Terminating the replication helicase

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A feature of the cell cycle is that the events of one cycle must be reset before the next one begins. New work shows that the replication machinery is removed from fully replicated DNA by a conserved ubiquitin- and Cdc48 (p97)-dependent pathway. This explains how eukaryotic chromosomes are returned to the unreplicated state.

All organisms must make a perfect copy of the genome during every cell division. For eukaryotes this involves separating the steps of DNA replication into distinct cell cycle phases. Although great strides have been made in understanding how DNA replication is initiated we understand very little about what happens when two replication forks converge to terminate this process. Clarifying the final steps of replication is important to appreciate how DNA is fully replicated and segregated in a timely manner. Recent work now identifies an evolutionarily conserved pathway involving the E3 ubiquitin ligase complex $CRL2^{Lrr1}$ that is required for the removal of the replication apparatus when DNA synthesis is complete[1, 2]. These studies begin to explain how DNA replication is irreversibly reset before the next division.

The tight control of DNA replication within the eukaryotic cell cycle centres on the state of the six-subunit replicative helicase Mcm2-7 (Figure 1). For the first step in genome duplication, the DNA must be ‘licensed’ to replicate through the loading of Mcm2-7 onto double stranded (ds) DNA as an inactive head-to-head double hexamer (Figure 1a). This licensing reaction is restricted to late mitosis / early G1

phase, due to the inhibition of this process by multiple mechanisms in other phases of the cell cycle [3].

Activation of Mcm2-7 double hexamers only occurs following the G1-S transition due to the accumulation of Cyclin-dependent kinase (CDK) and Dbf4-dependent kinase (DDK) activities. These two kinases cause major structural changes in the Mcm2-7 double hexamers, resulting in separation of the two hexamers, DNA melting and strand passage and recruitment of the helicase cofactors Cdc45 and GINS, to form the CMG complex (Cdc45, Mcm2-7, GINS) [4]. The exact mechanism by which DDK and CDK induce all of these changes in the helicase are not fully understood but the outcome is that the active form of the replicative helicase is the CMG complex encircling the single leading strand template (Figure 1b).

The separation of licensing and helicase activation into distinct phases of the cell cycle (Figure 1a and b) ensures that DNA cannot be re-replicated within a single cell cycle, but how are CMG complexes removed when DNA synthesis is complete to reset the cycle? Experiments in *Xenopus laevis* egg extracts [5] and in budding yeast [6] have shown that at the end of S-phase the CMG complex is actively removed from chromatin. This pathway involves ubiquitylation of the helicase subunit Mcm7, in yeast by the ubiquitin ligase SCF^{Dia2} [6]. The polyubiquitylated CMG complex becomes a substrate for the segregase Cdc48 (also called p97 or VCP) [5, 6]. Cdc48 is an ATPase that can remodel and potentially unfold ubiquitylated and sumoylated substrates [7], although exactly how Cdc48 removes CMG from chromatin is not yet known.

A key question arising from these studies is how this CMG removal pathway is specific for terminated complexes. Premature unloading of licensed (Figure 1a) or actively replicating (Figure 1b) Mcm2-7 complexes could be lethal. Furthermore, as the yeast Mcm7 ubiquitin ligase subunit Dia2 is not conserved beyond fungi, how is

this pathway regulated in metazoans? Recent studies, using the power of reverse genetics in *C.elegans* and the complete replication of DNA *in vitro* in *Xenopus* egg extracts [1, 2, 8] shed light on the final events of DNA synthesis in metazoa, completing the cycle of Mcm2-7 helicase activation and deactivation (Figure 1).

A major bottleneck in studying the termination of DNA replication in eukaryotes is that termination events happen asynchronously. Elegant work in *Xenopus* egg extracts has solved this problem using a system that can conditionally arrest the replication of a plasmid just before termination [8]. Relief of this arrest allows the synchronous convergence and termination of replication forks. This *in vitro* system was used to demonstrate that when two forks converge, the CMG complex transitions from unreplicated ssDNA onto the newly synthesised dsDNA [8] (Figure 1c). To understand what happens to this CMG complex at termination the authors performed a proteomic analysis of factors associated with chromatin during termination. This revealed that an E3 ubiquitin ligase complex $CRL2^{Lrr1}$ and Cdc48/p97 (together with cofactors including Npl4 and Ufd1) are recruited to chromatin, causing Mcm7 ubiquitylation and CMG disassembly specifically after termination of DNA replication [1]. Similar results were achieved through the analysis of the replication of sperm chromatin in *Xenopus* egg extracts [2]. Significantly, unloading the CMG complex is sufficient to trigger the unloading of other replication factors still bound to the helicase, such as DNA polymerases, suggesting that it is the topological linkage between CMG and DNA that is key for the stability of the replisome on chromatin [1].

To study metazoan CMG unloading *in vivo*, Sonneville et al analysed the chromatin binding of GFP-tagged CMG components in *C. elegans* embryos by live microscopy [2]. Using RNAi against all Cdc48 adaptors and Cullin-dependent E3 ligases, they showed that Mcm7 ubiquitylation and CMG unloading is caused specifically by the *C.elegans* orthologues of $CRL2^{Lrr1}$ ($CUL\text{-}2^{LRR\text{-}1}$ in *C.elegans*) and Cdc48/Npl4/Ufd1.

Importantly knock-down of components of the CMG complex can rescue the sterility phenotype of both LRR-1 and CUL-2 mutants in *C.elegans* [9], suggesting that unloading of the CMG complex is an important function of CUL-2^{LRR-1}. Together these studies, from yeast to nematodes to vertebrates [1, 2, 5, 6], demonstrate a striking evolutionary conservation of an Mcm7 polyubiquitination and Cdc48/p97-dependent CMG unloading pathway at termination (Figure 1).

Whereas down-regulation of Cdc48/Npl4/Ufd1 is sufficient to prevent CMG unloading throughout prophase during early embryonic cycles in *C.elegans*, $CRL2^{Lrr1}$ down-regulation did not prevent CMG unloading in late prophase, suggesting the existence of other CMG removal pathways *in vivo* [2]. Through further targeted RNAi screening, Sonneville et al showed that the Cdc48 partner protein UBXN-3 is required for the late prophase removal of CMG from chromatin (Figure 2). This is consistent with previous observations that depletion of UBXN-3 in *C. elegans* causes Cdc45 and GINS accumulation on chromatin [10]. Interestingly, depletion of UBXN-3 alone has a minor effect on CMG persistence, suggesting that $CRL2^{Lrr1}$ is the primary mechanism for CMG removal *in vivo* [2]. As the UBXN-3 pathway is Cdc48-dependent, but $CRL2^{Lrr1}$ independent it is possible that Mcm7 ubiquitylation is not required for this pathway (Figure 2). Intriguingly, inhibition of the SUMO protease ULP-4 mimics the phenotypes of UBXN-3 downregulation [2], although more work needs to be done to understand this pathway in mechanistic detail (Figure 2).

By studying CMG unloading pathways *in vivo*, Sonneville et al were able to address the physiological importance of this process. For this they combined RNAi of both CMG removal pathways, $CRL2^{Lrr1}$ together with either UBXN-3 or ULP-4. These assays showed that these two pathways are synthetically lethal in *C. elegans* [2], which suggests that both pathways for CMG removal are critical *in vivo* (Figure 2). An important area of future study will be to understand why failing to remove the CMG complex is lethal. One possibility is that CMG persistence results in re-

replication, which would have significant consequences for the onset of genomic instability. Indeed depletion of Faf1 (the human orthologue of UBXN-3) leads to replication stress in human cells [10] and multiple mutations in Faf1 have been found associated with a wide variety of cancers [11]. Whether these effects of loss of Faf1 are linked to defects in CMG unloading are not known.

A key outcome of the *in vitro* studies [1, 2, 5] together with work in budding yeast [6] is that this ubiquitination and Cdc48/p97-dependent removal of Mcm2-7 from chromatin is specific for CMG complexes post-termination (Figure 1). This pathway does not remove double hexamers in G1 phase or stalled/active CMG complexes before termination. The specificity of this pathway for terminated CMG complexes is critical to ensure that DNA replication is allowed to go to completion before the Mcm2-7 complexes are removed from DNA. Understanding how this specificity is achieved may lie in a structural understanding of the differences between the Mcm2-7 complexes during the cell cycle [4].

The post-termination ubiquitin/Cdc48-dependent removal of CMG forms an irreversible switch to end the replication cycle (Figure 1), but is there more to Mcm2-7 helicase regulation? It is possible that given the importance of the strict control of the helicase complex throughout the cell cycle (Figure 1) there will be multiple pathways that regulate Mcm2-7 unloading in different contexts. For example, it has been shown that Mcm2-7 double hexamers are removed from origins in cells that enter quiescence [12] and it is not known what happens to double hexamers that are not activated during S-phase. These studies have closed the Mcm2-7 ring cycle, but opened a new aspect of eukaryotic replication control.

Figure legends

Figure1. The MCM helicase cycle within the cell cycle. In late mitosis/early G1 phase DNA is ‘licensed’ to replicate through the loading of the Mcm2-7 helicase onto double

stranded (ds) DNA as an inactive double hexamer (a). In S-phase, inactive double hexamers are activated to form the CMG complex encircling single stranded (ss) DNA (b). At termination, the CMG complex transitions onto replicated dsDNA (c). Only the terminated CMG complex is a substrate for the E3 ubiquitin ligase $CRL2^{Lrr1}$ (pink arrows). Ubiquitylated CMG complex is a substrate for Cdc48 (together with cofactors Ufd1 and Npl4), which removes CMG from DNA, returning chromosomes to their unlicensed state, ready for a new cell cycle.

Figure 2. At least two pathways target terminated CMG complexes in *C.elegans*. The primary pathway for the removal of CMG complexes in *C.elegans* is $CRL2^{Lrr1}$-dependent, as in *Xenopus*, but a second pathway functions in late prophase if $CRL2^{Lrr1}$ is inhibited. This back-up CMG removal pathway is UBXN-3-dependent, but the mechanism of action of this pathway is not clear.

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Figure 1.

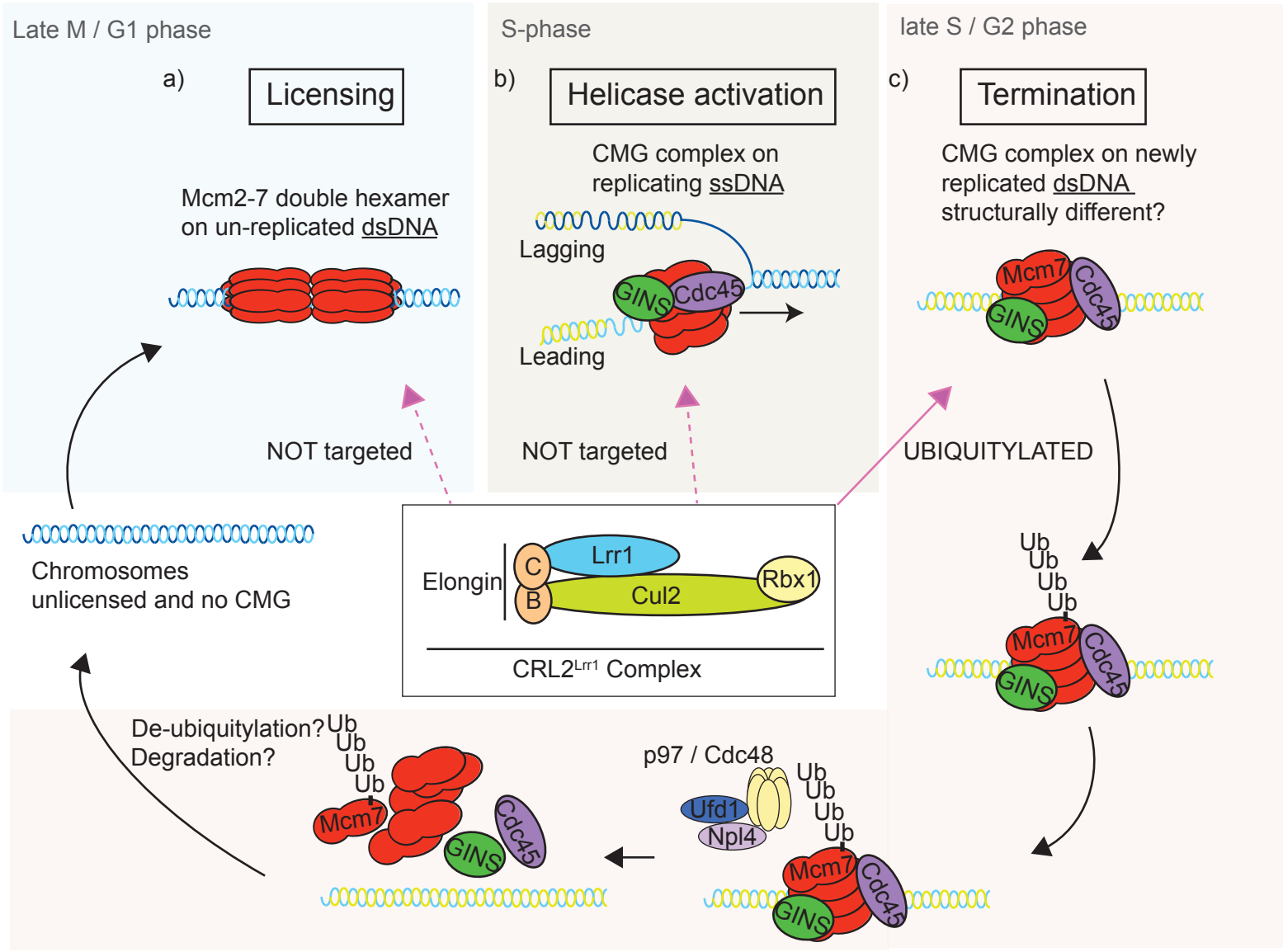
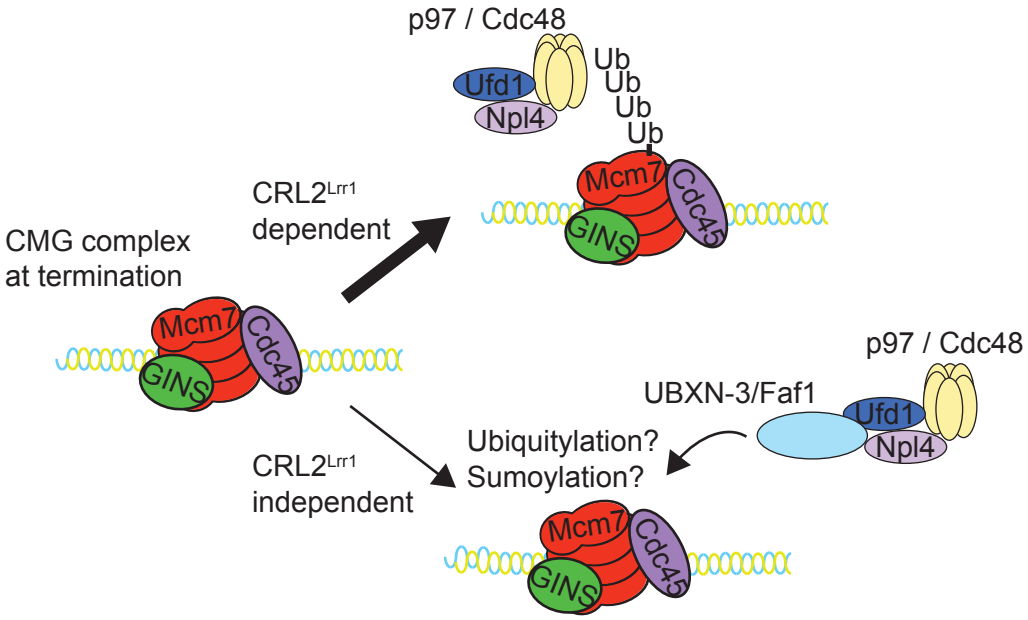

Late M / G1 phase
a)
Licensing
Mcm2-7 double hexamer
on un-replicated dsDNA
NOT targeted
Chromosomes
unlicensed and no CMG
S-phase
b)
Helicase activation
CMG complex on
replicating ssDNA
Lagging
Leading
GINS
Cdc45
NOT targeted
late S / G2 phase
c)
Termination
CMG complex on newly
replicated dsDNA
structurally different?
Mcm7
UBIQUITYLATED
Ub
Elongin
C
B
Lrr1
Cul2
Rbx1
CRL2Lrr1 Complex
De-ubiquitylation?
Degradation?
p97 / Cdc48
Ufd1
Npl4

Figure 2.


p97 / Cdc48
Ufd1
Npl4
Ub
Ub
Ub
Ub
CRL2Lrr1
dependent
Mcm7
Cdc45
GINS
CMG complex
at termination
Mcm7
Cdc45
GINS
p97 / Cdc48
UBXN-3/Faf1
Ufd1
Npl4
Ubiquitylation?
Sumoylation?
CRL2Lrr1
independent
Mcm7
Cdc45
GINS