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Eukaryotic Replisome Components Cooperate to Process Histones During Chromosome Replication

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SUMMARY

DNA unwinding at eukaryotic replication forks displaces parental histones, which must be redeposited onto nascent DNA in order to preserve chromatin structure. By screening systematically for replisome components that pick up histones released from chromatin into a yeast cell extract, we found that the Mcm2 helicase subunit binds histones cooperatively with the FACT (facilitiates chromatin transcription) complex, which helps to re-establish chromatin during transcription. FACT does not associate with the Mcm2-7 helicase at replication origins during G1 phase but is subsequently incorporated into the replisome progression complex independently of histone binding and uniquely among histone chaperones. The amino terminal tail of Mcm2 binds histones via a conserved motif that is dispensable for DNA synthesis per se but helps preserve subtelomeric chromatin, retain the 2 micron minichromosome, and support growth in the absence of Ctf18-RFC. Our data indicate that the eukaryotic replication and transcription machineries use analogous assemblies of multiple chaperones to preserve chromatin integrity.

INTRODUCTION

The transcription and replication of eukaryotic chromosomes both involve complex molecular machines that must traverse chromatin rapidly without causing its permanent disruption. The packaging of chromosomal DNA into nucleosomes is a critical determinant of gene expression (Yuan et al., 2005), and parental histones must be redeposited immediately behind RNA polymerase in order to preserve nucleosome density along with the epigenetic pattern of histone modifications across each chromosome. During chromosome replication, the replisome at eukaryotic DNA replication forks faces a similar challenge (Alabert and Groth, 2012).

It now seems clear that multiple evolutionarily conserved factors associate specifically with the elongating form of RNA polymerase II (Mayer et al., 2010) and play a key role in preserving nucleosome density during transcription. Work with budding yeast showed that mutating the components of the Spt4-Spt5 complex (Swanson et al., 1991), the FACT (facilitates chromatin transcription) complex comprising Spt16 and Pob3 (Malone et al., 1991; Rowley et al., 1991), or the Spt6 protein (Clark-Adams and Winston, 1987; Kaplan et al., 2003) leads to the initiation of short transcripts from cryptic promoters that are present within about 15% of yeast genes (Cheung et al., 2008) but are normally repressed by nucleosome occupancy.

Spt6 can deposit histones H3 and H4 onto DNA (Bortvin and Winston, 1996; Jamai et al., 2009), and FACT is a histone chaperone that binds nucleosomes as well as free histones (Belotserkovskaya et al., 2003; Orphanides et al., 1999). FACT associates preferentially with histones H2A and H2B in solution (Belotserkovskaya et al., 2003; Winkler et al., 2011), but it can also associate with H3-H4 complexes (Stuwe et al., 2008). Budding yeast genetics highlighted the importance of FACT's interaction with histones H3-H4 (Duina et al., 2007; Formosa et al., 2002; Myers et al., 2011), and FACT was shown to be critical for the redeposition of parental H3-H4 tetramers behind RNA polymerase during transcription (Jamai et al., 2009), as was Spt6 (Kaplan et al., 2003).

Chromosome replication causes much more disruption to chromatin than transcription does, because the DNA duplex is unwound to a much greater extent, but nucleosomes are still restored extremely quickly behind DNA polymerases within a couple of hundred nucleotides of the junction of the fork (McKnight and Miller, 1977; Smith and Whitehouse, 2012; Sogo et al., 1986). This involves both the transfer of parental histones and the deposition of new histones, which can then be modified according to their neighbors (Alabert and Groth, 2012).

The assembly of new nucleosomes at replication forks is understood relatively well and involves the concerted action of histone chaperones such as Asf1 and CAF1, which bind specifically to dimers of H3 and H4 and promote their deposition on DNA, leading first to the assembly of H3-H4 tetramers and then to histone octamers after the recruitment of H2A-H2B (Alabert and Groth, 2012). Previous work suggested that Asf1 might

also transfer parental histones at replication forks, given that, in extracts of human cells, Asf1 was found to form histone-dependent complexes with the Mcm2-7 proteins (Groth et al., 2007), form the catalytic core of the essential DNA helicase at eukaryotic forks. However, these complexes lack Cdc45 and the GINS (go ichi ni san) complex, which associate stably with Mcm2-7 during the initiation of chromosome replication to form the active Cdc45-MCM-GINS helicase complex and thus are unlikely to be derived from replication forks. Moreover, it seems clear that the H3-H4 tetramer is transferred as an intact unit at eukaryotic DNA replication forks (Katan-Khaykovich and Struhl, 2011; Prior et al., 1980; Vestner et al., 2000; Yamasu and Senshu, 1990), whereas Asf1 can only bind to H3-H4 dimers (English et al., 2006; Natsume et al., 2007). This indicates that other factors at replication forks must be able to bind to parental H3-H4 tetramers, which are released when the Cdc45-MCM-GINS helicase unwinds the parental DNA duplex.

In addition to its known role during transcription, a possible role for FACT at replication forks was suggested by its ability to bind to DNA polymerase alpha, which initiates each Okazaki fragment during lagging-strand DNA synthesis, in vitro (Wittmeyer and Formosa, 1997). Moreover, FACT was identified as part of the replisome progression complex that assembles around the Cdc45-MCM-GINS DNA helicase at replication forks (Gambus et al., 2006). These findings suggested that FACT might collaborate with other replisome components to process histones at forks, analogous to the combined action of FACT, Spt6, and Spt5-Spt4 during transcription. Here, we identify the yeast Mcm2 helicase subunit as the first such factor. We show that Mcm2 orthologs contain a conserved motif in their amino terminal tail that binds histones together with FACT and contributes to the efficacy of chromatin replication in vivo. Importantly, FACT is incorporated into the replisome independently of histone binding and uniquely among histone chaperones. These findings suggest that there is a fundamental similarity in the mechanisms by which the transcription and replication machineries preserve chromatin integrity in eukaryotic cells.

RESULTS

A Systematic Screen for Replisome Factors that Bind Histones Released from Chromatin

The eukaryotic replisome assembles at nascent DNA replication forks by association of the Cdc45-MCM-GINS helicase complex with a set of regulatory factors to form the replisome progression complex, which then associates with the enzymes responsible for synthesizing the leading and lagging strands, namely DNA polymerases epsilon and alpha (De Piccoli et al., 2012; Gambus et al., 2009; Yao and O'Donnell, 2010).

To screen systematically for replisome components that are able to bind to histone complexes released from chromatin, we generated a series of yeast strains in which the genes encoding a representative member of each replisome subcomplex were modified to introduce nine copies of the c-MYC epitope (in each case, we confirmed that the tagged proteins were functional; see Extended Experimental Procedures). Then, cell extracts were generated in the presence or absence of Benzonase nuclease, which degraded chromosomal DNA and released histone complexes into the extract, before the immunoprecipitation of the tagged replisome component. Because the various replisome components are all associated with each other at forks and, thus, in extracts of S phase cells, we initially had to synchronize cells outside of S phase for the purpose of the screen in order to distinguish the contribution of each individual replisome component to histone-binding activity (all the results were confirmed subsequently with extracts of S phase cells).

First, we examined the FACT complex and found that the Pob3 subunit copurified specifically with Spt16-9MYC regardless of whether chromatin had been digested in the extract (Figure 1A). In contrast, chromatin digestion greatly stimulated the specific association of Spt16 with all four histones, whereas Spt16 only associated with a very small amount of H2A and H2B in the absence of chromatin digestion, reflecting the very low level of free histones in yeast extracts.

Strikingly, immunoblotting for other replisome subunits showed that the Mcm2 component of the replicative DNA helicase also copurified with Spt16-9MYC (Figure 1A). This occurred only after chromatin digestion, suggesting that the interaction was dependent upon the release of histones into the cell extract. Consistent with this view, Mcm2-9MYC copurified with FACT and all four histones in analogous experiments, but only upon the release of histone complexes by chromatin digestion (Figure 1B). To show that Mcm2 and FACT have the ability to pick up histone complexes that have been released from chromatin, we grew "donor" cultures of yeast cells expressing tagged histone H3 in parallel with "recipient" cultures expressing Mcm2-TAP, Spt16-TAP, or neither tagged protein. After cultures were mixed as shown in Figure 1C, chromatin was digested in the resultant cell extracts before the immunoprecipitation of TAP-tagged proteins with IgG beads. Both Spt16-TAP and Mcm2-TAP were specifically able to pick up tagged histone H3 that had been released into the extract in vitro from donor chromatin. To confirm that FACT and Mcm2 were associating with parental histones released from chromatin in our assay, we showed that the released histones lacked acetylation of H3-K56, a marker of newly synthesized histones in budding yeast (Figure 1D; we confirmed the specificity of the antibody to H3-K56Ac using *hst3Δ hst4Δ* cells, in which almost all histone H3 in chromatin is acetylated on lysine 56).

Altogether, these experiments indicate that Mcm2 and FACT are able to bind together to parental histone complexes that have been released from chromatin. Additional experiments showed that this activity is not shared with the other Mcm2-7 proteins or with other subunits of the replisome progression complex (Figures S1A–S1C), though DNA polymerases alpha and epsilon copurified with a small amount of FACT and histones after chromatin digestion (less efficiently than Mcm2; see Figure S1D). Overall, these data indicate that the replisome contains multiple factors that are able to pick up histones released from chromatin, and FACT and Mcm2 are the most important of such activities. Thus, the situation is analogous to the RNA polymerase machinery, which contains multiple chaperones that are able to pick up histones released from chromatin. In contrast, the factors that chaperone histones en route to chromatin assembly, such as Asf1, CAF1, and HIR1, bind to dimers of histones H3 and H4 (English et al., 2006; Natsume

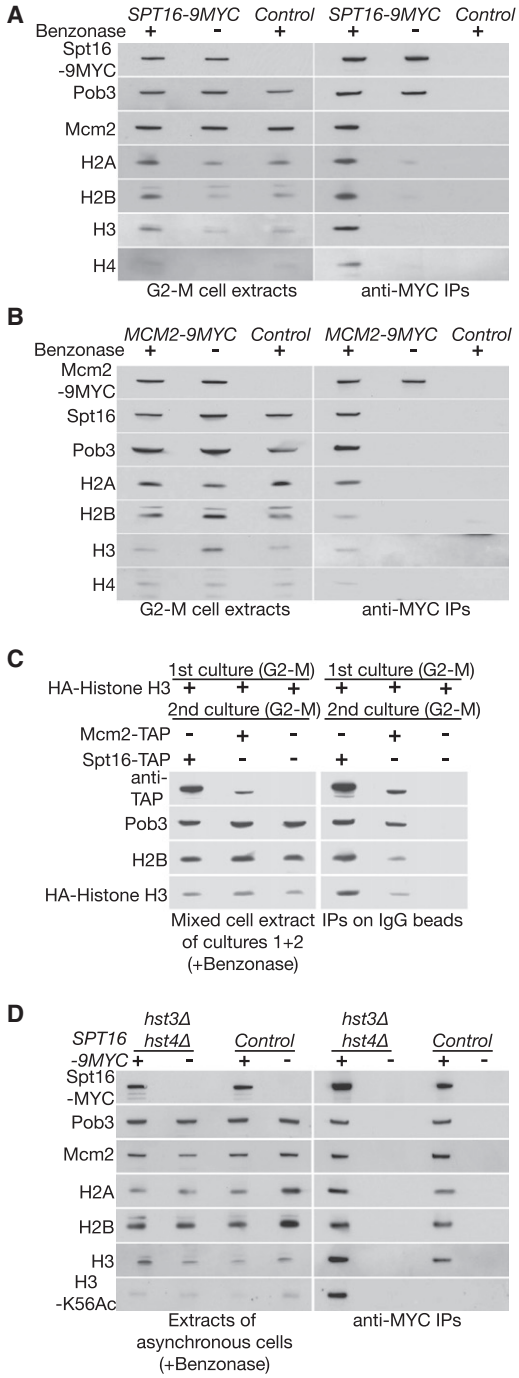

Figure 1. Mcm2 and FACT Bind Together to Histone Complexes Released from Chromatin

(A) *SPT16-9MYC* (YMP177) and control cells (YSS3) were grown at 30°C and arrested in G2–M phase by adding nocodazole to the medium. Extracts were incubated with or without Benzonase nuclease, as shown before immunoprecipitation with anti-MYC beads and detection of the indicated proteins by immunoblotting.

(B) An equivalent experiment was performed with *MCM2-9MYC* (YMP154-1) or control cells.

(C) Parallel cultures of cells expressing the tagged histone H3 (HA-Hht2; YMP442), Mcm2-TAP (YMP347-7), or Spt16-TAP (YMP419-1) were grown in parallel at 30°C and arrested in G2–M phase, as above. Then, cultures were mixed as indicated before the generation of cell extracts, treatment with Benzonase, and immunoprecipitation on IgG beads.

(D) Asynchronous cultures of the indicated strains were processed as above. See also Figure S1.

et al., 2007; Tagami et al., 2004) and were not able to pick up histone complexes released from chromatin in our assay (Figures S1E–S1G).

The Amino Terminal Tail of Mcm2 Is Conserved and Mediates Complex Formation with Histones and FACT

Earlier studies showed that human Mcm2-7 proteins could interact with histones in vitro (Ishimi et al., 1996), and work with the mouse orthologs then indicated that a small region in the amino terminus of the Mcm2 subunit mediated this interaction by binding to the globular domain of histone H3 (Ishimi et al., 1998). The histone-binding region of mouse Mcm2 is located within a long and flexible tail that is present at the amino terminus of all eukaryotic Mcm2 orthologs, analogous to the tails of Mcm4 and Mcm6. All three tails contain multiple phosphorylation sites for kinases, such as Cdc7, that drive the initiation of chromosome replication (Sheu and Stillman, 2006, 2010). The tails of Mcm4 and Mcm6 are very rich in Serine because of the abundance of phosphorylation sites but otherwise are not conserved (e.g., Figure S4A). In contrast, the Mcm2 tail has a highly acidic character (30% aspartate and glutamate), and BLAST searches with the tail of yeast or human Mcm2 revealed significant, though limited, homology with the corresponding tails of all eukaryotic Mcm2 orthologs (Figures 2A, 2B, and S4B) but not with other proteins. Notably, the region of highest conservation corresponds to the minimal segment of mouse Mcm2 that was found to bind histone H3 (Figure 2A, dotted red line).

To test whether the amino terminal tail mediates the interaction of Mcm2 with histones and FACT, we expressed the first 200 amino acids of yeast Mcm2 as a fusion to protein A, and we also made an analogous fusion involving the first 186 amino acids of yeast Mcm4 as a control. After digesting chromatin in cell extracts, the tail of yeast Mcm2 formed a complex with FACT and histones as seen for full-length Mcm2, but the tail of Mcm4 lacked this activity (Figure 2B). Importantly, we found that the tail of human Mcm2 containing the H3 binding site behaved just like yeast Mcm2 in an equivalent experiment (Figure 2B; Hs Mcm2 1–188) despite the very limited identity between the primary sequence of the Mcm2 tails, probably reflecting the fact that histone H3 is 85% identical between the two species. As predicted, the amino terminal tail of yeast Mcm2 was essential for the remainder of the protein to interact with histones and FACT (Figure 2C). Overall, these findings indicate that the Mcm2 tail contains a conserved histone-binding activity that is able to pick up histones released from chromatin in a cooperative manner with FACT. The Mcm2-histone-FACT complexes are resistant to the DNA intercalating agent ethidium bromide as well as to repeated treatment with a large excess of DNase (Figure 2D).

The Mcm2 histone-FACT complexes were stable up to 700 mM salt and lacked other stoichiometric components (Figures 2E and 2F; note that a small amount of the Dbf4

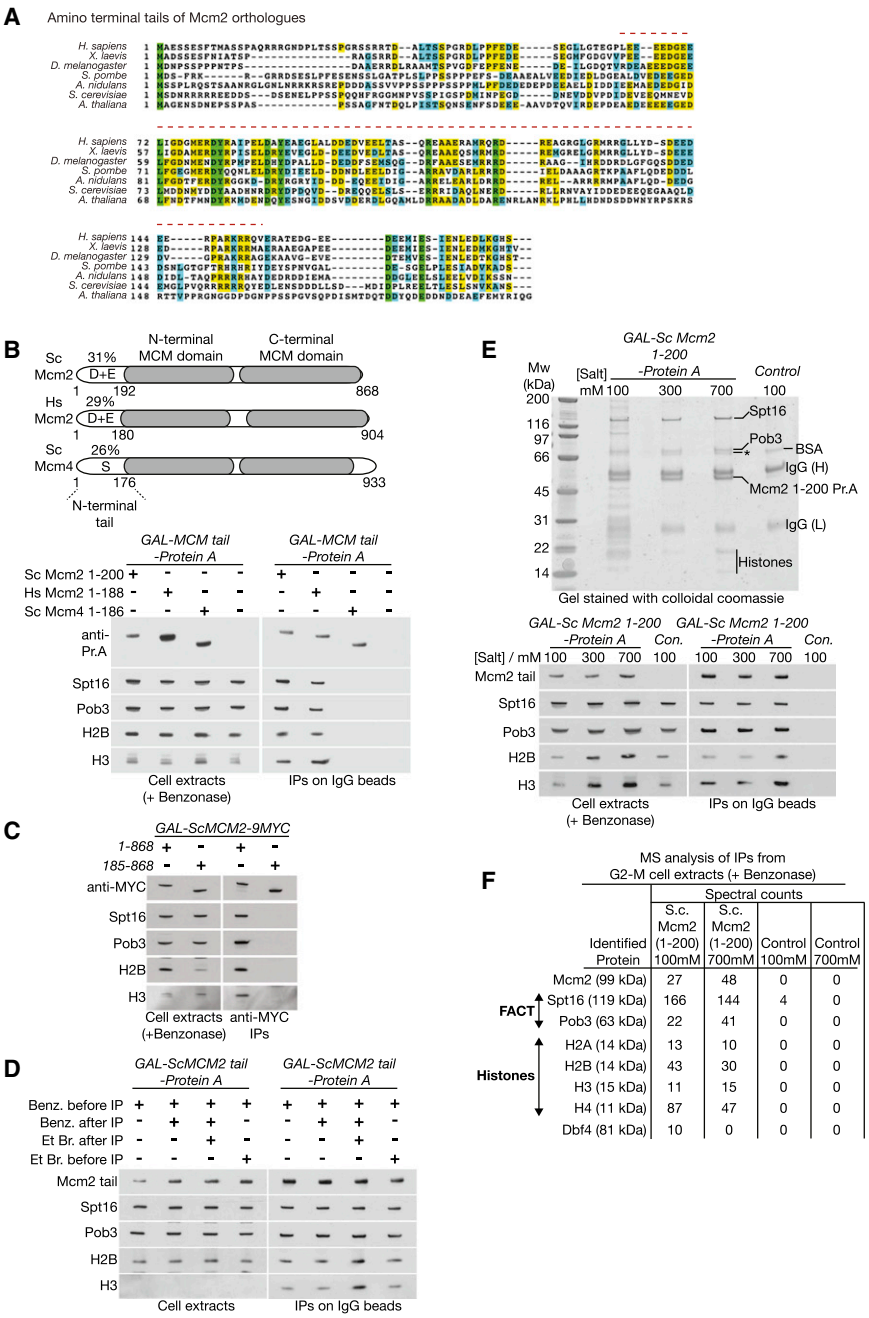

Figure 2. The Amino Terminal Tail of Eukaryotic Mcm2 Contains a Conserved Motif that Binds Histones Together with FACT

(A) Alignment of the amino terminal tails of Mcm2 from the indicated species, generated with the use of ClustalW and BOXSHADE software. The dotted line indicates the minimal region in human Mcm2 that binds to histone H3.

(B) The tails of yeast Mcm2 (ScMcm2 1–200; YMP287), human Mcm2 (HsMcm2 1–188; YMP296), and yeast Mcm4 (ScMcm4 1–186; YMP289) were expressed from the *GAL* promoter. Cells were grown in parallel to a control strain (YSS3) and treated as in Figure 1. Cell extracts were treated with Benzonase before immunoprecipitation on IgG beads.

(C) The amino-terminal tail of Mcm2 mediates association with histones and FACT.

(D) Extracts of cells expressing *GAL ScMCM2 1–200* (YMP287) were treated with Benzonase and Ethidium bromide as indicated before and after immunoprecipitation on IgG beads.

(E) *GAL ScMCM2 1–200* (YMP287) and control cells (YSS3) were treated as in Figure 1C, and cell extracts were then adjusted to 100 mM, 300 mM, or 700 mM potassium acetate before treatment with Benzonase and immunoprecipitation on IgG beads. Samples were separated by SDS-PAGE and gels were stained with colloidal Coomassie blue (upper panel) or used for immunoblotting (lower panels).

(F) Summary of mass spectrometry data for the samples in (E).

See also Figure S2.

partner of Cdc7 kinase copurified with the Mcm2 tail at 100 mM salt). Thus, it appears that FACT has a unique ability to join Mcm2 in binding to histone complexes that have been released from chromatin into yeast cell extracts. The resistance of the complexes to high salt levels indicates that they do not simply involve electrostatic interactions between positively charged histones and negatively charged regions of the Mcm2 tail and FACT but also involve specific hydrophobic interactions, as observed previously for the interaction of the Asf1 chaperone with histones H3-H4 (Bowman et al., 2011; Ishikawa et al., 2011).

Along with previous work, these data suggested that FACT might participate in mutually exclusive assemblies that involve either replication or transcription factors as well as histones. We confirmed this by isolating *SPT16-9MYC* in analogous experiments after chromatin was digested in cell extracts (Figure S2A). Mass spectrometry analysis showed that, in addition to histones and Mcm2, Spt16-9MYC copurified in a specific fashion with other components of the transcription elongation machinery, including Spt5, RNA polymerase II, Spt6, Elf1, and the Paf1 complex, along with a small amount of casein kinase II (Figure S2A). The association of FACT with the Paf1 complex and casein kinase II was observed even in the absence of chromatin digestion (Figure S2B), but complex formation with Spt5, RNA polymerase II, Spt6, and Elf1 was seen only after the release of histones into the extract (Figures S2B–S2C) and could be reproduced in vitro by mixing donor and recipient cultures as described above (Figure S2D). These data indicate that FACT can cooperate with specific components of the transcription or replication machineries to bind histones that have been released from chromatin.

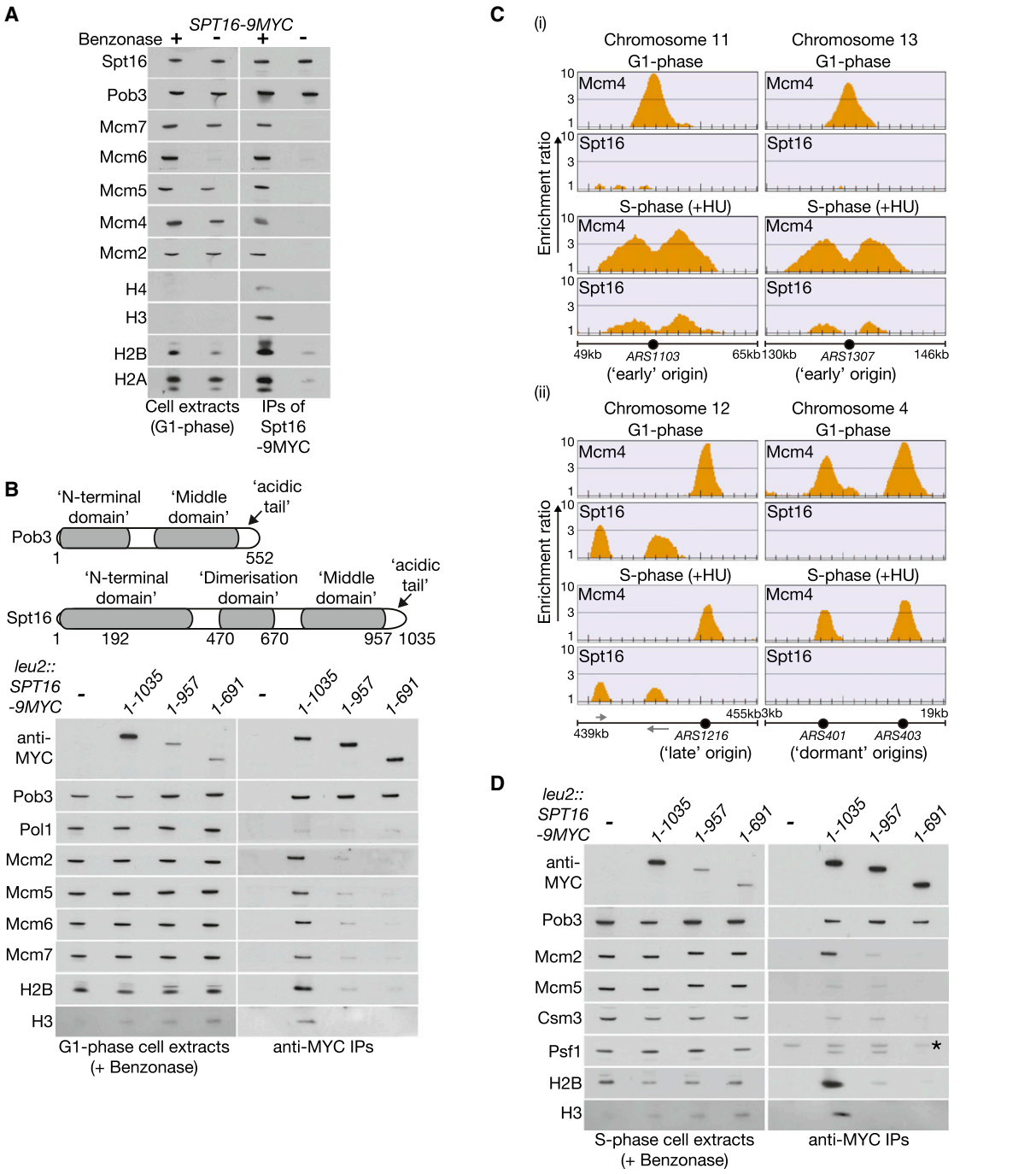

Figure 3. FACT Is Specifically Recruited to the Replisome and Does Not Associate with the Mcm2-7 Helicase at Replication Origins before Initiation

(A) *SPT16-9MYC* cells (YAG436-3) were grown at 30°C and synchronized in G1 phase by the addition of a mating pheromone. Cell extracts were incubated with or without Benzonase (digestion of chromosomal DNA liberated histones and loaded Mcm2-7 helicase from chromatin) before immunoprecipitation of Spt16-9MYC.

(B) Cells expressing the indicated 9MYC-tagged versions of *SPT16*, present as a second copy at the *leu2* locus, were synchronized in G1 phase at 30°C. Extracts were treated with Benzonase before immunoprecipitation on anti-MYC beads.

(C) ChIP-seq analysis of Mcm4 and Spt16 at origins and replication forks. *MCM4-5FLAG SPT16-9MYC* (YCE27) was arrested in G1 phase at 24°C and released into S phase for 60 min in the presence of 0.2 M hydroxyurea. After crosslinking with formaldehyde, Mcm4-5FLAG or Spt16-9MYC were isolated from sonicated cell extracts and the associated DNA was analyzed by massive DNA sequencing. The figures show the "enrichment ratio" of the DNA sequence reads at each genomic location, comparing the immunoprecipitates with extract samples, calculated as described in Experimental Procedures. Examples are shown for two early origins (i) and three late origins (ii). The indicated region of chromosome 12 also illustrates that Spt16 peaks at two active genes (denoted by arrows).

(legend continued on next page)

FACT Is Recruited Specifically to the Replisome but Does Not Associate with the Inactive Mcm2-7 Helicase at Replication Origins

Previously, it was thought that FACT is incorporated into the replisome by direct interaction with the Mcm2-7 helicase core, given that human FACT was found to copurify with Mcm2-7 proteins (Tan et al., 2006) and yeast FACT copurified with the Mcm2-7 complex not just during S phase but even in extracts of G1 phase yeast cells (Gambus et al., 2006). However, we have not been able to detect a direct association of FACT with Mcm2-7 using recombinant proteins expressed in *E. coli* (M.F. and K.L., unpublished data), and the association of FACT with Mcm2-7 in extracts of G1 phase cells is completely dependent upon chromatin digestion (Figure 3A). This could either mean that FACT associates specifically with the loaded double hexamer of Mcm2-7 at replication origins (Evrin et al., 2009; Remus et al., 2009), but not with free Mcm2-7 proteins, or reflect the ability of Mcm2 and FACT to bind together to histone complexes released from chromatin, either in vivo or in vitro, without involving a direct interaction between FACT and Mcm2-7.

To determine whether histone binding by FACT is important for its association with Mcm2-7 in extracts of G1 phase cells, we removed the short acidic tail from the carboxyl terminus of Spt16, which is crucial for FACT to bind nucleosomes or free histones in vitro (Belotserkovskaya et al., 2003; Winkler et al., 2011). In addition to the endogenous *SPT16* gene, 9MYC-tagged versions of full-length Spt16 (1–1035), Spt16 lacking the acidic tail (1–957), or Spt16 lacking both the tail and the preceding "middle domain" (1–691) were expressed as a second copy at the *leu2* locus (Figure 3B). After digesting chromatin in extracts of G1 phase cells, full-length Spt16 copurified with Pob3, histones, and the Pol1 catalytic subunit of DNA polymerase alpha, as well as with the Mcm2-7 proteins (Figure 3B; Spt16 1–1035). In contrast, the truncated versions of Spt16 interacted equally as efficiently as full-length Spt16 with Pob3 and Pol1, but very inefficiently with histones and Mcm2-7 (Figure 3B). Along with the data described above, these findings indicate that FACT and Mcm2 bind to histone complexes released from chromatin without requiring a direct interaction of Mcm2-7 with FACT. This is consistent with the fact that the histone H3-binding tail of human Mcm2 is just as efficient as the very distantly related tail of yeast Mcm2 at forming ternary complexes with yeast FACT and histones, (Figure 2B).

To analyze systematically the interaction of FACT with the chromosome replication machinery across the entire yeast genome, we performed ChIP-seq analysis (chromatin immunoprecipitation with massive DNA sequencing) of cells expressing both Mcm4-5FLAG and Spt16-9MYC. In cells synchronized in G1 phase, Mcm4-5FLAG was detected at all replication origins, as seen previously (De Piccoli et al., 2012). Strikingly, however, Spt16 was absent from origins (Figure 3C; n = 352 origins with Mcm4 peaks that lacked Spt16; sites that were contiguous with active genes were not counted) and, instead, was detected only at actively expressed genes (e.g., Figure 3C (ii)), including genes that are induced during the G1 phase of the cell cycle (Figure S3A). Then, we released cells into S phase in the presence of 0.2 M hydroxyurea so that replication forks moved slowly away from early origins, but the S phase checkpoint response blocked the activation of later origins (Santocanale and Diffley, 1998; Shirahige et al., 1998). Spt16 was again detected at active genes, including those induced by DNA replication defects (Figure S3B). Importantly, however, Spt16 also colocalized with Mcm4 at 98% of active replication forks (Figures 3C (i) and S3C (i); n = 98; sites that were contiguous with active genes were not counted). In contrast, Spt16 did not colocalize in the same samples with Mcm4 at inactive late origins (Figures 3C (ii) and S3C (ii)). These data indicate that FACT is not recruited to the loaded Mcm2-7 helicase at replication origins before initiation but is subsequently present at DNA replication forks.

Consistent with these findings, we found that Spt16 1–957 copurified with the Cdc45-MCM-GINS helicase from S phase cell extracts just as efficiently as full-length Spt16 (Figure 3D) despite not being able to form ternary complexes with histones and Mcm2. The Spt16 1–691 allele is similarly defective to Spt16 1–957 in histone binding but did not copurify with replisome material, indicating that the middle domain of Spt16 is required for replisome association. Spt16 1–691 still associates with DNA polymerase alpha (Figure 3B), indicating that FACT has at least one additional mode of interaction with the replisome, probably via some component(s) of the replisome progression complex. Along with the above data, these findings indicate that FACT has a unique ability to interact with the replisome independently of histone binding, which is in contrast to other chaperones, such as Asf1.

The Histone-Binding Activity of Mcm2 Is Mediated by a Conserved Motif that Is Important for the Efficacious Replication of Chromatin

Secondary structure analysis of the amino termini of multiple eukaryotic Mcm2 orthologs indicated the presence within the otherwise flexible tail of a single alpha helix (Figures 4A and S4B) with conserved residues that were found to be important for the association of the yeast Mcm2 tail with FACT and histones (Figure 4A; 5A and 7A alleles). Shortly upstream of the predicted alpha helix, charged amino acids flank two tyrosine residues that are absolutely conserved in all species (Figures 4A and S4B). These represent attractive candidates for the sites of hydrophobic interactions between Mcm2 and histones. All these features are contained within the minimal segment of mouse Mcm2 that was found to bind to histone H3 (Figure S4B). Importantly, mutation of the two conserved tyrosines in the tail of budding yeast Mcm2, along with a third tyrosine just upstream, abolished all detectable association of the isolated Mcm2 tail with histones and FACT without altering the

See also Figure S3.

(D) The same strains as in (B) were released from G1 phase into S phase for 20 min at 30°C. Extracts were treated with Benzonase before immunoprecipitation on anti-MYC beads.

See also Figure S3.

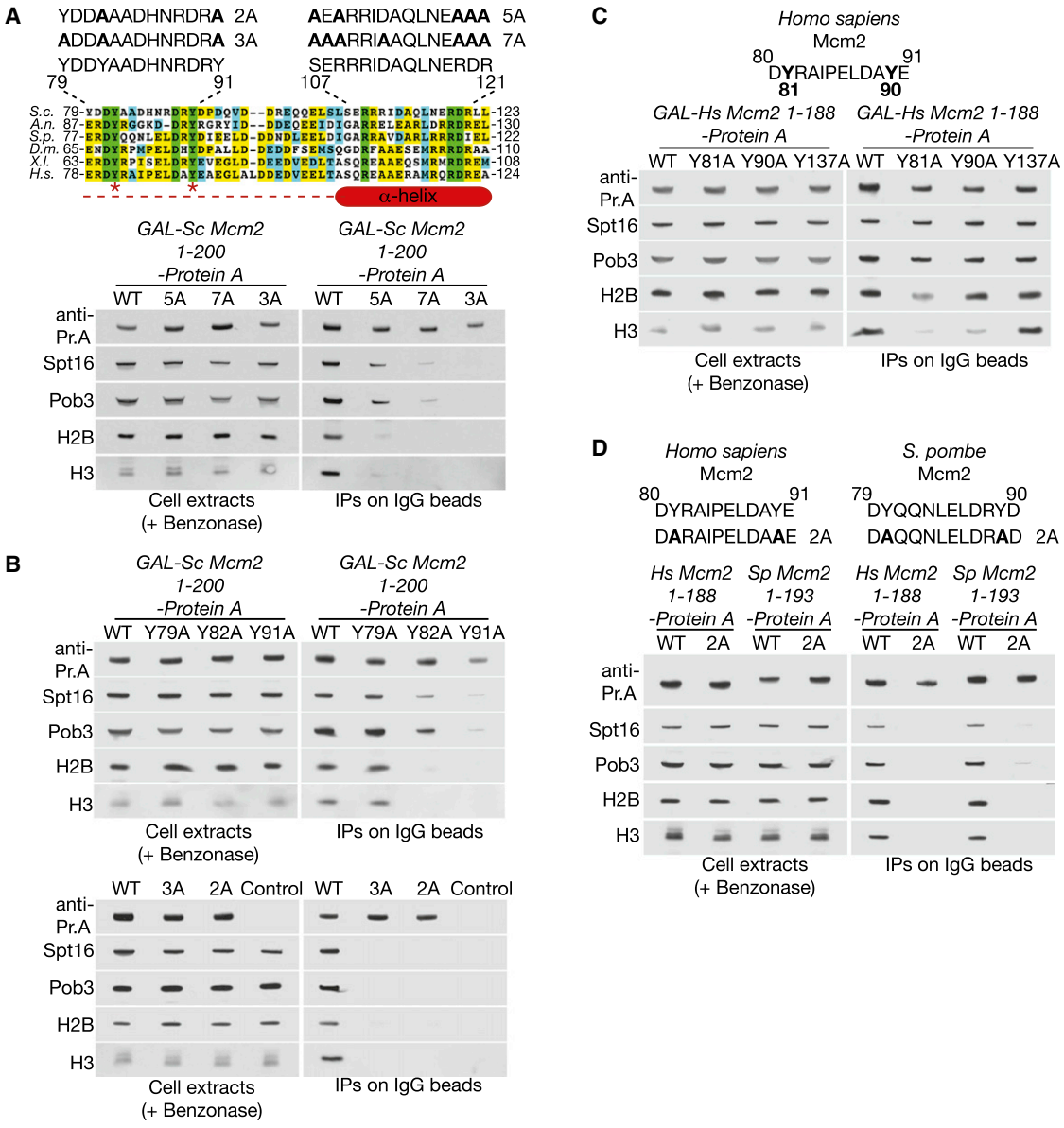

Figure 4. Conserved Tyrosines in the Amino Terminal Tail of Mcm2 Are Essential for Binding to Histones Together with FACT

(A) Conserved features of the amino terminal tail of Mcm2, as in Figure S4B. The sites of mutations within the predicted alpha helix (5A and 7A) or conserved tyrosines (2A and 3A) are indicated. Cells expressing wild-type or mutated versions of *GAL ScMCM2 1-200* were grown as in Figure 2. Cell extracts were treated with Benzonase before immunoprecipitation on IgG beads.

(B) Analogous experiments showed that the two invariant tyrosines both contribute to the binding of the ScMcm2 tail to histones with FACT.

(C–D) Similar experiments indicated that the two conserved tyrosines are also essential for the tail of human or fission yeast Mcm2 to bind histones released from chromatin together with FACT.

See also Figure S4.

negatively charged character of the tail or the predicted secondary structure element (Figure 4A; compare WT and 3A alleles). Each of the two conserved tyrosines contributes to the affinity of the Mcm2 tail for histones (Figure 4B, upper panels), and simultaneous mutation of the two conserved tyrosines causes a similar defect to the mutation of all three tyrosines (Figure 4B, lower panels). These data suggested that the amino terminal tail of eukaryotic Mcm2 proteins contains a conserved histone-binding motif, within which the two conserved tyrosines play a critical role.

Consistent with this view, the individual mutation of the two conserved tyrosines in the tail of human Mcm2 again caused a partial defect in histone binding (Figure 4C), whereas simultaneous mutation of both tyrosines abolished association with histones and FACT, as predicted (Figure 4D). Correspondingly, in similar experiments, we showed that the tail of fission yeast

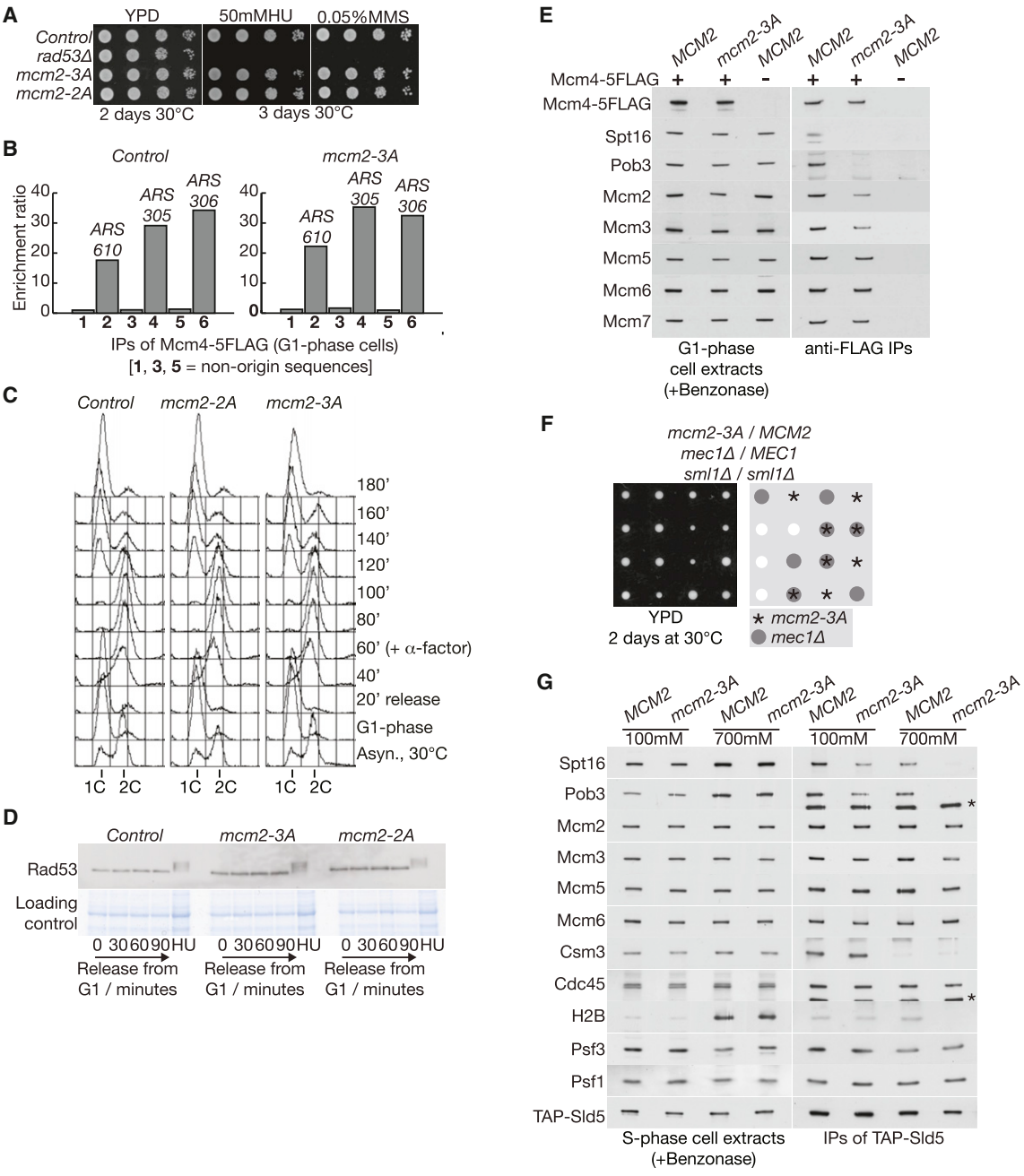

Figure 5. Mutation of the Histone-Binding Motif of Mcm2 Does Not Perturb DNA Synthesis, but Causes a Partial Defect in the Histone-Binding Activity of the Replisome

(A) Shown are 10-fold serial dilutions of the indicated strains from 50,000 cells to 50 cells placed on the indicated media and grown for 2 to 3 days at 30°C before imaging.

(B) Chromatin immunoprecipitation of Mcm4-5FLAG in G1 phase at the loci described in Figure S7. See Extended Experimental Procedures for further details.

(C) Cell-cycle progression of the indicated strains was monitored by flow cytometry after release from G1 arrest. A mating pheromone was added again 60 min after release from G1 arrest so that completion of cell division could be monitored by the disappearance of cells with a 2C DNA content and the re-appearance of 1C cells.

(D) In a similar experiment to that in (C), cell extracts were generated and used to monitor the activation of the Rad53 checkpoint kinase by immunoblotting (the activated form of Rad53 upon replication stress becomes hyperphosphorylated). As a control for activation of the S phase checkpoint, each strain was also released from G1 arrest in the presence of 0.2 M hydroxyurea (HU).

(E) Control (YSS3), *MCM4-5FLAG* (YSS170), and *mcm2-3A MCM4-5FLAG* (YMP586) were synchronized in G1 phase. Extracts were treated with Benzonase before immunoprecipitation with anti-FLAG beads.

(F) Tetrad analysis of the meiotic progeny of the indicated diploid strain.

(legend continued on next page)

Mcm2 was also able to pick up histones released from chromatin, along with budding yeast FACT, and the mutation of the two conserved tyrosines again abolished these interactions (Figure 4D). Overall, these data indicate that the amino terminal tail of eukaryotic Mcm2 proteins contains a histone-binding motif that has been conserved during the course of evolution.

To explore the biological significance of the histone-binding activity of Mcm2, we generated budding yeast strains in which the *MCM2* locus was converted into the *mcm2-2A* or *mcm2-3A* alleles (see Extended Experimental Procedures). Both *mcm2-2A* and *mcm2-3A* were viable and were not sensitive to exogenous sources of DNA replication stress (Figures 5A and S5A). Moreover, the Mcm2-7 helicase was loaded normally at origins of DNA replication in *mcm2-3A* (Figure 5B) and the kinetics of DNA replication were equivalent to control cells (Figure 5C) without any apparent activation of the S phase checkpoint response (Figure 5D). Despite the mutated Mcm2 proteins not being able to form ternary complexes with FACT and histones (Figures S5B and 5E), these data indicated that DNA synthesis was normal per se, and we confirmed this by showing that the viability of *mcm2-3A* was independent of the Mec1 checkpoint kinase (Figure 5F), which becomes essential even after very slight defects in DNA replication (e.g., Kilkenny et al., 2012).

Importantly, FACT was still able to associate with the replisome in *mcm2-3A* cells (Figure 5G) even in the absence of the acidic tail of Spt16 (Figure S5C), confirming that FACT is recruited to the replisome by a mechanism that is independent of its ability to pick up histone complexes together with Mcm2. This indicated that the replisome in *mcm2-3A* cells would have only a partial defect in its ability to process parental histones, given that it lacks the contribution of the conserved Mcm2 tail but still has FACT (and perhaps other factors). Consistent with this view, we found that histones still copurified with replisome material in extracts of *MCM2-3A* cells (Figure 5G; 100 mM salt). However, both FACT and histones could be displaced from isolated replisome material in extracts of *mcm2-3A* cells (but not control cells) by an increased salt concentration (Figure 5G; 700 mM salt). Thus, it appears that the replisome has at least two histone-binding subunits: FACT, which is tethered by salt-sensitive interactions to an unknown component of the replisome progression complex (as well as binding to DNA polymerase alpha), and Mcm2, which is able to form salt-stable ternary complexes with histones and FACT (the latter activity is missing from *mcm2-3A* cells).

The partial defect in histone binding in the replisome of *mcm2-3A* might cause subtle defects in chromatin, though these are balanced by the de novo deposition of nucleosomes at replication forks and perhaps also by the ability of the transcription machinery to help restore any chromatin defects experienced during chromosome replication. We searched systematically for synthetic growth defects that were caused by combining *mcm2-3A* with mutations in other factors that are known to be important during chromosome replication (Figure S6; M.F., unpublished data). Interestingly, only one such combination was found to produce a synthetic growth defect, which resulted from mutations in any of the three unique components of the "alternative clamp loader" complex Ctf18-RFC (Figure 6). Any combination of *mcm2-A* or *mcm2-3A* with *ctf18Δ*, *ctf8Δ*, or *dcc1Δ* produced very slowly growing colonies of sick cells (Figures 6A and 6B; M.F. and K.L., unpublished data).

Replication factor C is a 5 subunit complex that acts at replication forks to load the PCNA "clamp," which serves as a processivity factor for DNA polymerase delta but also recruits many other factors, including proteins involved in sister chromatid cohesion and chromatin assembly. The molecular action of Ctf18-RFC is poorly understood, but its three unique subunits (Ctf18, Ctf8, and Dcc1, which replace Rfc1 in conventional RFC) are all required for the activation of the Mrc1 branch of the S phase checkpoint (Crabbé et al., 2010; Kubota et al., 2011) and contribute to the establishment of sister chromatid cohesion (Mayer et al., 2001). Consequently, cells lacking Ctf18-RFC have a high rate of chromosome instability, as reflected by subnuclear foci of the recombination factor Rad52 (Naiki et al., 2001). Strikingly, none of these phenotypes seem to be shared with *mcm2-3A* (Figures 5D, 6C, 6D, and S6C), suggesting that the synthetic defect produced by combining mutations of Mcm2 and Ctf18-RFC reflects an additional role of the latter.

Interestingly, Ctf18-RFC has also been found to be important for chromatin inheritance, given that subtelomeric heterochromatin is abnormal in cells lacking Ctf18, Ctf8, or Dcc1 (Hiraga et al., 2006). Moreover, the viability of *spt16* alleles at semirestrictive temperatures was reduced upon deletion of the *CTF18* gene but not by the absence of the Mec1 checkpoint kinase (Formosa et al., 2001). These findings suggested that the synthetic growth defect between *mcm2-3A* and deletions of *CTF18*, *CTF8*, or *DCC1* might reflect chromatin defects.

Yeast chromosomes have been found to contain several classes of repressive chromatin, including subtelomeric regions (Gottschling et al., 1990), the silenced HMR and HML loci containing the mating type information (Pillus and Rine, 1989), and elements of the endogenous 2 micron yeast plasmid that are important for its maintenance (Grünweller and Ehrenhofer-Murray, 2002; Papacs et al., 2004). To assay for defects in the proper maintenance of such chromatin, we used strains in which the *ADE2* marker gene was inserted either into the *HMR* locus on chromosome 3 that contains the silenced mating type genes or into subtelomeric heterochromatin at the right end of chromosome 5 (Iida and Araki, 2004). Efficient expression of the *ADE2* gene on rich medium produced white colonies, whereas a failure to express *ADE2* produced red colonies. Control cells with *ADE2* at *HMR* formed a mixture of dark red colonies, white colonies, and sectored colonies (Figure 7A), reflecting a heritable

(G) To assess the ability of the replisome to associate with histones released from chromatin in *MCM2* and *mcm2-3A*, we generated extracts of S phase cultures in the presence of 100 mM or 700 mM potassium acetate. After treatment with Benzonase, the Sld5 component of GINS was isolated by immunoprecipitation, and the associated factors were monitored by immunoblotting.
See also Figure S5.

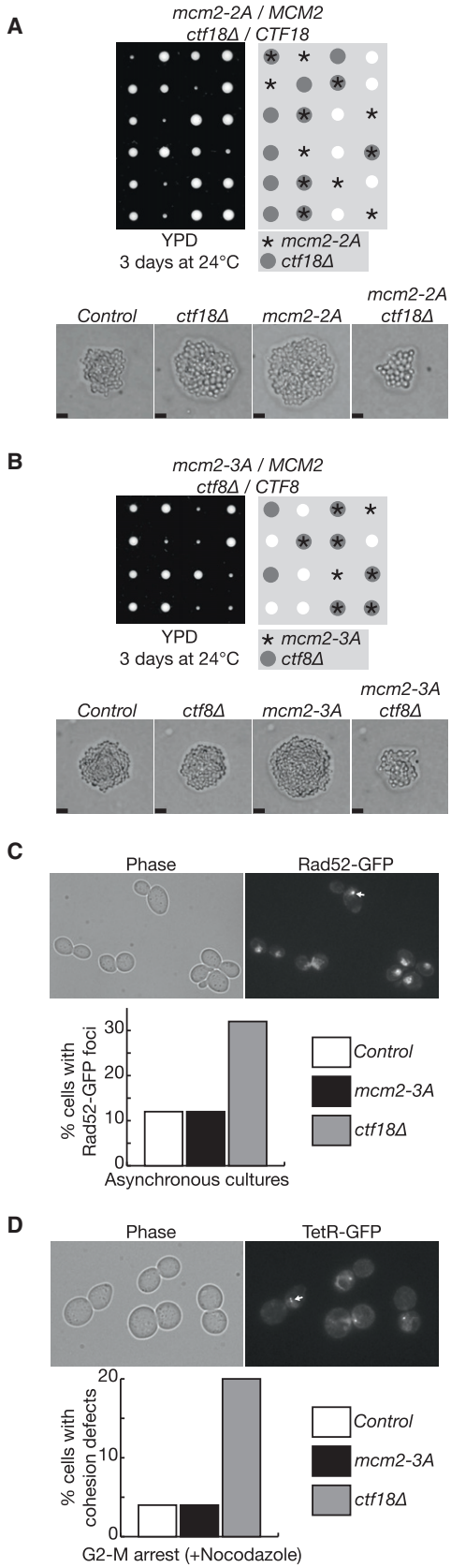

Figure 6. Mcm2 Shares an Important Role with Ctf18-RFC that Is Not Related to Checkpoint Activation or Cohesion

(A–B) Tetrad analysis of the indicated diploid strain indicates that the growth of *mcm2-2A* or *mcm2-3A* is compromised in the absence of the unique components of Ctf18-RFC (Ctf18, Ctf8, or Dcc1). Microscopy images are shown of the indicated strains after 20 hr of growth at 30°C (scale bars represent 10 μm).

(C) The percentage of cells with subnuclear foci of Rad52-GFP (the arrow marks an example) was determined for control, *ctf18Δ*, and *mcm2-3A* by examining 100 cells in asynchronous cultures.

(D) Cohesion defects were monitored with the use of strains expressing the Tet-repressor fused to GFP and an array of Tet-operator sites at the *ura3* locus. Cells were arrested in G2–M phase at 30°C by the addition of nocodazole. For each strain, 100 cells were examined to determine the percentage with separated sister chromatids (two dots of TetR-GFP instead of one; an example is indicated by the arrow), reflecting a defect in cohesion.

See also Figure S6.

variation in gene expression within heterochromatin. Subtelomeric *ADE2* produced a similar range of colonies, except that the repression was milder and, therefore, the red color was paler. Both *mcm2-2A* and *mcm2-3A* behaved like control cells when *ADE2* was present at HMR (Figure 7A, left panels). In contrast, however, all colonies were pure white when subtelomeric *ADE2* was introduced into *mcm2-2A* or *mcm2-3A* (Figure 7A, right panels). Telomere length was not altered in the *mcm2-A* cells (A. Calzada and M.F., unpublished data), indicating that the histone-binding activity of Mcm2 is important for the preservation of aspects of subtelomeric heterochromatin but is not needed for telomere elongation. Chromatin immunoprecipitation showed only subtle differences in histone occupancy in subtelomeric regions of *mcm2-3A* arrested in G1 phase (Figure S7), though transient defects during chromosome replication in the mutant might be subsequently masked by de novo nucleosome deposition.

We also used microarray analysis to compare global patterns of gene expression in control cells and *mcm2-3A* (see Extended Experimental Procedures). Although most transcripts did not show significant changes in *mcm2-3A*, the expression of the four genes encoded by the endogenous 2 micron plasmid was reduced more than 200-fold (Figure 7B (i)). We used tetrad analysis to derive new clones of control, *mcm2-2A*, and *mcm2-3A* from heterozygous diploid cells and used quantitative PCR analysis to find that 2 micron plasmid DNA was lost specifically when Mcm2 was unable to bind histones (Figure 7B (ii)). Thus, it appears that the maintenance of the 2 micron minichromosome is defective in *mcm2-2A* and *mcm2-3A*. Along with the defect in subtelomeric chromatin described above, these findings suggest that the conserved histone-binding motif of Mcm2 contributes to the efficacious replication of chromatin in budding yeast.

DISCUSSION

Our data indicate that both the eukaryotic replication and transcription machineries use analogous histone-binding modules to process parental histones (Figure 7C), and it seems likely that FACT contributes to both assemblies. Mcm2 and FACT jointly produce the ability of the replisome progression complex

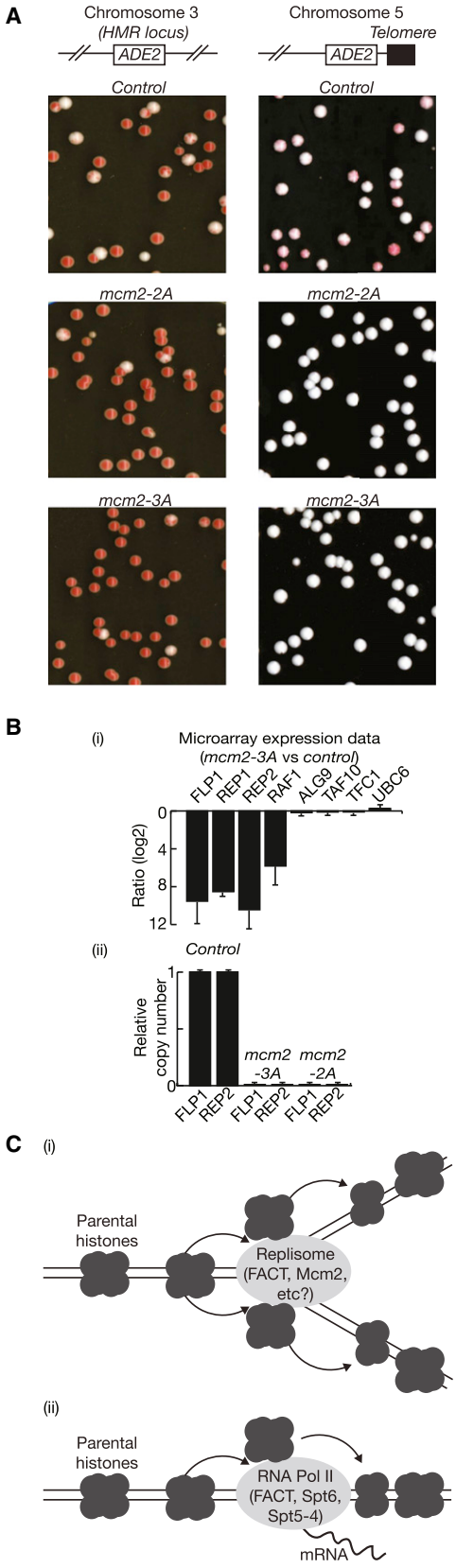

Figure 7. Subtelomeric Heterochromatin Is Defective in *mcm2-2A* and *mcm2-3A* and the Endogenous 2 Micron Minichromosome Is Lost

(A) The indicated strains were generated with the *ADE2* gene inserted at the *HMR* locus or next to the right telomere of chromosome 5. Cells were grown on rich medium (YPD) for 3 days and then incubated at 4°C for 3 days before imaging to show the formation of sectored colonies (red cells have a low expression of *ADE2*, white cells have a higher expression of *ADE2*). The *mcm2-2A* and *mcm2-3A* strains formed sectored colonies when *ADE2* was present at *HMR* but formed only white colonies with *ADE2* next to the right telomere of chromosome 5, indicating a specific defect in subtelomeric heterochromatin.

(B) (i) Gene expression of control cells (W303-1a) and *mcm2-3A* (YMP531) was monitored by microarray analysis in three independent experiments. Expression of the four genes of the 2 micron plasmid was seen to be greatly defective. (ii) Genomic DNA was prepared from control cells (W303-1a), *mcm2-2A* (YMP514), and *mcm2-3A* (YMP531). Then, real-time PCR was used to monitor the copy number of the 2 micron genes *FLP1* and *REP2* relative to the *TFC1* gene on chromosome 2. The results of two independent experiments were averaged. The error bars correspond to the SD from the mean value.

(C) A model for the transfer of parental histones during replication (i) and transcription (ii) based on the cooperation of FACT with specific components of the replisome and RNA polymerase II machinery. See text for details.

See also Figure S7.

to pick up histones that have been released from chromatin (Figure 5G). These findings suggest that FACT and Mcm2 help to retain parental histones transiently at the fork before deposition onto nascent DNA just behind the replisome. In this regard, it is interesting to note that the amino terminal tail of Mcm2 emerges from the rear face of the Cdc45-MCM-GINS helicase and is thus orientated toward the nascent DNA (Costa et al., 2011).

It now seems clear that FACT is a bona fide component of the budding yeast replisome and, therefore, is unique among the known histone chaperones. The same is likely to be true in other eukaryotic species, given the high conservation of replisome subunits across evolution. Indeed, very recent work has shown that FACT is enriched together with other replisome components on nascent chromatin isolated from human cell nuclei (C. Alabert, J.-C. Bukowski-Wills, J. Rappsilber, and A. Groth, personal communication).

FACT still contributes to the histone-binding activity of the replisome in *mcm2-3A* cells (Figure 5G). It will be important in future studies to learn how FACT associates with the replisome progression complex, in order to mutate the interaction and assess the phenotype, without disrupting the analogous role of FACT during transcription. It will also be interesting to explore how other replisome components might also contribute to the transfer of parental histones at replication forks. Histones were previously found to copurify with the components of DNA polymerase epsilon after the digestion of chromatin (Tackett et al., 2005), and we made similar observations for both DNA polymerase epsilon and DNA polymerase alpha (Figure S1D). In both cases, we found that a small amount of FACT also copurified with polymerase and histones, echoing the previous observation that FACT can bind directly to DNA polymerase alpha (Wittmeyer and Formosa, 1997), though it is not yet known whether this interaction can occur in the context of the fork. It remains possible that multiple FACT complexes might associate

with the replisome by binding to the Cdc45-MCM-GINS helicase as well as to DNA polymerases. As with the much more well-characterized transcription machinery, it now seems very likely that the eukaryotic replisome will also use multiple histone-binding modules to process parental histones and, thus, preserve nucleosome density and epigenetics during chromosome replication.

EXPERIMENTAL PROCEDURES

Details of yeast strains and growth, along with all methods not described below, are given in Extended Experimental Procedures.

To monitor the association of Spt16 or other chaperones with histones, we used 250 ml samples (2.5×10^9 cells), whereas 1 l samples (10^{10} cells) were used to screen for the association of Mcm2 or other replisome components with histones. Frozen cell pellets were ground in the presence of liquid nitrogen with a SPEX SamplePrep 6850 Freezer/Mill, as described previously (De Piccoli et al., 2012), and then incubated for 30 min at 4°C with or without the addition of 800 units of Benzonase (Merck, 71206-3) for the digestion of chromosomal DNA, as indicated in the Figures 1–5.

We isolated tagged proteins by immunoprecipitation with magnetic Dynabeads M-270 Epoxy (Invitrogen) coupled at 4°C to rabbit anti-sheep IgG (Sigma-Aldrich, S-1265), M2 anti-FLAG monoclonal antibody (Sigma-Aldrich, F3165), or 9E10 anti-MYC monoclonal antibody. We detected the indicated replisome proteins in each figure by immunoblotting replisome components with previously described polyclonal antibodies (Gambus et al., 2006) or by using M2 monoclonal anti-FLAG antibody, polyclonal anti-FLAG antibody (Sigma-Aldrich, F-7425), 9E10 (anti-MYC), or 12CA5 (anti-HA). Histones were detected with the following polyclonal antibodies: H2A (Active Motif, 39235), H2B (Active Motif, 39237), H3 (our own sheep polyclonal), H3 Ac-K56 (Active Motif, 39281), and H4 (Abcam, ab10158). To detect Spt16 and Pob3, we raised sheep polyclonal antibodies to 25 kDa portions of each protein, which were expressed as His-tagged recombinant proteins in *E.coli* and purified in a denatured form.

For mass spectrometry analysis of protein content, samples were run for about 2 cm in a single gel lane, which was then cut into ten bands before digestion with trypsin. The digested peptides were then analyzed by nano liquid chromatography tandem mass spectrometry with an Orbitrap Velos (Thermo Fisher Scientific).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, seven figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.02.028.

LICENSING INFORMATION

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