The MCM helicase: linking checkpoints to the replication fork

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Abstract
The MCM (minichromosome maintenance) complex is a helicase which is essential for DNA replication. Recent results suggest that the MCM helicase is important for replication fork integrity, and may function as a target of the replication checkpoint. Interactions between MCM proteins, checkpoint kinases, and repair and recovery proteins suggest that MCMs are proximal effectors of replication fork stability in the cell and are likely to play an important role in maintaining genome integrity.

MCM (minichromosome maintenance) proteins: an essential role in DNA replication
The MCM proteins are a family of highly conserved AAA (ATPase associated with various cellular activities) proteins that form a heterohexameric protein complex essential for DNA replication (reviewed in [1]). MCM2–MCM9 proteins are defined by a distinct core of homology in the ATPase domain, and different N and C termini that determine their subfamily identity (reviewed in [1]). The MCM complex is well characterized in its role as an assembly factor at the replication origin that is activated during replication initiation to become the primary replicative helicase at the replication fork (reviewed in [1]). Briefly, the replication origin is bound by the ORC (origin recognition complex), and activated by Cdc6 (cell division cycle 6) (Sp (Schizosaccharomyces pombe) Cdc18) and Cdt1 to load the MCM hexamer. Multiple cycles of MCM loading occur, allowing the MCMs to spread beyond their loading point (reviewed in [1]). The abundance of MCMs is well in excess of the number of replication origins, a puzzle that is dubbed ‘the MCM paradox’. The additional MCMs may help distribute origins (see, e.g., [2]), or mark unreplicated chromatin for replication, as proposed in the original ‘licensing factor’ model [3]. However, MCMs are linked to diverse additional functions including transcription, silencing, genome stability and response to DNA damage [1,4], which could indicate any number of functions for the ‘remote’ MCMs.

The concerted actions of the CDK (cyclin-dependent kinase) and DDK (Dbf4-dependent kinase) (SpHsk1, Sc (Saccharomyces cerevisiae) Cdc7) leads to activation of the MCMs at the origin. This allows loading of Cdc45 and the GINS (Go, Ichi, Nii, San) complex, which together convert the MCM complex from an assembly factor into a replicative helicase, which begins unwinding to produce short regions of ssDNA (single-stranded DNA) ([5,6], reviewed in [1]). The ssDNA-binding protein RPA (replication protein A) is loaded to protect the ssDNA and moves along with the replication fork; thus assembly of RPA is a marker of initiation [7,8]. Following initiation, Cdc6 and Cdt1 are inactivated, and MCMs are lost from the chromatin as replication proceeds (reviewed in [1]). MCMs cannot reload on to the chromatin until the next cell cycle, when Cdc6 is again activated to bind ORC, and this ensures origins fire just once per cell cycle (reviewed in [1]). Significantly, MCM function is required throughout S-phase, as expected for a central component of the replication fork (see, e.g., [9]).

In S. pombe most mcm temperature-sensitive alleles accumulate approx. 2C (diploid) DNA and arrest the cell cycle [10–13]. Significantly, this arrest depends on Chk1 (checkpoint kinase 1), the DNA damage checkpoint kinase, which suggests that these alleles promote DNA damage despite their apparently complete DNA content [10–14]. On return to the permissive temperature, the mcm mutants cannot recover and the cells are inviable [11,14] (J.M. Bailis, D.D. Luche, T. Hunter and S.L. Forsburg, unpublished work). This may reflect the export of wild-type MCM proteins from the nucleus during arrest [16], or that MCMs cannot be reassembled on to the chromatin in the absence of a pre-replication complex (reviewed in [1]), or simply that continuous MCM association with the chromatin is required for normal replication [9]. Because of the replicated DNA content, we call these late-blocking or elongation alleles.

In contrast, a few mcm alleles block with a 1C (haploid) DNA content, which would be expected if their primary defect were in initiation [10,17,18]. This has led to the suggestion that initiation actually requires a relatively low level of MCM activity, but completion of S-phase and maintenance of genome integrity requires substantially higher amounts [1,10–14]; this may provide some insight into the MCM paradox. We refer to these as early-blocking or initiation alleles.

The apparently replicated genome content in the late-blocking S. pombe mutants prompts the question of why...
the cells cannot complete S-phase and proceed through the cell cycle. What has happened that allows such substantial DNA accumulation, yet results in S-phase arrest and cell death? The **mcm** mutants with this phenotype have increased rates of mitotic recombination and chromosome loss, suggesting a loss of genome stability [11]. Consistent with the requirement for **Chk1**, which suggests DNA damage had occurred, we observed that the late-blocking **mcm** mutants undergo double-strand breaks at the restrictive temperature, but the early-blocking initiation alleles do not (J.M. Bailis, D.D. Luche, T. Hunter and S.L. Forsburg, unpublished work). We suggest this results from replication fork collapse when MCMs are inactivated. Consistent with this hypothesis, **mcm2** temperature-sensitive mutants are synthetic lethal with **Δswi1** (W.P. Dolan, M.D. Green, J.-P. Yuan and S.L. Forsburg, unpublished work), which is part of a previously defined replication fork protection complex [20]. Thus we suggest that MCMs are likely to be proximal effectors of replication fork stability.

### Responses to replication fork stalling

When DNA is damaged or DNA synthesis is blocked, S-phase cells activate checkpoints (reviewed in [21,22]). These pathways ensure that cells arrest at S-phase, protect replication structures, repair any defects and finally restart DNA synthesis and recover. Checkpoint proteins are not essential for viability in *S. pombe*, although recent results suggested that they are actively involved in repairing damage even during unperturbed DNA replication, as well as being involved in the response to external insults [22]. Cells during S-phase have a higher tolerance for damage than cells elsewhere in the cell cycle (see, e.g., [23]), which makes sense since low levels of damage are likely to occur as a normal consequence of DNA synthesis. There are two major checkpoint responses during S-phase (reviewed in [21,22,24,25]). In *S. pombe*, the damage checkpoint kinases **Rad3** (**ScMec1**) and **Chk1** are activated by DNA lesions caused by irradiation or DNA-damaging drugs, which may include nicks, nucleotide adducts or double-strand breaks (reviewed in [21]).

If replication forks are stalled, for example by starving the cells for ribonucleotides, by treating with HU (hydroxyurea), or blocking polymerases with aphidicholin, the replication checkpoint is activated (reviewed in [22,24,25]). In *S. pombe*, the kinases responsible are **Rad3** (**ScMec1**) and **Cds1** (**ScRad53**). Fork stalling causes accumulation of ssDNA regions that remain with the stalled replication fork [5,6,26]. This extended ssDNA occurs because MCM-driven unwinding is uncoupled from DNA synthesis [5,6,27,28]. The ssDNA recruits additional RPA, which activates the checkpoint signal [29,30]. Normally, however, this uncoupling of MCMs from synthesis does not dissociate the replisome, because cells can recover from the fork stalling, complete S-phase and return to cycling. Therefore there must be some limiting mechanism that maintains replisome structure while permitting extended unwinding that activates the checkpoint.

*S. pombe* **Δcds1** mutant cells treated with HU arrest cell cycle progression and undergo catastrophic replication fork collapse and DNA damage, and are unable to recover [20,31–34] (J.M. Bailis, D.D. Luche, T. Hunter and S.L. Forsburg, unpublished work). In checkpoint mutants that lack **SpCds1** or **ScRad53** kinase, the ssDNA regions are elongated relative to wild-type, and generate regressed forks and other aberrant and toxic structures which activate the damage checkpoint [5,6,20,26,33–35]. This indicates that, in the absence of Cds1/Rad53, cells lack a necessary mechanism that protects the replication fork, restrains unwinding and restricts aberrant structures. Interestingly, polymerases apparently remain at the forks in **rad53** mutants ([36], although see [26]), but MCMs are lost [36].

These results suggest that MCM unwinding is limited by Cds1/Rad53 kinase activity to allow sufficient unwinding for a checkpoint signal, but not enough to decouple the replisome completely. One suggestion is that MCMs are a target of the replication checkpoint, which maintains their association with the replisome and exerts a protective effect on the fork. This prevents collapse or excessive unwinding, and protects against double-strand breaks. Consistent with this, MCM proteins undergo ATM (ataxia telangiectasia mutated)/ATR (ATM- and Rad3-related)-dependent phosphorylation in metazoans [37–40], consistent with a possible role as a target of the checkpoint. Additionally, we have recent results which suggest that **SpMcm4** is a target of the Cds1 kinase (J.M. Bailis, D.D. Luche, T. Hunter and S.L. Forsburg, unpublished work). These results present the possibility that MCMs may be a crucial effector of the replication checkpoint.

### Replication fork recovery: the role of Cds1/Rad53 and Mrc1

Successfully arresting the cell cycle and maintaining replisome structure is only the first part of the problem. The replisome must be reactivated to allow replication forks to restart and recover, and any aberrant DNA structures must be appropriately resolved. This process is called recovery, and is distinct from the initial arrest. The **Mrc1** protein is a non-essential component of the replication fork that contributes to Cds1 activation [41–46]. Mrc1 may be phosphorylated by **Rad3/Mec1** in order to recruit Cds1/Rad53 to the fork [41,43,46]. However, the phenotype of **Δmrc1** is distinct from that of the **Δcds1/Δrad53** mutants, as replication forks do not collapse, but cannot recover [41–46]. ChIP (chromatin immunoprecipitation) analysis, both genome-wide and at individual origins, suggests that MCMs and polymerases are delocalized from the replication fork in **Δmrc1** mutants, in contrast with **Δrad53/Δcds1** mutants, which retain polymerases but lose MCMs [28,36,47–49]. In *S. cerevisiae* **rad53** mutants, excessive unwinding occurs when the MCMs are lost from the stalled replication forks, and asymmetric gaps and hemicatenanes are formed [36]. In **Δmrc1** mutants, both the MCMs and the polymerase migrate away from regions of newly synthesized DNA, although they maintain their chromatin association, and hyper-unwinding occurs [28,36,47,48].
Figure 1 | Model for replication fork maintenance

(A) The normal replication fork has tight associations with components of the replisome. Leading (δ) and lagging (α) strand polymerases are indicated. M, Mrc1. (B) On HU treatment, DNA synthesis stalls, but the helicase continues to unwind. This generates additional ssDNA and RPA loading that activates the checkpoint signal. Rad3 is the Mec1 homologue in S. pombe, and Cds1 is the Rad53 homologue. (C) In the absence of Mrc1, Cds1 is not activated appropriately. The helicase and polymerase are uncoupled from the replication fork. They move away from the region of synthesized DNA, generating additional unwinding, but they remain associated with the chromatin. (D) In the absence of Cds1 (the Rad53 homologue), the MCMs are uncoupled from the replisome, generating excessive unwinding, but Mrc1 maintains the polymerases adjacent to the fork. However, in the absence of MCMs, the replisome is disrupted, and abnormal structures are formed, causing the replication fork to collapse (jagged bolt). (E) In the absence of MCMs, similarly to (D), Mrc1 maintains the polymerases adjacent to the fork. However, in the absence of MCMs, the replisome is disrupted, and abnormal structures are formed, causing the replication fork to collapse (jagged bolt).

This suggests that it is not the presence or absence of proteins at the fork that causes the collapse of replication forks in rad53 mutants, but rather the accumulation of excessive amounts of ssDNA and abnormal adducts that form when MCMs are unlinked from the other replisome components, perhaps asymmetrically. SpΔswi1 mutants, which have defects in the fork protection complex, show an increase in ssDNA even in normal S-phase [50] and are defective in Cds1 activation [50,51]. Interestingly, Mrc1 is required for efficient replication fork progression, independently of its role in checkpoint response [44,45,52], which suggests that one role of Mrc1 may be to act as a processivity factor to keep the replisome properly assembled and linked to the DNA fork.

These results suggest a model in which Mrc1 and Cds1/Rad53 play distinct roles in protecting the replication fork, and that the link between MCMs and the replisome is the critical feature for fork protection (Figure 1). Cds1 enforces the stability of the protein components of the replisome, which prevents the helicase from disengaging and causing excessive unwinding. In contrast, Mrc1 is required to maintain proper
association of the entire replisome with DNA. In wild-type cells, the normal response to replication fork stalling is replication fork arrest. The MCMs continue to unwind the DNA, generating ssDNA that will recruit RPA and activate the ATR checkpoint signal. ATR in turn activates the Cds1/Rad53 kinase and other substrates. This results in phosphorylation of MCMs, perhaps preventing further unwinding, and prevents the replisome from dissociating from the replication fork or from the chromatin. If Cds1 is missing, the increase in ssDNA suggests that MCMs are unable to restrain unwinding, freed from the constraint of their association with the polymerases. This generates excessive amounts of ssDNA, which allows the DNA to form asymmetric structures and regressed forks. In contrast, in the absence of Mrcl, the MCMs and the replisome maintain their association with one another, but migrate away from the newly synthesized DNA. This does not appear to be accompanied by the dramatic DNA damage and fork collapse of a Δcds1 mutant, but rather, because the replisome is not longer properly coupled to the replication fork, it prevents restart of DNA synthesis, resulting in a failure to complete replication and proceed through the cell cycle. If the MCMs are inactivated, as in an mcm-ts mutant, we posit that the breakdown of the replisome caused by disruption of the MCM helicase again exposes unwound or ssDNA and allows fork regression and abnormal structures to form. An important question is whether these effects are specific to arrest of the leading or lagging strand polymerase.

Contributions from other factors
Resolution of some DNA structures after checkpoint-mediated replication fork arrest requires recombination proteins (reviewed in [22,24]). Fork restart in prokaryotes is known to require recombination (reviewed in [53]). In S. pombe, immediately following release from HU, the SpRad22 (SrRad52) protein is recruited to nuclear foci, and Δrad22 or SpΔhrp31(Rad51) mutants show defects in recovery from HU treatment [34] (J.M. Bailis, D.D. Luche, T. Hunter and S.L. Forsburg, unpublished work). In S. cerevisiae, Rad51 is required to maintain DNA polymerase ε on the forks on HU treatment and also shows defects in recovery [54]. Rad51 is the RecA homologue that forms a strand-invading filament (reviewed in [55]). Interestingly, in both human cells and S. pombe, Rad51 protein interacts with MCMs [56] (J.M. Bailis, D.D. Luche, T. Hunter and S.L. Forsburg, unpublished work), which could provide one way for Rad51 to be recruited to the replication fork.

However, not all recombination is healthy for recovering cells, and there are also mechanisms to suppress inappropriate or untimely recombination events. For example, the checkpoint may directly inactivate recombination proteins (reviewed in [22]). Another mechanism to suppress inappropriate recombination relies on helicases that oppose recombination functions, such as Srs2, which antagonizes Rad51 filament formation [55], or the Bloom’s syndrome helicase SpRqh1/ScSgs1, which acts on recombination products downstream of Rad51 [57]. Sgs1 is also required in a parallel pathway to Mrcl to maintain polymerase association with the fork [47,49]. Finally, a combination of post-replication repair responses may also be required to complete repair of the genome following replication fork restart, and the links between checkpoints and downstream error-prone and error-free pathways are only just being explored (see, e.g., [38]; reviewed in [22,24,53,59]).

The studies described here suggest that the MCM complex has a crucial role to play in genome integrity. This is likely to be significant in the clinic as well as in the laboratory. Replication stress has been associated with development of cancer [60,61]. Dysregulation of MCMs and other replication proteins is observed in cancer cells (reviewed in [62]). This dysregulation is not simply a marker of increased proliferation in cancer cells. Two recent examples specifically link MCMs directly to cancer formation. In one study, increased expression of Mcm7 increased tumour formation and malignant conversion of skin cancer cells treated with a chemical carcinogenesis protocol [63]. In a second study, an allele of mouse Mcm4 was isolated that is specifically associated with development of mammary cancer; this same study also showed chromosome instability in yeast Mcm4 with the same mutation [64]. These studies indicate that analysis of the mechanisms by which the MCM complex contributes to genome stability in model experimental systems is directly relevant to human health and disease.

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