Mcm10 interacts with Rad4/Cut5^{TopBP1} and its association with origins of DNA replication is dependent on Rad4/Cut5^{TopBP1}.

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ABSTRACT

Initiation of DNA replication in eukaryotes is a highly conserved and ordered process involving the co-ordinated, stepwise association of distinct proteins at multiple origins of replication throughout the genome. Here, taking *Schizosaccharomyces pombe* as a model, the role of Rad4^{TopBP1} in the assembly of the replication complex has been examined. Quantitative chromatin immunoprecipitation experiments confirm that Rad4^{TopBP1} associates with origins of DNA replication and, in addition, demonstrate that the protein is not present within the active replisome. A direct interaction between Rad4^{TopBP1} and Mcm10 is shown and this is reflected in the Rad4^{TopBP1}-dependent origin association of Mcm10. Rad4^{TopBP1} is also shown to interact with Sld2 and Sld3 and to be required for the stable origin association of these two proteins. Rad4^{TopBP1} chromatin association at stalled replication forks was found to be dependent upon the checkpoint protein Rad9, which was not required for Rad4^{TopBP1} origin association. Comparison of the levels of chromatin association at origins of replication and stalled replication forks and the differential requirement for Rad9 suggest functional differences for Rad4^{TopBP1} at these distinct sites.

INTRODUCTION

In order to achieve the faithful and complete replication of the genome eukaryotic cells initiate a single round of DNA replication per cell cycle at multiple sites, DNA replication origins, within the genome. DNA replication per se requires the activity of a number of DNA polymerases, the initial activity of which is confined to these origins (1). The co-ordination of DNA replication with cell cycle progression requires exquisite regulation of the initiation of DNA replication by the cell cycle control machinery. This control is partly manifest as a stepwise, ordered assembly of a number of different factors at each individual origin of replication (2). The hexameric origin recognition complex (ORC), consisting of the related Orc1-6 subunits is assembled first. Cdc6 and Cdt1 can then mediate recruitment of the MCM (mini-chromosome maintenance) heterohexamer Mcm2-7 to the replication origin through interaction with ORC. Formation of this pre-replication complex (pre-RC) prior to S phase licenses origins for replication, but initiation of DNA replication requires the activity of two protein kinases, CDK (cyclin dependent kinase) and DDK (Dbf4 dependent kinase), and the assembly of other factors including Cdc45, GINS, Mcm10, Sld2 and Sld3. In budding yeast Cdc45 and Sld3 associate with early firing origins in G1, prior to CDK and DDK activity, and with late firing origins in S phase (3, 4). Evidence from budding yeast has demonstrated that Sld2 and Sld3 recruitment are pivotal events in replication initiation at origins and the two proteins are critical targets of the S phase CDK (5,6,7,8). Upon phosphorylation by CDK, Sld2 and Sld3 interact with distinct BRCT domains of a third protein, Dpb11, the *Saccharomyces cerevisiae* orthologue of human TopBP1, and that interaction enables recruitment to the pre-RC (6,7,8). Phosphorylation of Sld2 promotes formation of a pre-loading complex containing Dpb11, the heteromeric GINS complex, DNA Polε and Sld2 itself (9). The metazoan orthologue of Sld3, Treslin/Ticrr, has been shown to interact with TopBP1 and to be required for DNA replication (10,11,12). Cdc45 recruitment appears to require direct interaction with Mcm complex and the *mcm5-bob1-1* mutation results in DDK-independent loading of Cdc45 and the bypass of the DDK requirement for initiation of DNA replication (13,14,15). Cdc45 loading

exhibits interdependence with Sld3, which in the light of the data discussed above, offers a compelling mechanism for combining the CDK and DDK activities in the initiation of DNA replication (3). Sld3 interacts with and is required for loading of the GINS complex in budding yeast (16). In turn the GINS complex has been reported to be necessary for Cdc45 S phase chromatin association suggestive of an intimate, conserved functional relationship between Sld3, Cdc45 and GINS (16,17). More recent evidence demonstrates that GINS is dispensable for Cdc45 origin loading but is required for its interaction with the active replisome whilst Sld3 is required for Cdc45 and GINS origin loading but does not move with the replisome (4). Mcm10 is also required for the initiation of DNA replication and is recruited to origins in a Mcm4-dependent manner, travels with the progressing replication fork and acts in part to maintain DNA polymerase α stability (18,19,20).

The picture of pre-replication complex assembly in the fission yeast, *Schizosaccharomyces pombe* is similar to that described above. One apparent difference is that fission yeast Sld3 loading is independent of Cdc45 rather than the loading of the two factors being interdependent (21). Chromatin association of Cdc45 has been shown to be dependent upon Mcm10, DDK activity and Rad4/Cut5^{TopBP1} (hereafter Rad4^{TopBP1}) function (22, 23). DNA replication origin association of Rad4^{TopBP1} and Cdc45 were found to be GINS dependent in contrast to Sld3 loading (24). Apart from these observations there has been comparatively little investigation into the role of Rad4^{TopBP1} at DNA replication origins in fission yeast. A number of studies have demonstrated that Rad4^{TopBP1} has dual functionality in the initiation of DNA replication and DNA damage checkpoint responses (25, 26, 27, 28,29). In addition to the observations described above the protein has been shown to interact with DNA polymerases δ and α (29). Separation of DNA replication and checkpoint function mutations have been described and Rad4^{TopBP1} shown to be required for the activation of checkpoint kinases, Cds1 and Chk1 (28, 29, 30, 31) Rad4^{TopBP1} interacts with phosphorylated Rad9p, part of the 9-1-1 checkpoint clamp complex. This association is required for checkpoint-dependent Chk1 activation, as well as a number of other checkpoint proteins (29, 31). Human

TopBP1 has also been shown to function in both DNA replication and the response to DNA damage and recent data mirror the requirement for interaction between hRad9 and TopBP1 for checkpoint signalling (32, 33). In *Xenopus*, human and budding yeast systems TopBP1 has also been shown to directly interact with and activate the ATR-ATRIP protein kinase complex and in *Xenopus* regulation of this process is 9-1-1 dependent (34, 35, 36, 37). Recent evidence has demonstrated that chromatin association of TopBP1 following inhibition of DNA replication is reduced in the absence or compromise of Rad17 function and a model for the sequential loading of the checkpoint proteins proposed (38).

Here we have re-examined the role of $Rad4^{TopBP1}$ in the assembly of pre-replication complexes. Using a chromatin immunoprecipitation assay (ChIP) we report that $Rad4^{TopBP1}$ associates with origins of DNA replication in a cell cycle-specific manner consistent with a role in assembling a replication complex. We identify a direct protein/protein interaction between $Rad4^{TopBP1}$ and Mcm10 and demonstrate that association of Mcm10 with origins of DNA replication is dependent on $Rad4^{TopBP1}$. Although $Rad4^{TopBP1}$ is present at origins at the replication initiation stage, in contrast to Mcm10, it does not migrate with the replication fork. The recruitment of Sld2 and Sld3 to origins of replication was found to be $Rad4^{TopBP1}$-dependent. $Rad4^{TopBP1}$ recruitment at stalled replication forks was also tested and found to be Rad9-dependent and, in contrast to the data cited above (38), Rad17 independent.

MATERIALS AND METHODS

Cell growth and media. Cells were grown in YES (0.5% Yeast Extract, 3% glucose supplemented with 150 mg/l of adenine, leucine, lysine, uracil and histidine) except prior to elutriation where 2% peptone was added to the YES. Hydroxyurea (HU) was added to exponentially growing cells at 10 mM.

Elutriation. Centrifugal elutriation of six litres of exponentially growing cells was carried out using a JE-5.0 (Beckman Coulter) centrifuge. Following elutriation G2 phase cells were grown in YES at either 26°C or 37°C. Samples were taken to measure cell cycle progression every 15 min,

and for Chromatin immunoprecipitation (ChIP) at 30 min (G2) and at three subsequent points coincident with increasing (G1), peak (S) and decreasing (late S) septation indices. $1x10^8$ cells were fixed by the addition of formaldehyde to 1% in the culture medium for 30 min at room temperature before the addition of glycine to 125 mM. Cells were washed in 10 ml of PBS then frozen in liquid nitrogen and stored at -80°C.

***cdc25-22* mediated synchrony.** Cells were grown to mid log phase at 26°C then the temperature raised to 37°C for 4 h by the addition of an equal volume of media pre-warmed to 48°C. Release from cell cycle arrest was achieved by the addition of chilled fresh medium, to 25°C. A sample of $2x10^8$ cells was removed at this point (time 0) and the remaining culture returned to batch growth at 25°C. Further samples of $2x10^8$ cells were taken at the indicated intervals. Cell samples were fixed and frozen as above.

Chromatin immunoprecipitiation (ChIP). ChIP was performed as in (39) with the exception that carrier DNA was added to a concentration of 2.5ng/ml prior to the immunoprecipitation step (40). qPCR was carried out using an ABCAM 7000 and SYBR Green Jump Start ready mix in accordance with manufacturer's instructions (Sigma-Aldrich UK Ltd.). Sets of PCR primers were designed to amplify a 100bp product using the Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Fold enrichments were calculated according to (41). Briefly, since the number of molecules doubles in each cycle, fold enrichment was calculated according to the formula: $2^{(dCt-dCtcontrol)}$. dCt is the difference between the number of cycles required to go above background between Input and IP at the site of interest and dCt control is the same calculation at the site 20 kb distal to *ars2004*. Appropriate controls using untagged strains and omission of the antibody were performed in parallel for all batches of affinity beads and antibodies and no signal enrichment was ever observed. Except where stated the data represent qPCR analysis of three independent experiments normalized to the zero time point. Error bars represent the standard error.

Yeast two-hybrid analysis. pGADT7 and pGBKT7 plasmids (Clontech) were used to make Rad4/Mcm10/Sld2/Sld3 and Gal4 activation domain (AD) or Gal4 DNA-binding domain (BD) fusion constructs. The two-hybrid assay was undertaken using the Clontech Gal4 Matchmaker 3 Two-Hybrid system. The fusion constructs were introduced into *S. cerevisiae* AH109 and plated on to synthetic dropout medium (SD) lacking leucine (leu) and tryptophan (trp) to select for cotransformants; cotransformants were replica plated on to SD medium containing 10 mM 3-amino-1,2,4-triazole and lacking leu, trp, adenine (ade) and histidine (his) to test for interaction. Co-transformants were streaked on to SD -leu -trp, grown for 1-2 days then replica plated on to SD -leu -trp for selection and SD -leu -trp -ade -his plates to test for interaction, or alternatively 10-20 cotransformant colonies were grown in SD to A_{595}=1.0, serially diluted fourfold and spotted (5 μl) on to SD -leu -trp and SD -leu -trp -ade -his plates.

***In vitro* translation and immunoprecipitation.** Rad4 and Mcm10 were transcribed and translated *in vitro* from the two-hybrid plasmids pGBKT7-Rad4 (c-Myc epitope) and pGADT7-Mcm10 (HA-epitope) in the presence of ^{35}S-methionine to generate radiolabelled products Myc-Rad4 and HA-Mcm10 using the TNT coupled reticulocyte lysate system (Promega). Following translation, immunoprecipitation was carried out using anti-Myc monoclonal antibody or anti-HA polyclonal antibody using the Matchmaker co-IP kit (Clontech) according to the manufacturer's instructions. After elution from Protein A beads 10 μl of the immunoprecipitate was fractionated on a 4-12% polyacrylamide gel, transferred to PDVF membrane and autoradiographed.

RESULTS.

Rad4^{TopBP1} and Mcm10 associate with origins of DNA replication:

In order to establish whether Rad4^{TopBP1} and Mcm10 were associated with origins of DNA replication at the point of initiation we synchronised cells in G2 by incubating *cdc25-22* mutants, expressing the relevant functional GFP fusion proteins at the wild type locus, at the restrictive temperature and subsequently releasing them at the permissive temperature to undergo synchronous

passage through mitosis, septation and initiation of DNA replication. Control Mcm4-GFP associated with three distinct origins of DNA replication, *ars1*, *ars2004* and *ars3005*, with the peak occurring at 90 min and this pattern was found to be highly reproducible (Fig. 1A) and no enrichment was observed in the absence of the a GFP epitope (Fig. 1D). Similar results were obtained for Mcm10 and Rad4^{TopBP1} (Figs. 1B and C). Enrichment of Mcm10-GFP and Rad4^{TopBP1}-GFP was much less than for Mcm4-GFP, but was observed repeatedly with a similar temporal profile to Mcm4. Haploid cells carrying the *cdc25-22* allele arrest at the G2/M transition with a fully replicated genome (2C DNA content). Upon release at the permissive temperature all strains initiate DNA replication approximately 60 minutes after release from G2 arrest, progressing through nuclear division and DNA replication at 90 minutes (4C DNA content) prior to cell division seen by the re-appearance of a 2C DNA content. The peak ChIP enrichment occurs at 90 minutes which correlates well with the DNA content analysis (Fig.1). The differences in the fold enrichment of the three proteins presumably reflect differences in protein level or turnover at the site of chromatin association and the accessibility of the GFP epitopes in the chromatin bound fraction. The growth characteristics of all three epitope tagged alleles were indistinguishable from a wild type control (Fig. S1A) consistent with earlier reports (22, 29, 42, 43).

Mcm10 but not Rad4^{TopBP1} association with the active replisome:

The association of Mcm4, Rad4^{TopBP1} and Mcm10 with *ars2004* and sites distal to the origin was examined in synchronous culture. Over time Mcm4 was progressively enriched at regions further from the origin indicating that it moves with the replication fork (Fig. 2B). Association with the origin was maximal at 90min but peaked 1 kb away at 110min and 2 kb away at 120min. Relative enrichment at these loci was less than at the *ars*. Since ChIP results represent an average of a population of cells this is consistent with replication proceeding at different rates in individuals cells. Mcm10 exhibits the same temporal and spatial pattern of association as Mcm4, consistent with it forming part of the replisome (Fig. 2C). In contrast whilst Rad4^{TopBP1} associated with origin DNA at the point of DNA replication initiation it was never found associated with DNA distal to

this site (Fig. 2D). One interpretation of this result is that Rad4^{TopBP1} is not part of the active replisome complex. Alternatively it might be that, as a result of the low levels of Rad4^{TopBP1} found at origins of replication and the heterogeneity of the timing of DNA replication initiation and rate of replication fork movement within the population, that the effective concentration of Rad4^{TopBP1} falls below the level of detection. The distinction between these two possibilities is discussed further, below. A further possibility is that the GFP moiety on Rad4 is inaccessible within the replisome and we have been unable to address this experimentally through the unavailability of appropriate research materials.

Rad4^{TopBP1} and Mcm10 physically interact:

A two-hybrid analysis utilizing a Rad4-Gal4 activation domain fusion plasmid demonstrates growth on selective media only in the presence of a Mcm10-Gal4 DNA binding domain fusion plasmid, indicative of a direct interaction between the two proteins. The *rad4-116*-Gal4 AD plasmid, containing the temperature sensitive allele, rendered the interaction conditional on growth temperature (Fig. 3A). Importantly interaction between the mutant Rad4 ^{TopBP1} protein and Sld2 was retained at the restrictive temperature, indicating that the mutant form of the protein was stable at this temperature. This demonstrates a degree of specificity for the interaction between Rad4^{TopBP1} and Mcm10 and offers a potential explanation for the temperature sensitive phenotype of *rad4-116* strains. Co-expression and radio-labelling of Rad4^{TopBP1} and Mcm10 *in vitro*, followed by immunoprecipitation provides biochemical support for the existence of a direct interaction between the two proteins (Fig. 3B). Deletion mapping of Mcm10 indicated that the C-terminus of Mcm10, amino acids 385-503, was dispensable for the interaction with Rad4^{TopBP1}, a further indication of specificity in the interaction (Fig. 3C). Attempts to co-immunoprecipitate or affinity isolate a Rad4^{TopBP1} –Mcm10 complex from cell extracts, analysed both by immunoblotting and mass spectrometry were not successful, which we attribute to a lack of complex stability in cell extracts.

Mcm10 association with origins of DNA replication is dependent on Rad4^{TopBP1}:

The fact that Rad4^{TopBP1} interacts with Mcm10 suggests that Mcm10 origin loading could require Rad4^{TopBP1}. In order to test this we used centrifugal elutriation to isolate G2 cells carrying the temperature sensitive *rad4-116* allele. Strains carrying this allele are unable to initiate DNA replication at the restrictive temperature (25, 44, 45). Fig 4A and B demonstrate that Mcm4 was able to associate with *ars2004* independently of the presence of a functional Rad4^{TopBP1}. However the level of Mcm4 association with the origin of DNA replication was reduced at the restrictive temperature and the protein levels were similarly decreased (Fig. S1B). In contrast Mcm10 was bound to *ars2004* at 26ºC but unable to associate with the same DNA replication origin at the restrictive temperature, 37ºC. Levels of Mcm10 were broadly similar at both 26ºC and 37ºC (Fig. S1B).

Sld2 and Sld3 origin loading are both Rad4^{TopBP1}-dependent:

In order to establish the conservation of Rad4^{TopBP1} function in the initiation of DNA replication we tested for interaction with the replication initiation factors Sld2 and Sld3 by two hybrid analysis. Interaction was observed between Rad4^{TopBP1} and both Sld2 and Sld3 (Fig. 3A and S2A). Representatives of three independent transformations are shown. We next, briefly, examined the dependency of *ars2004* association of Sld2 and Sld3 on Rad4^{TopBP1} function. Origin association of both proteins was found to be Rad4^{TopBP1}-dependent, origin binding being observed at 26ºC but not at 37ºC in *rad4-116* cells, the one caveat being that we did not test origin association at 37ºC in wild type cells (Fig. S2B and C). Both Sld2 and 3 are present at similar levels at 26ºC and 37ºC (Fig. S1B). Representative FACS profiles for the elutriated strains are presented in Supplementary Fig. 3, where the peak septation point can be seen to coincide with initiation of DNA replication in all strains except *rad4-116* at the restrictive temperature, 37^{o}C, when replication is abolished. Taken together these data suggest a mechanistic conservation between the two divergent yeast species with Rad4^{TopBP1} interacting directly with Sld2 and Sld3 and this interaction is likely to be required for stable origin association of Sld2 and Sld3.

Checkpoint proteins accumulate at stalled replication forks but not at origins of DNA replication:

Rad4^{TopBP1} acts in both the initiation of DNA replication and the DNA integrity checkpoint pathways and these two activities can be separated genetically (28, 29). This raises the question of whether Rad4^{TopBP1} associates with stalled replication forks and if so whether this exhibits distinct requirements from those determining origin association. Cells synchronized in G2 were released into the presence of hydroxyurea, HU, an inhibitor of DNA replication and profile of origin and replication fork association assessed by ChIP assays. Control experiments presented in Fig. 5A and B demonstrate that the replication fork associated Mcm4 accumulated at the origin of DNA replication and additionally at sites 1 and 2 kb distal to the origin of replication. In contrast the checkpoint proteins, Rad3 and Rad17, were never observed at the origin of replication in the either the presence or absence of HU (Fig. 5C, D and 5E, F respectively). Both Rad3 and Rad17 accumulate at the distal sites after 60 min in the presence of HU, Fig. 5D and F. Together these data strongly suggest that replication forks stall within 1 to 2 kb of the origin and that stalled forks are characterised by the accumulation of both replication fork associated proteins and checkpoint proteins. Mcm4 was also enriched 4kb and 9kb distal to the origin at the later time point and this potentially represents the presence of slow moving DNA replication forks that have escaped arrest following attenuation of the checkpoint signal.

Rad4^{TopBP1} accumulation at stalled DNA replication forks is Rad9 dependent:

Stalled replication forks are stabilized through the action of the intra-S phase checkpoint. The previously reported phosphorylation-dependent interaction between Rad4^{TopBP1} and the checkpoint protein Rad9 led us to test the dependency of Rad4^{TopBP1} association with the stalled replisome on Rad9 function. In an unperturbed S phase Rad9 is never found at either the origin of replication or the adjacent sites (Fig. 6A). Consistent with its role in checkpoint activation, Rad9 is chromatin associated in HU only at the origin distal sites, with a similar distribution to the other checkpoint proteins, Rad3 and Rad17 (Fig. 6B). In Fig. 6C Rad4^{TopBP1} is shown to associate with the origin of

replication at 60 minutes post release in the presence of HU. The protein also accumulates to higher levels at the distal sites than are observed at the replication origin, peaking at 2 kb after 60 and 90 minutes. Rad4^{TopBP1} is never observed at these sites in the absence of HU (see Fig. 2D). The overall physical and temporal distribution of sites of Rad4^{TopBP1} accumulation in the presence of HU is consistent with its presence at stalled or slow moving replisomes. The enrichment of Rad4^{TopBP1} observed 2 kb distal to the origin of DNA replication exhibits a clear Rad9 dependency (Fig. 6D). In contrast Rad4^{TopBP1} DNA replication origin association in the presence and absence of HU was found to be Rad9 independent (Fig. 6D and E). Finally the role of Rad17 in the recruitment and maintenance of Rad4^{TopBP1} at stalled replication forks was examined. The data in Fig. 6F clearly show that Rad4^{TopBP1} associated with sites distal to the origin of replication in the presence of HU, implying that Rad17 function is dispensable for Rad4^{TopBP1} association with stalled replication forks.

DISCUSSION

In budding yeast Mcm10 had previously been shown to associate with origins of DNA replication and the active replisome (18, 20) and our observations extend and confirm these data for *S. pombe*. If Rad4^{TopBP1} is to act as a loading factor for Mcm10 at origins of DNA replication then it must associate with those same DNA sequences and this is observed (24; Fig. 1). Predictably Mcm4 (replicative helicase) and Mcm10 associate with the replisome as it moves outward from the origin of DNA replication (Fig. 2A and B). In contrast the data for Rad4^{TopBP1} suggest that it does not associate with the active replisome once DNA replication has initiated (Fig. 2C). However caution is required in accepting this interpretation as the level of enrichment of Rad4^{TopBP1} at the origin is relatively low and as heterogeneity in the population will increase as cells proceed through S phase the amount Rad4^{TopBP1} associated with the active replisome may fall below the level detection. Two-hybrid studies demonstrate that Rad4^{TopBP1} interacts with Mcm10 and that this interaction is lost at the restrictive temperature for the *rad4-116* allele (Fig. 3A), supporting previously reported genetic interactions between *rad4* and *mcm10* (46; K. Moore and S. J. Aves, unpublished data).

The specificity of the interaction between Rad4^{TopBP1} and Mcm10 is further supported by the deletion analysis and *in vitro* interactions shown in Fig. 3B and C. These data demonstrate for the first time a direct interaction between Rad4^{TopBP1} and Mcm10 and are consistent with Rad4^{TopBP1}-dependent loading of Mcm10 at origins of DNA replication (Fig. 4). These observations raise additional questions about the order of assembly of additional pre-initiation factors at origins of DNA replication. In budding yeast the Rad4^{TopBP1} homologue Dpb11 has been shown to interact with both Sld2 and Sld3 and those events have been shown to be CDK-dependent and similar data have recently been reported in metazoans (6, 7, 8, 10, 11, 12, 49). Chromatin association of Cdc45 has been previously shown to be dependent upon Mcm10, Rad4^{TopBP1}, Sld3 and the GINS complex (22, 23, 24, 47). The data presented in Supplementary Fig. 2A suggest that Rad4^{TopBP1} interacts with Sld2 and Sld3. In addition the association of Sld2 and Sld3 with a DNA replication origin were found to be dependent upon functional Rad4^{TopBP1} (Fig. S2B and C). Interestingly the Sld3 result contrasts with earlier data indicating that Sld3 can associate with the pre-RC in the absence of detectable Rad4^{TopBP1} origin association when CDK levels are reduced (24). Additionally a mutated form of Sld3 impaired in its interaction with Rad4^{TopBP1} shows increased levels of origin association relative to wild type Sld3 and that association occurs in the absence of Rad4^{TopBP1} association (48). Origin association of Sld3 is also shown to occur in a *drc1*$^{-}$ (*sld2*$^{-}$) mutant in the absence of Rad4^{TopBP1} association. One possible explanation for these contrasting observations might be that in the wild type situation the stability of preRC association of Sld3 and Rad4^{TopBP1} is mutually dependent and that the experimental interventions employed in the different studies perturb this relationship. The phosphorylation-dependent Rad4^{TopBP1} interaction with Sld2 and Sld3 is conserved between budding and fission yeast (48). Indeed the conservation of the TopBP1-Sld3 interaction has recently been extended to human cells (49). Taken together, we conclude from these data that Rad4^{TopBP1} associates with origins of DNA replication where it is required for the assembly of the replication pre-initiation complex, specifically for the stable incorporation of Sld2, Sld3 and Mcm10. Cdc45 failed to interact with Rad4^{TopBP1} but was not observed at origins of DNA

replication in the absence of Rad4^{TopBP1} function (unpubl. obs.; 23). This suggests that Cdc45 origin loading is likely to be indirect with respect to Rad4^{TopBP1}, occurring via physical interaction with Mcm10 and GINS (22, 24). A model for the order of recruitment of these factors is presented in Fig. 7. The GINS complex is omitted from this picture for reasons of clarity but it would be anticipated to occur in parallel with Rad4^{TopBP1} recruitment (24).

In an effort to resolve the issue of Rad4^{TopBP1} association with the active replisome and to address its S phase checkpoint role we have examined Rad4^{TopBP1} chromatin association in the presence of HU, an inhibitor of DNA replication. The data presented in Figure 5 demonstrate that the replisome-associated Mcm4 and the checkpoint proteins, Rad3 and Rad17, all accumulate at sites distal to the origin of DNA replication as time progresses in the presence of HU as would be predicted if sites represented stalled replication forks. Rad9 is also shown to behave as Rad3 and 17, accumulating at stalled replication forks but never at the origin, irrespective of the presence of the replication inhibitor (Fig. 6A and B). In contrast Rad4^{TopBP1}, like Mcm4, is observed at origins of DNA replication and at the distal sites in the presence of HU. However origin association is Rad9 independent whilst accumulation distal to the origin, in the presence of HU, is Rad9 dependent. The Rad9-dependency of Rad4^{TopBP1} accumulation at stalled replisomes is consistent with earlier data demonstrating that Rad4^{TopBP1} acts downstream of Rad9 and Hus1 as judged by phosphorylation of these two checkpoint proteins, and the fact that interaction between Rad4^{TopBP1} and Rad9 requires DNA damage-dependent phosphorylation of Rad9 (30, 31, 33). In addition it bears upon Rad4^{TopBP1} replisome association. Active replication forks move slowly in the presence of HU in wild type cells and in checkpoint deficient cells the replisome is further hindered (50). If Rad4^{TopBP1} was associated with the replisome it might be assumed that at slow moving forks it might be more readily detected as synchrony is likely to be maintained, at least in the early stages of S phase progression. The fact that Rad4^{TopBP1} does not associate with the regions distal to the origin in HU treated *rad9*$^{-}$ deficient cells implies that it is not associated with the replisome. Taken together with the data presented in Fig. 2 we favour the idea that Rad4^{TopBP1} is not associated with

the moving replisome although we accept that the data remain inconclusive on this point due to the relatively low level of detection at the replication origins. A further intriguing possibility is that the low level of detection of Rad4^{TopBP1} at replication origins, in contrast to increased accumulation at the stalled forks in HU, might reflect distinct activities of the protein and the dynamics of Rad4^{TopBP1} turnover at the relevant sites within the genome. Formaldehyde cross-linking of proteins to DNA *in vivo* is a time limited process and in mammalian cells is able to capture only those interactions that extend beyond 5 seconds (51). It might then be that low levels of Rad4^{TopBP1} at origins in the absence of HU result from rapid turnover of the protein associated with a role as a loading factor for pre-initiation complex formation. In contrast at stalled replication forks it might function as a structural component of a more stable complex.

The paradigmatic view of checkpoint protein recruitment holds that the RFC-related Rad17 complex is required to load the PCNA-related 9-1-1 complex. As Rad9 is required for Rad4^{TopBP1} association with stalled forks then it would be predicted that Rad17 is also required. Intriguingly this was not case and Rad4^{TopBP1} behaved similarly in wild type and *rad17*$^{-}$ deficient cells in the presence of HU, clearly demonstrating that Rad17 is not required for the association of Rad4^{TopBP1} with stalled DNA replication complexes (Figs. 6C and F). TopBP1 is known to interact directly with vertebrate and budding yeast ATR-ATRIP complex (Rad3-Rad26 in *S. pombe*) and this interaction is required for activation of the kinase function (34, 35). Moreover this interaction is dependent on a TopBP1/Rad9 interaction (31, 33, 37). The data presented here and summarised in Fig. 7 reflect this predicted order of events and are consistent with an earlier model predicting the existence of a multimeric complex between these proteins in fission yeast (30). The fact that Rad17 is not required for Rad4^{TopBP1} is also consistent with this earlier model that proposed that Rad4^{TopBP1} served to integrate a bifurcated pathway. The lack of Rad17 dependency does however contrast with previous work in *Xenopus* demonstrating a strict requirement for Rad17 to effect TopBP1 chromatin loading following checkpoint activation (38, 52). One possible explanation for this dichotomy might be the fact that there are additional Rfc1-like proteins in yeast (53, 54) and the

observed Rad17 independence might then reflect functional redundancy in this organism, but this remains to be tested. A second possibility might lie in the fact that the *Xenopus* assay relies on bulk chromatin association whereas here TopBP1 association with mapped stalled replication forks is assessed. Alternatively the distinction between Rad9 and Rad17 requirement may reflect distinct chromatin structures at stalled, collapsed or restarted replication forks in the two mutants in the presence of HU (55). It has recently been shown that the *S. cerevisiae* homologue, Dpb11, forms Rad24 (Rad17 homologue)-dependent foci in the presence of DNA damage. Interestingly though, these foci are distinct from DNA replication-associated Sld3 foci and the authors were able to isolate a *dpb11* separation of function mutation that retained DNA replication checkpoint function whilst lacking DNA damage checkpoint function (56). It is possible then that Dpb11/Rad4^{TopBP1} exhibits distinct functional dependencies in DNA replication and damage checkpoint pathways. Whatever the explanation for the observation the exact role of Rad17 in the checkpoint pathways in fission yeast remains obscure and for this reason the protein is omitted from the model presented in Fig. 7.

In summary the *S. pombe* Rad4^{TopBP1} is required for the assembly of the DNA pre-initiation complex and as in budding yeast acts to load Sld2 and 3. In addition Mcm10 association with the replication origin is also dependent upon Rad4^{TopBP1} and the two proteins interact directly. It is likely, but not proven, that Rad4^{TopBP1} is not present in the active replisome, perhaps reflecting a role as loading factor for the assembly of the pre-initiation complex. When replication is inhibited Rad4^{TopBP1} is recruited to stalled replication complexes in a manner that is Rad9 dependent but Rad17 independent.

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FIGURE LEGENDS

Figure 1. **Enrichment of Mcm10 and Rad4^{TopBP1} at DNA replication origins**. ChIP was performed on cell samples taken at time 0, 30, 60, 70, 80, 90, 100, 110 and 120 min after release from *cdc25-22* arrest. Samples were analysed by qPCR using primers designed to amplify DNA fragments within *ars1*, *ars2004* and *ars3005* and at a point 20 kb distal to *ars2004*. Relative enrichment was calculated as the fold difference between the qPCR threshold crossing points recorded with two sets of primers (ARS and 20 kb) and normalised to time-point 0. The histograms show the enrichment at each ARS relative to that at the site 20 kb from *ars2004*. The data derive from six independent qPCR reactions and error bars indicate standard error of the mean. The DNA content profile measured by flow cytometry of a representative synchrony experiment is shown for all four strains. The arrows represent 2C (left) and 4C (right) DNA content. **A**. SPSC548 (Mcm4-GFP). **B**. SPSC556 (Mcm10-GFP) strain. **C**. SPSC573 (Rad4-GFP) strain. **D.** SPSC57 (WT control lacking GFP)

Figure 2. **Mcm10 associates with the active replisome whilst Rad4^{TopBP1} association is not observed**. Samples for ChIP analysis were taken at 0, 30, 60, 70, 80, 90, 100, 110, 120 and 140 min following release from *cdc25-22* arrest. Deoxyribonucleotide primers for qPCR were designed to amplify products at *ars2004* (0) and 1, 2, 4, 9 and 20 kb away from this origin as represented in **A**. The levels of enrichment at each position along the chromosome at the different time intervals are calculated and presented as described above. **B**. SPSC548 (Mcm4-GFP). **C**. SPSC556 (Mcm10-GFP). **D**. SPSC573 (Rad4-GFP).

Figure 3. **Rad4^{TopBP1} interacts directly with Mcm10. A.** Two-hybrid interactions between Rad4^{TopBP1}/Rad4-116 with Mcm10 and Sld2 at 28°C and 34°C. **B**. Rad4^{TopBP1}-myc and Mcm10-3HA proteins were expressed and S^{35}-labelled *in vitro* and then co-immunoprecipitated with protein specific antibodies. Lanes 1 and 2 show S^{35}-labelled Mcm10-HA protein precipitated with a monoclonal antibody raised against either the HA epitope (lane 1) or c-myc epitope (lane 2). Lanes 3 and 4 show S^{35}-labelled Mcm10-HA and Rad4-myc proteins co-precipitated with antibodies

against HA or c-myc respectively. Lanes 5 and 6 present S^{35}-labelled Rad4-myc protein precipitated with antibodies against HA or c-myc respectively. **C**. The Mcm10 interaction domain was mapped by two-hybrid analysis of truncated versions of Mcm10 as bait against $Rad4^{TopBP1}$ and interaction scored as +/- growth on selective media. Indicated on the Mcm10 protein diagram (left to right) are nuclear localisation signal (stippled box), Walker A motif (black line), oligonucleotide/oligosaccharide binding (OB)-fold (light grey box) containing an Hsp10 box (black line), CCCH zinc finger motif (dark grey box), and nucleotide transfer domain (black box).

Figure 4. **Mcm10 requires the presence of $Rad4^{TopBP1}$ to load at origins**. ChIP was performed on samples taken following centrifugal elutriation of each strain. Cell samples were taken 30 min after elutriation then at increasing, peak and decreasing septation index (indicated by the bold line in each histogram) following growth at 26°C and 37°C, the permissive and restrictive temperatures for the *rad4-116* allele. Samples were processed and the data presented as described above for *ars2004* association. **A**. Mcm4-GFP *ars2004* association in $rad4^+$ (WT, SPSC593) and *rad4-116* (SPSC581) cells grown at 26°C (left) and 37°C (right). **B**. Mcm10-GFP *ars2004* association in $rad4^+$ (WT, SPSC593) and *rad4-116* (SPSC581) cells grown at 26°C (left) and 37°C (right).

Figure 5. **Enrichment of Rad3-HA, Rad17-*myc* and Mcm4-GFP at stalled replication forks**. ChIP data is presented in these histograms for three strains released from *cd25-22* arrest into media containing either 0 or 10 mM HU. Data is shown for two time-points, 0 and 60 minutes following release, at sites 0, 1, 2, 4 and 9kb relative to *ars2004*. **A**. Mcm4-GFP is enriched at the origin at 60 minutes in the absence of HU. **B**. Mcm4-GFP shows greatest enrichment 2kb distal to *ars2004* in the presence of HU 60 minutes after release. **C**. No enrichment of Rad3-HA was detected at or near *ars2004* in the absence of HU. **D**. Rad3-HA was enriched 1-2kb distal to *ars2004* in the presence of HU, 60 min post-release. **E**. Chromatin association of Rad17-*myc* was not observed in the absence of HU. **F**. Rad17-*myc* was enriched at sites 1-2kb distal to the origin of DNA replication in the presence of HU, 60 min post-release.

Figure 6. **Rad4-GFP recruitment to stalled replication forks is Rad9-dependent.** The histograms show ChIP data from samples taken following release from *cdc25-22* arrest into media containing either 0 or 10 mM HU as indicated. Samples were taken at 0 (no HU), 30, 60, 90 and 120 min after return to growth temperature. All epitope tagged proteins were expressed in otherwise wild type strains with respect to checkpoint function except where stated. **A**. Rad4-GFP (SPSC573). Rad4-GFP was enriched at *ars2004* and sites 1-2kb distal to the origin of DNA replication in the presence of HU, 60 and 90 min post-release. **B**. Rad9-*myc* was enriched at sites distal to the origin of DNA replication in the presence of HU beyond the 60 min post-release time point. **C.** Rad9-myc (SPSC592). Chromatin association of Rad9-*myc* was not observed in the absence of HU. **D**. Rad4-GFP +HU in a *rad9Δ* background (SPSC587). Enrichment of Rad4GFP was observed only at the origin of replication in the absence of Rad9 function, despite the presence of HU. **E**. Rad4-GFP presence at *ars2004* was independent of Rad9 in the absence of HU. **F**. Rad4-GFP +HU in a *rad17Δ* background (SPSC588). Rad4-GFP association at sites distal to the origin of replication was observed in the presence of HU despite the lack of functional Rad17.

Figure 7. **Order of recruitment of Rad4^{TopBP1} to origins of replication and stalled replication forks.** The panel on the left illustrates the recruitment of Rad4^{TopBP1} to the pre-replication complex following MCM origin association. Subsequently Sld2, Sld3 and Mcm10 are stably recruited in a Rad4^{TopBP1}-dependent manner through direct protein-protein interaction. Cdc45 is then recruited and recruitment is indirectly dependent on Rad4^{TopBP1}. Rad4^{TopBP1} then dissociates before initiation of DNA replication. The right hand panel illustrates the probable order of recruitment of the checkpoint complexes, Rad3/Rad26, 9-1-1 and Rad4^{TopBP1} to stalled replication forks. Rad3/Rad26 and 9-1-1 are recruited independently and following Rad9 phosphorylation Rad4^{TopBP1} associates with the stalled replication fork complex through direct association with phosphorylated Rad9.

Supplementary Figure 1. **Growth rate and protein levels of the epitope tagged strains are not altered relative to wild type controls. A**. Growth curves for exponential cultures of the indicated strains at 26°C and 32°C in liquid YES medium. Standard errors are shown but are generally obscured by the data markers. **B.** Western blots following separation of whole cell extract using SDS-PAGE (10%acrylamide gel). Exponential cell cultures were grown at 26°C and 37°C for each of the indicated epitope tagged proteins. Primary antibodies were the same as used in the ChIP experiments at 1:4000 dilution in 5% semi skimmed milk/TBS (0.1%) Tween. Secondary antibodies were goat anti mouse HRP antibodies from DAKO at 1:5000 in 5% semi skimmed milk/TBS (0.1%) Tween. Epitope tagged proteins were visualised using ECL Plus (GE Healthcare) Western Blotting Reagent Pack and detected on Amersham ECL Hyperfilm (GE Healthcare).

Supplementary Figure 2. **Origin loading of Sld2 and Sld3 requires functional Rad4^{TopBP1}.** A. Two-hybrid interaction analysis between Rad4^{TopBP1} and Sld2 and Sld3, as indicated. B and C. ChIP analysis to monitor *ars2004* origin association of Sld2 and Sld3 in *rad4-116* mutant background. The strains shown are: B. SPSC586 (*Sld2-GFP rad4-116*). C. SPSC562 (*Sld3-FLAG rad4-116*).

Supplementary Figure 3. **DNA content profiles of cells following synchronization by centrifugal eluriation.** Fixed elutriated cells of the indicated strains were stained with propidium iodide and the DNA content was assessed by flow cytometry. The profiles of each strain at 26 ^{o}C and 37 ^{o}C are shown and the peak septation point is marked by arrows and is coincident with the initiation of DNA replication. The wild type strains, with respect the *rad4* allele, are presented in the left two panels and the *rad4-116* strain carrying the indicated epitope alleles of the target genes are shown in the rightward two panels and the growth temperatures are indicated. Note that the point of peak septation varies within strains dependent upon the growth temperature and that the *rad4-116* strains

fail to initiate DNA replication at the restrictive temperature of 37^{o}C. *The rad4-116* strains continue through septation at the restrictive temperature due to their lack of a checkpoint response.

Table 1. Strain list

Strain number	**Genotype**	**Source**
SPSC57	*ura4-D18 leu1-32 ade6- M216 h⁻*	Lab collection
SPSC548	*mcm4-GFP::URA3 cdc25-22 ura4-D18 leu1-32 ade6-M216 h⁻*	A.M. Carr
SPSC581	*mcm4-GFP::URA3 rad4-116 ura4-D18 leu1-32 ade6-M216 h⁻*	This study
SPSC593	*mcm4-GFP::URA3 ura4-D18 leu1-32 ade6-M216 h⁻*	This study
SPSC573	*rad4-GFP::kanMX cdc25-22 ura4-D18 leu1-32 ade6-M216 h⁻*	This study
SPSC556	*mcm10-GFP::kanMX cdc25-22 ura4-D18 leu1-32 ade6-M216 h⁻*	This study
SPSC563	*mcm10-GFP::kanMX rad4-116 ura4-D18 leu1-32 ade6 h⁻*	This study
SPSC555	*mcm10-GFP::kanMX ura4-D18 leu1-32 ade6 h⁻*	This study
SPSC550	*sld3::URA3 sld3-5FLAG ura4-D18 h⁻*	Yamada *et al*. 2004
SPSC562	*sld3::URA3 sld3-5FLAG rad4-116 ura4-D18 h⁻*	This study
SPSC585	*sld2-GFP::natMX cdc25-22 ura4-D18 leu1-32 ade6 h⁻*	This study
SPSC586	*sld2-GFP::natMX rad4-116 ura4-D18 leu1-32 ade6 h⁻*	This study
SPSC587	*rad4-GFP::kanMX rad9Δ::URA3 cdc25-22 ura4-D18 leu1-32 ade6 h⁻*	This study
SPSC588	*rad4-GFP::kanMX rad17Δ::URA3 cdc25-22 ura4-D18 leu1-32 ade6 h⁻*	
SPSC592	*rad9-myc::kanMX cdc25-22 ura4-D18 leu1-32 ade6 h⁻*	A.M. Carr
SPSC671	*rad17-myc::kanMX cdc25-22 ura4-D18 leu1-32 ade6 h⁻*	This study
SPSC676	*rad3-myc cdc25-22 ura4-D18 leu1-32 ade6 h⁻*	This study

Figure 1

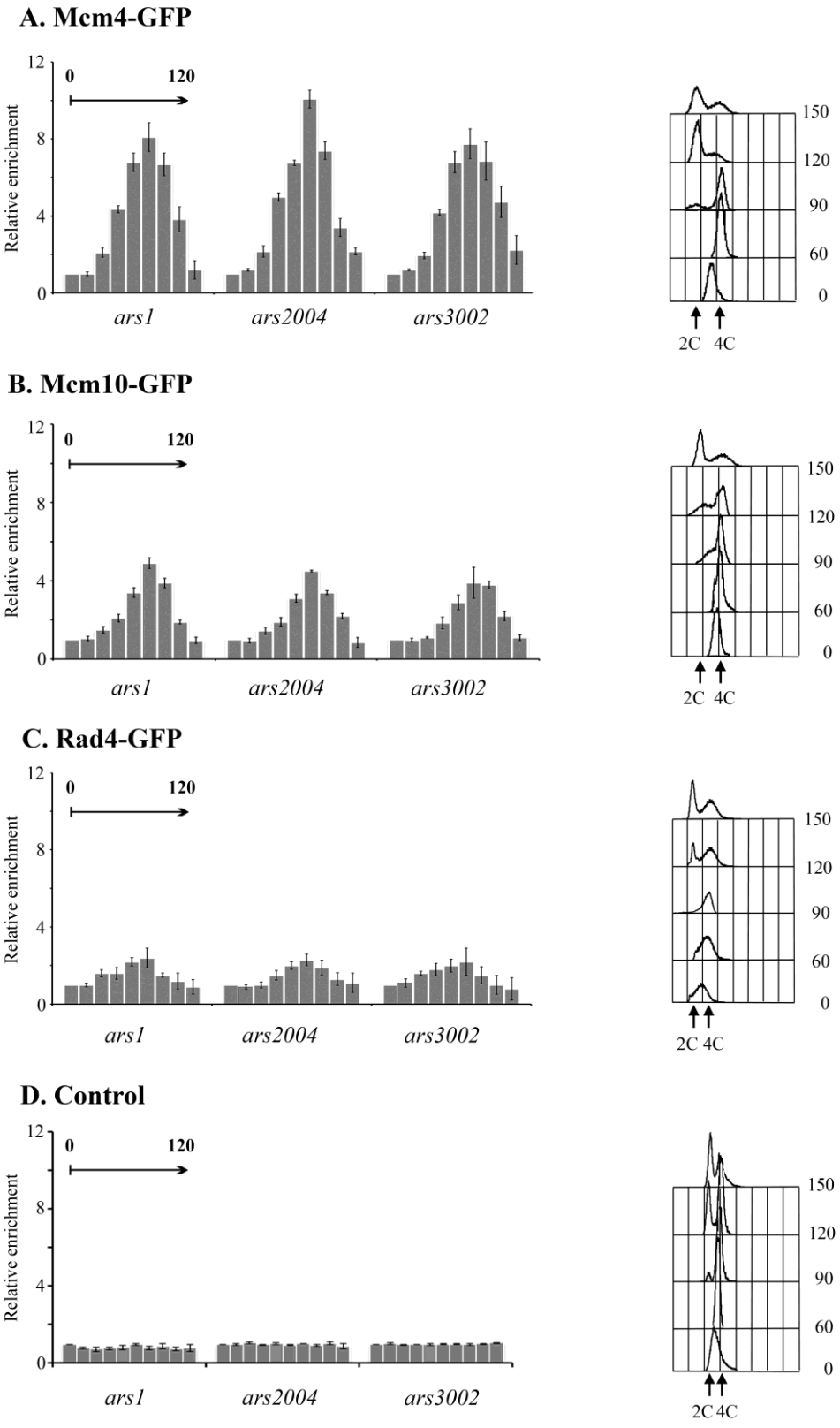

A. Mcm4-GFP
B. Mcm10-GFP
C. Rad4-GFP
D. Control
Relative enrichment
0
120
ars1
ars2004
ars3002
2C
4C
150
120
90
60

Figure 2

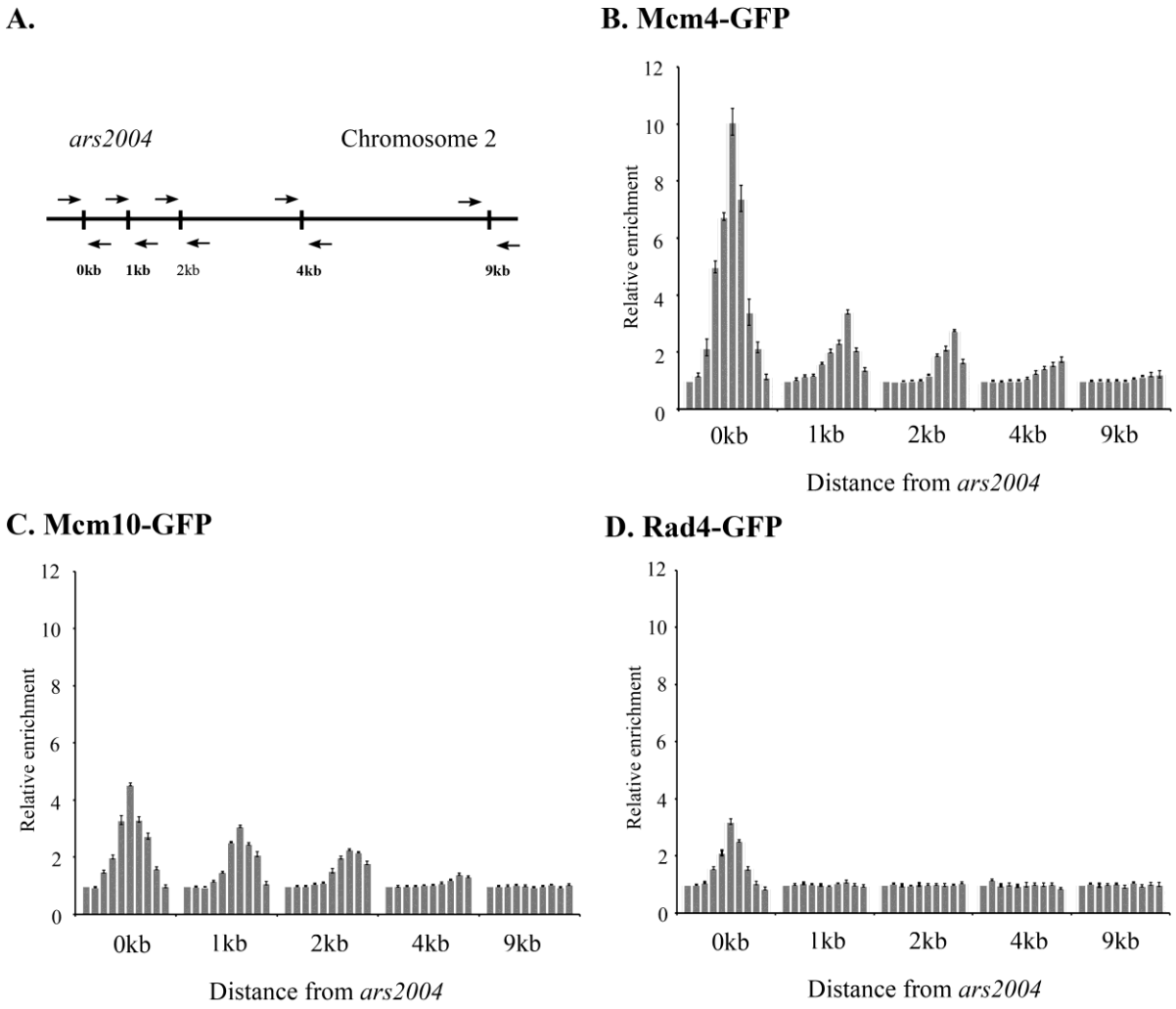

A.
ars2004
Chromosome 2
0kb
1kb
2kb
4kb
9kb
B. Mcm4-GFP
Relative enrichment
Distance from ars2004
C. Mcm10-GFP
Relative enrichment
Distance from ars2004
D. Rad4-GFP
Relative enrichment
Distance from ars2004

Figure 3

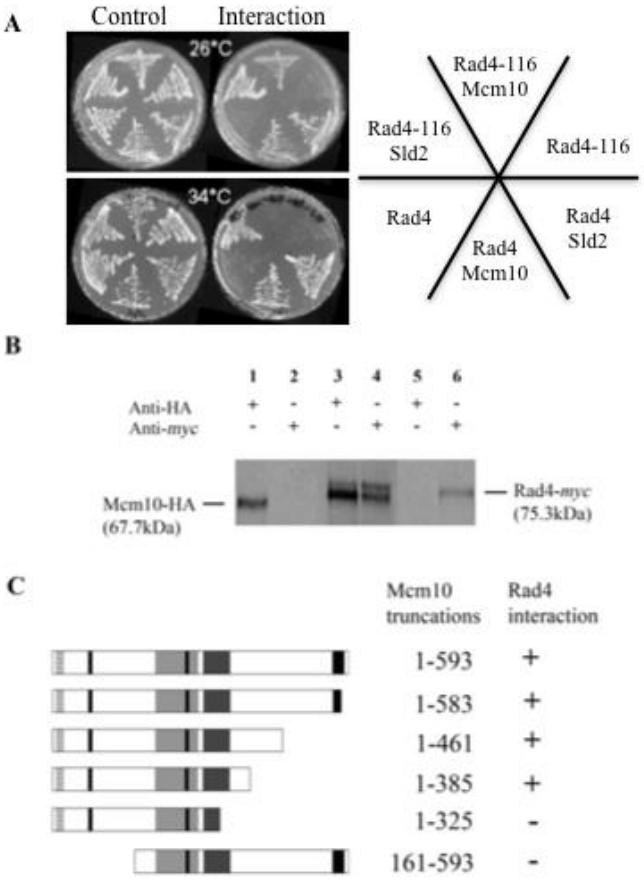

A
Control
Interaction
26°C
34°C
Rad4-116
Mcm10
Rad4-116
Sld2
Rad4-116
Rad4
Rad4
Sld2
Rad4
Mcm10
B
1 2 3 4 5 6
Anti-HA
Anti-myc
Mcm10-HA
(67.7kDa)
Rad4-myc
(75.3kDa)
C
Mcm10
truncations
Rad4
interaction
1-593 +
1-583 +
1-461 +
1-385 +
1-325 -
161-593 -

Figure 4

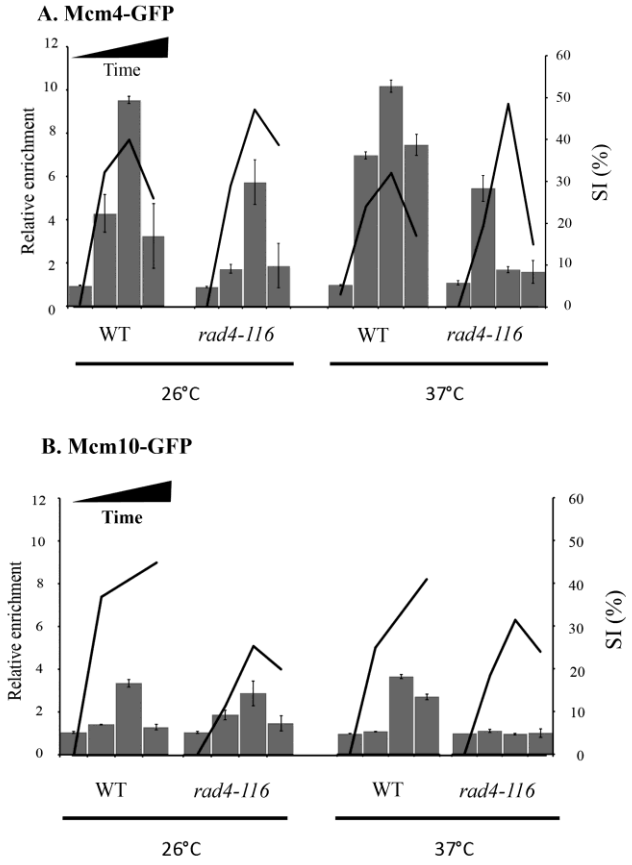

A. Mcm4-GFP
Time
Relative enrichment
SI (%)
WT
rad4-116
WT
rad4-116
26°C
37°C
B. Mcm10-GFP
Time
Relative enrichment
SI (%)
WT
rad4-116
WT
rad4-116
26°C
37°C

Figure 5

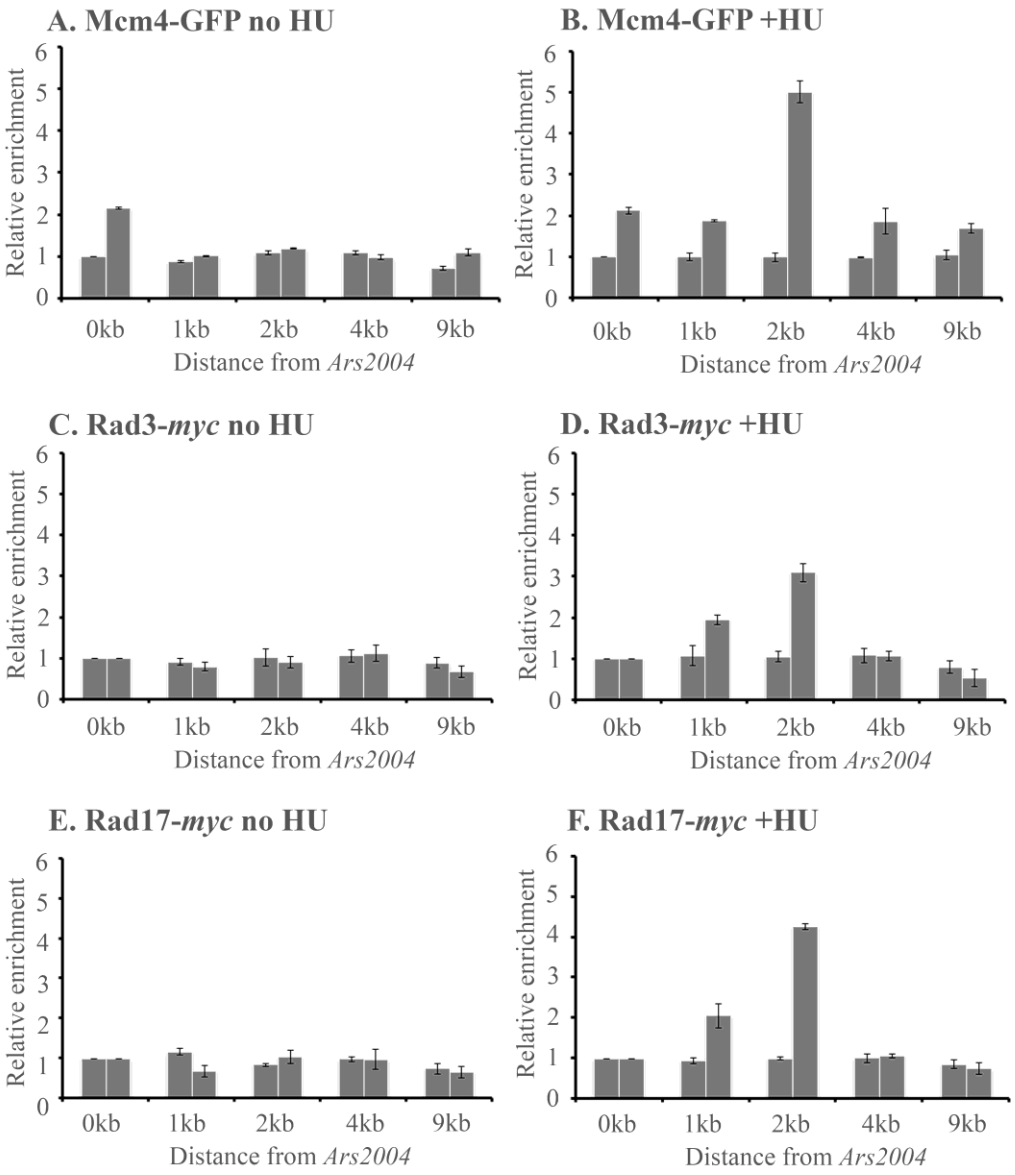

A. Mcm4-GFP no HU
B. Mcm4-GFP +HU
C. Rad3-myc no HU
D. Rad3-myc +HU
E. Rad17-myc no HU
F. Rad17-myc +HU
Relative enrichment
0
1
2
3
4
5
6
0kb
1kb
2kb
4kb
9kb
Distance from Ars2004

Figure 6

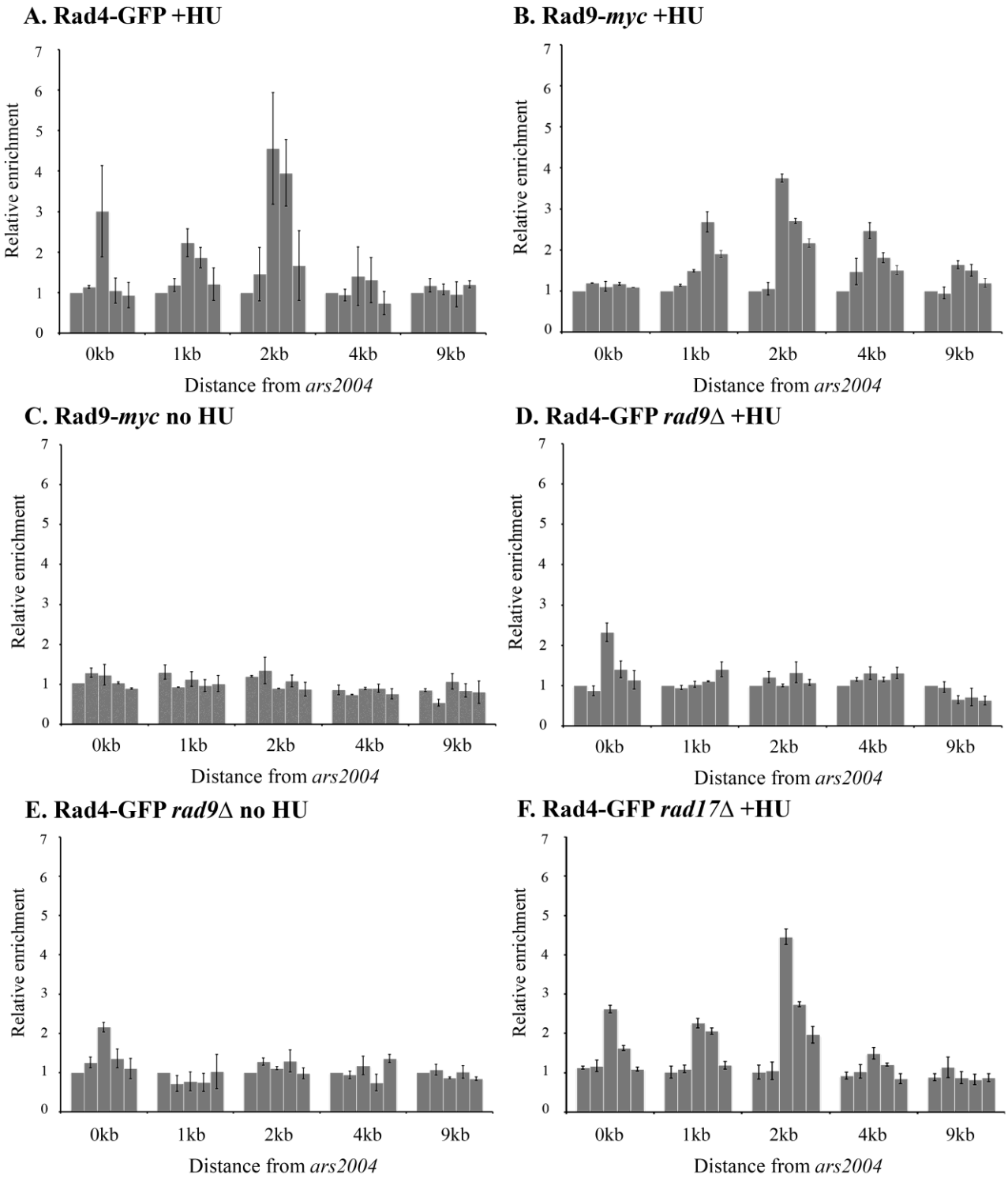
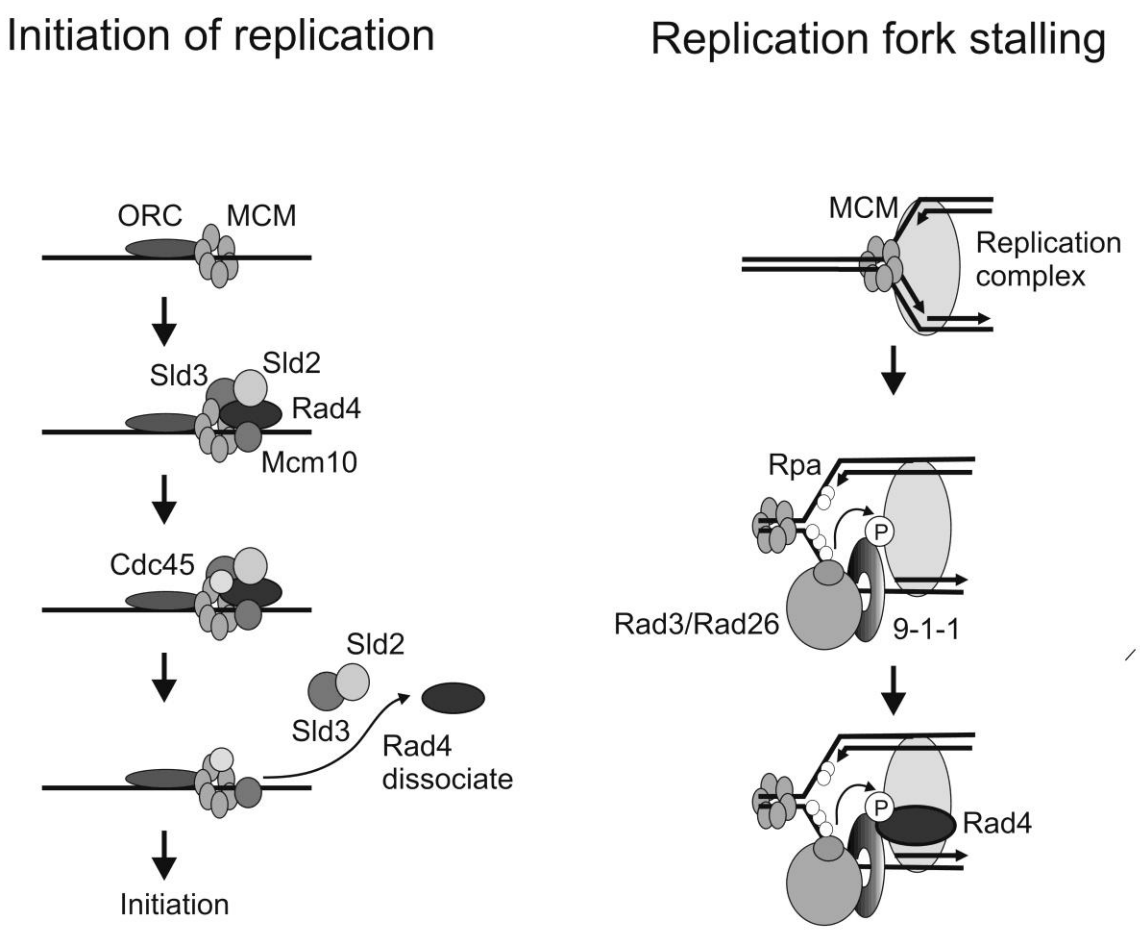
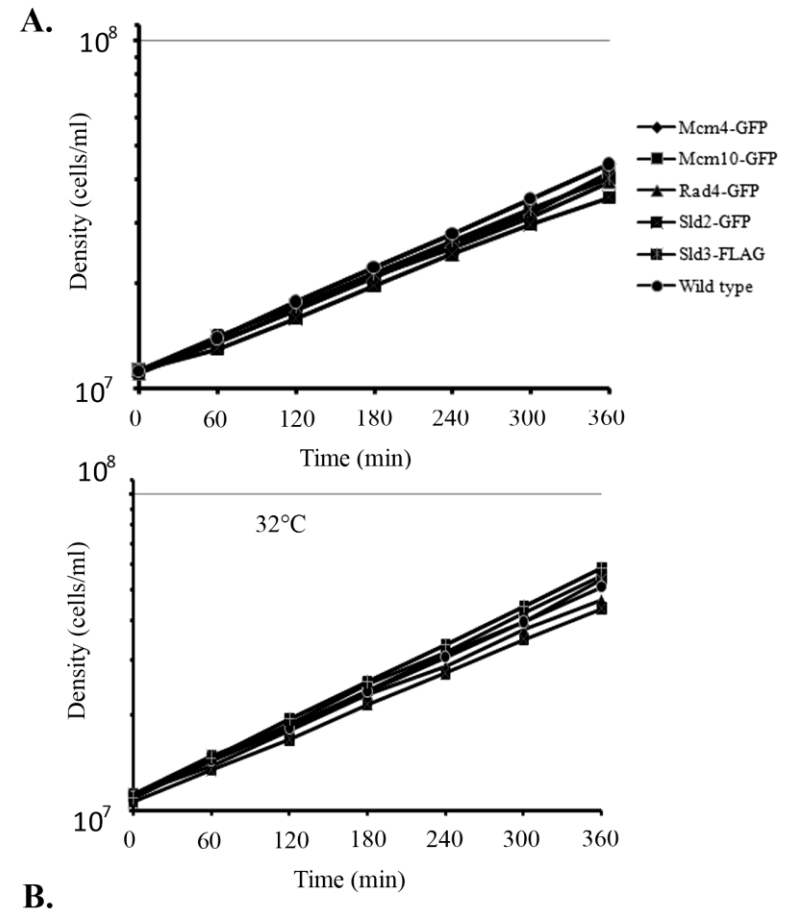

A. Rad4-GFP +HU
B. Rad9-*myc* +HU
C. Rad9-*myc* no HU
D. Rad4-GFP *rad9*Δ +HU
E. Rad4-GFP *rad9*Δ no HU
F. Rad4-GFP *rad17*Δ +HU
Relative enrichment
0
1
2
3
4
5
6
7
0kb
1kb
2kb
4kb
9kb
Distance from *ars2004*

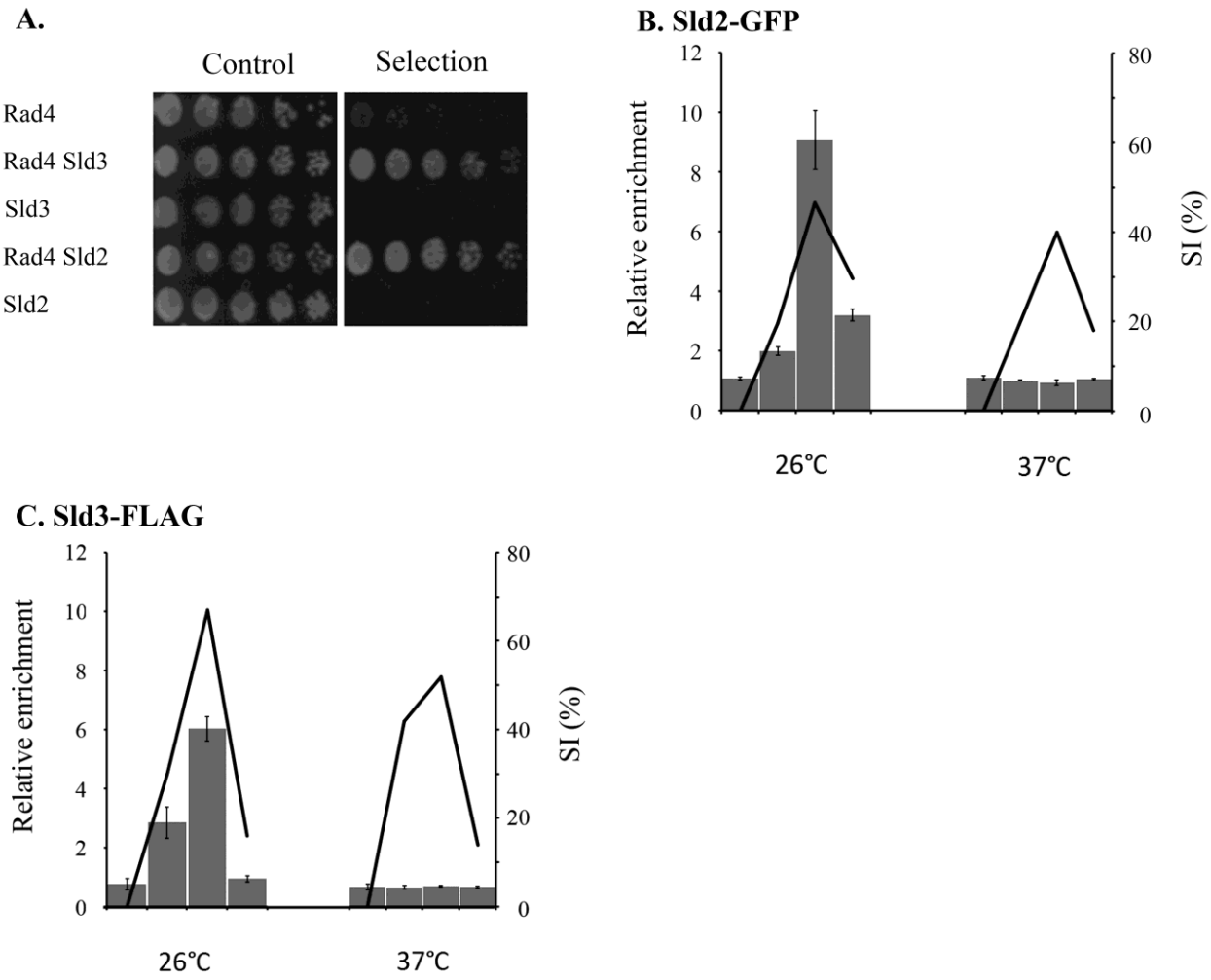
Figure 7


Initiation of replication
ORC
MCM
Sld3
Sld2
Rad4
Mcm10
Cdc45
Sld2
Sld3
Rad4
dissociate
Initiation
Replication fork stalling
MCM
Replication
complex
Rpa
P
Rad3/Rad26
9-1-1
P
Rad4

Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3