Replication stress in early S phase generates apparent micronuclei and chromosome rearrangement in fission yeast

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ABSTRACT DNA replication stress causes genome mutations, rearrangements, and chromosome missegregation, which are implicated in cancer. We analyze a fission yeast mutant that is unable to complete S phase due to a defective subunit of the MCM helicase. Despite underreplicated and damaged DNA, these cells evade the G2 damage checkpoint to form ultrafine bridges, fragmented centromeres, and uneven chromosome segregations that resemble micronuclei. These micronuclei retain DNA damage markers and frequently rejoin with the parent nucleus. Surviving cells show an increased rate of mutation and chromosome rearrangement. This first report of micronucleus-like segregation in a yeast replication mutant establishes underreplication as an important factor contributing to checkpoint escape, abnormal chromosome segregation, and chromosome instability.

INTRODUCTION DNA replication stress is a well-known source of genome instability and results in increased mutations, chromosome rearrangements, and missegregation (reviewed in Naim and Rosselli, 2009; Crasta et al., 2012; Holland and Cleveland, 2012; Hatch et al., 2013). Tempering replication stress by adding extra nucleosides (Burrell et al., 2013) or inducing a checkpoint response (Casper et al., 2002) can stabilize slowly replicated regions and diminish the effect on chromosome missegregation. Of importance, genome instability is also correlated with carcinogenesis (e.g., Bagley et al., 2012; Burrell et al., 2013; Hirsch et al., 2013), particularly within fragile regions of the genome that are unable to replicate efficiently (e.g., Chan et al., 2009; Lukas et al., 2011; Naim et al., 2013). Thus cellular ability to appropriately manage replication stress prevents malignant transformation (Bartkova et al., 2005; Gorgoulis et al., 2005; Halazonetis et al., 2008).

MCM4 is an essential subunit of the minichromosome maintenance (MCM) helicase that is required for DNA replication (reviewed in Forsburg, 2004; Bochman et al., 2008). Mice with minor mcm4 mutations show evidence of replication stress, including double-strand breaks, micronuclei, and increased formation of mammary tumors (Shima et al., 2007a) or leukemia (Bagley et al., 2012). Disruptions in replication correlate with chromosome fragile sites (reviewed in Debatisse et al., 2012), and the murine mcm4 phenotype is consistent with a failure to license dormant replication origins (reviewed in Kawabata et al., 2011; McIntosh and Blow, 2012). N-terminal truncation of Mcm4 is associated with chromosome breaks and DNA repair defects in an inbred human population (Casey et al., 2012; Gineau et al., 2012; Hughes et al., 2012). MCM overexpression has been correlated with hyperproliferation and carcinogenesis in tumors (Ishimi et al., 2003b; Guida et al., 2005; Lau et al., 2010; Majid et al., 2010; Suzuki et al., 2012). Thus changes in this single MCM4 subunit have profound consequences for genome stability.

We report a novel genome-instability phenotype in a specific allele of mcm4. In fission yeast cells, most conditional mcm mutations at the restrictive temperature show significant DNA accumulation, accompanied by activation of the DNA damage checkpoint and robust cell cycle arrest (e.g., Nasmyth and Nurse, 1981; Coxon et al., 1992; Liang and Forsburg, 2001) consistent with replication...
fork collapse generating DNA double-strand breaks. Cells with the mcm4-degron allele show DNA underreplication with accumulation of DNA damage markers at the restrictive temperature but are unable to maintain a checkpoint arrest. After release from replication stress, these damaged cells continue to divide. The divisions are abnormal, producing ultrafine DNA bridges, multipolar spindles, and uneven chromosome segregation accompanied by the formation of small, satellite nuclei. These apparent micronuclei retain DNA damage markers and frequently rejoin with the parent nucleus. Significantly, the cells that survive this stress show substantially increased rates of mutation and chromosome rearrangement. This phenotype is distinct from mutants that block replication initiation, which fail to undergo chromosome segregation. Our data suggest that underreplication is a critical factor associated with genomic instability and establish a genetic model to investigate the links between replication stress, disruptions in chromosome segregation, and genome rearrangements.

RESULTS
Most temperature-sensitive MCM helicase mutants duplicate the majority of their genome at 36°C (Figure 1A). However, these mcm-ts mutants accumulate DNA damage and trigger cell division cycle arrest (e.g., Nasmyth and Nurse, 1981; Coxon et al., 1992; Liang and Forsburg, 2001). On the other hand, temperature-sensitive mutants in other genes that bypass replication initiation do not replicate DNA but enter mitosis. This causes a cell untimely torn (cut) phenotype in which the unreplicated DNA is cleaved by the septum (e.g., cdc18\(\Delta\), rad4ts, or orp1-ts; Kelly et al., 1993b; Saka and Yanagida, 1993; Grallert and Nurse, 1996). Presumably, initiation mutants never begin DNA replication and do not generate signals to trigger the checkpoint (Kelly et al., 1993a).

We observe a novel phenotype in the temperature-sensitive mcm4-degron allele. This mutant has a degron cassette fused to the mcm4-ts allele (cdc21-M68, Mcm4\(\Delta\)) protein is <10% of the original level during incubation at 36°C (Supplemental Figure S1A). We monitored DNA synthesis by nucleoside analogue incorporation (5-ethynyl-2′-deoxyuridine [EdU]) and detected very little accumulation in mcm4-degron at the restrictive temperature (Figure 1A and Supplemental Figure S1B). In contrast, mcm4-ts at 36°C incorporates nearly wild type EdU amounts. Only early replication origins fire in mcm4-degron, whereas mcm4-ts cells activate a combination of early and late origins more efficiently (Supplemental Figure S1C). Surprisingly, despite these global replication defects, mcm4-degron cells divide multiple times at 36°C (Figure 1, B and C, and Supplemental Video S1), causing uneven DNA segregation in daughters and granddaughters.
When cells are returned to 25°C, wild-type cells robustly continue DNA synthesis and proliferation (Figure 1D and Supplementary Figure S1D). In contrast, neither mcm4-ts nor mcm4-degron cells incorporate much EdU after release to 25°C, a period that we call “recovery.” This low incorporation suggests that either there is limited, residual synthesis across the genome or just a few cells returned to the cell cycle. The mcm4-ts cells remain cell cycle arrested after release, consistent with persistent DNA damage (Balis et al., 2008). Surprisingly, mcm4-degron cells continue to divide (Supplementary Figure S1E and Supplementary Video S2). Spindle pole body (SPB) duplication and separation occurs with timing similar to that for wild type (Supplementary Figure S1F). However, the segregation of the DNA in mcm4-degron is highly abnormal (Figure 1E), forming lagging chromosomes, replication protein A (RPA)–labeled ultrafine bridges, and unequal DNA segregation into aneuploid and anucleate cells (Figure 1F and Supplementary Figure S1G).

**Apparent micronuclei form in underreplicated mcm4-degron cells**

We examined abnormal segregations in mcm4-degron more closely using live-cell video microscopy. The nuclear histone signal shows uneven segregation and fragmentation in >50% of mcm4-degron cells (Figure 2, A and B). These nuclear fragments form during mitosis and are enclosed in separate nuclear membranes, which is the definition of a micronucleus (Hatch et al., 2013; Figure 2, A–C, Supplementary Figure S2, A and B, and Supplementary Video S3). The proportion of fragmented histone masses was similar to the number of membrane-bound micronuclei, indicating that a membrane initially surrounds most wandering DNA fragments.

There are no obvious connections between the micronucleus and the parent nucleus. Some membrane stalks remain independently attached to the septum (Supplementary Figure S2B). When these membrane-enclosed fragments remain in the same cell, they frequently rejoin the mother nucleus (~60% of the time). Others segregate into a daughter cell during division, forming aneuploid cells. Subsequent divisions often show repeated segregation/fusion cycles (Supplemental Videos S4 and S5). Supplementary Video S4 shows delayed and failed mitosis followed by a later division, suggesting a dual spindle (e.g., Figure 2E and Supplementary Video S4). Thus these mis segregations may also be linked to mitotic defects such as multipolar spindle formations.

Another mitotic abnormality observed in mammalian cells after replication stress is ultrafine DNA bridges (UFBs) between fragile DNA regions (reviewed in Chan and Hickson, 2009). UFBs are not detected using DNA stains (e.g., 4′,6-diamidino-2-phenylindole [DAPI], histone; Chan et al., 2009) but can be visualized with RPA (Chan and Hickson, 2009). We observed twisting threads of RPA spanning unequal DNA masses in 20% of mcm4-degron divisions (Figure 2D, Supplementary Figure S2, C and D, and Supplementary Video S6). The RPA signal was often separate from the histone signal, suggesting that single-strand DNA (ssDNA) has pulled apart from the bulk chromatin.

These division anomalies resemble mitosis with unreplicated genomes (MUGs), which happens in replication-arrested human cells that bypass the G2 damage checkpoint (Wise and Brinkley, 1997). One MUG characteristic is centromere fragmentation (Beeharry et al., 2013), which we detected in strains expressing a tagged centromere-associated histone Cnp1–red fluorescent protein (RFP; CENPA homologue; Supplementary Figure S2E). Fission yeast centromeres replicate early (Zhu et al., 1992) and then cluster with the SPB, except briefly during metaphase-to-anaphase transition. We observed early centromere replication in mcm4-degron (Supplementary Figure S1C) as Cnp1 foci scatter, indicating that centromeres replicate, separate, and possibly fragment. These multiple mitotic abnormalities promote DNA missegregation after replication stress in mcm4-degron.

We examined evidence for chromosome rearrangement using a lacO array near centromere 1 (Figure 2F). Many mcm4-degron cells failed to separate lacO<sup>Cen1</sup> foci to both daughters, causing >2 green fluorescent protein (GFP) foci/nucleus or none at all. Because lacO arrays are potential fragile sites in *Schizosaccharomyces pombe* (Sofueva et al., 2011), a lacO<sup>Cen1</sup> rearrangement or duplication may occur after mcm4-degron replication stress, causing cells with greater than two separating foci. This is also consistent with evidence for centromere fragmentation and rearrangement.

**Increased mutations and rearrangements in surviving mcm4-degron cells**

We next asked whether the 10% of surviving mcm4-degron cells show lasting signs of genome instability after transient replication stress (Figure 3A). We tested surviving cells for forward mutations that cause canavanine resistance (can1<sup>ts</sup>, can1<sup>-</sup>; Figure 3B). The baseline mutation frequency in mcm4-degron cells is higher than for wild type and mcm4-ts and significantly increases after incubation at 36°C. We also saw high rates of marker loss at other loci, including loss of an integrated marker at his7 (Supplemental Figure S3).

To assess potentially catastrophic chromosome rearrangements, we introduced a nonessential minichromosome into the mcm4-ts and mcm4-degron strains. This minichromosome carries multiple genetic markers to maintain its overall stability or monitor its structural integrity (Figure 3C). A low level of chromosome rearrangement is observed in wild-type cells, including break-induced replication and isochromosome formation (Figure 3, D and E; Nakamura et al., 2008). Increased rearrangements are also observed in replication fork protection complex mutants (Li et al., 2013).

Consistent with the minichromosome maintenance (mcm) phenotype, we observed an increased rate of minichromosome loss in both mcm4-ts and mcm4-degron relative to wild type at 25°C (Figure 3E). Chromosome loss is modestly increased after incubation at 36°C in the mcm4-degron strain but shows no significant change in the mcm4-ts background.

*Both mcm4-degron and mcm4-ts strains show an increased rate of rearrangement relative to wild type at 25°C (Figure 3D). However, after a 4-h pulse at 36°C, the mcm4-degron mutant shows a dramatic increase in chromosome rearrangements that is not observed in mcm4-ts. Thus the division abnormalities observed in mcm4-degron are accompanied by increased mutations, chromosome rearrangements, and chromosome loss.*

**Damage persists in mcm4-degron mutants**

The ability of underreplicated mcm4-degron cells to divide repeatedly suggests either that there is little DNA damage or that the damage checkpoint is not activated. To address the first point, we examined DNA repair proteins during arrest and release by visualizing fluorescently tagged versions of the ssDNA-binding protein RPA, an early damage marker, and the Rad52 recombination protein. The mcm4-ts mutant forms many discrete RPA and Rad52 foci during arrest at 36°C, which coalesce into a bright, pannuclear signal upon release (Figure 4A and Supplemental Figure S4, A–C). This is consistent with earlier observations (Bailis et al., 2008) suggesting widespread late replication fork collapse at multiple sites, similar to the checkpoint mutant cds1<sup>A</sup> in *H. pombe* (Sabatinos et al., 2012). In contrast, mcm4-degron mutants form one or two large, distinctive RPA...
filaments that extend from the center forming cups and voids that contain Rad52 (Figure 4B). At this higher resolution, the RPA/Rad52 foci are not simple dots but instead highly structured patterns within a 0.2-μm diameter. The 3D-SIM images show that Rad52 and RPA fit together end to end and that RPA tendrils loop out into surrounding histone regions (Supplemental Figure S4E). Further, we observe that the megafocus occurs in histone-deficient nuclear regions (Supplemental Video S7).

"megafoci." RPA and Rad52 colocalization in both mcm4 mutants (Supplemental Figure S4D), coupled with their low viabilities, suggests that these are dominated by stalled or damaged replication forks (e.g., Lambert et al., 2010) and not stably stalled replication forks (Frmisch et al., 2009).

We used superresolution microscopy to examine the megafocus substructure. Three-dimensional (3D) structured illumination microscopy (SIM) images show that the megafocus is an RPA complex, with
The presence of Rad52 repair foci in ~15% of untreated mcm4-degron cells suggests that the cells suffer damage even under permissive conditions. Pulsed-field gels show that untreated mcm4-degron chromosomes migrate poorly and generate a low-molecular weight smear indicating DNA breaks (Supplemental Figure S4F). This genome instability may be due to Mcm4-degron protein instability compared with wild-type Mcm4 before temperature shift (Supplemental Figure S1A). These observations are consistent with our previous observation that reduced MCM levels cause genome instability before replication is noticeably affected (Liang et al., 1999).

Surprisingly, RPA and Rad52 foci persist in dividing mcm4-degron cells (Figure 4C and Supplemental Videos S2 and S8). We also see RPA and Rad52 foci in the apparent micronuclei (Figure 4D); these may be markers of ongoing DNA synthesis, stalled forks, or DNA damage. Consistently, we find that these signals appear later in the putative micronuclei than in the primary nucleus (Figure 4D, arrowhead vs. asterisk in the primary nucleus) and can be reincorporated into the parent nucleus (Figure 4E).

The phenotypes we observe with mcm4-degron are different from those seen in other replication initiation mutants. Orp1⁰ORC¹ marks replication origins (orp1-4; Grallert and Nurse, 1996), and Rad4¹TopBP1 is essential for replication initiation and also activation of the DNA damage checkpoint (rad4-116; Saka and Yanagida, 1993). These mutants enter a lethal mitosis with unreplicated DNA that is cleaved by the cell septum (cut). Both orp1ts and rad4ts formed some RPA and Rad52 foci, but the quantity and patterns were different from those for mcm4-degron (Supplemental Figure S5, A–C).

The rad4ts mutants are much shorter at division, typical of cut mutants, with a sub-1C DNA content and increased cell death (Supplemental Figure S5, D and E). Fewer chromosome missegregations occur in either orp1ts or rad4ts than in mcm4-degron, particularly during recovery at 25°C (Supplemental Figure S5F). Thus mcm4-degron defines a new class of early replication mutant.

mcm4-degron transiently activates the damage checkpoint and then escapes

RPA contributes directly to fork stability and damage checkpoint activation (Zou et al., 2003; Toledo et al., 2013). The checkpoint kinase, Chk1, is phosphorylated in asynchronous mcm4-degron cells. Chk1 activation, detected by a band shift Western blot
Consistent with these data, the inhibitory Cdc2 phosphorylation on threonine 15 that prevents mitosis (O’Connell et al., 1997) is reduced in mcm4-degron (Figure 5C). This checkpoint activation explains the robust cell cycle arrest of the mcm4-ts compared with mcm4-degron. Moreover, under these conditions, we Crb253BP1 levels drop sharply in mcm4-degron at 36°C, suggesting that the checkpoint signal is interrupted upstream of Chk1 (Figure 5B).
Without Chk1, the mcm-ts chk1Δ double mutants enter premature mitosis and cut at 36°C (Supplemental Figure S6, A and B; Liang et al., 1999). In mcm4-degron chk1Δ double mutants, the fraction of cut cells is only slightly higher than in mcm4-degron alone. This suggests that the Chk1 checkpoint transiently restrains division in mcm4-degron at 36°C. Once returned to 25°C, there is no difference between division numbers and morphology in mcm4-degron and mcm4-degron chk1Δ double mutants.

**Mus81 promotes checkpoint arrest during late-replication failure**

What is different about the late replication fork failure in mcm4-ts and the early collapse in mcm4-degron? Whereas mcm4-ts accumulates DNA breaks and robustly activates the damage checkpoint, mcm4-degron does not. The contrast in their RPA patterns and timing suggests that the two mutants generate different replication arrest structures. Because Mus81 endonuclease reportedly cleaves stalled replication forks in late S phase to promote fork restart (Froget et al., 2008; Saugar et al., 2013), we reasoned that Mus81 might cleave mcm4-ts arrested forks to form DNA breaks and generate a robust damage signal.

Consistent with this model, we found that a mcm4-ts mus81Δ double mutant showed a dramatic increase in dividing cells at 36°C compared with mcm4-ts alone (Figure 5D) and thus resembles the mcm4-degron. In contrast, mus81Δ did not change the proportion of mcm4-degron cells forming micronuclei or undergoing asymmetric divisions during release (Supplemental Figure S6, C–E). We infer that Mus81-dependent damage formed in mcm4-ts generates a signal for robust G2 checkpoint activation and cell cycle arrest. In contrast, the early-failing replication forks in mcm4-degron fail to activate fully or maintain the G2 checkpoint.

**DISCUSSION**

Mcm4 is an essential subunit of the MCM helicase, the primary replicative helicase of eukaryotic cells (e.g., Maiorano et al., 2000; Labib et al., 2001; Ishimi et al., 2003a; Yabuta et al., 2003). Disrupting Mcm4 function drives genome instability in many models. Mouse mcm4 mutations are associated with chromosome breaks, genome rearrangements, micronucleus formation, and breast or blood cancers (Shima et al., 2007a,b; Bagley et al., 2012). It has been proposed that this reflects a failure to license additional replication origins that allow rescue of a failed replication fork (Kawabata et al., 2011; McIntosh and Blow, 2012). In humans, MCM4 truncation mutations are associated with chromosome instability and DNA repair defects (Casey et al., 2012; Gineau et al., 2012; Hughes et al., 2012). MCM overexpression is correlated with hyperproliferation and carcinogenesis (e.g., Ishimi et al., 2003b; Guida et al., 2005). Therefore Mcm4 plays a fundamental role in maintaining genome stability.

We characterized a novel temperature-sensitive allele of mcm4 in fission yeast (mcm4-degron) that generates a distinct form of early replication stress in which early replication forks fire but undergo little DNA synthesis. This is accompanied by transient DNA damage checkpoint activation and then escape, suggesting that cells are unable to initiate or maintain a checkpoint response (Latif et al., 2004). Because there are low levels of checkpoint mediator protein Crb2 at 36°C, the checkpoint activation step in mcm4-degron may not be amplified (Lin et al., 2012); this agrees with recent work proposing that Chk1 activation is linked to the MCM complex (Han et al., 2014). Alternatively, mcm4-degron checkpoint maintenance might fail, allowing escape, as is the case at telomeres where Crb2 is absent (Carneiro et al., 2010).

This contrasts with mcm4-ts mutants, which syndrome almost a wild-type amount of DNA before undergoing robust checkpoint-dependent arrest. We observe that Mus81 endonuclease is required
to maintain activation of Chk1 in mcm4-ts cells. This suggests that Mus81 recognizes and acts upon a specific structure formed during late fork collapse in mcm4-ts, and this generates the robust checkpoint signal that maintains cell cycle arrest. This may be due to inactivation of Mus81 during early S phase, as is observed in Saccharomyces cerevisiae (Sauger et al., 2013). Alternatively, there may be no Mus81-susceptible substrates formed in mcm4-degron early replication arrest, preventing a strong G2 checkpoint activation.

The mcm4-degron cells show replication stress even without a temperature shift, as indicated by their constitutive DNA repair foci (Rad52), smeared chromosomes by pulsed-field gel electrophoresis (PFGE) analysis, higher mutation rates, and constitutively activated Chk1. This is consistent with other work showing that reduced MCM protein levels contribute to genome instability (Liang et al., 1999; Gineau et al., 2012). The mcm4-degron cells also acquire a novel RPA/Rad52 structure that is not seen in other replication-initiation mutants. We propose that this “megafocus” represents early-firing replication origins that are clustered during initiation (Knott et al., 2012) and then collapse as Mcm4\textsuperscript{degron} protein is lost. Our super-resolution analysis of the mcm4-degron megafocus shows that the structures of ssDNA and Rad52-bound DNA are intertwined. These megafoci of colocalized RPA and Rad52 do not stably activate the DNA damage checkpoint, similar to replication stress–induced foci of brc1a mutants (Bass et al., 2012). In our model, Mcm4\textsuperscript{degron} preassembled at early origins is protected from immediate inactivation at 36°C, and the protein becomes vulnerable during the transition to replication elongation, causing replication failure and ssDNA accumulation at an early stage. In contrast, the mcm4-ts mutants arrest with numerous dispersed RPA foci, consistent with late fork collapse detected by phosphorylated H2A(x) (Bailis et al., 2008). This may occur stochastically or at specific fragile sites.

Unexpectedly, we observe that despite underreplication, most mcm4-degron cells divide during both replication stress at 36°C and again after release (Figures 2 and 4). These abnormal mitoses produce UFBs marked with RPA and apparent centromere fragmentation. Cells undergo continued, abnormal divisions that generate small, membrane-bound bodies that contain a subset of the genome. These may segregate into separate daughter cells, generating aneuploidy, or remain in the mother cell, where they may rejoin the parent nucleus. These structures are intriguingly suggestive of micronuclei.

In mammalian cells, micronuclei form when a subset of the genome is separated into distinct membrane-bound bodies. These may form after irradiation (e.g., Kato and Sandberg, 1968) or when cells with replication defects enter mitosis (Kato and Sandberg, 1968; Shima et al., 2007a; Chan et al., 2009; Utani et al., 2010; Bagley et al., 2012). Micronuclei may containacentric genome fragments or whole chromosomes and may be associated with dicentrics and chromosome bridges. These data indicate that they may result from different forms of genetic stress or mitotic failure (e.g., Fenech et al., 2011).

Although micronuclei are common markers in cancer cells (e.g., Crasta et al., 2012; Hatch et al., 2013), the relationship between their formation, stability, and overall genome instability is not understood. For example, micronuclei clearly form in response to whole-genome damage and replication stress, as seen in mouse Mmc4 mutants (Shima et al., 2007a; Gineau et al., 2012), and yet spindle poisons that perturb mitosis also cause whole-chromosome missegregation and micronuclei (Crasta et al., 2012; Hatch et al., 2013; Zhang et al., 2015). In the latter studies, DNA replication is delayed in micronuclei compared with the parent nucleus (Crasta et al., 2012), leading to DNA damage. Indeed, there is long-standing evidence that chromosomes within micronuclei are severely damaged or pulverized (Kato and Sandberg, 1968; Crasta et al., 2012; Zhang et al., 2015). The resulting chromosome rearrangements may be incorporated into the genome if the micronuclear DNA merges with the parent nucleus during mitosis (reviewed in Forment et al., 2012; Holland and Cleveland, 2012; Zhang et al., 2013). These observations have led to the suggestion that aberrant micronuclear segregations are associated with the catastrophic chromosome rearrangements termed chromothripsis (Crasta et al., 2012; Holland and Cleveland, 2012; Zhang et al., 2015).

The events that generate micronuclei are likely linked to other cytogenetic abnormalities, including chromosome bridging, breakage-fusion-bridge cycles, and centromere fission (e.g., Fenech et al., 2011; Martínez and van Wely, 2011; Sorzano et al., 2013). In mammalian cells, caffeine-induced checkpoint bypass produces evidence of centromere fragmentation in underreplicated cells (Burrell et al., 2013). Centromere breaks and fission have been associated with micronuclear formation and chromosome rearrangements (Guerrero et al., 2010; Martínez and van Wely, 2011). Consistent with this, we previously described the fission yeast pericentromere repeats as vulnerable to rearrangement during replication stress (Li et al., 2013). The unusual mcm4-degron mutant phenotype establishes a yeast model to examine missegregation events in which we observe evidence for centromere fission, UFBs, and abnormal/aneuploid segregation.

We predicted that these abnormal segregations and apparent micronuclei should be associated with increased evidence of genome instability, and genetic studies showed this to be the case. The mcm4-degron strain is a mutator, with increased accumulation of forward mutations after incubation at 36°C. Using a nonessential minichromosome (e.g., Nakamura et al., 2008; Li et al., 2013), we observed a striking increase in chromosome rearrangements in the mcm4-degron cells that survive replication stress compared with wild-type or mcm4-ts cells, which maintain checkpoint arrest.

We infer that the abnormal divisions of mcm4-degron establish a source of continuing genome instability (model in Figure 4D). A fraction of the underreplicated genome is separated during mitosis and shows accumulation of RPA and Rad52 foci later than in the parent nucleus. This could reflect DNA damage or asynchronous DNA replication. Apparent nuclear fusion or rejoining between the separated body and the parent nucleus reincorporates the damaged DNA into the parent nucleus after mitosis. We hypothesize that this is one cause of enhanced mutation rate after transient mcm4-degron inactivation. Intriguingly, data in mammalian systems suggest that DNA damage that occurs during mitosis can be masked until the next cell cycle (e.g., Lukas et al., 2011). Of importance, we show that transient replication instability has long-reaching effects and that genome instability (persistent RPA/Rad52 foci, bridges, and apparent yeast micronuclei) is established and transmitted over multiple divisions during growth reestablishment (Supplemental Videos S4 and S5).

Of course, nuclear membrane dynamics differs in yeast and mammals. We observe that ~70% of fission yeast micronuclei fuse with the parent nucleus. This is similar to the frequency observed for micronuclear DNA rejoining the parent DNA during mitosis in microtubule-destabilized mammalian cells (Hatch et al., 2013). However, micronuclear membrane fusion is not reported in mammalian cells (Crasta et al., 2012). The open mammalian mitosis, with nuclear envelope breakdown, allows micronuclear DNA to rejoin the parent nucleus when the nuclear membrane is degraded during mitosis. In contrast, the fission yeast mitosis is closed, and the nuclear envelope

3446 | S. A. Sabatinos et al. Molecular Biology of the Cell
does not degrade. Therefore the mechanism of yeast micronuclear DNA rejoining may be different and appears to be postmitotic. Of importance, the abnormal segregation we observe is clearly mitotic in origin. It is led by centromeres and spindle pole bodies (Figure 2E and Supplemental Figure S2E) and is distinct from abnormal nuclear budding, such as that observed in fission yeast mutants with disrupted nuclear membrane dynamics (Sazer et al., 2014).

This is the first report of micronucleus-like divisions in fission yeast, and it is not observed in the other early replication mutants tested (orc1ts, rad4ts). Thus these divisions are a feature of a very specific early replication defect that allows some fraction of the genome to undergo segregation, evading the checkpoint by circumventing DNA breakage through Mss81.

Micronuclei induced by a yeast mcm4 mutation are particularly intriguing, given the association of micronuclei, chromosome breaks, and cancers in mouse Mcm4 mutants (Shima et al., 2007a; Bagley et al., 2012). It is possible that disruptions in the MCM4 subunit are particularly linked to damage that evades the checkpoint and promotes abnormal mitosis. Significantly, yeast genetic tools now allow a detailed investigation of contributing factors and description of outcomes. This provides a powerful genetic model to investigate the mechanisms of aberrant segregation and micronucleus formation caused by replication instability and the potential of large-scale genetic damage.

**MATERIALS AND METHODS**

**Cell growth and physiology**

Fission yeast strains are described in Supplemental Table S1 and were grown as in Sabatinos et al. (2012). Physiology experiments for viability, DNA synthesis, Chk1 protein, PFGE, and flow cytometry were performed in supplemented Edinburgh minimal medium (EMM). Live-cell imaging cultures were grown in fully supplemented EMM with 5 μM thiamine and photographed in the same medium. Septation and nuclear counts were performed on fixed samples. Briefly, cells were fixed with 70% ethanol, rehydrated, and then stained in 1 mg/ml aniline blue (M6900; Sigma-Aldrich) for 15 min. Stained cells were mounted on glass slides with SlowFade Gold antifade mount with DAPI (S36938; Invitrogen, ThermoFisher Scientific) and photographed. More than 200 cells were counted from two biological replicates and pooled, and then proportions and 95% confidence intervals (CIs) were calculated. Differences in proportions were assessed with a two-tailed Z test.

**Micronucleus measurement**

An initial assessment of the micronucleus-forming potential in cultures was made by incubating cultures at 36°C for 4 h and then imaging over 12 h at 25°C. Using a process similar to that of Hatch et al. (2013; Figure 1C), we monitored histone-RFP (hht1-RFP) in cells resolving division. The presence of smaller chromatin bodies away from the primary nucleus was scored as a “free chromatin body”; these were monitored to determine whether they rejoined the primary nucleus (resorbed).

To determine whether free chromatin bodies were membrane-enclosed micronuclei, the membrane marker crc1 N-terminal fragment (crc1N-GFP) was monitored with histone (hht1-RFP) in live cells after 4 h at 36°C. Cells were scored as micronucleus forming if they met three criteria: 1) the micronuclear histone mass was surrounded by membrane and separated from the parent nucleus; 2) the micronucleus formed after nuclear division, excluding rare spontaneous micronuclei; and 3) if micronuclei were retained after septation, to exclude fragmented bodies that formed transiently during mitosis. Videos from more than two biological replicates were used, and numbers were pooled. The combined proportions with 95% CI are presented. Deconvolved and projected images from a time course are shown in Figure 2A. A projected image of a single cell is shown in Supplemental Figure S2 and Supplemental Video S3.

**Protein methods**

Protein extracts were prepared from equal numbers of cells treated with 0.3 M sodium hydroxide. Cells were lysed by boiling for 5 min in acidic SDS–PAGE buffer (4% SDS, 60 mM Tris-HCl, pH 6.8, 5% glycerol, 4% 2-mercaptoethanol, 0.01% bromophenol blue, 0.1 M diithiothreitol). Samples were run on Tris-glycine gels and transferred to polyvinylidene fluoride membrane. Primary antibodies for Chk1HA (16B12 anti-hemagglutinin [HA]; Covance), phospho–Cdc2-Y15 (Cell Signaling Technology, Danvers, MA), and S. pombe Cdc2, Mcm4, and Crb2 (polyclonal antibodies) were incubated overnight. Blots were washed in phosphate-buffered saline (PBS)–TWEEN buffer, exposed to horseradish peroxidase–conjugated secondary antibody for 1 h, and then washed and exposed using enhanced chemiluminescence (Pierce). Quantitation of Mcm4 and Chk1-1HA (phosphorylated and unmodified forms) was performed using QuantityOne software (Bio-Rad) as in Furuya et al. (2010).

**DNA synthesis detection**

To monitor DNA synthesis by nucleoside analogue incorporation, cultures were treated with either 10 μM EdU or 10 μg/ml bromodeoxyuridine (BrdU) for appropriate times before harvest. EdU-treated cells were fixed with 70% ethanol and processed using the Click-IT EdU Alexa Fluor 488 Imaging Kit according to directions (C10337; Life Technologies, ThermoFisher Scientific). BrdU chromatin immunoprecipitation (IP) was performed as described in Knott et al. (2012) with the following modifications. Cells were pelleted, snap-frozen, and then stored at −80°C. After lysis in TES (100 mM Tris, pH 8.0, 50 mM EDTA, 1% SDS) with glass beads, chromatin was sheared by sonication, resulting in ~500–base pair fragments. DNA was phenol-chloroform extracted, isopropanol precipitated, and then resuspended in TE. Samples were diluted with IP buffer (1X PBS, 0.05% Triton X-100) before overnight incubation with anti-BrdU (RPN202; GE Healthcare, Sigma-Aldrich). Antibody-BrdU-DNA complexes were precipitated on magnetic protein A–Sepharose (Dynabeads, 10002D; Invitrogen, ThermoFisher Scientific), washed three times in IP buffer and once in TE, and then incubated in TES at 65°C (15 min). DNA was then purified using a Qiagen PCR purification kit and quantitatively amplified on a PerkinElmer HT9700 using origin-specific primers (Supplemental Table S2) and iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA). The Pfaffl method was used to determine percentage IP for each region relative to input DNA.

**Flow cytomtery and microscopy**

Cells were fixed in cold 70% ethanol for cell cycle analysis or microscopy. For DAPI/septa staining, cells were rehydrated in water and incubated for 10 min in 1 mg/ml aniline blue (M6900; Sigma-Aldrich). Cells in mount (50% glycerol, 1 μg/ml DAPI, and 1 μg/ml p-phenylenediamine) were photographed on a Leica DMR wide-field epifluorescence microscope using a 63× objective lens (numerical aperture [NA] 1.62 Plan Apo), 100-W Hg arc lamp for excitation, and a 12-bit Hamamatsu ORCA-100 charge-coupled device (CCD) camera. OpenLab version 3.1.7 (ImproVision, Lexington, MA) software was used at acquisition and ImageJ (National Institutes of Health, Bethesda, MD) for analysis.
Whole-cell SYTOX Green and EdU flow cytometry (fluorescence-activated cell sorting [FACS]) were performed as described in Sabatinos et al. (2012, 2013). DNA synthesis by EdU incorporation was assessed by adding 10 μM EdU to cultures before harvest. Whole-cell FACs for EdU were performed on rehydrated cells using Click-IT (Invitrogen, ThermoFisher Scientific) with Alexa Fluor 488.

Live-cell imaging

Medium for all live-cell imaging was EMM plus supplements plus thiamine. Live-cell videomicroscopy experiments at 36°C were performed on 2% agarose pads sealed with VaLaP (1/1/1 wt/wt/wt) Vaseline/lanolin/paraffin. Long-term videomicroscopy at 25°C was performed in CellAsics microfluidics plates (Y04C series; EMD Millipore), with constant temperature and medium flow. Fluorescent-tag images of live cells were acquired using a DeltaVision microscope (with softWoRx version 4.1; GE, Issaquah, WA) using a 60x (NA 1.4 PlanApo) lens, solid-state illuminator, and 12-bit CCD camera. Sections of static time points were eighteen 0.3-μm z-sections. Long-term time-lapse videos used nine z-steps of 0.5 μm. Images were deconvolved and maximum intensity projected (softWoRx). Transmitted light images were added to projected fluorescence images. Images were contrast adjusted using an equivalent histogram stretch on all samples. A threshold of signal 2σ over the average nuclear background was used for RPA-CFP and Rad52-YFP focus discrimination. Foci are presented as the proportion of nuclei per category of focus with ±95% CI. Significance was assessed with chi-squared tests and differences between proportions with two-tailed t tests.

Mutation analysis

The forward canavanine mutation rate at can1+ was determined as described (Sabatinos et al., 2013). Briefly, cultures were diluted in yeast extract with supplements (YES) medium and plated on 15-cm canavanine plates (70 μg/ml in pombe minimal medium with glutamate [PMG] plus supplements plus phloxine B). Plates were scored after 8 d at 25°C, for the number of can1− colonies compared with total cells plated, calculated from titer plates. Grouped experiments were performed independently, and then the mutation rate was calculated using the Mss-MLE algorithm in FALCOR (www.keshavisingh.org/protocols/FALCOR.html). Results were plotted in Delta Graph and compared by two-tailed t test.

The frequency of hsv-tk− loss and sectoring was scored in 500–2000 cells plated on YES, grown at 25°C, and then replica plated onto fluorodeoxyuridine (FUDr) plates (20 μg/ml in EMM plus supplements plus phloxine B). The number of FUDr-resistant and sectored colonies was counted per total number of colonies and the proportions assessed with Z tests (vassarstats.net/propdiff.ind.html). Grouped experiments were performed independently and pooled for analysis. A box plot of sectored data was made using BoxPlotR, showing median, 25th/75th percentile boundaries, and 1.5× interquartile whiskers (boxplot.tyerslab.com/).

The ChL minichromosome strains were grown as in Nakamura et al. (2008) and Li et al. (2013). Cultures were plated for viability and on PMG-HULA plates with 5-fluoroacetic acid (S-FOA; Zymo Research, Irvine, CA). Cultures were then incubated at 36°C for 4 h and then plated as at the start. All plates were grown at 25°C, and the number of Ura− colonies counted and compared with the total number of surviving cells. Ura− colonies were replica plated onto PMG-HUA and PMG-HUL with 5-FOA to assess Ura− and Ura− Ade− colonies, respectively. Ura− Leu− colonies were patched or replica plated onto PMG-HUL and YES-hygromycin to assess hygromycin and Ade status as in Li et al. (2013). FALCOR was used to calculate recombination/mutation rates, and a Mann–Whitney two-tailed U test was used to assess significance between observed sets.

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