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Genetic Studies of Genes Involved in the Initiation of DNA Replication in the Fission Yeast Schizosaccharomyces Pombe

Zhuo Wang
Wright State University

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GENETIC STUDIES OF GENES INVOLVED IN THE INITIATION OF DNA REPLICATION IN THE FISSION YEAST *SCHIZOSACCHAROMYCES POMBE*

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

By

Zhuo Wang

B.S., Shenyang Agricultural University

2010
Wright State University
I HEREBY RECOMMEND THAT THE thesis PREPARED UNDER MY SUPERVISION BY Zhuo Wang ENTITLED Genetic studies of genes involved in the initiation of DNA replication in the fission yeast *Schizosaccharomyces pombe* BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science

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ABSTRACT

Wang, Zhuo M.S., Department of Biochemistry and Molecular Biology, Wright State University, 2009. Genetic studies of genes involved in the initiation of DNA replication in the fission yeast *Schizosaccharomyces pombe*

1The initiation of DNA replication is a highly conserved process in all eukaryotes. However, the underlying mechanism is not well understood. Genetic studies in the fission yeast *S. pombe* have contributed greatly to and will continue to provide insights to our understanding of this important biological process.

In the first chapter, we have used a complementary method to test three recently identified human replication proteins DUE-B, Ticrr/Treslin, and GEMC1 as the candidate functional homologue of Sld3 in *S. pombe*. Sld3 is an essential replication initiation protein discovered in yeasts. Since no apparent sequence similarity can be found, its homologue in higher eukaryotes remains to be uncovered. In fact, among all yeast replication proteins, Sld3 is the only protein whose functional homologue has not been found in metazoan. Our preliminary results showed that all three human replication proteins failed to complement the function of Sld3 in fission yeast. Unlike DUE-B, whose expression does not perturb the cell cycle progression in fission yeast, overexpression of Ticrr and GEMC1 in *S. pombe* can suppress the cell growth.

In the second chapter, we have developed a screening strategy in *S. pombe* to discover new gene(s) that may function in the initiation of DNA replication. A yeast strain has been made in which the promoter of Chk1, the effector kinase of the DNA damage checkpoint in fission yeast, was replaced with a thiamine-repressive promoter. The Chk1 expression in this strain can be completely shut off by adding thiamine in the culture medium. It is known that defects in the replication initiation require the checkpoint function for cell survival. A mutation in the gene involved in the initiation of DNA replication is expected to be sensitive to the depletion of the Chk1 function. In a small-scale screening, we obtained 15 so-called Kds (Chk1 dependent survival) mutants. Characterization of one the mutants Kds15 in the preliminary study identified a structure-destructive mutation in Psf2, a subunit of the essential GINS complex required for the initiation of DNA replication. This result validates the strategy and shows that the screening can be productive.
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GENERAL INTRODUCTION

Maintenance of the genome integrity, a central concern for all eukaryotic cells, is dependent on faithful DNA replication, DNA repair and the checkpoint mechanism that responds to DNA damage or stalled replication forks. To achieve an efficient and precise DNA replication, the initiation of DNA replication in eukaryotic cells is highly regulated process that has three common features (Bell and Dutta, 2002): First, the initiation of the DNA replication is established at multiple sites of the genome called origins (Clyne and Kelly, 1995; Hayashi et al., 2007; Wu and Nurse, 2009). Second, the origin fires once, and only once, per cell cycle to avoid re-replication of DNA at the same origin (Gopalakrishnan et al., 2001; Yanow et al., 2001). Third, the onset of the DNA replication is strictly coordinated with the cell cycle, requiring the loading and activation of the DNA helicase at the origins at appropriate time during the cell cycle progression (Wu and Nurse, 2009). To coordinate the DNA replication with the cell cycle progression, a checkpoint mechanism has evolved to monitor the integrity of the genome and arrest cell cycle progression in the event of DNA damage or stalled replication (Nyberg et al., 2002). If undetected, stalled replication forks or damaged DNA can cause cell death or mutagenic chromosomal damage. Defects in the regulation of DNA replication initiation and the checkpoint control are linked to genome instability and cancer (Bell and Dutta, 2002; Masai et al., 2010; Nyberg et al., 2002)
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Table 1. Proteins involved in the replication initiation in S. pombe and their homologues in human.

Note: Sld3 is the only replication protein whose homolog has not been identified in higher eukaryotes.
REGULATION OF THE INITIATION OF DNA REPLICATION IN EUKARYOTES

The initiation of DNA replication involves multiple protein complexes that are conserved in all eukaryotes (Table 1) and regulated by at least two classes of protein kinase, the cyclin-dependent Kinases (CDKs) and Cdc7-Dbf4 Kinase (DDK). It is generally believed that two protein complexes, the pre-replication (pre-RC) (Figure 1) and the pre-initiation complex (pre-IC) (Figure 2) complexes, are assembled at origins in an orderly fashion before the initiation occurs. They assemble at replication origins during the late M to G1 phase of the cell cycle (Bell and Dutta, 2002; Masai et al., 2010). This introduction will focus on the replication initiation in fission yeast with a brief description of the same process in other model organisms.

Assembly of the pre-RC complex

The pre-RC complex includes the origin recognition complex (ORC) proteins, Cdc18/Cdc6, Cdt1, and the minichromosome maintenance complex (MCM) proteins that are conserved in all eukaryotes (Figure 1). These proteins are recruited sequentially at the replication origins during the late M to early G1 phase (Bell and Dutta, 2002). In fission yeast, the location and distribution of the pre-RC complex is highly reproducible in each cell cycle with an average separation of 26.7 kb. Pre-RC complexes are enriched at the centromeres and the subtelomeric regions (Hayashi et al., 2007). The main function of the pre-RC complexes is to serve as potential sites to be fired where the origins are equipped with MCM complex as the putative DNA helicase (Bell and Dutta, 2002).
**Figure 1. Assembly of the pre-RC complex in fission yeast.**

During the late M and early G1 phase, the replication origin is bound by the ORC complex followed by Cdc18 and Cdt1 and MCM complex (Kelly and Brown, 2000). See text for details.
Assembly of the pre-RC complex begins with the binding of ORC complex to the origins during the late mitosis (Chuang and Kelly 1999). ORC complex is a heterohexameric complex that recognizes and binds to DNA replication origins (Gavin et al., 1995). In contrary to the origins in budding yeast that has a high degree of sequence specificity (Clyne and Kelly, 1995), fission yeast origins consist of multiple AT-rich sequences of 20-50bp long with no defined sequence specificity. The recruitment of ORC complex specifically to the origins occurs through the “AT-hook” motifs of the subunit Orp4 (Chuang and Kelly, 1999; Moon et al., 1999). In addition to the local DNA AT rich sequences, binding of ORC to DNA can be promoted by negatively supercoiled DNA and the binding itself also induces topological changes in DNA (Houchens et al., 2008).

During the late G1 and early S phase, two MCM complex loading factors, Cdc18/Cdc6 and Cdt1, assemble at the replication origins (Nishitani et al., 2000; Tada and Blow, 1998). The ORC complex recruits Cdc18 (Chuang, Chretien et al. 2002), whose ATP hydrolysis activity may induce a conformational change in the ORC complex (DeRyckere, Smith et al. 1999; Neuwald, Aravind et al. 1999). Cdt1 may interact with Cdc18 to form a complex (Houchens et al., 2008; Nishitani et al., 2000). Both Cdc18 and Cdt1 are targets of regulation that prevents re-initiation of replication at the same origins (Gopalakrishnan et al., 2001). Cdc18 is negatively controlled by proteolysis after licensing the DNA replication (Jin et al., 2006). Fail to do so, cell re-initiates the DNA synthesis even in G2 phase of the cell cycle (Yanow et al., 2001) causing mutagenic chromosomal damage or heteroploidy of the cell.

With the cooperation of ORC complex, Cdc18 and Cdt1, MCM complex is loaded
at the origins to finish the last step of pre-RC complex formation. MCM complex is believed to form a ring structure with six conserved subunits and function as the DNA replication helicase (Bell and Dutta, 2002). However, when purified as a 560-kDa complex with stoichiometric amounts of all six MCM proteins, the complex does not have any helicase activity (Adachi et al., 1997). This may be due to lacking of activation factors (Lee et al., 2003; Uchiyama et al., 2001b) or missing some associated proteins (Akman and MacNeill, 2009) as Mcm4-Mcm6-Mcm7 heterotrimer possess a low processive DNA helicase activity in vitro (Ishimi, 1997). However, the heterotrimer losses this activity when interacting with Mcm2 (Ishimi et al., 1998). Mcm2 can be phosphorylated in vitro by DDK (Brown and Kelly, 1998). In addition, DDK may also target Mcm4 to release an inhibition effect from the amino terminal serine/threonine-rich domain (NSD) of Mcm4 in budding yeast (Sheu and Stillman, 2010). These indicate a possible control mechanism through DDK to activate DNA unwinding.

The pre-RC complex is normally assembled with an excess numbers at the origins (Hayashi et al., 2007). However, not all the pre-RC complexes are utilized for actual initiation. Origin firing may be affected by many factors such as DNA topology (Houchens et al., 2008), chromatin structures, transcription (Masai et al., 2010), the time of the pre-RC formation (Wu and Nurse, 2009) and so on. However, only those sites with successful formation of pre-IC complex are competent for unwinding the parental DNA duplex and initiating DNA replication (Wu and Nurse, 2009).

**Assembly of the pre-IC complex**
After the pre-RC complex is formed, pre-IC complex is assembled with the loading of additional replication proteins including Mcm10, Cdc45, Cut5/Dpb11/TopBP1,
Sld2/RecQL4, Sld3 and GINS complex. The assembly of pre-IC is regulated by at least two kinases, CDKs and DDK (Figure 2). One of the main purpose of the pre-IC complex formation is to activate the DNA helicase activity of the MCM complex (Masai et al., 2010). In fission yeast, Cdc45 is the key player to realize this activation (Kelly and Brown, 2000; Uchiyama et al., 2001b) and its loading to origins depends on other components such as Sld3 and Cut5/Dpb11/TopBP1 (Yabuuchih et al., 2006). The current model of the pre-IC formation in fission yeast is as follows (Yabuuchi et al., 2006):

During the G1/S transition, both CDKs and DDK are activated by binding to their “partner” proteins, which are only present in a specific phase of the cell cycle. For CDKs, the kinase Cdc2 of fission yeast is activated by binding to Cig2, the major cyclin during the G1/S transition and S phase (Fisher and Nurse, 1996). In a similar manner, activation of DDK (Hsk1 in fission yeast) depends on the association with Dfp1, whose presence is limited to G1/S transition (Brown and Kelly, 1999).

DDK acts to promote the association of Sld3 with the chromatin (Nakajima and Masukata, 2002; Yabuuchi et al., 2006). DDK may phosphorylate either pre-RC complex or Sld3 to facilitate this process (Nakajima and Masukata, 2002; Yabuuchi et al., 2006). The phosphorylation on pre-RC complex may target to the Mcm2 of the MCM complex to induce a conformational change, which facilitates the binding of other factors. Mcm10 has the ability to promote the phosphorylation of Mcm2 by DDK (Lee et al., 2003), which suggests that Mcm10 may be loaded ahead to assist the kinase activity of DDK.

After loading of Sld3, the kinase activity of CDK is required for the subsequent assembly of pre-IC (Yabuuchi et al., 2006). The CDK can also stimulate the degradation
Figure 2. Assembly of the pre-IC complex in fission yeast during G1/S transition and S phase.
of Cdc18/Cdc6 by phosphorylation to ensure the origin only fired once (Baum et al., 1998; Kominami and Toda, 1997). Recently, it was shown in budding yeast that CDK phosphorylates Sld2 and Sld3 to promote their binding with Dpb11 to establish the pre-IC complex (Zegerman and Diffley, 2007). In fission yeast, CDK may function in a similar way. Sld2 is phosphorylated by CDKs and this phosphorylation is required for its interaction with BRCT domains of Cut5 to establish the pre-IC complex (Masumoto et al., 2002; Noguchi et al., 2002). Although Sld3 is a potential target of CDKs and is hyperphosphorylated when it associates with Cdc45, mutations of three potential CDK phosphorylated sites of Sld3 do not affect cell viability (Nakajima and Masukata, 2002) suggesting redundant phosphorylation events may exist. Even so, Sld3 and the CDK activity are still required for loading Cut5 and the other factors (Yabuuchi et al., 2006; Yu et al., 2003).

Another protein complex required for Cdc45 loading is the GINS complex consisting of four subunits Sld5-Psf1-Psf2-Psf3. GINS complex is also involved in the DNA helicase activity of MCM complex (Takayama et al., 2003). Mutations in Psf3 impair the origin association of Psf2, Cut5 and Cdc45, but not Sld3. However, Sld3 and Cut5 are essential for the loading of Psf2, which suggests that the loading of GINS and Cut5 are mutually dependent (Yabuuchi et al., 2006). Thus, Cut5 may function as a “linker” protein, taking all other necessary factors Sld2 and GINS to the origins for loading Cdc45 (Yabuuchi et al., 2006). With these preparations, Cdc45 is assembled onto chromatin and stabilized to form the pre-IC complex (Yabuuchi et al., 2006). In addition, Cdc45 and GINS may facilitate loading of DNA polymerases and couple with the MCM complex (Kanemaki and Labib, 2006; Labib and Gambus, 2007; Uchiyama et al., 2001a).
As soon as the pre-IC is assembled, replication begins with DNA unwinding by the MCM complex (Bell and Dutta, 2002).

Besides their role in the activation of the MCM complex, Mcm10, Cdc45, and GINS move along with replication forks as part of the large replisome progression complex (RPC) to stabilize and stimulate MCM complex (Yang et al., 2005). It has been shown that in the presence of Cdc45 and GINS, the rate of ATP hydrolysis by MCM complex purified from Drosophila is elevated by two orders of magnitude (Ilves et al., 2010). The crystal structure and DNA-binding data also suggest a role of GINS at the replication forks where it may serve as a scaffold by interacting with MCM and DNA polymerase to coordinate DNA unwinding and synthesis (Boskovic et al., 2007; Chang et al., 2007). Other loading factors such as Cut5/Dpb11/TopBP1, Sld2/RecQL4, and Sld3 disassociate from the replication forks, which may explain why loading factors are evolved more rapidly than the core replisome proteins (Labib, 2010).

THE CHECKPOINT RESPONSE IN REGULATION OF THE CELL CYCLE

DNA is constantly exposed to insults of endogenous and exogenous sources, which causes various forms of DNA damage and stalled replication forks. To maintain a complete and faithful genome duplication during the cell divisions, eukaryotic cells have developed a conserved checkpoint response that can sense the DNA damage and stalled replication forks and pause the cell cycle progression to provide time for repairing the DNA damage (Nyberg et al., 2002). Mutations in the checkpoint pathways that fail to arrest the cell cycle cause genomic instability and cell death (Weinert and Hartwell, 1988).

It is generally believed that most, if not all, of the proteins that are essential for
the checkpoint response have been identified (Table 2). These proteins can be divided into three conceptual units as sensor, mediators and effectors. The sensor proteins detect DNA damage or stalled replication forks and activate effector kinases through the mediators (Nyberg et al., 2002). In fission yeast, Chk1 and Cds1 are the two major effector kinases that stimulate the cellular responses to DNA damages or stalled replication forks, respectively.

**DNA damage checkpoint**

In *S. pombe*, DNA damage outside of S phase activates the DNA damage checkpoint leading to the activation of Chk1 (Tapia-Alveal et al., 2009), the major effector kinase of the DNA damage checkpoint pathway. Activation of Chk1 requires its phosphorylation by Rad3 (Walworth and Bernards, 1996). It was found earlier that phosphorylation of serine 345 (S345) in Chk1 is essential for the activation of the kinase (Capasso et al., 2002), although the mechanism for Chk1 activation is still unclear. Activated Chk1 normally induces a cell cycle arrest in G2/M phase in the fission yeast (Carr et al., 1995; Martinho et al., 1998). However, activation of Chk1 has also been observed in G1 (Synnes et al., 2002), S (Francesconi et al., 1997) and M (Liu et al., 2000) phase of the cell cycle.

Activation of Chk1 requires an intact DNA damage checkpoint pathway (Nyberg et al., 2002). The sensor proteins involved in this pathway includes Rad3-Rad26 complex, “9-1-1” complex (Rad9-Rad1-Hus1), and Cut5 (Nyberg et al., 2002). Crb2 is the mediator protein that interacts with both Rad3 and Chk1 to promote Chk1 phosphorylation (Mochida et al., 2004; Nyberg et al., 2002). Earlier studies also showed that “14-3-3” proteins play a critical role in regulating Chk1 localization and
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<td>Chk1</td>
<td>hCHK1</td>
</tr>
<tr>
<td></td>
<td>Cds1</td>
<td>hCHK2</td>
</tr>
</tbody>
</table>

Table 2. The proteins involved in the checkpoint response in *S. pombe* and human.
The function of Tel1 in the DNA damage checkpoint response is not very clear in fission yeast as deletion of Tel1 does not sensitize the cells to various DNA damaging agents. In higher eukaryotes, DSB is detected by ATM and MRN complex (Uziel et al., 2003). The MRN complex contains three proteins Mre11, Rad50, and Nbs1, and has DNA-binding, nuclease, unwinding and ends-joining activities (Connelly and Leach, 2002). ATM can be recruited to the strand break site by interacting with an FXF/Y motif at the C terminus of Nbs1 (You et al., 2005).

Other forms of DNA damage can be detected by ATR-ATRIP (Rad3-Rad26) and the “9-1-1” complex that are believed to be loaded independently to sites of DNA damage (Edwards et al., 1999; Hartsuiker et al., 2001; Zou et al., 2002). Loading of Cut5 requires the phosphorylation of Rad9 in the “9-1-1” complex by ATR/Rad3 (Furuya et al., 2004). The recruitment of Crb2 to the DSBs requires both phospho-H2A by Tel1 and methylated H4K20 (Nakamura et al., 2004; Sanders et al., 2004). Phosphorylation of Chk1 by Rad3 is regulated by Crb2 and Cut5 through dynamic interactions (Mochida et al., 2004).

Activated Chk1 slows the cell cycle progression through regulation of the activities of the protein kinase Wee1 and the phosphatase Cdc25 (Raleigh and O'Connell, 2000). Wee1 and Cdc25 counteract with each other to control the phosphorylation status of a tyrosine residue Y15 on Cdc2 (Russell and Nurse, 1987), the only cell-cycle dependent kinase in the fission yeast (Simanis and Nurse, 1986). Dephosphorylation of Y15 by Cdc25 activates Cdc2 and promotes G2-M transition (Simanis and Nurse, 1986). When Cdc2 is phosphorylated during the checkpoint response, the cell cycle is arrested.
(Raleigh and O'Connell, 2000; Rhind et al., 1997). Chk1 phosphorylates Wee1 to up-regulate its activity (O'Connell et al., 1997; Raleigh and O'Connell, 2000). Chk1 may also regulate the activity and localization of Cdc25 through phosphorylation (Furnari et al., 1999; Furnari et al., 1997). Phosphorylated Cdc25 provides a binding site for 14-3-3 proteins, and is exported from nuclear (Lopez-Girona et al., 1999; Raleigh and O'Connell, 2000). Cdc2 can also be regulated by another kinase Mik1 because Mik1 can partially replace the function of Wee1 to arrest cell cycle (Baber-Furnari et al., 2000; O'Connell et al., 1997).

**DNA replication checkpoint**

Cds1 is the major effector kinase in the DNA replication checkpoint pathway in fission yeast that is activated when replication forks are stalled by nucleotide deprivation or physical impediments during the S phase (Murakami and Okayama, 1995). The replication checkpoint pathway shares most of the proteins in the DNA damage checkpoint pathway as listed in Table 2.

Similar to Chk1, Cds1 is also activated by Rad3 dependent phosphorylation (Martinho et al., 1998). Activation of Cds1 depends on the sensor proteins Rad3-Rad26 (Xu et al., 2006), “9-1-1” complex (Lindsay et al., 1998) and Cut5 (Harris et al., 2003) and the mediator protein Mrc1 (Xu et al., 2006). In response to hydroxyurea (HU), an inhibitor of ribonucleotide reductase, the replication fork stalls, which recruit the checkpoint sensor proteins (Edwards et al., 1999) to activate the effector kinase Cds1. Cds1 is activated by a two stage mechanism (Xu et al., 2006). In the first, or priming, stage, a binding site for the FHA domain of Cds1 is generated on Mrc1 by Rad3 dependent phosphorylation. Association of Cds1 via its FHA domain with Mrc1
facilitates the phosphorylation of Cds1 by Rad3 at threonine 11 site (Tanaka and Russell, 2004; Xu et al., 2006; Zhao et al., 2003). In the second stage, Cds1 is activated by autophosphorylation of threonine 328 in the kinase domain. Autophosphorylation of T328 occurs at a low level under normal condition but greatly promoted by dimerization of two inactive Cds1 molecules via phospho-specific interactions between the FHA domains and the phosphorylated T11 of Cds1 (Xu et al., 2006; Xu and Kelly, 2009). Once activated, Cds1 can arrest the cell cycle (Murakami and Okayama, 1995), stabilize the stalled replication forks (Noguchi et al., 2004), stimulate dNTP production, and inhibit the firing of the late origins (Kim and Huberman, 2001). The activity of Cds1 is negatively regulated through its autoinhibitory C-terminal 27-amino acid tail, which prevents spontaneous activation of the replication checkpoint during normal cell cycle (Xu and Kelly, 2009). Tel1 is not required for the activation of Cds1 (Zhao et al., 2003). Although Rad50 mutant is sensitive to HU, the function of MRN complex in DNA replication checkpoint is not very clear (Hartsuiker et al., 2001).

**USING FISSION YEAST S. POMBE AS THE WORKING MODEL ORGANISM**

The yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* are two powerful model organisms to study DNA replication and cell cycle control (Nurse et al., 1998) because of the conservation of the two mechanisms (Simanis et al., 1987) and the ease of their genetic manipulations (Hayles and Nurse, 1992). The fission yeast *S. pombe* is particularly useful for the current study because this organism shares some common features of human cells. For example, unlike that in budding yeast, the replication origins in fission yeast consist of multiple AT-rich sequences without a defined specific sequence (Clyne and Kelly, 1995) (Paixao et al., 2004; Wang et al., 2004), which is similar to that
in human cells. The first chapter of the thesis is to identify potential homologue of Sld3 in human that may be required for loading of Cdc45 to chromatin. It has been shown before that the loading of Cdc45 requires TopBP1 (the fission yeast Cut5) (Dolan et al., 2004; Schmidt et al., 2008; Yabuuchi et al., 2006) in both fission yeast and human cells while the loading of Cdc45 in budding yeast is independent of TopBP1 (the budding yeast homolog of TopBP1 is Dpb11) (Kamimura et al., 2001; Takayama et al., 2003). Clearly, the mechanism of Cdc45 loading appears to be conserved in S. pombe and metazoans and the results from the studies of Cdc45 loading in fission yeast will be more relevant to that in human cells.

This thesis contains two chapters. In the first chapter, I tested the hypothesis that Sld3 homolog exists in human cells by investigating whether all three newly discovered human replication proteins DUE-B, Ticrr/Treslin, and GEMC1 are the potential Sld3 homologues. Identification of human Sld3 homologues will greatly help the understanding of the loading mechanism of Cdc45, a key step in the initiation of DNA replication in human cells. The second chapter presents the identification of a Psf2 mutant whose survival strictly depends on the activity of Chk1. This result validated the strategy for screening new genes involved in maintenance of the genomic integrity and the initiation of DNA replication.
CHAPTER I

Complementary study of three candidate human replication proteins
DUE-B, Ticrr/Treslin, and GEMC1 as the functional homologue of Sld3
in fission yeast
INTRODUCTION

Most of the proteins involved in the initiation of DNA replication in yeasts share homologous protein in higher eukaryotes. However, the homologue of yeast Sld3 has not been found in metazoan by searching the sequence similarity. Considering the significant role of Sld3 in loading Cdc45 and the highly conserved mechanism of replication initiation, it is believed that homologous protein(s) of Sld3 exists in higher organisms (Bell and Dutta, 2002; Labib, 2010).

Function of Sld3 in the yeasts. Sld3 was first identified in a screening as one of the genes that is synthetically lethal with temperature sensitive mutant of dpb11-1 of budding yeast (Kamimura et al., 1998). The fission yeast homologue of Sld3 was identified based on the 24% similarity to that of budding yeast (Nakajima and Masukata, 2002). Sld3 is a protein of about 79kDa, and essential for DNA replication initiation in both fission and budding yeast (Kamimura et al., 2001; Nakajima and Masukata, 2002). Since Sld3 is an essential gene for yeast, deletion of Sld3 causes inviability. During the DNA replication initiation, Sld3 share the following functional properties in both fission and budding yeasts (Kamimura et al., 2001; Nakajima and Masukata, 2002). First, it is essential for Cdc45 association with chromatin (Kamimura et al., 2001; Nakajima and Masukata, 2002). Second, it may be phosphorylated by CDKs or DDK (Kamimura et al., 2001; Nakajima and Masukata, 2002). Finally, it may interact with Cut5/TopBP1 (Kamimura et al., 2001; Nakajima and Masukata, 2002). A functional homologue of Sld3 should have the three properties that can serve as the criteria to identify the functional homolog of Sld3. Since there is no apparent sequence similarity between Sld3 and any
metazoan proteins, I have been testing three newly discovered human replication proteins DUE-B, Ticrr/Treslin, and GEMC1 to see whether any of them can complement the functions of Sld3 in the fission yeast S. pombe.

**DUE-B** (DNA unwinding element binding protein) was identified in a yeast one-hybrid assay, which interacts with the DNA unwinding element (DUE) of the c-myc origin (Casper et al., 2005). Human DUE-B is a 23.4KDa protein with 209 amino acids. Sequence analysis showed that the N-terminus of DUE-B has a strong homology to D-tyrosyl-tRNA(tyr) deacylase of bacteria and yeast, which is believed to hydrolyze the D-Tyr-tRNA\textsuperscript{Tyr} to avoid a harmful effect of D-tyrosine (Soutourin a et al., 2000). Interestingly, there are additional 59 amino acids in the C-terminus of human DUE-B that is absent in its homologue in yeast. The C-terminus of DUE-B shares some sequence similarity to the C-terminus sequence of Sld3 (Chowdhury et al., 2010). DUE-B is preferentially loaded to c-myc origins and Lamin B2 origins after the pre-RC formation (Chowdhury et al., 2010; Moaddel et al., 2005). Immuno-depletion of DUE-B prevents the association of TopBP1 and Cdc45 with chromatin (Chowdhury et al., 2010) and inhibits DNA replication in Xenopus egg extracts (Casper et al., 2005). These effects can be rescued by adding back recombinant DUE-B from HeLa cells (Casper et al., 2005; Chowdhury et al., 2010). DUE-B can be phosphorylated by casein kinase 2 (CK2), which is dependent on its C-terminus (Chowdhury et al., 2010). Moreover, it has been shown that DUE-B can co-immunoprecipitate with both TopBP1 and Cdc45 in HeLa cell extracts or in Sf9 insect cells when they are overexpressed (Chowdhury et al., 2010). These interactions have been shown to depend on the C-terminus of DUE-B (Chowdhury et al., 2010). These results suggest that DUE-B may be the homolog of Sld3 in human.
**Ticrr** (for TopBP1-interacting, checkpoint, and replication regulator) is recently characterized as a protein that interacts with TopBP1 and is required for DNA replication initiation (Kumagai et al., 2010; Sansam et al., 2010). Ticrr was found in a zebrafish mutant in which the G2/M checkpoint is abrogated (Sansam et al., 2010), whereas Treslin (TopBP1-interacting, replication-stimulating protein), the Xenopus homologue of Ticrr, is identified as a TopBP1-interacting protein in Xenopus extract (Kumagai et al., 2010). In addition to its ability to interact with TopBP1 (Kumagai et al., 2010; Sansam et al., 2010), Ticrr/Treslin appears to meet all other criteria as the functional homologue of Sld3. DNA replication is impaired in Treslin immuno-depleted Xenopus egg extracts (Kumagai et al., 2010) or Ticrr disrupted or silenced Hela cells (Kumagai et al., 2010; Sansam et al., 2010). Immuno-depletion of Treslin also abolishes the association of Cdc45 with chromatin, but not the loading of Mcm2 and TopBP1 (Kumagai et al., 2010). Treslin is phosphorylated during the cell cycle and the phosphorylation is critical for its interaction with TopBP1. Remarkably, some conserved sequences between the N-terminus of Ticrr/Treslin and the yeast Sld3 it has also been identified, although no significant similarity can be found in full length of Ticrr/Treslin (Sanchez-Pulido et al., 2010).

**GEMC1** (geminin coiled-coil containing protein 1) is the third human protein as the potential homolog of Sld3. As the name of the protein suggests, GEMC1 contains a region similar to the coiled-coil domain of Geminin (Balestrini et al., 2010). Although GEMC1 does not share the sequence homology with Sld3, it is required for the DNA replication and the loading of Cdc45, the essential functions of Sld3 (Balestrini et al., 2010). The DNA replication is blocked when GEMC1 is immuno-depleted from Xenopus
egg extracts, or silenced by siRNA in mouse (Balestrini et al., 2010). Importantly, immuno-depletion of GEMC1 accompanies with the loss of the association of Cdc45 to the chromatin (Balestrini et al., 2010). Moreover, GEMC1 interacts with Cdc45, TopBP1, and Cdk2-CyclinE complex (Balestrini et al., 2010) and the interaction between GEMC1 and TopBP1 seems dependent on the phosphorylation of GEMC1 by Cdk2-CyclinE complex (Balestrini et al., 2010).

In this study, a temperature sensitive Sld3 (sld3-10ts) mutant was used for testing the three human candidate proteins mentioned above as the functional homologues of fission yeast Sld3 (Figure 1). Growth of sld3-10ts mutant cells is maintained at permissive temperatures (25°C or 30°C) but stopped by raising the temperature to 37°C (Figure 1 A). Any of these three proteins that can promote the survival of the sld3-10ts mutant at 37°C would be a functional homologue of Sld3 in fission yeast (Figure 1 B) (Osborn and Miller, 2007). Our preliminary results showed that none of the proteins has a significant effect in promoting the cell growth of the sld3-10ts mutant at the restrictive temperature. Interestingly, overexpression of Ticrr and GEMC1 can adversely affect the cell growth of the mutant even at permissive temperatures. It is clear that identification of the functional homolog of Sld3 still need further investigations.
Figure 1. Strategy for identifying the functional homologue of Sld3 in fission yeast. A) The phenotype of the Sld3 temperature mutant (sld3-10⁵). B) The complementary test to identify the homologue of fission yeast Sld3 in the sld3-10⁵ mutant.
MATERIAL AND METHOD

Strains

*S. pombe* strains and culture media were made following standard methods (Moreno et al., 1991). The following three *S. pombe* strains were used for this study: HM512, *h- sld3-10* (Nakajima and Masukata, 2002); TK8, *h+ leu1-32 ura4-D18 ade6-M216* (lab storage), WZ2, *h+ sld3-10 leu1-32*.

Plasmids

All plasmid vectors used in this study contain a LEU2 marker and *nmt* promoter for induced expression with various levels (Basi et al., 1993). Thiamine was used at 20 µg/ml to turn off the promoter and repress the protein expression. The vectors pYJ54, pYJ55, and pYJ56 were used to add an N-terminal 3HA tag to Ticrr and GEMC1. The vectors pRCE27, pRCE28, and pRCE29 were used to attach 3HA tag to Sld3 and Treslin at their C-termini. The cDNA of TopBP1 was cloned into the pREP1, pREP41, and pREP81 vectors without a tag. Since the N-terminus of DUE-B contains several ATG codons, the cDNA of DUE-B was cloned into a modified vector in which a redundant ATG codon has been deleted to ensure accurate translation.

Cloning of the cDNAs by PCR

The cDNAs of all genes were amplified by PCR with appropriate template and primers (see the primer list). Specifically, the cDNAs of Ticrr and Treslin were cloned using the plasmid from Dr. Dunphy’s lab (Kumagai et al., 2010) as the template. The
cDNA of DUE-B was cloned from the plasmid from Dr. Leffak’s lab (Casper et al., 2005). The cDNAs of Sld3 and GEMC1 were amplified from the fission yeast cDNA library (lab storage) and from the Quick-Clone cDNAs prepared from human fetal kidney (BD), respectively. The primers with restriction enzyme sites used in this study are listed in the following:

Sld3(BglII)f 5’- TGGTAGATCTATGAATAACGACCATGCTTCCAAG -3’
Sld3(NotI)b 5’- AAGTGCGGCCGCAAGGACTGGCTGATTGTTTTTTTACAG -3’
DUE-B(Sall)f 5’- CCGGGCGTCGACATGAAGGCCGTGGTGCAGCG -3’
DUE-B(BamHI)b 5’- GCCTGGATCCCTACGGCTCCCGTTCAGAGGACAC-3’
TopBP1(Ndel)f 5’- GTTCACATATGTCCAGAAATGACAAAGAAC -3’
TopBP1(BamHI)b 5’- GGGTGGATCCGATTAGTGTACTCTAGGTGC -3’
Ticrr(NotI)f 5’- GCCAGCGGCCGCGATGGCATGCTGTCACAAAGTAATG -3’
Ticrr(Sall)b 5’- TAATGTCGACGGCTATAAAGTCCTCCAGCCAG -3’
Treslin(Sall)f 5’- CGCAGTCGACATGGCTCCCTTCGCACAATG -3’
Treslin(NotI)b 5’- ATATGCAGCCGCGATGTAATGATTGTGTTGTCG -3’
GEMC1(NotI)f 5’- TTCCCGCGCCGCGATGACACCATTTCCTGGCTTG -3’
GEMC1(Sall)b 5’- AGAGGTCGACCTAAAGACTGCTTAGGGACCC -3’

The reactions contained in each sample (50 µl):

10x pfu ultra buffer: 5µl
dNTP: 200µM
Template DNA: 25ng
5’ primer: 0.5µM
3’ primer: 0.5µM
pfu ultra polymerase: 2.5U (Stratagene)

dH₂O to 50µl

The PCR Cycle was performed as below:

a. 95°C 1 min
b. 95°C 1min
c. 55°C 1 min (temperature varies)
d. 68°C 2 min/kb

Repeat b to d for 12 to 18 times
e. 68°C 10 min

**Manipulations of PCR products**

PCR products were usually purified by the 1% agarose gel electrophoresis (BioRad) followed by gel purification kit (Qiagen). The PCR products were digested with the restriction enzymes (New England Biolabs) and ligated into the vectors in a standard ligation using T4 DNA ligase (New England Biolabs) as recommended by the manufacturer:

10X T4 DNA ligase Buffer: 1µl

Vector DNA: 50ng

Insert DNA: 3X molar excess of the vector DNA

T4 DNA Ligase: 1µl (New England Biolabs):

dH₂O to 10µl

16°C: 6 hrs

Ligation products were transformed into competent cells of *E. coli* DH5α by using heat shock method. The transformed bacteria were incubated on plates containing LB
medium with 50µg/ml carbenicillin (United States Biological) at 37°C for ~12 hours until colonies appear. Single colonies were transferred into liquid cultures for preparing the plasmids with the miniprep kit (Qiagen). Prepared plasmids were normally digested with restriction enzymes to confirm the presence of cloned DNAs. The sequences of the cloned DNAs were confirmed by sequencing (Retrogen, Inc).

**Electroporation transformations**

Exponentially growing fission yeast cells were harvested from liquid culture with 3000 rpm at 4°C for 5 minutes, washed once with 25 ml ice-cold water and once with 25ml 1M D-sorbitol, and resuspended in ice cold 1 M D-sorbitol at 50 OD/ml. For electroporation, 25µl of the cell suspension were mixed with 100 ng DNA in a 30µl volume. After incubation on ice for 5 minutes, the mixture of cells and DNA was transferred to a pre-cooled 0.2 cm sterile electroporation cuvette and electroporated immediately. The electroporator (Life Technologies) is set as 400 voltages for the transformation. After the electroporation, the cells were spread on selective plates and incubated at 30°C.

**Western blotting**

The samples used for SDS-PAGE and western blot were whole cell lysates from the sld3-10ts mutant with indicated expression vectors. The cells were grown in the medium containing 20 µg/ml of thiamine. To induce the expression of the proteins, cells were washed extensively with sterilized water and then transferred into fresh medium without thiamine and incubated at for about 3 to 4 generations. The cells were fixed by 15% TCA (Sigma) at 4°C for 2h or overnight to make whole cell lysates.
The whole cell lysate were made by breaking up the cells with mini bead-beater (BioSpec) and analysed by SDS-PAGE. The concentrations of acrylamide SDS-PAGE gel used for different proteins were: 8% for Ticrr and Treslin, 10% for Sld3 and GEMC1, and 12% for DUE-B. Proteins were then transferred to nitrocellulose membranes (Whatman) 90 minutes at a constant current of 280mA, then probed with appropriate antibodies, which were used at dilutions of 1:5000 in 5% nonfat dry milk in Tris buffered saline with Tween-20T (TBST). The polyclonal antibodies of DUE-B, TopBP1 were from Dr. Leffak’s lab (Casper et al., 2005; Chowdhury et al., 2010). HA antibody was used to detect Sld3, Ticrr, Treslin, and GEMC1. The horseradish peroxidase-conjugated secondary antibodies were used at dilutions of 1:5000 in 5% nonfat dry milk.

**Spot Assay**

Exponentially growing cells were harvested and resuspend in sterilized water at 1 OD/ml. A serious 10 fold dilutions were spotted in 3 µl onto plates containing the indicated media and incubated at appropriate temperatures.
RESULT

Wild type Sld3 can rescue the temperature sensitive phenotype of the sld3-10\textsuperscript{ts} mutant

The temperature sensitive mutant strain HM512 was kindly provided by Dr. Masukata (Yamada et al., 2004). To confirm the temperature sensitivity of the strain was indeed caused by the mutation in sld3, we tried at the beginning to rescue the phenotype with wild type sld3. The sld3\textsuperscript{+} gene was cloned into vectors with various nmt promoters of increasing strength (Figure 2) (Kumar and Singh, 2006). The protein of the expected size was expressed and the expression was under the control of thiamine as confirmed by Western blotting. Since nmt1\textsuperscript{+} is the strongest promoter, suppression of the expression by thiamine was not as complete as the two weaker promoters (Compare lane 5 with lane 1 and 3 in Fig. 2). In the spot assay, the WZ2 strain with vector alone was temperature sensitive as expected (Figure 3) indicating that the mutant strain cannot grow at the non-permissive temperature of 37\textdegree C. The temperature sensitivity can be rescued by the expression of wild type Sld3 at 37\textdegree C confirming the WZ2 strain contains the temperature sensitive mutation in sld3 (Figure 3). Sld3 expressed under the control of weaker promoters nmt81 and nmt41 promoters showed a better rescuing effect than that of the strong promoter nmt1 when there was no suppression from thiamine (Figure 3). However, when suppressed with thiamine, the nmt1-sld3 exhibited a significant rescuing effect consistent with the low level expression of Sld3 in this situation (Figure 2 and 3). This result suggests that the mutant can be rescued only when Sld3 is expressed at an
Figure 2. Expression of Sld3 under the control of nmt promoters (nmt81, nmt41, and nmt1) of increasing strength in sld3-108ts mutant.

Thiamine was added to the cultures at 20 µg/ml to suppress the expression of Sld3. The 3HA-tag was added on the C-terminus of Sld3 for detection by immuno-blotting with anti-HA antibody. The predicted size of Sld3-3HA is about 80kDa. A section of the membrane stained with ponceau S was shown in the lower panel as a loading control.
Figure 3. Wild type Sld3 can rescue the ts phenotype of the \textit{sld3-10ts} mutant.

The mutant strain with Sld3 expression plasmids were grown in log phase in EMM6S[Leu-] liquid medium in the presence or absence of thiamine. The cells were spotted onto EMM6S[Leu-] plates with or without thiamine and incubated at 25\degree C for 4 days or at 30\degree C and 37\degree C for 3 days and photographed.
appropriate level. Over expression of Sld3 can instead inhibit the cell growth.

**DUE-B cannot complement Sld3 in fission yeast**

To test whether human DUE-B is the functional homologue of Sld3 in fission yeast, we expressed DUE-B in the *sld3 ts* mutant strain. Since the expression level of the protein may have an effect, the DUE-B gene was cloned in to the vectors with *nmt* promoters. The expression of DUE-B was confirmed by immuno-blotting similar to that described for Sld3 (Figure 4). *Sld3-10^ts* mutant cells with DUE-B expression plasmids were spotted on the plates to test whether DUE-B can complement Sld3. As shown in Figure 5, expression of DUE-B at various level failed to rescue the ts phenotype of the mutant although the expression did not affect the cell growth at permissive temperature. As the positive control, the mutant cells carrying wild type Sld3 grew well at non-permissive temperature.

**Human Ticrr and Xenopus Treslin failed to complement Sld3.**

Similarly, the open reading frame of human Ticrr and Xenopus Treslin were constructed into the expression vectors with *nmt* promoters. An HA tag was fused to Ticrr and Treslin at the N- and C-terminus, respectively, for detection of the expressed proteins. The predicted molecular weights of 3HA-Ticrr and Treslin-3HA are 214.6kDa and 225.2kDa, respectively. The expression of Ticrr and Treslin of the expected sizes were detected with antibody against the HA epitope (Figure 6 A and B). However, Treslin-3HA was detectable only when expressed under the strong promoter *nmt1*. As expected, expression of the two proteins was suppressed by thiamine (Figure 6 A and B). However, expression of either Ticrr or Treslin the *sld3-10^ts* mutant did not promote
Figure 4. The expression of DUE-B under increasing strength of nmt promoters in sld3-10s mutant. Polyclonal antibody against human DUE-B was used to detect the protein. The predicted size of DUE-B is 23.4 kDa, consistent with the expression of DUE-B in fission yeast.
Figure 5. The complementary test of DUE-B in sld3-10⁶ mutant.
The sld3-10⁶ cells with DUE-B expression plasmids were tested similarly as described in Figure 3 at the indicated temperature for 3 days.
Figure 6. The expression of human Ticrr (A) and Xenopus Treslin (B) in the sld3-10ts mutant.

The nmt81, nmt41, and nmt1 promoters are marked on the top of the blots. The expressed protein of expected sizes in whole cell lysates were detected by antibody against the tagged HA epitope. Mrc1-3HA and Sld3-HA were included as positive controls. * indicates the cross reaction bands.
the cell growth at the non-permissive temperature (Figure 7 A and B). Interestingly, over expression of Ticrr can suppress the cell even at permissive temperature (Figure 6 and 7). But this inhibition effect was released by suppressing the protein expression with thiamine (Figure 6 and 7). Similarly, the growth of the cells was also restricted by the expression of Treslin, but was not as serious as that induced by Ticrr (Figure 8 and 9). Thus, Ticrr/Treslin inhibited the growth of \( sld3-10^{ts} \) at permissive temperature, and cannot rescue the \( sld3-10^{ts} \) mutant at restrictive temperature.

**Human GEMC1 cannot complement the function of Sld3 in fission yeast**

Similarly, the human GEMC1 was cloned into the vectors with various \( nmt \) promoters. The thiamine-regulated expression of GEMC1 was shown in Figure 8. However, the expression of GEMC1 did not show a complementary effect in the \( sld3-10^{ts} \) mutant (Figure 9). Like Ticrr, over expression of GEMC1 slightly inhibits the cell growth at permissive temperature (Figure 8 and 9).

**Suppression of cell growth by over expressed Ticrr, GEMC1 and TopBP1.**

Unlike DUE-B and Treslin, over expression of Ticrr and GEMC1 can suppress the cell growth at permissive temperature suggesting the expressed proteins are not inactive proteins inside the fission yeast. We found most of the cells with the overexpressed Ticrr overexpression become inviable, suggesting the Ticrr is toxic to the fission yeast when overexpressed. In contrast, the suppression of cell growth caused by GEMC1 overexpression is reversible and cells can recover after incubated for 5 days at 30°C (data not shown). This result is enforced by the morphological study. Unlike the
Figure 7. The complementary test of Ticrr (A) and Treslin (B) in sld3-105 mutant. The sld3-105 strain with Ticrr or Treslin expression plasmids were grown up in EMM6S[Leu-] medium in the presence of absence of thiamine and spotted onto EMM6S[Leu-] plates with or without thiamine respectively. The plates were incubated at 30°C or 37°C for 3 days.
Figure 8. The expression of human GEMC1 in the sld3-10x mutant.
The predicated size of the 3HA-GEMC1 is about 42kDa, consistent with the induced expression of GEMC1. Sld3-3HA is used as a positive control.
Figure 9. The complementary test of GEMC1 in $sld3^{-10^A}$ mutant.
cells with over expressed DUE-B and Treslin that show a “normal” cell shape, over expression of Ticrr and GEMC1 dramatically elongated the cells (Figure 10). Interestingly, overexpression of another human replication protein TopBP1, as confirmed by Western (data not shown), also suppressed the cell growth although at a much lower level.
Figure 10. The morphology of the $sld3-10^{ts}$ cells with overexpressed proteins at the permissive temperature 30°C. Cells growing at 30°C for 3 days on the non-thiamine plate were examined under a microscope and photographed. TopBP1 is a human replication protein that can form complex with DUE-B and Cdc45.
DISCUSSION

Sld3 plays an important role in the loading of Cdc45, a key step during the initiation of eukaryotic DNA replication. So far, Sld3 is the only replication protein whose homologue has not been identified in higher eukaryotes. Since the DNA replication mechanism is highly conserved in all eukaryotes, it is believed that a functional homologue of Sld3 must exist in mammals. Recently, three human replication proteins, DUE-B, Ticrr/Treslin, and GEMC1 have been discovered that exhibit some similar properties of Sld3. To test whether any of these proteins is a homologue of Sld3, we devised a simple testing method in which exogenous proteins were expressed in sld3-10°ts mutant to determine their ability to complement the functions of Sld3. However, all three human proteins failed to rescue the ts phenotype of the sld3-10°ts strain suggesting that they are either non-functional when expressed in fission yeast or are not the functional homologues of Sld3 (Figure 11). Interestingly, overexpression of Ticrr and GEMC1 inhibited the cell growth even at permissive temperature suggesting that they are not “dead” proteins when expressed in the fission yeast, but have some unknown function inside S. pombe. We believe that the functional homologue of Sld3, if it exists, remains to be identified.

Although the three human proteins shared some similarities with yeast Sld3, some differences do exist that challenge the notion that one of them is the Sld3 homologue. For example, phosphorylation of budding yeast Sld3 by CDK is essential for its interaction with Dpb11 (Tanaka et al., 2007a; Tanaka et al., 2007b). Although DUE-B
Figure 11. Summary of the complementary test of DUE-B, Ticrr/Treslin, and GEMC1 in sld3-10s mutant.
can be phosphorylated by casein kinase 2 (CK2), its interaction with TopBP1 seems independent of the phosphorylation (Chowdhury et al., 2010). It has been shown in both budding and fission yeasts that the loading of DPB11/Cut5 requires the pre-loading of Sld3. Without Sld3, the Dpb11/Cut5 cannot associate with the chromatin (Kamimura et al., 2001; Nakajima and Masukata, 2002). However, the loading of Treslin is not required for the association of TopBP1 with the chromatin in Xenopus extract (Kumagai et al., 2010) although the loading of Cdc45 is affected (Kumagai et al., 2010). As for GEMC1, its loading to chromatin is different from that of Sld3. Since GEMC1 can be loaded to chromatin even by inhibiting the MCM complex loading with geminin (Balestrini et al., 2010), the loading of the protein can start before the formation of pre-RC complex. This is different from Sld3 in which the association of Sld3 with chromatin occurs after the pre-RC formation in both yeasts (Kamimura et al., 2001; Nakajima and Masukata, 2002). While the loading of GEMC1 requires the presence of TopBP1 (Balestrini et al., 2010), the loading of Sld3 is before the loading of Cut5/Dpb11 (Kamimura et al., 2001; Yabuuchii et al., 2006).

However, we cannot completely exclude the possibility that one of these proteins is indeed the Sld3 homologue. First, the mutant Sld3 may partially functional that inhibit the complementary function of the homologous protein in yeast by blocking their access to the targets of action. One possible method to test this possibility is to do the plasmid shuffle test in which potential homologous protein can be expressed in Sld3 deletion background. Second, the fission yeast may not be an appropriate model organism for testing the Sld3 homologue. In budding yeast, Sld3 binds to the early-firing origins in G1 phase after the formation of pre-RC complex and to late-firing origins in late S phase.
(Kamimura et al., 2001). In fission yeast, however, the association of Sld3 with the origins requires the kinase activity of DDK, but not CDKs during the G1/S phase (Nakajima and Masukata, 2002; Yabuuchi et al., 2006). Moreover, Sld3 has different roles on Cdc45 loading in the two yeasts. In budding yeast, the origin associations of Sld3 and Cdc45 are mutually dependent (Kamimura et al., 2001). In fission yeast, the recruitment of Sld3 to the pre-RC is independent of Cdc45 loading (Yamada et al., 2004), and pre-loaded Sld3 is required for Cdc45 loading as well as the assembly of GINS and Cut5 to the origins (Nakajima and Masukata, 2002; Yabuuchi et al., 2006). Last but not least, the expression level of these exogenous human proteins may be critical for the success of complementary study. The growth of the cells were suppressed even when wild type Sld3 was overexpressed in the \textit{sld3-10ts} mutant, suggesting the yeast cells may not require much Sld3 protein for survival (Figure 2 and 3). Thus, the expression level of these exogenous human proteins may not meet the requirement to complement the function of Sld3 at restricted temperature. And overexpression of Ticrr and GEMC1 even suppress the growth of the \textit{sld3-10ts} mutant at permissive temperature. One solution would be to express these exogenous human proteins under the promoter of Sld3 in the mutant.

In summary, we have tested three candidate human replication proteins DUE-B, Ticrr/Treslin, and GEMC1 as the potential Sld3 homologue in the fission yeast \textit{S. pombe}. Our results strongly suggest that none of the three proteins are the functional homologues of Sld3 when tested in \textit{sld3-10ts} mutant. We believe the functional homologue of Sld3 remains to be identified in higher eukaryotes. This conclusion, however, also raises an interesting question: if these three newly identified human replication proteins are not the
Sld3 homologues, what are their counterparts in the yeasts? It remains possible that new genes involved in the initiation of DNA replications will be discovered in yeasts.
CHAPTER II

Genetic screening for new genes that are involved in the initiation of DNA replication
INTRODUCTION

Understanding how eukaryotic genome integrity is maintained over generations - during which time the genome has to be accurately duplicated in each cell cycle - is one of the fundamental problems of modern biology. It is also a critical aspect of the more general problem of understanding the mechanisms that control cellular proliferation and prevent oncogenesis. The stability of the genome depends upon the precise operation of the DNA replication and repair machineries. It is also depends on the DNA structure checkpoint mechanism that arrest cell cycle progression when DNA damage is repaired (Feng and D'Urso, 2001; Yin et al., 2008).

Results from the studies in Chapter I of this thesis suggest that we still do not fully understand the replication initiation mechanism and unknown protein(s) involved in this process may remain unidentified. In fission yeast, several mutations in the initiation proteins are synthetic lethal with the deletion of Chk1, the effector kinase of the DNA damage pathway. For example, mutations in DNA replication initiation proteins Orc1 (Synnes et al., 2002; Yin et al., 2008) and Mcm7 (Liang and Forsburg, 2001), and DNA replication elongation protein Cdc6 (DNA polymerase delta catalytic subunit) (Yin et al., 2008). It appears that defects in the initiation of DNA replication may generate noncanonical DNA structures that are detected by the DNA damage checkpoint for the stimulation of DNA repair activities to fix the abnormal DNA structures. Interestingly, most mutations in the on-going replisomes require Cds1, the effector kinase of the
replication checkpoint, for survival.

To discover new genes that are potentially involved in the initiation of DNA replication, we devised a screening strategy looking for genes whose mutations are synthetic lethal to depletion of Chk1 in the fission yeast (Yin et al., 2008). We reasoned that by turning off the Chk1 function, defects of a mutated gene involved in replication initiation, should cause cell lethality that are otherwise “normal” when checkpoint is functional. We have modified the promoter of Chk1 so that its expression can be shut off by adding thiamine to the medium (Figure 1). The screened mutants were therefore named Kds (Chk1 dependent survival).

In a small-scale screening, we obtained 15 so-called Kds (Chk1 dependent survival) mutants (Figure 2). To identify the mutated genes in the screened Kds mutants, the mutants will be backcrossed several times with a wild type strain and then transformed with a genomic DNA expression library made from Δchk1 strain. For details, please see Figure 2.

Preliminary characterization of the screened Kds mutants showed that the mutated gene in Kds15 mutant is psf2, a subunit of the GINS complex that is required for the initiation of DNA replication in eukaryotes. This result validates the screening strategy and suggests that the screening can be productive. Future work will focus on characterization of the rest of the screened Kds mutants and a large-scale screening for more Kds mutants. We hope to discover new genes or new functions of known genes that function in the maintenance of genome integrity.
Figure 1. Strategy of the Kds screening. Endogenous or exogenous genotoxins can generate mutations in the genes that are involved in the maintenance of genome integrity. These mutations cause DNA damage and genetic instability, which generates checkpoint signal to activate Chk1, the major effector kinase in the signaling pathway. Activated Chk1 slows down the cell cycle and stimulates DNA repair so that damage caused by the mutations is repaired and genome integrity is thus maintained under normal conditions. However, cell may not survive well if the checkpoint function is turned off by suppression of Chk1 expression.
Figure 2. **Strategy to identify the mutated genes in Kds mutants.**
Mutants that cannot grow without chk1 expression on thiamine plate will be selected after treating the wild type strain with mutagen (N-methyl-N'-nitro-N-nitrosoguanidine/MNNG). Mutants will be back-crossed several times with a wild type strain to remove unrelated mutations in the genome and then transformed with a genomic DNA expression library that lacks Chk1 gene. Colonies that can grow like wild type cells, when expression of Chk1 is suppressed, will be selected. Plasmids from the selected colonies will be recovered and sequenced to identify the candidate genes on the plasmids. Each candidate gene on the recovered plasmids will be cloned and introduced back into the Kds mutants to verify the rescuing effect. If a gene can rescue the Kds phenotype, the same gene will be amplified by PCR from the genomic DNA prepared from the Kds mutants and sequenced to determine the nature of the mutations.
MATERIALS AND METHODS

Yeast Strains and Media

The wild type strains used in this study are listed in Table 1. Strains YJ843 was used as the parental strain to generate the Kds mutants. The auxotrophic markers ade6-210 and ade6-216, which generate dark red and pick colonies on low adenine plates, respectively, were utilized for the confirmation of crosses. Wild type strains and its derivative mutants were maintained on thiamine free Edinburgh minimal media (EMM).

Screening for Kds mutants

To generate random mutations in the fission yeast genome, YJ843 strain was treated with 0.3μg/μl N-methyl-N'-nitro-N- nitrosoguanidine (MNNG) (Loechler et al., 1984; Preston et al., 1986) for 90 minutes at room temperature to achieve 90% killing of the cells. After washing with phosphate buffered saline (PBS) buffer for two times, the cells were resuspended in EMM6S medium to recover for 3 hours at room temperature. The recovered cells were spread on EMM6S plates and grew at 30˚C for 3 to 5 days. Colonies were replicated onto plates containing thiamine and the lethality dye Phloxin B. Thiamine sensitive mutants were then isolated.

Electroporation transformations

The genomic DNA expression library or recovered plasmids were transformed into Kds15 mutant by eletroporation as described in Chapter I. The transformants were plated on EMM plates lacking uracil at 30˚C for 3 days. Colonies were replicated onto
<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
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<tbody>
<tr>
<td>YJ843</td>
<td>h+, nmt81+Chk1-3HA, leu1-32, ura4-D18, ade6-M210</td>
<td>Our stock</td>
</tr>
<tr>
<td>YJ846</td>
<td>h-, nmt81+Chk1-3HA, leu1-32, ura4-D18, ade6-M210</td>
<td>Our stock</td>
</tr>
<tr>
<td>YJ856</td>
<td>h+, nmt81+Chk1-3HA, leu1-32, ura4-D18, ade6-M216</td>
<td>Our stock</td>
</tr>
<tr>
<td>YJ857</td>
<td>h-, nmt81+Chk1-3HA, leu1-32, ura4-D18, ade6-M216</td>
<td>Our stock</td>
</tr>
</tbody>
</table>

Table 1. The wild type strains used in this study.
thiamine plates to select for rescuing colonies. The selected colonies were streaked out twice on EMM lacking uracil before the plasmids are recovered.

**Recovery of plasmids**
10 OD cells from a saturated culture were collected and treated with 1mg/ml Zymolyase (United States Biological) and 2mg/ml Lysing enzyme (Sigma) at 37˚C for 1 hour to digest the cell wall. Spheroplasts were resuspended in 300µl TE buffer and disrupted by adding 10% SDS and incubating at 65˚C for 5 min. After 100µl 5 M potassium acetate was added, the sample was incubated on ice for 30 min and centrifuged in a benchtop centrifuge for 30 min at 4˚C and maximum speed. The plasmid DNAs in the supernatant was purified with a PCR purification kit (Qiagen) and transformed into DH5α competent cells by the heat shock method. Plasmid DNA from a single colony was prepared with the miniprep kit (Qiagen).

**Purification of yeast genomic DNA**
50 OD cells from a saturated culture were collected and resuspended in citrate/phosphate/sorbitol (CSE) buffer with 1mg/ml Zymolyase and 2mg/ml Lysing enzyme. The cell suspension was incubated at 37˚C for 1 hour to digest the cell wall. The spheroplasts were resuspended in 495µl TE buffer. The cell membrane was broke by adding 55µl 10% SDS and incubating at 65˚C for 10 min. The supernatant containing the genomic DNA were mixed with equal amount of ice-cold isoprpanol, and incubated on ice for 10min to precipitate the genomic DNA. The pellet were washed with 1 ml 70% ethanol, and resuspended with 350µl of TE containing 50µg/ml RNase A and incubated at 65˚C for 10 min. The genomic DNA was extracted with 300µl phenol
(Roche)/chloroform (Sigma) (1:1) twice and the precipitated by adding 30μl of 3M NaOAC and 750μl ethanol and incubating on ice for 10min. The purified genomic DNA pellet was resuspended in 50μl of 1/10 TE buffer.

**PCR and Sequence analysis**

PCR was performed as described in Chapter I. The sequencing results were analysis with the Blast on NCBI database and DNAMAN software. And the Chimera software was used to analysis the structure of human Psf2 protein.
RESULT

Small-scale screening for the Kds mutants

A modified wild type strain carrying *nmt-chk1* in the chromosomal locus was used as the parental strain in which the expression of Chk1 can be completely turned off by adding thiamine to the culture medium. (Figure 3A) This strain was viable on minimal medium plates with or without thiamine and is resistant to the staining by phloxin B, a non-toxic dye that stains dead or sick cells (Figure 3B). MNNG, which causes G-C to A-T (Loechler et al., 1984), or T-A to C-G mutations (Preston et al., 1986), is used to generate random mutations in the genome. In a small-scale screening, we isolated 15 Kds mutants that can be stained by Phloxin B only in the presence of thiamine, indicating that viability of these mutants depends on the expression of Chk1. After back-crossed 5 times with the wild type strain, the Kds phenotype remains in most of the mutants, but three mutants Kds10, Kds11, and Kds14 lost some of their sensitivity to thiamine after the crosses, which may be due to the mutant phenotypes were contributed by multiple mutated genes (Fig3. B).

We also tested the temperature sensitivity of all the mutants at 37°C. Seven mutants, Kds2, Kds3, Kds4, Kds6, Kds9, Kds13, and Kds15, were found sensitive at higher temperature even when the Chk1 is expressed, suggesting the mutated genes are essential in these mutants. Interestingly, Kds10 and Kds14 mutants exhibited temperature sensitivity at 37°C only when the expression of Chk1 was turned off.
Figure 3. The sensitivity of wild type strain and the Kds mutants.
A. Expression of Chk1 in strain YJ843 is completely suppressed by adding 20 µg/ml thiamine to the medium. B. Thiamine and temperature sensitivity of the 15 screened Kds mutants. Non-toxic dye phloxin B is used in this experiment as an indicator of the cell death. Kds15 with moderate thiamine sensitivity is randomly picked for further characterization.
The morphology of the mutants grown at 37°C (Figure 4) was examined under a microscope. All the temperature sensitive Kds mutants showed an elongated cell shape, a typical terminal phenotype in fission yeast. Three mutants, Kds7, Kds10 and Kds12 are also “abnormal” in morphology although they are not temperature sensitive at 37°C.

**Characterization of the Kds15 mutant**

Kds15 with a moderate thiamine and temperature sensitivity is chosen for further characterization. To identify the mutated gene in Kds15 mutant, we transformed the mutant with a genomic DNA library and screened for genes that can rescue the Kds phenotype of the mutant. 12 colonies were isolate in the primary screening that showed various levels of rescuing effect (Figure 5). Of these, 7 colonies exhibited notable improvement of cell growth in the presence of thiamine, but the number 1 colony showed a significant rescuing effect (Figure 5).

We recovered two plasmids from each of the 7 colonies and digested the recovered plasmids with MluI and SphI to confirm the presence of genomic DNA and to remove the redundant plasmids (Figure 6A). It appears that no inserted DNA in the plasmid from colony 5. Plasmids from colony 8 and 10 appear to be redundant (Figure 6A). Two plasmids recovered from colony 12 may contain a long piece of genomic DNA with low copy numbers (Figure 6A). Five non-redundant plasmids were transformed back to confirm their ability to rescue Kds15 mutant phenotype. Interestingly, plasmid from colony 1 showed a significant rescuing effect while the rest four plasmids seem lost the effect (Figure 6B). The plasmid recovered from colony 1 was then sent for sequencing and the sequencing result showed that five genes exist in the plasmid. They are psf2,
Figure 4. The morphology of the Kds mutants growing at 37°C. The mutants were incubated on plate in the absence of thiamine for 2 days and examined under a microscope.
Figure 5. Isolated colonies that can rescue the Kds phenotype of the Kds15 mutant. Kds15 mutant cells were transformed with a genomic DNA expression library and 18 colonies that showed rescuing effect were selected for detailed analysis by spot assay. Colony 1 grows almost like the wild type cell.
Figure 6. Test of the recovered plasmids.
A. Diagnostic digestion of the recovered plasmids. Two recovered plasmids from each colony were digested with MluI and SphI and then analyzed by agarose gel electrophoresis. The backbone of the vector is 5,913bp. B. Confirmation of the rescuing effect of the recovered plasmids in Kds15.
Among these genes, *nbl1* and *psf2* seem related to genome instability, which would cause the activation of Chk1 (Yin et al., 2008), while the functions of the other genes appear to be remotely related with the maintenance of genome stability. We cloned the *psf2* gene and tested its ability to rescue the mutant phenotype of Kds15. Indeed, the phenotype of Kds15 can be rescued by the cloned *psf2* gene (Figure 7). We cloned the *psf2* gene from Kds15 mutant. Sequencing identified a single G to A mutation in the open reading frame of the *psf2* gene. The mutation changed the glutamic acid 103 to lysine (Figure 8A).

The amino acid E103 is highly conserved in Psf2 in all eukaryotes. The human Psf2 has been co-crystallized with other subunits in the GINS complex (Figure 8B) (Chang et al., 2007). In human Psf2, E94 is the conserved amino acid of fission yeast E103. The E94 is located in the joint area of multiple structures, and with high density of polar amino acids, suggesting this residue is important for the structure (Figure 8C). The E103K mutation is believed to cause a structure change in fission yeast Psf2 and the Kds phenotype in Kds15 mutant.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>php2</em></td>
<td>Gene-specific transcription from RNA polymerase II promoter</td>
</tr>
<tr>
<td><em>nbl1</em></td>
<td>Chromosome Segregation &amp; Meiosis</td>
</tr>
<tr>
<td><em>psf2</em></td>
<td>DNA Replication Initiation Factor</td>
</tr>
<tr>
<td><em>arg6</em></td>
<td>Arginine biosynthetic process</td>
</tr>
<tr>
<td><em>ura5</em></td>
<td>De novo' pyrimidine base biosynthetic process</td>
</tr>
</tbody>
</table>

**Table 2.** The five genes carried on plasmid 1.
Figure 7. Psf2 can rescue the phenotype of Kds15 mutant.
Wild type psf2 gene is cloned, transformed into Kds15, and tested for its rescuing effect.
Figure 8. The mutation of Psf2 in Kds15 mutant. 
A. Sequencing result of the psf2 gene in Kds15 mutant. The G to A mutation causes a change from glutamic acid to lysine at position 103. B. Human Psf2 structure in GINS complex. Psf2 is highly conserved in eukaryotes and the human Psf2 has been co-crystallized with other subunits in the GINS complex. C. The amino acid E94 in human Psf2, which is the conserved amino acid of E103 of fission yeast Psf2, is highlighted in yellow. It is located in the joint area of multiple structures, and with high density of polar amino acids. It is believed that E103 is important for the structure of fission yeast Psf2. Mutation of this residue to a positively charged residue may make the Psf2 protein unstable.
DISCUSSION

In response to the instability of genomic DNA, the checkpoint pathways are activated to block the cell cycle progression in all eukaryotes (Nyberg et al., 2002). In this chapter, we take the advantage of the checkpoint mechanism to identify genes that cause genomic instability when they are mutated. We isolated 15 mutants whose survival depends on the expression of Chk1. The mutated gene in one of the screened mutants, Kds15, was identified to be psf2, a subunit of GINS complex required for the replication initiation. This result suggests that defect in replication initiation can cause genomic instability that requires Chk1 to block the cell cycle or stimulate the DNA repair activities (Figure 9).

Malfunction in DNA replication initiation machineries has been found before to cause genomic instability and cell death. A fraction of the psf3-1 mutant cells, another subunit of GINS, exhibiting <1C DNA cells during the cell cycle (Yabuuchi et al., 2006). A similar phenotype has also been seen in other DNA replication initiation mutants, such as sld3-10 (Nakajima and Masukata, 2002), cut5-T401(Saka et al., 1994) and hskl-89 (Takeda et al., 2001). In the psf3-1 mutant, the formation of GINS complex was impaired, which affected its function in loading Cdc45 and DNA polymerase during DNA replication initiation (Yabuuchi et al., 2006). It is possible that the Psf2 (E103K) mutation we identified in Kds15 may also affect the function of GINS complex in replication initiation. Psf2 may also function in cell division and cytokinesis since a high copy of psf2 plasmid can suppress the defect of the bir1/cut17, the homolog of human...
Figure 9. The proposed mechanism for the Kds phenotype in mutants with defect in DNA replication initiation.
Survivin protein required for chromosome segregation and spindle attachment. Defect in this function may also contribute to the mutant phenotype of Kds15 (Huang et al., 2005).

Mutations in other initiation proteins such as Orc1 (Synnes et al., 2002; Yin et al., 2008), Orc2 (Yin et al., 2008), Orc5 (Kato et al., 2008), Mcm4 (Yin et al., 2008), Mcm7 (Liang and Forsburg, 2001), and Cdc18 (Francesconi et al., 1995; Yin et al., 2008) also have the Kds phenotype (Synnes et al., 2002; Yin et al., 2008). Without a functional Chk1, cell may go to mitosis without finishing DNA replication, which would result in severe genomic instability and cell deaths (Synnes et al., 2002; Yabuuchi et al., 2006).

In summary, we obtained 15 Kds (Chk1 dependent survival) mutants to identify new genes that may involve in the initiation of DNA replication. Primary characterization of the mutants identified psf2 in the mutant Kds15. This result proves the screening strategy and suggests the possibility to uncover new genes that are involved in replication initiation. In the future, we will characterize the rest of the Kds mutants we have already screened and then screen for more Kds mutants.
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