in Xenopus egg extract supplemented with 100 μM aphidicolin and 5 mM caffeine. 50 μM PHA-767491 (PHA) ± 1 μM tautomycetin (Tauto) was added to induce a further 25 min. Chromatin-bound proteins were immunoprecipitated with antibodies against Cdc45 or pre-immune sheep IgG. Samples were immunoblotted for Cdc45, Mcm4, Mcm2-P, S40, Mcm2-P-SS3, total Mcm2, and pS2.

(B) Xenopus egg extract were supplemented with demembranated sperm nuclei and 100 μM aphidicolin. After 60 min, p27kip1 was added and aliquots were supplemented with 50 μM PHA-767491 ± 5 mM caffeine (CAFF). After a further 25 min, chromatin was extracted and expressed as normalized values against the incorporation at 1 min.

(C) HeLa cells were treated with control or Rif1 siRNA, synchronized by double thymidine block, and released 2 hr before either treatment with hydroxyurea (HU; 5 mM), aphidicolin (APH; 1 μg/mL), methyl methanesulfonate (MMS; 0.02%), camptothecin (CPT; 5 μM), etoposide (ETO; 5 μM), or UV (-45 J/m²). EdU was added in the last 30 min of treatment, and 2 hr after the start of the treatments, cells were harvested, fixed, and analyzed by flow cytometry.

(D) Model for the role of Rif1 and Cdc7 in controlling replication initiation and replisome stability. Replication initiation is regulated by the phosphorylation of Mcm2-7 by Cdc7, opposed by Rif1/PP1. Active replisomes are also phosphorylated by Rif1/PP1, which requires ATR/Chk1 to stabilize them in response to replicative stresses. See also Figure S6.

DISCUSSION

We show here how the interplay between DDK-dependent phosphorylation and Rif1-PP1-dependent dephosphorylation of MCM proteins regulates S-phase progression and replisome stability, DDK-dependent hyper-phosphorylation of Mcm4, but not Mcm2, correlates well with replication initiation in both Xenopus and humans. Rif1-directed PP1 dephosphorylates DDK-dependent phosphorylation on all MCMs. Loss of Rif1 accelerates progression through the S phase due to increased initiation, disrupting the normal replication timing program independently of the ATR-dependent checkpoint pathway. We also show that Rif1 is required to destabilize replication forks that have stalled in the absence of ATR-Chk1 activity.

MCM4 Phosphorylation Correlates with Replication Initiation

We have shown that DDK-dependent phosphorylation sites on different MCMs are differentially regulated, with slight variations between Xenopus and human systems. Mcm4 hyper-phosphorylation, but not Mcm2 phosphorylation, at S40 and S53...
correlates well with replication initiation. This is in agreement with studies in yeast where phosphorylation of the N terminus of Mcm4 is required to relieve an inhibitory function on replication initiation (Sheu and Stillman, 2010).

Unexpectedly, we found that XL413 is a poor inhibitor of replication in comparison to PHA-767491 in Xenopus egg extracts. This could be explained by XL413’s poor inhibition of DDK activity on Mcm4. In vitro, XL413 is much more specific and has more extensive van der Waals interactions within Cdc7’s active site than PHA-767491 (Hughes et al., 2012). However, extended XL413 treatment in HeLa cells seems to merely delay progression through S phase rather than completely shut down replication. This perhaps provides a mechanistic explanation for why XL413 fails to induce apoptosis efficiently in cancer cells (Sasi et al., 2014). It is also important to note that several drug screens have been carried out using Mcm2 S40 phosphorylation as a marker of DDK activity. This phosphorylation can be observed off chromatin and outside of S phase, and it is possible that there is a structurally different form of DDK that phosphorylates MCMs off chromatin.

**Rif1 Controls S-phase Progression and Replisome Stability**

We have established that vertebrate Rif1 interacts with PP1. We show that, consistent with data from yeast for why Rif1 controls S-phase progression and replisome stability, we have established that vertebrate Rif1 interacts with PP1. When replication fork progression is inhibited, ATR/Chk1 becomes essential to prevent irreversible replication fork inhibition (Luciani et al., 2004), become unstable when combined with DDK inhibition. In HeLa cells, inhibition of Rif1-PP1 prevents the inactivation of replication forks that occurs when the ATR/Chk1 pathway is inhibited. The simplest explanation for this fork stability function of Rif1 is that it is associated with Rif1’s ability to drive MCM dephosphorylation, though it is also possible that replisome stability depends on the phosphorylation of other replisome proteins.

In this model (Figure 7D, bottom), DDK-dependent MCM phosphorylation of the active CMG helicase helps maintain it in an active conformation. Only once dephosphorylated by Rif1-PP1 does the replisome become susceptible to destabilization, and then the fork stability function of ATR/Chk1 becomes particularly important. This second role of Rif1 has interesting implications for the use of DDK inhibitors as anti-cancer agents. Since replisome stalling is likely to be particularly difficult to reverse, combining replication stresses with DDK inhibitors is likely to prove significantly more toxic to cancer cells lacking proper checkpoint controls.

### EXPERIMENTAL PROCEDURES

#### Xenopus Egg Extract and DNA Templates

Metaphase-arrested Xenopus laevis egg extract and demembranated Xenopus sperm nuclei were prepared and used as described previously (Gillespie et al., 2016). Briefly, extracts were released into interphase from metaphase arrest once incubated with 0.3 mM CaCl2 for 15 min at 23 °C. For DNA synthesis reactions, sperm nuclei were incubated at 3–10 ng DNA/μL in extract. DNA synthesis was assayed by measuring incorporation of [γ-32P]dATP into acid-insoluble material following scintillation counting (Gillespie et al., 2016).

#### Chromatin Isolation from Egg Extract

Chromatin isolation from egg extract was carried out as described previously (Gillespie et al., 2012). Briefly, extract was diluted with ice-cold nuclear isolation buffer (NIB) (50 mM KCl, 50 mM HEPES-KOH [pH 7.6], 5 mM MgCl2, 0.5 mM spermidine, 0.15 mM spermine, and 2 mM DTT) containing phosphatase inhibitors, under-laid with 20% sucrose (w/v) in NIB, and centrifuged in a swinging-bucket rotor at 2,100 g for 5 min at 4 °C. Following a cushion wash, chromatin was spun down at 13,000 g for 2 min in a fixed-angle rotor. The resulting pellet was resuspended in SDS loading buffer.

To isolate intact nuclei for transfer experiments, Triton X-100 was omitted from all buffers. Extracts were diluted as before, under-laid with a double cushion of NIB + 20% sucrose and NIB + 30% glycerol (v/v in NIB), and centrifuged in a swinging bucket rotor. Following a cushion wash, nuclei were resuspended in the glycerol cushion and added to the second extract at a final DNA concentration of 10 ng/μL.

#### Cell Culture and Synchronization

HeLa cells were obtained from the American Type Culture Collection (ATCC) and maintained in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Invitrogen) and antibiotics at 37 °C in 5% CO2. For mitotic shake-off experiments, HeLa cells were synchronized in early S phase via double thymidine block, washed with PBS, and released into media for 6 hr before treatment with nocodazole at 100 ng/mL for 4 hr. Flasks were knocked onto the benchtop to release mitotic cells and then were washed with pre-warmed PBS and replated.

#### Protein Preparation and Isolation of Chromatin Proteins in Human Cells

Whole-cell extracts were obtained from HeLa cells by extraction in RIPA buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% IGEPAL CA-630, 0.5% sodium deoxycholate, 1 mM EDTA, 1 mM PMSF, 1 mM sodium orthovanadate,
PhosSTOP (04906845001, Roche). For chromatin fractionation, cells were harvested from plates, washed with PBS, and resuspended in cytoskeleton (CSK) buffer (10 mM HEPES [pH 7.4]; 300 mM sucrose; 100 mM NaCl; 3 mM MgCl₂; 0.5% Triton X-100; 1 mM PMSF; 0.1 mM sodium orthovanadate; PhosSTOP [04906845001, Roche]; and 1 μg/mL each of leupeptin, aprotinin, and pepstatin) for 15 min on ice. Tubes were centrifuged at 5,000 × g for 5 min at 4 °C, and the non-chromatin bound supernatant was frozen in liquid nitrogen. The chromatin pellet was washed twice using CSK buffer without Triton X-100, centrifuged at 5,000 × g for 5 min at 4 °C, and frozen in liquid nitrogen. Protein was prepared from chromatin pellets by extraction in RIPA buffer.

FACS Analysis
At 30 min before collecting cell samples, EdU was added to cells (final concentration, 40 μM), after which cells were harvested and fixed with ice-cold 70% ethanol. EdU detection was achieved using the Click-IT Plus EdU Alexa Fluor 647 Flow Cytometry Assay Kit (C10635, Life Technologies), and DNA content was measured with propidium iodide. EdU incorporation and DNA content were measured using BD FACSanto, and data were analyzed using the FlowJo program.

Immunoblotting
For immunoblotting, samples were separated on either 3%–8% Tris-acetate gel or 12% SDS-PAGE, transferred to either PVDF (GE Healthcare, RPN303F) or nitrocellulose membranes (GE Healthcare, 10600001) using a wet transfer system, blocked in PBS with 0.2% Tween-20 and 5% non-fat milk. After incubation with primary and secondary antibodies, either membranes were developed using enhanced chemiluminescence detection (SuperSignal West Pico Chemiluminescent; Thermo Scientific, 34087), or signals were acquired using Odyssey bio-systems (LI-COR Biosciences) where LI-COR secondary antibodies were used.

Immunodepletion and Immunoprecipitation
To deplete Rif1 from Xenopus egg extract, metaphase-arrested extract was activated to release it into interphase, and subsequently, 1 vol of extract was incubated twice with 0.6 vol of protein A agarose beads conjugated to anti-Rif1 antibody for 45 min at 4 °C. To precipitate Rif1 from Xenopus egg extract, metaphase-arrested extract was released into interphase and then diluted 5-fold in LBF (licensing factor buffer) 1/50 (40 mM HEPES-KOH [pH 8.0]; 20 mM KH₂PO₄/K₂HPO₄ [pH 8.0]; 2 mM MgCl₂; 1 mM EGTA; 2 mM DTT; 10% [w/v] sucrose; 1 mM EDTA) plus protease inhibitor cocktail (Roche) on ice for 20 min. The diluted extract was incubated for 1 hr at 4 °C with protein A agarose beads covalently conjugated to anti-Rif1 antibody with bis(sulfosuccinimidyl)suberate (21580, ThermoFisher Scientific). The beads were then washed five times with filtered PBS before resuspension in loading buffer. Cdc45 was immunoprecipitated according to Gambus et al. (2011), with modifications. Briefly, chromatin samples were isolated in 10 vol of ice-cold NIB containing 100 mM KCl and were underlaid with NIB buffer containing 30% sucrose and spun for 10 min at 2,500 × g at 4 °C. PHA-767491 or tautomycetin were added optionally to the buffers. Proteins were released after DNA digestion with 2 U/μL Benzonase for 10 min at room temperature. After resuspension, KCl was adjusted to 100 mM, and chromatin was incubated for 5 min at room temperature before the final spin. Cdc45 antibody was covalently conjugated to Dynabeads Protein G (10003D, Thermo Fisher Scientific) with bis(sulfosuccinimidyl)suberate for 2 hr at 4 °C. Chromatin-bound Rif1 was immunoprecipitated from HeLa cells according to a procedure modified from Folt et al. (2018). HeLa cells were washed twice in ice-cold PBS before adding 5 mM bis(sulfosuccinimidyl)suberate to cross-link surface protein and were incubated on ice for 30 min. The reaction was quenched with 20 mM Tris-HCl (pH 7.5) for 15 min. Cells were washed twice with PBS and lysed in hypotonic buffer (25 mM Tris [pH 7.4], 2 mM MgCl₂, 50 mM KCl, and 1 mM EDTA) plus protease inhibitor cocktail (Roche) on ice for 20 min. Nuclei were washed twice in hypotonic buffer and then resuspended in RIPA buffer plus inhibitor cocktail and incubated at room temperature for 30 min with 50 U/mL benzonase. After clarification by centrifugation, lysates were subjected to co-immunoprecipitation (co-IP) using anti-Rif1 antibody covalently conjugated to Dynabeads Protein A (10008D, Thermo Fisher Scientific) with bis(sulfosuccinimidyl)suberate for 1 hr at 4 °C. Beads were then washed (five times) with filtered PBS before resuspension in SDS loading buffer.

RNAi and Transfections
The following siRNA duplexes were used: siCtrl 5’-UAGCGACUAACACAU CAA-3’ (#D0012100120, Fisher Scientific) and siRif1 (sc-62944, Santa Cruz Biotechnology). Cells were transfected using RNAiMax (#13778150, Invitrogen). Briefly, siRNA was mixed with Lipofectamine in Opti-MEM medium (#15185070, Invitrogen) and added to ~50% confluent cells grown in DMEM without antibiotics. Cells were transfected ~48 hr before each experiment.

RIPA Extraction
Cells were washed with PBS and incubated on ice for 5 min in RIPA buffer plus 1 μg/mL each of leupeptin, aprotinin, and pepstatin. Tubes were briefly sonicated and then centrifuged at ~21,000 × g for 10 min at 4 °C. Supernatants were quantified by Bradford assay before LDS (lithium dodecyl sulfate) sample buffer (NP0007, Invitrogen) and DTT were added to a final concentration of 1 X, and 50 mM, respectively. Samples were heated to 70 °C for 5 min, centrifuged at maximum speed for 5 min, and loaded onto SDS-PAGE gels.

Drug and Replication Stress Treatments
All experiments with HeLa cells used drugs at the following final concentrations: XL413 (10 μM), PHA-767491 (10 μM), caffeine (5 mM), CHIR-124 (5 μM), tautomycetin (225 mM), hydroxyurea (5 mM), aphidicolin (1 μg/mL), methyl methanesulfonate (0.02%), camptothecin (5 μM), and etoposide (5 μM). UV damage was induced by removing media from the tissue culture plate and irradiating (~45 J/m²). Media were added back, and cells were incubated for ~2 hr before harvesting.

DNA Fiber Analysis
Cells were treated with 20 μM chlorodeoxyuridine (CiDu) and 100 μM iododeoxyuridine (iD) for the indicated times, harvested, counted, and resuspended with an equal number of unlabelled cells at a concentration of 1 × 10⁶ total cells per milliliter. ~2,000 cells (2-μL cell suspension) were pipetted onto a glass slide and incubated in a humid chamber for 4 min. Cells were lysed with 8 μL digestion buffer (200 mM Tris [pH 7.4], 50 mM EDTA, 0.3% SDS), incubated for 7 min in a humid chamber, and then tilted 15° to allow the DNA to spread down the length of the slide. CiDu was detected with BioRad CBT0030CX antibody, and iDu was detected with Bo 357580 antibody. Images were acquired using a charge-coupled device (CCD) camera connected to an Olympus IX70 deltalvision deconvolution microscope under a 40x oil immersion objective. >25 fields were captured for each experiment, and 100 fibers per condition were measured using Velocity 3D Image Analysis software (PerkinElmer).

Microscopy
Cells were seeded into 10-cm dishes containing glass cover slips. Under indicated conditions, cover slips were removed from the dish, washed with PBS, fixed with 4% formaldehyde, permeabilized with 0.2% Triton X-100, washed, and blocked in 0.5% fish gelatin in PBS containing 0.1% Tween 20. EdU was detected using the Click-it EdU reaction (Thermo Fisher Scientific), and cover slips were washed, incubated with DAPI (D9542, Sigma), and mounted using Vectashield (H-1000; Vector Laboratories). Images were acquired using a CCD camera connected to an Olympus IX70 deltalvision deconvolution microscope under a 100× oil immersion objective. >25 fields were captured for each experiment, and 100 fibers per condition were measured using Velocity 3D Image Analysis software (PerkinElmer). Briefly, each nucleus was outlined as the region of interest utilizing the DAPI signal, and this was used to determine total nuclear EdU. After setting the nuclear background intensity, the number of nuclear EdU foci and intensity of EdU signal were obtained by running the following program: (1) find objects using intensity; (2) clip to region of interests (ROIs); (3) remove noise from objects (fine filter); (4) separate touching objects (object size guide, 0.02 μm³); (5) exclude objects by size (exclude objects < 0.015 μm³).

Recombinant Proteins, Reagents, and Antibodies
Full-length p27KIP1 was expressed from pGEX-p27KIP1 plasmid (a gift of J. Walter, Harvard Medical School) as described previously (Poh et al., 2014).
Chemicals were acquired as follows: PHA-767491, from the Division of Signal Transduction Therapy, University of Dundee; XL413, Axon Medchem (#2268) and a gift from Cancer Research Technology; taumolycin, Tocris (#2305); CHIR-124, Axon Medchem (Axon 1636); and KUS5933, Axon Medchem (Axon 1367). Antibodies against Xenopus proteins were as described: Mcm3, Mcm4, and Mcm7 (Prokhorova and Blow, 2000); Cdc45 and Paf1 (Gambus et al., 2011). Xenopus Rif1 antibody was raised in rabbit against the C-terminal 100 amino acids expressed in bacteria; the antisera were purified over protein A beads (see Figure S2). Antibodies against human Mcm4 (sc-22779), Mcm5 (sc-136336), PCNA (sc-56), PP1x (sc-6104), and PPTγ (sc-6108) were from Santa Cruz Biotechnology. Anti-Mcm2 was from BD Biosciences (#610701). Lamin B (ab16048), Mcm2 (phospho S53) (ab109133), Rif1 (ab140464 and ab133243), Mcm2 (phospho S50) (ab133243), Mcm2 (phospho S53) (ab109133), Rif1 (ab140464 and ab70254), and Cdc7 (ab1053) were from Abcam. Tubulin (T6199) was from Sigma-Aldrich. Antibodies raised against human Mcm2 and phospho-Mcm2 recognize the cognate proteins in Xenopus (Figures S2B–S2D; note that the Xenopus orthologs of human Mcm2 Ser40 and Ser53 are Ser25 and Ser38, respectively).

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2017.02.042.

AUTHOR CONTRIBUTIONS

R.C.A. performed all the cell-culture experiments. G.S.C. performed all the Xenopus experiments and Rif1 IP from HeLa cells. P.J.G. prepared and J.J.B. performed all the cell-culture experiments. G.S.C. performed all the experiments and Rif1 IP from HeLa cells. P.J.G. prepared and J.J.B. performed all the experiments. R.C.A., G.S.C., P.J.G., and J.J.B. wrote the article online at http://dx.doi.org/10.1016/j.celrep.2017.02.042.

ACKNOWLEDGMENTS

We would like to acknowledge the Flow Cytometry and Cell Sorting Facility at the University of Dundee, Dr. Sara ten Have and Kelly Hodge of the GRE Proteomics Support Team at the University of Dundee (Wellcome Trust Strategic Award 097945/2/11/2), and Dr. Sara Buonomo and her lab for helpful discussions. This work was funded by Cancer Research UK programme grant C303/14301, Wellcome Trust Senior Investigator Award WT096598MA, and Wellcome Trust Strategic Award 097945/B/11/2.

Received: September 16, 2016
Revised: January 5, 2017
Accepted: February 14, 2017
Published March 7, 2017

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