The initiation of DNA replication is a highly regulated event in eukaryotic cells to ensure that the entire genome is copied once and only once during S phase. The primary target of cellular regulation of eukaryotic DNA replication initiation is the assembly and activation of the replication fork helicase, the 11-subunit assembly that unwinds DNA at a replication fork. The replication fork helicase, called CMG for Cdc45-Mcm2–7, and GINS, assembles in S phase from the constituent Cdc45, Mcm2–7, and GINS proteins. The assembly and activation of the CMG replication fork helicase during S phase is governed by 2 S-phase specific kinases, CDK and DDK. CDK stimulates the interaction between Sld2, Sld3, and Dpb11, 3 initiation factors that are each required for the initiation of DNA replication. DDK, on the other hand, phosphorylates the Mcm2, Mcm4, and Mcm6 subunits of the Mcm2–7 complex. Sld3 recruits Cdc45 to Mcm2–7 in a manner that depends on DDK, and recent work suggests that Sld3 binds directly to Mcm2–7 and also to single-stranded DNA. Furthermore, recent work demonstrates that Sld3 and its human homolog Treslin substantially stimulate DDK phosphorylation of Mcm2. These data suggest that the initiation factor Sld3/Treslin coordinates the assembly and activation of the eukaryotic replication fork helicase by recruiting Cdc45 to Mcm2–7, stimulating DDK phosphorylation of Mcm2, and binding directly to single-stranded DNA as the origin is melted.

Introduction to DNA Replication and the Cell Cycle

DNA replication is restricted to the S phase of the eukaryotic cell cycle. During S phase, the parental genomic DNA is replicated precisely once to provide an additional copy of DNA for each daughter cell. It is important that replication is restricted to S phase, and that the parental is only duplicated just once. Over-replication of DNA, under-replication of DNA, or replication of DNA outside of S phase can lead to genome instability and possibly cell death or cancer.

The core enzymes of DNA replication are the DNA polymerases, which copy the parental duplex DNA. Pol α-primase supplies the initial nucleic acid primer for subsequent elongation by Pol δ and Pol ε. Pol ε is devoted to replication on the leading strand, while Pol δ is devoted to replication on the lagging strand, although recently this division of labor has been questioned. Importantly, Pol α-primase, Pol δ, and Pol ε each require single-stranded DNA templates (primed for Pol δ and Pol ε, or unprimed for Pol α-primase) for activity. Since the parental duplex is double-stranded, the replication fork helicase is required to melt the parental duplex to form single DNA strands (ssDNA). The replication fork helicase therefore functions before the replicative polymerases, and therefore the replication fork helicase is the physiologic target for cell cycle regulation.
The Mcm2–7 heterohexameric ring complex is the motor of the CMG helicase. The 6 homologous Mcm subunits contain an N-terminal DNA-interacting and oligomerization domain and a C-terminal AAA+ motor domain. The Mcm2–7 complex has very weak helicase and ATPase activity on its own, but the assembly with Cdc45 and the tetrameric GINS complex stimulates Mcm2–7 activity. Together, the CMG is very efficient in hydrolyzing ATP and processively unwinding DNA.

The Mcm2–7 complex loads as an inactive head to head double hexamer to encircle double-stranded DNA during G1 phase in a reaction known as replication licensing. This reaction is ATP-dependent and catalyzed by Orc, Cdc6, and Cdt1. The details of this reaction have recently been elucidated by a number of labs. Since Mcm2–7 complexes cannot load during S phase, this restriction of Mcm2–7 loading to G1 phase prevents re-replication or over-replication of the parental duplex DNA.

An excess of Mcm2–7 complexes load onto double-stranded DNA relative to the Mcm2–7 complexes that are activated to unwind DNA at a replication fork. This reaction is restricted to G1 phase in the cell cycle. Since Mcm2–7 complexes cannot load during S phase, this restriction of Mcm2–7 loading to G1 phase prevents re-replication or over-replication of the parental duplex DNA.

The studies that conclude that DDK phosphorylation of Mcm2 is only important during replication stress were based upon a genomic insertion of the kinase-dead mutant of mcm2 at the genomic locus, under regulation by native promoter. We recently acquired the yeast strain that harbored the genomic insertion of the kinase-dead mutant of mcm2. We overexpressed the kinase-dead mutant of mcm2 in wild-type yeast strain and in the yeast strain harboring the kinase dead mutant. Whereas overexpression of the kinase-dead mutant of mcm2 in wild-type cells results in a severe growth defect, overexpression of the same kinase dead mutant of mcm2 resulted in no defect in the cells harboring the genomic copy of kinase-dead mcm2. These data suggest that the strain with the genomic copy of kinase-dead mcm2 harbored a suppressor mutation. Thus, this suppressor mutation explains the discrepancy between the conclusions from our different laboratories, and the data now point to the conclusion that DDK phosphorylation of Mcm2 is important for the recruitment of Cdc45 to Mcm2–7. Furthermore, phosphorylation of Mcm4 by DDK alleviates an inhibitor activity present in the N-terminal region of Mcm4. The role of DDK phosphorylation of Mcm6 is currently under investigation.
Mcm2 is required for DNA replication under normal growth conditions.\textsuperscript{39,42,43} The interaction between Mcm2 and Mcm5 in the Mcm2–7 ring may dissociate under certain conditions, acting as a ‘gate’ for the movement of dsDNA into the Mcm2–7 ring during G1.\textsuperscript{8,44} Work from our laboratory showed how DDK phosphorylation of Mcm2 may have a critical role in modulating this gate. The phosphorylation of the N-terminal of Mcm2 by DDK may open the Mcm2–7 ring at the Mcm2-Mcm5 interface, allowing for ssDNA extrusion.\textsuperscript{42} The generation of ssDNA at origins of replication is critical for the timely assembly of the CMG helicase.\textsuperscript{42} Furthermore, expression of mcm5-bob1 (mcm5-P83L) bypasses the requirement for DDK in the cell,\textsuperscript{45} and expression of mcm5-bob1 also partially suppresses the growth inhibition induced by overexpression of kinase-dead mutant of mcm2.\textsuperscript{32} Mcm3-bob1 is also inhibited from binding Mcm2 relative to wild-type Mcm5, suggesting that the Mcm2-Mcm5 interface is a target of regulation by DDK.\textsuperscript{42}

**Roles of Sld2, Sld3, and Dpb11 in Helicase Assembly and Activation**

The roles of the essential initiation factors, Sld2, Sld3, and Dpb11, are beginning to be elucidated. For example, recent structural data shows that the N-terminal region of Sld3 binds to Sld7, and Sld3 and Sld7 from a tetramer of 2 subunits each of Sld3 and Sld7 (Fig. 1).\textsuperscript{23,24} Furthermore, the middle domain of Sld3 binds to Cdc45, and structural data on this interaction is available as well.\textsuperscript{46} Moreover, Sld3 recruits Cdc45 to Mcm2–7 during S phase in a manner that is dependent on DDK.\textsuperscript{32,47} Dpb11 is believed to be a scaffolding protein that attaches to CDK-phosphorylated Sld2 and Sld3.\textsuperscript{33,24} Each of these proteins, Sld2, Sld3, and Dpb11, bind directly to Mcm2–7 and single-stranded DNA (ssDNA).\textsuperscript{48,50} These data suggest that Sld2, Sld3, and Dpb11 may play an active role in assembly or activation of the helicase.\textsuperscript{48,50} Furthermore, the ssDNA-binding properties of Sld2 and Sld3 are conserved in their human counterparts, RecQL4 and Treslin, respectively.\textsuperscript{50,51} In addition, the region of Sld3 that binds to ssDNA and Mcm3 and Mcm5 subunits is the C-terminal domain of Sld3.\textsuperscript{50}

What could be the functional role of Sld2, Sld3, and Dpb11 binding to ssDNA? Work from other labs suggests that single-stranded DNA is formed at an origin of replication during the process of replication initiation, in a process known as origin melting.\textsuperscript{52} Furthermore, in one model of replication elongation, the steric exclusion model, the lagging single-stranded DNA template is excluded from the central channel of the CMG replication fork helicase.\textsuperscript{53} Other models for replication fork helicase mechanism involve a ploughshare at the back of the helicase,\textsuperscript{54} or an exit channel for single-stranded DNA extrusion through the CMG.\textsuperscript{55}

While details regarding CMG mechanism remain to be resolved, one theme in each of the models is for the separation of the 2 single DNA strands during helicase activation. Thus, during replication initiation, an ssDNA-binding platform is potentially generated for Sld2, Sld3, and Dpb11. Since the Sld2, Sld3, and Dpb11 proteins bind to one another in a CDK-dependent mechanism,\textsuperscript{23,24} and since Sld2, Sld3 or Dpb11 alone has considerable affinity for ssDNA,\textsuperscript{48,50} the Sld2-Sld3-Dpb11 complex likely has a very tight affinity for ssDNA.

Recent data from our lab suggest that Sld2, Sld3, and Dpb11 attachment to ssDNA at an origin of replication is required for DNA replication.\textsuperscript{48,50} In addition, we found that Sld2, Sld3, and Dpb11 interaction with ssDNA may be important for GINS attachment to Mcm2–7.\textsuperscript{48,50} This conclusion follows from the observation that Sld2, Sld3, and Dpb11 compete with GINS for Mcm2–7 in a manner that is influenced by ssDNA addition.\textsuperscript{48,50} In other words, Sld2, Sld3, and Dpb11 each interact with Mcm5 and Mcm3, the subunits of Mcm2–7 that contact GINS.\textsuperscript{48,50} Furthermore, addition of Sld2, Sld3, and Dpb11 to Mcm2–7 can dislodge GINS from Mcm2–7.\textsuperscript{48,50} However, single-stranded DNA also competes with Sld2, Sld3, and Dpb11, but not GINS, for Mcm3/Mcm5 interaction.\textsuperscript{48,50} In addition, the addition of single-stranded DNA releases Sld2, Sld3, and Dpb11 from Mcm3 and Mcm5, allowing GINS to bind to Mcm2–7 by a passive sequestration mechanism.\textsuperscript{48,50} There is also a recent manuscript from the Speck lab demonstrating that Sld2 and Sld3 exhibit an anti-cooperative mechanism for recruiting Cdc45 to loaded Mcm2–7 complexes.\textsuperscript{56} In other words, Sld2 and Sld3 release from Mcm2–7 once Cdc45 is recruited to Mcm2–7.\textsuperscript{56} Further details about the process of origin melting and helicase activation are not yet resolved, but the development of an \textit{in vitro} replication initiation assay by the Diffley lab holds promise that many of these details will soon be revealed.\textsuperscript{52}

Dpb4, Sld2, Sld3, and Dpb11 are present in low amounts in the cell.\textsuperscript{30} Thus, they can only bind to certain Mcm2–7 complexes, marking them for subsequent CMG helicase assembly and activation. But what is to prevent DDK from phosphorylating one Mcm2–7 complex, while Sld3, for example, binds to a different Mcm2–7 complex? A potential answer to this question has recently been revealed by work from our lab.\textsuperscript{43} DDK phosphorylation of Mcm2 is a very weak reaction \textit{in vitro}, and only a fraction of Mcm2 proteins are phosphorylated by DDK in a kinase assay using purified DDK and Mcm2.\textsuperscript{40} In spite of this weak activity \textit{in vitro}, we demonstrated an \textit{in vivo} DNA replication function for DDK phosphorylation of Mcm2.\textsuperscript{42,43} Thus, the data suggested that a critical factor was missing from our \textit{in vitro} phosphorylation assay.\textsuperscript{43,44}

Is Sld3/Treslin a Conductor of Helicase Assembly and Activation?

We examined Sld2, Sld3, and Dpb11 for their effect on DDK phosphorylation of Mcm2 \textit{in vitro}, and found that Sld3 exerts an 11-fold stimulation of DDK phosphorylation of Mcm2.\textsuperscript{43} In contrast, the ability of Sld2 and Dpb11 to stimulate DDK phosphorylation of Mcm2 is very slight.\textsuperscript{43} Furthermore, Treslin, the human ortholog of Sld3, stimulates human DDK phosphorylation of human Mcm2 by 15-fold.\textsuperscript{43} Thus, the ability of Sld3/Treslin to substantially stimulate DDK
phosphorylation of Mcm2 is conserved from yeast to human. 43,50

This observation may help explain the question asked above, what keeps Sld3/Treslin bound to Mcm2–7 and activates DDK phosphorylation of Mcm2 at the same time. 43,50 Thus, Sld3/Treslin is a conductor of replication initiation. Sld3/Treslin performs several functions upon binding to Mcm2–7: 1 Sld3/Treslin recruits Cdc45 to Mcm2–7 in a manner that depends upon DDK, 47,2 Sld3/Treslin stimulates DDK phosphorylation of the Mcm2–7 complex, 43,3 Sld3/Treslin binds to ssDNA, binds to Mcm3/Mcm5, and stimulates DDK phosphorylation of Mcm2.

**The Role of Mcm10 in DNA Replication Initiation**

Mcm10 is also required for the initiation of DNA replication. 57-59 Some early reports suggest that Mcm10 is involved in the recruitment of Cdc45 to Mcm2–7, 60, 63 while recent reports suggest that Mcm10 is involved in a step following CMG assembly. 64-66 These recent reports suggest that Mcm10 plays a role in activating the fully-assembled CMG. It may be that both models are correct; in other words, Mcm10 may have multiple functions in replication initiation. Future work may help resolve this controversy. There are also reports concluding that Mcm10 is a component of the CMG during replication elongation, providing stability to the Pol α-prime. 67,68 Thus, Mcm10 is required for initiation, and it is also required for replication elongation.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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