

Helicase activation mechanisms during initiation of DNA replication

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Declaration of Originality

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Abstract

This thesis provides new insights into the recruitment mechanisms of factors to replicative origins that are important for helicase activation during initiation of DNA replication in budding yeast.

DNA replication origins are recognised by the Origin Recognition Complex (ORC), which recruits, during late M-phase, Cdc6, Cdt1 and MCM2-7 to form a pre-replication complex (pre-RC). MCM2-7 represents the core of the eukaryotic replicative helicase, but it is inactive within the pre-RC. In early S-phase, the pre-RC is converted into the pre-initiation complex (pre-IC), leading to stable Cdc45/GINS/MCM2-7 (CMG) complex formation and helicase activation. This process depends on the recruitment of a series of factors (Sld3, Cdc45, Sld2, Sld7, Dpb11...) and is regulated by kinases (DDK and S-CDK) but their specific function and mechanism of action is largely unknown.

A systematic pairwise interaction analysis was performed using purified and *in vitro* translated proteins from *Saccharomyces cerevisiae* in order to understand how these factors interact with each other and MCM2-7, allowing helicase loading and activation.

During this study a new network of interactions was identified, which is required for the stable recruitment of Cdc45 to MCM2-7. This was concomitant with the *in vitro* reconstitution of Cdc45 loading with purified proteins, which described Sld2, in addition to Sld3, as Cdc45 loading factor. Moreover, the Mcm2 subunit of the MCM2-7 hexamer was identified as a central player in Cdc45 recruitment, representing a binding surface for Sld2, Sld3 and Cdc45. The interaction sites in these proteins were mapped and mutants were generated to disrupt the formation of the complex containing Cdc45.

The results obtained in this work constitute a comprehensive overview on the interactions occurring during pre-RC, and CMG formation. Additionally, they provide important details on the mechanisms leading to helicase activation and contribute to the understanding of this still poorly defined step of DNA replication.

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Abbreviations

A	Adenine or Alanine
aa	Amino acid
ACS	ARS consensus sequence
APC	Anaphase promoting complex
ARS	Autonomously replicating sequence
ATM	ABC transporter, mitochondrial
ATP	Adenosin triphosphate
ATR	Aminotriazole resistance
BRCT	BRCA1 C-terminal
BSA	Bovin serum albumin
C	Cytosine
Cdc6	Cell division cycle protein 6
CDK	Cyclin-dependent kinase
Cdt1	Cdc10-dependent transcription factor 1
CMG	Cdc45/MCM2-7/GINS complex
Cps	Cycles per second
CR	Conserved region
Ctf4	Chromosome transmission fidelity 4
CV	Colum volume
D	Aspartic acid
Da	Dalton
DDK	Dbf4-dependent kinase
DDR	DNA damage response
DEAE	Diethylaminoethyl
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
Dpb11	DNA polymerase B possible subunit 11
dsDNA	Double stranded DNA
DTT	Dithiothreitol
E	Glutamic acid

E. coli	<i>Escherichia coli</i>
g	Gram or gravity (relative centrifugal force)
G	Guanine
GINS	Go ichi ni san
GST	Glutathione S-transferase
h	Hour or human
H	Histidine
HA	Haemagglutinin
His	Histidine
IP	Immunoprecipitation
IPTG	Isopropyl β -D-1-thiogalactopyranoside
k	Kilo
K	Lysine
Kb	Kilobase
L	Litre
LB	Luria-Bertani broth
Leu	Leucine
μ	Micro
m	Milli or meter
M	Molar
MBP	Maltose-binding protein
MCM	Mini chromosome maintenance protein
Mec1	Mitosis entry checkpoint 1
Min	Minute
Mrc1	Mediator of the replication checkpoint
n	Nano
OD600	Optical density at 600 nm
ORC	Origin recognition complex
P	Proline
PAGE	Polyacrylamide gel electrophoresis
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PIC	Protease inhibitor cocktail

Pol	Polymerase
Pre-IC	Pre-Initiation complex
Pre-LC	Pre-Loading complex
Pre-RC	Pre-Replication complex
PS	PreScission protease recognition sequence
Psf	Partner for Sld5
R	Arginine
RFC	Replication factor C
Rif1	Rap1-interacting factor 1
RPA	Replication protein A
rpm	Revolutions per minute
S	Serine
S-CDK	S-phase cyclin-dependent kinase
sc	<i>Saccharomyces cerevisiae</i>
SDS	Sodium dodecyl sulphate
SFC	Skp, Cullin, F-box containing complex
Sld	Synthetically lethal with Dpb11
ssDNA	Single stranded DNA
T	Thymidine
T(number)	Threonine
TB	Terrific broth
TBS-T	Tris-buffered saline with Tween 20
td	Degron strain
TopBP1	Topoisomerase II binding protein 1
Trp	Tryptophan
Ura	Uracil
UV	Ultraviolet
V	Volts
V(number)	Valine
v/v	Volume in volume
W	Watts
w/v	Weight in volume
wt	Wild type

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Chapter 1 – Introduction

DNA replication is the central process of duplication of the genetic material and it is essential for the survival of all living cells. DNA replication involves the phases of initiation, elongation and termination and these basic mechanistic steps are conserved among all domains of life (Bacteria, Archaea and Eukarya) (O'Donnell et al, 2013).

The accurate copying of the double helix of DNA is required for inheritance of information that defines the phenotype of cells and ultimately organisms. Errors in DNA replication in higher eukaryotes are associated with a variety of serious consequences including birth defects, genetic abnormalities and cancer (Jackson et al, 2014).

The details of chromosome replication are better understood in the budding yeast *Saccharomyces cerevisiae* than in any other eukaryotic organism, principally because its replicative origins contain a well defined consensus sequence, contrarily to other species, and because of the simplicity of its genetic manipulation.

1.1 Initiation of DNA replication

In budding yeast, the initiation process is divided in two temporally distinct steps (Diffley et al, 1994). Firstly, in late mitosis and early G1-phase of the cell cycle, a large complex of initiator proteins called the pre-Replication Complex (pre-RC) assembles at the replication origin. Secondly, in early S-phase when cells have committed to DNA replication, the pre-RC is transformed into the pre-Initiation Complex (pre-IC). During this reaction more than 30 factors are assembled on origins (Gambus et al, 2006; Zou & Stillman, 1998) leading to the establishment of active replication forks. Two kinases are crucial for pre-IC formation, cyclin-dependent kinase (CDK) and Dbf4-dependent kinase (DDK), as they promote complex assembly and coordinate the timing of replication initiation at hundreds of origins (Siddiqui et al, 2013). Pre-IC formation culminates in the activation of the replicative helicase, which is composed of MCM2-7 and two accessory factors Cdc45 and GINS that together form a holoenzyme, the Cdc45/MCM2-7/GINS (CMG) complex (Costa et al, 2011; Gambus et al, 2006; Moyer et al, 2006). After the

unwinding of origin DNA, the synthesis machinery is loaded. Once the pre-IC starts forming, pre-RC assembly is inhibited and its reassembly is prevented until the next G1-phase. As a result, each origin is used once, and only once, per cell cycle.

1.2 Replicative origins

The eukaryotic origin in *S. cerevisiae*, called autonomously replicating sequence (ARS) has been defined using genetic methods. It does contain a 12-17 bp ARS consensus sequence (ACS) constituted of a A element and three B elements (Marahrens & Stillman, 1992). The A element is indispensable for origin function, while the individual B elements are important but essential all together. In contrast with the rest of the genome, the sequence at replicative origins is AT rich and therefore allows for easier strands separation. The Origin Recognition Complex (ORC) binds to the A and B1 elements (Bell & Stillman, 1992; Speck et al, 2005) and, crucially, the replication factor Cdc6 can only form a stable complex with ORC on the correct DNA template. In budding yeast therefore Cdc6 functions to select for the correct origins, otherwise inducing ATP-hydrolysis and ORC/Cdc6 complex disassembly (Speck & Stillman, 2007). It is currently not known if this is true for higher eukaryotes, where sequence specificity has not been identified yet but additional initiation factors, DNA structures (G-quadruplex) and epigenetic signals co-operate to stimulate initiation of replication in specific genomic regions (Mechali et al, 2013). Interestingly, replication origins are frequently found in proximity of promoter regions (Cadoret et al, 2008; Cayrou et al, 2011; Sequeira-Mendes et al, 2009) and ORC binding sites are linked with patterns of gene expression (MacAlpine et al, 2010; Mechali et al, 2013). One possible explanation is that both transcriptionally active promoters and DNA replication origins require a stretch of nucleosome-free DNA (Eaton et al, 2010; Papior et al, 2012; Xu et al, 2012). Additionally, origins are enriched for specific histone variants (H3.3 or H2AZ) and chromatin modifiers (histone acetylase HBO1, histone methyl transferase PR-Set7, chromatin-remodelling complex SNF2H) can facilitate pre-RC assembly in higher eukaryotes (Mechali et al, 2013). DNA structures were also recently linked to initiation of replication in *Drosophila*, where DNA supercoiling can promote ORC recruitment (Remus et al, 2004), although human ORC also has affinity for G-quadruplex structures (Hoshina et al, 2013).

Remarkably, the recognition of the origin is not crucial for replication. In fact, in mammalian cells the recruitment of ORC to non-origin DNA creates an artificial origin (Takeda & Dutta, 2005) and, consistent, the budding yeast ORC/Cdc6 can use non-origin DNA for helicase loading *in vitro* (Evrin et al, 2009; Remus et al, 2009). This suggests the possibility that any DNA sequence could support replication and that the specific recruitment of ORC to defined genomic regions only assures even spacing of replication origins. Finally, not all origins fire every cell cycle, leaving some to the possibility to be activated in case the replication fork is blocked due to DNA damage (Blow et al, 2011).

1.3 Pre-RC formation

Pre-RC formation is initiated by the binding of the Origin Recognition Complex (ORC) to the replication origins, where it stays attached throughout the cell cycle (Bell & Stillman, 1992; Liang & Stillman, 1997). During late M-, early G1-phase, the ORC-DNA complex recruits Cdc6 to the origins (Weinreich et al, 1999). The binding of ORC and Cdc6 is ATP dependent (Speck et al, 2005) and it involves interactions between Cdc6 and the Orc1 and Orc2 subunits (Huo et al, 2012). The ORC/Cdc6 complex in turn recruits Minichromosome maintenance (MCM2-7) helicase to origins with the help of Cdt1 (Coleman et al, 1996; Donovan et al, 1997; Evrin et al, 2009; Randell et al, 2006; Remus et al, 2009). Cdt1 is required *in vivo* for the nuclear import of MCM2-7 (Tanaka & Diffley, 2002) and the interaction of Cdt1 with Mcm6 alleviates an inhibitory activity in the C-terminus of Mcm6 (Fernandez-Cid et al, 2013), promoting the ORC/Cdc6/Cdt1/MCM2-7 (OCCM) complex formation (Evrin et al, 2013). The OCCM is an unstable intermediate and Orc1 and Cdc6 ATP hydrolysis is quickly activated as a consequence of its formation, resulting in the expulsion of Cdt1 from the complex and leaving on origins the ORC/Cdc6/MCM2-7 (OCM) complex (Fernandez-Cid et al, 2013; Randell et al, 2006). The OCM is competent for the recruitment of a second MCM2-7 hexamer to DNA, leading to the formation of a stable MCM2-7 double-hexamer (Evrin et al, 2014). The structure of the double-hexamer is in fact ideally suited to set up bidirectional replication forks in S-phase, with each fork inheriting one MCM2-7 hexamer. A model of the pre-RC formation is presented in Figure 1.1.

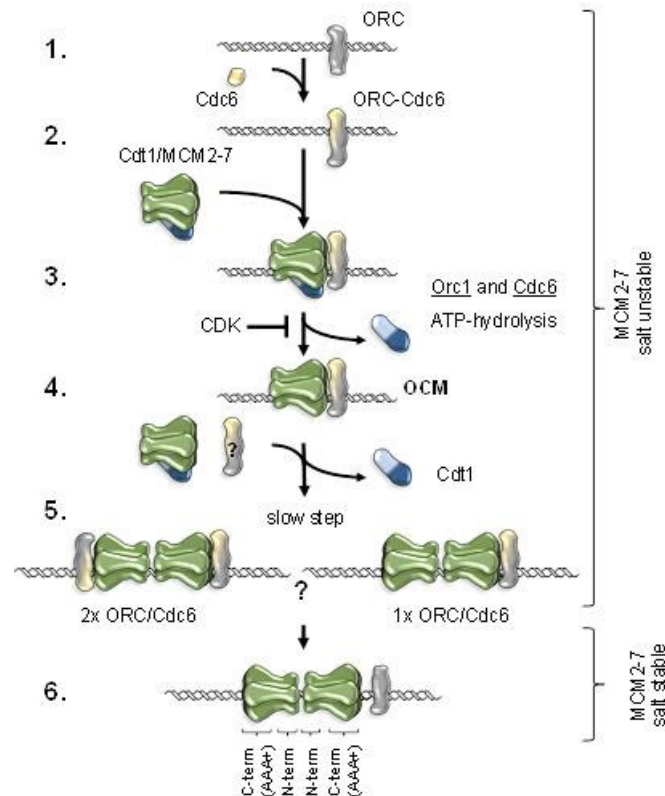


Figure 1.1: Pre-RC assembly model

1) ORC binds DNA origins. **2)** Cdc6 associates with ORC. **3)** Cdt1/MCM2-7 is recruited by ORC/Cdc6. **4)** Rapid ATP-hydrolysis by Cdc6 and Orc1 leads to Cdt1 release and OCM formation. This reaction is blocked by CDK. **5)** OCM formation results in a structural change in MCM2-7 to generate a dimerization competent state of the MCM2-7 helicase and recruitment of a second MCM2-7 complex. The recruitment of the second MCM2-7 hexamer is a slow reaction and may require another ORC/Cdc6 complex. **6)** The MCM2-7 loading finishes with the formation of a stable double-hexamer encircling dsDNA. (Riera et al, 2014)

It was found that all pre-RC intermediates are salt unstable, whereas the MCM2-7 double-hexamer that encircles dsDNA is salt resistant (Donovan et al, 1997; Evrin et al, 2009; Evrin et al, 2014; Fernandez-Cid et al, 2013; Remus et al, 2009). The stability of the MCM2-7 double-hexamer on chromatin is crucial to prevent loss of the helicase during the G1/S-phase transition, which would lead to inefficient DNA replication and genome instability because no reloading can occur during S-phase. Additionally, the MCM2-7 double-hexamer is able to slide on dsDNA in an ATP-hydrolysis independent manner (Evrin et al, 2009; Remus et al, 2009). Multiple MCM2-7 double-hexamers are loaded at replication origins in each cell cycle and the additional copies can potentially establish new forks in case of terminal fork collapse due to severe DNA damage (McIntosh & Blow, 2012). Although MCM2-7 double-hexamer formation is crucial for DNA replication, this complex has no DNA

unwinding activity (Evrin et al, 2009; Ishimi, 1997; Remus et al, 2009) and MCM2-7 needs to be activated in S-phase with the interaction with Cdc45 and GINS.

1.4 Pre-IC formation ¹

Pre-IC formation is divided into two discrete steps according to the requirements for DDK and CDK kinases. First, during late G1-phase DDK activity promotes the association of Sld3, Sld7 and Cdc45 with replication origins (Heller et al, 2011; Kamimura et al, 2001; Kanemaki & Labib, 2006; Tanaka et al, 2011a; Tanaka et al, 2011b). Subsequently, Sld2, Dpb11, DNA Polymerase ϵ (Pol ϵ) and the tetramer constituted by Sld5/Psf1/Psf2/Psf3 (GINS) associate with the origins in a CDK dependent manner (Heller et al, 2011; Masumoto et al, 2002; Muramatsu et al, 2010; Tanaka et al, 2007b; Zegerman & Diffley, 2007). Sld2, Dpb11, Pol ϵ and GINS form a weak complex in solution that is called pre-loading complex (pre-LC) (Muramatsu et al, 2010) and a CDK dependent interaction between Sld3 and Dpb11 facilitate the recruitment of GINS to the replication origin. In consequence the MCM2-7 double-hexamer is transformed into the CMG, which functions as the active helicase. Within the pre-RC MCM2-7 encircles dsDNA, but the CMG encircles single stranded DNA (ssDNA)(Fu et al, 2011), indicating that the MCM2-7 complex undergoes large conformational changes during pre-IC formation, which must involve MCM2-7 ring opening. Once the CMG is formed Mcm10 is likely required to activate the complex, since in the absence of Mcm10 the CMG cannot unwind DNA and stays associated with the origin (Kanke et al, 2012; van Deursen et al, 2012; Watase et al, 2012). Finally, with the help of Ctf4, the DNA polymerase α (Pol α) is recruited (Gambus et al, 2009; Kang et al, 2013; Simon et al, 2014), it binds ssDNA and initiates DNA synthesis by producing RNA primers prior to processive DNA synthesis. Pol ϵ primarily functions for leading strand DNA synthesis, while Pol δ serves for lagging strand DNA synthesis (Kunkel & Burgers, 2008). Additional factors are taking part in the replisome formation and they include Replication Protein A (RPA) (Alani et al, 1992; Longhese et al, 1994), a trimeric highly conserved ssDNA binding protein, and Replication Factor C (RFC), a DNA binding protein and ATPase

¹ As in Tognetti, Riera and Speck, submitted

that acts as a clamp loader of the Proliferating Cell Nuclear Antigen (PCNA) processivity factor for Pol δ and Pol ϵ (Yao et al, 2003).

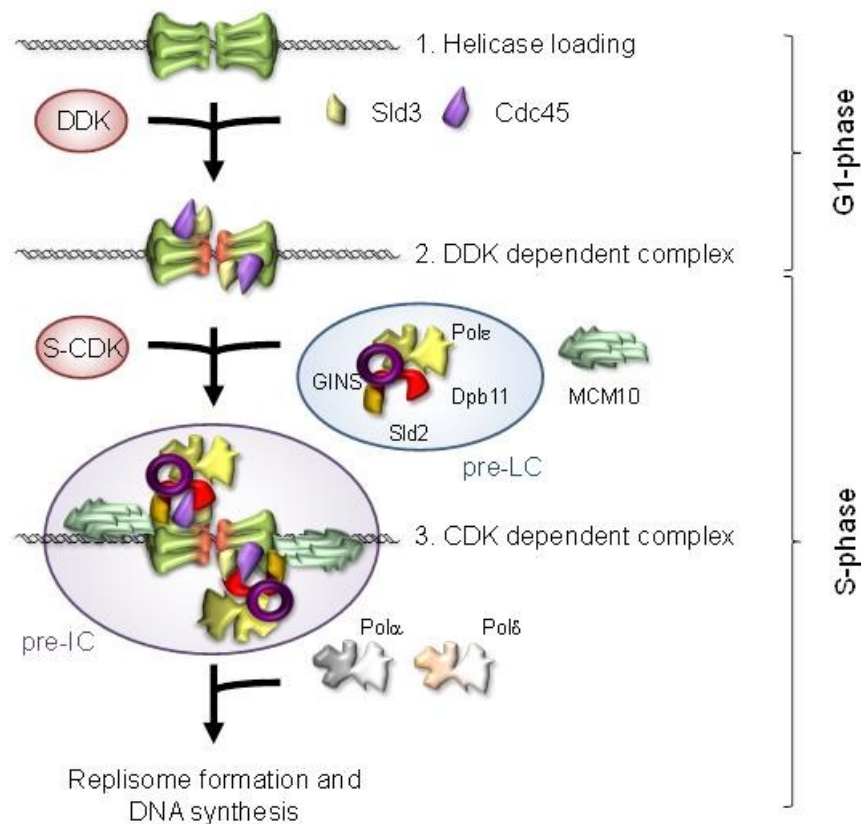


Figure 1.2: Schematic representation of the initiation of replication ¹

In late G1-phase DDK promotes the recruitment of Sld3 and Cdc45 to origins where MCM2-7 double-hexamer is loaded. Subsequently, in S-phase, Sld2, Dpb11, Pol ϵ , GINS and Mcm10 associate with the origins in a CDK dependent manner. The complex formed (pre-LC) is competent for polymerases recruitment and replisome formation.

1.5 The Cdc45/MCM2-7/GINS (CMG) complex ¹

MCM2-7 represents the core of the eukaryotic helicase, but this complex has little helicase activity on its own (Bochman & Schwacha, 2008; Ishimi, 1997; Lee & Hurwitz, 2000). However, once Cdc45 and GINS are associated with MCM2-7, the complex has a strong helicase activity (Ilves et al, 2010). Crucially, some structural information on the CMG complex has been obtained, giving insights into the organisation and function of the helicase. Moreover, as MCM2-7, Cdc45 and GINS are highly conserved, data obtained from various systems can be integrated,

¹ As in Tognetti, Riera and Speck, submitted

resulting in a more comprehensive understanding of the complex. The low resolution three dimensional reconstruction of the *Drosophila melanogaster* CMG identified the arrangement of its proteins (Costa et al, 2011). The complex assumes a planar form, with Cdc45 and GINS binding to the side of a closed circular MCM2-7 complex, touching the Mcm2, Mcm5 and Mcm3 subunits. However, the structure of MCM2-7 in the CMG appears to differ from the structure of the MCM2-7 hexamer by itself. *Drosophila melanogaster* MCM2-7 assumes in fact two different conformations: a notched planar form and a gapped spiral form; in each case the MCM2-7 ring is broken, which was not observed within the CMG (Costa et al, 2011). This indicates that the MCM2-7 hexamer is unstable and that the binding of Cdc45 and GINS to MCM2-7 promotes stabilization of the complex, which is likely important for its function. Additionally, it was found that the binding of Cdc45 and GINS to MCM2-7 generates a large channel through the CMG complex that stretches from the centre of the MCM2-7 pore to the inner surface of the Cdc45/GINS proteins. Moreover, within the CMG Cdc45 and GINS make extensive contact with each other, both seen in the EM structure and reported in protein-protein interaction analysis (Costa et al, 2011; Ilves et al, 2010; Im et al, 2009). One side of Cdc45 interacts with the N-terminal α -helical domain of Psf2, while the other side appears to contact the N-terminus of Mcm2. On the other hand, GINS seems to interact with Mcm5. The structure of the CMG suggests that the complex cannot associate with dsDNA, as the central pore is not wide enough (Costa et al, 2011). Moreover, the CMG contains only a single MCM2-7 hexamer suggesting that the splitting of the double hexamer has to occur prior to or during CMG formation. Indeed, a biochemical analysis of the CMG, using strand-specific DNA roadblocks and *Xenopus* egg extracts, has suggested that the CMG is a 3' to 5' ssDNA translocase (ssDNA passing through the central channel), suggesting that the helicase unwinds DNA via "steric exclusion" (Fu et al, 2011). Moreover, during DNA damage GINS can temporarily dissociate from the CMG complex, demonstrating that the complex is more dynamic than previously assumed (Hashimoto et al, 2012).

In contrast to bacteria, where a hexameric helicase unwinds DNA, eukaryotes have evolved a more complex holo-helicase, which contains in addition to the MCM2-7 hexamer also Cdc45 and the four subunit GINS complex. However, how these helicase accessory factors contribute to processive DNA unwinding and what the exact path of both DNA strands through the CMG complex is, are key

unanswered questions. Nevertheless, models that will contribute to answering these questions can be generated with current data. For example, it was reported that Cdc45 interacts tightly with ssDNA, both for budding yeast (Bruck & Kaplan, 2013) and human Cdc45 (Szambowska et al, 2014). Then again, in particular human Cdc45 seems to have a preference for 3' protruding strands and single-strand/double strand DNA junctions, and the protein appears to be able to slide on DNA with 3'-5' polarity (Szambowska et al, 2014). Additionally, a similarity was found between Cdc45 and RecJ, a prokaryotic ssDNA exonuclease (Krastanova et al, 2012; Sanchez-Pulido & Ponting, 2011). These features of Cdc45 suggest that ssDNA passes through Cdc45 and that it might be involved or responsible for the process of strands separation. Szambowska and colleagues proposed that Cdc45 in the CMG could act as a wedge for the separation of the two DNA strands, being situated in front of the complex, but this theory is not fully consistent with the three dimensional structure of the CMG complex (Costa et al, 2011; Krastanova et al, 2012). Based on this model, only a restricted path of DNA through the CMG complex is possible.

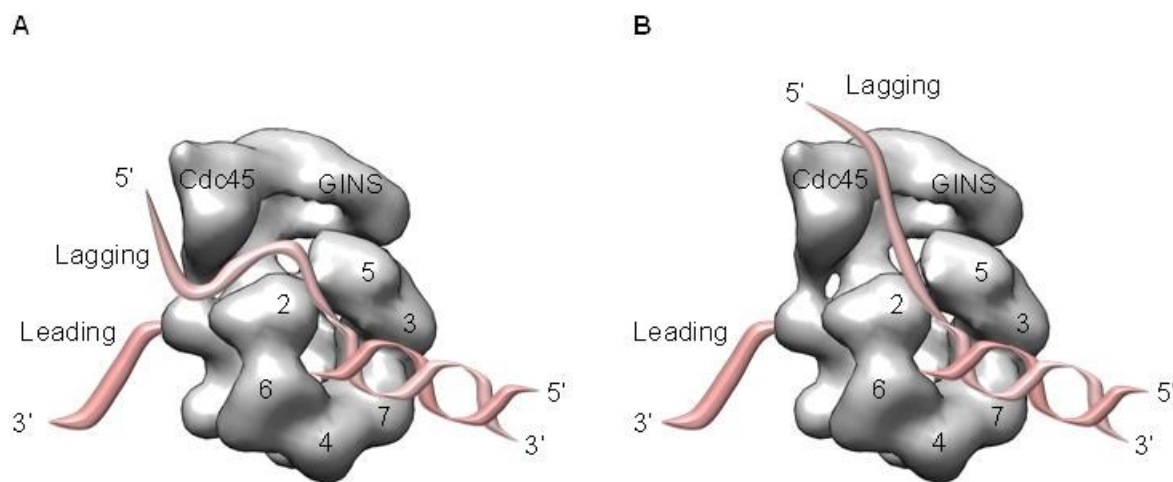


Figure 1.3: Models showing the path of DNA through the CMG ¹

C-terminal view of MCM2-7 in complex with Cdc45 and GINS. **A)** The leading strand passes right through the central channel of the CMG, whereas the lagging strand inserts in the lateral channel at the Mcm2/Mcm5 interface, contacting at this point Cdc45 or the Cdc45/GINS interface. **B)** The leading strand passes through the central channel of the CMG, while the lagging strand makes minor contact with MCM2-7 on its outer C-terminal surface or the Cdc45/GINS interface. The EM map of the CMG complex is based on EMDB-1832 (Costa et al, 2011).

¹ As in Tognetti, Riera and Speck, submitted

One option is that the leading strand of DNA passes through the central hole of MCM2-7, whereas the lagging strand inserts in the lateral channel produced between the N-terminal and C-terminal parts in the Mcm2/Mcm5 interface, possibly contacting at this point Cdc45 or Cdc45/GINS interface (Figure 1.2, model A). The other option is that the lagging strand does not pass through MCM2-7 at all, but contacts MCM2-7 on its outer C-terminal surface and/or Cdc45/GINS (Figure 1.2, model B).

Both models are consistent with a recent analysis of the DNA binding properties of an archaeal MCM homologue and budding yeast MCM2-7, which revealed that residues on the surface of the N-terminal MCM2-7 OB-fold are crucial for interaction with the leading strand (Froelich et al, 2014), consistent with the idea that MCM2-7 encircles ssDNA. Mutations of these residues in MCM2-7 did not have an effect on Cdc45 association with origins, but surprisingly impacted on GINS and RPA association. This could suggest that Cdc45 plays a role in the initial unwinding, whereas GINS is recruited as a consequence of this process. Additionally, further replicative factors could take part in the process of strand extrusion during CMG establishment or initial unwinding, such as Mcm10 and Sld2 which are reported to be ssDNA binding proteins (Kanter & Kaplan, 2011; Warren et al, 2008). Nevertheless, many more studies will be required to validate/invalidate these models and to conclude how the two strands of DNA pass through the CMG complex.

1.6 DDK dependent regulation of DNA replication ¹

Helicase activation becomes initiated by DDK, which is targeting the MCM2-7 double-hexamer. This reaction involves recruitment of the kinase to replication origins, which begins already in G1-phase of the cell cycle (Katou et al, 2006; Natsume et al, 2013). Here, Dbf4 interacts with Mcm2 (Ramer et al, 2013), whereas Cdc7 associates with both Mcm4 and Mcm5 (Ramer et al, 2013; Sheu & Stillman, 2006). Thus, several docking sites for DDK are available within the helicase. However, as these sites are spread over a large surface of the MCM2-7 complex, it appears also possible that multiple DDK complexes are operating in the context of the MCM2-7 double-hexamer. Purified DDK was found to phosphorylate the Mcm2, Mcm4 and Mcm6 subunits, with preference for MCM2-7 in its double-hexameric form

¹ As in Tognetti, Riera and Speck, submitted

(Francis et al, 2009). DDK target sequences for phosphorylation (S/T-D/E) are characterised by an acidic residue at the +1 position, which can be either an acidic amino acid or negative charge provided by a phosphoserine or phosphothreonine (Charych et al, 2008; Cho et al, 2006; Montagnoli et al, 2006). Indeed, the checkpoint kinase Mec1, and also CDK, phosphorylate chromatin-associated MCM2-7 generating priming sites for DDK dependent phosphorylation, which contributes to efficient helicase activation and genomic stability (Randell et al, 2010). DDK has likely a large number of targets in the cell, but in budding yeast it was shown that MCM2-7 represents the essential target of the kinase, as mutations in subunits of the helicase can bypass the DDK requirement of the cell (Hardy et al, 1997; Sheu & Stillman, 2006). In particular the Mcm4 N-terminus contains a domain with an inhibitory activity that becomes alleviated upon phosphorylation (Sheu & Stillman, 2010). Then again, a point mutation in the N-terminus of Mcm5 promotes also DDK independent DNA replication (Hardy et al, 1997). The structural change associated with DDK dependent phosphorylation of the MCM2-7 double-hexamer is unclear, although recently it was shown that it does not involve separation of the MCM2-7 double-hexamer (On et al, 2014). On the other hand it appears possible that DDK phosphorylation of MCM2-7 generates binding sites for proteins, because Sld3, Sld7 and Cdc45 only bind to origins upon DDK activation (Heller et al, 2011; Natsume et al, 2013; Tanaka et al, 2011a).

To establish temporal control of DNA replication, the activity of DDK must be regulated. The Cdc7 kinase subunit is stable during the cell cycle, however the regulatory Dbf4 subunit becomes ubiquitinated by the APC/C and targeted for proteasome mediated degradation during late mitosis and G1-phase of the cell cycle (Ferreira et al, 2000; Oshiro et al, 1999; Weinreich & Stillman, 1999). Subsequently, phosphorylation of the APC/C activator protein Cdh1 by G1-CDKs prevents Cdh1 from binding and activating the APC/C, therefore allowing S-phase kinases to accumulate (Jaspersen et al, 1999; Zachariae et al, 1998). This regulatory loop guarantees that Dbf4 activity is kept low during G1-phase of the cell cycle.

However, this does not explain how the replication timing of individual chromosome domains is regulated. Several recent studies showed that DDK becomes modulated in a loci specific form: for example, it was found that the Ctf19 kinetochore complex can recruit Dbf4 as early as telophase to the kinetochores to promote early DNA replication in S-phase (Natsume et al, 2013). Then again, origins

that fire late during S-phase, e.g. at the telomere, have been shown to contain a negative regulator of DDK activity. The Rap1-interacting factor 1 (Rif1) protein binds to late origins and recruits protein phosphatase 1 (PP1), which facilitates the dephosphorylation of the MCM complex and blocks helicase activation (Dave et al, 2014; Hiraga et al, 2014; Mattarocci et al, 2014; Renard-Guillet et al, 2014).

1.7 CDK dependent regulation of DNA replication ¹

The cell cycle of eukaryotes is controlled by the activity of CDKs, a family of threonine-serine protein kinases that phosphorylate specific target proteins promoting the activation or inhibition of cell cycle phases. The catalytic subunit of the kinase is regulated by cell cycle dependent association with a regulatory subunit, a cyclin, whose expression and/or stability is itself cell cycle dependent. Association of a cyclin with CDK controls the specific cell cycle transitions. For instance, G1-CDK promotes S-phase entry, S-CDK inhibits origins licensing and promotes DNA replication and finally M-CDK regulates chromosome segregation during mitosis (Humphrey & Pearce, 2005). Similar as for DDK, the S-CDK concentration is regulated by APC/C mediated degradation and therefore follows an analogous dynamic as described above for DDK (Ang & Wade Harper, 2005).

1.7.1 Regulation of pre-RC formation

S-CDK acts on initiation of replication in multiple ways. Firstly, relatively low levels of the kinase phosphorylate Orc2 and Orc6 (Nguyen et al, 2001), which inhibits the interaction of ORC with Cdt1 (Chen & Bell, 2011; Fernandez-Cid et al, 2013; Frigola et al, 2013). Secondly, S-CDK dependent phosphorylation of Cdc6 induces its degradation by the SCF complex (Drury et al, 1997; Elsasser et al, 1999; Perkins et al, 2001). Thirdly, phosphorylation of Mcm3 results in MCM2-7/Cdt1 export from the nucleus (Labib et al, 1999; Liku et al, 2005; Nguyen et al, 2001). All these redundant mechanisms prevent pre-RC formation and thus origin licensing during the G1-S transition, to guarantee that no origin becomes licensed after it has been already fired.

¹ As in Tognetti, Riera and Speck, submitted

1.7.2 Regulation of pre-IC formation

The most important role of S-CDK is to promote helicase activation. It was found that Sld2 and Sld3 are the two essential targets of CDK in budding yeast (Masumoto et al, 2002; Tanaka et al, 2007b; Zegerman & Diffley, 2007), however if this is also true in higher eukaryotes is not known. S-CDK phosphorylated Sld2 and Sld3 bind to Dpb11 involving two pairs of BRCT domains that have phosphopeptide binding activity. The N-terminal pair of BRCT domains bind Sld3-P and the C-terminal pair of BRCT domains bind Sld2-P, forming a trimeric complex. Moreover, recently it was shown that a flexible linker in between these two pairs of BRCT domains binds GINS, which is important for efficient replication (Tanaka & Araki, 2013). In particular the binding of Sld2-P to Dpb11 is crucial for pre-LC formation, GINS recruitment to the replication origin and ultimately CMG formation (Muramatsu et al, 2010). Importantly, Sld2 becomes phosphorylated on multiple sites; however the phosphorylation of T84 is essential for Dpb11 binding (Tak et al, 2006). T84 phosphorylation requires pre-phosphorylation of several other sites in Sld2, thus T84 becomes only phosphorylated at high CDK activity. This regulatory principle could be important to ensure that pre-RC formation is inhibited completely at low CDK concentrations prior to helicase activation at high CDK concentrations. Moreover, hierarchical multisite-phosphorylation is predicted to allow a switch-like activation of Sld2 and helicase activation (Brummer et al, 2010), however how this regulatory principle is coordinated with the replication timing program of the cell is currently unclear.

Remarkably, simultaneous bypass of the CDK and DDK pathways in G1-phase, using a Sld3–Dpb11 fusion (SD fusion), overexpression of a phospho-mimetic Sld2-T84D mutation and a *mcm4*Δ74–174 allele allows extensive DNA synthesis in α -factor arrested, G1 cells (Sheu & Stillman, 2010).

Importantly, in case of DNA damage, Rad53 functions to inhibit both CDK- and DDK dependent activation pathways. Rad53 acts on DDK directly by phosphorylating Dbf4, whereas the CDK pathway is blocked by Rad53-mediated phosphorylation of the essential CDK substrate, Sld3 (Duch et al, 2011; Lopez-Mosqueda et al, 2010; Zegerman & Diffley, 2010). This allows CDK to remain active during S-phase in the presence of DNA damage, which is crucial to prevent re-licensing of origins that have already fired.

1.8 Proteins relevant to this study

This study focuses its attention on the recruitment of Cdc45 to the loaded MCM2-7 complex as the initial step in the process of helicase activation. Several factors were known to be required (MCM2-7, Sld3, Cdc45) or implicated (Sld7) in this process, whereas a new function was identified for Sld2 during this study. An overview of the features of these crucial proteins is presented below.

1.8.1 MCM2-7

MCM2-7 forms the engine of the eukaryotic replicative helicase (Labib et al, 2000), but it has little helicase activity on its own (Bochman & Schwacha, 2008; Ishimi, 1997; Lee & Hurwitz, 2000) and it has to be activated by the interaction with Cdc45 and GINS (Araki, 2010). The MCM2-7 complex consists of six AAA+ ATPase proteins that assume a toroidal two-tiered structure, where the smaller ring is made up of the N-terminal section and the larger ring of the C-terminal ATPase domains (Riera et al, 2013). All Mcm subunits are highly homologous, containing a less conserved C-terminal AAA+ and an N-terminal domain, and their disposition in the heterohexamer is fixed (Mcm2/6/4/7/3/5). Only properly assembled hexamers can in fact enter the nucleus at the end of M-phase, where they stay throughout G1-phase before being released during S-phase (Labib et al, 1999; Nguyen et al, 2000). MCM2-7 lacks intrinsic affinity for DNA and its association with origins is ORC/Cdc6/Cdt1-dependent (Mendez & Stillman, 2003).

Individual Mcm subunits have specialised functions. Mcm6 C-terminus contains an inhibitory domain that blocks OCCM formation in the absence of Cdt1 and deletion of the conserved Mcm3 C-terminus (Sun et al, 2013) or even a C-terminal V972R point mutation in Mcm3 abrogates binding of Cdt1/MCM2-7 to ORC/Cdc6 (Frigola et al, 2013; Sun et al, 2013). Additionally, Mcm2, Mcm3, Mcm4, Mcm6 and Mcm7 carry N-terminal extensions, which *in vitro* get phosphorylated by DDK (Kihara et al, 2000; Weinreich & Stillman, 1999). It has been shown that a deletion of the Mcm4 N-terminus bypasses the requirement of DDK activity during S-phase (Sheu & Stillman, 2010) and a mutation of Mcm5 called bob-1 (P83L) allows DNA replication and cell growth in the absence of DDK as well (Hardy et al, 1997; Jackson et al, 1993). Since Mcm5 is the only MCM2-7 subunit that does not become

phosphorylated by DDK, it is likely that it mimics a DDK-mediated structural change in the helicase. Importantly, it was shown that DDK phosphorylation of MCM2-7 does not by itself promote separation of the double-hexamer, but it is required for the recruitment of firing factors and replisome components to origins (On et al, 2014). Additionally, pre-phosphorylation of the MCM2-7 is required for DDK to bind and phosphorylate the loaded MCM2-7 complex (Francis et al, 2009) and Mec1, facilitated by Mrc1, is one of the kinases that prime MCM2-7 for DDK phosphorylation (Randell et al, 2010).

1.8.2 Sld3

The replicative factor Synthetically Lethal with Dbp11-1, Sld3, (Kamimura et al, 1998) is considered to be a key protein for the pre-IC formation in yeast, since its association with origins is a prerequisite for the initiation of DNA replication. It is a limiting factor, as it is present with only 300 copies/cell whereas the number of replication origins in the genome is around 700-1000 (Tanaka et al, 2011b). It is essential *in vivo* (Kanemaki & Labib, 2006; Tanaka et al, 2007b) and it is required for Cdc45 association with origins (Kamimura et al, 2001). Sld3 and Cdc45 recruitment to origins indeed follows the same dynamics and it is weak during late G1-phase and stable in S-phase (Kamimura et al, 2001; Kanemaki & Labib, 2006). Work from Kaplan lab employing purified proteins from budding yeast showed that Sld3 binds MCM2-7, allegedly facilitating Cdc45 interaction with MCM2-7. Additionally, Sld3 shows ssDNA binding properties and its interaction with the nucleic acid impairs binding to MCM2-7, indicating that ssDNA and MCM2-7 could compete for Sld3 binding. Finally, it was found that Sld3 competes with GINS for binding to MCM2-7 and ssDNA facilitates GINS-MCM2-7 interaction, possibly promoting Sld3 release from origins and CMG formation (Bruck & Kaplan, 2011a). This is consistent with the finding that in the absence of GINS, Sld3 release from chromatin is slower *in vivo* (Kanemaki & Labib, 2006). Unfortunately this *in vitro* data is not conclusive, because MCM2-7 is not loaded on DNA, but the experiments were performed with purified MCM2-7 in solution. Moreover, it is believed that purified MCM2-7 can assume different conformations, closed circular and spiral shaped complexes (Costa et al, 2011), therefore the experiments by the Kaplan lab are not conclusive on its own.

Sld3 is also reported to bind the non-essential factor Sld7 throughout the cell cycle, which is probably involved in its stability and functionality (Tanaka et al, 2011b). Importantly, Sld3, together with Sld2, are the essential CDK targets. The CDK mediated phosphorylation of Sld3 generates a binding site for the N-terminal BRCT repeat in Dpb11 (Tanaka et al, 2007b; Zegerman & Diffley, 2007). 12 CDK-phosphorylation sites have been mapped in Sld3 and the phosphorylation of T600 and S622 by CDK were shown to be crucial for the binding of Dpb11 (Tanaka et al, 2007b; Zegerman & Diffley, 2010). Interestingly, a Cdc45 mutant (*Cdc45-Jet1*), which bypasses the requirement for S-CDK, allows Sld3-Dpb11 interaction in the absence of phosphorylation of T600 and S622, indicating that Cdc45 can bridge and stabilise the binding (Tanaka et al, 2007b). In the context of purified proteins in solution, S-CDK is not required for Sld3 interaction with MCM2-7 and Cdc45 (Bruck & Kaplan, 2011a) but the CDK-phosphorylation mediated interaction of Sld3 with Dpb11, which is part of the pre-LC (Muramatsu et al, 2010), could function to facilitate GINS recruitment to origins and helicase activation via CMG formation.

Additionally, during DNA damage, initiation of DNA replication needs to be blocked to maintain genomic integrity. In budding yeast this block is operated by a Rad53-mediated phosphorylation of Sld3 and Dbf4 (Zegerman & Diffley, 2010), suggesting that Sld3 represents a key regulatory protein during pre-IC formation. Rad53 has the function to reduce DNA replication in response to DNA damage and, during the S-phase checkpoint, this is exerted by inhibiting late origin firing. Rad53 phosphorylation of Sld3 impairs its interaction with Dpb11 and Cdc45, even when Sld3 had already been phosphorylated by S-CDK (Lopez-Mosqueda et al, 2010; Zegerman & Diffley, 2010). This mechanism appears crucial to prevent Cdc45 and GINS recruitment to loaded MCM2-7 on origins.

It was recently found that Sld3 gets phosphorylated during G1-phase in a DDK dependent manner. Interestingly, the modification increases in the presence of Rif1 (a telomere-binding factor implicated in DNA replication control) mutants (Mattarocci et al, 2014), providing further indication that Sld3 is subject to temporal and locus specific control during the cell cycle.

Based on this analysis it is clear that Sld3 is a central player in mediating initiation of DNA replication. The findings that the association of Sld3 with each origin is a prerequisite for initiation of DNA replication and its role in promoting the correct temporal order of chromosomal DNA replication, make Sld3 a particularly interesting

protein for detailed studies. Very little is known about the mechanism of its association with origins and MCM2-7 and about the molecular details of its function in promoting helicase activation.

1.8.3 Cdc45

Cdc45 is an essential replication factor and its function is conserved among species (Aparicio et al, 1997; Owens et al, 1997). It was found to associate weakly with origins already in late G1-phase together with Sld3, and their recruitment in budding yeast is mutually dependent (Kamimura et al, 2001). Cdc45 in fact interacts directly with Sld3 (Bruck & Kaplan, 2011a; Kamimura et al, 2001) and this interaction is believed to promote Cdc45 recruitment to the pre-RC, ultimately facilitating the CMG formation. Cdc45 is operating, together with GINS, for the activation of the MCM2-7 helicase (Costa et al, 2011; Ilves et al, 2010), but the initial association of Cdc45 with origins does not require the presence of GINS (Kanemaki & Labib, 2006). Cdc45 becomes part of the CMG complex and it travels with the replisome. Additionally, Cdc45 has ssDNA binding properties, both in budding yeast (Bruck & Kaplan, 2013) and human (Szambowska et al, 2014), and this feature could be required for the separation of the two strands of DNA during CMG establishment or it could facilitate processive DNA unwinding of the CMG helicase. Consistently, a similarity was found between Cdc45 and RecJ, a prokaryotic ssDNA exonuclease (Krastanova et al, 2012; Sanchez-Pulido & Ponting, 2011). In addition to the helicase activation function, Cdc45 is required for the association of DNA polymerases with origins (Aparicio et al, 1997) and it enhances GINS and Mcm10 recruitment (Heller et al, 2011).

1.8.4 Sld2

The replication factor Synthetically Lethal with Dbp11-1, Sld2 (Kamimura et al, 1998) binds to Dpb11 during S-phase in a CDK dependent manner (Tanaka et al, 2007b; Zegerman & Diffley, 2007). Sld2 has 11 motifs suitable for phosphorylation by S-CDK (S/T-P) but the phosphorylation of T84 is crucial for the binding to the C-terminal BRCT domain of Dpb11 (Araki, 2010; Tanaka et al, 2007b; Zegerman & Diffley, 2007). Despite the requirement of phosphorylation of one individual amino

acid, the phosphorylation of multiple additional residues appears to be occurring *in vivo* prior to modification of T84 (Araki, 2010; Tanaka et al, 2007b). Nevertheless, the introduction of a Sld3-Dpb11 fusion in addition to a S-CDK bypass mutant of Sld2 (Sld2T84D) allows replication *in vivo* in the absence of S-CDK (Zegerman & Diffley, 2007).

The phosphorylation of Sld2 and its interaction with Dpb11 are essential for the formation of the pre-LC complex, composed by Sld2, Dpb11, Pol ϵ , GINS (Muramatsu et al, 2010). Consistently, Sld5 (GINS subunit) and Dpb11 form a complex *in vitro* when Sld2 is phosphorylated by S-CDK (Tanaka et al, 2007a; Tanaka et al, 2007b). Additionally, Sld2 and Dpb11 were found to interact with the C-terminal portion of Pol2 (catalytic subunit of Pol ϵ) in yeast two hybrid assays (Muramatsu et al, 2010). Importantly, S-CDK dependent phosphorylation of Sld2 enhances its binding to Pol ϵ and GINS interacts with Sld2 only in the presence of Pol ϵ . The deprivation of DDK does not impair pre-LC formation, indicating that only S-CDK activity is involved in this step.

Sld2, similarly to Sld3 and Dpb11, does not move with the replication fork, therefore it appears possible that its function is to facilitate the recruitment of GINS via establishment of the pre-LC, which itself needs to be disassembled prior to initiation of replication (Muramatsu et al, 2010). Interestingly, Sld2 does not interact with GINS nor with Cdc45 (Muramatsu et al, 2010), therefore its role in promoting CMG formation is indirect. Consistently, the overexpression of GINS compensates for the individual depletion of Sld2, Dpb11 and Pol ϵ (Muramatsu et al, 2010) but in the absence of kinases or additional factors, Sld2, Sld3 and Dpb11 alone are unable to support CMG formation *in vivo* (Heller et al, 2011). It was recently found that a mutant of Sld2, which cannot bind to MCM2-7 (Sld2-m1,4), impairs DNA replication and stimulates CMG association already in G1-phase (Bruck & Kaplan, 2014), suggesting that a possible function for Sld2 is to prevent the association of GINS in G1-phase. Sld2 can interact *in vitro* with MCM2-7 in the context of purified proteins in solution, both in its CDK-phosphorylated form (P-Sld2) as well in the unmodified, but the binding of P-Sld2 and Sld3 to MCM2-7 appears to be competitive (Bruck & Kaplan, 2011a). Additionally, the interaction of P-Sld2 to MCM2-7 blocks *in vitro* the direct binding of GINS to MCM2-7 in a concentration dependent manner (Bruck et al, 2011). Interestingly, (Sld2T84D), which is the phosphomimetic form of S-CDK phosphorylated Sld2, binds directly to ssDNA at early firing origins (ARS1 and

ARS305) (Kanter & Kaplan, 2011) and ssDNA inhibits Sld2T84D and Sld3 interaction with MCM2-7 but, conversely, promotes GINS binding to MCM2-7. Crucially, unphosphorylated Sld2 blocks GINS-MCM2-7 interaction even in presence of ssDNA (Bruck et al, 2011) but, once again, the results were obtained in the context of purified MCM2-7 in the absence of DNA, and are not conclusive on their own. All these findings define Sld2 as a crucial player in CMG establishment and helicase activation but the mechanisms underlying its interactions and activity remain unclear.

1.8.5 Sld7

Following the discovery of Sld2, Sld3 and Sld5 (GINS subunit) in a screen for synthetically lethal mutants with Dpb11 (Kamimura et al, 1998) the Araki lab performed a second screen, identifying a new replicative factor, Sld7 (Tanaka et al, 2011b). Sld7 appears to bind to the N-terminal (amino acids 60-120) of Sld3 throughout the cell cycle and to reduce Sld3 affinity for Cdc45. It is not required for Sld3 interaction with origins but, in the absence of Sld7, Sld3 levels in the cells are reduced and there is a delay in origin firing, which is revealed by an extended association of GINS with origins. The role of Sld7 is not yet clear but it has been proposed that it helps in the stabilisation of Sld3 (Tanaka et al, 2011a; Tanaka et al, 2011b).

1.8.6 Protein interactions network

Based on the current data, the processes of helicase loading and activation depend on an intricate network of protein-protein interactions. While the *in vitro* pre-RC reconstitution has led to a detailed understanding of the mechanisms of helicase loading over the last few years, much has still to be discovered on the progression towards pre-IC and CMG formation and helicase activation. This is mainly due to the technical difficulties encountered attempting to reconstitute the pre-IC *in vitro*; the great number of factors involved and the strict kinase-mediated regulation of the process have been challenging the possibility to gain insights in the helicase activation step. Most of the recent work in this direction employed yeast extracts instead of purified proteins (Gros et al, 2014; Heller et al, 2011; On et al, 2014),

limiting the possibility to obtain information on the mechanistic details of the process. Nevertheless, some direct protein interactions involved have been studied over the years with both the two hybrid assay and immunoprecipitation with purified factors (full list in Appendix I, Table VI) and a schematic summary can be found in Figure 1.4.

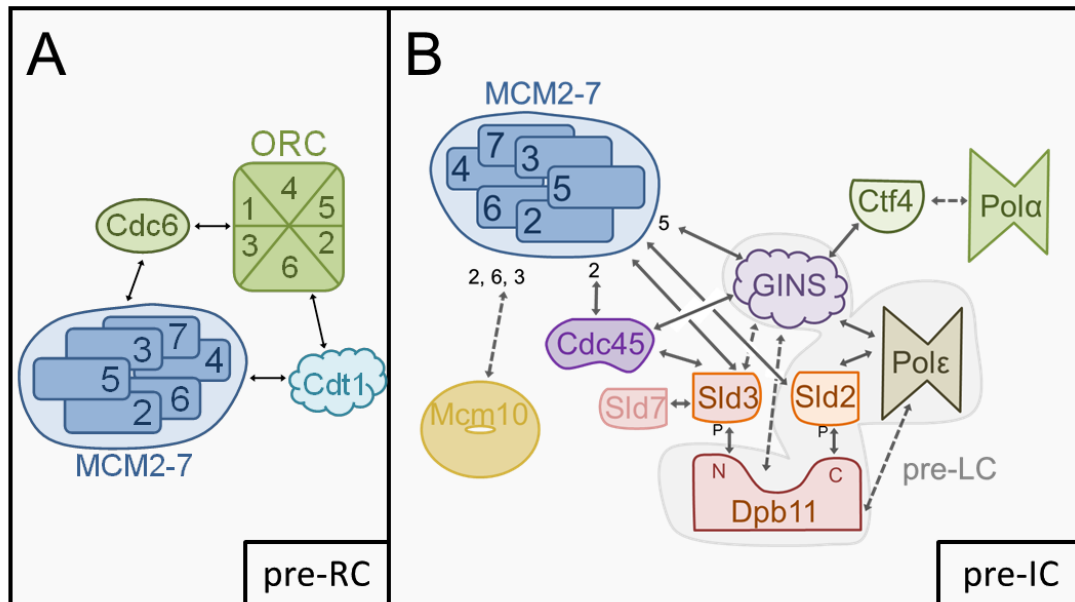


Figure 1.4: Protein-protein interactions in helicase loading and activation ¹

Schematic representation of the interactions involved in the helicase loading **A)** and activation **B)** processes. Solid lines represent direct physical interactions whereas dashed lines stand for interactions detected with two hybrid system.

A multitude of replicative factors are shown to interact with MCM2-7, remarking the crucial role that this complex plays during initiation of replication. In general, it is evident that most of the proteins involved in pre-IC formation are establishing contact with multiple other factors. The great number of elements involved and the apparent redundancy of some of the mechanisms can be explained with the necessity of strict regulation of DNA replication in order to avoid errors during the duplication of the genome and to generate a highly efficient DNA replication system. This is also reflected in the complicated helicase activation mechanism which, in eukaryotes, led to the establishment of a more sophisticated machinery compared to Archaea.

¹ As in Tognetti, Riera and Speck, submitted

1.8 Differential regulation of pre-IC formation in *S. pombe* and vertebrates¹

The core factors of the replicative helicase are conserved between Archaea and Eukarya (MCM2-7, Cdc45, GINS), while the pre-IC proteins (Dpb11, Sld3, Sld7, Sld3, Mcm10, Ctf4) have no apparent ortholog in prokaryotic or viral systems. Moreover, the latter factors have further evolved in higher eukaryotes and acquired new protein domains or functions (Tanaka & Araki, 2013). A summary of the replicative factors in *S. cerevisiae*, *S. pombe* and Metazoa and their function can be found in Appendix I, table VII.

S. pombe and *S. cerevisiae* helicase activation factors have a fairly high sequence similarity; however their regulation is partially different. DDK is required for Sld3 recruitment at origins, but Sld3 association, other than in budding yeast, is Sna41/Cdc45 independent. Instead Sna41/Cdc45 associates with chromatin in a CDK dependent manner (Fukuura et al, 2011; Yamada et al, 2004). Nevertheless, it was found in *S. pombe* that the Sld3-origin interaction is essential for the subsequent recruitment of Cut5/Dpb11, Drc1/Sld2, Sna41/Cdc45 and GINS (Yabuuchi et al, 2006). Similarly as in budding yeast, DDK is essential for the process of helicase activation; though, in fission yeast DDK-bypass mutants in the MCM2-7 helicase have not been identified. However, the deletion of Mrc1, which is required for maintenance of replication fork integrity, or of Rif1 (discussed in paragraph 1.6), can bypass the requirement of DDK in *S. pombe* (Hayano et al, 2012; Matsumoto et al, 2011). Moreover, *S. pombe* CDK facilitates the interaction of Drc1/Sld2 and Sld3 with Cut5/Dpb11, similarly as in budding yeast (Fukuura et al, 2011; Nakajima & Masukata, 2002; Noguchi et al, 2002; Saka et al, 1994), but then again CDK dependent phosphorylation of Sld3 is not essential for the interaction with Cut5/Dpb11, probably because the Drc1/Sld2 interaction with Cut5/Dpb11 stabilizes the trimeric complex (Fukuura et al, 2011).

In vertebrates the predicted homologues of Sld3, Sld2 and Dbp11 are Treslin/Ticrr, RecQ4 and TopBP1 respectively, but their amino acid and domain organisation has diverged significantly (Tanaka & Araki, 2013). As in budding yeast, Treslin/Ticrr/Sld3 can bind Cdc45 and it is required for its association with origins (Im et al, 2009; Kumagai et al, 2011; Van Hatten et al, 2002). Furthermore, it binds the

¹ As in Tognetti, Riera and Speck, submitted

N-terminal BRCT domain of TopBP1/Dpb11 and it is phosphorylated by CDK on the same conserved residues (Boos et al, 2011; Kumagai et al, 2011). Finally, the checkpoint kinase 1 Chk1 can reduce the interaction of Treslin/Sld3 with TopBP1/Dpb11, in the same way to what was observed for Rad53 regulation of the interaction Sld3-Dpb11 in budding yeast (Boos et al, 2011; Kumagai et al, 2011; Lopez-Mosqueda et al, 2010; Zegerman & Diffley, 2010), indicating that Treslin and Sld3 function in an identical manner, although their sequence has diverged.

Likewise, RecQ4 has been suggested as the homologue of Sld2, despite the fact that the two proteins have very limited sequence conservation. The two proteins share only a weak similarity in the N-terminal portion, but this region is essential for DNA replication (Matsuno et al, 2006; Sangrithi et al, 2005). Moreover, RecQ4 association with origins is TopBP1/Dpb11 and pre-RC dependent, similarly as in budding yeast. However, RecQ4 differs from Sld2 for the following functional characteristics: unlike Sld2, RecQ4 contains a C-terminal helicase domain and is not required for TopBP1/Dpb11, Cdc45 or GINS interaction with origin DNA. Furthermore, RecQ4 and TopBP1/Dpb11 can bind in the absence of CDK phosphorylation, which is instead essential for the Sld2-Dpb11 interaction (Matsuno et al, 2006; Sangrithi et al, 2005). Finally, unlike Sld2, RecQ4 binds to Mcm10 and associates with the CMG in a Mcm10 dependent manner (Xu et al, 2009). In summary, although RecQ4 and Sld2 share some similarities, it is still an open question if RecQ4 is the functional homologue of Sld2.

Cut5/TopBP1 is thought to be the functional homologue of Dpb11, as the proteins share important structural similarities (Garcia et al, 2005; Rappas et al, 2011; Tanaka & Araki, 2013); however, some differences have been observed as well. Similarly to what is known for lower eukaryotes, the association of Cut5/TopBP1/Dpb11 with chromatin is CDK dependent. However, other than in yeast, this interaction is modulated by CDK concentration: at low CDK concentrations *Xenopus* Cut5/TopBP1/Dpb11 is recruited to chromatin via an interaction with ORC and at high CDK concentrations via a pre-RC interaction, but even the recruitment at low CDK concentration is sufficient for DNA replication (Hashimoto et al, 2012; Van Hatten et al, 2002). Moreover, *Xenopus* Cut5/TopBP1/Dpb11 and Cdc45 interact directly (Schmidt et al, 2008), which is probably important, as Cdc45 association with chromatin requires Cut5/TopBP1/Dpb11 (Kumagai et al, 2011; Van Hatten et al, 2002). TopBP1 and its

counterparts share BRCT domains, even though they differ in number (Garcia et al, 2005; Rappas et al, 2011) and only the first two pairs involved in Treslin/Sld3 binding are indispensable for replication (Kumagai et al, 2010), while Dpb11 has four essential BRCT domains. The additional BRCT domains of TopBP1, which do not exist in Dpb11, function in checkpoint control of DNA replication (Garcia et al, 2005), indicating that this function has become more important for vertebrates.

Homologues for Mcm10 and Pol ϵ have also been identified in vertebrates. While budding and fission yeast Mcm10 is believed to be involved in the CMG activation in a step subsequent to its assembly at the replication origins (Kanke et al, 2012; van Deursen et al, 2012; Watase et al, 2012), human and *Xenopus* Mcm10 are probably involved also in the CMG assembly process (Im et al, 2009; Wohlschlegel et al, 2002) together with Ctf4 and RecQ4 (Im et al, 2009). If metazoan Pol ϵ functions in a way similar to the yeast counterpart for pre-LC and CMG formation has yet to be discovered.

Among all replication factors involved in helicase activation in budding yeast, only Sld7 is currently lacking homologues in higher eukaryotes. However, very recently a newly discovered factor, named MTBP (MDM two binding protein) was proposed as the functional homologue of Sld7 in human cells. It is reported to interact with Treslin/TICRR throughout the cell cycle and its depletion inhibits DNA replication by preventing assembly of the CMG during origin firing (Boos et al, 2013). Moreover, two metazoan factors, GEMC1 and DUE-B (Balestrini et al, 2010; Chowdhury et al, 2010), both of which bind to TopBP1/Dpb11 and Cdc45, are required for the recruitment of Cdc45 to chromatin. However, they have no homologue in lower eukaryotes, indicating that activation of the replicative helicase in vertebrates is more complex than in yeast.

DDK and CDK regulation of pre-IC formation is also essential in metazoans and it appears to function similarly as in *S. cerevisiae* with DDK operating prior to CDK. Moreover, DDK dependent phosphorylation of MCM2-7 (Masai & Arai, 2000a; Masai & Arai, 2000b; Masai et al, 2000) facilitates Cdc45 binding of chromatin (Masai et al, 2006), indicating that the overriding DNA replication concepts are conserved between lower and higher eukaryotes.

1.9 DNA replication and relevance for human health

DNA replication is a fundamental biological process in all living organisms; mis-regulation and mutation of the factors involved cause genomic instability, growth defects and cancer. Given that the replicative machinery shows conserved features among species, the insights acquired studying any organism are useful to improve the understanding of basic notions that can ultimately be relevant for human health. Moreover, replicative proteins have proven to be good markers for detection of several common carcinomas and are potential targets for treatments, as their inhibition affects cancer cells viability (Jackson et al, 2014).

1.9.1 Mutation of replicative factors

Genes encoding for human DNA replication proteins can carry mutations resulting in a range of clinical disorders. These disorders share some common features, including reduced growth and increased risk of cancer and multisystem dysfunction of highly energy-dependent tissues for mitochondrial genes (Jackson et al, 2014).

Mutations in genes coding for Orc1, Orc4, Orc6, Cdc6 and Cdt1 have recently been identified in patients with Meier-Gorlin syndrome (Bicknell et al, 2011a; Bicknell et al, 2011b; Guernsey et al, 2011), characterised by short stature, microtia (reduced external ear size), hypoplasia/absence of the patella but usually normal intellect (Bongers et al, 2001; Gorlin et al, 1975). The general growth defect appears to be a result of problems during origin licensing, therefore these mutations could impair rather than abrogate pre-RC function. However, it has not to be forgotten that several replicative proteins operate also outside DNA replication therefore the defects caused by their mutation could impair other functionality of the cell. For example, in addition to regulating DNA replication, the Orc1 subunit of the human ORC controls centriole and centrosome copy number. Meier-Gorlin syndrome mutations cause in fact centrosome reduplication because of disruption of an Orc1 CDK inhibitory domain (Hossain & Stillman, 2012).

Finally, a specific mutation in the gene coding for Mcm4 has been linked with genome instability and adenocarcinomas in mice (Shima et al, 2007a; Shima et al, 2007b). In human, a homozygous splice-site mutation in the *MCM4* gene is

associated with a syndrome causing adrenal insufficiency, natural killer cell deficiency and chromosome instability (Gineau et al, 2012; Hughes et al, 2012).

1.9.2 Replicative stress and cancer

DNA replication must be strictly regulated to ensure accurate duplication of the genome during each cell cycle. Deregulation is a major cause of replicative stress and DNA damage, both occurring in tumorigenesis. Genome instability can be either cause or consequence of tumour progression and it has been additionally proposed that activated oncogenes are responsible for replicative stress (Bartkova et al, 2005; Gorgoulis et al, 2005).

Replicative stress causes stalling or collapsing of replication forks, leading to DNA damage which in turn activates a DNA damage response (DDR) and ultimately senescence and apoptosis (Bartkova et al, 2006; Di Micco et al, 2006). These programs of cell death have the specific function to protect the cell from insults but, whenever additional changes occur to down regulate the DDR, tumour progression can take place. Cells, in order to prevent replicative stress, must avoid origin under-usage as much as over-usage and re-usage. These crucial issues are circumvented by several mechanisms that prevent re-replication (i.e. oscillation of CDK activity, see paragraph 1.7), ensure temporally regulated origin licensing and avoid simultaneous firing of origins (i.e. limiting levels of firing factors) (reviewed in (Hills & Diffley, 2014)). Whenever some origins are not used for replication there can be both loss of the genetic information as well as DNA damage, possibly because of increased fork speed due to the greater distance that has to be covered (Burrell et al, 2013). Over-usage of origins also promotes DNA damage in different ways: directly by depletion of RPA (which coats newly replicated ssDNA, protecting it from damage), or because of fork stalling due to depletion of dNTPs or limiting replicative factors, as well as possible clashes of replication and transcription (Toledo et al, 2013). Re-replication can cause DNA damage via fork stalling (Green et al, 2010) or collapse (Neelsen et al, 2013), it can induce homologous recombination therefore altering the gene dosage and it has been proposed to play a role during cancer progression (Jones et al, 2013; Liontos et al, 2007; Tatsumi et al, 2006).

The finding that cancer cells are characterised by situations of replicative stress could be used for the development of cancer therapies. In advanced tumours

for example it is possible that DDR is bypassed and the active components of the DDR pathway are actually actively promoting fork stability and DNA repair, helping the malignant cell to cope with the replicative stress. In this sense, further studies on replication and its regulation can be helpful to improve the understanding in the mechanisms cancer cells employ to circumvent death and in the future specifically target the de-regulated factors involved (Hills & Diffley, 2014).

1.9.3 DNA replication and cancer diagnosis

Diagnosis, essential for successful treatment of the most common cancers, is based on the differentiation of normal and cancer cells. DNA replication proteins have proved to be good markers for early detection of several common carcinomas. MCM2-7, in particular, appears to be a better marker than the currently used PCNA (DNA clamp required for the processivity of Pol δ during replication) and Ki67 (a nuclear protein associated with ribosomal RNA transcription) (Jackson et al, 2014). MCM2-7 expression in fact is down regulated before normal cells are released from solid tissues into body fluids but this does not occur in cancer cell, therefore MCM2-7 can be found exclusively in body fluids containing malignant and premalignant cells (Freeman et al, 1999; Gonzalez et al, 2005; Hiraiwa et al, 1998; Tachibana et al, 2005; Todorov et al, 1998). This could be explained with the assumption that malignant cells may be constantly replicating DNA or that they have an extended G1/S-phase because of defects in helicase loading and activation.

The employment of replicative proteins as markers reduces the false-negative results associated with conventional cytological screening and allows sensitive and automated detection of immunopositive cells by microscopy. MCM2-7 appeared so far superior to other replicative factors (PCNA, Cdc6, Cdt1, ORC) because of its high nuclear abundance and expression in malignant cells, for the availability of specific antibodies for the detection and low variability upon staining (Celis & Celis, 1985; Gonzalez et al, 2004; Madine et al, 2000). For all these reasons, MCM2-7 has been used as marker in the screening of several cancer types including cervical cancer (Baldwin et al, 2003), colorectal cancer (Davies et al, 2002), bladder and prostate cancer (Stoeber et al, 1999; Stoeber et al, 2002) and cancers of the squamous epithelium of the oral cavity (Scott et al, 2006) and anal canal (Scarpini et al, 2008).

These evidences provide further proof of the crucial importance of studying the process of replication.

1.10 Summary and project aims

Over the last years, thanks to the *in vitro* reconstitution of the budding yeast pre-RC (Evrin et al, 2009; Remus et al, 2009), extensive work has provided insights in the helicase loading process but much remains to be discovered concerning helicase activation. How Sld3 and Cdc45, the pre-LC components, the polymerases and additional replicative factors are recruited to the origins, how the CMG complex and the replicative fork are established and the dsDNA is melted for the extrusion of one ssDNA, remain open questions. Studies *in vivo* could not, so far, address these questions for several reasons. The redundancy that characterises these strictly regulated fundamental processes makes it difficult to discriminate the contribution of individual factors. Many *in vivo* assays allow only limited insights into the exact molecular mechanisms that facilitate complex formation and moreover, it is difficult to detect transient events *in vivo*.

These problems can partially be overcome using *in vitro* reconstituted systems and purified proteins. For this reason, this project took advantage of the *in vitro* reconstitution system developed in the Speck group (Evrin et al, 2009) and aimed to recapitulate the initial steps following MCM2-7 double-hexamer establishment which lead to Cdc45 recruitment.

Additionally, a systematic pairwise interactions analysis using purified and *in vitro* translated proteins from *Saccharomyces cerevisiae* was performed in order to understand how these factors interact with each other and MCM2-7, allowing helicase loading and activation.

A new network of interactions required for the stable recruitment of Cdc45 to MCM2-7 was identified during this study. The interaction sites in these proteins were mapped and mutants were generated in order to disrupt the formation of the complex containing Cdc45 and finally tested *in vivo* in the attempt to validate the proposed model for Cdc45 recruitment at replicative origins.

Chapter 2 – Materials and Methods

2.1 Materials

2.1.1 General reagents

All reagents were of molecular biology grade and obtained from Sigma Aldrich, except for those listed below.

Category	Product	Brand
Chemicals	Dithiothreitol (DTT)	Thermo Fisher Scientific
Chemicals	Ethanol	Thermo Fisher Scientific
Chemicals	Formaldehyde	Thermo Fisher Scientific
Chemicals	Iodoacetamide	Thermo Fisher Scientific
Chemicals	Isopropyl β -D-1-thiogalactopyranoside (IPTG)	Thermo Fisher Scientific
Chemicals	Magnesium sulphate (MgSO_4)	Thermo Fisher Scientific
Chemicals	Methanol	Thermo Fisher Scientific
Chemicals	Propan-2-ol	Thermo Fisher Scientific
Chemicals	Silver nitrate	Thermo Fisher Scientific
Chemicals	Bidistilled glycerol	VWR
Chemicals	Dimethyl sulfoxide (DMSO)	VWR
Chemicals	Ethylene diamine tetraacetic acid (EDTA)	VWR
Chemicals	Ethylene glycol tetraacetic acid (EGTA)	VWR
Chemicals	Glycine	VWR
Chemicals	Potassium hydroxide (KOH)	VWR
Chemicals	Sodium chloride (NaCl)	VWR
Chemicals	Sodium dodecyl sulphate (SDS)	VWR
Chemicals	Sodium hydroxide (NaOH)	VWR
Chemicals	Sodium thiosulphate	VWR
Cloning/mutagenesis	Herculase II Fusion DNA polymerase	Agilent Technologies
Cloning/mutagenesis	QuickChange II site-directed mutagenesis kit	Agilent Technologies
Cloning/mutagenesis	PfuUltra II Fusion HS DNA polymerase	Agilent Technologies
Cloning/mutagenesis	Antarctic phosphatase	New England Biolab (NEB)
Cloning/mutagenesis	LongAmp polymerase	New England Biolab (NEB)
Cloning/mutagenesis	Quick ligation kit	New England Biolab (NEB)
Cloning/mutagenesis	Restriction enzymes	New England Biolab (NEB)
Cloning/mutagenesis	Polymerase chain reaction nucleotide mix	Roche
DNA purification	Plasmid MiniPrep kit	Qiagen
DNA purification	Plasmid MidiPrep kit	Qiagen

DNA purification	Plasmid MaxiPrep kit	Qiagen
DNA purification	PCR purification kit	Qiagen
DNA purification	Gel purification kit	Qiagen
DNA purification	Nucleotide removal kit	Qiagen
Agarose gel electrophoresis	1 kb ladder	Invitrogen/Life technologies
Agarose gel electrophoresis	Agarose	Invitrogen/Life technologies
Media	Amino acid dropout mix (Leu ⁻ , Ura ⁻ , Trp ⁻ , His ⁻)	ForMedium
Media	L-Histidine	ForMedium
Media	L-Leucine	ForMedium
Media	L-Tryptophan	ForMedium
Media	Uracil	ForMedium
Media	Yeast extract	ForMedium
Media	Yeast nitrogen base (without amino acids)	ForMedium
Media	Peptone	ForMedium
Media	Bacto-Agar	Invitrogen/Life technologies
Media	D (+) galactose	Thermo Fisher Scientific
Media	D (+) glucose monohydrate	VWR
Antibiotics	Ampicillin disodium salt	Thermo Fisher Scientific
Antibiotics	Cloramphenicol	Thermo Fisher Scientific
Beads biotinylation	Long arm photoprobe biotin	Vector laboratories
Protein purification	PreScission protease	GE Healthcare
Protein purification	Benzonase	Merck
Protein purification	Complete protease inhibitor cocktail (+/- EDTA) (PIC)	Roche
Protein purification	PhosSTOP cocktail	Roche
Protein detection	Bradford reagent	BioRad
Protein detection	PageRuler Plus Prestained protein ladder	Thermo Fisher Scientific
SDS-PAGE	SDS-PAGE standard broad range protein marker	BioRad
SDS-PAGE	Acrylamide 40 % solution	GE Healthcare
SDS-PAGE	Methylenbisacrylamide 2 % solution	GE Healthcare
SDS-PAGE	NuPAGE Novex 4-12% Bis-Tris Protein Gels	Invitrogen/Life technologies
SDS-PAGE	NuPAGE MES running buffer (20X)	Invitrogen/Life technologies
SDS-PAGE	NuPAGE MOPS running buffer (20X)	Invitrogen/Life technologies
SDS-PAGE	ProtoGel (30%)	National Diagnostics
Western blotting	Bovine serum albumin (BSA) fraction V	Roche
Western blotting	Restore Western Blot Stripping Buffer	Thermo Fisher Scientific
Western blotting	SuperSignal kit west Pico/Dura/Femto	Thermo Fisher Scientific
Buffer RC components	Adenosine-5'-triphosphate (ATP)	Roche
Buffer RC components	DNase I (recombinant RNase free)	Roche
Buffer RC components	Human topoisomerase I	Topogen
Radiolabeled	EasyTag™ L-[³⁵ S]-Methionine	PerkinElmer
Radiolabeled	γ- ³² P-ATP	PerkinElmer

2.1.2 Oligonucleotides

Oligonucleotides used for cloning were obtained from Sigma Aldrich whereas those for *in vitro* transcription and translation were from Eurofins. All oligonucleotides produced during this study are listed in Appendix I (Table II and III, respectively).

2.1.3 Plasmids

pESC yeast epitope tagging vectors (URA⁻, TRP⁻, LEU⁻, HIS⁻) were purchased from Stratagene, pGEX-6P-1 from GE Healthcare, pMALC2x and pRS416 from NEB.

The origin containing DNA substrate for the reconstituted pre-RC assays was pUC19-ARS1 (Evrin et al, 2009).

All constructs produced in the Speck group, prior or during this study, and used during this work are listed in Appendix I, Table I. Constructs listed below were produced by me, unless differently stated.

Generation of FLAG-tagged variants of MCM2-7

For the addition of the FLAG tag at Mcm2 N-terminus, the original plasmid for Mcm2/Mcm7 expression (pESC-LEU-Mcm2/Mcm7 (Evrin et al, 2009)) was modified during this work. The tag was subcloned from another plasmid available in the lab using BamHI.

MCM2-7 mutants for the N-terminal deletions of Mcm2 (Δ 1-200, Δ 1-238, Δ 1-251) were produced by PCR, in pESC-LEU-Mcm2/Mcm7 or pESC-LEU-FLAG-Mcm2/Mcm7, using the Quick Change site directed mutagenesis kit.

Hybrid mutants of MCM2-7 (Mcm2 h1-200 sc222-868 and h1-232 sc254-868) were produced via subcloning (SbfI/AsiSI) of the human fragment derived from a plasmid available in the lab into a modified versions of expression plasmid for Mcm2 where the AsiSI restriction site was inserted internally in the sequence (AsiSI modified Mcm2 plasmids produced by Samel).

Cloning of Sld3 in pESC-HIS (Zech and Speck, unpublished)

The coding sequence of Sld3 by PCR adding BamHI and Sall restriction sites and cloned it into pESC-HIS for protein expression in yeast. This construct was modified by insertion of an N-terminal MBP tag and amplified from pMALC2x. The primers

also encoded for a PreScission protease site, a myc tag and the BamHI restriction sites.

Cloning of Sld3 (wt and mutants) in pRS416

Sld3 mutants for *in vivo* analysis were produced during this study as following. In first place the wild type plasmid was generated by PCR amplification of the coding sequence of Sld3 from genomic DNA (AS499 strain) preceded by 1 kb in the promoter region, with the simultaneous addition of SacI/BamHI restriction sites, which were subsequently used for the cloning into pRS416. Sld3 mutants were produced via gene synthesis (Genescript) within the coding region of Sld3 and then the region was subcloned into the wild type Sld3 plasmid using AatII/SphI restriction sites.

For overexpression of the Sld3 mutants, the mutated region was subcloned via AatII/SphI restriction sites into pESC-HIS-Sld3.

Cloning of Cdc45 in pESC (Zech and Speck, unpublished)

For yeast expression, the coding sequence of Cdc45 (missing the stop codon) was amplified by PCR, adding NotI and SpeI restriction sites, and cloned in pESC-HIS, pESC-LEU, pESC-TRP and pESC-URA vectors. The stop codon was deleted in order to have a C-terminal FLAG tag.

Cloning of Cdc45 in pGEX-6P-1

The coding sequence of Cdc45 was amplified by PCR using an existing pESC-Cdc45 plasmid as template, adding BamHI and NotI restriction sites and cloned in pGEX-6P-1 vector to obtain GST-Cdc45.

Cloning of Dbf4 and Cdc7 in pESC-TRP (Zech, Clarke and Speck, unpublished)

MBP-PreScission-Dbf4 and Cdc7 were cloned into pESC-TRP for yeast expression. The *Dbf4* gene was amplified from genomic DNA (AS499 strain) and SmaI/NheI restriction sites were added at the same time. The MBP-prescission coding DNA was amplified from a plasmid available in the group and cloned in before the *dbf4* gene using a BamHI site. The *cdc7* gene was amplified from genomic DNA of the *S. cerevisiae* strain S288C and cloned via NotI/SpeI the vector.

Cloning of Dpb11, Reg α and MCM2-7 N-termini in pGEX-6P-1 C-terminal

Dpb11, Reg α and the N-terminal portions of the MCM2-7 genes were amplified from Dpb11, Reg α and MCM2-7 expression plasmids available in the Speck group and cloned using SphI/NotI restriction sites into a modified version of pGEX-6P-1 carrying the GST tag on the C-terminus (Herrera and Speck, unpublished). The N-terminal MCM2-7 truncations (covering amino acids 1-322 for Mcm2, 1-192 for Mcm3, 1-340 for Mcm4, 1-174 for Mcm5, 1-302 for Mcm6 and 1-253 for Mcm7) were designed based on the strategy of Sheu and Stillman (Sheu & Stillman, 2010) and taking the secondary structure prediction and the multiple alignment into account.

Cloning of Sld2 and Sld7 in pGEX-6P-1 (Herrera and Speck, unpublished)

The coding sequence of Sld2 was amplified by PCR with the addition NotI and EcoRI restriction sites. The PCR product was cloned into pGEX-6P-1 vector. The coding sequence of Sld7 was amplified by PCR, adding NotI and BamHI restriction sites and cloned into pGEX-6P-1.

2.1.4 Bacterial competent cells

DH5 α competent cells (Invitrogen) were used for plasmid amplification; XL10 Gold ultracompetent cells (Agilent Technologies) were used for site-directed mutagenesis; BL21 Codon+ RIL and (DE3) RIL competent cells (Stratagene) were used for protein overexpression.

2.1.5 Yeast strains

AS499 (*MAT α bar1 Δ leu2-3,112 ura3-52 his3- Δ 200 trp1- Δ 63 ade2-1 lys2-801 pep4*) and S288C (*MAT α SUC2 gal2 mal2 mel flo1 flo8-1 hap1 ho bio1 bio6*) wild type yeast strain were gifts by Luis Aragon (Clinical Sciences Centre, London, UK) whereas Sld3-7 td strain was a gift from Karim Labib (College of Life Sciences, Dundee, UK).

2.1.6 Chromatography resins and columns

All columns, magnetic beads and resins were from GE Healthcare except for EZview Red anti-HA affinity gel and Glutathione agarose (Sigma-Aldrich), Amylose resin (NEB), Dynabeads MyOne Streptavidin T1 and Dynabeads Protein G (Invitrogen/Life Technologies).

2.1.7 Peptides

Peptides were purchased from LifeTein. They were all 85% purity grade or higher with a N-terminal biotin and two aminohexanoic acid (Ahx) spacers between biotin and peptide. Peptides sequences can be found in Appendix I, Table IV.

2.1.8 Antibodies

All antibodies used in this work are listed in Appendix I, Table V.

2.2 Methods

2.2.1 Cloning

PCR was performed using PfuUltra II Fusion HS DNA polymerase, DNA digested with NEB restriction enzymes, vector dephosphorylation was carried out with Antarctic Phosphatase and ligation reactions were performed using the NEB Quick Ligation kit. PCR products and digested DNA were purified with Qiagen PCR purification kit and gel extraction kit respectively.

2.2.2 Mutagenesis

All mutagenesis reactions were performed using the QuickChange II Site-Directed Mutagenesis kit following the manufacturer's instructions. Oligonucleotides for the amplification were generated using the QuikChange Primer Design program (<http://www.genomics.agilent.com/primerDesignProgram.jsp>) and ordered PAGE purified. The oligonucleotides were designed in order to generate a unique restriction site using silent mutations in the sequence

(http://watcut.uwaterloo.ca/template.php?act=silent_new). The PCR product was digested with DpnI to cleave the methylated DNA template and subsequently transformed into XL10 Gold ultracompetent cells. The success of mutagenesis was verified by restriction digestion and confirmed by sequencing.

2.2.3 Agarose gel electrophoresis

DNA samples were diluted with Orange G sample buffer (0.1 % (w/v) Orange G dye, 50% (v/v) glycerol) and analysed by electrophoresis at 100 V for the required time in 0.8, 1 or 2 % (w/v) agarose gel according to the size. The gel was stained with ethidium bromide and the stained DNA was visualised by UV exposure on BioRad Gel Doc.

2.2.4 Bacterial competent cells preparation

Bacterial competent cells were streaked out onto a LB plate (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, 15 g/L bacto-agar, pH adjusted to 7, sterilised by autoclaving) with appropriate antibiotics as required. A single colony was grown into 500 ml LB broth at 22°C with shaking (200 rpm) until OD₆₀₀ = 0.6. When the density was reached, the flask was placed on ice bath for 10 min. The culture was harvested at 2600 g for 10 min, 4°C. The pellet was resuspended into 80 mL ice cold sterile TB buffer (10 mM HEPES/KOH pH 6.7, 250 mM KCl, 15 mM CaCl₂, 55 mM MnCl₂) and placed in ice bath for 10 min. The culture was harvested and the pellet resuspended in 10 mL ice cold TB buffer. While swirling, 1.4 ml fresh DMSO were added and the mixture placed on ice bath 10 min, aliquoted, frozen in liquid nitrogen and stored at -80°C.

2.2.5 Bacterial transformation and culture

Competent cells were thawed on ice, mixed with DNA, incubated for 30 min on ice, heat shocked for 90 sec at 42°C, diluted in SOC (20 g/L tryptone, 5 g/L yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, 20 mM glucose, pH adjusted to 7 and sterilised by autoclaving) and allowed to recover for 60 min at 37°C

while shaking (1000 rpm) before plating onto appropriate selective plates. Single colonies were isolated after 12 h and grown in selective liquid media.

2.2.6 Plasmid DNA purification

Plasmid DNA was purified from 5, 250 or 500 ml culture using Qiagen MiniPrep, MidiPrep or MaxiPrep kits respectively, following the manufacturer's instructions. DNA concentration was measured using the Nanodrop ND-1000 spectrophotometer and sequencing was performed by the genomics core facility at the MRC Clinical Sciences Centre.

2.2.7 DNA biotinylation and immobilisation on streptavidin beads

Circular pUC19-ARS1 plasmid was covalently coupled with biotin by exposure to UV light using the Vector Laboratories Long Arm Photoprobe Biotin kit. The plasmid was mixed with the photoprobe, exposed to UV light, purified with sec-butanol and precipitated with ethanol (Evrin et al, 2009). Linear ARS1 was otherwise amplified by PCR using biotinylated oligonucleotides (using one or both primers biotinylated). DNA was subsequently immobilised overnight on streptavidin beads.

2.2.8 Bacterial protein expression

BL21 Codon+ RIL or (DE3) RIL competent cells were grown in TB liquid media (12 g/L tryptone, 24 g/L yeast extract, 4 ml/L glycerol) supplemented with appropriate antibiotics at 37°C to an OD₆₀₀ of 1. Protein expression was then induced by addition of 0.5 mM IPTG for 5h at 16°C before harvesting.

2.2.9 Yeast competent cells preparation

Yeast cells were grown over night in 500 ml YPD (20 g/L peptone, 10 g/L yeast extract, 2% (w/v) glucose) to OD₆₀₀=0.5-0.7 at 30°C (or 24°C for temperature sensitive strains). The culture was harvested at 500 g for 5 min at room temperature and the pellet washed with 1/10 volumes (5 ml) of sterile water. The sample was spun again and the pellet was resuspended in 1/10 volumes (5 ml) of SORB (100

mM LiAc, 10 mM Tris/HCl pH 8, 1 mM EDTA/NaOH pH 8, 1 M sorbitol). After another centrifugation the pellet was resuspended in 360 μ l SORB. 40 μ l of carrier DNA (10 mg/ml salmon sperm DNA, denaturated at 100°C for 10 min and stored on ice) were added and mixed well. The mixture was aliquoted and stored at -80°C.

2.2.10 Yeast transformation and culture

30 μ l of competent cells were thawed on ice. 300 ng of each plasmid to transform and 6 volumes (180 μ l) of PEG solution (100 mM LiAc, 10 mM Tris/HCl pH 8, 1 mM EDTA/NaOH pH 8, 40% (w/v) PEG3350) were added and carefully mixed by pipetting. The mixture was incubated for 1h at 28°C (or 24°C for temperature sensitive strains). 1/9 volumes (3 μ l) of DMSO were added and the cells were incubated for 15 min on 42°C. After the heat shock, the cells were harvested (2000 rpm for 3 min), the pellet resuspended in 100 μ l of minimal media and plated onto Kaiser plates (6.7 g/L yeast nitrogen base without amino acids, 2 g/L supplement amino acids mix dropout, added with 2% (w/v) glucose with selection for the appropriate auxotrophies). After 3 days a single colony was picked and re-streaked onto a new plate. After 3 days a single colony was picked and grown in SC liquid media (6.9 g/L yeast nitrogen base without amino acids, 0.6 g/L supplement amino acids mix dropout, added with 2% (w/v) glucose and selection for the appropriate auxotrophies) for glycerol stock or *in vivo* assays. Glycerol stocks were made by resuspending the pellet of a culture at OD₆₀₀>1.5 in 50% (w/v) glycerol and stored at -80°C.

2.2.11 Yeast growth assays

A liquid culture grown from a single colony was diluted to OD₆₀₀=0.5 in 10 ml of media and harvested by centrifugation at 4000 rpm for 5 min. The pellet was washed with 10 ml of sterile water in order to remove all glucose and subsequently resuspended in 10 ml of SC without carbon source with selection for appropriate auxotrophies and grown for at least 2h. The culture was then harvested as above and a 5 fold serial dilution was prepared in order to have the following OD₆₀₀: 1×10^7 , 0.2×10^6 , 0.4×10^5 , 0.8×10^4 , 1.6×10^4 , 3.2×10^3 , 6.4×10^2 . 3 μ l of each dilution were dropped onto appropriate plates to monitor differential growth.

2.2.12 Yeast protein expression

A liquid culture was grown for 16 h from an isolated colony in SC liquid media added with 2% (w/v) glucose in the presence of appropriate auxotrophies. It was then diluted to $OD_{600}=0.5$ in 500 ml of the same media and grown for 12 h prior dilution to $OD_{600}=0.5$ in 12 x 500 ml cultures. After 12 h ($OD_{600}=1.4-1.9$) the cultures were diluted with one volume each (500 ml) of Rich Media (10 g/L yeast extract, 20 g/L peptone, 0.1 g/L glucose, 30 g/L glycerol, 20 g/L lactic acid, pH 5.5-6) for 3 h. Expression was then induced with the addition of 20 g/L galactose for 6 h before harvesting. Whenever G1-phase arrest was required, 2.5 mg/L α -factor was added to the culture after 3h of induction. All cultures were grown at 30°C with the exception of temperature sensitive strains, which were grown at the permissive temperature of 24°C. The harvested culture was resuspended in half a pellet volume of the appropriate purification buffer, frozen in liquid nitrogen and stored at -80°C.

2.2.13 Yeast cells lysis

Yeast cells were lysed using a SPEX SamplePrep 6870 freezer mill (Spex Certiprep). 50 g of popcorn were supplemented with 4x complete protease inhibitor cocktail tablets and milled by executing 15 cycles of 4 min pre-cool, 3 min grinding at 15 cycles per second (cps) and 3 min cooling. The resulting powder was thawed at 4°C, mixed with one powder volume of appropriate purification buffer and incubated for 2 h while mixing. The extract was then centrifuged at 28000 g for 1 h and the supernatant was collected and stored at -80°C for up to 3 days prior to purification.

2.2.14 Protein purifications

MCM2-7, CDK, DDK, Sld3, Cdc45, GST-tagged Mcm N-termini and all FLAG-tagged MCM2-7 versions were purified by me. I was also involved in the purification of ORC and Sld2, whereas all other proteins were available in the lab.

Proteins were purified as following:

ORC, Cdc6, Cdt1, MCM2-7, MCM2-7 Δ C6 and CDK were purified as previously reported (Evrin et al, 2009; Fernandez-Cid et al, 2013).

MBP-Cdt1 was purified by Fernandez-Cid and Riera similarly to Cdt1 with the exception that the PreScission Protease cleavage step was substituted with Maltose elution.

MCM2-7 FLAG-tagged mutants were purified similarly to MCM2-7 with the exception that the gel filtration step was substituted with incubation with anti-FLAG resin and elution with FLAG peptide (Genescript).

Sld3 purification from yeast cells

The lysed cells were resuspended in buffer 3A (50 mM HEPES/NaOH pH8.1, 0.5 M NaCl 10% (w/v) glycerol, 0.1% (w/v) NP40, 1mM DTT) plus complete protease inhibitor cocktail (PIC), extracted on a rotating wheel for 2h at 4°C and centrifuged at 27,600 g for 1 h at 4°C. The extract was incubated for 2h at 4°C with Amylose Resin. The beads were afterwards rinsed with 10 column volumes buffer 3A + PIC and washed twice with 20 cv buffer 3A + PIC for 30 min and once with 20 cv buffer 3A without PIC for 30 min. MBP-myc-Sld3 was eluted at that stage with buffer 3A + 10 mM Maltose. To elute myc-Sld3, instead, beads were incubated with 1 cv of buffer 3A (without protease inhibitors) and PreScission protease for 2 h at 4 °C and more protein was eluted afterwards with 1 cv buffer 3A. Elutions were diluted to 0.1 M NaCl, bound to SP-Sepharose for 30 min. The resin was rinsed once with 10 cv buffer 3B (50 mM HEPES/NaOH pH8.1, 0.1 M NaCl 10% (w/v) glycerol, 0.1% (w/v) NP40, 1mM DTT) and once with 10 cv buffer 3C (50 mM HEPES/NaOH pH8.1, 0.2 M NaCl, 10% (w/v) glycerol, 0.1% (w/v) NP40, 1mM DTT). The protein was eluted in buffer 3A (Herrera, Tognetti et al, under revision).

Cdc45 purification from yeast cells

The protein was purified as previously described (Heller et al, 2011). To remove the FLAG peptide the sample was incubated for 30 min with DEAE beads, the resin was extensively washed and the protein was eluted with bufferC45 (50 mM HEPES/KOH pH 7.5, 1 mM EDTA, 10% glycerol, 0.02% NP40, 0.5 mM DTT, PIC) containing 500 mM KCl (Herrera, Tognetti et al, under revision).

DDK purification from yeast cells

The lysed extract was incubated with amylose resin (NEB) pre-equilibrated with buffer DK (50 mM Hepes-KOH pH 7.5, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA,

10% (w/v) glycerol, 0.02% (w/v) NP40, 1 mM DTT) and supplemented with proteases and phosphatases inhibitors for 2 h at 4°C. The protein was eluted by cleaving the MBP tag via addition of PreScission protease for 2 h at 4°C. The eluate was incubated with Glutathione agarose for 2 h to remove the PreScission protease. The GST flow-through was collected and concentrated by centricon (Millipore) (Herrera, Tognetti et al, under revision).

For the isolation of GST tagged Mcm N-termini on Glutathione beads, cells were lysed by sonication in the following buffers: Mcm2 50 mM HEPES/KOH pH 7.5, 250 mM KCl; Mcm3/4/6 50 mM TRIS/HCl pH 8, 250 mM NaCl; Mcm5/7 50 mM HEPES/KOH pH 7.5, 500 mM NaCl. All buffers also contained 10% (w/v) glycerol, 5 mM ATP, 10 mM MgCl₂, 1 mM DTT, 1% (v/v) Triton X-100, 25 U/ml Benzonase, 2x PIC, 1 mg/ml lysozyme. Dpb11 and Regα were instead resuspended from ammonium sulphate pellets in a buffer containing 50 mM PIPES pH 6.5, 200 mM NaCl, 10 mM MgCl₂, 2 mM ATP, 1% (v/v) Triton X-100, 3 mM DTT, 10% (w/v) glycerol and 2x PIC. They were then incubated with pre-washed Glutathione beads and extensively washed with the appropriate buffer to remove the unbound material.

Sld2 purification from bacteria (purified by Herrera)

The cells were lysed by sonication in buffer 2A (50 mM PIPES (pH 6.5), 500 mM AS, 10 mM MgCl₂, 5 mM DTT, and 1% (v/v) Triton X-100, 10% (w/v) glycerol, 2 mM ATP). The fusion protein was bound to glutathione agarose in buffer 2A at 4 °C for 2 h, and then the protein was eluted by addition of PreScission Protease for 2 h at 4 °C. The eluate was diluted with 1 volume buffer 2A with no salt and bound to SP Sepharose. Sld2 was eluted with buffer 2B (50 mM PIPES (pH 6.5), 500 mM AS, 5 mM DTT, and 0.1% (v/v) Triton X-100, 10% (w/v) glycerol) (Herrera, Tognetti et al, under revision).

Sld7 purification from bacteria (purified by Herrera and Gardenal)

The cells were lysed by sonication in buffer 7A (50 mM HEPES-KOH (pH 7.5), 300 mM NaCl, 10 mM MgCl₂, 1 mM DTT, and 1% (v/v) Triton X-100, 10% (w/v) glycerol, 2 mM ATP). Ammonium sulphate was added to a final concentration of 250 mM to the extract. Nucleic acids are precipitated from the cell extract by the addition of 30 µl 10 % (w/v) polymin P/HCl (pH 6.5) per ml cell extract. GST-Sld7 was precipitated

by adding 0.4 g fine powdered ammonium sulphate per ml of extract. The fusion protein was bound to glutathione agarose in buffer C at 4 °C for 2 h, and then the was eluted by addition of PreScission Protease for 2h at 4°C. The eluate was concentrated by centricon (Millipore) (Herrera, Tognetti et al, under revision).

2.2.15 SDS-PAGE

Samples were denatured for 5 min at 95°C in sample buffer (100 mM Tris-HCl pH 6.9, 40 % (w/v) glycerol, 100 mM DTT, 6 % (w/v) SDS, 0.02 % (w/v) bromophenol blue) before loading onto the appropriate percentage (7.5-10-15-20%) polyacrylamide gel and running at 250 V at 4°C in Tris-Glycine buffer (3 % (w/v) Tris, 14 % (w/v) glycine, 1 % (w/v) SDS). Alternatively samples were run onto NuPAGE Novex 4-12% Bis-Tris Protein Gels in 0.5 % (v/v) MES or MOPS. *In vitro* transcribed and translated samples were denatured for 5 min at 65°C.

2.2.16 Protein staining of polyacrylamide gels

Protein samples resolved by SDS-PAGE and the proteins were visualised by Coomassie Brilliant Blue or Silver staining.

Coomassie staining was used for samples containing > 200 ng of protein. Gels were immersed in Coomassie Brilliant Blue solution (0.25 % (w/v) Coomassie Brilliant Blue, 45 % (v/v) methanol, 10 % (v/v) glacial acetic acid), heated for 1 min in a microwave at full power, incubated for 10 min at room temperature and then destained (5 % (v/v) methanol, 7.5% (v/v) glacial acetic acid) by heating for 1 min in a microwave at full power and then incubated at room temperature until destained as required.

For samples containing a lower protein amount silver staining was performed. Initially the proteins were fixed by incubating the gel in fixing solution (40 % (v/v) methanol, 13.5 % (v/v) formaldehyde) for 10 min. The gel was washed twice with ultrapure water and then with 0.02 % (w/v) sodium thiosulphate before staining with 0.1 % (w/v) silver nitrate. Proteins were revealed with a solution containing 2 % (w/v) sodium carbonate, 0.05 % (v/v) formaldehyde, 0.0004 % (w/v) sodium thiosulphate. The reaction was stopped when required with 3 % (w/v) citric acid.

2.2.17 Western blotting

Samples resolved by SDS-PAGE were transferred to nitrocellulose membrane in transfer buffer (6 mM sodium bicarbonate, 4 mM sodium carbonate, 20 % (v/v) methanol, pH 9.5 or 12 if from NuPAGE Novex 4-12% Bis-Tris Protein Gels) at 500 mA for 1 h at 4°C. Membranes were stained with Ponceau-S solution (1 % (w/v) Ponceau-S, 1 % (v/v) acetic acid) and destained in 0.1 % (v/v) acetic acid prior to blocking in appropriate blocking solution. This could be 5 % (w/v) milk or BSA in TBS-T (25 mM Tris-HCl pH 7.5, 140 mM NaCl, 3 mM KCl, 0.05 % (v/v) Tween 20). Primary antibodies were diluted in blocking solution and incubated for 1 h at room temperature or overnight at 4°C. Membranes were washed three times for 10 min with TBS-T before incubation with HRP-conjugated secondary antibody. Three 10 min washes preceded the detection of the signal with enhanced Chemiluminescence.

After immunodetection, whenever re-blotting was required, the membranes were washed three times for 5 min with TBS-T and incubated with Restore Western Blot Stripping Buffer for 15 min. Membranes were then washed three times for 10 min and re-probed.

2.2.18 Bead based pre-RC formation assay

Bead based pre-RC formation assays were performed as previously described (Evrin et al, 2009; Evrin et al, 2014; Evrin et al, 2013; Fernandez-Cid et al, 2013; Sun et al, 2013). Briefly, 40 nM ORC, 80 nM Cdc6, 40 nM Cdt1 and 40 nM MCM2-7 were incubated for 10 min on ice in buffer RC (50 mM HEPES-KOH pH 7.5, 100 mM KGlu, 10 mM MgAc, 50 µM ZnAc, 3 mM ATP, 5 mM DTT, 0.1% (v/v) Triton X-100, and 5% (w/v) glycerol) followed by 10 min at 24°C. 6 nM pUC19-ARS1 magnetic beads were added to the sample, incubated for 15-30 min and washed with low salt or high salt (buffer RC with 500 mM NaCl in addition of 100 mM KGlu). 40 nM of additional proteins were added for 10-30 min whenever required before low salt washes and DNase I elution of the complex.

2.2.19 Gel filtration based pre-RC formation assay

Pre-RCs were assembled in solution using a one-step reaction (Herrera, Tognetti et al, under revision). 50 µl reactions containing 40 nM ORC, 80 nM Cdc6, 40 nM Cdt1 and 40 nM MCM2-7 were pre-incubated for 10 min on ice and 10 min at 24°C in buffer RC. 6 nM pUC19-ARS1 plasmid and 1.5 U of human topoisomerase I were added to the reaction and incubated for 15 min at 24°C, 950 rpm. Afterwards the following components were added to a final concentration of: 5 nM DDK, 40 nM Sld3, 40 nM Sld2, 40 nM Sld7, 40 nM Dpb11 and 40 nM Cdc45, 5 mM ATP, 250 ng/µl BSA, 1.4 µM competitor DNA (40 bp). Competitor DNA was derived from two complementary oligonucleotides incubated in buffer M (100 mM Tris-HCl (pH 7.9), 100 mM NaCl, 1 mM DTT, 10 mM MgCl₂) for 5 min at 95°C and then slowly cooled down to RT over 30 min. The hybridized oligonucleotides were concentrated using Microcon (Millipore) to a concentration of 5 µM. Due to the addition of these factors the volume of the reaction increased by 15 µl. Then the reactions were incubated for 1-30 min at 27°C 950 rpm. Then, KAc was added to a final concentration of 200 mM followed by a 3 min incubation at 24 °C and mixing at 950 rpm. Samples were loaded on top of a spin column, filled with 0.5 ml Sephacryl S400 HR resin. After loading of the samples the columns were spun for 5 min at 1000 g. Prior to the gel-filtration step the spin columns were filled with equilibrated resin (buffer RC containing 200 mM KAc, but without Triton X-100 and glycerol) and pre-spun for 5 min at 850 g. The eluate was concentrated by speed vac prior to SDS-PAGE analysis.

2.2.20 *In vitro* transcription and translation

To generate ³⁵S-Methionine-labelled proteins, the coding sequences of the genes were amplified by PCR using sense and anti-sense primers, as suggested by the supplier's instruction (Promega). All sense primers included at the 5' end a spacer (ACTGATC), the T7 promoter sequence (TAATACGACTCACTATAGGG), a short spacer (CAT), the Kozac sequence (CCACCATG) and a 17-22 nucleotides hybridization region. The anti-sense primer included at the 3' end a 30 nucleotides poly-A tail and the stop codon in addition to a 17-22 nucleotides hybridization region. To compensate for different methionine content additional methionines were

incorporated into the anti-sense primer. 400 ng of the resulting PCR product (whose size was verified by agarose gel electrophoresis) was used as template in the TNT-T7 Quick for PCR DNA system (Promega), combined with 20 µl of TNT Quick Master Mix and 20 µCi of ³⁵S-Methionine (Perkin-Elmer) in a total volume of 25 µl and incubated for 90 min at 30°C with mixing.

2.2.21 Pull-down assays

For pull-down assays with GST-tagged Mcm N-termini, Dpb11 and Regα, the normalised samples were immobilised on glutathione agarose (see paragraph 2.2.14), resuspended in buffer RC and 1 µg purified Sld3 was added in buffer RC for 15 min at 24°C (1000 rpm) before washes. Whenever CDK (20 nM) or DDK (80 nM) were added to the sample, they were first pre-incubated with Sld3 for 30 min at 27°C.

To generate anti-MBP/HA/FLAG/GST antibody beads, 50 µl Dyanabeads Protein G were washed three times with PBS-T (1.37 M NaCl, 27 mM KCl, 100 mM Na₂HPO₄, 18 mM KH₂PO₄, 0.05 % (v/v) Tween 20). They were then resuspended with 10 µg of the appropriate antibody and incubated for 15 minutes at 24°C (1000 rpm) before washing to remove the unbound antibody and resuspension in 2 volumes PBS-T. Beads were washed before use with the appropriate reaction buffer.

MCM2-7 pull-down reactions

0.5 µg purified HA-MCM2-7 were immobilized on anti-HA antibody beads for 15 min at 24°C with mixing in 50 µl of buffer RC. *In vitro* translated proteins were incubated with HA-MCM2-7 or empty control beads for 2h with mixing at 4°C in 500 µl of buffer RC; afterwards the beads were washed three times and analysed by SDS/PAGE followed by phosphorimaging analysis.

MBP-MCM2-7 pull-down reactions: equimolar amounts of MBP and MBP-MCM2-7 were immobilized on anti-MBP antibody beads for 15 min at 24°C with mixing in 50 µl of buffer RC. *In vitro* translated proteins were incubated with MBP or MBP-MCM2-7 beads for 2h with mixing at 4°C in 500 µl of buffer RC; afterwards the beads were washed three times and analysed by SDS/PAGE followed by phosphorimaging analysis.

MBP-Cdt1 pull-down reactions

Equimolar amounts of MBP and MBP-Cdt1 were immobilized on anti-MBP antibody beads for 15 min at 24°C with mixing in 50 µl of buffer RC. *In vitro* translated proteins were incubated with MBP or MBP-Cdt1 beads for 2h with mixing at 4°C in 500 µl of buffer RC. For the reactions in presence of Cdt1, 1 µg purified protein was added to the beads during the conjugation of the bait with the beads and additionally 2 µg purified Cdt1 were added simultaneously with the *in vitro* translated mixture. Afterwards the beads were washed three times and analysed by SDS/PAGE followed by phosphorimaging analysis.

Sld3 pull-down reactions

Equimolar amounts of MBP and MBP-Sld3 were immobilized on anti-MBP antibody beads for 15 min at 24°C with mixing in 50 µl of Sld3 buffer 3A followed by three washes with 100 µl of buffer RC2 (buffer RC with 225 mM KGlu and 1% (w/v) BSA). *In vitro* translated proteins were incubated with MBP or MBP-Sld3 beads for 2h with mixing at 4°C in 500 µl of buffer RC2 afterwards the beads were washed three times with 100 µl of buffer RC3 (RC2 without BSA) and analysed by SDS/PAGE followed by phosphorimaging analysis. For pull-down reactions with purified protein instead of *in vitro* transcribed, 0.5 µg Sld2 was incubated for 1h with mixing at 4°C in 200 µl of buffer RC3; afterwards the beads were washed three times with 100 µl of buffer RC3 and analysed by SDS/PAGE followed by Western Blot. Alternatively when untagged Sld3 was used, 0.5 µg of Sld3 protein was immobilized on anti-Sld3 318 beads (protein G) for 15 min at 24°C with mixing in 50 µl of 3A and used for pull-downs as above, together with IgG control beads.

GST-Cdc45 pull-down reactions

The pellets of 50 ml of GST and GST-Cdc45 culture were resuspended in 2.5 ml of buffer C45 (50 mM KPO₄ pH 7.5, 250 mM KCl, 5 mM MgCl₂, 1 mM MnCl₂, 10 % (w/v) glycerol, 1 % (v/v) Triton X-100, 1 mM DTT, 2 x PIC) added with 0.1 mg/ml lysozyme and incubated on ice for 30 min. The extract was sonicated and the supernatant separated by centrifugation and aliquoted. Equimolar amounts of GST and GST-Cdc45 were immobilized on anti-GST antibody beads for 1h at 4°C with mixing in 100 µl of buffer C45. Beads were washed three times with 100 µl buffer RC2. *In vitro*

translated proteins were incubated for 2h with mixing at 4°C in 500 µl of buffer RC2 before washes and analysis by SDS/PAGE and phosphorimaging.

Sld2 pull-down reactions

The pellets of 50 ml of GST and GST-Sld2 cultures were resuspended in 2.5 ml buffer 2A, added with 0.1 mg/ml of lysozyme and 25 U/ml Benzonase and incubate on ice for 30 min. The extract was sonicated, the supernatant separated by centrifugation and aliquoted. Equimolar amounts of GST and GST-Sld2 containing extracts were immobilized on anti-GST antibody beads for 1h at 4°C with mixing in 100 µl of buffer 2A followed by three washes with 100 µl of buffer RC2. *In vitro* translated proteins were incubated with GST or GST-Sld2 beads for 2h with mixing at 4°C in 500 µl of buffer RC2; afterwards the beads were washed three times with 100 µl of buffer RC3 and analysed by SDS/PAGE followed by phosphorimaging analysis. 0.5 µg of purified proteins were instead incubated for 1h with mixing at 4°C in 200 µl of buffer RC2; afterwards the beads were washed three times with 100 µl of buffer RC3 and analysed by SDS/PAGE followed by Western Blot.

Sld3 peptides pull-down reactions

60 pmol of each biotinylated peptide (3 fold excess compared to beads binding capacity) were incubated with pre-washed streptavidin beads Dynabeads MyOne T1 in Peptide binding buffer (PBS + 0.1% (v/v) Triton X-100 + 1 mM DTT + 1x PIC) over night at 4°C (1400 rpm). The morning after beads were washed with buffer RC and incubated with *in vitro* transcribed and translated proteins for 2h at 4°C (1000 rpm) in buffer RC prior to washes and analysis by SDS/PAGE followed by autoradiography.

2.2.22 Cross-linking reaction

Equimolar amount of proteins were incubated in 10 µl of buffer CL (50 mM HEPES/KOH pH 7.5, 100 mM KAc, 5 mM MgAc, 5% (w/v) glycerol, 0.1% (v/v) Triton X-100, 5 mM CaCl₂) for 10 min on ice and 5 min at 24°C, at 950 rpm. The samples were then incubated with Glutaraldehyde for 10 min at 4°C, 950 rpm. The crosslinking reaction was blocked adding 1/10 volumes (1 µl) of 1M TRIS-HCl pH 8 and incubated for 10 min at 24°C, 950 rpm. Sample were analysed by SDS-PAGE followed by silver staining or western blot.

2.2.23 Kinase assay

40 nM of each protein was incubated with increasing concentrations of DDK (4 fold dilution series starting from 20 nM) in 10 µl final of phosphorylation buffer (50 mM HEPES/KOH pH 7.5, 100 mM KGlu, 10 mM MgAc, 0.05 mM ZnAc, 5% (w/v) glycerol, 0.1% (v/v) Triton X-100, 0.1 mM ATP, 5 mM DTT, 0.1 µCi/µl γ -³²P-ATP) at 27°C for 15 min shaking at 950 rpm. The samples were analysed by SDS/PAGE followed by autoradiography.

2.2.24 *In silico* sequence analysis

Amino acid sequence alignments were produced using the T-Coffee server (Di Tommaso et al, 2011). Proteins secondary structure predictions were generated using a SAM-T08, HMM-based protein structure prediction (Karplus, 2009), whereas helical wheel projections were generated with the following online tool: <http://rslab.ucr.edu/scripts/wheel/wheel.cgi?sequence=ABCDEFGHIJKLMNOP&submit=Submit>.

Chapter 3 - Results

Protein-protein interactions during pre-RC formation

In eukaryotes, ORC/Cdc6 loads, with the help of Cdt1, the MCM2-7 helicase onto DNA (Evrin et al, 2009; Remus et al, 2009). This reaction is initiated when the ORC/Cdc6 complex recruits a Cdt1-MCM2-7 to the DNA replication origin.

The work outlined in this chapter was undertaken prior to the publication of three papers (Fernandez-Cid et al, 2013; Frigola et al, 2013; Sun et al, 2013) that described the components of the Cdt1/MCM2-7 heptamer that are required for initial ORC/Cdc6/Cdt1/MCM2-7 complex formation. Little is still known about the subunits in ORC/Cdc6 that allow binding of Cdt1/MCM2-7. Initial work by the Bell lab investigated Orc6 and Cdt1 in this process. It was found that a degron mediated depletion of Orc6 inhibits pre-RC assembly *in vivo*, while *in vitro* work showed that ORCΔ6 (ORC complex lacking Orc6) was incapable of interacting with Cdt1 and forming of an ORCΔ6/Cdc6/Cdt1/MCM2-7 complex. In addition to a direct interaction of the N-terminal (aa 1-185) and C-terminal (aa 270-435) domains of Orc6 with Cdt1, Orc6 was reported to bind directly Orc3 and Orc5 (Chen et al, 2007). Furthermore, another study from the Bell lab (Takara & Bell, 2011) showed that the C-terminus of Cdt1 (aa 500-602) binds MCM2-7, whilst the N-terminus (aa 1-272) is required to load MCM2-7 complexes on origin DNA that are competent for CMG formation. These studies suggested that an Orc6-Cdt1 interaction is key to recruit Cdt1/MCM2-7 to ORC/Cdc6 and to facilitate MCM2-7 double-hexamer formation. However two recent publications (Fernandez-Cid et al, 2013; Frigola et al, 2013) challenged this view, as they found that ORCΔ6 is sufficient to promote ORCΔ6/Cdc6/Cdt1/MCM2-7 complex formation on origin DNA. Additional analysis of the interactions involved was needed to clarify how the pre-RC forms and identify the surfaces of contact between proteins.

3.1 ORC – MCM2-7 interaction analysis

At the beginning of the study no data was available on specific protein interactions of ORC and MCM2-7. Indeed an origin bound ORC complex cannot

interact with Cdt1/MCM2-7 (Evrin et al, 2009), suggesting that ORC and MCM2-7 adopt a specific conformation that inhibits direct protein interactions. In consequence it was tested if individual purified subunits could be used for complex formation, but poor solubility of these proteins resulted in non-specific interactions (Uhle, Tognetti, Fernandez-Cid and Speck; unpublished observations). Therefore the use of *in vitro* translated ORC subunits in interactions with purified MCM2-7 was investigated first.

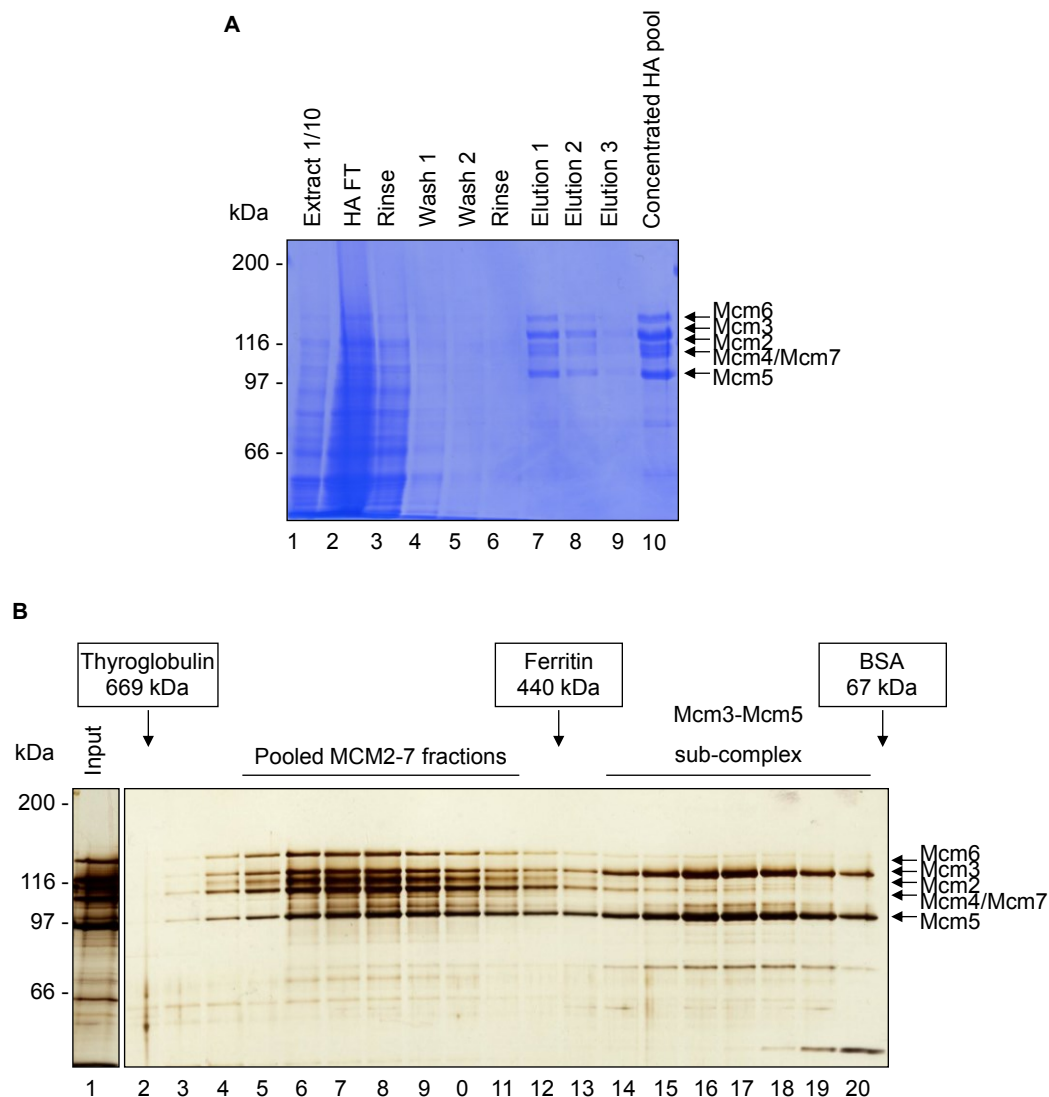


Figure 3.1: Purification of MCM2-7 from yeast

A) Coomassie blue stained gel for anti-HA purification step. Yeast extract produced via freezer milling was incubated with anti-HA beads, rinsed, washed twice and rinsed again before bound MCM2-7 (via HA-Mcm3) was eluted with HA peptide. The eluate was then concentrated. **B)** Silver stained gel showing the gel filtration fractions. The concentrated eluate was loaded into a Superdex 200 column and fractions corresponding to lanes 5-11 were pooled. The elution position of known molecular weights standards is represented in the boxes. Arrows indicate the position of the Mcm subunits.

MCM2-7 was overexpressed in budding yeast arrested in G1-phase with α factor, to ensure that it does not carry S-phase phosphorylations, using three expression plasmids each containing two Mcm subunits, including Mcm3 with a N-terminal HA tag, as previously described (Evrin et al, 2009). Mcm3 was tagged to enrich for MCM2-7 at the expense of other sub-complexes. MCM2-7 was then purified in a two-step purification process (Figure 3.1) where MCM2-7 was bound to anti-HA resin and eluted using HA peptide followed by gel filtration.

MCM2-7 was therefore used to immunoprecipitate *in vitro* transcribed and translated ORC subunits and this analysis showed that Orc2 can weakly interact with MCM2-7 (Figure 3.2).

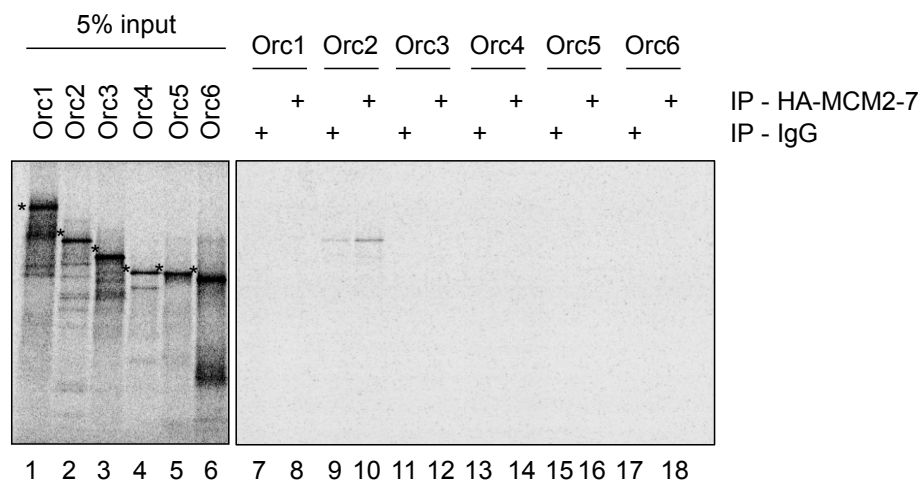


Figure 3.2: MCM2-7 interacts with Orc2

MCM2-7 interaction analysis with ORC subunits. HA-MCM2-7 immobilized on anti-HA magnetic beads or IgG control beads, were incubated with ^{35} S labelled *in vitro* transcribed translated ORC subunits, washed, separated by SDS-PAGE and analysed by autoradiography. Asterisks indicate full length proteins.

Since the Orc2 interaction was rather weak we tested Cdt1, which is known to bind MCM2-7 (Takara & Bell, 2011), as a positive control in this pull-down assay. Indeed we observed that Cdt1 interacts with MCM2-7 (Figure 3.3, lane 4), as previously reported. Instead, when Cdc6 was tested for direct binding to MCM2-7 no signal was detected (Figure 3.3, lane 7).

As the interaction of MCM2-7 with Cdc6 could be mediated by Cdt1, in this experiment the pull-down was also performed in the presence of purified Cdt1 as described paragraph 2.2.21. Purified Cdt1 competes with the *in vitro* translated Cdt1

as indicated by the reduced signal for MCM2-7 binding (Figure 3.3, compare lanes 4 and 5), indicating that the experiment works.

However, when purified Cdt1 was added into the Cdc6 pull-down assay, no increased signal was detected (Figure 3.3, compare lanes 7 and 8) indicating that MCM2-7 and Cdc6 do not interact directly nor in a Cdt1-mediated manner.

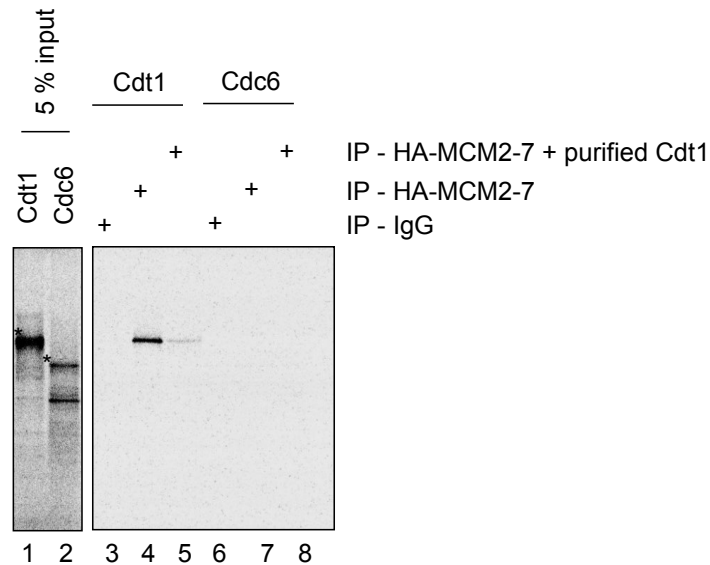


Figure 3.3: MCM2-7 interacts with Cdt1 but not with Cdc6

MCM2-7 interaction analysis with Cdt1 and Cdc6. HA-MCM2-7 immobilized on anti-HA magnetic beads or IgG control beads, were incubated with ³⁵S labelled *in vitro* transcribed translated Cdt1 and Cdc6, washed, separated by SDS-PAGE and analysed by autoradiography. For samples 5 and 8, 1 µg purified Cdt1 was added to the beads during the conjugation with HA-MCM2-7 and additionally 2 µg purified Cdt1 were added simultaneously with the *in vitro* translated mixture. Asterisks indicate full length proteins.

Based on the sequence alignment of the subunits Orc1-5 and Cdc6 (Figure 3.4) it is evident that they share a very similar structural organisation consisting of a AAA+ domain in the central portion, a N-terminal extension of variable length and a generally long C-terminal extension.

Orc6 was not included in this analysis because it is not related to the AAA+ family of ATPases (Erzberger et al, 2006; Speck et al, 2005) and therefore it does not show the same conserved elements.

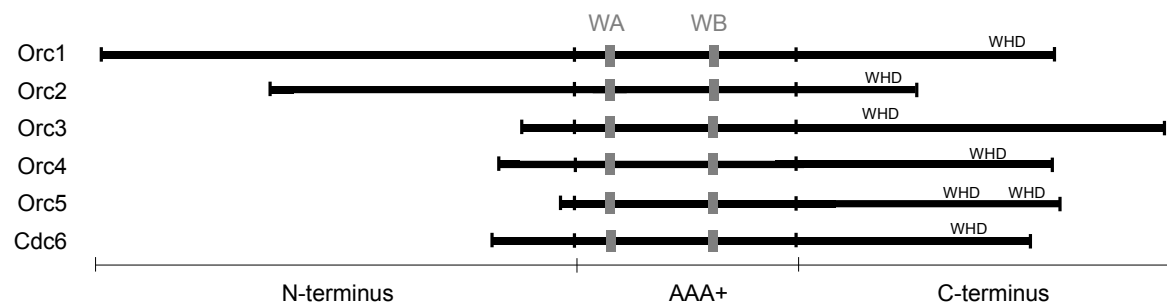


Figure 3.4: The domain architecture of the subunits Orc1-5 and Cdc6

Diagram of the structural domains based on sequence alignment. The functional elements are indicated as following: Walker A (WA), Walker B (WB) and winged-helix domain (WHD).

Given that the C-terminal extensions of Orc1-5 are of considerable length and structured in a winged-helix domain, which is known to function in protein-protein and protein-DNA interactions (Gajiwala & Burley, 2000; Teichmann et al, 2012), they became interesting in the study of the binding of ORC to MCM2-7. For this reason MCM2-7 was used to immunoprecipitate them, both in the absence and in the presence of purified Cdt1 (Figure 3.5). Their length was established according to sequence alignment and secondary structure predictions (Gardenal and Speck, personal communication). The full length Orc2 was used as positive control. The C-terminus of Cdc6 was also included in the assay despite the experiment with the full length protein showed no binding (Figure 3.3 lane 7) in order to exclude that an interaction site is present in this region but becomes exposed only under specific folding conditions. In the attempt of reducing the unspecific signal detected for the IgG control, MBP-MCM2-7 instead of HA-MCM2-7 was used in this assay. MBP-MCM2-7 presents a MBP tag internally fused to Mcm7 (Samel and Speck, unpublished) and was purified by Riera (unpublished result), similarly as described for the wild type.

None of the C-terminal extensions can interact with MCM2-7 under these conditions, not even Orc2 CT, suggesting that the interaction with the full length Orc2 (Figure 3.2, lane 10) is mediated by a different domain or that the folding of the subunit is important for the binding. Furthermore, Orc6 is immunoprecipitated by MCM2-7 in the presence of purified Cdt1 (Figure 3.5, compare lane 25 and 26), indicating that Cdt1 can bridge the interaction between these proteins. Finally, the interaction

occurring with full length Orc2 was strengthened by the addition of Cdt1 (Figure 3.5, compare lane 28 and 29).

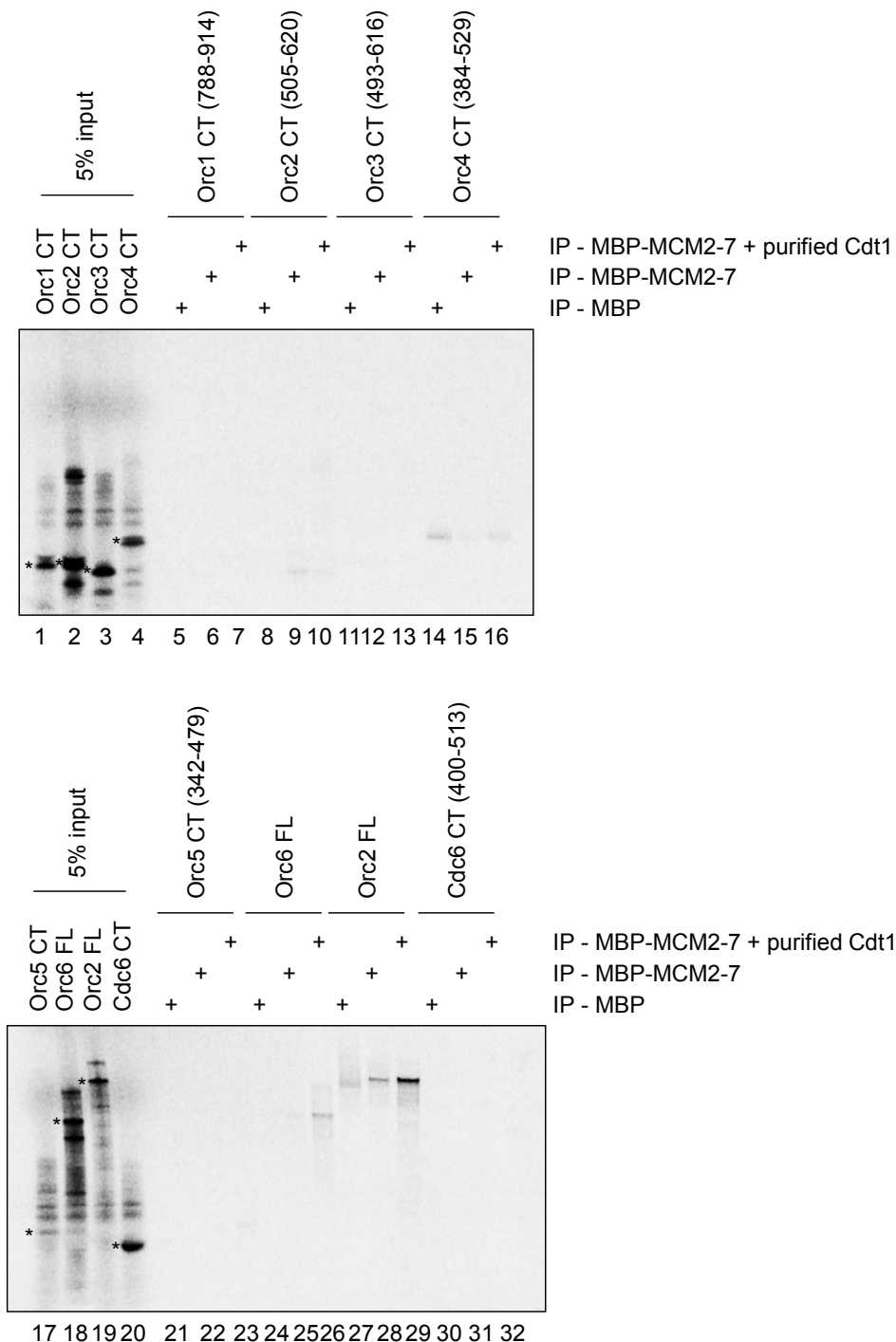


Figure 3.5: MCM2-7 interacts with Orc2 and Orc6 in the presence of Cdt1

MCM2-7 interaction analysis with Orc1-5 C-termini. MBP or MBP-MCM2-7 immobilized on anti-MBP magnetic beads, were incubated with ^{35}S labelled *in vitro* transcribed translated proteins, washed, separated by SDS-PAGE and analysed by autoradiography. For the reactions in presence of Cdt1, 1 μg purified protein was added to the beads during the conjugation of HA-MCM2-7 with the beads and additionally 2 μg purified Cdt1 were added simultaneously with the *in vitro* translated mixture. Asterisks indicate protein constructs.

MCM2-7 Δ C6 is a mutant of MCM2-7 which is defective for the Cdt1 binding domain at the C-terminus of Mcm6 (Fernandez-Cid et al, 2013). It was interesting to test how the interaction between MCM2-7 and ORC would change in the context of this mutant. For this purpose, MCM2-7 Δ C6 was used to pull-down ORC full length subunits, together with Cdt1 and Cdc6 as controls. Again, one sample of each was incubated also in the presence of Cdt1 (Figure 3.6). As this mutant was not available with an MBP-tag the HA-tagged variant was employed, which produced less specific interactions based on previous tests (data not shown).

The experiment showed similar results to the wild type MCM2-7: the only subunit of ORC that interacts is Orc2, which produces a very weak signal but becomes stronger in the presence of purified Cdt1 (Figure 3.6, compare lanes 13 and 14). This result indirectly shows that MCM2-7 Δ C6 is still capable of interacting with Cdt1, as previously reported (Fernandez-Cid et al, 2013), and indicates that the C-terminal domain of Mcm6 is not essential for the interaction with ORC.

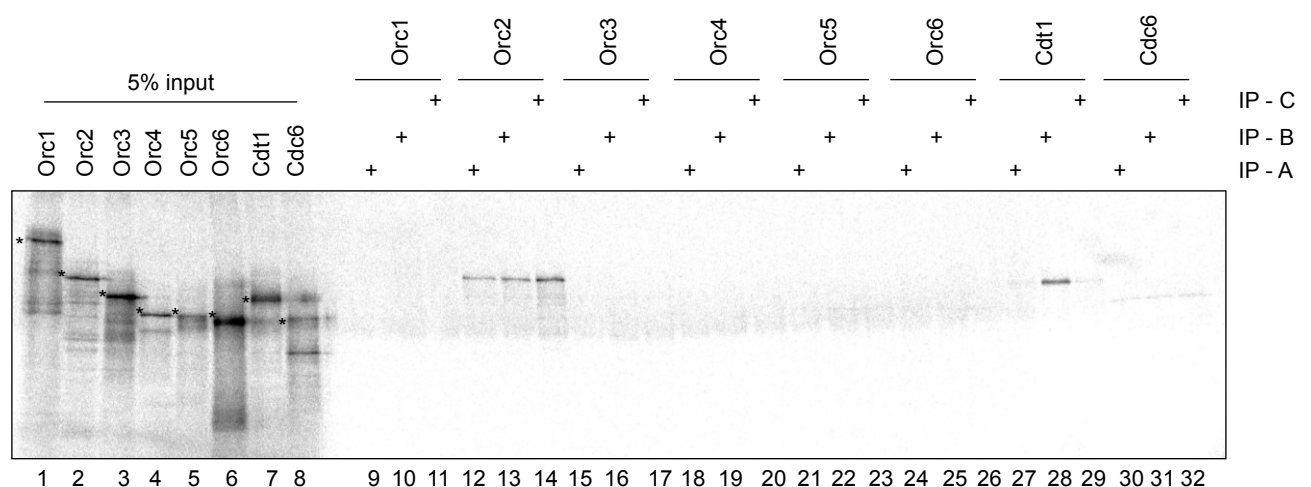


Figure 3.6: MCM2-7 Δ C6 interacts weakly with Orc2

MCM2-7 Δ C6 interaction analysis with ORC subunits. MCM2-7 Δ C6 immobilized on anti-HA magnetic beads (IP-B) or IgG control beads (IP-A), were incubated with ^{35} S labelled *in vitro* transcribed translated proteins, washed, separated by SDS-PAGE and analysed by autoradiography. For the reactions in presence of Cdt1 (IP-C), 1 μ g purified protein was added to the beads during the conjugation of HA-MCM2-7 Δ C6 with the beads and additionally 2 μ g purified Cdt1 were added simultaneously with the *in vitro* translated mixture. Asterisks indicate full length proteins.

3.2 ORC – Cdt1 interaction analysis

These results suggest that Cdt1 is contacting not only Orc6, as previously reported (Chen et al, 2007), but also Orc2, therefore it was interesting to further explore this interaction, especially as Orc6 is not required for initial ORC/Cdc6/Cdt1/MCM2-7 complex formation (Fernandez-Cid et al, 2013; Frigola et al, 2013).

Cdt1 was used to pull-down full length Orc2 and its C-terminus. The full length Orc6 was utilised as a positive control (Figure 3.7, lane 11). The experiment confirmed a direct binding only for the full length subunit of Orc2 and not for the C-terminus (Figure 3.7, lanes 1-6). Cdc6 full length and its C-terminus were also included in the experiment, in the eventuality that these two proteins could associate. Surprisingly, it was found that full length Cdc6 interacted with Cdt1 (Figure 3.7, lane 13).

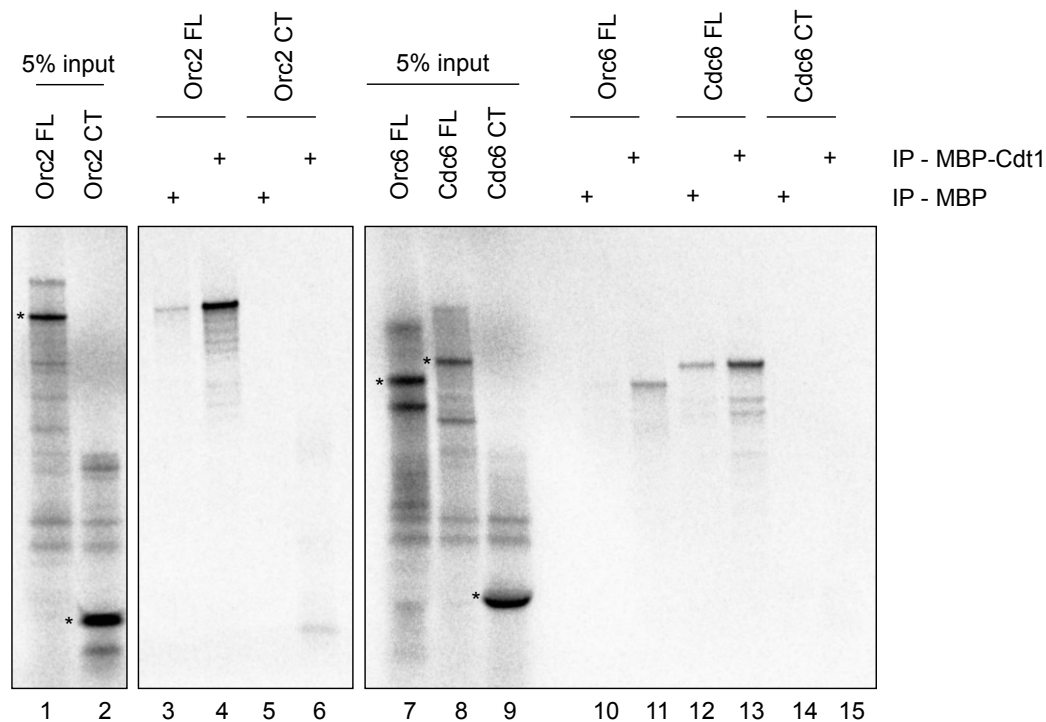
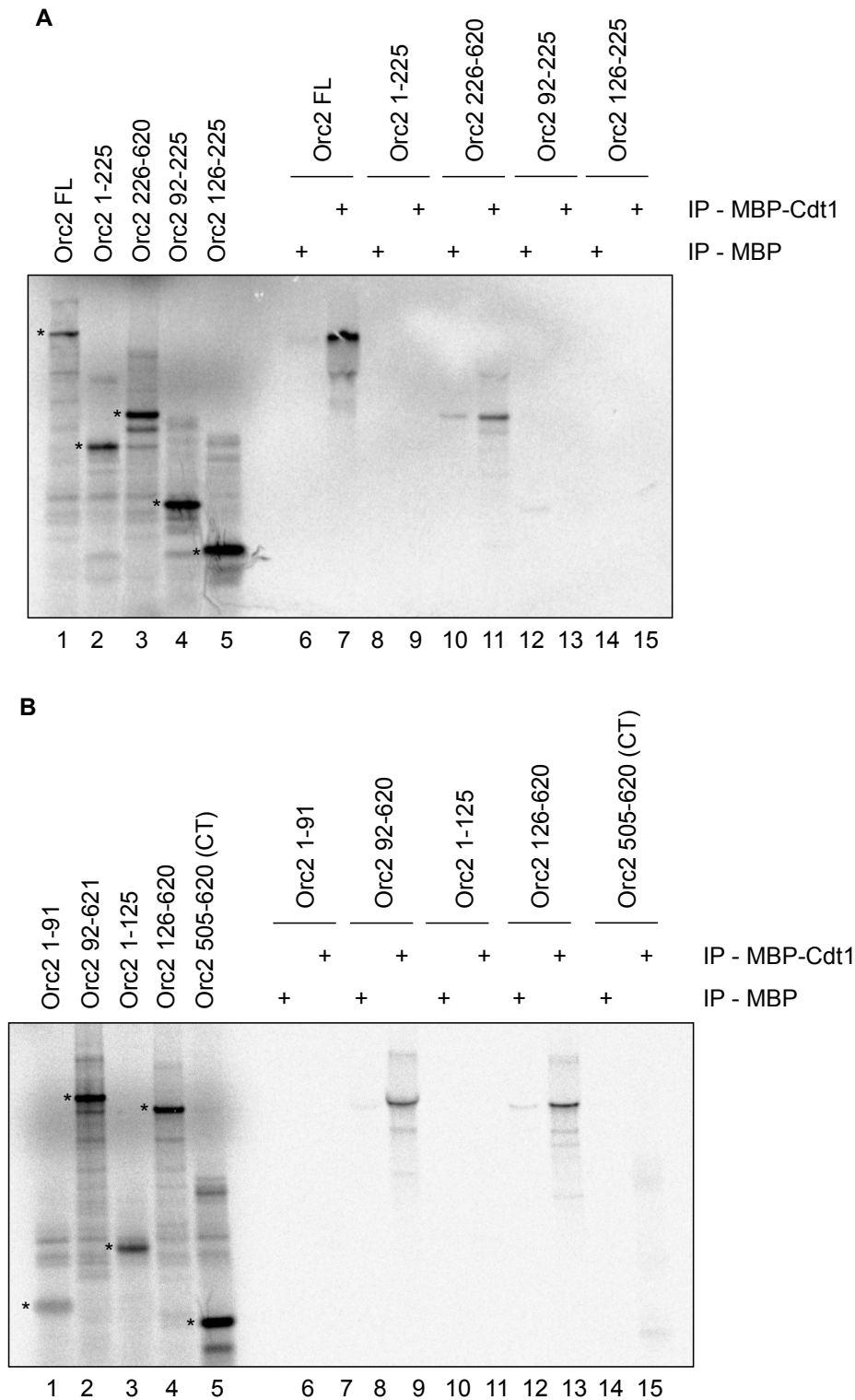


Figure 3.7: Cdt1 interacts with Orc2, Orc6 and Cdc6

Cdt1 interaction analysis with Orc2, Orc6 and Cdc6. MBP or MBP-Cdt1 immobilized on anti-MBP magnetic beads, were incubated with ³⁵S labelled *in vitro* transcribed translated proteins, washed, separated by SDS-PAGE and analysed by autoradiography. Asterisks indicate protein constructs.

To further explore the newfound interaction occurring between Cdt1 and Orc2, a series of experiments was designed to map the specific binding domain within Orc2, which could be used to produce mutants to better understand the organisation

of the pre-RC. For this purpose, several truncations of Orc2 were produced via *in vitro* transcription and translation after the analysis of the secondary structure prediction (Appendix II), in order to avoid disruption of the structural elements, and tested in Cdt1 pull-down reactions.



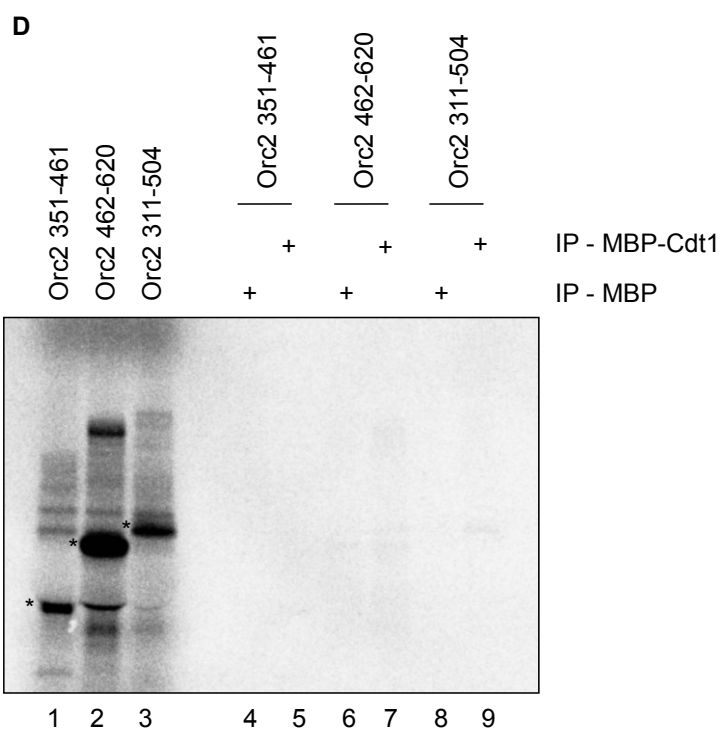
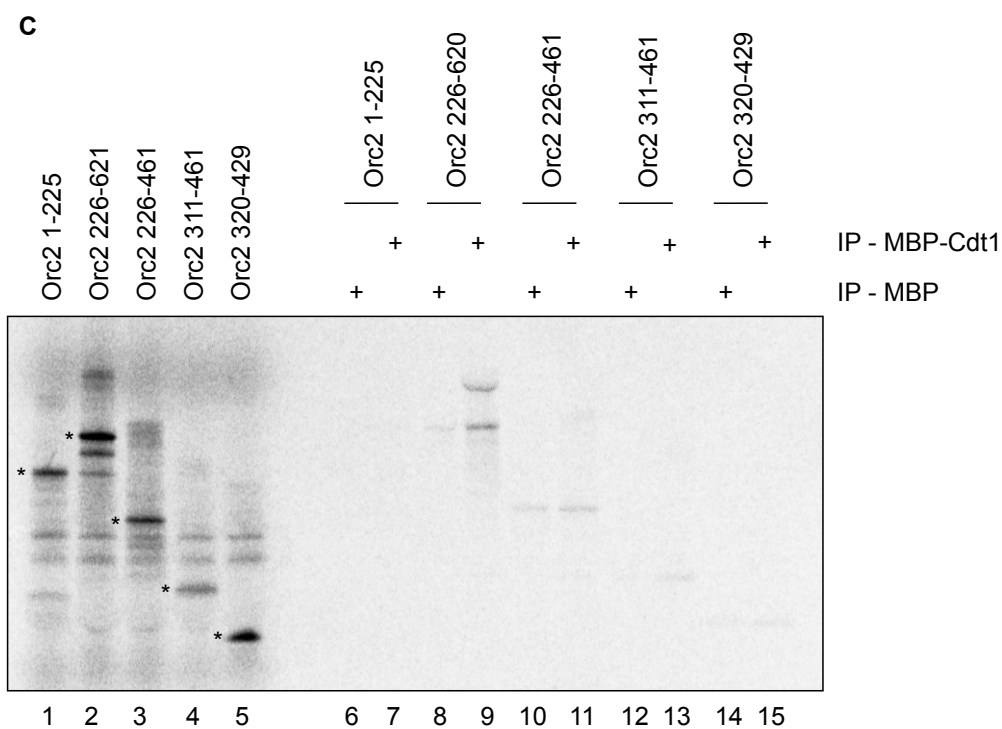


Figure 3.8: Cdt1 interaction with Orc2 truncations

MBP or MBP-Cdt1 immobilized on anti-MBP magnetic beads, were incubated with ^{35}S labelled *in vitro* transcribed translated Orc2 truncations, washed, separated by SDS-PAGE and analysed by autoradiography. Asterisks indicate protein constructs.

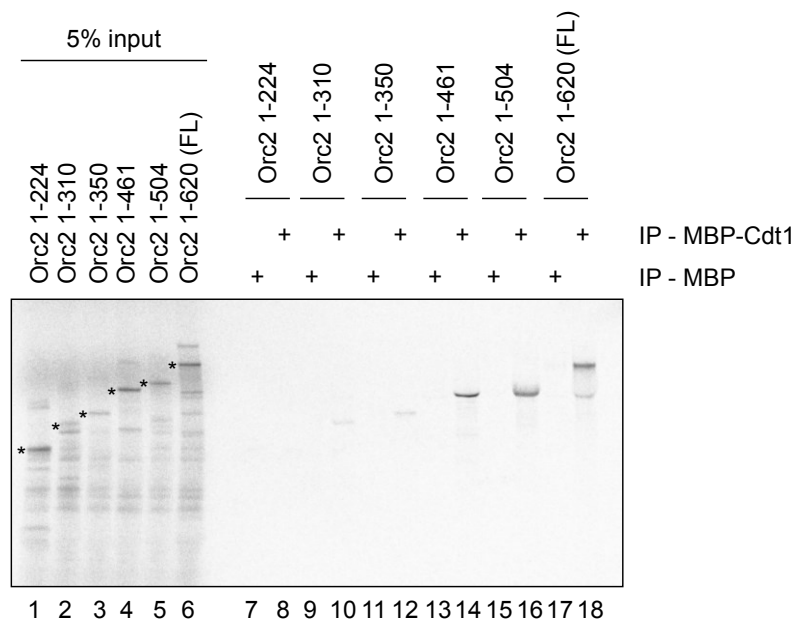


Figure 3.9: Cdt1 interaction with Orc2 N-terminal truncations

MBP or MBP-Cdt1 immobilized on anti-MBP magnetic beads, were incubated with ^{35}S labelled *in vitro* transcribed translated Orc2 N-terminal truncations, washed, separated by SDS-PAGE and analysed by autoradiography. Asterisks indicate protein constructs.

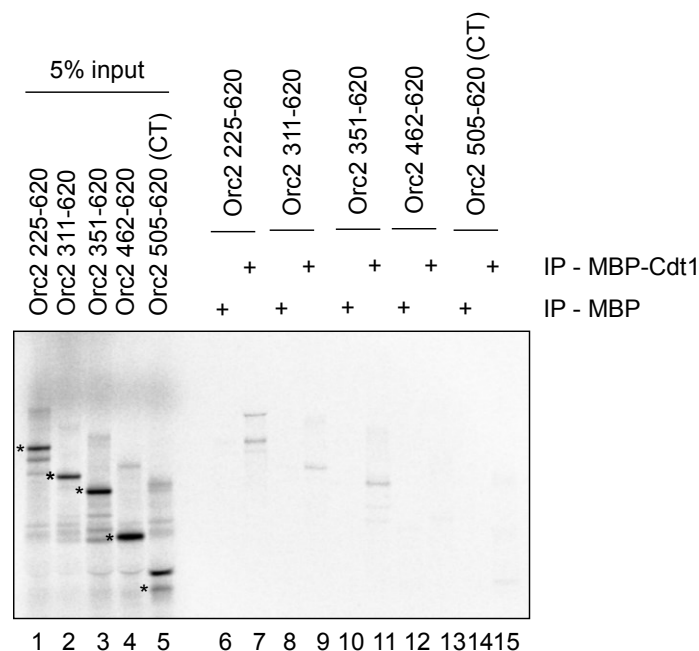


Figure 3.10: Cdt1 interaction with Orc2 C-terminal truncations

MBP or MBP-Cdt1 immobilized on anti-MBP magnetic beads, were incubated with ^{35}S labelled *in vitro* transcribed translated Orc2 C-terminal truncations, washed, separated by SDS-PAGE and analysed by autoradiography. Asterisks indicate protein constructs.

These experiments showed that only the long fragments with the very N-terminal portion deleted are still capable of binding Cdt1 (Figure 3.8 panel A lane 11,

panel B lanes 9 and 13, panel C lane 9), therefore a fine N-terminal (Figure 3.9) and C-terminal (Figure 3.10) mapping was performed under the same conditions. This would allow us to identify the minimal region required for the interaction, without completely disrupting the structure of the truncated fragments.

Finally, a scheme that summarises all the detected interactions is shown in Figure 3.11.

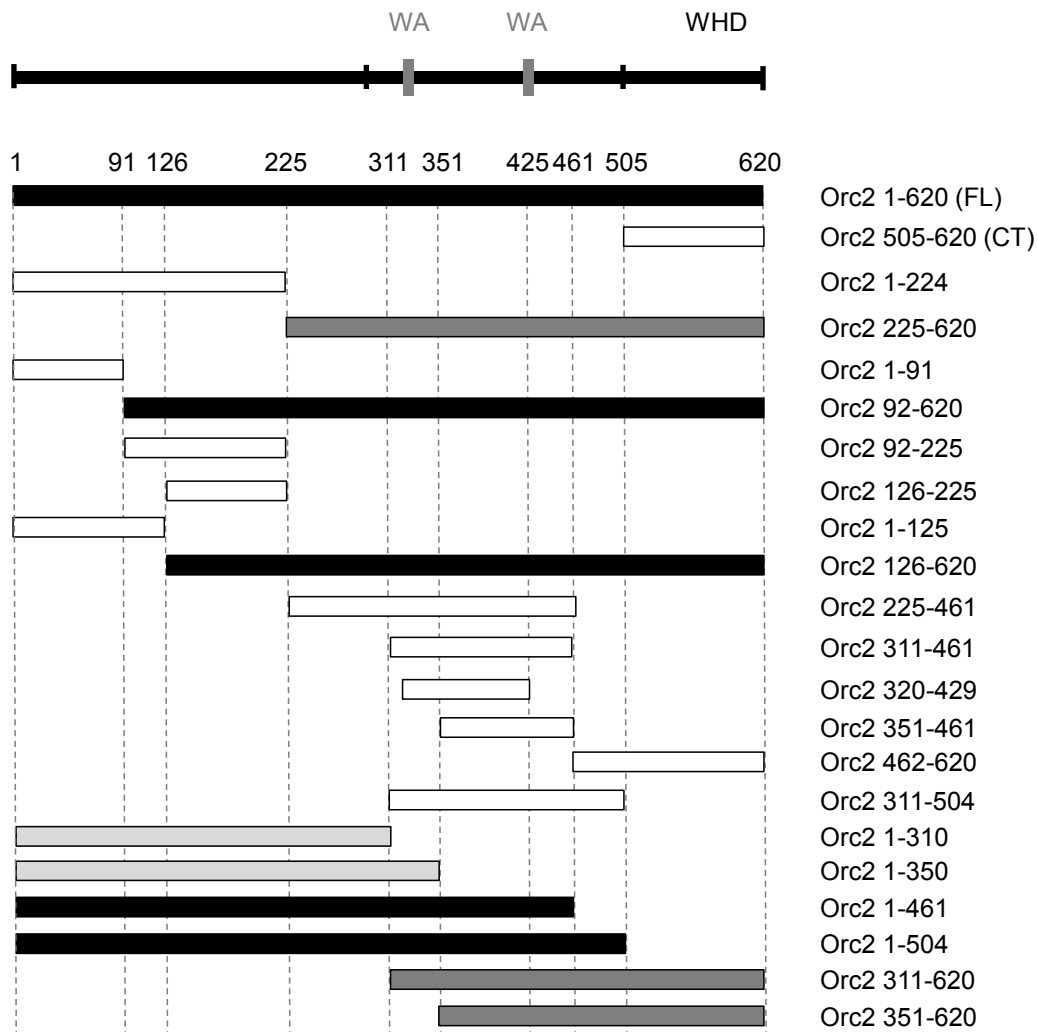


Figure 3.11: Summary of Cdt1 interaction with Orc2 truncations

The interactions showed in Figures 3.7 to 3.9 are summarised and distinguished based on the % of binding where grey stands for poor and black for good. The functional elements are indicated as following: Walker A (WA), Walker B (WB) and winged-helix domain (WHD).

The results show that no single motif in Orc2 is sufficient for the interaction with Cdt1. Instead it was found that the interaction is independent of the very N-terminal and C-terminal portions of Orc2. Any further shortening leads to a loss of binding. That could mean that a central region is required for binding or folding. To

explore this further, the fragment (Orc2 aa 126-620) could be shortened from the C-terminus and the fragment (Orc2 aa 1-461) could be shortened from the N-terminus.

3.3 Discussion

When this project started, very little was known on the interactions occurring during pre-RC formation and it was unclear how the ORC/Cdc6 complex, formed onto replicative origins, is contacting MCM2-7/Cdt1. The only details available were that Orc6 is crucial for the recruitment of Cdt1 (Chen et al, 2007) and that the C-terminus of Cdt1 binds MCM2-7 whilst the N-terminus is required to load pre-RC complexes that are competent for CMG formation (Takara & Bell, 2011). An impressive amount of work and publications led to an improved understanding of the pre-RC formation process (Fernandez-Cid et al, 2013; Frigola et al, 2013; Sun et al, 2013). These findings will be discussed in this paragraph together with the new results presented in this chapter.

During this study ORC and MCM2-7 were analysed at first. The isolated ORC subunits were used instead of the full length protein to maximise the chance of detecting an interaction, given that it seems plausible that ORC assumes a specific conformation upon DNA binding which could allow MCM2-7 binding. The use of *in vitro* transcribed and translated proteins was an important technical break-through, as it allowed the detection of specific interactions in a rapid process. This approach could lead to false positive results, exposing domains that are generally buried in the structure of the full length protein, but it is still a valuable tool for initial screening of interactions.

The pull-down proved to work, revealing the known interaction of MCM2-7 with Cdt1 (Figure 3.2, (Takara & Bell, 2011)). The full length MCM2-7 appears to interact exclusively with the Orc2 subunit (Figure 3.2), but it is also possible that the binding site is produced by the folding of more subunits other than Orc2. The interaction between MCM2-7 and Cdc6 was also analysed (Figure 3.3), but no binding was detected. This could suggest that the two proteins do not directly contact each other, or more likely that MCM2-7 has to be in a different conformation which could be achieved after a first contact with the DNA or ORC. The interaction would be then hindered by a change in MCM2-7 structure, similarly as observed for Cdc6 interacting with Mcm3 (Sun et al, 2013). Surely Cdt1 does not sufficiently alter the

structure of MCM2-7, as the pull-down of Cdc6 with Cdt1/MCM2-7 did not give any signal (Figure 3.3, lanes 7-8).

At the time of analysis it appeared reasonable to investigate the involvement of the C-terminal extensions of Orc1-5 and Cdc6 (Figure 3.4) in the binding to MCM2-7, as these represented one potential interaction surface. Sun and colleagues proved the hypothesis wrong; they indicated that the C-terminal part of the ORC subunits contact DNA, but are distal to MCM2-7 (Sun et al, 2013). The data on the interaction of MCM2-7 with Orc1-5 and Cdc6 C-terminal domains shows indeed no binding, both in absence and presence of Cdt1 (Figure 3.5). Interestingly, a signal was detected for the full length Orc6 in the presence of Cdt1, suggesting that these two proteins do interact as previously reported (Chen et al, 2007) and that Cdt1 could bridge an interaction with MCM2-7. However, in contrast to the initial report, Orc6 is required for OCM, but not OCCM, formation (Fernandez-Cid et al, 2013).

The relation between MCM2-7 and ORC was further analysed in the context of the MCM2-7 Δ C6 mutant, which has a defective Cdt1 binding domain (Takara & Bell, 2011), showing no significant difference compared with the wild type. The increased signal for the interaction of MCM2-7 Δ C6 with Orc2 in the presence of Cdt1 (Figure 3.6, compare lanes 13-14) is consistent with previous reports where it was shown that the mutant is still capable of interacting with Cdt1 and indicating that MCM2-7 has more than one binding site for Cdt1 (Fernandez-Cid et al, 2013).

The surface of contact between MCM2-7 and ORC surely deserves to be better investigated and the work of Sun and colleagues (Sun et al, 2013) provides some important hints on the direction to follow. In the current study though the interaction ORC-Cdt1 appeared more promising, therefore a series of Cdt1 pull-downs was performed. As already expected from MCM2-7 interacting with Orc2 stronger in the presence of Cdt1 (Figure 3.5, compare lanes 38 and 39), a direct interaction analysis confirmed the binding of Cdt1 to the full length Orc2 (Figure 3.7, lane 4), and consistently reproduced the literature for Cdt1 with Orc6 interaction (Asano et al, 2007; Chen et al, 2007). Surprisingly the assay also showed an interaction between Cdt1 and Cdc6 which has not been observed in before (Figure 3.7, lane 13) and will need further exploration.

An extensive number of different Orc2 truncations was examined for binding to Cdt1 (Figures 3.7 to 3.10, summarised in 3.11), indicating that the putative binding site must be in the central portion of Orc2, in the AAA+ region, with a minimum fragment

comprising amino acids 225-461. Unfortunately Cdt1 was not capable of pulling down this fragment, suggesting that the structural context is important as well.

Overall the data presented in this chapter provides some interesting contributions to the understanding of the pre-RC formation process.

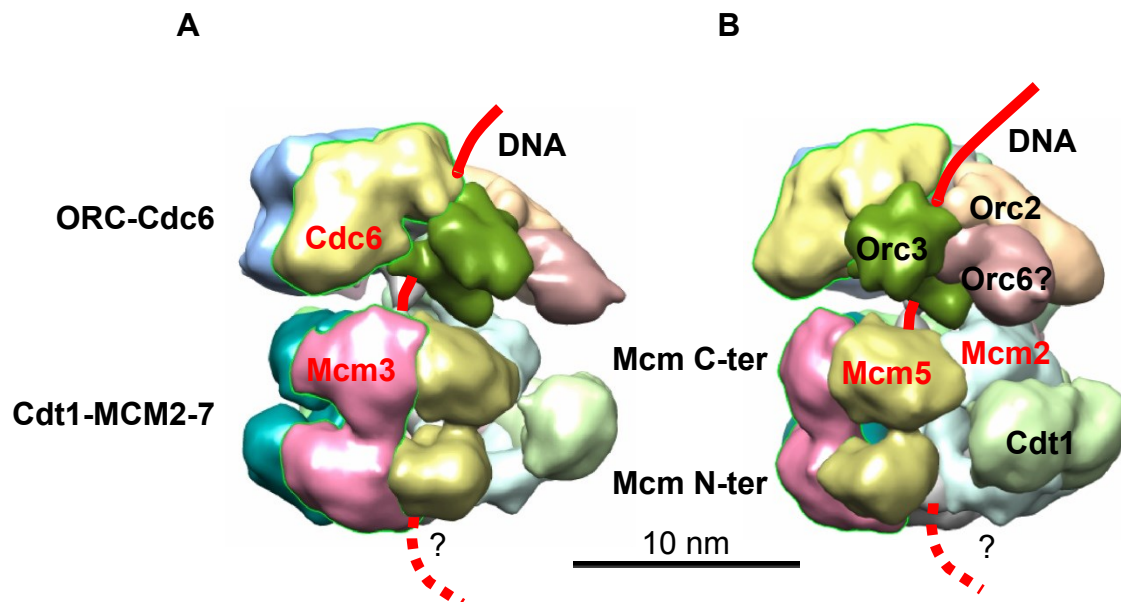


Figure 3.12: Cryo EM structure of the pre-RC complex

A) Proximity of Cdc6 and Mcm3, but no direct interaction. DNA appears loaded. **B)** Close up of the putative Mcm2/Mcm5 gate that could function for DNA entry. Adapted from Riera et al, 2013.

From the structure proposed by Sun and colleagues (Sun et al, 2013) and presented in Figure 3.12, it appears possible that the surface of contact between MCM2-7 and ORC involves Orc2, and also that Cdt1 and Orc2 can interact with each other, but that this interaction is potentially more important for initial ORC/Cdc6/Cdt1/MCM2-7 complex formation.

It is more difficult to find an explanation for the newfound interaction of Cdt1 with Cdc6, because the two proteins seem to be located far away from one other, at least in the structure of the OCCM. It is still possible to speculate that this interaction could find an importance during the recruitment of the second MCM2-7 hexamer for the formation of the double-hexamer. Additional studies are required to better understand the complicated and dynamic structure of the pre-RC complex.

Chapter 4 – Results

Analysis of the interaction of Sld3 with the pre-RC

The transition between pre-RC, which presents a loaded but inactive form of MCM2-7 helicase, and the pre-IC, a complex competent for initiation of replication, is believed to result in the formation of the CMG, where Cdc45 and GINS are recruited to MCM2-7 to activate the helicase (Costa et al, 2011; Ilves et al, 2010).

How the CMG is formed has been under analysis for several years and it is known that Cdc45 associates with origins before GINS, already in G1-phase, with the same timing as Sld3 (Kamimura et al, 2001).

Sld3 is an essential protein in yeast, it is a limiting factor present with only 300 copies/cell (Tanaka et al, 2011b) and it is required for Cdc45 recruitment to origins (Kamimura et al, 2001; Kanemaki & Labib, 2006). Its S-CDK dependent phosphorylation generates a binding site for Dpb11 and their interaction is essential for initiation of replication (Tanaka et al, 2007b; Zegerman & Diffley, 2007), but it is believed to be a recruiting factor since it does not move with the replicative fork (Kanemaki & Labib, 2006). Furthermore during DNA damage, a Rad53 mediated phosphorylation of Sld3 and Dbf4 blocks CMG formation (Lopez-Mosqueda et al, 2010; Zegerman & Diffley, 2007), thus origin firing is blocked until the damage has been repaired.

For all these reasons, Sld3 appears to be one of the most important factors during initiation of DNA replication and therefore its interaction with the pre-RC will be analysed in detail in this chapter.

4.1 Analysis of Sld3 recruitment to the pre-RC

Kamimura and colleagues showed that, *in vivo*, Sld3 and Cdc45 associate simultaneously with replication origin in G1-phase and with late-firing origins in late S-phase (Kamimura et al, 2001). A temperature sensitive mutant of Cdc45 was used to analyse the association of Sld3 with origins in chromatin immunoprecipitation assays, finding a reduction compared to the wild type strain, suggesting that Sld3 requires Cdc45 for stable recruitment. In fission yeast the recruitment of Sld3 to

origins does not depend on Cdc45 (Yamada et al, 2004). This indicates that Sld3 is potentially one of the first proteins binding to the pre-RC, which was tested in this project.

Sld3 was purified from budding yeast using a method developed in the group (Herrera, Tognetti et al, under revision). An MBP-PS-Myc-Sld3 construct was overexpressed in yeast from a galactose-inducible promoter, the culture was harvested and disruption with a cryogenic method prior to binding to amylose resin. Bound MBP-Myc-Sld3 was rinsed, washed and eluted with PreScission protease, whose recognition site was added between the MBP and Myc tags. The eluted material was incubated with SP sepharose to concentrate it and remove the protease (Figure 4.1) and eluted using 0.5 and 1 M NaCl. For simplicity, from now on MBP-PS-Myc-Sld3 will be called MBP-Sld3 whereas Myc-Sld3 just Sld3 because of the small size of the tag (203 Da). All purified Sld3 used in this work is Myc-Sld3.

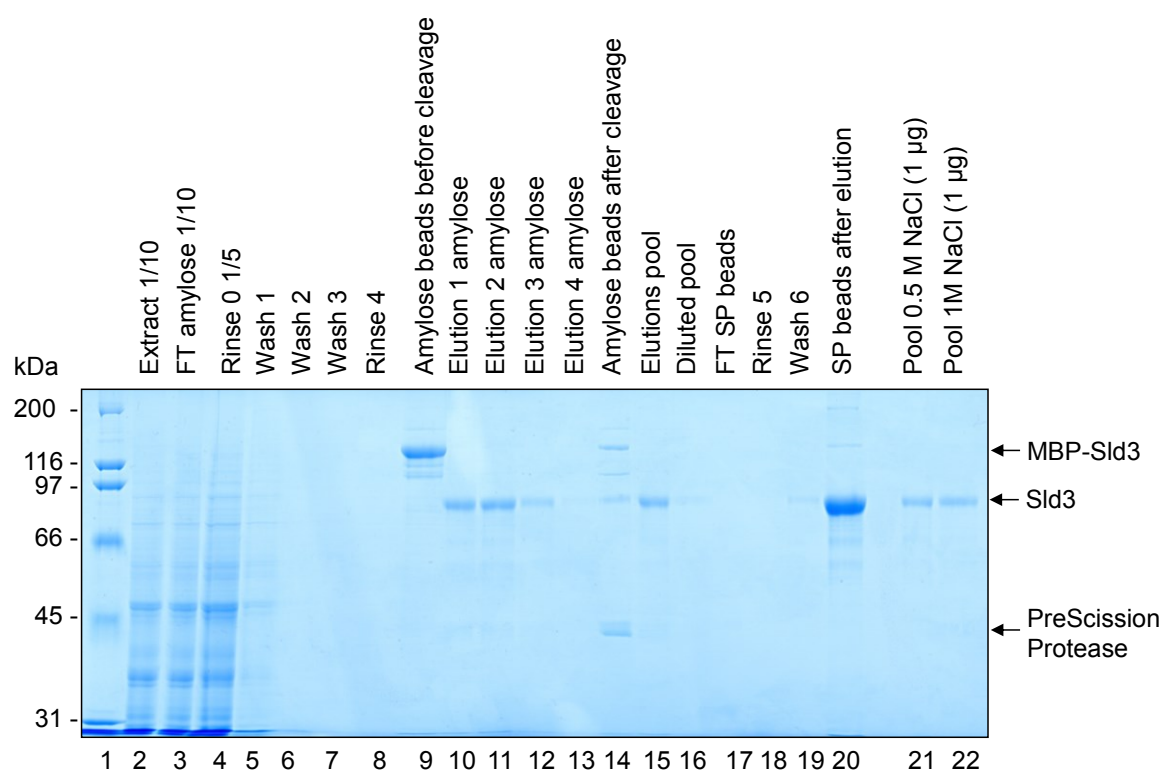


Figure 4.1: Purification of Sld3 from yeast

Coomassie stained gel showing the purification of Sld3 from yeast overexpressing MBP-PS-Myc-Sld3. Cells were lysed by freezer milling and the extract incubated with amylose resin. Bound MBP-Sld3 was washed three times and Sld3 eluted after cleavage with PreScission protease. The elutions were pooled, diluted to decrease the salt concentration and incubated with SP sepharose beads. Sld3 was then eluted using elution buffer containing 0.5 or 1 M NaCl. The relevant fractions determined by Bradford were pooled (lanes 21 and 22). Arrows indicate the position of MBP-Sld3 (120.3 kDa), Sld3 (77.3 kDa) and PreScission protease (46 kDa).

The pre-RC was initially reconstituted by incubating the purified pre-RC proteins with biotinylated origin DNA linked to streptavidin beads, as previously described (Evrin et al, 2009), the unbound proteins were then washed away and Sld3 added to the sample in a second step. The experiment was unsuccessful because of the high non-specific binding of Sld3 to the streptavidin beads used in the assay. A series of variations was performed in order to improve the amount of Sld3 available for the interaction with the pre-RC and a summary is presented in Table 4.1. Unfortunately none of these changes resulted in a sufficient decrease of the unspecific binding of Sld3 to the beads.

Parameter changed	Conditions tested	Result
Beads	Different streptavidin beads (Dynabeads, Magnalink, Nanolink, PEGylated) and neutravidin beads.	PEGylated streptavidin beads appeared promising
Beads blocking prior to pre-RC reconstitution	20 mg/ml BSA or milk	20 mg/ml milk improved
Beads blocking time	1 h or overnight	Overnight improved
Salt concentration washes	100, 200, 300 mM KGlu	No improvement
Additional proteins	DDK, Cdc45, MCM10, RPA	No improvement
Proteins ratio	Different Sld3/Cdc45 ratios	No improvement
Different Sld3	Sld3 purified from bacteria	No improvement

Table 4.1: Optimisation of the recruitment of Sld3 to the reconstituted pre-RC

Summary of parameters tested during the optimisation. Starting condition was pre-RC reconstituted as described in Evrin et al, 2009, added with 40 nM Sld3 for 15 min at 27°C before washes.

One of the unsuccessful experiments is presented in Figure 4.2. In this case the recruitment of Sld3 was tested in the presence of Cdc45 and DDK in the bead based assay. No Sld3 was detected bound to the Pre-RC in any of the conditions (Figure 4.2, panel A) but a very strong signal was instead sensed when the beads were analysed (Figure 4.2, panel B), suggesting that Sld3 is interacting non-specifically with the solid matrix. Cdc45 was behaving in a similar manner, despite a weak, non stoichiometric signal was detected as pre-RC bound (Figure 4.2, panel A), especially in the presence of Sld3 and DDK.

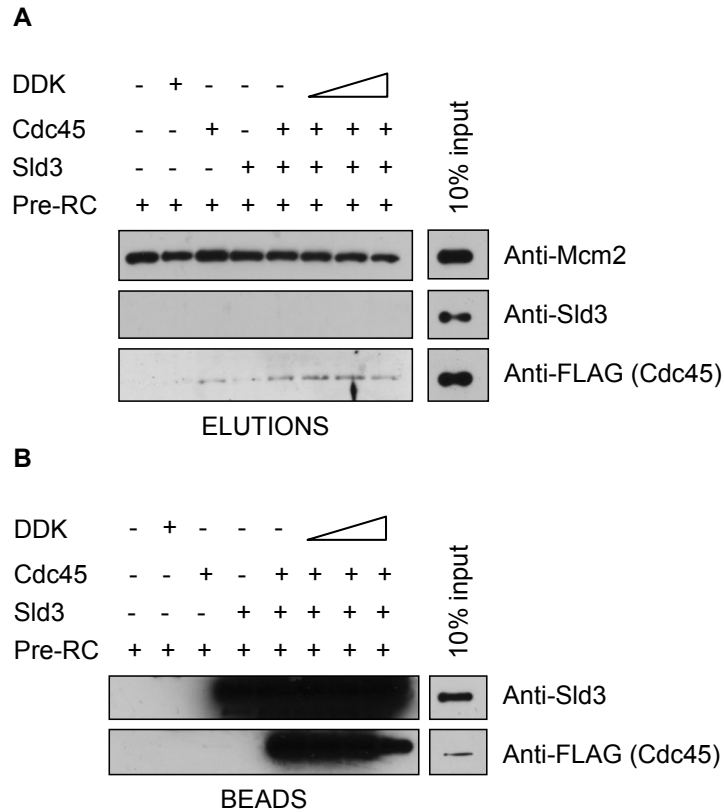


Figure 4.2: Bead based assay for Sld3 recruitment to the pre-RC

Analysis of the recruitment of Sld3 to the pre-RC reconstituted as previously described (Evrin et al, 2009). 40 nM Sld3, 40 nM Cdc45 and a 3 fold dilution (6-20-60 nM) DDK were added for 15 min at 27°C to the pre-RC prior to salt wash and elution with DNase I. The samples were analysed by SDS-PAGE followed by Western Blotting using specific antibodies against Mcm2, Sld3 and FLAG (Cdc45).

A novel method was developed in the lab for the reconstitution of the pre-RC in solution, followed by gel filtration (Herrera, Tognetti et al, under revision). This method, in contrast to the method involving a solid matrix(DNA coupled to streptavidin coated magnetic beads) which previously depleted the proteins from solution, allowed a successful analysis. In this assay, the pre-RC was assembled using a one-step reaction. Fifty microliter reactions containing 40 nM ORC, 40 nM Cdc6, 40 nM Cdt1 and 40 nM MCM2-7 were pre-incubated for 10 min on ice and 10 min at 24°C. 6 nM pUC19-ARS1 plasmid and 1.5 U of human topoisomerase I were added to the reaction and incubated for 15 min at 24°C 950 rpm. Afterwards the additional components were added (in this case Sld3 to a final concentration of 40 nM Sld3), together with 5 mM ATP, 250 ng/μl BSA, 1.4 μM competitor DNA (40 bp). Then the reactions were incubated for the required time at 27°C 950 rpm and finally

KAc was added to a final concentration of 200 mM followed by a 3 min incubation at 24°C and mixing at 950 rpm. Samples were loaded on top of a spin column, filled with 0.5 ml of resin (Sephacryl S400 HR, GE). After loading of the samples the columns were spun for 5 min at 1000 g. Prior to the gel-filtration step the spin columns were filled with equilibrated resin and pre-spun for 5 min at 850 g. The eluate was concentrated by speed-vac prior to SDS-PAGE analysis.

The pre-RC was therefore reconstituted with this method and is shown in Figure 4.3 (panel A, lane 4). In Figure 4.3, lane 2, the DNA was left out, whereas in lane 3 the pre-RC proteins (ORC, Cdc6, Cdt1 and MCM2-7) were not added into the reaction, showing that the signal detected in lane 4 is DNA dependent and corresponds to the reconstituted complex. The salt wash performed in this assay removes most ORC, Cdc6, Cdt1 and associated MCM2-7 leaving on DNA only loaded MCM2-7 complexes. This system was afterwards used to test Sld3 interaction with the pre-RC.

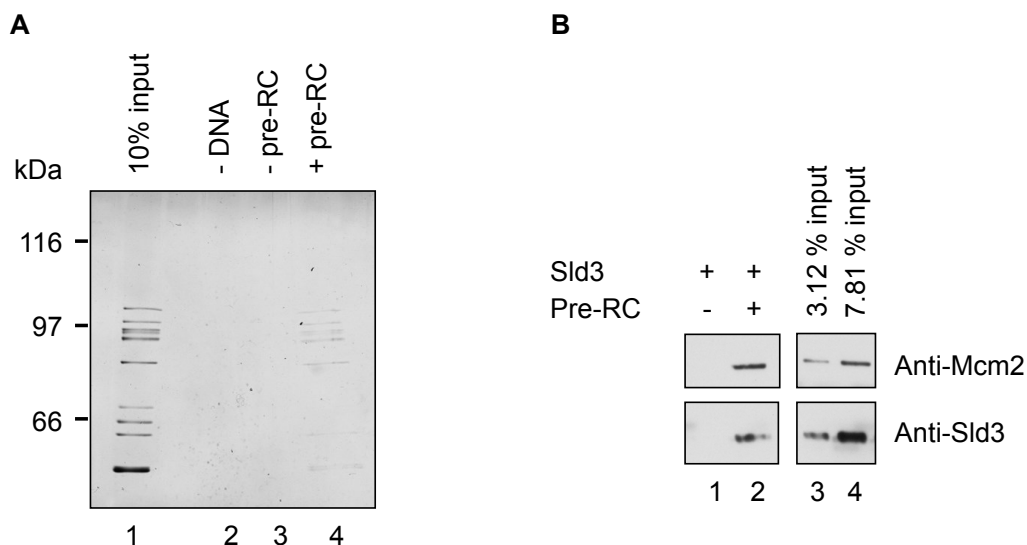


Figure 4.3: Sld3 recruitment to the pre-RC

A) Silver stained gel showing the reconstitution of the pre-RC using the gel filtration method. **B)** Analysis of the recruitment of Sld3 to the pre-RC reconstituted as in panel A. 40 nM Sld3 was added for 1 min at 27°C to the pre-RC reconstituted in solution prior to salt wash and gel filtration. After gel filtration, the samples were analysed by SDS-PAGE followed by Western Blotting using specific antibodies against Mcm2 and Sld3.

To analyse the recruitment of Sld3, 40 nM Sld3 was added to the pre-RC reconstituted in solution for 1 min at 27°C prior to salt wash and gel filtration. Under

these conditions, Sld3 was capable of binding to the DNA in a pre-RC dependent manner. In the absence of additional proteins a nearly stoichiometric complex between MCM2-7 and Sld3 was observed (Figure 4.3, panel B, lane 2).

4.2 Analysis of direct interaction between Sld3 and MCM2-7

Knowing that Sld3 and Cdc45 interact with one another (Kamimura et al, 2001), Cdc45 associates with MCM2-7 to form the CMG (Costa et al, 2011; Ilves et al, 2010) and Sld3/Cdc45/MCM2-7 can form a ternary complex *in vitro* (Bruck & Kaplan, 2011a), it was hypothesized that Sld3 directly binds to MCM2-7.

MBP-MCM2-7 was used for the interaction analysis because it performs in immunoprecipitations better than the wild type protein, as described in chapter 3. The interaction between purified Sld3 and MCM2-7 was tested first (Figure 4.4), showing a specific binding of Sld3 to MBP-MCM2-7, but not to the MBP control.

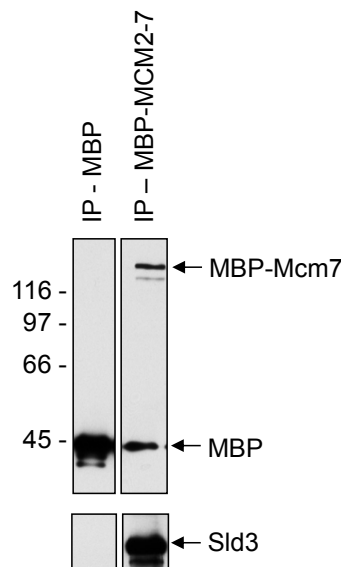


Figure 4.4: Direct interaction of Sld3 with MCM2-7

MCM2-7 interaction analysis with Sld3. MBP or MBP-MCM2-7 immobilized on anti-MBP magnetic beads, were incubated with 0.5 µg purified Sld3, washed, separated by SDS-PAGE and analysed by western blot against MBP (upper) or Sld3 (lower). The western blot probed against MBP in the immunoprecipitation for MBP-MCM2-7 reveals, together with a band corresponding to MBP-Mcm7 (137 kDa), one of the size of MBP (42.5 kDa) possibly due to C-terminal degradation of the protein.

One possible surface on MCM2-7 for interaction with Sld3 was hypothesised to be at the N-termini, where the MCM2-7 subunits contain non-conserved extensions of various lengths (Figure 4.5, in red).

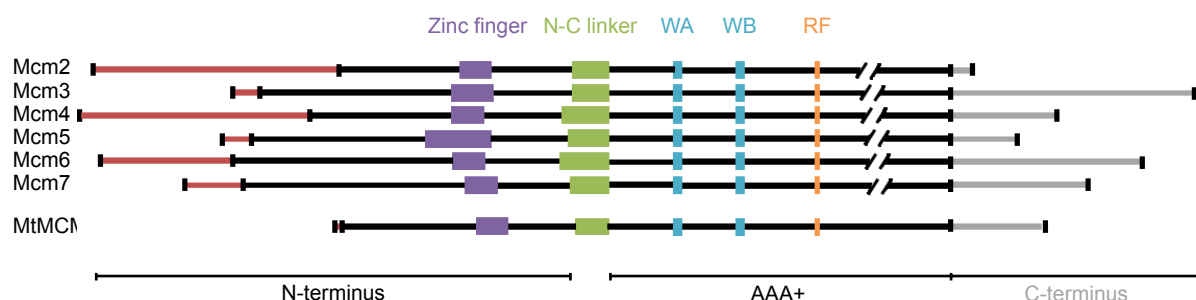


Figure 4.5: The domain architecture of the subunits of MCM2-7

Diagram of the structural domains based on sequence alignment. Archaeal MCM (MtMCM) is included. The functional elements are indicated as following: Walker A (WA), Walker B (WB) and Arginine Finger (RF).

A structure of the MCM2-7 hexamer comprising the non-conserved N-termini has yet to be defined, but these regions are believed to be flexible and therefore suitable for protein-protein interactions.

Before investing time into the cloning and isolation of tagged MCM2-7 N-termini, a test was performed with controls for Sld3 binding to check if the experimental controls could work. Dpb11, a known interacting partner of Sld3 (Zegerman & Diffley, 2007), was chosen as a positive control. *In vivo* though Sld3 and Dpb11 interact only after CDK dependent phosphorylation of Sld3, therefore their interaction *in vitro* needed to be tested. Regα was instead used as a negative control, being an unrelated protein involved in proteasome activation.

These proteins, GST-tagged at their N-terminus, were conjugated to Glutathione beads (Figure 4.5, panel E) and used in a pull-down assay with Sld3, alone or in combination with CDK, DDK or both (Figure 4.5, panel F). CDK is known to phosphorylate Sld3 and thus allows its binding to Dpb11 (Zegerman & Diffley, 2007); Whether DDK affects this interaction has not been tested, but as DDK is important for the transition of the pre-RC into pre-IC it was interesting to analyse (Heller et al, 2011). Initially, CDK was overexpressed in insect cells as HA-Clb5/Cdc28 (Fernandez-Cid et al, 2013), bound to anti-HA resin and eluted using HA peptide (Figure 4.6, panel A). The activity of CDK was then verified using Cdc6 as substrate for phosphorylation (Chen & Bell, 2011). Upon addition of increasing concentrations of CDK, a clear shift of Cdc6 is visible (Figure 4.6, panel B, lane 3 onwards), indicating that the kinase is active.

DDK was instead overexpressed in yeast as MBP-PS-Dbf4/Cdc7 from a galactose-inducible promoter. MBP-Dbf4/Cdc7 was bound to amylose resin, rinsed, washed and eluted with PreScission protease to cleave off the MBP tag. The eluted material was incubated with Glutathione resin to trap the GST-tagged protease and finally pooled and concentrated (Figure 4.6, panel C). DDK was then examined for its ability to phosphorylate loaded MCM2-7 (Kihara et al, 2000; On et al, 2014; Randell et al, 2010; Sheu & Stillman, 2010; Weinreich & Stillman, 1999), showing a clear shift of Mcm6 at the concentration tested (Figure 4.6, panel D).

In the absence of kinases, Sld3 was able to bind Dpb11 (Figure 4.5, panel F lane 3), suggesting that, either Sld3 is already phosphorylated by CDK when purified from asynchronous cells, or that Sld3 and Dpb11 can interact *in vitro* in the absence of modifications. Regα showed no interaction (Figure 4.5, panel F lane 2) as expected. The addition of CDK successfully resulted in a phosphoshift of Sld3 in the input (Figure 4.5, panel F, compare lane 4 to 1) and did not alter the binding of Sld3 to Dpb11 (Figure 4.5, panel F lane 6), whereas DDK slightly weakened the interaction (Figure 4.5, panel F lane 9).

Figure 4.6: Test of Sld3 interaction with controls and kinases purification and testing

A) CDK was overexpressed in insect cells as HA-Clb5/Cdc28, bound to anti-HA resin and eluted with HA peptide **B)** CDK activity test on Cdc6. 1.3 μ M of Cdc6 was incubated with increasing concentrations of CDK **C)** MBP-PS-Dbf4/Cdc7 was bound to amylose resin, rinsed, washed and eluted with PreScission protease. The eluted material was incubated with Glutathione resin, pooled and concentrated **D)** DDK activity test: reconstituted pre-RC was high salt washed to produce loaded MCM2-7 and incubated with 40 nM DDK for 15 min at 27°C. **E)** The *E. coli* extracts overexpressing GST-Reg α (54.7 kDa) and GST-Dpb11 (113 kDa) were incubated with Glutathione beads, washed, analysed on a SDS-PAGE gel together with 20% input of Sld3 and stained with Coomassie Brilliant Blue. They represent the loading controls for the immunoprecipitations in panel B. **F)** Samples prepared as described in panel A were incubated with 1 μ g of purified Sld3 alone or pre incubated with 20 nM CDK (lanes 4, 5, 6) or 100 nM DDK (lanes 7, 8, 9). The samples were analysed by SDS-PAGE followed by Western Blotting using a specific antibody against Sld3. The Ponceau-S stained membrane shows equal loading of GST-Dpb11 and GST-Reg α on Glutathione beads.

To analyse the interaction of Sld3 with the MCM2-7 N-termini, each N-terminal region preceding the Zinc finger (see Figure 4.5) was fused at its C-terminus with GST tag and overexpressed in bacteria. A range of different buffers, pH, and salt concentrations were analysed to elucidate a good condition for binding to Glutathione resin and the purification. Finally, a normalisation of the protein amount was performed, which is shown in Figure 4.7, panel A. The MCM2-7 N-termini are highly negatively charged therefore they separate in SDS-PAGE gel not at the expected molecular weight but significantly higher. The identity of the proteins was verified by Mass Spectrometry.

The Glutathione beads presenting the GST-fused N-termini, together with Dpb11 and Reg α as controls, normalised for the amount of protein bound, were then used to pull-down purified Sld3 (Figure 4.7, panel B).

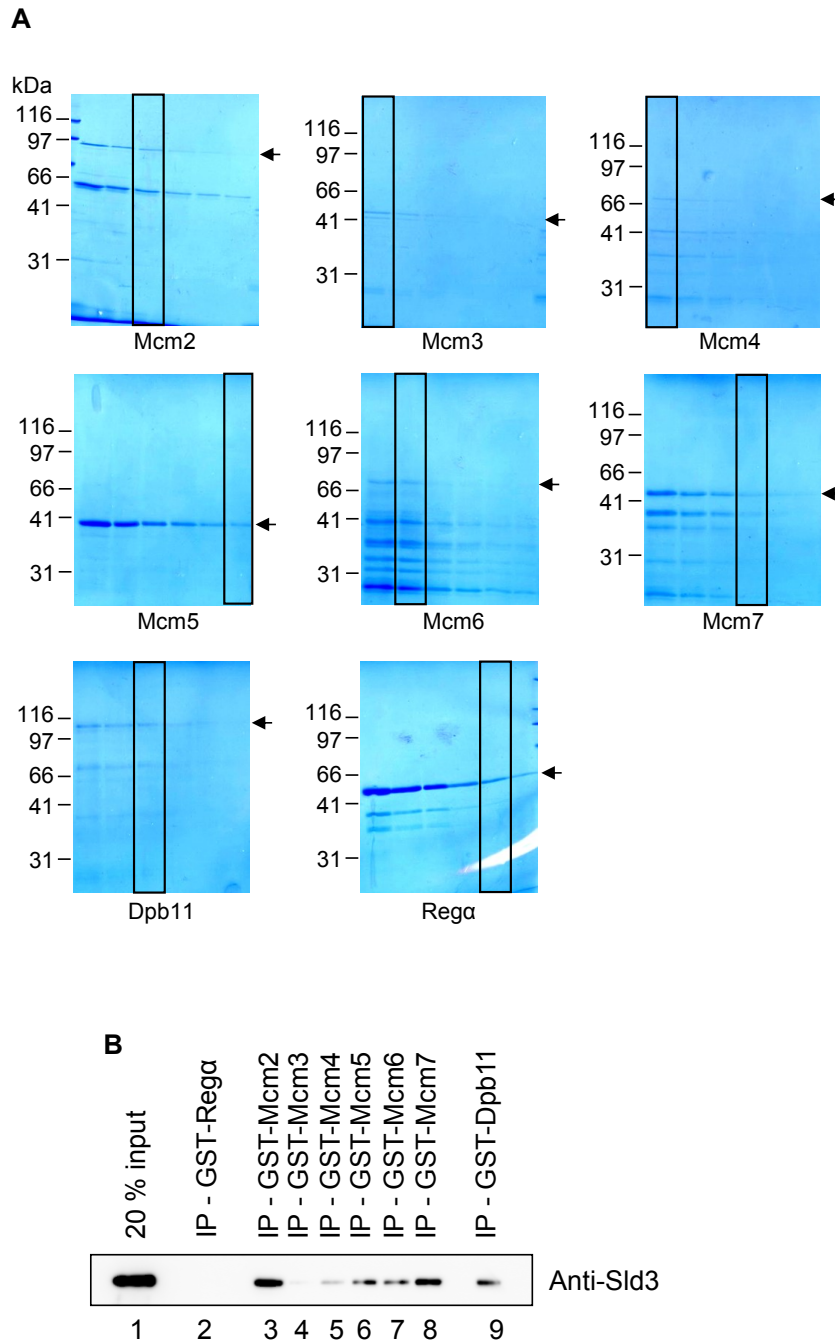


Figure 4.7: Interaction of Sld3 with GST-tagged MCM2-7 N-termini

A) The *E. coli* extracts overexpressing GST-tagged MCM2-7 N-termini, GST-Rega and GST-Dpb11 were bound to Glutathione beads, washed, analysed on a SDS-PAGE gel and stained with Coomassie Brilliant Blue. Dilutions that represent equal protein amount, and are used in panel B, are marked with black rectangles. All dilutions were run in the same gel and stained together. The identity of the proteins was verified by Mass Spectrometry; they are indicated with arrows. Molecular weights of the domains fused to GST: Mcm2 (aa 1-322) 65 kDa, Mcm3 (aa 1-192) 48 kDa, Mcm4 (aa 1-340) 65 kDa, Mcm5 (aa 1-174) 47 kDa, Mcm6 (aa 1-302) 60 kDa, Mcm7 (aa 1-253) 56 kDa, Dpb11 113 kDa, Rega 54.7 kDa. **B)** The samples indicated in panel A were incubated with 1 μ g of Sld3, washed and analysed by SDS-PAGE followed by Western Blotting using a specific antibody against Sld3.

The pull-down confirmed an interaction between Sld3 and Dpb11 (Figure 4.7, panel B lane 9) and none to Regα (lane 2), consistent with what was previously observed. When the GST-tagged MCM2-7 N-termini were used, instead, Sld3 was immunoprecipitated by Mcm2, Mcm7 and to a lesser extent by Mcm5 and Mcm6 (Figure 4.7, panel B).

Several optimisations were performed in order to decrease the non-specific interactions. The variations included a bigger volume and extended time for the washes, increased salt concentrations or addition of 0.1 mg/ml BSA to the buffer used for the washes. None of these conditions appeared to improve the quality of the assay as presented in Figure 4.7, panel B, in a consistent way. It was concluded that the difficulty in handling Glutathione agarose (see paragraph 4.6 for Discussion) could significantly affect the reproducibility of the experiment and, for this reason, it was decided to use a different approach to study this interaction, similarly to what is described in Chapter 3. MBP-Sld3 was therefore employed to immunoprecipitate *in vitro* transcribed and translated MCM2-7 subunits. In this case, the binding of the same protein (MBP-Sld3) to magnetic beads significantly reduces the variability among samples.

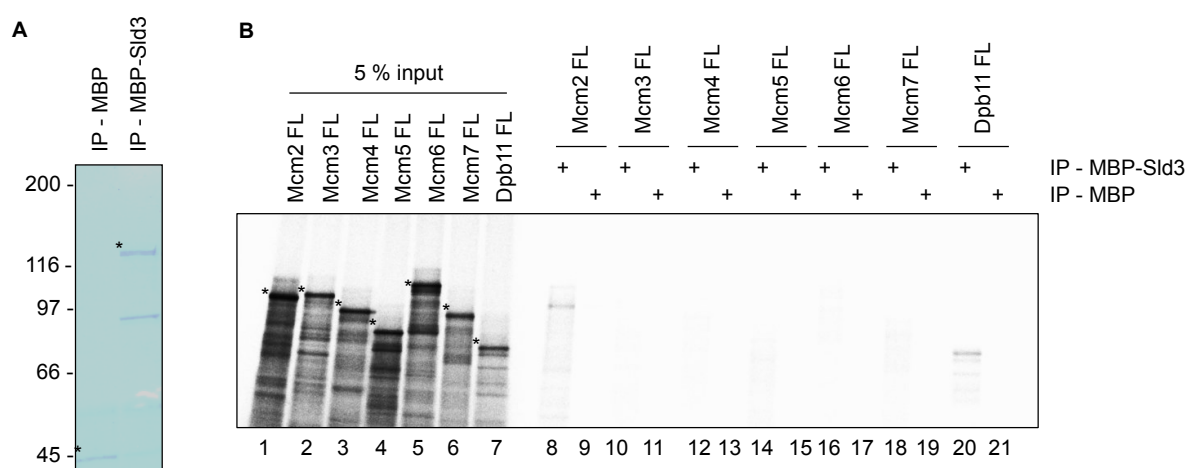


Figure 4.8: Sld3 interaction analysis with MCM2-7 subunits.

A) Coomassie stained gel showing MBP (42 kDa) and MBP-Sld3 (120.3 kDa) immobilized on anti-MBP magnetic beads. **B)** MBP or MBP-Sld3 immobilized on anti-MBP magnetic beads were incubated with ³⁵S labelled *in vitro* transcribed translated proteins, washed, separated by SDS-PAGE and analysed by autoradiography. Asterisks indicate full length proteins.

MBP-Sld3 was purified as described for Sld3 (Figure 4.1), with the exception that the protein was eluted with maltose instead of MBP cleaved with PreScission Protease. It was immobilised on anti-MBP beads and used to precipitate the full length subunits of MCM2-7. Once again, Dpb11 was used as a positive control while purified MBP was used as negative control during the immunoprecipitation assays (Figure 4.8).

The experiment confirmed the interaction of Sld3 with Dpb11 (Figure 4.8, lane 20) and also showed a specific interaction with the Mcm2 subunit of MCM2-7 (Figure 4.8, lane 8), consistent with the preliminary result obtained from the GST pull-downs of the N-termini (Figure 4.8, lane 3), but with the added advantage that the background was reduced.

Given that Mcm2 appeared as the only subunit of MCM2-7 to interact with Sld3 in this assay, it was decided to focus attention on this subunit. To confirm that the interaction involves the N-terminal domain of Mcm2, three truncations were produced after the analysis of the secondary structure prediction and tested in the pull-down with Sld3 (Figure 4.9).

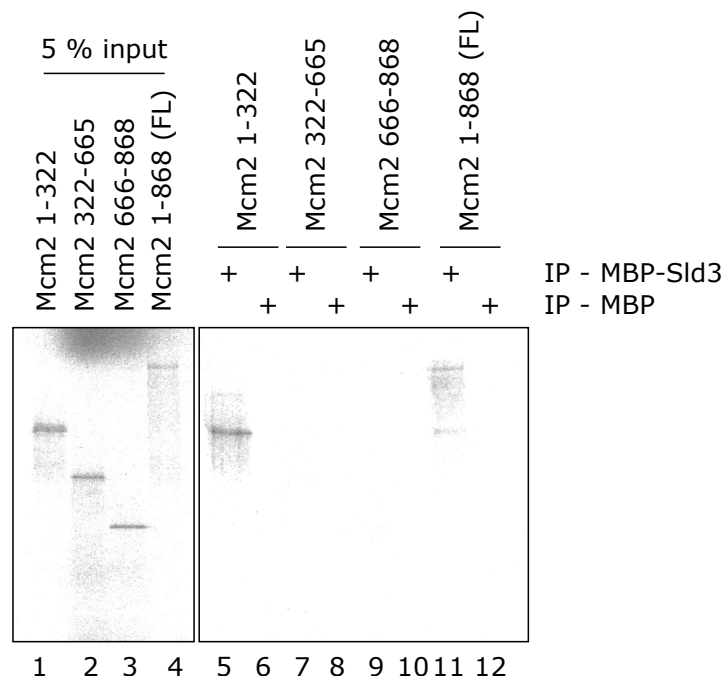


Figure 4.9: Sld3 interacts with the N-terminal of Mcm2

MBP or MBP-Sld3 immobilized on anti-MBP magnetic beads were incubated with ³⁵S labelled *in vitro* transcribed translated truncations of Mcm2, washed, separated by SDS-PAGE and analysed by autoradiography.

The prediction (Appendix II) would suggest the position of helices and sheets therefore the truncation could be produced in order to minimise disruption of these elements. The experiment confirmed the binding between MBP-Sld3 and Mcm2 full length (Figure 4.9, lane 11), as well as with the N-terminus of Mcm2 (Figure 4.9, lane 5). The central and C-terminal domains of Mcm2 were instead not precipitated by MBP-Sld3 (Figure 4.9, lanes 7 and 9 respectively).

MBP-Sld3 appears to interact with the N-terminal region of Mcm2 therefore it was interesting to map the minimal region of binding. A series of N-terminal and C-terminal truncations of the region of interest were produced and tested in the assay, while Mcm2 full length and aa 1-322 were used as positive controls.

Interestingly the fragment corresponding to aa 1-200 does not interact with MBP-Sld3 (Figure 4.10, panel A lane 11; panel B lane 12), whereas all other fragments tested are still immunoprecipitated. All C-terminal truncations, excluding 1-200, and all N-terminal truncations analysed can bind MBP-Sld3, suggesting that the minimal binding site is comprised between the amino acids 200-250 of Mcm2.

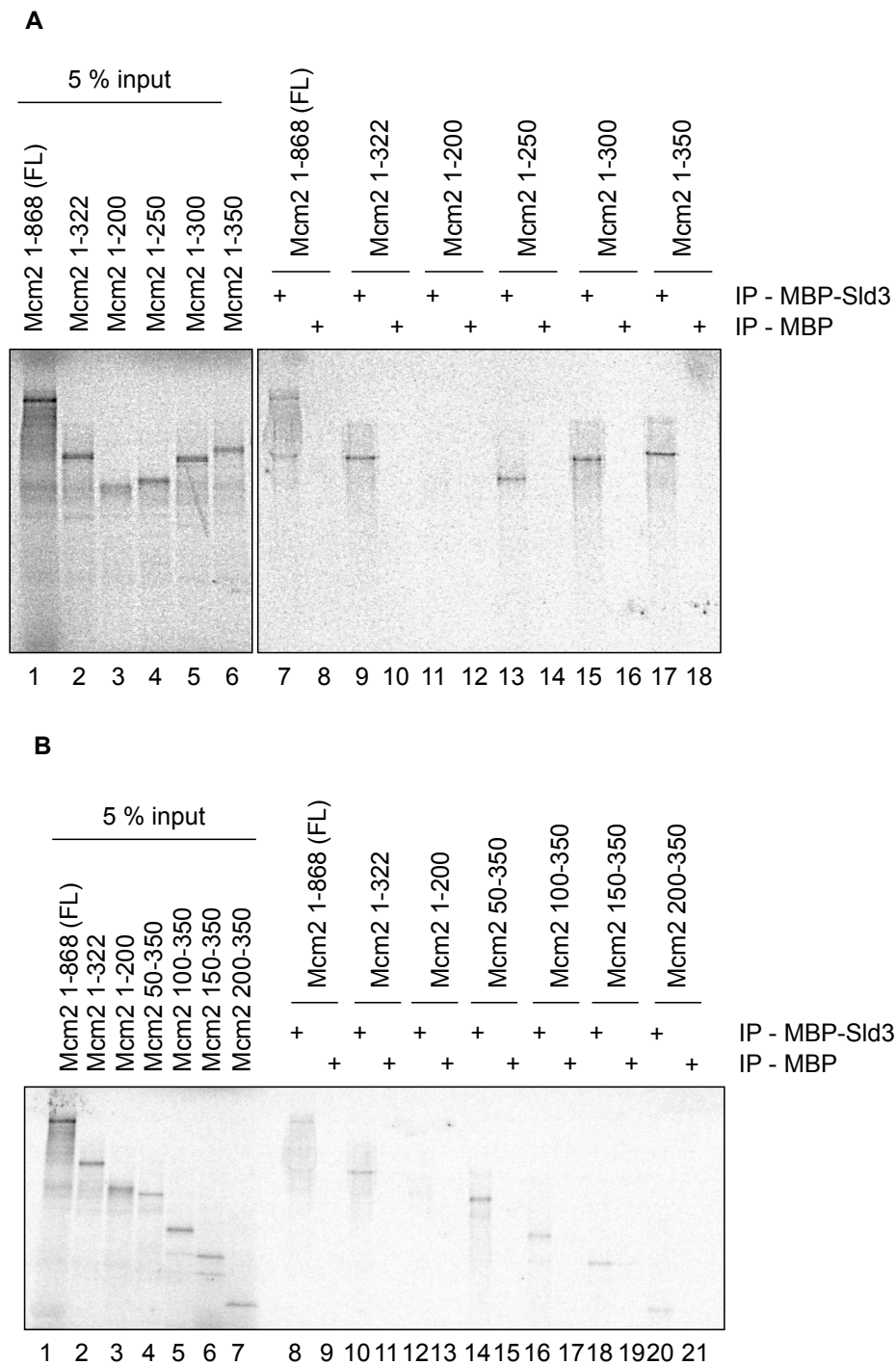


Figure 4.10: Sld3 interaction with Mcm2 truncations

MBP or MBP-Sld3 immobilized on anti-MBP magnetic beads were incubated with ^{35}S labelled *in vitro* transcribed translated truncations of Mcm2, washed, separated by SDS-PAGE and analysed by autoradiography.

In order to further characterise this potentially crucial region, the sequence comprised between amino acids 190-290 was submitted to the Quark server (Xu & Zhang, 2012) to predict the structure and then refined with ModRefiner (Xu & Zhang, 2011) (Riera, personal communication).

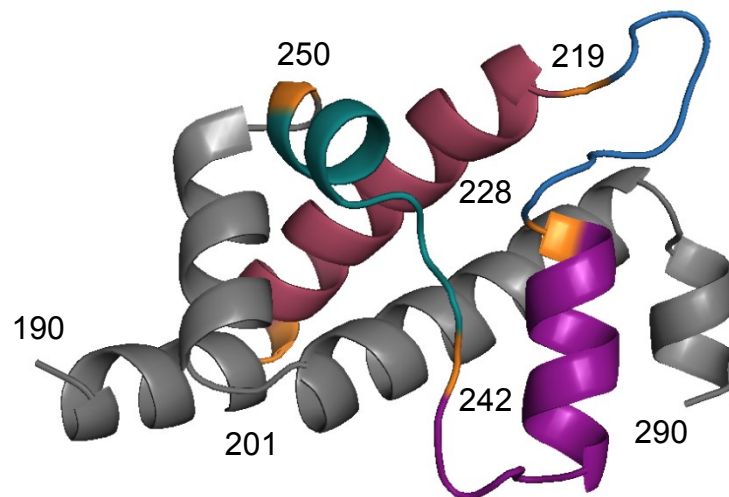


Figure 4.11: 3D structure prediction of amino acids 190-290 of Mcm2

The sequence comprised between amino acids 190-290 was submitted to the Quark server to predict the structure and then refined with ModRefiner.

In the prediction shown in Figure 4.11, several helices can be identified and this information was used to produce additional truncations in the region between aa 200-250. The logic was to avoid the disruption of the structure, and therefore affect the interaction with Sld3. These truncations were then tested in an immunoprecipitation assay with MBP-Sld3 (Figure 4.12).

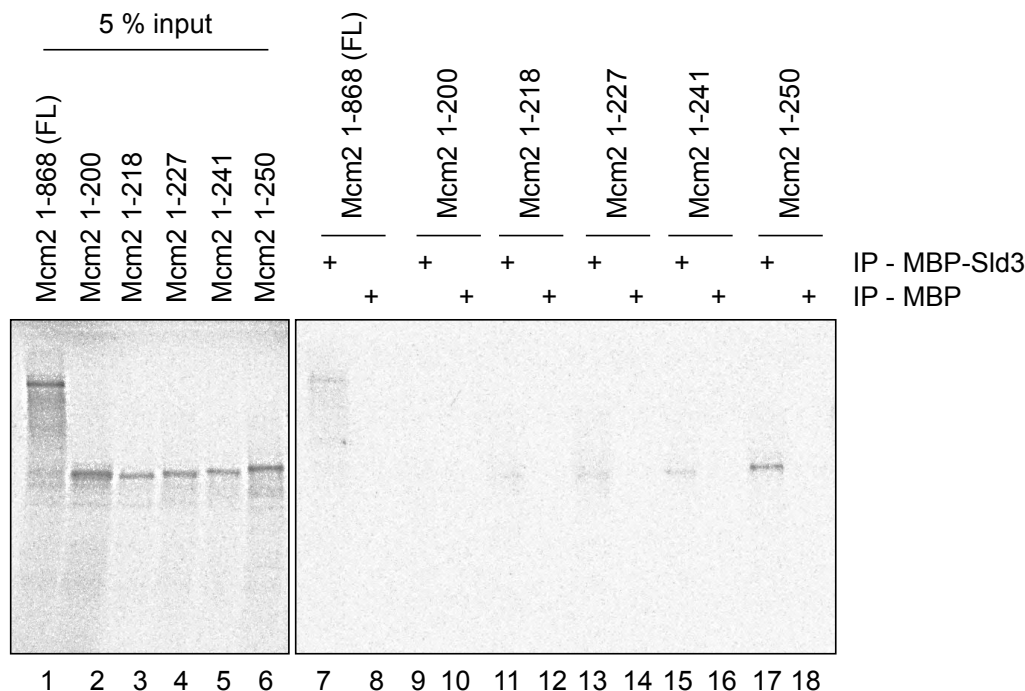


Figure 4.12: Fine mapping of the binding site on Mcm2 for Sld3

MBP or MBP-Sld3 immobilized on anti-MBP magnetic beads were incubated with ^{35}S labelled *in vitro* transcribed translated truncations of Mcm2, washed, separated by SDS-PAGE and analysed by autoradiography.

Mcm2 full length and 1-250 were used as positive controls, while Mcm2 1-200 was used as negative control. From this experiment it appears that the fragment 1-218 is already capable of interaction but the 1-250 is otherwise required to achieve a stronger binding. This suggests that a binding site is contained in the region 200-218 and another in 241-250 or that a defined structure is required for this interaction.

4.3 Analysis of Mcm2 N-terminal deletion mutants

In order to prove that the region 1-250 of Mcm2 is indeed responsible for the interaction of MCM2-7 with MBP-Sld3, a deletion mutant including these amino acids was cloned, expressed and purified employing the same strategy as for the wild type (Figure 3.1).

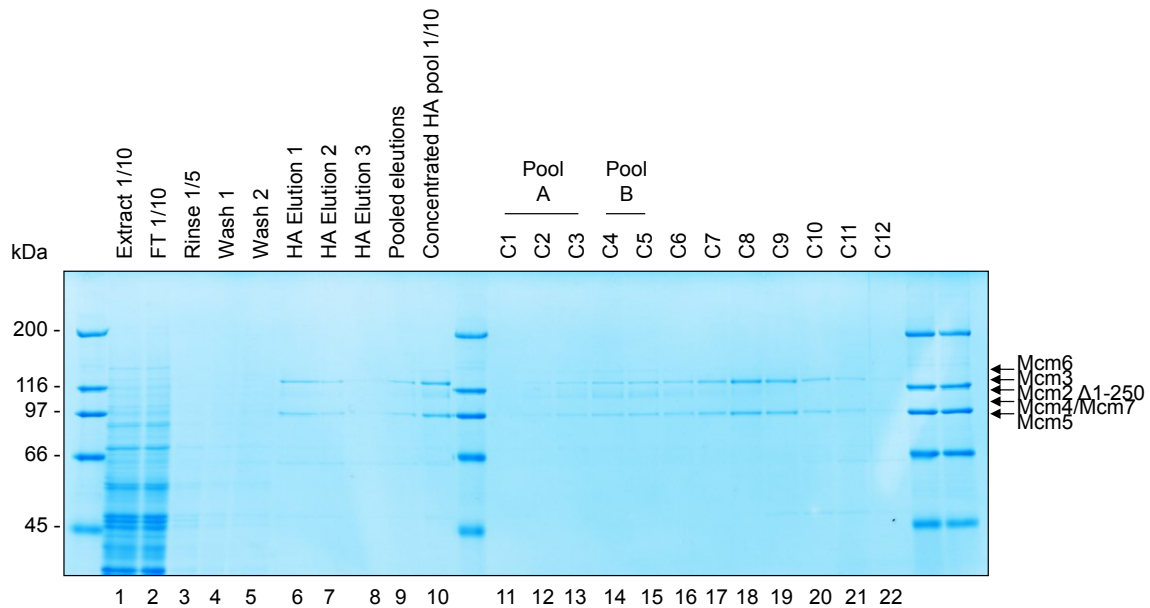


Figure 4.13: Purification of MCM2-7 2Δ1-250

Coomassie blue stained gel of the purification of the MCM2-7 deletion mutant. Yeast extract produced by freezer milling was incubated with anti-HA beads, rinsed, washed twice and rinsed again before bound MCM2-7 2Δ1-250 was eluted with HA peptide. The eluate was concentrated and loaded into a Superose 6 column for gel filtration and fractions corresponding to lanes 11-13 and 14-15 were pooled separately. Arrows indicate the position of the Mcm subunits.

The purified mutant was tested in first place for the ability to form a complex with the other components of the pre-RC on origin DNA and salt stable MCM2-7 loading. The pre-RC was reconstituted as previously described (Evrin et al, 2009) with either wild type MCM2-7 or MCM2-7 2Δ1-250 (Figure 4.14, panel A). Both forms of MCM2-7 appeared to be recruited onto DNA (Figure 4.14, panel A, lanes 6 and 8) and also to be stably loaded after high salt wash (Figure 4.14, panel A, lanes 7 and 9), although the deletion mutant appeared to be less efficient than the wild type in this condition. The high salt wash removes ORC, Cdc6 and Cdt1, leaving MCM2-7 encircling the DNA, with only very small amounts of ORC left behind.

MCM2-7 2Δ1-250 was produced following the strategy described in chapter 3, using three expression plasmids each containing two Mcm subunits, including Mcm3 with a N-terminal HA tag, therefore there is the possibility of contamination with wild type Mcm2, expressed from its genomic promoter. The purification using an anti-HA resin followed by gel filtration would in fact not be able to discriminate between the deletion mutant and contaminating wild type protein. Given the low yield of MCM2-7

complex obtained in the purification, it appears possible that wild-type Mcm2 could be contaminating the MCM2-7 Δ 1-250 complex. To exclude this possibility, wild type MCM2-7 and MCM2-7 Δ 1-250 were analysed by western blot, probed with a specific antibody anti-Mcm2. The epitope of this antibody is in the first 250 amino acids of the protein therefore it should not produce a signal for the deletion mutant. Nevertheless a signal at the same molecular weight as for the wild type could be detected in the case of the mutant (Figure 4.14, panel B), indicating that wild type Mcm2 is present in the sample. The loading obtained (Figure 4.14, panel A lane 9) could be therefore due to the contamination and not to MCM2-7 Δ 1-250 itself, rendering the result non-conclusive

To further analyse this mutant, a growth assay was performed comparing the wild type protein with the mutant (Figure 4.14, panel C), while the empty vectors were used to generate a control strain that does not express proteins. Each strain was spotted in a 5 fold serial dilution on a selective plate containing either glucose (no protein expression) or galactose (protein expression) and grown at 30°C. The strains grew in a comparable manner on the glucose plate and no dominant lethality was observed in galactose for the deletion mutant strain compared to the wild type.

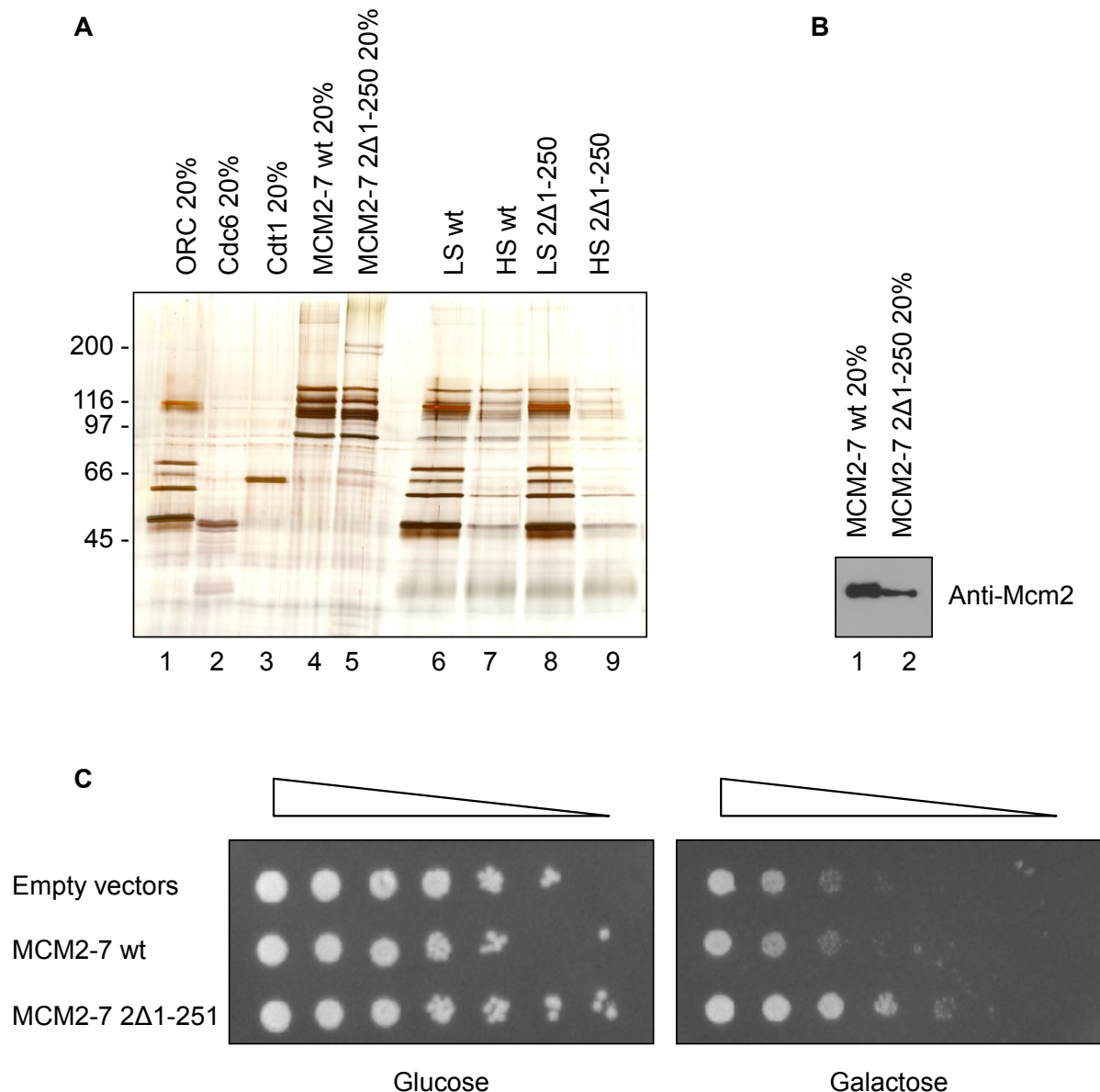


Figure 4.14: Activity test for MCM2-7 2Δ1-250

A) Silver staining gel showing the reconstituted pre-RC formed with MCM2-7 wild type or MCM2-7 2Δ1-250. The assay was performed as previously described (Evrin et al, 2009). **B)** 20% inputs of MCM2-7 wild type and mutant were analysed by SDS-PAGE followed by western blot and probed with a specific antibody anti-Mcm2. **C)** Yeast growth assay showing the effect of overexpression of the control strain (transformed with empty pESC-LEU, pESC-TRP and pESC-URA), wild type MCM2-7 or MCM2-7 2Δ1-250 mutant. A 5 fold serial dilution of cells (starting from 1×10^7) was spotted onto selective Kaiser plates with either 2% glucose or 2% galactose. Plates were incubated for 3 days at 30°C.

Taken all together, the results obtained with MCM2-7 2Δ1-250 suggest that the mutation could affect proper Mcm2 folding. This would explain the poor yield of the purification and the lack of an effect in the growth assay. It is still possible that the

mutant is indeed capable of loading and that the disruption of the interaction of Sld3 with Mcm2 1-250 is not dominant-lethal, as other Sld3 binding sites are present in MCM2-7. It is otherwise possible that this interaction is bypassed *in vivo* by the presence of other factors, or that the wild type MCM2-7 present in the strain is sufficient to support growth.

In order to produce a mutant protein complex that is not contaminated with the wild type, it was decided to add a FLAG tag to the Mcm2 subunit. In this way the FLAG-mediated purification would yield a sample that predominantly contains the deletion mutant. Furthermore, as the deletion of 250 amino acids could affect the stability of the mutant MCM2-7 complex, shorter deletion mutants were included as well, based on the findings shown in Figure 4.12.

Initially, a growth assay was performed to establish if the FLAG tag at the N-terminus of Mcm2 affects the protein function and whether dominant-lethality could be observed the $\Delta 1-200$ and $\Delta 1-238$ mutants (Figure 4.15).

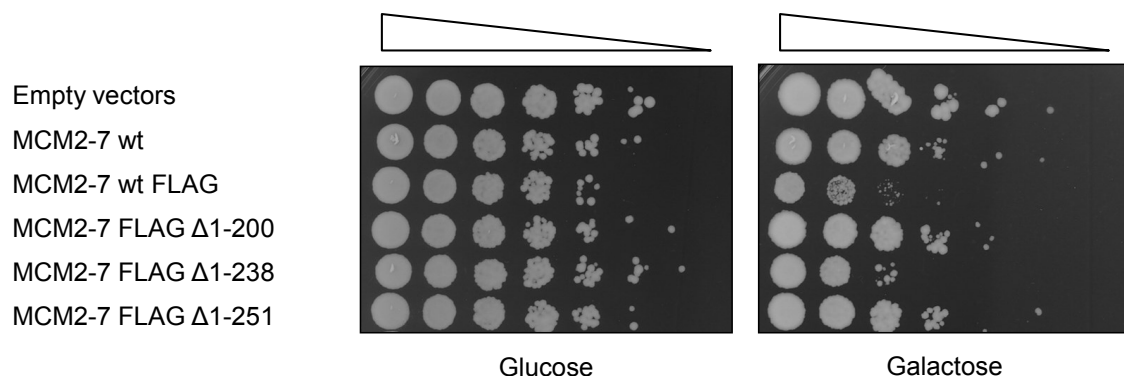


Figure 4.15: Growth assay for FLAG-tagged MCM2-7

Yeast growth assay showing the effect of overexpression of the control strain (transformed with empty pESC-LEU, pESC-TRP and pESC-URA), MCM2-7 wild type, wild type FLAG and FLAG-tagged deletion mutants. A 5 fold serial dilution of cells (starting from 1×10^7) was spotted onto selective Kaiser plates with either 2% glucose or 2% galactose. Glucose plates were incubated for 2 days whereas galactose plates for 3 days at 30°C.

The experiment indicates that the FLAG tag at the N-terminus of Mcm2 is causing dominant lethality, suggesting that the protein cannot fold or function in an optimal way. One possibility is that an N-terminal extension causes a specific defect, while the removal of the N-terminus is tolerated. It is interesting to note that 1-238 caused weak dominant lethality; this could suggest that this construct affects MCM2-7

function. Nevertheless, as the control wt FLAG was altered it was decided to analyse the mutants in more detail.

4.4 MCM2-7 hybrid mutant

The previous analysis of the deletion mutants suggests that the loss of the N-terminal portion of Mcm2 affects the stability of the MCM2-7 complex. It was hypothesised that the corresponding region of the human Mcm2 could stabilise MCM2-7, but that the human Mcm2 N-terminus would not support interaction with Sld3.

To test this possibility, the human and yeast sequences were aligned using the T-Coffee server (Di Tommaso et al, 2011), the portion of human sequence that aligned to the yeast Mcm2 1-250 (alignment in Appendix III) was used for *in vitro* transcription and translation and tested in a pull-down with MBP-Sld3. Also, a hybrid version of Mcm2 1-250 containing an important section of the yeast binding site for Sld3 was tested in this assay (Figure 4.16).

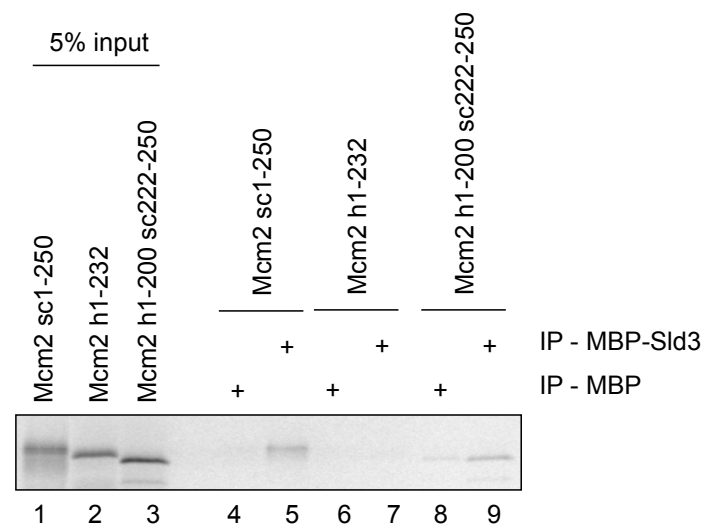


Figure 4.16: Test of human Mcm2 N-terminus for Sld3 binding

MBP or MBP-Sld3 immobilized on anti-MBP magnetic beads were incubated with ^{35}S labelled *in vitro* transcribed translated variants of Mcm2 N-terminus, washed, separated by SDS-PAGE and analysed by autoradiography.

Both, yeast Mcm2 1-250 and the hybrid human 1-200 fused to *S. cerevisiae* 222-250, were immunoprecipitated by MBP-Sld3 (Figure 4.16, lanes 5 and 9 respectively). Interestingly, human 1-232 did not interact with Sld3 (Figure 4.16, lane

7). This suggests that the interaction between Sld3 and MCM2-7 has diverged among species, consistently with the fact that Sld3 and Treslin show little homology in some stretches.

Remarkably, the hybrid h1-200 sc222-250 is immunoprecipitated by MBP-Sld3, confirming that the region 222-250 contributes significantly to the interaction (Figure 4.16, lane 9).

It was then decided to purify a FLAG-tagged version of MCM2-7 presenting the human N-terminus covering the binding site for Sld3 (MCM2-7 FLAG h1-232 sc254-868). MCM2-7 wild type FLAG-tagged was also purified to use as a control for the following experiments, keeping in mind that the tag appeared to cause a slight growth defect.

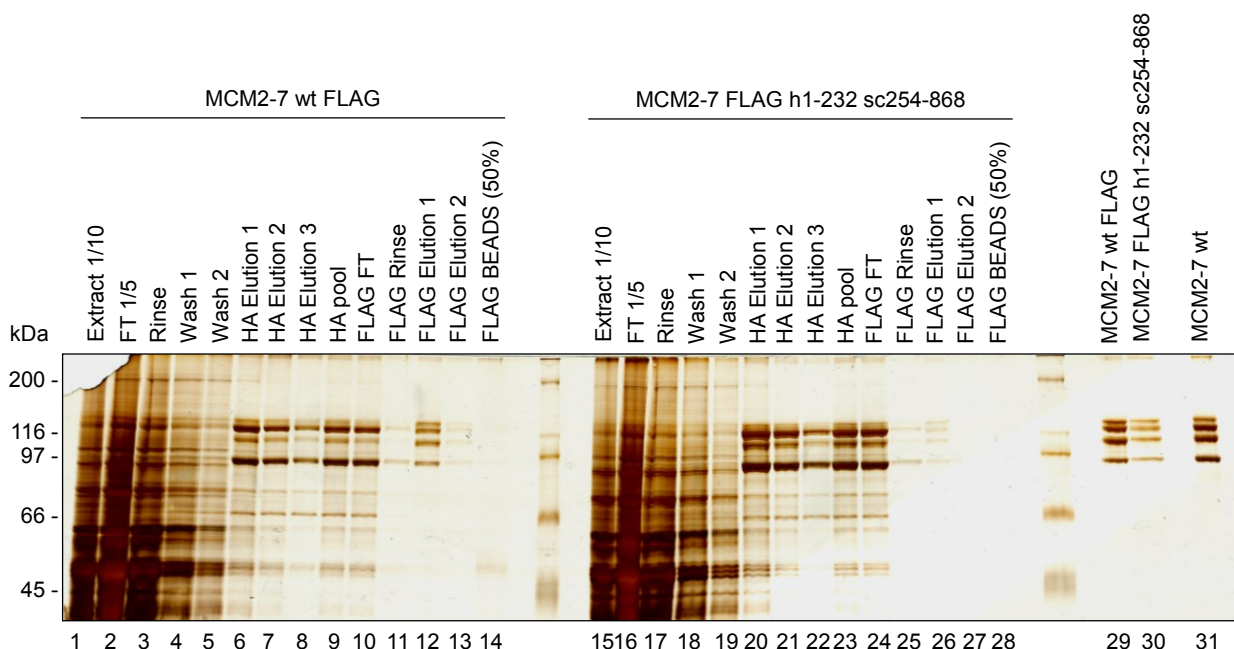


Figure 4.17: Purification of FLAG-tagged MCM2-7 wild type and human hybrid

Silver stained gel showing the purification of MCM2-7 wild type FLAG and hybrid mutant. Yeast extracts produced via freezer milling were incubated with anti-HA beads, rinsed, washed twice and rinsed again before bound MCM2-7 was eluted with HA peptide. The eluate was incubated with anti-FLAG beads, rinsed, eluted from the resin with FLAG peptide and concentrated. Final samples are shown in lane 29 and 30, together to the wild type purified with the standard protocol (lane 31).

The proteins were overexpressed in budding yeast cells arrested with α factor in G1-phase of the cell cycle, to ensure that the proteins are not carrying S-phase phosphorylations, using three expression plasmids each containing two Mcm subunits, including Mcm3 with a N-terminal HA tag and Mcm2 with a N-terminal

FLAG tag. They were then purified in a two-step purification process (Figure 4.17) where MCM2-7 was bound to anti-HA resin and eluted using HA peptide followed by a passage onto an anti-FLAG resin. The samples were finally eluted using FLAG peptide and concentrated. This protocol allowed the removal of a Mcm3/5 sub complex as well as any Mcm2 wild type contaminants.

These MCM2-7 complexes were then tested in the pre-RC assay for recruitment and loading onto origin DNA. A standard pre-RC reconstitution assay was performed as previously described (Evrin et al, 2009), using either wild type MCM2-7, the FLAG-tagged version or the hybrid mutant (Figure 4.18, panel A). The FLAG-tagged MCM2-7 appeared to be recruited and loaded similarly as the standard wild type version (Figure 4.18, panel A, compare lanes 9-10 with 7-8), indicating that the FLAG tag does not have an effect on pre-RC formation. This also indicates that the growth defect seen previously (Figure 4.15) must be due to the interference of the tag with other functionalities of the MCM2-7 protein. When the hybrid mutant MCM2-7 FLAG h1-232 sc254-868 was used, it showed poor recruitment and extremely weak loading compared to the wild type versions (Figure 4.18, panel A lanes 11 and 12), indicating that the human N-terminus of Mcm2 is not able to restore the functionality of the yeast counterpart.

This hybrid mutant, together with another one where a shorter portion of human Mcm2 is fused to the yeast Mcm2 preserving the binding site for Sld3, was also tested *in vivo* for dominant lethality (Figure 4.18, panel B).

Again MCM2-7 wild type FLAG showed a slight growth defect compared to its untagged version. Both hybrid mutants appeared to further impact the viability, suggesting that if they can form a stable MCM2-7 complex, it is not fully functional.

This data underlines how difficult it is to manipulate MCM2-7. The risk of disrupting the structure of the complex or affecting other interactions in addition to the one studied is very high.

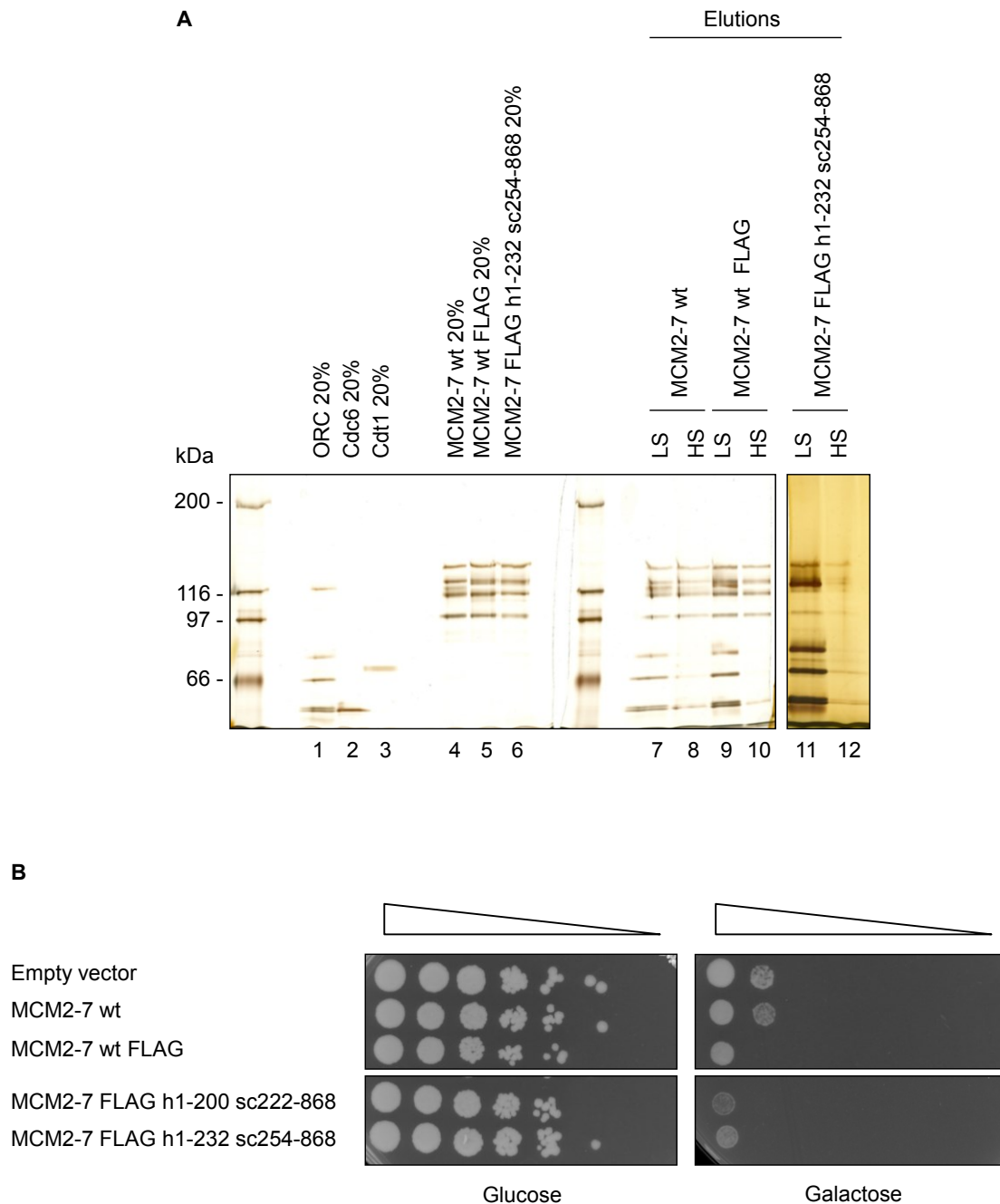


Figure 4.18: Analysis of the MCM2-7 hybrid mutants

A) Silver staining gel showing the reconstituted pre-RC formed with MCM2-7 wild type or FLAG-tagged versions. The assay was performed as previously described (Evrin et al, 2009). A longer staining was applied to lanes 11 and 12 in order to detect minimal amount of protein. **B)** Yeast growth assay showing the effect of overexpression of the control strain (transformed with empty pESC-LEU, pESC-TRP and pESC-URA), MCM2-7 wild type, FLAG-tagged wild type and two forms of hybrid mutants. A 5 fold serial dilution of cells (starting from 1×10^7) was spotted onto selective Kaiser plates with either 2% glucose or 2% galactose. Glucose plates were incubated for 2 days whereas galactose plates for 3 days at 30°C.

For these reasons it was decided to focus the attention on the residues in the region 200-250 of Mcm2 that differ between yeast and human and could be potentially crucial for the interaction with Sld3. As shown before in fact the human sequence 1-232, corresponding to the first 250 amino acids in yeast, could not interact with Sld3. An alignment was therefore performed with the two sequences (Figure 4.19 and Appendix III) and the most conserved residues in the fungal alignment but different from the human sequence were selected for mutation. All the amino acids chosen were mutated to the human counterpart. Some of them were also mutated to the opposite charge or to alanine (in case they were serine or threonine that could be phosphorylated), in order to maximise the effect.

Figure 4.19: Alignment of *S. cerevisiae* and *H. sapiens* Mcm2

The mutants were produced using *in vitro* transcription and translation system either with wild type *S. cerevisiae* Mcm2 aa 1-250 or with the hybrid version human 1-200 *S. cerevisiae* 222-250 as a template and tested in the pull-down assay with MBP-Sld3.

binding to MBP-Sld3 compared to the wild type, mainly in the context of the hybrid background (Figure 4.20, panel B, lanes 11 and 15 respectively).

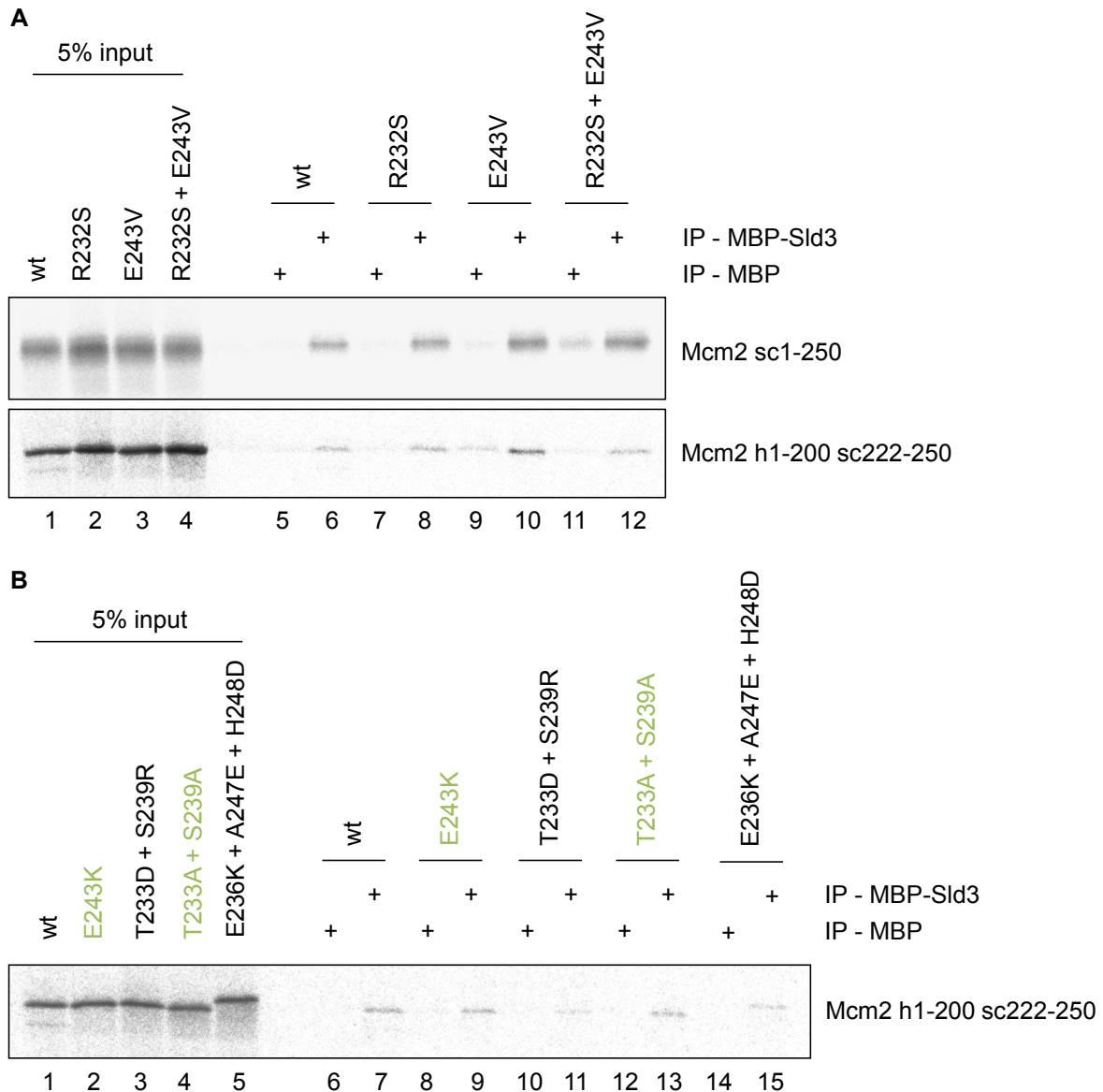


Figure 4.20: Analysis of the interaction between Sld3 and Mcm2 mutants

MBP or MBP-Sld3 immobilized on anti-MBP magnetic beads were incubated with ^{35}S labelled *in vitro* transcribed translated variants of Mcm2 N-terminus, washed, separated by SDS-PAGE and analysed by autoradiography (**A**). In green are labelled the mutants that were not changed to the human amino acid (**B**).

The single mutants of the most promising multiple mutants analysed in Figure 4.20 (T233D+S239R and E236K+R247E+H248D) were produced in the hybrid background and tested for binding to MBP-Sld3. The non-mutated version was again used as control. Unfortunately the mutant T233D was not efficiently transcribed and

translated (Figure 4.21, panel A lane 3), probably because of degradation of the DNA used in the reaction, therefore it was not possible to establish if it had an impact on the binding to Sld3. However, the mutant S239R alone was not able to produce a reduction comparable to the double mutant (Figure 4.21, panel A lane 12). The quantified radioactive signal of the double mutant T233D+S239R was around 30% of the wild type whereas S239R 45%, suggesting that T233D plays a role in the interaction.

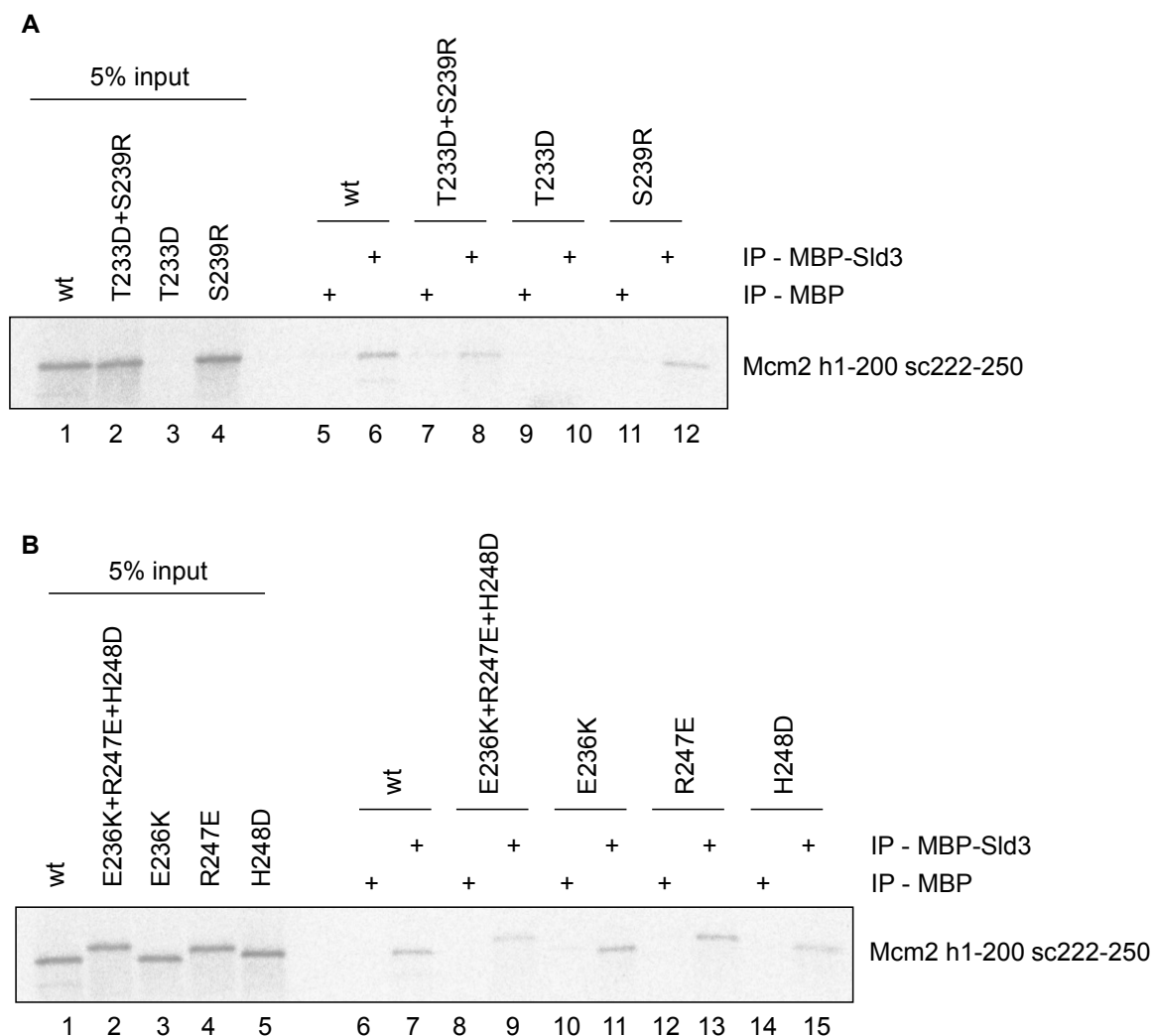


Figure 4.21: Test of the interaction of Sld3 with Mcm2 mutants

MBP or MBP-Sld3 immobilized on anti-MBP magnetic beads were incubated with ^{35}S labelled *in vitro* transcribed translated variants of Mcm2 N-terminus, washed, separated by SDS-PAGE and analysed by autoradiography.

When the residues modified in the mutant E236K+R247E+H248D were analysed separately (Figure 4.21, panel B) instead a contribution from H248D could be

detected. This mutation alone produced the same signal reduction detected as for the triple mutant.

This series of experiments resulted in the identification of three residues on the N-terminus of Mcm2 (T233, S239 and H248) that could be involved in the interaction of MCM2-7 with Sld3. These amino acids need to be analysed separately and in combination in the context of the MCM2-7 hexamer to confirm their importance.

4.5 Analysis of the binding site on Sld3

The previous paragraphs showed how challenging the handling of a big hetero hexamer such as MCM2-7 can be. One reason for the observed problems could be the instability of the complex as a result of manipulations. Therefore the study on the interaction between MCM2-7 and Sld3 proceeded with the analysis on the binding site of Sld3.

At first, the same approach employing a tagged form of MCM2-7 to pull-down *in vitro* transcribed and translated subunits of Sld3 was followed. After a series of non conclusive experiments, showing a very high non-specific interaction of all Sld3 domains tested with the control (both anti-IgG and anti-MBP were tried at this stage), it was decided that it was not the right approach to study this interaction. Sld3 in fact is predicted to lack a defined structure (secondary structure prediction in Appendix II), therefore it is possible that its domains mis-fold and become sticky.

To overcome this problem, the sequence of Sld3 was analysed in order to identify the most conserved regions in *fungi* (fungal alignment in Appendix III) and also in higher eukaryotes, taking advantage from the work of Sanchez-Pulido and colleagues (Sanchez-Pulido et al, 2010). This data was then integrated with a secondary structure prediction. As a result, seven conserved regions were identified which also contained structural elements that could be important for protein-protein interaction. They were used to generate a series of biotinylated peptides (Appendix I, Table IV) that cover the most relevant regions of Sld3 according to this analysis to be used to immunoprecipitate Mcm2 in order to identify minimal fragments of Sld3 that are responsible for the binding. This approach is similar to what previously described in few reports (Ball et al, 1997; Rolef Ben-Shahar et al, 2009). The purpose of this assay was to screen for putative regions of interaction of Sld3 therefore no mutated control peptides were included at this stage.

The biotinylated peptides were conjugated to streptavidin beads and then incubated with *in vitro* transcribed translated Mcm2 and two N-terminal domains (aa 1-250 and 1-322) that previously showed interaction with Sld3 (Figure 4.22). Three peptides appeared to bind to all three proteins examined and corresponded to amino acids 170-195 (CR1b), 196-226 (CR2) and 286-311 (CR3b) of Sld3, whereas peptides CR1a (152-175), CR3a (249-287), CR4 (333-355), CR5 (384-419) and CR6 (518-546) showed no interaction. Interestingly, all portions of Mcm2 tested interacted with CR3b which is conserved in lower eukaryotes but not in higher organisms (see Appendix III), consistently with the fact that the interaction of Sld3 with MCM2-7 may have diverged during evolution.

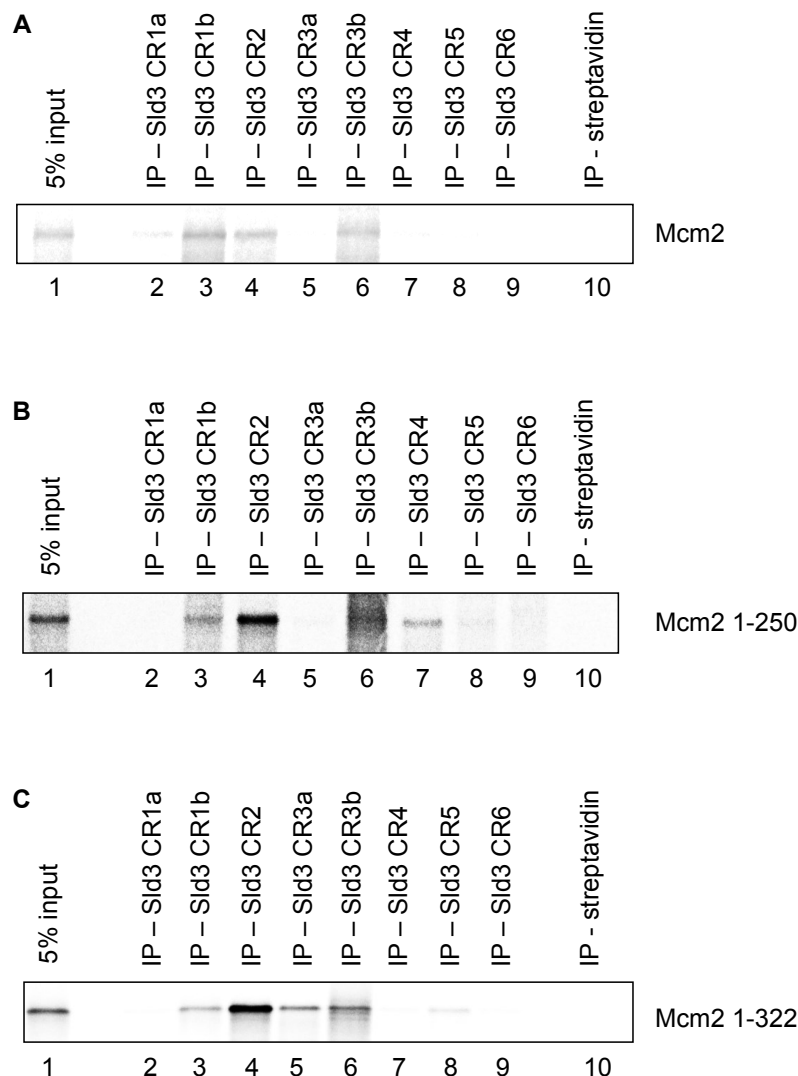


Figure 4.22: Interaction analysis of Sld3 peptides with Mcm2

Biotinylated peptides of Sld3 bound to streptavidin beads were incubated with ^{35}S labelled *in vitro* transcribed translated Mcm2 full length (A), aa 1-250 (B) and 1-322 (C), washed, separated by SDS-PAGE and analysed by autoradiography.

This approach provided a rapid and effective tool to screen a protein-protein interaction and identified a specific region of Sld3 that can bind Mcm2 and an *in vivo* analysis will be presented in chapter 6.

4.6 Discussion

Sld3 is believed to be one of the first factors recruited to the pre-RC essential for the progression into the pre-IC and CMG (Kamimura et al, 2001; Kanemaki & Labib, 2006). In this chapter the interaction occurring between Sld3 and MCM2-7 was analysed in detail.

All previous data on the matter had been produced via *in vivo* studies (Kamimura et al, 2001; Kanemaki & Labib, 2006; Tanaka et al, 2007b; Zegerman & Diffley, 2007) or employing yeast extracts that were used for *in vitro* assembly and DNA replication assays (Heller et al, 2011). However, these studies have the limitation of not being able to discriminate the contribution of each individual factor involved in the process and can therefore not uncover the detailed molecular mechanism of pre-IC assembly. The few studies performed *in vitro* on Sld3 (Bruck et al, 2011; Bruck & Kaplan, 2011a; Bruck & Kaplan, 2011b) were based on the analysis of purified proteins combined in solution in the absence of DNA and not in the context of the pre-RC, therefore the obtained results are not fully conclusive.

Consequently, it was tested whether Sld3 can be recruited *in vitro* to the pre-RC. The reconstitution of a complex containing Sld3 bound to the pre-RC proved to be challenging as Sld3 is predicted to be an unstructured protein with the tendency of becoming sticky on solid surfaces. Furthermore streptavidin (used for biotinylated-DNA conjugation) is negatively charged whereas Sld3 is a highly positively charged protein (the charge at pH 7 is around 40) and therefore the two are very likely to interact. The standard pre-RC reconstitution system (Evrin et al, 2009) was therefore not ideal for the purpose. Several attempts were made (Table 4.2) in order to overcome the non-specific binding of Sld3 to the beads, but they were not successful. The difficulty in optimising an *in vitro* assay capable of reconstituting complexes that are more elaborated than the pre-RC (i.e. pre-IC and CMG) is reflected in recent publications on the matter that employ yeast extracts instead of purified proteins (Gros et al, 2014; Heller et al, 2011; On et al, 2014).

A novel method was therefore developed in the lab for the reconstitution of the pre-RC in solution, followed by gel filtration, which separates proteins in solution from DNA bound complexes (Herrera, Tognetti et al, under revision). This system, despite presenting some limitations such as the impossibility of performing reactions that require multiple washes, has the advantage of significantly enhancing complex formation, as the proteins do not bind to the solid matrix.

The interaction of Sld3 with the pre-RC, thus, was reconstituted using this novel method and showed that Sld3 is in fact able to interact with the pre-RC in a nearly stoichiometric fashion and in the absence of additional factors. This finding appears to contradict the literature, where Sld3 was only recruited to the pre-RC upon DDK phosphorylation (Heller et al, 2011) and in a Cdc45-dependent manner (Kamimura et al, 2001). It seems reasonable to argue that the initial recruitment can be independent from other factors but it is too weak to be detected in *in vivo* studies, whereas it can still be sensed using a more sensitive *in vitro* assay. Alternatively, it appears possible that other factors binding to Sld3 might inhibit its interaction in the absence of Cdc45. The contribution of DDK and Cdc45 to this interaction is not in any way proved wrong and it will be analysed and discussed in chapter 6.

The study followed with an interaction analysis of Sld3 and MCM2-7. The direct binding of these two proteins *in vitro*, already shown by Bruck and Kaplan (Bruck & Kaplan, 2011a), was confirmed (Figure 4.4) and it was subsequently hypothesised to involve the non-conserved N-terminal extensions of MCM2-7 (Figure 4.5). These regions are in fact flexible, involve sites for post-translational modifications and are therefore suitable for protein-protein interactions.

The strategy used for the initial analysis involved the generation of the N-terminal MCM2-7 extensions, C-terminally fused to a GST tag, which can be conjugated to a Glutathione resin and employed in a pull-down with Sld3. The assay was validated with Dpb11 (binding reported in Zegerman & Diffley, 2007) as a positive control and Regα (an unrelated protein) as a negative control (Figure 4.6). However when the N-terminal sections of MCM2-7 were tested (Figure 4.7), the experiment identified some crucial difficulties with the approach. In the first place, extensive work was required to find the best condition for the purification and binding to the Glutathione resin for each subunit. Secondly, a careful normalisation of the beads, based on amount of bound protein, had to be carried out. Finally, the handling of the agarose Glutathione resin itself was more challenging than expected because the beads

could be easily lost. Obviously all these variables could impact very strongly on the reliability of the assay; therefore a new strategy was developed for further studies.

The reverse pull-down, using MBP-Sld3 conjugated to anti-MBP beads, was performed with *in vitro* transcribed and translated subunits of MCM2-7, showing a clear prevalence for interaction with Mcm2 (Figure 4.8).

Interestingly, Mcm2 is located on the putative “gate” of opening of MCM2-7 for the entry of the dsDNA during pre-RC formation and exit of ssDNA during helicase activation (Bochman & Schwacha, 2007; Bochman & Schwacha, 2008; Bochman & Schwacha, 2010; Costa et al, 2011; Samel et al, 2014; Sun et al, 2013) and it is shown that it interacts with Cdc45 in the CMG structure (Costa et al, 2011; Ilves et al, 2010). Sld3, which is known to interact with Cdc45 (Bruck & Kaplan, 2011a; Kamimura et al, 2001; Kanemaki & Labib, 2006), is therefore in the perfect position to take part in the binding with both Mcm2 and Cdc45. It is also possible to speculate that Sld3 executes the function of keeping the “gate” closed until GINS is recruited to the complex.

To confirm that its N-terminal portion was involved, Mcm2 was divided in three domains and tested in the same way (Figure 4.9), showing that the region comprised between aa 1-322 is necessary to support binding to Sld3. This is consistent with what reported in fission yeast, where the Mcm2 fragment containing aa 1-280, but not 1-204, was interacting with Sld3 (Fukuura et al, 2011) (see alignment in Appendix III).

A series of experiments was carried out at this point to map the binding site, which suggested that the region 200-250 is the most important for the interaction (Figure 4.10). This fragment was then further analysed, but it was not possible to effectively produce shorter domains that were still able to interact with Sld3 as much as the 1-250 (Figure 4.12). This suggests either that several important residues are contained between aa 200-250 or that the folding of the region is important.

A mutant version of MCM2-7, deleted of the first 250 amino acids was expressed, purified and tested in the pre-RC reconstitution assay, as well as tested *in vivo* for dominant lethality (Figure 4.13 and 4.14). The attempt of producing an MCM2-7 hexamer in the absence of such a long stretch of Mcm2, despite similar work was done on other subunits without a drastic effect on the stability (Sheu & Stillman, 2006), appeared to be too optimistic. MCM2-7 Δ 1-250 yielded in fact an extremely small amount of protein when overexpressed and purified, and the sample

seemed contaminated with the wild type Mcm2 subunit naturally expressed in *S. cerevisiae*. Furthermore, when the mutant was expressed *in vivo*, it did not show any dominant lethality, suggesting that the MCM2-7 Δ 1-250 complex does not form accurately and thus wild type MCM2-7 can complement the function of the mutant. The analysis of the N-terminal region of Mcm2 followed with the test *in vivo* of shorter FLAG-tagged deletion mutants for a growth effect, but these did not show differential growth compared to the control strain (Figure 4.15).

To overcome the potential instability of the MCM2-7 complex due to the deletion of a long stretch of Mcm2, another approach involved the design of versions of Mcm2 that were a hybrid between human and yeast. The N-terminal portion of human Mcm2, corresponding to *S. cerevisiae* aa 1-250 in fact was not able to interact with Sld3 *in vitro* (Figure 4.16). The mutant MCM2-7 FLAG h1-232 sc254-868, when overexpressed and purified, showed a defect in the recruitment and loading to DNA (Figure 4.17, panel A) *in vitro* and, when tested *in vivo*, appeared to slightly slow the growth compared to the control (Figure 4.18, panel B).

Taken together these findings indicate that first 250 amino acids at the N-terminus of Mcm2 play an important role in the stability of the complex and are required for protein-protein interactions.

Unfortunately this data did not validate in a conclusive way the hypothesis of Sld3 interacting with the region 200-250 of Mcm2, therefore it was decided to focus on the residues in the region 200-250 of Mcm2 that differ between yeast and human and could be potentially crucial for the binding. Several alignments were run (Figure 4.19 and fungal alignment in Appendix III) to identify the most important amino acids in the region of interest. They were subsequently mutated to the human counterpart in the yeast 1-250 peptide and tested for binding to Sld3 (Figure 4.20 and 4.21). Three amino acids (T233, S239 and H248) were identified for their role in the interaction with Sld3. It cannot be excluded that these amino acids are only contributing to the creation of the binding site for Sld3 and that other binding sites are required, but it would still be extremely interesting to test them separately and in combination, both *in vivo* and *in vitro*.

The analysis of the interface of contact on MCM2-7 for Sld3 required an extensive amount of work and did not end with a definitive result. It was still useful to explore different techniques for the analysis of a protein-protein interaction.

Another approach to prove that the interaction between MCM2-7 and Sld3 was occurring and it was essential for initiation of DNA replication, was to study the binding site on Sld3. For this purpose, the sequence of Sld3 was analysed for its conservation in *fungae* and in higher eukaryotes and this data was then integrated with a secondary structure prediction to identify stretches that could support protein-protein interactions. A series of peptides of Sld3 were thus designed to cover these interesting regions and tested for interaction with Mcm2, identifying the most important regions of Sld3 as 170-195 (CR1b), 196-226 (CR2) and 286-311 (CR3b). This is consistent with the work of Fukuura and colleagues in fission yeast (Fukuura et al, 2011), who identified the region comprises between aa 201 and 601 of Sld3 for the interaction with Mcm2 (see annotated alignment in Appendix III). This strategy was much faster and more promising than the ones adopted for the analysis of the binding site on Mcm2. The regions of interest are sufficiently small to allow careful analysis and systematic mutagenesis, which most likely would result in the generation of a mutant that is indeed defective for the binding. Other essential interactions for initiation of replication were tested in this work and a comprehensive *in vivo* analysis will be presented and discussed in chapter 6.

Chapter 5 – Results

Analysis of the interaction of Cdc45 with the pre-RC and Sld3

Cdc45 is a conserved protein required for the activation of the MCM2-7 helicase; together with GINS, it is involved in the formation of the CMG complex (Costa et al, 2011; Ilves et al, 2010) upon activation of the pre-RC by DDK and S-CDK (Labib, 2010), and it is essential for the progression of the replicative fork (Aparicio et al, 1997; Labib, 2010; Tercero et al, 2000). As explained in chapter 4, Cdc45 can be detected *in vivo* upon addition of a cross-linking reagent together with Sld3 as weakly chromatin bound already in late G1-phase (Aparicio et al, 1999; Kamimura et al, 2001) as one of the first factors to interact with the pre-RC. Nevertheless, its interaction with chromatin becomes more stable only during S-phase, concomitantly with GINS recruitment to origins (Kanemaki & Labib, 2006).

Cdc45 association with the pre-RC has been under investigation for several years. Kamimura and colleagues (Kamimura et al, 2001) reported that the recruitment of Cdc45 to origins is Sld3 dependent, whereas Kanemaki and Labib (Kanemaki & Labib, 2006) showed that GINS is required for its stable engagement with the nascent replisome. The Bell lab confirmed these findings using a new *in vitro* system, which employs a G1-phase extract for pre-RC formation and an S-phase extract, where a number of limiting factors are overexpressed, to promote helicase activation. Cdc45 or other factors were removed from the extract to understand the pre-RC assembly process (Heller et al, 2011). Using this method they proved that Cdc45 association to origin DNA is DDK dependent.

These systems have the disadvantage of not being able to discriminate the contribution of each individual factor involved in the process and even the depletion of one protein at the time could result in compensation by the others, as the limiting factors were overexpressed. For this reason the recruitment of Cdc45 to the pre-RC was here studied *in vitro* using purified proteins, allowing the analysis of interactions that could be obscure to more complex assays.

5.1 Analysis of the recruitment of Cdc45 to the pre-RC

To study the recruitment of Cdc45 to the pre-RC *in vitro*, Cdc45 was purified in the first place as a C-terminal FLAG-tagged fusion protein overexpressed in *S. cerevisiae* from a galactose-inducible promoter. The yeast extract was bound to anti-FLAG resin and the protein was eluted by competition with the FLAG peptide, pooled and concentrated (Figure 5.1). Whenever the FLAG peptide had to be removed (i.e. for immunoprecipitation using anti-FLAG beads), the sample was diluted and incubated with DEAE beads. The resin was then extensively washed and the protein was eluted with purification buffer containing high salt (protocol as in Herrera, Tognetti et al, under revision). Unless differently stated, all Cdc45 used in this work is Cdc45-FLAG.

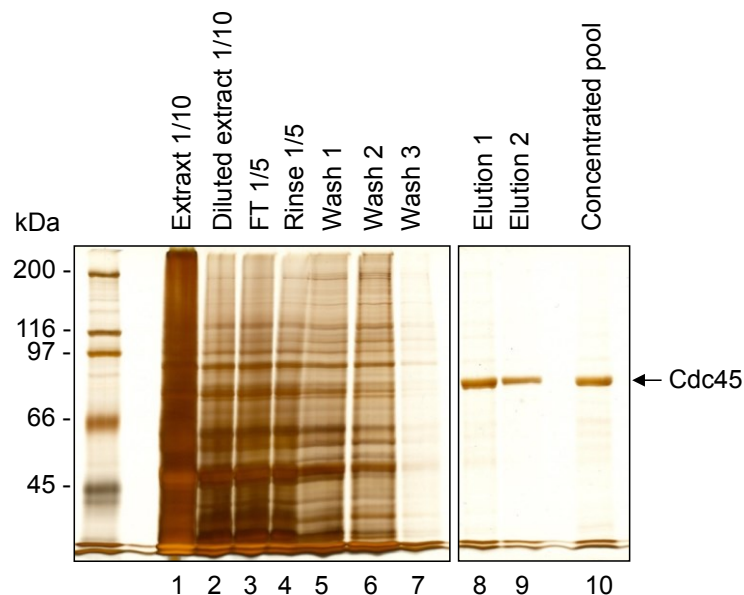


Figure 5.1: Purification of Cdc45-FLAG from yeast

Silver stained gel showing the purification of Cdc45 from yeast overexpressing Cdc45-FLAG. Cells were lysed by freezer milling and the extract incubated with anti-FLAG resin. Bound Cdc45-FLAG was washed three times and eluted using elution buffer containing FLAG peptide. The elutions were pooled (lanes 8-9). Arrow indicates the position of Cdc45-FLAG (75 kDa).

Initially, the bead based pre-RC assay was used to analyse if Cdc45 could associate on its own with the pre-RC. The pre-RC was formed as previously reported (Evrin et al, 2009) and 40 nM of Cdc45 (equimolar to the input of MCM2-7) were added for 30 minutes in a second step. A sub stoichiometric amount of Cdc45 was bound to the pre-RC if compared to MCM2-7 (Figure 5.2, panel A) indicating that

Cdc45 cannot directly interact with the pre-RC under these conditions. Several changes were performed to improve Cdc45 binding: Cdc45 concentration, time and temperature of incubation and salt concentration of the reaction buffer. None of these changes showed a better result. Cdc45 recruitment was also tested in the presence of Sld3 and DDK, which could chaperone the interaction (Heller et al, 2011; Kamimura et al, 2001; Kanemaki & Labib, 2006), as shown in Figure 4.2, panel A, proving unsuccessful.

In order to exclude the fact that Cdc45 gets depleted because of non-specific interactions with the beads, a series of experiments were performed by Carmen Herrera using the gel filtration based pre-RC assay. Initially 40 nM of Cdc45 were added to the pre-RC for either 1 or 30 minutes to capture both quick and transient or slow type of association (Figure 5.2, panel B). Only after 30 minutes of incubation, a weak association of Cdc45 was detected (Figure 5.2, panel B lane 4).

To exclude that the amount of Cdc45 used in the assay is suboptimal, and to test the influence of Sld3 on the reaction, which is known to bind to Cdc45 (Heller et al, 2011; Kamimura et al, 2001; Kanemaki & Labib, 2006), or that the purified protein is inactive because of the tag or the purification process, Cdc45 was titrated on top of the pre-RC where Sld3 was also present. Under these conditions though, only a small amount of Cdc45 was detected at the highest concentration tested (Figure 5.2, panel C).

The recruitment of Cdc45 to the pre-RC was finally tested in the presence of both Sld3 and DDK, reported to be essential *in vivo* (Heller et al, 2011; Kamimura et al, 2001; Kanemaki & Labib, 2006). Equimolar amount of Sld3 and Cdc45 were incubated with the pre-RC for 1 minute in the presence of DDK. Sld3 was interacting with the pre-RC both in the absence and in the presence of Cdc45 (Figure 5.2, panel D, compare lanes 2 and 4), consistent with what is shown in figure 4.3 (panel B). The signal of Sld3 appears slightly reduced in the presence of Cdc45 suggesting that the two proteins could interact in solution, diminishing the amount of Sld3 available for binding to the pre-RC. Cdc45 signal was extremely weak, both in the absence and in the presence of Sld3 (Figure 5.2, panel D, compare lanes 3 and 4).

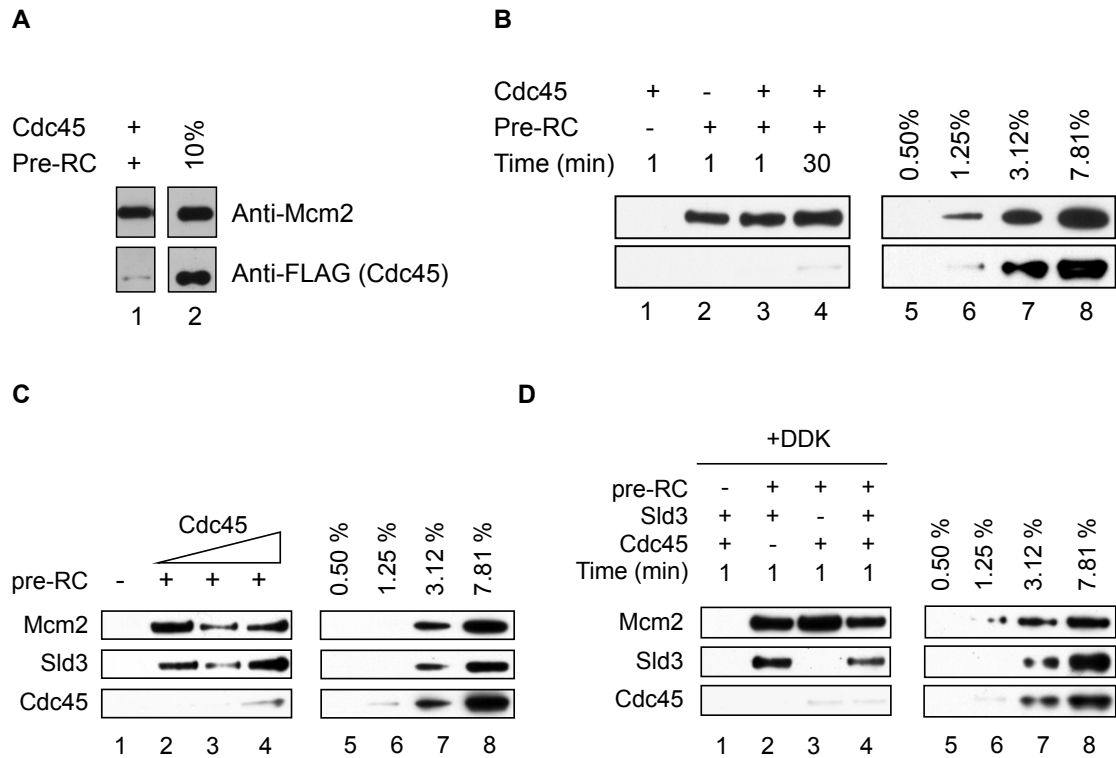


Figure 5.2: Analysis of Cdc45 recruitment to the pre-RC

A) 40 nM Cdc45 were added for 30 min at 27°C to the pre-RC reconstituted with the bead based assay. The samples were analysed by SDS-PAGE followed by Western Blotting using specific antibodies against Mcm2 and FLAG (for detection of Cdc45). **B)** 40 nM Cdc45 were added for 1 or 30 min at 27°C to the pre-RC reconstituted with the gel filtration system. The samples were analysed by SDS-PAGE followed by Western Blotting using specific antibodies against Mcm2 and FLAG. **C)** 40 nM Sld3 and 40-60-80 nM Cdc45 were added for 1 min at 27°C to the pre-RC reconstituted with the gel filtration system. The samples were analysed by SDS-PAGE followed by Western Blotting using specific antibodies against Mcm2, Sld3 and FLAG. **D)** 40 nM Sld3 and 40 nM Cdc45 were added in the presence of 5 nM DDK for 1 min at 27°C to the pre-RC reconstituted with the gel filtration system. The samples were analysed by SDS-PAGE followed by Western Blotting using specific antibodies against Mcm2, Sld3 and FLAG. Experiments shown in panels B, C and D were performed by Herrera (Herrera, Tognetti et al, under revision).

These experiments show that Cdc45 associates *in vitro* very poorly with the pre-RC and that Sld3 and DDK are not sufficient to support this interaction.

5.2 Analysis of direct interactions between Cdc45 and MCM2-7

Several publications suggested that Cdc45 and MCM2-7 can directly contact each other on origins (Gambus et al, 2009; Kamimura et al, 2001; Kanemaki & Labib, 2006; Sheu & Stillman, 2006; Zou & Stillman, 1998; Zou & Stillman, 2000) and form the CMG (Costa et al, 2011; Gambus et al, 2006; Ilves et al, 2010; Moyer

et al, 2006), but only Bruck and Kaplan used purified proteins to study the interaction in the context of Sld3 or GINS trimeric complexes (Bruck & Kaplan, 2011a).

In order to answer the question of whether Cdc45 and MCM2-7 can directly associate one with another in the absence of other factors and origin DNA, immunoprecipitations were performed using GST-Cdc45 or MBP-MCM2-7. GST-Cdc45 and GST (negative control) were overexpressed in bacteria and the lysate extract bound to anti-GST magnetic beads (Figure 5.3, panel A). The samples were used to immunoprecipitate purified MCM2-7 (chapter 3, Figure 3.1) but no interaction was detected (Figure 5.3, panel C). To exclude that the N-terminal GST tag of Cdc45 could impair the binding, purified MBP-MCM2-7 was used to immunoprecipitate Cdc45 (purified as in Figure 5.1, FLAG-tagged at the C-terminal) but once again, no signal was observed (Figure 5.3, panel D).

These interaction assays show that Cdc45 is not able to bind to MCM2-7 directly, suggesting that either other factors or the DNA are required to bridge the interaction or that the conformation assumed by these two proteins in solution does not support a direct contact. The latter hypothesis is reinforced by the knowledge that MCM2-7 undergoes several conformational changes during initiation of replication (Costa et al, 2011; Evrin et al, 2009; Remus et al, 2009). The discrepancy between this result and what reported by Kaplan lab (Bruck & Kaplan, 2011a), where an interaction was observed between Cdc45 and MCM2-7 that would be stimulated by the addition of Sld3 to the reaction, could be explained with the different sensitivity of the detection methods employed. The radiolabelled proteins employed in their work in fact are most likely easier to detect compared to the method used in this study.

Given that a functional assay is not yet available for Cdc45, it was not known if the tagged purified protein was folded in a physiological way and therefore if it was able to exert its roles. In the case of MCM2-7, it has been shown that the protein purified following the same protocol is capable of interacting with the DNA and to support pre-RC formation (Evrin et al, 2009; Evrin et al, 2014; Evrin et al, 2013; Fernandez-Cid et al, 2013; Sun et al, 2013).

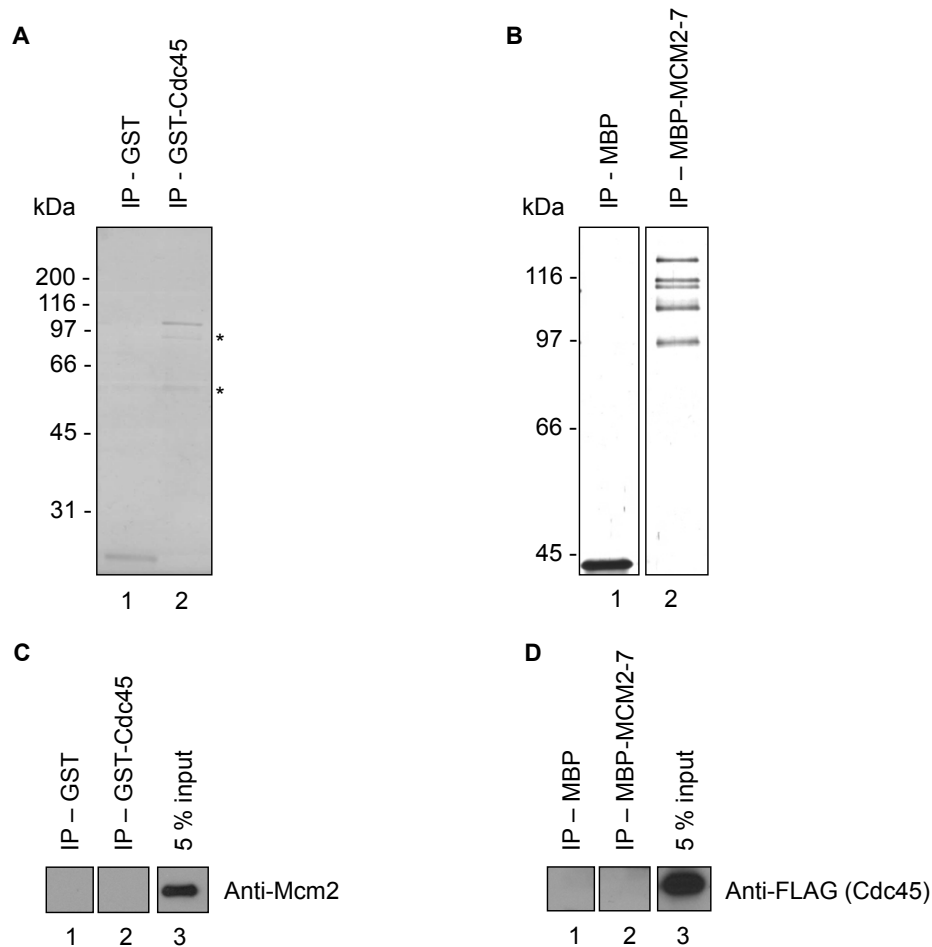


Figure 5.3: Cdc45 and MCM2-7 direct interaction analysis

A) Coomassie stained gel showing GST (26.6 kDa) and GST-Cdc45 (100.8 kDa) immobilized on anti-GST magnetic beads. Asterisks indicate contaminant proteins. **B)** Silver stained gel showing MBP (42 kDa) and MBP-MCM2-7 immobilized on anti-MBP magnetic beads. **C)** GST or GST-Cdc45 immobilized on anti-GST magnetic beads were incubated with purified MCM2-7, washed, separated by SDS-PAGE and analysed by western blot with a specific anti-Mcm2 antibody. **D)** MBP or MBP-MCM2-7 immobilized on anti-MBP magnetic beads were incubated with purified Cdc45, washed, separated by SDS-PAGE and analysed by western blot with a specific anti-FLAG antibody to detect Cdc45.

To exclude that the purified sample of Cdc45 was not functional, or that the binding site for MCM2-7 was masked by other parts of Cdc45 (auto-inhibited), MBP-MCM2-7 was used to immunoprecipitate *in vitro* transcribed and translated Cdc45 full length and individual domains. The truncations were designed after analysis of the secondary structure of Cdc45 (Appendix II) in order to minimise disruptions of the structural elements of the protein. Purified MBP was used as negative control (Figure 5.4). Full length Cdc45 did not bind to MCM2-7 (Figure 5.4, lane 12), consistent with what was previously seen with purified proteins (Figure 5.3, panel C), as well as none of the fragments analysed did bind MCM2-7.

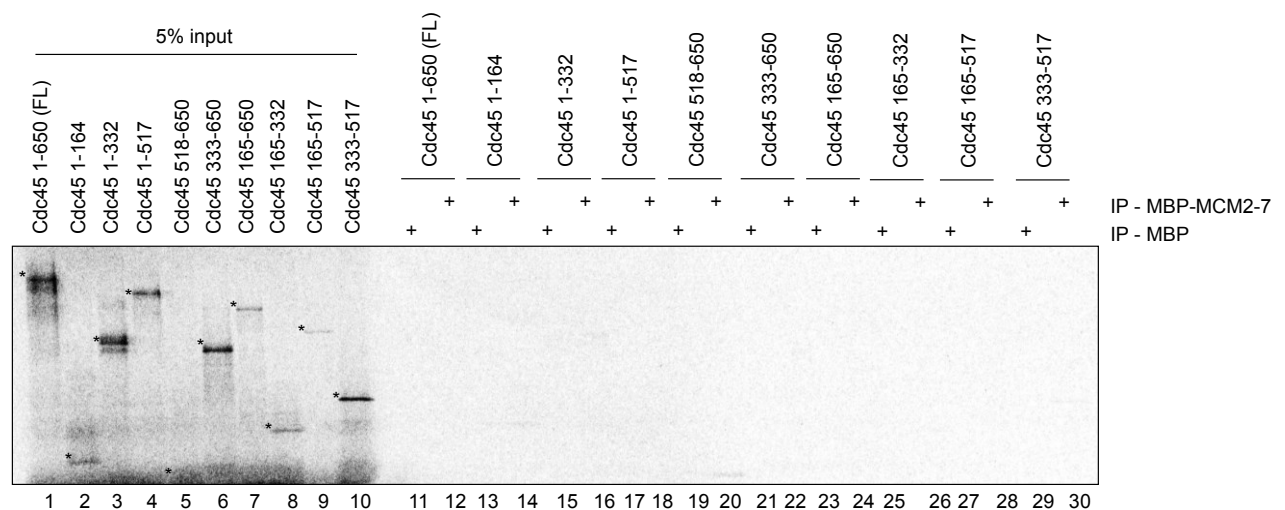


Figure 5.4: MCM2-7 interaction analysis with Cdc45 truncations

MBP or MBP-MCM2-7 immobilized on anti-MBP magnetic beads were incubated with ^{35}S labelled *in vitro* transcribed translated truncations of Cdc45, washed, separated by SDS-PAGE and analysed by autoradiography. Asterisks indicate protein constructs.

This result suggests that MCM2-7 and Cdc45 do not interact with each other, contrarily to what previously reported. However, as Cdc45 is known to interact with MCM2-7 in the context of the CMG, it is likely that the Cdc45 binding site in the purified MCM2-7 is hidden. In order to facilitate the exposure of the hidden domains, the reverse experiment was performed using GST-Cdc45 to pull-down *in vitro* transcribed and translated MCM2-7 subunits, similarly to what was previously shown for Sld3 (Figure 4.8, panel B). Interestingly, GST-Cdc45 immunoprecipitated weakly but specifically Mcm2, similarly to Sld3 (Figure 5.5, lane 8), supporting the idea that Sld3 and Cdc45 can form a complex with MCM2-7 (Bruck & Kaplan, 2011a). This also suggests that the binding site for Cdc45 on MCM2-7 is not exposed in the hexamer in solution and that a conformational remodelling is required prior to the interaction. A partial mis-folding of the MCM2-7 protein sample employed in Bruck and Kaplan's analysis could therefore explain their positive result for the interaction analysis of the full length MCM2-7 and Cdc45 (Bruck & Kaplan, 2011a).

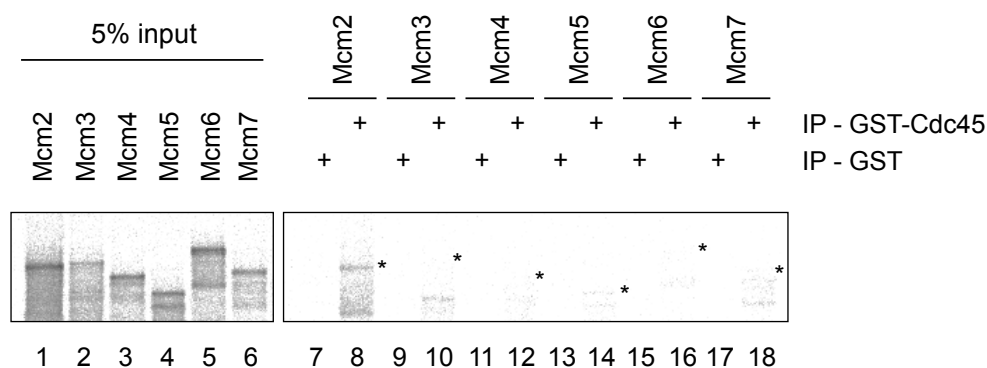


Figure 5.5: Cdc45 interaction analysis with MCM2-7 subunits

GST or GST-Cdc45 immobilized on anti-GST magnetic beads were incubated with ^{35}S labelled *in vitro* transcribed translated proteins, washed, separated by SDS-PAGE and analysed by autoradiography. Asterisks indicate full length proteins.

A series of experiments was performed at this point in order to map the binding site for Cdc45 in Mcm2, using N- or C-terminal truncations similarly to what was described in chapter 4 for Sld3, but the results were not conclusive. A weak non-specific interaction with GST was detected and all fragments analysed interacted to some extent with GST-Cdc45, indicating that either the immunoprecipitation with the GST-tagged protein produces too much non-specific signal or that the binding site on Mcm2 involves several regions that cannot be separated without impacting on the interaction.

Further studies would be required to try to determine the surface of interaction, but a different approach, such as peptide arrays, should be used in place of the pull-down approach.

This work instead continued with the characterisation of the interaction of Cdc45 with Sld3, which appeared equally relevant and less challenging to achieve.

5.3 Analysis of direct interaction between Cdc45 and Sld3

The interaction of Cdc45 with Sld3 was shown in several publications, both *in vivo* (Kamimura et al, 2001; Kanemaki & Labib, 2006; Tanaka et al, 2011b) and, while this project was already undertaken, also *in vitro* (Bruck & Kaplan, 2011a; Bruck & Kaplan, 2011b). Sld3 is considered a Cdc45 loading factor, as it is required for CMG formation, but not for CMG maintenance. The possibility to create mutants that interfere with the interaction with Cdc45 could offer precious insights into the

contribution of these two essential proteins for the following steps of replication initiation, especially when analysed with a reconstituted system.

The initial attempt was to determine which domain of Cdc45 was responsible for the interaction. MBP-Sld3 was therefore used to immunoprecipitate *in vitro* transcribed and translated Cdc45 full length and truncations (Figure 5.6). MBP was used as a negative control.

Consistent with the literature (Bruck & Kaplan, 2011a; Bruck & Kaplan, 2011b; Kamimura et al, 2001; Kanemaki & Labib, 2006; Tanaka et al, 2011b), MBP-Sld3 immunoprecipitated full length Cdc45 (Figure 5.6, lane 12). Two other Cdc45 truncations were specifically and reproducibly precipitated: aa 1-456 (lane 18) and 333-456 (lane 22). Fragments 165-456, 333-650 and 165-650, which all contain the 333-456 domain, gave inconsistent results with a weak signal or no signal in different experiments. This could indicate that the binding site has to be located in a specifically folded region or that some portions of the protein can mask the binding domain. Obviously it could also indicate that aa 333-456 can interact with Sld3 because of their charge (Cdc45 fragment is weakly negatively charged, whereas Sld3 is positively charged) and that this interaction would not occur physiologically when the protein is correctly folded.

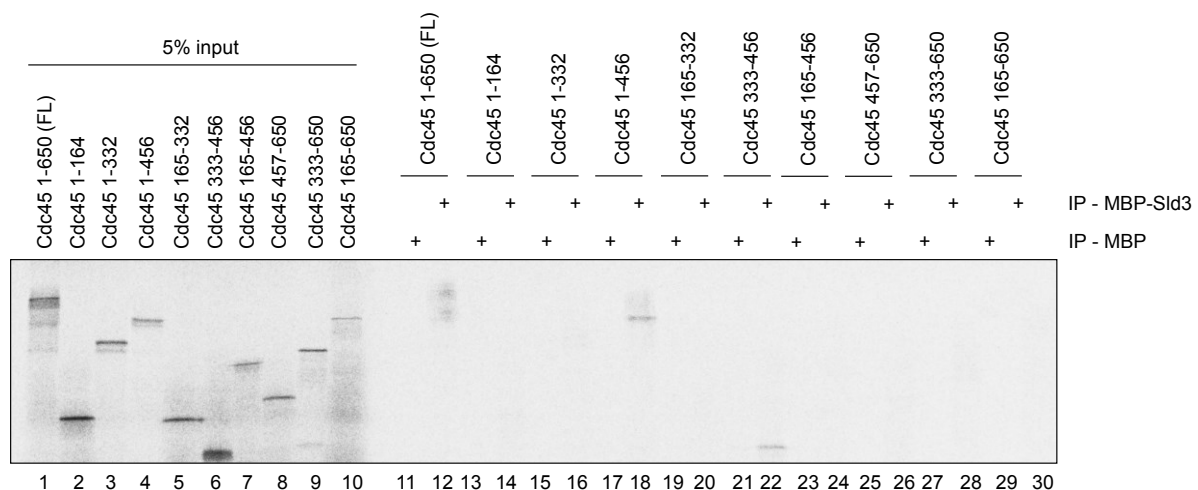


Figure 5.6: Sld3 interaction analysis with Cdc45 subunits

MBP or MBP-Sld3 immobilized on anti-MBP magnetic beads were incubated with ³⁵S labelled *in vitro* transcribed translated Cdc45 full length and truncations, washed, separated by SDS-PAGE and analysed by autoradiography.

Despite the partially discordant result of this interaction analysis, it would still be worthwhile trying to examine in detail the sequence of Cdc45 to define some

stretches in the fragment 333-456 that could be mutated in order to disrupt the interaction with Sld3 and then test these mutations both *in vitro* and *in vivo*. In particular, a sequence analysis of Cdc45 showed that the fragment 333-456 is fairly well conserved (see alignment in Appendix III), suggesting that the interaction between Sld3 and Cdc45 could also be evolutionary conserved.

This study instead followed with the characterisation of the binding site for Cdc45 on Sld3, employing the peptide strategy described in paragraph 4.5. An initial attempt involved the use of GST-Cdc45 to immunoprecipitate *in vitro* transcribed and translated Sld3 domains but proved to be unsuccessful because of the high non-specific signal detected for the GST control (similarly to what discussed in paragraph 5.2). Peptides were therefore designed to cover the most conserved regions of Sld3 and their biotinylated version was used to immunoprecipitate *in vitro* transcribed and translated Cdc45 (Figure 5.7).

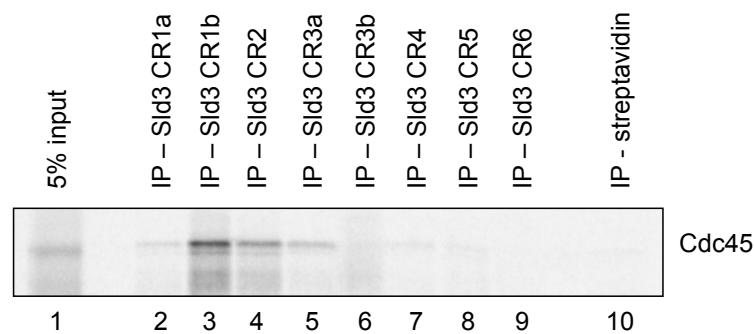


Figure 5.7: Interaction analysis of Sld3 peptides with Cdc45

Biotinylated peptides of Sld3 bound to streptavidin beads were incubated with ^{35}S labelled *in vitro* transcribed translated full length Cdc45, washed, separated by SDS-PAGE and analysed by autoradiography

Mainly three peptides of Sld3 resulted in a clear interaction with Cdc45: 170-195 (CR1b), 196-226 (CR2) and 249-287 (CR3a), but 170-195 (CR1b) bound best. This is consistent with the findings from the Araki lab (Tanaka et al, 2007b), where, using a two hybrid system, mapped the region 150-430 of Sld3 as responsible for interaction to Cdc45.

These regions are sufficiently small to allow for a careful sequence analysis which can result in the identification of mutants that are defective for the interaction. A more detailed *in vivo* analysis on these regions was performed and it will be described in chapter 6.

5.4 Discussion

In this chapter the recruitment of Cdc45 to the pre-RC, as well as its interaction with MCM2-7 and Sld3, was analysed. Cdc45 is a conserved protein, essential for the progression of the replicative fork (Aparicio et al, 1997; Labib et al, 2000; Tercero et al, 2000) and one of the first factors to interact with the pre-RC after its formation on origin DNA already in late G1-phase (Aparicio et al, 1999; Kamimura et al, 2001).

The direct recruitment of Cdc45 to the reconstituted pre-RC was attempted in the first place, using both bead based and gel filtration based methods. A reconstituted system offers in fact the possibility to conclusively prove the mechanism of the reaction. When Cdc45 was added alone to the pre-RC no interaction was detected (Figure 5.1, panels A and B). This is consistent with previous reports (Kamimura et al, 2001; Kanemaki & Labib, 2006), where Cdc45 could not stably associate with the origins in the absence of Sld3.

The contribution of Sld3 to the recruitment of Cdc45 to the pre-RC was therefore analysed, but once again no interaction was detected (Figure 5.1, panel C). Moreover, DDK was also added to the reaction, according to Heller's finding (Heller et al, 2011) that showed that Sld3 and Cdc45 recruitment to the origin is DDK dependent. Surprisingly DDK did not allow Cdc45 binding to the pre-RC, indicating that Sld3 and DDK are not sufficient to support stable interaction of Cdc45 with the pre-RC *in vitro*. The discrepancy of this result with the literature could be explained with the fact that all previous work was performed *in vivo* or using yeast extract. These experimental conditions are not fully controllable and mechanisms of compensation could occur without being able to detect them.

In the absence of any observed recruitment it was hypothesized that an interaction occurs *in vivo* as suggested in several publications (Costa et al, 2011; Gambus et al, 2006; Gambus et al, 2009; Ilves et al, 2010; Kamimura et al, 2001; Kanemaki & Labib, 2006; Moyer et al, 2006; Sheu & Stillman, 2006; Zou & Stillman, 1998; Zou & Stillman, 2000), but that something abrogates the stable Cdc45/MCM2-7 complex formation in other cases. Alternatively, it was possible that one of the proteins used in the reconstituted assay was not active, possibly because of the position of the tag, despite previous work showing that the fusion at the N-terminal of Cdc45 and Sld3 does not affect their interaction *in vitro* (Bruck & Kaplan, 2011a) nor in two hybrid

assay (Kamimura et al, 2001). It remains to be elucidated if the tags could affect Cdc45 recruitment to the pre-RC, maybe impairing its interaction with MCM2-7.

Purified Cdc45 and MCM2-7 were therefore tested for direct binding and they appeared to be unable to interact in solution (Figure 5.3, panels C and D), independently from the position of the tag on Cdc45 (C-terminal GST in panel C and N-terminal FLAG in panel D).

This result indicates that either they do not bind directly, require additional factors or DNA to bridge them, or that the binding site on MCM2-7 is hidden under these conditions. In recent work, Bruck and Kaplan suggested that Cdc45 and MCM2-7 directly interact and that this interaction is disrupted by the addition of ssDNA (Bruck & Kaplan, 2013). For their experiment they used GST-Cdc45 (N-terminal tag) and MCM2-7 purified from bacteria. It is possible to speculate that the expression in bacteria of a large complex such as MCM2-7 could result in mis-folding, causing the exposure of Cdc45 binding site.

It is known that MCM2-7 changes its conformation to adapt to the different steps of helicase recruitment and activation (Costa et al, 2011; Evrin et al, 2009; Remus et al, 2009) therefore it appears reasonable to think that the binding site of some MCM2-7 interaction factor could be hidden in the purified MCM2-7 hexamer. Indeed, MBP-MCM2-7 was unable to immunoprecipitate Cdc45 or Cdc45 truncations (Figure 5.4). This supports the idea that MCM2-7 adopts a conformation that cannot sustain the interaction with Cdc45.

To test this model further, individual MCM2-7 subunits were used for immunoprecipitation assays with Cdc45. In this case a weak but specific interaction with Mcm2 was detected (Figure 5.5). The fact that the subunit involved in this binding is Mcm2 is interesting for multiple reasons. In first place, Mcm2 was shown in this work to interact with Sld3 (Figure 4.8, panel B) and this is fully consistent with the fact that MCM2-7/Cdc45/Sld3 can form a trimeric complex in solution (Bruck & Kaplan, 2011a). Furthermore, Mcm2 is part of the putative “gate” for opening of MCM2-7 for DNA entry (Bochman et al, 2008; Bochman & Schwacha, 2007; Bochman & Schwacha, 2008; Bochman & Schwacha, 2010; Costa et al, 2011; Samel et al, 2014; Sun et al, 2013) and the CMG structure showed that Mcm2 interacts with Cdc45 in the context of the complex (Costa et al, 2011; Ilves et al, 2010).

It was not possible during this project to further map the binding site, possibly because of technical limitations conferred by the use of GST-tagged proteins such as protein amount variability during immobilisation and unspecific interactions with other proteins. The investment of time in the optimisation of an assay for the fine mapping of the binding site on Mcm2 appeared risky considering the difficulties encountered during the study of Sld3 association (chapter 4). The focus was therefore switched to the interaction of Cdc45 with Sld3, equally important and possibly easier to manipulate. The binding of Cdc45 with Sld3 is commonly accepted and it has been shown both *in vivo* and *in vitro* (Bruck & Kaplan, 2011a; Bruck & Kaplan, 2011b; Kamimura et al, 2001; Kanemaki & Labib, 2006; Tanaka et al, 2007b; Tanaka et al, 2011b). An attempt to identify a region of interaction small enough to be susceptible for analysis by point mutations was made at first using MBP-Sld3 to immunoprecipitate Cdc45 truncations (Figure 5.6). This experiment resulted in a partially conclusive result, given that a small fragment of Cdc45 (aa 333-456) was precipitated, but not all fragments were performing in a consistent way in this assay, indicating that the folding of some of them was not correct or optimal or that aa 333-456 can interact with Sld3 because of their charge. This region is still too large to allow the identification of residues that are important for the interaction but attempts can be made analysing the sequence conservation among species.

At the same time as this work was ongoing, the peptide approach was used to identify conserved domains in Sld3 that are involved in the interaction with Mcm2, and seemed an appealing and fast alternative to use. The biotinylated peptides of Sld3 were therefore used to immunoprecipitate *in vitro* transcribed and translated Cdc45 (Figure 5.7), resulting in the identification of three regions involved in the interaction: 170-195 (CR1b), 196-226 (CR2) and 249-287 (CR3a). An *in vivo* analysis on these regions and their mutations was performed and it will be described in chapter 6.

Chapter 6 – Results

Analysis of the interactions involved in Cdc45 recruitment to the pre-RC

At the time when the recruitment of Cdc45 to the pre-RC was under investigation my colleague Carmen Herrera was working on the reconstitution of the pre-IC and identified a salt stable complex containing Cdc45 (Herrera, Tognetti et al, under revision). The assay was performed differently from the standard bead based assay to enhance complex assembly. The pre-RC was assembled in solution and additional factors were added to promote Cdc45 assembly, prior to the addition of salt and gel filtration mediated purification of DNA associated complexes, as described in chapter 4 (Herrera, Tognetti et al, under revision). The newly identified complex formed in the presence of DDK and contained in addition to Cdc45, also Sld3, Sld7 and Sld2 (Figure 6.1, panel A). Given that Sld7 is a non-essential factor (Tanaka et al, 2011b), its contribution to the complex formation was analysed by Herrera, showing that Sld7 is dispensable for this reaction (Figure 6.1, panel B). In order to verify what components are essential for Cdc45 recruitment, an assay was performed where individual factors were left out from the reaction. The participation of Sld2 in recruitment of Cdc45 had not been observed before and therefore required further investigation.

The analysis of Cdc45 recruitment offered interesting insights. In the first place it was found that Cdc45 recruitment to the pre-RC required Sld3, Sld2 and DDK (Figure 6.1, panel C, lane 6). In the absence of individual factors much less Cdc45 can be detected (Figure 6.1, panel C, lanes 2,3 and 5), but a stoichiometric Sld2/Sld3/Cdc45-pre-RC complex is only observed upon the addition of all factors. In the absence of individual factors sub-stoichiometric amounts of Sld3 were detected (Figure 6.1, panel C, compare lane 6 to 3, 4 and 5). Sld2 instead appeared to interact better in the absence of Sld3 (Figure 6.1, panel C, lane 2) than in the absence of Cdc45 and DDK (Figure 6.1, panel C, lanes 4 and 5).

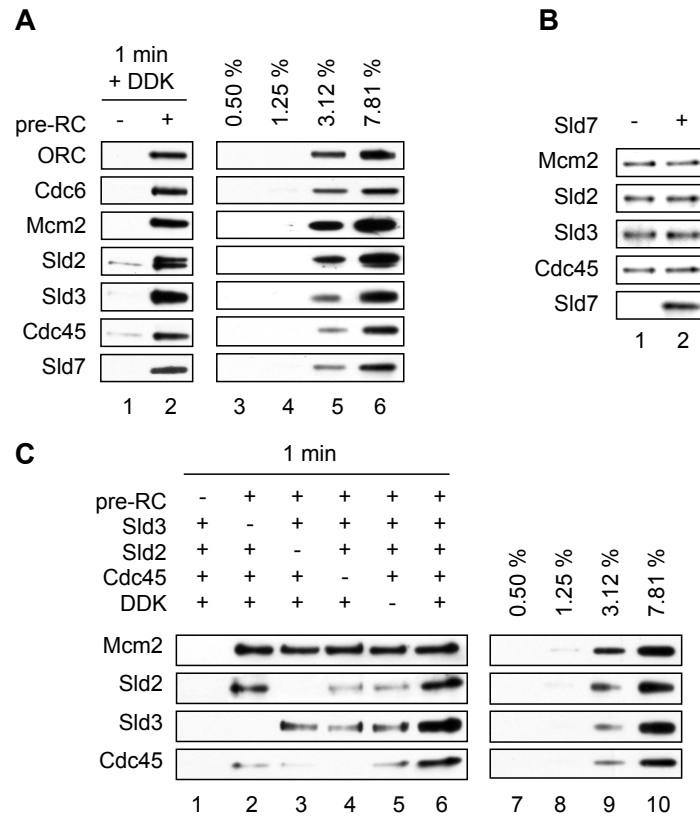


Figure 6.1: Reconstitution of Cdc45 recruitment to the pre-RC

A) Efficient Cdc45 recruitment to the pre-RC by Sld2, Sld3, Sld7 and DDK. **B)** Sld7 is not required for Sld2/Sld3/Cdc45-pre-RC complex formation. This experiment was performed in the presence of DDK. **C)** Sld2, Sld3 and DDK are required for optimal Cdc45 recruitment to the pre-RC. This experiment was performed in the presence of Sld7. In all experiments 40 nM of each protein was used, besides DDK (5 nM), and added for 1 minute at 27°C to the pre-RC reconstituted in solution. A salt wash was added before samples were purified by gel filtration, separated by SDS-PAGE and analysed by western blot. Dilution series represent % of total proteins (40 nM), which were added into the reaction. All experiments shown in this figure were performed by Herrera (Herrera, Tognetti et al, under revision).

These experiments provide a new and interesting way to study the transition of the pre-RC to the CMG and represent the first *in vitro* reconstitution of Cdc45 binding to the pre-RC using purified proteins. Thus, Herrera and I worked together in order to characterise the complex and analyse the mechanism of its formation.

6.1 Analysis of Sld2 interactions

Sld2 is an essential factor, whose phosphorylation by S-CDK creates a binding site for Dpb11 (Tanaka et al, 2007b; Zegerman & Diffley, 2007) and is required to facilitate the recruitment of GINS, and therefore CMG formation. Sld2 in

fact is a member of a transient complex called pre-Loading Complex (pre-LC), together with Dpb11, Pol ϵ and GINS (Muramatsu et al, 2010). Moreover, Sld2 interaction with purified hexameric MCM2-7 was shown by the Kaplan lab (Bruck et al, 2011). No function is reported for Sld2 outside S-phase in the absence of CDK activity, therefore the finding that it is required for the stable recruitment of Cdc45 to the pre-RC deserved further investigation.

The analysis of Sld2 involvement in the process under study started with the examination of its recruitment to the pre-RC. Herrera reconstituted the pre-RC in solution and added 40 nM of purified Sld2 (equimolar to MCM2-7) for either 1 or 30 minutes to capture both quick and transient or slow type of association, prior to salt addition and gel filtration (Figure 6.2, panel A). The association appeared efficient already at 1 minute (Figure 6.2, panel A, lane 3), increased over time (Figure 6.2, panel A, lane 4) and did not require any additional factor nor kinases.

Therefore the surface of contact between MCM2-7 and Sld2 was analysed in this work. GST-Sld2 and GST (negative control) were overexpressed in bacteria and the lysate extract was incubated with anti-GST magnetic beads (Figure 6.2, panel B). The beads were then used to immunoprecipitate *in vitro* transcribed and translated MCM2-7 subunits (Figure 6.2, panel C). Interestingly, GST-Sld2 immunoprecipitated weakly but specifically Mcm2, similarly as observed for Sld3 (Figure 4.8, lane 8) and Cdc45 (Figure 5.5, lane 8).

The finding that Sld2 interacts with Mcm2 supports the concept that Sld2 is involved in Cdc45 recruitment to the pre-RC. Sld2 could in fact directly interact with Cdc45 or alternatively remodel the pre-RC/Sld3 complex. However, the binding site for Sld3, Cdc45 and Sld2 could be only partially formed by Mcm2 and additionally involve adjacent subunits.

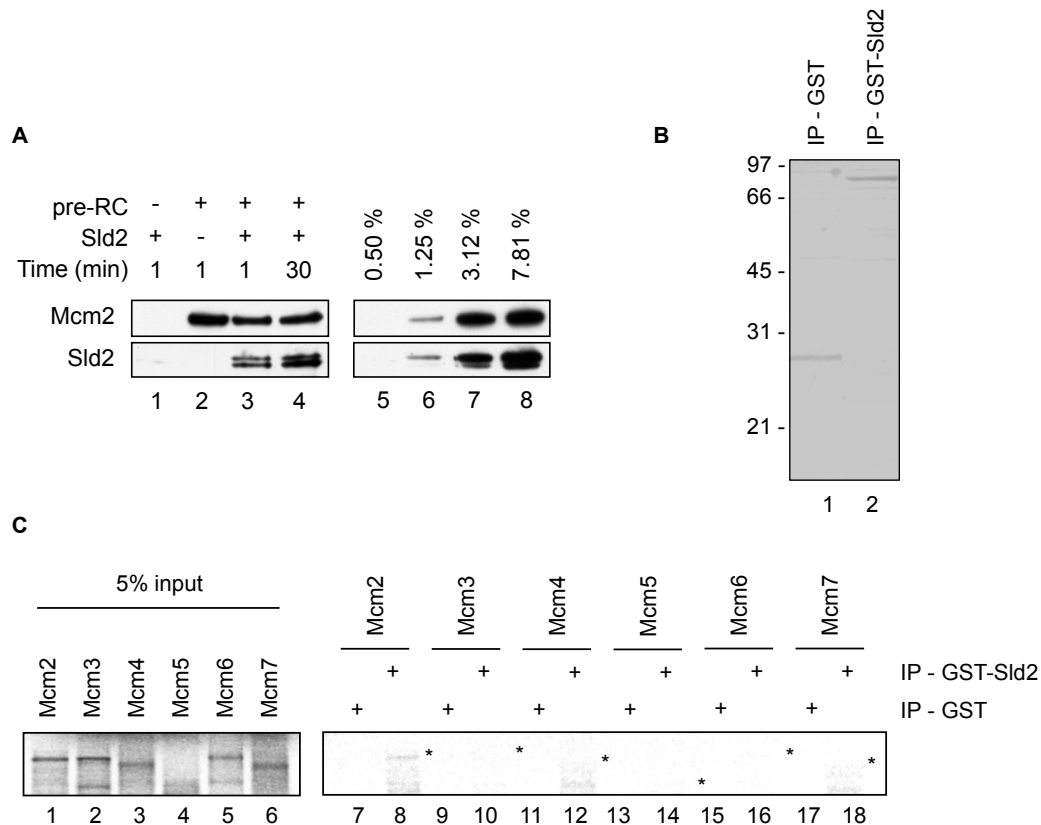


Figure 6.2: Sld2 recruitment to the pre-RC and interaction with MCM2-7

A) 40 nM Cdc45 were added for 1 or 30 min at 27°C to the pre-RC reconstituted with the gel filtration system. The samples were analysed by SDS-PAGE followed by Western Blotting using specific antibodies against Mcm2 and Sld2. This experiment was performed by Herrera. **B)** Coomassie stained gel showing GST (26.6 kDa) and GST-Sld2 (78.8 kDa) immobilized on anti-GST magnetic beads. **C)** GST or GST-Sld2 immobilized on anti-GST magnetic beads were incubated with ³⁵S labelled *in vitro* transcribed translated proteins, washed, separated by SDS-PAGE and analysed by autoradiography. Asterisks indicate full length proteins.

In addition, Herrera found that Sld2 and Sld3 by themselves become recruited to the pre-RC and, when added in combination, the interaction of both proteins did not improve, but the binding of Sld2 was decreased (Figure 6.3, panel A, compare lane 2 and 3 with 4). This indicates that the two proteins cannot bind simultaneously to the pre-RC and suggests that either the pre-RC undergoes a conformational change in the presence of Cdc45 and DDK (Figure 6.1 shows in fact that Sld3 and Sld2 are recruited in a stoichiometric fashion) or that Cdc45 stabilises the complex, perhaps bridging some interactions.

Interestingly, Herrera also found that DDK could resolve the competition occurring between Sld3 and Sld2 and the two proteins could be again stoichiometrically

recruited to the pre-RC when the kinase was added to the reaction (Figure 6.3, panel B).

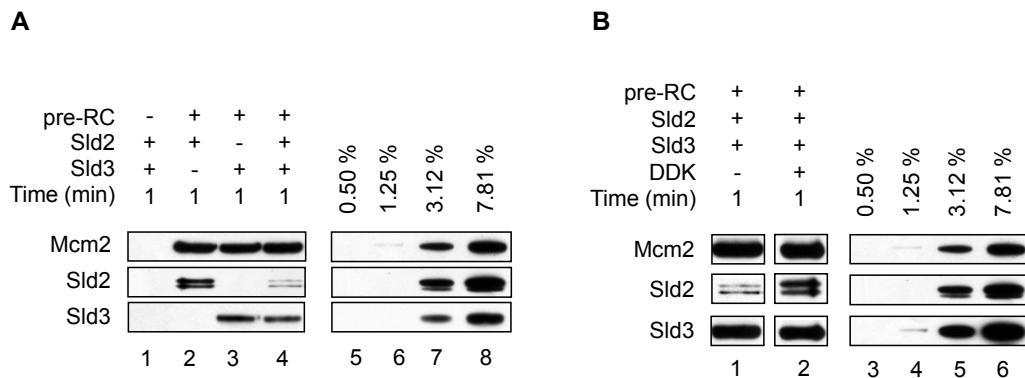


Figure 6.3: DDK regulates Sld2 and Sld3 interactions with the pre-RC

A) Sld2 and Sld3 binding to the pre-RC. Sld2, Sld3 or the combination of both was incubated with the pre-RC reconstituted in solution. **B)** DDK stabilizes Sld2 within the Sld2/Sld3-pre-RC complex reconstituted in solution. After the salt wash, the samples were purified by gel filtration and analysed by SDS-PAGE followed by Western Blotting using specific antibodies against Mcm2, Sld3 and Sld2. Both experiments were performed by Herrera (Herrera, Tognetti et al, under revision).

The intriguing finding that Sld3 and Sld2 can both interact with Mcm2 but cannot be simultaneously associated to the pre-RC unless DDK is added to the reaction warranted a more detailed analysis of the Sld2-Mcm2 interaction, especially as this could help to understand how the kinase could stabilise an Sld2/Sld3-pre-RC complex. If DDK phosphorylation would merely generate the binding site for Sld2, then Sld2 should not interact with the pre-RC when no kinase is added (Figure 6.2, panel A). To test if Sld3 and Sld2 are binding the same portion of Mcm2 or alternative sites, GST-Sld2 was used to immunoprecipitate *in vitro* transcribed and translated truncations of Mcm2. Considering that the binding site of Sld3 and Sld2 could overlap, and knowing that Sld3 binds the N-terminal region of Mcm2 (Figure 4.9), a series of C-terminal truncations were tested together with the three domains that cover the whole Mcm2 (Figure 6.4). As expected, GST-Sld2 weakly immunoprecipitated full length Mcm2 (Figure 6.4, lane 15), although the interaction appeared weaker than before (Figure 6.2, panel C). The *in vitro* transcribed and translated full length protein appears in fact degraded. This phenomenon was observed sometimes during this work, especially when full length proteins were *in vitro* transcribed and translated and then used in a pull-down.

Surprisingly, none of the Mcm2 fragments containing the N-terminus interacted with GST-Sld2 and, instead, a good interaction was observed with the C-terminal portion of the protein (aa 666-868, Figure 6.4, lane 24) and a weak signal was also observed with the central part of Mcm2 (aa 322-665).

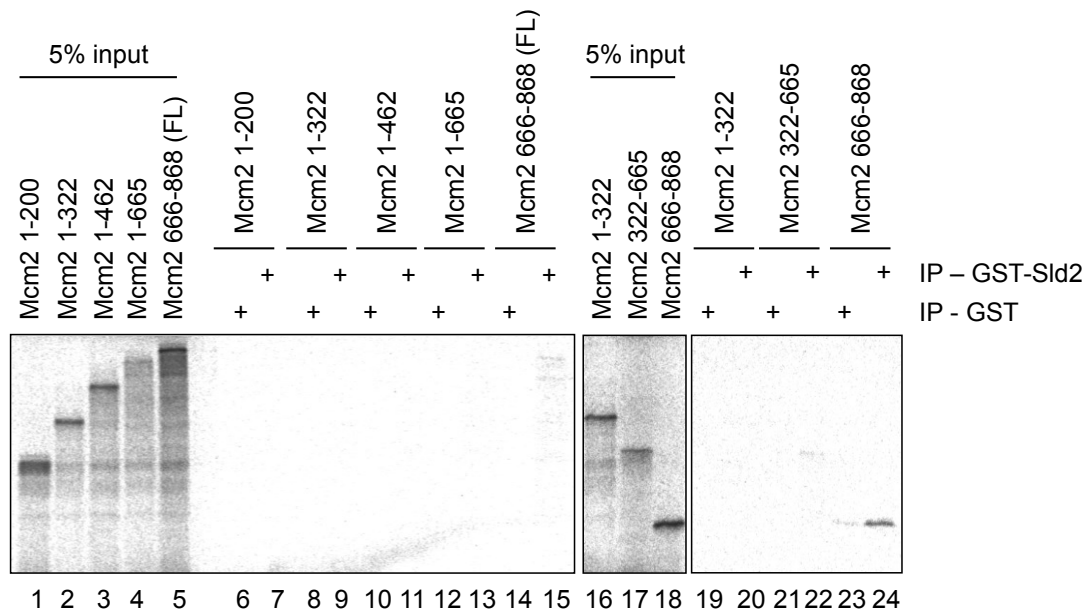


Figure 6.4: Sld2 interacts with Mcm2 C-terminus

GST or GST-Sld2 immobilized on anti-GST magnetic beads were incubated with ³⁵S labelled *in vitro* transcribed and translated truncations of Mcm2, washed, separated by SDS-PAGE and analysed by autoradiography.

The data suggest that Sld3 and Sld2 do not compete for the binding site on Mcm2. Nevertheless, Sld3 interaction with the Mcm2 N-terminus hindered Sld2 binding to the C-terminus. DDK dependent phosphorylation of MCM2-7 could therefore produce a conformational change in the complex allowing simultaneous binding of Sld2 and Sld3 (Figure 6.5).

It has to be pointed out that the N-termini of the MCM2-7 subunits are unstructured regions and therefore it seems reasonable to think that this region could be flexible and assume, upon Sld3 binding, a folding that obstructs the Sld2 binding site at the C-terminus. This finding allows us to draw a model where DDK regulates Cdc45 recruitment to the pre-RC in multiple ways, one of which being the simultaneous interaction of Sld3 and Sld2. Further structural analysis is required in order to prove this theory.

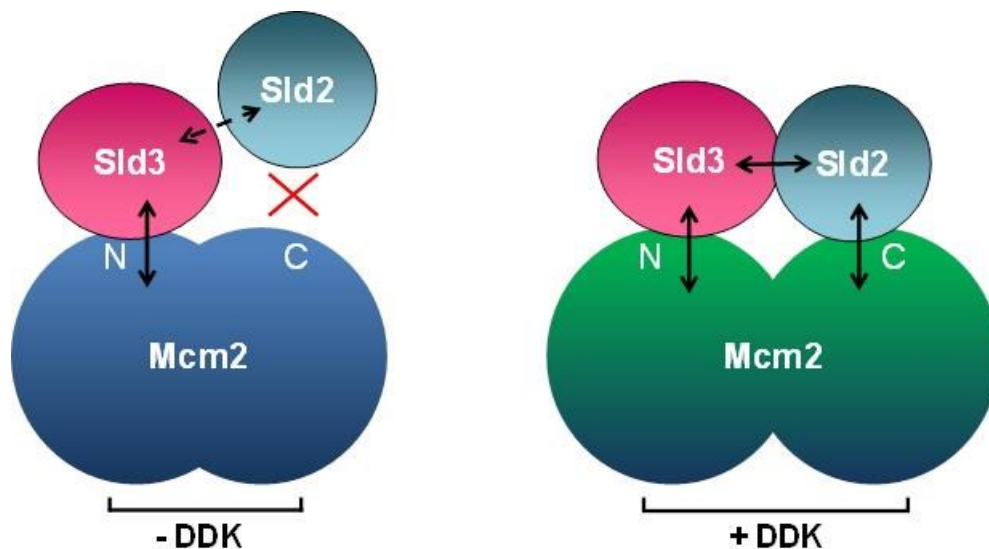


Figure 6.5: Model of Sld3 and Sld2 interaction with Mcm2

Sld3 interacts with the N-terminus of Mcm2 and, in the absence of DDK, the hindrance impairs Sld2 interaction with the C-terminus. Upon DDK phosphorylation, MCM2-7 undergoes a conformational change that exposes again Sld2 binding side and therefore Sld3 and Sld2 can be simultaneously associated with the pre-RC.

For the purpose of this study, however, the interaction of Sld2 with the other components of the Cdc45 containing complex was carried out. No data was available on Sld2 interaction with Sld3 or Sld7. *In vivo* work by the Araki lab (Muramatsu et al, 2010) indicated that Sld2 and Cdc45 do not interact but that Sld2, upon CDK dependent phosphorylation, interacts with Dpb11, as also shown by Zegerman and Diffley (Zegerman & Diffley, 2007).

GST-Sld2 was therefore used to immunoprecipitate Sld3, Sld7 as well as Cdc45. Moreover, it was tested if Dpb11 could bind to Sld2 *in vitro* in the absence of CDK kinase (Figure 6.6). Here a new interaction of Sld2 with Sld3 and Sld7 was observed (Figure 6.6, lanes 5 and 7 respectively). It was also confirmed that Sld2 and Cdc45 cannot interact (Figure 6.6, lane 10) and, surprisingly, a weak interaction was found with Dpb11, although previous data suggested that this interaction required CDK (Figure 6.6, lane 12).

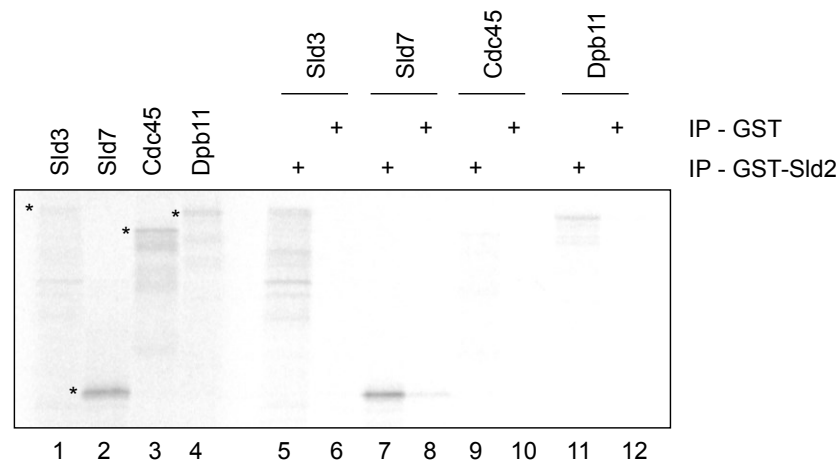


Figure 6.6: Sld2 interacts with Sld3, Sld7 and Dpb11 but not with Cdc45.

GST or GST-Sld2 immobilized on anti-GST magnetic beads were incubated with ^{35}S labelled *in vitro* transcribed and translated Sld3, Sld7, Cdc45 and Dpb11, washed, separated by SDS-PAGE and analysed by autoradiography. Asterisks indicate full length proteins.

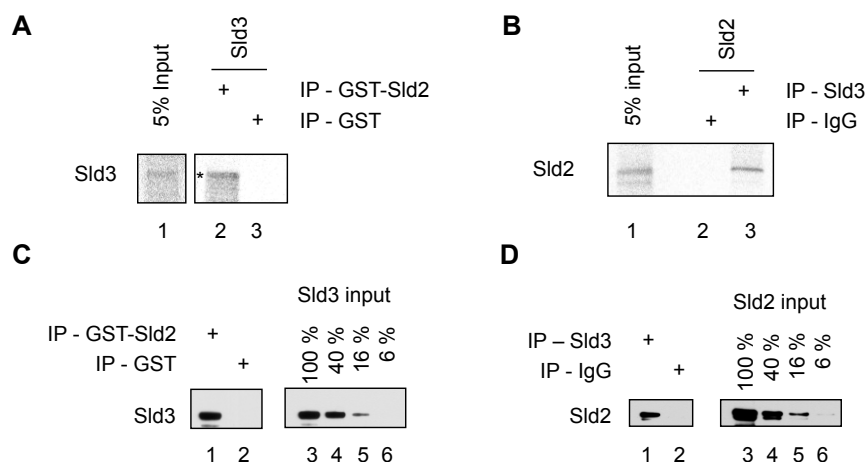


Figure 6.7: Sld2-Sld3 interaction analysis

A) *In vitro* transcribed translated Sld3 interacts with Sld2. GST-Sld2 (400ng) and an equimolar amount of GST, immobilized on magnetic beads, were incubated with ^{35}S labeled *in vitro* transcribed translated Sld3, washed, separated by SDS-PAGE and analyzed by autoradiography. **B)** *In vitro* transcribed translated Sld2 interacts with Sld3. Sld3 (150 ng) immobilized on anti-Sld3 magnetic beads or control beads, were incubated with ^{35}S labeled *in vitro* transcribed translated Sld2, washed, separated by SDS-PAGE and analyzed by autoradiography. **C)** Purified Sld3 interacts with Sld2. GST-Sld2 (400ng) and an equimolar amount of GST, immobilized on magnetic beads, were incubated with purified Sld3 (500 ng), washed, separated by SDS-PAGE and analyzed by western-blot. Lanes 3-6; a dilution series representing % of total Sld3 input is shown. **D)** Purified Sld2 interacts with Sld3. Sld3 (150 ng) immobilized on anti-Sld3 magnetic beads or IgG control beads, were incubated with purified Sld2 (500 ng), washed, separated by SDS-PAGE and analyzed by western-blot. Lanes 3-6; a dilution series representing % of total Sld2 input is shown.

Given that the interaction between Sld3 and Sld2 is of major importance in the context of this work, their binding was confirmed by immunoprecipitating purified Sld3 with GST-Sld2 (Figure 6.7, panel C) as well as using Sld3 to capture purified (Figure 6.7, panel D) and *in vitro* transcribed and translated Sld2 (Figure 6.7, panel C). This analysis proved an interaction between the two proteins and showed that the interaction is independent of the N-terminal GST tag on Sld2, or the N-terminal Myc on Sld3.

The analysis proceeded with the mapping of the binding site on Sld2 using MBP-Sld3 to immunoprecipitate *in vitro* transcribed and translated portions of Sld2. These truncations were produced after the analysis of the secondary structure prediction (Appendix II), which was performed in order to minimise disruption of α -helixes and β -sheets. Full length Sld2 bound to MBP-Sld3 (Figure 6.8, lane 17), but it appeared to be partially degraded similarly to what was previously observed (Figure 6.4). The very N-terminal region of Sld2 did not interact with Sld3 under these conditions (fragments 1-148 and 1-249, Figure 6.8, lanes 11 and 13 respectively). All truncations containing the amino acids 250-327, instead, appeared to bind, but some unspecific signal was also detected for the MBP control in some cases (lanes 14-15, 22-23 and 24-25).

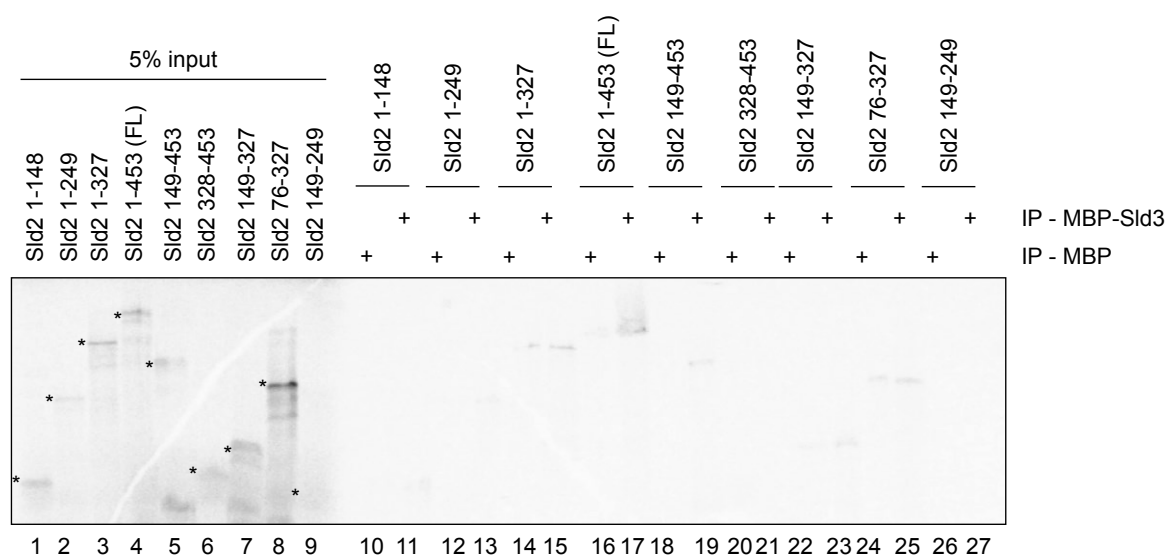


Figure 6.8: Sld2 interaction with Sld3

MBP or MBP-Sld3 immobilized on anti-MBP magnetic beads were incubated with ^{35}S labelled *in vitro* transcribed and translated truncations of Sld2, washed, separated by SDS-PAGE and analysed by autoradiography. Asterisks indicate protein constructs.

Unfortunately the fragment 250-327 was too short to be analysed under the same conditions because even the high percentage acrylamide gel used in the SDS-PAGE for these assays could not resolve peptides smaller than 100 amino acids. An alignment of fungal Sld2 amino acid sequences (Appendix III) was therefore prepared and a limited number of residues were found to be highly conserved in this small region. These amino acids could be mutated in the future in order to identify a version of Sld2, which is defective for the interaction with Sld3.

This analysis however continued with the study of the binding site for Sld2 on Sld3, employing Sld3 peptides as described in paragraph 4.5 and 5.3. The biotinylated peptides (Appendix I, Table IV) were conjugated to streptavidin beads and incubated with *in vitro* transcribed and translated Sld2. Five peptides appeared to bind to Sld2 and corresponded to amino acids 170-195 (CR1b), 196-226 (CR2), 249-287 (CR3a), 286-311 (CR3b) and 333-355 (CR4) of Sld3, while peptides CR1a (152-175), CR5 (384-419) and CR6 (518-546) did not show an interaction (Figure 6.9).

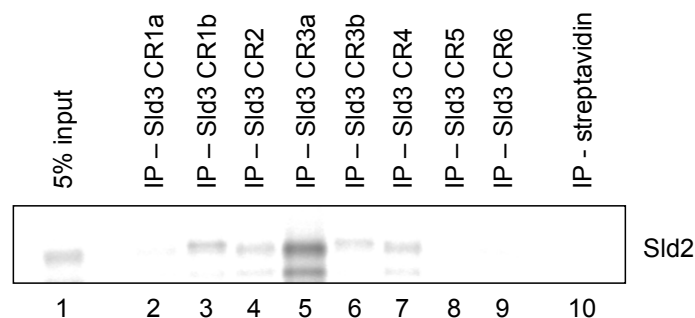


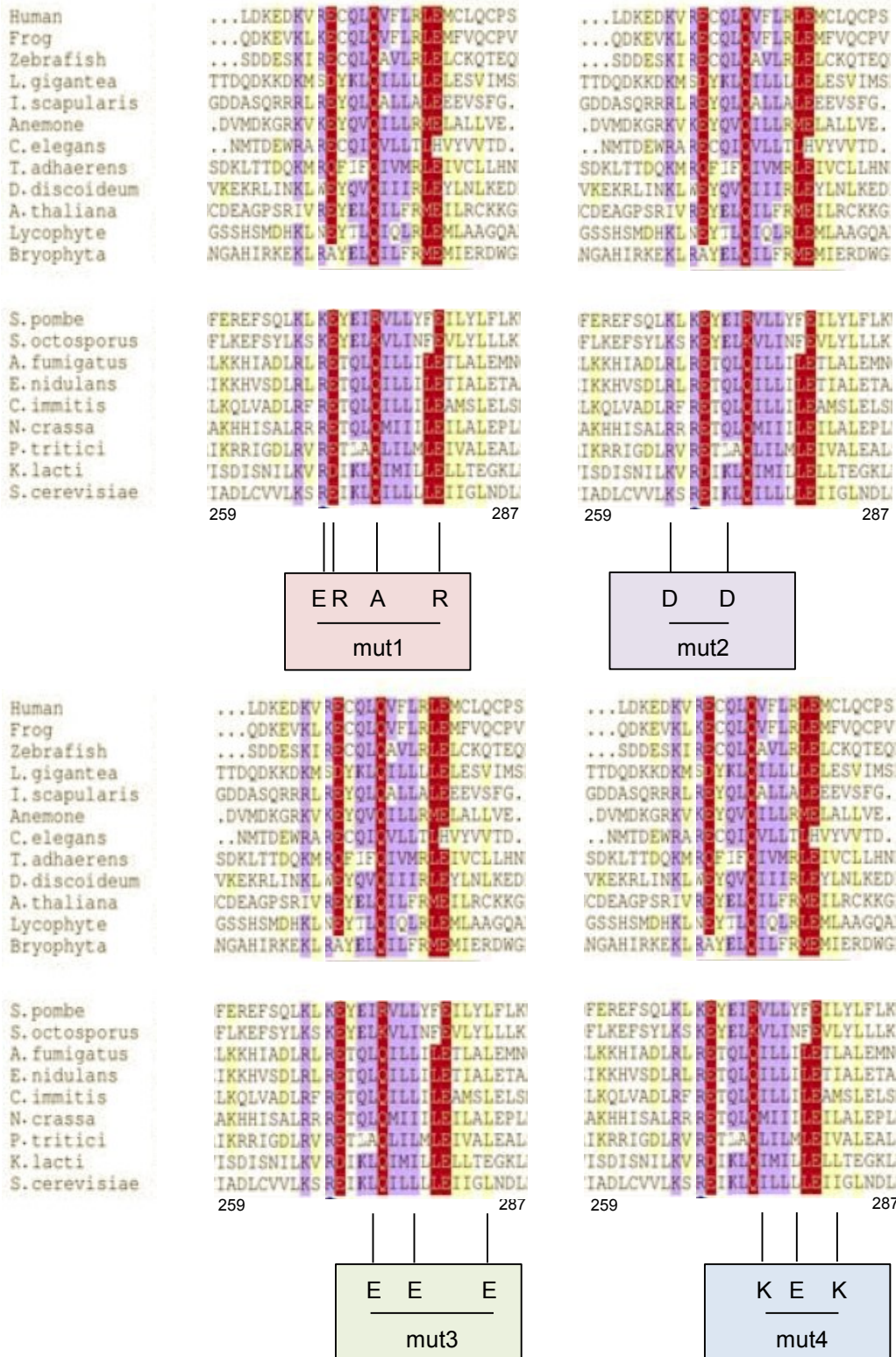
Figure 6.9: Interaction analysis of Sld3 peptides with Sld2

Biotinylated peptides of Sld3 bound to streptavidin beads were incubated with ^{35}S labelled *in vitro* transcribed translated full length Sld2, washed, separated by SDS-PAGE and analysed by autoradiography.

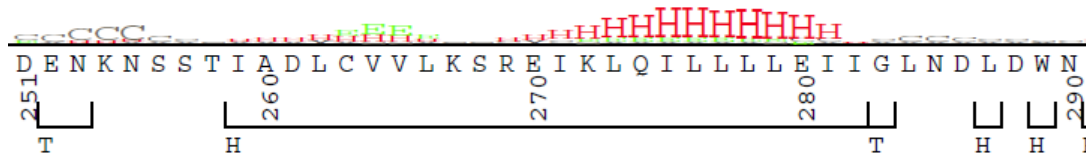
Contrarily to what was observed for Mcm2 and Cdc45, a greater number of peptides interacted with Sld2, suggesting that *in vitro* transcribed and translated Sld2 could interact non-specifically with some of them, or that the binding site on Sld3 involves multiple residues or regions covering amino acids 170-355 of Sld3. The Sld3 peptide CR3a (aa 249-287) however appeared to interact very well with Sld2, indeed stronger than with any other peptide, therefore it was decided to analyse its

sequence starting from the sequence alignment of Sanchez-Pulido and colleagues (Sanchez-Pulido et al, 2010) in order to identify conserved residues that could be responsible for the interaction. The region comprising amino acids 259 and 287 contains a cluster of very conserved residues (Figure 6.10, panel A). The sequence was analysed for a secondary structure prediction as well (Figure 6.10, panel B) and this analysis predicts a helix within the amino acids 269-282. A helical wheel projection of the sequence corresponding to CR3a was therefore performed (Figure 6.10, panel C), as it could help to identify clusters of amino acids with similar charge or hydrophobicity. Indeed, the most conserved residues obtained from the sequence alignment were clustered in four groups based on their characteristics and proximity in the helical projection. They were subsequently mutated following the strategy adopted by Frigola and colleagues (Frigola et al, 2013), where several amino acids were substituted simultaneously, in order to maximise the disruption. Positively charged amino acids were changed for negative charge and vice versa and the ones containing a long side chain were altered for a short side chain and vice versa. The mutations produced are indicated in Figure 6.10, panel A. Two mutants (mut3 and mut4) were produced altering the hydrophobic residues positioned in proximity, whereas mut2 targeted the conserved lysines, which were changed for aspartic acid, finally mut1 altered the remaining conserved amino acids.

A



B



C

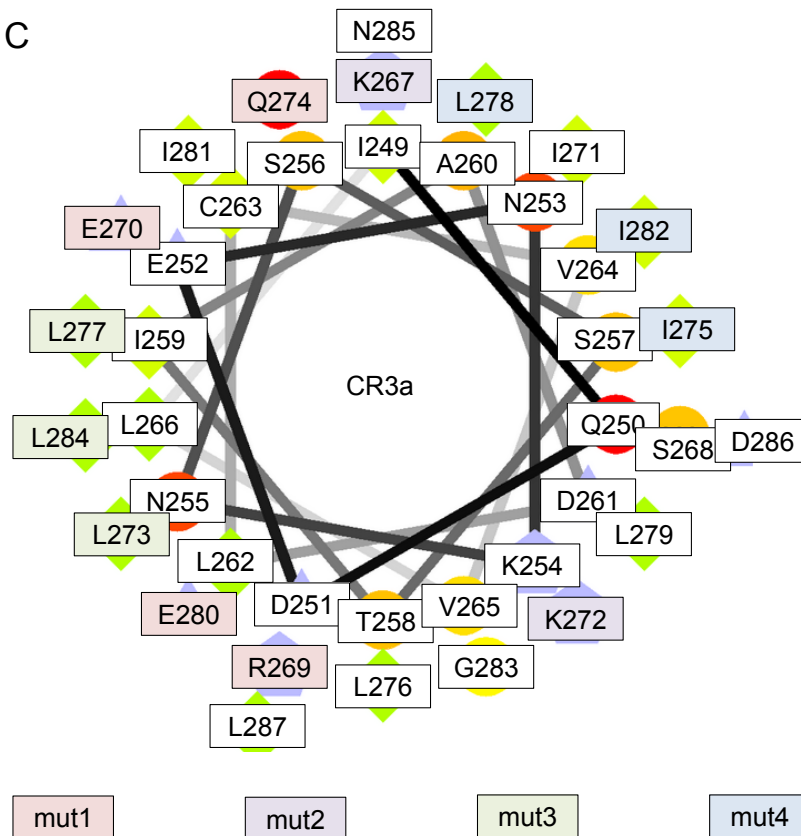


Figure 6.10: Sequence analysis of Sld3 CR3a (aa 249-287)

A) Representative multiple sequence alignments of Treslin/Sld3 family adapted from Sanchez-Pulido's work (Sanchez-Pulido et al, 2010) of the most conserved region of CR3a (amino acids 259-287). The mutated residues are marked. **B)** Secondary structure prediction of the region 250-290. A helix is predicted for the residues 269-282. **C)** Helical wheel projection for the sequence corresponding to CR3a. The most conserved residues were clustered based on their characteristics and proximity in four groups to produce the following mutants: mut1 (wild type mixed) R269E_E270R_Q274A_E280R, mut2 (wild type positively charged) K267D_K272D, mut3 (wild type hydrophobic) L273E_L277E_L248E, mut4 (wild type hydrophobic) I275K_L278E_I282K.

The indicated mutations were produced in the context of the peptide CR3a. The biotinylated peptides (Appendix I, Table IV) were conjugated to streptavidin beads and then incubated with *in vitro* transcribed and translated Sld2. It was found that

mut1 (R269E_E270R_Q274A_E280R) and mut3 (L273E_L277E_L248E) suppressed very strongly the interaction with Sld2 (Figure 6.11, lanes 4 and 6 respectively compared to lane 3). On the other hand, mut2 did not alter the binding compared to the wild type (Figure 6.11, compare lanes 5 and 3) and mut4 showed only a reduced signal (Figure 6.11, compare lanes 7 and 3).

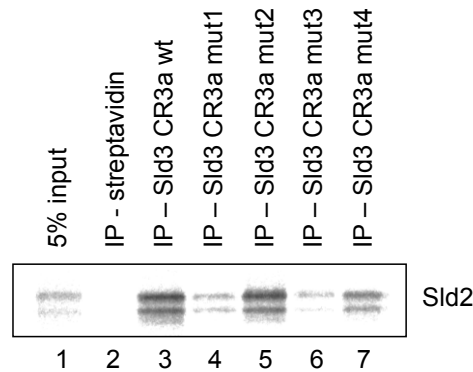


Figure 6.11: Interaction analysis of mutated Sld3 peptides with Sld2

Biotinylated peptides of mutated Sld3 bound to streptavidin beads were incubated with ^{35}S labelled *in vitro* transcribed translated full length Sld2, washed, separated by SDS-PAGE and analysed by autoradiography.

The result of this assay appeared promising, as mutations in a conserved region of Sld3 were suppressing the interaction of Sld2; therefore it was decided to test these mutations *in vivo*. It appears possible that the binding of Sld2 with Sld3 could be essential for its stable interaction with the pre-RC and could facilitate Cdc45 recruitment.

6.2 *In vivo* analysis of Sld3 mutants

The screen with the mutants of CR3a gave some promising results, as some mutants affected the interaction with Sld2 and, in order to perform a comprehensive *in vivo* analysis, more mutants were generated in the peptides CR1b and CR2 following the same strategy described in paragraph 6.1. The peptides covering these two regions in fact were found to interact with Mcm2 and Cdc45 (Figure 4.22 and 5.7 respectively). The CR3b peptide interacted with Mcm2 (Figure 4.22) but this region does not contain well conserved residues that could be targeted for mutation (see Appendix III) therefore it was decided to focus on the other regions during this analysis.

Three of the most conserved residues of CR1b were mutated to produce Sld3 F179R_K181D_R186E (Sld3 CR1b mut) whereas four residues in CR2 were changed to generate Sld3 L213R_F218R_R221E_H222D (Sld3 CR2 mut). The variations are shown in figure 6.12.

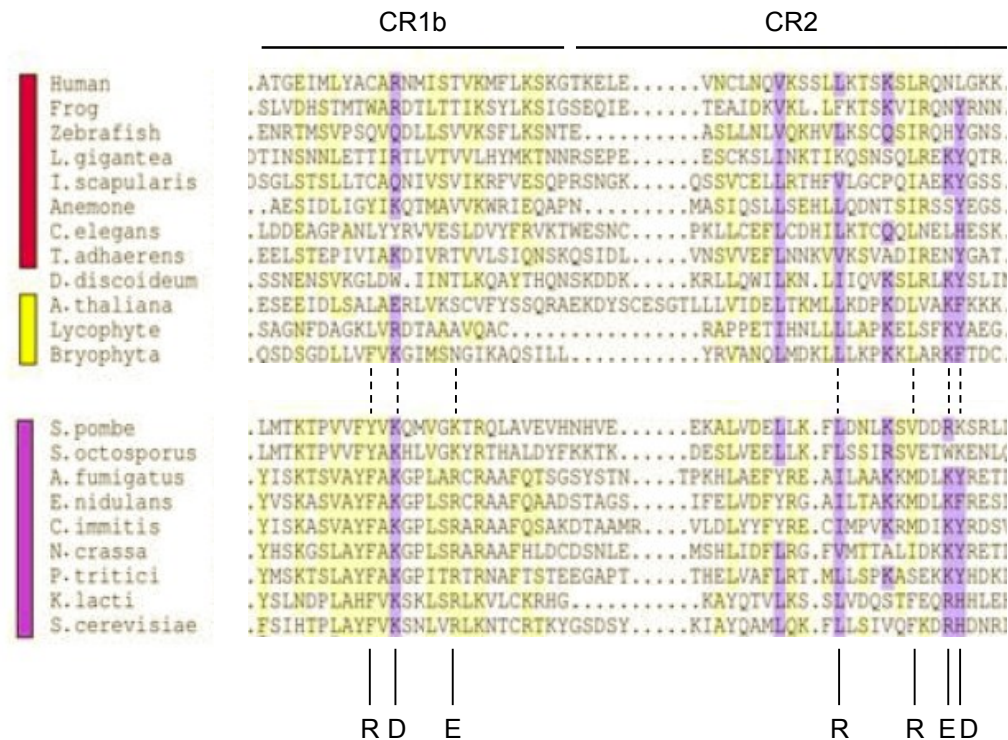


Figure 6.12: Representation of mutants of CR1b and CR2

Representative multiple sequence alignments of Treslin/Sld3 family adapted from Sanchez-Pulido et al (Sanchez-Pulido et al, 2010) for the most conserved region of CR1b (amino acids 170-195) and CR2 (amino acids 196-226). The mutated residues are marked.

To test the Sld3 mutants *in vivo* it was decided to use the Sld3-7td strain developed by the Labib lab (Kanemaki & Labib, 2006). Sld3-7 contains a temperature sensitive mutation that affects the ability of MCM2-7 and Cdc45 to interact during initiation (Kamimura et al, 2001). Cells with this mutation grow well at 24°C, but their growth is reduced at 37°C, therefore this mutant version of Sld3 was combined with the heat-inducible degron cassette (Kanemaki et al, 2003; Sanchez-Diaz et al, 2004) producing a strain that grows almost as well as the control strain at 24°C and exhibits severe growth defect at 37°C (Kanemaki & Labib, 2006). The addition of the degron cassette to wild type Sld3 on the contrary does not prevent growth at 37°C (Kanemaki & Labib, 2006). Indeed, Sld3 is a limiting factor for DNA

replication (Tanaka et al, 2011a), therefore it is possible that even a small amount of functional Sld3 in the cell is sufficient to support growth. For this reason a temperature sensitive allele of Sld3 was combined by the Labib lab with the degron technology.

The degron cassette is recognised by the Ubr1 protein that is associated with an ubiquitin-conjugating enzyme, and proteolysis is stimulated at higher temperatures, because the unfolding of the degron exposes lysines that are sites of ubiquitylation (Dohmen et al, 1994). Efficient proteolysis of the degron requires increased expression of the Ubr1 protein (Labib et al, 2000) therefore the only copy of the *UBR1* gene was expressed under the control of the galactose-inducible promoter CUP1. The CUP1 promoter additionally requires the addition of CuSO₄ to the medium. In this way the cells can be grown at the permissive temperature of 24°C with normal Ubr1 protein levels and after a temperature shift to the restrictive temperature (37°C) galactose addition triggers strong expression of Ubr1 and rapid degradation of the target protein (Sanchez-Diaz et al, 2004). This strain allows the phenotypic analysis of Sld3 mutants in the absence of its wild type copy.

For the expression of the mutant versions of Sld3 a yeast centromere plasmid was chosen (pRS416). These vectors have both a replication origin and a centromere sequence and are propagated at a very low copy-number, autonomously replicating due to the presence of a yeast replication origin and efficiently segregated as the plasmid contains a yeast centromere sequence, in addition to a selectable marker (Stearns et al, 1990).

The *SLD3* gene with mutations in CR1b, CR2, CR3a mut1 and mut3, preceded by the natural promoter, were cloned into the yeast centromere plasmid and transformed into the Sld3-7td strain described above. Moreover, the Sld3-7td strain transformed with the empty vector that does not express any protein served as a negative control, while the transformation of the plasmid containing non modified *SLD3* gene served as positive control, as this plasmid should effectively rescue the lethality conferred by the degradation of the endogenous protein.

The strains were grown in selective media and spotted in a 5 fold serial dilution series on a selective plate containing either glucose (no Ubr1 expression) or galactose (Ubr1 expression) in the presence of CuSO₄ and incubated at the permissive temperature of 24°C, a condition in which the Sld3-7 mutant does not show growth defects. The strains were also spotted on a selective plate containing

either glucose and CuSO₄ (no Ubr1 expression) or galactose (Ubr1 expression) and incubated at the restrictive temperature of 37°C, condition in which the Sld3-7 mutant shows severe growth defect. CuSO₄ is omitted from the galactose plate incubated at 37°C because it stimulates the transcription of the degron fused protein from the CUP1 promoter and in this condition the degradation of the Sld3-7 is instead wanted (Kanemaki et al, 2003; Sanchez-Diaz et al, 2004).

All strains analysed appeared to grow similarly well at the permissive temperature and with inactivated Ubr1 (glucose plate at 24°C, Figure 6.13 panel A). The strains expressing CR1b mut, CR2 mut and CR3a mut1 show a slightly reduced growth of colonies compared to the other strains. This could be due to a weak dominant lethal effect of these mutants. Indeed, overexpression of Ubr1 at the permissive temperature revealed a strong dominant lethality of the mutants CR1b mut, CR2 mut and CR3a mut1, suggesting that in these conditions the Sld3 concentrations dropped sufficiently to reveal the observed effect (galactose plate at 24°C, Figure 6.13 panel B). When the strains were analysed at the restrictive temperature with inactivated Ubr1 (glucose plate at 37°C, Figure 6.13 panel C), they all appeared to be able to grow. Strains expressing CR1b mut, CR2 mut and CR3a mut1 showed again slightly reduced growth under these conditions, consistent with what was observed at the permissive temperature.

At the restrictive temperature and in case of Ubr1 overexpression, namely when endogenous Sld3 is degraded, only the strain transformed with the plasmid containing wild type Sld3 could grow (galactose plate at 37°C, Figure 6.13 panel D). This indicates that the lethal effect of the degron (observed for the control strain transformed with the empty vector) can effectively be rescued by the addition of Sld3 and also, importantly, that all mutants analysed cannot complement the strain.

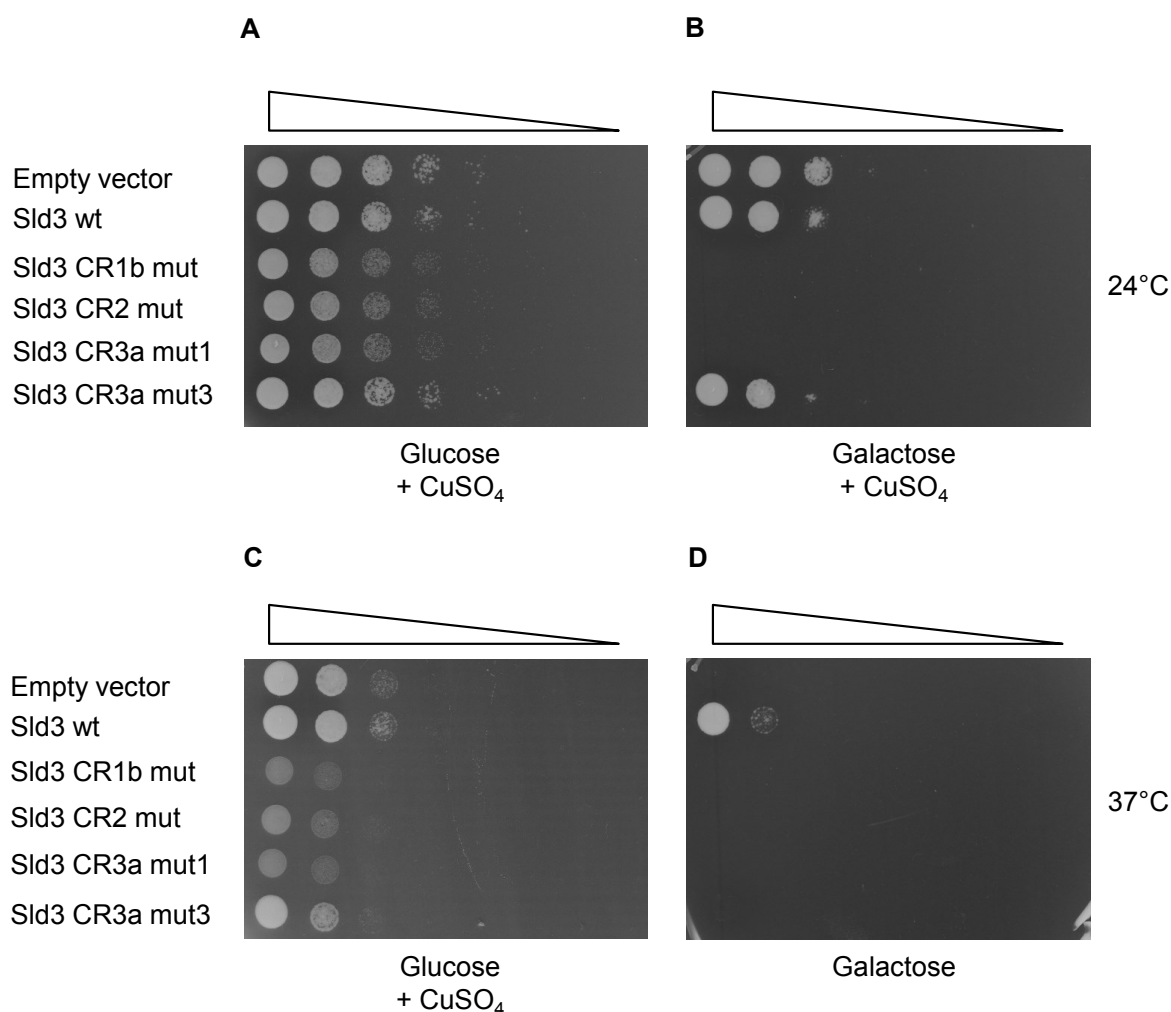


Figure 6.13: Growth assay for Sld3 mutants

Yeast growth assay showing the effect of expression of the indicated proteins in the Sld3-7td strain. A 5 fold serial dilution of cells (starting from 1×10^7) was spotted onto Kaiser plates lacking uracil with either 2% glucose or 2% galactose and 0.1 mM CuSO₄ as indicated. Plates were incubated for 3 days at the specified temperature.

This assay shows that the mutations affect an essential function of Sld3, likely involving DNA replication. Unfortunately, it is not possible to claim with this experiment only, that the growth defect observed is indeed due to disruption of one of the interactions studied in this project and described in this and the previous chapters.

To analyse the effect of the mutations on pre-IC assembly, these strains were therefore grown in liquid media and arrested in G1-phase before synchronous release into S-phase, chromatin fractionation of the samples and western blot analysis for the essential proteins involved in initiation of DNA replication (MCM2-7, Sld3, Sld2, Cdc45, GINS). The purpose of this experiment was to prove that

mutations in Sld3, which affect *in vitro* the interaction with Mcm2, Cdc45 or Sld2, had an impact on pre-IC assembly during initiation of replication *in vivo*.

The experimental setting was a variation of what was previously described by Kanemaki & Labib (Kanemaki & Labib, 2006) and Sheu & Stillman (Sheu & Stillman, 2006). Briefly, it included the growth of the culture in selective liquid media in the presence of raffinose as a carbon source (to facilitate the subsequent switch to galactose), the arrest in G1-phase with α factor in raffinose for 3 hours at the permissive temperature (to maintain the repression of Ubr1) followed by 40 additional minutes in galactose (to induce Ubr1 expression while still in G1-phase), the shift to the restrictive temperature in galactose for 1 hour in presence of α factor (to boost the degradation of the degron cassette and therefore of endogenous Sld3) before the release in S-phase in presence of hydroxyurea, which allows the firing of early replication origins, but then slows the cell cycle progression due to dNTPs starvation. A sample was collected before the release in S-phase and at 90 and 120 minutes into S-phase. The samples were spheroplasted to degrade the cell wall and finally the chromatin was isolated by centrifugation through a sucrose cushion. The chromatin bound and soluble fractions were analysed by SDS-PAGE followed by western blot and probed against the essential proteins for initiation of replication (MCM2-7, Sld3, Sld2, Cdc45, GINS).

Unfortunately the arrest/release conditions, as well as the chromatin fractionation, required some optimisation and a conclusive result was not obtained in the timeframe of this project. It remains a very valuable experiment to perform in the future in order to achieve a final proof of the relevancy of the newly identified mutants of Sld3.

6.3 Analysis of the trimeric complex Sld3/Sld2/Sld7

During the characterisation of the complex containing Cdc45 (Figure 6.1), Herrera observed that Sld3 and Sld2 could interact with the pre-RC in the presence of DDK and they were rapidly displaced from the complex when incubated longer (Figure 6.14, panel A). Interestingly, it was also found that the addition of Sld7 to this reaction would result in the lack of recruitment of Sld3, Sld2 and Sld7 to the pre-RC (Figure 6.14, panel B), suggesting that these three proteins could form a complex in solution that inhibits its interaction with the pre-RC. Finally, a striking recruitment of

Sld3, Sld2 and Sld7 in the presence of Cdc45 was observed (Figure 6.14, panel C) that was much more stable over time compared to what was observed for Sld3 and Sld2. Over time Sld3, Sld2 and Sld7 would get displaced from the complex, leaving only Cdc45 at this point stably bound to the pre-RC (Figure 6.14, panel C, lane 8).

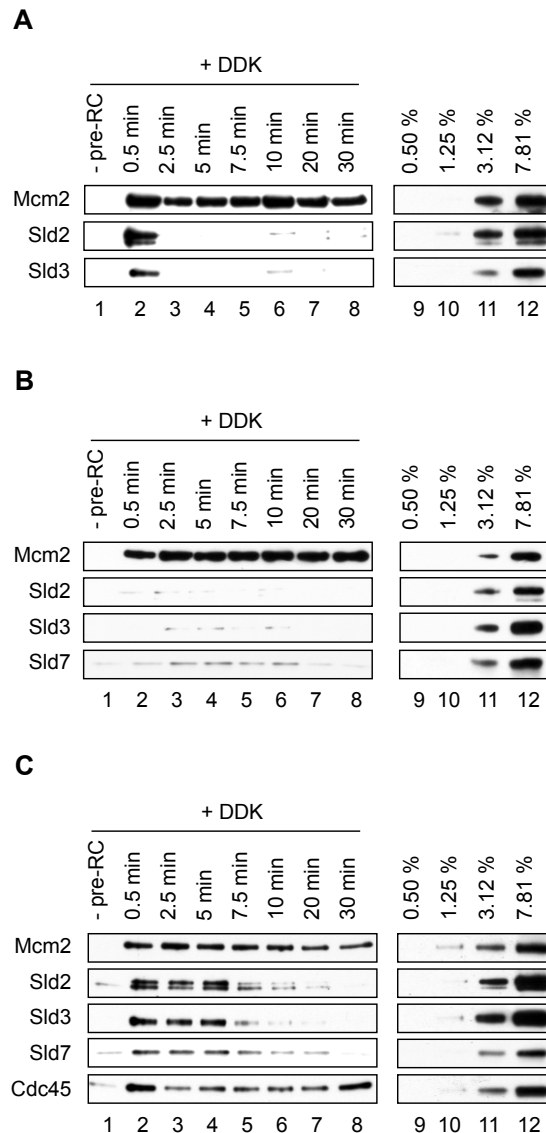


Figure 6.14: Sld3, Sld2, Sld7 release from the pre-RC

A) Time course analysis of Sld2 and Sld3 association with the pre-RC in the presence of DDK (5 nM). **B)** Time course analysis of Sld2, Sld3 and Sld7 association with the pre-RC in the presence of DDK (5 nM) **C)** Time course analysis of Cdc45, Sld2, Sld3 and Sld7 association with the pre-RC in the presence of DDK (5 nM). In all cases, after pre-RC formation the additional proteins were added and incubated for the indicated time points. The reaction omitting the pre-RC proteins (-pre-RC) was incubated for 1 minute. Lanes 9-12 show a dilution series representing % of total protein (40 nM), which were added into the reactions. All experiments in this figure were performed by Herrera.

This experiment provides evidence that Cdc45 is recruited as a consequence of Sld3, Sld2 and DDK action, given that Cdc45 could not be found in a complex with the pre-RC (Figure 5.2). An extensive discussion of these results from the point of view of Cdc45 recruitment will be found in Chapter 7.

The outcome of these assays, however, is suggesting that a trimeric complex between Sld3, Sld2 and Sld7 could form in solution, given that Sld7 blocked the recruitment of Sld3 and Sld2 to the pre-RC (Figure 6.14, panel B) and also that Sld3, Sld2 and Sld7 are released from the pre-RC in a synchronous manner (Figure 6.14, panel C).

It was tested whether Sld7 could interact with Sld3, Sld2 and Cdc45 in a similar fashion. The interaction of Sld7 with Sld3 was already described by the Araki lab (Tanaka et al, 2011a; Tanaka et al, 2011b) *in vivo* and with the two hybrid system. Sld2 and Sld7 were found to interact in this study (Figure 6.6) whereas the physical binding of Sld7 to Cdc45 was never reported.

The direct interaction of Sld7 with the other proteins was therefore tested. MBP-Sld3, GST-Sld2 and GST-Cdc45 were used to immunoprecipitate *in vitro* transcribed and translated Sld7, similarly to what was described in the previous chapters.

It was found that both Sld3 and Sld2 are immunoprecipitated very well by Sld7 (Figure 6.15, panels A and B respectively), whereas Cdc45 is interacting but to a lesser extent (Figure 6.15, panel C).

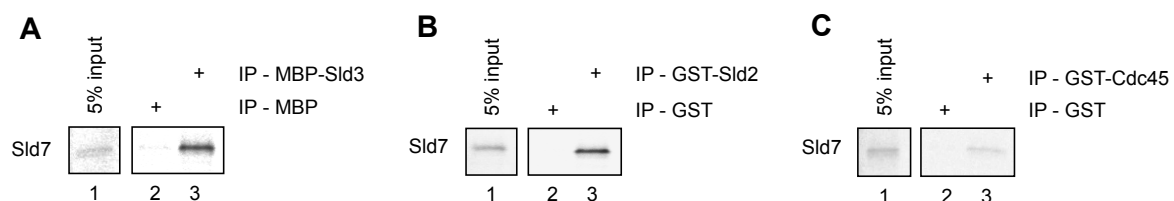


Figure 6.15: Interaction analysis of Sld7 with Sld3, Sld2 and Cdc45

A) *In vitro* transcribed and translated Sld7 interacts with Sld3. MBP-Sld3 (400 ng) and an equimolar amount of MBP, immobilized on magnetic beads, were incubated with ^{35}S labeled *in vitro* transcribed and translated Sld7, washed, separated by SDS-PAGE and analyzed by autoradiography. **B)** *In vitro* transcribed and translated Sld7 interacts with Sld2. GST-Sld2 (400 ng) and an equimolar amount of GST, immobilized on magnetic beads, were incubated with ^{35}S labeled *in vitro* transcribed translated Sld7, washed, separated by SDS-PAGE and analyzed by autoradiography. **C)** GST-Cdc45 (400 ng) and an equimolar amount of GST, immobilized on magnetic beads, were incubated with ^{35}S labeled *in vitro* transcribed and translated Sld7, washed, separated by SDS-PAGE and analyzed by autoradiography.

To test the possibility that Sld3, Sld2 and Sld7 could form dimeric or a trimeric complex in solution, the purified proteins were incubated with each other and cross-linked with glutaraldehyde, similarly as previously described for the pre-RC complex (Evrin et al, 2013).

Initially different concentrations of glutaraldehyde were tested in order to find an optimal condition for the treatment of the proteins analysed (Figure 6.16, panel A). For this experiment the four subunit GINS complex (containing Sld5, Psf1, Psf2 and Psf3) was used as a positive control to monitor the success of the crosslinking reaction (Figure 6.16, panel A, lanes 21-25). Sld3, Sld2 and Sld7 were analysed alone or all together, the samples were separated by SDS-PAGE and the proteins were visualized by silver staining. The experiment was then repeated under the best cross-linking condition (0.1% glutaraldehyde) for all protein combinations and analysed by Western Blot, probing with specific antibodies against the proteins tested (Figure 6.16, panels B-D).

Sld5, Psf1, Psf2 and Psf3 are known to interact forming the GINS complex (Takayama et al, 2003) and they were successfully cross-linked here with concentrations of 0.1% glutaraldehyde and upwards (Figure 6.16, panel A, lane 24). A weak high molecular weight complex can be already detected at 0.02% (Figure 6.16, panel A, lane 23), but then still uncross-linked subunits can be detected, suggesting that SDS can still denature the complex under those conditions. With higher concentrations of glutaraldehyde a large complex is detected. The addition of more glutaraldehyde results in a faster migration during SDS-PAGE, as the complex becomes stabilised in its very compact folded structure.

When Sld3 was analysed by itself in the presence of the cross-linking agent, two species could be observed, one with a molecular weight below 77 kDa expected for Sld3 monomer, and another one with a molecular weight considerably higher than 77 kDa, but well below 200 kDa. This high molecular weight species could be possibly a Sld3 dimer. Once the samples were analysed by western blot using a specific antibody against Sld3 (Figure 6.16, panel B), it was possible to confirm that the species with a molecular weight similar to the 66 kDa marker was indeed Sld3 (Figure 6.16 A, lane 9 and Figure 6.16 B, lane 4). No signal was instead detected for the higher molecular weight band observed in the silver staining gel (Figure 6.16, panel A, lane 9 in box), suggesting that either the epitope of Sld3 is masked in the

conformation of the dimer or that it was produced by contaminants present in the sample and it has nothing to do with Sld3.

The Sld2 protein used for this experiment showed some contaminant bands derived from the purification process (Figure 6.16, panel A, lane 11) and the addition of glutaraldehyde did not appear to result in the formation of new species (Figure 6.16 A, lanes 12-15). At the higher concentration of cross-linking reagent however a strong signal appeared, possibly due to a low molecular weight contaminant modified under these conditions. The western blot against Sld2 confirmed the absence of additional higher molecular weight species for the cross-linked sample compared to the untreated one (Figure 6.16 C, compare lane 5 to 2).

Sld7 was analysed in the same way and upon addition of glutaraldehyde, the appearance of a new high molecular weight species was detected in the silver stained gel (Figure 6.16 A, lanes 19 and 20), likely belonging to a Sld7 dimer. The identity of the complex was therefore confirmed by western blot (Figure 6.16 D, lanes 6).

The possible formation of dimers between two proteins, as well as the formation of a trimeric complex, was tested next.

The combination of Sld3 and Sld7 was tested first. No signal was detected with the Sld3 antibody, neither at the size of cross-linked Sld3 alone nor at a higher molecular weight (Figure 6.16 B, lane 7). For the same condition Sld7 antibody showed a reduction of the signal at the molecular weight of cross-linked Sld7 and the appearance of several high molecular weight species (Figure 6.16 D, lane 7). This suggests that the two proteins are indeed forming a complex which could be a Sld3/Sld7 dimer or some form of a multimer. Moreover, the absence of the Sld3 signal suggests that the epitope for Sld3 antibody is hidden in this structure.

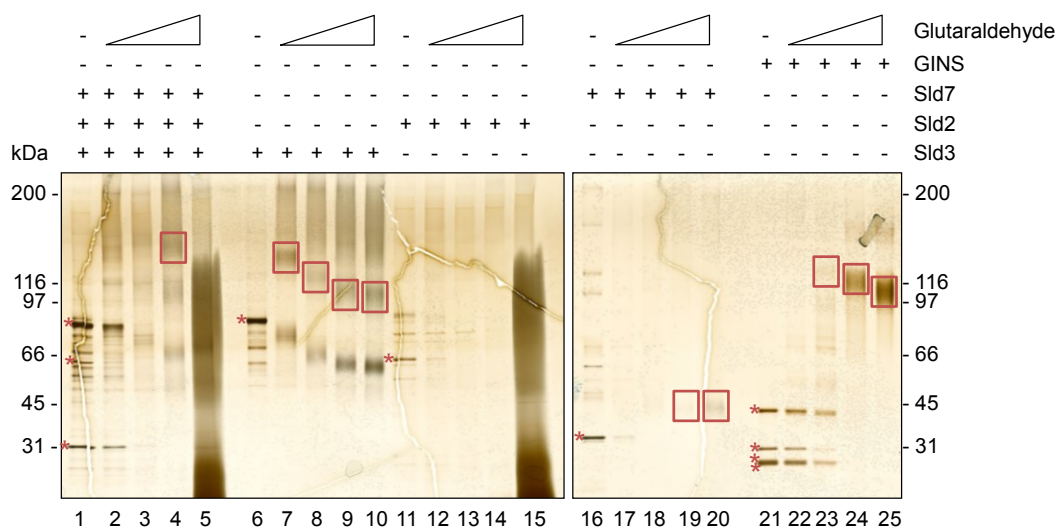
When Sld3 and Sld2 were cross-linked together, the signal detected for cross-linked Sld3 alone disappeared and a new high molecular weight band was detected instead (Figure 6.16 B, lane 8), possibly due to a Sld3/Sld2 dimer. The analysis with Sld2 antibody revealed two bands, which were absent in the sample of Sld2 cross-linked by itself. One complex weighs putatively around 80 kDa therefore it is unlikely to be Sld3/Sld2. The other signal is detected at very high molecular weight and it could either indicate an aggregate stuck in the well of the gel or a multimer of Sld2. Unfortunately this data does not conclusively indicate the nature of the complexes detected.

The combination of Sld2 and Sld7 was then tested. No new species were detected with the anti Sld2 antibody in comparison with the sample containing Sld2 alone (Figure 6.16 C, compare lane 9 to 5). The western blot against Sld7 revealed instead a very different profile compared to the sample containing only Sld7 (Figure 6.16 D, compare lane 9 to 6). In this case the signal belonging to Sld7 monomer reappears and several new higher molecular weight bands can be observed as well (two main signals and two weak above 66 kDa). This data suggests that Sld2 is indeed interacting with Sld7 or, alternatively, Sld2 induces Sld7 multimerisation. It is not possible to state that the two proteins form a dimeric complex because no signal was detected in the Sld2 western blot but, once again, the epitope for the antibody could be masked. It is quite likely though that Sld7 is in a complex either with other Sld7 molecules or with Sld2.

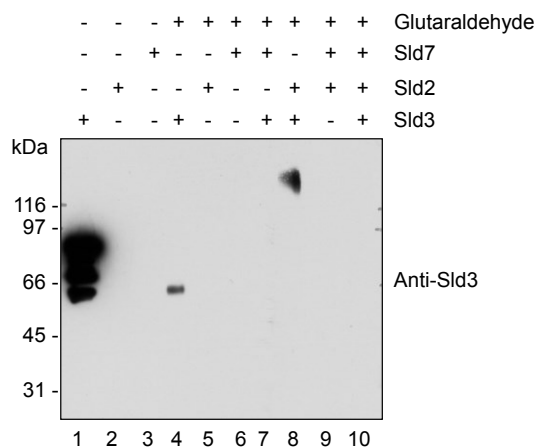
Finally the combination of Sld3, Sld2 and Sld7 was analysed. The silver staining reveals the formation of a new species at a molecular weight higher than 116 kDa (the trimer has an estimated size of 160 kDa) (Figure 6.16 A, lane 4). The western blot for Sld3 reveals no signal at all (Figure 6.16 B, lane 10), similar to what was observed for the sample with Sld3 and Sld7 (Figure 6.16 B, lane 7), suggesting that a complex was formed and that the epitope for Sld3 was hidden in this conformation. The western blot with Sld2 antibody (Figure 6.16 C, lane 10) showed a profile which was very similar to the one of the sample containing Sld3 and Sld2 (Figure 6.16 C, lane 8) with an additional band around 116 kDa. The analysis with anti Sld7 antibody showed a similar profile as with Sld7 and Sld2, but the signal was attenuated (Figure 6.16 D, lane 9) and a signal at higher molecular weight was detected, at a molecular weight of 116 kDa, similarly to what observed with Sld2 antibody (Figure 6.16 C, lane 10).

These data unfortunately are not sufficient to prove the formation of a trimeric complex in solution but strongly suggest that Sld3, Sld2 and Sld7 have the potential to associate with one another in different ways.

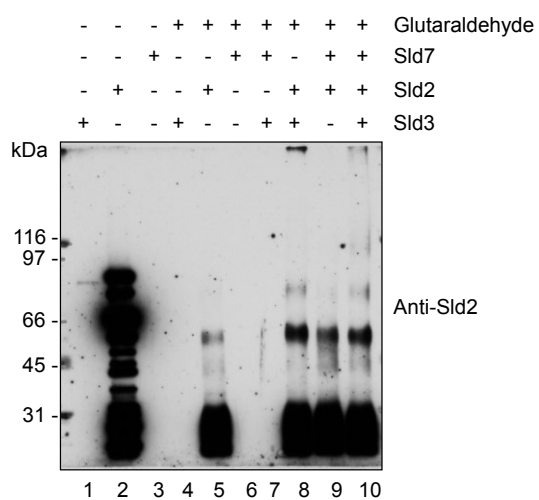
A



B



C



D

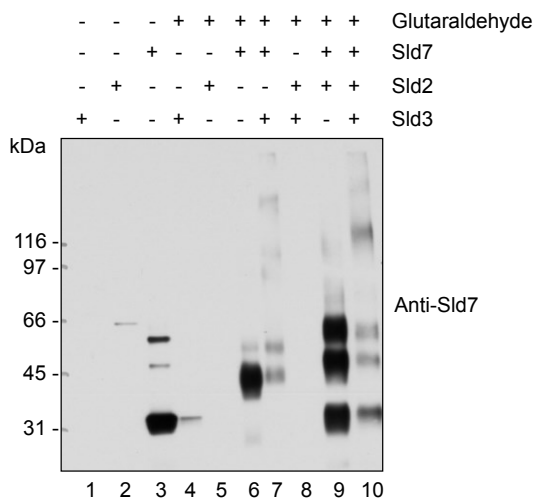


Figure 6.16: Sld3, Sld2 and Sld7 cross-linked in solution

A) 100 ng of Sld3 and equimolar amount of Sld2, Sld7 and GINS were incubated for 10 min on ice followed by 5 minutes at 24°C while shaking before the addition of glutaraldehyde (5 fold dilution: 0.004-0.02-0.1-0.5%) for 5 min at 4°C. The samples were analysed by SDS-PAGE followed by silver staining. Asterisks indicate full length proteins, boxes indicated high molecular weight species. **B-C-D)** 100 ng of Sld3 and equimolar amount of Sld2, Sld7 and GINS were incubated with 0.1% glutaraldehyde as described for panel A. The samples were analysed by SDS-PAGE followed by western blot against Sld3 (B), Sld2 (C) and Sld7 (D). Molecular weights: Sld3 77 kDa, Sld2 52 kDa, Sld7 29 kDa; subunit of GINS: Sld5 34 kDa, Psf1 24 kDa, Psf2 25 kDa, Psf3 22 kDa.

To prove conclusively that a trimeric complex is formed, other techniques need to be employed such as gel filtration or glycerol / sucrose gradient sedimentation, or immunoprecipitation of the complex using one of the proteins and probing for the other two.

6.4 DDK phosphorylation of the proteins involved

In this section, the role of DDK in modifying factors involved in Cdc45 recruitment to the pre-RC was investigated.

The activity of DDK in initiation of replication has been reported *in vivo* in several papers (Francis et al, 2009; Gros et al, 2014; Heller et al, 2011; Jackson et al, 1993; On et al, 2014; Randell et al, 2010; Sheu & Stillman, 2006; Sheu & Stillman, 2010), as well as the specific requirement of DDK for stable Cdc45 recruitment to the origins (Heller et al, 2011), which is consistent with the observations presented here (Figure 6.1, panel C).

So far the post-translational modification of MCM2-7 by DDK was extensively studied (Francis et al, 2009; Gros et al, 2014; On et al, 2014; Randell et al, 2010; Sheu & Stillman, 2006; Sheu & Stillman, 2010) but not much is known on DDK dependent phosphorylation of other replication factors. For this reason, a kinase assay was performed employing purified proteins. The purified proteins involved in Cdc45 recruitment to the pre-RC (Sld3, Sld2 and Sld7) were incubated with increasing amounts of DDK at 27°C in the presence of γ -³²P-ATP and analysed by silver staining and autoradiography. 20 nM of DDK, the highest concentration used in the assay, was tested by itself as well and served as negative control to identify the signal caused by autophosphorylation of Dbf4 (Lee et al, 2012).

Among the proteins tested, only Sld3 appeared to incorporate radioactive ATP upon addition of increasing amounts of DDK, specifically at the concentrations of 5 nM (lane 6) and 20 nM (lane 7) (Figure 6.17, panel A), where the band is additionally upshifted. In addition, a weak signal was detected for all DDK concentrations and even in the absence of kinase.

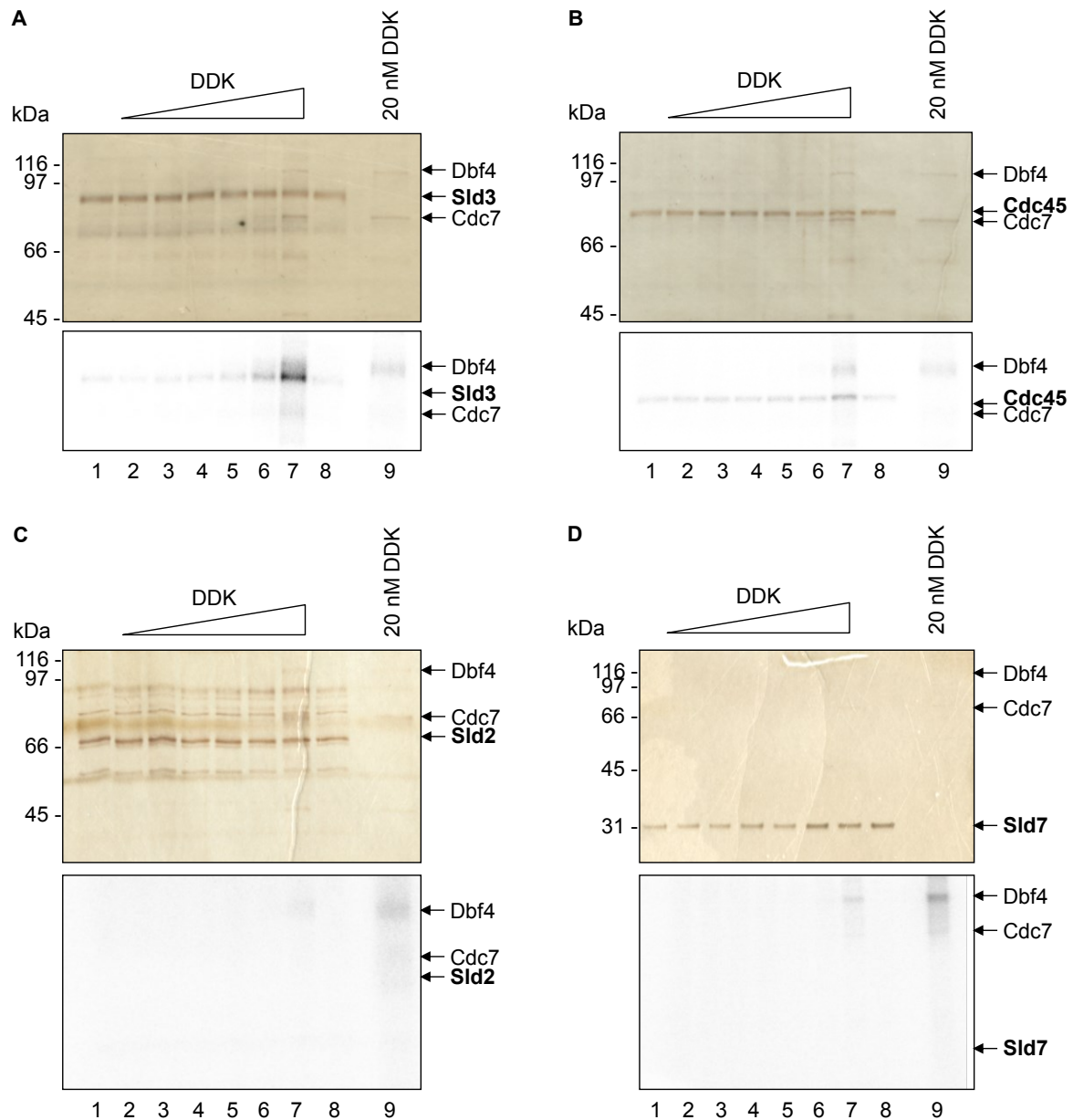


Figure 6.17: DDK phosphorylation assay for Sld3, Cdc45, Sld2 and Sld7

40 ng of (A) Sld3, (B) Cdc45, (C) Sld2, and (D) Sld7 were incubated with no DDK (lanes 1 and 8) or with a 4 fold dilution (0.02 to 20 nM) of DDK (lanes 2-7) for 15 min at 27°C in the presence of γ -³²P-ATP. The samples were separated by SDS-PAGE and analysed by silver staining and autoradiography. Lane 9 contains 20 nM of DDK in the absence of additional proteins.

Cdc45 as well exhibited a weak signal even in the absence of DDK, indicating that the protein was incorporating could incorporate γ -³²P-ATP by autophosphorylation or due to a contamination, but no change was observed upon addition of DDK (Figure 6.17, panel B). Sld2 and Sld7 did not show any incorporation of γ -³²P-ATP (Figure 6.17, panels C and D respectively), indicating that these proteins cannot be phosphorylated by DDK under these conditions.

DDK promotes the recruitment of Cdc45 to the pre-RC. Most likely DDK functions here by phosphorylating Mcm2, Mcm4 and Mcm6 of the loaded MCM2-7 double-hexamer, but it will be interesting to further explore the role of the newfound modification on Sld3 in this respect.

6.5 Discussion

The work described in this chapter started with the finding by a colleague, who showed that the recruitment of Cdc45 to the pre-RC is *in vitro* not only dependent on Sld3 and DDK, as previously described (Heller et al, 2011; Kamimura et al, 2001; Kanemaki & Labib, 2006), but also on Sld2 (Figure 6.1). Sld2 was never shown to have a role for Cdc45 recruitment. The only function proposed for this protein was in fact to bind Dpb11 upon CDK phosphorylation (Tanaka et al, 2007b; Zegerman & Diffley, 2007) and take part in the transient pre-LC complex (Muramatsu et al, 2010) together with Dpb11, GINS and Pol ϵ to facilitate the formation of the CMG. This intriguing finding led to an extensive investigation in order to obtain a substantial model of Cdc45 recruitment to the pre-RC. The work of Heller and colleagues (Heller et al, 2011) in fact appears to contradict this discovery. Heller employed an extract based system to assemble protein complexes, which depends on the overexpression of Sld2, Sld3, Cdc45 and Dpb11. Using this system they observed a DDK and Sld3 requirement for Cdc45 recruitment to the pre-RC, but the depletion of Sld2 had no effect on Cdc45. It is possible to argue that the overexpression of these limiting factors in the S-phase extract used by Heller is responsible for this discrepancy. Under these conditions the requirement of Sld2 could be bypassed by the large number of Sld3 and/or Cdc45 molecules available for interaction with limited pre-RC complexes. Indeed, overexpression of Cdc45 by itself is sufficient to bypass a Sld3 temperature sensitive allele (Kamimura et al,

2001; Tanaka et al, 2007b), indicating that overexpression of these factors could cause synthetic effects.

A number of experiments were therefore performed in order to analyse how Sld2 could function for Cdc45 recruitment to the pre-RC. No evidence was available that Sld2 would interact directly with the pre-RC at any point of the cell cycle, therefore the pre-RC reconstitution assay was used to determine whether Sld2 could interact with the pre-RC *in vitro*. It was observed that Sld2 can be recruited to the pre-RC rapidly and stably in the absence of additional factors (Figure 6.2 panel A).

These data in combination with the observation by the Kaplan lab, that showed Sld2 interaction with MCM2-7 in the context of purified proteins in solution (Bruck et al, 2011), prompted us to study which MCM2-7 subunit is interacting with Sld2. Cdc45 in fact cannot bind neither the pre-RC alone nor the pre-RC/Sld3 complex so it appears reasonable to think that Sld2 either directly bridges the interaction of Cdc45 or produces a conformational change on the pre-RC/Sld3 complex to facilitate the binding. Sld2 was therefore expected to interact with Mcm2 (subunit of interaction of Sld3 and Cdc45) or an adjacent subunit of MCM2-7 in order to be able to exert this function. GST-Sld2 was therefore used to immunoprecipitate *in vitro* transcribed and translated MCM2-7 subunits (Figure 6.2, panel C) and indeed it was found that it interacted weakly but specifically with Mcm2, similarly as observed for Sld3 (Figure 4.8, lane 8) and Cdc45 (Figure 5.5, lane 8).

The simultaneous binding of three proteins to a single Mcm subunit, namely Mcm2, appears difficult for spatial reasons and it is more reasonable to speculate that the binding site for these three proteins is only partially formed by Mcm2 and additionally involves one or more adjacent subunits.

In this regard it is interesting to note that Herrera observed competitive Sld2 and Sld3 interactions with the pre-RC (Herrera, Tognetti et al, under revision). Both proteins appeared to be efficiently recruited in the first case but, when added in combination, their signal, and especially that of Sld2, was decreased (Figure 6.3, panel A, compare lane 2 and 3 with 4). This suggested that the two proteins cannot be stably recruited to the pre-RC. However, upon addition of DDK and Cdc45, the pre-RC undergoes an apparent conformational change (Figure 6.1), as now a stable complex was observed. Indeed, Cdc45 could stabilise the complex, perhaps bridging some interactions. Interestingly, Herrera also found that DDK could negate competitive Sld2 and Sld3 interactions to the pre-RC and induce formation of a

stoichiometric pre-RC/Sld2/Sld3 complex (Figure 6.3, panel B). To determine how this could happen, the binding site for Sld2 on Mcm2 was mapped, finding that Sld2 was precipitating the C-terminal Mcm2 (Figure 6.4), contrary to what observed for Sld3 (interaction with the N-terminus, Figure 4.9).

This result indicates that Sld3 and Sld2 do not compete for the binding site on Mcm2, but that the interaction of Sld3 with the Mcm2 N-terminus causes an hindrance that impairs Sld2 binding to the C-terminus. The N-termini of the MCM2-7 subunits are unstructured regions and therefore it seems possible to think that this region could be flexible and assume, upon Sld3 binding, a folding that would obstruct the Sld2 binding site at the C-terminus. A model was drawn where DDK dependent phosphorylation of MCM2-7 could produce a conformational change in the complex allowing Sld2 binding site on Mcm2 to be exposed again (Figure 6.5). The DDK dependent regulation of the simultaneous binding of Sld3 and Sld2 to the pre-RC could therefore represent a quality control mechanism that ensures strictly regulated Cdc45 recruitment to the pre-RC. Several mechanisms are in fact functioning during initiation of replication to prevent origin firing in case of suboptimal condition. Sld3, for example, was shown to be inactivated by Rad53 mediated phosphorylation in presence of DNA damage (Zegerman & Diffley, 2010). Additional structural studies would be extremely helpful to prove this model. Indeed samples of the double-hexamer incubated with part or all the proteins involved in Cdc45 recruitment (Sld3, Sld2, DDK, Sld7) were sent for analysis by electron microscopy to the Li group, but unfortunately they have not been analysed yet.

Moreover, the interaction of Sld2 with the other components of the Cdc45-containing complex was carried out. No data was available in the literature on Sld2 interaction with Sld3 and Sld7, while an *in vivo* analysis by Muramatsu and colleagues (Muramatsu et al, 2010) indicated that Sld2 and Cdc45 do not interact. Sld2 was instead reported to interact with Dpb11, upon CDK dependent phosphorylation (Zegerman & Diffley, 2007).

GST-Sld2 was used to immunoprecipitate Sld3, Sld7 and Cdc45. Dpb11 was also tested in order to verify if the two proteins could interact *in vitro* in the absence of CDK phosphorylation (Figure 6.6). This assay revealed two new interactions between Sld2 and Sld3 and Sld2 and Sld7 (Figure 6.6, lanes 5 and 7 respectively). Furthermore, it was also confirmed that Sld2 and Cdc45 cannot interact (Figure 6.6, lane 10) and, surprisingly, a weak interaction was found with Dpb11, even in the

absence of CDK (Figure 6.6, lane 12). The fact that Sld2 and Cdc45 do not interact directly raises the question how Sld2 can function for Cdc45 recruitment. A conclusive answer could not be found during the course of this study, but it appears reasonable to speculate that Sld2 interaction with the pre-RC remodels the MCM2-7 double-hexamer, making it suitable for Cdc45 binding. Once again the structural studies will help to prove or rule out this hypothesis.

The newfound interaction between Sld3 with Sld2 was confirmed (Figure 6.7) and studied in more detail at this point. Using MBP-Sld3 to immunoprecipitate *in vitro* transcribed and translated domains of Sld2 it was found that all truncations containing the amino acids 250-327, appeared to bind (Figure 6.8, lanes 15, 23 and 25), while the very N-terminal region of Sld2 did not interact with Sld3 (fragments 1-148 and 1-249, Figure 6.8, lanes 11 and 13 respectively). Unfortunately some unspecific signal was also detected for the MBP control in some cases (lanes 14, 22 and 24), affecting the quality of this result. A fungal alignment (Appendix III) was performed for Sld2 and a limited number of residues within the minimal region comprised between amino acids 250-327 were found to be highly conserved. These amino acids could be mutated in the future in order to produce a version of Sld2 which is defective for the interaction with Sld3.

During this project however it appeared reasonable to take advantage of the peptides containing conserved regions of Sld3 used to map Mcm2 and Cdc45 interactions (described in paragraph 4.5 and 5.3). Sld3 peptides were therefore used to immunoprecipitate *in vitro* transcribed and translated proteins and for five of the peptides interactions were detected. These five peptides corresponded to amino acids 170-195 (CR1b), 196-226 (CR2), 249-287 (CR3a), 286-311 (CR3b) and 333-355 (CR4) of Sld3, while peptides CR1a (152-175), CR5 (384-419) and CR6 (518-546) did not show any interaction (Figure 6.9). Peptide CR3a (aa 249-287) in particular appeared to interact very well with Sld2, therefore its sequence was analysed in greater detail. The conserved residues were identified starting from the sequence alignment of Sanchez-Pulido and colleagues (Sanchez-Pulido et al, 2010). In the region comprising amino acids 259-287 a cluster of highly conserved residues can be found (Figure 6.10, panel A). Moreover, the secondary structure of the sequence was predicted (Figure 6.10, panel B) and a helical wheel projection of the sequence corresponding to CR3a was performed (Figure 6.10, panel C). The most conserved residues obtained from the sequence alignment were clustered in four

groups based on their characteristics and proximity in the helical projection and subsequently mutated following the strategy adopted by Frigola and colleagues (Frigola et al, 2013), as described in paragraph 6.1.

The indicated mutations were produced in the context of the CR3a peptide to compare to results to wild type CR3a. The assay showed that mut1 (R269E_E270R_Q274A_E280R) and mut3 (L273E_L277E_L248E) suppressed very strongly the interaction with Sld2 (Figure 6.11, lanes 4 and 6 respectively compared to lane 3). Mut3 did not alter the binding compared to the wild type (Figure 6.11, compare lanes 5 and 3) and mut4 showed a slightly reduced signal (Figure 6.11, compare lanes 7 and 3).

The generation of a mutant version of Sld3, which is defective for Sld2 interaction, could be an extremely valuable tool in order to clarify the mechanism of recruitment of the initial replicative factors to the pre-RC. Furthermore, for the purpose of this study, and the observed Sld2 requirement for Cdc45 recruitment to the pre-RC, the mutant could conclusively prove the proposed model.

It was decided to test these mutations *in vivo* and, in order to perform a comprehensive *in vivo* analysis, mutants in CR1b and CR2 were also produced following the same strategy. The peptides covering these two regions in fact appeared to interact with Mcm2 and Cdc45 (Figure 4.22 and 5.7 respectively) and so it was attempted to produce mutants that are defective for their binding. The peptide for CR3b interacted with Mcm2 (Figure 4.22) but the region is devoid of well conserved residues that could be mutated (see Appendix III) therefore it was decided to focus on the other regions during this analysis.

To test these Sld3 mutants *in vivo* it was decided to use the Sld3-7td strain developed by the Labib lab (Kanemaki & Labib, 2006). Sld3-7 is a mutated version of Sld3 that reduces the ability of MCM2-7 and Cdc45 to interact during initiation (Kamimura et al, 2001). Cells with this mutation grow well at 24°C, but show reduced growth at 37°C, therefore this mutant version of Sld3 was combined with the heat-inducible degron cassette (Kanemaki et al, 2003; Sanchez-Diaz et al, 2004) producing a strain that grows similarly to the control strain at 24°C and exhibits severe growth defect at 37°C (Kanemaki & Labib, 2006). The addition of the degron cassette to wild type Sld3 on the contrary does not prevent growth at 37°C (Kanemaki & Labib, 2006). The degron cassette allows efficient proteolysis of the protein fused to it via recognition by Ubr1, which is targeted by a ubiquitin-

conjugating enzyme (Dohmen et al, 1994). Ubr1 overexpression is under control of the galactose-inducible promoter (Labib et al, 2000) and the proteolysis is more efficient at high temperatures (Sanchez-Diaz et al, 2004).

The use of this strain allows the analysis of the Sld3 mutations in the absence of its wild type copy. For the expression of the mutant versions of Sld3 a yeast centromere plasmid was chosen and it has both a replication origin and a centromere sequence. It is propagated as low-copy-number, autonomously replicating, stably segregated plasmid (Stearns et al, 1990)

Sld3-7td transformed with the empty vector that does not express any protein was used as a negative control. Sld3-7td was also transformed with the plasmid containing non modified *SLD3* gene to verify that the wild type Sld3 can effectively rescue the lethality conferred by the degradation of the endogenous protein.

The strains were grown at the permissive and restrictive temperature while the Ubr1 expression was repressed or induced (Figure 6.13).

All strains analysed appeared to grow similarly well at the permissive temperature, while Ubr1 expression was inhibited. A slightly reduced number of colonies was observed in case of the strains expressing CR1b mut, CR2 mut and CR3a mut1 in comparison to the other strains. This could suggest that these mutant versions of Sld3 are slowing down the growth of the cells even in the presence of wild the type Sld3 protein, thus exerting a dominant lethal effect.

At the permissive temperature, but with Ubr1 expressed, a growth defect was observed for the strains expressing CR1b mut, CR2 mut and CR3a mut1, indicating a dominant effect on cell growth for these mutants, whereas the controls and CR3a mut3 did not show lethality.

When the strains were analysed at the restrictive temperature with inactivated Ubr1, they all appeared to grow. Strains expressing CR1b mut, CR2 mut and CR3a mut1 showed slightly reduced growth, consistent with what observed at the permissive temperature.

At the restrictive temperature with Ubr1 expression, when all endogenous Sld3 is degraded, only the strain transformed with the plasmid containing wild type Sld3 could grow. This indicates that the lethal effect of the degron (observed for the control strain transformed with the empty vector) can effectively be rescued by the addition of Sld3 and also, importantly, that all mutants analysed are defective for the complementation, meaning that they do not function like wild type Sld3.

Unfortunately this experiment alone does not prove that the growth defect observed is indeed due to disruption of one of the interactions studied in this project, which was described in this and the previous chapters.

Therefore an experiment was attempted to identify the influence of the mutations on the chromatin binding of replication proteins. For this experiment cells were arrested in G1-phase and synchronously released in S-phase followed by chromatin fractionation of the samples (similarly to what previously reported (Kanemaki & Labib, 2006; Sheu & Stillman, 2006)). Unfortunately the arrest and release conditions for the Sld3-7td strain as well as the chromatin fractionation required some optimisation, and a conclusive result was not obtained in the timeframe of this project. It remains a very promising experiment to perform in the future in order to identify how the mutations affect complex formation *in vivo* and if the results are confirming the model.

Alternatively, the overexpression and purification of these mutant versions of Sld3 was initiated during this study, but the lengthy process of clone isolation, culture expansion, protein expression and purification could not be completed on time. The purified mutants should be employed in *in vitro* tests for direct interaction with the binding partners (MCM2-7, Cdc45 and Sld2) and in pre-RC reconstitution assays in order to conclusively prove their efficacy. In the case that one or more mutants would produce the hoped outcome, it would be interesting to separate the individual mutations that were present in each of the mutants tested.

The obtained data suggested that Sld3, Sld2 and Sld7 could form a trimeric complex in solution. In fact Herrera found that, in the presence of DDK, Sld3 together with Sld2 could interact only transiently with the pre-RC and they were rapidly displaced from the complex (Figure 6.14 A). It was also found that the addition of Sld7 to this reaction would result in inhibited recruitment of Sld3, Sld2 and Sld7 to the pre-RC (Figure 6.14 B), suggesting that these three proteins could form a complex in solution that would impede the interaction with the pre-RC proteins. Finally, it was observed that the recruitment of Sld3, Sld2 and Sld7 in the presence of Cdc45 was much more stable. Over time Sld3, Sld2 and Sld7 would get displaced from the complex, leaving only Cdc45 at this point stably recruited (Figure 6.14 C). To test the possibility that Sld3, Sld2 and Sld7 could form dimeric or trimeric complexes in solution, the purified proteins were cross-linked with glutaraldehyde as previously described (Evrin et al, 2013). The data obtained with this assay are

interesting (Figure 6.16) and in summary they suggest that Sld3 could form a dimer (species revealed by silver staining but not by western blot) and it shows conclusively that Sld7 can form a dimer (species revealed by western blot at a higher molecular weight compared to the non cross-linked sample). When Sld3 and Sld7 were cross-linked together a signal that could correspond to a dimer or a multimer of Sld3 and Sld7 was detected by an anti-Sld7 western blot. Unfortunately, the Sld3 antibody did not confirm this finding, but this is likely due to epitope masking. The combination of Sld3 and Sld2 also showed a different profile compared to the proteins cross-linked by themselves, suggesting that a complex could form but a high molecular weight species could not be detected with neither of the antibodies. Sld7, when added together with Sld2, showed a different profile compared to the proteins crosslinked by themselves but once again the Sld2 antibody did not confirm a complex. Finally, when all three proteins were cross-linked together a signal could be detected by silver staining for a complex with a molecular weight higher than 116 kDa (the trimer has an estimated size of 160 kDa). Unfortunately Sld3 antibody did not confirm the identity of this complex nor could Sld2 and Sld7 antibody reveal the formation of a complex containing both proteins.

In conclusion, this assay is not sufficient to prove the formation of a trimeric complex in solution, but strongly suggests that Sld3, Sld2 and Sld7 can associate with one another in different ways. This method was used because it allows a rapid analysis of complex formation and it requires a very limited amount of protein. It has however a big limitation: the recognition of the proteins after cross-linking can be difficult due to epitope masking. Other techniques can be employed for the same purpose, such as separation via gel filtration or glycerol / sucrose gradient sedimentation, or immunoprecipitation of the complex using one of the proteins and probing for the other two.

To conclude the analysis presented in this chapter, the role of DDK in the modification of the factors involved in Cdc45 recruitment to the pre-RC was investigated.

The activity of DDK in initiation of replication has been extensively studied *in vivo* (Francis et al, 2009; Gros et al, 2014; Heller et al, 2011; Jackson et al, 1993; On et al, 2014; Randell et al, 2010; Sheu & Stillman, 2006; Sheu & Stillman, 2010), as well as the specific requirement of DDK for Cdc45 stable recruitment to the origins (Heller et al, 2011), and this effect appeared confirmed *in vitro* (Figure 6.1 C).

The post-translational modifications of MCM2-7 by DDK have been broadly studied (Francis et al, 2009; Gros et al, 2014; On et al, 2014; Randell et al, 2010; Sheu & Stillman, 2006; Sheu & Stillman, 2010) but little is known on phosphorylation of other components of the complex studied by DDK. For this reason, a kinase assay was performed using purified Sld3, Sld2, Sld7 and Cdc45. Among the proteins tested, only Sld3 appeared to incorporate radioactive ATP upon addition of increasing amounts of DDK (Figure 6.17 A). This is consistent with what recently reported by Mattarocci and colleagues (Mattarocci et al, 2014), where Sld3, similarly to Mcm4, appeared to be target of DDK phosphorylation during G1-phase. It will be interesting to further characterise this modification and understand if it plays a role in the mechanism of recruitment/release of Sld3 to the pre-RC. Cdc45, instead, exhibited a weak signal even in the absence of DDK, indicating that the protein could incorporate γ - ^{32}P -ATP, but no increase was observed upon addition of DDK. This differs from what previously reported by Nougarede and colleagues (Nougarede et al, 2000). Sld2 and Sld7 did not show any γ - ^{32}P -ATP incorporation. The role of DDK on Sld3 phosphorylation will deserve to be better investigated because it could affect initiation of replication even though it appears more plausible that the contribution of DDK to the recruitment of Cdc45 is exerted by the phosphorylation of the MCM2-7 loaded double-hexamer. *In vitro* kinase assays are prone to non-specific phosphorylation events, therefore this result would need to be confirmed. An analysis of the sequence of Sld3 should be performed to look for residues that could be phosphorylated by DDK, as well as a mass spec analysis to determine whether a Sld3 sample treated with DDK would show new modifications.

Chapter 7 – Summary and conclusions

The large genomes of eukaryotic cells are replicated from multiple replication origins during S-phase of the cell cycle. Ensuring that each origin is efficiently activated once and only once during each S-phase is crucial for maintaining the integrity of the genome. This is achieved by a two-step mechanism (Diffley et al, 1994). First, in late mitosis and early G1-phase, a large complex of initiator proteins (pre-RC), assembles at origins. Second, in early S-phase, when cells have committed to DNA replication and cell division kinases are activated, the pre-RC is transformed into the pre-IC. After the unwinding of origin DNA, the DNA synthesis machinery is loaded on ssDNA. Once the pre-IC starts forming, the pre-RC disassembles, and its reassembly is prevented until the next G1. As a result, each origin is used once, and only once, per cell cycle (Morgan, 2007). Re-initiation from any origin would lead to unbalanced gene dosage and long-lived, potentially fragile replication forks whereas the use of an insufficient number of origins would leave regions of the genome unreplicated before mitosis, generating loss of genetic information (Diffley, 2011).

DNA replication is a fundamental, conserved and still poorly defined process. This project intended to contribute to its understanding through the *in vitro* and *in vivo* analysis of the transition from pre-RC formation to CMG assembly in *S. cerevisiae*.

7.1 Analysis of the interactions occurring during pre-RC formation

This work started with the study of the protein-protein interactions involved in pre-RC formation. Very little was in fact known on how the ORC/Cdc6 complex, formed onto replicative origins, how ORC could contact MCM2-7/Cdt1, with the exceptions that Orc6 was shown to be required for the recruitment of Cdt1 (Chen et al, 2007) and that the C-terminus of Cdt1 binds MCM2-7 whilst the N-terminus is required to load pre-RC complexes that are competent for CMG formation (Takara & Bell, 2011).

A series of experiments presented in chapter 3 aimed to identify new regions of interaction that could provide additional information on pre-RC formation and complement the ongoing research of other lab members on the subject.

Employing bait proteins to immunoprecipitate *in vitro* transcribed and translated binding partners it was found that MCM2-7 appears to interact exclusively with the Orc2 subunit of ORC, even though it is entirely possible that other subunits also contribute to the binding site. It was also found that isolated MCM2-7 and Cdc6 do not directly contact one another suggesting that an interaction does not occur or that MCM2-7 has to be in a different conformation (which could be achieved after a first contact with the DNA or ORC). Cdt1, regardless of its interaction with MCM2-7, could not produce a change in structure to allow Cdc6 interaction.

An interaction between Orc6 and Cdt1 was observed, consistent with what was previously reported (Chen et al, 2007).

It was also shown that MCM2-7 does not interact with the C-terminal domains of Orc1-5 and Cdc6, neither in absence nor presence of Cdt1, consistent with the model proposed by Sun and colleagues (Sun et al, 2013) where this part of the ORC subunits contact DNA, but are distal to MCM2-7. The relation between MCM2-7 and ORC was further analysed in the context of the MCM2-7 Δ C6 mutant, which has a defective Cdt1 binding domain, showing no significant difference compared with the wild type consistent with what was previously reported (Fernandez-Cid et al, 2013), where this mutant is still capable of interacting with Cdt1, indicating that MCM2-7 has more than one binding site for Cdt1.

Additionally, Cdt1 interacted with the full length Orc2, consistent with the literature (Asano et al, 2007; Chen et al, 2007). Surprisingly the assay also showed an interaction between Cdt1 and Cdc6 which has not been observed before and will need further exploration.

A number of different Orc2 truncations were examined for binding to Cdt1 and indicated that the putative binding site must be in the central portion of Orc2, in the AAA+ region, with a minimum fragment comprising amino acids 225-461.

Taking this data together with the structure proposed by Sun and colleagues (Sun et al, 2013), it appears possible that the surface of contact between MCM2-7 and ORC involves Orc2, and also that Cdt1 and Orc2 can interact with each other, and this interaction is potentially important for initial ORC/Cdc6/Cdt1/MCM2-7 complex formation. More work needs to be done in order to understand the interaction of Cdt1

with Cdc6, because the two proteins seem to be located far away one from the other, at least in the structure of the OCCM. This interaction could be however important during the recruitment of the second MCM2-7 hexamer for the formation of the double-hexamer.

Overall the results obtained in this part of the study provide some interesting contributions to the understanding of the pre-RC formation process. Additional analysis is required to better comprehend the complicated and dynamic structure of the pre-RC complex and the newfound interaction surfaces could significantly facilitate this work.

7.2 Analysis of the interaction of Sld3 with the pre-RC

Sld3 is believed to be one of the first factors recruited to the pre-RC with an essential role in assembling the pre-IC and CMG (Kamimura et al, 2001; Kanemaki & Labib, 2006). Therefore the study proceeded with the analysis of this crucial step in initiation of DNA replication.

The recruitment of Sld3 to the pre-RC was investigated *in vitro* with purified proteins, given that all previous data on the subject had been produced through *in vivo* studies (Kamimura et al, 2001; Kanemaki & Labib, 2006; Tanaka et al, 2007b; Zegerman & Diffley, 2007) or employing yeast extracts that were used for *in vitro* assembly and DNA replication assays (Heller et al, 2011). An *in vitro* study has in fact the advantage of being able to distinguish the contribution of each individual factor involved in the process and can provide conclusive details on the molecular mechanisms.

A lot of efforts were made in order to reconstitute the recruitment of Sld3 to the pre-RC employing the reconstituted system developed by Evrin and colleagues (Evrin et al, 2009) but they were not effective because of the intrinsic characteristic of Sld3 and its unspecific charge-mediated interaction with the streptavidin beads used in the assay. A successful recruitment of Sld3 to the pre-RC was however obtained with the use of a novel method for the reconstitution of the pre-RC in solution (Herrera, Tognetti et al, under revision). It was shown that Sld3 is in fact able to interact with the pre-RC in an almost stoichiometric fashion and in the absence of additional factors. This finding appears in contradiction with the literature, where Sld3 was only recruited to the pre-RC upon DDK phosphorylation (Heller et al,

2011) and in a Cdc45 dependent manner (Kamimura et al, 2001). It seems reasonable to argue that the initial recruitment can be independent from other factors but it is too weak to be detected in *in vivo* studies, whereas it can still be sensed using a more sensitive *in vitro* assay. Alternatively, it appears possible that other replication factors could bind to Sld3 and regulate its association with the pre-RC *in vivo*. *In vitro* the contribution of Cdc45 and DDK to the Sld3/pre-RC interaction was observed, which resulted in an enhanced recruitment of Sld3 to the pre-RC, as it was shown in chapter 6.

The interaction of Sld3 with MCM2-7 was analysed in great detail: Sld3 can bind the MCM2-7 hexamer in solution as well as the Mcm2 subunit.

Mcm2 is part of the putative “gate”, which is thought to promote opening of MCM2-7 for the entry/exit of the DNA during helicase activation (Bochman & Schwacha, 2007; Bochman & Schwacha, 2008; Bochman & Schwacha, 2010; Costa et al, 2011; Samel et al, 2014; Sun et al, 2013) and it is shown to interact with Cdc45 in the CMG structure (Costa et al, 2011; Ilves et al, 2010). Sld3, which is known to interact with Cdc45 (Bruck & Kaplan, 2011a; Kamimura et al, 2001; Kanemaki & Labib, 2006), is therefore positioned in a suitable place to interact with both Mcm2 and Cdc45 and to modify the gate for helicase activation.

The binding site was mapped to the residues 200-250 of Mcm2. Several attempts were made to produce mutant versions of MCM2-7 which were defective for Sld3 binding and they included a series of deletion mutants for the N-terminus of Mcm2 that were tested *in vitro* and *in vivo* as well as the design of hybrid versions of MCM2-7 where part of the yeast Mcm2 N-terminus was substituted with the human counterpart. This last strategy was designed after the finding that Sld3 cannot interact with the N-terminus of human Mcm2, suggesting that this surface of interaction has diverged among species. Unfortunately all these versions of MCM2-7 appeared to be unable to form a stable hexamer or did not produce any effect when analysed *in vivo*. Taken together these findings indicate that first 250 amino acids at the N-terminus of Mcm2 play an important role in the stability of the complex and are likely required for essential protein-protein interactions.

Unfortunately this data did not validate in a conclusive way the hypothesis that Sld3 is interacting with the region 200-250 of Mcm2, therefore it was decided to focus on the residues in this region of Mcm2 that differ between yeast and human and could be potentially crucial for the binding. A series of point mutations were produced,

which were tested in the context of *in vitro* transcribed and translated Mcm2 N-terminus in order to facilitate the screen and overcome the issue of protein instability. Three amino acids on Mcm2 (T233, S239 and H248) were identified for their role in the interaction with Sld3. It cannot be excluded that these amino acids are only contributing to the creation of the binding site for Sld3 and that other binding sites are required, but they provide an excellent starting point for a future analysis of this interaction.

An alternative approach was used to study the binding site on Sld3. For this purpose, the sequence of Sld3 was analysed for its conservation and secondary structure prediction to identify stretches that could support protein-protein interactions. A series of peptides were designed to cover those interesting regions and tested for interaction with Mcm2, identifying the most important regions of Sld3 as 170-195 (CR1b), 196-226 (CR2) and 286-311 (CR3b). This strategy was significantly faster and more promising than the ones adopted for the analysis of the binding site on Mcm2. The mutated CR1b and CR2 were then tested *in vivo* employing a temperature sensitive degon strain (Sld3-7td), as described in paragraph 6.2. The mutants in Sld3 were unable to rescue the lethality caused by the degradation of the endogenous proteins, demonstrating that they are not functional.

Unfortunately it was not possible during the timeframe of this project to prove conclusively that their deficiency is due to the disrupted interaction with Mcm2. Nevertheless, the data obtained on Sld3/Mcm2 interaction during this analysis is a substantial advancement to the knowledge on the matter and will provide excellent preliminary data for future work.

7.3 Analysis of the interaction of Cdc45 with the pre-RC and Sld3

After the successful reconstitution of a pre-RC complex containing Sld3 (chapter 4), the recruitment of Cdc45 was explored *in vitro*. Both bead and gel filtration based methods were used for this purpose but no interaction with the pre-RC was detected when Cdc45 was added by itself. The contribution of Sld3 and DDK was therefore tested since, according to the literature (Heller et al, 2011; Kamimura et al, 2001; Kanemaki & Labib, 2006), these factors are required for Cdc45 recruitment but, once again, no recruitment was detected. The discrepancy of

these results could be explained with the fact that all previous work was performed *in vivo* or using yeast extracts. These experimental sets are in fact not fully controllable and mechanisms of compensation could take part without being able to detect them. Another reason could be that an interaction occurs *in vivo* but that something abrogates a stable complex formation in this particular work.

Cdc45 and MCM2-7 are in fact both components of the CMG and their interaction is suggested in several publications (Bruck & Kaplan, 2011a; Costa et al, 2011; Gambus et al, 2006; Gambus et al, 2009; Ilves et al, 2010; Kamimura et al, 2001; Kanemaki & Labib, 2006; Moyer et al, 2006; Sheu & Stillman, 2006; Zou & Stillman, 1998; Zou & Stillman, 2000). Their direct interaction was therefore tested but the two purified proteins did not bind to each other in solution. This result indicates that isolated MCM2-7 is not in the correct conformation to support Cdc45 binding and suggests that a conformational change has to occur as a consequence of another event (i.e. DNA encircling, phosphorylation or other protein interaction). Furthermore, it was shown that Cdc45 interacts with the Mcm2 subunit of MCM2-7. This is consistent with the structure proposed by Costa and colleagues (Costa et al, 2011) and with the finding that MCM2-7/Cdc45/Sld3 can form a trimeric complex in solution (Bruck & Kaplan, 2011a), given that Sld3 also interacts with Mcm2.

It was not possible during this project to further map the binding site, probably because of technical limitation conferred by the use of GST-tagged proteins, but the interaction of Cdc45 with Sld3 was analysed instead. The binding of Cdc45 to Sld3 is commonly accepted and it has been shown both *in vivo* and *in vitro* (Bruck & Kaplan, 2011a; Bruck & Kaplan, 2011b; Kamimura et al, 2001; Kanemaki & Labib, 2006; Tanaka et al, 2007b; Tanaka et al, 2011b), therefore it was attempted to map the binding site in Sld3 and to identify point mutants that are defective for this interaction.

It was found that fragments comprising amino acids 333-456 of Cdc45 could be immunoprecipitated by Sld3. Moreover, using the peptide strategy, three regions of Sld3 were identified to interact with Cdc45: CR1b (aa 170-195), CR2 (aa 196-226) and CR3a (aa 249-287). The conserved residues in these regions were mutated and their effect tested *in vivo* (paragraph 6.2). All strains produced a phenotype. As described for Mcm2, this result cannot conclusively prove that the mutated residues are essential and sufficient to support the interaction but provide some useful insights into the complex formation.

7.4 Analysis of Cdc45 recruitment to the pre-RC

Herrera showed that the recruitment of Cdc45 to the pre-RC is not only dependent on Sld3 and DDK as previously described (Heller et al, 2011; Kamimura et al, 2001; Kanemaki & Labib, 2006), but also on Sld2, therefore this final part of the study was a collaboration aimed to analyse in detail how Sld2 could function in this process .

Firstly, it was shown that Sld2 can be rapidly recruited to the pre-RC and is stably bound in the absence of additional factors.

Sld2 interaction with MCM2-7 in the context of purified proteins in solution was suggested (Bruck et al, 2011) and, given that Sld2 appears required for Cdc45 recruitment, together with Sld3, the subunits of contact on MCM2-7 was analysed, observing an interaction with Mcm2. The proximity of Sld3, Cdc45 and Sld2 with the single Mcm2 subunit appears sterically difficult and it is more reasonable to think that the binding site for these three proteins is only partially formed by Mcm2 and additionally involves adjacent subunits.

Moreover, Herrera found that Sld2 and Sld3 by themselves were efficiently recruited to the pre-RC but, when added in combination, their binding, and especially the binding of Sld2, was decreased. Interestingly, it was also found that DDK could alter this interaction and induce cooperative binding, however this complex was only short-lived. This suggested that the two proteins cannot be stably recruited simultaneously in the absence of additional factors and indicated that either the pre-RC undergoes a conformational change in the presence of Cdc45 and DDK or that Cdc45 stabilises the complex, perhaps bridging some interactions.

When the Sld2 binding site on Mcm2 was mapped it was found, surprisingly, that it involved the C-terminus of Mcm2, contrarily to the N-terminus required for Sld3 interaction. This result indicates that Sld3 and Sld2 do not compete for the binding site on Mcm2, with Sld3 getting a better chance of interaction, but that the interaction of Sld3 with the Mcm2 N-terminus could induce a hindrance that impairs Sld2 binding to the C-terminus. The regulation of the simultaneous interaction of Sld3 and Sld2 to the pre-RC could therefore be an additional DDK induced quality control mechanism to strictly regulate Cdc45 recruitment to the pre-RC.

The interaction analysis of Sld2 with the other components of the Cdc45-containing complex was carried out in order to understand its functional role. Sld2

and Cdc45 did not show any binding, consistent with what reported by Muramatsu and colleagues (Muramatsu et al, 2010). However, two other new interactions were identified: Sld3/Sld2 and Sld2/Sld7.

It was not possible during this study to conclusively state how Sld2 can facilitate Cdc45 recruitment given that they do not interact directly, but it appears reasonable to speculate that Sld2 interaction with the pre-RC remodels the MCM2-7 double-hexamer, making the complex suitable for Cdc45 binding. Structural analysis will help to prove or rule out this hypothesis.

The newfound interaction between Sld3 and Sld2 was studied in more detail, identifying a minimal binding region in Sld2 that comprised amino acids 250-327. Using a peptide approach, a region of Sld3 involved in the binding of Sld2 was also identified (CR3a, aa 249-287). The most conserved residues in this region were mutated and two sets of modifications suppressed the interaction (R269E_E270R_Q274A_E280R and L273E_L277E_L248E). Interestingly, helical wheel analyses showed that both sets of mutants were located on the same side of a predicted alpha helix, suggesting that they both affect the same interaction surface. These mutants were subsequently analysed *in vivo* and it was found that they were defective for the complementation of a temperature sensitive degon strain. Because this result by itself cannot prove that the growth defect is due to the disruption of the interaction between Sld3 and Sld2, the effect of the mutations on the chromatin binding of Cdc45 was tested, but these experiments were not completed in the timeframe of this study.

Herrera found that Sld3 and Sld2 could be recruited to the pre-RC in the presence of DDK, but then they were rapidly displaced from the complex. The addition of Sld7 to this reaction did suppress the recruitment of Sld3, Sld2 and Sld7 but their binding in the presence of Cdc45 was much stabilised compared to what was observed for Sld3 and Sld2 alone. Over time Sld3, Sld2 and Sld7 would get displaced from the complex, leaving only Cdc45 stably recruited. This data suggests that Sld3, Sld2 and Sld7 could form a complex in solution, which is not competent for the interaction with the pre-RC proteins. Cross-linking experiments with glutaraldehyde were performed in order to characterise the potential Sld2/Sld3/Sld7 complex, but despite several indications of dimers and trimer formation, no conclusive result could be obtained due to detection problems. This data suggests

that a more detailed analysis with different techniques such as gel filtration or glycerol or sucrose gradient sedimentation is required.

To conclude this analysis, the role of DDK in modifying factors involved in Cdc45 recruitment to the pre-RC was investigated and it was found that only Sld3 was phosphorylated by DDK. This observation will require further characterisation, it would be interesting to understand if this modification plays a role in the mechanism of recruitment/release of Sld3 to the pre-RC.

Taken together, the results presented in chapter 6 and summarised in this paragraph, allowed us to draw a model for the recruitment of Cdc45 to the pre-RC (Figure 7.1).

The pre-RC is formed on origin DNA and its final product is the MCM2-7 double-hexamer, which represents a basis for pre-IC formation. Sld2, Sld3 and Sld7 all interact with each other in solution and could form a complex (Figure 7.1 A). When Sld3 and Sld2 are added to the pre-RC in the absence of additional factors, these proteins competes for pre-RC binding, possibly because the binding of Sld3 to the flexible N-terminus of Mcm2 produces an obstruction for Sld2 interaction with its binding to the C-terminus of Mcm2 (Figure 7.1 B), alternatively Sld3 acts as an allosteric negative inhibitor of Sld2. DDK activity alters these interactions and promotes cooperative Sld2/Sld3-pre-RC complex formation, most likely producing a conformational change that alters the spatial arrangement of Mcm2 (Figure 7.1 C).

In the absence of Cdc45, the Sld2/Sld3-pre-RC complex becomes rapidly destabilized, suggesting that this reaction functions as a quality control step to redistribute Sld2 and Sld3 to sites of preferred complex formation (Figure 7.1 D).

When Cdc45 is present, a Cdc45/Sld2/Sld3/Sld7-pre-RC complex is formed (Figure 7.1 E). Over time Sld3, Sld2 and Sld7 become destabilized from this complex. The release of these factors could allow the CDK dependent binding of Dpb11 or allow GINS interaction and CMG formation.

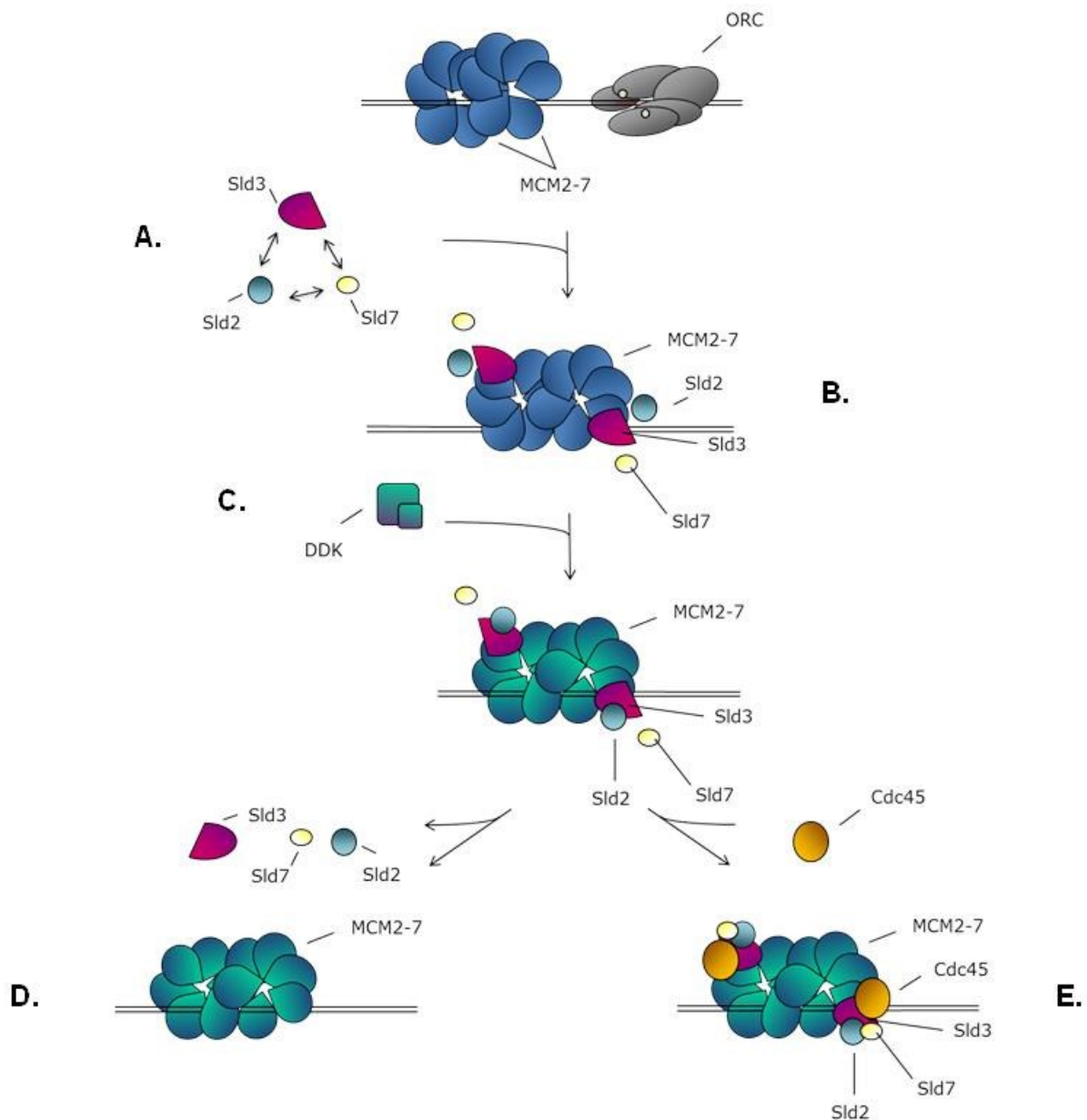


Figure 7.1: Summary of the Cdc45 recruitment process

The MCM2-7 double-hexamer is the landing platform for complex formation during S-phase. **A)** Sld2, Sld3 and Sld7 could interact with each other in solution. **B)** Sld2 and Sld3 compete in the absence of DDK for pre-RC interactions. **C)** DDK promotes cooperative Sld2/Sld3-pre-RC binding, but this complex is only short lived. Sld7 destabilizes this complex even further. **D)** In the absence of Cdc45 Sld2/Sld3/Sld7 cannot form a stable complex with the pre-RC and these limiting factors can be redirected to other locations where Cdc45 can induce complex assembly. **E)** Cdc45 recruitment to the pre-RC depends on Sld2, Sld3 and DDK and Cdc45 greatly stabilizes the unstable Sld2/Sld3/Sld7-pre-RC complex. After prolonged incubations Sld2/Sld3/Sld7 dissociate off and reveal a stable Cdc45/pre-RC complex.

7.5 Technical advancements

This study highlighted many of the difficulties that can be encountered during the analysis of protein-protein interactions. Firstly, it underlined how problematic the identification of the minimal binding site can be. Secondly, it showed that the manipulation of large protein complexes can result in their instability and consequent loss of function due to structural reasons independent of the analysis performed. Thirdly, it revealed that the process of identification of an interaction, mapping of the binding site, mutation of the putative residues involved and test of the mutants can be challenging to achieve in a limited amount of time.

Nevertheless, several important advancements were achieved during this project, both from a scientific and from a technical point of view. The issues encountered with the GST-tagged MCM2-7 subunits (paragraph 4.2) required to identify an alternative method for the mapping on the binding site. The use of *in vitro* transcribed and translated proteins was an important technical break-through, as it allowed the detection of specific interactions in a rapid process. This system, even though already used for the same purpose, was never employed before in such a systematic way. Moreover, the analysis of the interface between MCM2-7 and Sld3 required an extensive amount of work and did not end with a definitive result but was undoubtedly useful to explore several different approaches for the variation of a putative binding site: deletion mutants, hybrid mutants and point mutants were analysed, producing a useful set of data for further studies.

Finally, the use of peptides for the study of protein-protein interactions based on sequence analysis was a fairly uncommon approach in the field but it generated remarkably relevant results in a very limited time frame, which will be certainly used in the future for similar work by the group.

7.6 Conclusions

From a scientific point of view, the data produced during this study represent a considerable advancement in the field of DNA replication. Previous work had demonstrated that Sld3 functions in Cdc45 recruitment (Kamimura et al, 2001; Kanemaki & Labib, 2006). Additionally, Sld3 together with Sld2 represent the essential targets of CDK during S-phase and their phosphorylation enables binding of Dpb11, which promotes GINS recruitment, CMG formation and activation of the

replicative helicase (Muramatsu et al, 2010; Tanaka et al, 2007b; Zegerman & Diffley, 2007). An EM structure of the CMG has shown that Cdc45 and GINS contact MCM2-7 at the Mcm2/5 interface (Costa et al, 2011), a putative exit gate for ssDNA during helicase activation. However, if other factors besides Sld3 are involved in Cdc45 loading was not known prior to this study. Moreover, how Sld3 and others factors bind to the MCM2-7 helicase was also not known. Previous studies, exploring mechanism in Cdc45 recruitment to the replication origin, were based on *in vivo* experiments or extract based systems. However, these systems do not allow the analysis of individual components during complex formation or more complex reaction mechanisms. The extract based system (Heller et al, 2011) has the limitation that several factors involved in the process are overexpressed, which could alter complex assembly, e.g. it was shown that high-copy *CDC45* suppressed the growth defect conferred by some temperature sensitive mutants of Sld3 (Kamimura et al, 2001) and, additionally, overexpression of Cdc45 or Dpb11 can bypass the requirement of CDK dependent phosphorylation of Sld3 (Tanaka et al, 2007b). Finally, the extract based system did not allow so far a detailed analysis of each component or more complex reaction mechanisms.

The reconstitution of Cdc45 loading with purified proteins was a breakthrough, as it allowed a much more detailed analysis of complex assembly than previous systems, and described Sld2 as Cdc45 loading factor. The detailed analysis of protein interactions identified that the Mcm2 subunit of the MCM2-7 hexamer acquires a central role in Cdc45 recruitment, representing the landing pad for Sld2, Sld3 and Cdc45. This is particularly interesting, as Mcm2 is a substrate for DDK phosphorylation, which is essential for Cdc45 recruitment and, since Mcm2 is also part of a putative gate for DNA entry/exit. During helicase activation it is thought that the MCM2-7 ring must open at the Mcm2/5 interface and that one strand of DNA becomes extruded. Indeed, while the double-hexamer encircles dsDNA and has no helicase activity, the CMG encircles ssDNA and functions as a processive helicase. Currently it is unknown how the MCM2-7 ring opens during helicase activation; however the data obtained here in collaboration with my colleague suggests that DDK dependent conformational changes in Mcm2 promote cooperative interactions in Sld3/Sld2, which in turn allows Cdc45 recruitment and loading. Interestingly, in the absence of Cdc45, Sld3, Sld2 and Sld7 fail to form a stable complex with the pre-RC, suggesting that these limiting factors can be redistributed to other sites of

preferred complex formation. These redundant mechanisms of regulation guarantee that DNA replication is initiated only when the cell can support it, preventing helicase activation in case of suboptimal conditions. Such a highly controlled system is necessary to ensure that the DNA is correctly copied and no mistakes can be introduced, leading to genome instability (Green et al, 2010). In this sense, the investigation of the process of DNA replication is crucial for future therapeutic applications. All pre-RC proteins, as well as Sld3 and Cdc45, have a human homologue and it is reasonable to expect that the function of Sld2 is also conserved. Mutations on these proteins have been found in cancer and other human syndromes, indicating that the information acquired studying the process of replication *in vitro* or in species other than human could provide critical advancement in the understanding and treatment of human conditions.

Appendix I - Tables

Table I. Plasmids

Name	Gene	Plasmid	Reference
pCS372	ARS1	pUC19	(Evrin et al, 2009)
pCS160	Cdc6	pGEX-6P-1	(Speck et al, 2005)
pCS178	Cdt1	pGEX-6P-1	(Evrin et al, 2009)
pCS14	Mcm2/Mcm7	pESC-LEU	(Evrin et al, 2009)
pCS479	Mcm2/Mcm7-MBP	pESC-LEU	Samel and Speck, unpublished
pCS524	Mcm2Δ1-250/Mcm7	pESC-LEU	This study
pCS558	FLAG-Mcm2/Mcm7	pESC-LEU	This study
pCS572	FLAG-Mcm2Δ1-200/Mcm7	pESC-LEU	This study
pCS573	FLAG-Mcm2Δ1-238/Mcm7	pESC-LEU	This study
pCS574	FLAG-Mcm2Δ1-251/Mcm7	pESC-LEU	This study
pCS508	hMcm2	pESC-HIS	Schlott and Speck, unpublished
pCS576	FLAG-hMcm2(1-200) scMcm2(222-868)/Mcm7	pESC-LEU	This study
pCS577	FLAG-hMcm2(1-232) scMcm2(254-868)/Mcm7	pESC-LEU	This study
pCS15	Mcm6/Mcm4	pESC-TRP	(Evrin et al, 2009)
pCS496	Mcm6ΔC6/Mcm4	pESC-TRP	(Fernandez-Cid et al, 2013)
pCS232	Mcm3-HA/Mcm5	pESC-URA	(Evrin et al, 2009)
pCS319	Mcm2(1-322)-GST	pGEX-6P-1 C-term	This study
pCS320	Mcm3(1-192)-GST	pGEX-6P-1 C-term	This study
pCS321	Mcm4(1-340)-GST	pGEX-6P-1 C-term	This study
pCS322	Mcm5(1-174)-GST	pGEX-6P-1 C-term	This study
pCS323	Mcm6(1-302)-GST	pGEX-6P-1 C-term	This study
pCS324	Mcm7(1-253)-GST	pGEX-6P-1 C-term	This study
pCS313	Cdc7/MBP-Dbf4	pESC-TRP	Herrera, Tognetti et al, under revision
pCS245	MBP-HIS-Myc-Sld3	pESC-HIS	Herrera, Tognetti et al, under revision
pCS609	Sld3 wt + natural promoter	pRC416	This study
pCS610	Sld3 CR1b mut + natural promoter	pRC416	This study
pCS611	Sld3 CR2 mut + natural promoter	pRC416	This study
pCS612	Sld3 CR3a mut1 + natural promoter	pRC416	This study
pCS614	Sld3 CR3a mut3 + natural promoter	pRC416	This study
pCS8	Cdc45-FLAG	pESC-HIS	Herrera, Tognetti et al, under revision
pCS241	Cdc45-FLAG	pESC-LEU	Herrera, Tognetti et al, under revision
pCS242	Cdc45-FLAG	pESC-TRP	Herrera, Tognetti et al, under revision
pCS243	Cdc45-FLAG	pESC-URA	Herrera, Tognetti et al, under revision
pCS229	Cdc45-GST	pGEX-6P-1 C-term	Herrera, Tognetti et al, under revision
pSC290	GST-Sld2	pGEX-6P-1	Herrera, Tognetti et al, under revision
pSC486	GST-Sld7	pGEX-6P-1	Herrera, Tognetti et al, under revision
pSC300	GST-Dpb11	pGEX-6P-1	Herrera, Tognetti et al, under revision

Table II. Oligonucleotides for cloning

Name	Full name	Final product	Sequence
oCS1941	Mcm2Δ1-250_F	Mcm2Δ1-250/Mcm7	GGATGGCTTTGGACTCCATGGATCCGGGG TTT
oCS1942	Mcm2Δ1-250_R	Mcm2Δ1-250/Mcm7	AAACCCCGGATCCATGGAGTCCAAAGCCAT CC
oCS1859	FLAG link BamHI_F	FLAG-Mcm2/Mcm7	[PHOS]GATCC ATG GATTATAAAGATGATGATATAAA GGTGGTAGAGCTAATGGTAATAAT G
oCS1860	FLAG link BamHI_R	FLAG-Mcm2/Mcm7	[PHOS]GATCC ATTATTACCATAGCTCTACCACC TTTATCATCATCATCTTTATAATC CAT G
oCS2136	FLAG_Mcm2 Δ1-200_F	FLAG-Mcm2Δ1-200/Mcm7	CTAATGGTAATAATGGATCCATG CCTAATGTCTCAAGAACTATTGC
oCS2137	FLAG_Mcm2 Δ1-200_R	FLAG-Mcm2Δ1-200/Mcm7	GCAATAGTTCTTGAGACATTAGG CATGGATCCATTATTACCATTAG
oCS2182	FLAG_Mcm2 Δ1-238_F	FLAG-Mcm2Δ1-238/Mcm7	GCTAATGGTAATAATGGATCCATG TCTGAATCTTTGGAGTTAATTAT
oCS2183	FLAG_Mcm2 Δ1-238_R	FLAG-Mcm2Δ1-238/Mcm7	ATAATTAACCTCCAAAGATTGAGA CATGGATCCATTATTACCATTAGC
oCS2184	FLAG_Mcm2 Δ1-251_F	FLAG-Mcm2Δ1-251/Mcm7	GGTAATAATGGATCCATG TCCAAAGCCATCCTGGCA
oCS2185	FLAG_Mcm2 Δ1-251_R	FLAG-Mcm2Δ1-251/Mcm7	TGCCAGGATGGCTTTGGA CATGGATCCATTATTACC
oCS2158	SbfI_FLAG_hMcm2_F	hybrid plasmids	CAGTTGATCCTGCAAGGATGGATTATAAAGA TGATGATGATAAAGGTGGTAGAGCTAATGG TAATAAT ATG GCA TCC AGC CCG GC
oCS2159	AsiSI_hMcm2_232R	FLAG-hMcm2(1-200) scMcm2(222-868)/Mcm7	TCAGTCACGAT GCGATCGC CTC CCT GGC TGC CAA GTC C
oCS2160	AsiSI_hMcm2_200R	FLAG-hMcm2(1-232) scMcm2(254-868)/Mcm7	TCAGTCACGAT GCGATCGC GCT GTC GAC GTG AGT GCG C
oCS1364	Mcm2_F	Mcm2(1-322)-GST	CAGTTGAT GC ATGC TA ATGTCTGATAATAGAAGACGTAGACGTG
oCS1365	Mcm2 322_R	Mcm2(1-322)-GST	TCAGTCACGAT GCGGCCGC AGGGAAGACTCCTGTTCTTCTTGT
oCS1366	Mcm3_F	Mcm3(1-192)-GST	CAGTTGAT GC ATGC TA ATGGAAGGCTCAACGGGATTT
oCS1367	MCM3 192_R	Mcm3(1-192)-GST	TCAGTCACGAT GCGGCCGC TGGCCTGACCAACGAAGTCTTA
oCS1368	Mcm4_F	Mcm4(1-340)-GST	CAGTTGAT GC ATGC TA ATGTCTCAACAGTCTAGCTCTCCAA
oCS1369	MCM4 340_R	Mcm4(1-340)-GST	TCAGTCACGAT GCGGCCGC AGGGATAACCGGAGTTGATCT
oCS1370	Mcm5_F	Mcm5(1-174)-GST	CAGTTGAT GC ATGC TA ATGTCATTTGATAGACCGGAAA
oCS1371	Mcm5 174_R	Mcm5(1-174)-GST	TCAGTCACGAT GCGGCCGC GGAAGATAAACTGACGTGGATATT
oCS1372	Mcm6_F	Mcm6(1-302)-GST	CAGTTGAT GC ATGC TA ATGTCATCCCCCTTTCCAGCT
oCS1373	Mcm6 306_R	Mcm6(1-302)-GST	TCAGTCACGAT GCGGCCGC TGGACGGACCTCAGATGTTCTT
oCS1374	Mcm7_F	Mcm7(1-253)-GST	CAGTTGAT GC ATGC TA ATGAGTGCGGCACTTCCAT
oCS1375	Mcm7 253_R	Mcm7(1-253)-GST	TCAGTCACGAT GCGGCCGC TGGTTTGACATCAGAAACTCTGG
oCS2187	Sld3promoter_Fwd	Sld3 wt+natural promoter	CCTCCCCAAGAGCTCAGTTC
oCS2188	Sld3_BamHI_Rev	Sld3 wt+natural promoter	CGCGCG GGA TCC CTA TGT GGA TTC TGG AGC AAA TAA AC

Table III. Oligonucleotides for *in vitro* transcription and translation

Code	Name	Sequence	Reference
oCS1711	Orc1_Fwd	ACTGATC TAATACGACTCACTATAGGG CAT CCACCATG GCAAAAACGTTGAAGGATTAC	Gardenal and Speck, unpublished
oCS1712	Orc1_788_Fwd	ACTGATC TAATACGACTCACTATAGGG CAT CCACCATG TCTCATGTAATTACGTTTATGACGC	Gardenal and Speck, unpublished
oCS1713	Orc1_Rev_End	AAAAAAAAAAAAAAAAAAAAAAAAAAAA CTA TAA ATT TCT CAA TGT CTC ATC C	Gardenal and Speck, unpublished
oCS1714	Orc2_Fwd	ACTGATC TAATACGACTCACTATAGGG CAT CCACCATG CTAATGGGGAAGACTTTG	Gardenal and Speck, unpublished
oCS1715	Orc2_505_Fwd	ACTGATC TAATACGACTCACTATAGGG CAT CCACCATG AGTGGTGCTGAAGGTGCG	This study
oCS1771	Orc2_91_Rev+4Met	AAAAAAAAAAAAAAAAAAAAAAAAAAAA TTA CAT CAT CAT CAT CCT ATC AGT TAA TTC TTC CTG TAT C	This study
oCS1772	Orc2_92_Fwd	ACTGATC TAATACGACTCACTATAGGG CAT CCACCATG ATC AAG AAG GAT GAG AAA GAT ACA ATT	This study
oCS1773	Orc2_125_Rev+4Met	AAAAAAAAAAAAAAAAAAAAAAAAAAAA TTA CAT CAT CAT CAT GTT GTT AGA AGT CTT GCT TTC C	This study
oCS1774	Orc2_125_Fwd	ACTGATC TAATACGACTCACTATAGGG CAT CCACCATG AAG CAG GTG ATG GAA AAG ACG	This study
oCS1775	Orc2_225_Rev+2Met	AAAAAAAAAAAAAAAAAAAAAAAAAAAA TTA CAT CAT TTT ATT TTT CGG TAC AGG GGT TG	This study
oCS1776	Orc2_226_Fwd	ACTGATC TAATACGACTCACTATAGGG CAT CCACCATG AAG CTC TAC CAA ACT TCG GAA AC	This study
oCS1779	Orc2_311_Rev	AAAAAAAAAAAAAAAAAAAAAAAAAAAA TTA TTG AGT CAA TTC AAA CCA ATA CTG G	This study
oCS1780	Orc2_311_Fwd	ACTGATC TAATACGACTCACTATAGGG CAT CCACCATG GGA TTC TCC TTA TTA TTT TAT GGT G	This study
oCS1781	Orc2_319_Rev	AAAAAAAAAAAAAAAAAAAAAAAAAAAA TTA TAC ACC ATA AAA TAA TAA GGA GAA TCC	This study
oCS1782	Orc2_320_Fwd	ACTGATC TAATACGACTCACTATAGGG CAT CCACCATG GGT TCG AAA CGT AAT TTT TTG GAA G	This study
oCS1783	Orc2_350_Rev	AAAAAAAAAAAAAAAAAAAAAAAAAAAA TTA TTG TAA TTC ATT CTC ATA AGC CAG	This study
oCS1784	Orc2_351_Fwd	ACTGATC TAATACGACTCACTATAGGG CAT CCACCATG CAA AAC AAA CCT GTA AAT TCC ATC C	This study
oCS1785	Orc2_429_Rev+3Met	AAAAAAAAAAAAAAAAAAAAAAAAAAAA TTA CAT CAT CAT ACC ATC CAG ATT ATG CAC TAC AAG	This study
oCS1786	Orc2_430_Fwd	ACTGATC TAATACGACTCACTATAGGG CAT CCACCATG CCT AGC ATA AGG AAA AAC ACT TTT C	This study
oCS1787	Orc2_461_Rev+2Met	AAAAAAAAAAAAAAAAAAAAAAAAAAAA TTA CAT CAT AGC GTA AAT GTG GTC TGT AGA G	This study
oCS1788	Orc2_462_Fwd	ACTGATC TAATACGACTCACTATAGGG CAT CCACCATG CCG CTC CTC TGG GAC AAC	This study
oCS1789	Orc2_504_Rev	AAAAAAAAAAAAAAAAAAAAAAAAAAAA TTA GCT GGT ATC GCT TTT ACC CAT C	This study
oCS1716	Orc2_Rev_End	AAAAAAAAAAAAAAAAAAAAAAAAAAAA TTA TAG AGT ATT TAA AAC GGT TTT CAG	Gardenal and Speck, unpublished
oCS1717	Orc3_Fwd	ACTGATC TAATACGACTCACTATAGGG CAT CCACCATG AGCGACCTTAACCAATC	Gardenal and Speck, unpublished
oCS1718	Orc3_493_Fwd	ACTGATC TAATACGACTCACTATAGGG CAT CCACCATG AGT TGG GAG CAG GTG CTG	Gardenal and Speck, unpublished
oCS1719	Orc3_Rev_End	AAAAAAAAAAAAAAAAAAAAAAAAAAAA CTA AAT TCC TCT CCA GAC AC	Gardenal and Speck, unpublished
oCS1720	Orc4_Fwd	ACTGATC TAATACGACTCACTATAGGG CAT CCACCATG ACT ATA AGC GAA GCT CGT C	Gardenal and Speck, unpublished
oCS1721	Orc4_384_Fwd	ACTGATC TAATACGACTCACTATAGGG CAT CCACCATG TCCGATTAGAGTTAGCCATTTT	Gardenal and Speck, unpublished
oCS1722	Orc4_Rev_End	AAAAAAAAAAAAAAAAAAAAAAAAAAAA TCA	Gardenal and Speck,

		CAG TTG TGT CCA GGA GTA G	unpublished
oCS1723	Orc5_Fwd	ACTGATC TAATACGACTCACTATAGGG CAT CCACCATG AATGTGACCACTCCGG	Gardenal and Speck, unpublished
oCS1724	Orc5_342_Fwd+2M	ACTGATC TAATACGACTCACTATAGGG CAT CCACCATG ATG ATG GAACCTAGATACGATGCGAG	Gardenal and Speck, unpublished
oCS1725	Orc5_Rev_End	AAAAAAAAAAAAAAAAAAAAAAAAAAAA TCA TTC GTG AAT ATC GCT GAA G	Gardenal and Speck, unpublished
oCS1726	Orc6_Fwd	ACTGATC TAATACGACTCACTATAGGG CAT CCACCATG TCC ATG CAA CAA GTC CAA C	Gardenal and Speck, unpublished
oCS1727	Orc6_Rev_End	AAAAAAAAAAAAAAAAAAAAAAAAAAAA TTA TAA AGG TTC TGT TAA TGC CAA ATC	Gardenal and Speck, unpublished
oCS1728	Cdt1_Fwd	ACTGATCT AATACGACTCACTATAGGG CAT CCACCATG AGT GGC ACA GCT AAT AG	Gardenal and Speck, unpublished
oCS1730	Cdt1_Rev_End	AAAAAAAAAAAAAAAAAAAAAAAAAAAA TCA ATC TTG TTG TTT TGA TTT GTG	Gardenal and Speck, unpublished
oCS1731	Cdc6_Fwd	ACTGATC TAATACGACTCACTATAGGG CAT CCACCATG TCAGCTATACCAATAACTCC	Gardenal and Speck, unpublished
oCS1732	Cdc6_400_Fwd+2M	ACTGATC TAATACGACTCACTATAGGG CAT CCACCATG ATG ATG AATTCTACGAGAACGAGGATAGC	Gardenal and Speck, unpublished
oCS1733	Cdc6_Rev_End	AAAAAAAAAAAAAAAAAAAAAAAAAAAA CTA GTG AAG GAA AGG TTT CAA AAT TG	Gardenal and Speck, unpublished
oCS1670	Mcm2_Fwd	ACTGATC TAATACGACTCACTATAGGG CAT CCACCATG TCTGATAATAGAAGACGTAGAC	This study
oCS1799	Mcm2_50_Fwd	ACTGATC TAATACGACTCACTATAGGG CAT CCACCATG GAC GAT AAT GAA GTT GAC GAT G	This study
oCS1801	Mcm2_101_Fwd	ACTGATC TAATACGACTCACTATAGGG CAT CCACCATG CAACAAGAACTATCTTTAAGCGAAC	This study
oCS1803	Mcm2_151_Fwd	ACTGATC TAATACGACTCACTATAGGG CAT CCACCATG AGA CGA AGA AGG AGA AGG CAG	This study
oCS1805	Mcm2_200_Fwd	ACTGATC TAATACGACTCACTATAGGG CAT CCACCATG CAACCTAATGTCTCAAGAACTATTG	This study
oCS1673	Mcm2_322_Fwd	ACTGATC TAATACGACTCACTATAGGG CAT CCACCATG GGG GTG GTG ACA AGA AGA AC	This study
oCS1784	Mcm2_666_Fwd	ACTGATC TAATACGACTCACTATAGGG CAT CCACCATG AAT GTT AGT TTG ACC GAG CCT ATT	This study
oCS1800	Mcm2_200_Rev+1Met	AAAAAAAAAAAAAAAAAAAAAAAAAAAA TTA CAT TTG TGT TAT CCA TTC CGA GTA AC	This study
oCS1843	Mcm2_218_Rev	AAAAAAAAAAAAAAAAAAAAAAAAAAAA TTA ATA TTC CAG CAG GAA TGA TTT TAA C	This study
oCS1845	Mcm2_227_Rev	AAAAAAAAAAAAAAAAAAAAAAAAAAAA TTA ATA TAC AGA ACG ACC CGT TTC ATC	This study
oCS1847	Mcm2_241_Rev	AAAAAAAAAAAAAAAAAAAAAAAAAAAA TTA AGA TTC AGA ATT CAT TTC ACC TAA TG	This study
oCS2174	Mcm2_250_Rev+3Met	AAAAAAAAAAAAAAAAAAAAAAAAAAAA TTA CAT CAT CAT CGC TAA GTG TCT ATA ATT AAC CTC	This study
oCS1804	Mcm2_300_Rev+2Met	AAAAAAAAAAAAAAAAAAAAAAAAAAAA TTA CAT CAT AAA ATC AGA GAT TCT TAC GTG AAT TTC	This study
oCS1672	Mcm2_332_Rev	AAAAAAAAAAAAAAAAAAAAAAAAAAAA TTA CCC AGT GAC GCG TAC TAG	This study
oCS1806	Mcm2_350_Rev+3Met	AAAAAAAAAAAAAAAAAAAAAAAAAAAA TTA CAT CAT CAT TGG GCC CAA AAT GGA GCC A	This study
oCS1783	Mcm2_665_Rev	AAAAAAAAAAAAAAAAAAAAAAAAAAAA TTA CTG AGC TAA AGG CAA GGT TGA A	This study
oCS1785	Mcm2_Rev_end	AAAAAAAAAAAAAAAAAAAAAAAAAAAA TTA GTG ACC CAA GGT ATA AAT TGC	This study
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oCS2061	hMcm2_232_Rev	AAAAAAAAAAAAAAAAAAAAAAAAAAAA TCA TGC CAA GTC CTC ATA GTT CAC	This study
oCS2174	Mcm2_250_R232S_Rev	AAAAAAAAAAAAAAAAAAAAAAAAAAAA TTA CAT CAT CAT CGC TAA GTG TCT ATA ATT AAC CTC CAA AGA TTC AGA ATT CAT TTC ACC TAA TGT AGA AAT ACG	This study
oCS2175	Mcm2_250_E243V_Rev	AAAAAAAAAAAAAAAAAAAAAAAAAAAA TTA CAT CAT CAT CGC TAA GTG TCT ATA ATT AAC AAC CAA AGA TTC AGA ATT CAT TTC ACC TAA TGT TCT AAT ACG	This study

oCS2176	Mcm2_250_R232S+E243V_Rev	AAAAAAAAAAAAAAAAAAAAAAAAAAAA TTA CAT CAT CAT CGC TAA GTG TCT ATA ATT AAC AAC CAA AGA TTC AGA ATT CAT TTC ACC TAA TGT AGA AAT ACG	This study
oCS2177	Mcm2_250_E243K_Rev	AAAAAAAAAAAAAAAAAAAAAAAAAAAA TTA CAT CAT CAT CGC TAA GTG TCT ATA ATT AAC TTT CAA AGA TTC AGA ATT CAT TTC ACC TAA TG	This study
oCS2178	Mcm2_250_T233D+S239R_Rev	AAAAAAAAAAAAAAAAAAAAAAAAAAAA TTA CAT CAT CAT CGC TAA GTG TCT ATA ATT AAC CTC CAA AGA TTC TCT ATT CAT TTC ACC TAA ATC TCT AAT ACG TGC ACC	This study
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oCS2191	Mcm2_250_E236K_Rev	AAAAAAAAAAAAAAAAAAAAAAAAAAAA TTA CAT CAT CAT CGC TAA GTG TCT ATA ATT AAC CTC CAA AGA TTC AGA ATT CAT CTT ACC TAA TGT TCT AAT ACG TGC ACC	This study
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oCS1669	Mcm3_Rev_end	AAAAAAAAAAAAAAAAAAAAAAAAAAAA TCA GAC TCT CCA AAC TTT ATC G	This study
oCS1677	Mcm4_Fwd	ACTGATC TAATACGACTCACTATAGGG CAT CCACCATG TCTCAACAGTCTAGCTCTCCAA	This study
oCS1678	Mcm4_Rev_end	AAAAAAAAAAAAAAAAAAAAAAAAAAAA TCA GAC ACG GTT ATT CAG GCG	This study
oCS1681	Mcm5_Fwd	ACTGATC TAATACGACTCACTATAGGG CAT CCACCATG TCATTGATAGACCGGAAATATAC	This study
oCS1682	Mcm5_Rev_end	AAAAAAAAAAAAAAAAAAAAAAAAAAAA TCA TAC ACC ACT TCT GTA AAT ATT C	This study
oCS1685	Mcm6_Fwd	ACTGATC TAATACGACTCACTATAGGG CAT CCACCATG TCATCCCCTTTTCCAGC	This study
oCS1686	Mcm6_Rev_end	AAAAAAAAAAAAAAAAAAAAAAAAAAAA TTA GCT GGA ATC CTG TGG TTC	This study
oCS1689	Mcm7_Fwd	ACTGATC TAATACGACTCACTATAGGG CAT CCACCATG AGTGCGGCACTTCCATC	This study
oCS1690	Mcm7_Rev_end	AAAAAAAAAAAAAAAAAAAAAAAAAAAA TCA AGC GTC TTG TAG ATC GAT ATC AG	This study
oCS1784	Cdc45_Fwd	ACTGATC TAATACGACTCACTATAGGG CAT CCACCATG TAT TAT GGA ATC AGC CAG TTT AG	This study
oCS1896	Cdc45_164_Rev+3Met	AAAAAAAAAAAAAAAAAAAAAAAAAAAA TTA CAT CAT CAT TTC AAG CAA TTT ATA GTA TGC TTT TTT TTG	This study
oCS1897	Cdc45_165_Fwd	ACTGATC TAATACGACTCACTATAGGG CAT CCACCATG CTG GAC GAG GAG AGT GGT G	This study
oCS1898	Cdc45_332_Rev+4Met	AAAAAAAAAAAAAAAAAAAAAAAAAAAA TTA CAT CAT CAT CAT AGA ATG CCT CAG TAA GAA AAG G	This study
oCS1899	Cdc45_333_Fwd	ACTGATC TAATACGACTCACTATAGGG CAT CCACCATG TCA TTG TAC GAC AGT TTT TAT TAT TCT AAC	This study

oCS1900	Cdc45_517_Rev+1Met	AAAAAAAAAAAAAAAAAAAAAAAAAAAA TTA CAT TTT CTT CTC CAA TAT AGC AAC CCC	This study
oCS1901	Cdc45_518_Fwd	ACTGATC TAATACGACTCACTATAGGG CAT CCACCATG TTA ATC AAG CAT TTA AGA ATT TAT AGA TTA TG	This study
oCS1902	Cdc45_650_Rev+2Met	AAAAAAAAAAAAAAAAAAAAAAAAAAAA TTA CAT CAT TAA CAA TCC ACT CAA GGT CAG C	This study
oCS1785	Cdc45_Rev	AAAAAAAAAAAAAAAAAAAAAAAAAAAA TTA TAA CAA TCC ACT CAA GGT C	This study
oCS1766	Sld3_Fwd	ACTGATC TAATACGACTCACTATAGGG CAT CCACCATG GAA ACA TGG GAA GTC ATA G	This study
oCS1767	Sld3_Rev	AAAAAAAAAAAAAAAAAAAAAAAAAAAA CTA TGT GGA TTC TGG AGC AAA TA	This study
oCS2040	Sld7_Fwd	ACTGATC TAATACGACTCACTATAGGG CAT CCACCATG TCA CGG AAA TTA TGC ACA CTA AAT TTT	This study
oCS2041	Sld7_Rev	AAAAAAAAAAAAAAAAAAAAAAAAAAAA CTA CAT TCA TGA TTT GGT AAA GAG CTT CAG	This study
oCS2041	Dpb11_Fwd	ACTGATC TAATACGACTCACTATAGGG CAT CCACCATG AAG CCC TTT CAA GGA ATA AC	This study
oCS2043	Dpb11_Rev	AAAAAAAAAAAAAAAAAAAAAAAAAAAA CTA TCA AGA ATC TAA TTC CTT TGT CTG	This study
oCS2044	Sld2_Fwd	ACTGATC TAATACGACTCACTATAGGG CAT CCACCATG TAC TCA TTT GAA CTG GAC AAA TTG	This study
oCS2045	Sld2_148_Rev+1M	AAAAAAAAAAAAAAAAAAAAAAAAAAAA CTA CAT TGG TTC CGC CAC TAA CGT AC	This study
oCS2046	Sld2_149_Fwd	ACTGATC TAATACGACTCACTATAGGG CAT CCACCATG ATA TCC AGT GTG AAA CGC CAA TTA	This study
oCS2047	Sld2_327_Rev+3M	AAAAAAAAAAAAAAAAAAAAAAAAAAAA CTA CAT CAT CAT AAA TAT GTC CTT CAC AAC TTT TGG	This study
oCS2048	Sld2_328_Fwd	ACTGATC TAATACGACTCACTATAGGG CAT CCACCATG CAA GAG GAT GAT GAT AAT GAT GAT AG	This study
oCS2049	Sld2_Rev+4M	AAAAAAAAAAAAAAAAAAAAAAAAAAAA CTA CAT CAT CAT CAT TCA CCT TCT TCC CCA TCG TC	This study
oCS2052	Sld2_76_Fwd	ACTGATC TAATACGACTCACTATAGGG CAT CCACCATG GAC GAA GTC GTA GAG ATA GG	This study
oCS2053	Sld2_249_Rev+1M	AAAAAAAAAAAAAAAAAAAAAAAAAAAA CTA CAT AAT ATC CGC TTG ATC CTT TGA GG	This study
oCS2054	Sld2_250_Fwd	ACTGATC TAATACGACTCACTATAGGG CAT CCACCATG TCA ACT TCA TTT AGT CCG TCT CC	This study
oCS2055	Sld2_365_Rev+3M	AAAAAAAAAAAAAAAAAAAAAAAAAAAA CTA CAT CAT CAT CTC GGT TTC TGG ATC ATT GTC	This study
oCS2050	Sld2_Rev	AAAAAAAAAAAAAAAAAAAAAAAAAAAA CTA TCA CCT TCT TCC CCA TCG TC	This study

Table IV. Peptides

Name	Sld3 aa	Mutations	Sequence
CR1a	152-175	wt	DYYSDPKKEYIESKYYDALFSIHTP
CR1b	170-195	wt	FSIHTPLAYFVKSNLVRKNTCRTKY
CR2	196-226	wt	GSDSYKIAYQAMLQKFLLSIVQFKDRHDNRL
CR3a	249-287	wt	IQDENKNSSTIADLCVVVLSREIKLQILLLEIIGLNDL
CR3b	286-311	wt	DLDWNFDFEKKYKLKLRSLNLTK
CR4	333-355	wt	ITTSLDYCEQLDLYLDRACILDI
CR5	384-419	wt	KSKEASLVGFINYVLIPYFNKKVPHAVEFIIQKLKG
CR6	518-546	wt	RTKSDLTMNHLQKRQFSVSDLSTTRVPNS
CR3a_mut1	249-287	R269E_E270R_Q274A_E280R	IQDENKNSSTIADLCVVVLSREIKLAILLLEIIGLNDL
CR3a_mut2	249-287	K267D_K272D	IQDENKNSSTIADLCVVVLSREIDLQILLLEIIGLNDL
CR3a_mut3	249-287	L273E_L277E_L284E	IQDENKNSSTIADLCVVVLSREIKEQILELLEIIGENDL
CR3a_mut4	249-287	I275K_L278E_I282K	IQDENKNSSTIADLCVVVLSREIKLQKLLELEIKGLNDL

Table V. Antibodies

Antibody name	Antigen	Peptide Sequence		Source
Cdc6	Cdc6	-	mouse	Santacruz (SC53218)
ORC1	ORC1	-	mouse	(Bell et al, 1995)
MCM2	MCM2	-	mouse	(Zou & Stillman, 2000)
Sld3 (318)	Sld3	CTKKGLVRRRSKKKTSE	rabbit	Speck lab
Sld3 (426)	Sld3	CKLKGPSMRPKRALKKVND	rabbit	Speck lab
Sld2	Sld2	CRKRPKRRKVIRRLRDNDPETE	rabbit	Speck lab
Sld7	Sld7	THRDELKRISMGSGEVS	rabbit	Speck lab
Dpb11	Dpb11	CGSIQDKKRTASLEKPMRRQTRNQTK	rabbit	Speck lab
FLAG	FLAG epitope	-	mouse	Sigma (A8592)
MBP	MBP	-	mouse	NEB (E8032L)
GST	GST	-	mouse	Pierce (P08263)
HA	HA	-	mouse	Sigma (H3663)
Anti-mouse	gamma chain	-	goat	Sigma (A 3673)
Anti-mouse	kappa light chain	-	goat	Millipore (AP200P)
Anti-rabbit	IgG	-	goat	Sigma (A 6154)

Table VI. Published protein-protein interactions ¹

Interaction	Binding involves	Method	Reference
<i>pre-RC proteins</i>			
ORC - Cdc6	Orc1 and Orc2, Cdc6 FL	Two-hybrid	(Huo et al, 2012)
Orc6 - Cdt1	Orc6 aa 1-185, 270-435; Cdt1 FL	Purified proteins	(Chen et al, 2007)
	Full-length proteins	Purified proteins	(Chen et al, 2007)
Cdc6 - Mcm3	Full-length proteins	Purified proteins	(Sun et al, 2013)
Cdt1 - MCM2-7	Cdt1 aa 1-312, MCM2-7 FL	Purified proteins	(Fernandez-Cid et al, 2013)
	Cdt1 aa 306-604, MCM2-7 FL	Purified proteins	(Fernandez-Cid et al, 2013)
	Cdt1 aa 472-604, MCM2-7 FL	Purified proteins	(Takara & Bell, 2011)
<i>pre-IC proteins</i>			
MCM2-7 - DDK	Mcm4 and Cdc7	Purified proteins	(Sheu & Stillman, 2006)
	Mcm2 aa 204-278, DDK	Purified proteins	(Bruck & Kaplan, 2009)
	Mcm2 and Dbf4	Purified proteins	(Ramer et al, 2013)
	Mcm5 and Cdc7	Purified proteins	(Ramer et al, 2013)
MCM2-7 - Sld3	Full-length proteins	Purified proteins	(Bruck & Kaplan, 2011a)
Sld3 - Sld7	Sld3 aa 60-120, Sld7 FL	Two-hybrid and purified proteins	(Tanaka et al, 2011b)
Sld3 - Cdc45	Full-length proteins	Two-hybrid	(Kamimura et al, 2001)
	Sld3 aa 150-430, Cdc45 FL	Two-hybrid	(Tanaka et al, 2011b)
	Full-length proteins	Two-hybrid	(Zegerman & Diffley, 2010)
	Full-length proteins	Purified proteins	(Bruck & Kaplan, 2011a; Bruck & Kaplan, 2013)
Cdc45 - MCM2-7	Full-length proteins	Purified proteins	(Bruck & Kaplan, 2011a)
Sld3 - Dpb11	Sld3 aa 527-668, Dpb11 FL	Two-hybrid	(Tanaka et al, 2007b)
	Full-length proteins	Two-hybrid	(Zegerman & Diffley, 2010)
	Full-length proteins	Purified proteins	(Bruck et al, 2011)
	Full-length proteins	Purified proteins	(Wang et al, 2012)
Cdc45 - GINS	Full-length proteins	Purified proteins	(Bruck & Kaplan, 2011a)
Cdc45 - Rad53	Full-length proteins	Two-hybrid and purified proteins	(Aucher et al, 2010)
Sld2 - MCM2-7	Full-length proteins	Purified proteins	(Bruck et al, 2011)
Dpb11 - Sld2	Full-length proteins	Two-hybrid	(Kamimura et al, 1998)
	Full-length proteins	Two-hybrid	(Tak et al, 2006)
Dpb11 - Pol ε	Dpb11 FL and Dpb2 FL	Two-hybrid	(Edwards et al, 2002)
Dpb11 - GINS	Dpb11 FL and Psf1 FL	Two-hybrid	(Takayama et al, 2003)
Sld2 - Pol ε	Sld2 FL and Dpb2 FL	Purified proteins	(Muramatsu et al, 2010)
Pol ε - GINS	Dpb2 FL and Sld5 FL	Purified proteins	(Muramatsu et al, 2010)
GINS - Ctf4	Full-length proteins	Purified proteins	(Tanaka et al, 2009)
	Full-length proteins	Purified proteins	(Gambus et al, 2009)
Pol α - Ctf4	Pol1 FL and Ctf4 FL	Two-hybrid	(Zhou & Wang, 2004)
	Full-length proteins	Two-hybrid	(Gambus et al, 2009)
Mcm10 - MCM2-7	Mcm10 FL and Mcm2 FL	Two-hybrid	(Merchant et al, 1997)
	Mcm10 FL and Mcm2 FL	Two-hybrid	(Lee & Hurwitz, 2000)
	Mcm10 FL and Mcm3 FL	Two-hybrid	(Liachko & Tye, 2009)
	Mcm10 FL and Mcm6 FL	Two-hybrid	(Merchant et al, 1997)
	Mcm10 FL and Mcm6 FL	Two-hybrid	(van Deursen et al, 2012)

¹ As in Tognetti, Riera and Speck, submitted

Table VII. Orthologs of replication factors ¹

<i>S. cerevisiae</i>	<i>S. pombe</i>	Metazoa	Function
ORC (Orc1-6)	ORC (Orp1-6)	ORC	Origin Recognition Complex, pre-RC component
Cdc6	Cdc18	Cdc6	ATP-binding protein, pre-RC component
Cdt1	Cdt1	Cdt1	DNA replication licensing factor, pre-RC component
MCM2-7	MCM2-7	MCM2-7	Replicative DNA helicase, pre-RC and CMG component
-	-	Geminin	DNA licensing inhibitor
?	?	GEMC1	Required for recruitment of Cdc45 to chromatin
?	?	DUE-B	Required for recruitment of Cdc45 to chromatin
Sld3	Sld3	Treslin/Ticrr	Required for DNA helicase activation and pre-IC formation
Sld7	?	MTBP?	Non-essential factor, interacts with Sld3/Treslin
Cdc45	Cdc45/Sna41	Cdc45	Required for pre-IC formation, CMG component
Sld2	Drc1	RecQ4/ RecQL4?	Required for helicase activation and pre-IC formation, pre-LC component
Dpb11	Cut5/Rad4	TopBP1/Cut5/Mus101	Loads Pol ϵ and GINS into pre-IC, pre-LC component
GINS	GINS	GINS	CMG and pre-LC component
Pol ϵ	Pol ϵ	Pol ϵ	Leading strand polymerase, pre-LC component
MCM10	MCM10	MCM10	Required for CMG activation
RPA	RPA	RPA	Single-stranded DNA binding protein
Ctf4	Mcl1	AND-1	Required for sister chromatid cohesion; interacts with Pol α
PCNA	PCNA	PCNA	Sliding clamp of DNA polymerases
<i>Kinases involved in DNA replication</i>			
Dbf4	Dfp1/Him1	Dbf4	DDK regulatory subunit
Cdc7	Hsk1	Cdc7	DDK catalytic subunit
Clb5	Cig2	Cyclin E	Cyclin involved in S phase, CDK regulatory subunit
Cdc28	Cdc2	Cdc2/Cdk2	CDK catalytic subunit
Mec1	Rad3	ATR	Genome integrity checkpoint protein kinase
Rad53	Cds1	Chk2	DNA damage response protein kinase

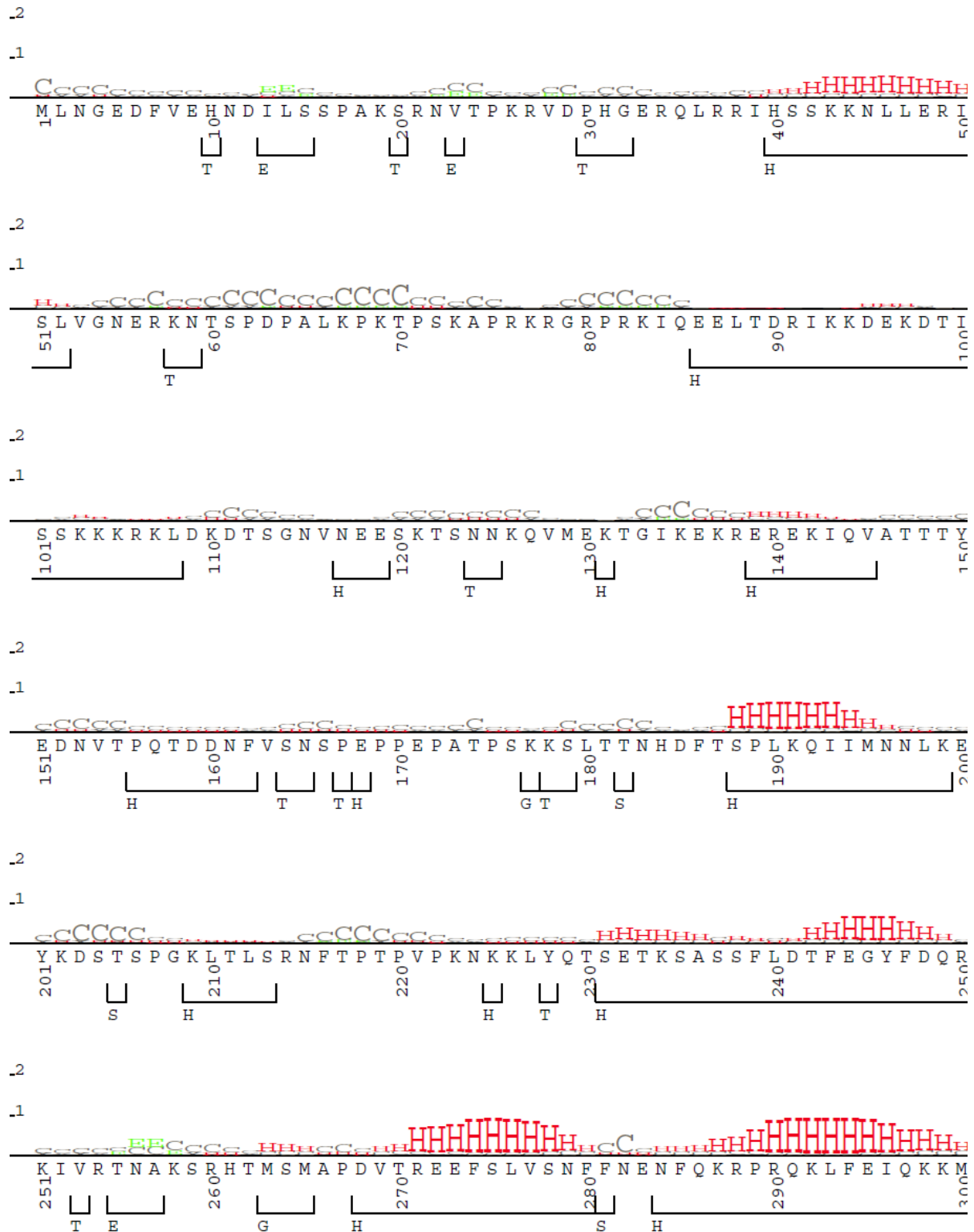
The replicative factors whose association with origins is kinases independent are shown in grey; the factors that associate in a DDK dependent manner are shown in red and in purple are shown the factors that require both DDK and CDK. No colour is shown whenever the kinase requirement has not been directly determined.

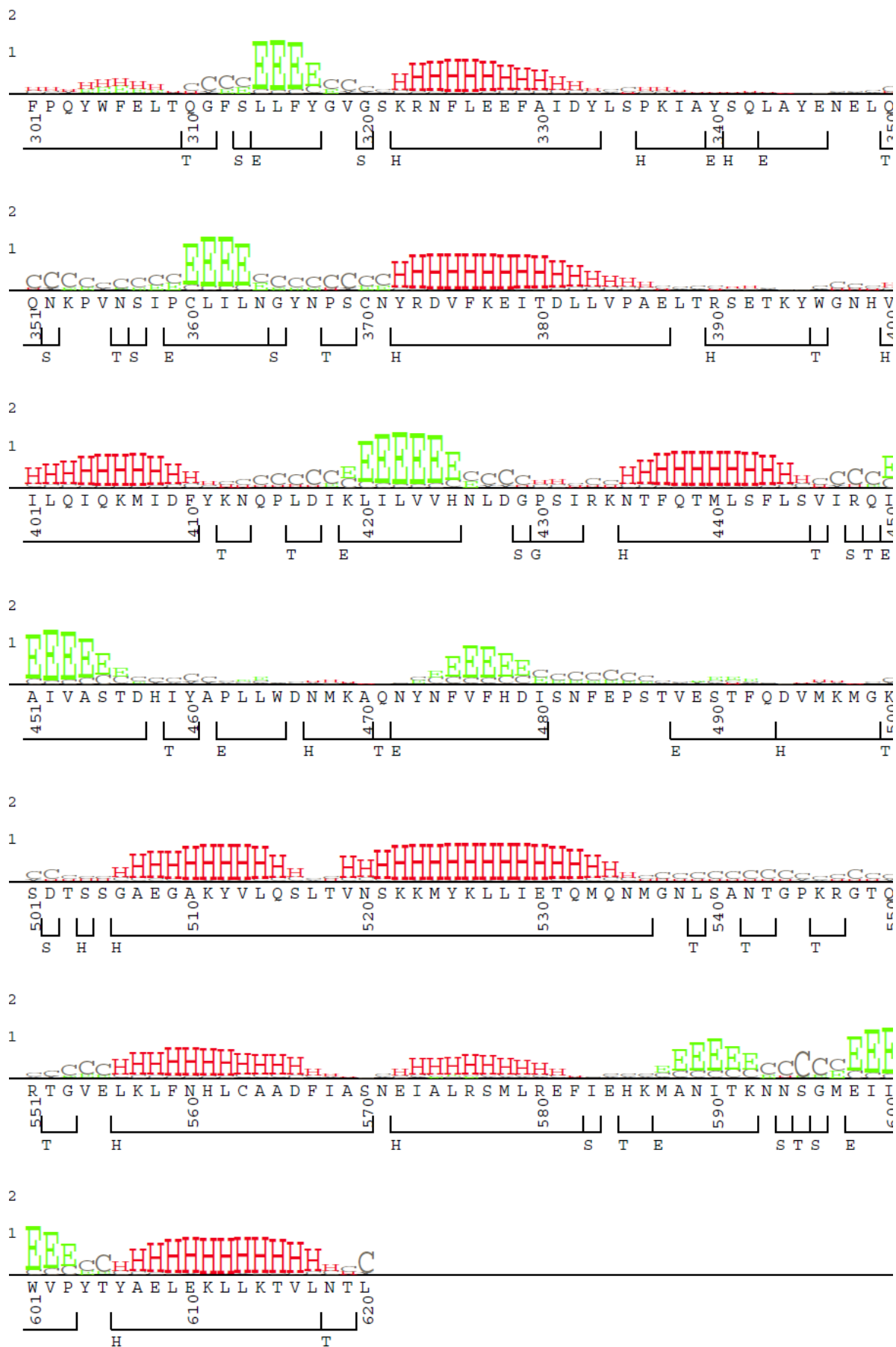
¹ As in Tognetti, Riera and Speck, submitted

Appendix II - Secondary structure predictions

Orc2

YBR060C.t2k DSSP-EHL2



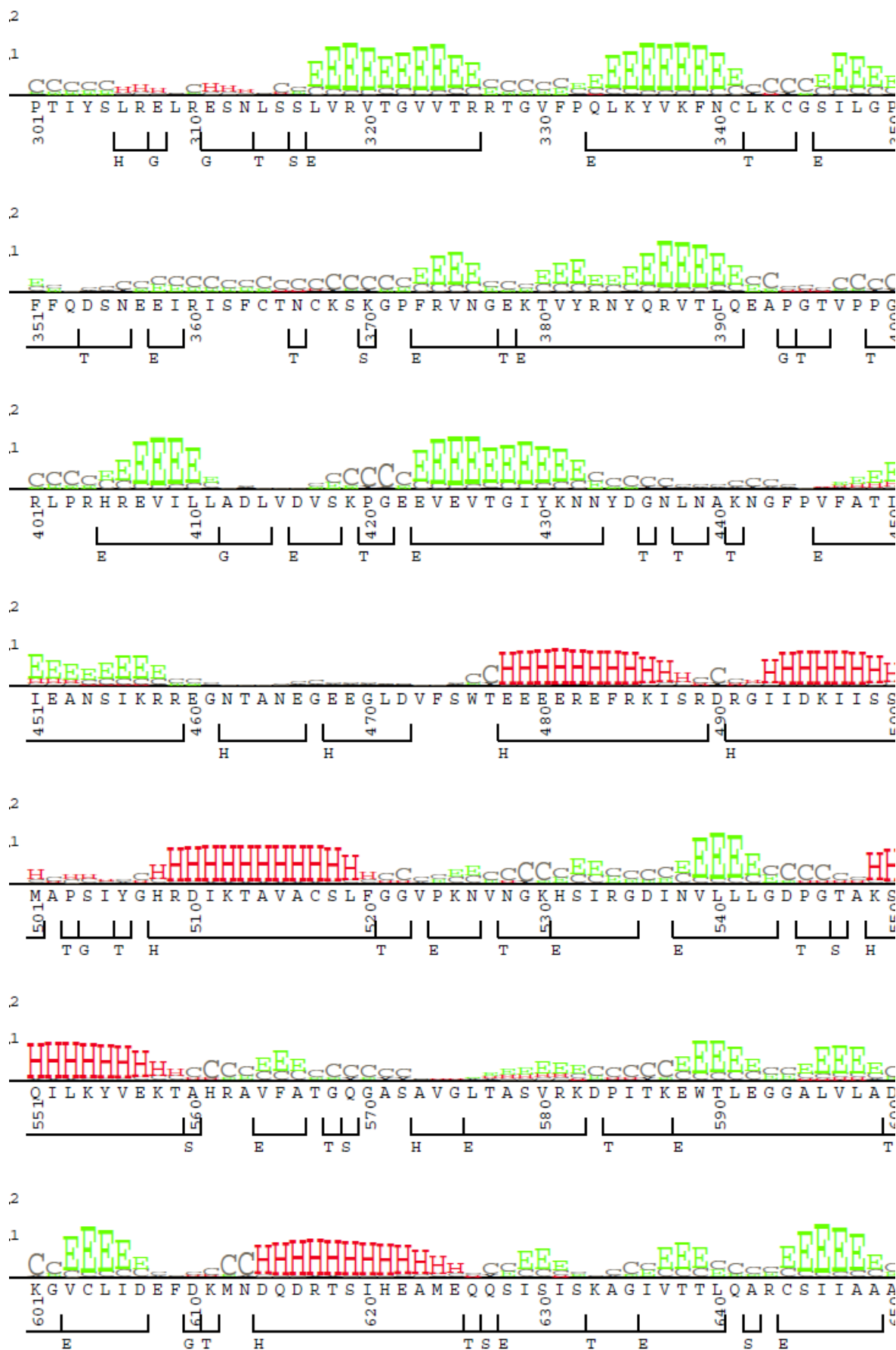


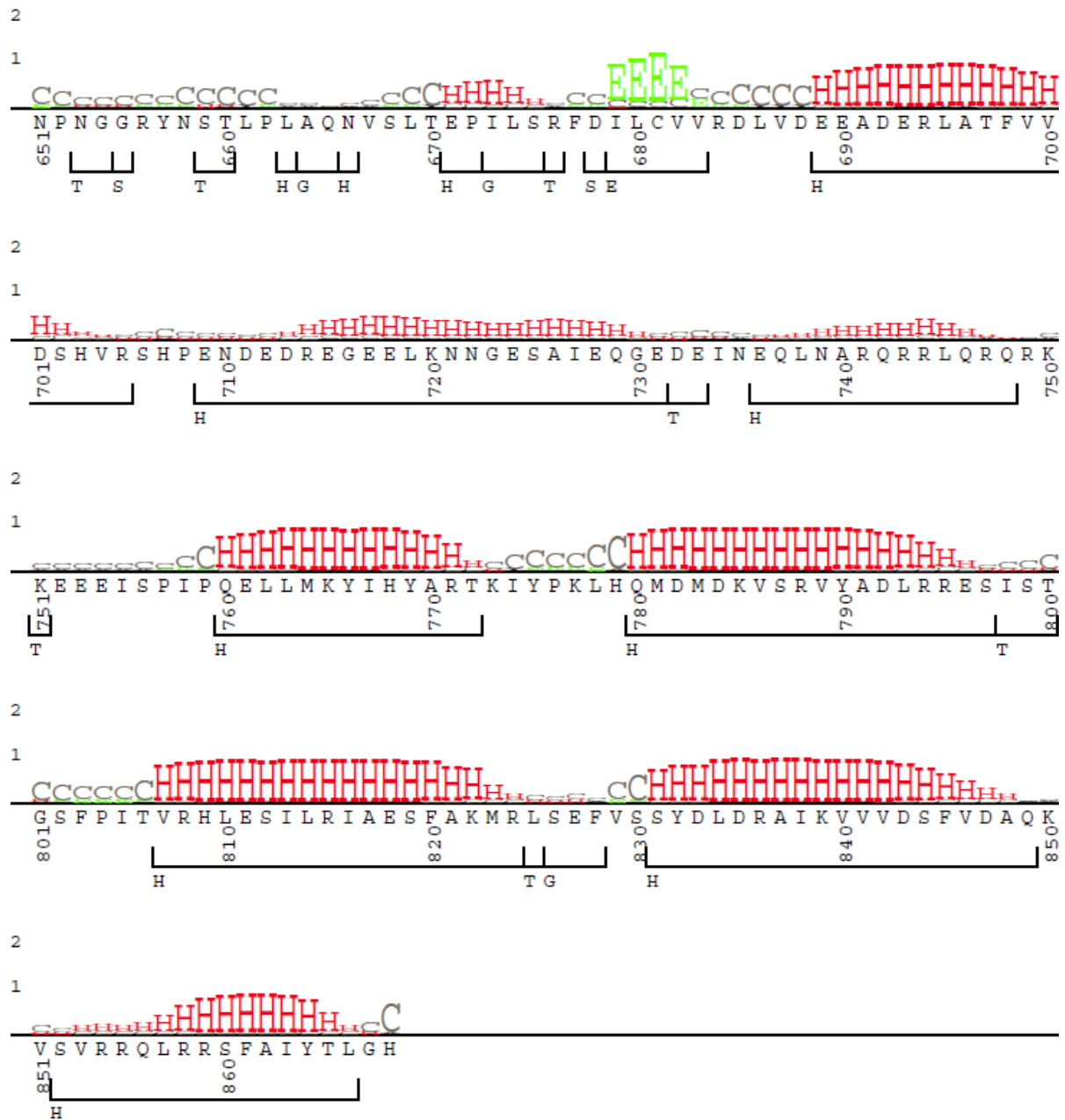
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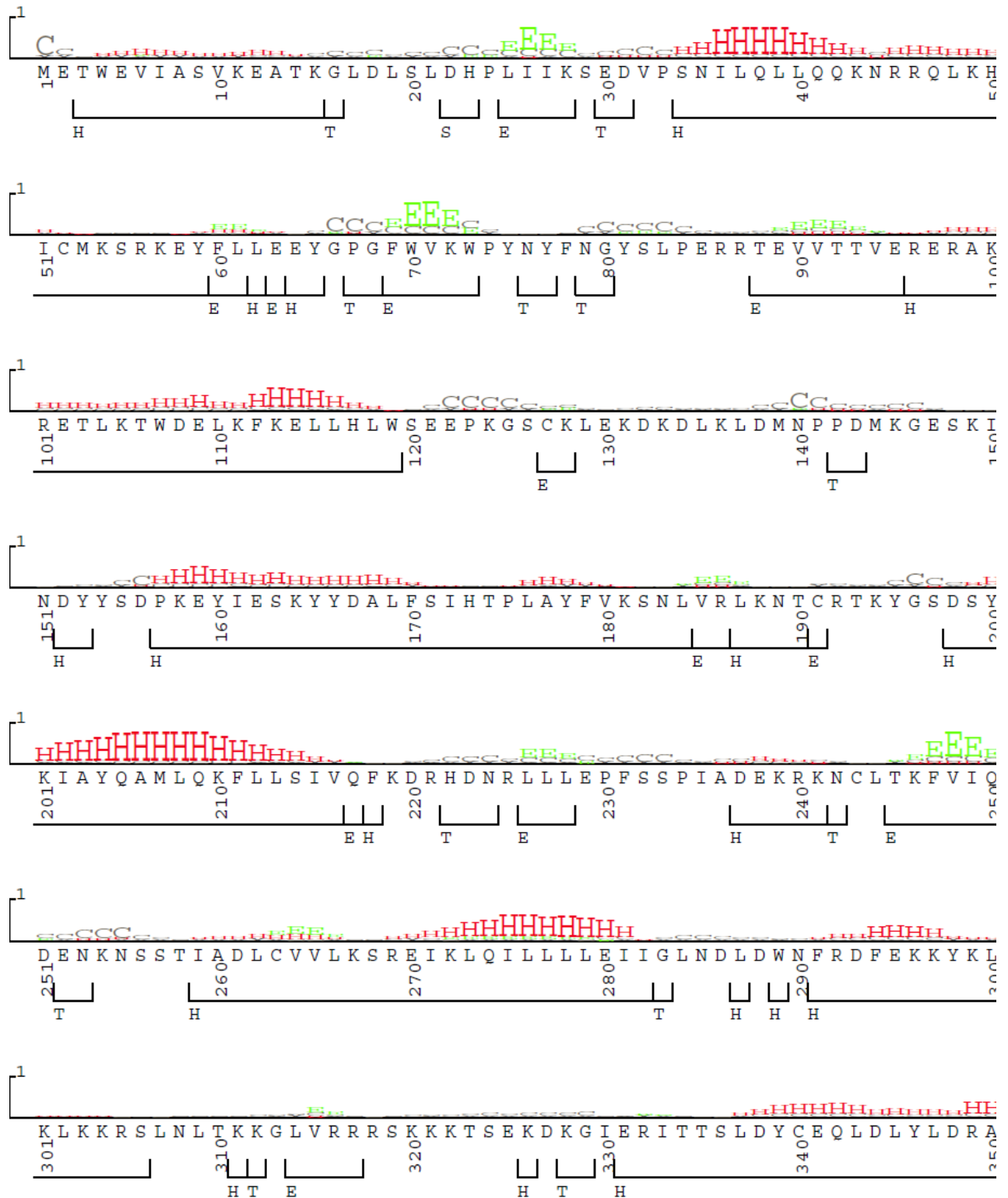


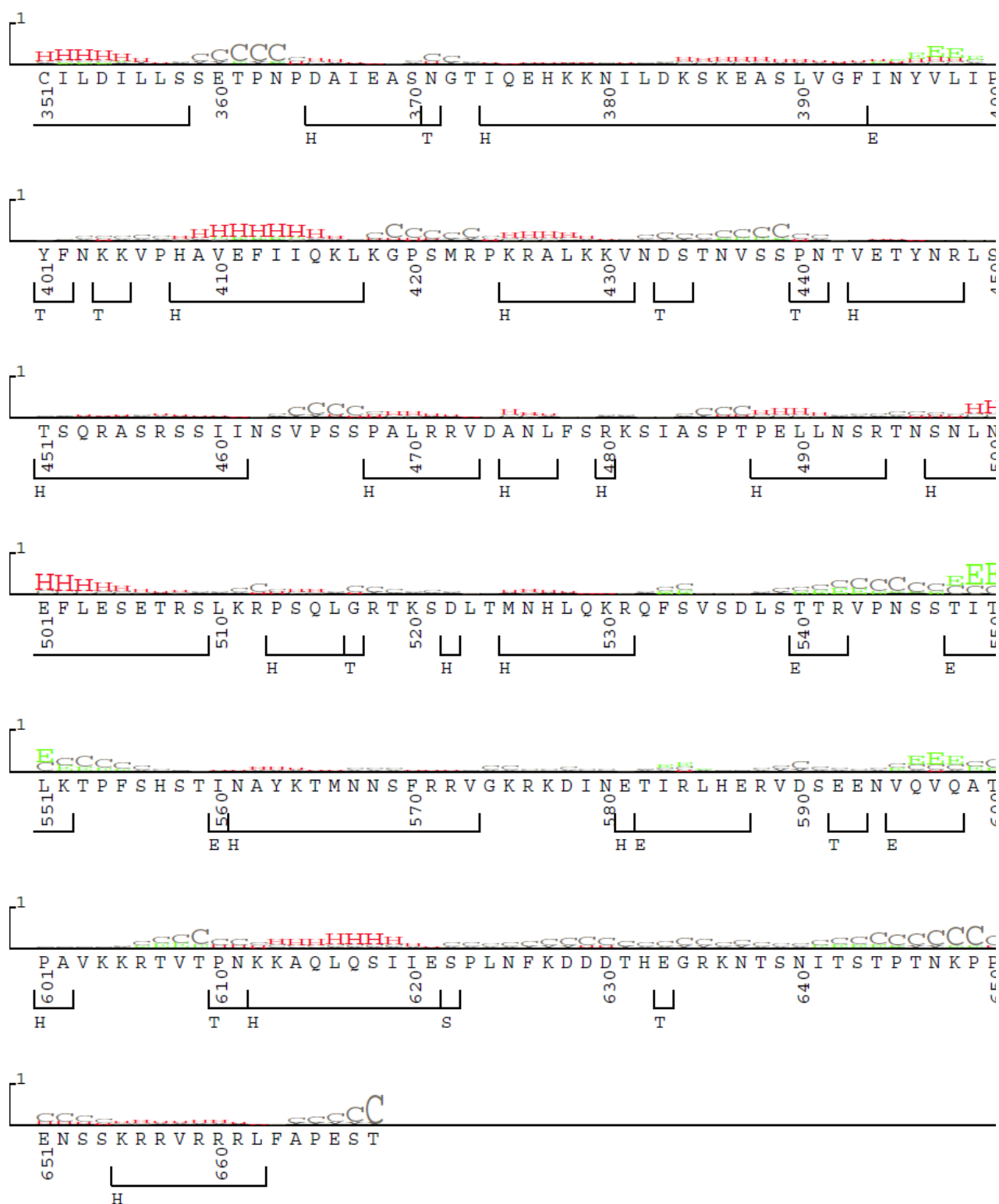




Sld3

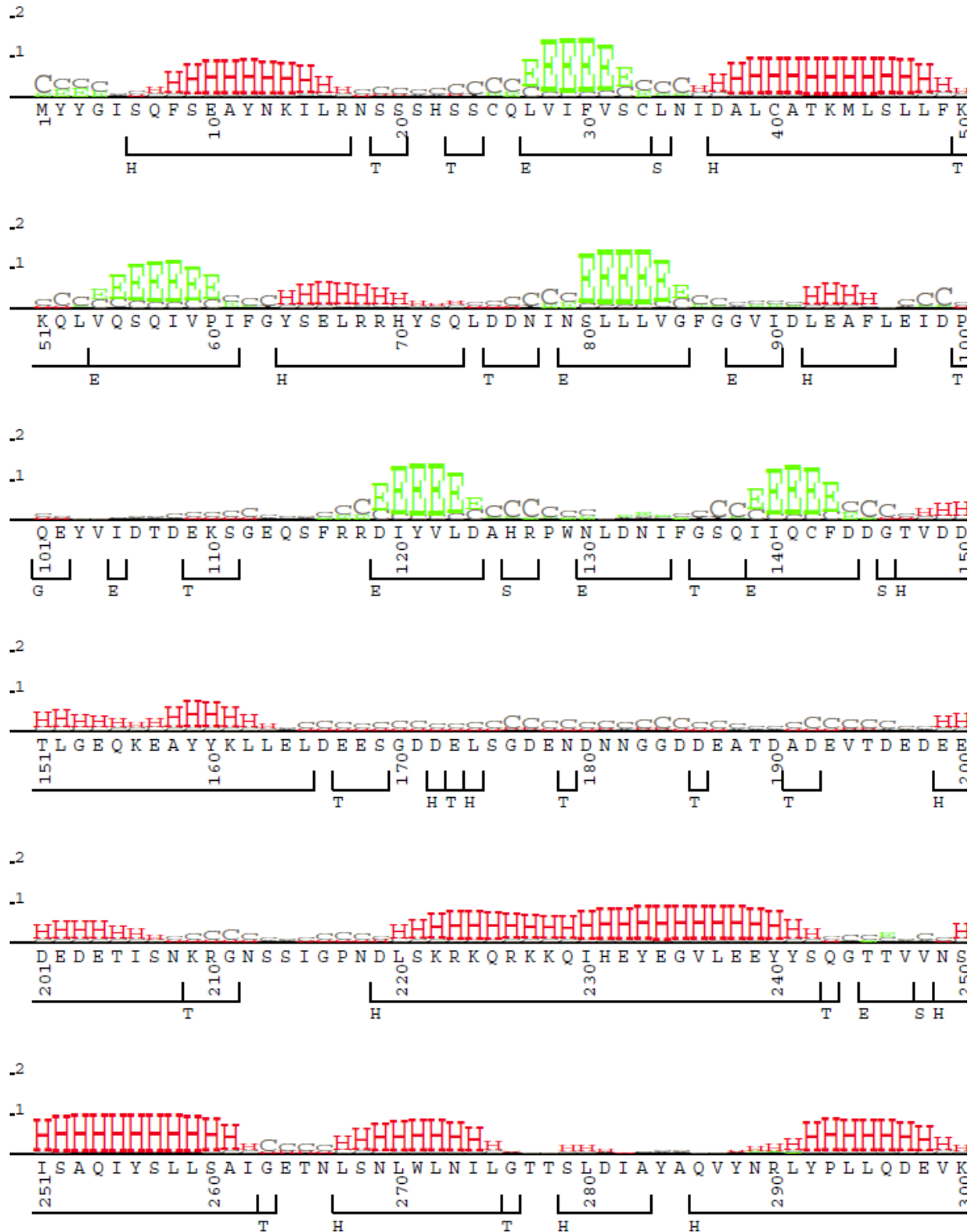
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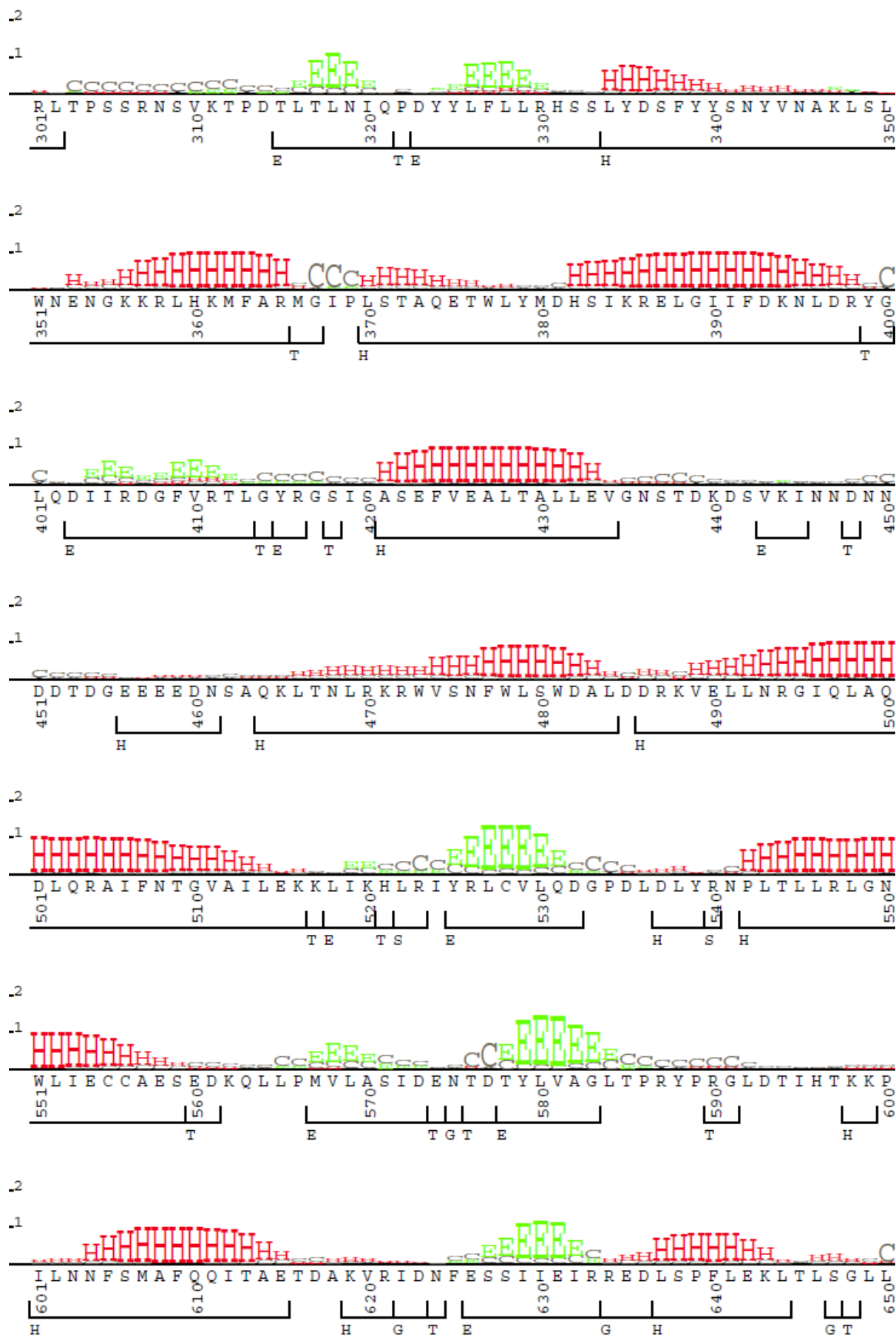




Cdc45

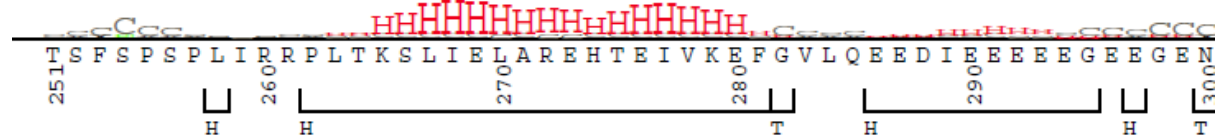
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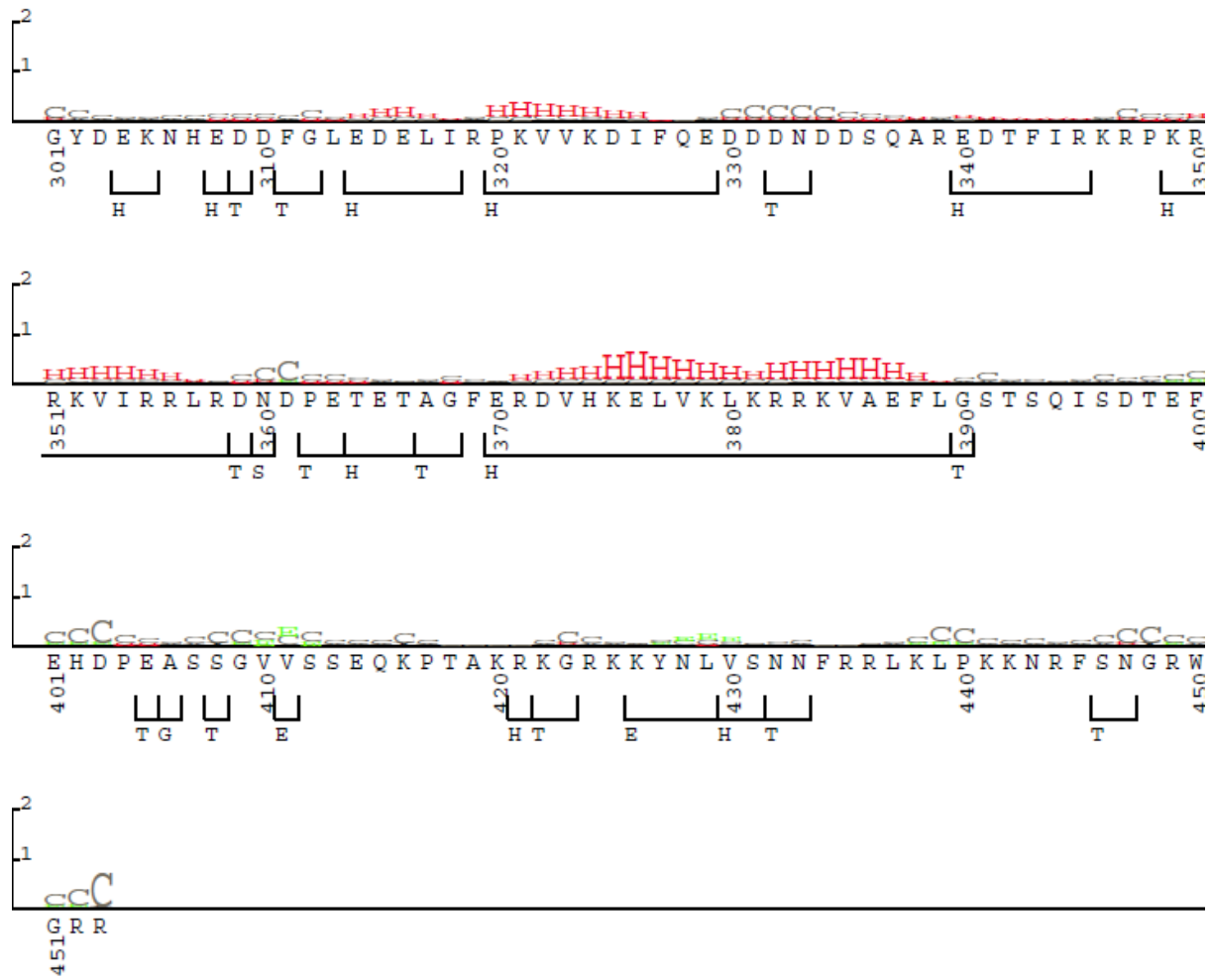




2.

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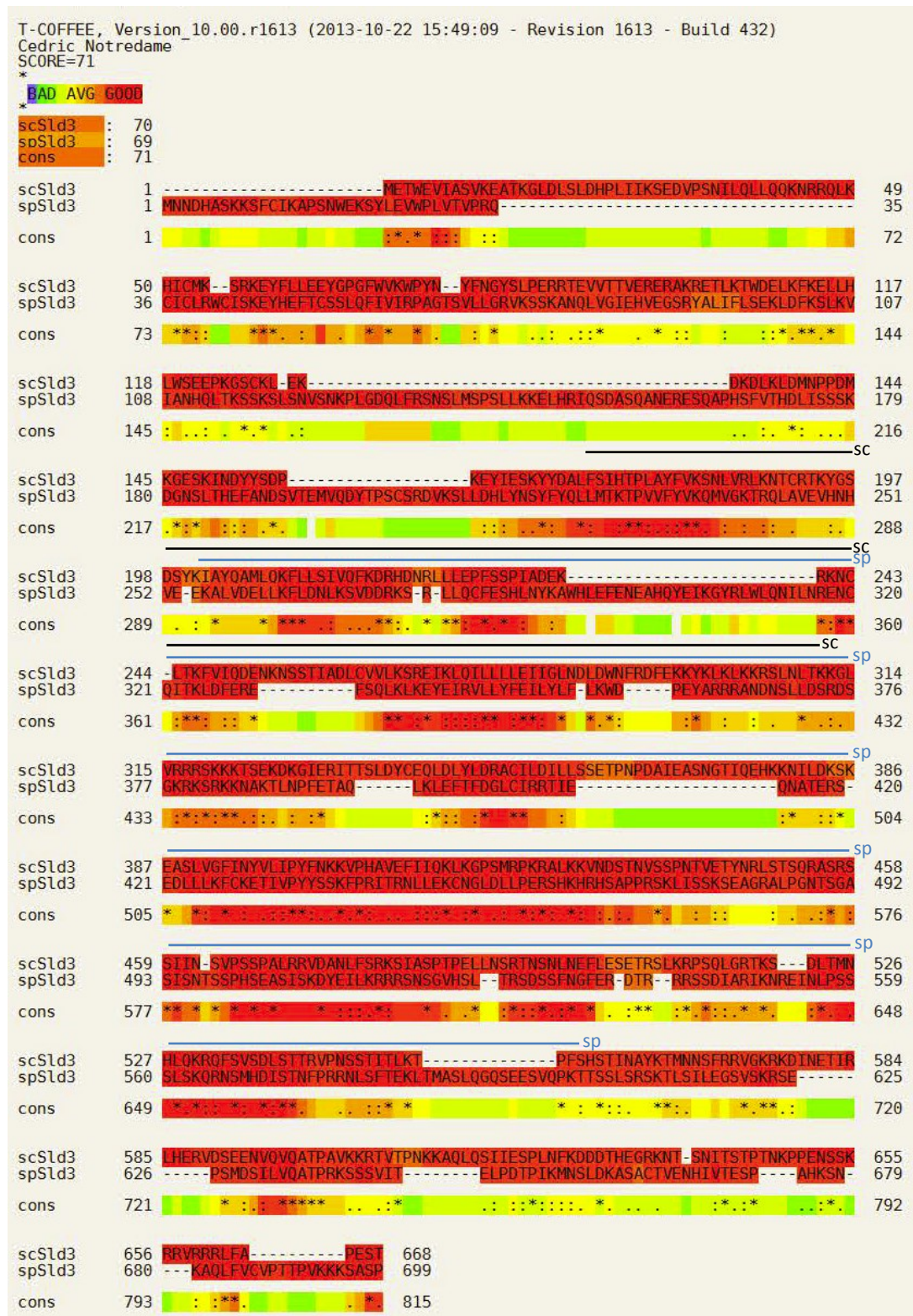
Alignment of *S. cerevisiae* / human Mcm2



Alignment of *S. cerevisiae* / *S. pombe* Mcm2

[illegible]

Alignment of *S. cerevisiae* / *S. pombe* Sld3



Alignment of *S. cerevisiae* / human Cdc45

[illegible]

Fungal alignment of Mcm2

Symbols:		* = identical		: = strong similarity		. = weak similarity	
SGD_Scer_MCM2/YBL023C	1	-----MSDNRRRRRREEDDSSENELPPSSPQQHFRGGMNPFVSSPIGS---	42				
MIT_Sbay_c829_557	1	-----MSDNRRRRRREEDDSSENELPPSSPQQPFRGGIDPISPIGA---	42				
MIT_Smik_c137_1400	1	-----MSDNRRRRRREEDDSSENELPPSSPQQPLRRGMNPFVSSPIGS---	42				
MIT_Spar_c365_850	1	-----MSDNRRRRRREEDDSSENELPPSSPQQPFRGMNPFVSSPIGS---	42				
WashU_Sbay_Contig650.28	1	-----MSDNRRRRRREEDDSSENELPPSSPQQPFRGGIDPISPIGA---	42				
WashU_Scas_Contig710.21	1	-----MADNRRRRRREEDDSSENELPPSSPQQTGRGFVPMSSPIGSPA	45				
WashU_Sklu_Contig2087.4	1	MSDNTSGRRRRRSSIGENDGRAGENDSDHEMQQALASSPQAGVLGSPDN	50				
Symbols		. .***				. * . . : *	
SGD_Scer_MCM2/YBL023C	43	PDMINPEGDDNEVDVDPDIDEVEEQMNEVDLMDDNMYEDYAADHNRRDRYD	92				
MIT_Sbay_c829_557	43	PDMINPEGDDNEVDVDPDIDEVEEQMNEVDLMDDNMYEDYAADHNRRDRYD	92				
MIT_Smik_c137_1400	43	PDMINPEGDDNEVDVDPDIDEVEEQMNEVDLMDDNMYEDYAADHNRRDRYD	92				
MIT_Spar_c365_850	43	PDMINPEGDDNEVDVDPDIDEVEEQMNEVDLMDDNMYEDYAADHNRRDRYD	92				
WashU_Sbay_Contig650.28	43	PDMINPEGDDNEVDVDPDIDEVEEQMNEVDLMDDNMYEDYAADHNRRDRYD	92				
WashU_Scas_Contig710.21	46	HDMINPEGADDEIQDDPDIDEVEEQMNEVDLMDDNMYEDYAADHNRRDRYD	95				
WashU_Sklu_Contig2087.4	51	IDFEDPDGATSGQRFPQDLDDLEEQMDEVDLLGDDMYRDYDPNAAEDAYD	100				
Symbols		* : : * * . : * : : : : : : : : : : : : : : : : . * * *					
SGD_Scer_MCM2/YBL023C	93	PDQVDDREQQELSLSERRRIDAQLNERDRLLRNVAIIDDEEEQEGAAQL	142				
MIT_Sbay_c829_557	93	PDQVDDREQQELSLSERRRIDAQLNERDRLLRNVAIIDDEEEQEGAAQL	142				
MIT_Smik_c137_1400	93	PDQVDDREQQELSLSERRRIDAQLNERDRLLRNVAIIDDEEEQEGAAQL	142				
MIT_Spar_c365_850	93	PDQVDDREQQELSLSERRRIDAQLNERDRLLRNVAIIDDEEEQEGAAQL	142				
WashU_Sbay_Contig650.28	93	PDQVDDREQQELSLSERRRIDAQLNERDRLLRNVAIIDDEEEQEGAAQL	142				
WashU_Scas_Contig710.21	96	LNQVDDREQQELSLSERRRIDAQLNERDRLLRNVAIIDDEEEQEGAAQL	144				
WashU_Sklu_Contig2087.4	101	LQQVDDREQQELSLSERRRIDAQLNERDRLLRNVAIIDDEEEQEGAAQL	150				
Symbols		: * * : * : * : : * : * : * : * : * : * : * : * : * : * : * : *					
SGD_Scer_MCM2/YBL023C	143	DEMGLPVQRRRRRRQYEDLENSDDDLSDMDIDPLREELTLESLSNVKAN	192				
MIT_Sbay_c829_557	143	DEMGLPVQRRRRRRQYEDLENSDDDLSDMDIDPLREELTLESLSNVKAN	192				
MIT_Smik_c137_1400	143	DEMGLPVQRRRRRRQYEDLENSDDDLSDMDIDPLREELTLESLSNVKAN	192				
MIT_Spar_c365_850	143	DEMGLPVQRRRRRRQYEDLENSDDDLSDMDIDPLREELTLESLSNVKAN	192				
WashU_Sbay_Contig650.28	143	DEMGLPVQRRRRRRQYEDLENSDDDLSDMDIDPLREELTLESLSNVKAN	192				
WashU_Scas_Contig710.21	145	DEMGLPIQRRRRRRQYEDLENSDDDLSDMDIDPLREELTLESLSNVKAN	194				
WashU_Sklu_Contig2087.4	151	QLDELGLPVQRRRRRRQYEDLENSDDDLSDMDIDPLREELTLESLSNVKAN	200				
Symbols		: * : : * : * : : * . . * : * : * : * : * : * : * : * : * : *					
SGD_Scer_MCM2/YBL023C	193	SYSEWITQPNVSRITIAELKSFLLLEYTDETGSRVYGARIRTLGEMNSES	242				
MIT_Sbay_c829_557	193	SYSEWITQPNVSRITIAELKSFLLLEYTDETGSRVYGARIRTLGEMNSES	242				
MIT_Smik_c137_1400	193	SYSEWITQPNVSRITIAELKSFLLLEYTDETGSRVYGARIRTLGEMNSES	242				
MIT_Spar_c365_850	193	SYSEWITQPNVSRITIAELKSFLLLEYTDETGSRVYGARIRTLGEMNSES	242				
WashU_Sbay_Contig650.28	193	SYSEWITQPNVSRITIAELKSFLLLEYTDETGSRVYGARIRTLGEMNSES	242				
WashU_Scas_Contig710.21	195	SYSEWITQPNVSRITIAELKSFLLLEYTDETGSRVYGARIRTLGEMNSES	244				
WashU_Sklu_Contig2087.4	201	SYSEWITQPNVSRITIAELKSFLLLEYTDETGSRVYGARIRTLGEMNSES	250				
Symbols		* * * * * : * * * * * : * : * * * * * : * : * * *					
SGD_Scer_MCM2/YBL023C	243	EVNYRHLAESKAILALFLAKCPEEMLKIFDLVAMEATELHYPDYARIHSE	292				
MIT_Sbay_c829_557	243	EVNYRHLAESKAILALFLAKCPEEMLKIFDLVAMEATELHYPDYARIHSE	292				
MIT_Smik_c137_1400	243	EVNYRHLAESKAILALFLAKCPEEMLKIFDLVAMEATELHYPDYARIHSE	292				
MIT_Spar_c365_850	243	EVNYRHLAESKAILALFLAKCPEEMLKIFDLVAMEATELHYPDYARIHSE	292				
WashU_Sbay_Contig650.28	243	EVNYRHLAESKAILALFLAKCPEEMLKIFDLVAMEATELHYPDYARIHSE	292				
WashU_Scas_Contig710.21	245	EVNYRHLAESKAILALFLAKCPEEMLKIFDLVAMEATELHYPDYARIHSE	294				
WashU_Sklu_Contig2087.4	251	EVNYRHLAESKAILALFLAKCPEEMLKIFDLVAMEATELHYPDYARIHSE	300				
Symbols		* * * * * : * * * * * : * * * * * : * * * * * : * * * * *					
SGD_Scer_MCM2/YBL023C	293	IHVRIISDFPTIYSLRELRESNLSSLVRVTGVVTRRTGVFPQLKYVKFNCL	342				
MIT_Sbay_c829_557	293	IHVRIISDFPTIYSLRELRESNLSSLVRVTGVVTRRTGVFPQLKYVKFNCL	342				
MIT_Smik_c137_1400	293	IHVRIISDFPTIYSLRELRESNLSSLVRVTGVVTRRTGVFPQLKYVKFNCL	342				
MIT_Spar_c365_850	293	IHVRIISDFPTIYSLRELRESNLSSLVRVTGVVTRRTGVFPQLKYVKFNCL	342				
WashU_Sbay_Contig650.28	293	IHVRIISDFPTIYSLRELRESNLSSLVRVTGVVTRRTGVFPQLKYVKFNCL	342				
WashU_Scas_Contig710.21	295	IHVRIISDFPTIYSLRELRESNLSSLVRVTGVVTRRTGVFPQLKYVKFNCL	344				
WashU_Sklu_Contig2087.4	301	IHVRIISDFPTIYSLRELRESNLSSLVRVTGVVTRRTGVFPQLKYVKFNCL	350				
Symbols		* * * * * : : . * * * * * . * * * * * : * * * * * : * * * * *					

SGD_Scer_MCM2/YBL023C	343	KCGSILGPFQDSNEEIRISFCTNCKSKGPFVNGEKTYYRNYQRVTLOE	392
MIT_Sbay_c829_557	343	KCGSILGPFQDSNEEIRISFCTNCKSKGPFVNGEKTYYRNYQRVTLOE	392
MIT_Smik_c137_1400	343	KCGSILGPFQDSNEEIRISFCTNCKSKGPFVNGEKTYYRNYQRVTLOE	392
MIT_Spar_c365_850	343	KCGSILGPFQDSNEEIRISFCTNCKSKGPFVNGEKTYYRNYQRVTLOE	392
WashU_Sbay_Contig650.28	343	KCGSILGPFQDSNEEIRISFCTNCKSKGPFVNGEKTYYRNYQRVTLOE	392
WashU_Scas_Contig710.21	345	KCGTILGPFQDSNEEIRISYCVNCKSKGPFVNGEKTYYRNYQRITLOE	394
WashU_Sklu_Contig2087.4	351	KCGSILGPFQDSNEEIRISFCTNCKSKGPFRTNAEKTLYRNYQRITLOE	400
Symbols		***:*****:***:***:*****:***:*****:*****:***	
SGD_Scer_MCM2/YBL023C	393	APGTVPFGRRLPRHREVILLADLVDVSKPGEEVEVTGIYKNNDGNLNAKN	442
MIT_Sbay_c829_557	393	APGTVPFGRRLPRHREVILLADLVDVSKPGEEVEVTGIYKNNDGNLNAKN	442
MIT_Smik_c137_1400	393	APGTVPFGRRLPRHREVILLADLVDVSKPGEEVEVTGIYKNNDGNLNAKN	442
MIT_Spar_c365_850	393	APGTVPFGRRLPRHREVILLADLVDVSKPGEEVEVTGIYKNNDGNLNAKN	442
WashU_Sbay_Contig650.28	393	APGTVPFGRRLPRHREVILLADLVDVSKPGEEVEVTGIYKNNDGNLNAKN	442
WashU_Scas_Contig710.21	395	SPGTVPFGRRLPRHREVILLADLVDVSKPGEEVEVTGIYKNNDGNLNAKN	444
WashU_Sklu_Contig2087.4	401	APGTVPFGRRLPRHREVILLADLVDVSKPGEEVEVTGIYKNNDGNLNAKN	450
Symbols		:*****:*****:*****:*****:*****:*****:*****:*****	
SGD_Scer_MCM2/YBL023C	443	GFPVFATIIIEANSIKRREG----NTANEGEEGLDVFWSWTEEEEREFRKIS	488
MIT_Sbay_c829_557	443	GFPVFATIIIEANSIKRREG----NSANDGEEGLDVFWSWTEEEEREFRKIS	488
MIT_Smik_c137_1400	443	GFPVFATIIIEANSIKRREG----NAANDGEEGLDVFWSWTEEEEREFRKIS	488
MIT_Spar_c365_850	443	GFPVFATIIIEANSIKRREG----NAANDGEEGLDVFWSWTEEEEREFRKIS	488
WashU_Sbay_Contig650.28	443	GFPVFATIIIEANSIKRREG----NSANDGEEGLDVFWSWTEEEEREFRKIS	488
WashU_Scas_Contig710.21	445	GFPVFATIIIEANSIKRREG----NSANEGEEGLDVFWSWTEEEEREFRKIS	490
WashU_Sklu_Contig2087.4	451	GFPVFATIIIEANSIKRREGGRISNPSIEDDEGLDVFWSWTEEEEREFRMS	500
Symbols		*****:*****:*****:*****:*****:*****:*****:*****	
SGD_Scer_MCM2/YBL023C	489	RDRGIIDKIISSMAPSIYGHRIKTAACSLFPGGVPKNVNGKHSIRGDIN	538
MIT_Sbay_c829_557	489	RDRGIIDKIISSMAPSIYGHRIKTAACSLFPGGVPKNVNGKHSIRGDIN	538
MIT_Smik_c137_1400	489	RDRGIIDKIISSMAPSIYGHRIKTAACSLFPGGVPKNVNGKHSIRGDIN	538
MIT_Spar_c365_850	489	RDRGIIDKIISSMAPSIYGHRIKTAACSLFPGGVPKNVNGKHSIRGDIN	538
WashU_Sbay_Contig650.28	489	RDRGIIDKIISSMAPSIYGHRIKTAACSLFPGGVPKNVNGKHSIRGDIN	538
WashU_Scas_Contig710.21	491	RDRGIIDKIISSMAPSIYGHRIKTAACSLFPGGVPKNVNGKHSIRGDIN	540
WashU_Sklu_Contig2087.4	501	RDRGIIDKIISSMAPSIYGHRIKTAACSLFPGGVPKNVNGKHSIRGDIN	550
Symbols		***:*****:*****:*****:*****:*****:*****:*****	
SGD_Scer_MCM2/YBL023C	539	VLLLGDPGTAKSQILKYVEKTAHRAVFATGQGASAVGLTASVRKDPITKE	588
MIT_Sbay_c829_557	539	VLLLGDPGTAKSQILKYVEKTAHRAVFATGQGASAVGLTASVRKDPITKE	588
MIT_Smik_c137_1400	539	VLLLGDPGTAKSQILKYVEKTAHRAVFATGQGASAVGLTASVRKDPITKE	588
MIT_Spar_c365_850	539	VLLLGDPGTAKSQILKYVEKTAHRAVFATGQGASAVGLTASVRKDPITKE	588
WashU_Sbay_Contig650.28	539	VLLLGDPGTAKSQILKYVEKTAHRAVFATGQGASAVGLTASVRKDPITKE	588
WashU_Scas_Contig710.21	541	VLLLGDPGTAKSQILKYVEKTAHRAVFATGQGASAVGLTASVRKDPITKE	590
WashU_Sklu_Contig2087.4	551	VLLLGDPGTAKSQILKYVEKTAHRAVFATGQGASAVGLTASVRKDPITRE	600
Symbols		*****:*****:*****:*****:*****:*****:*****:*****	
SGD_Scer_MCM2/YBL023C	589	WTLEGGALVLADKGVCLIDEFDKMNDQDRTSIHEAMEQQSISISKAGIVT	638
MIT_Sbay_c829_557	589	WTLEGGALVLADKGVCLIDEFDKMNDQDRTSIHEAMEQQSISISKAGIVT	638
MIT_Smik_c137_1400	589	WTLEGGALVLADKGVCLIDEFDKMNDQDRTSIHEAMEQQSISISKAGIVT	638
MIT_Spar_c365_850	589	WTLEGGALVLADKGVCLIDEFDKMNDQDRTSIHEAMEQQSISISKAGIVT	638
WashU_Sbay_Contig650.28	589	WTLEGGALVLADKGVCLIDEFDKMNDQDRTSIHEAMEQQSISISKAGIVT	638
WashU_Scas_Contig710.21	591	WTLEGGALVLADKGVCLIDEFDKMNDQDRTSIHEAMEQQSISISKAGIVT	640
WashU_Sklu_Contig2087.4	601	WTLEGGALVLADKGVCLIDEFDKMNDQDRTSIHEAMEQQSISISKAGIVT	650
Symbols		*****:*****:*****:*****:*****:*****:*****:*****	
SGD_Scer_MCM2/YBL023C	639	TLOARCSIIAAANPNGGRYNSTLPLAQNVSLTEPILSRFDILCVVRDLVD	688
MIT_Sbay_c829_557	639	TLOARCSIIAAANPNGGRYNSTLPLAQNVSLTEPILSRFDILCVVRDLVD	688
MIT_Smik_c137_1400	639	TLOARCSIIAAANPNGGRYNSTLPLAQNVSLTEPILSRFDILCVVRDLVD	688
MIT_Spar_c365_850	639	TLOARCSIIAAANPNGGRYNSTLPLAQNVSLTEPILSRFDILCVVRDLVD	688
WashU_Sbay_Contig650.28	639	TLOARCSIIAAANPNGGRYNSTLPLAQNVSLTEPILSRFDILCVVRDLVD	688
WashU_Scas_Contig710.21	641	TLOARCSIIAAANPNGGRYNSTLPLAQNVSLTEPILSRFDILCVVRDLVD	690
WashU_Sklu_Contig2087.4	651	TLOARCSIIAAANPNGGRYNSTLPLAQNVSLTEPILSRFDILCVVRDLVD	700
Symbols		*****:*****:*****:*****:*****:*****:*****:*****	

Fungal alignment of Sld3

Symbols:		* = identical	: = strong similarity	. = weak similarity
SGD_Scer_SLD3/YGL113W	1	----METWEVIASVKEATKGLDLSLDHPLIIKSEDEVESNIIQLQLQKQ--N	44	
MIT_Sbay_c393_8133	1	----METWEVLASIKEATKGLDLSLSPLIIKSEDLFVHVLLQLQLQKQ--D	44	
MIT_Smik_c276_7989	1	----METWEVIASVKEATKGLDLSLDHPLIIKSEELPESHILRLQLQKQ--N	44	
MIT_Spar_c22_8756	1	----METWEVIASVKEATKGLDLSLDPLIIKSEDEVESHILQLQLQKQ--S	44	
WashU_Sbay_Contig490.4	1	----METWEVLASIKEATKGLDLSLSPLIIKSEDLFVHVLLQLQLQKQ--D	44	
WashU_Scas_Contig652.4	1	----MDTWSYGLSLINIPKSIILNYDSVTVIQAETIPTVLTEHFG----N	42	
WashU_Skl_u_Contig2133.7	1	MLNAQNKTTLVGSSVKELPAGVVVLKNDFOVLDELIDPEILSLIQGKSVL	50	
WashU_Skud_Contig2026.5	1	----METWEVLASVKEATKGLDLSLSPFIIRSEDLFIHVLRLLQLQKQ--N	44	
Symbols		: . : . * : . . : . : : * : *	:	
SGD_Scer_SLD3/YGL113W	45	RRQLKHKICMKSRKEYFLLEEYGGPGFWVKWPYNFYNGYSIPERRTEVVTTV	94	
MIT_Sbay_c393_8133	45	RRHLKHICTNSKKEHFLLEGYGGPGFWVKWPYTYFNEYSIPKKSTEITSSL	94	
MIT_Smik_c276_7989	45	RRQLKHKICMESEKVFLLLEEGYGGPGFWVKWPFNCFNGYSIPERHEKAAASL	94	
MIT_Spar_c22_8756	45	RSQKHKICMKSEKEYFLLEEYGGPGFWVKWPYNFYNEYSIPERHTEVVTSL	94	
WashU_Sbay_Contig490.4	45	RRHLKHICTNSKKEHFLLEGYGGPGFWVKWPYTYFNEYSIPKKSTEITSSL	94	
WashU_Scas_Contig652.4	43	DPPTKHILEDTHRYHLVERYSQYIDWGVKELDLTTIQFDQKELNVAK	92	
WashU_Skl_u_Contig2133.7	51	LKHLVYECTNGVFQYAILKEYCQSKWIKWQIENLDLPEFKSDQ---ISST	97	
WashU_Skud_Contig2026.5	45	RRQLKHKICVKSEKDYFLLEEYGGPGFWVKWPYNFYNEYSIPKNGEIVASL	94	
Symbols		: . . : : * * : : * : : :	:	
SGD_Scer_SLD3/YGL113W	95	ERERAKRETLLKTWDELKFKELLHLWSEEPKSGSKLEKDKDKLDMNPP--D	143	
MIT_Sbay_c393_8133	95	KLERGKRETLLKTWDEMRFKELLHLWSEEPKTSKHIEKDTCTFKLDMKPP--D	143	
MIT_Smik_c276_7989	95	ERDTHKRETSKTWDELKFKELLHLWSEEPKGLLKSEKDKNLVLDKMP--D	143	
MIT_Spar_c22_8756	95	QREKAKRETSKTWDELKFKELLYLWSEEPKGSYKLEKNNDLKLDMKPP--D	143	
WashU_Sbay_Contig490.4	95	KLERGKRETLLKTWDEMRFKELLHLWSEEPKTSKHIEKDTCTFKLDMKPP--D	143	
WashU_Scas_Contig652.4	93	ISKVPYP--NPNINENFNKIFPPFWEEDSNP----EVEENIKLSMTTPPGD	136	
WashU_Skl_u_Contig2133.7	98	RLADYPEIKVQEWNKLDIPDFMELWKEDRLVPEGEVDEEAQLDMKPP--D	146	
WashU_Skud_Contig2026.5	95	ERDRAKRETSRTWNELKFKELLDFWSEESVSHLEKDKSKPLDMKPP--N	143	
Symbols		: : : : * * : : * : * : *	:	
SGD_Scer_SLD3/YGL113W	144	MKGES---KINDYYSDPKEYIESKYYDALFSIHTPLAYFVKSNLVRLLKNT	190	
MIT_Sbay_c393_8133	144	MKGQS---RVNVHSDPKKEYIENKYYDALFSIHVPLAYFVKSNLVRVKNT	190	
MIT_Smik_c276_7989	144	TKHES---KVNDYSDPKEYIESKYYDALFSIHTPLAYFVKSNLVRLLKNT	190	
MIT_Spar_c22_8756	144	MKGES---RVNDYSDPKEYIESKYYDALFSIHTPLAYFVKSNLVRLLKNT	190	
WashU_Sbay_Contig490.4	144	MKGQS---RVNVHSDPKKEYIENKYYDALFSIHVPLAYFVKSNLVRVKNT	190	
WashU_Scas_Contig652.4	137	KQSPV---KIEAEIANPRTYLETKYKYEALFSIQIPLAYFVKSNLIRFKNM	183	
WashU_Skl_u_Contig2133.7	147	ISTEVSLLKYKKDTMQDPNKYLKRYKYEALFSIRMPLAYFVKSNLVRLLKNT	196	
WashU_Skud_Contig2026.5	144	MKDES---RTNKDYSDPKEYIESKYYDALFSIRTPLAYFVKSNLVRLLKNT	190	
Symbols		. : : * : * * : * : * : * : * : *	:	
SGD_Scer_SLD3/YGL113W	191	CRTKYGSDSYKIAIYQAMLQKFLLSIVQFKDRHNRLLLEP---FSSPIAD	237	
MIT_Sbay_c393_8133	191	CRTKYGSGSYKIAIYQTLQKFLLSIAQFNDHRHNRLLLEP---FGSPIAD	237	
MIT_Smik_c276_7989	191	CRTKYGNDSYKIAIYQTLQKFLLSVQFKDRHNRLLLES---IPSSPIAD	237	
MIT_Spar_c22_8756	191	CRTKYGSHSYKIAIYQAILQKFLLSIAQFNDHRHNRLLLES---FSSPIAD	237	
WashU_Sbay_Contig490.4	191	CRTKYGSGSYKIAIYQTLQKFLLSIAQFNDHRHNRLLLEP---FGSPIAD	237	
WashU_Scas_Contig652.4	184	CRTTYNS-EFEMAYQATLLDMLLTIPKFDQRHDLNALVNSI-FTNPIAL	231	
WashU_Skl_u_Contig2133.7	197	CKTHGNP---ELAYRALISGMLLDIPEFDRHRDIANFGSLKYFPAQQIAE	243	
WashU_Skud_Contig2026.5	191	CRTKYGNDSYKIAIYQAILQKFLLSIVQFNSRHRHNRLLLEP---LSSPIAD	237	
Symbols		* : * . : : * : : : * : : * : * : * : *	:	
SGD_Scer_SLD3/YGL113W	238	EKRKNCLTKFVIQDENKN-SSTIADLCVVLKSRREIKLQIILLLEIIGLND	286	
MIT_Sbay_c393_8133	238	EKRNNCLTKFVIQGNNT-GSTITDLCVIFKSRREIKLQIILLLEIIGLNN	286	
MIT_Smik_c276_7989	238	EKRKNCLTKFAMKGGNK-TAITDLCVVLKSRREIKLQIILLLEIIGMNN	286	
MIT_Spar_c22_8756	238	EKRKNCLTKFVIQDENKT-GSTITDLCVVLKSRREIKLQIILLLEIIGLND	286	
WashU_Sbay_Contig490.4	238	EKRNNCLTKFVIQGNNT-GSTITDLCVIFKSRREIKLQIILLLEIIGLNN	286	
WashU_Scas_Contig652.4	232	DKDACLNKYHLSRNIEQPSQLSDLSFVLKARELKQIIVILLELIYLLNG	281	
WashU_Skl_u_Contig2133.7	244	LKDTILRDSYHISVQLDEVHVMKDLVAFKKIREIKLQIIMLLEIISMSN	293	
WashU_Skud_Contig2026.5	238	VKRKGCLTKFIIQINNKN-DSTAADLCVILKSRREIKLQIILLLEIIGLNN	286	
Symbols		: : : : * : : * : * : * : * : *	:	

SGD_Scer_SLD3/YGL113W	287	LDWNFRDFFKKYKLLKKRSLNLTKKGLVRRRSKKKTSEKDKGIERITTS	336
MIT_Sbay_c393_8133	287	LDCNFKDFEKRYKSKLKKRSLNLTQRLIRRRPKKAVNQNKDIEKIATP	336
MIT_Smik_c276_7989	287	LDWNFRDFFKKYKSKLKKRSLNLTKKGLVRRRSKKKTSEKDKGIERITTS	336
MIT_Spar_c22_8756	287	LDWNFRDFFKKYKSKLKKRSLNLTKKGLVRRRSKKKISEKGEIGRITTS	336
WashU_Sbay_Contig490.4	287	LDCNFKDFEKRYKSKLKKRSLNLTQRLIRRRPKKAVNQNKDIEKIATP	336
WashU_Scas_Contig652.4	282	RDDNFKNFERRYKSKLKKRALNVTRIATRNNKLNKEHKKDPNTKKDEEVS	331
WashU_Sklu_Contig2133.7	294	MDAKLKNFEKRYTSKFKARSINVTTRAGKFFRN-KKMNNHKKRPIGSEKPE	342
WashU_Skud_Contig2026.5	287	LDWNFKDFEKRYKSKLKKRSLNLTKKGLIRRRPKKIINEPDKNIAQAATS	336
Symbols		* : : : * : : * . : : * * : : * : . . : : . :	
SGD_Scer_SLD3/YGL113W	337	LDYCEQLDLYLDRACILDILLSSETNPDAIEASNGT----IQEHKKNIL	382
MIT_Sbay_c393_8133	337	LDYCEQLDLYLDRACILDILLSSETNPDAIEIPNGT----IQEHKKNIL	382
MIT_Smik_c276_7989	337	LDYCEQLDLYLDRACILDILLSSEAPNPDAIEGNGI----IKEHKKNIL	382
MIT_Spar_c22_8756	337	LDYCEQLDLYLDRACILDILLSSETNPDAIEASNGT----ILEHKKNIL	382
WashU_Sbay_Contig490.4	337	LDYCEQLDLYLDRACILDILLSSETNPDAIEIPNGT----IQEHKKNIL	382
WashU_Scas_Contig652.4	332	LDLCEQLDLYLDRACILDILLSSEVSNP-DLEADNNP----THEQKQNIL	376
WashU_Sklu_Contig2133.7	343	LDFCEQLDLYLDRACILDILLSSEVENIDKDKETFTTHDRITIEHKKNIL	392
WashU_Skud_Contig2026.5	337	LDYCEQLDLYLDRACILDILLSSETNPDAIENATNGT----IQEHKKNIL	382
Symbols		** * : : * : : * * * * . * : : . * * : : * * *	
SGD_Scer_SLD3/YGL113W	383	DKSKEASLVGFINYVLIPIYFNKKVPHAVEFIIQKLKGPSMRPKRALKKVN	432
MIT_Sbay_c393_8133	383	NKNKEASLVGFINYVLIPIYFTKKVPHAVDFIIQKLKGPSMRPKRAPKKIT	432
MIT_Smik_c276_7989	383	DKNKEASLVGFINYVLIPIYFKKKVPYTFEIIQKFKGPSMRPKTAPKKIN	432
MIT_Spar_c22_8756	383	DKSKEASLVGFINYVLIPIYFNKKVPHAVEFIIQKLKGPSMRPKRALKKVN	432
WashU_Sbay_Contig490.4	383	NKNKEASLVGFINYVLIPIYFTKKVPHAVDFIIQKLKGPSMRPKRAPKKIT	432
WashU_Scas_Contig652.4	377	NKNKEASLVGFINYVLIPIYFVKKLPNAVFFIISKLGPSLRSGKPSLRT	426
WashU_Sklu_Contig2133.7	393	NGGKEASLVGFINYVLIPIYFNKKVPYAVELITRKIKGPDLSALKAKRS	442
WashU_Skud_Contig2026.5	383	NRNKEASLVGFINYVLIPIYFTKKVPHAVEFIIQKLKGPSMRPKKAPKKIS	432
Symbols		: . * * * : * * . : : * : : * * : : * * : : : . .	
SGD_Scer_SLD3/YGL113W	433	DSTNVSSPNTVETYNRLSTSQR--ASRSSIINSVFPSSPALRRVDANLFS-	479
MIT_Sbay_c393_8133	433	DTTGGSSPSTAELYNKLSTSQR--TSRSSIINSVFPSSPALNGTDNMFS-	479
MIT_Smik_c276_7989	433	DSTHLSPPNNVDMYNNRFTTSQR--TSRSSIINSVFPSSPALRRTKNALC-	479
MIT_Spar_c22_8756	433	NSPNVSSPKTVDIYNRLPTTSQR--SSRSSIINSVFPSSPALRRMEANLFS-	479
WashU_Sbay_Contig490.4	433	DTTGGSSPSTAELYNKLSTSQR--TSRSSIINSVFPSSPALNGTDNMFS-	479
WashU_Scas_Contig652.4	427	SHNTQTDIAIQVALKSRDETSKEISQSTTASSIVSSPTRKISRPSLNR-	475
WashU_Sklu_Contig2133.7	443	GSSKVTSKILEEPELKHAPLSLPSLSRQNSLTSIASTLNSSTPSPCFVPP	492
WashU_Skud_Contig2026.5	433	NGTNETSHSTVDVYSKLPINQ--TSRSSIINSVFPSSPALRGTDSHVFN-	479
Symbols		. : : . : . . . * : : .	
SGD_Scer_SLD3/YGL113W	480	--RKSIASTPELNSRTNSNLNEFLESETRSLRRPSQLGRTKSDLTMMNH	527
MIT_Sbay_c393_8133	480	--RKSITTPPELNSRTNSNLNEFLESETRSLRRPSQLGRTKSDLTMMNH	527
MIT_Smik_c276_7989	480	--RKSITSPAPELNSRTNSNLNEFLESESRGLRHPSQLGRTKSDLTMMNH	527
MIT_Spar_c22_8756	480	--RKSIASTPELNSRTNSNLNEFLESETRSLRRPSQLGRTKSDLTMMNH	527
WashU_Sbay_Contig490.4	480	--RKSITTPPELNSRTNSNLNEFLESETRSLRRPSQLGRTKSDLTMMNH	527
WashU_Scas_Contig652.4	476	--TMTIGSLTSDLTNRNTNSNLTEFLESQSRLKPSLSRTKSDLVMMNH	523
WashU_Sklu_Contig2133.7	493	TIKRHNSTVLPALLNSRTNSNLGEFLESEVQVVRHPSLSRTKSDMSFNR	542
WashU_Skud_Contig2026.5	480	--RMSKTSPELNSRTNSNLNEFLESSTRSLRRPSQLGRTKSDLTMMNH	527
Symbols		: . * * * * * * * : : : : * : : * * * : : *	
SGD_Scer_SLD3/YGL113W	528	LQKRQFSVSDLSSTRVPNSSTITLKTFFSHSTIN-----AYKTMNNSFRF	572
MIT_Sbay_c393_8133	528	LQKRQFSVSDLSSTRVTSPTSSIPAFSRTVNG-----VHISTNNSFRF	569
MIT_Smik_c276_7989	528	LQKRQFSVSDLGTTTRPTLSATSLKTPFSRTAIN-----THAITNNSFRF	572
MIT_Spar_c22_8756	528	LQKRQFSVSDLSSTRVPNSSTIAPKTPFSYTAVN-----AHRTMNNNSFRF	572
WashU_Sbay_Contig490.4	528	LQKRQFSVSDLSSTRVTSPTSSIPAFSRTVNG-----VHISTNNSFRF	569
WashU_Scas_Contig652.4	524	LQKRQLSVNDLTASSTKDDFASRKSSIIIFDGSQSSVRKKIPSFQTSFRF	573
WashU_Sklu_Contig2133.7	543	LQKRQLSVNDLTVNGIQKKKPLASEKSQPMVAING-KSIKLASGTSFQR	591
WashU_Skud_Contig2026.5	528	LQKRQFSVSDLTSTRVPNPSAAPLKT-FSRTAV-----AHTITNNSFRF	571
Symbols		* * * : * * * .	
SGD_Scer_SLD3/YGL113W	573	VGKRKDINETIRHERVDSEEN-----VQVQATPAVKKRTVTENKKAQ	615
MIT_Sbay_c393_8133	570	VGKRKDINETVPSSESIDSEEN-----VQVQATPAVKNRRTTETNKRAQ	612
MIT_Smik_c276_7989	573	VGKRKDINETVHFEYENSEEN-----IQVQATPAVKKRTVTENKKAQ	615
MIT_Spar_c22_8756	573	VGKRKDINETVHFEHINPEEN-----VQVQATPAVKKRTTATENKKAQ	615
WashU_Sbay_Contig490.4	570	VGKRKDINETVPSSESIDSEEN-----VQVQATPAVKNRRTTETNKRAQ	612
WashU_Scas_Contig652.4	574	VGKFKSKTSPTIIPETSKHESPSNM---VQVQATPQIKGNKNTPRKRGL	619
WashU_Sklu_Contig2133.7	592	VGKRKSESTRGNITAMAAVQVMATPHGKKLSSSTTQDKNNTIVASPLVH	641
WashU_Skud_Contig2026.5	572	VGKRKDINETVHFESEINSEEN-----VQVQATPAVKKRTTATENKRAQ	614
Symbols		* * * . . . : .	

SGD_Scer_SLD3/YGL113W	287	LDWNFRDFFKKYKLLKKRSLNLTKKGLVRRSSKKKTSEKDKGIERITTS	336
MIT_Sbay_c393_8133	287	LDCNFKDFEKRYKSKLKKRSLNLTQRLIRRKPKKAVNQNKDIEKIATP	336
MIT_Smik_c276_7989	287	LDWNFRDFFKKYKSKLKKRSLNLTKKGLVRRSSKKSTNQTDKDFARITTS	336
MIT_Spar_c22_8756	287	LDWNFRDFFKKYKSKLKKRSLNLTKKGLVRRSSKKKISEKGEIGRITTS	336
WashU_Sbay_Contig490.4	287	LDCNFKDFEKRYKSKLKKRSLNLTQRLIRRKPKKAVNQNKDIEKIATP	336
WashU_Scas_Contig652.4	282	RDDNFKNFERRYKSKLKKRALNVTRIATRNNKLNKEHKKDPNTKKDEEVS	331
WashU_SklU_Contig2133.7	294	MDAKLKNFEKRYKSKFKARSINVTTRAGKFFRN-KKMNNHKKRPIGSEKPE	342
WashU_Skud_Contig2026.5	287	LDWNFKDFEKRYKSKLKKRSLNLTQRLIRRKPKKIINEPDKNIAQAATS	336
Symbols		* : : : : * : : * : : * : : * : : * : : * : : * : : *	
SGD_Scer_SLD3/YGL113W	337	LDYCEQLDLYLDRACILDILLSSETFPNDAIEASNGT----IQEHKKNIL	382
MIT_Sbay_c393_8133	337	LDYCEQLDLYLDRACILDILLSSETFPNDAIEIPNGT----IQEHKSNIL	382
MIT_Smik_c276_7989	337	LDYCEQLDLYLDRACILDILLSSEAFNPNAIEGNGI----IKEHKKNIL	382
MIT_Spar_c22_8756	337	LDYCEQLDLYLDRACILDILLSSETFPNDAIEASNGT----ILEHKKNIL	382
WashU_Sbay_Contig490.4	337	LDYCEQLDLYLDRACILDILLSSETFPNDAIEIPNGT----IQEHKSNIL	382
WashU_Scas_Contig652.4	332	LDLCEQLDVYLDKLNIVDILLGSEVSNP-DLEADNPN----THEQKQNIL	376
WashU_SklU_Contig2133.7	343	LDFCEQLDLMVDKLCIADILLSVESTENIDKDKETFTTHDRIIIEHKKNIL	392
WashU_Skud_Contig2026.5	337	LDYCEQLDLYLDRACILDILLSSETFPNDAIATNGT----IQEHKKNIL	382
Symbols		** * : : : : * : : * : : * : : * : : * : : * : : *	
SGD_Scer_SLD3/YGL113W	383	DKSKEASLVGFINYVLIPIYFNKKVPHAVEFIIQKLKGPSMRPKRALKKVN	432
MIT_Sbay_c393_8133	383	NKNKEASLVGFINYVLIPIYFTKKVPHAVDFIIQKLKGPSMRPKRAPKKIT	432
MIT_Smik_c276_7989	383	DKNKEASLVGFINYVLIPIYFKKKVPYTVFIIQKFKGPSMRPKTAPKKIN	432
MIT_Spar_c22_8756	383	DKSKEASLVGFINYVLIPIYFNKKVPHAVEFIIQKLKGPSMRPKRALKKVN	432
WashU_Sbay_Contig490.4	383	NKNKEASLVGFINYVLIPIYFTKKVPHAVDFIIQKLKGPSMRPKRAPKKIT	432
WashU_Scas_Contig652.4	377	NKNKEASLVGFINYVLIPIYFVKKLPNAVFFIISKLGPSLRSGKPSLSRT	426
WashU_SklU_Contig2133.7	393	NGGKEASLVGFINYVLIPIYFNKKVPYAVELITRKIKGPDLKSTALKAKRS	442
WashU_Skud_Contig2026.5	383	NRNKEASLVGFINYVLIPIYFTKKVPHAVEFIIQKLKGPSMRPKKAPKKIS	432
Symbols		: . * : : : : * : : * : : * : : * : : * : : * : : *	
SGD_Scer_SLD3/YGL113W	433	DSTNVSSPNTVETYNRLSTSQR--ASRSSIINSVPSSPALRRVDANLFS-	479
MIT_Sbay_c393_8133	433	DTTGGSSPSTAEYLNKLSTSQR--TSRSSIINSVPSSPALNGTDNMFS-	479
MIT_Smik_c276_7989	433	DSTHLSPPNNVDMYNNRFTTSQR--TSRLSIVDSVPSSPALRRTKNALC-	479
MIT_Spar_c22_8756	433	NSPNVSSPKTVDIYNRLPTQR--SSRSSIINSVPSSPALRRMEANLFS-	479
WashU_Sbay_Contig490.4	433	DTTGGSSPSTAEYLNKLSTSQR--TSRSSIINSVPSSPALNGTDNMFS-	479
WashU_Scas_Contig652.4	427	SHNTQTDIAIQVALKSRDETSKEISQSTTASSIVSSPTRKISRPSLNR-	475
WashU_SklU_Contig2133.7	443	GSSKVTSKILEEPELKHAPLSLPSLSRQNSLTSIASTLNSSTPSPCFVPP	492
WashU_Skud_Contig2026.5	433	NGTNETSHSTVDVYSKLPINQ--TSRLIISKVPSSPALRGTDHVFVN-	479
Symbols		. : : . : . : . : * : : *	
SGD_Scer_SLD3/YGL113W	480	--RKSIASTPELNLNRTNSNLNFEFLESETRSLRRPSQLGRTKSDLTMMH	527
MIT_Sbay_c393_8133	480	--RKSITTPPELNLNRTNSNLNFEFLESETRSLRRPSQLGRTKSDLTMMH	527
MIT_Smik_c276_7989	480	--RKSITSPPELNLNRTNSNLNFEFLESETRSLRRPSQLGRTKSDLTMMH	527
MIT_Spar_c22_8756	480	--RKSIASTPELNLNRTNSNLNFEFLESETRSLRRPSQLGRTKSDLTMMH	527
WashU_Sbay_Contig490.4	480	--RKSITTPPELNLNRTNSNLNFEFLESETRSLRRPSQLGRTKSDLTMMH	527
WashU_Scas_Contig652.4	476	--TMTIGSLTSDLNRTNSNLNFEFLESDSLSLRRPSQLGRTKSDLTMMH	523
WashU_SklU_Contig2133.7	493	TIKRHNSTVLPALLNRTNSNLNFEFLESEVQNVRRHPSLVSRKSDMSFNR	542
WashU_Skud_Contig2026.5	480	--RMSKTSPELNLNRTNSNLNFEFLESSTRSLRRPSQLGRTKSDLTMMH	527
Symbols		: . * * * * * : : : : * : : * : : * : : *	
SGD_Scer_SLD3/YGL113W	528	LQKRQFSVSDLSSTRVPNSSTITLKTFFSHSTIN-----AYKTMNNSFR	572
MIT_Sbay_c393_8133	528	LQKRQFSVSDLSSTRVTSPTSSIPAFSRTVNG-----VHISTNNSFR	569
MIT_Smik_c276_7989	528	LQKRQFSVSDLSSTRVTSPTSLTSATSLKTPFSRTAIN-----THAITNNSFR	572
MIT_Spar_c22_8756	528	LQKRQFSVSDLSSTRVPNSSTIAPKTPFSYTAVN-----AHRTMNNNSFR	572
WashU_Sbay_Contig490.4	528	LQKRQFSVSDLSSTRVTSPTSSIPAFSRTVNG-----VHISTNNSFR	569
WashU_Scas_Contig652.4	524	LQKRQLSVNDLTASSTKDDFASRKSSIIFDGSQSSVRKKIPSFQTSFR	573
WashU_SklU_Contig2133.7	543	LQKRQLSVNDLTAVNGIQKKKPLASEKSQPMAVING-KSIKLASGTSFQR	591
WashU_Skud_Contig2026.5	528	LQKRQFSVSDLSSTRVPNPSAAPLKT-FSRTAVS-----AHTITNNSFR	571
Symbols		***** : : * : : * : : * : : * : : *	
SGD_Scer_SLD3/YGL113W	573	VGKRKDIINETIRHERVDSEEN-----VQVQATPAVKKRTVTENKKAQ	615
MIT_Sbay_c393_8133	570	VGKRKDIINETVPSSESIDSEEN-----VQVQATPAVKNRRTTETNKRAQ	612
MIT_Smik_c276_7989	573	VGKRKDIINETVHFEYENSEEN-----IQVQATPAAKKRTVTENKKAQ	615
MIT_Spar_c22_8756	573	VGKRKDIINETVHFEHINPEEN-----VQVQATPAAKKRTATENKKAQ	615
WashU_Sbay_Contig490.4	570	VGKRKDIINETVPSSESIDSEEN-----VQVQATPAVKNRRTTETNKRAQ	612
WashU_Scas_Contig652.4	574	VGKFKSKTSPTIIPETSKHESPSNM---VQVQATPQIKGNKNTPRKRGL	619
WashU_SklU_Contig2133.7	592	VGKRKSESTRGNITAMAAVQVMATPHGKKLSSSTTQDKNNTIVASPLVH	641
WashU_Skud_Contig2026.5	572	VGKRKDIINETVHFEYENSEEN-----VQVQATPAVKNRRTATENKRAQ	614
Symbols		*** * : : : : * : : * : : * : : *	

SGD_Scer_SLD3/YGL113W	616	LQSIIESPLNFKDDDDTHEGR---KNTSNITSTPTNKPPENSS-----	654
MIT_Sbay_c393_8133	613	LQSIVESPLNIKPDTRHEDIENSQRNISPAAATLTNATSENNSS-----	655
MIT_Smik_c276_7989	616	LQSIIESPLNLKDD-THEEIGISQKHTSLSVSVSTNTSLESKP-----	657
MIT_Spar_c22_8756	616	LQSIIESPLNVKDDDDTHEGRGNAQKDTSHFTSTSTNTPESTS-----	658
WashU_Sbay_Contig490.4	613	LQSIVESPLNIKPDTRHEDIENSQRNISPAAATLTNATSENNSS-----	655
WashU_Scas_Contig652.4	620	LHQIVESPIDSVTILQTPRIPEVSPLSKAIKEAPDKDNNETIPP-----	663
WashU_Sklu_Contig2133.7	642	NTSLVSSSELPLDTIKATPIKYTNIQRYQSAPTQLGDTLMEGKMKVSPQLQ	691
WashU_Skud_Contig2026.5	615	LQSIVESPLNVKAD-PPKDAGNSQKNISPIVSVSINTSSEKSS-----	656
Symbols		. : . * :	

SGD_Scer_SLD3/YGL113W	655	KRRVRRRLFAPEST	668
MIT_Sbay_c393_8133	656	KRRVRRRLFAPE---	666
MIT_Smik_c276_7989	658	KRRVRRRLFAPEET	671
MIT_Spar_c22_8756	659	KRRVRRRLFAPEAT	672
WashU_Sbay_Contig490.4	656	KRRVRRRLFAPE---	666
WashU_Scas_Contig652.4	664	KKKIRRRRLFAPE---	674
WashU_Sklu_Contig2133.7	692	GKKVRRRLFAPE--	703
WashU_Skud_Contig2026.5	657	KKKVRRRLFAPEAT	670
Symbols		: : : * * * * *	

Symbols:	* = identical	: = strong similarity	. = weak similarity
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Fungal alignment of Cdc45

Symbols:		* = identical	: = strong similarity	. = weak similarity
SGD_Scer_CDC45/YLR103C	1	MYYGISQFSEAYNKILRNSSSSSSCQLVIFVSCNLIDALCATKMLSLFLFK		50
MIT_Sbay_c128_16060	1	MYYGISQFSEAYNKILRNSSSSSSCQLVIFVSCNLIDALCATKMLSLFLFK		50
MIT_Spar_c46_14436	1	MYYEISQFSEAYNKILRNSSSSSSCQLVIFVSCNLIDALCATKMLSQLFK		50
WashU_Sbay_Contig672.84	1	MYYGISQFSEAYNKILRNSSSSSSCQLVIFVSCNLIDALCATKMLSLFLFK		50
WashU_Scas_Contig566.8	1	MFYQVDQLSQAYRTILKNSSSSSSCQLVIFVSCNLIDALCATKILSQVFK		50
WashU_Sklu_Contig1208.1	1	MFLNIQQFRNAYEKIVRNSSSSSSCQLVIFVSCLSIDALCATKMLVRLFK		50
Symbols		*: .*: :*: :*: :*: :*: :*: :*: :*: :*: :*: :*: :*: :*		
SGD_Scer_CDC45/YLR103C	51	KQLVQSQIVPIFGYSELRRHYSQLDDNINSLLLVGFGGVIDLEAFLEIDP		100
MIT_Sbay_c128_16060	51	KQLVQSQIVPIFGYSELRRHYSQLDDNINSLLLVGFGGVIDLEAFLEIDP		100
MIT_Spar_c46_14436	51	KQLVQSQIVPIFGYSELRRHYSQLDENINSLLLVGFGGVIDLEAFLEIDP		100
WashU_Sbay_Contig672.84	51	KQLVQSQIVPIFGYSELRRHYSQLDDNINSLLLVGFGGVIDLEAFLEIDP		100
WashU_Scas_Contig566.8	51	KQLVQLQIVPVVGYSELRRHYLQLDSNINSVILVGFASVDLETFLDIEK		100
WashU_Sklu_Contig1208.1	51	KQLIQLQLVVFVGYSELREHFLKLDNINSVLLVGCGLMIDVSEFFEIDA		100
Symbols		***: **: :*: :*: :*: :*: :*: :*: :*: :*: :*: :*: :*: :*		
SGD_Scer_CDC45/YLR103C	101	QEYVIDTDEKSGEQSFRRDIYVLDAAHFPWNLDNIFGSQIIQCFDDGTVD		150
MIT_Sbay_c128_16060	101	DDYVIDTDQNPGRSFRRDYVLDAAHFPWNLDNIFGSQIIQCFDDGTVD		150
MIT_Spar_c46_14436	101	QEYVVDTDEKSGEQSFRRDIYVLDAAHFPWNLDNIFGSQIIQCFDDGTVD		150
WashU_Sbay_Contig672.84	101	DDYVIDTDQNPGRSFRRDYVLDAAHFPWNLDNIFGSQIIQCFDDGTVD		150
WashU_Scas_Contig566.8	101	-----DEATEDDPMKRLIYVLDAAHFPWNLDNIFGSQIIQCFDDGTVD		143
WashU_Sklu_Contig1208.1	101	QEYLVEEKPEP---KFKRNIYVLDTHREFPWNLDNIFGSQIIQCFDDGTVD		147
Symbols		. . :*: :*: :*: :*: :*: :*: :*: :*: :*: :*: :*: :*		
SGD_Scer_CDC45/YLR103C	151	TLGEQKEAAYYKLELEDEESG-----DDELSGDENDNNGGDDDEATDAD		192
MIT_Sbay_c128_16060	151	TLGEQKAAYYKLELENGREQDGDGDDGLSDDDDDDDDDDNDGEDMTDAD		200
MIT_Spar_c46_14436	151	ALSEQKEAAYYKLELDQESD-----NGEVSGDDDDDDGGEDDEATDAD		192
WashU_Sbay_Contig672.84	151	TLGEQKAAYYKLELENGREQDGDGDDGLSDDDDDDDDDDNDGEDMTDAD		200
WashU_Scas_Contig566.8	144	SLQEEQDAYMKLVLEQERE-----DSEDDGLSDSDDEE		177
WashU_Sklu_Contig1208.1	148	TLQEQRREAYYKLELEDEEQN-----SESDGDPEDNTE		180
Symbols		*: *: :*: :*: :*: :*: :*: :*: :*: :*: :*: :*: :*		
SGD_Scer_CDC45/YLR103C	193	EVTDE--DEEDEDE-TISNKRGNSSIGFPNDLSKRRQRRKQIHEYEGVLEE		239
MIT_Sbay_c128_16060	201	EAADEGEDNENQDDSIASSTRNSNDKNSNALPKKQRRKQIHESEGVLEE		250
MIT_Spar_c46_14436	193	EATDE--DEEGEGSGRISNKRNSSTGSNDPSKRRQRRKQIHEYEAVLEE		240
WashU_Sbay_Contig672.84	201	EAADEGEDNENQDDSIASSTRNSNDKNSNALPKKQRRKQIHESEGVLEE		250
WashU_Scas_Contig566.8	178	EEEEEDPTDEDEDEEDGPHKKKLSQDINPRKKQIKERRKLILQNERLLEE		227
WashU_Sklu_Contig1208.1	181	EDSDEKDSGDESDDDFPKKRVNQEDNP-KKQRRKLRLKLLHNCETAIMED		229
Symbols		* :* : :* :* :* :* :* :* :* :* :* :* :*		
SGD_Scer_CDC45/YLR103C	240	YYSQGTIVVNSISAQIYSLLSAIGETNLSNLWLNILGTTSLDIAAQQVYN		289
MIT_Sbay_c128_16060	251	YYSQGTIVVNSISAQIYSLLSAIGETNLSNLWLNILGATSLDTAYAAQVYN		300
MIT_Spar_c46_14436	241	YYSQGTIVVNSISAQIYSLLSAIGETNLSNLWLNILGTTSLDIAAQQVYN		290
WashU_Sbay_Contig672.84	251	YYSQGTIVVNSISAQIYSLLSAIGETNLSNLWLNILGATSLDTAYAAQVYN		300
WashU_Scas_Contig566.8	228	YYSTGSTVVNSVSVQVYSLSTIGGTNLQYLWLTILGATSLDTQHSQVYN		277
WashU_Sklu_Contig1208.1	230	YYSQGTITISNSLSVQVYSLSSIGETKQLQFLWLTILGASSLDTSPQQVYN		279
Symbols		*** *: :*: :*: :*: :*: :*: :*: :*: :*: :*: :*: :*		
SGD_Scer_CDC45/YLR103C	290	RLYFLLQDEVKRLTFSS--RNSVKTFPDTLTNLIQPDYFLFLRRHSSLYDS		337
MIT_Sbay_c128_16060	301	RLYFLLQDEVKRLTFPSN--RNSVKTFPDTLTNLIQPDYFLFLRRHSSLYDS		348
MIT_Spar_c46_14436	291	RLYFLLQDEVKRLTFPSN--RNSVKTFPDTLTNLIQPDYFLFLRRHSSLYDS		338
WashU_Sbay_Contig672.84	301	RLYFLLQDEVKRLTFPSN--RNSVKTFPDTLTNLIQPDYFLFLRRHSSLYDS		348
WashU_Scas_Contig566.8	278	RLFLLQDEVKRLSPSSISYSAKTFPDTLSLDIQPDYFLFLRRHSTLYDS		327
WashU_Sklu_Contig1208.1	280	KLRFLQDEVKRLSPEND---STKTFPDTLALDIQPDYFLFLRRHSSLYDS		325
Symbols		:* :*: :*: :*: :*: :*: :*: :*: :*: :*: :*: :*		

Symbols: * = identical : = strong similarity . = weak similarity

Fungal alignment of Sld2

Symbols:		* = identical	: = strong similarity	. = weak similarity
SGD_Scer_SLD2/YKL108W	1	-----MYSFELDKLKIELKTWEHDFIDKNKREPTRDD		32
MIT_Sbay_c259_14334	1	-----MYSLELDKLVKVELKTWEHKKFIDTHNREPTRDD		32
MIT_Smik_c702_13260	1	MNIHAIKKPVQRLDNCNSMFSFDLDKLVKVELKTWEHEFIEKNRREPTRDD		50
MIT_Spar_c321_13898	1	-----MFSFELQLKIELKTWEHDFINKNKRREPTRDD		32
WashU_Sbay_Contig593.12	1	-----MYSLELDKLVKVELKTWEHKKFIDTHNREPTRDD		32
WashU_Scas_Contig721.139	1	-----MSLPQLKIELKNWEHTFIKQNNRPPTKND		29
WashU_Skud_Contig2010.9	1	-----MHSLELDKLVKIELKTWEHGFIDKYSRREPTGDD		32
Symbols		.: * :*:***.*** ** . * ** :*		
SGD_Scer_SLD2/YKL108W	33	IKSLRTRVRQMYKQYSTLKKKQSLQRQKVDTSQSVLPAAHKKDHDVEVEIG		82
MIT_Sbay_c259_14334	33	IKGLRDVRQMYKQYSILKKKHGLQQGLLAQESTKMLVHIKDEDEITEIG		82
MIT_Smik_c702_13260	51	IKSLRNVVRQMYKQYSLKKKQSLQTQEPFVHKSEIAAHNNKNDMEGEIG		100
MIT_Spar_c321_13898	33	IKSLRDVRQMYKQYSILKKKHSLQQKLLAQESVEVPAHSKDNDEIVEIG		82
WashU_Sbay_Contig593.12	33	IKGLRDVRQMYKQYSILKKKHGLQQGLLAQESTKMLVHIKDEDEITEIG		82
WashU_Scas_Contig721.139	30	IKKNPHIKAMYKSYSLKSSSSSSSQPHETPQKTTTQDPDAGLVQLG		79
WashU_Skud_Contig2010.9	33	IKNLRNVVRQMYRQYSILKKKQSLQQKILEQESIEIPVYTGNDDEITEIG		82
Symbols		** : : *:*** ** . . . : : : . : : *		
SGD_Scer_SLD2/YKL108W	83	PTFQVYVGKAISIFDMNLSPIKPIYMTFTNNIDVNNDNSKTI SNESSPRKT		132
MIT_Sbay_c259_14334	83	PTFQVYVGKAISIFEMNLSPIKPVYMTTTNGFGADDDSKTISNEPSPQRT		131
MIT_Smik_c702_13260	101	PTFQVHVGKAISIFEMNLSPIKPIYMAFTNDINMNNDSKTI SNVSSPEKT		150
MIT_Spar_c321_13898	83	PTFQVYVGKAISIFDMNLSPIKPIYMTFTNNTDINNNDSKTI SNKSSPQKT		132
WashU_Sbay_Contig593.12	83	PTFQVYVGKAISIFEMNLSPIKPVYMTTTNGFGADDDSKTISNEPSPQRT		131
WashU_Scas_Contig721.139	80	PTFQIYVGKAMSIFEMMVSPKIQTPSDVATTPATDVT---DVESPPRED		125
WashU_Skud_Contig2010.9	83	PTFQVHVGKAISIFEMNVSPKPIYMTFANNIDINDDLTKTI SNQSPQKT		132
Symbols		****:***:***: * :**** : . : . . **..		
SGD_Scer_SLD2/YKL108W	133	ILLKSSPADRTLVAEPISSVVRQLNFQMLNASSSTRPTSSPCKNRNGKLV		182
MIT_Sbay_c259_14334	132	ISQSSSPANRTLVSDSISNVVRQLNFQMLKAPSSSHTPISSPCKKAELS		181
MIT_Smik_c702_13260	151	ISVNSSPANRTLVAESISNVVRQLNFQIPKVSLSRTPISSPCKKQDELHA		200
MIT_Spar_c321_13898	133	MSLKSSPEDRTLVAESISNVVRQLNFQILKASSTRTPISSPCKKCDGQSA		182
WashU_Sbay_Contig593.12	132	ISQSSSPANRTLVSDSISNVVRQLNFQMLKAPSSSHTPISSPCKKAELS		181
WashU_Scas_Contig721.139	126	HDQDQGGPMQVPSLSPPIVR-----		146
WashU_Skud_Contig2010.9	133	ISQSSSPANRTLVEFVSNVVRQLDFQVLKVPSSRTXTSSPCKKRGELS		182
Symbols		..* : . . . *		
SGD_Scer_SLD2/YKL108W	183	EIKKCSP---TINPPLES GKPSGYGNSPLKLDEENIHLNISLNSSTKR		229
MIT_Sbay_c259_14334	182	EGEKPDSELTVMKPSLEPSKPSRYYGNSPLKLEENIHLNISLESNTKR		231
MIT_Smik_c702_13260	201	ENKKFSP---TKPLLES DKSSRYYGNSPLRLDEENIHLNIPFNSKIKR		247
MIT_Spar_c321_13898	183	EIKKWSP---TIKPLLES GKPSRYYGNSPLKLDEENIHLNIPLNSNTKR		229
WashU_Sbay_Contig593.12	182	EGEKPDSELTVMKPSLEPSKPSRYYGNSPLKLEENIHLNISLESNTKR		231
WashU_Scas_Contig721.139	147	-----SKWGNNSPLKMN-DIVKISINLNKRIER		173
WashU_Skud_Contig2010.9	183	ESKKCTPTLAIVRPLVESNESSYYGNSPLKLEENIHLNISLSSNTKR		232
Symbols		:*****:: : : : * .. : *		
SGD_Scer_SLD2/YKL108W	230	RLQIAYPSLQKTPSKD-QADISTFSPSPLIRRLPLTKSLIELAREHTEIV		278
MIT_Sbay_c259_14334	232	RLQMAFPSSLQKTPSKDNHGGITTSFSPSPLIRRLPLSKSLIELAKEHIEIV		281
MIT_Smik_c702_13260	248	RLQMGYPSSLKTPSKDNHVDISNSFSPSPLIRRLPLTKSLIELAKEHIEIV		297
MIT_Spar_c321_13898	230	RLQMAYPSSLQKTPSKDNHVDISVSFSPSPLIRRLPLTKSLIELAREHTEIV		279
WashU_Sbay_Contig593.12	232	RLQMAFPSSLQKTPSKDNHGGITTSFSPSPLIRRLPLSKSLIELAKEHIEIV		281
WashU_Scas_Contig721.139	174	-----VNLTPNKMGV DANVESPSPIVKNLKFQKSLKQLDKEYKDIL		215
WashU_Skud_Contig2010.9	233	RLQMVYPSLQKTPSKDNHVDISNSFSPSPLIRRLPLTKSLIELAKEHTEIV		282
Symbols		: **.: * ** : *** : * : : : *		

SGD_Scer_SLD2/YKL108W	279	KEFGVLQ-----EEDIEEEEGEGEGENGYDEKNHEDDFGLDEDELI	318
MIT_Sbay_c259_14334	282	KEFSIVQ-----EEEEDDDDGEDDEN-----DGALEDGLV	311
MIT_Smik_c702_13260	298	KEFSILE-----EEGDRGENGEESDEGCNADKCEEEESKLEDGLI	339
MIT_Spar_c321_13898	280	KEFSTLQGEDIEEDIEEEEEEEEEEGEDDKDGRDEKDGEDESRLEDGLI	329
WashU_Sbay_Contig593.12	282	KEFSIVQ-----EEEEDDDDGEDDEN-----DGALEDGLV	311
WashU_Scas_Contig721.139	216	RELKLDK-----PNDNNNDEDK-----ETDVI	237
WashU_Skud_Contig2010.9	283	KEFGILE-----KEDREGEGEIDDNSHGQVDEDESKLEDGIV	321
Symbols		** : : : : *	

SGD_Scer_SLD2/YKL108W	319	RPKVVKDIFQEDDDNDDSQAREDTFIRKRPKRRKVIIRRLRDNDPETETAG	368
MIT_Sbay_c259_14334	312	RSTVMKDIFQEDD-NEDNQSKEGTFIKRPKRRKIIRRLRDGDPETENST	360
MIT_Smik_c702_13260	340	RSKVMADIFQEDE-VEGNQGREGAFIRKRPKRRKVIIRSRRENDLEDETSI	388
MIT_Spar_c321_13898	330	RRKVVKDIFQEDDDNEDNQARENTFIRKRPKRRKVIIRRLRDDDPETEPTA	379
WashU_Sbay_Contig593.12	312	RSTVMKDIFQEDD-NEDNQSKEGTFIKRPKRRKIIRRLRDGDPETENST	360
WashU_Scas_Contig721.139	238	SGVAIRDIFNEDE--EETTTTTLTYGPKPKRRKIIR--RLDVAENEQKV	283
WashU_Skud_Contig2010.9	322	RSRVVKDIFQEDD-NKVNQAKESTFIRKRPKRRKVIIRRLRDENPDTENSV	370
Symbols		: : *** : * : : : : * : *	

SGD_Scer_SLD2/YKL108W	369	FERDVHKKELVKLKKRKVAEFLGSTSQISDTEFEHDPEASSGVVSSEQKPT	418
MIT_Sbay_c259_14334	361	VRADVHKKELMKLKKRKVAEFLGSTSKLEDTESEDDDESANSSVKPEQKPA	410
MIT_Smik_c702_13260	389	VKRDVHKKELMKLKKRKVAEFLGSTSQLSETESENDDDEATGGVIP-KQKPA	437
MIT_Spar_c321_13898	380	VKRDVHKKELMKLKKRKVAEFLGSTSQLSDTEFENNDEATSGVVNLEQKPA	429
WashU_Sbay_Contig593.12	361	VRADVHKKELMKLKKRKVAEFLGSTSELFDTESEDDDESANSSVKPEQKPA	410
WashU_Scas_Contig721.139	284	VPKNIHKKELLKLLKKRQVSALLTEDENHSKTSDLEDETTSEEEKEDLPVK	333
WashU_Skud_Contig2010.9	371	ATKDVHKKELMKLKKRKVAEFLGSTSQLSDTESEDAGETAISNSTPEQKPA	420
Symbols		: : * * * * : * : * : * : * : *	

SGD_Scer_SLD2/YKL108W	419	AKRKGR-KKYNLVSNNFRRLKLPKKNRFSNGRWGRR	453
MIT_Sbay_c259_14334	411	AKRKGR-KKYNLVSNNFRRLKLPKKNRFPNRRWGRR	445
MIT_Smik_c702_13260	438	SKRKGR-KKYNLVSNNFRRLKLPKKNRFFNRRSGRR	472
MIT_Spar_c321_13898	430	AKRKGR-KKYNLVSNNFRRLKLPKKNRFPNRRWGRR	464
WashU_Sbay_Contig593.12	411	AKRKGR-KKYNLVSNNFRRLKLPKKNRFPNRRWGRR	445
WashU_Scas_Contig721.139	334	RQKKKRPKKYNLVSNNFRRLKLPKKNRRWPGRR--	367
WashU_Skud_Contig2010.9	421	AKRKGR-KKYNLVSNNFRRLKLPKKNRFPNRRWGRR	455
Symbols		: : * * * * * * * * * * * *	

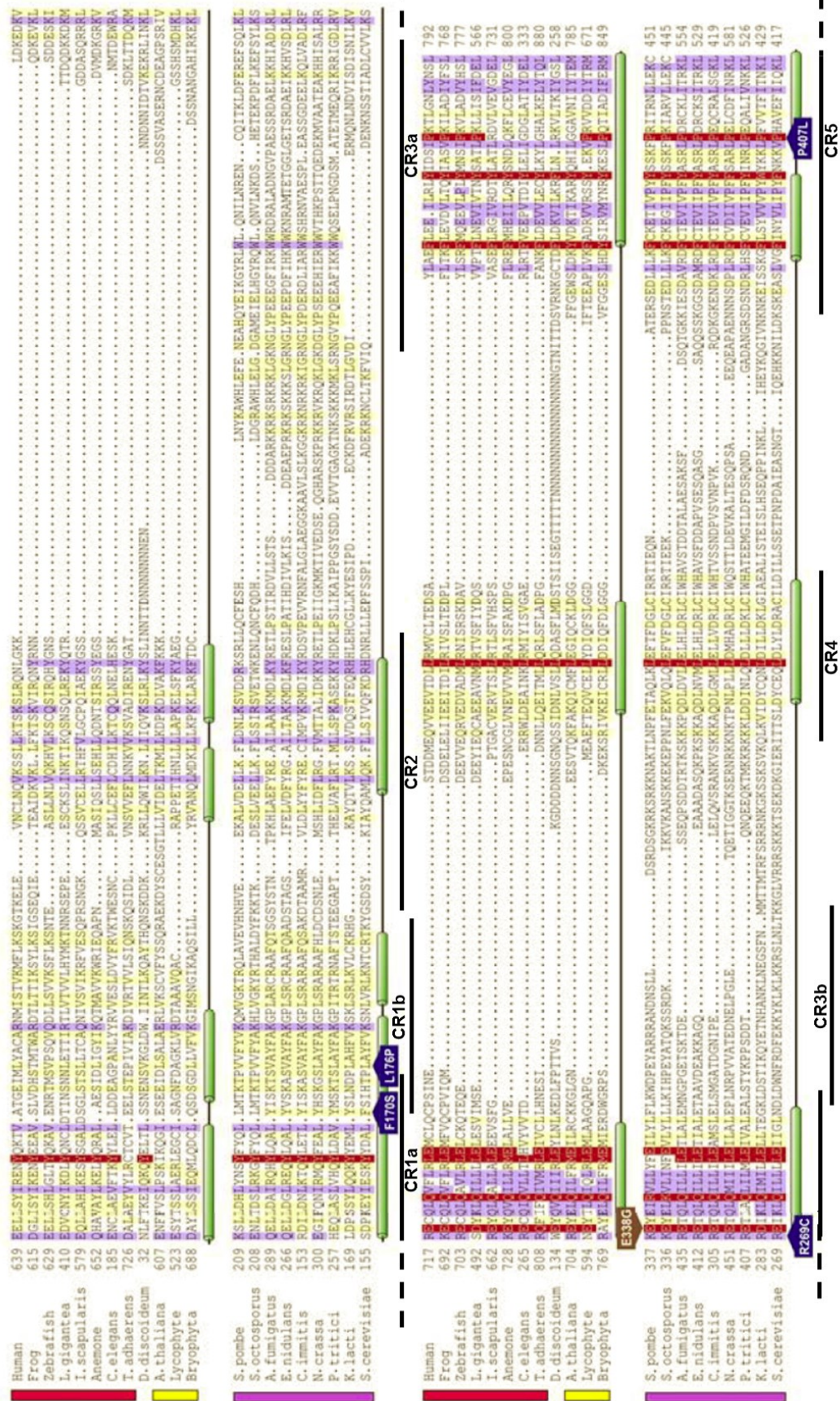
Symbols:

* = identical

: = strong similarity

. = weak similarity

Multiple alignment of Sld3 (Sanchez-Pulido et al, 2010)



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