STRUCTURAL AND FUNCTIONAL CHARACTERISATION
OF MCB1 AND THE MCM$^{Mcb1}$ COMPLEX IN
SCHIZOSACCHAROMYCES POMBE

Jasmin Schnick

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews

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Structural and functional characterisation of Mcb1 and the MCM$^{Mcb1}$ complex in *Schizosaccharomyces pombe*

Jasmin Schnick

A thesis presented for the degree of Doctor of Philosophy of the University of St Andrews

School of Biology

December 2013
Abstract

The MCM helicase plays an important role in eukaryotic DNA replication, unwinding double stranded DNA ahead of the replication fork. MCM is a hetero-hexamer consisting of the six related proteins, Mcm2-Mcm7. The distantly related MCM-binding protein (MCM-BP) was first identified in a screen for proteins interacting with MCM2-7 in human cells and was found to specifically interact with Mcm3-7 but not Mcm2. It is conserved in most eukaryotes and seems to play an important role in DNA replication but its exact function is not clear yet.

This study contributes to the understanding of the fission yeast homologue of MCM-BP, named Mcb1, but also of MCM-BP in general. Results presented in this thesis document the initial biochemical characterisation of the complex Mcb1 forms with Mcm proteins, the MCM$^{Mcb1}$ complex. Interactions of Mcb1 with Mcm proteins, potential interaction sites between the proteins and the size of the complex were analysed using a variety of methods, including tandem affinity purification, co-immunoprecipitation, sucrose gradients and in vitro pull-down assays. Sequence analysis and structure prediction were utilised to gain some insight into Mcb1 and MCM-BP ancestry and structure. Results presented here indicate that fission yeast Mcb1 shares homology with Mcm proteins and forms a complex with Mcm3-Mcm7 but not Mcm2 and thus replaces the latter in an alternative high molecular weight complex that is likely to have an MCM-like appearance.

Deletion of mcb1$^+$ showed that Mcb1 is essential in fission yeast. To examine the cellular function of the protein, temperature-sensitive mutants were generated. Inactivation of Mcb1 leads to an increase in DNA damage and cell cycle arrest in G2-phase depending on the activation of the Chk1 dependent DNA damage checkpoint. Similar observations were made when Mcb1 was overexpressed, indicating that certain levels of the protein are important for accurate DNA replication. Construction of truncated versions of Mcb1 suggested that almost the full-length protein is needed for proper function.
Declaration

I, Jasmin Schnick, hereby certify that this thesis, which is approximately 79000 words in length, has been written by me, that it is the record of work carried out by me, with the exception of the construction of plasmids containing Mcb1 pentapeptide insertion mutants, which was carried out by Justina Ray, the construction of the mcb1-Ts alleles, which was conducted by Juan-Juan Li, and the construction of a diploid S. pombe strain lacking one copy of mcb1+ (carried out by Dr Stuart MacNeill), and that it has not been submitted in any previous application for a higher degree.

I was admitted as a research student in March 2010 and as a candidate for the degree of PhD in April 2011; the higher study for which this is a record was carried out in the University of St Andrews between 2010 and 2013.

15th December 2013 signature of candidate

I hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropriate for the degree of PhD in the University of St Andrews and that the candidate is qualified to submit this thesis in application for that degree.

Date signature of supervisor

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First and foremost, I would like to thank my brilliant supervisor Dr Stuart MacNeill for his valuable guidance, support, advice and inspiration but also for giving me lots of freedom while working on this highly interesting project. You are exactly the supervisor I needed.

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I would like to thank past and present members of the MacNeill lab, especially Agnieszka Skowyra, Xavier Giroux, Antonia Evripoti and Kazim Ogmen for advice, many discussions, encouragement, thesis pep talks and nice evenings in the pub. You made the lab a nice place to work and brightened my time in St Andrews. Many thanks to Justina Ray for constructing the pentapeptide insertion mutants. I would also like to thank Liz King for all the delicious cake, the members of the White lab and all the other people who contribute to the nice working atmosphere in the BSRC.

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Publications

Some results from this thesis have been published:

*Joint first authors

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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>Δ</td>
<td>Gene deletion</td>
</tr>
<tr>
<td>3′</td>
<td>3 prime DNA end</td>
</tr>
<tr>
<td>5′</td>
<td>5 prime DNA end</td>
</tr>
<tr>
<td>AAA⁺</td>
<td>ATPase associated with a variety of cellular activities</td>
</tr>
<tr>
<td>ACL</td>
<td>Allosteric communication loop</td>
</tr>
<tr>
<td>AID</td>
<td>Auxin inducible degron</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>ATR</td>
<td>Ataxia telangiectasia and Rad3-related protein</td>
</tr>
<tr>
<td>Aux</td>
<td>Auxin</td>
</tr>
<tr>
<td>BiFC</td>
<td>Bimolecular fluorescence complementation</td>
</tr>
<tr>
<td>bp</td>
<td>Base pairs</td>
</tr>
<tr>
<td>Cdc</td>
<td>Cell division cycle</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin-dependent kinase</td>
</tr>
<tr>
<td>Cdt1</td>
<td>Cdc10-dependent transcript 1</td>
</tr>
<tr>
<td>CMG complex</td>
<td>Cdc45/ MCM2-7/ GINS complex</td>
</tr>
<tr>
<td>co-IP</td>
<td>Co-immunoprecipitation</td>
</tr>
<tr>
<td>Crm1</td>
<td>Chromosomal region maintenance 1</td>
</tr>
<tr>
<td>CTD</td>
<td>C-terminal domain</td>
</tr>
<tr>
<td>Cut5</td>
<td>Cell untimely torn 5</td>
</tr>
<tr>
<td>Dbf4</td>
<td>Dumbbell former 4 protein</td>
</tr>
<tr>
<td>DDK</td>
<td>Dbf4-dependent kinase (=Dbf4-Cdc7)</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxyribonucleotide triphosphate (A, T, C and G)</td>
</tr>
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<td>Dpb11</td>
<td>DNA polymerase B possible subunit 11</td>
</tr>
<tr>
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<td>Double strand break</td>
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<td>Double-stranded DNA</td>
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<tr>
<td>E</td>
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<td>EDTA</td>
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<td>FL</td>
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<td>Gram</td>
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<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
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<tr>
<td>GINS</td>
<td>Go ichi ni san complex, a component of the CMG complex</td>
</tr>
<tr>
<td>H2I</td>
<td>Helix-2-insert</td>
</tr>
<tr>
<td>HA</td>
<td>Human influenza hemagglutinin</td>
</tr>
<tr>
<td>HBD</td>
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<td>Hsk1</td>
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</tr>
<tr>
<td>HU</td>
<td>Hydroxyurea</td>
</tr>
<tr>
<td>IAA</td>
<td>Indole-3-acetic acid</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
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</tr>
<tr>
<td>IVT</td>
<td><em>In vitro</em> translation</td>
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</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>l</td>
<td>Litre</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Bertani medium</td>
</tr>
<tr>
<td>LCEA</td>
<td>Last common eukaryotic ancestor</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>mA</td>
<td>Millampere</td>
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<td>Minichromosome maintenance complex*</td>
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<td>Nanometre</td>
</tr>
<tr>
<td>NTD</td>
<td>N-terminal domain</td>
</tr>
<tr>
<td>OB fold</td>
<td>Oligonucleotide-binding fold</td>
</tr>
<tr>
<td>OCM</td>
<td>ORC/Cdc6/MCM2-7 complex</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>ORC</td>
<td>Origin recognition complex</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PBS-T</td>
<td>PBS-Tween</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PCNA</td>
<td>Proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PIP</td>
<td>PCNA-interacting peptide</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>Pol</td>
<td>Polymerase</td>
</tr>
<tr>
<td>PPI</td>
<td>Pentapetide insertion</td>
</tr>
<tr>
<td>preIC</td>
<td>PreInitiation complex</td>
</tr>
<tr>
<td>preLC</td>
<td>Preloading complex</td>
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<tr>
<td>preRC</td>
<td>Prereplication complex</td>
</tr>
<tr>
<td>PS1</td>
<td>Presensor 1</td>
</tr>
<tr>
<td>Psf</td>
<td>Partner of Sld five</td>
</tr>
<tr>
<td>RFC</td>
<td>Replication factor C</td>
</tr>
<tr>
<td>RPA</td>
<td>Replication protein A</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>SB</td>
<td>Sample buffer for protein gel electrophoresis</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>Skp1</td>
<td>S-phase kinase-associated protein 1</td>
</tr>
<tr>
<td>Sld2,3,5,7</td>
<td>Synthetically lethal with Dpb11 2,3,5,7</td>
</tr>
<tr>
<td>sso</td>
<td>Sulfolobus solfataricus</td>
</tr>
<tr>
<td>Sp</td>
<td>Schizosaccharomyces pombe</td>
</tr>
<tr>
<td>SSB</td>
<td>Single-strand DNA binding protein</td>
</tr>
<tr>
<td>ssDNA</td>
<td>Single-stranded DNA</td>
</tr>
<tr>
<td>TAP</td>
<td>Tandem affinity purification</td>
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<tr>
<td>T</td>
<td>Thiamine</td>
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<tr>
<td>TE</td>
<td>Tris EDTA</td>
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<tr>
<td>Tir1</td>
<td>Transport inhibitor response 1</td>
</tr>
<tr>
<td>Tris</td>
<td>2-Amino-2-hydroxymethyl-propane-1,3-diol</td>
</tr>
<tr>
<td></td>
<td>Tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>Ts</td>
<td>Temperature-sensitive</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>V</td>
<td>Volt</td>
</tr>
<tr>
<td>WB</td>
<td>Western blot</td>
</tr>
<tr>
<td>wt</td>
<td>Wild-type</td>
</tr>
<tr>
<td>YE4S</td>
<td>Yeast extract medium with four supplements</td>
</tr>
<tr>
<td>YFP</td>
<td>Yellow fluorescent protein</td>
</tr>
<tr>
<td>µg</td>
<td>Microgram</td>
</tr>
<tr>
<td>µl</td>
<td>Microlitre</td>
</tr>
<tr>
<td>µm</td>
<td>Micrometre</td>
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</table>

*As the conventions for nomenclature of genes and proteins vary in different organisms, a simplified version was used here for nomenclature of MCM/Mcm: Mcm indicates an individual protein (e.g. Mcm4 or ssoMcm); MCM and MCM2-7 stand for complexes. The yeast MCM complex however is Mcm2-7.*
Chapter 1 Introduction

1.1 Eukaryotic DNA replication

Complete and accurate duplication of the genome is crucial for genome stability and thus for the survival of the cell, ensuring that the genetic information in the daughter cells is identical to the parental genomic DNA. DNA replication is essential for all living organisms and defects in the process of DNA replication or in the components of the replication machinery can have serious consequences, including cell death and a variety of diseases such as cancer in higher eukaryotes. This is why gaining an understanding of this fundamental process is important: to be able to diagnose, treat and maybe even prevent problems emanating from disturbed DNA replication in the future.

The replication of genomic DNA is a complicated process that requires the accurate interplay of a variety of different protein factors. It is highly regulated to provide a precise course of events and ensure only once per cell cycle replication to maintain the integrity of the genome (Blow & Dutta 2005). DNA replication consists of three stages, initiation, elongation and termination. During initiation, which happens in M and G1-phase (reviewed in Bell & Dutta, 2002), the so-called prereplication complex (preRC) is formed at origins of replication. At the G1/S transition, the combined action of S-phase CDK and DDK and the recruitment of additional protein factors lead to the conversion of the preRC into the actual replicative helicase, the CMG complex (consisting of Cdc45, GINS and MCM2-7). The active CMG complex then starts unwinding of double-stranded DNA and the recruitment of the other components of the replication machinery leads to the start of replication. The actual chromosome replication (“elongation”), during which bidirectional replication forks synthesize new DNA in a semiconservative manner takes place in S-phase of the cell cycle. Events leading to initiation of replication are thought to take place mainly during G1 but as early as M-phase and termination of replication might even extend into G2-phase (Lygeros et al. 2008), so that one round of replication almost spans a full cell cycle.
1.1.1 Assembly of the pre-replication complex at the origins of replication

Initiation of eukaryotic DNA replication starts early in M/G1-phase, when there are only low levels of inhibitory CDK activity, with the assembly of the pre-replication complex (preRC) at special DNA sites termed origins of replication. This leads to the loading of the replicative helicase and “licensing” of the chromatin for replication (reviewed in Blow & Tanaka, 2005; Diffley, 2004; Remus & Diffley, 2009). The licensing model was originally developed in 1988 by Blow and Laskey who suggested that a “licensing factor” could only bind DNA when the nuclear envelope breaks down during mitosis (Blow & Laskey 1988).

1.1.1.1 Origins of replication

Bacteria usually have only one origin (oriC) as starting point for the replication of their single circular chromosome and need short specific DNA sequences for binding of different factors and subsequent melting of oriC. Conversely, the more complex and larger eukaryotic chromosomes have multiple origins. Initial work with budding yeast revealed the existence of well defined autonomously replicating sequences (ARS) (Stinchcomb et al. 1979): short (100-150 base pairs) DNA regions containing specific conserved elements, which are used as starting sites for replication (reviewed in Cvetic & Walter, 2005; Gilbert, 2001). In other eukaryotes replication origins seem to be less well defined and do not necessarily contain specific DNA sequences (Dai et al. 2005; Cvetic & Walter 2005; Legouras et al. 2006). In Xenopus and Drosophila embryonic systems, it appears that any DNA sequence can be used as an origin, while in mammalian cells a certain length of DNA is necessary to make an origin. However a specific sequence could not be identified that was indicative of metazoan origins (reviewed in Méchali 2010; Cvetic & Walter 2005; Gilbert 2001).

Fission yeast seems to have evolved an intermediate between completely random and extremely specific origins: their replication start sites are exclusively located in intergenic regions, longer than in budding yeast (500-1500 bp long), are AT-rich and lack a highly specific consensus as it is found in S. cerevisiae, but contain some of multiple different elements that contribute to origin activity (Dai et al. 2005; Segurado
et al. 2003; Legouras et al. 2006; Cvetic & Walter 2005). Of the many origins of replication in eukaryotic genomes only a subset fires in each round of the cell cycle. In addition, firing of each origin takes place with characteristic timing, leading to the classification of early, mid or late origins (reviewed in Méchali 2010).

1.1.1.2 PreRC assembly

The assembly of the preRC starts with the binding of the origin recognition complex (ORC) to the origins of replication. The hexameric ORC, consisting of Orc1-6, was originally found to specifically interact with ARS (autonomously replicating sequences) in budding yeast (Bell & Stillman 1992) and homologous forms of the complex were subsequently identified in other eukaryotes, including fission yeast (Moon et al. 1999), providing evidence for a conserved mechanism of replication initiation in eukaryotes (reviewed in Duncker et al. 2009; Li & Stillman 2012). The ORC complex has ATPase activity and its DNA association usually depends on ATP (Li & Stillman 2012). The S. cerevisiae complex was found to remain bound to chromatin throughout the whole cell cycle (Aparicio et al. 1997), which was also thought to be the case for the fission yeast ORC (Lygerou & Nurse 1999). However, recent data from ChIP experiments suggests that S. pombe ORC binding to origin DNA happens periodically and peaks in M/G1, as in metazoans (Wu & Nurse 2009; Li & Stillman 2012).

DNA-bound ORC serves as platform for the assembly of the other components of the PreRC. Cdc6 (Cdc18 in S. pombe) and Cdt1 are both recruited to ORC in G1-phase and are absolutely necessary for DNA replication initiation (Hofmann & Beach 1994; Nishitani et al. 2000; Nishitani et al. 2001), facilitating the initial loading of the MCM2-7 helicase. Despite Cdt1 and Cdc6 being able to interact directly, they are independently recruited to the origins (Coleman et al. 1996; Nishitani et al. 2000; Maiorano et al. 2000; reviewed in Bell & Dutta 2002). Cdc6 is an AAA+ ATPase related to Orc1 (Neuwald et al. 1999) and its ATPase activity is essential for the licensing reaction (Randell et al. 2006; Gillespie et al. 2001; Bell & Dutta 2002; Blow & Dutta 2005). Cdt1 forms heptameric complexes with the MCM2-7 helicase and its main role seems to be the recruitment of MCM2-7 to ORC-Cdc6 (Caillat & Perrakis 2012). The assembly of Cdt1/MCM2-7 with ORC/Cdc6 was initially thought to be mediated by direct interaction
between Cdt1 and Orc6 in budding yeast (Randell et al. 2006; Takara & Bell 2011), but there is new evidence that the C-terminus of Mcm3 might be important for MCM2-7 recruitment to the origins too, and that Cdt1 rather has a stabilising effect on MCM2-7 (Frigola et al. 2013). Cdt1 interaction with MCM2-7 is mediated through a conserved C-terminal motif of Mcm6, in yeast and also higher eukaryotes (Yanagi et al. 2002; Jee et al. 2010; Maki et al. 2011; Liu et al. 2012) and Cdt1 was also reported to interact with mouse Mcm2 \textit{in vitro} (You & Masai 2008).

Cdt1 is a highly controlled protein and its activity is limited to G1 and early S-phase by CDK-dependent regulation of transcription and degradation by ubiquitin-mediated proteolysis. Metazoans additionally regulate Cdt1 activity by geminin binding, which is thought to prevent Cdt1 function by blocking Cdt1-Mcm2 interaction (Tanaka & Diffley 2002; Nishitani et al. 2001; Wohlschlegel et al. 2002; Tada et al. 2001; Li & Blow 2005; Blow & Dutta 2005; Caillat & Perrakis 2012). The tight connection of Cdt1 regulation and preRC formation ensures that licensing of origins can only take place in G1-phase and makes an important contribution to prevent re-replication of DNA (Blow & Dutta 2005, see also 1.2).

The heterohexameric MCM2-7 is generally accepted to be the core of the eukaryotic replicative helicase, which unwinds double-stranded DNA ahead of the replication fork. Its six subunits are highly conserved in eukaryotes, are members of the AAA+ ATPase family and absolutely essential for DNA replication (Duderstadt & Berger 2008; reviewed Bochman & Schwacha 2009). Interestingly the number of MCM2-7 complexes found on chromatin greatly exceeds the number of initiation events (Laskey & Madine 2003) and MCM complexes can be found on unreplicated chromatin (Dimitrova et al. 1999), which is referred to as the “MCM paradox”. The function of excess MCM complexes on DNA is still unclear but they could be used to license dormant origins, which are only activated in case of replication stress to rescue stalled replication forks (Woodward et al. 2006). The structure and function of the MCM complex will be discussed in detail in 1.3.
Introduction

Figure 1-1 A simplified view of the Initiation of eukaryotic DNA replication. During late mitosis and G1-phase, when CDK is inactive, the pre-replication complex (preRC) is formed at origins of replication. This involves the loading of two MCM2-7 hexamers via the intermediate OCM (ORC, Cdc6, MCM), and licenses the chromatin for DNA replication. At the G1/S-phase transition, S-phase CDK and Cdc7-Dbf4 are activated. The combined action of the kinases and the recruitment of additional protein factors lead to the formation of the pre-initiation complex (preIC) and the activation of the Cdc45/MCM2-7/GINS (CMG) complex. DNA unwinding allows the establishment of two bidirectional replication forks and assembly of the replisomes for the elongation of DNA replication. Not all proteins which have been
Once an initial complex of ORC/Cdc6/Cdt1/MCM2-7 is formed, Cdt1 and ORC/Cdc6 act together to load MCM2-7 helicase onto chromatin, however the exact mechanism is still unknown (reviewed in Blow & Tanaka 2005; Blow & Dutta 2005). ATP hydrolysis in Cdc6 induces tight binding of MCM2-7 to DNA (possibly by opening the hexameric ring of MCM2-7 and allowing it to wrap around DNA; reviewed in Blow & Dutta 2005) and leads to the displacement of Cdt1 (Randell et al. 2006; Arias & Walter 2007).

Additional ATP hydrolysis in Orc1 and Orc4 is important to complete MCM2-7 loading (Bowers et al. 2004). Even though they are essential for helicase loading, Cdt1, ORC and Cdc6 are dispensable for subsequent steps of DNA replication, indicating that the primary role of the preRC is the loading of the MCM complex (reviewed in Blow & Dutta 2005; Donovan et al. 1997; Hua & Newport 1998; Rowles et al. 1999; Maiorano et al. 2000).

In vitro studies using purified budding yeast proteins and cryo-EM indicate that budding yeast MCM2-7 is loaded as a double hexamer in a concerted way (Evrin et al. 2009; Remus et al. 2009) by the recruitment of two Cdt1/MCM2-7 heptamers to one origin (reviewed in Labib 2011; Takara & Bell 2011). The double hexamer has a head-to-head configuration with the N-terminal domains of MCM2-7 touching and the C-terminal AAA+ ATPase domains pointing outwards (Remus et al. 2009). EM and biochemical analysis showed that the double hexamer encircles double-stranded DNA in the central channel (Remus et al. 2009; Evrin et al. 2009). How the initial ORC/Cdc6/Cdt1/MCM2-7 complex is transformed into an MCM2-7 double hexamer is an important question.

Recent in vitro studies with reconstituted S. cerevisiae loading assays report a novel and stable preRC intermediate, the OCM, consisting of ORC/Cdc6/MCM2-7. OCM formation depends on ATP-hydrolysis in Cdc6 and Orc1 and subsequent Cdt1 release from the initial ORC/Cdc6/Cdt1/MCM2-7 complex (Fernández-Cid et al. 2013). Interestingly, Mcm3-mediated binding of Cdt1/MCM2-7 to ORC/Cdc6 was recently reported to stimulate this ORC and Cdc6 ATPase activity. And, as mentioned above, the Mcm3 C-terminal domain seems to be important for the actual recruitment of both MCM2-7/Cdt1 heptamers to origins (Frigola et al. 2013). The same study also describes
that absence of Cdt1 leads to dissociation of MCM2-7 into MCM4/6/2 and MCM5/3/7 upon recruitment (Frigola et al. 2013). After loading of the first MCM2-7 complex, OCM could act as platform for the formation of a double hexamer by recruiting a second Cdt1/MCM2-7 heptamer (Fernández-Cid et al. 2013).

Based on these two very recent studies Samson and Bell suggested the following model for MCM2-7 loading: MCM2-7/Cdt1 is recruited to origins via Cdt1 and the Mcm3 C-terminus. Cdt1 has a stabilising effect on MCM2-7. ATP hydrolysis in Orc1 and Cdc6 leads to release of Cdt1 and formation of the OCM. Due to the instability of MCM2-7 in the absence of Cdt1, a remodelling occurs and MCM2-7 swings open into an open-book confirmation, where MCM4/6/2 and MCM5/3/7 are still connected via their N-terminal domains, and Mcm3 still interacts with ORC/Cdc6. Recruitment of a second hexamer could work in exactly the same way and push over the MCM5/3/7 of the first hexamer, effecting the closing of the book and leading to a head-to-head double hexamer (Samson & Bell 2013). Irrespective of whether the proposed model is correct, the MCM2-7 loading mechanism might be conserved in eukaryotes, as MCM2-7 double hexamers have been observed in *Xenopus* extracts as well (Gambus et al. 2011).

Loaded MCMs are free to slide along DNA, which explains how so many MCM complexes can be loaded from a very small number of active origins (Evrin et al. 2009; Remus et al. 2009; reviewed in Boos et al. 2012). However MCM2-7 in the double hexamers do not possess ATPase or helicase activity yet, as the firing of the origins and activation of the helicase occur only upon entry into S-phase and require other factors to bind (reviewed in Remus & Diffley 2009).

1.1.2 Formation of the CMG complex and activation of replication

While the archaeal MCM complexes have helicase activity (McGeoch et al. 2005; Chong et al. 2000; Kelman et al. 1999) the isolated eukaryotic MCM2-7 complex is only a weak helicase *in vitro* (Ilves et al. 2010; Bochman & Schwacha 2008). Bochman and Schwacha showed that purified *S. cerevisiae* Mcm2-7 can efficiently unwind dsDNA if the reaction conditions are appropriately modified. Such modifications might induce structural changes in Mcm2-7, leading to an activation of the helicase activity. *In vivo* the changes
are presumably induced by interaction with additional protein factors (Bochman & Schwacha 2008).

These factors are likely to include Cdc45 and GINS, which have been shown to be essential for DNA replication initiation and elongation in multiple studies (Aparicio et al. 1997; Kanemaki & Labib 2006; Kubota et al. 2003; Gambus et al. 2006; Labib & Gambus 2007; Tercero et al. 2000; Takayama et al. 2003; Moyer et al. 2006). They do not associate with Mcm2-7 in the preRC, but later, indicating a function after preRC formation (Labib & Gambus 2007).

Eukaryotic GINS is heterotetrameric complex and its name derives from the Japanese go ichi ni san, meaning 5 1 2 3 after its subunits Sld5, Psf1, Psf2 and Psf3 (Takayama et al. 2003). The four subunits are paralogues and highly conserved in all eukaryotes (Marinsek et al. 2006; Makarova et al. 2005). Archaea also have an equivalent of GINS, however most archaeal GINS homologues consist of two dimers of GINS15 (homologous to Psf1 and Sld5) and GINS23 (homologous to Psf2 and Psf3) and some archaea have a homotetrameric GINS (Oyama et al. 2011; Labib & Gambus 2007).

The actual function of GINS is unclear. It seems to be important to maintain the interaction between Cdc45 and MCM in the RPC complex (Gambus et al. 2006; Kanemaki & Labib 2006) and it might be involved in coupling MCM2-7 to Pol $\alpha$ (Gambus et al. 2009)

The eukaryotic Psf2 is essential for DNA replication, as its inactivation using a hormone binding domain fusion (Psf2-HBD) in fission yeast leads to an arrest of DNA replication and loss of Psf2 nuclear localisation. This causes delocalisation of Psf3, implying that the whole GINS complex falls off chromatin when Psf2 is inactivated (Pai et al. 2009). Psf3 also seems important for DNA replication as it is needed for Polymerase $\alpha$ binding to origins in S-phase (Yabuuchi et al. 2006). One probable role of Psf1 is recruitment of Cdc45 to chromatin (Kubota et al. 2003; Yabuuchi et al. 2006) and also its maintenance on DNA during elongation, which was shown using a hormone binding domain to inactivate Psf1 (Pai et al. 2009). It is also needed for the chromatin binding of the replicative Pol $\epsilon$, as Psf1 inactivation causes DNA replication arrest with loss of DNA association of Pol $\epsilon$, but does not seem to have an effect on priming Pol $\alpha$ binding to origins (Pai et al. 2009).
To date three crystal structures of the human GINS are available (Chang et al. 2007; Choi et al. 2007; Kamada et al. 2007) all suggesting a compact ellipsoid structure for the complex. The proposed structures are noticeably different from the horse-shoe shaped architecture suggested by an EM study (Boskovic et al. 2007), but the reason for the discrepancy is not clear (Onesti & Macneill 2013).

Cdc45 is required for replication fork progression and its interaction with a variety of protein factors including MCM2-7, GINS, Mcm10, RPA, Pol α, Pol δ and Pol ε has been shown (Kubota et al. 2003; Zou & Stillman 2000; Mimura & Takisawa 1998). However not much is known about its exact function. Only recently it has been suggested that eukaryotic Cdc45 is an orthologue of the archaeal and bacterial RecJ nucleases (Krastanova et al. 2012; Sanchez-Pulido & Ponting 2011; Makarova et al. 2012). In archaea the RecJ nuclease is speculated to play a role in Okazaki fragment maturation (Krastanova et al. 2012), but the nuclease function seems to be dispensable (Makarova et al. 2012). Therefore Cdc45 could have evolved from an ancestral 5’-3’ exonuclease, which lost its activity in eukaryotes.

Biochemical experiments indicate that Cdc45 and GINS can induce the ATPase activity of MCM2-7 and stimulate its helicase function (Moyer et al. 2006; Ilves et al. 2010). It was shown that in a complex with Cdc45 and GINS, Drosophila MCM2-7 has 300 fold enhanced ATPase activity and increased affinity for DNA, in comparison to MCM2-7 on its own (Ilves et al. 2010). This assembly is called the CMG complex (Cdc45/MCM2-7/GINS), and is likely to function as the replicative helicase in eukaryotes (Moyer et al. 2006; Ilves et al. 2010; reviewed in Vijayraghavan & Schwacha 2012). While MCM2-7 seems to be the motor of the CMG helicase, the exact functions of GINS and Cdc45 are not clear but they undoubtedly play a very important role for the activity of the replicative helicase (reviewed in Kamada 2012; Onesti & Macneill 2013). Furthermore the conservation of all CMG components in eukaryotes and also archaea emphasizes its importance for replication.

Structural studies using purified MCM2-7, GINS and Cdc45 from Drosophila and single particle EM 3D reconstruction gave insight into the structure of the CMG complex (Costa et al. 2011). It was found that MCM2-7 could adopt two different states: a planar, notched ring-like state and an open right-handed lock-washer state. Both
conformations have a gap between the Mcm2 and Mcm5 subunits referred to as “gate”. In the CMG complex MCM2-7 exclusively adopts the planar configuration and Cdc45 and GINS bridge the gate, by interacting with each other and MCM2-7 (Figure 1-2 A; Costa et al. 2011). Protein-protein interactions are mediated between Psf2, Psf3 and the conserved A domains in the N-terminal domain of Mcm3 and Mcm5. Cdc45 presumably interacts with the N-terminal domain of Mcm2 and the Psf2 subunit of GINS. ATP binding by MCM2-7 seems to tightly lock the ring, closing the gate (Figure 1-2 B; Costa et al. 2011). However the mere ring closing by Cdc45 and GINS does not seem to be sufficient for helicase activation; they are also thought to induce conformational changes in MCM2-7 affecting the ATPase centres (Boos et al. 2012).

![Model of Drosophila CMG](from Costa et al. 2011). Structures of human GINS (Chang et al. 2007) and archaeal MCM (Fletcher et al. 2003, only N-terminal hexamer of mthMCM) proteins were docked into the EM reconstructions of the CMG complex. The N-terminal (left), side (middle) and C-terminal views are shown. The different subunits of MCM2-7 are depicted in colours, GINS is shown in white. The unoccupied grey density is assigned to Cdc45 (no crystal structure available). A) CMG complex in nucleotide free (apo) state. The red arrow indicates the gate between Mcm2 (pink) and Mcm5 (yellow). B) CMG complex in its ADP-BeF₃ bound form with the gate closed.

The assembly of the CMG complex is initiated at the entry into S-phase and depends on the activity of S-phase CDK and Dbf4 dependent Cdc7 kinase (DDK) (reviewed in Remus & Diffley 2009).
Budding yeast Cdc7 was found to bind the MCM2-7 complex at the origins and phosphorylate Mcm2, Mcm4, Mcm6 and Mcm7 but not Mcm5 (Lei et al. 1997; Weinreich & Stillman 1999; reviewed in Labib 2010). Mcm4, Mcm2 and Mcm6 all seem to be phosphorylated at their N-terminal extensions and the hyperphosphorylated form of Mcm4 was found to be enriched in a MCM2-7/Cdc45 complex in S-phase cells, indicating that phosphorylation of Mcms by Cdc7 promotes the stable assembly of Cdc45 and MCM2-7 (Sheu & Stillman 2006; reviewed in Labib 2010). How exactly this Cdc7-mediated phosphorylation contributes to the activation of replication is unclear but it is likely to induce structural changes, which, together with the reorganisation of the protein components after CDK-mediated phosphorylation, lead to the formation of a stable CMG complex (reviewed in Labib 2010). Also, how exactly GINS and Cdc45 activate MCM2-7 in the CMG is unknown.

The targets of CDK phosphorylation in *S. cerevisiae* replication are Sld2 (Synthetically lethal with Dpb11 2) and Sld3 (Synthetically lethal with Dpb11 3; called Drcl in fission yeast) (Masumoto et al. 2002; Zegerman & Diffley 2007; Tanaka et al. 2007; Noguchi et al. 2002), however the N-terminal tail of Mcm4 seems to be phosphorylated by CDK (Devault et al. 2008), too. The Sld2 and Sld3 proteins are important for the initiation of replication but their molecular functions are still unknown (Kanemaki & Labib 2006; Labib 2010). Both proteins were found to have several CDK phosphorylation sites (Masumoto et al. 2002; Zegerman & Diffley 2007). CDK phosphorylation of Sld2 and Sld3 presumably promotes S-phase in budding yeast by causing the interaction of Sld2 and Sld3 with tandem BRCT repeats in the N-terminal and C-terminal domains of Dpb11 (DNA polymerase B possible subunit 11), thereby bridging Sld2 and Sld3 (Zegerman & Diffley 2007; Tanaka et al. 2007).

Cdc45 is associated with Sld3 in budding yeast during most of the cell cycle, which also mediates the recruitment of Cdc45 to origins of replication already in G1-phase, but a stable complex of MCM and Cdc45 is only found in S-phase and the formation depends on CDK and DDK activity (Kamimura et al. 2001; Sheu & Stillman 2006; Labib 2010). Recently Sld7 was also found to be associated with Sld3 in *S. cerevisiae* and, even though not essential, it is important for efficient and punctual replication (Tanaka et al. 2011). In budding yeast, GINS is found as part of a preloading complex (preLC Figure 1-1) together with Sld2, Dpb11 and DNA polymerase ε (Pol ε), which forms upon CDK-
phosphorylation of Sld2 (Muramatsu et al. 2010). It is suggested that this complex is recruited to origins via the Dpb11 bridge between Sld2 and Sld3, resulting in one large assembly, the pre-initiation complex (preIC Figure 1-1), containing MCM2-7, GINS and Cdc45 (Labib 2010). Loading of additional factors then turns the preIC into a larger assembly, called the replisome progression complex (RPC) (Gambus et al. 2006; Gambus et al. 2009; Tanaka et al. 2009), comprising the active CMG.

The RPC was found to contain only one copy of Mcm4 and thus only one MCM2-7 complex (Gambus et al. 2006). Further, recent results of some elegant single molecule studies suggest that MCM2-7 double hexamers uncouple during replication so that the active CMG contains only one MCM2-7 (Yardimci et al. 2010) and that the CMG encircles ssDNA and has a 3’ to 5’ directivity (Fu et al. 2011), while MCM2-7 is loaded onto double-stranded DNA in the preRC. Thus the activation of MCM2-7 does not only involve the simple loading of GINS and Cdc45 but also requires some structural changes to take place, including separation of the double hexamer into two MCM2-7 heterohexamers and opening of the ring to leave out one DNA strand (reviewed in Boos et al. 2012; Onesti & Macneill 2013).

One of the first proteins to be loaded onto chromatin after S-phase onset is Mcm10 (Wohlschlegel et al. 2002). Mcm10 is necessary for cell division in *S. cerevisiae* and *S. pombe* and it seems to be important for a variety of events during initiation and also elongation of DNA replication (Du et al. 2012).

Mcm10 is only distantly related to MCM2-7 proteins and does not have an AAA^+^ ATPase domain. To date its architecture is not clear and the data from studies with homologues from different organisms is controversial. While the homologues from *S. cerevisiae*, *S. pombe* and *Xenopus* have been reported to form asymmetric small complexes of two to three Mcm10 molecules (reviewed in Du et al. 2012), the human homologue was found to form a ringshaped hexamer (Okorokov et al. 2007). Whether this hexamer has a biological function remains unclear.

Studies in *S. cerevisiae* and *Xenopus* suggest that Mcm10 recruitment to the origins depends on preRC assembly (Ricke & Bielinsky 2004; Wohlschlegel et al. 2002) while *S. pombe* Mcm10 seems to be associated with chromatin during all cell cycle and does not
require loaded Mcm2-7 (Gregan et al. 2003). Nevertheless Mcm10 functions after preRC loading (Wohlschlegel et al. 2002; Gregan et al. 2003; Ricke & Bielinsky 2004) and is important for activation and correct assembly of the CMG in budding yeast and human cells (Gambus et al. 2009; Im et al. 2009). Results from fission yeast and human cells suggest that Mcm10 presence at the origins is important for CDK and DDK mediated phosphorylation events, e.g. of MCM2-7, Sld2 and its putative homologue in humans, RecQ4, to stimulate their activity (Lee et al. 2003; Xu et al. 2009; Du et al. 2012).

Recent studies in budding and fission yeast using an inducible degron approach suggested that Mcm10 is not necessary for CMG loading but required for a novel step in CMG activation and that it plays an essential role in origin unwinding (Watase et al. 2012; Kanke et al. 2012; van Deursen et al. 2012). Mcm10 also seems to be important for the recruitment of Pol α onto chromatin and its coupling to the replicative helicase during replication elongation (Fien et al. 2004; Gambus et al. 2009; Im et al. 2009; Ricke & Bielinsky 2004; Lee et al. 2010; Du et al. 2012) and has been found to be required to maintain Pol α on chromatin independent of Cdc45 (Ricke & Bielinsky 2004).

Unlike GINS, Cdc45 and Mcm10, the Sld2, Sld3 and Dpb11 components of the preIC do not move with replication forks and are not necessary for the completion of replication after it has started (Kanemaki & Labib 2006; Muramatsu et al. 2010).

Fission yeast also has homologues of Dbf4-Cdc7, Sld2, Sld3 and Dpb11, named Dfp1-Hsk1 (Homologue of Cdc seven kinase 1), Drc1 (DNA replication and checkpoint 1), Sld3 and Cut5 (Cell untimely torn 5) (Noguchi et al. 2002; Nakajima & Masukata 2002; Fenech et al. 1991) and the overall mechanism that leads to CMG formation is likely to be similar to that in budding yeast. Hsk1 was found to phosphorylate Mcm2 and Mcm4 and is required for the recruitment of Cdc45 in S-phase (Masai et al. 2006; reviewed in Labib 2010). Drc1 and Sld3 were both identified as targets of CDK and phosphorylation of Drc1 was found to stimulate the interaction with Cut5 (Noguchi et al. 2002). Further Sld3, CDK and DDK activity were found to be required for the recruitment of GINS and Cdc45, while Sld3 binds to origins in a DDK-dependent and CDK-independent manner (Yabuuchi et al. 2006; Kamada 2012; Labib 2010). However it remains unclear how the binding of GINS, Sld3, Cut5 and Cdc45 works in detail.
Even though Sld2, Sld3 and Dpb11 are conserved in yeasts, an Sld2 homologue in higher eukaryotes has not definitely been identified. The sequences of these proteins are not very much conserved, which complicates the identification of homologues, but it is speculated that RecQ4 could be the human orthologue of Sld2 (Sangrithi et al. 2005; Labib 2010). Nevertheless the overall mechanism leading to the activation of DNA replication is believed to be similar in all eukaryotes (reviewed in Labib 2010).

1.1.3 Replication elongation

Once the active CMG is formed, unwinding can be initiated and the many components of the replication machinery can be recruited to the nascent replication forks.

1.1.3.1 RPA

Replication protein A (RPA) is a single-strand DNA binding protein (SSB) and binds to the single-stranded DNA generated by the helicase activity of the CMG complex. SSBs have been identified in all three domains of life and structural similarities indicate that they have a common ancestor (Chédin et al. 1998; Prakash & Borgstahl 2012). Eukaryotic RPA is a heterotrimer consisting of RPA1, RPA2 and RPA3, which all contain oligonucleotide-binding (OB) folds, and each domain is involved in some specific function (like ssDNA binding, recognition and binding of damaged DNA, protein-protein interactions; reviewed in Prakash & Borgstahl 2012). One of RPAs main roles in eukaryotic DNA replication is the binding of ssDNA with high affinity to protect it and prevent its refolding (Oakley & Patrick 2010). However it also coordinates the assembly of other protein factors on replication forks during replication elongation and for DNA repair (Prakash & Borgstahl 2012). This includes the binding of some helicases, RFC (Replication factor C, the clamp loader) and Rad52 (Fanning et al. 2006) and the protein-protein interactions are important for the integrity of the replication fork (Prakash & Borgstahl 2012).

RPA has also been reported to be involved in the recruitment of the Pol α-primase complex to origins after initiation of replication (Tanaka & Nasmyth 1998; Zou & Stillman 2000) and seems to stimulate its activity and increase its processivity.
Introduction

(Dornreiter et al. 1992; Braun et al. 1997). Further RPA might be involved in a switch that occurs to replace Pol α-primase with a processive polymerase (Pol ε or δ) (Waga et al. 1994; Prakash & Borgstahl 2012).

During elongation a stimulating effect of RPA on Pol δ and Pol ε activity and processivity has been observed as well (Waga et al. 1994; Waga & Stillman 1994). This could be the result of RPA’s interaction with the sliding clamp PCNA (Loor et al. 1997; Dianov et al. 1999; Oakley & Patrick 2010; Prakash & Borgstahl 2012).

1.1.3.2 The replicative polymerases

The central components of the replisome are certainly the DNA polymerases, of which three have been found to be essential for eukaryotic DNA replication, namely Pol α-primase, Pol ε and Pol δ (Garg & Burgers 2005). They all consist of one large catalytical subunit and a number of additional smaller subunits, and are all members of the B family of DNA polymerases, based on the structure of their catalytic subunit (Bebenek & Kunkel 2004; Rothwell & Waksman 2005; Garg & Burgers 2005). The B family is one of seven families of DNA polymerases with different roles in DNA repair and replication, and also includes bacteriophage polymerases T4 and RB69 and the archaeal PolB enzymes (Garg & Burgers 2005). The catalytic subunits all fold into five domains that together resemble a right hand-like structure: N-terminal domain, 3’-5’ exonuclease domain, palm, fingers and thumb (Johansson & MacNeill 2010; Rothwell & Waksman 2005; Bebenek & Kunkel 2004). Polymerase active sites are found in the palm, while the fingers function in nucleotide binding and the thumb binds DNA (Johansson & MacNeill 2010). The orientation of the domains contributes to the high fidelity of the polymerases, as it simplifies the detection of nucleotide mis-incorporations and their removal by the exonuclease domain (Johansson & MacNeill 2010).

Replication of the two antiparallel strands of chromosomal DNA happens in a coordinated way. But as the DNA polymerases can only synthesize DNA in 5’-3’ direction, only one strand (the leading strand) can be replicated continuously, while replication of the other (the lagging strand) happens discontinuously by synthesis of Okazaki fragments, which have to be joined.
In eukaryotic cells after origin unwinding, the heterotetrameric Pol α-primase complex initiates replication on both DNA strands. Pol α-primase is thought to be loaded onto DNA as part of the replisome progression complex (RPC), which indicates a direct link between the primase and DNA unwinding mediated by the CMG complex (Gambus et al. 2009).

The Pri1 primase subunit has RNA polymerase activity, binds to single-stranded DNA and de novo synthesizes a short RNA primer. This is then handed over to Pol1 (Copeland & Wang 1993; Eki et al. 1991; reviewed in Pellegrini 2012), the subunit with the DNA polymerase activity, to extend the RNA primer to a RNA-DNA oligonucleotide of about 20 nucleotides (Pellegrini 2012; Johansson & MacNeill 2010; Garg & Burgers 2005). Afterwards a polymerase switch from Pol α-primase to Pol ε or Pol δ occurs, in which RPA, again, seems to be involved (Prakash & Borgstahl 2012; Waga & Stillman 1994).

Polymerases ε and δ are the two processive DNA polymerases responsible for the leading and lagging strand replication in eukaryotes. Their catalytic subunits exhibit polymerase activity and a proofreading 3′-5′ exonuclease activity allowing DNA replication with high fidelity (Morrison et al. 1993). Which polymerase is involved in replication of which DNA strand has been clarified in elegant genetic studies in budding yeast: using mutant Polymerase ε or δ, whose error rate for incorporation of one mismatch is higher than for the complementary mismatch, the template DNA strand was determined. Results suggest that Pol ε is the leading strand polymerase, while Pol δ serves to replicate the lagging strand (Nick McElhinny et al. 2008; Pursell et al. 2008).

Budding yeast Pol δ consists of the catalytic subunit Pol3 and two smaller subunits Pol31/Hys2 and Pol32 (Garg & Burgers 2005; Gerik 1998). Human and S. pombe Pol δ contain an additional subunit, which is thought to stabilise the complex (Garg & Burgers 2005; Podust et al. 2002; Liu et al. 2000; Zuo et al. 1997).

Budding yeast Pol ε is a heterotetrramer of Pol2, Dpb2 and the Dpb3/Dpb4 dimer (Hogg & Johansson 2012; Garg & Burgers 2005; Chilkova et al. 2003) Pol ε is likely to contain at least four subunits in other organisms as well (Garg & Burgers 2005). Pol2 is the catalytical subunit with the polymerase and exonuclease centres in the N-terminal domain and a large C-terminal domain that might have evolved from another polymerase domain but lost its activity (reviewed in Hogg & Johansson 2012).
Interestingly the N-terminal domain of Pol2 is dispensable in both yeasts, even though important for DNA replication, the C-terminal domain however is essential (Dua et al. 1999; Kesti et al. 1999). This indicates that the polymerase function could be replaced by another DNA polymerase and it is speculated that the C-terminus might be important for the loading of Pol ε at origins before primer synthesis (Garg & Burgers 2005; Tsubota et al. 2003). There is evidence, that Pol ε is loaded onto DNA as part of the preLC (in *S. cerevisiae* with Dpb11, Sld2 and GINS (Muramatsu et al. 2010)) its Dpb2 subunit interacting with the GINS subunit Psf2 (Takayama et al. 2003; Hogg & Johansson 2012).

### 1.1.3.3 PCNA

Another essential component of the replisome is PCNA (proliferating cell nuclear antigen), the sliding clamp in eukaryotic DNA replication, which confers processivity on the replicative polymerases. PCNA is a ringshaped homotrimeric complex, which encircles double-stranded DNA (Krishna et al. 1994), but is still able to slide along it freely. By tethering DNA polymerases it is thus able to prevent their dissociation from DNA and increases their processivity (Kelma & O’Donnell 1995; Maga & Huebscher 2003). Due to its ringshape, PCNA has to be opened and closed in order to be loaded onto DNA. This is mediated by the activity of a clamp loader called RFC (replication factor C) in an ATP dependent process in eukaryotes (reviewed in Tsurimoto 1999). RFC is a pentameric complex consisting of Rfc1-5, which are members of the AAA+ ATPase family (reviewed in O’Donnell & Kuriyan 2006).

PCNA loading triggers the displacement of the Pol α-primase complex from sites of DNA replication initiation and recruits Pol ε or Pol δ for processive DNA synthesis (Garg & Burgers 2005; Maga & Huebscher 2003). Additionally to its association with Pol ε and Pol δ and its role as processivity factor, PCNA serves as platform for the recruitment of a variety of DNA replication and repair proteins (Tsurimoto 1999). Many PCNA interacting proteins contain a conserved PCNA interacting peptide (PIP) motif (Dieckman et al. 2012; Maga & Huebscher 2003; Tsurimoto 1999). The consensus of this PIP motif is Qxx(h)xx(a)(a) with h being a moderately hydrophobic residue (leucine,
isoleucine, methionine) and a being an aromatic residue (phenylalanine, tyrosine) (Warbrick 1998; Maga & Huebscher 2003). Due to its interactions with many different proteins, PCNA plays a role in many different cellular processes: DNA replication, DNA repair (early steps of nucleotide excision repair (NER) and mismatch repair (MMR)), cell cycle regulation (through its interaction with cyclin-CDK complexes and CDK inhibitors) and apoptosis, to name a few (reviewed in Maga & Huebscher 2003).

1.1.3.4 Okazaki fragment maturation

While the leading strand is replicated in a continuous way, the lagging strand is built as an array of short Okazaki fragments synthesised by Pol δ alternating with RNA/DNA primers synthesised by Pol α-primase. To produce mature duplex DNA, the 5’ RNA/DNA primer needs to be removed and the Okazaki fragments need to be joined. The key players in this process are Fen1 (Flap endonuclease 1), Dna2 (DNA synthesis defective 2) and DNA ligase 1.

When Pol δ reaches the 5′ end of the previous Okazaki fragment it displaces a short piece of the RNA primer and continues to synthesize another 1-2 nucleotides of DNA, creating a short flap (Burgers 2009; Zheng & Shen 2011). Cleavage of the flap is mediated by Fen1, which can recognize branched structures with 5’ flaps and cut them off at the junction from single-stranded flap to double-stranded DNA (Zheng & Shen 2011). If more RNA primer is present, Pol δ and Fen1 might repeat this cycle until all RNA has been replaced with DNA and a DNA-DNA nick is formed. This nick is then closed by the activity of DNA ligase 1 (reviewed in Howes & Tomkinson 2012).

This model has been supported by several in vivo studies with yeast and mouse (Zheng & Shen 2011; Larsen et al. 2003; Kucherlapati et al. 2002), indicating that it is the major pathway for removal of RNA primers. Long single-stranded RNA/DNA flaps can be generated as a consequence of failure of the short flap pathway. These long flaps bind RPA, which inhibits the endonuclease activity in Fen1 (Chai et al. 2003; Bae et al. 2001). RPA binding was shown to recruit the Dna2 endonuclease in yeast, which can cleave a large part of the DNA/RNA flap (Bae et al. 2001), and the remaining short flap is then removed by Fen1 (reviewed in Kang et al. 2010).
Apart from removal of the RNA primer, the maturation of Okazaki fragments also provides an opportunity for editing of errors made by Pol α. This is likely to happen by interaction of Pol α with the 3’ exonuclease domain of Pol δ, leading to proofreading of Pol α errors already during primer synthesis (reviewed in Burgers 2009; Zheng & Shen 2011).

The recruitment and coordination of the different enzymes required for Okazaki fragment processing seems to be mediated by PCNA: Pol δ, Fen1 and Lig1 all have a PIP motif for interaction. Thus Okazaki fragment maturation is another process PCNA is involved in (Zheng & Shen 2011).

1.2 Mechanisms that control once per cell cycle replication

To maintain genome stability eukaryotic DNA replication has to be restricted to one single round per cell cycle. The separation of eukaryotic DNA replication initiation in two phases, licensing by MCM2-7 loading and helicase activation, allows for specific control mechanisms during different stages of the cell cycle. Cells have developed multiple overlapping mechanisms to control preRC assembly and prevent re-replication. Interestingly the specific regulation mechanisms vary between different organisms, although the components of the preRC are conserved (reviewed in Arias & Walter 2007).

The decrease of CDK activity at the end of M-phase allows the cell to get ready for a new round of replication and assemble preRCs at the origins, licensing them for replication. The increased CDK activity from G1-S transition until late M-phase allows initiation of replication and firing but also converts the origin from a pre- into a postreplicative state. This prevents the de novo assembly of preRCs and inhibits rereplication in the same round of the cell cycle (reviewed in Arias & Walter 2007; Nishitani & Lygerou 2002). In general, the regulation of DNA replication happens in different ways. Posttranslational modifications as phosphorylation or ubiquitination can lead to degradation or inhibition of a preRC component. Nuclear export of a preRC component makes it unavailable for re-licensing at origins, which can also be achieved by transcriptional downregulation.
In yeast, most control mechanisms to ensure once per cell cycle replication depend on CDK activity (reviewed in Li & Jin 2010; Arias & Walter 2007). Cdc6 and its fission yeast homologue Cdc18 are phosphorylated by CDK, which triggers ubiquitin-mediated degradation during S and G2-phase (Elsasser et al. 1999; Drury et al. 1997; Jallepalli et al. 1997). CDK further inhibits transcription of budding yeast Cdc6 (Moll et al. 1991) and can inactivate the protein by direct interaction during S and G2 (Mimura et al. 2004). ORC activity is also negatively regulated by CDK binding in fission yeast (Wuarin et al. 2002) or CDK-mediated phosphorylation in budding yeast (Nguyen et al. 2001). Mcm2-7/Cdt1 is exported from the nucleus in budding yeast upon CDK phosphorylation, preventing re-licensing (Arias & Walter 2007; Tanaka & Diffley 2002; Nguyen et al. 2000; Labib et al. 1999), while fission yeast Mcm2-7 is constitutively nuclear and Cdt1 is degraded by ubiquitin-mediated proteolysis from S-phase onset (Arias & Walter 2007; Ralph et al. 2006). In metazoans, Cdt1 is also degraded in an ubiquitin-mediated pathway, induced by CDK phosphorylation during S-phase (reviewed in Arias & Walter 2007). However there is also a mechanism that depends on chromatin bound PCNA during S-phase, which couples the inhibition of re-licensing directly to DNA replication (Diffley 2011; Li & Jin 2010; Arias & Walter 2007; Arias & Walter 2006; Nishitani et al. 2006). Metazoan Cdt1 activity is additionally regulated by Geminin outside of G1-phase. This factor inhibits preRC assembly by directly binding Cdt1, preventing its interaction with MCM2-7 (Tada et al. 2001; Diffley 2011). Geminin seems to be the main factor for inhibition of re-replication in some cell types (reviewed in (Blow & Dutta 2005; Arias & Walter 2007). ORC and Cdc6 are differently regulated in metazoans, but often CDK-mediated phosphorylation of Cdc6 leads to its nuclear export and phosphorylation of ORC prevents its association with DNA (Li & Jin 2010; Arias & Walter 2007).

1.3 The MCM2-7 complex

The MCM complex is the motor of the replicative helicase complex in eukaryotes and absolutely essential for DNA replication (Moyer et al. 2006; Labib et al. 2001). It is loaded onto origins of DNA replication and moves ahead of the replication fork unwinding the double-stranded DNA when elongation takes place.
1.3.1 Discovery, evolution and classification

The Mcm proteins Mcm2 Mcm3 and Mcm5 were initially discovered in a screen to identify genes necessary for the maintenance of a plasmid with a centromere and an origin of replication (minichromosome) in *S. cerevisiae* (Maine & Sinha 1984) and this is also where their name comes from: Mcm = minichromosome maintenance. Mcm4 and Mcm7 were identified as cell-division-cycle (cdc) mutants (*CDC54* and *CDC47* genes; Hennessy et al. 1991; Moir & Botstein 1982). Mcm6 was first discovered in a screen for temperature-sensitive mutants with high rates of minichromosome loss in *S. pombe* (Takahashi et al. 1994). To date eight Mcm proteins have been identified in eukaryotes, however only six homologues, Mcm2, 3, 4, 5, 6 and 7 are conserved and essential in all eukaryotes and form the MCM2-7 helicase (Aves et al. 2012). Archaea also possess at least one homolog, but there are some exceptions with two or more (e.g. *M. jannaschii*, *M. kandleri* and other Methanococcales, *Methanosarcina acetivorans, Thermococcus kodakaraensis*) (Pan et al. 2011; Walters & Chong 2010; Bochman & Schwacha 2009; Kelman & Kelman 2003). Archaeal and eukaryotic Mcm proteins are thought to have evolved from a common ancestor (Aves et al. 2012) and different Mcm proteins might have appeared as a consequence of multiple gene duplication events in the last common eukaryotic ancestor (LCEA) or ancestral archaeal species (Aves et al. 2012; Walters & Chong 2010; Liu et al. 2009).

Archaeal and eukaryotic Mcm proteins are all members of the AAA\(^+\) (ATPase associated with a variety of cellular activities) ATPase superfamily (Erzberger & Berger 2006; Neuwald et al. 1999; Koonin 1993), which includes many RNA and DNA helicases, proteases and DNA replication factors such as ORC, Cdc6 and RFC (Bochman & Schwacha 2009). AAA\(^+\) ATPases are often found to assemble into ringshaped oligomers, which are stabilised by nucleotide binding and can couple ATP hydrolysis to the remodelling of their substrate (Duderstadt & Berger 2008). The eukaryotic MCM complex consists of Mcm2 through 7, forming a heterohexameric toroid with a large central channel, wide enough to fit dsDNA or ssDNA (Costa et al. 2011; Bochman & Schwacha 2007). Similar observations were made for archaeal MCMs however they usually assemble into homohexameric rings (Bochman & Schwacha 2009; Pape et al.
MCM complexes use ATP hydrolysis to unwind dsDNA, but how exactly ATPase activity is converted into unwinding is still unclear. The hexameric complex exhibits ATPase activity but none of the single Mcm proteins hydrolyses ATP on its own (Davey et al. 2003; Lee & Hurwitz 2000).

1.3.2 Subunit composition

ATPase active sites in MCM complexes are formed at the interfaces between two subunits. One subunit contributes the cis-acting motifs, Walker A, Walker B and Sensor I. The other subunit holds the trans-acting arginine finger (Vijayraghavan & Schwacha 2012; Bochman & Schwacha 2009). Further there are a variety of β-hairpin fingers protruding into the central channel and at least some of them (PS1 and H2I) are likely to be important for the coupling of ATP hydrolysis and DNA unwinding (Vijayraghavan & Schwacha 2012; Bochman & Schwacha 2009; Brewster et al. 2008; Jenkinson & Chong 2006).

As MCM is ring-shaped, each subunit contributes to two active sites. The subunit architecture of eukaryotic MCM2-7 was first uncovered by reconstitution of Mcm pairs using purified *S. cerevisiae* proteins (Davey et al. 2003) and led to the model pictured in Figure 1-3. Interestingly a dimer of Mcm2 and Mcm5 has never been observed and results indicate that the interface between Mcm2 and Mcm5 is weak (Vijayraghavan & Schwacha 2012; Bochman & Schwacha 2009; Bochman et al. 2008; Davey et al. 2003). It is suggested that there is a discontinuity between the Mcm2 and Mcm5 subunits, which forms an ATP dependent gate in the toroid (Bochman & Schwacha 2009). Only recently EM reconstruction studies with *Drosophila* CMG components (Costa et al. 2011) and also the MCM2-7 complex of *Enzephalitozoon cuniculi* (EcuMCM; Lyubimov et al. 2012) provided visual evidence for the existence of this gate. It is suggested that the gap could serve as an entry or exit site for DNA loading or strand exclusion and that it is regulated by ATP binding and the association of GINS and Cdc45 with MCM2-7 (Costa et al. 2011).

The hexameric MCM seems to be the motor of the DNA unwinding machinery in replication, but it is loaded as a double hexamer. Archaeal and eukaryotic Mcm proteins can not only assemble into hexamers, but a variety of different MCM
subcomplexes were observed *in vitro*, including heptamers, dimers and helical arrangements (Slaymaker & Chen 2012). This variability might reflect the experimental conditions used, but it is also a hallmark of the great flexibility of Mcm proteins, which is necessary for their loading, activation and working mechanism.

One such MCM subcomplex is a trimer of MCM4/7/6, which forms a relatively stable core complex. It can also assemble into a hexameric ring (dimer of MCM4/7/6 trimers) and the fact that this hexamer exhibits helicase activity *in vitro*, in contrast to the MCM2-7 complex, has made it so interesting for research (Kanter et al. 2008; Davey et al. 2003; Lee & Hurwitz 2000; You et al. 1999). Addition of Mcm2 or MCM5/3 to this complex inhibited its helicase activity (Sato et al. 2000; Ishimi 1998) and led to the idea of two distinct groups of Mcm proteins: Mcms which exhibit helicase activity and Mcms which regulate helicase activity (Bochman & Schwacha 2009; Schwacha & Bell 2001).

![Figure 1-3 Simplified model of the hexameric MCM2-7 complex based on pairwise interactions and ATPase analyses (adapted from Davey et al. 2003). The model shows the contribution of each subunit to two ATPase sites. P-loop represents the cis acting Walker A and Walker B motifs, while SRF stands for the trans acting arginine finger.](image)

Even though in other hexameric helicases like papillomavirus E1 (Enemark & Joshua-Tor 2006) or SV40 LTTag (Gai et al. 2004) all active sites contribute equally to activity, the ATPase sites of the MCM2-7 heterohexamer are functionally different (Vijayraghavan & Schwacha 2012; Bochman & Schwacha 2009; Bochman et al. 2008). *In vitro* studies with *S. cerevisiae* proteins with mutations in the active sites indicate that the ATPase activities of the Mcm7/4, Mcm4/6 and Mcm3/7 active sites are essential for DNA unwinding and Mcm7/4 and Mcm3/7 also play an important role in ssDNA binding,
while the other sites rather have regulatory roles (Bochman & Schwacha 2010; Bochman et al. 2008; Kanter et al. 2008; Davey et al. 2003). In vivo, most ATPase sites are required for proper function and most mutations disturbing the active sites are lethal in budding and fission yeast, indicating that these sites still perform essential functions (Bochman & Schwacha 2008; Bochman et al. 2008; Gomez et al. 2002). Thus the six ATPase active sites might contribute differently to the activity of MCM2-7, however they function in an interdependent manner to coordinate or regulate ATP hydrolysis and DNA unwinding (Bochman & Schwacha 2009). Similar results were also reported for ssoMCM, indicating that ATP hydrolysis at only a subset of active sites is sufficient for DNA unwinding and that the different subunits act cooperatively for MCM helicase activity (Moreau et al. 2007).

1.3.3 Structure

Archaea and eukaryotes share many aspects of replication such as participating components and their regulation and sequence similarities suggest that all Mcm proteins have a similar domain structure and composition (Slaymaker & Chen 2012; Bae et al. 2009; Liu et al. 2008; McGeoch et al. 2005; Fletcher et al. 2003; Tye 1999). As the heterohexameric eukaryotic MCM2-7 complex has proven to be experimentally intractable a lot of the available information about MCM complexes has been inferred from studies with the less complex homohexameric archaeal MCMs. The best studied Mcm proteins so far come from Sulfolobus solfataricus (ssoMcm) and Methanothermobacter thermautotrophicus (mthMcm). Work with these proteins gave insight into mechanism and especially structure of the MCM helicases (Slaymaker & Chen 2012).

To date three archaeal MCM crystal structures have been solved: the N-terminal domain of M. thermautotrophicus Mcm (N-mthMcm), which crystallised as a double hexamer (Fletcher et al. 2003), the N-terminal domain of S. solfataricus Mcm (N-ssoMcm) (Liu et al. 2008) showed a single hexameric architecture, while the near full-length Mcm from S. solfataricus (ssoMcm) (Figure 1-4, Brewster et al., 2008), and an inactive full-length Mcm from M. kandleri (mkaMcm2) (Bae et al. 2009) crystallised as
monomers, but models based on the NTD hexamers were generated to predict the hexamer conformation of the full-length proteins (Brewster et al. 2008). Furthermore there are low-resolution EM reconstructions available, mostly of mthMcm (Costa et al. 2008; Costa et al. 2006; Gómez-Llorente et al. 2005; Pape et al. 2003) but also from fission yeast Mcm2-7 (Adachi et al. 1997), the *Drosophila* CMG complex (Costa et al. 2011) and *Encephalitozoon cuniculi* MCM2-7 (Lyubimov et al. 2012).

### Figure 1-4 Crystal structure of near full-length ssoMcm (adapted from Brewster et al. 2010; Brewster et al. 2008)

**A)** 3D model of ssoMcm monomer created with Pymol based on PDB 3F9V with individual domains highlighted in different colours: A domain (green), B domain (orange), C domain (yellow), N-C linker (red) α/β domain (cyan), α/β-α linker (blue), α-domain (purple). Hairpins and loops are highlighted in magenta. The arginine finger and external hairpin are located at the rear side of the model. The Zn ion coordinated by domain B is pictured as grey sphere (Brewster et al. 2008). **B)** Top view and side view of ssoMCM hexamer showing the β-hairpins NT-hp (red), H2I (yellow), PS1-hp (green), Ext-hp (blue) (Brewster et al. 2010).

Individual Mcm proteins can be separated into two large domains, an N-terminal domain (NTD) and a C-terminal domain (CTD). The NTD can be further divided into three subdomains, A, B and C, while the CTD contains the AAA$^+$ ATPase structural unit and a winged helix domain at its very C-terminus (see Figure 1-4, reviewed in Slaymaker & Chen, 2012). The model of the hexameric ssoMCM complex is dumbbell-shaped, with a narrow waist between the NTDs and CTDs. It has a wide positively
charged central channel and six side channels, which radiate away from the central channel (Brewster et al. 2008). This appearance has been confirmed by EM studies (Costa et al. 2008; Costa et al. 2006; Gómez-Llorente et al. 2005; Pape et al. 2003).

1.3.3.1 N-terminal domain

The NTD sequence is poorly conserved between the different Mcms, not only from different species but also within one organism. However there is some conservation of buried hydrophobic residues and charged residues lining the central channel of a hexamer, indicating a consistency of the overall fold and function of the NTD (Slaymaker & Chen 2012; Fletcher et al. 2003). This is supported by the observation that the N-mthMcm and N-ssoMcm structures are highly similar (Liu et al. 2008; Fletcher et al. 2003). The N-terminal domain is thought to not have catalytic activity but to exhibit a regulatory function. Deletion of the whole domain in archaeal MCM does not abrogate the unwinding activity of the helicase (Barry et al. 2009; Barry et al. 2007). It is speculated that the NTD could clamp the actual motor domain to DNA and confer processivity and substrate specificity to the helicase (Slaymaker & Chen 2012; Bochman & Schwacha 2009; Barry et al. 2007).

In the available structures, the α-helical subdomain A is attached to domain C and is located on the outer surface of the MCM hexamer (Figure 1-4; Brewster et al. 2008; Fletcher et al. 2003). It is assumed to be involved in the regulation of MCM via kinases: Mutation of a conserved proline residue in S. cerevisiae Mcm5 domain A allows bypass of Cdc7 phosphorylation for the activation of the helicase (bob1 mutant; Hardy et al. 1997; Sclafani et al. 2002). Structural analysis of mthMcm revealed that the conserved proline mediates the contact of domain A to domain C and mutation increases the flexibility of domain A and induces a domain shift or “domain-push”, which might be important for helicase activation (Slaymaker & Chen 2012; Sakakibara et al. 2009; Fletcher & Chen 2006; Fletcher et al. 2003). Interestingly this domain seems to be dispensable in some archaea, as deletion of mthMcm domain A only has minor effects (Kasiviswanathan et al. 2004) and some species (e.g. Methanococcus jannaschii) do not even possess such a domain (Sakakibara et al. 2009). Furthermore there is evidence that a portion of domain A contributes to DNA binding (Costa et al. 2008).
Domain B consists of several β-sheets and coordinates a Zn ion at its tip, using a motif of four cysteines or three cysteines and a histidine, which can also be found in eukaryotic Mcm proteins (Liu et al. 2008; Fletcher et al. 2003). B domains of Mcm monomers seem important for oligomerisation into hexamers and also double hexamers (Slaymaker & Chen 2012; Jenkinson et al. 2009; Fletcher et al. 2003; Fletcher et al. 2005). However there is also evidence that the B domain might contribute to DNA binding of the MCMs (Sakakibara et al. 2009; Jenkinson et al. 2009).

Subdomain C is the core of the Mcm-NTD and consists of five antiparallel β-sheets forming a β-barrel like structure that resembles an oligonucleotide-binding (OB) fold (Brewster et al. 2008; Liu et al. 2008; Fletcher et al. 2003). The C domain is thought to be important for hexamerisation (Kasiviswanathan et al. 2004). It contains a positively charged β-hairpin (the N-terminal hairpin or NT-hp Figure 1-5 A) that protrudes into the central channel (Fletcher et al. 2003). The crystal structures of N-mthMCM and N-ssoMCM hexamers show that the six NT-hairpins narrow the central channel and might contribute to DNA binding in the NTD (Slaymaker & Chen 2012; Liu et al. 2008; Brewster et al. 2008; Fletcher et al. 2003, also see Figure 1-4 B, based on ssoMCM).

The so-called allosteric communication loop (ACL) is also a feature of the C domain (Barry et al. 2009; Sakakibara et al. 2008). Structural analysis suggests that the ACL in one Mcm-NTD might interact with another hairpin in the adjacent Mcm-CTD, which is called PS1-hairpin (Bochman & Schwacha 2009; Brewster et al. 2008; Barry et al. 2009). The ACL seems to be important for the regulation of communication between the NTD and the catalytical AAA⁺ domain and regulate the positioning of the NT-hp (Barry et al. 2009; Sakakibara et al. 2008). Conservation of the loop residues suggests a similar role in eukaryotes and archaea (Sakakibara et al. 2008). Together the NT-hp and ACL might help adjust the C domain by interacting with the CTD (Slaymaker & Chen 2012).

1.3.3.2 C-terminal domain

The C-terminal domain is well conserved across species and between the different Mcm families. It contains the ATP binding pocket with the conserved AAA⁺ motifs and represents the motor of the MCM helicase. ATPase sites are formed at the interfaces
between two neighbouring Mcm proteins but there is no crystal structure for the C-terminal tier of an MCM hexamer or an ATP-bound CTD. Thus hypotheses about motifs involved in ATPase activity are based on structures of related AAA\(^+\) ATPases (Slaymaker & Chen 2012; Erzberger & Berger 2006) or based on the structure of the (near) full-length Mcms fitted into the hexameric structures of the N-MCM (reviewed in Bochman & Schwacha 2009; Brewster et al. 2008)

The C-terminal domain of ssoMcm contains a canonical α-helical/β-strand region (α/β domain) and an α-helical domain (α-domain) connected by an α/β-α linker. Furthermore there is a winged helix domain at the very C-terminus of the protein, which could not be built in the model due to low resolution (Brewster et al. 2008).

The Walker A motif of the P-loop is important for ATP binding, while Walker B coordinates the H\(_2\)O for the nucleophilic attack on the ATP γ-phosphate for ATP hydrolysis. This coordination is assisted by the Sensor I motif. Walker A, B and Sensor I are located within the α/β domain and contribute in \textit{cis} to ATPase function of MCM, while the arginine finger and Sensor II function in \textit{trans}. The arginine finger stabilises the ATP γ-phosphate and might act similar to the arginine finger in SV40 L-Tag, causing an ATP-hydrolysis dependent iris-like contraction of the hexameric ring, which could pump DNA through the channel (Slaymaker & Chen 2012; Gai et al. 2004).

The C-terminal domain also contains three hairpins, which are important for MCM function. The presensor 1 hairpins (PS1-hp) lie a bit recessed from the central channel in the side channels (Figure 1-5 A) and seem to contribute to MCM helicase activity, because mutations eliminate DNA unwinding (McGeoch et al. 2005). This hairpin seems to mediate communication of the CTD with the NTD via the N-terminal ACL loop, as mentioned above (Slaymaker & Chen 2012; Bochman & Schwacha 2009). The external hairpin (Ext-hp) is located on the outer surface of the hexamer just below the side channel exit and mutational analysis of ssoMCM suggests its involvement in ATPase activity and DNA binding (reviewed in Slaymaker & Chen 2012; Brewster et al. 2008; Brewster et al. 2010). The third hairpin (helix-2-insert or H2I) has a piece of an α-helix at its tip and protrudes into the central channel where it most likely contacts DNA or acts as a steric block for it (Bae et al. 2009; Brewster et al. 2008). Mutations of H2I in mthMCM were found to abolish helicase activity but not DNA binding. It is suggested that H2I has an important role in coupling ATPase activity to DNA unwinding (Jenkinson
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& Chong 2006). Poor sequence conservation of this loop has led to the idea that its interactions do not depend on the primary sequence but are steric (Jenkinson & Chong 2006). Together with PS1, H2I could serve as wedge to unwind dsDNA (Slaymaker & Chen 2012).

1.3.4 Modes of DNA unwinding

MCM2-7 was long thought to not have any helicase activity in vitro, before Bochman and Schwacha reported that special conditions are necessary to stimulate this activity (Bochman & Schwacha 2008). The discovery of the CMG complex as actual replicative helicase in eukaryotes and initial EM studies, which give insight into the CMG structure, suggest that the helicase activity of MCM2-7 is activated by ATP binding and association with GINS and Cdc45 in vivo, leading to (unknown) conformational changes within the complex (reviewed in Onesti & Macneill 2013; Costa et al. 2011). This also explains why the hexamer does not exhibit its function when isolated.

Despite extensive study over the past years, it is still not clear how exactly eukaryotic or archaeal MCM unwinds dsDNA and several models for unwinding, derived from mechanisms of other helicases, have been developed.

The steric exclusion model postulates that the MCM helicase binds and translocates along ssDNA. One strand passes through the central channel of the helicase while the other strand is sterically excluded, which leads to unwinding of the duplex (Brewster et al. 2008; Takahashi et al. 2005; Kaplan et al. 2003; Lee & Hurwitz 2001). This was suggested to be the mechanism of DNA unwinding by MCM4/6/7 hexamer on a forked substrate (Kaplan et al. 2003) and also papillomavirus E1 helicase (Enemark & Joshua-Tor 2006). Results from FRET studies further suggest that the excluded strand dynamically interacts with the surface of the helicase via “opposite strand interaction” (Rothenberg et al. 2007).

The ploughshare model suggests that the helicase encircles dsDNA. As it translocates away from the origin it drags along a “wedge” (like a horse pulls a plough), which physically separates the two strands. The wedge could either be part of the MCM complex itself or an associated protein like Cdc45 (Takahashi et al. 2005).
Other models assume that MCM encircles dsDNA and can actively rotate it. One such model is the rotary pump model, which suggests that two populations of MCM travel along DNA and become immobilised at some distance from the origin. They can pump or rotate DNA in opposite directions, resulting in the unwinding of the duplex between the two helicases. This theory would explain the fact that there are many MCM complexes loaded per origin (Bochman & Schwacha 2009; Laskey & Madine 2003).

Another model involving dsDNA binding double hexamers is based on the mechanism of DNA unwinding by the SV40 large T-antigen (L-Tag). The head-to-head coupled hexamers pump dsDNA through their central channels and ssDNA is extruded through the side channels, creating a structure described as “rabbit ears” (Bochman & Schwacha 2009; Takahashi et al. 2005; Wessel et al. 1992).

Figure 1-5 Models for dsDNA unwinding by ssoMCM (adapted from Brewster et al. 2008). A) Schematic representation of a cross-section of an ssoMCM hexamer. Black rectangles represent β-hairpins (external hairpin, PS1-hairpin, helix-2-insert and N-terminal hairpin) protruding into the central channel or side channels. Black lines indicate possible pathways for DNA. B) Steric exclusion model C) Side channel extrusion model, with one strand being extruded through the side channel.

Relatively recent results helped to narrow down the number of potential unwinding modes for MCM. Eukaryotic MCM2-7 seems to be loaded as a double hexamer onto dsDNA in the preRC, however the active MCM2-7 seems to travel along ssDNA as a single hexamer in the CMG complex in vitro (Fu et al. 2011; Yardimci et al. 2010; Remus et al. 2009) and the RPC seems to contain only one MCM complex (Gambus et al. 2006). This argues against models describing a double hexameric, dsDNA-binding
MCM2-7 as active helicase. Single molecule studies with an in vitro Xenopus DNA replication system did not see disruption of DNA replication when using a DNA substrate tethered at both ends, dismissing the dsDNA pump model (Vijayraghavan & Schwacha 2012; Yardimci et al. 2010). Further it was found that MCM complexes only unwind forked DNA substrates or substrates with a 3’ overhang (Bochman & Schwacha 2008; Lee & Hurwitz 2001; You et al. 1999). This indicates that dsDNA must be melted prior to unwinding and that some rearrangements need to take place in MCM, including splitting of the double hexamer and exclusion of one DNA strand (Boos et al. 2012). The availability of the crystal structure of a near full-length homologue of ssoMcm also gave some insights into possible unwinding modes (Brewster et al. 2008). It is suggested that unwinding could be mediated by dsDNA binding of the MCM hexamer ahead of the replication fork and threading one strand through the side channel. This would allow interaction of all four β-hairpins with ssDNA (Brewster et al. 2008; Figure 1-5 C side channel extrusion model).

An alternative would be the steric exclusion of one strand, while the other strand passes through the central channel. The external hairpin which is sticking out from the surface of the hexamer might help with the coordination of the extruded strand (Brewster et al. 2008, Figure 1-5 B), as proposed in the opposite strand interaction model. The steric exclusion and wrapping model (Graham et al. 2011) provides further refinement: FRET results indicate that the extruded strand wraps around defined paths on the surface of the helicase, which could contribute to MCM and ssDNA stabilisation.

1.3.5 Other MCMs and additional roles of Mcm proteins outside DNA replication

1.3.5.1 MCM in cancer

Misregulation of MCM levels was associated with genome instability and DNA damage in yeast (Forsburg 2004). Genome instability is a characteristic of cancer cells, but whether genome instability directly causes cancer has been subject of debate (reviewed in Sieber et al. 2003). However, mutations in mouse Mcm proteins were identified that cause impaired DNA replication, leading to genome instability and to cancer development. Female homozygous mice carrying the $MCM^\text{Chaos3}$ (chromosome
aberrations occurring spontaneously) allele are highly susceptible to develop mammary carcinomas (Shima, Buske, et al. 2007; Shima, Alcaraz, et al. 2007). A similar mutation in *S. cerevisiae* Mcm4 caused DNA replication defects and mouse cells homozygous for the mutation were prone to chromosome breaks (Shima, Buske, et al. 2007). The Chaos3 mutation leads to a single amino acid change of a conserved residue close to the Zn-finger domain in mouse Mcm4. It is assumed that the mutation interferes with Mcm-Mcm interaction but only causes mild defects in MCM function. However total levels of Mcm4 and also other Mcm proteins were reduced, presumably leading to a reduced amount of backup MCMs, which are important under replication stress. This causes genomic instability and might result in cancer development (Shima, Alcaraz, et al. 2007). Furthermore, an Mcm2 mutant was reported to cause lymphomas in mice. Homozygous mice exhibited reduced levels of Mcm2 and also Mcm7 and showed increased chromosome instability (Pruitt et al. 2007). This implies that minor misregulations in the levels of Mcm proteins might cause genome instability and cancer susceptibility (Chuang et al. 2010; Kunnev et al. 2010; Pruitt et al. 2007; Shima, Alcaraz, et al. 2007; Shima, Buske, et al. 2007).

Another Mcm4 mutation, which affects a conserved residue of the Walker B motif was related to leukaemia. This dominant *MCM4* allele presumably results in a stable but functionally inactive MCM helicase in mice, which causes chromosomal abnormalities and sensitivity to replication stress. Interestingly the total levels of MCM were not compromised, so the instability is not caused by a lack of backup MCMs. The corresponding mutation in *S. cerevisiae* is lethal (Bagley et al. 2012).

**1.3.5.2 MCM functions other than DNA unwinding**

Mcm proteins act as replicative helicase, however there is evidence that these proteins might also be involved in processes other than DNA replication. It is speculated that non-conserved divergent parts of Mcm proteins might have evolved to serve as docking stations for interaction with other proteins, involving MCM in various other functions (Bochman & Schwacha 2009).

Several studies suggest an interaction of replication and transcription machineries and a role of Mcm proteins in transcription (reviewed in Forsburg 2004). Mcm2 was shown
to interact with RNA Pol II in *Xenopus* (Yankulov et al. 1999). Mcm5 specifically interacts with the Stat1 (Signal transducers and activators of transcription) transcription factor (Zhang et al. 1998) and seems to be important for the transcription activation of Stat1 target genes (Snyder et al. 2005). Mcm7 is suggested to regulate its own expression in combination with the transcription factor Mcm1 in budding yeast (Fitch et al. 2003) and it was also reported to interact with the tumor suppressor Rb, which regulates E2F transcription factors (Sterner et al. 1998). Thus Mcm proteins seem to have a function in transcription.

Recent results suggest that the histone chaperone FACT (Facilitates chromatin transcription) binds to histones together with Mcm2 in budding yeast and this is important for the processing of parental histones during DNA replication (Foltman et al. 2013).

MCM subunits also seem to have a role in the regulation of centrosome duplication (Knuckleby & Lee 2010). Mcm proteins were found to localise at centrosomes (Stuermer et al. 2007) and downregulation of *Xenopus* Mcm5 leads to centrosome duplication indicating that Mcm proteins might help with regulation of this process (Ferguson & Maller 2008).

Many different factors associate with the CMG in a large assembly called the replisome progression complex (RPC). The RPC might consequently connect MCM2-7 to other proteins (Gambus et al. 2006; Gambus et al. 2009), among them Mrc1 (Mediator of the replication checkpoint 1), Tof1 (Topoisomerase I-interacting Factor 1) and Csm3 (Chromosome segregation in meiosis 3). When replication forks reach a DNA lesion, the forks are paused and stabilised by Mrc1/Tof1/Csm3 checkpoint proteins while the defect is being repaired (Bochman & Schwacha 2009). Interaction of Mrc1 with Mcm2-7 and also Pol ε in yeast might provide a way of communication between unwinding and replication in case of checkpoint activation. Interaction of Mrc1 and Mcm6 in *S. cerevisiae* was reported to be important for the activation of the DNA replication checkpoint (Komata et al. 2009). Other Mcms are speculated to interact with Tof1 or Csm3, which also seem to have a role in sister chromatid cohesion establishment (Tanaka et al. 2009; Mayer et al. 2004). It is speculated that the interaction of MCM2-7 with Tof1-Csm3 in *Xenopus* controls the helicase when RPCs pass cohesion rings (Tanaka et al. 2009). Mcm7 and Mcm6 were found to interact with the cohesion factor
Smc1 (Stability of minichromosomes), indicating a link between sister chromatid cohesion and the DNA replication machinery (Ryu et al. 2006). All this suggests that Mcm proteins have acquired additional roles beyond their function in DNA replication.

1.3.5.3 Mcm8 and Mcm9

Apart from Mcm2-Mcm7, Mcm8 and Mcm9 have been identified as members of the MCM family of AAA\(^+\) ATPases. They are only present in higher eukaryotes and were lost in fungi during evolution (Aves et al. 2012; Liu et al. 2009; Lutzmann et al. 2005; Kaplan et al. 2003). They usually co-occur, only Drosophila has a homologue of Mcm8 without Mcm9 (Aves et al. 2012). Initially human Mcm8 was reported to be involved in the assembly of the preRC (Volkening & Hoffmann 2005). Mcm8 and 9 from Xenopus were suggested to play a role in elongation of replication and loading of MCM2-7 onto origins (Lutzmann & Méchali 2008; Tada et al. 2001; Maiorano et al. 2005), indicating a role of Mcm8 and Mcm9 in replication. However Drosophila Mcm8 was associated with meiotic recombination repair (Blanton et al. 2005), and mouse Mcm9 was shown to be dispensable for replication but is needed for germline cell maintenance (Hartford et al. 2011). The two proteins were shown to form dimeric complexes in chicken, mouse and human cells. These complexes do not seem to be essential for cell viability and DNA replication but were shown to be involved in homologous recombination repair in chicken (Nishimura et al. 2012) and mice (Lutzmann et al. 2012). Recently, the Xenopus homologues were also reported to form complexes, which were not needed for preRC formation or DNA elongation, contrary to previous findings, but necessary in response to DNA damage (Gambus & Blow 2013). Thus, Mcm8 and 9 might not have a direct role in DNA replication but function in DNA recombination and repair in higher eukaryotes.

1.4 MCM-BP

1.4.1 Identification and subunit composition

The previously unstudied MCM-binding protein (MCM-BP) was first identified in a tandem affinity purification (TAP) experiment designed to study the composition of
MCM complexes in human cells (Sakwe et al. 2007). MCM-BP was found to specifically and stoichiometrically interact in a complex with MCM3-7. MCM-BP is conserved in eukaryotes but some species like *C. elegans* and *S. cerevisiae* do not have a homologue of the protein.

Mcm proteins and MCM-BP seem to interact continuously during all cell cycle, which was shown in synchronized *S. pombe* (Santosa et al. 2013) and human cells (Nguyen et al. 2012). Even though they interact with the same Mcm subunits, interactions between MCM-BP and Mcm2 were at first not found in most of the systems studied (Nishiyama et al. 2011; Ding & Forsburg 2011; Sakwe et al. 2007; Li et al. 2011). Consequently it was initially suggested that MCM-BP replaces Mcm2 in some alternative, presumably hexameric MCM complex (Sakwe et al. 2007), but direct evidence for a high molecular weight complex containing MCM-BP and MCM3-7 was not available at that time. Furthermore, differences in the interaction of MCM-BP with the Mcms in different species have been reported.

The *Xenopus* MCM-BP was described to associate with Mcm3 to Mcm7, but the main interaction partner seems to be Mcm7, with the interaction taking place via the Mcm7 AAA+ box but independently of its ATPase activity (Nishiyama et al. 2011). Etg1 (E2F target gene 1), the *Arabidopsis* homologue of MCM-BP, was found to interact with all Mcm proteins including Mcm2 in a TAP purification screen. It seems to interact most strongly with Mcm5 (Takahashi et al. 2008). Human MCM-BP can interact with the MCM4/6/7 core in a high molecular weight complex (Sakwe et al. 2007). Besides, *in vitro* pairwise interaction with all members of the MCM2-7 complex has been reported, with Mcm4 and Mcm7 association being the most stable (Nguyen et al. 2012). Recent results suggest that the *T. brucei* MCM-BP forms an unusual complex with MCM4-7 and Mcm8 (Kim et al. 2013). Interactions of MCM-BP with a different set of Mcm proteins in different species could be due to experimental conditions but it is also possible that the interaction of MCM-BP with Mcm proteins take place via different sites in different organisms. Even though these results all indicate a specific interaction of MCM-BP with Mcm proteins, the exact composition of the *in vivo* complex(es) is not clear yet.
1.4.2 Localisation

MCM-BP is present in the cells throughout the cell cycle at about the same level in fission yeast (Santosa et al. 2013; Ding & Forsburg 2011) and is a mainly nuclear protein, found in the soluble fraction as well as bound to chromatin in human cells, *Xenopus* and fission yeast (Ding & Forsburg 2011; Nishiyama et al. 2011; Sakwe et al. 2007). In human cells the highest chromatin association was observed in G1/S. This coincides with the chromatin binding of Mcm proteins and ChIP experiments also suggest an origin association of MCM-BP during this period (Sakwe et al. 2007). The *Arabidopsis* Etg1 and *T. brucei* MCM-BP are found in the nucleus as well and Etg1 was reported to contain a putative nuclear localisation sequence (Kim et al. 2013; Takahashi et al. 2008). The *Xenopus* protein is only imported into the nucleus in mid S-phase and only sparsely associated with chromatin. As it is cytoplasmic during most of the cell cycle (Nishiyama et al. 2011), this might point to a slightly different function or mechanism of *Xenopus* MCM-BP.

1.4.3 Other interaction partners

Human MCM-BP was identified to interact with the ssDNA binding proteins RPA1 and RPA2 when all three RPA subunits (RPA1 RPA2 RPA3) are present (Nakaya et al. 2010). RPA binds single-stranded DNA generated by the MCM helicase during replication, but is also involved in induction of checkpoint signalling when DNA replication elongation is disturbed (Fanning et al. 2006). Interaction of MCM-BP and RPA could mediate some kind of regulative function of MCM-BP during replication (Nakaya et al. 2010). Yeast two-hybrid assays, coexpression studies and immunoprecipitation also showed an interaction of human MCM-BP with the regulatory Dbf4 subunit of Cdc7 (Nguyen et al. 2012). Interestingly MCM-BP does not appear to be a substrate for Dbf4/Cdc7 phosphorylation *in vitro* unlike the Mcm proteins (Nguyen et al. 2012). But it was found that increasing amounts of MCM-BP interfered with DDK phosphorylation of MCM4/6/7 and MCM3-7 but not of Mcm2 (Nguyen et al. 2012). This indicates a role of MCM-BP in an early step of replication, during which regulation of DDK-mediated phosphorylation is important (e.g. origin activation at G1/S). MCM-BP was reported to bind oris at this time in human cells (Sakwe et al. 2007); however DDK-mediated
phosphorylation is also important during elongation stage, Chk1 signalling and fork restart.

1.4.4 MCM-BP function

1.4.4.1 Xenopus

In *Xenopus* egg extracts the effects of immuno-depletion of MCM-BP were studied. The absence of MCM-BP was reported to not have a dramatic effect on DNA replication initiation, as neither the assembly of the pre-replication complex (preRC) nor the loading of Cdc45 or PCNA were affected (Nishiyama et al. 2011). However dissociation of MCM2-7 from chromatin in S-phase was delayed in MCM-BP depleted cells, but could be rescued by addition of recombinant MCM-BP. No checkpoint activation (as judged by Chk1-phosphorylation) was observed in the absence of *Xenopus* MCM-BP and there was also no indication of defective DNA replication or accumulation of ssDNA (Nishiyama et al. 2011). It has to be considered that immuno-depletion often leads to incomplete removal of the target protein and that remaining levels of MCM-BP might be sufficient for a cell cycle without checkpoint activation.

Excess amounts of MCM-BP in turn were found to cause unloading of MCM2-7 from chromatin and disassembly of the complex into its subunits or low molecular weight complexes (Nishiyama et al. 2011). Thus it is suggested that *Xenopus* MCM-BP regulates replication-dependent MCM2-7 unloading from chromatin after ongoing DNA synthesis and also works to clear chromatin from all silent MCM2-7 before mitosis. This unloading might be induced by direct interaction between MCM-BP and Mcm7. As binding of MCM-BP to Mcm7 is suggested to immediately promote dissociation of MCM2-7 from chromatin, this would also explain why nearly no *Xenopus* MCM-BP was found to be chromatin associated (Nishiyama et al. 2011).

1.4.4.2 Human cells

Human MCM-BP was found to interact with the MCM core-helicase *in vitro*, but unlike Mcm2 it does not inhibit the MCM4/6/7 helicase activity in helicase assays (Sakwe et
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al. 2007). Initial attempts to silence MCM-BP in HeLa cells using siRNA showed a reduced association of Mcm4 with chromatin in G1 and S-phase and an increased amount of soluble Mcm4 was detected, which gave rise to the idea that MCM-BP might be important for MCM stabilization (Sakwe et al. 2007).

shRNA-induced silencing of MCM-BP (Jagannathan et al. 2012) caused severe changes in nuclear morphology of HeLa cells with doughnut shaped or multi-lobed nuclei. Similar observations were made when overexpressing the protein but not when knocking down MCM subunits, indicating an MCM-BP specific phenotype. The lack of MCM-BP was reported to increase the amount of apoptotic cells and cause centrosome amplification in mitotic cells. Thus the observed defects in nuclear morphology might be the consequence of passage through mitosis with multiple centrosomes (Jagannathan et al. 2012). It has to be noted that MCM-BP was not found to be associated with human centrosomes unlike Mcm 4, 6 and 7 (Jagannathan et al. 2012; Andersen et al. 2003), so that the centrosome amplification might be an indirect effect of MCM-BP depletion (Jagannathan et al. 2012).

Upon MCM-BP silencing, a slight increase in RPA foci was observed, indicating replication stress and DNA damage. However the G2 checkpoint was not permanently but only transiently activated upon depletion of MCM-BP and did not cause a cell cycle arrest (Jagannathan et al. 2012). Still, cells lacking MCM-BP showed a slower progression through G2-phase and delayed entry into mitosis (Jagannathan et al. 2012). This supports some of the results from Nishiyama et al, who reported accumulation in S and G2-phase and delayed entry into mitosis in siRNA MCM-BP depleted cells. They further observed a delayed dissociation of Mcm7 from chromatin, consistent with the findings in Xenopus that MCM-BP could regulate G2/M progression by controlling MCM2-7 dissociation from chromatin (Nishiyama et al. 2011). When shRNA was used for depletion, a more general effect on the MCM population was observed: not only chromatin-bound but also soluble Mcm levels were increased in the absence of MCM-BP during S and G2/M-phase, whereas other replication proteins such as PCNA were not affected (Jagannathan et al. 2012). It is hence thought that human MCM-BP affects the level of Mcm proteins during the whole cell cycle in a more general way and is not just needed for unloading in S-phase (Jagannathan et al. 2012).
Human MCM-BP was also reported to increase the \textit{in vitro} stability of Mcm4, Mcm5 and Mcm7 and it is suggested that MCM-BP might have a chaperone-like function, affecting the pool of soluble MCMs which might contribute to their regulation (Nguyen et al. 2012). This function might also be important for the stimulation of MCM2-7 dissociation: similar to the \textit{Xenopus} homologue, excess human MCM-BP was found to dissociate MCM2-7 complexes into smaller complexes or monomers in glycerol gradient centrifugation experiments (Nguyen et al. 2012).

1.4.4.3 Plants

Etg1 (E2F target gene 1) is the MCM-BP homologue in \textit{Arabidopsis thaliana}. Loss of function mutants, generated by tDNA insertion, show cell cycle delay and prolonged G2-phase. Bioinformatic screens revealed that Etg1 is co-expressed with DNA replication proteins (Mcms, PCNA, Pol α-primase etc.) and as it interacts with Mcm proteins and resides in the nucleus, Etg1 is thought to assemble into the replisome and to be necessary to maintain genome stability. In fact, Etg1 depleted plants show less efficient DNA replication, increased DNA damage and cell cycle inhibition, triggered by activation of the DNA replication checkpoint (Takahashi et al. 2008; Takahashi et al. 2010). Activation of the checkpoint was found to be crucial for the survival of Etg1 deficient plants, indicating an essential function of Etg1 for cell cycle progression (Takahashi et al. 2008). Furthermore plants lacking Etg1 showed an increased number of separated sister chromatids leading to the idea of Etg1 being important for the establishment of sister chromatid cohesion along chromosome arms. \textit{ETG1} expression seems to be co-regulated with other genes involved in cohesion establishment in plants, which makes it likely to function in that process as well (Takahashi et al. 2010). The involvement of MCM-BP in cohesion establishment was also analysed in human cells by silencing MCM-BP using siRNA or shRNA. While siRNA treatment also increased the levels of separated sister chromatids (Takahashi et al. 2010), Jangannathan \textit{et al} did not find an effect on sister chromatid cohesion after depleting MCM-BP with shRNA (Jagannathan et al. 2012). So it remains unclear whether human MCM-BP plays a role in sister-chromatid cohesion establishment.
1.4.4.4 T. brucei

The T. brucei MCM-BP homologue was identified in a forward genetic screen to isolate genes, responsible for maintenance of the repressed state of VSG loci in the T. brucei genome. VSGs are variant surface glycoproteins that cover the surface of the parasite and allow persistence of a T. brucei infection in the host. While the genome codes for an array of VSGs, only one is transcribed by RNA Pol II at each time and the others are repressed (Kim et al. 2013). TbMCM-BP was found to be required for the repression or silencing of these VSGs, as TbMCM-BP knock down induced de-repression. The protein was further identified to be essential in T. brucei and its inactivation leads to G2 cell cycle arrest, indicating that it also has an important function for cell cycle progression (Kim et al. 2013). However, as it co-purified in a rather unusual complex with Mcm4, Mcm5, Mcm6, Mcm7 and Mcm8 it might function differently in T. brucei than in other eukaryotes.

Even though the results of MCM-BP study in different organisms are somewhat controversial, there are some noticeable similarities. All homologues interact with Mcm proteins and the results from functional analyses in different species suggest a role of the protein in DNA replication and cell cycle progression.

1.5 Aims

This study aims to contribute to the understanding of the novel MCM-BP protein, which seems to play an important role in DNA replication. The genetically tractable yeast S. pombe will be used to characterise the fission yeast homologue of MCM-BP, Mcb1 (Mcm-binding protein 1), which was unstudied at the outset of this work.

In addition to biochemical characterisation of Mcb1 and the complex it forms with Mcm proteins, experiments were conducted to address the function of the protein in fission yeasts and attempts were made to gain structural information about Mcb1 and the complex it is part of. The results of this work will hopefully be applicable on other eukaryotic MCM-BP proteins, thereby validating fission yeast as a suitable model organism for the study of MCM-BP.
Chapter 2 Characterisation of the fission yeast MCM$^{Mcb1}$ complex

2.1 Introduction

As discussed in Chapter 1, MCM-BP was first discovered in a screen for proteins interacting with the human MCM2-7 complex (Sakwe et al. 2007). While human and Xenopus MCM-BP seem to interact with Mcm3-7 (Sakwe et al. 2007; Nishiyama et al. 2011), there is evidence that the plant MCM-BP protein (Etg1) binds to all members of MCM2-7 (Takahashi et al. 2008) and very recent results suggests a rather unusual complex of T. brucei McmBP containing Mcm8 and MCM4-7 (Kim et al. 2013). So different McmBP-containing complexes might exist in these species and could represent different mechanisms of regulation of the process MCM-BP is involved in.

To address the question of whether fission yeast Mcb1 binds Mcm proteins and to investigate its relationship with the Mcm2-7 complex in S. pombe, different strategies were pursued including TAP purification of the endogenous Mcb1-containing complex from fission yeast, analysis of pairwise interactions in vivo and in vitro and sucrose gradient centrifugation. Findings from these experiments were also related to the canonical Mcm2-7 complex by performing similar experiments using Mcm2.

The results of these approaches will be shown and discussed in this chapter. Some of the results shown here have been reported previously (J. Schnick, Master’s thesis, Ludwig Maximilians University Munich, 2009) and are included here for completeness, labelled appropriately, and parts of them have been published as (Li et al. 2011).

2.2 Characterisation of the Mcm$^{Mcb1}$ complex and comparison with canonical Mcm2-7

2.2.1 Identification of fission yeast Mcb1

BLAST searching of public databases with the sequences of human and Arabidopsis MCM-BP genes identified the previously unstudied S. pombe SPAC1687.04 gene as
encoding an *MCM-BP* homologue. The gene was later named *mcb1* (Ding & Forsburg 2011), and can be found on chromosome 1 (base pairs 906884-908433). The gene is 1550 base pairs in length and contains a single 44 bp long intron from bp 163-206. The encoded protein, Mcb1, is 501 amino acids long and has a predicted molecular weight of 56.6 kDa.

### 2.2.2 Initial purification of a Mcb1-containing complex from fission yeast

Tandem affinity purification (TAP) is a powerful method to purify endogenous protein complexes that was originally developed for the use in yeasts, and allows the identification of interaction partners of a tagged target protein (Rigaut et al. 1999; Gould et al. 2004). The method requires the target protein to be expressed as a fusion protein with a dual affinity tag consisting of a protein A domain (protein A from *Staphylococcus aureus*) and a calmodulin binding peptide (CBP), separated by a TEV (tobacco etch virus) protease cleavage site (Rigaut et al. 1999). The tagged target protein can be expressed from its endogenous promoter allowing for wild-type like protein levels. Complexes containing the target protein and its interaction partners can then be purified from cell extracts using a two-step protocol. In a first step, the target and interacting proteins are immobilised on IgG (Immunoglobulin G) via the IgG-binding domains in the protein A portion of the TAP-tag. After washing, elution from the IgG is mediated by TEV protease. The second purification step depends on the interaction of the remaining CBP part of the tag with calmodulin (immobilised on beads) in the presence of calcium ions. After washing, the target protein complex can be eluted with EGTA (Rigaut et al. 1999). Expressed from its endogenous promoter, the yield of purified tagged protein is low, but further concentration can be achieved by precipitation of the final eluate using trichloroacetic acid (TCA). Analysis of eluted proteins and identification of interaction partners can be performed by mass spectrometry either directly or after separation of the components by SDS-PAGE.

In order to analyse whether the fission yeast homologue of MCM-BP, Mcb1, also forms a complex with Mcm proteins, tandem affinity purification (TAP) was used to purify Mcb1-TAP containing protein complexes.
Characterisation of the MCM<sup>Mcb1</sup> complex

**Figure 2-1 Strategy of PCR based C-terminal gene targeting in S. pombe (adapted from Krawchuk & Wahls 1999)**

A) The pFA6a template plasmids carry an epitope tag and a selectable marker cassette between two universal primer binding sites (PBS a and b). The primers used for PCR contain 80 nucleotides homologous to the target sequence (gene-specific a and b) and 20 nucleotides homologous to the PBS (pFA6a-specific a and b). Because of the modular design of the plasmids, one pair of primers can be used to tag a protein with several different tags. B) PCR product used for transformation C) Alignment of the PCR product with the target gene (mcb1<sup>+</sup>) after transformation D) Integration of the PCR product by homologous recombination E) Fusion protein with C-terminal tag.

Therefore the <i>mcb1</i><sup>+</sup> gene had to be tagged at its 3’ end with sequences encoding for a TAP-tag. Tagging of genes in <i>S. pombe</i> can be easily achieved by a PCR based method referred to as PCR-mediated gene targeting (Bähler et al, 1998, see Figure 2-1). Using this modular approach, a gene of interest can be linked in frame to either N- or C-terminal tags (like affinity or immune- tags, but also promoters such as <i>p<sub>nmt1</sub></i>), which are usually complemented with an antibiotic resistance cassette for selection. A variety of pFA6a template plasmids are available (e.g. Bähler et al, 1998; Krawchuk & Wahls, 1999; Tasto et al, 2001; Van Driessche et al, 2005), carrying a tag and selective marker in between two universal primer binding sites (PBS). Customised 100 nt long primers, containing about 80 nt homologous to the 3’ end or 3’ UTR (for 3’ tagging) of the target gene sequence and 20 nucleotides homologous to the primer binding sites on the pFA6a plasmid can be used to link the tag and marker cassette to the target gene by PCR.
PCR products (containing the last 80 base pairs of the target gene and the sequences encoding the tag and marker as well as 80 base pairs of the 3’ UTR) were transformed into fission yeast cells to be integrated into the genome by homologous recombination, adding the tag and a marker to the gene of interest. Use of selective medium only allows growth of colonies from cells where the PCR product was successfully integrated into the genome. Several of these transformants were screened for the presence of the desired tagged gene by colony PCR. The integrity of the tag in positive transformants was confirmed by sequencing and by Western blot.

The tandem affinity purification to enrich a Mcb1-TAP containing complex was performed with soluble extract from 12 litres of exponentially growing mcb1-TAP
fission yeast cells: cells were frozen in buffer by dripping the cell suspension into liquid nitrogen to make pellet-beads. For soluble extracts, the beads were ground to a fine powder using a grinder under liquid nitrogen cooling. The purification was performed from extracts as described in Materials and methods. Final eluates from the calmodulin-resin were TCA-precipitated, separated on 10% polyacrylamide gels and silver stained. The mcb1-TAP containing sample showed several bands, which were all not present in the wild-type control (Figure 2-2 A). Proteins were identified by ESI-MS/MS spectrometry.

Table 2-1 Identification of proteins co-purifying with Mcb1-TAP by mass spectrometry. Samples were processed by ESI MS/MS on a Q-STAR pulsarXL and proteins identified by comparison to the MASCOT database. Mcb1-TAP asynchronous: results were obtained from an experiment with asynchronously growing cells. Mcb1-TAP HU: results from an experiment with cells synchronised in S-phase by hydroxyurea treatment. Mcm3, Mcm4 and Mcm6 were found in a band of a MW of 100 kDa (Figure 2-2 A or B), Mcm7 of 80 kDa (Figure 2-2 A), Mcm5 of 70 kDa (Figure 2-2 A). These results were presented in part in J. Schnick, Master’s thesis, LMU, 2009.

<table>
<thead>
<tr>
<th>Protein identified</th>
<th>Mcb1-TAP asynchronous</th>
<th>Mcb1-TAP + HU</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Score</td>
<td># peptides</td>
</tr>
<tr>
<td>Mcb1</td>
<td>589</td>
<td>118</td>
</tr>
<tr>
<td>Mcm2</td>
<td>Not found</td>
<td></td>
</tr>
<tr>
<td>Mcm3</td>
<td>Not found</td>
<td></td>
</tr>
<tr>
<td>Mcm4</td>
<td>852</td>
<td>28</td>
</tr>
<tr>
<td>Mcm5</td>
<td>1648</td>
<td>162</td>
</tr>
<tr>
<td>Mcm6</td>
<td>883</td>
<td>58</td>
</tr>
<tr>
<td>Mcm7</td>
<td>1143</td>
<td>137</td>
</tr>
</tbody>
</table>

As shown previously (J. Schnick Master’s thesis, Li et al, 2011,) Mcm 4, 5, 6 and 7 were clearly identified to interact with Mcb1 (Figure 2-2 A, Table 2-1 left side). Mcm3 was only identified in sufficient amounts and with good sequence coverage (meaning that the identified peptides emanated from the whole length of the protein) when repeating the experiment with cells synchronised in S-phase with 12 mM hydroxyurea for three hours (Figure 2-2 B, Table 2-1 right side). Mcm2 could not be identified to interact with Mcb1-TAP in this experiment.

To compare the members of a putative MCM\textsuperscript{Mcb1} complex and the canonical Mcm2-7 complex, the TAP purification was performed using cells carrying Mcb1-TAP or Mcm2-
TAP in parallel (Figure 2-3). Mass spectrometry identified Mcm2-TAP in the band just above 100 kDa, while Mcm4 and Mcm6 but not Mcm3 were identified to be present in the band at 100 kDa with high scores and good coverage. The corresponding band in the Mcb1-TAP lane contained Mcm4 and Mcm6. The presence of Mcm5 and Mcm7 was not analysed, even though there were bands on the gel corresponding to their size (below and above the 75 kDa MW marker). There was no evidence of the presence of any protein of the size of Mcb1 (55.6 kDa) in the Mcm2-TAP purification.

These results suggest that Mcb1-TAP and Mcm2-TAP both co-purify other Mcm proteins, presumably Mcm3-7, but not one another. Thus Mcb1 and Mcm2 might indeed be members of different protein complexes, with Mcb1 not present in the MCM$^\text{Mcm2}$ complex and Mcm2 not present in the MCM$^\text{Mcb1}$ complex.

Figure 2-3 TAP purification of mcb1-TAP and mcm2-TAP cell is parallel to compare the composition of their complexes. Exponentially growing asynchronous mcb1-TAP or mcm2-TAP cells were processed as described in Figure 2-2 and Materials and methods.
2.2.3 Co-IPs of Mcb1-TAP and Mcm2-TAP confirm results from TAP purification

In order to confirm the results of the TAP purification (in particular co-purification of Mcm3-7 but not Mcm2 with Mcb1-TAP), interactions of Mcb1-TAP and myc-tagged Mcm proteins were investigated by co-immunoprecipitation and compared to interactions of Mcm2-TAP with the other Mcm proteins and Mcb1-myc. Strains expressing Mcm2, 3, 4, 5, 6 or 7 with a C-terminal 13myc-tag from their native promoter were constructed by PCR-mediated gene targeting (Bähler et al. 1998) and crossed with mcb1-TAP to obtain the double tagged strains (see Materials and methods).

Soluble extracts of exponentially growing mcb1-TAP or mcb1+ cells expressing myc-tagged Mcm2, 3, 4, 5, 6 or 7 were incubated with IgG-Sepharose and the pulled down proteins were analysed by Western blot using α-myc and α-PAP antibodies for detection. The α-myc antibody is specifically directed against the myc epitopes in the tag. The TAP-tag is detected via its Protein A domain. This contains two IgG-binding regions, which can bind to the Fc (fragment crystallisable) region of immunoglobulins (Langdone 1982). Rabbit IgGs have a high affinity for Protein A (Richmann et al. 1982) and it was reported that any Protein A fusion can be detected with rabbit antibodies (Weser et al. 2006). The detection of the TAP-tag in Western blots is usually achieved using α-PAP (peroxidase anti-peroxidase) antibody from rabbit. This is directed against a complex of an antibody against horse radish peroxidase (HRP) bound to HRP. It is normally used for immunochemical staining and should not bind to endogenous proteins, which makes it useful as standard antibody for Protein A detection. However, also other rabbit antibodies can be used for the detection of TAP-tagged proteins, as e.g. α-Mcb1 antibody for the detection of Mcm4-TAP later in this study (e.g. Figure 3-8).

Analysis of the Western blots showed that Mcb1-TAP co-purified Mcm4-myc, Mcm5-myc, Mcm6-myc and Mcm7-myc while there was no co-purification of Mcm2-myc (Figure 2-4 A). The amounts of Mcm5-myc detected were quite low compared to the other Mcm proteins and Mcm3-myc showed non-specific interaction with the IgG resin. As the Mcm3/5 dimer has been reported to be only loosely attached to the Mcm2-7 complex (Lee & Hurwitz 2000; Sherman & Forsburg 1998; Sherman et al.
Characterisation of the MCM<sup>Mcb1</sup> complex

1998) and this could also be true for the MCM<sup>Mcb1</sup> complex, attempts were made to stabilise a potential interaction by adding ATP, Mg<sup>2+</sup> and Zn<sup>2+</sup> to the lysis buffer (used to purify MCM2-7 and should thus stabilize the complex; Evrin et al, 2009; Remus et al, 2009). The addition of 5 mM ATP did not increase the amount of Mcm5 pulled down with Mcb1-TAP, but the interaction was reproducible and specific (Figure 2-5). As the presence of ATP does not affect the interaction between Mcb1 and Mcm5, the binding is likely to happen without any participation of ATP.

Figure 2-4 Co-immunoprecipitation of Mcb1-TAP and MCM2-TAP with myc-tagged MCM proteins and Mcb1 to confirm Mcb1-MCM interactions. Soluble extracts were made in buffer containing 200 mM sodium glutamate (300 mM for mcm6-13myc samples). Small scale soluble protein extracts were prepared using the Fast Prep method as described in Materials and methods. TAP-tagged protein was precipitated on IgG-Sepharose. Soluble extracts (input) and precipitated protein (co-IP) were analysed by Western blot using α-myc and α-PAP antibodies. A) Co-immunoprecipitations with Mcb1-TAP and mcm2-13myc, mcm4-13myc, mcm5-13myc, mcm6-13myc or mcm7-13myc cells. To test for non-specific binding mcm2, 4, 5, 6, or 7-13myc cells were processed in the same way. Interaction with MCM3-myc was non-specific and is not shown here. B) Co-immunoprecipitation with mcm2-TAP and mcm4-13myc,
mcm5-13myc, mcm6-13myc, mcm7-13myc or mcb1-13myc cells. As control cells without mcm2-TAP were processed similarly. Interaction with Mcm3-myc was non-specific and is not shown here. Note that Mcm2, Mcm4 and Mcm7 pull-down from A) have already been presented as part of J. Schnick, Master’s thesis, LMU, 2009.

Mcm3-myc immunoprecipitation failed to reproducibly show an interaction with Mcb1-TAP, and strong non-specific interaction of the myc- tagged protein with the IgG resin complicated the analysis further (not shown). A more detailed analysis and discussion of Mcm3-myc interaction will be shown below.

Recapitulating, Mcm4, 5, 6 and Mmc7 were clearly co-purified with Mcb1-TAP and there was no interaction with Mcm2-myc observed, confirming the results from the TAP purifications. The pull-down of Mcm3-myc did not give consistent results so the protein cannot clearly be said to interact with Mcb1 in this experiment. Interestingly the amount of Mcm3 identified in the TAP purification (2.2.2) was also quite low compared to the other Mcm proteins.

To address the binding of Mcm2 in the Mcm2-7 complex similar co-immunoprecipitations were conducted using double tagged strains carrying mcm2-TAP and mcm3-myc, mcm4-myc, mcm5-myc, mcm6-myc, mcm7-myc or mcb1-myc. Again Mcm4-myc, Mmc6-myc and Mmc7-myc co-purified with Mmc2-TAP and no interaction with Mcb1-myc could be observed (Figure 2-4 B).

Thus Mcb1 does not co-precipitate with Mmc2-TAP and Mmc2 does not co-precipitate with Mcb1-TAP, indicating that the two proteins are not part of the same complex. Interestingly Mcm5-myc was not found to co-purify with Mmc2-TAP. Again stabilisation of a possible interaction was attempted using ATP and Mg$^{2+}$ in the lysis buffer. But this did not lead to an increased interaction of Mmc2-TAP and Mcm5-myc while Mcb1-Mmc5 interaction was detectable under these conditions (Figure 2-5).

The results for Mcm3-myc interaction with Mmc2-TAP were similar to those obtained for Mcb1-TAP interactions: co-IPs showed non-specific binding of Mcm3-myc to IgG and no reproducible pull-down with Mmc2-TAP (not shown). As Mcm3 is a known member of the Mmc2-7 complex it would be expected to pull down with Mmc2.
Characterisation of the MCM<sub>Mcb1</sub> complex

**Figure 2-5** Co-immunoprecipitation of Mcm5-myc with Mcb1-TAP and Mcm2-TAP in the presence and absence of ATP. Soluble extracts were made from exponentially growing cells as described before. Extracts were split and 5 mM ATP, 10 mM MgOAc, 5 μM ZnOAc were added to one part (indicated as ‘<sup>A</sup>’) before adding the IgG-Sepharose. Beads were washed and boiled in sample buffer. Soluble extracts and precipitated protein were analysed by Western blot using α-myc and α-PAP antibodies to detect Mcm5-myc and Mcb1-TAP or Mcm2-TAP respectively.

Spotting assays showed slower growth of cells carrying 13myc-tagged mcm3 compared to wild-type, which was also found for double tagged mcb1-TAP mcm3-13myc cells but not mcm2-TAP mcm3-13myc cells (Figure 2-6 A). When examined under the microscope, mcm3-13myc cells had an elongated morphology, which is indicative of problems with the cell cycle or replication: while wt cells had an average length of 14.8 μm, the mcm3-13myc cells were 18.8 μm in average. Mcb1-TAP mcm3-13myc were 22.4 μm and mcm2-TAP mcm3-13myc 20.4 μm long. Thus cells containing mcm3-13myc were significantly elongated when compared to wild-type (with mcm3<sup>3</sup>) or strains with only mcb1-TAP or mcm2-TAP. This leads to the conclusion that tagging mcm3 with 13myc leads to problems for the cells, maybe by interference of the tag with Mcm3 protein-protein interactions, as the 13myc-tag itself is quite long (180 AA, 20 kDa). This could also be the reason for the lack of a significant interaction of Mcm3 with Mcm2-TAP or Mcb1-TAP.

To circumvent this problem, Mcm3 was re-tagged with the much smaller V5 and FLAG tags by PCR-mediated gene targeting. The integrity of the new constructs was confirmed by sequencing and the cells had a wild-type like morphology. However the newly tagged protein could not be detected by Western blot (not shown), which is why the strains were not used to examine the interaction of Mcb1-TAP/Mcm2-TAP and Mcm3.
In summary, Mcm4, 6 and 7 were precipitated with both Mcm2-TAP and Mcb1-TAP, while Mcm5 only interacted with Mcb1-TAP. There were no reproducible results for an interaction of Mcm3-13myc with either Mcm2-TAP or Mcb1-TAP here. This could be caused by the large myc-tag, as cells carrying \textit{Mcm3-13myc} had growth defects. But \textit{mcm3} re-tagged with smaller V5 and FLAG was not detectable and thus was not suitable for detection of a Mcm3-Mcb1 or Mcm3-Mcm2 interaction. Most importantly Mcb1 and Mcm2 did not interact with each other in both sets of experiments, leading to the conclusion that they are not part of the same complex. These results again suggest that an Mcm\textsuperscript{Mcb1} complex contains Mcb1 and at least Mcm4-7.
2.2.4 Bimolecular fluorescence complementation does not confirm in vivo interactions of Mcb1 and Mcms

In addition to TAP and co-immunoprecipitation, bimolecular fluorescence complementation (BiFC) was used to visualise protein-protein interactions between Mcb1 and putative interaction partners in vivo. BiFC makes use of the production of a fluorescent signal when the N- and C-terminal parts (VN and VC) of a modified yellow-fluorescent protein called Venus (Nagai et al. 2002) form a complex. This happens, when two proteins, one fused to VN and the other fused to VC, are brought in close proximity, e.g. by interacting with each other (Figure 2-7 A). The genes encoding Mcb1, Mcm2 and Mcm4 were tagged with sequences encoding either the N-terminal or the C-terminal half of YFP-Venus using PCR-mediated gene targeting (Bähler et al. 1998). Strains were viable and appeared indistinguishable from wild-type. The single tagged strains were then crossed to obtain double tagged strains expressing one protein fused to Venus-NTD and another with Venus-CTD. Cells were then grown in EMM (Edinburgh minimal medium) to mid log phase, DNA stained with Hoechst 33342 and live cells were examined by fluorescence microscopy in the DAPI/Hoechst channel (excitation wavelength 350 nm) and the GFP channel (excitation wavelength 515 nm). A weak Venus fluorescence could be detected in all cells, even those carrying only one VC or VN tagged construct (Figure 2-7 B and C, Venus). In mcm4-VC cells for example, this fluorescence was particularly bright in the nucleus (Figure 2-7 B). But as there is only the C-terminal half of Venus expressed in this strain, which should not give a signal on its own, the fluorescence must be considered as background. When examining the VN/VC double tagged strains, mcm4-VC mcb1-VN (Figure 2-7 B bottom row), mcm4-VN mcb1-VC (Appendix 1, Figure 0-3) and mcm2-VN mcb1-VC (Figure 2-7 C bottom row), no intense signal arising from interaction of the two tagged proteins could be detected. There was only a weak and rather diffuse fluorescence. This was somewhat stronger in the mcm4-VC mcb1-VN and mcm4-VN mcb1-VC cells than in the mcm2-VN mcb1-VC strain and could possibly indicate an interaction of Mcm4 and Mcb1.
Characterisation of the MCM$^{Mcb1}$ complex

A

interaction of A and B $\rightarrow$ fluorescence

no interaction of A and B $\rightarrow$ no signal

B

Hoechst 33342  |  Venus  |  Merge: Hoechst Venus

mcm4-VC

mcb1-VN

mcm4-VC mcb1-VN
Characterisation of the $MCM^{Mcb1}$ complex

Figure 2-7 Bimolecular fluorescence complementation (BiFC)

A) Principle of BiFC
B) BiFC using $mcm4$-VC $mcb1$-VN: cells carrying $mcm4$-VC or $mcb1$-VN or both were stained with Hoechst 33342 and examined by fluorescence microscopy. Images of Hoechst-channel, Venus-channel (= YFP) and merge of both are shown.
C) BiFC using $mcm2$-VN $mcb1$-VC: images of cells carrying $mcm2$-VN or $mcb1$-VC or both of Hoechst-channel, Venus-channel and merge of both are shown. Images from BiFC experiments with $mcm4$-VN $mcb1$-VC can be found in Appendix 1, Figure 0-3. Scale bar = 10 µm.

Interestingly, while the single tagged strains often showed a more intense signal in the nucleus, the fluorescence seemed more evenly distributed (in cytoplasm and nucleus) in the double tagged strains.

But the lack of an intense fluorescent signal rather indicates that there is no bimolecular fluorescence complementation of Mcb1-VC and Mcm4-VN or Mcb1-VN and Mcm4-VC or Mcb1-VC and Mcm2-VN in the experiments described here. Thus the
BiFC does not confirm the interactions between Mcb1 and Mcm proteins described earlier in this chapter.

### 2.2.5 Mcb1 contributes to a high molecular weight complex in fission yeast

The previously described approaches all address the composition of a putative MCM\textsuperscript{Mcb1} complex and its subunit-subunit interactions but they do not give any information about the size of such a complex. Theoretically the findings from the co-immunoprecipitations and the TAP purification could result from several different Mcb1-Mcm dimers present at the same time but also from larger assemblies or mixtures thereof.

To address the size of the MCM\textsuperscript{Mcb1} complex, sucrose gradient centrifugation was used. This technique allows the separation of mixtures of large protein-complexes depending on their size and weight while running through a linear sucrose gradient. Soluble extracts from cells carrying immunodetectable Mcm or Mcb1 protein were layered onto a 5 ml gradient (method by Stone, 1974) containing 18% sucrose at the bottom and 5% at the top and centrifuged in a Sw55Ti rotor at 4°C and 32000 rpm for 22 hours. After piercing a hole into the bottom of the tube, five-drop fractions were collected and their protein content was analysed by Western blot.

To analyse the size of a putative MCM\textsuperscript{Mcb1} complex, cells expressing Mcb1-TAP were used. Extracts from cells with Mcm4-TAP and Mcm6-TAP served as controls, potentially being members of both the MCM\textsuperscript{Mcb1} complex and the known Mcm2-7 complex. A mixture of globular proteins usually used to calibrate gel filtration columns was processed similar to the protein extracts to act as molecular weight marker. A linear standard curve based on three of these standard gradients (Figure 2-8 A-C) was then used to determine the approximate size of the analysed protein complexes (Figure 2-8 D).

Western blot analysis showed that the tagged protein was never only present in one fraction but usually spread over about ten fractions corresponding to 40% of the gradient volume, however with clear peaks. Mcm4-TAP was found in fractions 8-16 with a peak in fraction 10-12 that corresponds to a size of about 400 kDa. Mcm6-TAP peaks at fraction 12-14 (330 kDa) and Mcb1-TAP around fraction 14 (300 kDa), and
both proteins are distributed between fractions 8-18 (Figure 2-8 D). Note that the second, lower band in the Westerns below Mcm4-TAP and Mcm6-TAP seems to be a non-specific signal of the PAP antibody, as it is also present in the blot containing Mcb1-TAP (Figure 2-8 D indicated by *).

**Figure 2-8 Mcb1 is part of a high molecular weight complex.** A-C) Calibration of sucrose gradients. BioRad Gel filtration standard containing 0.5 mg Thyroglobulin ("T" 660 kDa), bovine γ-globulin ("B" 158 kDa), chicken ovalbumin ("C" 44kDa), 0.25 mg equine myoglobin (17 kDa) and 0.05 mg vitamin B12 (1.35 kDa) was separated on an 18-5% sucrose gradient. Five drop fractions (≈ 200 µl) were collected and 10 µl subjected to SDS-PAGE. **A)** 10% SDS-PAGE gels with fractions of run 1 of 3. **B & C)** Peak-fractions containing proteins T, B and C from three different runs were plotted against the proteins’ molecular weights to obtain a standard curve. **D)** Separation of Mcm4, Mcm6 and Mcb1 containing complexes on gradients. Soluble extracts of mcm4-TAP, mcm6-TAP or mcb1-TAP cells were layered onto an 18-5% sucrose gradient and centrifuged at 32000 rpm for 22 hours. Five-drop fractions were collected and the protein contents of every second fraction was analysed by Western blot using an α-PAP antibody. Positions of marker bands, as determined in C) are indicated above the blots. * indicates a non-specific signal caused by the α-PAP antibody.
The monomer sizes of the proteins are 101.5 kDa (Mcm4), 99.5 kDa (Mcm6) and 56.6 kDa (Mcb1) plus 21 kDa of the TAP-tag, indicating that all three proteins are present in multimeric assemblies in the gradients, as expected, and not in their monomeric state. However, these multimers might not have the size of $\text{MCM}^{\text{Mcm2}}$ (557 kDa) or a hexameric $\text{MCM}^{\text{Mcb1}}$ (521 kDa), but of smaller complexes. The two hexameric species should theoretically be found in fraction 6 or 7, so if the size estimation is correct, none of the analysed proteins would definitely be part of these hexamers, as they only appear from fraction 8 onwards.

This data does not allow conclusion of the exact composition of a putative $\text{MCM}^{\text{Mcb1}}$ complex, but it shows that Mcb1’s in vivo state is clearly not monomeric. The estimated size of 300 kDa of the main Mcb1 species observed here could correspond to Mcb1 plus two Mcm proteins for example and the small amount of Mcb1 complex in fraction 8 could represent a hexamer considering the inaccuracy of size determination.

### 2.2.6 Mcb1 is as abundant as Mcm proteins

The results from the previous sections showed that Mcb1 interacts with Mcm proteins in vivo and seems to be part of a multimeric complex. Interaction of Mcb1 with Mcm proteins takes place during the whole cell cycle (Master’s thesis Jasmin Schnick) and can be observed under similar conditions and with comparable intensity as Mcm-Mcm interactions (see section 2.2.3). This suggests that Mcb1 is equally abundant as Mcm proteins in fission yeast. To determine the relative amount of Mcb1 present in fission yeast cells, total cellular levels of Mcm proteins and Mcb1 were compared by Western blot. Total protein extracts were prepared from cells expressing 13myc- tagged Mcms or Mcb1 and analysed by Western blot using α-myc antibody and a CCD camera (Fujifilm LAS 3000) for detection. At least three different total extracts were analysed per strain and the myc signal intensities were normalised against the intensity of the tubulin loading control.

Most analysed proteins were found to be present at comparable but not identical levels (Figure 2-9). Only in the case of Mcm5-myc a slightly weaker signal was
obtained. This was not due to a less concentrated extract as the signal of the loading control (tubulin) was as intense as for the other samples. Importantly, the amount of Mcb1 is in the same range as the amount of the analysed Mcm proteins indicating that Mcb1 is indeed as abundant as Mcm proteins in fission yeast.

**Figure 2-9 cellular levels of Mcb1 and Mcm proteins are comparable.** Denaturing protein extracts were prepared from exponentially growing wild type, mcb1-13myc, mcm2-13myc, mcm3-13myc, mcm4-13myc, mcm5-13myc, mcm6-13myc, mcm7-13myc or wt cells, adjusted by OD. Extracts were analysed by Western blot using α-myc and α-tubulin antibodies and a CCD camera (Fujifilm LAS 3000) for detection. Quantification of the signals was performed with AIDA image analyzer software (Raytest, Straubenhardt, Germany). A) Western blot with Mcb1-myc and Mcm2-7myc; tubulin serves as loading control B) relative levels of Mcm-13myc and Mcb1-13myc based on quantification of α-myc signal intensities normalized against α-tubulin signals. Per strain at least three independent extracts were analysed.
2.3 Characterisation of pairwise interactions of Mcb1 with Mcm proteins \textit{in vitro}

2.3.1 Pull-down of \textit{in vitro} translated Mcm constructs with recombinant Mcb1-NTD

The results described so far indicate that Mcb1 associates with Mcm3, Mcm4, Mcm5, Mcm6 and Mcm7 in high molecular weight complexes \textit{in vivo}. However, the exact subunit composition and neighbour-neighbour interactions of such complexes remain unresolved by these experiments. To identify direct interaction partners of Mcb1 and possibly clarify the subunit composition of Mcb1-Mcm complex(es) \textit{in vitro}, co-precipitations were performed using recombinant proteins.

As a purification strategy for recombinant Mcb1 N-terminal domain (rMcb1-NTD) had successfully been developed (see Chapter 5.2), this was available to be used as bait for co-precipitations. At this point, it was considered likely that Mcb1 interacts with the Mcm proteins in a similar way as the Mcm proteins interact with each other in Mcm2-7, i.e. aligning side by side (N-terminal domains with N-terminal domains and C-terminal domains with C-terminal domains) to form a hexameric ring. Consequently, for \textit{in vitro} expression and co-precipitation with Mcb1-NTD, only the N-terminal domains of Mcm2- Mcm7 and Mcb1 were used (Figure 2-10 A). Furthermore, it was thought that \textit{in vitro} expression of the relatively short N-terminal domains would be more straightforward than expressing full-length proteins but still allow the results to be valid for interaction of the full-length proteins. The boundaries for the N-terminal domains were determined from an alignment of the Mcm2- Mcm7 and Mcb1 proteins (see Appendix 3) and were defined to be located in the flexible linker connecting the N-terminal and the C-terminal halves of the proteins (compared to ssoMcm 3D structure; Figure 4-4).

Sequences encoding the N-terminal domains were cloned into pTNT (Promega), which is optimised for \textit{in vitro} transcription/translation, containing a T7 promoter and terminator as well as a 5’ β-globin leader sequence and a synthetic poly(A)tail for enhanced gene expression. The proteins were \textit{in vitro} synthesised and labelled with $^{35}$S methionine using a rabbit reticulocyte System (Promega TNT$^R$ Quick coupled transcription/translation rabbit system) before co-precipitation with recombinant
Mcb1-NTD-Strep immobilised on StrepTactin Sepharose (Figure 2-10 B). For analysis, samples of the \textit{in vitro} reactions and the pull-downs were subjected to SDS-PAGE and gels were analysed by autoradiography.

Some optimisation was necessary, as several of the reactions showed a high background with numerous non-full-length proteins/degradation products instead of one protein species and the co-precipitation samples contained many bands which could not be identified with confidence. Results were found to be clearest when using...
0.3 μg of template plasmid and including protease inhibitors chymostatin and leupeptin in the reactions. Most of the in vitro translations then resulted in one major protein species with approximately the expected size (compare Figure 2-10 A and D, IVT panel), however the Mcm2-IVT product migrated much more slowly, consistent with a molecular weight of 100 instead of 55 kDa.

A portion of each in vitro reaction was found to interact with rMcb1. In case of Mcm4-NTD, Mcm5-NTD and Mcm7-NTD the pulled-down protein co-migrated with the most prominent band in the total in vitro reaction so that it can be assumed that rMcb1-NTD can directly interact with the N-terminal domains of Mcm4, 5 and Mcm7 (compare lanes 4, 5 and 7 in IVT and Pull-down panels of Figure 2-10 D). In the Mcm3 and Mcm6 samples, a protein smaller than the most prominent one from the total reaction was pulled down with Mcb1-NTD (Figure 2-10 D, lanes 3 and 6 in IVT and * in pull-down panels). It is possible that these interacting proteins result from degradation or transcription initiation at alternative methionine codons. For Mcm6 for example, transcription start at M48 would lead to a 43 kDa protein, which is approximately the size of the pulled-down protein. Why the predominant Mcm3 and Mcm6 IVT products do not interact with rMcb1-NTD remains unclear. It is possible though that they do not fold properly, which could interfere with the binding.

The main Mcm2-NTD product had a size of 100 kDa, which is about double the actual size of Mcm2-NTD, and was followed by an array of bands (Figure 2-10 D, IVT panel, lane 2) indicating degradation of the in vitro synthesised protein or translation start at alternative ATG codons (M76 or M236). Several of these bands seemed to co-precipitate with rMcb1-NTD but none of them was dominant. Thus it is unclear whether Mcm2-NTD domain can interact with Mcb1-NTD as it was not possible to identify Mcm2-NTD and a clear pull-down was not observed either. Similar results were obtained when analysing the self-interaction of rMcb1-NTD with in vitro expressed Mcb1-NTD. While one clear Mcb1-NTD construct was visible after the translation reaction, there was no distinct band observed in the pull-down. Thus Mcb1-NTD does rather not self-interact. This was also confirmed by gel filtration (see chapter 5.2.1): no indication of Mcb1-NTD dimerization was apparent during purification.
In summary, rMcb1-NTD clearly co-precipitated in vitro translated Mcm4-NTD, Mcm5-NTD and Mcm7-NTD. Further some minor Mcm3-NTD and Mcm6-NTD species also seemed to interact, while Mcm2-NTD does not. Experiments with recombinant Mcm proteins that revealed the subunit composition of Mcm2-7 (Davey et al. 2003) showed that each Mcm subunit can only bind two others. Mcb1 in contrast seems capable of binding five of the Mcm proteins in vitro. To rule out the possibility that the observed interactions are indirect and mediated via proteins present in the rabbit reticulocyte lysate, pull-down experiments with purified proteins were performed, which will be discussed in 2.3.2.

The apparent binding of different Mcm proteins to Mcb1 could reflect a general ability of Mcb1 to bind Mcm proteins, but Mcb1 could still have preferred interaction partners in vivo. To test this, co-precipitations were performed with Mcm4-NTD, Mcm5-NTD and Mcm6-NTD at the same time to create a situation closer to in vivo conditions with different Mcm proteins competing for rMcb1-NTD binding. Initial experiments with equal amounts of labelled Mcm4-NTD, Mcm5-NTD and Mcm6-NTD did not lead to clear results, as the different proteins caused intersecting bands and also the background signal was quite strong (Figure 2-11 A, left side). In addition, labelling only one of the proteins to be pulled-down did not lead to a competition among Mcm4, 5 and 6 because presumably there was enough rMcb1-NTD (50 μg) present to bind all three proteins (Figure 2-11 A, right side).

Thus, smaller amounts (10 μg or 1 μg) of Mcb1-NTD were immobilised on StrepTactin (15 μl) and incubated with radio-labelled Mcm4-NTD only or equal volumes of labelled Mcm4-NTD and unlabelled Mcm6-NTD and Mcm5-NTD. In this way an observed interaction between rMcb1-NTD and Mcm4-NTD should be weakened if Mcm5-NTD or Mcm6-NTD compete for the binding. In the 10 μg assays (Figure 2-11 B) the signal of pulled-down Mcm4-NTD was much stronger than in the 1 μg assays indicating that the maximum Mcb1 binding capacity in the 1 μg assays is exceeded and a competition should be possible. However there was no clear reduction in the amount of co-precipitated Mcm4-NTD in the presence of Mcm5-NTD and Mcm6-NTD compared to the Mcm4-NTD only sample (Figure 2-11 compare pull-down 1 μg, lanes 4* and 4*56). Thus Mcb1 either preferentially binds Mcm4-NTD over Mcm6-NTD and Mcm5-NTD or it is able to bind all three (or two out of three) proteins at the same time.
Figure 2-11 Mcm4-NTD and Mcm6-NTD do not compete for Mcb1-NTD binding Co-precipitations with rMcb1-NTD-Strep were performed as before but with different combinations of in vitro translated Mcm4-NTD, Mcm5-NTD or Mcm6-NTD simultaneously. Per pull-down 8 µl of each indicated in vitro preparation were used. 4 indicates Mcm4-NTD, 5 = Mcm5-NTD, 6 = Mcm6-NTD, * marks 35S Methionine labelled preparations. Autoradiographs after 3 days exposure are shown. Load: IVT: 7.5% of amount loaded in pull-down, pull-down: 15 µl. A) Pull-downs of individual Mcm-NTDs and their combinations with 50 µg rMcb1-NTD on 30 µl StrepTactin. Note that the left/ middle panel originate from a different gel/ film as the right panel. B) Pull-downs of labelled Mcm4-NTD only or in combination with unlabelled Mcm5-NTD and Mcm6-NTD with 10 µg or 1 µg rMcb1-NTD. C) Pull-down of labelled Mcm4-NTD plus different amounts of unlabelled Mcm6-NTD or BSA with 1 µg of rMcb1-NTD.

The experiment was repeated with 1 µg of rMcb1-NTD and addition of labelled Mcm4-NTD only, equal amounts of labelled Mcm4-NTD and unlabelled Mcm6-NTD, or labelled Mcm4-NTD and double amounts of unlabelled Mcm6-NTD. To ensure that the interactions are specific, the co-precipitation was also conducted with labelled Mcm4-NTD in the presence of an excess of BSA (40 µg, as a random protein that might interfere with the binding). Again the signal intensity of Mcm4-NTD interaction with Mcb1-NTD did not change in the presence of Mcm6-NTD or excess BSA (Figure 2-11 C). Apparently the Mcm4 binding to Mcb1 is specific but not challenged by Mcm6, which means that either Mcm4 is the preferred interaction partner, or Mcb1 is indeed
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capable of binding several Mcm proteins at the same time. Similar experiments with other labelled Mcm subunits were not conducted, however it is likely that addition of an additional Mcm-NTD in excess would not change the interaction pattern so that the option of Mcb1 binding more than one Mcm subunit at once seems possible. To find out which option is correct requires more experiments to be conducted.

2.3.2 Interaction of rMcb1 and rMcm4 <i>in vitro</i>

The pull-down experiments with <i>in vitro</i> translated Mcm and recombinant Mcb1-NTD protein suggest that Mcb1-NTD is capable to directly interact with most of the Mcm proteins <i>in vitro</i>. To ensure that the observed interactions are direct and not mediated by proteins present in the translation lysate, pull-down experiments were conducted in an environment free of additional proteins using purified recombinant Mcm4 and Mcb1.

Fission yeast Mcm4-NTD (without the N-terminal extension, fused to an N-terminal 6His-tag) and Mcb1 N-terminal domain (fused to a C-terminal Strep-tag) were purified from <i>E. coli</i> (see chapter 5.4 and 5.2.1). Recombinant Mcb1-NTD or Mcm4-NTD were incubated with either StrepTactin or Ni-NTA resin to bind before addition of the respective other protein. After several washes the resin-associated proteins were analysed by SDS-PAGE and Western blot.

Mcm4-NTD interaction with immobilised Mcb1-NTD (on StrepTactin) was visible by SDS-PAGE and seemed stable in all salt concentrations tested (0-300 mM sodium glutamate; Figure 2-12 left side). The amount of Mcm4-NTD pulled down was possibly not more than 15 μg in total per reaction (Figure 2-12, left panel, compare Mcm4-NTD pull-down SDS-PAGE to Mcm4-NTD 15 μg), which still corresponds to 30% of the input of 50 μg. Western blot analysis with two-colour detection on the Licor Odyssey IR imaging system confirmed specific interaction of Mcm4-NTD with Mcb1-NTD (Figure 2-12).

The reciprocal experiment with His-Mcm4-NTD immobilised on Ni-NTA did not show interaction with Mcb1-NTD when analysed by SDS-PAGE. A pull-down of Mcb1 was visible on the Western blot but was not specific as similar amounts of the Mcb1-NTD interacted with the resin-control (Figure 2-12 right side). The non-specific binding of
Mcb1-NTD to the Ni-NTA resin could probably be reduced when adding imidazole to the binding buffer but that might also reduce the specific binding of His-Mcm4-NTD.

**Figure 2-12** *in vitro* interaction of recombinant fission yeast NTDs of Mcb1 and Mcm4 50 μg of rMcb1-NTD-Strep (left panels) or rHis-Mcm4-NTD (right panels) were incubated with 30 μl StrepTactin (IBA lifesciences) or Ni-NTA (GE) in binding buffer (pH 8, containing 0-300 mM sodium glutamate, 0.1% Tween) for one hour at 4°C. As negative control, rMcm4-NTD was also incubated with StreptTactin (control StreptTactin only) and Mcb1-NTD was incubated with Ni-NTA resin (control Ni-NTA only). The supernatant was removed and 50 μg of prey protein and binding buffer to a total volume of 400 μl were added and incubated for 1 hour at 4°C, before washing with binding buffer. The resin was boiled with sample buffer and samples were analysed by SDS-PAGE (12% gels) and Western blot. Proteins were detected using α-His antibody (for rMcm4-NTD) and α-Mcb1 (for rMcb1-NTD) and the LICOR OdysseyCLX infrared imager. Load SDS-PAGE: 10 μl per well (25%); Western blot: 5 μl per well (12.5%).

These experiments show that Mcb1-NTD can directly interact with Mcm4-NTD in the absence of any other protein. And although not tested here it is likely that Mcb1 is also able to interact with the other Mcm proteins *in vitro*, even those that might not be direct neighbours of Mcb1 in an *in vivo* complex.
2.4 Other interaction partners of Mcb1

Parts of this section have been reported previously (J. Schnick, Master’s thesis, LMU, 2009) and are labelled as such in the figure legends.

MCM2-7 plays a crucial role in DNA replication and interacts with many different proteins during this process like GINS and Cdc45 in the CMG complex (Moyer et al, 2006; RPC in budding yeast: Gambus et al, 2006; unwindosome in Xenopus: Kubota et al, 2003) but also others including Cdt1 and DDK (e.g. reviewed in Boos et al, 2012). It seems reasonable to speculate that Mcb1 might bind some of these factors as well; the existence of Mcb1-GINS, Mcb1-Cdc45 or Mcb1-Cdt1 interactions would allow to draw conclusions about the processes Mcb1 is involved in or the time its activity is needed. Cdt1, GINS and Cdc45 did not co-purify with Mcb1-TAP in the TAP purification, but the interactions between Mcb1 and these components could be quite weak (compared to Mcb1-Mcm) so that a putative complex would not survive the TAP purification under the conditions used.

2.4.1 Components of the CMG complex

To investigate if Mcb1 interacts with CMG components, co-immunoprecipitations of Mcb1-TAP with myc-tagged GINS subunits or Cdc45 were performed (Figure 2-13 A). The required strains expressing myc-tagged proteins from their endogenous promoter were made by PCR-mediated gene targeting and subsequent crossing with mcb1-TAP to produce the double tagged strains. In this way strains carrying psf2-myc, psf3-myc, sld5-myc and cdc45-myc alone or in combination with mcb1-TAP were obtained. Soluble extracts were prepared before isolation of Mcb1-TAP by immunoprecipitation on IgG-Sepharose. Co-precipitated myc-tagged proteins were subsequently analysed by Western blot.

While Psf2-myc clearly precipitated with Mcb1-TAP (Figure 2-13 A), Psf3-myc (Figure 2-13 A), Sld-5myc (Figure 2-13 A) and Cdc45-myc (Figure 2-13 B) did not associate. To assure that the lack of interactions was not due to sensitivity of detection, the Psf2/ Psf3 pull-down experiment was repeated with cells arrested in S-phase by hydroxyurea.
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The co-precipitation of Psf2 could be confirmed but there was still no signal of Psf3 found (Figure 2-13 C).

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**Figure 2-13 Mb1 does not interact with GINS or Cdc45.** Co-immunoprecipitations were performed using -13myc and Mcb1-TAP double tagged strains. Small scale soluble extracts were prepared in 200 mM sodium glutamate buffer using the Fast Prep machine. TAP-tagged proteins were precipitated on IgG-Sepharose for one hour, washed and boiled with sample buffer. Soluble extracts (input) and precipitated protein (co-IP) were analysed by Western blot using α-myc and α-PAP antibodies. 

A) Co-immunoprecipitation of GINS subunits Psf2-myc, Psf-3myc and Sld5-myc with Mcb1-TAP. 

B) Co-immunoprecipitation of Cdc45-myc with Mcb1-TAP and Psf1-TAP 

C) Co-immunoprecipitation of Psf2-myc and Psf3-myc with Mcb1-TAP from cells arrested in early S-phase by hydroxyurea. 

D) Co-immunoprecipitation of Mcb1-myc with Psf1-TAP and Psf2-TAP. 

Parts of A) B) and C) have been shown before in J. Schnick, Master's thesis, LMU, 2009.

The co-immunoprecipitation was also performed the other way around with Psf2-TAP or Psf1-TAP (made by PCR-mediated gene targeting) and Mcb1-myc to ensure the observed interaction is real and not an experimental artefact. GINS Psf1-TAP successfully pulled down Cdc45-myc (Figure 2-13 B), confirming that Psf1 and Cdc45 are members of the same large assembly, the CMG complex, and that the used
method is suitable to analyse this particular interaction. Mcb1-13myc on the other hand was not precipitated with either Psf1-TAP or Psf2-TAP (Figure 2-13 D).

In summary, Psf2-myc is pulled down with Mcb1-TAP, but Mcb1-myc is not pulled down with Psf2-TAP. It could be speculated that in the strain expressing Mcb1-TAP and Psf2-myc, the GINS complex is disturbed by Psf2’s 13myc-tag, which could lead to a partial dissociation of the complex. Dissociation products containing Psf2-13myc could interact with Mcb1-TAP. This might not happen in the psf2-TAP mcb1-13myc, psf1-TAP mcb1-13myc and mcb1-TAP psf3-13myc cells, so that an interaction of Psf2 and Mcb1 (and also Psf1 and Mcb1 or Psf3 and Mcb1) cannot be detected. This leads to the assumption that the previously observed interaction between Mcb1-TAP and Psf2-myc is unlikely to reflect a real binding situation.
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Figure 2-14 Bimolecular fluorescence complementation (BiFC) using psf2-VC mcb1-VN and psf1-VC mcb1-VN cells. Indicated strains were grown in EMM to mid log phase. Nuclear DNA was Hoechst 33342 stained and cells were examined by fluorescence microscopy using DAPI/ Hoechst and GFP/Venus filter sets. Mcb1-VN, psf1-VC and psf2-VC strains serve as control. Scale bar = 10 μm.

This is also suggested by results from BiFC studies to visualise putative Mcb1-GINS interactions in vivo. Background fluorescence was visible in single tagged psf1-VN, psf1-VC, mcb1-VN, mcb1-VC and psf2-VC cells, carrying only one half of the fluorescent Venus protein (Figure 2-14). This background could also be observed in double tagged psf1-VN mcb1-VC (not shown), psf1-VC mcb1-VN (Figure 2-14) and psf2-VC mcb1-VN cells (Figure 2-14), and was not stronger than in the single tagged controls, indicating that the signal is not specific.

Similar results were obtained for strains carrying cdc45-VC, mcb1-VN or cdc45-VC and mcb1-VN, with background fluorescence in single tagged strains but no strong signal indicative of Cdc45-Mcb1 interaction in the double tagged strain (Figure 2-15).
Thus the BiFC assays did not show any interaction between Mcb1 and Psf1, Psf2 or Cdc45, which is consistent with the results from the TAP purification and the co-IPs. These results suggest that there is no interaction between Mcb1 and GINS or Cdc45 in fission yeast.

2.4.2 Cdt1 interaction

The licensing factor Cdt1 is an important component of the preRC and primarily responsible for the loading of MCM2-7 onto origins of replication (Nishitani et al., 2000; reviewed in Caillat & Perrakis, 2012) presumably via direct interaction with Mcm2 and Mcm6 (Yanagi et al. 2002; You & Masai 2008; Liu et al. 2012). To investigate if Mcb1, like the Mcm proteins, can form a complex with Cdt1, co-immunoprecipitations were performed with Cdt1-TAP and Mcb1-myc or Mcm2-myc, the latter serving as a positive
control for Cdt1/Mcm2-7 interaction. Strains were made by crossing cdt1-TAP cells (a generous gift from the Kearsey lab, Oxford) to mcb1-myc or mcm2-myc.

\[
\begin{array}{ccc|ccc}
\text{Input} & \text{Co-IP on IgG} & \text{Mcb1-myc/Mcm2-myc} & \text{Cdt1-TAP} & \text{Mcm2-myc} & \text{Mcb1-myc} \\
1 & 2 & 1 & 2 & + & + \\
\end{array}
\]

\[
\begin{array}{ccc|ccc}
\text{Input} & \text{Co-IP on IgG} & \text{NaOGLu [mM]} & \text{Cdt1-TAP} & \text{Mcm2-myc} & \text{α-myc} \\
150 & 200 & 300 & 400 & + & + \\
\end{array}
\]

**Figure 2.16 Interaction of Cdt1 with Mcb1 remains unclear.** Co-immunoprecipitations were performed using strains expressing Cdt1-TAP, Nda3-311 and Mcb1-myc or Mcm2-myc. To enrich Cdt1, cells were synchronised in mitosis by incubation at 20°C for 4 hours and released for 20 minutes at 32°C prior to generating soluble extracts with the glass bead method in 200 mM sodium glutamate, 10 mM MgOAc, and 50 μM ZnOAc, 0.1% Triton. Cdt1-TAP was precipitated on IgG resin and total extracts (input) and co-IPs were analysed by Western blot with anti-myc antibody and anti-PAP antibody. As negative control, extracts from strains without cdt1-TAP were processed in the same way. **A** Co-immunoprecipitation of Mcb1-myc and Mcm2-myc with Cdt1-TAP is not specific. **B** Co-immunoprecipitation of Mcm2-myc with Cdt1-TAP using 150 mM-400 mM sodium glutamate and 1% Triton X-100 to reduce non-specific binding.

Initial experiments with asynchronously growing cells failed to detect Cdt1-TAP (not shown), presumably because endogenous fission yeast Cdt1 is synthesized only in M-G1-phase and subjected to proteolysis in S-phase to prevent preRC re-assembly after DNA replication initiation (Nishitani et al. 2001; Liu et al. 2004; Li & Blow 2005; Kim & Kipreos 2007).

To overcome this problem, cells were synchronized prior to S-phase using the cold sensitive nda3-311 mutation. Nda3 encodes a β-tubulin homologue in fission yeast and cells carrying the cold sensitive nda3-KM311 allele cannot form a mitotic spindle when
incubated at 20°C and arrest in prophase. This arrest is reversible and can thus be used for synchronisation of fission yeast cells in mitosis (Hiraoka et al. 1984). Immunoprecipitations were conducted with extracts from $cdt1$-$TAP \ mcb1$-$myc$ or $cdt1$-$TAP \ mcm2$-$myc$ cells, which had been released from M-arrest for 20 minutes (see Guarino et al., 2011) to ensure high Cdt1 levels. This still did not allow detection of Cdt1-TAP in total extracts, but when pulled down with IgG-Sepharose (Figure 2-16 A, co-IP $\alpha$-PAP, lane 5 and 6). A specific co-precipitation of Mcb1-myc or Mcm2-myc could not be observed however, as both proteins were found to bind non-specifically to the IgG-Sepharose resin (Figure 2-16 A, co-IP $\alpha$-myc, lanes 7 and 8). It could be argued that the band corresponding to Mcm2-myc pulled down with Cdt1-TAP is stronger than the band corresponding to the non-specific interaction of Mcm2-myc with IgG-Sepharose resin only (compare Mcm2-myc co-IPs in lanes 6 and 8 in Figure 2-16 A) and that Mcm2-myc does interact with Cdt1-TAP. Thus the co-immunoprecipitations with $cdt1$-$TAP \ mcm2$-$myc$ cells were repeated, applying more stringent buffer conditions with higher salt concentrations to reduce the non-specific interactions (Figure 2-16 B). As seen in lanes 13-16 (Figure 2-16 B, $\alpha$-myc panel), Mcm2-myc pulls down with Cdt1-TAP in comparable amounts at 150, 200, 300 and 400 mM sodium glutamate. But all these interactions are unspecific, as Mcm2-myc is also pulled down on IgG from extracts of $mcm2$-$myc \ cdt1^+$ control cells (lanes 9-12 Figure 2-16 B). Thus this attempt also failed to show a specific interaction of Mcm2-myc and Cdt1-TAP, even under 400 mM salt.

In conclusion, as Mcm2 is a known interaction partner of Cdt1 it should be possible to analyse the interaction by co-IP, which did not succeed here. It was refrained from testing further conditions for a specific Cdt1-Mcm2 interaction and also Cdt1-Mcb1 interaction. Thus an interaction of Mcb1 and Cdt1 could not be shown, which does not necessarily indicate an absence of such a complex or interaction.

2.5 Discussion and future work

Results from the experiments described in this chapter indicate that a multimeric complex of Mcb1 and Mcm proteins exists in fission yeast.
TAP purification showed that this MCM\textsuperscript{Mcb1} complex likely contains Mcm3-7 but not Mcm2. Accordingly Mcb1 was not found to co-purify with Mcm2-TAP in a similar experiment indicating that two different Mcm complexes occur in \textit{S. pombe}: MCM\textsuperscript{Mcb1} and MCM\textsuperscript{Mcm2} (which is Mcm2-7). Co-immunoprecipitation studies confirmed that Mcb1 is in a complex with Mcm4, 5, 6 and Mcm7 but not with Mcm2. Mcm2-TAP was confirmed to co-precipitate Mcm4, Mcm6 and Mcm7 but not Mcb1. Interestingly Mcm5 did also not co-precipitate with Mcm2-TAP. The MCM2-7 helicase was reported to have a discontinuity between Mcm2 and Mcm5 (referred to as "gate"), which might open or close depending on the conditions or the state of the CMG complex (Bochman et al. 2008; Costa \textit{et al.} 2011). Possibly the complex pulled down here with Mcm2-TAP has adapted an open confirmation which was not changed by the presence of ATP, magnesium and zinc. Consequently it is possible that Mcm5 is not precipitated with Mcm2, because there is no direct interaction and Mcm5 might be too far away for an efficient pull-down. Similar observations have also been made by others (Sherman \textit{et al}, 1998; Davey \textit{et al}, 2003; reviewed in Bochman & Schwacha, 2009). Between Mcb1 and Mcm5 however there might be a direct interaction or at least the proteins seem to be in close proximity so that a pull-down is possible.

An interaction of Mcm3 with Mcb1-TAP or Mcm2-TAP could not definitely be verified, as the co-IP of Mcm3-myc with Mcb1-TAP or Mcm2-TAP was unspecific. \textit{Mcm3-13myc} cells were found to have an elongated phenotype, indicating cell defects caused by the large tag. It is possible that the 13myc-tag on Mcm3 interferes with protein-protein interactions, which might also disturb potential Mcm2-Mcm3 or Mcb1-Mcm3 interaction. Use of smaller and presumably not disturbing V5 or FLAG-tags lead to undetectable proteins.

While this study was underway, Ding \textit{et al} also reported that fission yeast Mcb1 and Mcm2 do not interact with one another but with the other Mcm proteins and that consequently two different MCM complexes might exist in fission yeast (Ding & Forsburg 2011).

A very recent study with Mcb1 (Santosa \textit{et al} 2013) showed \textit{in vivo} interaction of Mcb1 with Mcm3-7, but they also found that fission yeast Mcm2 pulls down with Mcb1 in similar co-precipitations as presented here. The general amounts of Mcm2
found in these experiments were very low compared to the other Mcm proteins so that it remains unclear if Mcm2 is really a part of that complex. Interestingly Mcm2 can also interact with Mcb1 when the latter is overexpressed (Ding & Forsburg 2011).

BiFC experiments conducted with live cells failed to deliver evidence for an interaction of Mcm4 and Mcb1 or Mcm2 and Mcb1, as no specific fluorescent signal could be observed in any of the double tagged strains. The background fluorescence observed was quite high in single- and also double tagged strains, so it could possibly conceal a real BiFC signal. This background might be reduced when using growth medium which causes even less fluorescence than EMM, like EMM without ammonium chloride (for nitrogen starvation), or more extensive washing. It can only be speculated, why the BiFC system did not confirm an Mcb1-Mcm4 interaction, as indicated by other results described here. The approach has successfully been used to visualize a number of protein-protein interactions before, e.g. GINS-Mcm4 (Akman & MacNeill 2009). It is possible that topological constraints prevent the two tags from interacting so that Venus cannot be reconstituted. Further it is also possible that the observed diffuse signal in the double tagged strains is caused by Mcm4 and Mcb1 interacting with each other. Mcb1 is thought to be mainly nuclear but can also be found in the cytoplasm (Li et al. 2011; Ding & Forsburg 2011), however the dynamics of Mcb1 in nucleus and cytoplasm are still unknown. Mcb1 could possibly take Mcm4 along to the cytoplasm, as the VN-VC interaction, once formed is very tight and might prevent dissociation of the two interaction partners. This could lead to an equally distributed but rather weak fluorescence signal, whereas interaction in the nucleus would presumably cause a stronger signal due to the smaller space.

It has to be noted that an initial BiFC experiment with the double tagged strains indicated an interaction of Mcm4-VC and Mcb1-VN. This experiment was conducted during a visit in the Kearsey lab in Oxford. A nuclear BiFC signal was observed in live and methanol fixed cells. It is unclear why these results could not be reproduced. For the experiments in Oxford some of the single tagged control cells were not examined so that it is unclear whether background fluorescence contributed to the observed signal. However it is possible that the use of different growth medium and different hardware (microscope and camera) supported the reduction of background and
enhancement of a signal, which was not the case for the experiments shown in 2.2.4. For clarity the BiFC experiments could be repeated not only using low background medium but also in the presence of a positive control.

Results from sucrose gradient centrifugation suggest that Mcb1 is part of a high molecular weight complex, which is clearly larger than the Mcb1-monomer or a Mcm-Mcb1 dimer. Mcb1 was also found to co-migrate with Mcm4 or Mcm6 containing complexes, indicating that it might be part of a complex with a size comparable to the Mcm2-7 complex. However there was no evidence for a large amount of a putative hexameric MCM<sup>Mcb1</sup>, even though a small fraction of Mcb1 was found in a complex with a size of 500kDa. The gradients are very sensitive to disruption/movement especially after centrifugation so that the relative distribution of proteins inside is easily disturbed. The fraction collection, based on the count of drops, is rather imprecise and might lead to fractions of different volume. It has to be considered as well that the mixture used for calibration of the gradients contains globular proteins, which might behave ideally running through the gradient. The Mcm proteins and Mcb1 in the complexes observed here in contrast most likely do not have a globular structure and might run slower than a globular protein of the same weight. Further, the long centrifugation time and handling of the samples could lead to a dissociation of complexes and their stability might depend on many different conditions anyway. Thus the size estimations for observed protein-complexes are only approximate and it is possible that the species observed by Western blot are only fractions of the original protein-complexes or seem smaller than they really are. Mcm4 and Mcm6, which are both known members of the Mcm2-7 complex, are mainly found in fractions corresponding to lighter complexes than Mcm2-7, thus it becomes very likely that also Mcb1 seems part of a smaller complex than it really is. To investigate the composition of MCM<sup>Mcb1</sup> further, the complex(es) could be stabilised using crosslinkers before the centrifugation. This would require highly sensitive detection methods and a set of antibodies directed against the individual Mcm proteins.

To identify direct binding partners of Mcb1, binding assays using <em>in vitro</em> translated Mcm-NTDs and purified recombinant Mcb1 were conducted. Instead of indicating two
direct interaction partners, results of these assays suggest a general ability of Mcb1-NTD to directly bind to Mcm N-terminal domains \textit{in vitro}. This is consistent with results from another study, which reported that fission yeast Mcb1 can interact with all six members of Mcm2-7 \textit{in vitro} (Santosa et al. 2013). Results presented here do not clearly show an interaction with Mcm2 \textit{in vitro}, but this could be caused by a problem with the Mcm2-NTD construct, which appeared to be unexpectedly large. It is also possible that for Mcb1-Mcm interaction the full-length Mcm2 is necessary and not only the NTD as tested here.

A possibility that the observed interactions of rMcb1-NTD and the different Mcms are not direct but probably mediated via proteins present in the reticulocyte lysate was disproved in pull-down experiments with purified recombinant Mcb1-NTD and Mcm4-NTD. The results suggest that Mcm4-NTD can indeed interact directly with Mcb1-NTD in the absence of any other protein, which is likely to be extendable to the other Mcm proteins.

Thus it looks like Mcb1 might have a general ability to bind Mcm proteins \textit{in vitro}, which could be achieved by having either several different binding sites, each specific for one Mcm protein, or by having one or several more tolerant binding sites suitable to fit all Mcm proteins.

It was tested if Mcb1 preferably binds one Mcm over the others but no competition between Mcm4-NTD and Mcm5-NTD or Mcm6-NTD could be detected. This could indicate that Mcb1-Mcm4 interaction is preferred over other Mcm interactions so that Mcm5 or Mcm6 cannot replace Mcm4. But it is more likely that Mcb1 can bind several Mcm proteins at the same time, either directly via several Mcb1-Mcm interactions or indirectly (Mcb1-Mcm-Mcm).

To confirm simultaneous binding of several Mcm proteins at the same time, more experiments would be necessary, e.g. pull-down assays with labelled Mcm6 and unlabelled Mcm4. If this leads to similar results (i.e. Mcm4 not competing with Mcm6) both proteins might indeed bind at the same time. But to address whether this binding is direct or indirect would require a different experimental setup including tagged \textit{in vitro} translated proteins that can be precipitated. But this was not available for this study. Further, \textit{in vitro} precipitations with rMcm4-NTD could be performed to show if Mcm4-NTD can bind all Mcm-NTDs or just its nearest neighbours Mcm6 and Mcm7.
and if simultaneous binding of different Mcms is possible. Previous experiments with recombinant *S. cerevisiae* Mcm dimers suggest that each Mcm subunit only interacts with two others, i.e. its neighbours in the Mcm2-7 complex (Davey et al. 2003) so that Mcm4-NTD probably does not interact with all other Mcm-NTDs. But as fission yeast Mcm N-terminal domains only are studied here, this would have to be investigated.

Mcb1-NTD seems capable to bind all Mcm-NTDs *in vitro* and individual Mcm-NTDs do not seem to compete with each other for Mcb1 interaction. The *in vivo* binding situation might be different though. Not only are there many different proteins present, which could interfere with Mcb1-Mcm interactions, but Mcms and Mcb1 also have a C-terminal domain, which might have a considerable role in protein-protein interaction. Thus Mcb1 might have a preferred Mcm- interaction partner *in vivo* and other Mcm proteins or subcomplexes could simply assemble on that initial Mcb1-Mcm pair.

Mcb1’s next neighbours in the Mcm\textsuperscript{Mcb1} complex could be investigated further by crosslinking the TAP-purified complex with special labelled crosslinker pairs and subsequent MS/MS analysis. EM would be a suitable approach to verify the multimeric nature of a Mcb1 containing complex. An attempt to produce negative-stain pictures failed however, as the purified complex was not visible (either because of degradation or too low concentration; data not shown).

MCM2-7 interacts with a large quantity of protein factors not only for its regulation, including GINS, Cdc45 in the CMG complex, but also Cdc7-Dbf4 and Cdt1. Due to its likely similarity to Mcm2-7, an Mcm\textsuperscript{Mcb1} complex could also interact with some of these factors. There is evidence that human McmBP interacts with Dbf4 but is not a DDK substrate (Nguyen et al. 2012) and that RPA also binds MCM-BP (Nakaya et al. 2010). Here, co-IP studies with Mcb1-TAP and myc-tagged GINS-subunits and Cdc45 did not reveal clear interactions. Psf2-myc seemed to interact with Mcb1-TAP, however this could not be confirmed in experiments with Psf2-TAP and Mcb1-myc or by BiFC. Also is it difficult to imagine how only the Psf2 subunit of the GINS complex can be pulled down with Mcb1 while the other subunits are not, unless the complex breaks apart. Further there was no specific interaction of Mcb1 with Cdt1, which is
Characterisation of the MCM\textsuperscript{Mcb1} complex

important for the loading of MCM2-7 hexamers onto DNA before replication. But as an interaction of Cdt1 and Mcm2 could not be reproduced either, it cannot be ruled out that the absence of interaction between Mcb1 and Cdt1 was a result of the experimental conditions used. Still, these results imply that Mcb1 does not function in association with GINS, Cdc45 or Cdt1, suggesting that it is not part of the preRC or replicative helicase.

Mcb1 was found to be approximately as abundant in fission yeast cells as Mcm proteins. The detected protein levels were not identical but in the same range, with Mcm5 levels being the lowest and Mcm7 levels the highest. This is consistent with another study in fission yeast, reporting that Mcb1 and Mcm2 are equally abundant. They also give evidence that Mcb1 levels and mobility do not change during cell cycle (Ding & Forsburg 2011). Mcm proteins were reported to be present at around $10^4$ molecules/cell in fission yeast. Their amounts were found to be comparable, with Mcm4 being the most abundant (Namdar & Kearsey, 2006). Unfortunately the levels of Mcm5 were not tested in that particular study so it remains unclear whether or not Mcm5 would be present at lower levels than the other Mcm proteins. But this shows that small differences in the Mcm-protein levels can be expected. This could be due to different expression levels of different Mcm proteins resembling their involvement in other processes than replication. For example, Mcm7 and Mcm5 seem to be involved in transcription (Fitch et al. 2003; Snyder et al. 2005) and Mcm2 was reported to have a function in histone processing during replication (Foltman et al. 2013). It is speculated that there are pools of free Mcm proteins in the cells, able to perform these additional functions (reviewed in Forsburg 2004). Still the results indicate that Mcb1 is as abundant as the Mcm proteins. This provides a possibility that a putative MCM\textsuperscript{Mcb1} complex occurs as frequently as the Mcm2-7 complex so that Mcb1 would be able to regulate Mcm function or compete with Mcm2 for other Mcm proteins.

Concluding, it is still not clear what an Mcm\textsuperscript{Mcb1} complex looks like in terms of subunit composition and order. Results presented here indicate that Mcb1 interacts with Mcm3-7 but not with Mcm2. Further there is evidence that Mcb1 is part of a high
molecular weight complex, presumably including Mcm proteins. Thus evidence in favour of an abundant hexameric complex is accumulating and interaction with Mcm3, 4, 5, 6 and 7 has been shown not only in fission yeast but also other model organisms, while interaction with Mcm2 remains a controversy. Mcb1 could replace Mcm2 in an alternative kind of Mcm complex consisting of Mcb1 and Mcm3-7. A simplified model of such a MCM$^\text{Mcb1}$ complex in contrast to the canonical Mcm2-7 is shown in Figure 2-17.

![Figure 2-17 Model of MCM$^\text{Mcm2}$ and MCM$^\text{Mcb1}$ from C-terminal ends of the proteins](image)

Results presented here also suggest that Mcb1 has a general ability to bind all Mcms \textit{in vitro}, which might be subject to regulation \textit{in vivo} and lead to a certain subunit order in the Mcm$^\text{Mcb1}$ complex. However this general binding ability could serve as basis for a possible role of Mcb1 in regulation of Mcm activity, which will be discussed later.
Chapter 3 Functional analysis of Mcb1

3.1 Introduction

The function of MCM-BP is still unclear. Initial studies with human cells indicated a contribution of MCM-BP to loading or stabilisation of Mcm proteins on chromatin (Sakwe et al. 2007) while results from *Xenopus* suggested a role in MCM unloading from DNA after replication (Nishiyama et al. 2011) and the plant homologue Etg1 was suggested to play a role in establishment of sister chromatid cohesion (Takahashi et al. 2010).

One aim of the study presented here was to investigate the function of fission yeast Mcb1 by deleting the *mcb1*<sup>+</sup> gene. Further, other strategies should be pursued to gain some insight into the role of the protein.

Conditional inactivation can be very helpful to study protein function *in vivo*, especially when the protein of interest is essential and therefore cannot be deleted. Ideally the inactivation is quick, reversible and efficient so that the effects can be evaluated before any secondary effects complicate the analysis. The most common method for conditional inactivation is the use of temperature-sensitive mutants, which is quite easily achieved in the haploid *S. pombe*. In addition other systems have recently been developed to conditionally inactivate or degrade a target by tagging it with a binding domain for hormones (HBD; Mattioni et al. 1994; Bøe et al. 2008) or with a sequence targeting for degradation (temperature-sensitive degron, AID-system (Kanke et al. 2011)). These systems often allow for a rapid inactivation of the target but only work for some proteins, not others. It is also possible to regulate the level of protein by controlled gene expression using promoters such as the fission yeast *nmt1* promoter and its derivatives (Maundrell 1990; Basi et al. 1993).

Apart from conditional inactivation, the creation of insertion mutants or truncated versions of a protein can be useful to identify important functional regions or study loss of function effects. And of course the regulation of proteins at transcript level by RNA-based methods (RNAi) has become very important in recent years. However,
these strongly depend on the stability of the target protein, often leading to only incomplete knock-down of the target, and are prone to off-target effects. This chapter describes the results of different approaches to inactivate Mcb1 to study its function and the consequences for fission yeast cells. This includes the use of temperature-sensitive mutants, auxin-inducible degradation (AID), regulation of activity by a hormone binding domain (HBD) as well as controlled expression from the \textit{nmr1} promoter and its derivatives. Effects of insertion mutants and truncations will be discussed in Chapter 4.

Temperature-sensitive mutants (3.3) were isolated by Juan-Juan Li and Jacky Hayles (Cancer Research UK, London Research Lab) and given to us for further analysis. Sensitivity assays of \textit{Ts} mutants to HU, 4-NQO, CPT, MMS and UV radiation described in 3.3.1 and deletion of \textit{mcb1}+ (3.2) were conducted by Dr Stuart MacNeill.

\section*{3.2 Deletion - \textit{mcb1} is essential}
In order to test whether \textit{mcb1}+ is essential, one copy of the gene in an \textit{mcb1}/\textit{mcb1}+ diploid strain was replaced with the \textit{ura4}+ selectable marker using the standard PCR-mediated gene deletion method (Bähler et al. 1998). The resulting \textit{mcb1}/\textit{mcb1::ura4}+ diploid was then sporulated and the meiotic products (azygotic asci containing four spores) were analysed by tetrad dissection using a micromanipulator. Only two spores from each ascus were viable; the viable spores were all \textit{ura4}−, implying that the \textit{mcb1::ura4}+ spores were non-viable. Microscopic examination of the germinated spores after 18 hours showed elongation of the cells consistent with a cell cycle arrest, which will be discussed later in this study. Thus \textit{mcb1}+ is essential in \textit{S. pombe}. Fission yeast was the first organism in which \textit{MCM-BP} was found to be essential, as so far it does not seem to be in human cells, \textit{Xenopus} or \textit{A. thaliana}. However, recent data suggests that \textit{MCM-BP} is essential in the parasite \textit{T. brucei} as well (Kim et al. 2013).
3.3 Conditional/ Functional inactivation of Mcb1 using temperature-sensitive mutants

3.3.1 Construction and properties of temperature-sensitive mutants of Mcb1

For further analysis of Mcb1 function, temperature-sensitive mutants were isolated by Juan-Juan Li from J. Hayles’ lab using a previously described PCR-based method (Fong et al. 2010) with mcb1-YFP-natMX6 as the starting construct. A number of different temperature-sensitive mutants were isolated, of which two were used in this study, called mcb1-Ts1 (this encodes a protein with a single amino acid change, S465P) and mcb1-Ts6 (two single amino acid changes, S452P and S484P). All these mutations map to the very C-terminus of the Mcb1 segment of the Mcb1-YFP protein (see Figure 3-1 A).

When grown at the permissive temperature of 25°C, the Ts mutants behaved like the wild-type, showing similar growth rates and regularly shaped cells of comparable size. But the Ts mutants, unlike wild-type cells, were unable to form colonies at the non-permissive temperature of 35°C (Figure 3-1 B) and cells were found to arrest with a single nucleus and an elongated phenotype after seven hours at 35°C (Figure 3-1 C). mcb1-Ts6 was found to produce cells that were even more elongated than the mcb1-Ts1 mutants. Flow cytometric analysis of mcb1-Ts cells showed that the mutants arrest with a 2C DNA content when incubated at non-permissive temperature, indicating that bulk DNA replication is finished in these cells (Appendix 1 and Li et al. 2011).

Furthermore, the recovery of mcb1-Ts1 and Ts6 strains when incubated at non-permissive temperature for 2-8 hours was reduced in comparison with wild-type, and survival of mcb1-Ts1 and Ts6 cells began to drop after 4 hours (Figure 3-2 A). Again mcb1-Ts6 showed a stronger phenotype than mcb1-Ts1, with even less surviving cells. Thus the mcb1-Ts1 and Ts6 mutants appeared suitable to inactivate Mcb1 and were further used to investigate the effects of Mcb1 absence on fission yeast cells.
Figure 3-1 Functional inactivation of Mcb1 using temperature-sensitive mutations I. A) Schematic representation of the Mcb1 protein showing the location of the temperature-sensitive mutations in mcb1-Ts1 and mcb1-Ts6. The locations of AAA+ motifs characteristic for Mcm proteins (but not present in Mcb1) are indicated as well. B) Spotting assay of serial dilutions of wild-type, mcb1-Ts1 and mcb1-Ts6 strains on YE at permissive temperature of 25°C and non-permissive temperature of 35°C. C) Microscopy analysis of Hoechst 33342 stained wild-type, mcb1-Ts1 and mcb1-Ts6 cells following incubation at permissive or non-permissive temperature. Scale bar 20 μM.
The mutants’ sensitivity to the different replication stressing or DNA damaging agents hydroxyurea, camptothecin, MMS, MMC, 4-NQO or UV radiation was assessed by spotting assays.

Hydroxyurea (HU) inhibits the ribonucleotide reductase, which leads to an exhaustion of available dNTPs in the cells, causing fork stalling in early S-phase. In fission yeast this activates the DNA replication checkpoint (Lindsay et al. 1998). MMS (methyl methanesulfonate) is a DNA methylating agent, which methylates mainly N3 of deoxyadenosine and N7 of deoxyguanosine. This presumably leads to physical block of
replication forks in S-phase and also formation of double strand breaks during the attempt to repair the damaged forks (Groth et al. 2010). Homologous recombination repair (HR) but also nucleotide excision repair (NER) and base excision repair (BER) seem to contribute to the repair of defects caused by alkylation, and cells deficient in these mechanisms are sensitive to elevated levels of drugs as MMS (Memisoglu & Samson 2000; Kanamitsu & Ikeda 2011). Camptothecin (CPT) specifically poisons topoisomerase I, by stabilising a topoisomerase I-DNA complex (Hsiang et al. 1985; Porter & Champoux 1989; Hertzberg et al. 1989; Wan et al. 1999). Collision of active replication forks with this topoisomerase I-DNA complex is assumed to result in double strand breaks (reviewed in Pommier, 2006) and activation of the DNA damage checkpoint in fission yeast. Defects in checkpoint activation thus make cells hypersensitive to CPT treatment (Wan et al. 1999). 4-nitroquinoline 1-oxide (4-NQO) forms bulky adducts with DNA (Kohda et al. 1991; Tada 1976), which are usually processed by nucleotide excision repair (NER), similar to dipyrimidine photoproducts resulting from UV irradiation. Consequently, cells deficient in the NER pathway are hypersensitive to 4-NQO and UV light. Mitomycin C acts as a DNA crosslinking agent leading to replication block by fork stalling and triggers activation of the DNA damage checkpoint. The repair of such interstrand crosslinks is mediated by NER and HR in S. pombe but also in S. cerevisiae and mammals (Lambert et al. 2003).

Serial dilutions of wild-type, mcb1-Ts1 or mcb1-Ts6 cells were spotted onto YE supplemented with hydroxyurea (3, 6, 9, 12 mM), methyl methanesulfonate (0.006, 0.008, 0.010, 0.012 %), 4-NQO (0.2, 0.4, 0.6, 0.8 µg/ml), camptothecin (4, 8, 12, 16 µg/ml), or irradiated with UV-light (254 nm; 100, 200, 300 J/m²) before incubation at permissive temperature of 25°C (Figure 0-2 in Appendix 1; published in Li et al, 2011). Dilutions were also spotted onto YE containing mitomycin C (100 or 200 µg/ml Figure 3-2 B) and incubated at semi-permissive temperature of 30°C. However, the Ts mutants grew like wild-type in the presence of all tested drugs or after UV irradiation (Figure 3-2 B and Figure 0-2 in Appendix 1; Li et al, 2011). Thus mcb1-Ts1 and mcb1-Ts6 cells are not more sensitive to any of the tested DNA stressing agents than the wild-type.
3.3.2 Inactivation of Mcb1 leads to DNA damage

The presence of elongated cells when Mcb1 is inactivated can be a sign of checkpoint activation in G2-phase. Activation of such a checkpoint delays cell cycle progression into mitosis and gives the cell time to solve problems having occurred e.g. during DNA replication. If there are severe problems with DNA replication or extensive DNA damage, the cells might be unable to repair the defect and will permanently arrest their cell cycle, eventually leading to cell death. As cells do not stop growing in length during checkpoint activation or cell cycle arrest, elongation can serve as an indicator for checkpoint activation and hint at problems with DNA replication or DNA damage.
Figure 3-3 DNA damage and replication problems. Cells expressing Rad22-YFP in wild-type, mcb1-Ts1 or mcb1-Ts6 background (Sp611, Sp612, Sp613, Sp614, Sp617, Sp618, Sp620, Sp643) were grown in EMM at 25°C to mid log phase, diluted to OD 0.3 and incubated at non-permissive temperature of 36°C for 0-4 hours before examination by fluorescence microscopy. Cells and quantity of YFP-foci were counted from microscopy images. Per strain at least 200 cells were examined. Arrows indicate nuclei with Rad22-YFP foci. A) Temperature-sensitive mcb1- mutants exhibit a slightly increased number of Rad22-YFP foci even at permissive temperature. Cells expressing Rad22-YFP in wild-type or mcb1-Ts background were grown at 25°C and examined by fluorescence microscopy. To induce DNA damage, wild-type cells were treated with 0.0075% MMS for four hours (wt_MMS). B) and C) Incubation at non-permissive temperature rapidly increases the number of Rad22-YFP foci in Ts mutants. Strains were incubated at non-permissive temperature of 35°C for 0, 2 or 4 hours prior to analysis. Fractions of cells harbouring Rad22-YFP repair foci were determined from microscopy images (B). Microscopy images showing Hoechst stained nuclei and Rad22-YFP foci at indicated time points (C). Scale bar 20 µm.
DNA damage can be indirectly visualised by the appearance of Rad22-YFP foci in cells (Noguchi et al. 2003). Rad22 is the Rad52 homologue in *Schizosaccharomyces pombe* and is important for an early step of double strand break repair by homologous recombination (Kim et al. 2000). It is recruited to sites of DNA damage, binds ssDNA ends and stimulates strand exchange. Rad22 or Rad52 fusion proteins with GFP or YFP can thus be used as a fluorescent marker for DNA damage (Noguchi et al. 2009; Noguchi et al. 2003). Cells carrying *rad22-YFP* in *mcb1-Ts1* or *Ts6* background were examined for the formation of Rad22-YFP foci and compared to wild-type cells and cells where DNA damage had been induced by treatment with 0.0075% MMS for four hours.

At the permissive temperature of 25°C, the number of *mcb1-Ts* cells with at least one Rad22-YFP foci was increased compared to the wild-type (*Ts1*: 25% *Ts6*: 33% wt: 13%; see chart with cells at 25°C Figure 3-3 A). However, in wild-type cells where DNA damage had been induced by MMS treatment almost 50% of cells had Rad22-YFP foci, some of them multiple (Figure 3-3 A).

Incubation at non-permissive temperature for two hours increased the amount of *mcb1-Ts* cells with foci to 35% (*Ts1*) and 48% (*Ts6*) and even 66% (*Ts1*) and 68% (*Ts6*) after 4 hours while in the wild-type the proportion of cells with foci was constantly less than 15%. Noticeably after 4 hours at 36°C many of the *mcb1-Ts* cells displayed multiple Rad22-YFP foci, which was very rare in wild-type cells (Figure 3-3 B and C) but frequent in wild-type cells with MMS induced double strand breaks (compare Figure 3-3 A). Thus the *mcb1-Ts* mutants accumulate DNA damage when incubated at non-permissive temperature, which is comparable to the frequency of damage observed after treatment of cells with MMC, indicating that they are unable to repair the damage.

The cell cycle distribution and the distribution of cells with Rad22-YFP foci in temperature-sensitive and wild-type were examined. Cells were assigned to a particular cell cycle stage by looking at their morphology on microscopy images. A noticeable feature of the fission yeast cell cycle is its very short G1-phase and long G2-phase. Therefore, cytokinesis with formation of septa and final fission of the daughter
Functional analysis of Mcb1
cells does not finish in G1, but later in interphase. Thus, late M and G1-phase cells characteristically have two nuclei, while early S-phase cells can be recognized by the presence of a septum between the two nuclei, which is also the position where cells separate later in S-phase. Cells with one single nucleus were consequently assigned to G2-phase (Kim & Huberman 2001).

Figure 3-4 Cell cycle distribution of asynchronous wild-type and mcb1-Ts cells. Cells expressing Rad22-YFP in wild-type, mcb1-Ts1 or mcb1-Ts6 background were treated as described in Figure 3-3. Cell cycle stages were determined by cell morphology from microscopy images as in Figure 3-3. A) Distribution of cells having Rad22-YFP foci before and after 2 or 4 hours at 36°C. B) General cell cycle distribution of asynchronously growing cells before and after 2 or 4 hours at 36°C.

In asynchronous exponential fission yeast cultures the majority (~80%) of cells were found in G2-phase, while late M/G1 and S-phase cells were found to make around 10% of the population (Figure 3-4 B). This distribution did not change much in the Ts strains at permissive temperature or the wild-type strain even at high temperature (Figure 3-4 B). The mcb1-Ts6 mutant showed slightly increased levels of S-phase cells.
after two hours and both mutants had increased levels of G2 cells after four hours at non-permissive temperature. This increase indicates that the temperature-sensitive mutants activate a checkpoint and arrest their cell cycle in G2-phase when incubated at non-permissive temperature. It would thus be expected that at some point the entire population of \textit{mcb1-Ts} cells will be found to be in G2.

The distribution of Rad22-YFP foci (Figure 3-4 A) overall resembled the cell cycle distribution of cells with most foci appearing in G2 cells. In wild-type and Ts strains slightly more Rad22-YFP foci were found in S-phase than in G1-phase cells (15\% S, 5-10 \% G1) which could be explained by the fact that replication in S-phase is a major source for DNA damage. Most of the cells displaying YFP foci however were found in G2, where most of the damage is repaired. After two hours at non-permissive temperature there was a slight increase in Rad22 foci in S-phase cells (from 10 \% to 16\%) in the Ts mutants that dropped again after four hours, coinciding with an increase in G2. The increase of S-phase cells with Rad22-YFP foci in the Ts mutants indicates that S-phase cells start accumulating DNA damage after two hours at non-permissive temperature. When these cells pass on to G2-phase they apparently still have the Rad22- foci. If there is a G2 arrest in \textit{mcb1-Ts} cells, presumably all Rad22-YFP foci would be found in G2 cells at some point.

In general inactivation of Mcb1 using temperature-sensitive mutations seems to cause increased levels of DNA damage in cells, which could be associated with problems in DNA replication. Accumulation of such DNA damage further indicates that the cells have difficulties sufficiently repairing it, which might lead to cell death eventually. It has to be noted that the presence of the temperature-sensitive mutation in Mcb1 already leads to more sites of DNA damage than the wt at permissive temperature indicating at least a partial defect of the proteins. Further it seems that the phenotype caused by \textit{mcb1-Ts6} is more severe than in \textit{Ts1}, implying that the Ts6 mutant might be more efficient in inactivating the protein. The accumulation of DNA damage in the Ts mutants at non-permissive temperature is accompanied by an increase of the cell number in G2-phase. This all indicates that inactivation of Mcb1 causes DNA damage leading to an irreversible cell cycle arrest in G2-phase.
3.3.3 The Chk1 dependent but not the Cds1 dependent checkpoint is responsible for cell cycle arrest in mcb1-Ts mutants

As mentioned before, the elongated morphology observed when inactivating Mcb1 is characteristic of checkpoint activation leading to cell cycle arrest. In fission yeast there are two checkpoints controlling the integrity of S-phase and DNA replication. The DNA replication checkpoint is activated when problems occur very early in DNA replication, e.g. fork stalling by hydroxyurea induced shortage of deoxyribonucleotides, and depends on the Cds1 kinase (Lindsay et al. 1998). The DNA damage checkpoint in contrast is induced by different kinds of DNA damage (which can cause problems with replication) and results in the phosphorylation of the Chk1 (Checkpoint kinase 1) kinase (Walworth & Bernards 1996; Capasso et al. 2002). Both pathways are controlled by the upstream Rad3 kinase (Figure 3-5 A) and arrest the cell at the G2/M transition through phosphorylation of Cdc25 (Zeng et al. 1998).

To determine the checkpoint response in mcb1-Ts cells, the temperature-sensitive mutants were combined with deletions in cds1, chk1 or their common upstream sensor rad3 by crossing strains containing the respective alleles. The temperature-sensitive checkpoint mutants were examined by microscopy after different incubation times at non-permissive temperature (Figure 3-5 B and Figure 3-6).

After only four hours at 35°C the mcb1-Ts rad3Δ cells displayed abnormal morphology (fragmented nuclei, septated cells with unequally divided nuclei, abnormal nuclear morphology) due to abrogated checkpoint activation. The cds1Δ cells did not show a similar phenotype after incubation at 35°C but were elongated like the simple temperature-sensitive mutants, indicating an active checkpoint response even in the absence of Cds1 (Figure 3-5 B and Figure 3-6 row 3). Deleting chk1 however lead to a phenotype comparable to rad3Δ with nuclear abnormalities indicating checkpoint failure. Thus Chk1 is important for checkpoint activation in mcb1-Ts cells (Figure 3-5 and Figure 3-6 row 4). When comparing the different mcb1-Ts mutants it was found that the phenotype associated with chk1Δ and rad3Δ was more severe and started earlier in the Ts6 background (Figure 3-5 B) again indicating that the mutations in this strain inactivate the protein more efficiently.
**Figure 3-5**

A) Cds1 dependent DNA replication checkpoint and Chk1 dependent DNA damage checkpoint in fission yeast. (Adapted from Sabatinos, 2010).

B) Deletion of chk1 or rad3 but not cds1 in the mcb1-Ts6 background abrogates checkpoint activation. Microscopy analysis of indicated strains. Cells were grown to mid log phase at 25°C or at non-permissive temperature of 35°C for 5 hours before nuclear staining with Hoechst 33342. Arrows indicate aberrant nuclei. Scale bar 20 μm.
Functional analysis of Mcb1

Figure 3-6 Deletion of chk1 or rad3 in the mcb1-Ts1 background abrogates checkpoint activation. Microscopy analysis of indicated strains. Cells were grown to mid log phase at 25°C or at non-permissive temperature of 35°C for 5 hours before nuclear staining with Hoechst 33342. Arrows indicate aberrant nuclei. Scale bar 20 μm.

The survival rate of the mcb1-Ts checkpoint mutants when incubated at non-permissive temperature for two to eight hours followed by recovery on rich medium at permissive temperature was also examined (Figure 3-7 A). It was found that chk1Δ in the temperature-sensitive background leads to a severe drop in viability after six hours of incubation at 35°C, while the survival of mcb1-Ts alone or mcb1-Ts cds1Δ is less affected. As expected, in mcb1-Ts rad3Δ cells the recovery rates also dropped dramatically.
This data indicates that the Chk1 dependent DNA damage checkpoint but not the Cds1 dependent replication checkpoint response is triggered when Mcb1 is inactivated in the Ts mutants.

Chk1 is activated at a molecular level by Rad3 mediated phosphorylation at S345 (Figure 3-5 A, Capasso et al, 2002). This phosphorylation can be visualised as a mobility-shift by Western blot, as phosphorylated Chk1 migrates more slowly than the non-phosphorylated protein. The activation of the Chk1-checkpoint in the Ts mutants at non-permissive temperature was confirmed by Western blot using mcb1-Ts1 chk1-HA or mcb1-Ts6 chk1-HA cells and an anti-HA antibody. A Chk1-HA band-shift appeared in samples from both mcb1-Ts strains taken after incubation at 36°C, indicating phosphorylation of the protein, which could not be observed in wild-type samples (Figure 3-7 B).

Figure 3-7 The Chk1 dependent but not the Cds1 dependent checkpoint is responsible for cell cycle arrest in mcb1-Ts. A) Survival of cells expressing temperature-sensitive alleles mcb1-Ts1 or mcb1-Ts6 in combination with checkpoint deletions after 0-8 hours incubation at non-permissive temperature of 35°C. 600 cells were plated on YE per time-point. Data from two experiments per curve. B) Phosphorylation of Chk1 after inactivation of Mcb1 indicates activation of Chk1 dependent DNA damage checkpoint. mcb1-Ts1, mcb1-Ts6 or wild-type were grown at 25°C before shifting to 36°C for six hours. Soluble extracts were examined by Western blot.
3.3.4 Mcb1-Mcm4 interaction is not impaired in mcb1-Ts mutants

While this work was in progress, Santosa et al showed that a single amino acid change (L254P or L363P) in Mcb1 leads to temperature sensitivity accompanied by the inability to interact with Mcm proteins even at permissive temperature (Santosa et al. 2013). Hence it was investigated whether the interaction of Mcb1 with Mcm proteins would also be compromised in the mcb1-Ts1 and mcb1-Ts6 mutants. Mcm4 was chosen as representative of Mcm proteins because of the strong and clear results from previous interaction studies (see chapter 2.2.3).

Pull-down of Mcb1-Ts6 with Mcm4-TAP was investigated by co-immunoprecipitation on IgG-Sepharose. Cultures were grown at permissive or non-permissive temperature, soluble extracts prepared and the Mcb1 protein detected by Western blot and using an α-Mcb1 antibody and the LICOR Odyssey imaging system.

Mcb1-Ts6 co-immunoprecipitated with Mcm4-TAP in extracts from cells, which had been grown at permissive or non-permissive temperature for six hours, in similar quantities (Figure 3-8 left side, upper panel, compare lanes 5 and 6). Further the

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**Figure 3-8 Mcb1 interaction with Mcm4 is not impaired in mcb1-Ts6 mutants.** Co-immunoprecipitation of Mcb1-Ts6 with Mcm4-TAP. **Left panel:** Strains with mcb1-Ts6 (having a C-terminal YFP-tag) or mcb1-YFP and mcm4-TAP were grown to mid log phase at permissive (25°C) or non-permissive (36°C) temperature. Soluble extracts were made and Mm4-TAP was precipitated using IgG-Sepharose (GE). Input (7.5% lower panel) and co-immunoprecipitations (co-IP; 100%, upper panel) were analysed by Western blot and α-Mcb1 antibody using the Licor OdysseyCLX for detection. As control, mcm4- cells (without Mcm4-TAP) were processed in the same way. Mcm4-TAP can be detected by rabbit α-Mcb1, as rabbit IgG in general has a strong affinity to protein A in the TAP-tag (Weser et al. 2006). **Right panel:** Controls; mcb1-Ts6, mcb1-YFP or mcb1- cells with or without mcm4-TAP were processed as before. Mcb1 was detected by α-Mcb1 (red) and α-GFP (green; yellow in merge with α-Mcb1). Asterisks mark degradation products of mcb1-YFP.
amount of Mcb1-Ts6 protein pulled-down with Mcm4-TAP was comparable to the amount of Mcb1-YFP (lanes 7 and 8) at both temperatures and also to the amount of wt Mcb1 (Figure 3-8 right side, lane 12).

It might be possible that the 6 hours incubation time at non-permissive temperature were not enough to disrupt the interaction of the Ts mutant with Mcm4-TAP. However the temperature-sensitive cells had started to show an elongated phenotype by that time, indicative of Mcb1 inactivation. Thus the Ts6 mutant most likely does not interfere with the Mcb1- Mcm4 interaction, at least not under the tested conditions.

It has to be noted that the additional band at 55 kDa found in the pull-down of Mcb1-YFP with Mcm4-TAP (Figure 3-8 upper panels, lanes 7, 8, 13 and 14 marked by asterisk) is presumably caused by degradation of Mcb1-YFP and loss of the YFP-tag. This is possible as the linker between Mcb1 and YFP represents a weak point, likely being accessible for proteases. It is unclear why the temperature-sensitive mcb1 alleles (lanes 5, 6 and 15) are not affected in a similar way, however these mutants were constructed by others, and it cannot be ruled out that there are additional mutations in the linker or that the linker has a different length protecting it from proteolysis.

This does not impair the result, as even when adding up the band intensities (Figure 3-8 upper panels), the pull-down of the Mcb1-Ts6 is still comparable and by no means significantly reduced, so that the mcb1-Ts6 mutants do not show reduced interaction with Mcm4 at permissive temperature and also not after six hours at non-permissive temperature.

3.4 Inactivation of Mcb1 by fusion to an estradiol receptor hormone binding domain

Besides the isolation of conditional Ts mutants another method to functionally inactivate proteins in fission yeast is the fusion of a target protein to a hormone binding domain (HBD) of steroid receptors such as the estrogen receptor. This cassette can then be used to quickly and reversibly regulate the activity of the target protein by hormonal control (Mattioni et al, 1994; Bøe et al, 2008; see Figure 3-9 A). In the absence of estrogen hormones, the HBD is bound by the Hsp90 molecular chaperone,
which might inactivate the fused target protein by steric hindrance. Upon estradiol addition, the hormone is thought to displace the Hsp90 from the HBD leading to the re-activation of the fusion protein (Mattioni et al. 1994). This system has been used previously to efficiently and reversibly regulate the activity of e.g. Cdc13, Psf1 and Psf2 (Pai et al. 2012; Pai et al. 2009; Bøe et al. 2008).

mcb1 was tagged at its 3′ end with sequences encoding a hormone binding domain (HBD) using PCR-mediated gene targeting (Bähler et al. 1998). Transformants were selected on medium containing 250 nM estradiol and tagging was confirmed by PCR and sequencing. The successful transformants were viable and had a wild-type like appearance in the presence of estradiol.

\[ \text{Inactive} \quad \text{Protein X} \quad \text{HBD} \quad \text{Hsp90} \]

\[ \text{Active} \quad \beta\text{-estradiol} \quad \text{Protein X} \quad \text{HBD} \]

**Figure 3-9** Fusion to estrogen receptor hormone binding domain (HBD) does not efficiently inactivate **Mcb1 A)** Schematic representation of the regulation of an HBD fusion protein by estradiol. Adapted from (Pai et al. 2012). **B)** mcb1-HBD (Sp536) or wild-type (Sp347) cells were grown in YE in the presence of estradiol and serial dilutions were spotted onto YE, YE supplemented with 250 nM estradiol, YE with 8 mM hydroxyurea, or YE with 250 nM estradiol and 8 mM hydroxyurea and incubated at the indicated temperatures for 2 to 4 days.
It was analysed if the HBD was suitable to conditionally inactivate Mcb1. Therefore, serial dilutions of wild-type or mcb1-HBD cells were spotted onto YE in the presence or absence of estradiol at different temperatures to see if the fusion constructs, combined with the addition or deprivation of the hormone, had any effect on growth of the strain (Figure 3-9 B). However no growth retardation was observed for the mcb1-HBD cells in comparison to wild-type in the absence of estradiol, neither at 20°C nor at higher temperatures (Figure 3-9 B, compare panels +estradiol and −estradiol; Pai et al., 2012). This led to the conclusion that the fusion of Mcb1 to the HBD is not sufficient to completely inactivate the protein. But it seemed possible that the system leads to a partial inactivation or delocalisation of Mcb1, as Hsp90 is thought to be mainly cytoplasmic (Matsuyama et al. 2006), and could shift the localisation of some Mcb1-HBD to the cytoplasm, when bound. This might inactivate some of the protein, which could be manifested in sensitivity towards replication stressing agents, as Mcb1 is thought to function in replication. Thus spotting assays were conducted on YE supplemented with hydroxyurea and with or without estradiol. But the HBD-fusion constructs did not make the cells more sensitive to hydroxyurea at any of the tested temperatures (Figure 3-9 B, compare panels +hydroxyurea +estradiol, +hydroxyurea −estradiol). Thus the hormone regulation does not sufficiently inactivate the Mcb1-HBD fusion to produce a phenotype here, which has been observed for the regulation of other proteins as well (Pai et al. 2012; Bøe et al. 2008).

3.5 Depletion of Mcb1 using an auxin-inducible degron approach

The auxin-inducible degron (AID) system (Nishimura et al. 2009) and its improved versions (iAID and off-AID) (Kanke et al. 2011) have been shown to be powerful protein-depletion systems in fission yeast. Using these approaches, the efficient depletion of replisome components such as Mcm4, Cdc45 and Pol1 (Kanke et al. 2011) and Mcm10 (Kanke et al. 2012; Watase et al. 2012) could be achieved.

The system involves a plant specific mechanism, stimulated by the hormone auxin, leading to the ubiquitin-mediated degradation of Aux/IAA transcription repressors (Figure 3-10 A, described in Kanke et al. 2011). Auxin binds to the Tir1 (Transport inhibitor response 1) F-box protein, which leads to activation of the E3 ubiquitin ligase
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SCF (Skp1, Cullin, F-box protein; Skp1= S-phase kinase-associated protein 1). SCF\textsuperscript{Tir1} then binds Aux/IAA transcription repressors, leading to their poly-ubiquitination and proteasomal degradation (reviewed in Chapman & Estelle, 2009). As the SCF pathway and the ubiquitin-mediated degradation are conserved among eukaryotes it is possible to utilize the auxin-induced system in non-plant cells. Therefore it is necessary to tag the target protein with a plant IAA-degron, additionally express Tir1 or fusion proteins like Skp1-AtTir1 (fission yeast Skp1 and *Arabidopsis* Tir1) or Skp1-OsTir1 (with rice Tir1) and to add auxin (e.g. synthetic auxin 1-naphtaleneacetic acid NAA) (Nishimura et al. 2009; Kanke et al. 2011).

The *mcb1*\textsuperscript{+} and *mcm2*\textsuperscript{+} genes were tagged at their 3\textquotesingle end with sequences encoding the IAA-tag in strains expressing Skp1-AtTir1-2NLS or Skp1-OsTir1 and Skp1-AtTir1-2NLS from the *adh15* promoter (p\textsubscript{*adh15*-Skp1-AtTir1-2NLS or p\textsubscript{*adh15*-Skp1-OsTir1-natMX6-p\textsubscript{*adh15*-Skp1-AtTir1-2NLS; generous gifts from H. Masukata’s lab}). The expression of two different Tir1 protein constructs in one strain is supposed to increase total Tir1 levels and amplify the degradation effect (Kanke et al. 2011). The integrity of the strains was verified by PCR and sequencing. Cells were viable and had a wild-type like appearance. Initial tests if the presence of auxin had an effect on the Mcm2-IAA and Mcb1-IAA fusion constructs were conducted by patching cells onto YE supplemented with auxin. No severe growth retardation could be determined, but when observed under the microscope, the *mcm2*-IAA *skp1*-AtTir1-2NLS cells showed a slightly elongated morphology, while the cells expressing Mcb1-IAA were unaffected (not shown).

To see if there is an effect of auxin on the IAA-fusion constructs, the Mcb1 or Mcm2 protein content before and after auxin treatment was examined by Western blot. Unfortunately the proteins could not be detected at first, presumably due to the weak signal emanating from the 2HA-tag with only two copies of HA. Immunoprecipitation of HA-tagged protein from extracts of auxin treated/ untreated cells and optimisation of the Western blot procedure and detection (LICOR OdysseyCLX) finally allowed examination of Mcb1-IAA and Mmc2-IAA protein levels.

It was found that the auxin treatment did not significantly reduce the amount of Mcb1 or Mmc2 in the extracts (Figure 3-10 B). Interestingly Mcb1 appears as multiple bands which could be caused by a degradation of the protein or a stepwise loss of the tag,
but the combined signals were still equally strong in auxin treated and untreated samples.

**Figure 3-10** The auxin-inducible degron (AID) system does not cause a phenotype and does not decrease the amount of Mcb1 protein. Strains used express Mcb1-2HA-IAA or Mcm2-2HA-IAA either from its endogenous or the nmt41 promoter and Skp1-AtTir1-2NLS-9myc (iAID) or Skp1-OsTir1 and Skp1-AtTir1-2NLS (offAID) from the adh15 promoter. A) Schematic representation of auxin induced degradation of IAA-tagged target protein. B) Auxin treatment alone does not lead to reduction of Mcb1.
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or Mcm2 protein levels in offAID strains. Wild-type, mcb1-2HA-IAA-offAID (lanes 1) or mc当地区-2HA-IAA-offAID (lanes 2) were incubated with auxin for 3 hours. HA-IAA-tagged proteins were immunoprecipitated from soluble extracts (total protein content of 5 mg) and detected by Western blot. As control, Protein A beads with α-HA (lanes α-HA) and without α-HA (lanes beads) are shown. C) Auxin treatment does not lead to increased sensitivity towards DNA damage. Indicated strains were grown in YE to mid log-phase and serial dilutions were spotted onto YE, YE supplemented with auxin, auxin and 6 mM hyroxyurea or auxin and 0.01% MMS and incubated for 2.5- 4 days. D) Auxin treatment does not further reduce levels of Mcb1-2HA-IAA when Mcb1- expression is down regulated by 41ntt1. Mcb1-2HA protein from extracts of indicated strains was immunoprecipitated on α-HA resin and monitored by Western blot. * indicates the heavy chain of α-HA antibody.

This leads to the assumption that the simple auxin-induced degradation of Mcb1 or Mcm2 is not successful here. Kanke et al (2011) described that some proteins, like Mcm4, cannot be sufficiently degraded using only IAA fusion proteins and expression of Tir1, but need the application of an extended AID system, additionally repressing transcription of the target gene. As Mcm2 and Mcb1 are similar to Mcm4, they might also require this extended system for efficient degradation.

Therefore additional downregulation of Mcb1-IAA expression levels was attempted, by placing the gene construct under the control of the nmt41 promoter, a weaker derivative of the nmt1 promoter which can be used to efficiently reduce protein expression (Basi et al, 1993, also see 3.6). Strains expressing p_{nmtn}-mcb1-2HA-IAA in the p_{adh15}-skp1-AtTir1-2NLS-9myc background (referred to as iAID) or p_{adh15}-skp1-OsTir1-natMX6-p_{adh15}-Skp1-AtTir1-2NLS background (referred to as offAID) were made by PCR-mediated gene targeting using the previously described AID strains without nmt41 as starting material.

The new p_{nmtn}-mcb1-2HA-IAA and old mcb1-2HA-IAA strains (with iAID and offAID background) were grown in the presence of thiamine to repress the promoter and reduce expression of nmt41-controlled mcb1. Serial dilutions were spotted onto YE or YE supplemented with auxin to induce degradation. But none of the AID- strains (iAID and offAID) showed any different growth than the control strains (wt, 41ntt-mcb1, Figure 3-10 C). A slight downregulation of Mcb1 induced by the degron would possibly not have an effect on the growth of the cells under ideal conditions but would maybe show as growth defect under DNA stressing conditions. Therefore serial dilutions of the AID strains were also spotted onto YE supplemented with auxin and hydroxyurea or auxin and MMS. However the AID strains did also not exhibit an increased sensitivity towards the DNA damaging agents in comparison with the control strains.
here (Figure 3-10 C). Hence, the additional downregulation of Mcb1 in the AID background does not affect cell growth. Sometimes even a drastic reduction of the amount of a specific essential protein does not influence cell viability and if this is the case with Mcb1, auxin-induced degradation would not cause a visible growth reduction in spotting assays.

Thus Mcb1 protein levels in the new 41nmt controlled AID were also examined. $p_{nmt41}$-mcb1-2HA-IAA $p_{adh}$-skp1-AtTir1-2NLS-9myc (iAID) and $p_{nmt41}$-mcb1-2HA-IAA $p_{adh}$-skp1-OsTir1-$p_{adh}$-Skp1-AtTir1-2NLS (offAID) cells were grown in the presence of thiamine to repress the nmt41 promoter. Mcb1 protein levels of these cells, with and without auxin treatment, were then compared by Western blot (Figure 3-10 D). However no significant reduction in the amount of Mcb1 protein could be detected.

Noticeably, the $p_{nmt41}$-controlled expression of Mcb1 did not alter the appearance of the cells when grown in the presence of thiamine. Thus low level transcription from the promoter in its “repressed” state (with thiamine) must still allow for the expression of enough Mcb1 protein for the cells to survive (compare chapter 3.6).

Concluding, the auxin-inducible degron approach did not lead to a significant reduction in Mcb1 protein levels either by the auxin-induced degradation alone or in combination with promoter repression. Thus the system is not suitable to study the effects of Mcb1 absence in fission yeast.

### 3.6 Overproduction of Mcb1 is toxic for cells

Controlled overexpression of a protein is another possibility to study protein function. In fission yeast the most used promoter for controlled protein overexpression is the nmt1-promoter, which is repressed by thiamine (Maundrell 1990). Strong overexpression of nmt1-controlled genes is induced in the absence of thiamine. However a full induction of the promoter takes several cell cycles. Further even in the repressed state, expression is not completely switched off, as the promoter is leaky. Several weaker derivatives of the nmt1 promoter, nmt41 and nmt81, have been developed (with TATA box mutations) with reduced activity in repressed and induced states (Basi et al. 1993), allowing for a fine tuning of expression levels.
The $mcb1^+$ gene was tagged with sequences for the $nmt1$ promoter at its 5’ end by PCR-mediated gene targeting and transformants were selected on medium containing thiamine to repress the promoter. Initial experiments with patches of $p_{nmt1}$-$mcb1$ cells on EMM lacking thiamine showed that strains overexpressing Mcb1 failed to grow. Microscopic analysis of cells grown in liquid culture revealed that cells either arrested with an elongated morphology or were very small, indicating that overexpression of Mcb1 is toxic for fission yeast cells (Figure 3-11 A).

To investigate this toxicity, DNA damage was examined in Mcb1-overexpressing cells by the appearance of Rad22-YFP foci (Noguchi et al. 2009; Noguchi et al. 2003). When compared with wild-type cells it was found that $p_{nmt1}$-$mcb1$ cells, with the promoter in its repressed state (i.e. in the presence of thiamine), in general were misshapen (uneven shape, see Figure 3-11 A and B panels $nmt1$ off) and had a slightly increased number of Rad22-YFP foci (26%; Figure 3-11 B arrows, Figure 3-11 C). This indicates that the Mcb1 expression, even in presence of thiamine, stresses the cells and the protein levels are slightly too high but can be tolerated. The phenotype was amplified when overexpression was induced. After 16 hours the culture was a mixture of very elongated and very small cells of which 35% had Rad22-YFP foci indicating increased levels of DNA damage (Figure 3-11 B and C). Thus Mcb1 overexpression is toxic for the cells as it leads to DNA damage, which causes cell cycle arrest.
Figure 3-11 Overexpression of Mcb1 causes DNA damage and is toxic for the cells. A) Cells overexpressing Mcb1 are elongated and have abnormal morphology. Cells with mcb1 under control of the nmt1-promoter were grown in the presence of thiamine (promoter off) or without thiamine to induce overexpression for 14 hours. DNA was stained with Hoechst 33342 before analysis. B) Mcb1 overexpression leads to increased amounts of DNA damage. Cells with mcb1 under control of the nmt1-promoter and expressing Rad22-YFP were grown in the presence of thiamine (promoter off) or without
thiamine for 16 hours to induce overexpression. DNA was stained with Hoechst 33342 and examined by fluorescence microscopy for the appearance of Rad22-YFP foci (arrows). Scale bar 10 μm. C) Rad22-YFP foci were counted in wt cells or nmt1-mcb1 cells in the presence (off) or absence (on) of thiamine. At least 250 cells were counted per strain.

To achieve a moderate (over-)expression of Mcb1 or even a reduction of the protein amount to below wild-type levels, mcb1+ was also tagged with sequences encoding the nmt41 promoter or nmt81 promoter at its 5′ end. While many transformants for p_nmt41-mcb1 were obtained, none could be isolated for p_nmt81-mcb1, not even when keeping the promoter on (in the absence of thiamine). Presumably the leaky expression from this promoter is not sufficient for the survival of the cells while overexpression is still toxic (even though overexpression from p_nmt81 is supposed to be comparable to p_nmt1 repression (Basi et al. 1993)).

Figure 3-12 Overexpression of Mcb1 from the nmt41 promoter is lethal. A) Recovery from Mcb1 overexpression. nmt41-mcb1 cells were grown in the presence of thiamine (promoter off) or without thiamine to induce overexpression for 0, 6 or 18 hours. 500 cells were plated on EMM with thiamine after each time point and incubated at 30°C. Curve based on two experiments. B) Serial dilutions of cells from A) were spotted on EMM plus thiamine and incubated at 30°C.

Overexpression of Mcb1 from p_nmt41 was found to be toxic for cells and the phenotype was similar to overexpression from p_nmt1. Recovery of p_nmt41-mcb1 cells was examined after 0 to 18 hours of promoter induction (see Figure 3-12 A, recovery curve and Figure 3-12 B, spotting assays). After six hours most of the cells were still able to recover and there was no apparent difference to the control cells (p_nmt41 off). However, after 18 hours the recovery rate dropped significantly (Figure 3-12 A and B).
As Mcb1 is thought to replace Mcm2 in an alternative MCM complex (see Chapter 2), it seems possible that Mcb1 and Mcm2 compete with each other for binding of Mcm3-7. Mcb1 overexpression was found to be toxic for the cells, and it seems possible that this is caused by Mcb1 displacing Mcm2 from the canonical Mcm2-7, leading to an excess of $MCM^{Mcb1}$ and a lack of Mcm2-7. Quite often a phenotype associated with the overexpression of one protein can be overcome by overexpression of another protein.

**Figure 3-13 Overexpression of Mcb1 is toxic and cannot be compensated by Mcm2 overexpression.**
Cells expressing Mcb1, Mcm2 or both from pREP3X and pREP4X respectively were grown in the presence of thiamine ($nmt1\text{ off}$) or without thiamine for 16 hours to induce overexpression. **A)** Serial dilutions were spotted onto EMM plus thiamine and incubated at 32°C to recover. **B)** Total extracts from cells without thiamine were analysed by Western blot and the overexpressed proteins were identified by α-FLAG antibody. empty or e indicate control strain with empty pREP3X and pREP4X.

Here, an increase in Mcm2 levels could possibly compensate for the excess of Mcb1, if the two proteins compete. Thus it was attempted to simultaneously overexpress Mcm2 and Mcb1 from $nmt1$ promoters on two different plasmids. The full-length sequences of $mcb1^+$ and $mcm2^+$ were tagged with sequences encoding a C-terminal FLAG-tag by PCR-mediated gene targeting (Bähler et al. 1998), cloned into pREP4X ($mcm2$-$\text{FLAG}$) or pREP3X ($mcb1$-$\text{FLAG}$) respectively for $p_{nmt1}$-controlled overexpression (Forsburg 1993) and transformed into wild-type. Viability of cells after overexpression
of Mcb1 or Mcm2 or both was assessed by spotting assays. When the promoter was repressed, all strains were viable. The $p_{nmt1}$-mcb1 strain showed slightly reduced growth in comparison to $p_{nmt1}$-mcm2 or $p_{nmt1}$-mcb1 $p_{nmt1}$-mcm2 (Figure 3-13 A). When overexpression was induced for 16 hours, $p_{nmt1}$-mcb1 cells failed to grow due to the toxicity of Mcb1 overexpression, while $p_{nmt1}$-mcm2 was growing weakly but significantly better. Thus Mcm2 overexpression alone is not toxic for the cells. The simultaneous overexpression of Mcb1 and Mcm2 however seems to be toxic, as cells did not grow (Figure 3-13 B). Consequently Mcm2 overexpression cannot compensate the toxicity of Mcb1 overexpression.

### 3.7 Discussion

To study Mcb1 function different approaches were pursued. The protein was shown to be the first known essential MCM-BP homologue, as deletion is lethal in fission yeast. The essential character of Mcb1 was also confirmed by another study while this work was in progress (Ding & Forsburg 2011).

For conditional inactivation of the protein several temperature-sensitive mutants were isolated. These were sufficient to inactivate the Mcb1 protein, as cells carrying the mutation arrest their cell cycle with an elongated phenotype and the mutations are lethal after several hours at non-permissive temperature.

The $Ts$ mutants did not show increased sensitivity to several DNA stressing agents. A study with different $Ts$ mutants that shared most of the phenotypes described here, however reported sensitivity towards MMS, HU and CPT but not UV-irradiation (Santosa et al. 2013). The difference in the results could be explained with the different temperatures used. The $Ts1$ and $Ts6$ mutants presented in this study were analysed for their sensitivity towards MMS, HU, CPT, 4-NQO and UV only at permissive temperature. At this temperature, the sensitivity of the mutants described by Santosa et al towards CPT and MMS was barely visible. As their mutants in general seemed to have a more severe phenotype than our mutants, it is possible, that our (less efficient) $Ts1$ and $Ts6$ mutants would not show increased sensitivity at permissive temperature, as there might still be enough functional protein present. Consequently the sensitivity
would probably only show at a higher temperature (30°C). There was clearly no increased sensitivity towards MMC of the mutants described here. It is also possible that the wild-type like resistance of our Ts mutants to DNA stressing agents is the consequence of disabling a different functional aspect of Mcb1. So it is assumed that the temperature-sensitive mutants presented here are still able to repair DNA damage.

The Ts1 and Ts6 mutants exhibited high levels of DNA damage (even at permissive temperature), visualized by the formation of Rad22-YFP repair foci, indicating a partial protein defect even at low temperature. This is most likely responsible for the observed activation of the Chk1 dependent DNA damage checkpoint, which arrests the cell cycle in G2. Increased DNA damage susceptibility and Chk1 checkpoint activation were confirmed by the other study using different Mcb1-Ts mutants (Santosa et al. 2013).

Activation of the DNA damage checkpoint, as opposed to the DNA replication checkpoint, does not necessarily indicate an involvement of Mcb1 in DNA repair. It has been shown that the Chk1 dependent checkpoint is induced by replication stress (Groth et al. 2010; Walworth & Bernards 1996) as well, and that mutants defective in initiation of replication also activate this checkpoint (Yin et al. 2008), while the Cds1 dependent checkpoint is only activated when forks are stalled early in replication e.g. by HU treatment (Lindsay et al., 1998; reviewed in Rhind & Russell, 2010, 1998). So Mcb1 is possibly not a major component of a repair pathway but likely plays a role in DNA replication initiation or elongation.

Santosa et al described genetic interaction of Mcb1 with preRC and preIC components and also found genetic suppression of their mcb1-Ts mutants by CDK modulation. A prolonged G1-phase upon transient CDK inhibition might provide additional time for correct preRC assembly, which might be crucial in cells with preRC defects. This suggests an involvement of Mcb1 in preRC formation, which is also indicated by the increased sensitivity of their mutants to MMS and CPT, as cells defective in preRC formation have been reported to be hypersensitive to these drugs (Santosa et al. 2013; Maki et al. 2011).
Interaction of Mcb1 with Mcm proteins is undoubtedly one of its important features. The *Ts* mutants described by Santosa *et al* (2013) had significantly impaired interaction with all Mcm proteins already at permissive temperature, most likely causing the phenotype of the *Ts* mutants. The protein encoded by our *mcb1-Ts6* mutant in contrast did not show any change in the interaction with Mcm4 at permissive or non-permissive temperature or when compared to the wild-type protein. This is most likely expandable to *mcb1-Ts1*. Thus, either the incubation time at non-permissive temperature prior to the immunoprecipitations was not sufficient to significantly disturb the interaction with Mcm4, or the temperature sensitivity in our mutants is not caused by the impaired interaction with Mcm but by something else. This is possible as the mutations causing the temperature sensitivity in the different sets of mutants reside in different parts of the protein. For completeness the interaction of Mcb1-*Ts* with the other Mcm proteins would have to be analysed as well.

Interestingly, Slaymaker *et al*. show that ssoMcm is able to form a left-handed filament *in vitro* for which a bundle of α helices close to the C-terminus is important (Slaymaker *et al*. 2013). They also show evidence for the biological significance of interactions between these helices in fission yeast. The point mutations leading to the temperature sensitivity of our Mcb1-*Ts* reside in the analogous α-helices of Mcb1. Even though Mcb1 does probably not act like an Mcm protein it could still share some of their features and thus also exhibit this binding activity in the C-terminal α helices. Maybe this interaction between Mcb1 and an unknown binding partner is impaired in the *Ts* mutants.

Overexpression of Mcb1 was found to have quite similar effects as its inactivation. Cells usually died after long-term overexpression from the *nmt1* and also *nmt41* promoter. They exhibited increased amounts of DNA damage, were elongated and arrested their cell cycle. While this work was in progress, this was confirmed by another study, which also showed activation of the DNA damage checkpoint in Mcb1 overproducing cells (Ding & Forsburg 2011).

Mcm2 overexpression is not able to compensate Mcb1 overproduction, as tested here, Mcm4 overexpression however was reported to partially rescue the lethality associated with Mcb1 overexpression (Ding & Forsburg 2011). Mcm4 was found to be
toxic when overexpressed in wild-type (Maiorano et al. 1996; Forsburg et al. 1997), as was Mcm5 (Forsburg et al. 1997). So possibly these proteins and Mcb1 have the potential to disturb the architecture of Mcm2-7 when overexpressed but also compensate for imbalances in the levels of other Mcm proteins, while Mcm2 does not. Overexpression of Mcb1 was also reported to cause problems with initiation of DNA replication, as a portion of overproducing cells exhibited a sub 1C-DNA content (Ding & Forsburg 2011). This is caused by cells, which fail to initiate DNA replication but still undergo mitosis and thus tear apart their DNA. It also explains the two morphologies of Mcb1- overexpressing cells observed here: the very small cells were those with 1C-DNA content, while the elongated cells had managed to initiate DNA replication but also activated a checkpoint and cell cycle arrest.

Figure 3-14 Model for Mcm2-7 dissociation caused by Mcb1 overexpression. When overexpressed, Mcb1 might start to compete with Mcm proteins for Mcm-interaction. This might lead to the dissociation of free Mcm2-7 complexes as well as DNA loaded Mcm2-7 and result in disturbance of DNA replication.

Further evidence suggested that Mcm-Mcm and MCM-chromatin association is disturbed in Mcb1 overproducing cells while Mcm-Mcb1 interaction is increased (Ding & Forsburg 2011). Thus overexpression of Mcb1 seems to cause dissociation of the canonical Mcm2-7 complex in fission yeast by dispersing its individual subunits away. Consequently not enough Mcm2-7 is available for undisturbed complete DNA
replication, which leads to checkpoint activation and cell cycle arrest, or separation of unreplicated DNA and cell death.

The approach of using a hormone-binding domain fused to Mcb1 did not efficiently inactivate the protein. It is estimated that the regulation of proteins via the HBD system is comparable to current expression systems (Bøe et al. 2008). Mcb1 was not sufficiently down-regulated under control of the nmt1 or nmt41 promoter (in their off-state), hence it seems likely that the hormone induced regulation just does not reduce the amount of active protein enough to effect a phenotype, which does not necessarily mean that Mcb1-HBD is completely unaffected by the absence of estradiol.

The inactivation of HBD fusion constructs in the absence of estradiol was initially thought to happen through steric interference with Hsp90 (Mattioni et al. 1994). Recent results suggest that the inactivation in fission yeast could also be due to (mis-) regulation of the intracellular localisation of the target protein (e.g. HBD tagged GINS subunits Psf1 and Psf2 quickly delocalize in absence of estradiol which might be the reason for their inactivation; (Pai et al. 2012)). However it could be speculated that different localisation of the target and Hsp90 potentially prevents inactivation. Fission yeast Hsp90 (Swo1) is primarily found in the cytoplasm (Matsuyama et al. 2006) but Mcb1 was reported to reside mainly in the nucleus (Ding & Forsburg 2011). Thus the HBD construct might not be sufficiently bound by Hsp90 due to the different localisations or a mis-localisation of Mcb1-HBD might lead to a cleavage of the tag. Maybe a better inactivation could be achieved by expression of Hsp90 (Swo1) in the nucleus.

Further, the proteins, which could be regulated most efficiently using the HBD were subunits of the GINS complex. As GINS function is dependent on its interaction with other replication proteins, the GINS-subunits might be very sensitive to steric effects caused by the Hsp90 chaperone, which is not necessarily the case with Mcb1.

The attempt to control Mcb1 levels using an auxin-inducible degron (AID) approach did not result in a detectable reduction of Mcb1, neither when using the simple AID-system, nor when additionally repressing Mcb1 expression with the nmt41 promoter. Similar results were obtained for downregulation of Mcm2. This could at least partly
be explained with problems to detect the IAA-HA-tagged proteins in the first place. However as also no effect on cell appearance was found, apparently the system did not work here, as $mcb1^+$ and $mcm2^+$ are both essential proteins in fission yeast. It has to be considered that the inactivation of some proteins might require additional measures, as synchronisation, before inactivation (Kanke et al. 2011) what would lead to very complex systems and was not used here.

The failure to efficiently degrade Mcb1 could result from a localisation effect. The downregulation of the target protein requires its localisation in the nucleus, where Mcb1 is thought to mainly reside. However it is not known whether it would be accessible for the AID system, as the tag might be covered or the protein might be tightly bound to Mcm proteins, which could interfere with the IAA-SCF$^\text{Tir1}$ interaction. A fraction of Mcb1 can be found in the cytoplasm (Li et al. 2011; Ding & Forsburg 2011), where it would probably not be targeted by the AID system. Further the mobility of Mcb1 or its potential to travel between the nucleus and the cytoplasm have not been studied yet, so it is unclear how long the protein actually resides in the nucleus. It could be that it is not staying long enough for efficient degradation. Further Kanke et al. (2011) did not use the $nmt41$ but the $nmt81$ promoter for repression of target genes. This promoter represses expression even more than the $nmt41$ promoter, leading to even lower protein levels. But as described in 3.6, control of $mcb1$-expression from $p_{nmt81}$ does not result in viable transformants so that the AID system in combination with $nmt81$-controlled expression of Mcb1 was not applied here.

Results presented in this study show that fission yeast Mcb1 is essential but also toxic when overexpressed. So in general it seems that balanced levels of Mcb1 are crucial for cell survival as imbalances in either direction have severe consequences such as cell cycle arrest and cell death.
Chapter 4 Structure and function of fission yeast Mcb1

4.1 Introduction

Eukaryotic Mcm proteins are thought to have evolved by gene duplication events from one single Mcm-like protein, which was probably similar to today’s archaeal Mcm, leading to the paralogous Mcm2-Mcm7, Mcm8 and Mcm9 in the last common eukaryotic ancestor (LCEA). Diversification of the LCEA resulted in different eukaryotes, all carrying the six paralogues of MCM2-7, which are essential for eukaryotic DNA replication (Liu et al. 2009). Only a subset of today’s eukaryotes carries MCM-BP, Mcm8 and 9, indicating gene loss during evolution of some eukaryotic branches (Liu et al. 2009). This also suggests a non-essential function of these proteins for DNA replication or the presence of alternative pathways in some eukaryotes (Aves et al. 2012).

Initial sequence analysis of MCM-BP after it was first discovered in human cells revealed that the protein, despite its close proximity to Mcm proteins, only shares little homology with human Mcm proteins (Sakwe et al. 2007). But given that the fission yeast Mcb1 is smaller than the human orthologue and also less complex it could be speculated that it might be more closely related to the ancestor and also be more suitable to obtain information about homology to other proteins as the MCM family.

The previous chapters initially characterised the MCM$^{Mcb1}$ complex and its interaction partners and several approaches to inactivate Mcb1 to study its function in the MCM$^{Mcb1}$ complex were discussed. Still little is known about the composition and structure of the complex or what its exact function is. In this chapter the sequence of Mcb1 and its homologues in other organisms will be analysed to gain some insight into the role of the protein. Therefore theoretical methods like multiple sequence alignments and structure prediction will be used.

Further the results of experiments altering the sequence and thus structure and function of Mcb1 will be shown and analysed with respect to the structural information gained from alignments and structure prediction. This includes the
construction of different truncations of Mcb1 and a pentapeptide scanning mutagenesis. The pentapeptide insertion mutant plasmids described in 4.3.2 were made by Justina Ray during her Honours project in the MacNeill lab in 2012.

4.2 Theoretical approaches

4.2.1 Sequence analysis suggests limited homology of Mcb1 and Mcm proteins

BLAST searches with fission yeast Mcb1 show homologues of the protein in most eukaryotes. MCM-BP is widely distributed in eukaryotes (reviewed in Aves et al. 2012) and homologues appear in all clades, so not only in the Opisthokonts (animals and fungi) or Plantae supergroups (land plants, green algae, red algae, glaucophytes), which contain most of the popular model organisms used for research, but also the other supergroups of Chromalveolata (secondary endosymbionts like Dinoflagellates), Rhizaria (e.g. Foraminifera and Radiolaria), Excavata (mostly heterotrophic flagellates like trypanosomatids and parabasalids, which cause sleeping sickness and trichomoniasis) and Amoebozoa (Amoeba and slime molds) (Simpson & Roger 2004; Adl et al. 2005). However MCM-BP is missing in some lineages, just like Mcm8 and Mcm9, which might imply gene loss at some point during evolution (Aves et al. 2012).

Multiple sequence alignments were made with different MCM-BP proteins from different organisms, covering all eukaryotic clades. For the Opisthokonta, MCM-BP sequences from *H. sapiens*, *M. musculus*, *X. laevis*, *Danio rerio*, *Drosophila melanogaster*, *S. pombe*, *Schizosaccharomyces cryophilus* and *Schizosaccharomyces japonicus* were used. From Plantae *A. thaliana*, *Oryza*, *Physcomitrella patens*, *Ostreococcus lucimarinus* and *Ostreococcus tauri*. *Bigelowiella natans* was the representative for Rhizaria, while *Dictyostelium discoideum* represents Amoebozoa. The Chromalveolata MCM-PB sequences were those of *Plasmodium falciparum* and *Paramecium tetraurelia*, and *T. brucei* MCM-BP represented the Excavata.
Alignments of these protein sequences show that there is a high level of conservation between MCM-BP proteins, especially in the C-terminal domain (Figure 4-1 B and Appendix 4). This feature can also be found in eukaryotic MCM2-7 proteins, with the C-terminal half, which contains the AAA+ box, being highly conserved and the N-terminal half comprising just a low level of conservation (reviewed in Bochman & Schwacha, 2009). The three yeast species’ MCM-BPs alongside the *P. tetraurelia* homologue have the shortest sequences of the examined homologues with around 500 amino acids in length (*P. tetraurelia* MCM-BP has only 375 amino acids). They do not show insertions.
relative to the other MCM-BPs so that they probably represent the simplest MCM-BP homologues. Especially the homologues from higher eukaryotes like *H. sapiens* or *Xenopus* are long and more complicated having more than 600 amino acids and many insertions in the multiple sequence alignment (see Appendix 4).

![Figure 4-2 Multiple sequence alignment of Mcm and MCM-BP proteins from different eukaryotic and archaeal species.](image)

A conserved area from the C-terminal domain is shown, containing the Mcm H2I and Walker B motif. PPI13/ PPI18 mark the position of pentapeptide insertion mutants #13 and #18 (paragraph 4.3.2). Sequences included are: *Xenopus laevis* Mcm9 and Mcm8, *Homo sapiens* Mcm9 and Mcm8, *Danio rerio* Mcm9 and Mcm8, *Oryza sativa japonica* Mcm9 and Mcm8, *Archaeoglobus fulgidus* Mcm, *Haloferax volcanii* Mcm (*hfx* Mcm), *Methanothermobacter thermautotrophicum* Mcm (*mth* Mcm), *Sulfolobus solfataricus* Mcm (*ssOMcm*), *Schizosaccharomyces cerevisiae* Mcm2, 3, 4, 5, 6, 7, *Schizosaccharomyces pombe* Mcm2, 3, 4, 5, 6, 7, *Drosophila melanogaster* Mcm2, 3, 4, 5, 6, 7, *Mus musculus* Mcm2, 3, 4, 5, 6, 7, and the MCM-BP homologues from *Arabidopsis thaliana*, *Oryza sativa japonica*, *Physcomitrella patens*, *Ostreococcus lucimarinus*, *Danio rerio*, *Salmo salar*, *Homo sapiens*, *Mus musculus*, *Xenopus laevis*, *Drosophila melanogaster*, *Schizosaccharomyces pombe*, *Schizosaccharomyces japonicus*, *Bigelowiella natans*, *Dyctiostelium discoideum* and *Trypanosoma brucei*. The alignment was constructed using Seaview, the full alignment can be found in Appendix 4.

Alignments of different MCM-BP homologues with MCM2-7 as well as Mcm8 and Mcm9 proteins from multiple eukaryotic species and archaeal Mcm proteins were prepared as well (Figure 4-2 and Appendix 4). Interestingly they show homologous
areas of Mcms and MCM-BP spread over the whole length of the proteins. Again these homologies are more pronounced in the C-terminal halves of the proteins even though they are not as strong as within the MCM2-7 proteins. Generally the MCM-BP sequences seem to have insertions relative to the Mcm sequences in the N-terminal half, while in the C-terminal portion the Mcm proteins have insertions relative to the MCM-BP sequences.

Sequences of designated motifs important for MCM activity are not conserved in the MCM-BPs, including the cis-acting Walker A and Walker B motif and the trans-acting arginine- finger of the AAA+ box. However there is a short stretch of homology in the MCM-BPs with the conserved hydrophobic sequence just before the Walker B motif in MCM proteins (Figure 4-2, also see Sakwe et al. 2007). The homologous residues also include the conserved Walker B aspartate and glutamate residues, which are thought to be important for ATPase activity in AAA+ ATPases, either coordinating the H$_2$O molecule for the nucleophilic attack on ATP or contributing the actual catalytic base (Orelle et al. 2003). Interestingly the aspartate seems to be absolutely conserved among MCM-BPs and the following glutamate and threonine are present in all but one of the analysed homologues (T. brucei, see Figure 4-2), indicating that these residues might be important for MCM-BP function as well. However there is an insertion present in the putative Walker B motif in MCM-BPs, which could interfere with a potential function. Some of the conserved residues of the H2I motif are found in MCM-BP homologues as well, including a conserved aspartate and leucine (S. pombe Mcb1 D320 and L324; Figure 4-2), even though the motif seems to lack some amino acids in the MCM-BPs. The conserved serine of the arginine finger motif in Mcm proteins is also found in all MCM-BP homologues even though the rest of the motif is absent.

Due to the less pronounced homology in the N-terminal half it is sometimes difficult to align the MCM-BP homologues to the Mcm proteins: the B domain, which contains the Zn$^+$ coordinating cysteine residues does not seem conserved (see Appendix 4; MSA of MCM-BP and Mcm proteins). And while all four cysteine residues can be found in most Mcm homologues, some of them are missing in all MCM-BP homologues and they also do not align properly with the Mcm sequences.
All this indicates that MCM-BP proteins share homology with Mcm proteins even though their key motifs are missing or only represented by few conserved residues. Thus MCM-BP proteins are distant relatives of Mcm proteins. MCM-BPs might have evolved by a gene duplication event, followed by a gradual loss of the original MCM function and gain of a new function, which has not been clarified yet. Gene duplication is also thought to be the origin of the multiple different Mcm proteins in eukaryotes (reviewed in Bochman & Schwacha, 2009). Possibly the MCM-BP protein could have evolved similarly to Mcm8 and 9, which are homologues of Mcms, but do not act as members of the replicative helicase but have acquired other functions in eukaryotes (Park et al. 2013; Nishimura et al. 2012; Lutzmann et al. 2012; Gambus & Blow 2013). MCM-BPs might have lost their ATPase character but could still act in close proximity to Mcm proteins, maybe as an inactive placeholder with regulatory function.

### 4.2.2 Structure prediction and modelling indicate an Mcm-like structure

With MCM-BP being a relatively novel and not well-characterised protein no structural data is available. However the multiple sequence alignments (paragraph 4.2.1) suggest homology of MCM-BP and Mcm proteins and it is likely that MCM-BP evolved from the same ancestor as the Mcm proteins. Thus it is possible that MCM-BP adopts a similar structure as Mcm proteins. At present there is no high resolution structure for eukaryotic Mcm homologues available, but there are some crystal structures (NmthMcm: Fletcher et al. 2003, N-ss0Mcm: Liu et al. 2008, FL-ss0Mcm: Brewster et al. 2008, inactive mkaMcm2: Bae et al. 2009) and EM structures (Adachi et al. 1997; Costa et al. 2006; Costa et al. 2008; Gómez-Llorente et al. 2005) from the simpler archaean Mcm homologues. Using fission yeast Mcb1 as representative of eukaryotic MCM-BP proteins might be beneficial as the yeast MCM-BPs seem to be simpler than the higher eukaryotic MCM-BPs and might therefore still be closer to the ancestral protein.
4.2.2.1 PSI-BLAST search and secondary structure prediction

In order to get some information about the possible structure of Mcb1, Phyre2 (Protein Homology/analogY Recognition Engine V 2.0; Kelley & Sternberg 2009) was used for structure prediction.

In a first step Phyre2 conducts a PSI-BLAST search and creates a pseudo alignment. For the Mcb1 protein sequence this contained 721 homologous sequences (partly pictured in Appendix 5). Most of the homologues were Mcm proteins with sequence identities below 15% and very low E values (e.g. homologue 579 still has an E value of $9 \times 10^{-04}$), indicating extensive sequence diversity, which is necessary for an accurate secondary structure prediction (Kelley & Sternberg 2009).

![Secondary structure and disorder prediction for fission yeast Mcb1.](image)

Figure 4-3 Secondary structure and disorder prediction for fission yeast Mcb1. A section of the secondary structure prediction from amino acids 1-240 is shown. The full prediction can be found in Appendix 5.
The secondary structure prediction (Figure 4-3) suggests that Mcb1 consists of 33% α-helices, 24% β-strands and 22% disordered regions. In general there are more regions with lower confidence prediction in the N-terminal half of the protein (e.g. amino acids 49-59; 95-120; 151-172; 265-280 but also 395-405). Disordered regions are mainly depicted for amino acids 24-33, 137-147, 152-169, 218-229, 244-249, 407-416. The disorder in amino acids 137-147, 152-169 and 218-229 coincide with regions where no secondary structure elements were predicted and could possibly be flexible linkers between protein domains.

4.2.2.2 3D-model based on ssoMcm

The two top hits for the final Mcb1 structure prediction were the crystal structures of near full-length Sulfolobus solfataricus Mcm (FL-ssoMcm, PDB identifier 3f9v; Brewster et al. 2008) and full-length Mcm from Methanopyrus kandleri (mkmMcm2, PDB 3f8t; Bae et al. 2009). Even though the sequence identity between Mcb1 and the two archaeal proteins is only 17% and 16% respectively, the structure could be modelled with high confidence scores (i.e. probability that the match is a true homology) of 99.6 and 99.5. This means that the models might not be highly accurate in terms of positioning of individual residues, but as the confidence scores are very high the protein is very likely to adopt a fold similar to that shown in the model, and the data might still provide useful information about Mcb1 structure and maybe MCM-BP structure in general. The ssoMcm structure was the top hit and also covers a larger region of Mcb1 in the alignment (residues 4-496 while mkaMcm aligned only amino acids 174-496). Further the mkaMcm crystal structure is derived from an inactive Mcm monomer, which lacks the Zn-coordinating domain and contains non-canonical insertions in the AAA+ box (Bae et al. 2009) so that it is possibly not a suitable representative for Mcm structure. Thus analysis was consequently focused on the model derived from ssoMcm. A 3D-model of Mcb1 visualised with PyMol is shown in Figure 4-4 A, the superposition of ssoMcm and Mcb1 in Figure 4-4 B and the corresponding alignment of Mcb1 and ssoMcm sequences and secondary structures in Figure 4-5.
The ssoMcm protein consists of an N-terminal and a C-terminal domain (NTD and CTD) separated by a 40 amino acids long linker. The NTD can be further divided into three subdomains, A, B and C. The CTD contains the AAA+ domain that consists of a canonical α/β domain (five β-strands and five α-helices) and another domain consisting of three α-helices (α-domain), connected by a linker (α/β-α linker with two long α-helices) (Brewster et al. 2008).
Figure 4-5 Alignment of Mcb1 (query) and ssoMcm (template). Protein sequences as well as Mcb1 predicted secondary structure (based on PDB 3f9v) and ssoMcm predicted and known secondary structure are shown. Coloured horizontal lines above the alignment indicate the respective domains (based on ssoMcm). “Template Known Secondary Structure”: S= bend, T= hydrogen bonded turn, B= residue in isolated β-bridge, G= 3-turn helix (3_10 helix).

Overall the Mcb1 protein fits the ssoMcm model quite well. However the Mcb1 sequence only starts at a position corresponding to ssoMcm amino acid 58 (Figure 4-5), so that the first three α-helices of ssoMcm domain A (green in Figure 4-4 B) are missing in Mcb1. As amino acid 24-37 of Mcb1 represent an insertion relative to ssoMcm, they are not modelled and the α-helices before and after are joined to one
long α-helix in domain A. There is a gap between Mcb1 amino acid 51 and 52, where no structure is modelled, as ssoMcm has an insertion here. This region was already found to have poor confidence in the secondary structure prediction and links domain A and domain C in ssoMcm. The first part of Mcb1 domain C (yellow) is similar to ssoMcm. The sequence corresponding to the ssoMcm domain B (orange), which contains the Zn-coordinating motif in Mcm homologues is unstructured in Mcb1, consisting mainly of coils. The secondary structure for this region was predicted with low confidence (Figure 4-3), which might explain its unstructured nature. Amino acids 143-155 of Mcb1 again represent an insertion relative to ssoMcm and are missing in the model. For this part of the C domain an α-helix was predicted flanked by two disordered regions. The model however depicts a long β-strand. The rest of the C domain (yellow) fits the ssoMcm model, but the ACL loop (ssoMcm 202-208; Barry et al. 2009) and NT hairpin (amino acid 240-254) are altered, as ssoMcm has insertions relative to Mcb1 in these areas. As noticed in the secondary structure prediction, the region around amino acids 219-252 is indeed modelled as linker between the N-terminal and the C-terminal half of the protein.

The overall fold of ssoMcm αβ-domain (cyan) is adopted in the Mcb1 model; however, the β-sheets flanking the external hairpin (Ext-hp; ssoMcm amino acid 320-333) are not modelled properly as the confidence for this region is low. Further the characteristic β-α-β motif of the helix-2-insert is not modelled and ssoMcm has an insertion respective to Mcb1 here (ssoMcm amino acid 374-389). The reason for the lack of distinct motifs could be the low confidence for secondary structure and the predicted disorder in this region. For the Walker B motif (ssoMcm amino acids 403-409) an α-helical insert is found in Mcb1 structure prediction. The PS1-hairpin superimposes almost perfectly. At the end of the α/β domain, ssoMcm (Ca4, α5 and β5) has a long insertion of 22 amino acids relative to Mcb1. Together with the low confidence of the secondary structure prediction this leads to a long region of unstructured coils in the Mcb1 model (amino acids 395-408) very different from the ssoMcm structure. This region also includes what would be the arginine finger motif (amino acids 472/473 of ssoMcm) and about half of the α/β-α linker (blue). The three α-helices of the ssoMcm α domain (purple, ssoMcm Ca6-8) adopt a very similar structure in the Mcb1 model. Due to the winged helix domain of Mcm not being part
of the crystal structure (Brewster et al. 2008), it cannot be modelled for Mcb1 either. But as there are only five amino acids missing at the end of Mcb1, it is unlikely that Mcb1 contains a winged helix domain.

Some of the functionally important motifs found in Mcm proteins are modelled for the Mcb1 structure as well, but with certain differences. In most cases, there are parts of the sequence missing in Mcb1 (as for ACL, NT-hp, H2I) or insertions present (Walker B), while only PS1, Ext-hairpin and Walker A adopt a similar conformation. However, not only the 3D structure but also the sequence, especially conserved amino acids important for the function of a motif, has to be considered when looking for function inside the structure, which will be discussed further. Generally it is hard to predict from the model if MCM-BP homologues have functional motifs, which exhibit the same or a similar function as the corresponding regions in Mcms.

Using Phyre2, a high confidence 3D model of Mcb1 could be produced, which adopts the structure of ssoMcm, a simple archaeal Mcm protein. Due to low sequence similarity of only 17%, the model might not be highly accurate, but the high confidence score obtained makes it likely that Mcb1 has a structure similar to the model. Overall Mcb1 fits the model well, only some stretches cannot be modelled properly as they represent insertions or deletions relative to the ssoMcm sequence. Conclusions about the function of typical Mcm motifs in Mcb1 are difficult to make: firstly, because the important amino acid residues are not always conserved in Mcb1, and secondly because often insertions or deletions in the respective motifs are found. Thus the modelling data alone is not suitable to predict if Mcb1 contains functional motifs found in Mcm proteins. However it indicates that Mcb1 is not only distantly related to Mcm proteins but also that it is likely to adopt a very similar structure.

4.3 Functional analysis of Mcb1

Even though the structure prediction does not give a definite 3D structure of Mcb1, it is still a very useful feature. It could be used for functional analysis, for example to
explain the effects of mutations on the protein function as the position of single amino acids or short sequences becomes much clearer with a 3D model.

In order to find out about regions of Mcb1 needed for its function, two different approaches were pursued. Truncation constructs were made, deleting domains stepwise from either end and tagging the intact end with a FLAG tag. For controlled (over-) expression the truncations were put under control of the nmt1 promoter on the pREP3X plasmid. It was anticipated to find some domains that can be deleted without affecting Mcb1 function. Additionally for a narrower analysis, a set of pentapeptide insertion mutants was constructed using a transposon-based system with the aim to identify precise regions important for Mcb1 function.

4.3.1 Mcb1 truncation constructs

The start sites for N-terminal truncation constructs were chosen according to the position of conserved domains in a multiple sequence alignment of Mcm proteins from S. pombe and some archaeal species and different yeast MCM-BPs (Appendix 3). It has to be noted that the more extensive multiple sequence alignments described in 4.2.1, which were made later, define slightly different boundaries for the domains than those chosen for the truncation mutants.

In construct Mcb1_{18-501} amino acids 1-17 are deleted, corresponding to the absence of the first two α helices. Mcb1_{85-501} lacks the A domain and the first part of domain C, Mcb1_{149-501} is missing domain A, the first part of domain C and the whole domain B, and Mcb1_{239-501} is missing the whole N-terminal domain. The C-terminal deletions in turn were just lacking increasing regions from the C-terminus: Mcb1_{1-450} (roughly lacking the equivalent to the α-domain of Mcm-CTD in a multiple sequence alignment), Mcb1_{1-400} (lacking the α/β-α linker of the C-terminal domain), Mcb1_{1-350} (stops after the region corresponding to the Walker B motif in Mcm proteins) Mcb1_{1-300} and Mcb1_{1-238} (lacks the whole C-terminal domain of Mcb1) (see image Figure 4-6 A).

Expression of the constructs was confirmed by Western blot (Figure 4-6 B), using extracts from cells overexpressing the truncation constructs for 16 hours. The reason for inducing overexpression was that the amount of protein expressed from the nmt1
promoter in its off-state was not detectable despite the background levels of expression from $\rho_{nmt1}$, which should be comparable to wild-type $mcb1^+$ expression levels (not shown).

**Figure 4-6 Truncation constructs of Mcb1 expressed from pREP3X.**

A) Graphic representation of Mcb1-truncation constructs in the context of conserved domains in Mcb1 based on multiple sequence alignments. B) Overexpression of FLAG-Mcb1 and Mcb1-FLAG truncation constructs from pREP3X in wild-type background. Cells were grown at 25°C in the presence of thiamine ($nmt1$ repressed) to exponential phase, diluted to an OD of 0.03 and overexpression was induced for 16 hours in the absence of thiamine. Total extracts were subjected to SDS-PAGE, Western blotted and truncation constructs were detected using anti-FLAG antibody. FL = full-length Mcb1.

To examine the function of the different constructs in comparison to the full-length (FL) FLAG-tagged Mcb1, the plasmids were transformed into wild-type and strains with temperature-sensitive alleles $mcb1-Ts1$ and $mcb1-Ts6$. Functionality was evaluated by
the constructs’ ability to rescue the temperature sensitivity of \textit{mcb1-Ts1} and \textit{Ts6} strains at 35°C and by the toxicity of the overexpressed protein (in the absence of thiamine in the medium) in spotting essays. As controls, an empty plasmid and a plasmid expressing the untagged full-length Mcb1 (FL Mcb1) were processed in the same way as the truncation mutants.

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\caption{Structure and function of fission yeast Mcb1}
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Figure 4-7 Use of truncation mutants of Mcb1 as a screen for functionally important regions of the protein reveals that almost the full-length protein is needed for proper function. Spotting assays of wild-type (A; Sp208), mcb1-Ts1 (B; Sp578) or mcb1-Ts6 (C; Sp580) strains expressing different Mcb1-truncation constructs from pREP3X. Cells were grown at 25°C in EMM plus uracil in the presence of thiamine to 0.4-0.8x10^7 cells/ml. Fivefold serial dilutions (10000, 2000, 400, 80, 16 cells) were spotted onto EMM-uracil plates with thiamine (promoter off) or without thiamine (overexpression) and incubated at 25 or 35°C. Diagrams on the left represent the respective Mcb1-constructs.

In the wild-type background all constructs allowed normal growth when the promoter was repressed (thiamine present at 15 μM (5 μg/ml)) at 25°C and at 35°C. A slight reduction in the growth of the full-length constructs might be caused by already slightly too high levels of Mcb1 due to the leaky nature of the nmt1 promoter even in its off-state. When the nmt1 promoter was induced (in the absence of thiamine), only cells expressing full-length Mcb1 failed to grow, while the truncation constructs did not have an effect, showing that they are all not toxic (Figure 4-7 A).

When expressed in the Ts1 and Ts6 background, at permissive temperature (25°C), again all truncated constructs allowed normal cell growth independent of the promoter activity. Only the full-length Mcb1 was toxic when overexpressed (Figure 4-7 B and C). At 35°C, when the endogenous Mcb1-Ts is inactivated, most truncations failed to rescue the temperature sensitivity and no cell growth was observed. The full-length constructs however, and also the truncation lacking the first 17 amino acids...
Structure and function of fission yeast Mcb1

(Mcb1<sub>18-501</sub>), were able to rescue the phenotype and even cells overexpressing the latter were able to grow (Figure 4-7 B and C and summary Figure 4-8).

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<tr>
<td>450</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>400</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>350</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>300</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>238</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>FL Mcb1</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>18</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>85</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>149</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>FLAG tag</td>
<td>239</td>
<td>–</td>
</tr>
</tbody>
</table>

Figure 4-8 Almost the full-length Mcb1 protein is necessary for proper function. Summary of Mcb1-truncation constructs function based on their toxicity when overexpressed and ability to rescue the Ts phenotype. – does not fulfill criteria, +/-++ fulfills criteria

These results suggest that almost the full sequence of Mcb1 is necessary for the protein to be fully functional, as only the full-length protein, but none of the truncations is toxic when overexpressed. Only the first few amino acids of Mcb1 might be dispensable, as the construct lacking amino acids 1-18 can compensate the Ts phenotype. But this truncation leads to a non-toxic, and thus not fully functional version of the protein. Similar experiments with different truncation constructs resulted in similar conclusions (Ding & Forsburg 2011).

4.3.2 Pentapeptide scanning mutagenesis

Pentapeptide scanning mutagenesis is based on the random insertion and imperfect excision of the transposon Tn4430, originally isolated from Bacillus thuringiensis, and provides a straightforward way of gaining information about essential regions of a target protein (Hayes & Hallet 2000; Hallet et al. 1997). Unlike the truncations described in 4.3.1 this method allows for a more precise identification of important regions.
Justina Ray constructed the plasmids containing the pentapeptide insertion mutants described in this paragraph during her Honours project in the MacNeill lab in 2012.

The *S. pombe mcb1* coding sequence with an N-terminal FLAG tag on a plasmid (pBR322) was transformed into an *E. coli* strain (FH1046) containing the transposon Tn4430 on another plasmid, leading to random transposition of Tn4430, during which a 5 bp piece of target sequence is duplicated. Transconjugants were selected after crossing to a recipient strain (DS941) and insertion of the transposon into Mcb1, rather than the plasmid, was screened for by colony PCR. After removal of the bulk of Tn4430 by restriction digest with Acc65I, which cuts 5 bp from either end of the transposon, the now altered *mcb1*-sequences were cloned 3’ of the *nmt1* promoter in pREP3X for controlled expression in fission yeast. 26 pentapeptide insertion mutants were obtained, some of them identical, some with insertions in the *mcb1* intron or the region 3’ of the coding sequence (#6 and #24), so that 19 different mutants were available for further analysis. An overview can be found in Table 4-1, a schematic representation of Mcb1 (based on the structure prediction described in 4.2.2) with the different mutations in Figure 4-9.

**Table 4-1 Pentapeptide insertion mutants of Mcb1 showing mutant number, position and sequence of the insert as well as growth effects on wild-type or temperature-sensitive strains.** ** indicates frameshift mutations with 16 or 14 instead of 15 base pair- inserts, leading to a completely changed amino acid sequence after the insertion site. #11 thus has a premature stop codon after amino acid 242. #24 contained an insertion in the intron, #6 in the 3’ region. Identical mutants are summarised in the same row, the mutant used for the experiments underlined. The functional regions corresponding to the insertion sites were derived from the 3D structure prediction and multiple sequence alignment in 4.2.2 and are based on the structure and domains of ssoMcm.

<table>
<thead>
<tr>
<th>Mutant #</th>
<th>Insert-sequence</th>
<th>Between AA-AA</th>
<th>Functional region</th>
<th>Rescues Ts?</th>
<th>Overex. toxic?</th>
</tr>
</thead>
<tbody>
<tr>
<td>10/20</td>
<td>WGTPR</td>
<td>R_{14}^-S_{15}</td>
<td>Domain A</td>
<td>++</td>
<td>−</td>
</tr>
<tr>
<td>14</td>
<td>GVPPT</td>
<td>T_{48}^-E_{49}</td>
<td>Domain A</td>
<td>++</td>
<td>−</td>
</tr>
<tr>
<td>3/4/5</td>
<td>GGTPT</td>
<td>T_{48}^-E_{49}</td>
<td>Domain A</td>
<td>++</td>
<td>−</td>
</tr>
<tr>
<td>9**</td>
<td>GYPYR</td>
<td>E_{49}^-E_{50}</td>
<td>Domain A</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>15</td>
<td>RGTPR</td>
<td>R_{53}^-K_{54}</td>
<td>Domain A</td>
<td>++</td>
<td>−</td>
</tr>
<tr>
<td>23</td>
<td>GVPHL</td>
<td>L_{115}^-D_{116}</td>
<td>Domain B</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>8</td>
<td>GVPLD</td>
<td>D_{123}^-E_{124}</td>
<td>Domain B</td>
<td>++</td>
<td>−</td>
</tr>
<tr>
<td>16/17/19</td>
<td>RGTPV</td>
<td>V_{141}^-I_{142}</td>
<td>Domain B</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>12</td>
<td>GVPLE</td>
<td>E_{143}^-A_{144}</td>
<td>Domain C</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>2</td>
<td>GYPLK</td>
<td>K_{159}^-Y_{160}</td>
<td>Domain C</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>25</td>
<td>KGYPS</td>
<td>S_{161}^-N_{162}</td>
<td>Domain C</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>11**</td>
<td>GVPHQ</td>
<td>Q_{165}^-A_{166}</td>
<td>Domain C</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>22</td>
<td>GYPLQ</td>
<td>Q_{165}^-A_{166}</td>
<td>Domain C</td>
<td>++</td>
<td>−</td>
</tr>
</tbody>
</table>
Noticeably most of the obtained mutants, 18 out of 26, cluster in the N-terminal domain of Mcb1 (amino acids 1-238). This indicates that the transposition is not completely random as initially claimed (Hallet et al. 1997), which was also found in other studies with pentapeptide insertion mutagenesis (Sanchez Garcia et al. 2009; Gray et al. 2009). The reason for the observed clustering of mutations in the N-terminal half of the protein might not be the Mcb1 sequence itself, but rather changes in the DNA structure at the 5’ end, which could be caused by the replacement of the endogenous promoter.

Three insertion mutants (#9, 11 and 21) led to a completely changed amino acid sequence due to insertion of 16 or 14 instead of 15 base pairs and will be referred to as frameshift mutants.

Figure 4-9 Location of pentapeptide insertions in the Mcb1 protein. Schematic representation of the fission yeast Mcb1 protein showing the location of pentapeptide insertions. Light grey box: C-terminal domain of Mcb1 (with indication of position of Walker A, B and RF from Mcm proteins). N-terminal domain of Mcb1 with subdomain A (white box), subdomain B (dark grey box) and subdomain C (grey boxes). White circles: frameshift mutations, grey circles: partly functional mutant proteins, black circles: functional mutant proteins.
Structure and function of fission yeast Mcb1

The position of the insertions was compared to the predicted 3D structure and secondary structure alignment of Mcb1 (described in 4.2.2), to get an idea which functional domains of Mcb1 were affected. Eight of 26 inserts were found in the putative A domain of Mcb1, five in the C domain, five in the B domain and only six in the entire CTD. #13 and #18 were found to be located just after the region corresponding to the H2I hairpin, N-terminal of the Walker B motif, and #21 is in close proximity to where the Walker B motif would be found in Mcm proteins.

4.3.2.1 Analysis of PPI mutant function in S. pombe

In order to analyse their function, the plasmids containing the pentapeptide insertion mutants of mcb1 were transformed into wild-type and a strain with the temperature-sensitive allele mcb1-Ts6. Serial dilutions were spotted onto medium with or without 15 mM thiamine to repress or activate the nmt1 promoter at permissive and non-permissive temperature. The functionality of the mutants was again assessed regarding their ability to rescue the temperature-sensitive mutation at non-permissive temperature (i.e. to replace the wild-type protein) and their toxicity when overexpressed.

When overexpressed at permissive temperature, most of the PPI mutants as well as the wild-type version of Mcb1 (FL-Mcb1) were toxic for the cells and did not grow. The empty plasmid, mutants #13 and #18 and the frameshift mutants (#9, 11, and 21 which are expected to be non-functional) did not kill the cells, so were not toxic. These results were obtained in wild-type and also Ts background (compare Figure 4-10 A and B).

Growth of the Ts cells at permissive temperature, in the presence of thiamine (to repress the nmt1 promoter), led to spots almost indistinguishable from wild-type. However a slight growth reduction of the strain containing FL-Mcb1 and mutants #2, 12, 17 and 25 was observed and is likely due to the leakage of the promoter, which leads to enhanced, and slightly toxic levels of Mcb1.

When grown in the presence of thiamine at non-permissive temperature all mutants were able to rescue the temperature sensitivity in mcb1-Ts6 background. Mutants #1,
3, 7, 8, 9, 10, 11, 13, 14, 15, 21 and 22 (Figure 4-10 B, panel “promoter off” 36°C) showed efficient growth, comparable to the results at permissive temperature, indicating that these mutants can compensate for the loss of Mcb1. Interestingly this includes the frameshift-mutants, which were expected to be non-functional. #2, 12, 17, 18, 23 and 25 were growing as well as the FL construct but less than the previously mentioned mutants. Wt Mcb1 is toxic for the cells when overexpressed and there is always a low level of expression from the nmt1 promoter even in its repressed state, which might be higher than the endogenous expression and thus slightly toxic. So constructs that are as functional as the wild-type might cause a growth reduction while still compensating for the loss of Mcb1 in the temperature-sensitive background. Another reason for the weak growth could be that the respective mutant is not as functional as the wild-type protein and thus does not rescue as well (e.g. #18, which is not toxic when overexpressed and thus presumably not functional). This also indicates that the mutants supporting growth at non-permissive temperature might not necessarily be 100% functional.

**Figure 4-10 Spotting assays of Mcb1-pentapeptide insertion mutants.** Plasmids containing Mcb1-PPI mutants (1-26) full-length Mcb1 (FL) or an empty MCS (E) were transformed into wild-type or mcb1-Ts6 cells. Serial dilutions were spotted onto medium containing thiamine (promoter off) or lacking thiamine (overexpression) and incubated at 25°C or 36°C or 30° for wild-type controls. Function of the mutants
was assessed by their ability to rescue the temperature sensitivity of \textit{mcb1-T6} and their toxicity when overexpressed.

Overexpression of most of the mutants at non-permissive temperature in the Ts strain was toxic (Figure 4-10 B panel “overexpression 35°C), as expected. Surprisingly #13 and 18 but also the frameshift mutants (#9, 11, and 21) were not toxic when overexpressed but still able to rescue the temperature-sensitivity. All of these were also found to be not toxic when overexpressed at permissive temperature.

Thus, the pentapeptide screening produced only two PPI mutants, #13 and 18 and the frameshift mutants, with an altered/reduced Mcb1 function. The insertions apparently disrupt the toxicity and also interfere with the function to some level but not enough to completely abolish it, as the mutants are still able to rescue. This might indicate that the toxicity and the unknown function of Mcb1 might be two different traits. The majority of pentapeptide insertion mutants behaved similarly to the wild-type (able to rescue the temperature sensitivity of \textit{mcb1-Ts} and toxic when overexpressed), leading to the conclusion that these insertions do not disturb Mcb1 function and toxicity. The frameshift mutants should produce non-functional proteins, due to the completely altered sequence after the frameshift. However they were still able to rescue the Ts phenotype, indicating the presence of functional Mcb1. So the observed effects of the mutants must have different reasons than only the pentapetide insertion, which will be discussed further.

\textit{4.3.2.2 Analysis of interaction with Mcm4 in the PPI-mutants}

Severe temperature-sensitive mutations disturbing the function of Mcb1 were reported to abolish its interaction with Mcm proteins (Santosa et al. 2013). To investigate the Mcm4-binding of partly-functional PPI mutants #13 and #18 alongside the non-mutated Mcb1 (FL), the functional insertion mutant #14 and the truncated insertion mutant #11, the PPI-plasmids were transformed into a strain expressing Mcm4-TAP from its endogenous promoter. Pull-down assays of the FLAG-tagged PPI-mutants with Mcm4-TAP were analysed by Western blot.
As the single FLAG-tagged Mcb1-PPI-mutants could not be detected by Western blot when the \textit{nmt1} promoter was repressed, overexpression of the constructs was induced such that the toxic effects of FL-Mcb1 would not yet kill the cells and that the level of overexpression would still be low enough to allow for a specific interaction with Mcm4. This level was found to be reached after 15 hours overexpression at 25°C (data not shown).

While the non-mutated full-length Mcb1 and the functional insertion mutant \#14 were pulled down with Mcm4-TAP in comparable amounts (Figure 4-11 co-IP FL and 14), the truncated insertion mutant \#11 did not show any interaction (consistent with (Ding & Forsburg 2011)) but was expressed and visible (Figure 4-11 input 11, indicated by the asterisk). The Mcm4 interaction in the partly functional mutants \#13 and \#18 was significantly reduced when compared to FL or \#14, especially in the mutant \#18. This leads to the conclusion that in the partly functional Mcb1-PPI mutants \#13 and \#18 the interaction with Mcm4 is compromised, which might explain the disturbed function described earlier.

\textbf{Figure 4-11 Interaction of Mcb1-PPI mutants \#13 and \#18 with Mcm4 is impaired.} Wild-type or \textit{mcm4-TAP} cells carrying pREP3x with full-length Mcb1 or Mcb1-PPI mutants 11, 13, 14 or 18 were grown at 25°C without thiamine to induce overexpression of the constructs for 15 hours. Soluble extracts were made and Mcm4-TAP was precipitated on IgG-Sepharose. Total extracts (input) and co-IPs were analysed by Western blot using \textit{\alpha-Mcb1} (red) and \textit{\alpha-FLAG} (green) antibodies and the Licor OdysseyCLX for detection. Mcm4-TAP was detected using \textit{\alpha-Mcb1}. The asterisks mark the truncated Mcb1-PPI \#11 protein.
Thus only two out of 26 isolated pentapeptide insertions lead to an impaired Mcb1 function. These two were also found to have reduced interaction with Mcm4 in co-immunoprecipitation experiments. This indicates that Mcb1-Mcm interaction in general is closely linked to Mcb1 function.

4.4 Conclusions

4.4.1 Multiple sequence alignments and structural models

Multiple sequence alignments of Mcm and MCM-BP proteins and structure prediction for the simple MCM-BP homologue Mcb1 indicate that MCM-BPs share homology with Mcm proteins and adapt a similar 3D-structure. The sequence alignment shows homologies between Mcm and MCM-BPs over the whole length of the proteins. However this is more pronounced in the C-terminal domain than in the N-terminal domain. The homologous areas are also reflected in the predicted 3D model of Mcb1, as the overall structure of Mcb1 appears similar to that of ssoMcm, including all the bigger domains. The designated motifs characteristic for Mcm proteins however cannot clearly be identified in MCM-BPs. The structure prediction for Mcb1 roughly shows most of the motifs, but fails to model the hairpins and loops in detail. The multiple sequence alignments suggest that most of the motifs are not sufficiently conserved in MCM-BP proteins to perform a function.

It can be assumed that a motif is non-functional if the important residues are completely missing. The Zn-coordinating motif in domain B of the Mcm proteins is quite unstructured in the Mcb1 model. Additionally the multiple sequence alignment does not detect conserved stretches in this area, and while all Mcm sequences contain the four cysteine/histidine residues for coordination of Zn, they are partly or completely missing in most of the MCM-BPs. This indicates that MCM-BP, unlike the Mcm proteins, might not be able to bind Zn.

Mcb1 and other MCM-BPs presumably do not have a functional Walker A motif, as the conserved sequence (GDPXXA/SQXL, Forsburg, 2004) is missing in MCM-BP homologues, even though Mcb1 might adapt a very similar structure in that region. The arginine finger is not modelled for Mcb1, as the corresponding region is
completely unstructured. However the multiple sequence alignment suggests that the serine in the motif is conserved in Mcms and MCM-BPs and also a stretch of hydrophobic residues just before the motif, so that the predicted structure of the model in that part is quite likely not correct. Still, MCM-BPs presumably do not have a functional arginine finger. The H2I motif of Mcms protrudes into the central channel and is thought to be crucial for coupling of ATP hydrolysis and helicase activity in MCM helicases and it could be used to mediate the mechanical separation of dsDNA in the central channel (Jenkinson & Chong 2006). The motif is not modelled in detail for Mcb1, so the β-α-β is not shown and four of the amino acids are missing in Mcb1. However there are some conserved residues present in all MCM-BPs (Mcb1 D320 and L324) in a stretch corresponding to the Mcm H2I. It has to be noted that a generally poor sequence conservation for H2I among Mcms has led to the idea that the interactions mediated by H2I might not be sequence specific but steric (reviewed in Brewster & Chen 2010; Jenkinson & Chong 2006). Thus it could be speculated that the H2I corresponding residues in MCM-BP and Mcb1 could presumably contribute to a function.

The PS1-hairpin seems to be partly conserved. The 3D model of Mcb1 superimposes with the ssoMcm structure perfectly and comparison with the MCM-BP and Mcm alignment in Appendix 4 shows that a conserved Q (Mcb1 Q367) and a hydrophobic residue (L, I or V; L369 in Mcb1) are present in Mcb1 and other MCM-BP homologues as well.

Mcb1 is lacking some amino acids in the sequences corresponding to ACL and NT-hp in Mcm proteins, and the 3D model also depicts shorter loops than for ssoMcm. The consequence of the absence or addition of a stretch of amino acids (as in H2I, NT-hairpin and ACL loop) in a particular loop is hard to predict. Not all residues of these motifs are conserved among Mcm proteins and variations in the length of hairpins occur, nevertheless the motifs are most likely still functional in Mcms. So it cannot be concluded that Mcb1 has any of these motifs.

In case of the Walker B motif, the conserved D and E residues, which are crucial for the ATPase activity in Mcm proteins (by coordinating the water molecule for the nucleophilic attack (Bochman & Schwacha 2009)) are present in all MCM-BP homologues. The 3D model shows, that Mcb1 contains an insertion, forming an
additional α-helix after the conserved DE. The effect of this rather bulky insert is unclear. A partial function of the motif seems possible, which could include the coordination of water or ATP, even though it is unlikely that MCM-BP proteins could hydrolyse it because of the lacking Walker A and Arginine finger motifs. But as these motifs adopt a similar structure in Mcb1 and Mcms, even though the conserved residues are missing, Mcb1 could possibly act as an inactive placeholder. Of course all these assumptions are based on theoretical methods and are mere speculations. However ATP binding of Mcb1 could be examined, as well as the DNA binding properties of the protein. To investigate the importance of the structural domains and motifs, mutants in some of the conserved residues or domains could be made and their functionality and binding to Mcm proteins could be studied. The random pentapeptide insertion mutagenesis described in this study produced two such mutants, which might influence conserved regions in Mcb1.

4.4.2 PPI mutants

Pentapeptide-insertion scanning mutagenesis to identify functionally important regions of Mcb1 produced 19 different mutants, of which #13, #18 and three frameshift constructs (#9,11,21) had an effect on the protein function. While they were able to rescue the temperature sensitivity of \textit{mcb1-Ts6} in a wild-type-like manner, they were not toxic when overexpressed, even at the non-permissive temperature. For a semi-functional protein this could possibly be expected; however it is difficult to imagine how a frameshift mutation would be converted into a functional protein. #11 which carries a premature STOP-codon was detected by Western blot (see Figure 4-11), showing a real truncation of the protein and thus dismissing the possibility that the sequencing results were interpreted in a wrong way.

A simple concept would be that non-functional PPI-mutations somehow stabilize the \textit{Ts} mutant but do not kill the cells, as they are not toxic. However this is rather unlikely, as e.g. expression of non-toxic Mcb1-truncation constructs from the \textit{nmt1} promoter (chapter 4.3.1) did not rescue the \textit{Ts} phenotype. Gene conversion between the insertion mutants on the plasmid and the genomic \textit{mcb1-Ts} allele has to be considered though. The full \textit{mcb1} coding sequence is present
on all the PPI-mutant plasmids, even on those leading to a truncated protein. Thus a cross over event to cure the temperature-sensitive mutation is possible, even if quite unlikely to happen in all the insertion-mutants. We also cannot rule out the possibility that the whole plasmid integrated into the genome (see Figure 4-12).

In case of the frameshift mutations, depending on the positions of the insertion and the cross-over, this would lead to one non-functional Mcb1-copy with a frameshift (and a non-relevant Ts mutation) and one fully functional copy (see Figure 4-12 way B). As only the non-functional Mcb1 would be controlled by the nmt1-promoter, overexpression would not compromise the viability of the cells.

Figure 4-12 Model of gene conversion between the pentapeptide insertion mutants (dark grey) and the endogenous copy of Mcb1 (light grey). Depending on the crossover happening before (A) or after the site of the insertion (B) two different products can be expected of which only B leads to a reasonable explanation for the observations described in Figure 4-10.

If this happened with the pentapeptide insertion mutants without frameshift there would be a wild-type like copy followed by a copy with insertion and Ts mutation controlled by the nmt1 promoter. The consequence of Mcb1-Ts overexpression was not examined here, but is likely to be toxic at permissive temperature, maybe also at
non- or semi-permissive temperature. Granted that the particular insertion does not compromise Mcb1 function, and overexpression of the Ts-allele is toxic for the cells, most of the pentapeptide-insertion mutants would lead to cell death. Only the insertions that impair the function would not be toxic, which is consistent with the results presented here.

Generally it is possible that this conversion does not happen with the presumably functional pentapeptide insertion mutants, but only with the non-functional ones (and the frameshift mutations) because there is a huge benefit from incorporating the extra-chromosomal DNA in that case.
So gene conversion happening between non-functional insertion-mutants and the endogenous mcb1-Ts allele can explain why a subset of the mutants was able to rescue the temperature sensitivity while being not toxic when seemingly overexpressed: In fact the previously Ts cells would now have a wild-type mcb1, while overexpressing a simply non-functional and thus not toxic protein from nmt1. This indicates that in addition to the obviously non-functional frameshift mutants, PPI #13 and #18 must also have compromised Mcb1 function. To verify this, mutants could be made, expressing PPI#13 or #18 from the endogenous promoter.

These two mutants were also found to have reduced interaction with Mcm4 in co-immunoprecipitation experiments, indicating that Mcb1-Mcm interaction in general is closely linked to Mcb1 function and that the disruption leads to the defects observed in our mutants.

The insertion sites of #13 (amino acid 326) and #18 (amino acid 331) are located just after what would be the H2I insert in Mcm proteins and are also just N-terminal to the Walker B-similar region on Mcb1. Both motifs seem to be partly conserved in MCM-BP homologues (Figure 4-2). Further the #18 insertion site lies in a stretch of hydrophobic residues (mainly A, M, V and L), which is conserved in Mcm and MCM-BP proteins as well (Figure 4-2).

So maybe the pentapeptide insertion mutants #13 and #18 could disturb the function of an Mcb1-H2I or putative Mcb1-Walker B motif by interfering with their orientation. If these sites are important for Mcb1-Mcm interaction, insertions are likely to affect
the interaction with Mcm. This might be the case with PPI mutants #13 and #18, so that their interaction with Mcm4 is impaired, which was shown here. This might also be true for the interaction with other Mcm proteins in fission yeast. One of the Ts mutants described by (Santosa et al. 2013) has a point mutation close to the Walker B similar sequence (L363P) and exhibits reduced Mcm interaction as well. The insertion sites are located at the interface between NTD and CTD of the modelled protein, so they could also disturb interaction between the subdomains or the flexibility of the protein, which might also have an effect on its interaction with Mcm proteins.

This indicates that interaction of Mcb1 and Mcm-proteins is an important functional trait of the Mcb1 protein. Only the interaction with Mcm4 was tested here, however it seems likely that also other Mcm interactions are affected.

4.4.3 Truncations

Expression of truncated Mcb1 constructs from plasmids under the control of the nmt1 promoter showed that almost the full-length protein is necessary for viability and function. Only a construct missing the first 17 amino acids was able to rescue the Ts phenotype but was not toxic when overexpressed (so not fully functional). This mutant protein is missing the majority of the first modelled α-helix of Mcb1 (Figure 4-4) which is about half of the domain A.

Regarding the results from the pentapeptide scanning mutagenesis it has to be considered that gene conversion between the Mcb1_{18-501} construct and the endogenous mcb1-Ts can happen. In that case a wild-type like product of the crossover could be responsible for the rescue of the Ts phenotype, which would also indicate that the Mcb1_{18-501} is possibly non-functional (as it is non-toxic when overexpressed). As the other constructs did not lead to an integration of the plasmid it remains questionable if it actually happened in case of Mcb1_{18-501}.

Interaction of the truncation constructs with Mcm proteins was not tested. Firstly there were problems detecting the non-overexpressed proteins by Western blot and secondly, while this study was in progress Ding et al reported the construction of a
similar set of truncation mutants and their interaction with Mcm4 (Ding & Forsburg 2011) so that no new results were to be expected. Consistent with the results shown here, only full-length or near full-length constructs missing the first 54 amino acids (corresponding to the majority of domain A) were able to complement Δmcb1+ and only the full-length proteins were toxic when overexpressed, while the deletion of amino acids 1-54 resulted in reduced toxicity (Ding & Forsburg 2011). So apparently at least parts of domain A are dispensable for the essential function of Mcb1. The truncations do not exhibit the same level of toxicity as the full-length protein when overexpressed, indicating that domain A must somehow contribute to the full function of Mcb1. The effect of the removal of the whole domain A (amino acids 1-83) has not been studied though.

Ding et al also showed that their full-length constructs, the N-terminal domain and the construct missing the first exon are still able to interact with Mcm4, as is the C-terminal domain lacking the last 100 amino acids. Mutants lacking the N-terminal domain or N-terminal domain and the first part of the C-terminal domain are not able to interact with Mcm4 anymore (Ding & Forsburg 2011). Interestingly this first part of the C-terminal domain would contain the AAA+ box (if Mcb1 had any). This provides further evidence for the importance of this domain in Mcb1-Mcm interaction.
Chapter 5 Expression, purification and applications with recombinant protein

5.1 Introduction

Biochemical assays, production of antibodies, crystallisation screens and other applications often require large amounts of pure protein, which is usually difficult to achieve in the host. To produce large quantities of protein, other systems can be utilised, the most widely used ones being bacterial expression systems, which can quickly produce high yields of proteins. However, complex eukaryotic multi-domain proteins are often non-functional when expressed in bacteria, as bacterial cells cannot ensure proper folding and fail to provide the necessary post-translational modifications. For these cases eukaryotic expression systems, like insect cell systems or even mammalian cells are available, but these usually give a lower yield at higher costs and are a lot more time-consuming than the use of *E. coli* expression systems.

For small scale applications, cell free systems for the in vitro synthesis of protein are available. Whole cell extracts (e.g. from rabbit reticulocytes or wheat germ), containing all the components for protein transcription and translation can be programmed to produce small amounts of protein, which can also be labelled with modified amino-acids in the translation process. This can provide quick access to relatively small amounts of functional protein (reviewed in Bernhard & Tozawa, 2013).

A variety of affinity tags, like His, GST, StreptII and FLAG have been developed that have proven to be powerful tools allowing purification of recombinant fusion-proteins from cell extracts under mild conditions (Lichty et al. 2011). Using special expression vectors, tagging with these affinity tags and subsequent controlled expression of almost any protein coding sequence can be achieved in a standardised way.

The polyhistidine-tag (His-tag) is probably the most popular tag for protein purification (Hochuli et al. 1988). The system depends on the challengeable interaction of histidine with nickel (or cobalt) ions, immobilised by chelation on chromatography resin. $\text{Ni}^{2+}$
Expression, purification and applications with recombinant protein

resins have a high binding capacity and allow the purification of large amounts of reasonably pure protein at relatively low cost.

The Strep-tag II consists of the eight-amino-acid sequence WSHPQFEK (Schmidt et al. 1996) and selectively and strongly binds to StrepTactin, an engineered Streptavidin (Skerra & Schmidt 2000). Recombinant Strep-tag II fusion proteins can be purified from crude \textit{E. coli} extracts (but also yeast or mammalian cells), theoretically in a one-step protocol, because of the highly specific interaction between Strep-tag II and StrepTactin resin under a variety of physiological conditions. Elution of the target protein can be accomplished by very low concentrations (2.5 mM) of desthiobiotin. In combination with the \textit{tetA}-promoter system (Skerra 1994), the expression of Strep-tag II fusion proteins can be tightly controlled: Strep-tag II vectors (IBA technologies) carry the \textit{tetA} promoter/ operator, which can be fully induced by low, not antibiotically active, concentrations of anhydrotetracycline (Skerra 1994; Skerra & Schmidt 2000). This leads to very high purity proteins, however with lower yields and at a higher price than use of His-tags. For simplicity the Strep-tag II will be referred to as “Strep” in the following.

In this chapter expression and purification of several Mcb1- and Mcm4- N-terminal-con structs, using different \textit{in vivo} and \textit{in vitro} expression systems and purification strategies, will be described. In addition the results of different applications of these recombinant proteins, including production of an \textalpha-Mcb1 antibody, crystallisation screens and \textit{in vitro} interaction assays will be presented.

5.2 Purification of recombinant Mcb1 domains

5.2.1 Expression and purification of Mcb1-NTD and CTD from pASK-IBA3+/5+

5.2.1.1 Expression of Mcb1-NTD and CTD from pASK-IBA3+/5+

Using a multiple sequence alignment of fission yeast Mcb1 and archaeal and fission yeast Mcm proteins (Appendix 3) the boundary between the N-terminal and the C-terminal half of Mcb1 was defined to be close to the C-terminal end of the linker.
connecting both halves of Mcb1, so that the N-terminal domain comprises AA 1-238 and the C-terminal domain consists of AA 239-501.

The gene sequences encoding for the N-terminal or C-terminal domain of the Mcb1 protein were PCR amplified and cloned into pASK-IBA3+ or pASK-IBA5+ (IBA technologies) for fusion of a 3’ or 5’ Strep-tag to the constructs, making the plasmids pASK-IBA-Mcb1_{1-238}\text{-Strep} and pASK-IBA-Strep-Mcb1_{239-501}. Integrity of the constructs was confirmed by sequencing before transformation into \textit{E. coli} Rosetta2.

To find suitable conditions for expression of soluble protein, test expressions were performed at different temperatures and times: 20°C for 4.5 hours and overnight, 25°C for 4 hours, 30°C for 4 hours and 37°C for 4 hours (Figure 5-1). Expression of constructs was induced with 200 µg/l anhydrotetracycline (AHT) and soluble extracts were prepared.

Mcb1-NTD-Strep was found to be expressed in high yields from pASK-IBA-Mcb1_{1-238}\text{-Strep} at 20°C overnight and good solubility was achieved in 100 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.1% Tween (Figure 5-1 D).

Strep-Mcb1-CTD was not clearly expressed from pASK-IBA-Strep-Mcb1_{239-501} at all temperatures tested (Figure 5-1 A-D).

Small scale Strep-purifications using soluble extracts from cells overexpressing Mcb1-CTD at 37°C or 20°C were conducted but did not result in a clear band either, indicating that Mcb1-CTD is not expressed or not soluble (Figure 5-1 E, F). However it was expected for this construct to be more difficult to solubilise as the C-terminal domains of the Mcm proteins were reported to be difficult to purify (reviewed in Bochman & Schwacha 2009; Xu et al. 2013). Therefore Mcb1-CTD was cloned into pEHisTEV and pEHisGFPTEV to see if these allow for expression of soluble protein (see chapter 5.3).
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Figure 5-1 Test expression of Mcb1-NTD from pASK-IBA 3+ and Mcb1-CTD from pASK-IBA 5+ Rosetta2 cells carrying pASK-IBA-Mcb1\textsubscript{1-238}-Strep, pASK-IBA-Strep-Mcb1\textsubscript{239-501} or empty pASK-IBA3+/5+ were grown to mid log phase at 37°C before induction of overexpression with anhydrotetracycline and incubation at indicated temperatures for the indicated times. Total protein extracts or samples of pellet or soluble extracts were subjected to SDS-PAGE. U = uninduced; I = induced; 3+ indicates pASK-IBA 3+ plasmid; 5+ indicates pASK-IBA 5+ plasmid; N = Mcb1-NTD; C = Mcb1-CTD; asterisks indicate Mcb1-NTD

A) Total samples of cells with empty vectors and vectors expressing Mcb1-NTD or Mcb1-CTD before (U) and after induction (I) at 37°C. B)-D) Samples of pellets and soluble extracts of pASK-IBA 3+/5+ expressing Mcb1-NTD/CTD for 4 h at 37°C (B), 4 h 25°C (C), 4.5 h 30°C (D), overnight 20°C (D). Soluble extracts were prepared in buffer W (100 mM Tris pH8, 150 mM NaCl, 1 mM EDTA, 0.1% Tween-20, complete protease inhibitor). E, F) Small scale Strep purifications of Mcb1-CTD on 15% gels. Soluble extract was prepared from cells overexpressing Mcb1-CTD from pASK-IBA 5+ for 4 h at 30°C (E)
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or 4.5h/ overnight at 20°C (F) in buffer W. Soluble extracts were incubated with StrepTactin resin for 1 hour. P = pellet, U = uninduced, S = soluble extract, SN = unbound, B = boiled resin.

5.2.1.2 Purification of rMcb1-NTD-Strep

rMcb1-NTD-Strep was purified with a two-step protocol including a StrepTactin affinity column (5 ml StrepTactinHP, GE) and a Superdex 200 gel filtration column (GE) using the Äkta purifier as described in Materials and methods. Per run soluble extract from 2 litres of E. coli culture was loaded onto a StrepTactinHP column. The amount of Mcb1-NTD-Strep protein present exceeded the binding capacity of the column, as unbound protein was found in the flow through and wash fractions (Figure 5-2 A, SDS-PAGE lane FT). After extensive washing the Strep-tagged protein was eluted with a linear gradient of binding buffer containing increasing concentrations of desthiobiotin (0-2.5 mM). A protein eluted at around 40-50% elution buffer (corresponding to 1 mM desthiobiotin; Figure 5-2 A) in one very sharp and high peak. Analysis of the peak fractions (Figure 5-2 A, SDS-PAGE) confirmed that the eluted protein had the right size to be Mcb1-NTD and also showed the purified protein to be present in high amounts with only weak impurities (faint bands at 35 kDa and 70 kDa) most likely representing E. coli proteins interacting with Mcb1.

Pooled eluate-fractions from several runs with the StrepTrap column were subjected to gel filtration using a HiLoad 16/60 Superdex 200 pg gel filtration column (GE). Mcb1-NTD-Strep eluted as one sharp peak at 210 ml (Figure 5-2 B). Analysis of the protein containing fraction by SDS-PAGE indicated high purity and mass spec analysis confirmed the identity of Mcb1(-NTD). Protein fractions were pooled and concentrated for further use. The average yield was about 1 mg of pure Mcb1-NTD per litre of E.coli culture. Purified recombinant Mcb1-NTD was subsequently used for antibody production and pull-down assays with in vitro translated Mcm-NTD constructs as well as in vitro interaction studies with Mcm4-NTD (chapter 5.5.5) and for crystal trials (chapter 5.5.2).
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Figure 5-2 Two-step purification of rMcb1-NTD-Strep. Soluble extract from 2 litres of Rosetta2 overexpressing Mcb1-NTD at 20°C overnight was loaded on a StrepTrapHP 5 ml column. After extensive wash with buffer W, the protein was eluted with a gradient of buffer W containing up to 2.5 mM desthiobiotin. Samples from each step of the purification were subjected to SDS-PAGE using 12% gels. Pooled and concentrated elution fractions from two runs were loaded onto a Superdex 200 column pre-equilibrated with buffer W. Protein containing fractions were analysed by SDS-PAGE. A) StrepTrap purification step showing a graph with UV-trace and elution gradient, as well as an SDS-PAGE with samples of all steps. U= uninduced cells, T= induced cells, S= soluble extract, P= pellet (insoluble), FT= unbound, W1= first 50 ml of wash fraction; peak = peak fractions from the elution gradient; load 5µl each; elution fractions: 10 µl. B) Gel filtration using a Superdex 200 column. The graph shows the UV trace during the purification. The gel contains fractions from the indicated peaks. L= sample of concentrated pool before gel filtration. Load: 10 µl per lane.

5.2.2 Expression and purification of full-length Mcb1 from pASK IBA3+

As the purification of rMcb1-NTD worked well, purification of the full-length protein was attempted. The mcb1 coding sequence without the intron was cloned into pASK-IBA3+ to make pASK-IBA-Mcb1-Strep and the plasmid was transformed into Rosetta2. To find suitable expression/lysis conditions several temperatures and buffer compositions were tested (see Figure 5-4: 22°C and 27°C with 150, 300, 450 mM NaCl; 37°C with 150 and 300 mM NaCl; 32°C with 150 and 300 mM NaCl).
The yield of soluble protein was found to be highest when the expression was induced at 22°C overnight and 300 mM NaCl were added to the lysis buffer. However the majority of the protein was still found to be insoluble in the pellet (Figure 5-3D).

**Figure 5-3 Test expression of FL-Mcb1-Strep** Rosetta2 cells with pASK-IBA-Mcb1-Strep were grown at 37°C to mid-log phase before induction of overexpression with anhydrotetracycline overnight (22°C and 27°C) or for four hours (32°C and 37°C). Soluble extracts were made in buffer W containing 150-450 mM NaCl. For better detection of soluble protein, extracts were incubated with StrepTactin resin (IBA technologies). Samples were analysed by SDS-PAGE using 10% gels. U= uninduced, T= total induced, P= pellet (insoluble), S= soluble extract, B= boiled StrepTactin resin. Arrow indicates size of FL-Mcb1-Strep. A) Overexpression at 37°C; 150 or 300 mM NaCl in lysis buffer. B) Overexpression at 32°C; 150 or 300 mM NaCl in lysis buffer. C) Overexpression at 22°C and 27°C; 150 mM NaCl in lysis buffer. D) Overexpression at 22°C and 27°C, 300 mM NaCl in lysis buffer. Asterisks mark highest yield of soluble FL-Mcb1-Strep.

Purification was conducted with a two-step protocol using a gravity flow StrepTactin column (1.5 ml IBA) at 4°C and a highprep 16/60 Sephacryl 500 HR column (GE healthcare) for gel filtration. FL-Mcb1 eluted from the gravity flow StrepTactin column with the first three elution fractions (Figure 5-4 A). But there was also an almost equally strong contaminating band present in elutions 3-6, most likely an *E. coli* protein interacting with Mcb1. Wash fractions 4 and 5 and elution fractions 1-3 were pooled,
concentrated to 2 ml and loaded onto a highprep 16/60 Sephacryl 500 HR column (GE) for further purification.

**Figure 5-4 Expression and purification of rFL-Mcb1-Strep from pASK-IBA 3+** FL-Mcb1-Strep was overexpressed at 22°C over night. Soluble extracts were made in buffer W containing 300 mM NaCl. A) Affinity purification using a 1.5 ml gravity flow StrepTactin column at 4°C. Soluble extract was incubated with StrepTactin, washed and bound protein was eluted with buffer containing 2.5 mM desthiobiotin in 6 fractions. Samples were analysed by SDS-PAGE: P= pellet, S= soluble extract, FT= flow through. B) Gel filtration using a highprep 16/60 sephacryl column. Peak fractions and a sample of the input (L) were analysed by SDS-PAGE. Mcb1 is marked as #. Asterisks indicate co-purified protein identified as DnaK by mass spec.

The protein eluted in one small peak of only 5 mAU at about 100 ml. Analysis of the protein containing fractions by SDS-PAGE showed a contaminating protein of about 75
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kDa in size (Figure 5-4 A and B, marked by asterisk) to co-purify with rMcb1. The contamination was identified by mass spec as *E. coli* chaperone DnaK, which could probably assist with proper folding of Mcb1.

The total yield of rFL-Mcb1 purification was only 0.1 mg per litre of *E. coli* culture and concentration of the purified protein using concentrators resulted in precipitation. To optimise the purification and increase the yield, the expression temperature was increased to 44°C over three hours. It was speculated that expression at high temperature might lead to an elevated DnaK activity in *E. coli*, presumably assisting with protein folding. However this did not result in higher yields, as the majority of the protein was still not soluble (not shown).

Furthermore, the protein seemed to be sensitive to temperature and long sonication, as an attempt to purify it in higher scale using the StrepTrap 5 ml column on the Åkta at room temperature resulted in very low solubility and almost no recovery of purified protein (not shown).

In conclusion rFL-Mcb1 proved to be difficult to purify, as the solubility of the protein is quite low in the tested conditions when compared to e.g. rMcb1-NTD. In addition the protein is sensitive to temperature, as purification at room temperature leads to drastically reduced yields. Thus the entire purification should be carried out at 4°C. In this study only the affinity step using StrepTactin could be conducted at 4°C while the gel filtration was performed at room temperature.

5.3 Expression of rMcb1-CTD from pEHisTEV and pEHisGFPTEV

As the expression of Mcb1-CTD (amino acids 239-501) as Strep-fusion (from pASK-IBA5+) did not result in soluble protein, the construct was cloned into pEHisTEV (Liu & Naismith 2009) using the *Eco*RV and *Hind*III sites, to express a His-fusion protein. The plasmid was transformed into Rosetta2 and overexpression of the protein was induced by 0.5 mM IPTG. Test expressions were performed at 20°C overnight, and 30°C and 37°C for 4 hours. Soluble extracts were made in buffer with 200 mM NaCl and to recover soluble protein, small scale Ni-NTA purifications were performed. Analysis of samples by SDS-PAGE showed that the protein is expressed at 30°C and 37°C, but most of it is found in the pellet (Figure 5-5 A, band at 35 kDa in P lanes). No soluble protein
was recovered on the Ni-NTA agarose (B lanes). To ensure that this was not due to the buffer conditions (EDTA in the buffer might interfere with protein binding) small scale purifications were repeated with extracts from expression at 20°C and 25°C, using 300 mM NaCl, 0.1% Tween, 20 mM imidazole. Mcb1-CTD was clearly expressed, especially in the 25°C test, but the protein was insoluble again and could not be recovered on Ni-NTA (Figure 5-5 B).

Addition of a domain can sometimes improve the folding and thus the solubility of a protein. Thus Mcb1-CTD was cloned into pEHisGFPTEV (Liu & Naismith 2009), which adds a GFP-tag between the His-tag and the protein. The expression of a GFP-fusion should also allow for a rapid identification of soluble protein by fluorescence of the soluble extracts (Liu & Naismith 2009). Initial transformation of the plasmid into Rosetta2 and BL21(DE3) did not result in any transformants, possibly due to toxicity of the protein product. Thus C43 cells were used. This strain was derived from BL21(DE3) but contains mutations conferring tolerance to toxic proteins (Miroux & Walker 1996) and could successfully be transformed with His-GFP-Mcb1-CTD. Test expressions were performed at 16°C, 22°C and 37°C and soluble extracts were further incubated with Ni-NTA agarose to test for soluble protein. The expected size of HisGFP-Mcb1-CTD is around 60 kDa (30 kDa Mcb1-CTD + 28 kDa GFP). However no soluble fusion-protein was detected and there was no prominent band at 60 kDa in the total or pellet fractions of all test expressions, indicating that the HisGFP-Mcb1-CTD construct is not expressed (Figure 5-5 C).

Weak green fluorescence of the total lysates could be detected, most likely emanating from GFP-only expression. Thus the band detected at 35 kDa in the pellet fractions from the 16°C expression (Figure 5-5 C, empty plasmid and overexpression of HisGFP-Mcb1-CTD at 16°C) could correspond to a GFP-tag. But the Mcb1-CTD construct was not expressed.
Figure 5-5 Test-expression of Mcb1-CTD from pEHisTEV and pEHisGFPTEV Rosetta2 cells with pEHis-Mcb1-CTD (A and B) or C43 cells with pEHisGFP-Mcb1CTD (C) were grown to mid log phase at 37°C before induction of overexpression with 0.5 mM IPTG and incubation at indicated temperatures. Soluble extracts were made and incubated with Ni-NTA agarose (GE) for 1 hour at 4°C. Samples were analysed by SDS-PAGE on 15% gels. U= uninduced, T= total induced, P= pellet (insoluble), S= soluble extract, B= boiled Ni-NTA resin. A) Soluble extracts were made in 100 mM Tris pH 8, 200 mM NaCl, 1 mM EDTA, 0.1% Tween-20. Note that EDTA can chelate Ni$^{2+}$ from the resin and interfere with protein binding. B) 50 mM Na-phosphate pH 7.5, 300 mM NaCl, 0.1% Tween, 20 mM imidazole was used for soluble extracts with His-Mcb1-CTD. C) 50 mM Na-phosphate pH 7.5, 300 mM NaCl, 0.1% Tween, 20 mM imidazole was used for soluble extracts with HisGFP-Mcb1-CTD.
5.4 Purification of His-Mcm4-NTD

The interaction of recombinant Mcb1-NTD with the \textit{in vitro} translated N-terminal domains of all fission yeast Mcm proteins but Mcm2 (described in 2.3) raised the question whether this interaction is not bridged by some protein present in the rabbit reticulocyte lysate. To address this, it was attempted to test these interactions in a system free of any contaminating proteins, and therefore a recombinant Mcm protein in addition to recombinant Mcb1-NTD was required. Mcm4-NTD was chosen as it was not supposed to be a direct neighbour of Mcb1 in the working model (see 2.5) but showed strong interaction with rMcb1-NTD in the IVT pull-downs. Mcm4 has a long N-terminal extension domain (AA 1-152), which could possibly mediate interactions with non-adjacent proteins in the Mcm2-7 complex but also in a putative Mcm\textsuperscript{Mcb1}. To prevent this, and to be able to test for interactions between the main- parts of Mcb1-NTD and Mcm4-NTD, the sequence coding for this N-terminal extension was removed from the cloning-template. This also ensured that Mcm4-NTD could serve as model for other Mcm proteins, which are also not neighbours of Mcb1 in the working model like Mcm3 and Mcm7.

The DNA sequence encoding fission yeast Mcm4-NTD without its N-terminal extension was cloned between the \textit{NcoI} and \textit{HindIII} site of pEHisTEV making pEHisTEV-Mcm4NTD. The plasmid was transformed into Rosetta2 and test expressions were performed at 22 and 30°C for five hours. Soluble extracts were made in phosphate or Tris buffer containing 300 mM NaCl and incubated with Ni-NTA resin to capture soluble His-Mcm4-NTD protein. The protein was well expressed at both tested temperatures and soluble in both tested buffers, indicated by the appearance of strong bands at about 50 kDa in the lanes with soluble extract and Ni-NTA resin (Figure 5-6).
Figure 5-6 Expression of His-Mcm4-NTD from pEHisTEV. Expressions were induced with 0.5 mM IPTG and conducted at 22°C and 30°C for five hours. Soluble extracts were made in phosphate or Tris buffer containing 300 mM NaCl. Soluble extracts were incubated with Ni-NTA resin to pull down soluble His-Mcm4-NTD. Samples were subjected to SDS-PAGE: T=total, P= pellet (insoluble), S= soluble, B= pull-down on Ni-NTA. Arrow indicates His-Mcm4-NTD.

For the large-scale purification, expression was conducted at 30°C and extracts were prepared in Tris buffer. The purification was performed in two steps using the Äkta purifier. In the first step, soluble extract from two litres of *E. coli* culture was loaded onto a HisTrap FF 5 ml column. After washing, elution of His-Mcm4-NTD was accomplished with a linear gradient of increasing imidazole concentrations (20-500 mM). The protein eluted at around 150 mM imidazole in one symmetrical sharp peak (Figure 5-7 A, diagram). The height of this peak of 350 mAU indicated a high protein yield for this purification step. Analysis of the fractions by SDS-PAGE confirmed a high concentration of Mcm4-NTD in the peak fractions. However there were multiple bands visible below the main Mcm4-NTD band, most likely originating from protein degradation, and also some contaminating proteins above the Mcm4-NTD band. (Figure 5-7 A, SDS-PAGE). To improve the purity, a stepwise gradient could be run in future attempts.
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Figure 5-7 Purification of His-Mcm4-NTD from two litres of *E. coli* culture. Expression was performed at 30°C for five hours and soluble extracts were made in Tris buffer with 300 mM NaCl as described in Materials and methods. A) Affinity purification using a HisTrap FF 5 ml column (GE). The protein was eluted with a linear gradient of 0-100% buffer B containing 500 mM imidazole (secondary axis). Samples of all purification steps and peak fractions were analysed by SDS-PAGE: U= uninduced, T= total, P= pellet, S= soluble, FT= flow through, W2= wash fraction after 75 ml. B) Gel filtration with Superdex 200 column (GE). The protein eluted in multiple peaks (A, B and C) between 110 and 210 ml. Peak fractions were analysed by SDS-PAGE: L= pooled protein fractions from HisTrap column; A, B, C every third fraction of indicated peaks.
For further purification, the peak fractions from two His-Trap runs were pooled, concentrated and loaded onto a Superdex 200 gel filtration column (GE). Interestingly the UV-profile of this gel filtration did not show one peak, but a series of peaks starting at 110 ml and ending at 210 ml (Figure 5-7 B). Analysis of the corresponding fractions by SDS-PAGE revealed that they all contain the same 50 kDa protein-species, His-Mcm4-NTD (Figure 5-7 C, SDS-PAGE). Thus it is assumed, that the observed peaks are derived from multimeric complexes of Mcm4-NTD. As the column has not been calibrated, the sizes corresponding to the peaks can only be estimated, but the peak at 180 ml most likely represents a monomer (peak C), while the 160 ml peak could be caused by a dimer (peak B) and the plateau between 110 and 150 ml might represent different multimers (plateau A) which are not resolved.

The fractions corresponding to the Mcm4-NTD monomer were pooled and concentrated for further use.

### 5.5 Applications with purified recombinant Mcb1- NTD

#### 5.5.1 Antibody production

2 mg of purified rMcb1-NTD were used for immunisation of two rabbits by Dundee Cell Products Ltd as described in Materials and methods. In total four immunisations were conducted over three months and the serum was tested for detection of endogenous Mcb1 following each immunisation. 1:100 dilutions of the serum were used in Western blots of samples from cells with endogenous levels of Mcb1 (wt or tagged) or overexpressing Mcb1 constructs (as in chapter 4.3.1). The signal was very weak though: endogenous Mcb1 levels could not be detected (Figure 5-8 A, B, C) and strong signals were only obtained when using extracts from fission yeast strains overexpressing (FLAG-tagged) Mcb1 (Figure 5-8 B, C).

However, the detection was specific and no major contaminating bands, emanating from proteins other than Mcb1 were observed. In general the serum from rabbit 45 seemed to allow a more efficient Mcb1 detection, resulting in stronger signals (compare rabbit 44 and 45 in Figure 5-8). The antibody in the final bleed of rabbit 45 was affinity purified and concentrated using NTD Pierce NHS-Activated Agarose Dry
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resin as described in Materials and methods. The purified antibody was used for
detection of endogenous and also tagged Mcb1 in 1:350 dilutions (e.g. Figure 4-11).

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over night exposure, film

7 minutes, CCD camera

Figure 5-8 Production of polyclonal α-Mcb1 antibodies in rabbit by Dundee Cell Products LTD. Two rabbits (44 and 45) were immunised with rMcb1-NTD. The serum was tested for Mcb1 detection after immunisations 2, 3 and 4. A) Pre- serum and serum after the second immunisation were tested for detection of endogenous and YFP-tagged Mcb1. B) and C) Serum after the third/ fourth immunisation was used to detect different FLAG-tagged truncation constructs of Mcb1, overexpressed from the nmt1 promoter or endogenous levels of wt Mcb1.
5.5.2 Crystallisation screens with Mcb1-NTD

As rMcb1-NTD-Strep could easily be purified in high yields, it was attempted to set up crystallisation screens in order to find conditions for the growth of crystals suitable for X-ray diffraction and structure determination. Purified rMcb1-NTD in 100 mM Tris-HCl pH 8, 150 mM NaCl, 1 mM EDTA, 0.1% Tween-20 was concentrated to 5 mg/ml. Sitting drop vapour diffusion crystal trials were set up using a crystallisation robot (Hamilton-Thermo Rhombix system) and different crystallisation screens. After some days many of the drops looked dry, presumably due to the presence of Tween in the protein concentrate.

**Table 5-1 Conditions that supported the growth of needle shaped crystals in Mcb1-NTD crystallisation screens**

The screen identifiers and the composition of original conditions that supported the growth of micro crystals are given. The rows “refinements” show the composition of the refinement-screens, which were set up to enhance the quality of the initial crystals. Conditions in which crystals were observed are highlighted in bold.

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However some conditions showed thin, long needle-like crystals. These conditions were WizardI #15, WizardII #20 and CryoII #9 and their compositions are listed in Table 5-1. Refinements of these conditions were set up manually and monitored daily. In some of the refinements again long needle-shaped crystals grew (see Table 5-1). However it was not possible to clearly distinguish if the observed crystals were protein or salt crystals, as the needles were so thin that incorporation of Izit dye was very hard to see. Izit is a dye that penetrates the solvent channels of macromolecular crystals and colours them blue, while salt crystals cannot absorb it and stay colourless.
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(hamptonresearch.com). A possible cause for the extremely thin needles (and also the observed drying of the crystallisation drops) could be the Tween-20 in the concentrated protein solution.

For further crystallisation trials, consequently attempts were made to remove the Tween from the protein solution. Mcb1-NTD in 100 mM Tris pH 8, 150 mM NaCl, 1 mM EDTA, 0.1% Tween-20 was dialysed against a buffer without Tween and the product was concentrated using a protein concentrator (9K MWCO, Thermo Scientific).

Unfortunately Mcb1-NTD precipitated when the concentration exceeded 3.5 mg/ml. So apparently the Tween-20 is needed for stabilisation of the protein. Attempts to purify Mcb1-NTD using buffers without Tween but different detergents (e.g. 0.1% NP-40, 10% Glycerol) resulted in lower solubility and very low yields (0.2 mg/l with 0.1% NP40), precipitating again when concentrated too much.

The simultaneous addition of the charged amino acids L-arginine and L-glutamic acid at low concentration (50 mM) has been reported to increase the solubility and stability of a purified protein and prevent its degradation (Golovanov et al. 2004). It was found that the presence of an increased number of arginine and glutamic acid molecules around the target protein can improve protein stability. Additional molecules lead to increased crowding, which prevents protein association, which can be the cause of precipitation and degradation (Shukla & Trout 2011).

Even though the addition of 50 mM glutamic acid and arginine increased the solubility and stability of rMcb1-NTD, the protein-concentration of at least 5 mg/ml, necessary for crystallisation screens, could not be obtained without Tween-20 in the buffer. Thus efforts to crystallise Mcb1-NTD failed as the protein could not be concentrated enough in the absence of detergent.

5.5.3 Interaction of rMcb1 with ivt NTDs of Mcm proteins

In order to test the ability of Mcb1 to interact with Mcm proteins in vitro, recombinant Mcb1-NTD was immobilised on StrepTactin and incubated with in vitro translated N-terminal domains of Mcm2, Mcm3, Mcm4, Mcm5, Mcm6 and Mcm7. The results of these experiments are shown in 2.3.1.
5.5.4 Interaction of rMcb1-NTD and rMcm4-NTD

Recombinant purified Mcb1-NTD and Mcm4-NTD were used to investigate whether the two protein-subdomains are capable of interacting in the absence of any other protein. This was of interest, as in vitro synthesised NTDs of Mcm proteins, which are not neighbours to Mcb1 in the model of a putative MCM$^{Mcb1}$ complex, were found to be able to interact with Mcb1-NTD. To exclude the possibility that the observed interaction was caused by proteins in the rabbit reticulocyte lysate, recombinant Mcm4-NTD and Mcb1-NTD were used for in vitro interaction studies, the results of which are described in 2.3.2.

5.5.5 Interaction of rMcb1 with ivt Mcm4 constructs

Results from interaction studies of rMcb1-NTD with in vitro translated NTDs of Mcm proteins indicated that fission yeast Mcb1 is able to bind all Mcm proteins, the ability to bind Mcm2 being not absolutely clear (see 2.3.1).

To obtain information about how the interaction of Mcb1 and Mcm proteins is mediated and to get an idea about a possible arrangement of an MCM$^{Mcb1}$ complex, it was attempted to identify regions of the Mcm proteins essential for these interactions. As Mcm proteins share homology with each other (and Mcb1) it is possible that these binding regions are conserved among the different Mcms. To address which regions in the Mcm-NTDs are needed for interaction with rMcb1-NTD, several truncation constructs of Mcm4-NTD for in vitro translation were made. Mcm4-NTD was chosen because of its clear interaction with rMcb1 in the results presented in Chapters 2.2.2 and 2.2.3 and its 11 internal methionines, which allow the construction of many different short products that can still be labelled with $^{35}$S methionine for visualisation. For eukaryotic Mcm proteins no crystal structures have been reported yet but it is likely that they adopt a structure similar to archaeal Mcms, for which structures are available (Bae et al. 2009; Liu et al. 2008; Brewster et al. 2008; Fletcher et al. 2003). Structure prediction with Phyre2 was used to obtain a possible structure for fission yeast Mcm4. As for Mcb1 structure prediction, the structure derived from ssoMcm
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(PDB identifier 3f9v) was the top hit with a confidence score of 100 (indicating that Mcm4 is a true homologue of ssoMcm) and a sequence identity of 38%, covering 75% of the Mcm4 sequence.

**Figure 5-9 Predicted 3D structure of *S. pombe* Mcm4.** Structure was predicted with Phyre2 and modelled based on ssoMcm (3f9v). Models were visualised using Pymol. **A)** SpMcm4 only. Different functional domains are highlighted. Domain A (gold), domain B (grey, with Zn-coordinating cysteines in orange), domain C (black), NT-hairpin (green, sticking out to the side) and ACL (green, pointing upwards), Walker A and Walker B (magenta). **B)** Superposition of SpMcm4 and ssoMcm.

Mcm4 superimposes with ssoMcm almost perfectly (Figure 5-9). However the first 167 amino acids of Mcm4 are not modelled. Most of this part of Mcm4 represents the N-terminal extension domain, which is not present in archaean Mcm proteins, and explains its absence in the model. The structure prediction was used in combination with an alignment containing Mcm proteins from multiple species (alignment of Mcms and MCM-BPs in Appendix 4), to select some Mcm4 domains and motifs, suitable for deletion, and determine their boundaries, in order to make different truncations. These truncations should then be tested for their capability to interact with Mcb1-NTD *in vitro*. A schematic representation of the domains in Mcm4, focussing on the N-terminal half of the protein, can be found in Figure 5-10. The N-terminal extension was defined to include amino acids 1-152. This domain is not modelled in the 3D structure,
but it can be imagined to extend over the top of an MCM complex and thus be suitable for interactions with other proteins, which makes it a good target for deletion.

**Figure 5-10** Schematic representation of Mcm4 and Mcm4 truncation constructs for analysis of Mcb1-Mcm4 interaction. Boundaries of the constructs were determined from a multiple sequence alignment ([Appendix 4](#)) and a structure prediction for Mcm4 ([Figure 5-9](#)). The table indicates the size of the expected products, as well as the number of internal methionines for labelling.

Domain A contains amino acids 153-287 ([Figure 5-9 A](#), highlighted in gold), and a construct lacking this domain and the N-terminal extension was made. Domains B and C include amino acids 288-496. The N-terminal hairpin (NT-hp) was located at amino acids 423-431. As it is sticking out to the side of the protein ([Figure 5-9](#)), it might make a good site for interaction with Mcb1, even though there is evidence that the motif is involved in DNA binding ([McGeoch et al. 2005](#)). The allosteric communication loop (ACL), which was described to be important for interaction of the NTD of one Mcm protein with the CTD of the adjacent Mcm in the hexameric complex ([Barry et al. 2009](#)) was located at amino acids 377-389, and could also represent a suitable site for Mcb1-interaction. Deletion of one or both motifs was attempted to see if there is an effect on Mcm4-Mcb1 interaction. Further the N-terminal domain of Mcm proteins contains a Zn-finger located in domain B (with Zn-coordinating cysteine residues highlighted in orange in [Figure 5-9](#)), which is thought to be involved in ssDNA binding ([Kasiviswanathan et al. 2004](#); [Poplawski et al. 2001](#)). Still, mutants were constructed to see if there is an effect on the Mcm4-Mcb1 interaction when inactivating the Zn-finger.
Therefore three of the four Zn-coordinating cysteines were mutated to alanine (C326A, C329A and C332A). Sequences coding for the different Mcm4-constructs were amplified by PCR and cloned into pTNT using *Mlu*I and *Xba*I as described in Materials and methods for *in vitro* translations. The integrity of the inserts was confirmed by sequencing. A diagram of all the constructs generated is shown in Figure 5-10.

*In vitro* translation of the constructs, labelling of the products and pull-down assays with rMcb1 were conducted as described in Materials and methods and 2.3.1. The *in vitro* translation reaction of all twelve Mcm4 constructs was successful, resulting in one major band of the expected size for each (Figure 5-11 input). The products of the translation reactions with constructs 1, 3 and 7 were accompanied by an array of weaker bands, possibly caused by degradation of the main product (Figure 5-11 input lanes 1, 3, 7). Furthermore the products of constructs 8 and 9 co-migrate with proteins from the reticulocyte lysate (possibly ribosomal proteins) leading to the appearance of blotches instead of clean bands (Figure 5-11 input lanes 8 and 9).

![Figure 5-11](image)

**Figure 5-11** *In vitro* translation of Mcm4-NTD truncation constructs and pull-down with rMcb1-NTD. *In vitro* translation reactions, using pTNT plasmids carrying the sequences for the Mcm4-NTD constructs (1-12) as templates, were conducted as described in 2.3.1 and Materials and methods. Synthesized protein was incubated with 50 μg rMcb1-NTD immobilised on Strep-Tactin for pull-down. As negative
control, \textit{in vitro} translated protein was incubated with Strep-Tactin only. Samples were boiled with 5xSB and subjected to SDS-PAGE. Gels were dried and exposed to Kodak Bio Max MR film for 24 hours. For constructs 1-7, 12% gels were used, for 8-12 15% gels. Input: 1-7: 6.5\% of pull-down, 8-12: 13\% of pull-down.

Analysis of the pull-down reactions of rMcb1-NTD with the twelve Mcm4-constructs revealed that all constructs, apart from the N-terminal extension on its own (Figure 5-11 lane 2), could interact with Mcb1-NTD under the conditions used. For each \textit{in vitro} translation, the major product was also the species pulled-down by Mcb1-NTD. This means that the constructs lacking the N-terminal extension can interact with Mcb1-NTD. This is also the case for domain A on its own (Figure 5-11 lane 12), and the constructs lacking domain A. Thus, constructs, which do not overlap (as domain A and the constructs lacking domain A) are able to interact with Mcb1-NTD independently. The ACL loop and the sequence downstream of it seem to be redundant for Mcm4-Mcb1 interaction, as constructs missing this motif were still pulled down with Mcb1-NTD. The same was found for the NT-hp and constructs lacking both, domain A and the ACL (lanes 10 and 11) or the NT-hp (lanes 8 and 9). This indicates that the N-terminal half of Mcm4 contains several binding sites, able to mediate the interaction with Mcb1: at least one site in domain A, and at least another one in the remainder of Mcm4-NTD. In this regard it would be interesting to find out if the short stretch of domain C containing the ACL loop and the NT-hp is sufficient for Mcm4-Mcb1 interaction \textit{in vitro} and contains a binding site as well.

Interestingly the mutation of the Zn-coordinating cysteine residues did not abolish or weaken the interaction of the respective Mcm4 constructs with Mcb1-NTD. If at all, the products carrying the mutation seemed to be pulled down more efficiently than their non-mutated counterparts (Figure 5-11 compare lanes 8 and 9, 10 and 11). However, as the protein amounts were not quantified, this remains speculative.

Summarising, pull-down assays of different Mcm4 truncations with rMcb1-NTD showed that apart from the Mcm4-N-terminal extension all analysed products were able to interact with Mcb1-NTD. Designated motifs present in Mcm4, like the ACL and NT-hp were not found to be important for interaction, as was the Zn-finger in domain B. As non-overlapping constructs were pulled down with Mcb1, it is concluded that
Mcm4 contains at least two sites for interaction between Mcb1 and Mcm4, one in the domain A and another in domain B and C.

5.6 Discussion

In this chapter the expression and purification of different Mcb1 and Mcm4 constructs was described. Strategies to successfully purify Mcb1-NTD-Strep and His-Mcm4-NTD were developed using a two-step protocol consisting of an affinity-step (StrepTrap HP column in case of Mcb1-NTD and HisTrap in case of Mcm4-NTD) and a final gel filtration.

The elution of the Mcb1-NTD off the gel filtration column in one sharp peak suggests that the protein binds to the column as a uniform monomeric population. This is a first indicator that Mcb1-NTD, unlike Mcm-NTDs from *Methanthermobacter thermautotrophicus* (Fletcher et al. 2003) and *Sulfolobus solfataricus* (Liu et al. 2008), does not multimerise, at least not under the conditions used here. Mcm4-NTD in contrast, elutes as a series of peaks from the gel filtration column indicating that Mcm4-NTD is present as monomer but also as different multimers. The nature of the multimers was not determined. However it is likely that the observed high molecular weight complexes are rather the result of random association of several Mcm4-NTD monomers than structured hexamers, as the fission yeast Mcm4 protein is usually part of a heterohexamer and not a homohexamer like the archaeal Mcm proteins. Still it cannot be ruled out that the N-terminal domain of Mcm4 has a function in oligomerisation and might also form homo-oligomers. Experiments to test self-interaction of Mcm4-NTD (e.g. using *in vitro* translated Mcm4 and rMcm4-NTD) were not conducted but could be attempted. Interestingly, Xu et al report that different constructs of fission yeast Mcm4-NTD, Mcm6-NTD and Mcm7-NTD all purify as monomers or dimers but they did not observe the pattern found here (Xu et al. 2013).

The purification strategies described allowed the production of pure protein in quite high yields: about 1 mg Mcb1-NTD could be obtained per litre of *E. coli* culture. In case of Mcm4-NTD 2.4 mg monomeric protein was purified per litre of culture. Additionally another 2 to 3 mg of different multimers were obtained per litre of *E. coli*. The yield of
Mcb1-NTD could be improved further by loading less of the initial soluble extract per affinity column to not exceed the binding capacity of the StrepTactin and execute more runs instead.

Despite the straightforward expression and solubility of Mcb1-NTD, the full length protein appeared to be difficult to purify as only small amounts of soluble protein were recovered. Furthermore no suitable conditions for the expression of soluble Mcb1-CTD were found. The protein domain was not expressed from pASK-IBA 5+ (N-terminal Strep-tag) and pEHisGFPTEV (N-terminal His-tag followed by GFP tag) at different temperatures. When using pEHisTEV (for N-terminal His-tag), good expression could be detected but the protein was not soluble and could not be recovered on Ni-NTA resin in small scale Ni-purifications. Problems with the solubility of fission yeast Mcm-CTDs have been reported earlier (Bochman & Schwacha 2009; Xu et al. 2013) and a crystal structure derived from Mcm-CTD only is not available yet, possibly also due to the difficult solubilisation of the protein. However purification of smaller domains from Mcb1-CTD corresponding to Mcm-α/β or α-domains might be possible. Further, parts of the N/C linker could be added to the Mcb1-CTD construct used here to provide some stability.

Purified Mcb1-NTD was used for a variety of applications. Polyclonal antibodies were raised in rabbit. The serum proved to be specific for the detection of Mcb1, but only if the protein was present in large quantities. Endogenous levels of Mcb1 were not detected. This was improved by affinity purification (using a column with immobilised Mcb1-NTD) and concentration of the antibody. Still, low levels of the protein (as in small scale protein extracts from fission yeast) cannot be detected.

It was also aimed to run some crystal trials to possibly grow Mcb1-NTD crystals suitable for X-ray analysis. Despite the presence of Tween-20 in the protein concentrate, which could have a negative effect on crystallisation, crystal trials were set up. Indeed some very thin needle shaped crystals formed in some of the tested conditions, which were used for further refinements. But these did not result in any bigger crystals and it could not be assured that the observed micro crystals consisted of protein and not salt.
Interestingly the observed crystals formed in conditions containing either Zn$^{2+}$ or Li$^+$ as additives. This could indicate a stabilising effect of the ions on the protein. The effect of Zn is interesting, as Mcm proteins contain a Zn binding motif in the B-subdomain of the NTD. But structure prediction and sequence analysis of Mcb1 and its homologues suggest that MCM-BPs are presumably unable to bind Zn, as the necessary coordinating cysteine residues are not conserved in the proteins. However this is based only on theoretical methods and it might be possible that Mcb1 can bind Zn. The attempt to remove or replace Tween-20 from the protein concentrate failed, as the protein showed to be highly unstable and insoluble in the absence of the detergent, so that further trials with Tween-free protein concentrate could not be started here. However it seems possible to find a condition, which allows a sufficient concentration for crystallisation, possibly by trying other detergents and additives. Further, co-crystallisation with e.g. Mcm4-NTD could be attempted.

Pull-down assays with \textit{in vitro} synthesised Mcm4 truncation constructs and rMcb1-NTD were conducted to obtain information about how the interaction of Mcb1 with Mcm proteins is mediated and which domains of Mcm proteins are necessary. The results of the pull-downs suggest that all tested constructs apart from the N-terminal extension of Mcm4 on its own can interact with Mcb1. The inability of the N-terminal extension to interact with Mcb1 was unexpected, as this domain could be imagined to stretch out over the N-terminal end of an Mcm2-7 hexamer or putative MCM$^{Mcb1}$ to contact proteins, which are not direct neighbours. The N-terminal extension of human and fission yeast Mcm4 has been reported to be important for the activation of MCM2-7, as it is a target for Cdc7 phosphorylation and subsequent interaction with Cdc45 (Masai et al. 2006; Devault et al. 2008). It might not be involved in interaction with Mcb1. However it might also be possible that this N-terminal extension can only mediate interactions with other proteins in intact hexamers. Here, only the \textit{in vitro} interaction of Mcb1-NTD and Mcm4-NTD constructs was tested and maybe the N-terminal extension on its own is unable to fold properly. Thus it cannot be ruled out completely that the extension might have a role in Mcm4-Mcb1 interactions.
Some of the domains, which were tested positive for Mcb1-NTD interaction, do not overlap, suggesting the presence of several sites in the Mcm4-NTD, which could contribute to Mcb1-NTD binding. One of these sites would be found in domain A. The role of this domain in Mcm proteins is not entirely clear yet but it has been suggested to have regulatory function in helicase activity of eukaryotic MCM2-7 ("domain-push hypothesis" Sclafani et al. 2002; Fletcher et al. 2003) and could be involved in DNA binding (Kasiviswanathan et al. 2004; reviewed in Sakakibara et al. 2009). Interestingly domain A has been reported to be non-essential for some archaeal Mcm proteins (Kasiviswanathan et al. 2004). Further, at least a part of the putative domain A of Mcb1 was found to be dispensable for Mcb1 function in fission yeast (see 4.3.1). Maybe this domain contributes to interaction of Mcm4 (and also other Mcm proteins) and Mcb1 in fission yeast. Another interaction site is suggested to reside in the remaining part of Mcm4-NTD before the NT-hp and the ACL (AA 288-420). This is mainly the B domain and some part of the C domain.

The presence of the ACL loop and N-terminal hairpin was found to be not necessary for Mcb1-NTD Mcm4-NTD interaction. The ACL has been reported to be important for communication between adjacent Mcm proteins, so it might not have a role in NTD-NTD interaction.

A more accurate localisation of these binding sites is not possible just with the results presented here but could potentially be achieved using different constructs. Further it has to be noted that these studies were conducted \textit{in vitro} and are not suitable to make a statement about the \textit{in vivo} situation of respective Mcm4 mutants in fission yeast. Most likely, none of them would be viable \textit{in vivo}. 
Chapter 6 General discussion

6.1 Principal conclusions

This study aimed to characterise Mcb1, the fission yeast homologue of MCM-BP. Biochemical analysis showed that Mcb1 forms a complex with Mcm proteins. It was found to interact with Mcm3 through Mcm7 but not with Mcm2. Fission yeast Mcb1 was discovered to be essential. Inactivation using temperature-sensitive mutants, but also overexpression of Mcb1 cause DNA damage, checkpoint activation and cell cycle arrest, indicating that distinct levels of the protein are necessary for proper cell cycle progression. Structure prediction and sequence analysis suggest that Mcb1 is related to Mcm proteins and has a similar structure. Construction of different Mcb1 truncations indicated that for complete function almost the full-length protein is necessary. Mcb1 is likely to behave similarly to Mcm proteins concerning complex formation and interaction between Mcb1 and individual Mcm proteins is presumably mediated via multiple interaction sites, however the exact locations could not be identified here.

6.2 Mcb1 and other MCM-BP homologues interact with Mcm proteins

TAP purification and co-immunoprecipitations presented in Chapter 2 indicate that fission yeast Mcb1 interacts with Mcm3-7 but is not in a complex with Mcm2 \textit{in vivo}. This is consistent with other results from fission yeast (Ding & Forsburg 2011), human cells (Sakwe et al. 2007) and \textit{Xenopus} (Nishiyama et al. 2011), where MCM-BP was found to interact with Mcm3-7 but not with Mcm2. A very recent study reporting an interaction of Mcb1 and Mcm2 in fission yeast, shows only small amounts of Mcm2 when compared to other Mcm proteins (Santosa et al. 2013). Interestingly, some species seem to exhibit a preferred interaction of MCM-BP with one particular Mcm protein. In \textit{Xenopus} this is Mcm7 (Nishiyama et al. 2011), while \textit{Arabidopsis} Etg1 interacts most strongly with Mcm5 (Takahashi et al. 2008). Whether this preference is
an experimental artefact or resembles the real binding situation remains to be elucidated.

Furthermore, results from sucrose gradients described in Chapter 2 suggest that Mcb1 is part of a high molecular weight complex, consistent with the size of an assembly of Mcb1 with multiple Mcm proteins up to a size of about 500 kDa. A study with *Xenopus* extracts, which was published while this work was in progress, suggests that MCM-BP mainly forms smaller, probably dimeric complexes mainly with Mcm7. But it is speculated that this might be due to dissociation of a bigger complex during the long experimental incubation time (Nishiyama et al. 2011). Nguyen *et al* showed evidence that human MCM-BP co-sediments with Mcm proteins and a fraction is part of a bigger complex that could be a hexamer (Nguyen *et al*. 2012).

Thus it is likely that fission yeast Mcb1 participates in an alternative high molecular weight MCM complex, MCM\textsuperscript{Mcb1}, in which it replaces Mcm2. The same might be true for other MCM-BP homologues; although it cannot be excluded that MCM-BP might form different complexes in different organisms, resembling differences in the function or regulation of MCM-BP. This might explain the preferred interaction with certain Mcm proteins observed in some organisms. In this respect it has to be mentioned that *T. brucei* MCM-BP was found to co-purify with Mcm4-8 but not Mcm2 and Mcm3 (Kim *et al*. 2013). This is rather unusual, as previously no interaction of MCM-BP homologues with Mcm8, which is absent in several eukaryotes, has been reported.

Analysis of pairwise interactions of rMcb1-NTD with the N-terminal domains of the members of Mcm2-7 revealed that Mcb1-NTD can interact with most Mcm proteins *in vitro* (Chapter 2). For interaction with Mcm2-NTD the results were not absolutely clear but rather argue against an interaction. The results from other studies, which were published while this work was underway, give evidence that human MCM-BP is able to interact with all Mcm proteins *in vitro*, including Mcm2 (Nguyen *et al*. 2012) which was also reported in another fission yeast Mcb1 study (Santosa *et al*. 2013).

The results described here also suggest that Mcb1 can interact with several different Mcm proteins at the same time, indicating that it is indeed capable of forming high molecular weight complexes with Mcm proteins. In support of this, human MCM-BP
was reported to be able to interact with the three subunits of the core helicase (MCM4/6/7) in one big complex (Sakwe et al. 2007).

Even though the results presented here cannot provide definite evidence for it, a scenario where Mcb1(-NTD) binds one Mcm(-NTD), which then binds to another Mcm(-NTD), seems likely. This would allow an ordered structure, like in MCM2-7 or NssoMCM hexamers (Liu et al. 2008). Concordantly, Mcb1 and other MCM-BP homologues could have preferred direct interaction partners in a complex as it was observed in *Xenopus* (Nishiyama et al. 2011) and *Arabidopsis* (Takahashi et al. 2008) but it is also possible that MCM-BP interacts with a random Mcm protein first and other Mcm proteins just assemble onto this primary complex. The differences in *in vitro* interaction of Mcb1/MCM-BP with Mcm2 might be explained with the fact that in the experiments described here, only the N-terminal domain of Mcm2 was used for interaction with Mcb1-NTD, while the other studies all used full-length proteins. It is possible that for an interaction between Mcb1 and Mcm2 the CTD has to be present as well.

Concluding, it is still unclear what an MCM\textsuperscript{Mcb1} or MCM\textsuperscript{MCM-BP} complex looks like in terms of subunit composition and direct interaction partners of MCM-BP. Present evidence suggests that a high molecular weight complex of MCM-BP and Mcms exists and that MCM-BP can interact with more than just one Mcm protein, however it is unclear if this is a hexamer or heptamer or rather a smaller complex and if MCM-BP interacts in an Mcm-Mcm manner or sticks to the outside of a complex. Data presented here favours a hexameric assembly of Mcb1 with Mcm3-7. To reveal the subunit composition of MCM\textsuperscript{Mcb1}, the TAP-purified complex could be analysed by EM. Alternatively, crosslinkers (e.g. BS3-d0/d4 pair) could be used to stabilise the complex and MS analysis would possibly allow the identification of interaction sites.

It is assumed that the putative MCM\textsuperscript{MCM-BP} complex functions in replication. Like MCM2-7, it might interact with different replication factors. Here the interaction of fission yeast Mcb1 with the GINS subunits and Cdc45 were examined, proteins closely associated with MCM2-7 in the CMG complex, the replicative helicase in eukaryotic replication. However, no interaction could be detected. Furthermore Mcb1 did also not interact with Cdt1, which functions in MCM2-7 recruitment to the origins of replication and preRC assembly. Recent studies indicate that human MCM-BP interacts
with RPA and the regulatory Dbf4 subunit of Cdc7 kinase (Nakaya et al. 2010; Nguyen et al. 2012), both of which act very close to the replication process. Thus there might be more replication factors, which interact with MCM-BP. As the plant homologe Etg1 was reported to have a role in cohesion establishment (which is also a process MCM2-7 might be involved in) it seems possible that MCM-BP interacts with Tof1-Csm3 or cohesion subunits and this might be worth some investigation.

6.3 Mcb1 and other MCM-BP homologues function in replication and regulate the MCM2-7 helicase

Results presented in Chapter 3 suggest that fission yeast Mcb1 is essential, which was confirmed by another study (Ding & Forsburg 2011). So far only one other essential MCM-BP homologue is known, the T. brucei MCM-BP that was described only recently (Kim et al. 2013).

Mcb1 function was examined using temperature-sensitive mutants to inactivate the protein. Inactivation of Mcb1 caused high levels of DNA damage and activation of the Chk1 dependent DNA damage checkpoint, accompanied by an elongated cdc phenotype. While increased DNA damage as a consequence of MCM-BP inactivation has been observed in most studied organisms so far (Takahashi et al. 2008; Li et al. 2011; Jagannathan et al. 2012; Kim et al. 2013; Santosa et al. 2013), checkpoint initiation was only observed in plants, fission yeast and T. brucei (Takahashi et al. 2008; Li et al. 2011; Kim et al. 2013; Santosa et al. 2013), but was only transient in human cells (Jagannathan et al. 2012) and not observed in Xenopus (Nishiyama et al. 2011). Furthermore a delay in cell cycle progression was not only observed in fission yeast but also plants and human cells when MCM-BP was inactive. This indicates that lack of MCM-BP can be tolerated in organisms, where MCM-BP was not found to be essential, but they still suffer consequences from MCM-BP absence.

DNA damage and checkpoint activation indicate problems with DNA replication in MCM-BP deficient cells. Depletion of Arabidopsis Etg1 was reported to cause defects in sister chromatid cohesion, which might be an indirect effect of impaired DNA replication (Takahashi et al. 2010). Components of the replisome and MCM2-7 seem to be involved in cohesion establishment (Tanaka et al. 2009) through their interaction with Tof1-Csm3 (Tanaka et al. 2009; Mayer et al. 2004) and Smc1 (Ryu et al. 2006) and
misregulation of MCM2-7 could possibly lead to problems with cohesion. In human cells MCM-BP depletion disrupted nuclear morphology, leading to doughnut shaped nuclei. It is assumed that this is caused by chromosome missegregation due to amplified centrosomes, which were also observed in cells lacking MCM-BP (Jagannathan et al. 2012). Mcm proteins and also other replication factors were shown to be associated with centrosomes and the regulation of their duplication, (Andersen et al. 2003; Stuermer et al. 2007; Ferguson & Maller 2008; Knockleby & Lee 2010) even though MCM-BP was not. It is likely that centrosome amplification is an indirect effect of problems with replication or impaired MCM2-7 function in the absence of MCM-BP. Furthermore, centrosome amplification could also be a consequence of G2 checkpoint activation, caused by MCM2-7 malfunction.

Results from other studies with fission yeast Mcb1 reported decreased Mcm7 loading onto origins when Mcb1 is inactivated. Furthermore genetic interactions of Mcb1 and preRC components were observed and mcb1-Ts mutants were suppressed by CDK modulation, which indicates problems specifically with preRC formation in Mcb1 deficient cells (Santosa et al. 2013). Interestingly studies with Xenopus extracts reported that MCM2-7 unloading off chromatin was delayed in MCM-BP depleted cells, while preRC assembly was not compromised and also Cdc45 and PCNA loading occurred normally (Nishiyama et al. 2011). However it is possible that MCM-BP in Xenopus has a slightly different role than in other eukaryotes.

Interestingly, the mcb1-Ts mutants described by Santosa et al showed significantly reduced interaction with the MCM complex compared to the wild-type protein (Santosa et al. 2013), which was not observed with the mcb1-Ts mutants used in the study presented here. Moreover, it was reported that nuclear localisation of Mcm proteins was impaired when Mcb1 was inactivated and Mcm proteins were found to be transported to the cytoplasm in a Crm1-dependent process (Santosa et al. 2013). Crm1+ (Chromosomal region maintenance 1) is highly conserved in eukaryotes and encodes a nuclear export receptor, which controls relocalisation of nuclear proteins (Fukuda et al. 1997). Nuclear export of Mcm proteins was also indicated as a consequence of Mcb1 overexpression (Ding & Forsburg 2011). In fission yeast Mcm localisation in the nucleus requires formation of the Mmc2-7 complex. Consequently, when Mmc2-7 assembly is disturbed, the subunits are exported to the cytoplasm (Pasion & Forsburg 1999). However this is likely to be a fission yeast specific effect, as
reduction of MCM-BP levels did not cause nuclear export of Mcms in any other system studied. Studies with human cells indicated that upon MCM-BP depletion general levels of Mcm proteins, especially the soluble fraction, were increased (Jagannathan et al. 2012; Sakwe et al. 2007).

How the observed de-silencing of VSG encoding loci in MCM-BP depleted T. brucei relates to this is unclear (Kim et al. 2013). However it might be possible that the MCM2-7 complex is involved in repression of these regions and that its regulation by MCM-BP contributes to the silencing.

The consequences of MCM-BP overexpression were also studied. Results described here indicate that overexpression of Mcb1 is toxic and is accompanied by similar symptoms as Mcb1 inactivation, such as an elongated cell phenotype, increased DNA damage and checkpoint activation. This was confirmed in an extensive study by Ding et al, who further reported problems with replication initiation in Mcb1 overproducing cells (Ding & Forsburg 2011), again pointing at a role of Mcb1 in this process. Mcb1 overexpression was shown to disturb interactions between Mcm proteins while Mcb1-Mcm interaction was enhanced. Interestingly, Mcb1 also clearly interacted with Mcm2 under these conditions. This indicates that Mcb1 toxicity Is caused by its amplified interaction with individual Mcm subunits, leading to dissociation of MCM2-7 (Ding & Forsburg 2011). In Xenopus and human cells excess MCM-BP caused unloading of MCM2-7 from chromatin (Nishiyama et al. 2011), impaired Mcm-Mcm interaction and dissociation of MCM2-7 (Nishiyama et al. 2011; Nguyen et al. 2012), confirming the results from fission yeast. Consequences of Mcb1 overexpression, like DNA damage and checkpoint arrest are thus likely to be caused by a lack of functional MCM2-7. Interestingly excess MCM-BP seems unable to dissociate MCM2-7 that is already part of the replication fork, as a small fraction of MCM2-7 remained intact in human and Xenopus cell extracts incubated with excess MCM-BP (Nishiyama et al. 2011; Nguyen et al. 2012). Thus MCM-BP might only dissociate MCM2-7 before it is activated in the CMG or even before its loading in the preRC.

The different processes MCM-BP homologues have been associated with, like cohesion establishment, MCM2-7 unloading, MCM export, nuclear morphology and centrosome duplication, might be different consequences of one central role of MCM-BP. All these processes are connected to MCM2-7 and replication. The actual cause behind all these
errors is most likely the disturbance and misregulation of MCM2-7, which could result in problems already early in replication during preRC assembly. This indicates that MCM-BP has an important role in the regulation of MCM complexes during replication initiation or even earlier. While fission yeast and *T. brucei* MCM-BP homologues are essential, it seems that higher eukaryotes have evolved partly redundant mechanisms for the function of MCM-BP and attenuate the effects of its depletion. Results from Chapter 3 in agreement with other studies show that defined levels of Mcb1 or MCM-BP are important for undisturbed cell cycle progression. Excess MCM-BP leads to dissociation of MCM2-7, while depletion causes multiple phenotypes associated with replication problems. The requirement for certain MCM-BP levels might reflect the need for the formation of specific complexes between MCM-BP and Mcm proteins.

**6.4 Mcb1 is related to Mcm proteins and might have a similar structure**

Multiple sequence alignments with different MCM-BP homologues shown in Chapter 4 indicate that MCM-BP proteins are related to Mcm proteins. Structure prediction for Mcb1 suggests that Mcb1 adopts a structure similar to that of ssoMcm. Due to sequence conservation it is likely that all MCM-BPs have a similar structure. An Mcm-like structure possibly allows for Mcb1 to interact with Mcm proteins in the same way as Mcm-Mcm interactions are mediated in MCM2-7. Even though some of the structural motifs found in Mcm proteins seem to be partly conserved in Mcb1, it is not possible to predict if they are functional. Several of these motifs and their exact contribution to MCM function are not well understood (reviewed in Bochman & Schwacha 2009), thus it is impossible to predict their function in Mcb1 based on structure modelling. However it is unlikely that Mcb1 (and probably other MCM-BP homologues) contributes to a functional ATPase site. Most likely they cannot bind and hydrolyse ATP, as only the Walker B motif of the AAA⁺ box is partially present, while Walker A and arginine finger are missing. To be sure, ATP binding and hydrolysis could be assayed however.
6.5 Analysis of Mcb1 mutants Indicates that Mcb1-Mcm interaction is a hallmark of Mcb1 function

Out of 19 different pentapeptide insertion mutants described in Chapter 4, two mutants were identified to have an effect on Mcb1 function. Both mutated protein products were not toxic when overexpressed and showed significantly reduced interaction with Mcm4, which is probably true for the interaction with other Mcm proteins as well but was not tested here. This provides more evidence that interaction of Mcb1 with Mcm proteins is an important hallmark of its function. Loss of interaction in the PPI mutants and also some Ts mutants (Santosa et al. 2013) seems to induce misregulation of Mcm2-7, which is also caused by excessive Mcb1-Mcm interaction upon wild-type Mcb1 overproduction.

The insertions in both PPI-mutants are located in a region at the interface between the NTD and CTD of Mcb1, sitting between the H2I and Walker B motifs. Mcm proteins form an ATP binding site using the Walker A and B motif of one binding partner and the arginine finger of the other binding partner, indicating that the Walker B motif is involved in Mcm-Mcm interaction (Bochman & Schwacha 2009). Furthermore, it was suggested that MCM-BP/Mcm7 interaction is mediated via the Mcm7 AAA$^+$ box (Nishiyama et al. 2011). And a truncation construct corresponding to an AAA$^+$ box of Mcb1 was shown to interact with Mcm4 (Ding & Forsburg 2011). This all indicates that the region where H2I and Walker B are located plays a role in interaction of MCM-BP and Mcms. A temperature-sensitive allele of mcb1 (Santosa et al. 2013) leads to a single amino acid change close to the Walker B similar sequence and the resulting protein also exhibits reduced interaction with Mcm proteins, which is similar to the observations with our PPI mutants.

The structural details of how these mutations or insertions influence the interaction between Mcb1 and Mcm proteins is unclear. It could be speculated that the insertions could disturb H2I or Walker B orientation, which might interfere with the binding to Mcm proteins. Another possibility is that the PPI-insertions have an effect on the flexibility of the protein, as they are located at the interface between the NTD and CTD subdomains and could change their conformation.

The region around the putative Walker B motif in Mcb1 and MCM-BP seems to be important for the function of the protein and involved in Mcm interaction. Not only do mutations or insertions lead to inactivation of the protein, presumably by disturbing its
interaction with Mcm proteins, but also the high level of sequence conservation indicates some importance of this particular area. Some residues, including the signature aspartic acid and glutamic acid of the Walker B motif are conserved throughout Mcm and MCM-BP homologues. They are without doubt crucial for MCM function. Mutations of these two amino acids in Mcm proteins are lethal in yeasts (Bochman & Schwacha 2009); however, a dominant mouse MCM4 allele with a mutation of the aspartic acid is viable but causes leukaemia presumably due to a defective MCM2-7 helicase (Bagley et al. 2012). It would be interesting to see the effects of equivalent mutations in MCM-BP homologues.

6.6 Full-length Mcb1 is needed for complete function

Experiments with different truncation constructs indicate that only the full-length Mcb1 protein exhibits full function, e.g. is able to rescue the Ts phenotype and is toxic when overexpressed. A short truncation of the first 17 amino acids of the N-terminus is viable even though not toxic. Equal observations were made with similar truncation constructs (Ding & Forsburg 2011). Reduced toxicity of a truncation lacking the first 54 amino acids was reported, but the construct was viable and sufficient to complement the Δmcb1. This truncation was still able to interact with Mcm4 and could still promote some dissociation of Mcm2-7 (Ding & Forsburg 2011).

Both of these N-terminal deletion constructs lack parts of domain A. Thus it seems likely that this domain is at least partly dispensable for viability. Interestingly it has been reported, that removal of the full domain A in mthMcm has only limited effects on its function and some archaeal Mcms contain only a truncated version of this domain (Kasiviswanathan et al. 2004). Several studies in fission yeast however indicate that the domain A of eukaryotic MCMs has an important function in the activation of the helicase upon Cdc7/Dbf4 phosphorylation. The mcm5-bob1 mutation of a conserved residue in domain A bypasses the requirement for Cdc7/Dbf4 in MCM activation (Hardy et al. 1997). The mutant presumably has an increased flexibility in domain A, which can lead to premature helicase activation (reviewed in Slaymaker & Chen 2012).

Thus it is likely that the domain A of fission yeast Mcb1 is not essential but still contributes to the function of the protein.
6.7 Different sites mediate Mcb1-Mcm interaction

Binding studies of rMcb1-NTD with different in vitro translated constructs of Mcm4-NTD indicate that the interaction between the two NTDs is mediated by different sites. One such interaction site is located in the A domain of Mcm4, while the other is found in domain B or C, but before the ACL.

As Mcb1 also interacts with other Mcm proteins, which supposedly have a similar structure as Mcm4, the interactions might be mediated by equivalent sites on the other Mcm proteins. Furthermore Mcb1 seems to have an Mcm like structure as well and could consequently also use these particular sites for interaction with Mcm proteins. Here only interactions between the NTDs were examined, but *Xenopus* Mcm7 was reported to interact with MCM-BP via the Mcm7 AAA⁺ box (Nishiyama et al. 2011). Results from the study of pentapeptide insertion mutants described here also indicate that the area corresponding to the AAA⁺ box on Mcb1 might be important for the interaction with Mcm4. This was confirmed by reduced interaction of a temperature-sensitive Mcb1 mutation with all Mcm proteins (Santosa et al. 2013). Further a truncation of Mcb1, corresponding to the AAA⁺ box, was found to interact with Mcm4 (Ding & Forsburg 2011).

In case Mcb1/MCM-BP and Mcm proteins use corresponding areas for their interaction with each other, there are consequently at least three interaction sites spread over the whole length of MCM-BP and Mcm proteins: one in domain A, one in domains B or C and one in the C-terminal AAA⁺ box. The exact location of the binding sites remains unclear. Each of these sites might have a different significance in the interaction of MCM-BP with a particular Mcm protein. And while parts of Mcm4 or Mcb1 where shown to be sufficient for interaction in vitro (5.5.5; Ding & Forsburg 2011), it is likely that in vivo interactions are controlled differently. The Mcb1 pentapeptide mutations in the AAA⁺ box described here for example, show significantly reduced interaction with Mcm4, even though only one possible Mcm4 interaction site is compromised.

Complex formation between MCM-BP and Mcms seems possible in several ways: MCM-BP could participate as a subunit in an MCM2-7-like complex or it could also simply stick to the outside of the MCM2-7 complex.
Biochemical analysis of Mcb1-Mcm interaction (Chapter 2) suggests that Mcb1 might replace Mcm2 in an alternative hexameric complex. The data from structure predication and sequence analysis indicates that Mcb1 could fit in such a hexamer as it is likely to adopt an Mcm like structure to mediate the necessary interactions in a similar way as among Mcm proteins. Thus it would make a suitable placeholder for Mcm2, which was not found to interact with Mcb1 here. Interaction studies of MCM-BP and Mcm constructs from fission yeast and *Xenopus* indicate that there are multiple interaction points present in both groups of proteins, spread along their entire length. This makes it likely that MCM-BP forms MCM2-7 like complexes with Mcm proteins. Of course instead of forming a hexamer, there is also the possibility that Mcb1 mainly interacts with individual Mcm proteins or smaller Mcm assemblies. Since Mcb1 seems capable to interact with all individual Mcm proteins, this option appears reasonable. However, as no real structural or EM data for Mcb1 or a MCM\textsuperscript{Mcb1} complex is available, this remains speculation. It must be considered as well that Mcb1 could also form complexes with Mcm2-7 or Mcm proteins by sticking to the outside of the Mcm2-7 complex. To elucidate the precise locations of interaction between Mcb1 and Mcm proteins, crosslinking and subsequent MS analysis could be conducted.

### 6.8 Model for Mcb1 function

Observations described in this study and reported by others, lead to the following model for Mcb1 function, which is also based on suggestions by Santosa *et al*. Mcb1 acts as a chaperone to control Mcm proteins. It binds free Mcms and also smaller complexes of several Mcm proteins, stabilises them but keeps them inactive, too. Mcb1 does not only bind one specific Mcm subunit but can associate with most Mcm proteins. In this way Mcb1 helps with the assembly of complete Mcm2-7 complexes. As Mcm2 is usually not found in complex with Mcb1 it is suggested that Mcm2 is the last subunit to be added to the complex, an event that could cause the dissociation of Mcb1 from the complex. Binding of free Mcms and subcomplexes by Mcb1 prevents the nuclear export of individual subunits but also their uncontrolled interaction with other factors or interference with complete Mcm2-7 hexamers. Furthermore it ensures the correct stoichiometry of the Mcm2-7 while under construction. Thus complete and
active Mcm2-7 can function as helicase while incomplete subcomplexes are contained in a complex with Mcb1 until they are ready.

For the interaction with individual Mcm proteins, Mcb1 uses several binding sites, which fit for the binding of all Mcms and are distributed across the protein. The Mcm proteins have equal binding sites, which are similar but not identical in the different homologues. Thus Mcb1 can bind all Mcm proteins but has an increased affinity for certain subunits, which is why some Mcms seem to interact stronger with Mcb1 than others.

Certain levels of Mcb1 are important to ensure a balance between binding of free Mcms and dissociation of Mcm2-7. Excess protein leads to interference with Mcm-Mcm interactions within complete Mcm2-7 complexes, resulting in the dissociation of the hexamer. Lack of Mcb1 affects the assembly of complete Mcm2-7, so that not enough hexamers are available for loading onto DNA and activation as helicase. This causes problems with faithful replication and leads to DNA damage, checkpoint activation and cell cycle arrest. In fission yeast the Mcm monomers are transported to the cytoplasm, while they probably stay in the nucleus in other organisms, which could also account for the increase of soluble Mcm4 found in human cells upon MCM-BP depletion (Jagannathan et al. 2012; Sakwe et al. 2007).

Apparently fluctuations in the Mcb1 amounts can be tolerated up or down to a certain level. In the mcb1-Ts mutants described here, Mcb1 is still able to interact with Mcm4 but somehow fails to function properly, while the mutant described by Santosa et al cannot interact with Mcm proteins anymore and thus only limited amounts of Mcm2-7 are available for proper replication. This also explains the increased sensitivity of the Ts mutants by Santosa et al towards replication stressing agents, which was not observed in the Ts mutants used for this study: the Santosa mutants are already sick at permissive temperature and only deliver the minimal required amount of Mcm2-7. However under DNA stressing conditions abundance of preRC complexes becomes important (Maki et al. 2011), which are not available in this mutant. The mutant used for the study presented here has almost normal activity at permissive temperature and might consequently provide enough Mcm2-7 to survive replication stress.

Mcm proteins undoubtedly play a very important role in eukaryotic DNA replication, but also other cellular processes. Mutations in single Mcm subunits have been
Discussion

associated with genome instability and cancer development presumably due to misregulation of MCM2-7. Results presented here and also in other studies indicate that MCM2-7 is not the only complex containing Mcm proteins, showing evidence for the existence of an MCM

$^{\text{MCM-BP}}$

complex, which is important for undisturbed eukaryotic DNA replication. Thus it has to be considered that mutations in Mcm proteins might not only affect MCM2-7 but also the novel MCM

$^{\text{MCM-BP}}$

. Therefore it appears likely that genome instability in Mcm mutants is not only a consequence of MCM2-7 misregulation but could also result from MCM

$^{\text{MCM-BP}}$

 misregulation. Thus, additionally for its function in DNA replication, a role of MCM-BP in cancer development in higher eukaryotes seems possible. Indeed, MCM-BP was found to be overexpressed in human breast cancer, brain cancer and seminoma (Jagannathan et al. 2012).

The study of MCM-BP could contribute to a better understanding of DNA replication and cancer progression and also lead to the development of new treatments of health issues emanating from these processes.

Fission yeast as a simple unicellular eukaryote has proven to be a suitable organism for the study of MCM-BP. The fact that Mcb1 is essential allows the investigation of the protein function in a much more direct way than in more complex metazoan systems. And with the Mcb1-Ts mutants described here, suitable tools for this task are available. Furthermore the high degree of conservation of MCM-BP sequence and the processes it is involved in allows conclusions from studies with fission yeast to be valid for higher eukaryotes as well.
Chapter 7 Materials and methods

The PPI mutant-plasmids were constructed by Justina Ray. The Mcb1-Ts mutants were made and generously provided by J.-J. Li (J. Hayles lab in London). The diploid *S. pombe* strain lacking one copy of *mcb1* was made by Dr Stuart MacNeill.

7.1 Materials

7.1.1 Chemicals

Most chemicals used for this study were supplied by Melford Chemicals, Fisher Scientific or Sigma-Aldrich, ingredients for growth-medium were obtained from Formedium. Media compositions can be found in Table 7-5.

Centrifugations of samples in 15 ml or 50 ml Falcon tubes were performed in a MSE Mistral 2000 centrifuge. Centrifugations of sample-sizes of less than 2 ml were performed in a MSE Micro Centaur table-top centrifuge at room temperature or Heraeus cooling centrifuge at 4°C.

7.1.2 *E. coli* strains

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<th>Use (Supplier)</th>
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<td>DH5-α</td>
<td>F-endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Δ80dlacZΔM15 Δ(lacZYA-argF)U169, hsdR17(rK mK, λ–)</td>
<td>Cloning (Fermentas)</td>
</tr>
<tr>
<td>Rosetta 2(DE3) [pLysS]</td>
<td>Δ(ara-leu)7697 ΔlacX74 ΔphoA PvuII phoR araD139 ahpC galE galK rpsL (DE3) F’[lac+ lacIq pro] gor522::Tn10 trxB pLysSRARE (CamR, StrR, TetR)</td>
<td>Expression of recombinant protein with rare codons (Novagen)</td>
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<td>Stbl3</td>
<td>F mcrB mrrhsdS20(ρ6, m6) recA13 supE44 ara-14 galK2 lacY1 proA2 rpsL20(Str+) xyl-5 λ/leumtl-1</td>
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<td>C43</td>
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<td>Derived from BL21(DE3), tolerance to toxic proteins (Miroux &amp; Walker 1996)</td>
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<tr>
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<td>F ompT hsdS2 (r6 m6) gal dcm (DE3)</td>
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<td>Pentapeptide screening (Hallet et al. 1997)</td>
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7.2 Fission yeast strains

Table 7-2 *S. pombe* strains used in this study

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<td><strong>Sp571</strong></td>
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<td><strong>Sp572</strong></td>
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<td><strong>Sp587</strong></td>
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<td><strong>Sp588</strong></td>
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<tr>
<td><strong>Sp591</strong></td>
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<td><strong>Sp595</strong></td>
<td>mcm2-cTAP-natMX6 mcm3-13myc-kanMX6 h&lt;sup&gt;s&lt;/sup&gt;</td>
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<td><strong>Sp596</strong></td>
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<td><strong>Sp633</strong></td>
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<td><strong>Sp634</strong></td>
<td>mcm3-FLAG-kanMX6 h&lt;sup&gt;s&lt;/sup&gt;</td>
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<td><strong>Sp635</strong></td>
<td>mcm3-V5-kanMX6 h&lt;sup&gt;s&lt;/sup&gt;</td>
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<td><strong>Sp663</strong></td>
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### Material and methods

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<td>Sp677</td>
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<td>Sp721</td>
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<td>Sp727</td>
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### Microscopy

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<td>Sp602</td>
<td>rad22-YFP-kanMX6 h&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Stuart MacNeill</td>
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<td>Sp611</td>
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<td>Stuart MacNeill</td>
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<td>Sp612</td>
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<td>Sp643</td>
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<td>Sp625</td>
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<td>Stuart MacNeill</td>
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<tr>
<td>Sp626</td>
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<td>Stuart MacNeill</td>
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<tr>
<td>Sp627</td>
<td>mcb1-Ts6-YFP-natMX6 cds1::ura4+ ura4-D18 leu1-32 h&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Stuart MacNeill</td>
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<tr>
<td>Sp628</td>
<td>mcb1-Ts6-YFP-natMX6 chk1::ura4+ ura4-D18 leu1-32 h&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Stuart MacNeill</td>
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### Ts mutants

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<th>Sp578</th>
<th>mcb1-Ts1-YFP natMX6 leu1-32 ura4-D18 h&lt;sup&gt;+&lt;/sup&gt;</th>
<th>J.-J. Li</th>
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<tr>
<td>Sp580</td>
<td>mcb1-Ts6-YFP natMX6 leu1-32 ura4-D18 h&lt;sup&gt;+&lt;/sup&gt;</td>
<td>J.-J. Li</td>
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### AID strains

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<tr>
<th>Sp 707</th>
<th>ade6::ade6+-Padh15-Skp1-AtTir1-2NLS-9myc ura4-D18 h&lt;sup&gt;+&lt;/sup&gt;</th>
<th>H. Masukata</th>
</tr>
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<tr>
<td>Sp708</td>
<td>ade6::ade6+-Padh15-Skp1-AtTir1-2NLS-Padh15-Skp1-OsTir1-natMX6 ura4-D18 h&lt;sup&gt;+&lt;/sup&gt;</td>
<td>H. Masukata</td>
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<td>Sp717</td>
<td>mcb1-2HA-IAA-natMX6 ade6::ade6+-Padh15-Skp1-AtTir1-2NLS-9myc ura4-D18 h&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
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<td>Sp718</td>
<td>mcm2-2HA-IAA-natMX6 ade6::ade6+-Padh15-Skp1-AtTir1-2NLS-9myc ura4-D18 h&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
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<td>Sp723</td>
<td>mcb1-2HA-IAA-natMX6 ade6::ade6+-Padh15-Skp1-AtTir1-2NLS-Padh15-Skp1-OsTir1-natMX6 ura4-D18 h&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>This study</td>
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</table>
Material and methods

7.2.1 Primers

Most primers used in this study were synthesised by Eurofins MWG or IDT (Integrated DNA Technologies). Long primers for PCR-mediated gene targeting were ordered from DNA Technology A/S. Primer sequences can be found in Appendix 2.

7.2.2 Antibodies

Table 7-3 Antibodies used in this study

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<tr>
<th>Primary antibodies</th>
<th>Type</th>
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<th>Supplier</th>
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<tr>
<td>Anti-myc 4A6</td>
<td>Mouse monoclonal</td>
<td>1:2500</td>
<td>Millipore</td>
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<tr>
<td>Anti-PAP</td>
<td>Rabbit polyclonal</td>
<td>1:500</td>
<td>Sigma</td>
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<tr>
<td>Anti-FLAG</td>
<td>Mouse monoclonal</td>
<td>1:1000</td>
<td>Sigma</td>
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<tr>
<td>Anti-V5</td>
<td>Mouse monoclonal</td>
<td>1:1000</td>
<td>Lab stock</td>
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<tr>
<td>Anti-Tubulin</td>
<td>Mouse monoclonal</td>
<td>1:1000</td>
<td>Lab stock</td>
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<tr>
<td>Anti-Mcb1 (NTD)</td>
<td>Rabbit polyclonal</td>
<td>1:500</td>
<td>Dundee cell</td>
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<tr>
<td>Anti-HA</td>
<td>Mouse monoclonal</td>
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<td>Lab stock</td>
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<td>Anti-GFP</td>
<td>Mouse monoclonal mix</td>
<td>1:1000</td>
<td>Roche</td>
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Secondary antibodies

| NA931VS ECL HRP anti-mouse          | Goat monoclonal       | 1:20000  | Sigma          |
| Anti-rabbit HRP                     | Goat monoclonal       | 1:50000  | Sigma          |
Material and methods

7.2.3 Plasmids

Table 7-4 plasmids used in this study

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Source</th>
<th>Comments and use</th>
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<tr>
<td><strong>Template plasmids for PCR-mediated gene targeting</strong></td>
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<tr>
<td>pFA6a-13myc-natMX6</td>
<td>(Van Driessche et al. 2005)</td>
<td>C-terminal 13 copies of human cmyc;</td>
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<td>pFA6a-VN173-kanMX6</td>
<td>(Akman &amp; MacNeill 2009)</td>
<td>C-terminal VN; for BiFC</td>
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<tr>
<td>pFA6a-VC155-natMX6</td>
<td>(Akman &amp; MacNeill 2009)</td>
<td>C-terminal VC; for BiFC</td>
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<td>pFA6a-3HA-natMX6</td>
<td>(Van Driessche et al. 2005)</td>
<td>C-terminal 3HA-tag</td>
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<tr>
<td>pFA6a-YFP-natMX6</td>
<td>(Van Driessche et al. 2005)</td>
<td>C-terminal YFP fusion</td>
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<tr>
<td>pFA6a-ura4+</td>
<td>(Bähler et al. 1998)</td>
<td>For gene deletion (replaces gene with ura4+)</td>
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<tr>
<td>pFA6a-CTAP2-natMX6</td>
<td>(Tasto et al. 2001)</td>
<td>C-terminal TAP-tag for tandem affinity purification</td>
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<tr>
<td>pFA6a-6xGLY-FLAG-kanMX6</td>
<td>(Funakoshi &amp; Hochstrasser 2009)</td>
<td>Single FLAG tag</td>
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<tr>
<td>pFA6a-6xGLY-V5-kanMX6</td>
<td>(Funakoshi &amp; Hochstrasser 2009)</td>
<td>Single V5 tag</td>
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<td>pFA6a-AID-natMX6</td>
<td>Stuart MacNeill</td>
<td>C-terminal IAA cassette, for auxin mediated degradation</td>
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<td>pFA6a-HBD-natMX6</td>
<td>(Bøe et al. 2008)</td>
<td>C-terminal hormone binding domain</td>
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<td>pFA6a-kanMX6-pnmt1</td>
<td>(Bähler et al. 1998)</td>
<td>N-terminal nmt1 promoter overexpression (high)</td>
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<tr>
<td>pFA6a-natMX6-pnmt1</td>
<td>(Van Driessche et al. 2005)</td>
<td>N-terminal nmt1 promoter overexpression (high)</td>
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<td>pFA6a-kanMX6-pnmt41</td>
<td>(Bähler et al. 1998)</td>
<td>For N-terminal nmt41 overexpression (medium)</td>
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<tr>
<td>pFA6a-kanMX6-pnmt81</td>
<td>(Bähler et al. 1998)</td>
<td>For N-terminal nmt81 promoter overexpression (low)</td>
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<td><strong>Plasmids for regulated expression of Mcb1 and Mcm2 in S. pombe</strong></td>
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<tr>
<td>pREP3X</td>
<td>S. Forsburg</td>
<td>nmt1-controlled expression</td>
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Goat anti-Mouse IRDye 800CW Goat monoclonal 1:15000 Licor
Goat anti-Rabbit IRDye 680RD Goat monoclonal 1:20000 Licor
**Material and methods**

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<thead>
<tr>
<th>Plasmids with Mcb1 truncation constructs and pentapeptide insertion mutants</th>
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<td>Plasmids for <em>in vitro</em> translation</td>
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<td>pTNT-Mcm4_{288-496}</td>
<td>This study</td>
<td>Mcm4 NTD domains B and C</td>
</tr>
<tr>
<td>pTNT-Mcm4_{153-377}</td>
<td>This study</td>
<td>Mcm4 NTD- before ACL loop</td>
</tr>
<tr>
<td>pTNT-Mcm4_{153-420}</td>
<td>This study</td>
<td>Mcm4 NTD- before NT hairpin</td>
</tr>
<tr>
<td>pTNT-Mcm4_{153-496Zn}</td>
<td>This study</td>
<td>Mcm4-NTD ZnF mutation</td>
</tr>
<tr>
<td>pTNT-Mcm4_{288-377}</td>
<td>This study</td>
<td>Mcm4 NTD domain B- before ACL loop</td>
</tr>
<tr>
<td>pTNT-Mcm4_{288-377Zn}</td>
<td>This study</td>
<td>Mcm4 NTD domain B- ACL loop, ZnF mutation</td>
</tr>
<tr>
<td>pTNT-Mcm4_{288-420}</td>
<td>This study</td>
<td>Mcm4 NTD domain B- NT hairpin</td>
</tr>
<tr>
<td>pTNT-Mcm4_{288-420Zn}</td>
<td>This study</td>
<td>Mcm4 NTD domain B- NT hairpin, ZnF mutation</td>
</tr>
<tr>
<td>pTNT-Mcm4_{153-287}</td>
<td>This study</td>
<td>Mcm4 domain A only</td>
</tr>
</tbody>
</table>

7.2.4 Media

Media were made with distilled water and sterilised by autoclaving.

7.2.4.1 *S. pombe growth media*

Fission yeast was grown in YE4S (Yeast extract with four supplements) or EMM (Edinburgh minimal medium) liquid medium or on agar plates. For sporulation of cells malt extract agar (MEA) was used. Compositions of the different media can be found in table Table 7-5. Antibiotics used for selection were G418 at 100 mg/l and ClonNat at 100 mg/l. EMM was supplemented with uracil (112 µg/ml from 3.75 mg/ml stock) or leucine (112 µg/ml from 7.5 mg/ml stock) as required and for repression of the *nmt1* promoter and its derivatives thiamine was added at 5 mg/l (5 g/l stock).

Cell cycle arrest in early S-phase was induced with 12 mM hydroxyurea (0.5 M stock).

Table 7-5 Media composition

<table>
<thead>
<tr>
<th>Fission yeast media</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>YE4S</td>
<td>5 g/l Yeast extract (BD)</td>
</tr>
<tr>
<td></td>
<td>30 g/l D-Glucose</td>
</tr>
<tr>
<td></td>
<td>L-leucine, uracil, adenine hemisulphate, L-histidine, 250 mg/l each</td>
</tr>
<tr>
<td></td>
<td>ad 1 l H$_2$O adjust to pH 5.6, for YE4S agar 8 g of Agar (Formedium) were added per 400 ml</td>
</tr>
<tr>
<td>EMM</td>
<td>3 g/l potassium hydrogen phthallate</td>
</tr>
</tbody>
</table>
**Material and methods**

<table>
<thead>
<tr>
<th>2.2 g/l Na₂HPO₄</th>
<th>5 g/l NH₄Cl₂</th>
<th>20 g/l D-glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 ml/l Salts (from 50x stock)</td>
<td>1 ml/l Vitamins (from 1000x stock)</td>
<td>0.1 ml/l Minerals (from 10000x stock)</td>
</tr>
<tr>
<td>ad 1 l H₂O adjust to pH 5.6; for EMM Agar 8 g of Agar (formedium) were added per 400 ml</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>MEA</th>
<th>30 g/l malt extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 g/l glucose</td>
<td></td>
</tr>
<tr>
<td>20 g/l agar</td>
<td></td>
</tr>
</tbody>
</table>

| 1000x Vitamins Stock | 1 g/l pantothenic acid, 10 g/l nicotinic acid, 10 g/l inositol, 10 mg/l biotin |

| 10000x Minerals Stock | 5 g/l H₃BO₃, 4 g/l MnSO₄, 4 g/l ZnSO₄ x7H₂O, 2 g/l FeCl₂ x6H₂O, 0.4 g/l Molybdic acid, 1 g/l KI, 0.4 g/l CuSO₄ x5H₂O, 10 g/l citric acid |

### 7.2.4.2 E. coli growth media

*E. coli* cells were grown in LB (Luria Bertani) made in-house. For selection kanamycin (50 µg/ml from a 50 mg/ml stock), ampicillin (100 µg/ml from a 100 mg/ml stock) or chloramphenicol (34 µg/ml from a 34 mg/ml stock) were added.

Protein expression was induced either by IPTG (0.5-1 mM; 1 M stock) or anhydrotetracycline (200 ng/ml; 200 µg/ml stock)

### 7.2.5 Fission yeast buffers and solutions

**Transformation**

**10x LiAc:** 1 M LiAc; adjusted to pH 7.5 with acetic acid

**10x TE:** 0.1 M Tris HCl, 0.01 M EDTA, adjusted to pH 7.5

**LiAc/ TE:** 0.1 M LiAc pH 7.5, 10 mM Tris-HCl pH 7.5, 1 mM EDTA

**PEG/LiAc/TE:** 40% (w/v) PEG 4000, 1x LiAc, 1x TE

**Standard lysis buffer:** 10 mM sodium phosphate, 1% (v/v) Triton X100, 1 mM EDTA, mini Complete, 200 mM sodium glutamate
Material and methods

TAP purification

**IPP**<sub>150</sub>: 10 mM Tris-HCl pH 8.0, 150 mM NaOGlu, 0.1% (v/v) NP-40

**IPP**<sub>300</sub>: 10 mM Tris-HCl pH 8.0, 300 mM NaOGlu, 0.1% (v/v) NP-40

**CBB**<sub>150</sub>: 10 mM Tris-HCl pH 8.0, 150 mM NaOGlu, 1 mM MgOAc<sub>2</sub>, 1 mM imidazole, 2 mM CaCl<sub>2</sub>, 10 mM β-mercaptoethanol, 0.1% / 0.02% (v/v) NP-40

**CBB**<sub>300</sub>: 10 mM Tris-HCl pH 8.0, 300 mM NaOGlu, 1 mM MgOAc<sub>2</sub>, 1 mM imidazole, 2 mM CaCl<sub>2</sub>, 10 mM β-mercaptoethanol, 0.1% / 0.02% (v/v) NP-40

**TEV cleavage buffer**: 10 mM Tris-HCl pH 8.0, 150 mM NaOGlu, 0.1% (v/v) NP-40, 0.5 mM EDTA, 1 mM DTT

**10x lysis buffer**: 60 mM Na<sub>2</sub>HPO<sub>4</sub>, 40 mM NaH<sub>2</sub>PO<sub>4</sub>, 20 mM EDTA, 500 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>

**2x lysis buffer**: 1/5 10x lysis buffer, 400 mM sodium glutamate, 2% (v/v) NP-40, 20 μl/ml 0.1 M PMSF in isopropanol, 40 μl/ml 50x complete,

**Calmodulin elution buffer (CEB)**: 10 mM Tris-HCl pH 8.0, 150 mM NaOGlu, 20 mM EGTA, 0.02 % (v/v) NP-40, 1 mM MgAc<sub>2</sub>, 1 mM imidazole, 10 mM β-mercaptoethanol

**Solution to make PCR template**: 200 mM lithium acetate, 1% SDS

**Mounting solution**: 90% (v/v) Glycerol, 10% PBS-A, 0.1% (w/v) phenylenediamin

7.2.6 Other buffers and solutions

**TE buffer**: 1 mM EDTA, 10 mM Tris-HCl pH 7.5

**TAE buffer**: 40 mM Tris-HCl pH 7.5, 20 mM acetic acid, 1 mM EDTA

**5x Laemmli buffer** (10 ml): 5 ml Glycerol, 1.5g SDS, 2.0 ml Tris pH 6.8 (1M), 2.5 ml β-mercaptoethanol, bromophenol blue

**FIX solution**: 20% (v/v) ethanol, 7.5% (v/v) acetic acid

**Stain solution**: 0.1% Page Blue, 4% perchloric acid

**SDS running buffer**: 5x SDS-PAGE (1 l) 72g glycine, 15g Tris, 5g SDS

**Western blot transfer buffer**: 3.0 g/l Trizma; 14.4 g/l glycine; 15% methanol

**TSB buffer**: LB broth with 10% (w/v) PEG, 5% (v/v) DMSO, 20-50 mM magnesium salts (MgSO<sub>4</sub> and MgCl<sub>2</sub>)

Buffers for purification of recombinant protein
**Material and methods**

**Buffer W**: 100 mM Tris pH 8.0, 150 mM/ 300 mM NaCl, 0.1% Tween, complete, PMSF

**Elution buffer**: buffer W containing 2.5 mM desthiobitoin

**Buffer A**: 100 mM Tris-HCl pH 7.5, 300 mM NaCl, 0.1% Tween, 20 mM imidazole, 1 mM β-mercaptoethanol, 1 mM PMSF

**Buffer B**: 100 mM Tris-HCl pH7.5, 300 mM NaCl, 0.1% Tween, 500 mM imidazole, 1 mM β-mercaptoethanol

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**7.3 S. pombe methods**

**7.3.1 Growth and maintenance of fission yeast strains**

*S. pombe* growing conditions used in this study have been described previously (Moreno et al. 1991). Cells were grown in liquid culture in a shaking water bath or on agar plates in an incubator. The growth medium used was either rich YE4S liquid medium/ agar or selective EMM medium/ agar if not indicated otherwise. Growing temperature for wild-type like strains was usually 32°C, while temperature-sensitive strains were grown at 25 or 35°C. For most experiments exponentially growing cells were used, having an OD$_{600}$ of 0.5-1.0 (0.5-1.0x10$^7$ cells/ml). Cell density was determined by measuring the absorbance of 1 ml of cell culture at 600 nm (OD$_{600}$) in a Sanyo SP50 spectrophotometer. If needed, cell numbers were counted using a Coulter Z2 cell counter.

For short term storage strains were patched on YE4S agar plates and kept at room temperature. For long-term storage a dense overnight culture was mixed with equal amounts of Yeast freezing medium (YE4S with Glycerol) and frozen at -80°C in Nunc Cryo Tubes™. Liquid cultures were started by inoculating 10 ml of medium with one freshly grown single colony and overnight incubation to obtain a dense pre-culture which was then used to inoculate larger volumes.

**7.3.2 Genetic crosses and random spore analysis**

To cross two haploid strains with opposite mating type, a fresh patch of each strain was grown on EMM with supplements overnight. Then a loop full of each was mixed in
10 µl of water and spotted onto a malt agar plate. The plate was incubated at 32°C (or 25°C in case of temperature-sensitive alleles) for 1 day and appearance of tetrads was observed under the light microscope.

For random spore analysis a loop full of sporulating cells was incubated with 490 µl H₂O and 10 µl of helix pomatia juice overnight at 32°C (or 25°C for temperature-sensitive product) to break down the asci walls. The spores were washed with water, and resuspended in 50 µl water. Serial dilutions (10⁻² –10⁻⁵) were plated onto YE and incubated at 32°C until single colonies formed. Plates with 50-100 single colonies were replicaplated onto selective agar plates. Some colonies with the designated antibiotic resistance or marker combination were patched and analysed further by PCR or Western blot to confirm their integrity.

### 7.3.3 *S. pombe* spotting assays

The fission yeast culture to be analysed was grown to log phase (0.5-1.0 x 10⁷ cells/ml) in YE4S or EMM plus supplements. 1x10⁶ cells were pelleted by centrifugation, washed twice with medium and resuspended in 500 µl of EMM. This stock (10000 cells/ 5 µl) was further diluted to 2000, 400, 80 and 16 cells/ 5 µl. 5 µl of each dilution were spotted onto plates containing supplements and thiamine (5 mg/l) if appropriate and incubated for 4-6 days at 25°C or 36°C. Images of the grown spots were obtained by scanning the plates face down with a Epson Perfection 2450 Photo scanner against a black background.

### 7.4 *E. coli* methods

*E. coli* strains used in this study can be found in table Table 7-1. Strains were usually stored on LB plates containing appropriate antibiotics at 4°C for short time and as glycerol stocks at -80°C. Transforming strains were stored in dense liquid cultures for up to three weeks at 4°C.

Liquid cultures were started by inoculating 10 or 20 ml of LB medium plus appropriate antibiotics with a single colony or 100-200 µl dense pre-culture and grown in a shaking
incubator at 37°C. Transformation of *E. coli*, isolation of plasmid DNA and protein expression in *E.coli* are described on page 198 and page 210 respectively.

### 7.5 DNA methods

#### 7.5.1 PCR-Polymerase chain reaction

All PCR reactions were performed in a Techne TC-4000 or TC-4712. Products were analysed by agarose gel electrophoresis on 1 % agarose gels and purified if required using the Thermo Scientific DNA purification kit.

**Table 7-6 PCR reaction conditions**

<table>
<thead>
<tr>
<th></th>
<th>Temperature</th>
<th>Time</th>
<th># cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fermentas long PCR</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Initial denaturation</td>
<td>95°C</td>
<td>3 min</td>
<td>1</td>
</tr>
<tr>
<td>Amplification cycles</td>
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</tr>
<tr>
<td></td>
<td>55°C</td>
<td>30 sec</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>68°C</td>
<td>2 min 30 sec</td>
<td></td>
</tr>
<tr>
<td></td>
<td>95°C</td>
<td>30 sec</td>
<td></td>
</tr>
<tr>
<td></td>
<td>55°C</td>
<td>30 sec</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>68°C</td>
<td>2 min 30 sec (1&lt;sup&gt;st&lt;/sup&gt;)</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>6 min 40 sec (last)</td>
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<tr>
<td>Final elongation</td>
<td>68°C</td>
<td>10 min</td>
<td>1</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th></th>
<th>Temperature</th>
<th>Time</th>
<th># cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Q5</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Initial denaturation</td>
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<td>1</td>
</tr>
<tr>
<td>Amplification cycles</td>
<td>98°C</td>
<td>10 sec</td>
<td></td>
</tr>
<tr>
<td></td>
<td>56-65°C</td>
<td>20 sec</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>72°C</td>
<td>50 sec</td>
<td></td>
</tr>
<tr>
<td>Final extension</td>
<td>72°C</td>
<td>2 minutes</td>
<td>1</td>
</tr>
</tbody>
</table>
Material and methods

### MyTag programme 5a

<table>
<thead>
<tr>
<th>Step</th>
<th>Temp/Time</th>
<th>Reps</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
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<td>1</td>
</tr>
<tr>
<td>Amplification cycles</td>
<td>94°C 45 sec</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>55-65°C 45 sec</td>
<td></td>
</tr>
<tr>
<td></td>
<td>72°C 50 sec</td>
<td></td>
</tr>
<tr>
<td>Final extension</td>
<td>72°C 4 min</td>
<td>1</td>
</tr>
</tbody>
</table>

**Standard PCR for cloning and PCR-mediated gene targeting**

For amplification of long templates for cloning purposes Fermentas long PCR enzyme was used in reaction-volumes of 50 µl (or 500 µl for PCR-mediated gene targeting in *S. pombe*). The concentration of oligonucleotide primers was 0.5-1 µM and 200 µM of dNTPs. The “Fermentas long PCR” programme was used (see Table 7-6).

Another enzyme for amplification of long fragments was NEB Q5 Polymerase with programme “Q5” (see Table 7-6).

For diagnostic *S. pombe* PCR and *E. coli* colony PCR, the Bioline RedMix PCR kit was used. Therefore ½ reaction-volume of RedMix was mixed with 1 µl of template and 4 µl of primer mix at 1 pmol/µl. The cycle for this enzyme was “MyTag programme 5a” (see Table 7-6).

*S. pombe* genomic DNA extracts were made as described in 7.5.5 and 1 µl was used as template. For *E. coli* colony PCR a single colony was resuspended in 100 µl of water and 1 µl was used as PCR template.

### 7.5.2 Restriction digest

DNA digests were usually performed as double digests in 60 µl reactions using 1 µg of DNA and standard restriction enzymes supplied by NEB, Promega or Thermo Scientific (Fermentas) according to the manufacturer’s instructions for not more than 2 hours at 37°C, followed by purification of the constructs using a DNA cleanup kit (thermo scientific). Plasmids were additionally de-phosphorylated during the last 30 minutes of the restriction digest by NEB Antarctic Phosphatase according to the manufacturer’s instructions. For diagnostic digest the reaction was set up in a total of 20 µl.
7.5.3 DNA ligation

Purified digested DNA was ligated with the suitable digested and dephosphorylated plasmid using Quick ligase (NEB) according to the manufacturer’s instructions in 20 µl reactions for 20 minutes at room temperature. Usually 50 ng of plasmid and a five-fold excess of insert were used for ligation and directly used for transformation into *E. coli*.

7.5.4 Isolation of plasmid DNA

For plasmid DNA preparation from *E. coli*, the thermo scientific miniprep kit (GeneJET) was used according to manufacturer’s instructions. Usually 10 ml of *E. coli* overnight culture grown in the presence of the appropriate antibiotic were used per preparation. If necessary, the presence of the correct insert was confirmed by diagnostic digest in 20 µl reactions and agarose gel electrophoresis.

7.5.5 Isolation of *S. pombe* genomic DNA

Extraction of fission yeast genomic DNA for PCR analysis was based on the approach of Looke *et al* (2011). In an eppendorf tube, a big colony or loop full of freshly grown fission yeast cells was suspended in 100 µl of 200 mM lithium acetate, 1% SDS solution, vortexed and incubated at 70°C for 10 minutes. 300 µl of ethanol were added to precipitate DNA. The DNA was pelleted by centrifugation (13000 rpm, 4°C 5 minutes) washed with 500 µl of 70% ethanol and then dried at 37°C. Finally 100 µl of TE were added to dissolve DNA. Cell debris was pelleted and the supernatant was used as template for PCR.

7.5.6 Sequencing

DNA-sequencing was performed by Dundee sequencing service.

7.5.7 Analysis of DNA by agarose-gelectrophoresis

DNA samples to be analysed were mixed with 10x Laemmli loading dye and loaded onto a 1% agarose gel (1% agarose in 1x TAE and 0.001% ethidium bromide) alongside
Material and methods

0.5 µg fermentas 1 kb DNA ladder. The gel was run at 120 V for 45 minutes. DNA was visualised using a UV transilluminator (Syngene U Genius).

7.5.8 DNA Transformation

7.5.8.1 E.coli transformation-TSB method

20 ml of E.coli cell culture (DH5α or Rosetta 2) were grown at 37°C in the presence of appropriate antibiotics to an OD_{600} of 0.4-0.6, harvested, resuspended in 1 ml TSB buffer (5% DMSO, 1 mM Mg-Salts, 10 % PEG in LB) and stored on ice for at least 30 minutes prior to use.

10 µl of a ligation-reaction or 1:100 dilution of plasmid were mixed with 100 µl of cell suspension and incubated on ice for 30 minutes. After adding 200 µl of TSB-buffer cells were incubated 1 h at 37°C before plating 150 µl onto selective LB agar. If necessary, the presence of the right insert in transformants was confirmed by colony-PCR.

7.5.8.2 S. pombe transformation – lithium acetate method

Transformation of S. pombe cells with PCR products for gene targeting or plasmid DNA was performed according to Bähler et al (Bähler et al. 1998, based on the LiAc method of Keeney & Boeke, 1994). Per transformation 20 ml of cell culture (at 1x 10^7 cells/ ml) were pelleted, washed with an equal amount of water, resuspended in 1 ml of water and washed again with 1 ml of LiAc/TE. The cells were pelleted again and resuspended in LiAc/TE to a final concentration of 2x 10^9 cells/ml.

100 µl of this cell suspension were mixed with 2 µl of carrier DNA (sheared herring testes DNA, 10 mg/ml; Clontech Laboratories) and 10 µl of the transforming DNA and incubated at room temperature for 10 minutes. 260 µl of 40% PEG/LiAc/TE were added, mixed gently and the suspension was incubated at 32°C (or 25°C for temperature-sensitive strains) for 60 minutes. 43 µl of DMSO were added and the cells were heat shocked for 5 minutes at 42°C. The cells were washed with 1 ml of water, resuspended in 0.5 ml of water and plated onto two YE plates using 250 µl per plate. After 24 h incubation at 32°C, the plates were replicaplated onto selective medium.
The replicaplates were incubated at 32°C till single colonies formed (3-5 days). Plasmid transformants were directly plated onto selective medium to avoid loss of the plasmid.

7.5.9 PCR-mediated gene targeting of *S. pombe* genes

**Strategy**

A PCR based approach (Bähler et al. 1998) was used to tag proteins in haploid *S. pombe* cells with different C-terminal tags like TAP, 13myc, 3HA, V5, VN and VC or for insertion of *nmt1*, *nmt41* or *nmt81* promoters upstream of the gene sequence. pFA6a plasmids Table 7-4 were used as templates, carrying the desired tag and a *kanMX6* or *natMX6* module for selection of G418 or nourseothricin (nat) resistance. The tag and selective marker are set in between two universal primer binding sites (PBS) linking the whole cassette in-frame to the targeted gene. The 100 nt long primers contain about 80 nt homologous to the 3’ end/ 3’ UTR of the target gene sequence and 20 nucleotides homologous to the primer binding sites on the pFA6a plasmid. PCR products were transformed into fission yeast cells to be integrated into the genome by homologous recombination, adding a tag and selective marker to the gene of interest.

**PCR**

PCR was performed according to Fermentas Long PCR Enzyme kit. pFA6a template plasmids are listed in Table 7-4, primer sequences (P1-P22) in Table 0-1 Appendix 2). Per construct 500 µl of PCR reaction were prepared with 1 µM 5’ and 3’ oligonucleotides and 10 ng of template.

**DNA precipitation**

The DNA of the pooled PCR reactions was extracted with an equal volume of phenol/chloroform/isoamylalcohol and precipitated with 2½ volumes of ethanol and 1/10 volume 3 M NaOAc pH 4.6 for at least 1 hour at -20°C. The DNA pellet was washed once with 70 % ethanol and dried in a speed vac (15 min 30°C) or in a heating block at 40°C and dissolved in 10 µl of TE buffer before using it for transformation (Bähler et al. 1998).
Transformation of fission yeast cells

Transformation of *S. pombe* cells with the PCR products was performed according to Bähler *et al* (see 7.5.8). Successful transformants were identified on selective medium and further screened by PCR if applicable.

7.5.10 Cloning

7.5.10.1 Cloning of Mcb1-NTD and CTD into pASK IBA3+ and pASK IBA5+

The cloning of Mcb1-NTD and CTD into pASK IBA3+ and 5+ was carried out by Dr Stuart MacNeill.

The sequence encoding Mcb1-NTD (amino acids 1-238) was cloned into pASK IBA3+ for addition of a C-terminal Strep-tag, and the sequence encoding Mcb1-CTD (amino acids 238-501) was cloned into pASK IBA5+ for addition of an N-terminal Strep-tag, using *Bsa*I restriction sites. Ligated plasmids were transformed into DH5α and the inserts were sequenced before transformation into Rosetta 2.

7.5.10.2 Cloning of full-length Mcb1 into pASK IBA3+

To make recombinant protein, the DNA sequence coding for full-length Mcb1 was cloned into the *Bsa*I sites of pASK-IBA3+. As a template pREP3x-Mcb1-a was used and the intron (base pairs 163-206) was removed by overlap extension PCR: In a first round of PCR two overlapping products were created using primers P61 and P64 or P63 and P62. These products were then used as template for a second PCR using P61 and P62. The digested PCR product and pASK-IBA 3+ were ligated and transformed into DH5α and the insert was confirmed by sequencing. A positive isolate was then transformed into Rosetta 2 for expression.

7.5.10.3 Cloning of Mcb1-CTD into pEHis and pEHisGFP

The sequence encoding the C-terminal domain of Mcb1 was cloned into pEHisTEV and pEHisGFPTEV between the *EcoR*V and *HindIII* sites using pREP3x-Mcb1-a as template and primers P59 and P60 for PCR amplification. Digested PCR product and plasmids
were ligated and transformed into DH5α before re-isolating plasmid DNA and sequencing of the insert.

7.5.10.4 Cloning of full-length Mcm2 into pREP4X

*S. pombe* mcm2 was cloned into pREP4X using the Sall and BamHI sites. The intron (base pairs 234-452) was removed by modified overlap extension PCR: PCR products of the sequence prior and after the intron with overlapping ends were created using primers P69 and P68 or P67 and P70 and genomic DNA as template. Due to the length of the 3’ product (nt 452-2712), a shorter product using an additional reverse primer binding nt 637-662 just downstream of the internal XhoI site (base pairs 602-607) was made (using P67 and P71) and used for the overlap extension PCR. The intron free 5’ overlap-product was digested with Sall and XhoI and the long 3’ product was digested with XhoI and BamHI. The long 3’ product was ligated with appropriately digested and dephosphorylated pREP4X and transformed into DH5α. Transformants were screened by PCR for the presence of the insert. Plasmids from positive transformants were isolated and checked again by digest with XhoI and BamHI prior to sequencing. The short 5’ product was then ligated with XhoI cut plasmid (XhoI and Sall are complementary) containing the longer part of insert and transformed into DH5α. Transformants were screened by colony-PCR, and, following isolation of plasmid DNA, various digests to confirm the right insertion of the smaller piece.

7.5.10.5 Cloning of Mcm2-7 N-terminal domains, Mcb1 N-terminal domain and various Mcm4 domains into pTNT for in vitro translation

The TNT plasmid was purchased from Promega. Sequences encoding the N-terminal domains of Mcb1, Mcm2, Mcm3, Mcm4, Mcm5, Mcm6, Mcm7 and various domains and truncations of Mcm4 were PCR amplified using primers designed to insert a *Mlul* restriction site 5’ of the respective *mcm* or *mcb1* sequence and a STOP codon and Xbal site after the 3’ end (primers P73-P86 and P89-P96 in Table 0-1). As template for *mcm3*, 4, 5, 6 and 7 *S. pombe* genomic DNA was used, pREP4X-mcm2 containing the full-length (intron-free) *mcm2* sequence served as template for Mcm2-NTD, and pASK-mcb1-Strep (for FL Mcb1 without intron) for Mcb1-NTD. For the Mcm4-domains
Material and methods

pTNTMcm4NTD was used as template. The end of the NTDs was determined by the position of the N-C linker in a multiple sequence alignment of Mcm proteins from different eukaryotic and archaeal species (Appendix 4).

The purified PCR products and pTNT were digested using *Mlu*I and *Xba*I and the plasmid was dephosphorylated before ligation. The ligated products were transformed into DH5α, and the plasmids were verified by sequencing using primers P87 and polyT rev (IDT).

7.5.10.6 Construction of Mcb1 truncation mutants and cloning of full-length FLAG-Mcb1 and Mcb1-FLAG into pREP3X

Truncated, FLAG-tagged versions of *mcb1* were constructed by PCR and cloned into pREP3X under the control of the inducible *nmt1* promoter. The aspired protein constructs were full-length FLAG-Mcb1 (amino acids 1-501), FLAG-Mcb1<sub>1-450</sub>, FLAG-Mcb1<sub>1-400</sub>, FLAG-Mcb1<sub>1-350</sub>, FLAG-Mcb1<sub>1-300</sub>, FLAG-Mcb1<sub>1-238</sub>, full-length Mcb1-FLAG, Mcb1<sub>18-501</sub>-FLAG, Mcb1<sub>85-501</sub>-FLAG, Mcb1<sub>149-501</sub>-FLAG, Mcb1<sub>239-501</sub>-FLAG.

As a template for PCR amplification, pREP3x-mcb1-FLAG and pREP3x-FLAG-mcb1 were used. To make these, *mcb1* was tagged with a FLAG tag on either the 5’ or the 3’ end, using primers P54b and P54 (to add a FLAG-tag, a STOP codon and an *Bam*HI site to the 3’ end) or P48 and P48b for 5’ end tagging also introducing an *Xho*I site. Digested PCR products were ligated with cut pREP3X, transformed into DH5α and verified by sequencing.

Plasmids with the full-length FLAG-mcb1 or mcb1-FLAG were then used as templates for the construction of truncation mutants. PCR was performed with Fermentas long enzyme.

For C-terminal truncations, pREP3X-FLAG-*mcb1* and primers P54b plus reverse primers P49 to P53 were used. Primers for N-terminal truncations were P55 to P58 plus P48b and pREP3X-mcb1-FLAG as template. Digested products were cloned between the *Xho*I and *Bam*HI sites in pREP3X and the ligated insert and plasmid were transformed into DH5α, sequenced to verify the correct insert and then transformed into fission yeast (Sp347, Sp578 and Sp580).
7.5.10.7 Cloning of Mcm4-NTD and Mcm6-NTD into pEHisTEV

The sequence encoding the N-terminal domain of Mcm4 lacking the N-terminal extension (amino acids 153-496) was cloned into pEHisTEV using the Ncol and HindIII restriction sites and primers P97 and P98. For cloning of sequences encoding Mcm6-NTD (amino acids 2-413) into pEHisTEV, Ncol and Sall and primers P99 and P100 were used.

PCR products and the plasmid were digested with Ncol and HindIII, ligated and transformed into DH5α. The integrity of the insert was confirmed by sequencing.

7.5.11 Isolation of temperature-sensitive mutants of fission yeast Mcb1

The temperature-sensitive mcb1-mutants used in this study were made by J.-J. Li from J. Hayles’ lab using a previously described PCR based method (Fong et al. 2010). mcb1-YFP-natMX6 genomic DNA was used as a template and PCR-amplified with error prone Z-Taq Polymerase (Takara Bio). PCR fragments were ethanol-precipitated and transformed into wild-type. Transformants were screened for temperature sensitivity at 35°C. From several identified Ts mutants, only mcb1-Ts1 and mcb1-Ts6 were used in this study.

7.5.12 Pentapeptide scanning mutagenesis of mcb1

The pentapeptide insertion mutants (PPI-mutants) of Mcb1 were made by Justina Ray during her honours project in the MacNeill lab in 2012.

Pentapeptide scanning mutagenesis was carried out based on the approach by (Hallet et al. 1997; Hayes & Hallet 2000).

The coding sequence of mcb1 was cloned into pBR322 using the Sall and BamHI restriction sites and the plasmid was transformed into E. coli strain FH1046. FH1046 contains the transposon Tn4430 on plasmid pHT385 and successful transformants were screened by resistance to ampicillin and kanamycin. This donor strain was then mated with the recipient strain DS941 carrying a gene for Strep resistance. This allowed for the selection of transconjugants with cointegrates between pHT385 and pBR322 (i.e. transposon-plasmid has integrated into the target-plasmid). Colonies where the Tn4430-transposon had integrated into the mcb1-sequence were identified.
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using colony PCR. Plasmids were isolated by miniprep and digested with Acc651 to remove the bulk of the transposon followed by religation and transformation into DH5α.

The mutated mcb1-sequences were then transferred back into the fission yeast compatible plasmid pREP3X, using restriction enzymes XhoI and BamHI and subsequent ligation with appropriately digested pREP3X and transformed into DH5α.

The mcb1-inserts on the re-isolated plasmids were then sequenced to determine the site and sequence of the PPI-mutation.

7.6 Protein methods

7.6.1 SDS-PAGE and Western blot

For SDS-PAGE, 10 or 15 well gels with 10% 12% or 15% Acrylamide were used in this study. Gels were cast in a Biorad PSIII system and ran at 200 V for approximately 50 minutes. Gels were fixed for 10 minutes in 7.5 % acetic acid and 20 % ethanol prior to staining (10 minutes in 0.1 % Page Blue, 4 % perchloric acid) and destaining in water.

After SDS-PAGE the gels to be blotted were equilibrated in transfer buffer (25 mM Tris, 200 mM glycine, 20 % methanol) for about 15 minutes. PVDF membranes were activated according to the manufacturer’s instructions (BioRad PVDF or Millipore immobilon FL). Transfer was performed at 100 V for 60 minutes using ice packs to cool the tank.

Following transfer the membranes were either directly subjected to blocking (in 5% milk in PBS when using BioRad PVDF) or rinsed with water and allowed to dry before re-wetting and blocking (in Licor Odyssey blocking buffer when using Millipore immobilon FL for IR-detection). Blocking was usually performed for one hour at room temperature or over night at 4°C, depending on the expected signal and antibodies used. After blocking, the membranes were incubated with the first antibody in appropriate dilution in either PBS with 0.05 % Tween or Odyssey blocking buffer and 0.2 % Tween for 1.5 hours at room temperature or over night at 4°C.
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The membranes were washed five times in 100 ml PBS-T (0.05- 0.1 %) and incubated with the secondary antibody diluted in PBS-T (0.05 %) or Odyssey blocking buffer with 0.2 % Tween and 0.01 % SDS for one hour at room temperature. Detection was performed with the LICOR Odyssey system when IR labelled secondary antibodies were used. For detection of chemiluminescence, ECL substrate (Pierce Femto or Pico West) was applied to the membrane for five minutes and the signal was captured on film or by a CCD-camera (Cooled charge coupled devices camera, LAS-3000 Fujifilm).

7.6.2 Fission yeast total protein extract (denaturing conditions)

For protein extracts for diagnostic purposes, approximately 10 ml exponentially growing cells (OD$_{600}$ 0.2-0.8), or as indicated, were used. Different samples were adjusted to the same OD to allow a direct comparison later. Cells were harvested by centrifugation (2000 rpm 4 min). The pellet was washed with 1 ml PBS-A in a 1.5 ml Eppendorf tube. After discarding the supernatant, the pellet was heated to 95˚C for 5 minutes, spun down, resuspended in 150µl PBS-A and transferred to a pre-chilled Ribolyser tube. The samples were ribolysed twice for 30 seconds using a FastPrep FP120 machine (Thermo scientific) at 6.5 m/s with a 2 minutes interval on ice between the ribolysing steps. After, a hole was pierced into the bottom of the ribolyser tubes, and the liquid spun into 1.5 ml Eppendorf tubes by centrifugation for 1 minute at 1000 rpm. 100 µl of lysate were mixed with 100 µl of 2x SDS-PAGE sample buffer and heated for 5 minutes to 95˚C.

7.6.3 Fission Yeast native protein extracts

Approximately 5-10 x 10$^7$ cells (cell numbers of samples were adjusted), were pelleted at 2000 rpm for 4 minutes and resuspended in 5 ml cold PBS-A. The cells were spun down again, the pellet was drained, resuspended in 100 µl ice cold lysis-buffer and added to a pre-chilled Ribolyser tube and vortexed 2 seconds. The samples were processed in the FastPrep machine at 4 m/s for 20 seconds. Then 100 µl of the lysis buffer were added directly to the bottom of the tube and vortexed to mix. The tube was inverted, the bottom punctured with a needle, clipped into a fresh 1.5 ml tube and
spun for a minute at 1000 rpm to collect the liquid extract. The extract was centrifuged for 2 minutes at 4°C to get rid of debris and 150 µl of the clear extract were removed to a fresh tube. For analysis by PAGE and western blot, sample buffer was added to the extract, boiled at 95°C for 5 minutes and spun down before applying to an SDS gel.

For larger sample volumes of 20-100 ml (for sucrose gradient analysis e.g.), exponentially growing yeast cultures were harvested at 2000 rpm for 4 minutes, pellets washed with 20 ml ice cold PBS-A. The pellet was then resuspended in 20 µl cold lysis buffer and transferred to a snap cap tube. Then 1 ml of acid washed glass beads were added and the tubes were vortexed 2 times for 1 minute. After, 500 µl of lysis buffer was added and as much as possible of the extract was captured using a P1000 pipette. The extract was cleared by centrifugation prior to analysis.

7.6.4 Co-immunoprecipitation/ pull-down

For co-immunoprecipitation soluble extracts of strains carrying a tagged protein to be precipitated and another tagged protein to be co-precipitated were prepared, either using the fast prep machine or the glass bead method for larger culture volumes. When using the fast-prep machine, culture volumes used for the extracts were adjusted by OD, when using the glass-bead method the total protein concentration of the extracts was determined by BCA assay (Pierce) and a total of 2-5 mg was used. Extracts from strains lacking the tag to be precipitated served as negative controls and were processed in the same way. A sample of native extract was kept as loading control.

TAP-tagged proteins were precipitated with IgG-Sepharose (GE healthcare). 150 µl (fast prep) or as indicated (for glass bead extracts) of cleared extract was added to 30 µl of prepared IgG resin and incubated at 4°C for 1 h. The beads were then washed 4 times with 600 µl of buffer and resuspended in buffer and 5xSB to a total volume of 50 µl.

For precipitation of HA or YFP-tagged proteins the extract was incubated with 5 µg anti-HA or anti-GFP antibody over night at 4°C in a total volume of 500 µl. Then 100 µl protein A slurry were added to bind the antibody and incubated for 2 hours at 4°C. Beads were washed 4 times with 600 µl buffer before resuspending in buffer and 5xSB and boiling for 5 minutes.
Samples were analysed by Western blot using appropriate antibodies for the detection of the respective tag or protein.

**7.6.5 BCA assay**

To determine the total protein concentration of protein extracts or the yield of protein purification, the Pierce™ BCA Protein Assay Kit (Thermo Scientific) was used. This is based on the biuret reaction, the protein mediated reduction of Cu$^{2+}$ to Cu$^{1+}$ in an alkaline environment and the subsequent colorimetric detection of Cu$^{1+}$ by bicinchoninic acid (BCA). The complex formed by two molecules of BCA and one Cu$^{1+}$-cation has a purple colour and absorbs at 562 nm. This absorption is linear for a protein concentration from 20-2000 µg/ml. Spectrometric readouts can be converted into a protein concentration by comparison to a known standard of Albumin (BSA).

A small amount of protein containing solution (1-20 µl) or BSA standard was mixed with 1 ml of working solution (50 parts solution A and 1 part solution B) in an eppendorf tube and incubated at 37°C for 30 minutes. Contents were transferred to a cuvette and the absorption at 562 nm was measured in a photospectrometer zeroed with water. A sample with buffer only was processed in the same way to serve as blank. To increase the accuracy of the result, usually at least two different samples were measured per essay.

The blank absorption was subtracted from the sample measurements to obtain the real absorption.

A standard curve was plotted with the absorptions at 562 nm corresponding to 0 µg, 2 µg, 4 µg, 8 µg, 16 µg and 32 µg of BSA. Five of these standard curves were pooled to one average curve with the equation \[ A = \left( \frac{0.0306}{\mu g} \right) \times x \] (A= absorption at 562 nm; x= protein amount in µg) and an R$^2$ value of 0.9968.

To determine the protein concentrations of the samples, the real absorption values were inserted in the equation \[ x = \left( \frac{A}{0.0306/\mu g} \right) / V \] (with A= absorption, V= initial volume of protein solution in the sample in µl and x= protein concentration in µg/µl).
7.6.6 TAP purification

TAP purification was performed according to Gould et al. (Gould et al. 2004) under high salt conditions (200 mM sodium glutamate, 300 mM IPP).

Production of cell pellet

12 litres of *S. pombe* cells were grown at 32°C overnight to an OD of ~0.8. The cells were pelleted in a JLA 8.1000 rotor at 5000 rpm at 4°C for 15 minutes, resuspended in 1 ml lysis buffer each and transferred to a pre-weighed 30 ml centrifuge tube before spinning in a JA 25.50 rotor at 12000 rpm 4°C for 10 minutes. The supernatant was discarded and the pellet was weighed and resuspended in half of its weight of 2x lysis buffer. The suspension was frozen by dripping it into a flask of liquid nitrogen, using a 10 ml pipette. After pouring off excess nitrogen, the pellet beads were transferred to a 50 ml tube and stored at -80°C.

Cell lysis

Lysis of the pellet beads was performed using a Retsch grinder under liquid nitrogen cooling. The cells were ground to a fine powder in 15-20 minutes. During that time the pestle setting was gradually increased from 0 to 8 and the pellet was kept close to a paste by adding liquid nitrogen constantly to avoid heating of the cells. The lysate was thawed on ice and then centrifuged at 12000 rpm 4°C for 10 minutes. The supernatant was transferred to a fresh tube and spun again.

Purification

400 µl of activated IgG-Sepharose were added (sepharose was washed with 3 bed volumes of 0.5 M acetic acid and then with TST pH 7.5. Both washes were repeated and then washed with TST until the pH was about 7.5 and the beads were resuspended in one bed volume of lysis buffer) to the supernatant and incubated 1 h at 4°C with rotation. The slurry was centrifuged at 3000 rpm for 1 minute at RT. After removing the supernatant, the beads were resuspended in 5 ml IPP300 and transferred to a Bio-Rad polyprep column and washed with 4x 10 ml IPP300 in total, followed by a wash with TEV (*Tobacco etch virus*) cleavage buffer. The bottom of the column was closed, 1 ml of TEV cleavage buffer and 300 U (30 µl) TEV-protease were added and incubated
overnight with rotation at 4°C to cleave off the remaining portion of TAP-tag and interacting proteins. The supernatant was eluted into a new column and 6 ml of CBB$_{300}$ (0.1% NP-40), 6 µl CaCl$_2$ and 300 µl of washed Calmodulin affinity resin (washed in CBB$_{300}$ (0.1% NP-40) and resuspended in an equal volume of CBB$_{300}$ (0.1% NP-40) were added and the column was incubated 1 h at 4°C with rotation.

The resin was washed twice with CBB$_{300}$ (0.1% NP-40) and once with CBB$_{300}$ (0.02% NP-40). Elution was performed by adding 2x 0.5 ml CEB (with EGTA) to the column with closed bottom and incubating 10 minutes at 4°C before draining into an Eppendorf tube. A second elution with one more ml of CEB was performed in the same way.

Both elution samples were TCA precipitated: 1/3 Volume of 100% TCA was added to each sample and incubated on ice for 30 minutes with periodic vortexing. The samples were spun at 13000 rpm 4°C for 30 minutes and the pellets washed once with ice cold acetone containing 0.5 N HCl and once with ice cold pure acetone. The pellets were dried in a speedvac for 15 minutes and stored at –20°C.

Analysis by SDS-PAGE and MS/MS

The pellets were resuspended in sample buffer, analysed by SDS-PAGE (Invitrogen System) and silver stained with the Biorad silver stain Plus Kit according to the manufacturer. Bands of interest were excised and identified by mass spectrometry (MS/MS) using a Q Star Pulsar XL quadrupol time of flight instrument (applied Biosystems) and electrospray ionisation.

7.6.7 Sucrose gradient centrifugation

A linear 5-18% sucrose gradient was made based on the method described by Stone (Stone 1974). 2.4 ml of a solution of 18 % sucrose in lysis buffer (1x TAP purification lysis buffer with 200 mM sodium glutamate) were pipetted into a 5 ml polyallomer tube, which was held upright in a suitable rack. 2.4 ml of a 5% sucrose solution were carefully layered on top of the 18% solution using a pipette with a 200 µl tip stacked on top of a 1000 µl tip. The tube was sealed with a rubber stopper and the rack with the tube was then slowly tipped to the side (in about 45 sec) and left in this position for about 3 h for the gradient to form. Then the rack was slowly brought back in the upright position.
100 µl of soluble yeast protein extract were carefully layered on top of a gradient. Tubes were balanced and spun in an Sw55Ti rotor at 4°C and 32000 rpm for 22 h. After the run the gradient-tubes were carefully removed from the rotor. Fractions of the gradient were collected by piercing the bottom of the tube with a needle and releasing 5 drops (approximately 200 µl) each into eppendorf tubes containing 5x sample buffer. The samples were boiled for 5 minutes at 95°C and analysed by Western blotting.

7.6.8 Expression and purification of recombinant proteins

Protein purification was conducted on an Äkta purifier (GE) equipped with a FRAC-950 fraction collector. For loading of columns either the pump P1 (GE) or a loop (5 ml or 10 ml) was used.

7.6.8.1 Expression and purification of rMcb1-NTD-Strep

pASK-IBA3+ containing the sequence encoding for the N-terminal domain of Mcb1 (amino acids 1-238) with a C-terminal Strep-tag was transformed into Rosetta 2. For expression a culture derived from a single colony was grown in LB supplemented with ampicillin (100 µg/ml) and chloramphenicol (34 µg/ml) to an OD of 0.5 at 37°C. The culture was cooled down to 21°C and overexpression was induced by adding AHT (final concentration 200 µg/l) the cells were grown shaking at 21°C over night. The culture was harvested by centrifugation (5000 rpm 15 min) and pellets were frozen in aliquots corresponding to 2 litres of culture. Soluble protein was extracted from thawed cell-pellets by sonication (large probe at 12 micron amplitudes, 30 second bursts with 30 seconds on ice, for 10 minutes total sonication time) in buffer W (100 mM Tris pH 8.0, 150 mM NaCl, [1 mM EDTA], 0.1% Tween, complete, PMSF) and subsequent centrifugation of the lysate (40000 rpm for 45 min at 4°C) and filtration through a 0.22 µm syringe-filter. Mcb1-NTD-Strep was purified from soluble extracts in a two-step purification. In a first step the filtered lysate was loaded onto a Strep Trap 5 ml column (GE, according to manufacturer’s instructions) pre-equilibrated with buffer W using pump P1. Following
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the loading, the column was connected to an Äkta purifier and washed extensively with buffer W until the UV trace was stable (80-100 ml). Elution was performed by running a gradient of 0-100% elution buffer (buffer W containing 2.5 mM desthiobiotin) over 10 column volumes at 4 ml/min and collecting 1.5 ml fractions. Protein containing fractions were subjected to SDS-PAGE and fractions containing the target protein were pooled and concentrated to less than 5 ml for size exclusion chromatography.

The pooled protein containing fractions after Strep-purification were loaded onto a HiLoad 16/60 Superdex 200 pg column (GE) equilibrated in buffer W. The column was run at 2 ml/min with buffer W and protein containing fractions were screened by SDS-PAGE to identify those containing the target protein. Fractions were pooled and concentrated using a Millipore concentrator column with a cut-off of 9 kDa.

7.6.8.2 Expression and purification of rFL-Mcb1-Strep from pASK-IBA

pASK-IBA-Mcb1-Strep was transformed into Rosetta 2. A culture derived from a single colony was grown to an OD_{600} of 0.5 at 37°C in the presence of ampicillin (100 µg/ml) and chloramphenicol (34 µg/ml). Overexpression was induced at 22°C over night by adding 200 µg/l AHT. Cells were collected by centrifugation (5000 rpm 15 min) and frozen in aliquots corresponding to 2 litres of culture.

Soluble protein was extracted from thawed cell-pellets by sonication (large probe 12 micron amplitudes, 30 second bursts with 30 seconds on ice, for 10 minutes total sonication time) in five volumes buffer W (100 mM Tris-HCl pH 8.0, 300 mM NaCl, 1 mM EDTA, 0.1% Tween) supplemented with PMSF and complete protease inhibitor cocktail, followed by centrifugation of the lysate (40000 rpm for 45 min at 4°C).

The soluble extract was applied to a gravity flow StrepTactin-column (column volume 1.5 ml), unbound protein was washed off with five times 2 ml buffer W and the target protein was eluted with 6 times 0.5 ml elution buffer (buffer W containing 2.5 mM desthiobiotin). All fractions were analysed by SDS-PAGE and target-protein containing
fractions were pooled and further purified using a highprep 16/60 Sephacryl 500 HR column (GE healthcare).

7.6.8.3 Expression and purification of rMcm4-NTD from pEHisTEV

pEHisTEV-Mcm4NTD encoding His-tagged Mcm4-NTD without its N-terminal extension (AA153-496) was transformed into Rosetta 2. A culture derived from a single colony was grown to an OD<sub>600</sub> of 0.5 at 37°C in the presence of kanamycin (50 µg/ml) and chloramphenicol (34 µg/ml). Overexpression was induced by addition of 0.5 mM IPTG for five hours at 30°C. Cells were collected by centrifugation (5000 rpm 15 min) and frozen in aliquots corresponding to 2 litres of culture.

Soluble protein was extracted from thawed cell-pellets by sonication (large probe 12 micron amplitudes, 30 second bursts with 30 seconds on ice, for 10 minutes total sonication time) in five volumes buffer A (100 mM Tris-HCl pH 7.5, 300 mM NaCl, 0.1% Tween, 20 mM imidazole, 1 mM β-mercaptoethanol, 1 mM PMSF) and subsequent centrifugation (SS34 18000 rpm for 45 min at 4°C) of the lysate and filtering through a 0.22 µm filter.

His-Mcm4-NTD was purified from soluble extracts using a two-step protocol. First, the cleared extract from two litres of <i>E. coli</i> culture was loaded onto a HisTrap FF 5 ml column (GE, according to manufacturer’s instructions) pre-equilibrated with buffer A followed by extensive washing of the column (15 CV buffer A) using the Äkta purifier. The protein was eluted with a linear gradient with increasing imidazole concentrations from 100% buffer A – 100% buffer B (100 mM Tris-HCl pH 7.5, 300 mM NaCl, 0.1% Tween, 500 mM imidazole, 1 mM β-mercaptoethanol) over 15 CV. Protein containing fractions were screened for the presence of Mcm4-NTD by SDS-PAGE and fractions containing the target-protein from two HisTrap runs were pooled, concentrated to 3 ml and further purified by size exclusion chromatography.

The protein fractions were loaded onto a superdex 200 column (GE) pre-equilibrated with 100 mM Tris-HCl pH 7.5, 300 mM NaCl, 0.1% Tween-20 using a 10 ml loop on the Äkta purifier. The column was run at 2.0 ml per minute collecting 2 ml fractions. Target protein was identified by SDS-PAGE and mass-spec, fractions were pooled and concentrated further using a Millipore concentrator with a cut-off size of 9 kDa.
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7.6.9 Protein concentration

Protein containing solution after purification or dialysis was concentrated using Pierce protein concentrators (7 ml or 20 ml, 9K MWCO) at 3500 rpm and 4°C.

7.6.10 Protein crystallisation

For crystal trials, purified protein was concentrated to at least 5 mg/ml. Crystal trials were carried out at 20°C using the sitting drop vapour diffusion method and a crystallisation robot (Hamilton-Thermo Rhombix system). Drops were usually 150 nl in volume. For initial screens WizardI and II (Emerald Bio), Cryol and II (Emerald Bio), JCSG+ (Quiagen) and PACT premier (Quiagen) crystallisation screens were used. Plates were examined daily for the appearance of crystals for the first week, then once a week.

Refinements were set up in 96 well plates with 1 μl drops containing 0.5 μl protein (at 5 mg/ml) and 0.5 μl reservoir solution.

Salt crystals were differentiated from protein crystals using Izit-dye (Hampton research).

7.6.11 In vitro transcription/translation using Promega TNT

7.6.11.1 IVT reactions

In vitro translations were carried out using Promega TNT® Quick Coupled Transcription/Translation rabbit reticulocyte System according to the manufacturer’s instructions. 20 μl reactions were prepared containing 16 μl master-mix, 0.3 μg of template-plasmid (pTNT optimized for in vitro transcription/translation) 8 μCi of 35S-methionine to label the newly synthesized protein and protease inhibitors leupeptin (1 μg/ml) and chymostatin (6 μg/ml).

Reactions were incubated at 30°C for 90 minutes. For analysis, 2.5 μl samples were taken, 5xSB buffer and water added to 50 μl and boiled at 80°C for 10 minutes.
7.6.11.2 **IVT pull-down assays with rMcb1-NTD**

Pull-down assays of *in vitro* translated Mcm-NTD with Strep-tagged rMcb1-NTD were carried out in 50 mM Tris HCl pH 8, 250 mM sodium glutamate, 5% Glycerol, 0.1% Tween-20, 2 mM EDTA and complete protease inhibitors (“binding buffer”). StrepTactin resin (IBA technologies) was washed with binding buffer three times. 50 µg rMcb1-NTD-Strep (or as indicated) were added per 30 µl of resin slurry and binding buffer was added to a volume of 200 µl. Control resin was prepared without Strep-tagged protein but buffer only. For assays with 10 µg or 1 µg of rMcb1-NTD-Strep only 15 µl of StrepTactin were used. rMcb1-NTD-Strep was allowed to bind for 45 minutes. Then the supernatant was removed and 8 µl of IVT-mix and 150 µl of binding buffer was added. In competition experiments with different Mcm-NTDs 8 µl of each indicated Mcm-NTD preparation were added. The tubes were incubated at 4°C, rotating for 1 hour. The supernatant was removed and the resin was washed five times with 500 µl binding buffer and boiled with sample buffer at 80°C for 10 minutes. Total in-vitro translation reactions and pulled-down protein were applied to SDS-PAGE, the gels were dried in a gel slab dryer and subsequently exposed to film (Kodak Bio Max MR) for three to five days.

7.6.12 **Production of polyclonal α-Mcb1 antibody in rabbit**

About 2 mg of purified rMcb1-NTD-Strep protein were used for the immunisation of two rabbits by Dundee Cell Products Ltd. Detection of endogenous protein by the serum was tested after each immunisation. Following the final immunisation the sera were affinity purified using Pierce NHS-Activated Agarose resin with immobilised rMcb1-NTD according to the manufacturer’s instructions. Briefly 2.5 mg of rMcb1-NTD was coupled to 330 mg dry resin over night. Unoccupied active sites were blocked. Then about 5 ml of rabbit antiserum diluted 1:1 with PBS were incubated with the prepared column for two hours. The supernatant was removed and the incubation repeated with another 5 ml of antiserum plus PBS. After washing the column, the antibody was eluted in 1 ml fractions with 0.15 M glycine-HCl pH 2.7 and neutralized with 1 M phosphate pH 9 (50 µl per ml). Fractions were tested on the presence of heavy/ light chains by SDS-PAGE. Antibody containing fractions were pooled, concentrated and stored at 4°C with 0.02% azide.
7.6.13 Mass spectrometry

MS/MS analysis was performed in-house by the BSRC mass spectrometry and proteomics facility using a Q-Star Pulsar XL QTOF instrument (Applied Biosystems) and electrospray ionisation. The MS/MS data was compared to the MASCOT database to identify peptides and proteins.

Individual gel bands were excised and cut into 1 mm cubes. These were then subjected to in-gel digestion using a ProGest Investigator in-gel digestion robot (Genomic Solutions). The gel cubes were destained with acetonitrile and reduced and alkylated before digestion with trypsin at 37°C. The peptides were extracted with 10% formic acid.

MALDI TOF/TOF analysis

0.5 ml of the digest solution was applied to the MALDI target along with alpha-cyano-4-hydroxycinnamic acid matrix (0.5 ml, 10 mg/ml in 50:50 acetonitrile: 0.1% TFA) and allowed to dry. MALDI MS was acquired using a 4800 MALDI TOF/TOF analyser (Applied Biosystems) equipped with a Nd:YAG 355 nm laser and calibrated using a mixture of peptides. The most intense peptides (up to 15) were selected for MS/MS analysis and the combined MS and MS/MS data was analysed, using GPS Explorer (Applied Biosystems) to interface with the Mascot 2.1 search engine (Matrix Science), against the UniProt (Swiss-Prot and TREMBL combined) database. The data was searched with tolerances of 100 ppm for the precursor ions and 0.5 Da for the fragment ions, trypsin as the cleavage enzyme, assuming up to one missed cleavage, carbamidomethyl modification of cysteines as a fixed modification and methionine oxidation selected as a variable modification.

nLC-ESI-MSMS analysis

The peptides were concentrated down to 20 μl using a SpeedVac (ThermoSavant). They were then separated using an UltiMate nanoLC (LC Packings) equipped with a PepMap C18 trap and column, using a 30 min or 60 min gradient (depending on molecular weight of sample being analysed) of increasing acetonitrile concentration, containing 0.1 % formic acid (5-35% acetonitrile in 18 min or 35 min respectively, 35-
50% in a further 7 or 20 min, followed by 95% acetonitrile to clean the column). The eluent was sprayed into a Q-Star Pulsar XL tandem mass spectrometer (Applied Biosystems) and analysed in Information Dependent Acquisition (IDA) mode, performing 1 sec of MS followed by 3 sec MSMS analyses of the 2 most intense peaks seen by MS. These masses are then excluded from analysis for the next 60 sec. MS/MS data for doubly and triply charged precursor ions was converted to centroid data, without smoothing, using the Analyst QS1.1 mascot.dll data import filter with default settings. The MS/MS data file generated was analysed using the Mascot 2.2 search engine (Matrix Science) against UniProt (Swiss-Prot and TREMBL combined), with no species restriction. The data was searched with tolerances of 0.2 Da for the precursor and fragment ions, trypsin as the cleavage enzyme, one missed cleavage, carbamidomethyl modification of cysteines as a fixed modification and methionine oxidation selected as a variable modification.

7.7 Cell biological methods

7.7.1 Survival essays of mcb1-Ts1/6 and Δchk1 Δcds1 Δrad3 and nmt41-mcb1 cells

Cells carrying mcb1-Ts in the Δchk1, Δcds1 or Δrad3 background were grown in YE4S at permissive temperature of 25°C to an OD of 0.2 before shifting them to the restrictive temperature of 35°C. Cell numbers were counted using a Coulter counter and 600 cells were plated onto two YE plates before the temperature shift or after 2, 4, 6 or 8 hours at 35°C. Plates were incubated at 25°C until colonies formed.

7.7.2 Fluorescence microscopy

Cells to be examined by fluorescence microscopy were grown to exponential phase, collected by centrifugation and washed with PBS. Cells were stained with a 1:1000 dilution of Hoechst 33342 in PBS in the dark for 15 minutes at room temperature to visualize DNA, before washing with PBS for three times and resuspending in 20 μl of PBS. 2 μl of cell suspension were placed on a glass slide with a drop of mounting solution (90% v/v glycerol, 10% v/v PBS, 0.1% w/v p-phenylenediamine) to prevent
bleaching before adding a cover slip and sealing the slide with nail varnish. Fluorescent signals (Hoechst 33342 or YFP) were detected using a fluorescence microscope (Zeiss Axiovert 10 or Zeiss Axioplan 2) with a 63x objective (numerical Aperture 1.4) and DAPI/ Hoechst (excitation wavelength 350 nm) or GFP/YFP (excitation wavelength 515 nm) filters. Images were taken using a coupled camera (Zeiss AxioCam ICc1) and Axio Vision programme 4.8 for processing.

7.8 Bioinformatics

7.8.1 Multiple sequence alignments

Multiple sequence alignments were made with Clustal2W and Seaview (Gouy et al. 2010). Seaview can drive the newer Clustal Omega algorithm, which gives improved quality alignments. Seaview was also used for drawing phylogenetic trees, which were pictured with Dendroscope (Huson & Scornavacca 2012). Output files were created from the alignments using ClustalW2.

7.8.2 Structure prediction and 3D modelling

For prediction of 3D protein structures, protein sequences were entered into the Phyre2 (Protein Homology/analogY Recognition Engine V 2.0; (Kelley & Sternberg 2009) structure prediction engine. PDB result files were displayed with PyMOL Molecular Graphics System, Version 1.5.0.4 (Schrödinger, LLC), which was also used to create images.


Bowers, J.L. et al., 2004. ATP hydrolysis by ORC catalyzes reiterative Mcm2-7 assembly at a defined origin of replication. *Molecular Cell*, 16(6), pp.967–78.


References


Dua, R., Levy, D.L. & Campbell, J.L., 1999. Analysis of the Essential Functions of the C-terminal Protein/Protein Interaction Domain of *Saccharomyces cerevisiae* Pol epsilon and Its Unexpected Ability
References


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Gambus, A. & Blow, J.J., 2013. Mcm8 and Mcm9 form a dimeric complex in *Xenopus laevis* egg extract that is not essential for DNA replication initiation. *Cell cycle*, 12(8), pp.1225–32.


Groth, P. et al., 2010. Methylated DNA causes a physical block to replication forks independently of damage signalling, O(6)-methylguanine or DNA single-strand breaks and results in DNA damage. *Journal of molecular biology*, 402(1), pp.70–82.


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Im, J.-S. et al., 2009. Assembly of the Cdc45-Mcm2-7-GINS complex in human cells requires the Ctf4/And-1, RecQL4, and Mcm10 proteins. PNAS, 106(37), pp.15628–32.


References


Komata, M. et al., 2009. The direct binding of Mrc1, a checkpoint mediator, to Mcm6, a replication helicase, is essential for the replication checkpoint against methyl methanesulfonate-induced stress. *Molecular and cellular biology*, 29(18), pp.5008–19.


References


References


References


Sanchez Garcia, J. et al., 2009. Functional mapping of the fission yeast DNA polymerase delta B-subunit Cdc1 by site-directed and random pentapeptide insertion mutagenesis. *BMC molecular biology*, 10(82).


References


Takara, T.J. & Bell, S.P., 2011. Multiple Cdt1 molecules act at each origin to load replication-competent Mcm2-7 helicases. The EMBO journal, 30(24), pp.4885–96.


References


References


Appendices

Appendix 1

Analysis of mcb1-Ts1 and mcb1-Ts5 mutants by flow cytometry

Analysis of the DNA content of mcb1-Ts1 and mcb1-Ts5 at permissive and non-permissive temperature shows that the temperature-sensitive mutants undergo S-phase and replicate bulk DNA before arresting in G2-phase. The FACS analysis shown in Figure 0-1 was conducted by J.-J. Li.

Figure 0-1 Flow cytometry of mcb1-Ts1 and mcb1-Ts5 cells from (Li et al. 2011). Wild-type, mcb1-Ts1 and mcb1-Ts5 cells were grown to ~1.5x10^6 cells/ml at the permissive temperature of 25°C before incubation at the restrictive temperature of 36°C for 6 hours (or incubation at 25°C for the same period). Samples were taken hourly and cells fixed with cold 70% ethanol, washed in 50 mM sodium citrate,
RNase treated (0.1 mg/ml for 2 h) and stained with propidium iodide (2 µg/ml). Cells were analysed using a Becton Dickinson FACScan.

**Drug sensitivity of Ts mutants**

*Mcbl-Ts1* and *mcb1-Ts6* temperature-sensitive mutants do not exhibit increased sensitivity to DNA stressing agents.

**Figure 0-2 Sensitivity to DNA damaging agents** from (Li et al. 2011). Wild-type, *mcb1-Ts1* and *mcb1-Ts6* cells were grown to mid-exponential phase at the permissive temperature of 25°C and serial dilutions spotted onto YE plates and incubated at 25°C or 35°C for three days (upper panel), or onto YE plates supplemented with 4NQO, CPT, HU or MMS at the concentrations shown, or spotted and irradiated with UV-C (254 nm) light at the doses shown, before being incubated for three days at 25°C (lower panel). No hypersensitivities are apparent with the *mcb1-Ts1* and *mcb1-Ts6* strains.
Bimolecular fluorescence complementation (BiFC) using \textit{mcm4-VN mcb1-VC} cells

\begin{figure}
\centering
\includegraphics[width=\textwidth]{biomolecular_fluorescence_complementation.png}
\caption{BiFC using \textit{mcm4-VN mcb1-VC}: cells carrying \textit{mcm4-VC} or \textit{mcb1-VN} or both were stained with Hoechst 33342 and examined by fluorescence microscopy. Images of Hoechst-channel, Venus-channel (=YFP) and merge of both are shown.}
\end{figure}

\section*{Appendix 2 Primers and gene sequences}

\begin{table}
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Primer name & Sequence \tabularnewline \hline
\textbf{PCR-mediated gene targeting and sequencing}; sequences annealing pFA6a template plasmid are underlined & \tabularnewline P1 & Mhi1-CTAG-5 \tabularnewline & 5’-\tabularnewline & CGAAGAGTTTCGGTCGTA\underline{ACTGGTTTCAAGAATTGATT}TT\underline{TTGAGGCTGCGTAGTCTAATCAATTGGACTGTCAACCGGATCCC CGGTTAAATTAA-3’} \tabularnewline P2 & Mhi1-CTAG-3 \tabularnewline & 5’-\tabularnewline & TGAGCTTTACTTG\underline{TAGTAATAATTAATAAACAATATAAAT}ATGAACT \underline{TAAAATGGAGGAAATTAAGAAT}TCCAG & \tabularnewline \hline
\end{tabular}
\caption{Primers used in this study. Primers are listed based on experiments they were used in. Comments in “note” column: Seq. indicates sequencing primers; “Intron” indicates primer which was used for removing introns from coding sequences.}
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**Mcb1 truncations with N-terminal FLAG tag**

| P48  | Mcb1 Nterm for | 5’-TAGACACTCGAGATGGACTACAAGGACGATGACGATAAGGTAAT TGCTTTATCTGATT-3’ |
| P48b | nmt3’          | |
| P49  | Mcb1 283stop rev | 5’-GTGCACACCGATCCCTATAATATTTTTGTGCAAT-3’ |
| P50  | Mcb1 300stop rev | 5’-GTATCGACGGATCTCCTACATTCTTTTTATCAAGG-3’ |
| P51  | Mcb1 350stop rev | 5’-ATTCATATGGATCCCCTAGTTAAGGTTCCACTCGA-3’ |
| P52  | Mcb1 400stop rev | 5’-CTTGAATGATCGATCTTGAGACGATCGGCAAAT-3’ |
| P53  | Mcb1 450stop rev | 5’-ATGCGCTTAGATCCTTTACATATGTAGATTGAT-3’ |

**Mcb1 truncations with C-terminal FLAG tag** (random XhoI START ....)

| P54  | Mcb1 C term rev | 5’ -AGTATAGGATCTCCTACTTTAGCTTGATCGCTCTTGAGCAGTCCAATGATT-3’ |
| P54b | nmt5’          | |
| P55  | Mcb1 start18   | 5’-CATGACAGCTCAGATGCAAAGATTCTTCTGACGATTGCA-3’ |
| P56  | Mcb1 start85   | 5’-CATGACAGCTCAGATGCAAAGATTCTTCTGACGATTGCA-3’ |
| P57  | Mcb1 start149  | 5’-CATGACAGCTCAGATGCAAAGATTCTTCTGACGATTGCA-3’ |
| P58  | Mcb1 start 238 | 5’-CGCGCCAGCTCGAGATGTTAAATATTTTTGAAAAAA-3’ |

**Cloning of Mcb1-CTD into pEHisTEV**

| P59  | HisTEV Mcb1CTDfor | 5’-GCATCGCCGGATATCGTATAATAATTTTTGAAAAAAGT-3’ |
| P60  | HisTEV Mcb1CTDrev | 5’-ACTATTATAAGACGCTTTAGTACGCTCGTCAATGAT-3’ |

**Cloning of Mcb1-FL into pASK-IBA3+**

| P61  | Mhi1-N5 Bsal for  | 5’-GTGGTGGTTGATCCTTACATGGTATAATTTGCTTTATCTG-3’ |
| P62  | FLMcb1-STREP Bsal Rev | 5’-CATTACATATGGTCTCAGGCGCGCTGTGAGCAGTCCAATGATTGAT-3’ |
| P63  | SpMhi1-INV For | 5’-GAGGAAAGAATAAGAAAGATATTCAAAACTATCGTGAC-3’ | Intron |
### Appendices

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#### Cloning of mcm2 into pREP4x (remove intron)

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#### Cloning of Mcb1-NTD and Mcm-NTDs into pTNT

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### Mcm4 truncation constructs from pTNT

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### Cloning of Mcm4-NTD and Mcm6-NTD into pEHisTEV

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### Other primers

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### Some gene and protein sequences used in this study

\[ mcb1^+ \] coding sequence

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ATGGTAAATTTGCTTTATCTCGTTTTTATCGAAAAATCAGATCGTTTTCTCAAAGGATTTCGAGGATGACACTGTCGCTGGTGGGT-3'
TGCAATGGTTTTTCGAGGATCTAAGCAGCATTACACGCAACTTTAGGCACTGAAAGGAATTTTCAATATATTTTGCTGACATATGAATGCTC
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```
Appendices

Mcb1 protein sequence (NTD underlined)
MVIALDSFIENPRSFLQRFQDALFAGSKPDLQGTLIGEESNFATEERIRKIPYNLDCKWSELT
GQLRLOQGMVQDTNFHEFGAGAVEVENIWRGCRYILDFSEDEMHLDESKIVLDERVEYNLFNTV
PGERTLPVEALGNWSKLSRLYKSNLQASNDTGVCPKCYGGMETQVQCAIDVGIYEEP
SEYSDGLPLMLFKDYSATQAPSPQAAIEPPIKLYFKELVGENIAAESLMLALSNNVHKT
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LIVLDETESSHGTNDVGCRRNVQFLSSLSQDQDFTFYFSSFVHSVNRIIILSHGRLSPADVGCRCR
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RLYAKSFGQRLVSIDFEAARSLINHWTVN

Mcm2 protein sequence (NTD underlined)
MDSFRKRGDRDSELPFLFESENSLLGATPSLLPSSPPEFSEAAEALVEEDIEDLDGEALDEEED
EDLFGEGMERDYQONLLEQDYEELDDNDELDIIGARRAVDLRRLRRDIELDAAGRTKPAAP
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Mcm3-NTD protein sequence
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GVLLKPLEYVPEFDEALRNVSTLIDPVVHSDKDKLFLYGFGRSGDHVNPRTLAMHLNMISSL
Appendix 3 Multiple sequence alignments I

A multiple sequence alignment of fission yeast Mcb1 proteins with Mcm proteins from fission yeast and archaea for the determination of domain boundaries in S. pombe Mcb1 for the construction of truncation mutants.
Figure 0-4 Multiple sequence alignment of archaeal Mcms, *S. pombe* Mcm2-7 and Mcb1 from different fission yeasts. *Schizosaccharomyces cryophilus* ScMcb1, *Schizosaccharomyces octosporus* SoMcb1, *S. pombe* SpMcb1, *Schizosaccharomyces japonicas* SjMcb1 and archaeal Mcm proteins from *Haloferax volcanii* HfxMcm, *Methanothermobacter thermotrophicus* MthMcm, *Sulfurobacter solfataricus* ssoMcm for the determination of domain boundaries. The alignment was created with ClustalW.
Appendix 4 Multiple sequence alignments II

A multiple sequence alignment of MCM-BP proteins from different species was constructed using SeaView (Gouy et al. 2010). The output file in Figure 0-5 was created with ClustalW.
## Appendices

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**NTD** and **CTD** annotations shown. Patterns indicate homologous regions.
MCM-BP proteins from different species were also aligned to Mcm proteins from different species including archaeal Mcms.
Appendix 5 Structure prediction additional data

Phyre2 was used for structure prediction for *S. pombe* Mcb1. In a first step, a PSI-BLAST search is conducted and a pseudo alignment with homologous sequences is created. For Mcb1, this contained 721 sequences mostly of Mcm proteins (Figure 0-7).

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Figure 0-6 Complete multiple sequence alignment of Mcm proteins and MCM-BP proteins from different species.
Figure 0-7 Excerpt of pseudo alignment created by Phyre using PSI-BLAST. 721 sequences homologous to Mcb1 were used.

Based on this pseudo alignment, the secondary structure and disorder of Mcb1 were predicted (Figure 0-8).
Phyre2 secondary structure and disorder prediction for *S. pombe* Mcb1

Figure 0-8 Secondary structure and disorder prediction for fission yeast Mcb1.