Mms1 and Mms22 stabilize the replisome during replication stress

Jessica A. Vaisica\textsuperscript{a,b}, Anastasija Baryshnikova\textsuperscript{b,c}, Michael Costanzo\textsuperscript{b,c}, Charles Boone\textsuperscript{b,c}, and Grant W. Brown\textsuperscript{a,b}

\textsuperscript{a}Department of Biochemistry, University of Toronto, Toronto, ON M5S 1A8, Canada; \textsuperscript{b}Donnelly Centre for Cellular and Biomolecular Research, University of Toronto, Toronto, ON M5S 3E1, Canada; \textsuperscript{c}Banting and Best Department of Medical Research and Department of Cell and Developmental Biology, University of Toronto, Toronto, ON MSG 1L6, Canada

ABSTRACT Mms1 and Mms22 form a Cul\textsuperscript{Ddb1-Bub1}-like E3 ubiquitin ligase with the cullin Rtt101. In this complex, Rtt101 is bound to the substrate-specific adaptor Mms22 through a linker protein, Mms1. Although the Rtt101\textsuperscript{Mms1-Mms22} ubiquitin ligase is important in promoting replication through damaged templates, how it does so has yet to be determined. Here we show that mms1\textsuperscript{Δ} and mms22\textsuperscript{Δ} cells fail to properly regulate DNA replication fork progression when replication stress is present and are defective in recovery from replication fork stress. Consistent with a role in promoting DNA replication, we find that Mms1 is enriched at sites where replication forks have stalled and that this localization requires the known binding partners of Mms1—Rtt101 and Mms22. Mms1 and Mms22 stabilize the replisome during replication stress, as binding of the fork-pausing complex components MrC1 and Csm3, and DNA polymerase \(\varepsilon\), at stalled replication forks is decreased in mms1\textsuperscript{Δ} and mms22\textsuperscript{Δ}. Taken together, these data indicate that Mms1 and Mms22 are important for maintaining the integrity of the replisome when DNA replication forks are slowed by hydroxyurea and thereby promote efficient recovery from replication stress.

INTRODUCTION

Faithful transmission of the genome from one generation to the next requires the accurate and timely replication of the DNA. Accurate DNA replication requires a complex of proteins that localize to the replication fork, collectively referred to as the replisome (Aparicio et al., 1997; Tercero et al., 2000; Calaza et al., 2005). Key components of the replisome include Cdc45-MCM-GINS (CMG) which likely compose the replicative DNA helicase (Aparicio et al., 1997; Tercero et al., 2000; Zou and Stillman, 2000; Takayama et al., 2003; Gambus et al., 2006) Polex-primase, which primes the leading strand and Okazaki fragments (Plevani et al., 1984; Singh and Dumas, 1984), the leading and lagging-strand polymerases (Pol\(\varepsilon\) and Pol\(\alpha\); Purcell et al., 2007; Nick McElhinny et al., 2008), Ctf4 and Mcm10, which interact with Polex-primase (Ricke and Bielinsky, 2004; Gambus et al., 2009; Tanaka et al., 2009), and the replication fork-pausing complex (FPC), which comprises MrC1, Csm3, and Tof1 (Katou et al., 2003; Noguchi et al., 2004; Calzada et al., 2005; Nedelcheva et al., 2005; Szyjka et al., 2005; Bando et al., 2009).

The timely progression of DNA replication forks can be challenged by a number of conditions, including nucleotide base lesions, abasic sites, inter- and intrasubstrate cross-links, DNA secondary structures, sites of strong protein–DNA interactions, transcription complexes, depletion or inhibition of DNA polymerases, and depletion of dNTPs. These challenges are collectively referred to as replication stress. Replication stress is readily induced experimentally by the ribonucleotide reductase inhibitor hydroxyurea (Krakoff et al., 1968; Slater, 1973; Alvino et al., 2007), which results in a depletion of deoxyribonucleotide triphosphates (dNTPs), thereby causing a large decrease in replication fork rate. Hydroxyurea (HU) also activates a cell cycle checkpoint (Weinert et al., 1994), which delays cell cycle progression until S phase is complete, stabilizes replication fork proteins at the stalled or slowed forks, and promotes the ability of replication forks to complete DNA synthesis (Desany et al., 1998; Alcasabas et al., 2001; Lopes et al., 2001; Sogo et al., 2002; Cobb et al., 2003; Katou et al., 2003; Osborn and
Elledge, 2003; Tercero et al., 2003; Lucca et al., 2004; Bjergbaek et al., 2005; Feng et al., 2006; Naylor et al., 2009; Tittel-Elmer et al., 2009). Although the mechanisms by which the checkpoint stabilizes replisomes under conditions of replication stress and promotes DNA replication are not entirely known, there are a number of connections between replisome components and the checkpoint. Replisome destabilization has been noted in deletion mutants of several genes with roles in maintaining genome integrity. Deletion of MEC1, RAD53, or SGS1 decreases the association of DNA polymerases with stalled forks (Cobb et al., 2005), as does deletion of MRC1 and genes encoding components of the MRX complex (Lou et al., 2008; Tittel-Elmer et al., 2009). In addition, deletion of MRC1, CSM3, TOF1, DIA2, and CTF4 each causes replisome migration to become uncoordinated from the nascent DNA chain (Katou et al., 2003; Bando et al., 2009; Mimura et al., 2009; Tanaka et al., 2009). These data indicate that a complex network of proteins functions to maintain the integrity of the replisome at the DNA replication fork when cells encounter replication stress.

In budding yeast the genes MMS1 and MMS22 are important for resistance to replication stress, either from dNTP depletion by HU or by DNA damage induced by methyl methanesulfonate (MMS) or camptothecin (Bennett et al., 2001; Chang et al., 2002; Hryciw et al., 2002; Araki et al., 2003; Baldwin et al., 2005; Dovey and Russell, 2007; Dovey et al., 2009; Yokoyama et al., 2007; Roberts et al., 2008). Genetic interaction data suggest that Mms1 and Mms22 function with the cullin Rtt101 (Pan et al., 2006; Collins et al., 2007; Costanzo et al., 2010; Koh et al., 2010). Consistent with these data, rtt101Δ shares many phenotypes with mms1 and mms22 deletion mutants. Similar to mms1Δ and mms22Δ strains, rtt101Δ cells are sensitive to agents that perturb replication and induce DNA damage (Chang et al., 2002; Luke et al., 2006; Roberts et al., 2008). Deletion mutants of rtt101Δ also have increased rates of spontaneous DNA damage during a normal cell cycle (Luke et al., 2006), accumulate at the G2/M transition (Michel et al., 2003), and display an abnormal nuclear morphology (Michel et al., 2003; Luke et al., 2006), much like mms1Δ and mms22Δ (Dovey and Russell, 2007; Dovey et al., 2009; Yokoyama et al., 2007; Duro et al., 2008; Roberts et al., 2008).

Protein–protein interactions also suggest that Mms1 and Mms22 function in concert with Rtt101 (Ho et al., 2002; Pan et al., 2006; Suter et al., 2007; Zaidi et al., 2008; Ben-Aroya et al., 2010; Mimura et al., 2010), and sequence conservation further suggests that Rtt101ΔMms1/Mms22 is similar to the mammalian Cul4ΔDdb1 complex (Zaidi et al., 2008). Cul4ΔDdb1 is a ubiquitin ligase with a number of roles in genome maintenance (for a review see Jackson and Xiong, 2009), including degradation of the replication factors CDT1 and CHK1 (Higa et al., 2003; Leung-Pineda et al., 2009) and ubiquitination of histones and XPC under conditions of DNA damage (Sugasaki et al., 2005; Kapetanaki et al., 2006; Wang et al., 2006). As is the case for the Cul4ΔDdb1 ubiquitin ligase, Rtt101ΔMms1 forms multiple complexes with several substrate specific adaptors, and these are expected to dictate function (Zaidi et al., 2008; Fujii et al., 2009; Mimura et al., 2010).

The precise role for this Cul4ΔDdb1-like complex, Rtt101ΔMms1/Mms22, in maintaining genome integrity in response to replication stress has yet to be determined. Here we show that Mms1 localizes to regions adjacent to replication origins when replication stress is present, suggesting that Mms1 is recruited to stalled replication forks. We find that deletion of MMS1 or MMS22 reduces the association of replisome proteins with stalled replication forks, and that, in the absence of MMS1 or MMS22 replication forks progress and recover inefficiently in the presence of HU. Together our results indicate that Mms1 and Mms22 are important for the stabilization of protein components at the replication fork during replication stress and thereby function to prevent fork collapse and promote the resumption of DNA synthesis.

RESULTS

Deletion of MMS1 or MMS22 causes an abnormal cell cycle following recovery from replication stress

Deletion of MMS1 or MMS22 renders cells sensitive to DNA-damaging agents (Bennett et al., 2001; Chang et al., 2002; Hryciw et al., 2002; Araki et al., 2003; Baldwin et al., 2005; Dovey and Russell, 2007; Dovey et al., 2009; Yokoyama et al., 2007; Roberts et al., 2008) and to replication stress caused by chronic exposure to HU (Chang et al., 2002; Araki et al., 2003; Dovey and Russell, 2007; Dovey et al., 2009; Yokoyama et al., 2007; Roberts et al., 2008) (Figure 1A). To delineate the role of MMS1 and MMS22 in HU resistance, we assessed the viability of mms1Δ and mms22Δ over a 24 h period of HU exposure (Figure 1B). Samples were collected at the indicated times from wild-type, mms1Δ, and mms22Δ cultures treated with 200 mM HU. Samples were collected at the indicated times from wild-type, mms1Δ, and mms22Δ cultures treated with 200 mM HU, and viability was measured following plating on media lacking HU. Replication stress began to affect viability in mms1Δ and mms22Δ after 6 h of HU treatment, indicating that even during short-term exposure Mms1 and Mms22 are required for HU resistance. Consistent with previous assays of MMS sensitivity, mms22Δ was more sensitive to HU than was mms1Δ (Roberts et al., 2008; Zaidi et al., 2008; Dovey et al., 2009).

We next examined cell cycle progression in mms1Δ and mms22Δ strains during treatment with HU for 90 min, and during a subsequent 3 h recovery period, by flow cytometry and by
Deletion of MMS1 results in abnormal replication fork progression during replication stress

The lengthy mitotic delay in mms1Δ and mms22Δ strains following replication stress suggested that DNA replication in the absence of Mms1 or Mms22 might be defective when HU-induced replication stress is present. We examined replication dynamics in wild-type and mms1Δ cells in the presence of replication stress. G1-arrested cells were released into media containing both bromodeoxyuridine (BrdU), to label newly synthesized DNA, and HU, to stall replication. The wild-type and mms1Δ cultures were sampled at 45 and 90 min, and BrdU-labeled DNA was hybridized to tiling microarrays to identify replicated regions genome wide. The wild-type strain exhibited narrow peaks of BrdU incorporation at early-firing replication origins after 45 min in HU (Figure 3A, top). These peaks broadened after 90 min (Figure 3A, bottom), consistent with the observation that HU slows, but does not arrest, DNA synthesis in wild-type cells (Alvino et al., 2007). By contrast, DNA synthesis in mms1Δ cells was defective in several ways. First, the BrdU peaks were broader at 45 min compared with wild-type cells (Figure 3B, top), indicating that
Mms1 and Mms22 stabilize the replisome

replication forks traveled further in the mutant relative to wild type when replication stress was present. Second, the BrdU peak height at 45 min was reduced in mms1Δ compared with wild type, which might indicate greater variation in the distances traveled by individual replication forks in the population. Third, the BrdU peaks in mms1Δ cells were largely unchanged at 90 min (Figure 3B, bottom), suggesting the absence of significant DNA replication in the mutant between 45 and 90 min. We conclude that DNA synthesis during replication stress in mms1Δ is initially advanced but then halts as replication forks arrest. We were unable to analyze replication in mms22Δ mutants, as deletion of mms22 in the multicopy thymidine kinase strain that allows incorporation of BrdU resulted in a synthetic sick phenotype (unpublished data).

Mms1 binds origin-proximal regions when replication forks stall

Several studies have suggested that Mms1 and Mms22 function to maintain the stability of the genome (Bennett et al., 2001; Chang et al., 2002; Hryciw et al., 2002; Araki et al., 2003; Baldwin et al., 2005; Dovey and Russell, 2007; Dovey et al., 2009; Yokoyama et al., 2007; Duro et al., 2008; Roberts et al., 2008; Zaidi et al., 2008; Ben-Aroya et al., 2010; Mimura et al., 2010); however, it remains unclear how they exert this effect. Our data indicated that absence of Mms1 caused defects in DNA replication when replication fork stress was present, suggesting that Mms1 might act directly at stalled replication forks. We examined the association of Mms1 with sites of stalled replication forks using chromatin immunoprecipitation (ChIP) (Figure 4, A–C). Cells were arrested in G1 phase and released into S phase in the presence of HU. Following cross-linking to preserve protein–DNA interactions, Mms1–DNA complexes were isolated, and the enrichment of an early-firing origin of replication, ARS607, versus that of a late-firing origin, ARS609, was measured using quantitative PCR (qPCR). We observed a twofold enrichment at the early-firing replication origin in the mutant relative to wild type when replication stress was present, suggesting that Mms1 might act directly at stalled replication forks. We were unable to analyze replication in mms22Δ mutants, as deletion of mms22 in the multicopy thymidine kinase strain that allows incorporation of BrdU resulted in a synthetic sick phenotype (unpublished data).

FIGURE 3: mms1Δ cells have an irregular pattern of fork progression in HU. BrdU IP-chip analysis was performed following synchronous release of wild-type (A) and mms1Δ (B) strains into 200 mM HU for 45 min (top) or 90 min (bottom). Enrichment of DNA fragments in the BrdU sample relative to an unreplicated control is shown along chromosome VI. The signal intensity ratio on a log2 scale is shown on the y-axis and the chromosome coordinate is shown on the x-axis. Positive signal represents regions that are replicated, and regions where the positive signal is statistically significant over 300 base pairs are shown in light blue and over 900 base pairs are shown in dark blue. Replication origins (ARSs) are indicated. Early-firing origins are colored red.

Volume 22    July 1, 2011

Mms1 and Mms22 stabilize the replisome | 2399
these data indicate that Mms1 associates with early-firing origins of replication when replication forks stall. We next looked at Mms1 dynamics by conducting an Mms1 ChIP/qPCR time course with the ARS607/ARS609 probe pair. If Mms1 functions at replication forks, the levels of Mms1 protein detected at these sites may change with time as forks accumulate at the probe region. We detected a modest but significant increase in the amount of early-origin DNA in Mms1 immunoprecipitates between 60 and 120 min (Figure 4B).

Mms1 functions as part of a complex with Rtt101 and Mms22 (Zaidi et al., 2008; Mimura et al., 2010). We examined whether binding of Mms1 at stalled replication forks required either of the Mms1-binding partners. Using ChIP/qPCR, we found that enrichment of the early-origin DNA in Mms1 chromatin immunoprecipitates was abolished in rtt101Δ and in mms22Δ strains (Figure 4C). Thus the presence of Mms1 at an active replication origin when replication stress is present depends on the known Mms1-binding partners Mms22 and Rtt101.

Because DNA synthesis is defective in mms1Δ strains during replication fork stress and Mms1 associates with replication forks, we performed parallel BrdU immunoprecipitation (IP)-chip and ChIP-chip experiments to examine the localization of fork proteins during DNA replication in mms1 mutants (Figure 4D). We tagged Dpb3, a subunit of DNA polymerase epsilon (Polε), in wild-type and mms1Δ strains to assess protein–DNA association and DNA synthesis concurrently. As the leading-strand polymerase, Polε localizes to origins of replication and colocalizes with sites of DNA synthesis (Aparicio...
Mms1 and Mms22 stabilize replication proteins at stalled replication forks

Our data indicate that replication forks arrest in mms1Δ and mms22Δ cells in the presence of replication stress and that replication does not resume efficiently during recovery from replication stress. Furthermore, our data suggest that replication fork proteins likely dissociate from forks in the absence of Mms1 or Mms22. We mined high-throughput genetic interaction data sets to identify candidate genes whose function might be influenced by the absence of mms1, mms22, and rtt101 (Costanzo et al., 2010; Koh et al., 2010). The genes encoding members of the Rtt101-Mms1-Mms22 complex exhibited positive genetic interactions with genes encoding proteins that reside at the replication fork, including Mrc1, Csm3, and Tof1, and DNA Pol (Figure 5A). Mrc1-Csm3-Tof1 form a replication fork–pausing complex (FPC) that tethers the MCM helicase to the leading-strand polymerase, Polε, when forks are stalled (Katou et al., 2003; Calzada et al., 2005; Nedelcheva et al., 2005; Szajka et al., 2005; Lou et al., 2008). Although positive interactions often connect members of the same nonessential complex, analysis of genetic interaction networks showed that the vast majority of positive interactions occur between different protein complexes, which may belong to a common biological pathway (Baryshnikova et al., 2010; Costanzo et al., 2010). Thus these data suggest that the fork-pausing complex or the leading-strand polymerase may be targets of Rtt101-Mms1-Mms22 action.

We assessed whether deletion of MMS1 or MMS22 altered the association of the FPC with forks when replication stress is applied. Steady-state levels of the replication fork proteins Mrc1, Csm3, and Dpb3 were not reduced by deletion of either MMS1 or MMS22, indicating that any observed effects were not due to decreased protein abundance (Figure 5B). We examined the recruitment of Mrc1 to stalled forks genome wide by ChIP-chip in wild-type, mms1Δ, and mms22Δ strains (Figure 6, A and B). As reported, Mrc1 localizes to origin proximal regions in HU-treated cells (Katou et al., 2003). However in mms1Δ and mms22Δ strains, the enrichment of origin proximal regions was decreased (Figure 6, A and B, bottom). Although the signal intensity ratio was lower in the mutants, Mrc1 localization occurred at the same chromosome coordinates, suggesting that there is less Mrc1 at these sites and not a spreading of Mrc1 to adjacent regions. This effect was observed genome wide in both mms1Δ and mms22Δ mutants. Consistent with our HU sensitivity data, according to which mms22Δ mutants display a more severe growth defect under conditions of replication stress than mms1Δ mutants, deletion of MMS1 had a more subtle effect on Mrc1 localization than deletion of MMS22. To corroborate this finding, we analyzed the signal intensity peaks at 12 early-firing origins of replication and compared the distribution in wild-type to that in mms1Δ and mms22Δ strains. The median peak area for Mrc1 was significantly decreased (p < 0.05) in mms1Δ and mms22Δ strains compared with their respective wild-type controls (Figure 6C). Again, this effect was stronger in the mms22Δ strain. Together, the ChIP-chip data indicate that deletion of MMS1 or MMS22 reduces Mrc1 localization to stalled replication forks under conditions of fork stress. We quantified the reduction in Mrc1 localization to replication origins using qPCR to measure enrichment of the early-firing origin ARS607 compared with the inactive (in HU) origin ARS609 in at least two independent Mrc1 chromatin immunoprecipitates from each of the wild-type, mms1Δ, and mms22Δ strains. The enrichment of the early-firing origin in the Mrc1 immunoprecipitates was reduced to approximately half of the wild-type level in both the mms1Δ and the mms22Δ mutants (Figure 6D). To confirm that this effect is not unique to Mrc1, we examined the localization of Csm3, a second component of the fork-pausing complex, to these same chromosome coordinates by qPCR. Similar to Mrc1, mms1Δ and mms22Δ strains exhibit a twofold reduction in early origin enrichment in the Csm3 immunoprecipitates (Figure 6E). These data indicate that Mms1 and Mms22 promote the stable binding of the FPC at replication forks under conditions of replication fork stress.

Given that Mrc1 links Polε to the replicative helicase when forks stall (Lou et al., 2008), and since Polε exhibits positive genetic interactions with the Rtt101-Mms1-Mms22 pathway/complex, we assessed the association of Dpb3, a subunit of DNA Polε, with stalled forks. Deletion of MMS1 or MMS22 resulted in a significant reduction in the amount of Dpb3 at early-firing origins, genome wide, by ChIP-chip (Figure 7, A–C). As was observed for Mrc1, deletion of...
FIGURE 6: Deletion of MMS1 or MMS22 reduces the association of the FPC with stalled replication forks.

(A, B) ChIP-chip analysis was performed following synchronous release of MRC1::flag, mms1Δ, MRC1::flag, or mms22Δ MRC1::flag cells into S phase in the presence of 200 mM HU for 90 min at 23°C. After cross-linking and DNA fragmentation, Mrc1 was precipitated. Enrichment of DNA fragments in the Mrc1-bound fraction relative to the unbound fraction is shown along chromosome VI, as in Figure 4D. Replication origins (ARSs) are indicated in red for early-firing origins that are active in HU and in black for late-firing origins that are inactive in HU. (C) The distributions of signal intensity peak areas in the ChIP-chip data for 12 replication origins for the indicated strains is shown as a boxplot, with the median indicated by the horizontal bar. Peak areas were determined by extracting the signal intensity ratio values plotted in A and B at 50-nucleotide (nt) intervals for 5000 nt on either side of each origin and summing those values. Significant differences in distributions between each mutant and its wild-type control as calculated using a Mann–Whitney test are indicated. (D) Enrichment of early-origin DNA fragments relative to late-origin DNA in the Mrc1-bound and input fractions was quantified by quantitative PCR for the indicated strains. The ratio of early-origin DNA (ARS607) to late-origin DNA (ARS609) was quantified for the input and the Mrc1-bound fractions. The average of at least two experiments is plotted, and the SD is shown. (E) Enrichment of DNA fragments in the Csm3-bound fraction from chromatin immunoprecipitations of wild-type, mms1Δ, and mms22Δ strains was quantified by quantitative PCR. The ratio of early-origin DNA (ARS607) to late-origin DNA (ARS609) was quantified for the input and the Csm3-bound fractions. The average of at least two experiments is plotted, and the SD is shown.
FIGURE 7: Deletion of MMS1 or MMS22 reduces the association of DNA Polε with stalled replication forks. (A, B) ChIP-chip analysis was performed following synchronous release of DPB3::flag, mms1Δ DPB3::flag, or mms22Δ DPB3::flag cells into S phase in the presence of 200 mM HU for 90 min at 23°C. After cross-linking and DNA fragmentation, Dpb3 was precipitated. Enrichment of DNA fragments in the Dpb3-bound fraction relative to the unbound fraction is shown along chromosome VI, as in Figure 4D. Replication origins (ARSs) are indicated in red for early-firing origins that are active in HU and in black for late-firing origins that are inactive in HU. (C) The distributions of signal intensity peak areas in the ChIP-chip data for 12 replication origins for the indicated strains is shown as a boxplot, with the median indicated by the horizontal bar. Peak areas were determined by extracting the signal intensity ratio values plotted in A and B at 50-nt intervals for 5000 nt on either side of each origin and summing those values. Significant differences in distributions between each mutant and its wild-type control as calculated using a Mann–Whitney test are indicated. (D) Enrichment of early-origin DNA fragments relative to late-origin DNA from the Dpb3-bound and input fractions was quantified by quantitative PCR for the indicated strains. The ratio of early-origin DNA (ARS607) to late-origin DNA (ARS609) was quantified for the input and the Dpb3-bound fractions. The average of at least two experiments is plotted, and the SD is shown.
We have characterized the function of \textit{MMS1} and \textit{MMS22} during replication stress induced by HU. Following short-term exposure to HU, \textit{mms1}Δ and \textit{mms22}Δ strains have decreased viability and are unable to complete mitosis efficiently, accumulating with an anaphase-like morphology. DNA replication in the \textit{mms1}Δ mutant is defective, and replication forks stall in the presence of replication stress. Finally, we find that the association of replisome components with the stressed replication forks decreases by twofold. Together our data suggest a model in which the function of Mms1, and perhaps Mms22, is to promote the stable association of the fork-pausing complex, the leading-strand polymerase, and likely other replisome components with the replication fork when replication stress is present. In the absence of Mms1 (and by extension Mms22) the dissociation of fork components results in replication fork stalling and perhaps collapse, leading to inefficient DNA synthesis during recovery from replication stress (Figure 8).

**DISCUSSION**

We have characterized the function of \textit{MMS1} and \textit{MMS22} during replication stress induced by HU. Following short-term exposure to HU, \textit{mms1}Δ and \textit{mms22}Δ strains have decreased viability and are unable to complete mitosis efficiently, accumulating with an anaphase-like morphology. DNA replication in the \textit{mms1}Δ mutant is defective, and replication forks stall in the presence of replication stress. Finally, we find that the association of replisome components with the stressed replication forks decreases by twofold. Together our data suggest a model in which the function of Mms1, and perhaps Mms22, is to promote the stable association of the fork-pausing complex, the leading-strand polymerase, and likely other replisome components with the replication fork when replication stress is present. In the absence of Mms1 (and by extension Mms22) the dissociation of fork components results in replication fork stalling and perhaps collapse, leading to inefficient DNA synthesis during recovery from replication stress (Figure 8).

**DNA synthesis in the presence of replication stress is perturbed in \textit{mms1}Δ and \textit{mms22}Δ cells**

Our data show that DNA synthesis at replication forks in the presence of HU is defective in \textit{mms1}Δ mutants. Unexpectedly, \textit{mms1}Δ displayed broader replication peaks than did the wild-type strain after 45 min in HU. Because the replication peaks were shallower than in wild type and there was little difference evident in the flow cytometry profiles, we infer that there is considerable diversity in the amount of DNA synthesis at individual replication forks in individual cells in the population analyzed. This could reflect some degree of HU-resistant DNA synthesis, as we have observed in mutants in elg1 and pol30 (M.B. Davidson, Y. Katou, A. Keszthelyi, J. Ou, T.L. Sing, J.A. Vaisica, A. Chabes, K. Shirahige, and G.W. Brown, unpublished data), or an asynchronous or advanced entry into S phase in \textit{mms1}Δ. A similar advanced DNA synthesis in HU was seen in ctf18Δ mutants, although in contrast to \textit{mms1}Δ this was accompanied by checkpoint activation defects (Crabbe et al., 2010). Despite this initial burst of DNA synthesis, \textit{mms1}Δ showed little change in replication peaks between 45 and 90 min, suggesting that forks had completely arrested. This contrasts with wild-type cells, which continue to synthesize DNA in the presence of HU but at lower rates (Figure 3) (Alvino et al., 2007). Furthermore, in \textit{mms1}Δ strains, we found that Polε does not colocalize with the BrdU signal after 90 min in HU, and in particular origin-distal forks appear to lack Polε (Figure 4D). Given the replication profile of the mutant, we suggest that during replication fork stress in \textit{mms1}Δ strains, replication at a subset of origins initially proceeds more rapidly than in wild type, resulting in DNA synthesis at more origin-distal sites. These forks ultimately arrest and lose replisome components, failing to synthesize DNA even after recovery from replication stress (Figure 8). Consistent with the arrest and/or collapse of a subset of replication forks in HU in \textit{mms1}Δ, we found that completion of S phase remained defective in the mutant even after the removal of HU, as evidenced by the persistence of mitotic forms in the mutant. Together our data are consistent with a loss of replisome integrity in \textit{mms1}Δ.

**Mms1 and Mms22 are important for the stabilization of the replisome during replication stress**

We find that Mms1 and Mms22 are important mediators of Mrc1 localization to sites of stalled replication. Under conditions of replication stress, checkpoint activation is modulated by the phosphorylation of the mediator protein Mrc1 (Alcasabas et al., 2001; Naylor et al., 2009). In addition to its role in checkpoint signaling, Mrc1 is present at replication forks and forms a complex with Csm3 and Tof1 called the FPC (Katou et al., 2003;
Mms1 and Mms22 stabilize the replisome

Is a twofold decrease in fork residence of these proteins likely to have a biological consequence? Short HU exposures have little effect on the viability of cells lacking Mms1 or Mms22, but it is clear that even modest depletion of replisome components can have severe consequences in terms of genome integrity. For example, moderate depletion of DNA Polε causes genome instability without obvious effects on growth rate (Lemoine et al., 2008). Our data indicate that absence of Mms1 or Mms22, and the depletion of Mcr1, Csm3, and Dpb3 at stalled forks that results, causes a defect in DNA replication in the presence of HU and during recovery from HU.

MATERIALS AND METHODS

Yeast strains and media

Yeast strains used in this study are derivatives of BY4741 (Brachmann et al., 1998) or W303 and are listed in Table 1. Standard yeast media and growth conditions were used (Sherman, 1991).

Hydroxyurea sensitivity assays

To measure sensitivity to continual exposure to HU, cells were grown in 5 ml of YPD overnight at 30°C, serially diluted 10-fold, and spotted onto YPD plates containing 0, 50, 100, 150, and 200 mM HU. Plates were incubated at 30°C for 2–3 d.

To measure sensitivity to acute HU exposure, cells were grown overnight at 30°C in 30 ml of YPD. Cells were then diluted and grown to early-log phase and treated with hydroxyurea at a final concentration of 200 mM. Samples were collected at the indicated time points and plated onto YPD to determine the number of colony-forming units. The average of two independent experiments was plotted.

Flow cytometry and mitotic index

Examination of DNA content in the presence of replication fork stress and during recovery from fork stalling was performed on a Guava flow cytometer (Millipore, Billerica, MA). Data were analyzed using FlowJo Flow Cytometry Analysis Software, version 9.0 (Ashland, OR). Histograms represent the cell cycle distribution of the indicated samples. The y-axis of each graph has been scaled to represent the percentage of the maximum bin contained in that profile. Samples were also processed for parallel analysis of nuclear morphology (Roberts et al., 2006). In brief, log-phase cells were blocked in 2 μg/ml α-factor for 3 h at 23°C. Cells were then washed and resuspended in media containing 100 μg/ml pronase (Sigma-Aldrich, St. Louis, MO) and 200 mM HU (Sigma-Aldrich) for 90 min at 23°C with sampling every 30 min. Cells were then washed to remove HU, resuspended in YPD, and incubated for an additional 3 h at 23°C with sampling every 30 min. The 1-ml samples were fixed in 70% ethanol and stored at 4°C. For morphology analysis, 50 μl of cells were harvested, resuspended in 10 μl of VECTASHIELD mounting media with 4,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA), and imaged on a Zeiss Axiovert inverted microscope.

BrdU IP-chip, ChiP-chip, and qPCR

BrdU IP-chip and ChiP-chip experiments were performed essentially as described (Katou et al., 2003, 2006; Roberts et al., 2008). Signal intensity peak area was used to assess differences in ChiP-chip profiles between mutants and their respective wild-type controls. For each experiment, the log2 signal intensity values for probes 5000 base pairs on either side of each origin were extracted at 50-nucleotide intervals and the values summed as a measure of peak area. Twelve early-firing origins were selected, including 10 origins that were randomly chosen from the BrdU data (ARS305, ADE1, and ARS327).
TABLE 1: Strains used in this study.

| Strain | Source |
|--------|--------|
| ABY8   | MATa DPB3-6HIS-10FLAG-KanMX mms22Δ::NatMX leu2Δ0 his3Δ1 ura3Δ0 met15Δ0 | This study |
| BY4741 | MATa leu2Δ0 his3Δ0 ura3Δ0 met15Δ0 | Brachmann et al. (1998) |
| CSM3-3FL | MATa CSM3-6HIS-3FLAG-loxP-KanMX-loxP leu2Δ0 his3Δ0 ura3Δ0 met15Δ0 | Katou et al. (2003) |
| E1670  | MATa ade2-1 trp1-1 can1-100 his3-11, 15 leu2-3112, RAD5+ GAL psi+ ura3::URA3/GPD-TK(7x) | This study |
| JOY128 | MATa DPB3-6HIS-10FLAG-KanMX leu2Δ0 his3Δ1 ura3Δ0 met15Δ0 | This study |
| JYY39  | MATa MMS1-6HIS-10FLAG-KanMX leu2Δ0 his3Δ0 ura3Δ0 met15Δ0 | This study |
| JYY54  | MATa MMS1-6HIS-10FLAG-KanMX rtt101Δ::NatMX leu2Δ0 his3Δ0 ura3Δ0 met15Δ0 | This study |
| JYY56  | MATa MMS1-6HIS-10FLAG-KanMX mms22Δ::NatMX leu2Δ0 his3Δ0 ura3Δ0 met15Δ0 | This study |
| JYY65  | MATa mms1Δ::NatMX ade2-1 trp1-1 can1-100 his3-11, 15 leu2-3112, RAD5+ GAL psi+ ura3::URA3/GPD-TK(7x) | This study |
| JYY72  | MATa mms1Δ::NatMX leu2Δ0 his3Δ1 ura3Δ0 met15Δ0 | This study |
| JYY73  | MATa mms22Δ::NatMX leu2Δ0 his3Δ1 ura3Δ0 met15Δ0 | This study |
| JYY76  | MATa MRC1-6HIS-3FLAG-loxP-KanMX-loxP mms1Δ::NatMX leu2Δ0 his3Δ1 ura3Δ0 met15Δ0 | This study |
| JYY77  | MATa MRC1-6HIS-3FLAG-loxP-KanMX-loxP mms22Δ::NatMX leu2Δ0 his3Δ1 ura3Δ0 met15Δ0 | This study |
| JYY80  | MATa CSM3-6HIS-3FLAG-loxP-KanMX-loxP mms1Δ::NatMX leu2Δ0 his3Δ0 ura3Δ0 met15Δ0 | This study |
| JYY101 | MATa CSM3-6HIS-3FLAG-loxP-KanMX-loxP mms22Δ::NatMX leu2Δ0 his3Δ0 ura3Δ0 met15Δ0 | This study |
| JYY134 | MATa DPB3-6HIS-10FLAG-KanMX mms1Δ::NatMX leu2Δ0 his3Δ1 ura3Δ0 met15Δ0 | This study |
| JYY157 | MATa DPB3-6HIS-10FLAG-KanMX ade2-1 trp1-1 can1-100 his3-11, 15 leu2-3112, RAD5+ GAL psi+ ura3::URA3/GPD-TK(7x) | This study |
| JYY158 | MATa mms1Δ::NatMX DPB3-6HIS-10FLAG-KanMX ade2-1 trp1-1 can1-100 his3-11, 15 leu2-3112, RAD5+ GAL psi+ ura3::URA3/GPD-TK(7x) | This study |
| MRC1-3FL | MATa MRC1-6HIS-3FLAG-loxP-KanMX-loxP leu2Δ0 his3Δ1 ura3Δ0 met15Δ0 | Katou et al. (2003) |

ARS306, ARS315, ARS416, ARS606, ARS719, ARS737, ARS920, ARS1015, ARS1211, and ARS607 and ARS1018, which were used for qPCR analysis. The distributions of the signal intensity sums were subjected to the Mann–Whitney test to determine whether the distributions were significantly different (p < 0.05) and are presented as box plots. For quantification of signal by qPCR, input and IP samples of DNA from at least two independent chromatin immunoprecipitations were collected using the same procedure as for ChIP-chip. Following purification of the DNA using a QIAquick PCR Purification Kit (Qiagen, Valencia, CA), samples were quantified using the DyNaMo HS SYBR Green qPCR Kit (Finnzymes, Thermo-Fisher Scientific, Waltham, MA) in an Applied Biosystems (Foster City, CA) 7500 Real-Time PCR System. Primers for amplification of ARS607 and ARS609 have been previously described (Lengronne et al., 2006). Primer sequences were designed for the amplification of DNA proximal to ARS1018 (forward, 5′-TAA CAC AAA ATC CAG ATT TGT ACA GAA AGA AGA-3′; reverse, 5′-ATA TGT AAC CGC AAC AGT AGC CAA-3′) and the interorign region between ARS1009 and ARS1010 (forward, 5′-TGA ATT AGA TGC TCT TCT GTA TAC TTT CTT-3′; reverse, 5′-GGT ACA TTC ACC TTG GTT TTC AAC TAC GT-3′). Averages of at least two independent experiments were plotted with the SD.

Genetic Interaction Data
Quantitative genetic interactions were identified by synthetic genetic array (SGA) analysis (Costanzo et al., 2010) and measured as described elsewhere (Baryshnikova et al., 2010; Costanzo et al., 2010). Protein complexes were assessed for biases in either positive or negative genetic interactions among its members by using a monochromatic purity (MP) score, described elsewhere (Baryshnikova et al., 2010). Complexes exhibiting only positive genetic interactions will have an MP score of +1, whereas an MP-score of -1 corresponds to complexes composed exclusively of negative interactions. Complexes whose interactions reflect the background ratio of positive to negative interactions have an MP score equal to 0. The ratio of positive to negative interactions (monochromacy) occurring between pairs of protein complexes was assessed in a similar manner. Protein complex pairs were also assessed for overall enrichment of genetic interactions using a hypergeometric p value.

Immunoblotting
Five optical density units of mid-log-phase cells were fixed with 10% trichloroacetic acid (Sigma-Aldrich), and extracts were prepared as described (Roberts et al., 2008). For treatment with hydroxyurea, cells were grown to early log phase at 23°C, arrested in G1 with 2 μg/ml α-factor for 3 h, and synchronously released into 100 μg/ml pronase and 200 mM HU (Sigma-Aldrich) for 90 min before fixation and extract preparation. Proteins were resolved on SDS–PAGE and subjected to immunoblot analysis with monoclonal ANTI-FLAG M2-peroxidase antibody (Sigma-Aldrich) or anti–phosphoglycerate kinase monoclonal antibody (Molecular Probes, Invitrogen, Carlsbad, CA). Immunoblots were developed using SuperSignal ECL (Pierce Chemical, Rockford, IL).
ACKNOWLEDGMENTS
We thank Yuki Katou and Katsu Shirahige for analysis of BrdU IP-chip and ChIP-chip data and Attila Balint for strain construction. This work was supported by Grant 020254 from the Canadian Cancer Society to G.W.B., by Genome Canada through the Ontario Genomics Institute as per research agreement 2004-0G1-3-01, the Canadian Institutes of Health Research as per research agreement MOP-57830, and the Natural Sciences and Engineering Research Council of Canada as per research agreement RGPIN 204899-06 to C.B., and by a Canadian Graduate Scholarship from the Natural Sciences and Engineering Research Council of Canada to J.V.

REFERENCES
Alcasabas AA et al. (2001). Mcr1 transduces signals of DNA replication stress to activate Rad53. Nat Cell Biol 3, 958–965.
Alvino GM, Collingwood D, Murphy JM, Delrow J, Brewer BJ, Raghuraman MK (2002). Replication in hydroyxura: it’s a matter of time. Mol Cell Biol 22, 6396–6406.
Aparicio OM, Weinstein DM, Bell SP (1997). Components and dynamics of DNA replication complexes in S. cerevisiae: redistribution of MCM proteins and Cdc45p during S phase. Cell 91, 59–69.
Araki Y, Kawasaki Y, Sasanuma H, Tye BK, Sugino A (2003). Budding yeast DNA polymerases and other proteins during grace fork remodelling and fork bypass. Proc Natl Acad Sci USA 99, 16934–16939.
Bando M, Katou Y, Komata M, Tanaka H, Itoh T, Suzuki T, Shirahige K (2009). S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. Yeast 14, 115–132.
Banyashtikova A et al. (2010). Quantitative analysis of fitness and genetic interactions in yeast on a genome scale. Nat Methods 7, 1017–1024.
Ben-Aroya S, Agmon N, Yuen K, Kwok T, McManus K, Kupiec M, Hieter P (2010). Proteosome nuclear activity affects chromosome stability by controlling the turnover of mms22, a protein important for DNA repair. PLoS Genet 6, e1000952.
Bennett CB, Davies A, Cost GJ, Caputo E, Li J, Hieter P, Boeke JD (2004). Checkpoint-mediated control of replisome-fork association in yeast on a genome scale. Nat Methods 7, 1017–1024.
Bennett CB, Lewis LK, Karthikeyan G, Lobachev KS, Jin YH, Sterling JF, Bando M, Katou Y, Komata M, Tanaka H, Itoh T, Sutani T, Shirahige K (2009). Saccharomyces cerevisiae containing tandem repeats of the RAD53 open reading frame as a tool for analyzing the DNA damage response. Mol Genet Genom 278, 22828–22837.
Bendo M, Katou Y, Komata M, Tanaka H, Itoh T, Suzuki T, Shirahige K (2009). S-phase checkpoint proteins Tof1 and Mrc1 form a heterotrimetric mediator complex that associates with DNA replication forks. J Biol Chem 284, 34355–34365.
Bryshkina A et al. (2010). Mechanistically distinct roles for Sgs1p in checkpoint activation and replication fork maintenance. EMBO J 24, 405–417.
Brachmann CB, Dale A, Cost GJ, Caputo E, Li J, Hieter P, Boeke JD (1998). Designer deletion strains derived from Saccharomyces cerevisiae S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. Yeast 14, 115–132.
Calzada A, Hodgson B, Kanemaki M, Bueno A, Labib K (2005). Molecular anatomy and regulation of a stable replisome at a paused euayotic DNA replication fork. Genes Dev 19, 1905–1919.
Chang M, Bellouai M, Boone C, Brown GW (2002). A genome-wide screen for methyl methanesulfonate-sensitive mutants reveals genes required for S phase progression in the presence of DNA damage. Proc Natl Acad Sci USA 99, 16934–16939.
Cobb JA, Schleker T, Rojas V, Bjergbaek L, Tercero JA, Gasser SM (2005). Establishment of sister chromatid cohesion at the C. elegans X chromosome centromere. Nucleic Acids Res 33, 2786–2792.
Cobb JA, Bjergbaek L, Shimada K, Frei C, Gasser SM (2005). Mechanically distinct roles for Sgs1 in checkpoint activation and replication fork progression. Nat Cell Biol 7, 942–951.
Collins SR et al. (2007). Functional dissection of protein complexes involved in yeast chromosome biology using a genetic interaction map. Nature 446, 806–810.
Costanzo M et al. (2010). The genetic landscape of a cell. Science 327, 425–431.
Dovey CL, Russell P (2007). Mms22 preserves genomic integrity during DNA replication in Schizosaccharomyces pombe. Genetics 177, 47–61.
Duro E, Vaisca JA, Brown GW, Rouse J (2008). Budding yeast Mms22 and Mms1 regulate homologous recombination induced by replicase blockage. DNA Repair (Amst) 7, 811–818.
Feng W, Collingwood D, Boeck ME, Fox LA, Alvino GM, Fangman WL, Raghruraman MK, Brewer BJ (2006). Genomic mapping of single-stranded DNA in hydroxyurea-challenged yeasts identifies origins of replication. Nat Cell Biol 8, 148–155.
Fujii K, Kitabatake M, Sakata T, Miyata A, Ohno M (2009). A role for ubiquitin in the clearance of nonfunctional rRNAs. Genes Dev 23, 1663–1674.
Gambus A, Jones RC, Sanchez-Diaz A, Kanemaki M, van Deursen F, Edmondson RD, Labb K (2006). GINS maintains association of Cdc45 with MCM in replisome progression complexes at euayotic DNA replication forks. Nat Cell Biol 8, 358–366.
Gambus A, van Deursen F, Polychronopoulous D, Foltman M, Jones RC, Edmondson RD, Calzada A, Labb K (2009). A key role for Ctf4 in coupling the MCM2-7 helicase to DNA polymerase alpha within the euayotic replisome. EMBO J 28, 2992–3004.
Higa LA, Mihaylov IS, Banks DP, Zheng J, Zhang H (2003). Radiation-mediated proteolysis of CDT1 by CUL4-ROC1 and CSN complexes constitutes a new checkpoint. Nat Cell Biol 5, 1008–1015.
Hiraga S, Higahara-Hayashi A, Ohya T, Sugino A (2005). DNA polymerases alpha, delta, and epsilon localize and function together at replisomes in Saccharomyces cerevisiae. Genes Cells 10, 297–309.
Ho Y et al. (2002). Systematic identification of protein complexes in Saccharomyces cerevisiae by mass spectrometry. Nature 415, 180–183.
Hryciw T, Tang M, Fontanie T, Xiao W (2002). MMS1 protects against replication-dependent DNA damage in Saccharomyces cerevisiae. Mol Genet Genomics 266, 848–857.
Jackson S, Xiong Y (2009). CRL4s: the CUL4-RING E3 ubiquitin ligases. Trends Biochem Sci 34, 562–570.
Kapetanaki MG, Guerrero-Santoro J, Bisi DC, Hsieh CL, Rapic-Otrin V, Levine AS (2006). The DDB1-CUL4A-DDB2 ubiquitin ligase is deficient in xeroderma pigmentosum group E and targets histone H2A at UV-damaged DNA sites. Proc Natl Acad Sci USA 103, 2588–2593.
Katou Y, Kaneshiro K, Aburatani H, Shirahige K (2006). Genomic approach for the understanding of dynamic aspect of chromosome behavior. Methods Enzymol 409, 389–410.
Katou Y, Kanoh Y, Bando M, Noguchi H, Tanaka H, Ashikari T, Sugimoto K, Shirahige K (2003). S-phase checkpoint proteins Tof1 and Mrc1 form a stable replication-pausing complex. Nature 424, 1078–1083.
Koh JL, Ding H, Costanzo M, Banyashtikova A, Toufighi K, Bader GD, Myers CL, Andrews BJ, Boone C (2010). DYGRIN: a database of quantitative genetic interaction networks in yeast. Nucleic Acids Res 38, DS50–507.
Kroeko LF, Brown NC, Reichard P (1968). Inhibition of ribonucleoside diphosphate reductase by hydroxyurea. Cancer Res 28, 1559–1565.
Lemoine FJ, Degtyarneva KP, Kokosja R, Petes TD (2008). Reduced levels of DNA polymerase delta induce chromosome fragile site instability in yeast. Mol Cell Biol 28, 5357–5368.
Lengronne A, McIntyre J, Katou Y, Kanoh Y, Hopfner KP, Shirahige K, Uhmann F (2006). Establishment of sister chromatid cohesion at the S. cerevisiae replication fork. Mol Cell 23, 787–799.
Leung-Pineda V, Huh J, Pwncna-Worms H (2009). DBD1 targets Chk1 to the CUL4 E3 ligase complex in normal cycling cells and in cells experiencing replication stress. Cancer Res 69, 2630–2637.
Lopes M, Cotta-Ramusino C, Pellicioli A, Liberi G, Plevani P, Musi-Falconi M, Newlon CS, Frioni M (2001). The DNA replication checkpoint response stabilizes stalled replication forks. Nature 412, 557–561.
Lou H, Komata M, Katou Y, Guan Z, Reis CC, Budd M, Shirahige K, Campbell JL (2008). Mcr1 and DNA polymerase epsilon function together in linking DNA replication and the S phase checkpoint. Mol Cell 32, 106–117.
Luke B, Versini G, Jaquenoud M, Foltman M, Jones RC, Peter M (2006). The cullin Rtt101p promotes replication fork progression through damaged DNA and natural pause sites. Curr Biol 16, 748–752.
Michel JJ, McCanvile JF, Xiong Y (2003). A role for Saccharomyces cerevisiae CUL8 ubiquitin ligase in proper anaphase progression. J Biol Chem 278, 22828–22837.
