Make your proposal easy to read.

Use figures (including models).

Use 13 or 14 point spacing between lines (instead of 12).

Include spacing between paragraphs.

Use frequent headings.

Indent new paragraphs.

Avoid long text!

Easy to read

be invaluable to interpreting the results of the studies described in specific aims 2 and 3 that investigate the functions of individual proteins during pre-RC formation and their regulation.

D.2 Determine the Role of ATP Binding and Hydrolysis During Pre-RC Formation.

Nucleotide binding and hydrolysis are frequently coupled to the regulated formation of multi-protein complexes and the steps coupled to nucleotide hydrolysis frequently represent key decision points. Perhaps the most well understood example of this is protein translation, in which all of the key steps are coupled to a round of GTP binding and hydrolysis (Ramakrishnan, 2002). Importantly, for many translation factors the decision to hydrolyze GTP is intimately linked to the order, specificity, and regulation of translation (e.g. GTP hydrolysis by EF-Tu is linked to correct codon-anti-codon recognition). Similarly, the assembly of sliding DNA clamps around a primer-template junctions is coupled to ATP binding and hydrolysis (O'Donnell et al., 2001). Again, the specificity of this reaction is controlled by a primer-template junction dependent activation of ATP hydrolysis. Current evidence indicates that formation of the pre-RC is also controlled by ATP binding and hydrolysis. Ten of the fourteen proteins required for pre-RC formation contain consensus ATP binding and hydrolysis motifs (Orc1, 4, 5, Mcm2-7 and Cdc6) and in most cases mutating these sites eliminates their function *in vivo* (Klemm et al., 1997; Schepers et al., 2001; Schwacha et al., 2001). Assembly of the *in vitro* pre-RC requires ATP and is inhibited by the addition of ADP or ATP-γ-S (Gillespie et al., 2001; Seki et al., 2000). Finally, three ORC subunits (1, 4, and 5) and Cdc6 are closely related to subunits of the sliding clamp loader. This has led to the suggestion that ORC and Cdc6 could work in a manner analogous to the AAA+ proteins in sliding clamp and/or helicase loaders to assemble the proposed ring shape of the Mcm2-7 complex around origin DNA (Fig. 10). If so, we might expect an ATP hydrolysis dependent switch in Mcm2-7 association during pre-RC assembly and that hydrolysis defects could capture these intermediates. In this aim, we propose to use a combination of *in vivo* and *in vitro* assays to determine the steps in pre-RC formation that are coupled to ATP binding and hydrolysis.

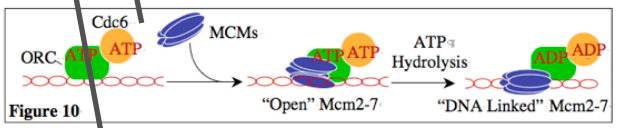

Figure 10

D.2A What is the role of ORC ATP hydrolysis during pre-RC formation

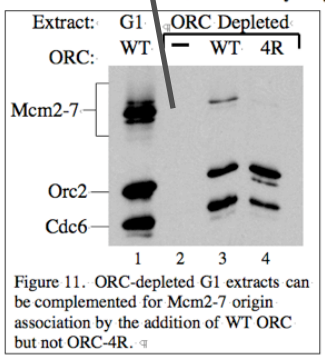

Figure 11. ORC-depleted G1 extracts can be complemented for Mcm2-7 origin association by the addition of WT ORC but not ORC-4R.

The role of ORC in pre-RC assembly remains unclear. ORC could act primarily as a scaffold to tether other proteins to the origin. Alternatively, ORC could play a more active role as part of an ATP-dependent pre-RC assembly machine. Analysis of *ORC1* mutants that are defective in the ORC ATPase suggest that ORC needs to be bound to ATP to interact with Cdc6 (Klemm et al., 2001), suggesting at least two roles for ORC ATP binding during pre-RC assembly (including origin binding). Despite this evidence, our understanding of the role of ATP hydrolysis in ORC function remains elusive. The experiments in this aim seek to distinguish between a "scaffold" and a "machine" model for ORC function by analyzing the function of ORC ATP hydrolysis mutants during pre-RC formation.

i. Determine the role of ORC ATP hydrolysis during *in vitro* pre-RC formation. These studies will exploit G1 extracts that have been biochemically depleted of ORC (by fractionation

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irradiation). We will also use a simple plasmid-based assay we have recently developed to determine if this protein alters DNA replication timing.
It is possible that the only proteins that will meet the required criteria will be known components of the pre-RC (although we have found that the addition of purified preparations of the four known components is not sufficient to direct pre-RC formation). However, even this finding would be useful as there is some controversy about the involvement of proteins in pre-RC formation. For example, Noc3 has been proposed to be part of the pre-RC (Zhang et al., 2002), however we have failed to find independent evidence for this. It is also possible that proteins will be identified by the mass spec approach that are not required for pre-RC formation but are nonetheless associated with a component of the pre-RC. Although not the primary target of these studies, their association with a pre-RC component could suggest a role in a downstream step in DNA replication, a checkpoint response, or in the control of replication timing. Finally, fractionation will lead to a more reconstituted assay that will allow more precise analysis of the process of pre-RC formation. Because this assay uses DNA with only a single origin (unlike the Seki and Diffley assay, which used repeated DNA) we will be interested in developing assays to look at the pre-RCs formed by footprinting and electron microscopy. In addition, a more refined assay will allow a rigorous determination of the stoichiometry of the proteins in the pre-RC, paying particular attention to the number of Mcm2-7 complexes loaded per origin.
D.2 Determine the nature of Mcm2-7 association with origin DNA. The assembly of a pre-RC is likely to extend beyond a series of protein-protein and protein-DNA associations. *In vivo* experiments support this concept as both ORC and Cdc6 are thought to be unnecessary to maintain Mcm2-7 in a state able to direct initiation (Piatti et al., 1996; Shimada et al., 2002). This set of experiments is aimed at determining the nature of the association of the pre-RC components with the origin DNA. Is the Mcm2-7 complex topologically linked to the DNA? Are proteins in the extract beyond the pre-RC components required for its stability? Is nucleotide hydrolysis required only for pre-RC assembly or also for its maintenance? These experiments will focus on the Mcm2-7 complex, as this is the current marker for pre-RC formation. Nevertheless, these same or related experiments will also be informative concerning the stable association of other components of the pre-RC.
To address this question, we will first determine the stability of Mcm2-7 association with origin DNA once it is removed from the G1 extract. After removal from the extract, the bead-bound DNA will be washed to remove unbound proteins and then transferred to buffer alone or buffer with 3 mM ATP or ATP-γ-S. We will determine the extent of protein association that remains over time by western blot. We will test for Mcm protein association using our monoclonal antibody that recognizes all six proteins and epitope tagged Mcm proteins to detect specific Mcm proteins (we will test a member of the "catalytic" and "regulatory" Mcm protein class by this method). Complexes will be removed from the extract at several different times (5, 15, 30 and 60 min) during the assembly process to determine if there are intermediates with reduced stability.
We will compare the off-rates observed in buffer alone (+/-ATP) to those obtained in G1 extracts. To this end we will exploit two strains that have the Mcm4 protein epitope-tagged with either HA or Myc epitopes (both strains will over-express ORC and Cdc6). We will prepare G1 extracts from each strain after inducing expression of both Cdc6 and ORC. We will then use extracts from the HA tagged strain to assemble pre-RCs on bead-bound DNA for 5, 15, 30 and 60 minutes. At the appropriate times, the beads will be isolated from the Mcm4-HA extract and transferred to the Mcm4-myc extract. We will then determine the relative levels of Mcm4-HA and Mcm4-myc association with the bead bound DNA at the same intervals as above. This will determine the off-rate of Mcm4 when the G1 extract is present and whether new Mcm4-myc loading is still occurring. The reverse experiment starting with the Mcm4-myc extract will also be performed to make sure that the results are not due to tag-dependent changes in Mcm4 function.
Many current models for pre-RC formation suggest that ORC and Cdc6 act to topologically link a ring shaped Mcm2-7 complex around dsDNA (Davey et al., 2003). If this is true then forming the pre-RC on longer, circular, or DNA with bulky adducts at its ends could stabilize the association of the Mcm2-7 complex after purification from extracts. In addition, we are also interested in determining how the DNA template influences pre-RC formation. We have already observed that DNA molecules longer than 300 bases significantly improve the level of Mcm2-7 association. This could be due to a need for additional DNA for the assembly process or a stabilizing effect.
To address the above issues, we will perform both assembly and off-rate experiments (as described above) using a variety of different DNA templates containing a single origin of replication. We will test different lengths of the DNA in three different forms: linear with biotin at one end, linear with biotin at one end and a bulky adduct at the other end, and circular. The bulky adduct will be created using an EcoRI site and a mutant EcoRI protein that has a half life for dissociation from this site of >20 hours (Randell et al., 2001). The circular DNAs will be made by ligation of the same linear DNAs followed by limited biotinylation of the DNA with