Characterization of a Novel MMS-Sensitive Allele of 
Schizosaccharomyces pombe mcm4⁺

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ABSTRACT The minichromosome maintenance (MCM) complex is the conserved helicase motor of the eukaryotic replication fork. Mutations in the MCM4 subunit are associated with replication stress and double strand breaks in multiple systems. In this work, we characterize a new temperature-sensitive allele of Schizosaccharomyces pombe mcm4⁺. Uniquely among known mcm4 alleles, this mutation causes sensitivity to the alkylation damaging agent methyl methanesulfonate (MMS). Even in the absence of treatment or temperature shift, mcm4-c106 cells show increased repair foci of RPA and Rad52, and require the damage checkpoint for viability, indicating genome stress. The mcm4-c106 mutant is synthetically lethal with mutations disrupting fork protection complex (FPC) proteins Swi1 and Swi3. Surprisingly, we found that the deletion of rif1⁺ suppressed the MMS-sensitive phenotype without affecting temperature sensitivity. Together, these data suggest that mcm4-c106 destabilizes replisome structure.

The MCM helicase comprises six related proteins (Mcm2–7) that form a highly conserved heterohexameric ring functioning as the primary unwinding activity in the eukaryotic replisome (reviewed in Abid Ali and Costa 2015; O’Donnell and Li 2016). The loading of the MCM complex specifies potential replication origins. Replication initiation occurs with assembly of active replicative helicase known as CMG (Cdc45-Mcm-GINS). The helicase travels with other replisome components (Gambus et al. 2009). Recent studies examining the structure of the active CMG complex have provided insight into its mechanism. Cdc45 and GINS are activating agents (Moyer et al. 2006; Ilves et al. 2010) that bind at the Mcm2-Mcm5 “gate” where the MCM ring opens and closes around DNA (Costa et al. 2011, 2014; Sun et al. 2015). CMG makes direct contacts at the leading C-terminal side of the MCM ring with DNA polymerase α, which is the processive leading strand polymerase (Langston et al. 2014; Sun et al. 2015). The DNA polymerase α/primase complex that initiates lagging strand synthesis is coupled to CMG via a trimeric protein called Mc1 (Sc Clp4, Hs AND-1) (Simon et al. 2014). Another conserved protein, Mrcl (Hs Clapsin), is thought to help maintain the coupling with DNA polymerase Epsilon (Liu et al. 2008). The Swi1-Swi3 complex (Hs Timeless-Tipin, Sc Tof1-Csm3), called the Fork Protection Complex (FPC), also acts with Mrcl and travels with the replisome (reviewed in Leman and Noguchi 2012). The FPC is not essential for viability in the yeasts, but in its absence, cells show uncoupling of the replisome and increased ssDNA formation, disruption in activation of the replication checkpoint, sensitivity to DNA damaging agents, and defects in cohesion (reviewed in Leman and Noguchi 2012).

The Mcm4 subunit resides on the opposite side of the MCM ring from the Mcm2–5 gate that binds Cdc45 and GINS, near the proposed lagging strand template (reviewed in O’Donnell and Li 2016). Interestingly, numerous mutations in this subunit have been linked to genome instability in mammalian systems. The point mutation F345I (chaos3), located downstream of the Zn-finger motif of Mcm4 in mouse, is associated with mammary carcinoma (Shima et al. 2007). The mcm4-D573H mutation is associated with T cell lymphoblastic leukemia/lymphoma in a mouse model (Bagley et al. 2012), and mcm4-G364R in humans is associated with skin cancer (Ishimi and Irie 2015). All these mutations are related with increased double strand breaks, and in some cases formation of micronuclei. N-terminally truncated Mcm4 (Δ1–50) is linked to glucocorticoid deficiency and defective DNA repair in humans (Hughes et al. 2012; Gineau et al. 2012). Although the primary sequence of the Mcm4 N-terminus is neither conserved nor essential, this domain appears to be a common substrate for the DDK kinase required to initiate replication (Masai et al. 2006; Sheu and Stillman 2006). In budding yeast, deletion of the N-terminus bypasses a requirement for DDK, suggesting
that DDK overcomes an inhibitory function (Sheu and Stillman 2010). The N-terminus is also important for regulating fork progression when cells have depleted nucleotide pools during hydroxyurea (HU) treatment (Devault et al. 2008; Sheu et al. 2014). C-terminal truncations of Mcm4 also cause HU sensitivity, and fail to restrain single-stranded DNA accumulation (Nitani et al. 2008).

The fission yeast mcm4+ (edc21+) gene was originally identified in a screen for temperature-sensitive cdc mutants that arrest as elongated cells with undivided nuclei at the restrictive temperature (Nasmith and Nurse 1981; Coxon et al. 1992). These mcm4-M68 cells accumulate approximately 2C DNA content, and show evidence of DNA damage including DNA double strand breaks and generating a robust checkpoint-dependent arrest (Nasmith and Nurse 1981; Coxon et al. 1992; Liang et al. 1999; Bailis et al. 2008; Sabatinos et al. 2015). Viability is low upon return to permissive temperature, suggesting that this damage is irreversible (Liang et al. 1999; Bailis et al. 2008). The 2C DNA content observed in mcm4-M68 suggests that cells are competent for replication initiation and the bulk of DNA replication at the restrictive temperature. A second temperature-sensitive allele was constructed by fusing a degron cassette to mcm4-M68 (Lindner et al. 2002). This enhances protein turnover leading to a rapid inactivation at restrictive temperature, and cells arrest with a 1C DNA content (Lindner et al. 2002; Bailis et al. 2008; Sabatinos et al. 2015). However, despite evidence of DNA damage including large RPA and Rad52-containing megafoci, mcm4-dg cells continue to divide, indicating that they have evaded the damage checkpoint (Sabatinos et al. 2015). Survivors show dramatic evidence for chromosome mis-segregation, abnormal nuclear structure, causing genome instability.

MATERIALS AND METHODS

Cell growth and cultures

Fission yeast strains are listed in Table 1. All strains were maintained according to standard protocols (Sabatinos and Forsburg 2010). Strains were grown in YES or Edinburgh minimal medium (EMM) with ammonium chloride as the nitrogen source, supplemented with the required nutrients at 25°C unless otherwise stated. For all experiments, cultures were grown in 5 ml of liquid media from a single colony at 25°C overnight and released to fresh media and grown at 25°C to midlog phase. Serial dilutions and plating assays were performed in cultures grown in YES, while the imaging experiments were performed in cultures grown in EMM.

Serial dilution assays and relative viability

For serial dilutions, cell cultures were grown in 5 ml of YES from a single colony at 25°C overnight, to mid-exponential phase. Cells were counted and fivefold serial dilutions were spotted onto plates to assess drug or temperature sensitivity. Drug plates were allowed to grow for 3–5 d at 25°C before scanning on a flatbed scanner. The experiments were repeated at least twice. For relative viability, cells at OD_{595} ~0.3 were treated with 0.01% MMS for 4–6 hr or shifted to 36°C for 4–6 hr (as indicated in the figure legend). Samples were collected every 2 hr and fixed in 70% ethanol for Flow Cytometry (FACS) and DNA staining with DAPI. Serial dilutions of equal volumes were plated at selected time points and allowed to grow at 25°C for 5 d before counting viable colonies.

Protein extractions

Western blot analysis was performed using cultures grown to early log phase (OD_{595} ~0.3) in YES at 25°C. Cultures were split into equal volumes and treated with 0.01% MMS or left untreated for 4 hr at 25°C. 10 × stop buffer containing 2% sodium azide was added and cultures were incubated on ice for 10 min before harvesting the cells. Cells were subsequently washed twice with 1 × PBS and whole cell proteins were extracted using trichloric acid (TCA) (Foiani et al. 1994). The extractions were quantified using a Pierce BCA Kit. 80–100 μg of protein was loaded on 8% SDS-PAGE gels. Primary antibody for Chk1HA (16B12 anti-HA; Covance or anti-HA; Abcam) were used in 1:1500 dilutions overnight at 4°C. Mcm4 protein levels were detected with antibody purified from rabbit serum S898 diluted 1:5000 (Sherman et al. 1998) incubated at 4°C overnight. After washing with PBST, anti-mouse-IgG-HRP secondary antibody (Sigma) was used to detect HA in a 1:5000 dilution, while a 1:5000 dilution of anti-rabbit-HRP (BD Biosciences) incubated for 1 hr at room temperature was used in Mcm4 detection. 1:1500 PCNA anti-mouse (Santa Cruz) was used as the loading control.

Pulse field gel electrophoresis (PFGE)

PFGE was performed to separate full-length chromosomes using a BioRad Chef II Pulse Field Machine. 50 ml cultures grown to early log phase (OD_{595} ~0.3–0.4) were shifted to 36°C for 4 hr and released to permissive temperature 25°C for 2 hr. For the MMS treatment, the cells were treated with 0.01% MMS for 4 hr and released to media lacking MMS for 2 hr for recovery after washing out the drug from the cultures. Cells were treated with 10 × stop buffer containing 2% sodium azide and placed on ice for 5 min before harvesting the cells. Harvested cells were washed with 1 × PBS and CSE buffer (20 mM Citric Acid, 20 mM Na_{2}HPO_{4}, 40 mM EDTA, and 1.2 M sorbitol; at pH 5.6, sterilized, and stored at room temperature). Each culture was digested with 0.2 mg/ml 20T Zymolase and 0.45 mg/ml lysing enzyme (Sigma) in CSE. Digested cells were used to prepare plugs that were resuspended in 1 × TSE (10 mM Tris pH 7.5, 45 mM EDTA pH 8.0, and 0.9 M Sorbitol). Plugs were treated with 5 ml of 1 mg/ml protease K Sarkosyl/EDTA at 55°C for 48 hr (1% Sarkosyl and 0.5 M EDTA pH 9.5). The buffer was changed after 24 hr of incubation and washed four times (30 min each) with 1 × TE. Plugs were washed with 1 × TAE prior to running the gels. Gels were run for 48 hr using 2 V/cm, 1200–1800-sec switch time, and a 106° angle. DNA was visualized via ethidium bromide staining.

FACS

FACS was performed as described in (Sabatinos and Forsburg 2015). Briefly, cells were fixed in 70% ice cold ethanol, washed with 50 mM sodium citrate, and resuspended in 50 mM sodium citrate with 0.1 mg/ml RNase. Samples were next stained with 1 μM Sytox Green (Invitrogen) in 50 mM sodium citrate, and sonicated at 200 amplitude for 5 sec. Samples were analyzed by running on a Becton Dickinson FACScan flow cytometer.

Microscopy

Cultures were grown in EMM supplement with ammonium chloride. Agar pads were prepared as described in Green et al. (2015). Images of live cells were acquired with a DeltaVision Core (Applied Precision, Issaquah, WA) microscope using a 60 × N.A. 1.4 PlanApo objective lens and a 12-bit Photometrics CoolSnap HQII CCD. The system
### Table 1 Strains used in this study

| Strain     | Genotype                                                                 | Source          |
|------------|---------------------------------------------------------------------------|-----------------|
| FY 7       | h- 972                                                                   | Our stock       |
| FY 527     | h-his3-D1 ade6-M216 ura4-D18 leu1-32                                       | Our stock       |
| FY 528     | h+ his3-D1 ade6-M210 ura4-D18 leu1-32                                      | Our stock       |
| FY 261     | h+ can1-1 leu1-32 ade6-M214 ura4-D18                                      | Our stock       |
| FY 784     | h+ cdc21-106:kan                                                         | Takuro Nakagawa |
| FY 4241    | h+ cdc21-106:kan                                                         | This work       |
| FY 4311    | h- cdc21-106:kan ura4-D18 his3-D1 ade6-M210                               | This work       |
| FY 4310    | h- cdc21-84:kan Ura4-D18 his3-D1                                         | This work       |
| FY 5942    | h- cdc21-106::HphMX ura4-D18 his3-D1 ade6-M210                            | This work       |
| FY 3395    | h- cdc21-106::HphMX ts-dg::ura4+ ura4-D18                                 | This work       |
| FY 6126    | h+ cdc21-106::kan ura4-D18 his3-D1 ade6-M210                              | This work       |
| FY 6038    | h- pcn1-K164R::ura4 cdc21-106::kan ura4-D18 ade6-M210                     | This work       |
| FY 6039    | h+ kbp1::bleMX6 cdc21-106::kan his4-239/his3-D1 ade6-M26 (kbp1)           | Our stock       |
| FY 6040    | h+Δhph18::ura4 cdc21-106::kan ura4-D18 leu1-32 ade6-M210                  | This work       |
| FY 6041    | h+Δreb1::kanMX cdc21-106::HphMX his 3-D1                                  | This work       |
| FY 6043    | h+ cdc21-106::kan cyc17::ura4 his3 D-1                                     | This work       |
| FY 6044    | h+ cdc21-106::kan cyc17::URA4 his3 D-1                                    | This work       |
| FY 6052    | h+ Δrev1::ura4+ cdc21-106::kan ura4-D18 his3-D11 ade6-M216/210?ura4-D18  | This work       |
| FY 6053    | h+Δrev1::ura4+ cdc21-106::kan ura4-D18 his3-D12 ade6-M216                 | This work       |
| FY 6054    | h- eso1-eta::kanMX cdc21-106::HphMX ura4-D18 his3-D11 ade6-M210           | This work       |
| FY 6055    | h- eso1-eta::kanMX cdc21-106::HphMX ura4-D18 his3-D11 ade6-M210 Leu1-32  | This work       |
| FY 6077    | h+Δrad8::HphMX cdc21-106::kan ura4-D18 his3-D11 ade6-M216 leu1-32         | This work       |
| FY 6078    | h+Δorc::ura4+ cdc21-106::kan ura4-D18 ade6-M210                           | This work       |
| FY 6079    | h+ Δorc::ura4+ cdc21-106::kan ura4-D18 his 3-D12 ade6-M210                | This work       |
| FY 6080    | h+ Δubc13::ura4+ cdc21-106::kan his3-D1 ura4-D18 ade6-210                 | This work       |
| FY 6123    | h+ cdc21-106::kan rev3::hphMX6 ura4-D18 his3-D12 ade6-M210                | This work       |
| FY 6146    | h+rns2::kan cdc21-106::HphMX ade6-M210 ura4-D18 his3-D12                  | This work       |
| FY 6147    | h+rns2::kan cdc21-106::HphMX ade6-M210 ura4-D18 his3-D12                  | This work       |
| FY 6238    | h+ cdc21-106::HphMX ura4-D18 his3-D11                                     | This work       |
| FY 6248    | h+Δ mms2::leu2 cdc21-106::kan ura4-D18 leu1-32 his3-239 ade6-M26          | This work       |
| FY 6266    | h+ cdc21-106::kan rad11-Cereulean::HphMX rad22-YFP-natMX ura4-D18 leu1-32| This work       |
| FY 6268    | h+ cdc21-106::kan chk1HAE(ep) ade6-M216 ura4-18 leu1-32 his3-D1            | This work       |
| FY 6308    | h+ cdc21-106::kan Δcds1::ura4+ ura4-D18 leu1-32 his3-D1 ade6-M210         | This work       |
| FY 6309    | h+ cdc21-106::kan Δcds1::ura4+ ura4-D18 his3-D1 ade6-M210                 | This work       |
| FY 6751    | h+ cdc21-106::kan mad2D::ura4+ ade6-M210 ura4-D18 leu1-32 ura4-D18        | This work       |
| FY 6777    | h+ cdc21-106::HphMX Δrnfl::kanMX6-Bioneur ura4-D18 ade6-M210 his3-D1      | This work       |
| FY 6778    | h+ cdc21-106::HphMX Δrnfl::kanMX6-Bioneur ura4-D18 ade6-M210 his3-D1      | This work       |
| FY 6779    | h+ cdc21-106::kan exol::ura4 ura4-D18 ura4-D18 ade6-M210                  | This work       |
| FY 6780    | h+ cdc21-106::kan exol::ura4 ura4-D18 ura4-D18 ade6-M210                  | This work       |
| FY 6961    | h+Δ swi6::ura4+ cdc21-106::kan ade6-12 ura4- (DS or D18?) ade6-M210 "can1-1" | This work       |
| FY 7045    | h+ cdc21-106::kan flm1::natMX4 ura4-D18 his3-D11 leu1-32 ade6-M210/216   | This work       |
| FY 7047    | h+ cdc21-106::kan flm1::natMX4 ura4-D18 his 3-D-1 leu1-32 ade6-M210/216   | This work       |
| FY 7048    | h+ cdc21-106::kan flm1::natMX4 ura4-D18 his3-D11 leu1-32 ade6-M210/216   | This work       |
| FY 7165    | h+Δ mus81::kanMX cdc21-106::HphMX ura4-D18 his3-D1 ade6-M210              | This work       |
| FY 7166    | h+Δ mus81::kanMX cdc21-106::HphMX ura4-D18 his3-D1 ade6-M210              | This work       |
| FY 7461    | h+ mcl1-11 cdc21-106::kan ade6-M210 ura4-D18 ade6-M210 his3D-1             | This work       |
| FY 7462    | h+ mcl1-11 cdc21-106::kan ade6-M210 ura4-D18 ade6-M210 his3D-1             | This work       |
| FY 7611    | h+ rhp5::ura4+ cdc21-106::kan ade6-M210 ade6-M210 ura4-D18                | This work       |
| FY 7802    | h+Δchfl::kanMX6-Bioneur cdc21-106::HphMX his3-D11 leu1-32 ura4-D18 ade6-M210/216 | This work       |
| FY 7922    | h+ arg3::cccN1-mCherry(D817 aa1-275):his5+ cdc21-106::kan rad11-Cereulean::hphMX rad22-YFP-NATMX ura4-D18 his5D leu1-32 ade6-M210 | This work       |
| FY 7923    | h+Δrnfl::ura4+ Δswi6::kanMX ura4-D18 leu1-32 ade6-M210 his3-D1             | This work       |
| FY 7924    | h+Δrnfl::ura4+ Δswi6::kanMX ura4-D18 leu1-32 ade6-M210 his3-D1             | This work       |
| FY 7925    | h+Δrnfl::ura4+ Δswi6::kanMX ura4-D18 leu1-32 ade6-M210 his3-D1             | This work       |
| FY 7926    | h+Δrnfl::ura4+ Δswi6::kanMX ura4-D18 leu1-32 ade6-M210 his3-D1             | This work       |
| FY 3664    | h+ mcm4::cmpr::ura4-D18 leu1-32 ade6-M210                                  | Our Stock       |
| FY 8000    | h+ Δhht1-mRFP::kanMX his7::lacGFP lys1::lacO cdc21-106::HphMX leu1-32 ura4-D18 | This work       |

(continued)
Table 1, continued

| Strain  | Genotype                                      | Source            |
|---------|-----------------------------------------------|-------------------|
| FY 8015 | h+ Δctf8::kanMX6-Bioneer cdc21-c106::HphMX his 3-D1 ura4-D18 Leu1-32 ade6-M210 | This work         |
| FY 8016 | h- Δctf8::kanMX6-Bioneer cdc21-c106::HphMX his 3-D1 ura4-D18 Leu1-32 ade6-M210 | This work         |
| FY 8017 | h+ Δctf8::kanMX6-Bioneer cdc21-c106::HphMX ura4-D18 Leu1-32 ade6-M210 | This work         |
| FY 8018 | h+Δct8::kanMX6-Bioneer cdc21-c106::HphMX ura4-D18 Leu1-32 ade6-M210? | This work         |
| FY 7689 | h+ Δctf8::kanMX6-Bioneer ura4-D18 Leu1-32 ade6-M210? | Our stock/Bioneer derived |
| FY 7690 | h+ Δctf8::kanMX6-Bioneer ura4-D18 Leu1-32 ade6-M210? | Our stock/Bioneer derived |
| FY 8107 | h+Δ? cdc20-M10 cdc21-c106::kan ura4-D18 ade6-M210 leu1-32 his3-D1 (pol1) | This work |
| FY 7808 | h+ rad21-K1::ura4+ cdc21-c106::kan ura4-D18 leu1-32 ade6-M216 his7-366/3 D1-1 | This work |
| FY 8108 | h+Δ?Δrad22::ura4+cdc21-c106::kan ura4-D18 leu1-32 his3-D1 arg3-D4 | This work |
| FY 5014 | h+ pcn1-K164R::ura4 ura4-D18 Leu1-32 ade6-M210 | Our stock |
| FY 5214 | h+ kpa1Δ::bleMX6 his4-239 ade6-M26 | Our stock |
| FY 5324 | h+Δhph18::ura4+ ura4-D18 Leu1-32 ade6-704 | Our stock |
| FY 4415 | h+Δreb1::kanMX ade6-M216 ura4-D18 leu1-32 | Our stock |
| FY 277  | h+ cycl7::ura4 ade6-M216 leu1-32 ura4 cycl17 is allelic to cig2 | Hiroto Okayama |
| FY 5401 | h+Δrev1::ura4+ ura4-D18 his4-239 ade6-M26 | Our stock |
| FY 4937 | h+ eso1::kanMX6 ura4-D18 leu1-32 ade-m210 | Our stock |
| FY 5142 | h+Δbrc1::ura4+ ura4-D18 leu1-32 ade6-M210 | Our stock |
| FY 4938 | h+ rev3::hphMX ura4-D18 leu1-32 ade6-M210 | Our stock |
| FY 2050 | h+Δsrs2::kan ade6-M210 Leu1-32 ura4-D18 | Our stock |
| FY 5260 | h+ Δmms2::leu2 leu1-32 his4-239 ade6-M26 | Our stock |
| FY 5625 | h+Δrad8::hphMX leu1-32 ura4-D18 ade6-M216 his3-D1 | Our stock/Bioneer derived |
| FY 4742 | h- rad11-Cerulean::hphMX rad22-YFP::natMX leu1-32 ade6-M210 ura4-D18 | (rad11 = ssb1) |
| FY 4611 | h- chic1-K1A(ep) ade6-M216 ura4-D18 leu1-32 | Our stock |
| FY 1163 | h- rad12::ura4+ ade6-M210 leu1-32 ura4-D18 | Our stock |
| FY 3845 | h- leu1-32::HENT-leu1+(pJAH29) his7-366::hsv-tk- his7+(pJAH31) ura4-D18 ade6-M216 | Our stock |
| FY 1257 | h+Δmad2::ura4+ ade6-M210 leu1-32 ura4-D18 | Shelly Sazer |
| FY 5583 | h+Δrif1::kanMX6-Bioneer leu1-32 ura4-D18 ade6-M210 his3-D1 | Our stock/Bioneer derived |
| FY 3884 | h- exo1::ura4 ura4-D18 | Mathew O’Connell |
| FY 2389 | h- leu1-32 ura4-D18 Δrif1::ura4+ | Junko Kanoh |
| FY 5555 | h-Δmfl1::matMX ura4-D18 his3-D1 leu1-32 | Our stock |
| FY 4581 | h- cfp1::kanMX6-Bioneer leu1-32 ura4-D18 ade6-M216 his3-D1 | Our stock |
| FY 44159| h+Δmus81::kanMX | Our stock |
| FY 11911| h+ mcl1-11 ade6-704 ura4-294 leu1-32 (ts) | Dwight Williams |
| FY 1203 | h+ rhp51::ura4+ ura4-D18 leu1-32 ura4-D18 | Greg Freyer |
| FY 1318 | h+ rec8::ura4+ ura4-D18 leu1-32 ade6-M210 | Our Stock |
| FY 1159 | h- rad21-K1::ura4+ ura4-D18 leu1-32 ade6-M216 his7-366 | Our stock |
| FY 3588 | h- arg3::::ccr1N-mCherry(D817 aa1-275)::his5+ ura4-D18 his5D | Zach Cande/Xie Tang |
| FY 3227 | h+ Δswil::kanMX ura4-D18 leu1-32 ade6-M210 his3-D1 | Our Stock |
| FY 3228 | h+Δswi3::kanMX ura4-D18 leu1-32 ade6-M210 his3-D1 | Our Stock |
| FY 7995 | h- arg3::::ccr1N-mCherry(D817 aa1-275)::his5+ rad11-1-Cerulean::hphMX rad22- YFP::kanMX ura4-D18 his5D leu1-32 | This work |
| FY 5787 | h+ hht1-mRFP::kanMX his7::lacI-GFP lys1::lacO leu1-32 ura4-D18 | Our stock |
| FY 7653 | h+ Δctf8::kanMX6-Bioneer his 3-D1 ura4-D18 Leu1-32 ade6-M210? | Our stock/Bioneer derived |
| FY 8017 | h+ Δctf8::kanMX6-Bioneer cdc21-c106::HphMX ura4-D18 Leu1-32 ade6-M210 | This work/Bioneer derived |
| FY 8110 | h+psf2-209 cdc21-c106::kan ura4-D18leu1-32 ade6-M216 | This work |
| FY 8111 | h+Δpsf2-209 ura4-D18 ade6-M216 leu1-32 | This work |
| FY 2797 | h+ Δpsf2-209 ura4-D18 ade6-M216 leu1-32 | Our stock |
| FY 3999 | h+ Δpsf2-209 allelic to dp1 | Our stock |
| FY 8197 | h+ pol-1Δ::ccr1N::kan ura4-D18 leu1-32 ade6-M210 his3-D1 | This work |
| FY 1110 | h+ pol-1Δ::ura4 ura4-D18 leu1-32 ade6-M210 | Our stock |

x-y pixel size is 0.109 μm. SoftWoRx v4.1 (Applied Precision, Issaquah, WA) software was used at acquisition. The image acquisition consisted of 13 Z-stacks with 0.5 μm for visualizing Rad11 and Rad52 foci at 36°C and MMS. Cells were visualized at the asynchronous stage; 4 hr post-treatment and 2 hr postrelease from the treatment. Movies were captured to look at the replication dynamics in real time. Eighteen Z-stacks with 0.5 μm were acquired 10 min apart for the length of the experiment. The temperature was controlled at 25°C if not specified. For still imaging, CFP was excited and detected with an (ex)438/24, (em)470/24 filter set and a 0.5 sec exposure excitation intensity attenuated to 10%; and YFP was excited and detected with an (ex)513/17, (em)595/38 filter set and a 0.5 sec exposure excitation intensity attenuated to 32%. Suitable polychroic mirrors were used. Ten 0.5 μm serial z-sections were captured. 3-D stacks were deconvolved with manufacturer-provided OTFs using a
RESULTS

Identification of an MMS-sensitive allele of mcm4+ 

Mutants with defects in replisome components often show sensitivity to DNA damaging agents, but not all mutants are sensitive to all drugs. For example, in a recent study, we showed that cells deleted for nonessential helicases have distinct patterns of genotoxin sensitivity that establish a fingerprint for their roles in DNA replication and repair (Ding and Forsburg 2014). Here, we analyzed a panel of strains with different mutations in the essential mcm4+ gene for their sensitivity to different damaging agents, including: HU, which depletes nucleotide pools and causes fork stalling (Thelander and Reichard 1979); MMS, which is an alkylating agent that generates diverse lesions that block DNA polymerase (Lundin et al. 2005); and camptothecin (CPT), a topoisomerase inhibitor that leads to S-phase chromosome breaks (Liu et al. 2000).

We examined the known temperature-sensitive alleles mcm4-M68, mcm4-dg, and mcm4-c106 (Nasmyth and Nurse 1981; Lindner et al. 2002; Nitani et al. 2008). The remaining mutants that we tested included a deletion of the N-terminal residues 2–73, a single point mutation (F346I) corresponding to the chaos allele in mouse (J. P. Yuan and S. L. Forsburg, unpublished data; Shima et al. 2007), and mcm4-4S4A, which contains mutations in putative damage-specific phosphorylation sites S30A, S38A, S81A, and T95A (Figure 1A). As observed previously, some mcm4 mutants show sensitivity to HU, including the temperature-sensitive degren allele mcm4-dg, (Supplemental Material, Figure S1A) and the C-terminal truncation alleles mcm4-c84 and mcm4-c106 (Nitani et al. 2008). We did not observe CPT sensitivity in any of the strains (Figure S1B). Unexpectedly, however, we observed that the temperature-sensitive mcm4-c106 truncation is also sensitive to MMS exposure at the permissive growth temperature, which is not seen for any other mcm4 alleles (Figure 1B). Importantly, temperature-sensitive and MMS-sensitive phenotypes were not observed for the mcm4-c84 truncation, which contains a shorter truncation (Nitani et al. 2008). These results indicate that a larger C-terminus of Mcm4 is necessary for a proper response to MMS.

In general, sensitivity to MMS is observed in strains defective in checkpoint response or repair, and mutations that disrupt a distinct subset of replisome components. These include mutations affecting the fork protection complex (FPC) proteins Swi1 and Swi3, and the MCM kinase Hsk1/DDK that interacts with FPC (Mimosoglu and Samson 2000; Noguchi et al. 2003, 2004; Kumar and Huberman 2004; Sommariva et al. 2005; Dolan et al. 2010). Given that mcm4-c106 shows sensitivity to MMS as well as to higher temperatures, we investigated both these phenotypes.

mcm4-c106 cells have a unique replication phenotype

There is no obvious difference in growth rate between wild-type and mcm4-c106 cells at permissive temperature (Figure 1A). To assess whether there is a subtle replication defect, we performed a classic minichromosome maintenance assay (Tye 1999). We transformed the wild-type, mcm4-M68, and mcm4-c106 strains with a plasmid (pUR19N; Barbet et al. 1992) containing a single copy of Schizosaccharomyces pombe ars1 and compared the number of transformants/μg DNA (transformation efficiency) and plasmid stability (colony size). We found that both the number of transformed cells and the size of the colonies were reduced in mcm4-c106 compared to either mcm4-M68 or the wild type at permissive temperature (Figure 2). Additionally, we examined a plasmid with an additional ars (pDBet), which is intrinsically more stable (Brun et al. 1995). These transformants were larger than when transformed with a single ars plasmid (data not shown). These data suggest that mcm4-c106 suffers a defect in replication, even at permissive temperature.

At 36°C, the canonical mcm4-M68 allele loses viability rapidly, with signs of DNA damage (Liang and Forsburg 2001; Bailis et al. 2008; Sabatinos et al. 2015). We examined the relative viability of mcm4-c106 following a shift to the restrictive temperature, and found that the loss of viability was more modest compared to mcm4-M68 (Figure 3A). We examined DNA accumulation using flow cytometry on cells that were arrested in G1 by nitrogen starvation, and released to the permissive temperature (25°C) and the restrictive temperature (36°C) (Figure 3B). We observed DNA accumulation to approximately 2C DNA content in both wild-type and mcm4-c106 cells, even at restrictive (36°C) and non-restrictive (25°C) temperatures. This is similar to observations for the original mcm4-M68 temperature allele, which has a late S phase arrest (Nasmyth and Nurse 1981; Coxon et al. 1992; Bailis et al. 2008; Sabatinos et al. 2015).

However, the chromosome profiles observed in PFGE were strikingly different between these two mcm4 alleles. Typically, the chromosomes from cells with replication defects do not migrate normally at restrictive
temperature, either due to unresolved replication or recombination intermediates that preclude migration, or due to chromosome breakage (e.g., Liang and Forsburg 2001; Waseem et al. 1992). Thus, as seen previously, the mcm4-M68 chromosomes do not migrate at their normal position during a 36° temperature shift or upon release to 25°, but are replaced by a smear (Figure 3C, middle lanes). This is consistent with unresolved replication intermediates retarding gel migration, and the accumulation of double strand breaks as reported previously (Liang and Forsburg 2001; Bailis et al. 2008; Sabatinos et al. 2015). In contrast, mcm4-c106 showed intact chromosomes under all conditions (Figure 3C, right lanes). This, along with the maintenance of viability and the ability to recover from temperature arrest, suggests that the nature of the temperature-sensitive defect in mcm4-c106 is different from that of the well-studied mcm4-M68.

We observed loss of viability of mcm4-c106 cells treated with MMS (Figure 3D) consistent with the MMS sensitivity observed in plate assays (Figure 1B). The loss of viability was relatively modest compared to a repair-defective allele of PCNA, pcm1-K164R (Frampton 2006). During MMS treatment in liquid culture at the permissive temperature, both wild-type and mcm4-c106 mutants showed an S phase delay, as indicated by the intermediate peak that is observed in the FACS profiles (Figure 3B). In both wild-type and mcm4-c106 cells treated with MMS and following release, we observed little if any migration of intact chromosomes into a PFGE gel (Figure S2).

Finally, we examined Mcm4 protein levels in the mutant. Loss of Mcm4 protein has been correlated with genomic instability (Liang and Forsburg 2001; Bailis et al. 2008; Sabatinos et al. 2015). However, we saw no change in Mcm4 protein levels during MMS treatment or at 36° in mcm4-c106 (Figure 4, C and D), suggesting that its temperature sensitivity and MMS phenotypes are not related to protein stability.

**Chromosome segregation is normal in mcm4-c106**

Recently, we showed that mcm4 dg mutants undergo division despite their replication defects, and this is accompanied by aberrant nuclear division, abnormal chromosome segregation, and reduced viability (Sabatinos et al. 2015). In contrast, we saw no evidence for abnormal mitosis in mcm4-c106 cells. We determined segregation of chromosome I, using a lacI-GFP fusion in a strain with a lacO array at centromere I to generate a centromere proximal signal (Nabeshima et al. 1998). We observed no evidence for lagging chromosomes, or chromosome mis-segregation, indicating no substantial mitotic defects in mcm4-c106 (Figure S3).

**mcm4-c106 requires an intact damage checkpoint**

The mcm4-c106 cells elongate following treatment at 36° or in MMS, which suggests successful activation of the damage checkpoint. We verified this by monitoring the checkpoint kinase Chk1, which undergoes an activating phosphorylation that results in a mobility shift in SDS-PAGE (Walworth and Bernards 1996). We observed a shift in Chk1 in both wild-type and mcm4-c106 cells treated with MMS (Figure 4A), and in wild-type and mcm4-c106 cells at the restrictive temperature (Figure 4B), consistent with successful activation of Chk1 under both conditions in the mutant.
We observed no evident Chk1 phosphorylation in asynchronously growing cultures at 25°C in the absence of treatment (Figure 4, A and B). Despite this, we found that double mutants between the mcm4-c106 and either rad3Δ or chk1Δ were inviable. However, double mutants with the S phase checkpoint mutant ads1Δ were viable (Table 2). We conclude that, even though we do not observe shifted mobility of Chk1 at permissive temperature, there is sufficient stress even in unperturbed mcm4-c106 cells to cause them to depend upon the damage checkpoint for viability.

**Repair foci accumulate in mcm4-c106**

Previously, we examined replication stress by examining the accumulation of repair foci corresponding to the single strand DNA binding protein RPA (labeled with CFP), or the recombination protein Rad52 (labeled with YFP), and have observed differences in their distribution, pattern, and intensity in different conditions (Bailis et al. 2008; Sabatinos et al. 2012, 2015). There are dramatically different phenotypes between two different temperature-sensitive alleles. mcm4-M68 forms multiple small foci and robustly arrests division at permissive temperature, while mcm4-c106 forms a single large megafocus and undergoes continued division (Bailis et al. 2008; Sabatinos et al. 2015). Therefore, we examined repair foci fluorescence in both wild-type and mcm4-c106 cells either shifted or released from 36°C, or from MMS (Figure 5).

At 25°C, mcm4-c106 shows a modest increase in cells with both foci compared to the wild type, consistent with an increased basal level of stress (Figure 5, A–C). Following a shift to 36°C, mcm4-c106 accumulated numerous small foci of RPA-CFP and Rad52-YFP and these remain after 2 hr of release, whereas the foci in wild-type cells decline by 4 hr at 36°C (Figure 5, A–C) (Sabatinos et al. 2015). The mcm4-c106 cells did not divide in the first 2 hr following release. This suggests multiple dispersed damage sites, similar to mcm4-M68.

In wild-type cells during a 4 hr treatment with 0.01% MMS or at 2 hr after release from MMS, there is modest increase in cells with RPA or Rad52 foci (Figure 5, A, D, and E). This increase is measured from approximately 20% in untreated cells to about 50% in treated cells, but most of these have just one or two foci. In contrast, while mcm4-c106 cells have similar overall levels of focus formation in untreated cells, up to 80% of the cells have at least one focus in treated cells, and a strikingly large fraction contains multiple bright signals, which persist through the period of release. The majority of the RPA foci observed overlap with Rad52 foci (Figure 5, A, D, and E). Therefore, there is evidence for constitutive repair foci in mcm4-c106 cells and these are dramatically increased upon exposure to MMS.

**rif1Δ rescues mcm4-c106 MMS phenotype**

One response to replication stress is to activate dormant origins (reviewed in Alver et al. 2014). The mcm6-S1 mutant, which affects another subunit of the MCM complex, is the only other MCM allele that displays MMS sensitivity (Maki et al. 2011). Deletion of the S-phase cyclin cig2 rescues this sensitivity, presumably by delaying G1/S phase and allowing additional licensing of origins (Maki et al. 2011). Therefore, we examined a double mutant of cig2Δ mcm4-c106. In contrast to the results reported for mcm6-S1, we observed only a very slight suppression of MMS sensitivity (Figure S4).

Unexpectedly, a double mutant rif1Δ mcm4-c106 showed a dramatic rescue of MMS sensitivity (Figure 6B). Rif1 has recently been identified as an antagonist of DDK kinase-mediated phosphorylation of MCM, and regulates timing of origin firing (Hayano et al. 2012; Yamazaki et al. 2013; Davé et al. 2014; Hiraga et al. 2014; Mattarocci et al. 2014). Despite this dramatic rescue of the MMS phenotype, however, rif1Δ does not rescue the temperature sensitivity of mcm4-c106 (Figure 6A).

**Mcm4-c106 requires fork protection complex for viability**

There is a central replisome scaffold that links the leading and lagging strand polymerases and the MCM helicase, including Mci1(ScCtf4), Mcr1, the fork protection complex (FPC) Swi1(ScTof1), and Swi3(ScCsm3) (reviewed in Aze et al. 2013; Leman and Naguchi 2012; Errico and Costanzo 2012). Mutants defective in these nonessential proteins are all sensitive to MMS, indicating that robust coupling of the helicase and polymerase is required for proper response to MMS. (Figure 3)
alkylation stress. We observed that, similar to mcm4-c106, the MMS sensitivity associated with swi1Δ and swi3Δ is suppressed in rif1Δ double mutants (Figure 6C and Table 2), suggesting a related function for the C-terminus and Swi1 and Swi3. Therefore, we tested epistasis between mcm4-c106 and FPC components. We found that double mutants between mcm4-c106 and swi1Δ, swi3Δ, or mrc1Δ are all inviable, even at 25°C. Deletion of rif1Δ did not rescue the inviability of swi1Δ mcm4-c106 or swi3Δ mcm4-c106 strains. A double mutant between the mcl1-1 temperature-sensitive strain (Sc CTF4) (Williams and McIntosh 2002) and mcm4-c106 was isolated, but grew so poorly that it was impossible to assess its MMS sensitivity (Table 2).

The DDK kinase Hsk1 (ScCdc7) is essential for DNA replication, in part due to its phosphorylation of MCM proteins (Masi et al. 2000, 2006; Sheu and Stillman 2006). It also interacts with the fork protection complex (Sommariva et al. 2005; Matsumoto et al. 2005) and antagonizes Rif1 (Hayano et al. 2012; Davé et al. 2014). Previously, we showed that the temperature-sensitive mutant hsk1-1312 is sensitive to MMS and that wild-type Hsk1/DDK persists on the chromatin during MMS treatment, which depends upon the regulatory subunit Dfp1 (Dolan et al. 2010). Consistent with Hsk1 working in concert with FPC, we observed that the double mutants of mcm4-c106 hsk1Δ 1312 and mcm4-c106 dfp-r35 formed microcolonies that could not be propagated. Thus, FPC and Hsk1, which are important for the MMS response, are also essential in the absence of the Mcm4 C-terminus.

The Ctf18 protein is part of an alternative replication factor C complex RFC clamp loader (Mayer et al. 2001; Hanna et al. 2001) that is associated with DNA polε (García-Rodríguez et al. 2015). In budding yeast and humans, Ctf18 associates with two additional subunits, Dcc1 and Ctf8, to form a heptameric complex that has been shown to have a role in sister chromatid cohesion (Mayer et al. 2001; Xu et al. 2007; Gellon et al. 2011) and a role in facilitating genomic stability (Gellon et al. 2011); this complex is also required for the replication checkpoint (Kubota et al. 2011). Additionally, in fission yeast, ctf18Δ is lethal with swi1Δ and swi3Δ (Ansbach et al. 2008). We find that double mutants mcm4-c106 ctf18Δ and mcm4-c106 ctf8Δ are viable, but with a modestly reduced permissive temperature (32°C) (Figure 7A). They show a little change in MMS sensitivity relative to their parents (Figure 7B and Table 2). This suggests that the Ctf18 complex may be part of a common epistasis group with the Mcm4 C-terminus.

Chl1 is a helicase linked to the lagging strand that is a high copy suppressor of swi1Δ damage sensitivity (Ansbach et al. 2008), and it further has a role in sister chromatid cohesion in mitosis (Petronczki et al. 2004). Chl1Δ is lethal when combined with ctf18Δ (Ansbach et al. 2008). Double mutants with mcm4-c106 and chl1Δ show increased sensitivity to MMS compared to their parents, but no effects on temperature (Figure 7C).

S. pombe Swi1 and Swi3, Ctf18 complex, and Hsk1 have all been linked to defects in chromosome cohesion (Bailis et al. 2003; Ansbach et al. 2008; Rapp et al. 2010). A temperature-sensitive mutation affecting the cohesin subunit rad21-K1 combined with ctf18Δ shows increased sensitivity to MMS compared to the parents (Ansbach et al. 2008). Therefore, we examined a double mutant mcm4-c106 rad21-K1. This strain is viable and shows a similar MMS sensitivity to the parent rad21-K1 (Figure 7D), suggesting that Rad21 also falls in an epistasis group with the Mcm4 C-terminus.

We tested several other mutations affecting proteins associated with the core replisome. Both temperature-sensitive mutations cdc20-M10 and pol1-1, encoding the leading strand DNA polymerase ε (D’Urso and Nurse 1997) and polymerase α (D’Urso et al. 1995), respectively, were viable in combination with mcm4-c106, while a temperature-sensitive allele cdc6-23 affecting the lagging strand DNA polymerase δ (Iino and Yamamoto 1997) is lethal (data not shown).

Mcm4-c106 interactions with repair pathways

Finally, we examined genetic interactions with other mutants in the MMS response pathway. The post replication repair (PRR) pathway includes both error free and error prone branches that facilitate the bypass of base lesions (reviewed in Huang and D’Andrea 2006; Ulrich and Walden 2010). These are regulated by levels of ubiquitylation on PCNA (Frampton 2006). We examined double mutants of mcm4-c106 with repair mutants mms2Δ, ubc13Δ, pcn1-K164R, and rad8Δ. The double mutants were significantly more MMS sensitive than either single mutant, consistent with the mutants working in different pathways. None of these mutants showed a growth defect in the absence of MMS (Figure S5A). Double mutant error prone polymerases rev3Δ, rev1Δ, polkΔ, or esol-Δ eta (Figure S5B) were all more sensitive than the single TLS mutant parents. Interestingly, while the double mutants with polk or rev1Δ were more sensitive than mcm4-c106, double mutants with esol-Δ eta or rev3Δ were slightly less sensitive. The basis for this difference is unknown.

Effects of mcm4-c106 in recombination defective mutants

Replication fork stability and fork restart depend on proteins associated with recombination (reviewed in Lambert and Carr 2013; Neelsen and
Table 2: Sensitivity to MMS of double mutants of genes involved in different aspects of the cell cycle

| Category                          | Mutant         | Function                                | Phenotype with c106 | MMS Phenotype |
|-----------------------------------|---------------|-----------------------------------------|--------------------|---------------|
| Checkpoint                        | *cds1Δ*       | Kinase; S phase/replcation checkpoint   | Viable             | >mcm4-c106    |
| Checkpoint                        | *chk1Δ*       | Kinase; G2/damage checkpoint            | Synthetic lethal   | ND            |
| Checkpoint                        | *rad26Δ*      | Checkpoint protein                      | Synthetic lethal   | ND            |
| Checkpoint                        | *rad3Δ*       | Kinase                                  | Viable             | =rad21-K1     |
| Cohesion                          | *chl1Δ*       | Helicase                                | Viable             | >mcm4-c106    |
| Cohesion                          | *rad21-K1*    | Cohesion protein                        | Viable             | =mcm4-c106    |
| Replication                       | *mcfl-1 ts*   | Part of the FPC/cohesion                | ND                 | ND            |
| Replication-FPC                   | *mrc1Δ*       | Replication mutants/FPC                 | Synthetic lethal   | ND            |
| Replication-FPC                   | *swiΔ*        | Replication mutants/FPC                 | Synthetic lethal   | ND            |
| Replication-FPC                   | *swi3Δ*       | Replication mutants/FPC                 | Synthetic lethal   | ND            |
| Replication                       | *hsk1-1312*   | DDK kinase                              | Synethetic lethal  | ND            |
| Replication                       | *dpfl-r35*    | DDK kinase                              | Viable             | ND            |
| Replication, genome stability     | *rif1Δ*       | Rif1 antagonist of DDK                  | Viable             | rescued       |
| Genome stability                  | *brc1Δ*       | Genome stability                        | Viable             | =mcm4-c106    |
| Replication clamp loader          | *ctf8Δ*       | Cohesion-specific clamp loader          | Viable < temp      | =mcm4-c106    |
| Genome stability                  | *ctf18Δ*      | Cohesion-specific clamp loader          | Viable < temp      | =mcm4-c106    |
| Genome stability                  | *fml1Δ*       | Helicase; genome stability              | Viable             | =fmi1Δ        |
| Genome stability                  | *mus81Δ*      | Holiday junction resolvase              | Sick               | =mus81        |
| Genome stability                  | *rpg1Δ*       | Helicase; recombination antagonist       | Viable             | >mcm4-c106    |
| Genome stability                  | *snz2Δ*       | Helicase; recombination regulator       | Viable             | >mcm4-c106    |
| Repair                            | *eso1-Δeta*   | Eso1- Polγ fusion; deletes polymerase   | Viable             | ≤mcm4c106     |
| Repair                            | *exo1Δ*       | Exonuclease I                           | Viable             | = eso1Δ       |
| Repair                            | *mms2Δ*       | Ubiquitin ligase; error free repair     | Viable             | >mms2Δ        |
| Repair                            | *pcn1-K164R*  | PCNA; ubiquitin site mutant             | Viable             | = pcn1-K164R  |
| Repair                            | *polkΔ*       | Polk; error prone repair                | Viable             | >mcm4-c106    |
| Repair                            | *rad51Δ*      | Homologous recombination                | Viable             | =rad51Δ       |
| Repair                            | *rad8Δ*       | Ubiquitin ligase-helicase; error free repair | Viable             | >rad8Δ        |
| Repair                            | *rev1Δ*       | Deoxycytidyl transferase; error prone repair | Viable             | ≥mcm4-c106    |
| Repair                            | *rev3Δ*       | Polγ Error prone repair                 | Viable             | <mcm4-c106    |
| Repair                            | *rhp18Δ*      | PCNA ubiquitin ligase                   | Viable             | =rhp18Δ       |
| Repair                            | *ubc13Δ*      | Ubiquitin ligase; error free            | Viable             | >ubc13Δ       |
| Repair                            | *cig2Δ-cycl17*| S phase cyclin                          | Viable             | ≤mcm4-c106    |
| Repair                            | *reblΔ*       | Transcription termination               | Viable < temp      | =mcm4-c106    |
| Repair                            | *swiΔ*        | Heterochromatin protein                 | Viable             | =mcm4-c106    |
| Repair                            | *rad22Δ*      | Viable/slow growing                     | ND                 | ND            |
| Repair                            | *cdc20- M10(polk)* | Component of the GINS replication complex | Viable             | >mcm4-c106    |
| Repair                            | *pol1-1(polk)*| Synthetic lethal                        | Viable             | ND            |
| Repair                            | *rad50Δ*      | Synthetic lethal                        | NA                 | NA            |

Double mutant phenotypes between *mcm4-c106* and other mutants in the indicated classes. MMS, methyl methanesulfonate; ND, Not Determined; FPC, fork protection complex; DDK, Dbf4-dependent kinase; PCNA, proliferating cell nuclear antigen; NA, Not Applicable.

*Phenotype: viable means no change in temperature sensitivity. Synthetic lethal is dead at temperatures. Sick shows reduced growth rate at all temperatures. <temp has reduced maximum growth temperature.*

*MMS phenotype is determined relative to the most sensitive parent (indicated) being > (greater sensitivity), < (less sensitive), or = (equal sensitivity).*
We constructed double mutants between mcm4-c106 and mutations that disrupt recombinational repair of damaged forks including: mus81Δ (endonuclease; Boddy et al. 2001), rad50Δ (MRN complex; Bressan et al. 1999), rad51Δ (homologous recombination regulator; Muris et al. 1993), rqh1Δ (RecQ helicase; Stewart et al. 1997), and srs2Δ (helicase; Wang et al. 2001; Maftahi et al. 2002). We find that rad50Δ mcm4-c106 is synthetic lethal, indicating that the constitutive damage of mcm4-c106 requires an active MRN complex for survival. The mus81Δ double mutant had an extreme growth defect even at 25°C, with slow growth and elongated cell morphology, suggesting the formation of structures that require Mus81 for successful resolution. In contrast, rad51Δ, rqh1Δ, or srs2Δ double mutants showed no growth defects at permissive temperature, but were sensitive to MMS at a level similar or greater than the most sensitive parent (Figure S6, A and B). These data suggest that these proteins function in a pathway separate from the C-terminus of Mcm4.

**DISCUSSION**

Fission yeast Mcm4 is an essential subunit of the MCM helicase, which is a critical component in the response to replication stress. Previous studies have shown that the Mcm4 C-terminal domain (CTD) is important for the efficient recovery of HU-stalled replication forks, and a C-terminal truncation mcm4-c84 causes excessive formation of ssDNA when replication is inhibited by hydroxyurea (Nitani et al. 2008). That study also identified a larger CTD truncation mutant, mcm4-c106, as HU-sensitive and temperature-sensitive, but did not characterize it further.

Our initial examination of the temperature-sensitive phenotype of mcm4-c106 shows three distinct phenotypes that we characterized. First, their temperature-sensitive phenotype has important differences from those in other alleles of mcm4+. Second, they are MMS-sensitive. Finally, they have distinct replication defects at permissive temperature, including a novel spectrum of genetic interactions.
Similar to the original mcm4-M68 strain (Bailis et al. 2008; Sabatinos et al. 2015), we observe that mcm4-c106 cells accumulate a 2C DNA content at restrictive temperature, indicating substantial bulk DNA synthesis (Figure 3B). Cells elongate and do not divide, demonstrating successful activation of the checkpoint, confirmed by a phosphorylation-induced shift of the Chk1 kinase (Figure 4B). The cells also show increased RPA and Rad52 foci during normal growth, temperature shift, and following release, with small punctate morphology similar to that observed in mcm4-M68 (Figure 5A; Sabatinos et al. 2015). Strikingly, however, mcm4-c106 chromosomes enter a pulsed-field gel normally, both at restrictive temperature and following release, without the chromosome breaks and or structural intermediates that impair chromosome migration in mcm4-M68. We previously suggested that the breaks in mcm4-M68 reflect distinct structures targeted by the Mus81 nuclease; (Sabatinos et al. 2015) the intact chromosomes of mcm4-c106 suggest that whatever structures are formed at restrictive temperature are not the same. The temperature-sensitive phenotype may reflect either Mcm4 protein unfolding (although the protein remains detectable) or some intrinsically temperature-sensitive activity involved in replisome coupling that renders the C-terminus essential at high temperatures. In any case, some fractions of the cells are competent to restart the cell cycle, indicating that the damage they suffer is not irreversible and that the MCM complex remains largely intact and located correctly in the nucleus, which is not seen for mcm4-M68 (Pasion and Forsburg 1999).

In contrast to the original mcm4-M68 allele, the mcm4-c106 cells have clear deficiencies even under permissive conditions. Efficiency of plasmid transformation is a metric for replication efficiency (Clyne and Kelly 1997; Tye 1999), and we observe a substantial reduction in plasmid transformation efficiency and colony size, consistent with a defect in replication in mcm4-c106 but not in mcm4-M68 at 25°C. In addition, the mcm4-c106 cells are synthetically lethal with rad3Δ or chk1Δ checkpoint kinase mutants, and have an increased level of RPA and Rad52 foci at permissive temperature, which suggests that the cells suffer some form of constitutive DNA damage present even at 25°C. This is not observed in mcm4-M68. We also found synthetic lethality between mcm4-c106 MRN component rad50Δ, and severe synthetic sickness with mus81Δ, which implicates fork processing and restart in the recovery from innate stress. The absence of a synthetic phenotype associated with rad51Δ suggests that typical homologous recombination mechanisms are not required.

Interestingly, we found that mcm4-c106 is also sensitive to alkylation damage caused by MMS treatment at the permissive temperature, unlike other mcm4 alleles (Figure 1B). Previously, the only MMS-sensitive MCM identified was an allele of mcm6 (mcm6-S1) that is defective in pre-RC assembly (Maki et al. 2011). Because mcm4-c106 cells showed increased sensitivity to MMS in combination with mutations that directly affect downstream repair, including homologous recombination, error free, and error prone postreplication repair pathways, we propose that its defect is not in repair but in fork stability, or restart by template switching.

MMS sensitivity has been observed in mutants affecting a subset of additional replisome components including the fork protection complex (swi1Δ, swi3Δ, and mrc1Δ), scaffolding protein mcl1, and the DDK kinase subunits hsk1-1312 or dpf1-r35 (Fung et al. 2002; Williams and McIntosh 2002; Sommariva et al. 2005; Dolan et al. 2010). MMS treatment in fission yeast results in slowing of the replication fork (Chahwan et al. 2003; Kumar and Huberman 2004; Willis and Rhind 2009). Although there have been reports that MMS generates DNA double strand breaks (Wyatt and Pittman 2006), this breakage is likely an artifact of the procedure used to extract DNA (Lundin et al. 2005), so the PFGE results are likely uninformative. Rather, a major form of recovery is fork arrest, template switching, and repriming (reviewed in Branzel and Foiani 2010), which leads to accumulation of single-stranded DNA and increased recombination intermediates (Willis and Rhind 2009; Koulintchenko et al. 2012). Formation of these MMS recombination structures is disrupted in swi1Δ and swi3Δ, and also in rad2Δ mutants lacking the FEN1 flap endonuclease (Noguchi et al. 2004; Koulintchenko et al. 2012). In budding yeast, DNA
polymerase α and the Mc11 ortholog Ctf4 are required for template switching, and evidence indicates that this requirement is not limited to lagging strand lesions (Fumasoni et al. 2015). These observations suggest that the scaffolding proteins that link CMG and polymerases are key players to allow successful template switching. The sensitivity of Mcm4-c106 is unlikely to reflect disruptions in the scaffold itself, as models of CMG suggest that Mcm4 lies on the ring face opposite to Cdc45, GINS, Ctf4, and polymerases (O’Donnell and Li 2016).

We found that rif1Δ rescues the MMS sensitivity of mcm4-c106, but not its temperature sensitivity. rif1Δ also rescues the MMS sensitivity of swi1Δ or swi3Δ. Rif1 in budding yeast has a role in replication timing, via the recruitment of Glc7 phosphatase (Hayano et al. 2012; Davé et al. 2014; Mattarocci et al. 2014; Hiraga et al. 2014; Peace et al. 2014). This antagonizes the DDK-mediated phosphorylation of Mcm4 that activates replication. Intriguingly, Rif1 is also proposed to modify the response to ssDNA that activates the checkpoint (Xu et al. 2010), as well as contributing to resection in break repair (Martina et al. 2014). A new study also links Rif1 to resolution of chromosome entanglements (Zaaier et al. 2016). In fission yeast, rif1Δ rescues temperature sensitivity of hsk1-89 (Hayano et al. 2012; Davé et al. 2014) and hsk1-1312 (J. P. Yuan and S. L. Forsburg, unpublished data). However, Rif1 is not essential for cellular responses to replication stress (Hayano et al. 2012; Peace et al. 2014).

There are several models by which this rescue could occur. One way would be by activating otherwise dormant origins, as rif1Δ is proposed to deregulate origin timing by antagonizing DDK (Hayano et al. 2012). Origin activation has been proposed to rescue the MMS sensitivity of mcm6-S1, another MCM subunit (Maki et al. 2011). However, unlike mcm6-S1, the MMS sensitivity of mcm4-c106 was not notably rescued by deletion of the S phase cyclin cig2, suggesting that additional pre-RC formation is not a mechanism for rescue. Alternatively, rescue may be linked to Rif1 antagonism of the Hsk1/DDK kinase, which is localized to the chromatin in MMS and potentially recruited by the FPC (Sommariva et al. 2005; Matsumoto et al. 2005; Dolan et al. 2010). Hsk1 is known to phosphorylate Mrc1 (associated with FPC) and the checkpoint clamp Rad9 during replication stress (Matsumoto et al. 2010; Furuya et al. 2010). In the absence of FPC, therefore, loss of localized Hsk1 activity may be balanced by the absence of the Rif1 antagonist.

The similarity of rescue of the MMS phenotype of mcm4-c106 and swi1Δ or swi3Δ led us to investigate whether they are in a common epistasis group. Contrary to that model, we observed synthetic lethality between mcm4-c106 and FPC mutants swi1Δ, swi3Δ, and mrc1Δ, as well as DDK mutants hsk1-1312 and dfp1-r35, indicating that an intact fork-protection complex including DDK must be present in the absence of the Mcm4 C-terminus. A similar synthetic lethal phenotype with the FPC was not observed with mcm4-c84, which harbors a shorter truncation of the C-terminus (Nitani et al. 2008), so this is unlikely to be related to the HU-ssDNA phenotype observed in that study.

Because we see no phenotype in double mutants with the replication checkpoint kinase cdc1Δ, we conclude that this synthetic lethality is not related to the S phase checkpoint activation by FPC (Noguchi et al. 2003), but rather to the FPC’s structural role linking the helicase to the polymerase (Noguchi et al. 2004; Lou et al. 2008; reviewed in Aze et al. 2013).

Mcm1 (ScCtf4) links the helicase to the lagging strand through the interaction with polymerase α and direct interaction with the GINS subcomplex (Gambus et al. 2009; Simon et al. 2014). At least one arm of the helicase coupling system is required for viability, because mcl1-1 swi1Δ double mutants are synthetically lethal (J. P. Yuan and S. L. Forsburg, unpublished results). Therefore, one possibility is that mutation of mcm4-c106 may lead to defects in coupling to the lagging strand side of the replisome, making it dependent upon the FPC and the leading strand coupling for viability even at permissive temperature.

FPC mutants are also synthetically lethal, with mutations affecting Ctf18 and Ctf8 forming an alternative RFC clamp loader complex (Ansbach et al. 2008). Long linked to sister chromatid cohesion (Hanna et al. 2001; Mayer et al. 2001), Ctf18 has more recently been associated with DNA damage response and replication checkpoint

Figure 7 mcm4-c106 interactions with alternative replication factor C (RFC). (A) mcm4-c106 combined with RFCΔCtf18Δ and RFCΔCtf8Δ. Representative response to temperature was assayed by serial dilutions. Strains were grown overnight at 25°C, 1:5 serially diluted, and plated on YES (rich media) as the control, and 32°C and 36°C to observe the temperature effect. (B) Representative response to MMS assessed by serial diluted samples plated on the indicated concentrations of MMS. (C) mcm4-c106 and (D) cohesion subunit rad21-K1 mcm4-c106 effects on MMS. MMS, methyl methanesulfonate; YES, yeast extract + supplements.
 Particularly intriguing, Mcm4 has also been identified as a partner of mammalian Rad21 in two separate proteomics studies that suggest that it is in a common epistasis group at this temperature. Double mutants between mcm4-c106 and the mcl1-1 temperature-sensitive allele were viable, although significantly sicker than either parent. Our genetic analysis offers further tantalizing evidence that this phenotype may be linked to cohesion. FPC, Mc11, polc, DDK, and Ctf18 are all associated with sister chromatid cohesion in S. pombe and other systems (Hanna et al. 2001; Williams and McIntosh 2002; Bails et al. 2003; Edwards et al. 2003; Xu et al. 2007; Ansbach et al. 2008; Rapp et al. 2010). We observed no evidence for chromosome segregation errors in mcm4-c106 that would indicate a sister chromatid cohesion defect. A temperature-sensitive mutation affecting the cohesin subunit rad21-K1 is synthetic lethal with either swi1Δ (Ansbach et al. 2008; Dolan et al. 2010) or hsk1-1312 (Smith et al. 2000). In contrast to the substantial genetic interactions in other double mutants, we did not observe growth defects in rad21-K1 mcm4-c106. Additionally, the MMS sensitivity of rad21 mcm4-c106 is similar to that of the rad21-K1 parent. We propose that the C-terminus of Mcm4 may facilitate the recruitment of cohesin to promote a distinct fork maintenance or restart function.

As it’s name implies, the S. pombe cohesin rad21 gene was first identified by its repair defect (Birkenbihl and Subramani 1992). Mutations in rad21 are MMS-sensitive, and lie in an epistatic pathway with rad50 (Hartsuiker et al. 2001). More recently, studies suggest that cohesin may influence replication origin activity by affecting 3-D genome organization (Guillou et al. 2010; Yun et al. 2016). Cohesin has been shown to be required for efficient template switching in budding yeast in a pathway that includes Ctf4 (Sp Mcd1) (Fumasconi et al. 2015). Particularly intriguing, Mcm4 has also been identified as a binding partner of mammalian Rad21 in two separate proteomics studies (Guillou et al. 2010; Panigrahi et al. 2012). We suggest that the extended C-terminus of Mcm4 collaborates with cohesin to promote fork stability during replication stress.

Together, these results indicate that mcm4-c106 has a novel replication defect, likely to do with replisome uncoupling, that is distinct from that in other mcm4 conditional alleles. Along with our previous study (Sabatinos et al. 2015), this suggests that physiological inspection of conditional mutant phenotypes is likely to identify new domains and interactions that assemble and maintain the replicative helicase.

ACKNOWLEDGMENTS

We thank Jiping Yuan for help with strain construction and Sarah Sabatinos for technical help and advice on the project. We thank Oscar Aparicio, and members of the Forbsurg lab, for helpful comments on the manuscript. This work was supported by National Institutes of Health grants R01 GM081418, R01 GM111040, and R35-GM118109.

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