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Genetic analysis of the fission yeast Spd2 protein

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ABSTRACT

Ribonucleotide reductase (RNR) catalyzes the rate-limiting step of dNTP production. The availability of finely tuned dNTP pools is very important to maintain genome stability during DNA synthesis for replication and repair. Therefore the activity of RNR is tightly regulated via various mechanisms. In yeast species RNR inhibitors proteins prevent the RNR activation in the G1 and G2 phases. Upon entry into S phase or in response to DNA damage where the DNA synthesis is required, these proteins are degraded through distinct mechanisms. Comparative genomic studies in fission yeast identified the *spd2* gene. The amino acid sequence of the Spd2 protein shows high similarity to the previously characterized RNR inhibitor protein, Spd1. Spd2 protein shows homology to Spd1 in distinct domains that are proven to be important for the function and regulation of Spd1. Using site directed mutagenesis I disrupted these domains in Spd2. I used two different assays to analyse the phenotypes corresponding to these mutations. In the first assay, I screened for the ability of these mutation to rescue the growth of *Δddb1* strains in the absence of *rad3* gene, a key component of DNA structure-dependent checkpoint. In the second assay I looked at the ability of these mutations to restore meiosis and spore formation in *Δddb1* strains. The Ddb1 is a component of $CRL4^{Cdt2}$ E3 ubiquitin ligase that is involved in the degradation of RNR inhibitor proteins in fission yeast. Furthermore, I looked at the stability of different Spd2 mutant proteins. Mutations in the PIP degron of the Spd2 were able to restore growth in strains that lack both active proteasome and checkpoint. Mutations in the PIP degron were able to restore meiosis and spore formation in *Δddb1* strains. Result of the SDS gel, suggest that the PIP degron is important for the stability of the protein. Mutations in the PIP degron and Helix II made the protein less stable compared to the mutations in the C terminus. From these results I conclude that the PIP degron of the Spd2 is important for its function and regulation.

LIST OF ABBREVIATIONS AND SYMBOLS

˚C	Degrees celcius; unit of temperature
5-FOA	5-Fluoroorotic Acid
A	Adenine
ADP	Adenosine 5'-diphosphate or atomic displacement factor
ADP	Adenosine 5'-diphosphate
Ala	Alanine
Arg	Arginine
Asn	Asparagine
ATP	Adenosine triphosphate
C	Cytosine
Cdc22	Large subunit of ribonucleotide reductase
cDNA	complementary DNA
CDP	Cytidine 5‟-diphosphate
CRL4	CRL4 ubiquitin ligases
Cys	Cysteine
dADP	2'-deoxyadenosine 5'-triphosphate
dCDP	2'-deoxycytidine 5'-triphosphate
DDB	DNA damage-binding protein
dGDP	2'-deoxyguanosine 5'-triphosphate
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide
DSC1	DSC E3 ubiquitin ligase complex subunit 1
dTTP	2'-deoxythymidine 5'-triphosphate
E. coli	*Escherichia coli*
FRET	Fluorescence resonance energy transfer
G	Guanine
G force	The unit of measure of acceleration in the International System of Units (SI) is m/s^2
GDP	Guanosine 5'-diphosphate
Gln	Glutamine
Glu	Glutamate
Gly	Glycine
HU	Hydroxyurea
IDP	Intrinsically disorder protein
kDa	Kilo dalton; measurement of molecular weight equal to one thousand Daltons
Leu	Leucine
LiAc	Lithium acetate

Lys	Lysine
MBF	MCB binding factor
MCB	MluI cell-cycle box
Mg/ml	Milligrams per milliliter
mRNA	Messenger RNA
NER	Nucleotide excision repair
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
Phe	Phenylalanine
PIP degron	PCNA interaction protein
Pro	Proline
RNA	Ribonucleic acid
RNR	Ribonucleotide reductase
S. cerevisiae	*Saccharomyces cerevisiae*
S. pombe	*Schizosaccharomyces pombe*
Ser	Serine
Spd1	S phase delayed 1
Spd2	S phase delayed 2
Suc22	Small subunit of ribonucleotide reductase
T	Thymine
Trp	Tryptophan
Tyr	Tyrosin
UDP	Uridine 5'-diphosphate
UV	Ultra violet
Val	Valine
YEL	Yeast extract liquid
YES	Yeast extract solid
α	Alpha; type of three-dimensional protein helical structure or crystallographic unit cell constant or large subunit of ribonucleotide reductase
β	Beta; type of three-dimensional protein sheet structure or crystallographic unit cell constant or small subunit of ribonucleotide reductase

Table of Contents

1. GENERAL INTRODUCTION

Cell cycle is the essential mechanism for reproduction and it takes place in all living cells. It is an orderly sequence of events in which a cell duplicates its contents and then divides into two. In general, the cell cycle is divided into four different phases: G1 phase, S phase, G2 phase and M phase. DNA synthesis/replication and chromosome duplication occurs during the S phase. Four deoxyribonucleotides (dNTPs), the building blocks of the DNA are required for DNA synthesis in S phase or in response to DNA damage. In majority of organisms, the dNTPs are generated by the enzyme ribonucleotide reductase (RNR) (Reichard, 1988). Optimal supply of dNTPs during replication and repair is vital and imbalanced dNTP pools are mutagenic and can lead to genome instability and cell death (Kunz, 1982). For this reason the activity of RNR is tightly regulated by several mechanisms. In yeast species a special group of inhibitor proteins inhibit the RNR activity when DNA synthesis is not required. Degradation of these proteins is necessary for RNR activation (Nielsen, 2003; Zhao *et al*., 2001). In fission yeast (*Schizosaccharomycec pombe*) such a protein, Spd1 is identified and well characterized (Woollard *et al*., 1996; Nestoras *et al*., 2010; Salguero *et al*., 2012). Recently, via the comparative genomic studies in *S. pombe*, *spd2* gene was identified (Rhind *et al*., 2011). The Spd2 protein sequence shows high similarity to Spd1. Preliminary studies suggests that Spd2 also is a potential RNR inhibitor and might function in the same pathway as the Spd1 (Vejrup-Hansen, unpublished data). The aim of this study is to characterize the function of different domains that are similar between Spd1 and Spd2 and proved to be important for the function and regulation of Spd1 protein (Nestoras *et al*., 2010; O. Nielsen, personal communication). Using oligonucleotide

mutagenesis, mutations in these domains are generated and two informative assays are used to analyze the role of these domains in Spd2 function.

2. BACKGROUND

2.1. Yeast

Yeasts are unicellular fungi, with a very well characterized cell cycle that is remarkably conserved in all eukaryotes. This feature makes yeasts, in particular two species: the fission yeast, *Schizosaccharomyces pombe* and the budding yeast, *Saccharomyces cerevisiae* great model organisms to study cell cycle and its constituents (Bähler, 2005).

2.1.1. Fission Yeast (Schizosaccharomyces pombe)

Fission yeasts are rod-shaped cells that grow by elongation at their ends and divide via medial fission. In general, the fission yeast cells spend around 70% of their life cycle in G2 phase, 10% in M phase, 10% in G1 and 10% in S phases (fig. 1) (Sabatinos and Forsburg 2010). Fission yeast is haploid through its life cycle and the haploid cells are either heterothallic or homothallic. The heterothallic haploid cells have two mating types. They are either *Plus* (h^+) or *Minus* (h^-) according to the different alleles that are present at the mating type locus mat1: *mat1-P* for h^+ and *mat1-M* for h^- (Beach, 1983; Kelly *et al.*, 1988). These genes code for two different types of pheromone and receptors that are essential for mating (Willer *et al.*, 1995). In contrast to heterothallic cells, homothallic (h^{90}) are able to switch between h^+ and h^- mating type every second generation and can mate with each other within a single colony (Egel, 1977). In an h^{90} population, approximately half of the cells are h^+ and half are h^- (Klar,

1992). The sexual differentiation and mating between different mating types is triggered by depletion of nutrients in the environment and in particular nitrogen. Nitrogen starvation promotes mating via two systems: it synchronizes cells in G1 and prompts the transcription of genes that code for the component of the pheromone communication system (Nielsen, 2004). Under normal conditions diploids cells are not stable and zygotes immediately undergo meiosis and generate haploid spores (zygotic ascospores). As mating and meiosis are coupled in *S. pombe*, it is possible to simply cross two strains and let them proceed to sporulation and there is no need for isolation of diploids in advance.

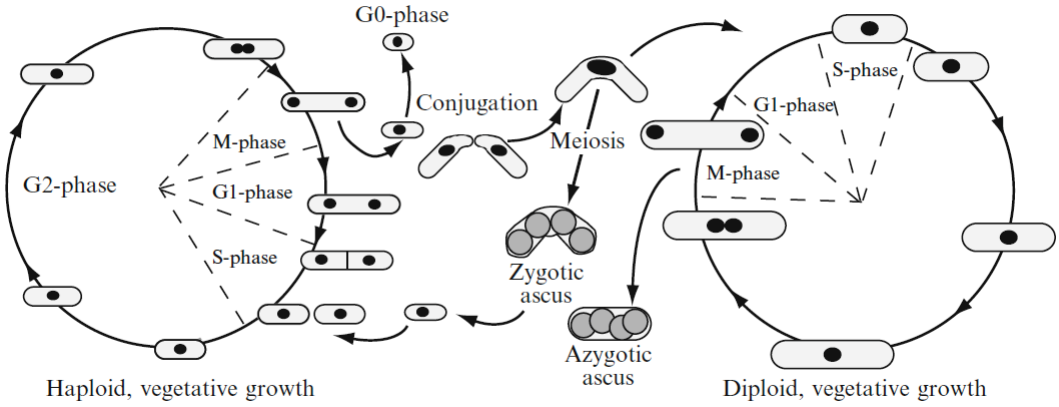


Figure 1. The different stages of mitotic and meiotic life cycle of fission yeast (*S. pombe*). Nutrient starvation promotes conjugation and switch to meiosis life cycle. For more detailed explanation read the text (figure from Sabatinos and Forsburg 2010).

In order to promote effective mating it is necessary to arrest the cell cycle in the G1 phase via nitrogen starvation. Mating and sporulation are essentially temperature sensitive. The optimal temperature for mating and sporulation is between 25°C to 30°C (O. Nielsen, personal communication). Temperature higher than 30°C arrest the cell cycle at G2 phase and disrupt mating (Sabatinos and Forsburg 2010).

2.2. Ribonucletoide reductase

Four deoxyribonucleoside triphosphates (dNTPs) are the building blocks of the DNA and their availability is vital during replication and repair in all organisms. Ribonucleotide reductase (RNR) is an important enzyme that catalyzes the rate-limiting step of *de novo* synthesis of dNTPs. It mediates the conversion of ribonucletides, the building blocks of RNA, into deoxyribo-nucleotides, the building block of DNA. In this reaction the 2´-OH of a ribonucleoside di- or triphosphate substitutes by a hydrogen atom (fig. 2). All deoxyribonucleotides, dADP, dCDP, dTTP and dGDP are generated directly from the action of RNR and they are ready for DNA synthesis after phosphorylation by nucleoside diphosphate kinase (NDK). In addition, in majority of organisms dNTPs are generated through a second pathway that is called salvage pathway. In this pathway kinases catalyze the phosphorylation of nucleosides and reutilize material obtained from the degradation of DNA or RNA (Reichard 1988).

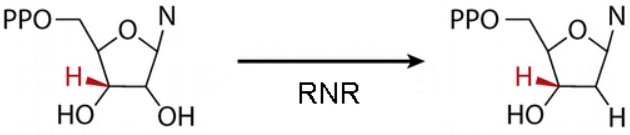


Figure 2. Conversion of ribonucletides to deoxyribonucleotide by RNR. In this reaction the 2´-OH of a ribonucleotide di- or triphosphate substitutes by a hydrogen atom. N= A, T, C or G.

2.2.1. Classification of Ribonucletide reductase

Based on their interaction with oxygen and the pathways that they generate their thiyl radicals, RNRs are divided into three classes.

Class I RNR enzymes are oxygen dependent. The active RNR is a tetrameric complex with α2β2 structure and consists of two large (R1) and two small (R2)

subunits (fig. 3). All eukaryotic organisms, from yeast and algae to plants and mammals, and some prokaryotes and viruses possess Class I RNR. Class I RNR requires oxygen for the generation of a stable tyrosyl radical by a Fe-O-Fe center in the smaller R2 subunit. When the RNR is active, the radical is constantly transferred from R2 to a cysteine within the R1 subunit, which eventually leads to generation of thiyl radical that is required for the activation of the substrate. The large subunits contain the catalytic sites for the reduction of the ribonucleotides and sites for the regulation of RNR activity. According to their polypeptide sequence homologies and their overall allosteric regulation, Class I is further divided into two subclasses, Class Ia and class Ib (Nordlund and Reichard, 2006).

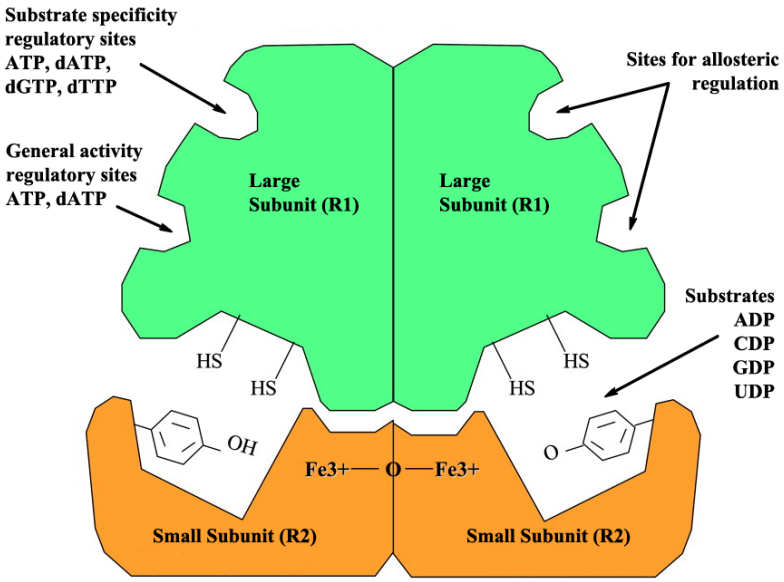


Figure 3. An illustration of Class I RNR with two large and two small subunits and sites for allosteric regulation (see the text, regulation of RNR) (from Yen, 2003).

Class II RNRs are present in some prokaryotes and they are independent of oxygen; they neither require it nor are inhibited by it. They use adenosyl-cobalamin in order to generate their thiyl radical.

Class III RNRs are anaerobic and cobalamin dependent enzymes and they exist mainly in prokaryotes. They are inactivated in the presence of oxygen and they form a glycyl radical through the iron-sulfur cluster (Reichard 1988; Nordlund and Reichard, 2006).

2.2.2. Ribonucletide reductase in yeast

Similar to the other eukaryotes, yeast species possess the class Ia RNR that consists of two large, and two small subunits. In *S. cerevisiae*, the large subunits of the RNR are encoded by two genes, *RNR1* and *RNR3* (Elledge and Davis 1990), while *RNR2* and *RNR4* genes encode the small subunits (Elledge and Davis 1987; Huang and Elledge 1997; Wang *et al*. 1997). The *RNR1* is cell cycle regulated and it is an essential gene. In contrast, *RNR3* is not essential during the normal cell cycle but its expression is up-regulated in response to DNA damage (Elledge and Davis 1990). The Rnr2 posses the typical eukaryotic R2 protein sequence. The amino acid sequence of Rnr4 lacks the certain elements that are essential for binding of iron and generation of tyrosyl radical (Wang *et al*., 1997). It is believed that Rnr4 has either structural role in RNR or acts as chaperon delivering iron to Rnr2 subunits (Chabes *et al*., 2000; Ge *et al*., 2001). Both Rnr4 and Rnr2 are required in order to form the ββ' heterodimer and together with the large subunits, form the active α_2ββ' heterotetramer of the *S. cerevisiae* RNR (Elledge and Davis 1990).

In *S. pombe*, the large subunits of the RNR are encoded by the *cdc22* gene and the small subunits are encoded by the *suc22* gene (Sarabia *et al*., 1993). Expression of the *cdc22* is highly induced at the G1 to S phase transition. The *cdc22* open reading frame encodes a protein that consists of 811 amino acids.

The amino acid sequence shows high similarity to the large subunit of RNR from several species. There are seven short sequence elements similar to the recognition sequence for *MluI* up stream of the *cdc22* coding region. Presence of *MluI* sequence is one of the characteristics of the genes that their expression is under regulation of *cdc10* and the transcription factor DSC1 (also referred as MCB binding factor or MBF).

The *suc22* gene codes for the small subunits of RNR that consist of 391 amino acids and it shows high similarity to the small subunits of *S. cerevisiae* Rnr2 and mouse M2 subunits. Two mRNA with different sizes are transcribed from the *suc22* locus: 1.5 kb and 1.9 kb transcripts. The 1.5 kb transcript is expressed constitutively during the normal cell cycle but in contrast the expression of 1.9 kb is highly induced in response to DNA replication block (Sarabia *et al*., 1993).

2.3. Regulation of Ribonucletide reductase

Optimal supply of dNTPs is vital for accurate DNA replication and repair to maintain genome stability (Kunz 1982; Reichard 1988; Chabes *et al*., 2003). In general, imbalanced dNTP pools cause genome instability and eventually cell death (Oliver *et al*., 1996; Holmberg *et al*., 2005). An increase in dNTP pools is toxic and leads to increase in spontaneous mutation rate via incorporation of incorrect dNTP's into the DNA during the replication and repair (Chabes *et al*., 2003; Holmberg *et al*., 2005). On the other hand, a decrease in dNTP pools inhibits replication fork and DNA synthesis, increases the rate of spontaneous mutation and frequency of mitotic recombination, accumulation of single or double strand break in DNA and eventually chromosomal abnormalities and cell

death (Collins *et al*., 1988; Roguska and Gudas 1984; Holmberg *et al*., 2005; Moss *et al*., 2010; Poli *et al*., 2012).

From the statements above we can easily conclude that the misregulation of RNR have dramatic effects over the organisms fate and one of the primary characteristics of all RNR enzymes is their ability for providing a finely balanced dNTP pool (Meuth 1989; Nordlund and Reichard, 2006). Cells that have low dNTP pools due to reduced RNR activity are hyper-sensitive to DNA damaging agents such as UV light (Collins *et al*., 1988; Holmberg *et al*., 2005; Moss *et al*., 2010). The activity of RNR is cell cycle regulated and its regulation involves multiple mechanisms at different levels (Nordlund and Reichard, 2006). Existence of several distinct mechanisms for regulation of RNR activity reflects the importance of balanced dNTP pools (Chabes *et al*., 2003). RNR activity is up-regulated during G1 to S phase transition and in response to DNA damage (Nordlund and Reichard, 2006; Sarabia *et al*., 1993).

2.3.1. Transcriptional regulation

Through the different stages of the cell cycle relevant protein complexes need to be assembled and dissembled and this is tightly regulated through the expression of corresponding genes at the right time. Similar to the other component of the cell cycle, assembly of DNA replication complex and RNR is tightly regulated and coordinated with the cell cycle and in particular S phase. Expression of the genes that codes for the different subunits of replication machinery are up-regulated in the late G1 and upon entry to S phase. The promoter of these genes contain the *MluI* consensus sequence, which is the common characteristic of the genes that are up regulated during G1 to S phase

transition by transcription factor DSC1 (Bähler, 2005). In yeast, transcription of *cdc22*^{R1} increases several folds at G1 to S phase transition, after the start, and in response to genotoxic stress. In contrast, expression of *suc22*^{R2} is constant during the normal cell cycle or in response to DNA damage (Sarabia *et al*., 1993; Elledge *et al*., 1992).

2.3.2. RNR inhibitor proteins

Up to now, the RNR inhibitor proteins are only discovered in yeast species. Through the cell cycle, these proteins prevent the RNR activation and increase in dNTP pools whenever DNA synthesis is not required. The RNR inhibitor proteins regulate the RNR activity at least via two mechanisms. First, they mediate and prevent the formation of RNR complex by localizing the small subunits into the nucleus away from large subunits when dNTP synthesis is not required (Liu *et al*., 2003; Nielsen, 2003; Lee *et al*., 2008).

Further more, the RNR inhibitor proteins inhibit RNR activity via binding to the large subunits (Zhao *et al*., 2000; Håkansson *et al*., 2006). Prior to activation of RNR, these proteins are degraded via distinct mechanisms (Homberg *et al*., 2005; Zhao *et al*., 2001). After degradation of RNR inhibitor proteins (See below), the allosteric mechanisms regulate the dNTP levels. Degradation of RNR inhibitors is essential for activation of RNR and increase in dNTP pools prior to entry into S phase or to repair damaged DNA (Liu *et al*., 2003 and Yao *et al*. 2003). Inability to degrade RNR inhibitors during DNA synthesis, prevent the increase in dNTP pools to the optimal concentration required for accurate DNA synthesis and blocks the replication fork (Poli *et al*., 2012). Low dNTP pools and

stalled replication fork in turn activate the DNA structure-dependent checkpoint and thereby causing cell cycle arrest (Lindsay *et al*., 1998; Hu *et al*., 2012).

2.3.3. Allosteric regulation

The RNR activity must be tightly coordinated with DNA synthesis. Allosteric regulations provide the balanced supply of dNTPs after the activation of RNR via two systems: substrate specificity and overall activity. The large subunits of the RNR contain two allosteric sites: the specificity site and the activity site. Substrate specificity regulation provides balance among the four dNTPs via the binding of effectors to the specificity site. Binding of ATP and dATP increase the reduction of CDP and UDP, whereas binding dTTP and dGTP increase the reduction of GDP and ADP respectively (Logan, 2011).

The overall activity regulates the total dNTP pools through the dATP/ATP ratio. Binding of ATP increases the activity of RNR while binding of dATP inhibits its activity through feedback inhibition. However, the exact mechanism of this regulation is not clear yet (Nordlund and Reichard, 2006).

2.4. DNA structure-dependent checkpoint

In general, checkpoints monitor the status of the DNA and prevent progression through the different stages of the cell cycle whenever DNA damage or other perturbations occur. In a situation where genome is at risk with lethal damages, checkpoints communicate with cell cycle machinery to temporarily arrest the cell cycle progression. In particular they delay mitosis and meiosis to prevent the onset of M phase before DNA damage is repaired or replication is completed. In yeast checkpoints are not essential during the normal cell cycle but they are crucial when DNA is damaged or during replication stress. Two distinct

DNA structure-dependent checkpoint pathways, The intra-S phase and DNA damage checkpoint can respond to several distinct signals and arrest the cell cycle and onset of M phase, until the replication or repair of the DNA become complete (Palermo and Walworth, 2007).

In fission yeast, the set of six Rad proteins (Rad1, Rad3, Rad9, Rad17, Rad26 and Hus1) are activated and participate in response to the signals that detect incomplete DNA replication and DNA damage as well as recovery pathways (Al-Khodairy *et al*., 2004). Rad3 is the key component of the DNA-structure dependent checkpoint pathway and it is required for both DNA replication and DNA damage checkpoints (Bentley *et al*., 1996). In response to replication stress in S phase or DNA damage, Rad3 become activated and phosphorylates the Cds1 or Chk1 kinases respectively (Martinho *et al*., 1998). During the S phase, depletion of dNTPs or loss of DNA polymerase function signal to DNA structural checkpoint. In response to dNTP depletion or replication stress, Rad3 becomes activated and phosphorylates the Cds1 kinase (Lindsay *et al*., 1998). Activation of Cds1 maintains and arrests the replication fork in a way that it can resume DNA synthesis after the resolution of the blockage. Inability to maintain the fork in stalled configuration results in fork collapse, a situation in which the recovery pathways cannot function anymore and result in cell death (Lindsay *et al*., 1998; Hu *et al*., 2012). Activation of Cds1 also prevents the onset of mitosis and meiosis through the interaction with proteins that regulate the Cdc2 activity (Lindsay *et al*., 1998).

2.5. Intrinsically Disordered Proteins (IDP's)

The small inhibitor proteins that regulate the RNR activity belong to a distinct group of proteins termed intrinsically disordered proteins (IDP's). These groups of proteins possess distinct characteristics that are reflected in their amino acid sequence and structure. These proteins have low content of order-promoting hydrophobic amino acids that include Ile, Leu, Val, Trp, Tyr, Phe, Cys, and Asn as these amino acids form the core of folded globular proteins. In contrast, IDPs are enriched in charged and disorder-promoting amino acids such as Ala, Arg, Gly, Gln, Ser, Glu, Lys, and Pro (Dyson and Wright, 2005). IDP's interact with multiple proteins and they serve as hubs in protein interaction networks. Most of the IDP's are involved in recognition, signaling, regulation, and control pathways where high-specificity and low affinity interactions with several targets are essential (Uversky, 2011).

Most of these proteins have basic regulatory roles in key cellular processes such as transcription, translation, signal transduction and the cell cycle (Tompa, 2002).

Many IDP's are stabilized *in vivo* via binding and interaction with specific targets and ligands such as nucleic acids, cofactors, membrane bilayers, other proteins, small molecules and metal ions (Uversky *et al*., 2000). Based on their effects on their targets, IDP's are categorized into six functional classes that consist of entropic chains, effectors, scavengers, assemblers, display sites and chaperones (Tompa, 2002; Tompa and Csermely. 2004). The intrinsic disorder regions are associated with protein-protein interactions as well as assembly of large complexes (Hegyi *et al*., 2007).

2.6. RNR Inhibitor proteins

So far three RNR inhibitor proteins, Sml1 and Dif1 in *S. cerevisiae* (Zhao *et al*., 2000; Basrai *et al*., 1999; Lee *et al*., 2008) and Spd1 in *S. pombe* (Nestoras *et al*., 2010) have been characterized. All these protein share similarity within conserved domains that are referred to Hug, Sml and Rnr1 (R1) domains (fig. 4). The Hug domain plays an important role in the function and regulation of RNR inhibitors. The Sml domain is a Phosphodegron and it is required for the phosphorylation and degradation and represents cell cycle and DNA damage regulation. The R1 domain mediates binding to the large subunits and inhibition of RNR (Lee *et al*., 2008).

Figure 4. Conserved domains between Dif1, Sml1, Hug1 and Spd1 (From Lee *et al*., 2008).

Sml1 and Dif1 regulate the activation of RNR via two different mechanisms. The Dif1 binds to Rnr2 and Rnr4 via the Hug domain and promote their localization into the nucleus away from the Rnr1 subunits during G1 and G2. However, Sml1 inhibits RNR activity via binding to R1 and R3 subunits (Zhao *et al*., 1998; Chabes *et al*., 1999). During S phase and DNA damage, which require elevated dNTP pools and increased RNR activity, Dif1 and Sml1 are inactivated and degraded via the function of Dun1, Mec1 and Rad53 that phosphorylate certain residues in the Sml domain (Zhao *et al*., 2001).

The function of Hug1 is not clear. The gene expression is up-regulated in response to DNA replication arrest and DNA damage through the activity of *MEC1*, *RAD53* and *DUN1* check point pathway. It can negatively mediate the *MEC1* pathway (Basrai *et al*., 1999). In *S. cerevisiae*, the Dif1, Hug1 and Sml1 possibly derived from the splitting of an ancestral gene by early duplication divergent event (Lee *et al*., 2008).

2.6.1. Spd1 (S phase delayed 1)

In *S. pombe*, *spd1* was identified in a screen for genes that blocks the cell cycle when overexpressed. The *spd1* gene expression is up-regulated during G2 to M phase transition (Rustici, 2004) and codes for a 14kDa Spd1 protein (Borgne and Nurse 2000). Spd1 arrest the cell cycle in both G1 and G2. The G1 arrest is post-Start at G1 to S phase transition and during G2, Spd1 prevents the entry into mitosis and meiosis (Borgne and Nurse 2000; Holmberg *et al*., 2005). The G2 block requires Wee1p kinase activity and depends on *rad* genes and *chk1/cds1*, the components of DNA replication checkpoint (Borgne and Nurse, 2000).

Spd1 arrests the cell cycle at least via two mechanisms. Using indirect immuno-fluorescence microscopy it has been observed that Spd1 promotes localization of $Suc22^{R2}$ into the nucleus away from the $Cdc22^{R1}$ when the RNR activity is not necessary (Liu *et al*., 2003). However, the exact mechanism by which Spd1 sequester $Suc22^{R2}$ into the nucleus is not clear. Spd1 does not act as an anchor to hold $Suc22^{R2}$ in the nucleus (Nestoras *et al*., 2010). On the other hand, using *in vitro* assays and a Biosensor technique, Håkansson *et al*. observed that Spd1 binds only to Cdc22 and inhibits the RNR activation with very high

affinity compared to Suc22 towards which it shows no affinity (Håkansson *et al*., 2006).

However, Spd1 might contribute to RNR structure via promoting the interaction between $Cdc22^{R1}$ and $Suc22^{R2}$ causing formation of inactive RNR (Nestoras *et al*., 2010). Recent studies suggest that Spd1 is stable and translocates between the nucleus and the cytoplasm during unperturbed cell cycle outside the S phase. During S phase and in response to DNA damage, the nuclear Spd1 accumulates into the nucleus to interact with chromatin bound PCNA in order to be degraded. Through this interaction, together with Spd1, RNR may translocate into the nucleus to the sites of DNA synthesis (Salguero *et al*., 2012).

Spd1 is ubiquitinated and degraded upon entry into S phase and after DNA damage or replication stress by the activity of $CRL4^{Cdt2}$ E3 ubiquitin ligase. Degradation of Spd1 by $CRL4^{Cdt2}$ ubiquitin ligase requires increased Cdt2 level (Liu *et al*., 2005) and interaction with chromosome-bound PCNA (Salguero *et al*., 2012). During the G1 to S phase transition of unperturbed cell cycle, transcription of *cdt2* gene is up regulated by Cdc10/DSC1 transcription factor (Liu *et al*., 2005). However, in response to DNA damage or replication stress, up-regulation *cdt2* gene transcription and Spd1 degradation depends on the function of DNA-structural checkpoint pathway (Liu *et al*., 2005; Salguero *et al*., 2012).

Following degradation of Spd1, $Suc22^{R2}$ translocate from the nucleus into the cytoplasm where it can interact with $Cdc22^{R1}$ subunits to form active RNR (Liu *et al*., 2003). Inability to degrade Spd1 causes slow S phase progression and promotes genome instability through increased sensitivity to DNA damaging

agent, mutation rate and minichromosome loss (Liu *et al*., 2003; Bondar *et al*., 2004; Holmberg *et al*., 2005; Salguero *et al*., 2012) due to a decrease in dNTP pools and stalled replication fork (Holmberg *et al*., 2005).

2.7. CRL4^{Cdt2} E3 ubiquitin ligase and PIP degron

In eukaryotes attachment of ubiquitin to proteins by E3 ubiquitin ligase target them for degradation by proteasome. In fission yeast, CRL4^{Cdt2} ubiquitin ligase regulates cell cycle, gene expression and DNA replication via degradation of target proteins (Havens and Walter, 2011). The CRL4^{Cdt2} ubiquitin ligase consist of cullin scaffold (Cul4), an adaptor protein Ddb1 (DNA damage-binding protein 1), and a substrate receptor Cdt2 or DCAF (DDb1- and Cul4-associated factor) that binds directly to Ddb1. Cdt2 is an adapter protein for CRL4^{Cdt2} ubiquitin ligase that interacts with Ddb1 and directs the Spd1 to proteasome (Liu *et al*., 2005). Fission yeast Ddb1 isolated together with COP9 signalosome complex and it is an essential component of CRL4^{Cdt2} E3 ubiquitin ligase (Liu *et al*., 2003). Fission yeast Ddb1 is the ortholog of human p127 DDB1 that is a component of UV-damaged DNA-binding heterodimer (UV-DDB) involve in nucleotide excision repair (NER). However fission yeast Ddb1 prevent mutation and DNA damage in a pathway separate from NER (Holmberg *et al*., 2005). Ddb1 acts as a linker to attach the substrate(s) to CUL4 (He *et al*., 2006). Deletion of *ddb1* is synthetically lethal with lose of either S phase or G2 DNA-damage checkpoint (Holmberg *et al*., 2005). Strains, which lack the *ddb1*, are not able to degrade Spd1 and they have reduced dNTP pools (Holmberg *et al*., 2005).

High affinity binding of CRL4^{Cdt2} substrates to PCNA is essential for efficient proteolysis (Havens and Walter, 2011). All the studied proteins that are

degraded via CRL4^{Cdt2} E3 ubiquitin ligase contain a PIP degron with consensus sequence (fig. 5A). The PIP degron is necessary for the interaction with both CRL^{Cdt2} and chromatin-bound PCNA. The PIP degron contains two essential elements. First is a PIP box that is essential for binding to chromatin-bound PCNA with high affinity. In most of but not all CRL4^{Cdt2} substrates, the PIP box contains a TD motif at positions 5 and 6. Presence of the TD motif in substrates indicates high affinity binding to PCNA (Havens and Walter 2009). Second essential element is the B+4 element downstream of PIP box. It consists of aromatic residues and a positively charged amino acid downstream (fig 5A). This element is conserved in all known CRL4^{Cdt2} substrates and it is essential for docking of CRL4^{Cdt2} onto PCNA-substrate complex and further degradation of the target protein (Havens and Walter, 2009).

The Hug domain of Spd1 contains a PIP degron, which does not exactly match the distinctive PIP degron (fig. 5B). It contains a semi-conserved TD motif and a true B+4 element. This PIP degron is essential for binding to the DNA-associated PCNA and degradation of Spd1 (Salguero *et al.,* 2012).

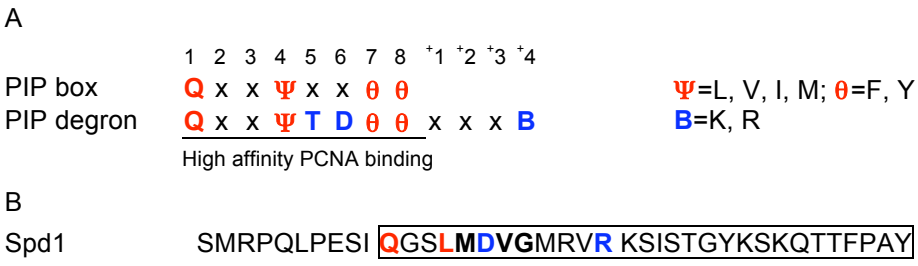


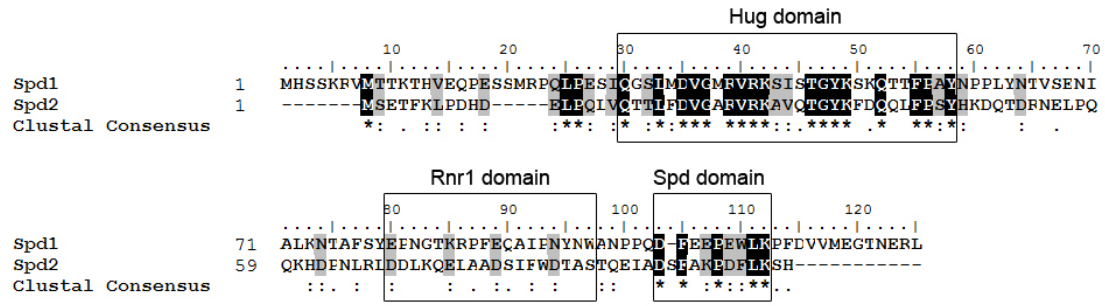
Figure 5. CRL4^{Cdt2} degrons .A) PIP box and PIP degron consensuses sequence. PIP box residues are shown in Red, and PIP degron-specific residues are shown in blue. The TD motif supports high-affinity binding to PCNA, whereas the basic residue four amino acids downstream from the PIP box is required for docking of CRL4^{Cdt2} onto the PCNA–PIP degron complex (from Havens and Walter, 2011). B) Spd1 Hug domain and the internal PIP degron.

2.8. Spd2 (S phase delayed 2)

The *spd2* gene is identified through the comparative genomic studies in *S. pombe* (Rhind *et al.*, 2011). Its amino acid sequence suggests that it belongs to RNR inhibitor protein family and it is an intrinsically disordered protein. Using theonline disorder predictors, PONDR (Romero *et al.*, 2001) suggests that 35.92% of the protein is disorder. The 3 regions of protein that are disordered include amino acid sequences 1–12, 53–68 and 94–102.

Spd2 possess similarity to Spd1 within Hug domain and C-terminus, which is referred to SPD domain. The Hug domain of Spd2 shows 72.4% similarity and 58.6% identity to the Spd1 Hug domain (fig 6A). The Spd2 Hug domain contains a PIP degron similar to Spd1 (fig 6B). Presence of this PIP degron suggests that Spd2 degradation is mediated by $CRL4^{Cdt2}$ E3 ubiquitin ligase and it might bind and interact with chromosome-bound PCNA.

A



B

Spd2 DELPQLV QTT LFDVGARVRK AVQTGYKFDQ QLFPSY

Figure 6. A) Sequence alignment between *S. pombe* Spd1 and Spd2. The three conserved domains, the Hug domain, the R1 domain, and the SPD domain, are boxed. B) Spd2 Hug domain and the internal PIP degron.

Preliminary studies show that unlike Spd1, the level of Spd2 protein does not fluctuate significantly through the cell cycle (C. Holmberg, personal communication). The Spd2 protein is degraded when cells are treated with hydroxyurea. Hydroxyurea inhibits the RNR, which in turn leads to inhibition of DNA replication by starving the DNA polymerase at the replication fork for dNTPs (Slater, 1973). However the protein is stabilized in cells that lack the Ddb1. These results suggest that Spd2 might function through inhibition of RNR and its degradation is more likely mediated via the activity of $CRL4^{Cdt2}$ E3 ubiquitin ligase. However Spd2 is not involved in accumulation of $Suc22^{R2}$ in nucleus (Vejrup-Hansen, unpublished data). Cells in which Spd2 is overexpressed are elongated. In addition deletion of *spd2* in *Δddb1* strains improves growth to some extent (Vejrup-Hansen, unpublished data). These two phenotypes suggest that Spd2 inhibits the cell cycle during the S phase (Bondar *et al*., 2004). Deletion of Spd2 to some extent improves the sporulation of *Δddb1* strains and it rescues the synthetic lethality of *ddb1 rad3* double mutants (Vejrup-Hansen, unpublished data). Results of the FRET analysis (Fluorescence resonance energy transfer) show that similar to Spd1, the Spd2 can also promote the interaction between $Cdc22^{R1}$ and $Suc22^{R2}$ and deletion of the Spd2 reduces the FRET signal (Vejrup-Hansen, unpublished data). The FRET signals associate with the interaction between two proteins (Kenworthy, 2001). When there is an interaction between $Cdc22^{R1}$ and $Suc22^{R2}$ the strong signals can be detected but in the absence of interaction between these subunits no signal can be perceived. However, the positive signal does not represent the active RNR. It can imply the formation of inactive RNR tetramer (Nestoras *et al*., 2010).

3. AIM OF THE PROJECT

The aim of the project is to disrupt the structure and function of Spd2 in order to characterize the contribution of specific residues to its function using scanning mutagenesis. The targets of this mutagenesis include residues in Hug domain, helix II, helix III and Spd domain (O. Nielsen, personal communication) (table 1; fig 7).

Table 1. List of mutations within different domains of Spd2.

Target Domains	Mutation number and Amino acid changes	Reason and effects
Hug domain (PIP box)	m18: Gln18 to Ala m21: Gln 21 to Ala	Decrease affinity and disrupt the interaction of PIP box with PCNA
	m28: Val28 to Pro m29: Arg29 to Pro	Recognition helix, PIP degron, Interaction with $CRL4^{Cdt2}$, Helix I and PIP degron destruction, Loss of PIP box specificity
Helix II/Helix III	m73: Glu73 to Pro m90: Ala90 to Pro	Helix II destruction Helix III destruction
SPD domain	m93: Phe93 to Ala	Conserved

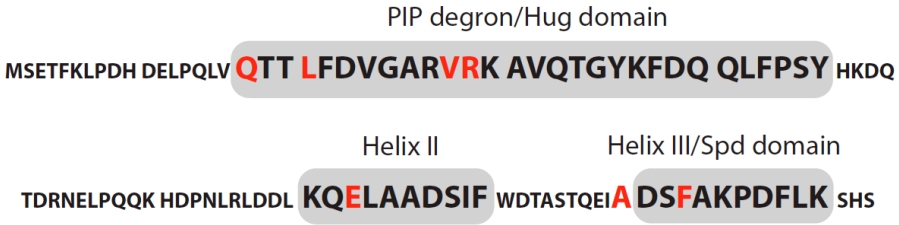


Figure 7, Spd2 amino acid sequence. Red letters indicate residues that are targeted for mutagenesis and substitution.

In this study, I looked at the ability of mutations to stabilize the protein using western blot. I used two assays in order to analyze the effect of individual mutations on growth of strains that lacks proteasome and check point functions and the ability of mutations to restore meiosis and spore formation in strains that lacks the proteasome function. In the first assay, I test the ability of each

mutation to suppress the synthetic lethality associates with loss of both *ddb1* and *rad3* checkpoint gene in two strains with different genotypic background (See table 2 materials and methods). In both strains the *ddb1* gene was deleted and they harbor the *rad3-ts* gene that code for the temperature sensitive Rad3 protein. Survival of the *Δddb1* cells depends on the activity of the *rad* genes. The checkpoint of the cells carrying the *rad3-ts* allele is functional at 25°C but is inactive at both 33°C and 36°C. One of the strains carries the mutated version of *cdc22* gene (*cdc22-D57N*). In this strain the allosteric regulation of RNR is damaged in a way that the dATP feedback inhibition is non-functional. In these cells dNTP concentration increases around 4 times compared to the wild type cells in response to DNA damage (C. Holmberg, personal communications). In *Δddb1* cells if the Spd2 inhibitory function is impaired, the checkpoint protein Rad3 is not essential and cells can survive in 33°C or higher temperature.

In the second assay, I looked at the ability of each mutation to restore meiosis and improve spore formation in homothallic *Δddb1* strains. In the absence of *ddb1*, cells are not able to degrade Spd2 and this results in reduced dNTP pools and inability to progress through the meiosis and spore formation. In homothallic (h^{90}) *Δddb1* strains if the Spd2 is not functional, cells can proceed through the meiosis and produce spores.

4. MATERIAL and METHODS

List of strains that are used in this study are available in supplementary material (Table S.1).

4.1. Oligonucleotide-directed mutagenesis and Cloning

Different *spd2* mutations were generated using oligonucleotide-directed mutagenesis Quichchange™ II protocol and each was confirmed by sequencing. Plasmid containing *spd2* cDNA plus 700 flanking sequence (fig. 8) and *S. pombe* strain in which genomic *spd2* is replaced by *ura4* kindly provided by Rasmus Vejrup-Hansen. List of primers for generation of different mutations and the PCR program are available in supplementary material. The PCR products were treated with the *Dpn1* endonuclease in order to digest the template plasmids. The plasmids were propagated in *E. coli* DH5-α cells and purified using Promega plasmid preparation kit according to the manufacturers instructions. The purified plasmids were cleaved with two restriction enzymes: *Xho1* and *Spe1* to linearize vectors harboring individual *spd2* mutations and digestion were confirmed by gel electrophoresis.

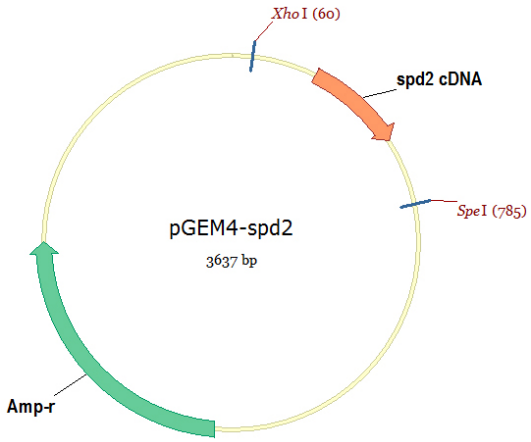


Figure 8. plasmid harboring *spd2* cDNA with two flanking sides and restriction sites for isolation of linear plasmid.

4.2. Transformation

The linearized vectors were used to transform the *spd2::ura4* strain where *ura4*$^{+}$ had replaced the *spd2* open reading frame (ORF) (Bähler *et al.*, 1998). Transformation was carried out according to rapid and simple procedure for high-efficiency lithium acetate transformation of cryopreserved *S. pombe* cells (Suga and Hatakeyama 2005). In brief, cells were grown on yeast extract liquid (YEL) medium to a density 1 x 10^7 cells/ml at 30°C. The culture was placed on ice for 15 minute prior to harvesting. The cells were collected by centrifugation at 1600 x G for 5 minutes and washed three times with ice-cold sterile water. The cell pellet was re-suspended in ice-cold 30% glycerol and 0.1 M LiAc (pH 4.9) to a concentration of 10^9 cells/ml. Aliquots of the cell suspension were placed on ice for 30 minutes prior to placing into a –80°C freezer. The frozen cells were quickly thawed in a water bath at 40°C for 2 minutes. The cell suspension was mixed directly with carrier DNA (10 mg/ml) linear vector and 145 µl 50% PEG-4000. The mixture was then immediately heat-shocked at 43°C for 15 minutes. The cell suspensions were diluted and spread on yeast extract solid (YES) plates and were incubated at 30°C for two days. Transformants were selected by isolating *ura4*$^{-}$ colonies growing on 5-Fluoroorotic Acid Monohydrate (5-FOA) plates and PCR with extracted genomic DNA.

4.4. Mating

Strains that harbor different *spd2* mutations were crossed with strains with different genotypic background to create new strains according to the purposes of this study (table 2). Cells from two different strains with different mating types were mixed in 20 to 30 µl sterile dH_2O and 10µl of mixture were used to

spot on Malt plates and incubated at 25°C for two days. After two days formation of zygotic asci were checked under the microscope.

Table 2, List of phenotypic analyses and desired genotype for different assays.

Phenotypic analyze	**Genotype**
Sporulation test	*h^{90} Δddb1 spd2^{m}*
	h^{90} Δddb1 Δspd1 spd2^{m}
Temperature sensitivity test	*rad3-ts Δddb1 spd2^{m}*
	rad3-ts Δddb1 spd2^{m} cdc22-D57N

4.4.1. Random spore analysis

A streak from the mixture of zygotic ascus and cells from the Malt plates were incubated on 30% ethanol for 20 minute to disrupt the cell wall of vegetative cells. Hundred to eighty microliter of this mixture was spread on the YES plates that contain antibiotics as selection marker for *Δddb1* cells and allowed to germinate.

After formation of colonies, the desired strains were selected by replica plating single colonies on different YES plates that contain distinctive antibiotics as selection marker.

4.4.2. Isolation of the temperature sensitive allele of rad3

To screen for temperature sensitive *rad3-ts* strains, cells from crosses were replica plated to a YES plate and two YES plates that contain 10mM hydroxyurea (HU). Colonies that were able to grow on YES plat and YES+10mM HU at 25°C but died at 33°C were selected as *rad3-ts* strains (Martinho and Carr, 1999; O. Nielsen, Personal communication).

4.4.3. Isolation of cdc22-D57N allele

PCR reaction with genomic DNA as template was used to isolate strains that contain the *cdc22-D57N* allele. Reaction results in generation of ~600 bp fragments. The *cdc22-D57N* contains a site that can be cleaved by the *Dde1* restriction enzyme. Cleavage with this enzyme generates two fragments around 300 bp each. In contrast, recognition site for the *Dde1* is not present in wild type *cdc22* allele and cannot be digested by the restriction enzyme (fig. S1 supplementary material).

4.5. Protein Extraction and Western blotting

Protein extracts were prepared from different strains using TCA extract method. Cells were grown in YEL media and harvested when the cultures were at exponential growth with the cell concentration around $5x10^6$/ml. The cell pellets were washed once with 1ml ice-cold 20% TCA and re-suspended in 200 µl 20% ice-cold TCA. Cells were broken with glass beads with FastPrep (Bio101) 3x 15 second at high speed. The crude extracts were recovered by washing glass beads with 400 µl 5% TCA. The cell pellets washed with 500 µl 80% acetone (-20°C). Cell pellets were re-suspended in 100 µl 2x Laemmli sample buffer and boiled for 3–5 minutes, and 20 µl of protein were run on 15% SDS-polyacrylamide gel (Laemmli, 1970).

Proteins were then blotted to Immobiolon™-P membrane (Millipore) and detected using ECL™ (Amersham). Antibodies used were the polycolonal anti-Spd2 diluted 1:500 and anti-α-tubulin diluted 1:2000 (R. Vejrup-Hansen, personal communication).

4.6. Temperature sensitivity test

Streak of cells was used to inoculate 5 ml YEL media and incubate at 25°C overnight. Next day, cultures were diluted to the concentration 3.5 x 10^6 cells/ml in YEL media and grown for ~one generation until the cell concentration reach 4 to 7 x 10^6 cells/ml. One milliliter of each culture was centrifuged and re-suspended in 400 to 700 µl in order to obtain the 1 x 10^7 cells/ml and 10-fold serial dilutions were made to a density of 10^3 cell/ml. From each dilution series 5 µl were spotted on YES plates and incubated on 25°C, 33°C or 36°C for 2 days.

4.7. Sporulation Test

To evaluate the sporulation, 10 ml YEL media were inoculated with cells from homothallic strains and grown to the concentration between 5–7 x 10^6 cells/ml. Cells were harvested and re-suspended in 200 µl dH_2O. From this mixture 10 µl were spotted on malt extract agar plates and incubated for 3 days at 25°C and after this period, cells were assessed under microscope for zygotes/asci with zero to four spores.

5. RESULTS

The *spd2* gene was identified by comparative genomic studies in fission yeast (Rhind *et al*., 2011). Its amino acid sequence shows high similarity to the first characterized RNR inhibitor, Spd1 in fission yeast. Spd2 shows high homology with Spd1 in Hug domains, helix II and the C-terminus, which is termed Spd domain (O. Nielsen, personal communication). Using alanine-scanning mutagenesis it has been shown that these domains are important for the function of the Spd1 (Nestoras *et al*., 2010). The Hug domain of the Spd1 and Spd2 contains a PIP degron, which is important for the protein function and regulation (Havens and Walter, 2011; Salguero *et al*., 2012). Mutations in PIP degron, helix II and C–terminus of Spd1 abolishes the inhibitory function of Spd1 in *Δddb1* background strains (Nestoras *et al*., 2010). Ddb1 is a component of $CRL4^{Cdt2}$ ubiquitin ligase that is required for the Spd1 proteolysis (Liu *et al*., 2003; Holmberg *et al*., 2005). In G1 to S phase transition degradation of Spd1 is essential to allow RNR activation and dNTP synthesis during both mitosis and meiosis cell cycles (Holmberg *et al*., 2005; Nestoras *et al*., 2010). Preliminary studies suggest that Spd2 function negatively regulates the cell cycle at the onset or during the S phase and Ddb1 is required for its degradation (Vejrup-Hansen, unpublished data). Using oligonucleotide mutagenesis different *spd2* mutations are generated within Hug domain and in particular PIP degron, helix II, helix III and Spd domain in order to analyze their contribution to the function and regulation of Spd2 in *Δddb1* strains. Different *spd2* mutations are introduced into a strain where wild type *spd2* has been replaced by *ura4* gene. Combination of these mutations with *Δddb1 rad3-ts* and *Δddb1* using genetic crosses were

generated to screen for mutations that are able to suppress the temperature sensitivity and inability for spore formation in *Δddb1* background strains.

5.1. Generation of desired strains

Using oligonucleotide-directed mutagenesis, different *spd2* mutations were generated within the Hug domain, helix II, helix III and Spd domain in order to disrupt the structure of these domains and each were confirmed by sequencing. Transformation of *Δspd2::ura4* strains with linearized vectors that harbours different *spd2* mutations resulted in integration of mutations in *spd2* locus (fig. 9). Different strains for desired purposes are generated via genetic crosses.

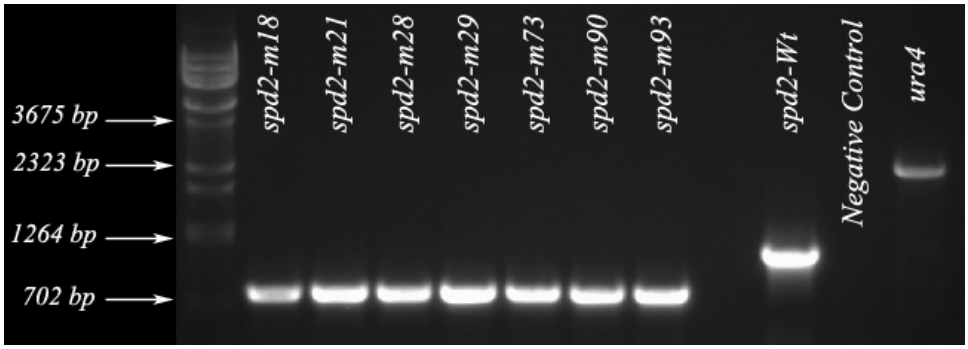


Figure 9. Presence and integration of different *spd2* mutant into *spd2* genomic locus after transformation confirmed by PCR using primers ONP 777 and 778. The primers bind to the flanking sites out side the *spd2* open reading frame. The wild type *spd2* contain introns and it will generate fragments with 1163 bp. The *spd2* cDNA that is used for site directed mutagenesis is 309 bp and it lacks the introns. Integration of *spd2* mutations generates fragments with 779 bp. Presence of the *ura4* generates 2670 bp fragments.

5.2. Effect of different mutations on Spd2 stability

The spd2 expression does not fluctuate significantly during the cell cycle (C. Holmberg, personal communication). The stability of Spd2 protein with different mutations is assessed and compared it with wild type Spd2 using western blot. Results of western blot with un-synchronised cells confirmed the expression of different *spd2* mutations after transformation of a strain in which the wild type

spd2 was replaced by *ura4* gene (fig. 10). The Spd2 antibody recognises a band just above the position of 10 kDa marker. The highest concentration of Spd2 was detected in wild type, *spd2-m90* and *spd2-m93* compared to the other mutations. Mutations within the PIP box and B+4 elements: *spd2-m21*, *spd2-m29* and *spd2-m18* were able to stabilize the protein to some extent but this was still lower compared to *spd2-m90* and *spd2-m93* and wild type strains. The lowest concentration of protein was detected in *spd2-m28* and *spd2-m73* and the band is absent in deleted *spd2* strains.

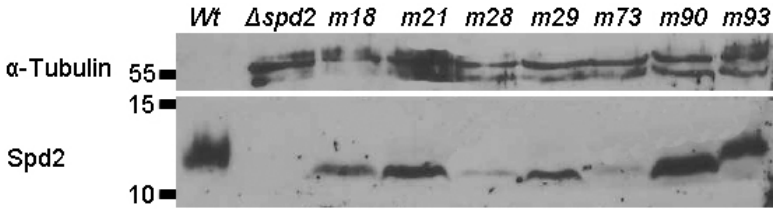


Figure 10. The Spd2 protein from different mutations in unsynchronised cells. Mutations in PIP degron (*m18*, *m21*, *m28* and *m29*) and Helix II (I) reduced the stability of the Spd2 protein.

5.3. Ability of different spd2 mutations to rescue the strains that lack the functional proteasome and Rad3 checkpoint.

During S phase depletion of the dNTP pools inhibits the activity of the replication fork. Stalled replication fork signals, activates intra-S-phase checkpoint proteins that include Rad3 (Al-Khodairy *et al.*, 1994). Rad3 is a central component of the DNA structural dependent checkpoint (Bentley *et al.*, 1996). Activated Rad3 phosphorylates and activates the Cds1 protein. The Cds1 activation stabilizes the stalled replication fork, slows S phase and prevents progression through the mitosis or meiosis until the DNA replication is complete (Lindsay *et al.*, 1997; Martinho *et al.*, 1998; Hu *et al.*, 2012). Cells that lost their components of intra-S phase checkpoint proceed into mitosis and meiosis with

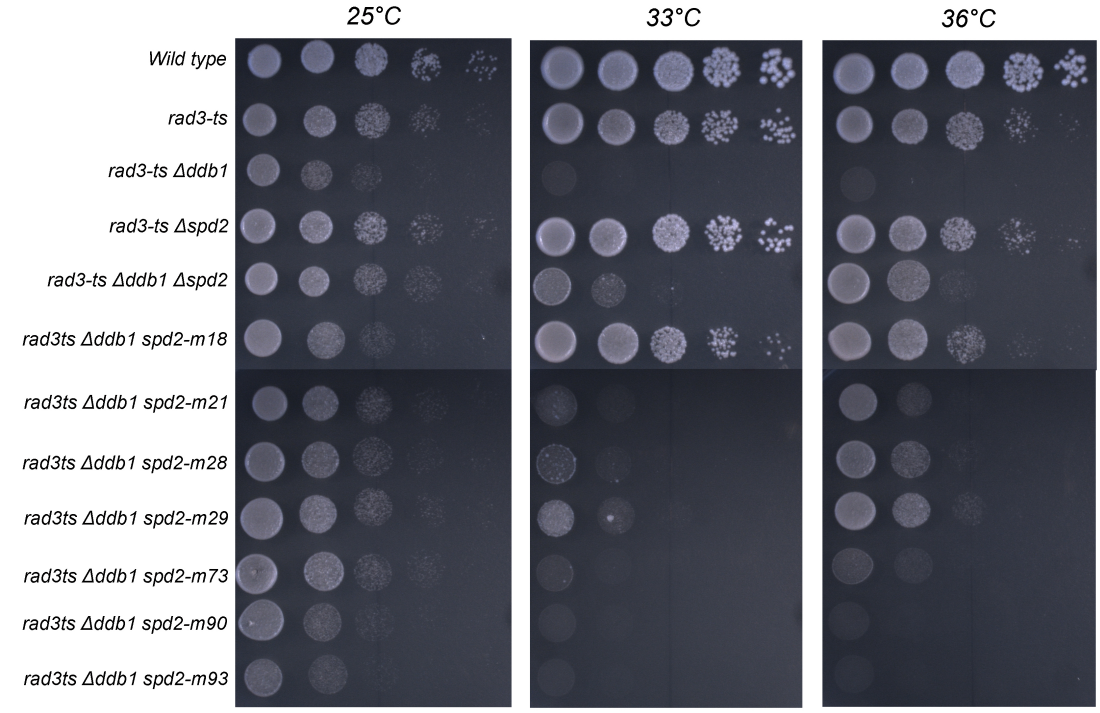
incompletely replicated DNA. Since the non-replicated or partially replicated daughter chromosomes cannot separate from each other, mitosis prior to complete DNA replication leads to chromosome breakage and cell death (Enoch and Nurse, 1990; Murakami and Nurse 1999). Moreover, cells that lost their Rad3 function are not able to recover from stalled replication after the resolution of the obstacle (Hu *et al*., 2012).

In fission yeast the Ddb1 is a component of $CRL4^{Cdt2}$ ubiquitin ligase that is required in order to link the CRL4 to the substrates and promote their degradation (He *et al*., 2006). Strains that lack the *ddb1* gene are not able to degrade the RNR inhibitor proteins. Inability to degrade RNR inhibitors and RNR activation leads to decrease in dNTP pools and replication block, which in turn result in activation of intra-S-phase checkpoint, Rad3 and cell cycle arrest (Holmberg *et al*., 2005; Nielsen 2003). The *Δddb1* cells grow slowly and spend longer time in S phase. Survival of these cells is dependent on Rad proteins and inactivation of both $CRL4^{Cdt2}$ and Rad3 is synthetically lethal (Holmberg *et al*., 2005). The *rad3-ts* allele encodes the Rad3 protein that is functional at 25°C but not at temperature over the 33°C (Martinho *et al*., 1998). Deletion of *spd2* in *rad3-ts Δddb1* background, partially suppress the synthetic lethality at 33°C or 36°C (fig 11A). In order to discover and analyze the functional domain of Spd2 and its role in regulation of RNR and cell cycle, different *spd2* mutants were tested for their ability to rescue the *Δddb1 rad3-ts* at restrictive temperature. In *Δddb1* cells if the Spd2 inhibitory function is impaired, the checkpoint protein Rad3 is not essential and cells can survive in 33°C or higher temperature. In general, mutations in PIP degron of the Hug domain enhanced the survival of these cells at 33°C or 36°C (fig 11A and B). As it is shown in figure 11A,

mutations in the PIP degron of *spd2*: *spd2-m18*, *spd2-m29*, *spd2-m28* and *spd2-m21* rescued *rad3-ts Δddb1* cells at restrictive temperature respectively. Interestingly the *spd2-m18* improves the growth to the level even higher than *rad3-ts Δddb1 Δspd2*. This is followed by *spd2-m29*, which improves the temperature sensitivity to the level comparative to *rad3-ts Δddb1 Δspd2* (fig 11A). The lower level of rescue at restrictive temperature observed with *spd2-m21* and *spd2-m73*. The *spd2-m90* and *spd2-m93* were unable to suppress the temperature sensitivity at all.

In another assay with *rad3-ts Δddb1 cdc22-D57N* background, deletion of *spd2* partially rescued the temperature sensitive at restrictive temperature (fig. 11B). Similar to previous test, mutations in Hug domain suppress the synthetic lethality of *rad3-ts Δddb1*. However in contrast to the previous test the *spd2-m18* was unable to rescue these cells at all. The *spd2-m29* rescued the temperature sensitivity of *rad3-ts Δddb1 cdc22-D57N* similar to the level when *spd2* was deleted in these cells. In addition, mutations *spd2-m21* and *spd2-m28* were also able to rescue the temperature sensitivity in *rad3-ts Δddb1 cdc22-D57N* background. Similar to the previous assay, *spd2-m90* and *spd2-m93* were unable to rescue the temperature sensitivity.

A



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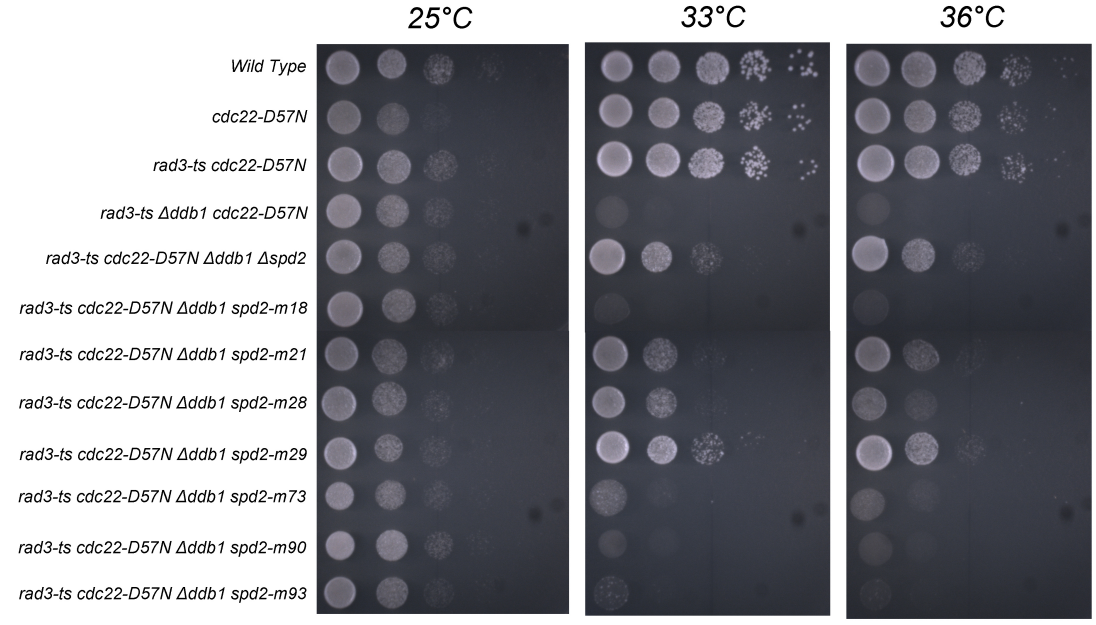

Figure 11. The temperature sensitivity test with different *spd2* mutation in A) *rad3-ts Δddb1* B) in *rad3-ts cdc22-D57N Δddb1* background at 25°C, 33°C and 36°C. 10-fold serial dilutions of each strains on YES plates. Mutations in PIP degron: *spd2-m21*, *spd2-m28* and *spd2-m29* except *spd2-m18*, were able to rescue growth at restrictive temperature. Mutations in C terminus, spd2-m90 and spd2-m93 were unable to rescue the temperature sensitivity at restrictive temperature.

5.4. Ability of different mutations to restore meiosis and sporulation of proteasome defective strains.

Nutrient starvation in *S. pombe* triggers mating and entry into the meiotic cell cycle. Meiosis starts at G1 phase followed by one round DNA replication, two nuclear divisions and eventually formation of asci containing four haploid spores (Harigaya and Yamamoto, 2007). During meiotic S phase, Ddb1 is required for degradation of Spd1 in order to activate the RNR and promote dNTP synthesis prior to DNA replication. Cells that lack the *ddb1* gene cannot degrade Spd1 to activate RNR and increase their dNTP pools to the optimal level. These cells fail to complete meiosis and form asci with four spores (Holmberg *et al*., 2005). However deletion of Spd1 in *Δddb1* strains enhanced the spore formation almost close to the level of wild type cells (Nestoras *et al*., 2010). In this assay we examined the contribution of different domains of Spd2 for regulation of meiotic cell cycle and in particular meiotic S phase. Using site directed mutagenesis, different residues within the PIP degron of Hug domain, Helix II, Helix III and Spd domain were substituted in order to disrupt these functional domains. In homothallic (h^{90}) *Δddb1* strains if the Spd2 is not functional, cells can proceed through the meiosis and produce spores.

Deletion of *spd2* was able to partially restore the meiosis and spore formation in a *Δddb1* background (fig. 12). We looked at the ability of various *spd2* mutations to restore meiosis in two different backgrounds: *Δddb1* and *Δddb1 Δspd1*. Around 98% of the *Δddb1* homothallic h^{90} strains mate and form zygotes but they were not able to proceed toward meiosis to form spores. However, deletion of *spd2* partially improves the spore formation. The amount of empty spore sacs are reduced to 64% compared with 98% in *Δddb1* cells (fig 12).

But the number of asci with four spores was significantly low or they were absent. Most of the asci contain one or two spores (fig 12). Mutations in PIP degron, except *spd2-m18*, were able to restore meiosis to the level comparable to *Δddb1 Δspd2.* Among these mutations *spd2-m29* and *spd2-m21* improved the spore formation slightly better than *spd2-m28*. The other mutations, *spd2-m73*, *spd2-m90* and *spd2-m18* improved the spore formation respectively, but this enhancement was lesser than mutations in PIP degron. The *spd2-m93* was unable to restore meiosis at all and the number of empty spore sacs was similar to *Δddb1* cells.

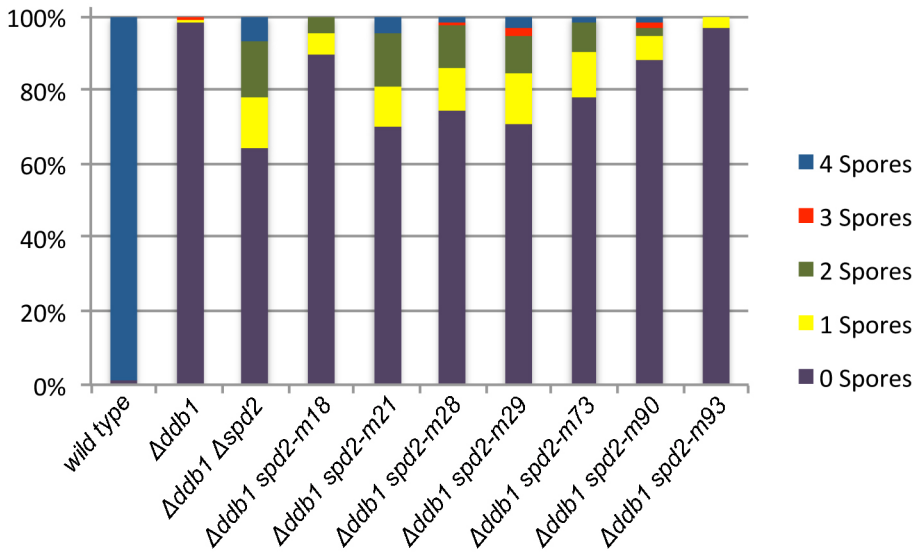

Figure 12. The ability of different *spd2* mutations to restore meiosis and spore formation in homothallic (h^{90}) with genotypic background *Δddb1 Δspd2*. Mutations *spd2-m21*, *spd2-m28* and *spd2-m29* restore spore formation to the level close to the *Δddb1 Δspd2*. Among these mutations, *spd2-m93* was unable to restore meiosis.

In the second sporulation assay with the *Δddb1 Δspd1* background, *spd2-m18* and *spd2-m29* did not affect the sporulation significantly compared to *Δddb1 Δspd1 Δspd2*. Reinsertion of *spd2-m29* slightly (10%) decreases the sporulation compared to the triple mutant. Reinsertion of *spd2-m93*, *spd2-m90*, *spd2-m21*

and *spd2-m28* decreases the ability of spore formation respectively by around 10% to 20% increase in the number of empty spore sacs. Interestingly *spd2-m73* disrupts the sporulation close to the level of *Δddb1* strains with around 80% empty asci (fig. 13).

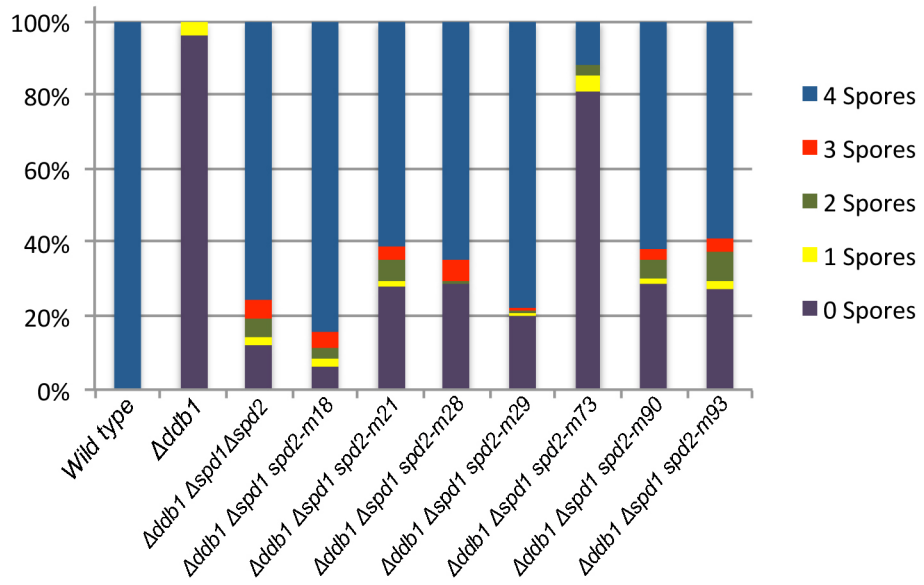

Figure 13, The ability of different *spd2* mutations to restore meiosis and spore formation in homothallic (h^{90}) with genotypic background *Δddb1 Δspd1 Δspd2*. Mutation *spd2-m18* and *spd2-m29* reduced the number of empty asci close to the original strain *Δddb1 Δspd1 Δspd2*. Surprisingly, mutation spd2-m73 disrupt the spore formation significantly.

6. DISCUSSION

The *spd2* gene was identified via comparative genomic studies (Rhind *et al*., 2011). The amino acid sequence of Spd2 shows high similarity to the Spd1 protein. The sequence alignment between Spd1 and Spd2 reveals that the functional domains are highly conserved in both proteins. This suggests that Spd2 might be involved in RNR regulation. Preliminary studies shows that deletion of *spd2* partially improves the growth and spore formation in *Δddb1* strains. The Ddb1 is a component of $CRL4^{Cdt2}$ ubiquitin ligase and it is essential for degradation of Spd1 in order to activate RNR and dNTP synthesis. Inability to degrade Spd1 leads to inhibition the activation of RNR, which leads to reduction in dNTP pools and replication block (Liu *et al*., 2003; Holmberg *et al*., 2005). With this background, in this study I used mutagenesis to analyze the contribution of different domains of Spd2 and their ability to rescue temperature sensitivity and spore formation in *Δddb1* background. The Spd2 contains a Hug domain and PIP degron very similar to Spd1 and also the C terminus of both proteins show high similarity. Mutagenesis studies in Spd1 underlined the residues in PIP degron and other domains that are important for Spd1 function and regulation (Nestoras *et al*., 2010; Salguero *et al*., 2012). From the results of this study we found that similar to Spd1, the Hug domain and the PIP degron of Spd2 are important for its function.

6.1. The PIP degron is important for regulation of Spd2 turn over

Degradation of $CRL4^{Cdt2}$ substrates requires their interaction with chromosome-bound PCNA. This interaction is mediated through a PIP degron, which is present in all studied $CRL4^{Cdt2}$ substrates (Havens and Walter, 2011; Salguero *et al*., 2012).

Single amino acid changes or deletion of Spd1 PIP degron did not stabilise the Spd1 protein *in vivo* (Nestoras *et al*., 2010; Salguero *et al*., 2012). This suggests that a single amino acid change may disrupt the protein function but it cannot prevent the interaction with DNA-bund PCNA and/or its degradation by $CRL4^{Cdt2}$ ubiquitin ligase. The other possibility is that other PCNA-interacting domains exist (Salguero *et al*., 2012). Single amino acid substitutions in PIP degron of Spd2 may disrupt its function but not its interaction with PCNA and its rapid turn over. In contrast, mutations in the C-terminus of Spd2 reduced its degradation and maintained its inhibitory function as they maintain their functional Hug domain and PIP degron.

Results of the Western blot suggest that the PIP degron of Spd2 is important for the regulation of its turn over. Lower level of Spd2 protein was detected in strains with *spd2-m28* and *spd2-m73*. Mutations in the PIP box and B+4 element: *spd2-m18*, *spd2-m21* and *spd2-m29* stabilized the protein. All $CRL4^{Cdt2}$ substrates interact with DNA-bound PCNA through the PIP box. In addition the B+4 element is important for docking of the proteasome onto DNA-bound PCNA and further degradation of the substrates (Havens and Walter, 2009). In strains that harbor *spd2* with mutations in C-terminus or wild type, the level of Spd2 was much higher and they possess an intact PIP degron. This

protein assay need be repeated using synchronized cells to reach more precise conclusion.

6.2. The PIP degron of Spd2 is important for its inhibitory function

6.2.1. Mutation in the PIP degron rescued the temperature sensitivity of rad3-ts Δddb1 strains

Deletion of *spd2* partially rescue the *rad3-ts Δddb1* synthetic lethality at 36°C compared to *Δspd1* that improve the growth to the higher extent. In the absence of *ddb1* survival of *Δddb1* cells highly depends on the activity of Rad3 a component of DNA structure-dependent checkpoint. In this study I used the temperature sensitivity assay to determine the functional domains of Spd2 protein and its contribution to the protein activity. In both assays with *rad3-ts Δddb1* backgrounds, mutations in PIP degron, except *spd2-m18*, were able to rescue the temperature sensitivity at restrictive temperature. Among these mutations, cells carrying *spd2-m29* showed better growth compared to other mutations. In the case of *spd2-m18* the results were completely contradictory in the two tests. In the assay using *rad3-ts Δddb1* background the *spd2-m18* rescued the temperature sensitivity even higher than *rad3-ts Δddb1 Δspd2*. But in the second test, with *rad3-ts Δddb1 cdc22-D57N* background the *spd2-m18* cells are extremely sensitive at 33°C and 36°C. The *cdc22-D57N* mutation disrupts the allosteric regulation of RNR in a way that the dATP feedback inhibition is non-functional. In these cells dNTP concentration increases around 4 times compared to the wild type cells in response to DNA damage (C. Holmberg, personal communication). In both tests disruption of helix II and Helix III did not improve the temperature sensitivity at all. In Spd1 also, mutations in PIP degron rescue the temperature sensitivity of *rad3-ts Δddb1* strains. Substitution of glutamine,

leucine, valine and arginine at position 1, 4, 8 and 9 of the PIP degron with alanine, rescued the temperature sensitivity of *rad3-ts Δddb1* strain (Nestoras *et al*., 2010). In concordance with results in Spd1, in this study mutation in B+4 elements suppress the temperature sensitivity to the higher extent compared to the other mutations.

6.2.2. Mutations in the PIP degron partially improve the sporulation of Δddb1 cells

Meiosis begins with meiotic S phase, DNA replication and chromosome duplication. Prior to meiotic S phase, activation of RNR is essential in order to provide a balanced concentration of dNTPs for DNA replication. Addition of HU to the nitrogen free media (Murakami and Nurse 1999) and deletion of *ddb1* (Holmberg *et al*., 2005) abolish the ability of cells to proceed through the meiosis and spore formation due to inhibition of RNR activity. However, this is mediated through the activation of DNA replication checkpoints, which arrest the cell cycle in prophase when cells are not able to complete pre-meiotic S phase (Murakami and Nurse 1999). Deletion of *spd1* in *Δddb1* cells restores meiosis and sporulation similar to the wild type strains (Holmberg *et al*., 2005; Nestoras *et al*., 2010). Deletion of *spd2* in *Δddb1* strains moderately restores meiosis and spore formation.

In the second assay I looked at ability of different mutation to restore meiosis and spore formation in *Δddb1* and *Δddb1 Δspd1* background. In general mutations within the PIP degron, except *spd2-m18*, has the higher ability to restore meiosis and spore formation in *Δddb1* background. These results are comparative to the results of the similar assay with *spd1* (Nestoras *et al*., 2010).

Corresponding mutations in PIP degron of *spd1* has the higher ability to restore meiosis compared to the other domains. Similar to *spd2-m18*, substitution of glutamine with alanine in Spd1 moderately improves the spore formation compared to other mutation in PIP degron (Nestoras *et al*., 2010). Among other mutations, disruption of helix II was also able to restore meiosis but this was lower compared to the mutation in PIP degron. Mutations in Helix III and C terminus of the Spd2 showed a weak ability to restore sporulation. Especially in the case of *spd2-m93* the sporulation was comparable to the level of *Δddb1* and suggest that Spd2 is completely functional. In contrast, in Spd1, mutations in Helix II and C-terminus of Spd1 significantly improved the sporulation in *Δddb1* background (Nestoras *et al*., 2010).

In another sporulation assay I looked at the effect of the different spd2 mutants in *Δddb1 Δspd1 Δspd2* background. Previous studies showed that there is no significant different between *Δddb1 Δspd1 and Δddb1 Δspd1 Δspd2*. However, I observed that the number of spore sacs with 2 and 3 spores are slightly higher in the later strain. Reinsertion of *spd2-m18* and *spd2-m29* did not affect the sporulation in the second assay but reinsertion of *spd2-m93*, *spd2-m90*, *spd2-m21* and *spd2-m28* decreases the ability of spore formation. One explanation for this result is that in *spd2-m18* and *spd2-m29* the inhibitory affect of Spd2 is impaired while in other mutations the Spd2 retains its inhibitory function. This is in agreement with the temperature sensitivity assays in which *spd2-m29* improve growth at restrictive temperature to the higher extent compared to the other mutations. However, in *Δddb1 Δspd1* double mutants in which the wild type Spd2 is present the rate of spore formation is similar to *Δddb1 spd1 Δspd2*. The question is, why reinsertion of mutations, *spd2-m-21*,

spd2-m28, *spd2-m90*, *and spd2-m93* especially *spd2-m73* should disrupt the spore formation? Via studying the interaction between $Cdc22^{R1}$ and $Suc22^{R2}$ it has been suggested that Spd2 can promote the interaction between these subunits and further formation of RNR complex (Vejrup-Hansen, unpublished data), a function that has been suggested for Spd1 as well (Nestoras *et al*., 2010). If this is the case we can conclude that that the wild type Spd2 might also plays a structural role in the formation of RNR complex and this function is impaired in those mutations specially mutation in Helix II.

7. CONCLUSION

In the two assays that are used in this study, mutations in C terminus showed phenotypes similar to that of wild type spd2 and they did not affect the inhibitory function of Spd2. The levels of protein were also comparable between these strains. In contrast mutation in PIP degron, in particular B+4 elements abolish the inhibitory function of Spd2 to the level of *Δspd2*. The level of Spd2 protein was lower compared to mutations in C terminus. However, this protein level was not similar among these mutations. From this results we can conclude that in fission yeast Spd2 negatively regulate the cell cycle during the S phase and Ddb1 is essential in order to remove the Spd2 inhibitory function through the protein turn over. Temperature sensitivity assays using a strain that harbours *cdc22-D57N* suggest that Spd2 inhibits the RNR prior to its activation. In these cells allosteric regulation of RNR and the dATP feedback inhibition is non-functional and the dNTP pools are 4 times higher compared to wild type if RNR is active (C. Holmberg, personal communication).

In the *Δddb1* background strains, mutations in the PIP degron suppress the temperature sensitivity and defective sporulation phenotype, as the Spd2 is not functional. We found that the PIP degron; in particular PIP box and the B+4 elements of Spd2 are important for the function and degradation of Spd2 (fig. 14). From results of this study we can suggest that the inhibitory function and proteolysis of Spd2 is mediated through its PIP degron. Furthermore, $CRL4^{Cdt2}$ ubiquitin ligase might regulate the inhibitory function of Spd2 via the net reduction in protein level but this needs to be proven by further studies.

MSETFKLPDH DELPQLV**Q**TT **L**FDVGAR**VR**K AVQTGYKFDQ QLFPSYHKDQ

TDRNELPQQK HDPNLRLDDL KQ**E**LAADSIF WDTASTQEI**A** DS**F**AKPDFLK SHS

Figure 14. The amino acid sequence of Spd2. The PIP degron that contain a PIP box and the B+4 element is boxed. The bold letters represent the residues that are mutated in this study. The red letters highlight the residues within the PIP box and blue letters highlight the amino acid residues within B+4 elements. The green letters represent residues in Helix II, helix III and Spd domain. The PIP box and the B+4 elements are important for the inhibitory function of the Spd2 protein.

8. FURTHER STUDIES

The spd2 was identified through the comparative genomic studies. Its amino acid sequence shows significant similarity to the Spd1. From this and previous studies we can conclude that Spd2 negatively affect the cell cycle during the onset of S phase, but the mechanism of its inhibition is not clear yet. In *Δddb1* background deletion of *spd2* shows similar phenotypes to that of *Δspd1* but to lesser degree. The Ddb1 is required for the degradation Spd1. Deletion of *ddb1* stabilizes Spd2 when cell are treated with HU in contrast to wild type cells in which spd2 is disappeared after treatment with HU. This suggests that the Ddb1 more likely mediates the degradation of Spd2. It will be interesting to analyse the effects of different mutations on Spd2 degradation.

Do mutations in different Spd2 domains affect its degradation pattern?

In this study I did western blot with protein extracts from un-synchronised cells and the result was informative. In order to confirm these results and come up with a conclusion, this experiment must be repeated. Cell cycle analysis and Western blot using synchronised cells can give us more information about the effect of different mutations and involvement of different domains on Spd2 turnover. Western blot with combination of different spd2 mutations and *Δddb1* strain can be made and compared with strain that retain their wild type *ddb1* gene. This will give us a more clear degradation pattern in various mutations and combine these results with results of this study and a cell cycle assay. Moreover, by using cycloheximide we can inhibit the protein synthesis (Obrig *et al*., 1971). In this way we can obtain more clear results regarding the Spd2 degradation *in vivo* (R. Vejrup-Hansen, personal communication).

Does Spd2 interact with DNA-bound PCNA to mediate its function and degradation?

If the mediates the degradation of Spd2, the next question is if the Hug domain of Spd2 is important for its function and degradation or other domains are involved? In general, interaction with Chromosome-bound PCNA is essential for the proteolysis of $CRL4^{Cdt2}$ substrates. Similar to the other proteins that are degraded by $CRL4^{Cdt2}$ pathway, Hug domain of Spd2 contains a PIP degron similar to that of Spd1 and it is essential for interaction with DNA-bound PCNA. Presence of a PIP degron suggests that Spd2 interacts with DNA-bound PCNA. I think in the next step we can test this interaction in wild type and mutated *spd2* and compare the affect of each mutation in this interface and its degradation. It is

possible to test this hypothesis using inactivated factor C that is required for the loading of PCNA on to DNA and analyse the Spd2 degradation.

There is increase in evidence that Spd1 plays a role in formation of inactive RNR and coupling dNTP synthesis to replication. Coupling dNTP synthesis to replication via a multi-protein complex, replitase is not a new concept (Reichard, 1988; Murthy and Reddy, 2006). Recently it has been suggested that during the S phase, Spd1 might translocate into nucleus to interact with DNA-bound PCNA to promote its degradation. However, it's binding to the RNR will result in translocation of RNR together with Spd1 to the nucleus and to the site of DNA synthesis (Salguero *et al*., 2012).

Which domains of the Spd2 are important to promote FRET between Cdc22^{R1} and Suc22^{R2}?

Using FRET assay, it was suggested that Spd1 promotes the formation of inactive RNR. FRET analyses with different mutant Spd1 revealed that mutations in the Hug domain, the helix II but not the helix III abolish the FRET signal between Cdc22^{R1} and Suc22^{R2}. This suggests that these domains are important to promote interaction between Cdc22^{R1} and Suc22^{R2}. Using FRET analysis, it has been observed that deletion of *spd2* eliminate the FRET signal between Cdc22^{R1} and Suc22^{R2} and suggest that Spd2 might have a functional role in RNR structure. It will be interesting to test the FRET signal in different *spd2* mutations and compare with results obtained with *spd1* mutations.

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SUPPLEMENTAL INFORMATION

Nucleotide sequence of wild type Spd2

ATGTCTGAAACGTTCAAGCTTCCCGATCATGACGAACTTCCCCAGCTAGTTCAAACTACT
CTTTTTGATGTTGGAGCCAGAGTGTAAGTTTAATCCAATTTTTTACTTTTAAACGTTGAATT
ATCCAAAATGCTGATTGTTCTGCTATCTTTTTTTGTAAGAGACAGGGCTTAAAGGTCTAAG
TACTTTTTTGAAAACTTATAATACAATTGTACTGCAGCACGGCATTTGATGATTTAACTCTT
TTTTAGTCTTTTTGTATAATAACTAACAATCATTTAGTCGCAAAGGTACGTGAATCAAATTT
AAAAAAAAAAAATGAGTGGATTTGTTTATTAATACCTTAATCAAAAATTTCTATAAAGCACT
CTTTCTTCATGTTTATACTTTTAATGGAAAACTGTTTTTATAGAAACTACTTCCTTTCTACC
TTTTTTCCTGTTTAGATGAAAGCAGTTTTTACTAACTTTAGCAGCTGTTCAAACTGGTTAC
AAATTTGACCAACAACTTTTCCCTTCTTACCACAAGGATCAAACTGATAGAAATGAGCTTC
CTCAGCAAAAACATGATCCTAATCTTCGTCTCGATGATTTGAAGCAAGAATTGGCTGCTG
ATTCTATTTTCTGGGATACCGCCTCCACTCAAGAAATCGCCGATTCTTTTGCCAAGCCTG
ATTTTCTCAAGTCTCATTAA

Nucleotide sequence of Spd2 cDNA

ATGTCTGAAACGTTCAAGCTTCCCGATCATGACGAACTTCCCCAGCTAGTTCAAACTACT
CTTTTTGATGTTGGAGCCAGAGTTCGCAAAGCTGTTCAAACTGGTTACAAATTTGACCAA
CAACTTTTCCCTTCTTACCACAAGGATCAAACTGATAGAAATGAGCTTCCTCAGCAAAAA
CATGATCCTAATCTTCGTCTCGATGATTTGAAGCAAGAATTGGCTGCTGATTCTATTTTCT
GGGATACCGCCTCCACTCAAGAAATCGCCGATTCTTTTGCCAAGCCTGATTTTCTCAAGT
CTCATTAA

Amino Acid sequence of the Spd2

MSETFKLPDH DELPQLVQTT LFDVGARVRK AVQTGYKFDQ QLFPSYHKDQ
TDRNELPQQK HDPNLRLDDL KQELAADSIF WDTASTQEIA DSFAKPDFLK SHS

Primers and the PCR programs

Primer	Sequence 5´–3´
spd2-m18	TTCCCCAGCTAGTTGCAACTACTCTTTTTG CAAAAAGAGTAGTTGCAACTAGCTGGGGAA
spd2-m21	GCTAGTTCAAACTACTGCTTTTGATGTTGGAGCC GGCTCCAACATCAAAAGCAGTAGTTTGAACTAGC
spd2-m28	TGTTGGAGCCAGACCTCGCAAAGCTGTT AACAGCTTTGCGAGGTCTGGCTCCAACA
spd2-m29	TTGGAGCCAGAGTTCCCAAAGCTGTTCAAA TTTGAACAGCTTTGGGAACTCTGGCTCCAA
spd2-m73	GATGATTTGAAGCAACCATTGGCTGCTGATTC GAATCAGCAGCCAATGGTTGCTTCAAATCATC
spd2-m90	CCACTCAAGAAATCCCCGATTCTTTTGCCA TGGCAAAAGAATCGGGGATTTCTTGAGTGG
spd2-m93	This mutation provided by DNA 2.0 company and generated by gene synthesis: ATCGCCGATTCTGCTGCCAAGCCTGATT
ONP 429	GCCGTTTGCTCTACTTCC
ONP 430	GCAACTTTGCCGTCGATACGC
ONP 771	CTGGGATACCGCCTCCACTC
ONP 774	GGGAGAGTGGTACGGTGTTAGC
ONP 777	TGATCTACAATCGCAACCCC
ONP 778	CAAACTATAGCAGATGAGCGAGC

Primers ONP 429 and 430 are used in order to screen for *cdc22-D57N* allele. Primers ONP 771, 774 and 777, 778 are used to confirm the integration of different spd2 mutations into the *spd2* genomic locus.

PCR programs

Table S1. The PCR programs that are used in this study

Oligonucleotide mutagenesis using KOD high fidelity DNA polymerase			**Screen for *cdc22-D57N* allele using Fermentas High fidelity DNA polymerase**		
95°C	2 min		97°C	3min	
95°C	20 sec	Repeat for 35 cycle	97°C	30 sec	Repeat for 35 cycle
60°C	20 sec		54°C	30 sec	
68°C	2 min		72°C	1 min	
68°C	5 min		72°C	10 min	

Results of gel electrophoresis

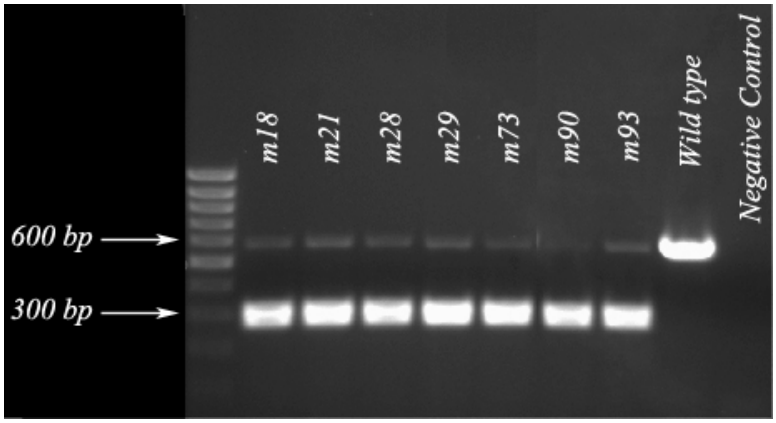

Figure S2. Shows the presence of *cdc22-D57N* allele in strains carrying different spd2 mutations. After the PCR with primers ONP429 and 430, the products are treated with *Dde1* restriction enzyme. The *cdc22-D57N* allele contains the site for the digestion by *Dde1*, which is absent in the wild type *cdc22*.

Table S1, List of strains that are used in this study

Strain	Genotype	Source
Eg545	*h+ Δmat2/3::leu2*	*O. Nielsen*
Eg1624	*h- cdc22-D57N*	"
Eg2835	*h+ Δ2/3 ddb1Δ::NAT spd2Δ::kanMX6*	"
Eg3032	*h+ Δmat2/3 Δspd2::NAT ura4D18*	"
	h+ Δmat2/3 spd2-m18	*This study*
	h+ Δmat2/3 spd2-m21	"
	h+ Δmat2/3 spd2-m28	"
	h+ Δmat2/3 spd2-m29	"
	h+ Δmat2/3 spd2-m73	"
	h+ Δmat2/3 spd2-m90	"
	h+ Δmat2/3 spd2-m93	"
Temperature sensitivity test		
Eg2713	*h- rad3-ts leu l-32*	*O. Nielsen*
Eg2736	*h- rad3-ts Δddb1::NAT*	"
Eg2826	*h- rad3-ts cdc22-D57N Leu1-32*	"
Eg2915	*h- rad3-ts ddb1Δ::kanMX6 cdc22-D57N*	"
Eg2924	*h+ Δ2/3 rad3-ts spd2::NAT*	"
Eg2967	*h- rad3-ts Δddb1::NAT Δspd2::kanMX6*	"
	h- rad3-ts Δddb1::NAT spd2-m18	*This study*
	h- rad3-ts Δddb1::NAT spd2-m21	"
	h- rad3-ts Δddb1::NAT spd2-m28	"
	h- rad3-ts Δddb1::NAT spd2-m29	"
	h- rad3-ts Δddb1::NAT spd2-m73	"
	h- rad3-ts Δddb1::NAT spd2-m90	"
	h- rad3-ts Δddb1::NAT spd2-m93	"
	h- rad3-ts cdc22-D57N Δddb1::NAT Δspd2::kanMX6	"
	h- rad3-ts cdc22-D57N Δddb1::NAT spd2-m18	"
	h- rad3-ts cdc22-D57N Δddb1::NAT spd2-m21	"
	h- rad3-ts cdc22-D57N Δddb1::NAT spd2-m28	"
	h- rad3-ts cdc22-D57N Δddb1::NAT spd2-m29	"
	h- rad3-ts cdc22-D57N Δddb1::NAT spd2-m73	"
	h- rad3-ts cdc22-D57N Δddb1::NAT spd2-m90	"
	h- rad3-ts cdc22-D57N Δddb1::NAT spd2-m93	"
Sporulation test		
Eg325	*h90 ura4-D18*	*O. Nielsen*
Eg1404	*h90 ddb1Δ::kanMX6 ura4-D18*	"
Eg2895	*h90 ddb1Δ::kanMX6 spd1Δ::hygro spd2Δ::NAT*	"
Eg2920	*h90 ddb1Δ::kanMX6 spd2Δ::NAT*	"
	h90 ddb1Δ::kanMX6 spd2-m18	*This study*
	h90 ddb1Δ::kanMX6 spd2-m21	"
	h90 ddb1Δ::kanMX6 spd2-m28	"
	h90 ddb1Δ::kanMX6 spd2-m29	"
	h90 ddb1Δ::kanMX6 spd2-m73	"
	h90 ddb1Δ::kanMX6 spd2-m90	"
	h90 ddb1Δ::kanMX6 spd2-m93	"
	h90 ddb1Δ::kanMX6 spd1Δ::hygro spd2-m18	"
	h90 ddb1Δ::kanMX6 spd1Δ::hygro spd2-m21	"
	h90 ddb1Δ::kanMX6 spd1Δ::hygro spd2-m28	"
	h90 ddb1Δ::kanMX6 spd1Δ::hygro spd2-m29	"
	h90 ddb1Δ::kanMX6 spd1Δ::hygro spd2-m73	"
	h90 ddb1Δ::kanMX6 spd1Δ::hygro spd2-m90	"
	h90 ddb1Δ::kanMX6 spd1Δ::hygro spd2-m93	"