CHARACTERIZATION OF RPA2 N-TERMINAL FUNCTION IN THE DNA

DAMAGE RESPONSE IN *SACCHAROMYCES CEREVISIAE*

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ABSTRACT

In response to DNA damage, two general but fundamental processes occur in the cell: (1) a DNA lesion is recognized and repaired, and (2) concomitantly, the cell halts the cell cycle to provide a window of opportunity for repair to occur. A key factor involved in the DNA damage response is the heterotrimeric protein complex Replication Protein A (RPA), which is not only essential for the repair of damaged DNA, but also is post-translationally modified on at least two of the three subunits in response to DNA damage by checkpoint kinases. Of particular interest is the 32-kDa subunit, called Rpa2, which is hyper-phosphorylated on its serine/threonine-rich N-terminus following DNA damage in human cells. This unstructured N-terminus is often referred to as the phosphorylation domain (PD) and is conserved amongst eukaryotic Rpa2 subunits, including Rfa2 in *Saccharomyces cerevisiae*. In this work we aim to characterize the function of Rfa2 N-terminus (Rfa2 NT) in DNA damage response and develop yeast as a tool to study human RPA.

With the help of mutagenesis we developed Rfa2 NT extreme mutants, which showed that the phosphorylation of Rfa2 NT is dispensable in DNA damage response. However, the presence of Rfa2 NT is essential for cells to survive in stressed condition indicating an uncharacterized function. We further discovered seven S/T sites are responsible for the damage sensitive phenotype of Rfa2 NT extreme mutants. And the phosphorylation affects protein interaction of RFA complex.

Although, the phosphorylation event of Rfa2 NT is dispensable in *S. cerevisiae* the cells have conserved the ability to phosphorylate Rfa2 N terminus. With the help Rfa2 NT fusion mutants we showed that *S. cerevisiae* could phosphorylate N terminus

from seven different eukaryotic species. Hence, we successfully developed yeast as a tool to study Rpa2 phosphorylation amongst various eukaryotic species.

ACKNOWLEDGMENTS

DEDICATION

To,

MOM

for being constant inspiration source

To,

Poornima

for being my support system

In loving memory of **DAD**

"We miss you!"

TABLE OF CONTENTS

x

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

5-FOA .... 5-fluoroorotic acid

A.. Alanine

A. thaliana ....*Arabidopsis thaliana*

aa ....amino acid

Ala ....Alanine

Amp^r...Ampicillin resistant

Asp .... Aspartic Acid

ATM... Ataxia Telangiectasia Mutated

ATR................................Ataxia Telangiectasia Mutated and Rad3-related protein

ATRIP .... ATR interacting protein

BER..base excision repair

C. Albicans ....*Candida Albicans*

Cdc6.. Cell division cycle 6 protein

CDK.. cyclin dependent kinase

cDNA ....complimentary DNA

Cdt1...................................... Chromatin licensing and DNA replication factor 1

CPT ....captothecin

CT ....carboxy (C)-terminus

D.. aspartic acid

D. melanogaster...*Drosophila melanogaster*

DBD .... DNA-binding domain

DDR .... DNA damage response

DNA .... Deoxy-ribonucleic acid

DNA-PK .... DNA dependent protein kinase

DSB .... double stranded break(s)

dsDNA .... double stranded DNA

e.g., ... example

G1 phase .... gap 1 phase

G2 phase .... gap 2 phase

G418 .... geneticin

Gal1 .... Galactose inducible promoter 1

GFP .... green fluorescent protein

H. sapiens .... *Homo sapiens*

His .... Histidine

HR .... Homologous recombination

hr .... hour

HU .... hydroxy-urea

Kan^r .... kanamycin resistant

Leu .... Leucin

M phase .... mitosis

M. musculus .... *Mus musculus*

M. thermautotrophicus .... *Methanothermobacter thermautotrophicus*

MAT .... Mating type locus

MCF7 .... Michigan cancer foundation-7 (breast cancer cell line)

Trp...Tryptophan

Ura ....Uracil

UV...ultraviolet radiation

X-gal 5-bromo-4-chloro-3-indolyl-β-D-galacto-pyranoside

X. laevis...*Xenopus laevis* (frog)

XP ....xeroderma pigmentosum

Y2H...yeast two-hybrid

YPD .... yeast extract, peptone, and dextrose (rich non-selective yeast media)

YPG ....yeast extract, peptone, and glycerol (mitochondrial function media)

β-gal ....β-galactosidase

Δ ....Deletion

CHAPTER 1. REPLICATION PROTEIN A (RPA): A KEY PLAYER IN DNA METABOLISM

<u>Introduction</u>

Replication Protein A (RPA) is a single-stranded DNA (ssDNA) binding protein conserved in all eukaryotes. It is a heterotrimeric complex composed of Rpa1 (70 kDa), Rpa2 (32 kDa) and Rpa3 (14 kDa). All the three subunits are essential, as deletion of any one subunit renders cells nonviable in *Saccharomyces cerevisiae* (BRILL and STILLMAN 1991). This indicates the importance of this complex and the necessity for maintaining the integrity of the complex for cell survival. Replication Protein A was first discovered as a factor essential for *Simian* Virus 40 (SV40) replication *in vitro* (WOLD and KELLY 1988). It is noteworthy that all three subunits were required for the *SV*40 DNA replication (KENNY *et al.* 1990; UMBRICHT *et al.* 1993). Since its discovery, RPA is known to have role in numerous cellular processes. These processes include DNA replication, repair, recombination, cell cycle regulation and telomere maintenance (OAKLEY and PATRICK 2010a). These various roles indicate that RPA is central to all DNA metabolic processes and is touted as the regulator of "DNA metabolic traffic".

By virtue of its indispensable role in DNA metabolism, RPA is linked to have a role in cellular diseases arising from genetic mutations (*e.g.* cancer). Elevated RPA expression has been linked to many cancers; like cervical, esophageal, and colon cancer (PETERS *et al.* 2001; TOMKIEL *et al.* 2002; GIVALOS *et al.* 2007; KANG *et al.* 2009). When RPA expression was knocked down in esophageal cancer cells, the previously radioresistant cells showed enhanced radiosensitivity (DI *et al.* 2014). In esophageal cancer, an elevated RPA expression was observed in tumor cells compared to the

adjacent non-tumor tissues (DAHAI *et al.* 2013). The RPA overexpression is reported as a prognostic indicator in many cancers (LEVIDOU *et al.* 2011).

Although no naturally occurring RPA mutations have been linked to human cellular diseases, the RPA complex has been directly implicated in maintaining genomic integrity to prevent cancer formation in mammals. In mice, a missense mutation in Rpa1 (L221P) leads to lymphoid tumors (WANG *et al.* 2005). In humans, when *rpa1-L221P* mutation was tested it caused defects in replication and repair and does not allow the cell to progress through the cell cycle (HASS *et al.* 2010). Since the RPA complex is central to all DNA processes known to be important in maintaining the genomic integrity, further understanding of its cellular roles furthers understanding of its cellular roles and may provide information as to how RPA might serve as a therapeutic target for cancer.

RPA structure and conservation amongst species

RPA structure

The RPA complex binds single-stranded DNA (ssDNA) via six oligonucleotide /oligosaccharide binding fold (OB fold) domains to protect it from nucleases and secondary structure formation (Figure 1.1). The 70 kDa (largest) Rpa1 consists of four DNA-binding domains (DBDs), while the 32 kDa Rpa2 subunit and the 14 kDa Rpa3 subunit each contain one DBD (FANNING *et al.* 2006a). Rpa2 also has a winged-helix domain at its carboxyl-terminus (C-terminus) and is shown to be important for protein-protein interactions (NAGELHUS *et al.* 1997a; PARK *et al.* 2005; ARUNKUMAR *et al.* 2005). Structural analysis has revealed that Rpa1 DBD-C, Rpa2 DBD-B and Rpa3 (DBD-E) are important for heterotrimerization of RPA complex (BOCHKAREVA *et al.* 2000). The Rpa2 N terminus is unstructured and appears to have co-evolved with Rpa1 N terminus, as both
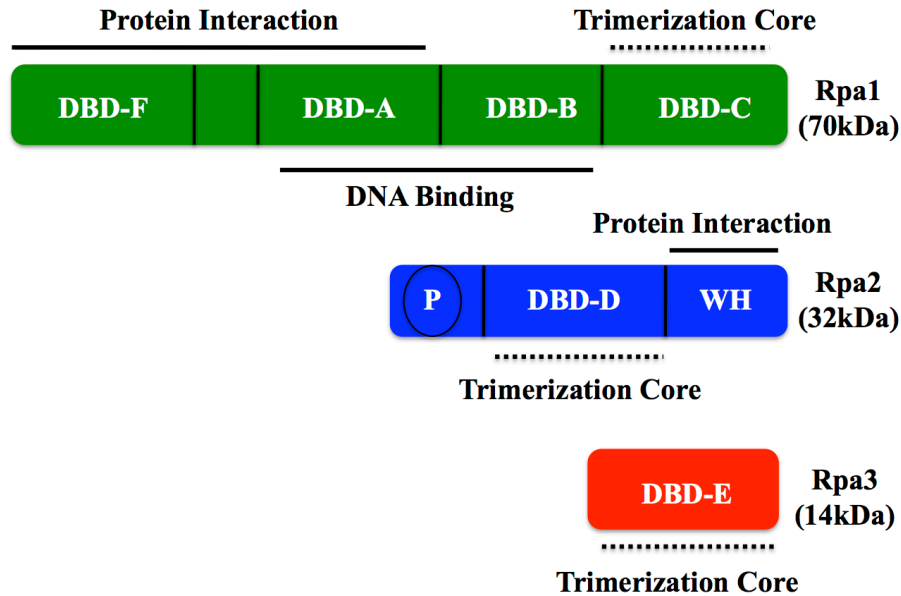
Figure 1.1: A block diagram showing structural features of the RPA complex. The RPA complex has six OB-folds represented here as the DBD A-F. The trimerization core represents the region important fo heterotrimeric complex formation. The most important regions in ssDNA-binding are DBD-A and DBD-B. The DBD-F, DBD-A and is involved in protein-protein interactions; similarly the C-terminus of Rpa2 is also thought to participate in interactions with other proteins. The N- terminus of Rpa2 (represented by P) is S/T rich domain and undergoes hyper-phosphorylation in response to DNA damage in human.

domains are missing in archaeal (CHÉDIN *et al.* 1998).The DBD-E participates in the trimerization of the complex and not yet known to be involved in any other function of RPA (WOLD 1997; FANNING *et al.* 2006b).

RPA forms a highly stable complex. Human RPA is stable in up to 6M Urea and 2M HCl (WOLD 1997). Soluble RPA has only been obtained as the complete trimeric complex or as a sub-complex of Rpa2 and Rpa3. Rpa1 does not form a soluble complex when expressed with either Rpa2 or Rpa3 alone (WOLD 1997). Similar results were observed when the *Schizosaccharomyces pombe* subunits were co-expressed in *Escherichia coli* (ISHIAI *et al.* 1996).

RPA across species

  The non-eukaryotic ssDNA-binding proteins (SSBs) are generally monomers or homo-oligomers (LOHMAN and FERRARI 1994). The eukaryotic SSB (*i.e.* RPA) differs in that it is a heterotrimer, yet SSB functions are conserved. Dr. Marc Wold reviews the sequence comparisons of RPA subunits in detail (WOLD 1997). This review states that, Rpa1 is better conserved across species as it shows more sequence similarity as compared to the other two subunits.

  RPA exists as two different complexes in human cells. These have been termed as the canonical and alternative RPA complexes. In the alternative RPA complex, Rpa2 subunit is substituted by its homologue Rpa4 (KESHAV *et al.* 1995; KEMP *et al.* 2010). The canonical RPA supports both *in vitro* and cellular (*in vivo*) DNA replication whereas alternative RPA does not (HARING *et al.* 2010). This indicates that RPA subunit homologs in the same species can drive the RPA complex to have differential functions.

  Plants are somewhat unique in that they often have multiple RPA subunit homologs. In *Arabidopsis thaliana*, there are five homologs of Rpa1 and two each for Rpa2 and Rpa3 and sub-compartmentalization of various homologs is observed (CHANG *et al.* 2009; ESCHBACH and KOBBE 2014). These homologs form different RPA complexes and each displays differential function. In *Oryza Sativa* there are three homologs of Rpa1 and Rpa2 each, while only one Rpa3 exists. These form various complexes and have different subcellular distribution in plant tissues (ISHIBASHI *et al.* 2001, 2006). Thus far, plants and mammals are the only eukaryotic species where multiple RPA homologs have been identified to function in distinct RPA complexes that may be necessary for different cellular functions in cells.

DNA-binding properties of RPA

RPA binds ssDNA as a heterotrimeric complex with an affinity of 10^9-10^{11} M^{-1} (KIM *et al.* 1994). RPA complex has the ability to bind double stranded DNA (dsDNA) and RNA but with three folds lower affinity as compared to ssDNA binding affinity (KIM *et al.* 1992). Both human and yeast RPA binds poly-pyrimidine rich sequences and binds with a defined 5'-3' polarity (KIM *et al.* 1992; IFTODE and BOROWIEC 2000). However, the sequence specificity is dispensable for RPA function.

The dsDNA-binding property of RPA is important for interaction with transcription regulation elements like 5'UTR sequences, the importance of which is unknown. The dsDNA-binding activity is important for helix destabilization by RPA (LAO *et al.* 1999). Also the RPA complex binds damaged ds DNA, which may have a role in repair (BURNS *et al.* 1996; PATRICK and TURCHI 1998). Both these activites suggest that the RPA binds damaged DNA and destabilizes the helix, so the ssDNA is available for processing. Interestingly, dsDNA-binding activity is reduced in presence of hyper-phosphorylated Rpa2 NT (OAKLEY *et al.* 2003). RPA binds the ssDNA utilizing domains A-D, with the DBD-A and DBD-B being the most important (BOCHKAREVA *et al.* 2001a).The four aromatic amino acids important for Rpa1 binding to ssDNA are located in these domains, two of which are conserved in eukaryotes and *E. coli* (PHILIPOVA *et al.* 1996; WALTHER *et al.* 1999) .

The binding of RPA is dependent on the length of ssDNA. In fact there are four ssDNA-binding modes of RPA proposed (Figure 1.2): (a) binding of RPA to ssDNA is initiated by DBD-A; and binds around five nucleotides. (b) As the binding progresses DBD-A and B bind around 8-10 nucleotides. (c) DBD-C is thought to bind next, but if it

binds with DBD-D simultaneously is unclear. It was shown that the DBD-C and DBD-D

contribute in ssDNA-binding synergistically (BOCHKAREVA *et al.* 2000). (d) RPA

complex can bind around 27-30 nucleotide sequences and requires all four DNA-binding

domains A to D. (BOCHKAREVA *et al.* 2001b, 2002; BASTIN-SHANOWER and BRILL 2001;

FANNING *et al.* 2006a). The N-terminus of both Rpa1 and Rpa2 do not appear to

participate in ssDNA-binding activity, but are important in protein binding and

interaction with DNA substrates (e.g., 5'ssDNA/dsDNA junctions). The N terminus of

Rpa2 is modified by phosphorylation (FANG and NEWPORT 1993; LIU *et al.* 2006).
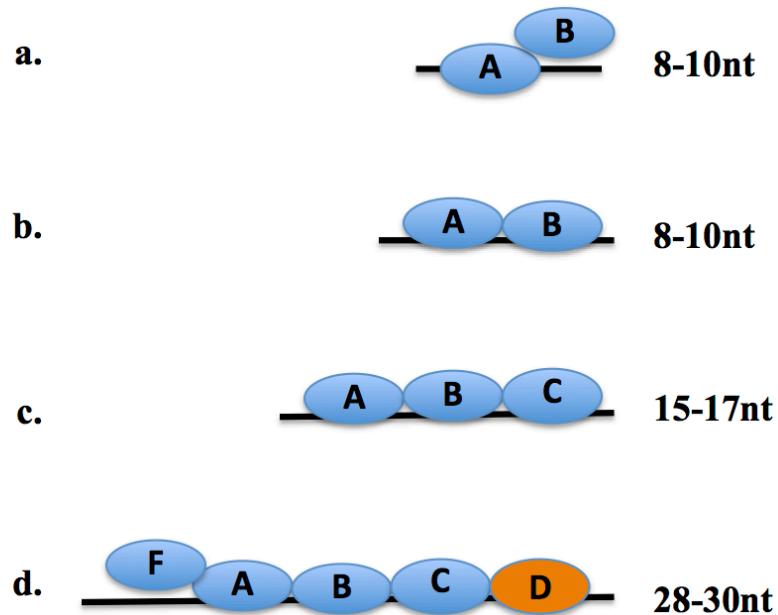
Figure 1.2: Different modes of ssDNA-RPA interaction. The ssDNA-binding of RPA
is initiated through DBD-A of Rpa1. DBD-A and DBD-B bind 8-10 nucleotide sequence.
DBD-C of Rpa1 and DBD-D of Rpa2 contribute to 28-30 nucleotide sequence binding.
The N-terminal regions of both Rpa1 and 2 do not contribute detectably to ssDNA-
binding by RPA.

RPA-protein interactions and cellular function

Single-stranded DNA is an intermediate product of all the DNA processes involving duplication and repair of DNA. RPA binds ssDNA with high affinity, so in order to access the ssDNA and complete a particular DNA process, one of the two things must happen: (1) the RPA complex must be displaced from the ssDNA in order to facilitate interaction of ssDNA with another protein(s) and /or (2) RPA must interact with the incoming protein to recruit it to ssDNA site. In both cases, the interaction of RPA with other protein partners is essential to complete the ongoing DNA metabolic process. In this section, known RPA-protein interactors are discussed.

Protein interactions in replication

RPA was discovered as a factor essential for *SV*40 DNA replication. The human RPA interacts with *SV*40 large T antigen and DNA polymerase alpha (pol α) (DORNREITER *et al.* 1992). Thus RPA assists in DNA unwinding initiating pol α activity. In mouse, RPA interacts with MCM complex components (Mcm2, Mcm4 and Mcm6) as well as Orc2, all proteins important in replication initiation (KNEISSL *et al.* 2003). RPA also plays a role in other stages of DNA replication. Dna2 is an ATP dependent helicase/nuclease important in Okazaki fragment processing. RPA interaction with Dna2 occurs via Rpa1 NT and is required for maximum activity of Dna2 (BAE *et al.* 2003).

Protein interactions in repair

RPA interacts with xeroderma pigmentosum damage-recognition protein (XPA), but only Rpa1 is necessary for the interaction. The RPA interaction enhances the ability of XPA to bind damaged DNA (LI *et al.* 1995). RPA also binds the endonuclease XPG involved in damage recognition and repair and XPF-ERCC1 (HE *et al.* 1995; FISHER *et*

7

al. 2011). Loss of RPA interaction with XPF-ERCC1 leads to mislocalization of XPF and loss of nucleotide excision repair (NER) activity. RPA plays an important role in nucleotide excision repair presumably through the coordination of a number of proteins involved in NER *via*.

Base excision repair (BER) is a pathway whereby specific chemical changes in the DNA structure are recognized. These include the 8-OxoG, a product of oxidative stress (G pairs with A instead of C). Or when cytosine is converted to uracil by hydrolysis it can pair with Adenine causing mutation. As compared to the NER pathway BER acts on smaller adducts and repairs short patches. Base excision repair requires DNA glycosylase activity. Incidentally RPA interactions with DNA glycosylases UNG2 and hMYH have been reported, indicating a role of RPA in BER (NAGELHUS *et al.* 1997b; MER *et al.* 2000; PARKER *et al.* 2001). The importance of these interactions is unclear in cells.

After recognition of a mismatched basepair(s), the base is removed and the DNA from one strand is removed by exonucleases. This sequence is then filled in to repair the DNA lesion. The resection of DNA is carried out by EXOI exonuclease. RPA functions in inhibiting the exonuclease activity thus regulating MMR. This activity of RPA was thought to be dependent on MutLα but later was dismissed due to lack of evidence (GENSCHEL and MODRICH 2003, 2009).

RPA interacts with a variety of DNA repair proteins especially helicases. RPA is reported to interact with BLM, WRN and RecQ helicases to enhance their helicase activity (BROSH *et al.* 1999, 2000; SOMMERS *et al.* 2005). These interactions are important to unwind the DNA duplex and have a role in replication and repair. The

SMARCAL1 is an 'annealing helicase', meaning it stabilizes the replication fork through the reformation of dsDNA. SMARCAL1 interacts with RPA1; this interaction is thought to be important in removal of RPA from ssDNA and activation fork stabilization (BHAT *et al.* 2015).

Protein interactions in recombination

One of the ways to fix double stranded break is through homologous recombination (HR). Once the DNA break is recognized, the DNA strands are resected in a 5'→3' direction generating 3' ssDNA overhangs that are bound by RPA. To facilitate HR, RPA is displaced by Rad52, which assists in the loading of Rad51 onto (and displacement of Rad52 from) the ssDNA. This Rad51-ssDNA filament then invades homologous DNA resulting in the formation of a Holliday junction (HJ). Once the homology is established the damaged strand is fixed by DNA synthesis and resolution of HJs.

RPA interacts with proteins involved in homologous recombination. This includes proteins of the MRN (Mre11-Rad50-Nbs1) complex which is required for 5'→3' resection of broken DNA (SHIBATA *et al.* 2014). Specifically, RPA interacts with Mre11 and Nbs1 through Rpa1 NT. These interactions were characterized in vitro, and the molecular mechanism for how MRN-RPA interaction facilitates homologous recombination is unclear. A potential explanation is discussed in following sections. The interaction of RPA with Rad52 has been demonstrated to require both the Rad52 C-terminus and both the Rpa2 NT and Rpa1 DBD-A and DBD-B (JACKSON *et al.* 2002). Rad51 interacts with Rpa1 DBD-A, an interaction important in exchanging ssDNA between RPA and Rad51 (STAUFFER and CHAZIN 2004). In addition to DBD-A and

DBD-B, the Rpa1 NT has also been demonstrated to be involved in HR through studies of the *rfa1-t11* mutation located in DBD-F. This mutant is recombination deficient and this deficiency is proposed to be due to the slow displacement of RPA from ssDNA by Rad51 observed in *rfa1-11* (KANTAKE *et al.* 2003).

Other protein interactions

The p53 protein is a tumor suppressor protein. In response to DNA damage, p53 is activated and it triggers a cell cycle checkpoint, replication arrest, and sometimes even induces apoptosis. p53 (aa 38-58) interacts with RPA (Rpa1 aa 1-120) and inhibits RPA activity in *SV*40 replication (DUTTA *et al.* 1993). The sequestration of p53 by RPA results in down-regulation of p53 mediated transcription resulting in down-regulation of apoptosis (KAUSTOV *et al.* 2006; RAJAGOPALAN *et al.* 2010).The hyper-phosphorylated RPA does not interact with p53, releasing it to activate damage response (ABRAMOVA *et al.* 1997). Another tumor suppressor protein; BRCA2 also interacts with RPA only in humans. This interaction is of interest, because the cancer-predisposing mutation Y42C in Brca2 disrupts interaction with RPA (WONG *et al.* 2003). This indicates that RPA interaction with Brca2 has important role in prevention of cancer formation, presumably through regulation of regulation of DNA replication and repair.

RPA is phosphorylated by phosphatidylinositol-3 kinase-related kinase (PIKK) family of kinases (*e.g.,*ATM, ATR, and DNA-PK) in response to DNA damage (LIU *et al.* 2012). The ATM and ATR are well known checkpoint kinases involved in the DDR to cause cell cycle arrest (*i.e.,*checkpoint activation). RPA physically interacts with these kinases. Since RPA is the first responder to bind ssDNA generated in response to DNA damage, RPA activation is one of the early steps in checkpoint activation. The ATR

interacting protein (ATRIP) is important in recognition of ssDNA coated with RPA and mediates the ATR-ATRIP interaction with RPA. This is true in both yeast and human cells. Another set of proteins necessary for normal cellular checkpoint response is Rad17-Rfc2-5 complex and Rad9-Rad1-Hus1 (9-1-1) complex (MAJKA *et al.* 2006).The RPA recruits the 9-1-1 damage clamp to DNA through interaction with Rad17-RFC. RPA further interacts with Rad9 and Hus1to establish proper checkpoint activation (WU *et al.* 2005; JANKE *et al.* 2010).

Post-translational modification of RPA

RPA phosphorylation

RPA phosphorylation is extensively studied; especially in human cells. However, the role of this post-translational modification still remains obscure. The human Rpa2 N-terminus (Rpa2 NT) consists of nine serine/ threonine (S/T) residues within first 35 amino acids (>25%). The Rpa2 NT is phosphorylated in cell cycle-dependent manner in humans, *S. cerevisiae*, *Schizosaccharomyces pombe* and *Xenopus laevis*. This phenomenon appears to be conserved in all eukaryotes, although the importance of this is unclear, phosphorylated human Rpa2 had no effect on in vitro *SV*40 DNA replication (PAN *et al.* 1995).

The damage response of cells can be broadly divided into four steps (1) DNA recognition and initial processing of DNA lesion, (2) Checkpoint activation to halt the cell cycle and DNA replication until the damage is repaired, which includes replication, (3) recruitment of factors necessary to further process and repair the damage, and (4) the resumption of cell cycle following repair. As mentioned previously, RPA participates in and/or recruits factors for all of the steps following lesion recognition. In response to

DNA damage Rpa2 NT becomes hyper-phosphorylated in human cells. The kinases

ATM, ATR and DNA-PK have been identified to phosphorylate Rpa2 NT in response to

DNA damage, and these reactions require priming (LIU *et al.* 2012). The Rpa2 NT

phosphorylation sites, the kinases involved and priming patterns summarized by Liu et al

are shown in Figure 1.3. Hyper-phosphorylation of Rpa2 NT regulates cellular RPA

function, potentially through regulation of RPA interactions with many factors necessary

to carry out DNA replication, repair and cell cycle regulation.
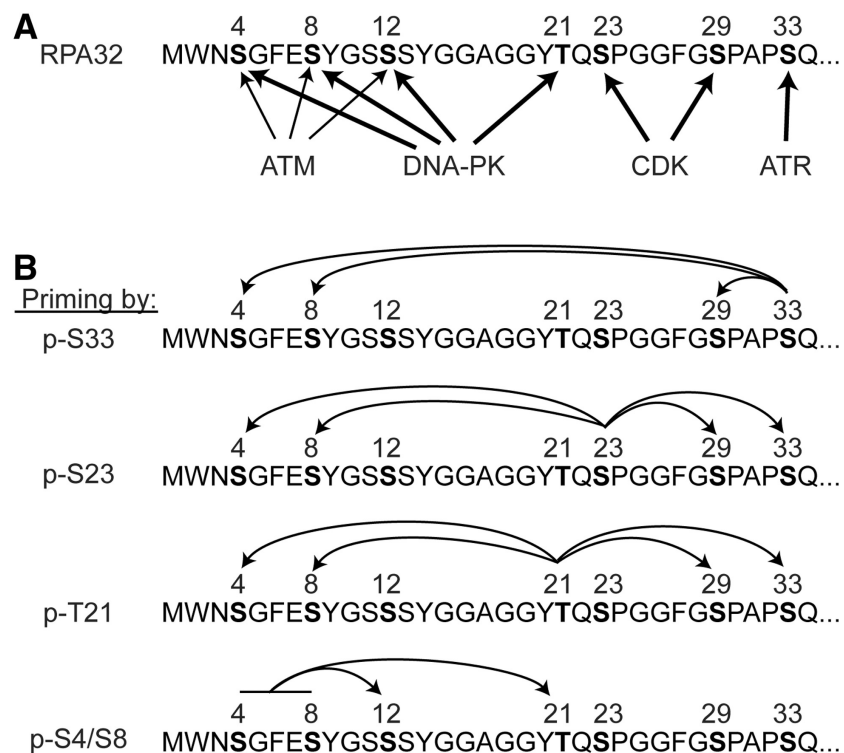
Figure 1.3: A figure from Liu et al 2012 showing the Rpa2 NT phosphorylation pattern. Reprinted (Figure-9) from Nucleic Acid Research 40 (11) Liu et al. 2012, 10780- 10793, with permission from Oxford journals (License number 3603810892063) A) The PIKKs/ CDK phosphorylate subset of sites on Rpa2 NT B) The priming pattern of Rpa2 NT phosphorylation lead by specific sites.

With respect to checkpoint activation, it has been demonstrated that RPA hyper-

phosphorylated on Rpa2 NT does not interact with p53, presumably releasing p53 to

activate the DNA damage response (ABRAMOVA *et al.* 1997). Also, both ATR-ATRIP

and 9-1-1 (Rad9- Rad1-Hus1) complex are important for the ATR mediated checkpoint

response. Although Rpa1 NT is important for interaction with both ATRIP and Rad9

(WU *et al.* 2005; BALL *et al.* 2007). The Rad9 interaction is also dependent on Rpa2 and

is modulated by Rpa2 NT hyper-phosphorylation. In fact, Rad9 precipitation was only

observed in presence of hyper phosphorylated Rpa2 (WU *et al.* 2005). This indicates a

role of Rpa2 NT hyper-phosphorylation in establishing checkpoint arrest.

Once the cells are arrested from progressing through the cell cycle, the DNA

damage intermediate must be further processed and repaired. RPA has been shown in

yeast to not only control initial processing of a DNA lesion (e.g., regulates resection

length after DSB), but also to control how an ssDNA intermediate is processed.

Homologous recombination is one to repair DNA damage that arises from DSB. As

mentioned previously, Rad51 and Rad52 are important for displacing RPA from ssDNA

and performing strand invasion to repair the DNA. Rpa2 NT hyper-phosphorylation is

reported to enhance the interaction of RPA with these proteins in humans (DENG *et al.*

2009).

Once the damage is repaired the cell cycle is resumed. Recent studies indicate a

probable role of Rpa2 NT in recovery after DNA damage. In humans, the hyper-

phosphorylated Rpa2 recruits partner and localizer of BRCA2 (PALB2) to the replication

fork to stabilize them after replication stress (MURPHY *et al.* 2014). In *Candida albicans*

it was demonstrated that the de-phosphorylation of Rfa2 in by PPH3 is important in

normal G1 cycle, as well as in response to replication stress (WANG *et al.* 2013). It has

also been shown that RPA, and specifically Rpa2 N-terminus may be important for release from a checkpoint.

Rpa2 hyper-phosphorylation has been associated with decreased replication, in UV irradiated cells (OLSON *et al.* 2006). A hyper-phosphorylation mimetic mutant of Rpa2 NT recruits RPA away from the replication centers (VASSIN *et al.* 2004).

A phospho-mimetic Rpa2 NT peptide was shown to interact with the Rpa1 N-terminus peptide, in solution *via* NMR data (BINZ *et al.* 2003). Given that many proteins for which the location of the interaction has actually been mapped to interact with Rpa1 N-terminus, this provides a convenient model for how Rpa2 hyper-phosphorylation regulates protein interactions. Mre11 interacts with Rpa1 through its N-terminus, and this interaction is decreased in presence of phosphorylated Rpa2 (OAKLEY *et al.* 2009). Similarly, Rpa1 N-terminus is required for Dna2 interaction and this interaction is thought to get affected by phosphorylation of Rpa2 NT leading to inhibition of Okazaki fragment processing (OAKLEY and PATRICK 2010b). The NBS1 mutant deficient in RPA interaction fails to support proper recovery of replication fork (SHIOTANI *et al.* 2013). These data suggest that hyper-phosphorylated Rpa2 NT aids in replication arrest.

Other post-translational modifications of RPA

Rpa1 is sumoylated *in vivo* at lysine 449 and 577 (DOU *et al.* 2011). The Rpa1 subunit is sumoylated by small ubiquitin-like modifier 2/3 (SUMO-2/3) in response to replication stress. The Rpa1 sumoylation facilitates the Rad51 recruitment, initiating homologous recombination repair (DOU *et al.* 2010). Both Rpa1 and Rpa2 are ADP-ribosylated however whether this modification affects the function of RPA is unknown (EKI and HURWITZ 1991).

Specific aims of this study

A common intermediate (ssDNA) is formed during most DNA metabolic processes (i.e. replication, repair and recombination). This makes the ssDNA-binding protein RPA central to each of these processes, and the importance of this complex is reflected to the fact that it is essential for cell survival. The field has progressed towards understanding the regulatory role of RPA, and many studies have focused on the hyper-phosphorylation of Rpa2 as a means to enact the regulatory mechanism. However, RPA is involved in numerous cellular processes, and it is still relatively unclear how a single protein complex regulates coordinates these processes simultaneously is still relatively unclear. In the studies described in this dissertation we have attempted to decipher how RPA function in regulated through the use of genetics, molecular and cellular biology, and biochemistry studies in the model organism *S. cerevisiae*. The specific aims /hypotheses of each study in this thesis is as follows:

1. **Modifying yeast as a tool to study human RPA**: Human RPA is of utmost interest, since further understanding its role may open new avenues for drug development against diseases like cancer. Currently, the best models available are the human cell lines derived mostly from cancerous cells are the best models available. These are expensive and hard to maintain. Furthermore human genome manipulation is difficult and tedious. Budding yeast on the other hand, are easy to maintain, non-pathogenic, and their genome is easily manipulated. We aimed to develop yeast cells as a tool to study human RPA in a simpler system (Chapter 2).

2. **Understanding the antagonistic function of Rpa2 and Rpa4**: Rpa4 is a homolog of Rpa2 in human cells. These two genes form two separate complexes,

15

which have antagonistic functions at least with respect to DNA replication. We aimed to study the contradictory function of these two genes utilizing siRNA and yeast two-hybrid assays. We hypothesized that the 'Rpa2/Rpa4 ratio decides the fate of cell cycle progression or arrest'.

3. **Yeast Rfa2 N-terminus phosphorylation**: The Rpa2 NT is hyper-phosphorylated in response to DNA damage. This post-translational modification of Rpa2 is extensively studied, but its function is not well understood in humans. Rfa2 in *S. cerevisiae* (yeast Rpa2) is not well studied, although it has many putative phosphorylation sites on its N-terminus. In this study we hypothesized that Rfa2 NT is also phosphorylated and is important in inducing the DDR in *S. cerevisiae*. Mutation analyses were used to characterize the role of Rfa2 NT in yeast cells.

4. **Protein interactions of RPA complex**: It is evident from literature that RPA coordinates cellular processes through various protein interactions. In order to understand the role of RPA complex in cells we need to gain insight into RPA-protein interactions. We employed a yeast two-hybrid assay to uncover potential protein interactions of RPA. We aimed to determine if: (1) Rpa2 and Rpa4 utilize different protein network to establish opposing cellular functions, and (2) the protein-protein interactions of RPA complex are affected by phosphorylation state of yeast Rpa2 NT.

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21

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CHAPTER 2. STUDYING EUKARYOTIC RPA FUNCTION IN HUMAN TISSUE CULTURE AND IN BUDDING YEAST[1]

Introduction

Single-stranded DNA is a common intermediate generated in all DNA metabolic processes such as replication, repair, and recombination. The stabilization and protection of this intermediate is the basic biochemical function of RPA (FANNING *et al.* 2006). Any error in these processes can lead to DNA lesions which if untreated can lead to cellular diseases like cancer. To treat such diseases it is important to understand how these processes are regulated. In order to understand the molecular mechanisms of these processes, it is important to understand the function of each of the proteins involved in them. Our lab focuses on understanding the function of the RPA complex.

Multiple forms of the RPA complex have different cellular functions

As mentioned in chapter 1, RPA is a heterotrimeric complex consisting of Rpa1, Rpa2, and Rpa3. The complex is conserved in all eukaryotes. This complex although conserved in terms of function, does not show a lot of sequence similarity amongst wide variety of species (WOLD 1997). The major conserved protein is Rpa1 which was shown to be conserved in 36 different eukaryotic species (AVES *et al.* 2012). The most conserved regions are the DBDs, which are essential for ssDNA interaction. However, regions that appear to be important in RPA 'regulatory functions' (*e.g.*, the Rpa1 N-terminus; DBD-F), are not identifiable in lower eukaryotes (*e.g.*, *Crithidia fasciculata*; BROWN *et al.* 1994).

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[1] Approximately 50% of this work is published in Exp Cell Res 2015, 331(1): 183-99

The RPA complex can exist in a variety of forms that are proposed to perform a variety of functions. For example, in the plant species *Arabidopsis thaliana*, there exist five different homologs of Rpa1, two homologs of Rpa2, and two homologs of Rpa3 (ESCHBACH and KOBBE 2014). In humans, Rpa4; a homolog of Rpa2 forms an alternative RPA complex (KESHAV *et al.* 1995a). The canonical form supports DNA replication while the alternative form does not (MASON *et al.* 2009; HARING *et al.* 2010a). But the alternative RPA complex is functional in repair and recombination (KEMP *et al.* 2010a). The antagonistic functions of these two complexes are not well understood. In this chapter I describe my attempts to develop tools to study the antagonizing function of canonical and alternative RPA complexes and the species-specific function of the Rpa2 N-terminus.

Yeast – an ideal model to study RPA

Due to the complex nature of human cells and intrinsic difficulties in studying mutant forms of a protein and its long-term effects (beyond 1-2 generations), it is somewhat difficult to study RPA in human cells. Budding yeast on the other hand is easy to maintain and has a number of advantages as a model system, including:

- Rapid growth rate, non-pathogenic nature, and comparatively smaller genome size.

- Growth as dispersed cells facilitating replica plating and mutant isolation.

- Highly efficient recombination machinery that makes yeast cells a great vehicle for gene cloning and manipulation of the genome.

- Cells that exist as haploids or diploids, making them a good tool for genetic studies and recombination analysis.

- A large-range of genetic techniques developed in yeast, such as yeast two-hybrid analysis, that can help to understand protein function better.

History and Hypothesis

We wanted to develop a model yeast system in which the human RPA functions in yeast cells as a sole source of the ssDNA-binding complex. This system would be ideal to study human RPA, as it would provide a molecular toolbox not available for tissue culture, and many of the results could be extrapolated to human cells. It is evident from literature that such model system has been very useful to study certain proteins; an example would be studies with mitochondrial DNA polymerase (QIAN *et al.* 2014).

However this was not the first attempt to clone, express, and examine function of a human RPA subunit in yeast. Human Rpa2 has been expressed in yeast cells where the endogenous *RFA2* gene had been deleted (*rfa2Δ::TRP1*); however, the human protein did not allow for cell viability (*i.e.*, human Rpa2 did not complement the *rfa2* defect) (BRILL and STILLMAN 1991). A similar attempt has been made by replacing yeast *RFA1* with human Rpa1; this was not successful (PHILIPOVA *et al.* 1996). Although these attempts indicate that individual human RPA subunits may not be able to support yeast cellular processes, we hypothesize that this is due to lack of formation of full RPA complex containing both yeast and human RPA proteins.

The canonical and alternative forms have antagonistic functions to one another. Furthermore, given that Rpa4 is intronless and lies on the X chromosome, it has been proposed that Rpa4 and Rpa2 may have important functions in different stages of development. Expression of canonical RPA is important in cell proliferation. In fact increased expression of Rpa1 and Rpa2 is correlated to various cancers (KANAKIS *et al.*

27

2011) (LEVIDOU *et al.* 2011). On contrary, Rpa4 is expressed at different levels in different tissues examined and its expression is reduced in cancerous tissues (KESHAV *et al.* 1995b; KEMP *et al.* 2010b).Hence we hypothesize that the cellular ratio of Rpa2/Rpa4 is a deciding factor towards cell proliferation or quiescence (Figure 2.1).
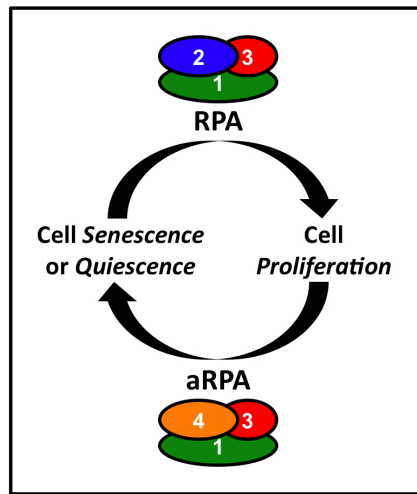
Figure 2.1: Model of ratio Rpa2/Rpa4 determination cellular fate. The canonical RPA complex consists of Rpa2 (represented by blue) while the alternative RPA complex (aRPA) consists of Rpa4 (orange). The canonical RPA supports cell proliferation while aRPA supports quiescence.

An effective technique to examine the importance of expression levels of a gene is through manipulation of its expression. In one such study, depletion of RPA in HeLA cells has been shown to slow S phase progression, G2/M cell cycle arrest through ATM activation, and apoptosis (DODSON *et al.* 2004). Rpa2 was found to be up-regulated in response to DNA damage by MMS in yeast (KIM *et al.* 2011). Elevated Rpa2 expression were correlated with survival of BRCA1 tumors (BOULEY *et al.* 2010). These data indicate that the manipulation of RPA expression is useful in depicting its function. In order to study our central hypothesis (Figure 2.1) we decided to manipulate RPA

28

expression by developing siRNA to target RPA subunits. We also worked on a TET-ON system to target overexpression of RPA subunits.

This chapter discusses development of tools to study effect of the RPA subunit expression on cancerous human cell lines and yeast. We also studied the compatibility of the human RPA complex in budding yeast cells.

Materials and Methods

Strains, cell lines, and media

The yeast strains used in these experiments are RMY122-A and EGY48. The RMY122-A (Appendix A) is a haploid strain with a chromosomal deletion of *RFA1* and *RFA2*. These deletions are supported by supplementary plasmids expressing wild-type copies of *RFA1* and *RFA2* driven from their native promoters. This strain was utilized in plasmid shuffle assays. The EGY48 (Appendix A) strain is utilized in yeast two-hybrid assay and was also used for *in vivo* cloning by homologous recombination. YPD media is rich non-selective growth media that contains 2% dextrose, 1% yeast extract, and 2% peptone. Synthetic complete media contained 2% sugar (dextrose or galactose; SD or SG), 0.5% ammonium sulfate, and 0.17% yeast nitrogen base without amino acids. This media was then supplemented with the appropriate dropout amino acid supplement. YPG media was similar to YPD except it contained 3% glycerol instead of 2% dextrose.

The different types of human cell lines utilized in the development and study of human RPA subunit expression were HEK293T, HT29, and DLD1. The HEK293T cell line was maintained in DMEM medium (10% FBS) in 5% CO_2 at 37°C. The HT29 cell line was maintained in McCoy's 5A medium (10% FBS) in 5% CO_2 at 37°C. The DLD-1 cell line was maintained in RPMI medium (10% FBS) in 5% CO_2 at 37°C.

Polymerase chain reaction and cloning

The primers designed for amplifying different RPA genes from the template pEGFP-RPA plasmid series were unique to incorporate ends homologous to pJM132 to develop a vector expressing all RPA genes (primers O-1 to O-10 Appendix B). Two micrograms (2 μg) of pJM132 plasmid DNA was digested with an appropriate restriction enzyme in a total of 20μl reaction. About 1/3 of the confirmed PCR product and 10 μL of digested DNA were co-transformed into EGY48 cells. The cells were then plated onto SD-Ura (lacking uracil) media and allowed to grow at 30°C for 3 days. A scrape of multiple colonies from these plates was used to start a culture in SD-Ura liquid media. The liquid culture was grown at 30° C overnight and was utilized to make genomic prep by glass bead disruption method.

The pEGFP-RPA plasmid series was also used as a template to amplify RPA genes with or without GFP tag to clone into pLVX-Tight-Puro1 using traditional cloning. The shRNA oligos (primers O-101 to O-114 Appendix B) were ordered from Integrated DNA Technologies (IDT; Coralville, IA) and were allowed to anneal to form a double-stranded insert by mixing the two strands at equimolar concentrations and in STE buffer (100 mM Tris•Cl pH 7.5, 500 mM NaCl, 10 mM EDTA pH 8.0). The shRNA for Rpa1 and Rpa2, and Rpa3 were the same as used in previous studies (HARING *et al.* 2010b). Four different shRNA sequences were designed for Rpa4 knockdown using the online tool available through Dharmacon. These were then cloned in pLVX-shRNA1 or pLVX-shRNA2 from Clontech Laboratories (containing a ZsGreen gene to allow for detection in cells) by traditional ligation cloning.

Construction and isolation of RPA containing plasmid

pJM132, a plasmid containing the yeast *RFA1*, *RFA2*, and *RFA3* genes was stepwise digested and used to clone in each of the corresponding human RPA subunit genes. The first step was to replace *RFA2* with either *Rpa2* or *Rpa4*. To do this, pJM132 was linearized with *Hpa*I and co-transformed with *Rpa2* PCR fragment consisting of ends homologous to pJM132. The successful recombination events were selected on SD-Ura media and genomic/plasmid DNA was isolated from multiple yeast colonies. The DNA was then transformed into DH10B cells. Using colony cracking followed by restriction digestion with *Bgl*II, we screened bacterial colonies and selected appropriate positive constructs. The constructs were further confirmed by DNA sequencing (Eton Biosciences).

Table2.1: A list of restriction enzymes used to linearize and confirm constructs in the construction of pJM132 with the RPA subunit genes.

Gene	Plasmid	RE to Linearize	Gene Replaced	Diagnostic RE
Rfa1	pJM132	AatII	Rpa1	XhoI
Rfa2	pJM132	HpaI	Rpa2	BglII
Rfa3	pJM132	MscI	Rpa3	MscI
Rfa2	pJM132	HpaI	Rpa4	EcoRI
Ura3	pJM132	SbfI and NsiI	G418^{r}	BstZ17I

This plasmid was used as vector backbone to replace yeast *RFA3* with human *Rpa3*, followed by replacement of yeast *RFA1* with human *Rpa1*. An example of colony cracking followed by *Xho*I digest to confirm Rpa1 insertion in pJM132-Rpa2+3 is shown

31

in Figure 2.2. All of the enzymes used for linearizing and diagnosis of correct insertions

are listed in Table 2.1. Finally, once all three yeast genes had been swapped with their

corresponding human genes, the G418 selectable marker was inserted to replace the

URA3 marker originally on the plasmid. This helped us switch from screening for the loss

of pJM132 to being able to select for it. This stepwise replacement method helped us

build a plasmid expressing either the canonical or alternative RPA complex with G418 as

a selectable marker.
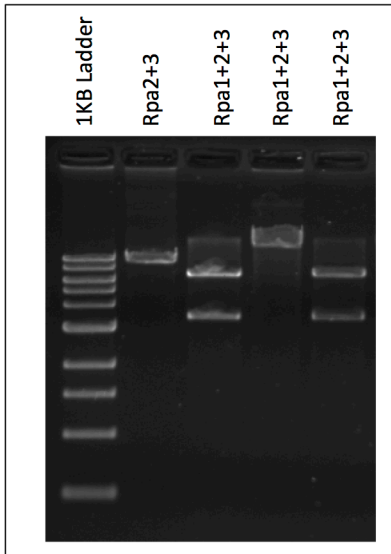
Figure 2.2: Agarose gel XhoI digest confirmation of Rpa1 insert in pJM132-Rpa2+3 vector. The Rpa2+3 is the control lane whereby the linearized plasmid is ≈ 11Kbp. The expected pattern after Rpa1 insertion is a ≈ 3.5Kbp and 8.5Kbp fragment. Hence candidates in lane 3 and 5 are successfully cloned.

Construction of human-yeast *rfa2* N-terminal mutants

Human-yeast *rfa2* N-terminal mutants were constructed by *in vivo* homologous

recombination of synthesized gene fragments into the plasmid vector pRS315-*rfa2-ΔN$_x$*.

This plasmid expresses an *rfa2* gene that is missing the N-terminal coding sequence. This

vector was linearized using restriction enzyme *Hpa*I. The Rfa2 N-terminal (2NT) coding

sequences from various organisms were commercially ordered as gBlocks (IDT). These gBlocks not only had the required 2NT sequences from various organisms, but also flanking regions homologous to the vector to facilitate homologous recombination cloning. About 250 ng of vector and 50 ng of gBlock were co-transformed into EGY48 cells. Cells where the appropriate recombination event had potentially occurred were recovered on SD-Leu (lacking leucine) plates.

A scrape of multiple colonies was used to inoculate overnight yeast culture to isolate yeast genomic DNA. One microliter (1μL) of genomic DNA was transformed in to DH10B cells using electroporation. Cells were plated on LB+Amp plates. The plasmids were isolated from bacterial cells (colony cracking), and correct clones were confirmed using diagnostic restriction digest with restriction enzyme (*Ssp*I). The clones were further confirmed by sequencing from Eton Biosciences (San Diego, CA).

Detection of correct clones by colony cracking method

Individual colonies were used to inoculate overnight bacterial cultures. One milliliter (1 mL) of this culture was pelleted, and the pellet was dissolved in 50 μL of 1x lysis buffer (2 mg/mL lysozyme, 15% sucrose, 0.2 mg/mL DNase-free RNase A, 10 mM Tris•Cl pH 8.0, 1 mM EDTA pH 8.0, 0.1 mg/mL BSA). This cell/lysis buffer mixture was boiled at 100°C for 90 sec, followed by centrifugation at 15,000 RPM for 10 min. The clarified supernatant was used to set up a diagnostic restriction digest using appropriate restriction enzyme (Table 2.1). The correct inserts were further confirmed by sequencing (Eton Bioscience).

Plasmid shuffle assay using RPA expression vectors

A plasmid containing all human RPA subunits and a G418 selectable marker was transformed with LiAc/PEG method into RMY122-A cells (GIETZ and WOODS 2002). The cells were plated on YPD+G418 media to select for transformants containing this plasmid. A YPD+G418 overnight culture was inoculated with scrape of these cells and grown at 30°C. The culture was again plated on YPD+G418 plate to isolate single colonies. A master plate was picked from these plates onto a YPD+G418 plate, and this master plate was used for replica plating onto YPD, YPG, and 0.8 μg/μl of 5-FOA (to select for cells that can lose the original pJM132 plasmid).

DNA damage assays

The colonies that have lost pJM132 were used to inoculate an overnight YPD culture incubated at 30°C. The next day cultures were sonicated five times at 20% power (0.5 sec on, 1 sec off) and were counted counted using a cellometer (Nexcelom). The concentration was adjusted to $1(10)^7$ cells/mL followed by 1:10 serial dilutions. Five microliters (5 μL) of each of the dilutions were spotted in a row on different DNA damaging agent-containing plates to test sensitivity (Refer Table 2.2)

Table 2.2: DNA damaging agents used in studies

DNA damaging agent	Concentrations used	Mode of DNA damage
Methyl methanesulfonate (MMS)	0.015%-0.12%	Alkylating agent
Camptothecin (CPT)	0.2-25 μg/mL	Topoisomerase I inhibitor
Phleomycin (PHL)	0.2-25 μg/mL	DNA intercalating agent
Hydroxyurea (HU)	40-320 mM	Depletion of dNTPs causes replication fork stalling resulting in replication stress

Yeast two-hybrid assay

Human Rpa1 was cloned into pEG202 (Appendix C) to express lexA N-terminally tagged Rpa1. This is referred to as the 'bait' construct. This cloning was performed using homologous recombination where pEG202 was linearized with *Nco*I and Rpa1 was amplified using primers O-260 and O-261 (Appendix B). All three yeast subunits and all four human RPA subunits were cloned into pJG4-5 vector to express B42 N-terminally tagged proteins. These are referred to as "prey" constructs and were generated by *in vivo* homologous recombination by Erica Mueller. About 200 ng of bait, prey, and reporter/supplementary plasmid were co-transformed into EGY48 cells and isolated on SD-HTU (lacking histidine, tryptophan, and uracil) plates to select for transformation of all three plasmids. Individual colonies were picked as patches on SD-HTU media. These patch plates were then replica plated onto YPD, YPG, SD-HTU, SG-HTU, SD-HLTU, SG-HLTU and SG-HTU+X-gal plates. Protein interaction was determined by growth on SG-HLTU plates after replica plating. The development of blue color on media containing X-gal was used as indicator of positive interaction.

Transfection of cell lines and flow cytometry analysis

Three micrograms (3 μg) of the shRNA vector was mixed with 15 μL of Lenti-X HT Packaging mixand was transfected into HEK293T cells using LentiPhos (Clontech), as per manufacturer's instructions. Lipofectamine 2000 (Invitrogen) was also used for transfection of 3 μg of vector into HEK293T cells, as per manufacturers instructions. Lentivirus was collected 2 days post-transfection.

Different volumes of the virus (0 μL, 25 μL, and 500 μL) were used for transduction (with 2 μg/μL of polybrene) into HEK293T or HT29 cell lines. The cells

were observed for 6 days for increase in the number of green cells and on the 6th day, the cells were harvested for flow cytometry. Cells were collected by trypsinization, rinsed with 1x PBS, centrifuged, and treated with RNase A for one hour. The cells were divided into two aliquots: one was used for propidium-iodide (PI) staining and the other was used for detection of ZsGreen protein. The samples were examined through an Accuri flow cytometry using the FL1-A channel for green fluorescence and the FL2-A channel for red fluorescence (PI staining for DNA content). About 50,000 events were counted, and only healthy cells (as determined by forward and side scatter (FSC and SSC, respectively) were analyzed for ZsGreen expression or DNA content.

Protein extraction, gel electrophoresis, and western blotting

The protein expression of RPA and RFA subunits was measured using western blot analysis. The RMY122-A strain (with pJM132) served as control for this experiment. The RMY122-A (control) cells were grown for 16 hr in YPD at 30°C, while the RMY122-A cells expressing canonical RPA complex (+123) and alternative RPA complex (+143) were grown in YPD media containing G418 to retain the RPA-expressing plasmids. Cultures were maintained in log (exponential growth) phase and protein was isolated using the Kushirnov method (REF). Cell concentrations were measured using a cellometer (Nexcelom), and approximately $5(10)^7$ cells were collected. The collected cells were treated with NaOH for 10 min, pelleted, and the supernatant was removed. The cell pellet was then dissolved in Kushnirov (modified SDS sample loading) buffer, followed by incubation at 100°C for 3 min. Cellular debris was pelleted, and the supernatant was collected. Five microliters (5 μL) of each sample was loaded for detection of RFA and RPA proteins.

For detection of Rpa2 and Rfa2 a 12% SDS PAGE gel was used, while for Rfa1 and Rpa1 protein a 6% SDS-PAGE gel was used (both percentage gels were run at constant current). The proteins were transferred to a 0.4 μm nitrocellulose membrane at 40 mA for 15 hr. The Rfa1 and Rfa2 protein were detected using rabbit polyclonal antibody (1:40,000 and 1:20,000, respectively). These antibodies were kindly provided by Dr. Steve Brill (Rutgers University). Rpa2 was detected by rabbit polyclonal antibody (N2.2; kindly provided by Dr. Marc Wold) to human Rpa2 using a 1:5,000 dilution. Rpa1 was detected using 1:1,000 dilution of mouse monoclonal antibody against human Rpa1 (ab176476; Abcam).

Detection of RPA and RFA mRNA expression using quantitative PCR

For the isolation of total cellular mRNA, RMY122-A cells were grown in YPD for 16 hrs at 30°C. RMY122-A expressing canonical RPA or alternative RPA were grown in YPD+G418 media. The exponentially growing 1.5 mL culture was pelleted, and the mRNA is isolated using YeaStar RNA Kit (Zymo Research) followed by genomic DNA cleanup using DNA-free RNA kit (Zymo Research). The isolated RNA was then quantitated using a Nanodrop (Thermo), and 1 μg of RNA was used to synthesize cDNA utilizing the AMV First-Strand cDNA Synthesis Kit (New England BioLabs). The primers to detect RFA subunit expression were designed using PrimerQuest (IDT) and ordered from IDT, and primers for detection of RPA subunit were designed using Primer Express (ABI) and ordered from IDT. The *UBC6* gene was used as normalizing control for qPCR, and the primer sets are as shown in Table 2.2. The PCR was performed using PerfeCTa SYBR Green FastMix Low ROX (Quanta Biosciences), and the reactions were

carried out on AB7500 Real-Time PCR (Applied Biosystems) machine. The data were

analyzed using Sequence Detection System software v1.2 (Applied Biosystems).

Results

RPA complex is unable to support normal RFA cellular function
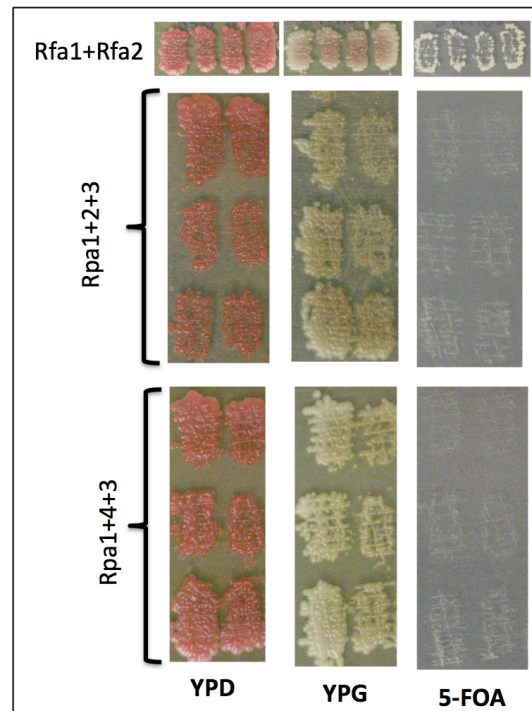
Figure 2.3: A replica-plating assay testing survival of candidates expressing canonical and alternative RPA complex in yeast. The WT RFA expressing cells are shown at the top row, the middle row shows six candidates expressing canonical RPA complex and the last row shows six candidates expressing alternative RPA complex.

The aim of this study was to replace the yeast RFA complex with human

canonical RPA or human alternative RPA, so that the yeast cells might serve as a tool to

study human RPA function. The RMY122-A cells containing either canonical RPA or

alternative RPA complex were grown in YPD+G418 media to saturation at 30°C. The

cells containing *RFA1* (*HIS3* plasmid) and *RFA2* (*LEU2* plasmid) served as positive

control for the ability of cells to lose pJM132 (*URA3* plasmid). The cells were patched

onto YPD+G418 plates and were replica plated to YPD, YPG, and 5-FOA media. Survival on 5-FOA plates was indicator of loss of pJM132, and only cells where the substituted form of RPA can take the place of yeast RFA will be able to lose pJM132.

As shown in Figure 2.3, the cells containing canonical RPA and alternative RPA complex were unable to survive on 5-FOA plates. This indicates that the cells were not able to lose pJM132 containing the yeast RFA complex. The cells were able to grow on both YPD and YPG indicating that the inability to grow on 5-FOA was not due to defective cellular function or defective mitochondria, respectively.

RPA is expressed at slightly-to-moderately lower levels compared to RFA in yeast cells

The success of the shuffle out reactions depends on two things. First, the human genes should be expressed at the mRNA level similarly to the yeast RFA genes. In order to optimize the likelihood that they are expressed at similar levels, we cloned the human RPA genes downstream of the native yeast RFA promoters. Second, the translation of this mRNA must occur similarly for both human and yeast RPA genes. It was possible that levels of translation could be affected due to codon bias in yeast *vs*. humans.

Using qPCR, we quantified the mRNA expression of all RPA subunits present in the yeast cells. The RMY122A cells served as the negative control for this experiment, as these cells did not express any human RPA genes. The 'canonical RPA' expressing cells (denoted cRPA) and the 'alternative RPA' expressing cells (denoted aRPA) express human *Rpa1* and *Rpa3*, with the difference being that cRPA cells express *Rpa2* and aRPA cells express *Rpa4*. The RNA isolation and cDNA synthesis was performed as described, and the amount of RNA used for the production of cDNA was the same for all

samples. As expected, the RMY122-A cells do not show any measurable human RPA

subunit expression. As seen in Figure 2.4, only cRPA cells express *Rpa2* and only aRPA

cells express *Rpa4*. In either cRPA- or aRPA-expressing cells, the middle subunit (*Rpa2*

or *Rpa4*) is expressed at high levels as compared to the *Rpa1* or *Rpa3*. Also, the

expression of all genes was higher in cRPA cells; we suspect this was partially due to

genomic DNA contamination as indicated in the (-) reverse transcriptase control (not

shown).
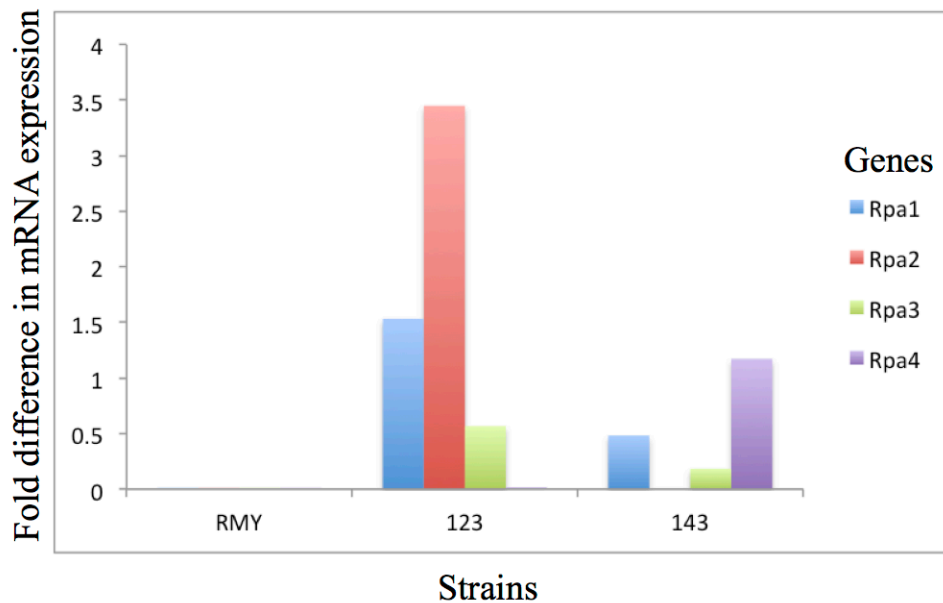
**Figure 2.4: A chart showing the quantification of expression of various human RPA
subunits in various strains using qPCR.** The RMY cells serve as a negative control and
only express yeast RFA subunits. The 123 cells express canonical RPA and hence do not
show any Rpa4 expressed (purple). The 143 cells should only express 'alternative RPA
complex' and hence do not show expression of Rpa2 (red).

We next tested the expression of yeast RFA genes, and as expected all cell types

showed expression of *RFA1*, *RFA2,* and *RFA3* mRNA (Figure 2.5). The *RFA3* expression

observed is probably higher than in other yeast strains due to these cells having two

copies of this gene (one on pJM132 plasmid and one chromosomal copy). Also, Rfa2

expression is higher compared to *RFA1*, indicating that these cells naturally produce

more *RFA2* mRNA. Therefore, it is not surprising that for our human RPA subunit gene

expression, we also always observed increased *Rpa2* or *Rpa4* expression compared to the

other human RPA genes, as *Rpa2* and *Rpa4* are driven by the native *RFA2* promoter

(Figure 2.4).
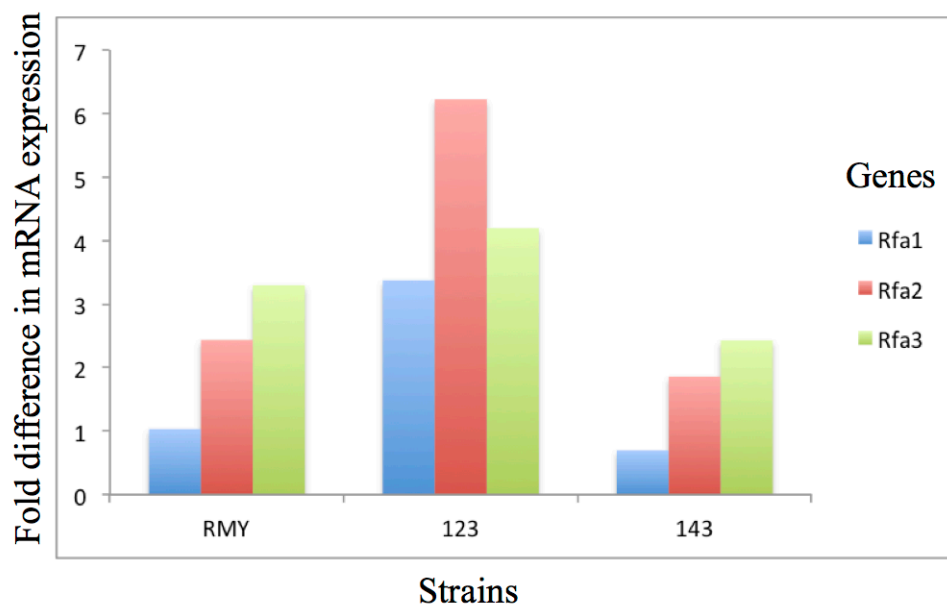
Figure 2.5: A chart showing quantification of expression of RFA subunits in various cells using qPCR. This chart shows that all the three strain types used in this experiment show expression of RFA subunits. The Rfa2 is always expressed more than Rfa1 subunit.

We further calculated whether the human RPA subunit genes are expressed at

similar to the yeast RFA subunit genes. In Figure 2.6, human RPA subunit mRNA

expression is compared to its yeast homolog. *Rpa1* and *Rpa2* are expressed at ~50% of

the levels measured for *RFA1* and *RFA2*, respectively. The lowest relative level was

measured for human *Rpa3* mRNA, which was expressed at about 10% compared to yeast

RFA3. Considering that the cells also have chromosomal copy of *RFA3*, we can estimate that the comparative expression level of human *Rpa3* to yeast *RFA3* mRNA is about 20%. Given that human subunit mRNA expression levels are only 20-50% of the yeast subunit mRNA expression levels, it still remains possible that lack of complementation (function) of human RPA in yeast cells is due to reduced expression.
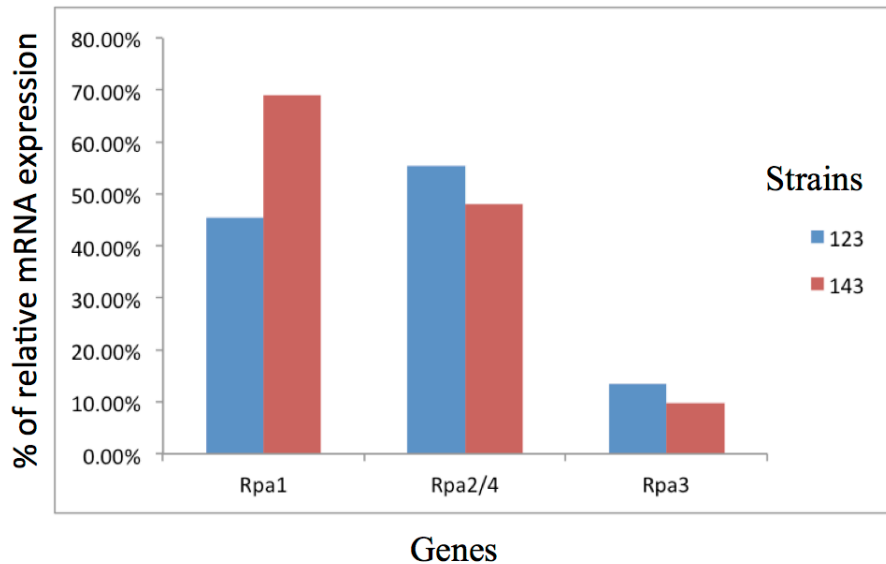
Figure 2.6: A chart displaying the percentage of human RPA subunit expression as compared to its yeast counterpart. The chart here displays the expression of RPA subunits in RPA1+2+3 and RPA1+4+3 cells compared to expression of RFA complex subunits in RMY122A cells. The Rpa2/4 is showing expression of Rpa2 in 1+2+3 cells compared to Rfa2 expression and the expression of Rpa4 in comparison to Rfa2 expression.

The human Rpa1 and Rpa2 proteins are translated and stable in yeast

As described previously, codon bias in yeast *vs.* human cells might interfere in the overall translation and stability of human RPA proteins in yeast. To determine if human RPA subunits were translated and stable in yeast cells, western blotting was utilized. Yeast Rfa1 and Rfa2 proteins are detected at similar levels in all three strains (without or with human cRPA or aRPA) utilized in this assay. This demonstrated that *RFA* mRNA

translation into protein or yeast protein stability is not affected by the presence of human

proteins. Furthermore, human Rpa1 was detected in both cRPA- and aRPA-expressing

yeast cells (Figure 2.7), indicating that the protein is translated and that it is stable.

Although appearing slightly lower in cRPA-expressing cells, human Rpa1 protein is still

observed. A more quantative assessment of protein expression was not performed.
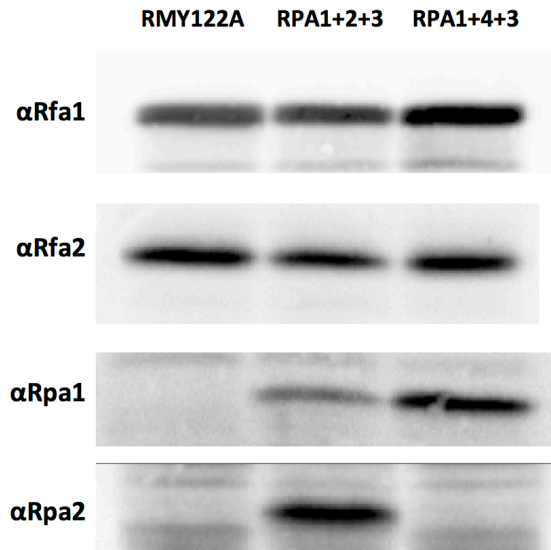
**Figure 2.7: A western blot assay showing expression of different RFA and RPA
subunit proteins in various yeast strains used in this study.** The first column
represents protein from WT cells (RFA only). The second column shows proteins from
1+2+3 cells (RFA+ cRPA). The third column shows proteins from 1+4+3 cells (RFA+
aRPA). The α stands for 'anti' and represents anti-protein antibody.

It has been demonstrated in human cells that Rpa1 is not stable without Rpa2 protein

expression. Therefore, one would predict that Rpa2 (or Rpa4) protein expression would

also be observed. Rpa2 is expressed only in the cRPA-expressing strain as expected, and

the protein appears stable. Rpa3 and Rpa4 protein expression could not be confirmed via

western blotting, as there are currently no reliable antibodies available for either. An

Rpa3 antibody was purchased from Abcam (ab 37679); however, reliable detection of

Rpa3 protein was not observed (data not shown). At this point, we cannot state whether

the failure of pJM132 to shuffle out is due to lack of proper RPA subunit expression or lack of proper protein translation (and folding).

The Rpa1 protein is unable to interact with yeast RFA subunits in the two-hybrid assay

Replication Protein A is involved in vital processes and it forms a strong complex that is stable in 6 M urea (WOLD and KELLY 1988) (FAIRMAN and STILLMAN 1988) (BRILL and STILLMAN 1989). This stability indicates that the complex needs all the subunits of the complex to carry out its function. This is why simultaneous replacement of all three subunits was attempted. Thus far, we have determined that the human RPA is unable to replace the yeast RFA complex (Figure 2.3). Another approach mentioned previously to study human RPA in yeast was to replace only one subunit and study its function, and as mentioned, these attempts have been unsuccessful (PHILIPOVA *et al.* 1996). The question of complex formation between yeast and human RPA subunits has been examined somewhat examined *in vitro* by the ability to purify a soluble hybrid RPA complex (MSW, unpublished results). We asked whether inter-species interactions could occur in yeast cells using the two-hybrid assay. In this assay we used Rpa1 as bait and tested its ability to interact with yeast RFA subunits. We also tested Rpa1 interaction with other human RPA subunits to understand complex formation and as a positive control for two-hybrid assay results.

Rpa1 is unable to interact with any of the yeast RFA subunits (Figure 2.8). In this assay we see normal growth SG-HTU indicating all plasmids are present in the cells and expression does not affect the viability of the cells. However, the lack of growth on SG-HLTU indicates lack of interaction between Rpa1 and yeast RFA subunits. Also, there

44

was no β-galactosidase activity detected on SG-HTU+Xgal plates, further supporting lack

of interaction.
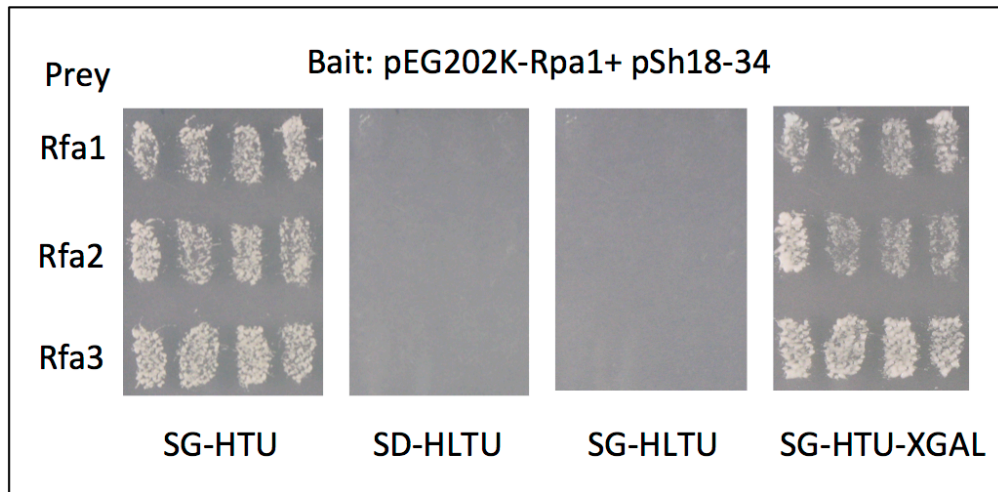
Figure 2.8: Replica plating assay with BD-Rpa1 to test interaction with the RFA complex subunits. Rpa1 does not interact with Rfa1, Rfa2 or Rfa3 in yeast two-hybrid assay. The replica plating is performed using dextrose or galactose (SD or SG) containing media lacking His (H), Trp (T), Ura (U) and Leu (L). The β-galactosidase activity is tested on media containing xgal (SG-HTU+XGAL).

Rpa1 does not interact with Rpa3 in two-hybrid assay

We also tested the Rpa1 interaction with human RPA subunits. Generally this

complex is isolated as a soluble heterotrimer; however, it can also be purified as an Rpa2-

Rpa3 heterodimeric subcomplex. Yeast two-hybrid analysis yields similar results. Rpa1

can interact with Rpa2 and Rpa4 (as indicated by blue color on SG-HTU+X-gal plates)

and it does not form a dimer with itself (Figure 2.9). The interesting observation was the

lack of detectable interaction with the Rpa3 subunit. As discussed earlier although Rpa1

and Rpa3 are part of the same complex, it is possible that this is not a direct interaction

and requires a mediator (Rpa2 or Rpa4) for complete complex formation. To test this

idea, we examined the Rpa1 and Rpa3 interaction in presence or absence of Rpa2 or

Rpa4 (mediator) protein.
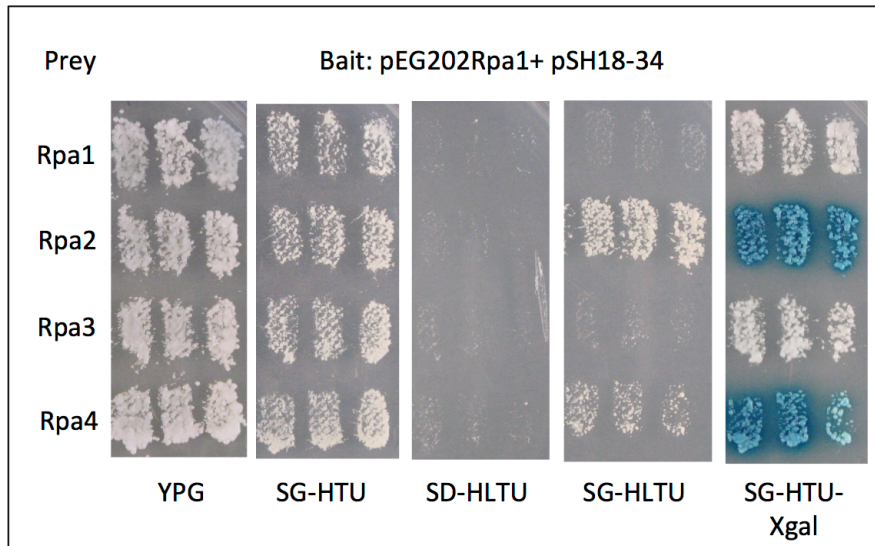
Figure 2.9: Replica plating assay with BD-Rpa1 to test interaction with the RPA complex subunits. Rpa1 bait interacts with Rpa2 and Rpa4 as prey in yeast-two hybrid assay. Rpa1 is not able to interact with Rpa3. The replica plating is performed using dextrose or galactose (SD or SG) containing media lacking His (H), Trp (T), Ura (U) and Leu (L). The β-galactosidase activity is tested on media containing xgal (SG-HTU+XGAL).

Rpa1 and Rpa3 are do not interact with each other in the absence of Rpa2 (or Rpa4)

When a supplementary plasmid is introduced to express either Rpa2 or Rpa4, growth on SG-HLTU (indicating interaction) was observed (Figure 2.10). This is complicated somewhat by the observation of autoactivation when Rpa2 or Rpa4 are expressed (Figure 2.10; rows 2 and 3). Rpa2 and Rpa4 have both shown autoactivation (data not shown) when present in the bait construct, and we propose that activation here might be a consequence of Rpa2 or Rpa4 interacting with BD-Rpa1. However, when Rpa2 and Rpa3 (or Rpa4 and Rpa3) are both expressed from the supplementary plamid, autoactivation is reduced (lack of growth on SD-HLTU), yet growth is observed on SG-HLTU. This would appear to indicate that Rpa1 and Rpa3 interact, but only when Rpa2 or Rpa4 are present.

46
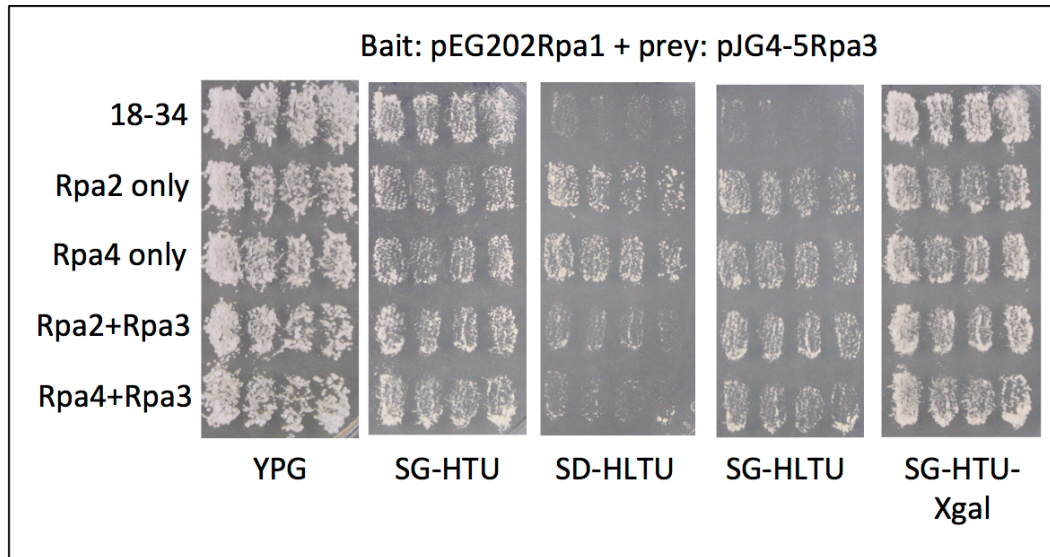
Figure 2.10: Replica plating assay for determination of Rpa1 and Rpa3 interaction in absence or presence of supplementary plasmid expressing either Rpa2 or Rpa4. The replica plating is performed using dextrose or galactose (SD or SG) containing media lacking His (H), Trp (T), Ura (U) and Leu (L). The β-galactosidase activity is tested on media containing xgal (SG-HTU+XGAL).

The Rpa2 N-terminus is interchangeable between species

The yeast two-hybrid data indicated that the reason behind the inability of any individual RPA subunits to support cell survival was a lack of inter-species interactions. While this study has also indicated that the full RPA complex is not able to support cell survival, it was shown in previous studies that individual domains (subregions) of yeast RFA (DBD-A and DBD-B, Figure 1.1) were replaceable with the human equivalent domains (PHILIPOVA *et al.* 1996). As discussed earlier, Rfa1 is the largest subunit with the ssDNA-binding properties and shows the most sequence similarity amongst species (WOLD 1997). Apart from Rfa1, Rfa2 is the next major subunit showing sequence similarity amongst species (ISHIAI *et al.* 1996).

A major focus of our lab is on the function of the Rfa2 N-terminus phosphorylation in DDR (described more in chapter 3). In chapter 3, I will describe how

47

the Rfa2 N-terminus is not detectably phosphorylated in response to DNA damage, unlike the human Rpa2 N-terminus in both human and yeast cells. Since we could measure human Rpa2 N-terminal phosphorylation in yeast cells (GHOSPURKAR *et al*. 2015b), we asked whether yeast might be a useful tool for measuring phosphorylation of other eukaryotic Rpa2 N-termini. To do this, we developed hybrid Rfa2-expressing constructs, where the N-terminus of the Rfa2 is replaced with the Rpa2 N-terminal sequence from other eukaryotic species. These will be referred to as Rpa2 N-terminus (2NT)-Rfa2 hybrids. In this study we have cloned in the Rpa2 N-termini from six different eukaryotic species (Table 2.3), including: *Mus musculus* (*Mm*), *Xenopus laevis* (*Xl*), *Arabidopsis thaliana* (*At*), *Orzya sativa* (*Os*), *Candida albicans* (*Ca*), and *Schizosaccharomyces pombe* (*Sp*). The plants *A. thaliana* and *O. sativa* have multiple Rpa2 subunits, which are referred to as *Rpa32A* and *Rpa32B* or *Rpa32-1*, *Rpa32-2*, and *Rpa32-3* in both organisms, respectively.

Table 2.3: A list of species whose Rpa2 NT phosphorylation was studied

Fungi	Plants	Animals
S. pombe	*A. thaliana* (Rpa2 A)	*X. laevis*
S. cerevisiae	*A. thaliana* (Rpa2 B)	*M. musculus*
C. albicans	*O. sativa* (Rpa2 1)	*H. sapiens*
	O. sativa (Rpa2 2)	

First, we tested the ability of these mutants to support cell survival in the unstressed condition (on rich media). In Figure 2.11, it is observed that all of the mutants can support viability of yeast cells; however, cells expressing the *Oryza sativa* (*Os*) Rpa2-2 N-terminus showed the lowest shuffle out rate (indicating some lack of function).

The overall shuffle out rate (ability to support cell growth) of the different *rfa2* hybrid 2NT mutants in increasing order is as follows:

Os2-2 < *Sp* < At2A and *Xl* < At2B and *Os*2-1 < *Ca* and *S. cerevisiae* (*Sc*).

It is notable that the N-terminus of homologs from the same species has a different effect on cell survival. For example, *Os*2-2 shows a defect in the ability for yeast cells to grow, while *Os*2-1 shows growth indistinguishable from WT budding yeast Rfa2. As mentioned in the introduction (chapter 1), there is the hypothesis that different homologs of RPA may participate in different cellular functions, and it is possible that this is a reflection of inhibition of Rfa2 function depending on the N-terminus that is present.
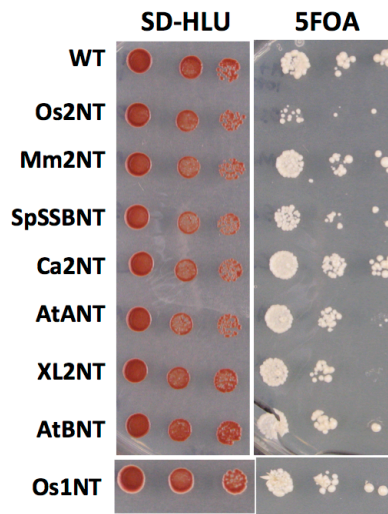
Figure 2.11: The shuffle out assay of 2NT-Rfa2-Hybrid mutants. The first column is to test the viability of cells with all three plasmids an SD-HLU (lacking his, leu and ura). The second column is to test ability of cells to lost WT Rfa2. The Os1NT and Os2NT represent two homologs from O. sativa. Similarly, the two homologs from A. thaliana are represented as AtANT and AtBNT

49
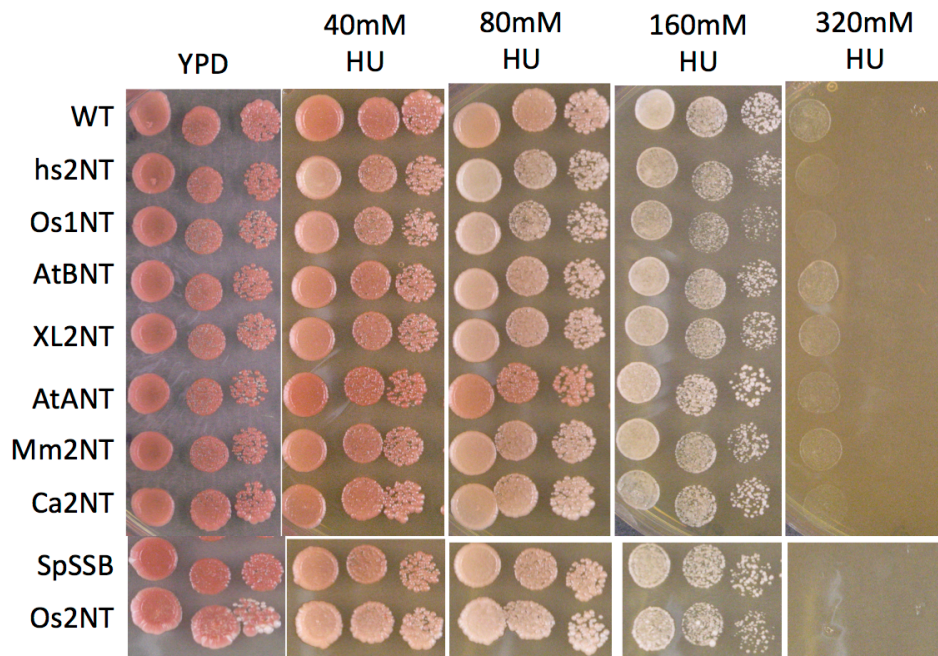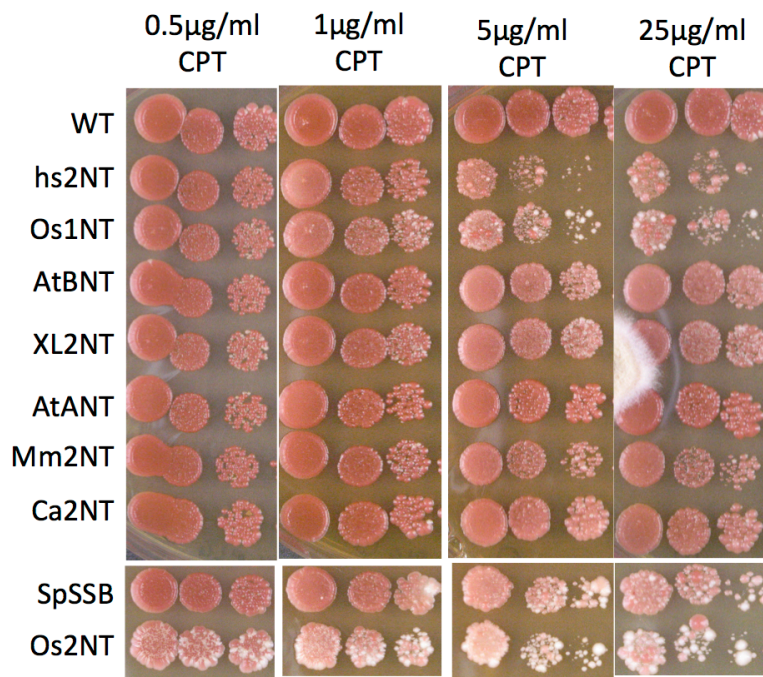
Figure 2.12: The DNA damage assay of various *rfa2* hybrid 2NT mutants. A) The DNA damage assay of *rfa2* 2NT hybrid mutants with increasing HU concentration B) The DNA damage assay of *rfa2* 2NT hybrid mutants with increasing CPT concentration.
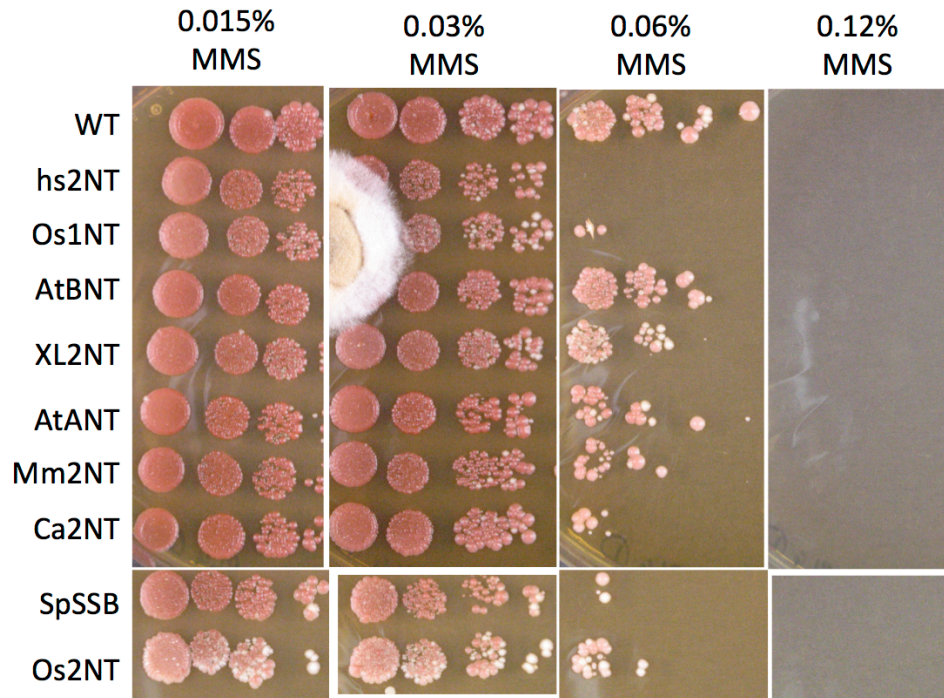
Figure 2.13: The DNA damage assay of *rfa2* hybrid 2NTmutants with increasing MMS concentration. The highest MMS concentration was toxic to cells and we did not recover any colonies however the difference in sensitivity is seen on the 0.06% MMS containing plates.

The *rfa2* hybrid 2NT mutants show varying responses to DNA damage

The Rpa2 N-terminus is phosphorylated in response to DNA damage in human cells (DUTTA and STILLMAN 1992) (DIN *et al.* 1990)**.** This leads to the proposed recruitment of RPA complex away from replication sites and to sites of DNA damage (VASSIN *et al.* 2004). Hence, the phosphorylation of Rpa2 N-terminus is an important event in DDR. A different study in our lab showed that the Rfa2 N-terminus phosphorylation in yeast appears to be at least partially dispensable DDR (chapter 3; GHOSPURKAR *et al.* 2015a). However, the Rfa2 N-terminus is required for the DDR.

Here we studied the *rfa2* hybrid 2NT mutants in presence of DNA damaging agents. We tested four different agents as listed in Table 2.2. As shown in Figure 2.12,

the Os1NT mutant was the most sensitive, although all of them, except *At*2B, show some degree of sensitivity to higher concentration of damaging agents (0.06% MMS and 25 μg/mL CPT). The human N-terminal hybrid (Hs2NT) served as a positive control in this assay. The order of sensitivity in increasing order is as follows

At2B< XL < At2A and Mm < Ca< Os2-1, Os2-2 and Sp <Hs2NT

The above order was based on the results observed in presence of MMS. The Os1NT, Os2Nt, SpSSB, Ca2NT, and Mm2NT also show sensitivity on the HU and CPT plates.

The yeast cells are able to phosphorylate the N-terminus region of different species

Phosphorylation of the yeast Rfa2 N-terminus was undetectable (chapter 3) in response to DNA damage. However, we have shown that when the Rfa2 N-terminus was swapped for the human Rpa2 N-terminus, this N-terminus was now detectably phosphorylated in response to DNA damage (GHOSPURKAR *et al.* 2015b). The significance of this remains undetermined. What this did demonstrate is that although the yeast cells do not detectably phosphorylate the Rfa2 N-terminus in response to DNA damage, they do possess the capability (*i.e.*, kinases) to phosphorylate an amino acid sequence on the N-terminus. Therefore, yeast cells are a potential tool for studying Rpa2 N-terminal phosphorylation.

As shown in Figure 2.14, yeast Rfa2 from WT cells does not show any detectable phosphorylation in presence of phleomycin. However, the yeast cells are able to phosphorylate the human Rpa2 N-terminus (hs2NT). When we examined protein isolated from the *rfa2* hybrid 2NT mutants in the absence or presence of DNA damaging agents, the observation of slower migrating species indicated that except for *Os*2NT, the

remainder of the hybrid proteins are phosphorylated in response to DNA damage. When homologs from the same species are not phosphorylated, it is consistent with the idea that these homologs may be carrying out different functions in cells. The data presented here is from two of three independent experiments with three independent colonies. We found similar results in all three sets. Since RPA antibodies detecting RPA subunits from different species often do not cross react (WOLD 1997), this system allows for anti-Rfa2 polyclonal antibody, to recognize the yeast Rfa2 portion of the *rfa2* hybrid 2NT proteins.. As observed in Figure 2.14, the intensity of phosphorylation and number of phosphorylated species produced varies from species to species. Also, in *Os*1NT and *At*BNT samples we observe that the undamaged sample displays a detectable amount of phosphorylation. This is not unusual as human Rpa2 is phosphorylated during the cell cycle, in addition to after DNA damage. The intensity of phosphorylation often increases in response to DNA damage in most of these samples. The Hs2NT served as a positive control, and the Mm2NT samples were expected to show phosphorylation similar to human Rpa2 NT, as mouse and human Rpa2 NT have highest sequence similarity (95%) (NAKAGAWA *et al.* 1991).

A)

B)

C)
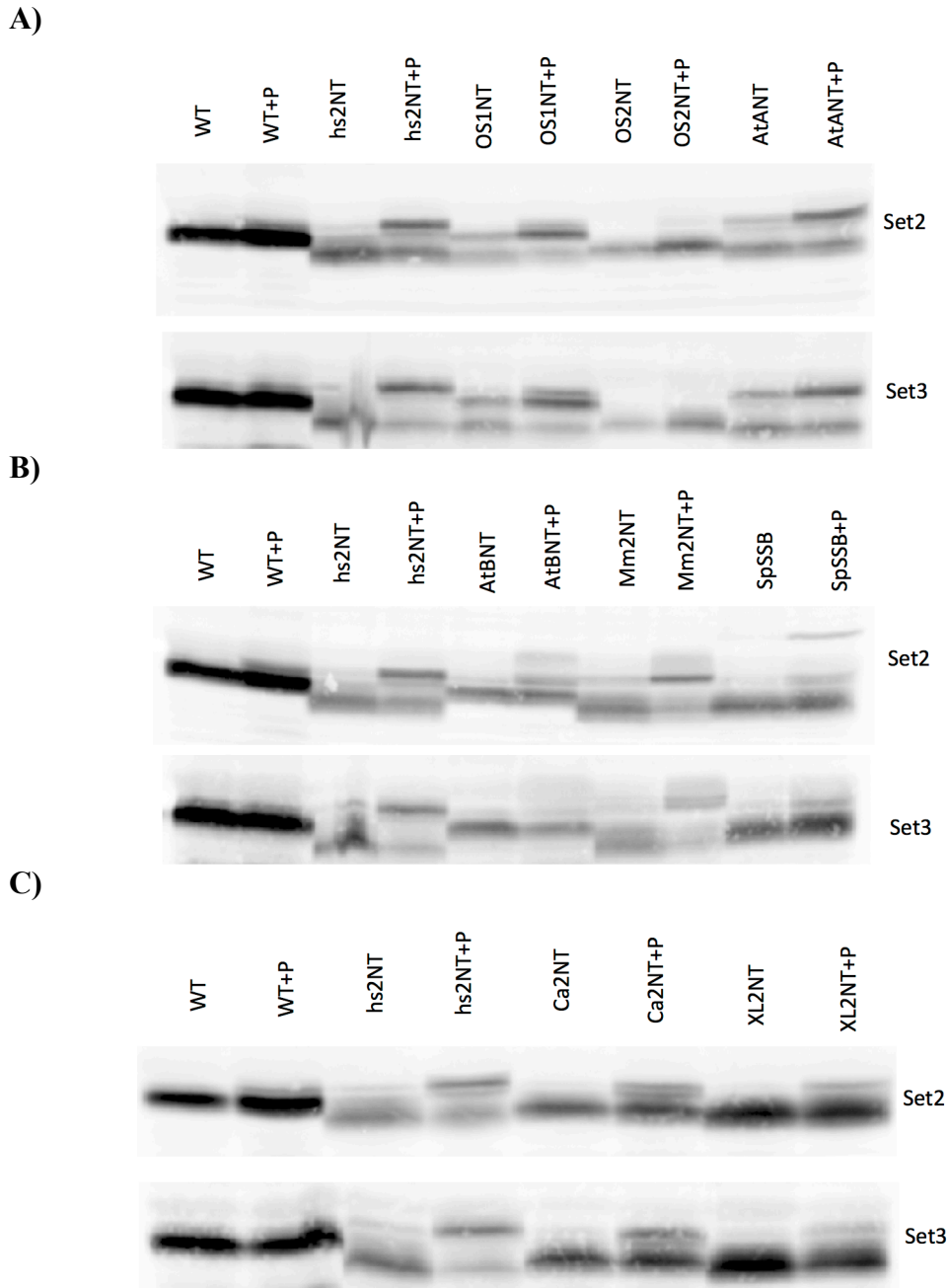
Figure 2.14: Western blots to detect the phosphorylation pattern of *rfa2* hybrid 2NT mutants. A) The western blot of 2NT-Rfa2-Hybrid mutants showing the phosphorylation of two different homologues in *Oryza Sativa* and *Arabidopsis thaliana*. B) The western blot of 2NT-Rfa2-Hybrid mutants showing the phosphorylation of *Arabidopsis thaliana Mus Musculus* and *Schizosaccharomyces Pombe*. C) The western blot of 2NT-Rfa2-Hybrid mutants showing the phosphorylation Rpa2 N terminus of *Candida Albicans* and *Xenopus Laevis* in the yeast cells.

Lipofectamine 2000 increases transfection efficiency of Rpa4-shRNA lentivirus

Attempts were made to study human Rpa2, and particularly Rpa4, in the context of human cells. The first step towards testing the effect of Rpa4 knockdown on cells was the production of Rpa4 shRNA-expressing lentivirus. We tested two different transfection reagents to transfect HEK293T cells: (1) LentiPhos and (2) Lipofectamine 2000. Generally, we observed that Lipofectamine 2000 was more efficient for transfection of cells as compared to LentiPhos (Figure 2.15). Non-transfected cells served as a negative control for this experiment.
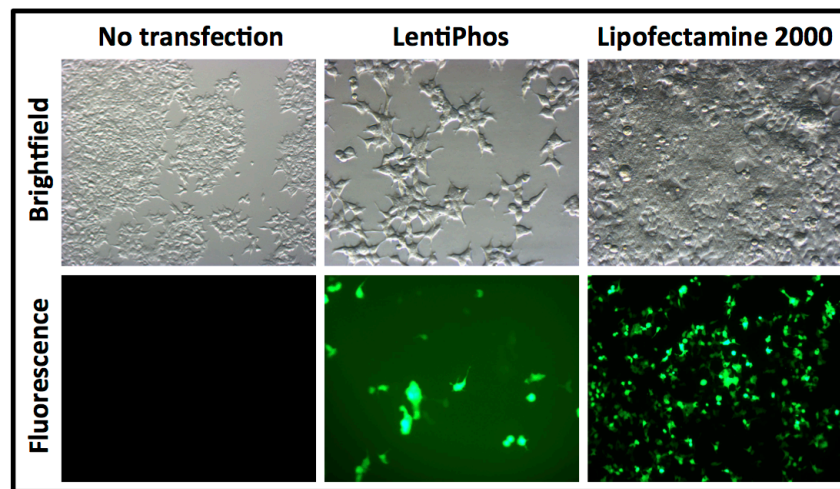
Figure 2.15: Transfection of HEK293T cells with Rpa4 shRNA containing lentivirus using different transfection reagents. The transfection efficiency of the Rpa4 shRNA containing lentivirus was increased with use of Lipofectamine 2000.

The transduction efficiency of Rpa4-shRNA lentivirus varies with different cell lines

To test the transduction efficiency of Rpa4 shRNA-expressing lentivirus, we used three different cell lines DLD1, HT29 both derived from human colorectal adenocarcinoma and HEK293T cell lines derived from human embryonic kidney cells. The DLD1 cells did not show much green cells after 6 days via fluorescence microscopy, and therefore flow cytometry was not performed on those samples. Three different

55

volumes (0 μL, 25 μL, and 500 μL) were used to examine the transduction efficiency into

HT29 and HEK293T cells. It was observed that the increasing amount of virus did not

correlate with the transduction efficiency that is addition of 20 times more volume did not

increase transduction by 20-fold (Figure 2.16). HEK293T cells were found to be more

receptive to viral transduction, and the highest transduction efficiency observed was

36.1% (Figure 2.17). Experiments were not performed further due to an inability to

achieve the transduction efficiency necessary to elucidate potentially subtle differences in

cell growth and replication.
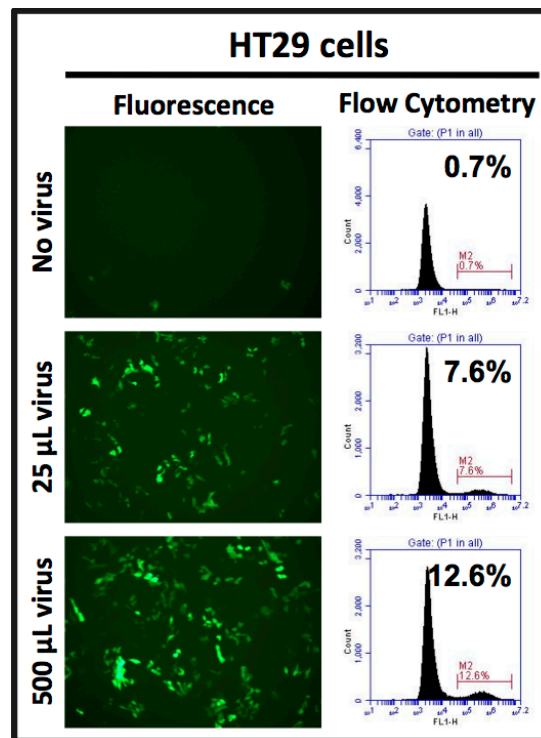
Figure 2.16: Transduction efficiency of Rpa4-shRNA containing lentivirus in HT29 cells. The transdunction was tested with increasing volume of virus.
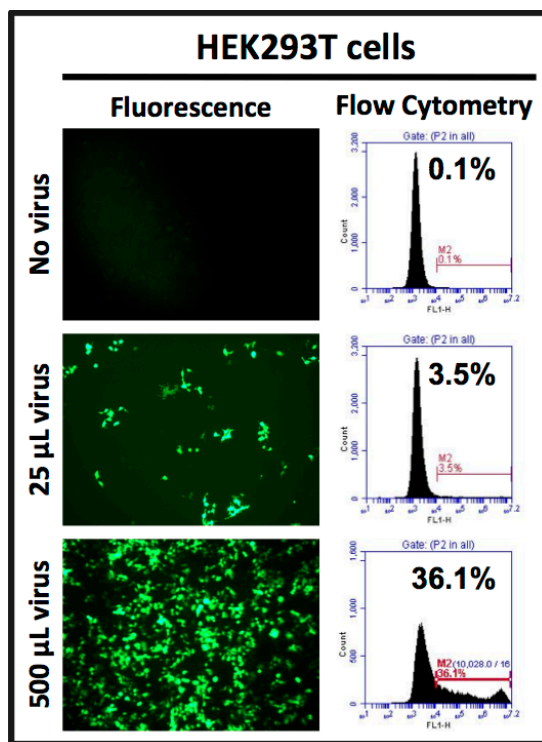
Figure 2.17: Transduction efficiency of Rpa4-shRNA containing lentivirus in HEK293T cells. The transduction efficiency was tested with increasing volume of virus.

Discussion

The inability of the RPA complex to substitute for RFA complex in yeast suggests a conservation of species-specific interactions

RPA complex is a well-conserved complex in all species with respect to cellular function and complex formation. In previous attempts to replace RFA by RPA, species-specific interactions were suggested to be responsible for non-complementation (PHILIPOVA *et al.* 1996). However, in all these experiments only a single subunit was replaced and tested for complementation. Lack of interaction between the BD-Rpa1 and subunits of RFA complex (Figure 2.8) in our experiments clearly supports these observations and extend them to demonstrate how the subunits might interact. When specific domains of Rpa1 (DBD-A and DBD-B) were swapped into *RFA1* in yeast cells,

these domains successfully complemented the yeast RFA. It is noteworthy that when swapped individually (*i.e.*, DBD-A or DBD-B), these domains did not complement (PHILIPOVA *et al.* 1996). These two domains were found to be essential for the ssDNA-binding activity of the RPA complex. To eliminate any species-specific inability to form a complete complex, we swapped all the three subunits and tested for survival on 5-FOA (Figure 2.3). It is clear from these experiments that the RPA complex cannot support yeast cellular functions. The human and yeast proteins are shown to have similar binding affinity and activity, but differences in *in vitro* properties have also been observed. For example, human RPA was found to bind deoxycytidine polymers more tightly while yeast RFA bound deoxythymidine polymers better (KIM *et al.* 1992). These minute differences can be attributed as the reason why there was a need to swap both the domains together.

As evident from the examples above if we consider that swapping the subunits or proteins according to their simple biochemical function of binding ssDNA, one would have predicted that human RPA should be able to substitute for yeast RFA in yeast cells. The failure of this experiment is more likely explained by considering two factors: (1) species-specific regulation of RPA and (2) protein interactions with RPA. There can be certain modifications that RPA undergoes in human cells to regulate its function, which are potentially missing from yeast cells (*i.e.*, RPA phosphorylation). This might render human RPA non-functional in yeast. Also, if we consider just the DNA metabolic pathways, in human cells there might exist some additional/different protein components, which are missing from the yeast cells. In order to study the full human RPA complex one also needs to introduce these necessary components in yeast cells. Alternatively, it

58

could be that the mechanism(s) by which proteins associate are different in human *vs.* yeast cells. Therefore, studying the individual domains and their effect on yeast cells might be a better approach to studying human (or other eukaryotic) RPA in yeast.

Secondly, from our qPCR and western blot data it is observed that human RPA genes are not expressed at similar levels as the yeast genes (Figures 2.4-2.7). This could be contributing factor to the failure of shuffle out assay. Hence, our next goal would be to increase expression (or cause over-expression) of these genes and determine if that improves the ability of cells to lose the pJM132 plasmid. However, it must be taken into consideration that in our hands, overexpression of *RFA1* was found to be toxic to cells (unpublished data).

Yeast cells are able to phosphorylate the Rpa2 N-terminus from various species

The goal of this study was to develop yeast cells, which can express human RPA complex and serve as a tool to study human RPA function. So far, we were not able to successfully develop a strain, which can exclusively express the human RPA complex. In the past, small parts of the complex have been replaced by the corresponding human region successfully. In this study we successfully developed hybrid protein-expressing cells where the Rfa2 N-terminus was replaced by its homolog from different species. These mutants were able to support cell survival in unstressed conditions, and supported cell growth to varying degrees after a DDR was established. Interestingly, the phosphorylation of the yeast Rfa2 N-terminus is not observed in response to DNA damage in yeast cells. From an evolutionary perspective this could mean that the yeast cells lack the ability to phosphorylate the Rfa2 N-terminus. However, since in our assay almost all of the hybrid mutants were phosphorylated in response to DNA damage

59

(Figure 2.14), we would suggest that there is purpose to the lack of phosphorylation observed for yeast Rfa2 in yeast cells.

This study indicates that the ability of the Rpa2 N-terminus phosphorylation exists from single-celled eukaryotes to multicellular organisms. However, the implication of this event in DDR is unclear from species to species. This data suggests that the Rpa2 NT phosphorylation evolved over time as a mechanism to regulate DNA damage response. This model system can potentially serve as a useful tool to study Rpa2 phosphorylation, especially in response to DNA damage. For example, a homolog from rice is not phosphorylated in response to DNA damage (*Os*2NT) may indicate that it is not involved in repair function in rice, compared to another rice homolog (*Os*1NT). Another important study will be to express these hybrid proteins in cells containing kinase deletions to determine which kinase(s) is important for phosphorylation of Rpa2 in the DDR.

Rpa3 does not interact with Rpa1 directly, but is essential for stability of Rpa1 and Rpa2 interaction

RPA complex was originally isolated as an essential component in *SV40* DNA replication (FAIRMAN and STILLMAN 1988; WOLD and KELLY 1988). It was isolated as a complex of four different polypeptides of which the 53 kDa peptide was found to be the cleavage product of Rpa1 (70 kDa) (WOLD and KELLY 1988). The RPA complex is soluble only when all three subunits are expressed (WOLD 1997). The individual subunits are difficult to isolate, and the only sub-complex that is able to be purified is the Rpa2 and Rpa3 heterodimer (HENRICKSEN *et al.* 1994).

Our data clearly supports the observations that Rpa1 bait can interact with Rpa2 or Rpa4, but does not interact with Rpa3 (Figure 2.9). This suggests that Rpa2 and Rpa3 or Rpa4 and Rpa3 must form a sub-complex before interacting with Rpa1 either directly through Rpa2 or Rpa4. Alternatively, Rpa2 or Rpa4 may be necessary to stabilize Rpa3 and allow it to interact with Rpa1.

The Rpa1 and Rpa3 proteins interact only in presence of either Rpa2 or Rpa4 (Figure 2.10). This clearly indicates that in the complex formation Rpa2 acts as a mediator between these two proteins. Since there is no direct interaction between Rpa1 and Rpa3, the role of Rpa3 is thought to be important in stabilizing the complex. It is possible that Rpa2 and Rpa3 sub-complex can perform certain cellular function without Rpa1. In fact, only Rpa2 was found to be associated with chromosomes in M phase of cell cycle (ADACHI and LAEMMLI 1992).

It can be hypothesized that the Rpa2+Rpa3 complex can participate in cellular functions independent of Rpa1. In our assay when we co-express Rpa2+Rpa3 as a supplementary plasmid we detect some auto activation (Figure 2.10). This auto activation is observed when the protein in question can act as a transcriptional activation domain. Rpa2 and Rpa4 were shown to auto activate in our lab when used as baits. But in this experiment Rpa1 is the bait and it can auto activate when the full complex is expressed. This phenomenon was never reported before.

The transduction of Rpa4-shRNA is not as efficient as desired

The Rpa4 protein, exclusively characterized in humans (and found in primates) is a homolog of Rpa2. However, Rpa2 is found to support DNA replication, while Rpa4 does not. Rpa4 was also reported to be expressed in tissue specific manner (KESHAV *et al.*

1995c) and is thought to be found in differentiated cells, perhaps providing a genome maintenance function without providing a cell proliferation function. Rpa2 is also found to have increased expression in cancer or proliferative cells (GLANZER *et al.* 2014) (BANERJEE *et al.* 2013). These observations lead us to hypothesize that the ratio of Rpa2: Rpa4 might help determine whether a cell is proliferative or quiescent (Figure 2.1).

In order to test this hypothesis, an undergraduate student, Kit Wong, carried out experiments whereby Rpa2 and Rpa4 mRNA expression were measured by qPCR in different tissue types. In his studies he found that Rpa4 was expressed in all tissues studied. Rpa4 expression was always lower than Rpa2, but there was no definitive correlation with the ratio and whether a cell was proliferative or not (Figure 2.18).

The reason that our experiments were not very successful was we needed better transfection and transduction efficiency. A future option might be to switch to an electroporation technique or pursue further optimization. Using different cell line(s) may also be helpful since different cell lines may have different effect with respect to viral transfection efficiencies.

Rpa4 is expressed in quiescent cells and often not so much in commonly used tissue culture cell lines. If this observation displays generality, then using cancerous cell lines may not be very useful in studying Rpa4 knockdown. Instead, these cell lines should be used to study the effect of overexpression of Rpa4 to determine if its overexpression can slow down/halt cell growth. Another effective method to study Rpa4 would be to use primary cell line and determine if its knockdown can make these divide for more generations or become immortalized.
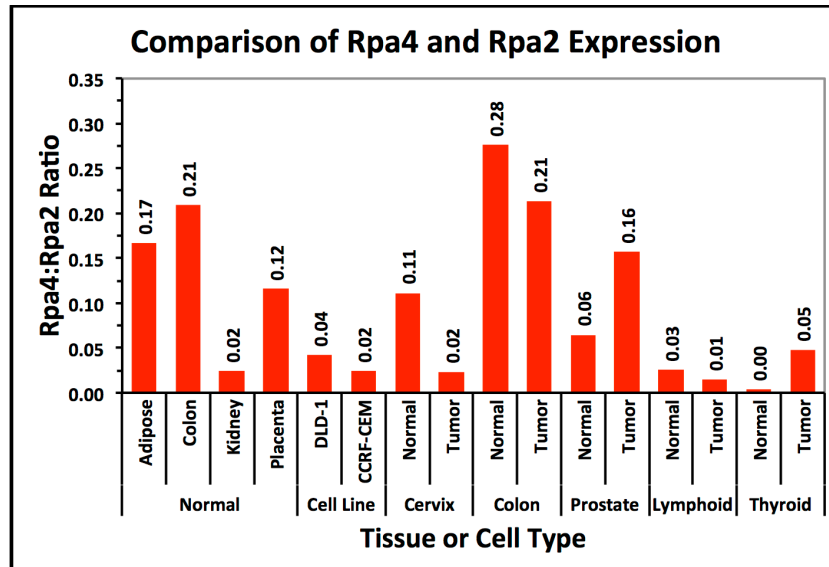
Figure 2.18: Comparison of Rpa2 and Rpa4 expression in various tissue types. This chart shows mRNA expression of Rpa2 and Rpa4 various human tissues.

Future Directions

1. In order to improve cell survival with a sole copy of RPA, we need to increase the expression of these genes in yeast cells.

2. With the help of *rfa2* hybrid 2NT proteins, we can identify which organisms potentially display Rpa2 NT phosphorylation and perhaps define the importance of this event from an evolutionary perspective.

3. Also, extension of eukaryotic Rpa2 NT examination might help define the role of various homologs in species containing multiple forms of the RPA complex.

4. With the help of genetic assays where we combine the kinase deletion mutant and the *rfa2* hybrid 2NT mutants we will be able to identify kinases responsible to establish the DDR, or at least phosphorylate Rpa2 NT in response to DNA damage.

5. If efficiency of DNA delivery can be optimized, studies examining the importance of Rpa4 to human cells might elucidate whether it plays a regulatory role in the decision for a cell to proliferate or quiesce.

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CHAPTER 3. THE N-TERMINUS OF RFA2 IS ESSENTIAL BUT ITS PHOSPHORYLATION IS DISPENSABLE IN *SACCHAROMYCES CEREVISIAE*[2]

Introduction

Cells are continuously exposed to environmental stresses that can lead to DNA lesions. These DNA lesions or errors in DNA metabolic processes, if not recognized and repaired properly by the cell, can lead to mutations. The accumulation of mutations has been implicated as a major cause of many cellular diseases, including cancer. These lesions can be generalized in two types (1) a nick in DNA strand, whereby one DNA strand is affected (*e.g.*, pyrimidine dimers) or (2) double stranded break, when both the strands are affected at the same time. Cells have the ability to recognize and repair DNA lesions through a number of mechanisms. These include nucleotide excision repair (NER), base excision repair (BER), or double-strand break repair (DSBR), which can occur via non-homologous end joining (NHEJ) or homologous recombination (HR) (SRIVASTAVA and RAGHAVAN 2015). Once the DNA lesion is recognized, a signal(s) is transduced to halt the cell cycle and allow for time to repair the lesion. This includes the recruitment and activation of cell cycle checkpoint proteins (*e.g.*, ATM and ATR) that further transduce the signal through activation of downstream proteins (COOPER *et al.* 2014). Generally, the activation of downstream targets is achieved through post-translational modification(s) that include phosphorylation and sumoylation.

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[2] Approximately 75% of this work was published in *Genetics* (2015) 199: 711-727.

RPA is a substrate of kinases responsible for activating the DNA damage response (DDR)

A protein essential in NER, BER and DSBR processes and a target of Phosphoinositide 3-kinase (PI3) family of kinases is Replication Protein A (RPA; RAMILO *et al.* 2002; DEMOTT *et al.* 1998). In humans, the amino (N)-terminus of Rpa2 (Rpa2 NT) is phosphorylated in a cell cycle-dependent manner (*i.e.*, during DNA replication and mitosis) and hyper-phosphorylated in response to DNA damage (WANG *et al.* 2001). Hyper-phosphorylated RPA changes interactions with both single-stranded DNA (ssDNA) and proteins (OAKLEY *et al.* 2003) and is conjectured to regulate RPA's cellular function in the DNA damage response. Therefore Rpa2 NT hyper-phosphorylation is touted as an important player in DDR.

Rpa2 is a direct substrate of ATR kinase, and its phosphorylation results in poor localization of RPA at replication sites; halting replication and mediating repair process (LIU *et al.* 2006; OLSON *et al.* 2006). The S4, S8, T21 and S33 located in N-terminus of human Rpa2 (Figure 3.1) are phosphorylated by DNA-PK in response to UV irradiation (ZERNIK-KOBAK *et al.* 1997). Phosphorylation at S4 and S8 of Rpa2 by DNA-PK helps suppression of sister chromatid exchange during mitosis to facilitate DNA repair (LIAW *et al.* 2011). ATM also phosphorylates Rpa2 in response to UV irradiation (WANG *et al.* 2001) and plays a role in helping cells in mitotic exit (ANANTHA *et al.* 2008). It is proposed that phosphorylation of Rpa2 by ATR is important when lesions occur during DNA replication (*i.e.*, replication stress), and phosphorylation by ATM is important when lesions occur during G2 and M phase. Distinct from its role in NHEJ, DNA-PK also assists in the DDR during both S and G2/M phase.

69

The cell cycle-dependent phosphorylation of RPA occurs by the action of cyclin-dependent kinases. Rpa2 is phosphorylated during S-phase by cyclin A-Cdk2 and during mitosis by cyclin B-Cdk1 (OAKLEY and PATRICK 2010).S23, S29 and S33 are constitutively phosphorylated during S phase . These phosphorylation events in the unstressed cells are thought to be priming for phosphorylation that occurs during the DDR.

```
              3           1112 14         21 23      27   30 32 34      38
Rfa2  MATYQPYNEYSSVTGGGFENSESRPGSGESETNTRVNTLT
Rpa2  MWNSGFESYGSSSYGGAGGYTQSPGGFGSPAPSQAEKKSR
         4           111213        21  23        29       33          39
```

Figure 3.1: Sequence comparison between amino acid 1-40 of Rfa2 (*S. cerevisiae*) andRpa2 (*H. sapiens*). Serine (S) and threonine (T) residues are highlighted, and their locations are denoted above or below the amino acid sequence.

RPA phosphorylation regulates interaction with single-stranded DNA and proteins

Rpa2 phosphorylation not only acts as a signal for DNA damage, but also can help initiate repair by changing interaction with DNA and proteins. The Rpa2 phosphorylated protein binds pyrimidine rich long sequences with affinity similar to WT RRA, but it shows decreased affinity towards short purine rich sequences (PATRICK *et al.* 2005). The N-terminus of Rpa1 and hyper-phosphorylation mimicking Rpa2 were shown to interact with each other showing a functional link between these domains (BINZ *et al.* 2003a). This interaction is thought to modulate the interaction of the complex with single-stranded DNA (ssDNA) and other proteins. The interaction between human RPA and Rad9 increases, whereas RPA interaction with the MRN complex decreases when the Rpa2 N-terminus is hyper-phosphorylated (OAKLEY and PATRICK 2010). Homologous recombination is one of the pathways to fix the DNA double-strand breaks, and two of

the proteins necessary for HR are Rad51 and Rad52. Hyper-phosphorylated Rpa2 was shown to interact more efficiently with Rad51 and Rad52 *in vitro* (Wu *et al.* 2005) and assist the transfer of ssDNA to Rad52 to facilitate recombination (DENG *et al.* 2009).

RPA is modified in single-celled eukaryotes to regulate cellular function

Rpa2 phosphoryaltion is not extensively studied in single-celled eukaryotes as compared to humans. However some studies indicate the role of Rpa2 phosphorylation in cell cycle regulation and repair. A recent study in *C. albicans* showed that the phosphorylation state of Rfa2 regulates the DNA damage response under genotoxic stress. Under stress, Mec1 and the Clb2-Cdc28 phosphorylate the Rfa2 N-terminus at T11, S18, S29 and S30, which leads to DNA repair. While the recovery of cells after repair is initiated by the dephosphorylation of Rfa2 by PPH3 and its counterparts (GAO *et al.* 2014). In *Candida albicans* Rfa2 is dephosphorylated by Pph3 phosphatase during G1 phase and under replication stress. Phosphorylation sites under these two conditions were different in Rfa2, indicating specific form of phosphorylated Rfa2 was key in regulating these events (WANG *et al.* 2013). While deletion of Pph3 phosphatase or Psy2 (regulatory subunit of Pph3) results in increased sensitivity to MMS, indicating that de-phosphorylation of Rfa2 may be important in some cases (HANWAY *et al.* 2002).

The S. cerevisiae is a commonly used model organism in genetics. The Rfa2 subunit in *S. cerevisiae* is also phosphorylated during S and G2 phase of cell cycle by Mec1 kinase (DIN *et al.* 1990; BRUSH *et al.* 1996).The phosphorylation of Rfa2 is thought to play role in recombination (BRUSH *et al.* 2001). However, the importance of Rfa2 phosphorylation in *S. cerevisiae* is unclear. In *S. cerevisiae* Ime2 kinase specifically phosphorylates Rfa2 at S27 during meiosis (CLIFFORD *et al.* 2004). The Ime2 is required

for normal meiotic progression, and the Rfa2 phosphorylation is thought to be important for proper initiation of meiotic progression (CLIFFORD *et al.* 2005).

Goal of this study

Overall, studies in human cells indicate that Rpa2 phosphorylation acts as switch to regulate DNA repair. In *S. cerevisiae*, two damage-dependent target sites for phosphorylation have been identified (Rfa1-S178 and Rfa2-S122); however, the mutation of known Mec1 phosphorylation sites in Rfa1 (S178) and Rfa2 (S122) do not result in any cellular sensitivity to DNA damage (GHOSPURKAR *et al.* 2015). This indicated that although these sites are targeted by a damage-dependent checkpoint kinase, they do not have an important role in the DDR in yeast. Studies indicate that phosphorylation of the Rfa2 N-terminus can occur in a pathogenic yeast. In Figure 3.1, a sequence comparison between the budding yeast Rfa2 and human Rpa2 N-termini show very similar locations for potential phosphorylation sites, indicating a potential role of this domain in DDR. Unlike its human counterpart, the specific sites involved in Rfa2 phosphorylation in response to DNA damage are relatively unexplored. We hypothesized that the Rfa2 N-terminus is phosphorylated and may regulate RPA function in budding yeast. The focus of this chapter is on the role of the budding yeast Rfa2 N-terminus, and this chapter describes the following avenues of research by:

1. Examining the role of the Rfa2 N-terminus and its phospho-state in yeast cell survival.

2. **Examining the role of the Rfa2 N-terminus and its phospho-state in DNA damage repair and cell cycle regulation.**

72

3. Identifying important region(s)/site(s) located in Rfa2-N-terminus and their role in cell survival and the DDR.

4. Examining the role of Rfa2 N-terminus and its phosphorylation in suppression of an Rfa1 mutant phenotype.

Materials and Methods

Strains and plasmids

RMY122-A, RMY122-A-*mre11Δ* (deletion of the *MRE11* gene), EGY48, RM26-26C (M163) and RM-K264-10D (M403) yeast strains (Appendix A) were used to test cell viability and DNA damage survival of Rfa2 extreme mutants. RM26-26C and RM-K264-10D are commonly used for meiotic analyses; however, in these studies, they provided for analysis of Rfa2 extreme mutant phentoypes in independent strain backgrounds. The RMY122A cells have a chromosomal deletion of the *RFA1* and the *RFA2* genes, which are complemented with two plasmids: (1) pRS313-*RFA1* (*HIS3* selectable marker) and pRS315-*RFA2* (*LEU2* selectable marker) (Appendix A). All plasmids containing *rfa2* extreme N-terminal mutants are derivatives of pRS315-*RFA2*.

Plasmid Shuffle Assay

Cells were typically grown on non-selective rich media, YPD (2% dextrose, 1% yeast extract, and 2% peptone), unless requiring dropout media to retain a plasmid(s). For plasmid shuffle assays to test for cell viability, various synthetic complete dropout media were required. The plasmid pRS313-*RFA1* and appropriate pRS315-*RFA2* mutant was co-transformed with LiAc/PEG method into RMY122A cells (GIETZ and WOODS 2002). . Transformants were recovered on synthetic complete media (0.5% ammonium sulfate and 0.17% yeast nitrogen base without amino acids) containing 2% dextrose (SD) and

lacking the amino acids histidine, leucine, and uracil (-HLU). An overnight culture in

SD-HLU was inoculated with a colony and grown at 30°C (220 RPM) in an incubator

shaker. The next day the cultures were pulse sonicated five times at 20% power (0.5 sec

on, 1 sec off) and were counted using a cellometer Nexcelom. The concentration was

adjusted to $1(10)^7$ cells/mL followed by 1:10 serial dilutions. Five microliters (5 µL) of

each of the dilutions were spotted in a row on YPD, YPG (2% glycerol, 1% yeast extract,

and 2% peptone), SD-HLU, and (SD+)5-FOA (0.8 µg/mL) plates to test viability. YPG

media is used to assess whether cells have functional mitochondria, SD-HLU selects for

cells that have retained all plasmids and 5-FOA selects for cells that have lost the *URA3*-

containing plasmid pJM132 (which also contains wild-type copies of *RFA1* and *RFA2*). If

the cells can survive with mutated *rfa1* and *rfa2* genes, then the cells will lose pJM132

and survive on 5-FOA plates. Simultaneously 50 µL of $1(10)^6$ cells/mL was plated onto

5-FOA plates to recover colonies that have lost the pJM132 plasmid. In this assay we are

examining the ability of cells to lose pJM132 and survive on 5-FOA, and in the process,

we are also recovering mutant cells.

DNA damage assays

The colonies that have lost the pJM132 plasmids were used to inoculate an

overnight YPD culture incubated at 30°C. The next day the cultures were pulse sonicated

five times at 20% power (0.5 sec on, 1 sec off) and were counted using cellometer

(Nexcelom). The concentration was adjusted to $1(10)^7$ cells/mL followed by 1:10 serial

dilutions. Five microliters (5 µL) of each dilution were spotted in a row on different YPD

plates containing various concentrations of DNA damaging agents (Table 2.2) to assay

cell sensitivity to DNA damage.

***In vitro* site-directed mutagenesis to generate *rfa2* N-terminus mutants**

The Rfa2 extreme mutant plasmids were generated by Dr. Andre Walther (Cedar Crest College, Allentown, PA) and described in GHOSPURKAR *et al.* (2015). In order to generate Rfa2 N-terminus single and multi-mutants, primers were designed to be 60 nucleotides (nt) in length, contain the desired missense mutation (S/T to either D or A), and in most cases, contain a silent mutation that either generated or removed a diagnostic restriction site. Colony cracking (crude plasmid DNA isolation) followed by restriction digestion was used to identify correct clones. Each mutagenesis reaction utilized a single mutagenic primer as described in http://www.molecularstation.com/forum/protocols-methods-forum/19707-site-directed-mutagensis.html. The components of the reaction were: (1) 50 ng template DNA (pRS315-*RFA2*), (2) 0.2 μM single mutagenic primer, (3) 0.2 mM dNTP mix, (4) 1x Phusion DNA polymerase buffer, (5) 3 mM MgCl$_2$ and high fidelity Phusion DNA polymerase (New England BioLabs). The mutagenic primers are listed in Appendix C. Following mutagenesis, the reaction was digested with *Dpn*I digestion at 37°C for 2 hr. A half of a microliter (0.5 μL) of the reaction was then transformed into DH10B *E. coli* cells via electroporation, and cells were plated onto LB plates containing 100 μg/mL ampicillin (LB+Amp) to select for transformants. Correct mutagenic plasmids were verified by DNA sequencing (Eton Biosciences).

***In vitro* site-directed mutagenesis to generate *rfa1* aromatic mutants**

We used a unique strategy to generate the *rfa1* aromatic mutants. There are two aromatic amino acids in DBD-A and two in DBD-B, which are important for interaction between Rfa1 and ssDNA. To mutagenize each of these sites required four individual mutagenic primers (Appendix C). However, to generate all possible mutant combinations

(Table 3.1), all four mutagenic primers were added to the same mutagenesis reaction

using pRS313-*RFA1* as the template. Again, this was followed by *Dpn*I digestion at 37°C

for 2 hr and transformation of the reaction into DH10B cells. Mutations were identified

by restriction digestion using diagnostic enzymes *Bgl*II ($rfa1$-A^1), *Blp*I ($rfa1$-A^2), *Nae*I

($rfa1$-B^1) and *Eco*RV ($rfa1$-B^2) .All possible combinations of *rfa1-aro* mutants were

recovered and verified by DNA sequencing (Eton Biosciences).

Table 3.1: Potential combinations of *rfa1* aromatic mutants arising from *in vitro* site-directed mutagenesis using all four mutagenic primers in one reaction

Number of *rfa1* mutations	Potential mutant combinations
0 (no mutation)	WT
1 (single mutation)	A^1, A^2, B^1, B^2
2 (double mutation)	A^{12}, A^1B^1, A^1B^2, A^2B^1, A^2B^2, B^{12}
3 (triple mutation)	$A^{12}B^1$, $A^{12}B^2$, A^1B^{12}, A^2B^{12}
4 (quadruple mutation)	$A^{12}B^{12}$

DBD-A and DBD-B are denoted as A and B, respectively. Superscripts indicate the location of the aromatic residue in DBD-A or DBD-B (A^1 = F238A; A^2 = F269A; B^1 = W360A; B^2 =F385A).

Detection of correct clones by colony cracking

One μL of muatagenesis reaction was transformed in to DH10B cells using

electroporation. Cells were plated on LB+Ampicillin plates. Individual colonies were

used to inoculate overnight LB+Ampicillin cultures. 1mL of this culture was pelleted and

the pellet was dissolved in 50μL of 1X lysis buffer (2mg/ml lysozyme, 15% sucrose,

0.2mg/ml RNase A, 10mM Tris Cl pH 8.0, 1mM EDTA pH 8.0, 0.1mg/mL BSA). This

mixture was boiled at 100°C for 90sec, followed by centrifugation at 15,000 RPM for 10

min. The supernatant is used to set up a reaction using appropriate restriction enzyme. The pRS315-Rfa2 mutants were further confirmed by sequencing from Eton Biosciences.

Protein extraction, SDS-PAGE and Western Blotting

The *rfa2* extreme mutants were grown overnight in YPD at 30°C. The next day cultures growing in logarithmic phase were divided into two cultures, and one set of each culture was treated with 0.03% MMS. The cultures were allowed to grow for an additional 3 hr. Following the 3 hr growth period, the concentration of cells was determined using a cellometer, and approximately 5-6(10)^{7}cells were harvested. Protein was isolated using the method of Kushnirov (2000). The separation of protein was carried out using standard Laemlli SDS-PAGE at constant current (30 mAmp). For detection of Rad53, an 8% (37.5:1 acrylmono:bis) SDS-PAGE gel was used. Rfa2 was detected using a 12% (37.5:1 mono:bis) SDS-PAGE gel containing 0 μM, 25 μM, or 50 μM Phos-Tag (Wako Pure Chemical Industries). The proteins were transferred to 0.45 μm nitrocellulose membrane at constant current 40 mAmp for 13 hr at 4°C.

Dr. Steve Brill provided the rabbit polyclonal Rfa2 antibody. The rabbit polyclonal Rad53 antibody was ordered from Abcam (ab104232). Blots were blocked using 10% non-fat dry milk in 1x TBS-T followed by primary antibody incubation with a 1:20,000 dilution of anti-Rfa2 antibody in 10% milk in 1x TBS-T overnight. After the primary antibody incubation, blots were washed 3 times with 1x TBS-T and incubated for 1 hr with goat anti-rabbit IgG (1:40,000) by Abcam (ab6721). The blots were again washed 3 times using 1x TBS-T and developed using Pierce ECL kit. To detect Rad53, blots were incubated overnight with anti-Rad53 antibody diluted 1:6,000 in 10% milk in

1x TBS-T. The rest of the procedure was followed similar to Rfa2 blot development as described above.

Results

The *rfa2* N-terminus extreme mutants are viable but the *rfa2-D$_x$* and *rfa2-ΔN$_x$* are sensitive to DNA damage

The *rfa2* N-terminus extreme mutants (Figure 3.3) were co-transformed with WT Rfa1 and cells were recovered on SD-HLU. The plasmid shuffle assay was performed, and the loss of pJM132 (containing Rfa1 and Rfa2) was assessed by growing cells on 5-FOA plates. If the mutant *rfa2* can support cell growth as a sole copy of *RFA2* in cells, then cells will lose pJM132 plasmid and survive on 5-FOA plates. The shuffle out assay showed that all of the Rfa2 N-terminus extreme mutants are able to support cell growth in unstressed condition (Figure 3.2). The *rfa2-ΔN$_x$* mutant was shown to survive in a deletion mutation study carried by Philipova (1996). In this study, we observed the same phenotype by *rfa2-ΔN$_x$*. The shuffle out frequency of the extreme mutants was very similar to WT cells, as shown in the Figure 3.2.
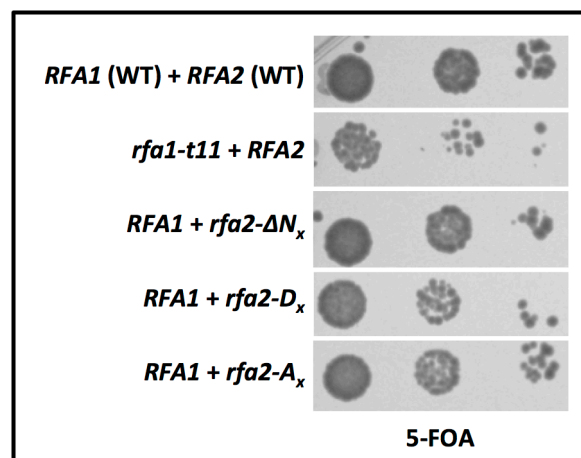
Figure 3.2: Plasmid shuffle assay of Rfa2 extreme mutants. Growth on 0.8 µg/mL 5-FOA indicated viability, and the *rfa1-t11* mutant serves as a control in this assay.

An interesting observation about the phenotype of the RMY122A cells with Rfa2 N-terminus mutants is the difference in growth rates (Table 3.2). The growth rate here represents the ability of these cells to grow under unstressed condition and reflects ability of cells to replicate and divide. The WT cells were used as the positive control while *rfa1-t11* cells were used as a negative control reference. As represented in the Table 3.2 we can see that *rfa2-A$_x$* has a slightly better growth rate than WT cells, which further asserts that *rfa2-A$_x$* does not interfere with the cellular function (Figure 3.4). The *rfa2-D$_x$* and *rfa2-ΔN$_x$* show similar growth rate. Although these mutants do not grow as slow as *rfa1-t11*, they grow slightly slower than WT. This indicates that rfa2-Dx and *rfa2-ΔN$_x$* are growth deficient (or sick) and indicates that the presence of the Rfa2 N-terminus is essential for cell growth. It is noteworthy, that these two mutants represent completely different phospho-states, yet show a similar phenotype.

Table 3.2: Growth rates for *rfa2* extreme mutants in the RMY122-A background

rfa mutant	Generation time (min)
WT	105.6 ± 11.9
rfa1-t11	161.9 ± 16.6
rfa2-D$_x$	112.1 ± 6.0
rfa2-A$_x$	102.4 ± 11.9
rfa2-ΔN$_x$	111.5 ± 6.0

We also tested Rfa2 N-terminus extreme mutants in multiple strain backgrounds to measure damage sensitivity and to determine if phenotypes observed were generally due to the *rfa2* mutation or are strain-specific. We also tested different types and concentrations of DNA damaging agents. This tests if the phosphorylation state of Rfa2

N-terminus affects the different types of DNA damage response and helps to elucidate subtle differences between the mutants. For example, the camptothecin inhibits Topoisomerase Type-I while Hydroxyurea stalls replication fork. Although these two agents cause double stranded breaks, the mechanism is different and possibly they might use different response mechanism.
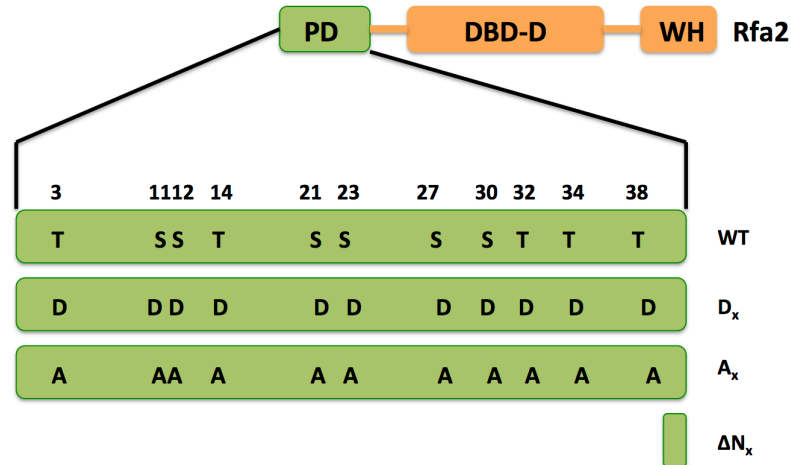
Figure 3.3: A diagrammatic representation of *rfa2* N-terminus extreme mutants. The WT Rfa2 shows the different Ser and Thr sites. In the mutants all the sites are mutated at the same time to Asp (D_x), or to Ala (A_x). The mutant where aa 1-38 are deleted is known as *rfa2-ΔN_x* and represents absence of domain.

As shown in Figures 3.4-3.7, irrespective of the strain background or type/concentration of DNA damaging agent, the *rfa2-D_x* and *rfa2-ΔN_x* are the most sensitive mutants to DNA damage. For example in Figure 3.4, in the presence of 0.06% MMS, the WT and *rfa2-A_x* cells continue to survive up to 4th dilution the *rfa2-D_x* and *rfa2-ΔN_x* mutant show growth up to the 2nd dilution. This was somewhat surprising, as the *rfa2-ΔN_x* mutation is more similar to *rfa2-A_x* with respect to phosphorylation potential (*i.e.*, neither can be phosphorylated at S/T residues). However, *rfa2-A_x* showed a phenotype nearly indistinguishable to WT. The *rfa2-D_x* was predicted to be DNA-damage resistant, as it is mimicks the phospho-state that occurs in response to DNA

damage; however, it is DNA-damage sensitive. We propose that this might be due to this mutant mimicking a constitutively phosphorylated state, and that the inability to be "dephosphorylated" might lead to this phenotype.
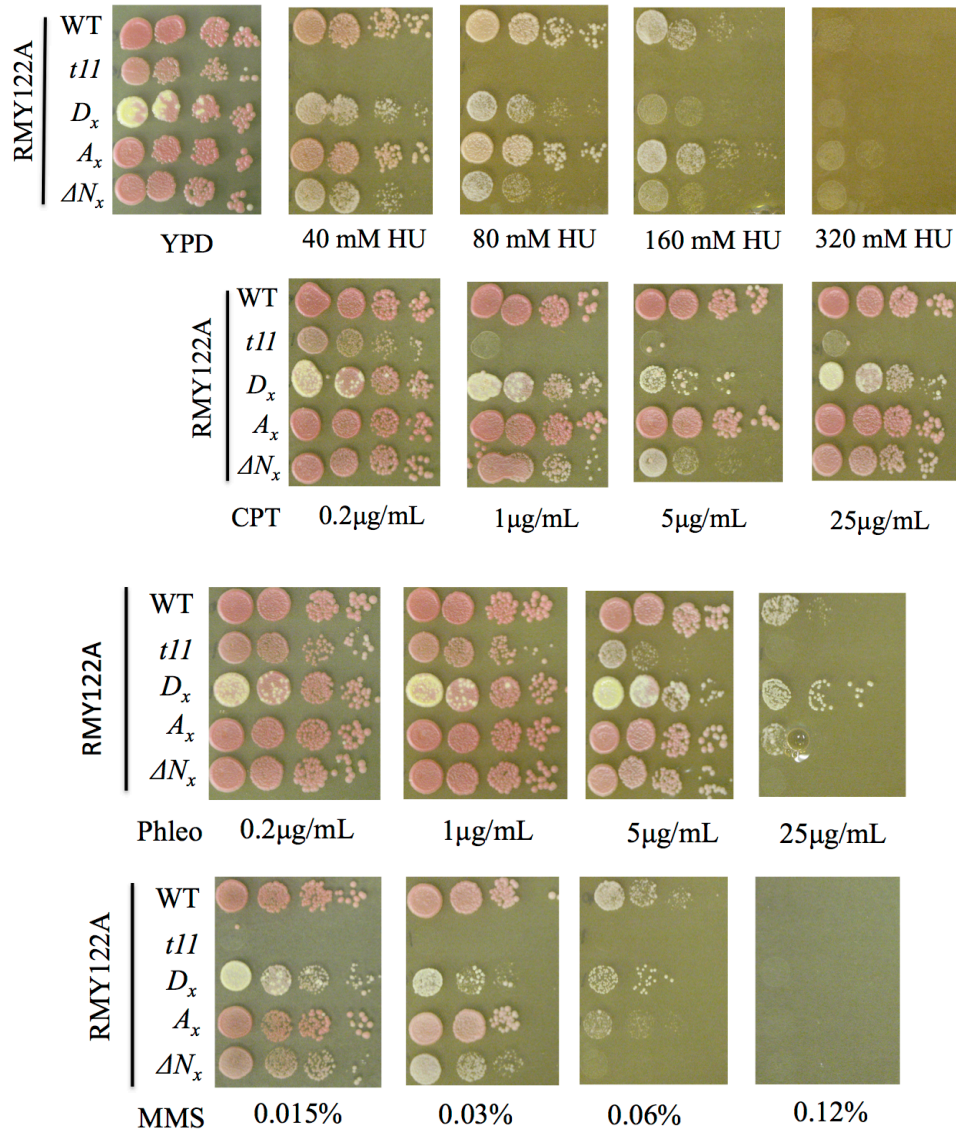
Figure 3.4: DNA damage assay of rfa2 extreme mutants in RMY122A strain background. The various damaging agents are denoted at the bottom left of each row. The *rfa1-t11* served as a negative control in these experiments.
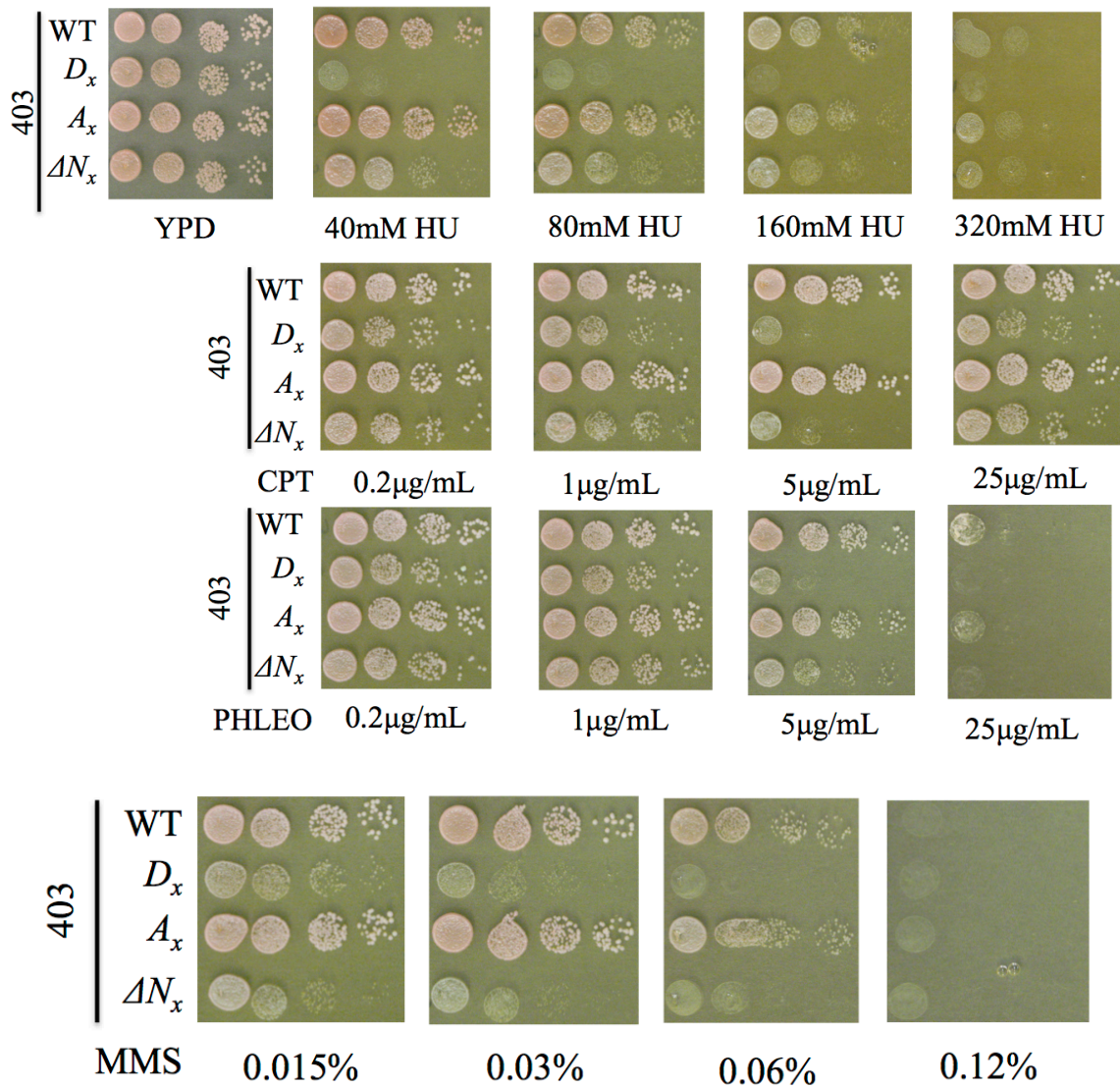
Figure 3.5: A picture showing DNA damage assay of *rfa2* N-terminus extreme mutants in a 403 strain background. The various damaging agents are denoted at the bottom left of each row. The *rfa1-t11* served as a negative control in these experiments. In this strain the *rfa2-D_x* and *rfa2-ΔN_x* are sensitive in stressed condition irrespective of the type and concentration of damaging agent.
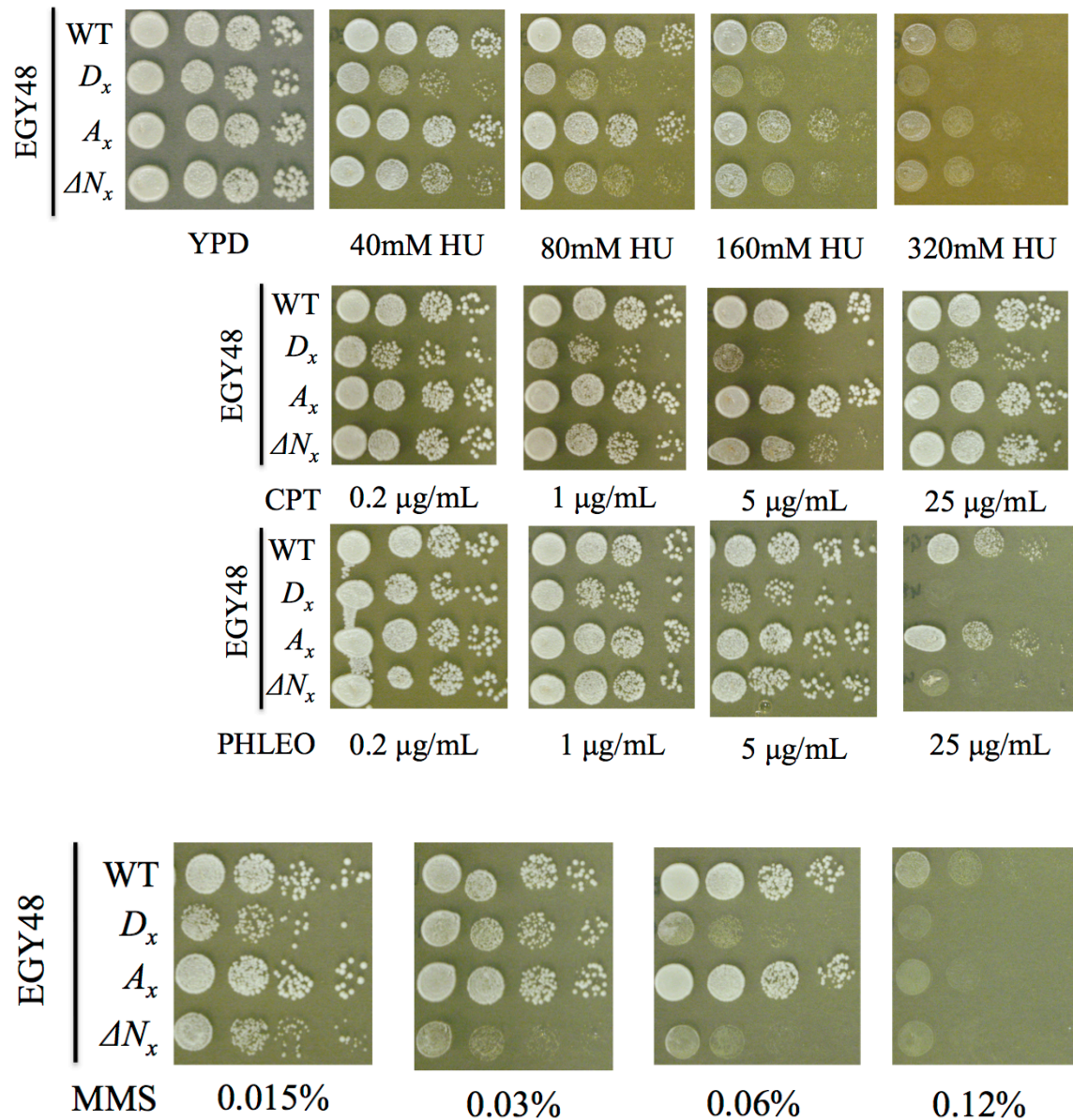
Figure 3.6: A picture showing DNA damage assay of *rfa2* N-terminus extreme mutants in an EGY48 strain background. The various damaging agents are denoted at the bottom left of each row. The *rfa1-t11* served as a negative control in these experiments. As seen in the picture all the mutants are able to survive in unstressed condition (YPD) and the most sensitive mutants are *rfa2-D$_x$* and *rfa2-ΔN$_x$* in both strains and all DNA damaging conditions.
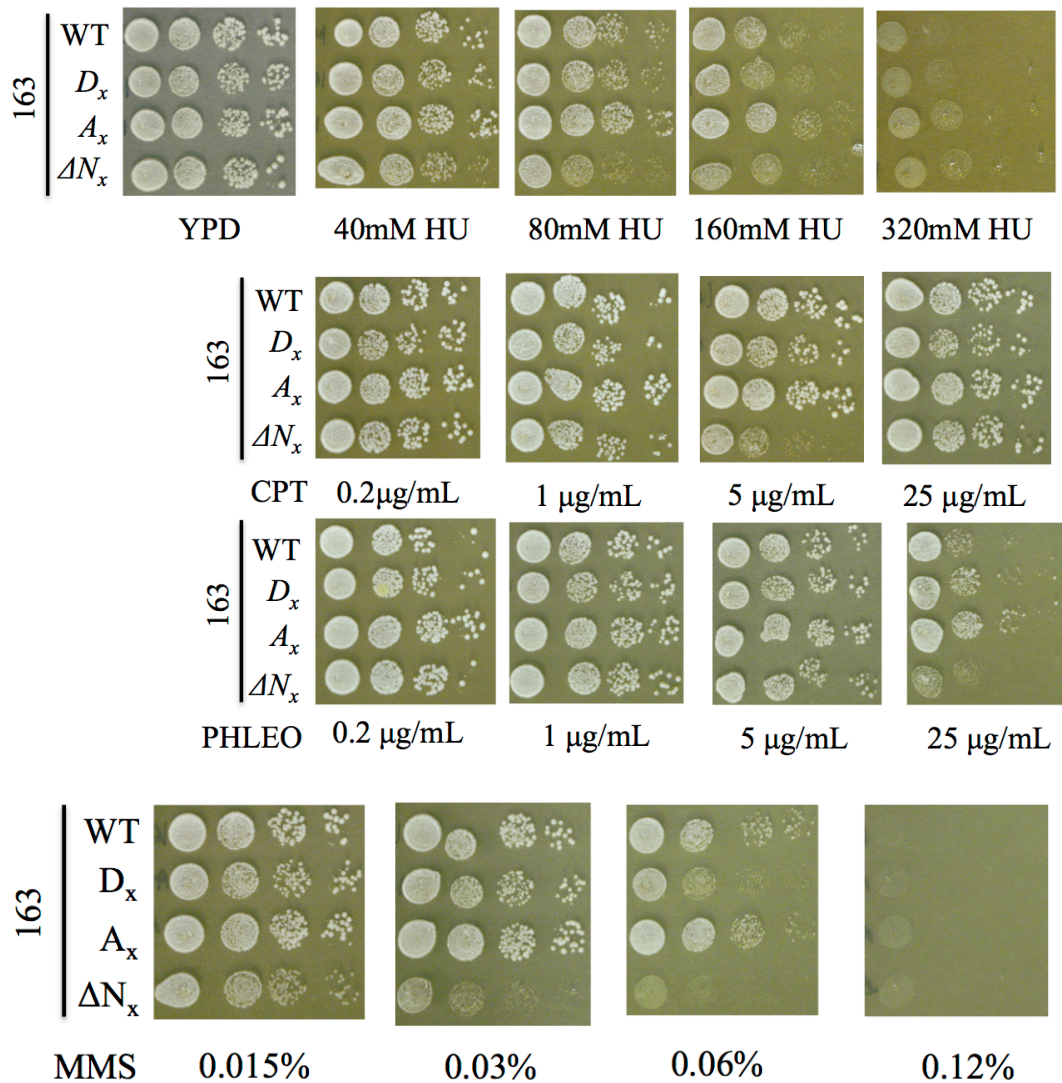
Figure 3.7: A picture showing DNA damage assay of *rfa2* N-terminus extreme mutants in 163 strain background. The various damaging agents are denoted at the bottom left of each row. The *rfa1-t11* served as a negative control in these experiments.

The Rfa2-N-terminus single mutants are viable but are not affected by DNA damage

A study carried in our lab showed that the known Mec1 phosphorylation sites in Rfa1 and Rfa2 when mutated to Alanine, do not show damage sensitivity (GHOSPURKAR *et al.* 2015). However, the *rfa2* extreme mutants tested in this study show DNA damage sensitivity. This indicates that phosphorylation of Rfa2 might be involved in damage response like its human counterpart. In humans, it is known that the phosphorylation at

one site in N-terminus triggers phosphorylation of the other sites. For example, the phosphorylation of S33 by ATR in meiotic cells triggers phosphorylation at S23 and S29 by CDK (ANANTHA *et al.* 2007). We hypothesized that like its human counterpart, the phosphorylation of Rfa2 in yeast is also sequential and is useful to amplify the DNA damage response signal. To test this hypothesis we developed Rfa2 single mutants, whereby we mutated one site at a time to either Asp (*rfa2-D$_i$*) or Ala (*rfa2-A$_i$*).

The first step was to test the ability of the single mutants to support the viability of cells in unstressed condition and lose the pJM132 plasmid. In the shuffle out assay we observed that all the *rfa2-D$_i$* and *rfa2-A$_i$* mutants were able to support cell viability in unstressed condition (Figure3.8). Also the frequency of shuffle out was found to be similar to the WT cells. These cells were recovered on 5-FOA plates and were then subjected to DNA damage assay. In this assay we tested CPT, HU and MMS in the optimal concentrations. As seen in Figure 3.9 the *rfa1-t11 rfa2-D$_x$* double mutant shows "no growth" phenotype and serves as a negative control. This shows that these concentrations are enough to induce damage sensitivity. We observed that the *rfa2-A$_{i3}$* mutant has a high sectored colony phenotype (red and white colonies); this is indication of high mutation rate, which can be effect of *rfa2-A$_{i3}$* mutant. When tested further, it was found to be a defect in the particular patch and had no direct connection with the mutant (data not shown). However as shown in Figure 3.9 A and B, both the *rfa2-D$_i$* and *rfa2-A$_i$* set of mutants do not show any sensitivity and have a phenotype closer to WT cells. This suggests that phosphorylation at individual sites may not be enough to trigger DNA damage sensitive phenotype in *S. Cerevisiae*.
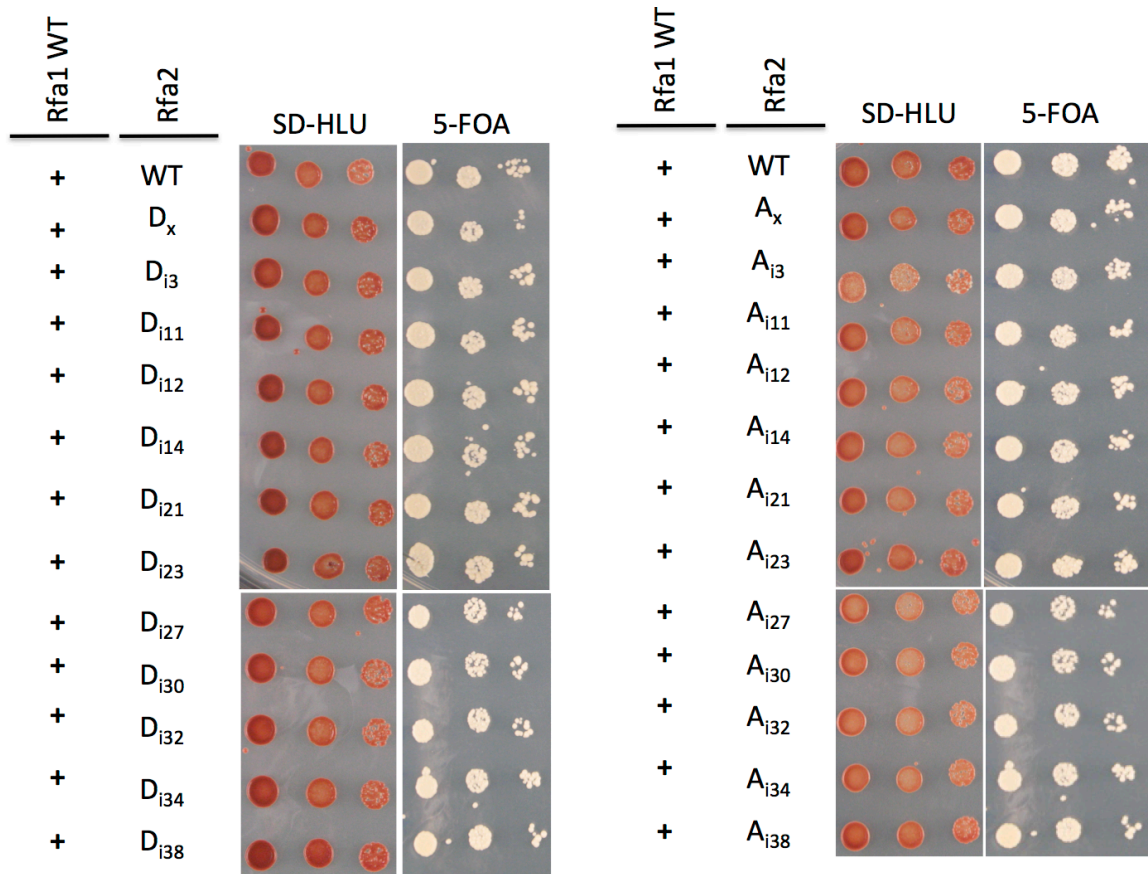
Figure 3.8: The shuffle out assay of *rfa2-D_i* and *rfa2-A_i* mutants. The Aspartic mutants are denoted by D_i and Alanine mutants are denoted by A_i. The symbol 'i' stands for the amino acid site in Rfa2 NT. Both types of mutants support the cell viability. And the frequency of shuffle out is observed similar to the WT cells.

A.
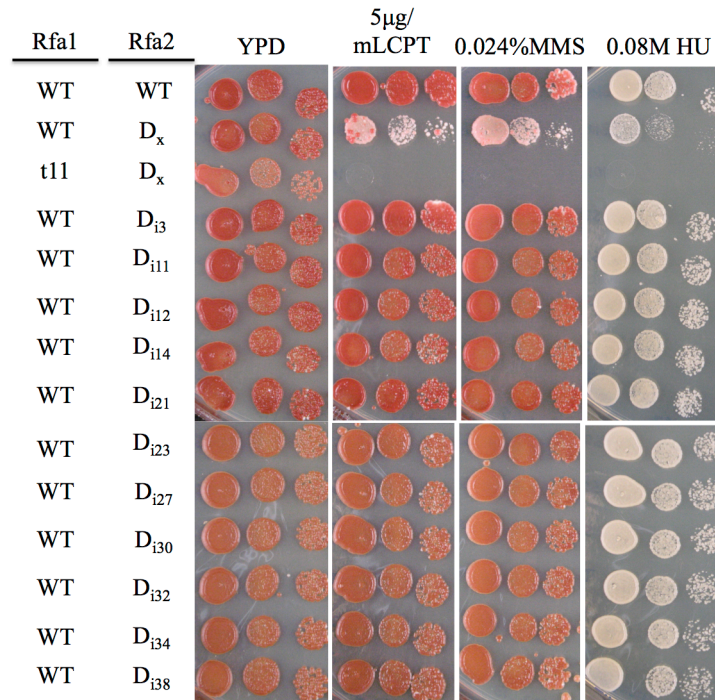
B.
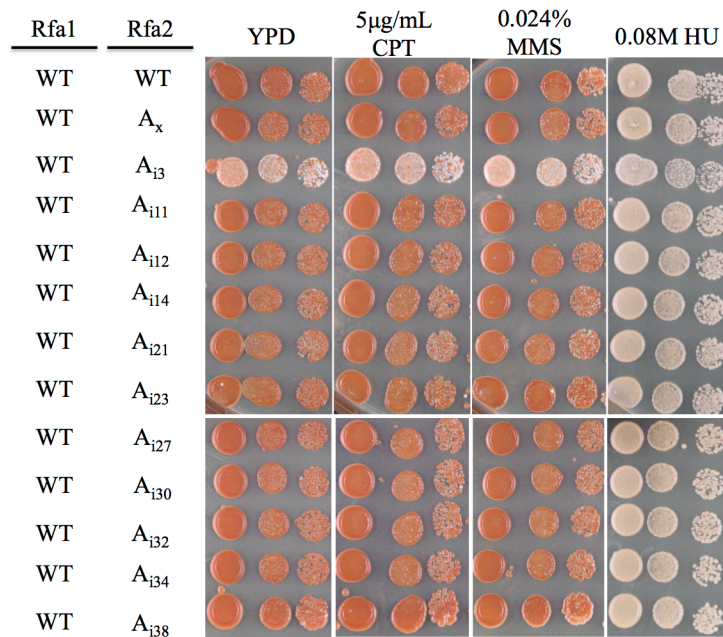
Figure 3.9: The DNA damage assay *of rfa2* NT single mutants. A) The DNA damage assay of *rfa2-D_i* mutants shows that all the individual mutants are resistant to DNA damage unlike the *rfa1-t11+rfa2-D_x*, which is sensitive. B) The DNA damage assay of *rfa2-A_i* mutants show that all the individual mutants are resistant to DNA damage.

The Rfa2-multi-mutants are viable and decipher the important regions responsible for DNA damage sensitivity

The Rfa2 extreme mutants in this study show that the phosphorylation domain's presence and its phosphorylation state affects the DDR pathway. However, we could not impart this sensitivity to any particular phosphorylation site in this domain. Therefore, we developed mutants by dividing them into three different groups. These groups consist of 3-4 phosphorylation sites (Figure 3.10); and will be referred as multi-mutants (D_m, A_m or N_m). The multi-mutants were tested for supporting cell viability in shuffle out assay. All the *rfa2-D_m* and *rfa2-A_m* multi-mutants were found to shuffle out the pJM132 plasmid supporting the cell growth. The frequency was found to be similar to WT cells and better than the extreme mutants *rfa2-D_x* and *rfa2-A_x* Figure 3.11 A. The *rfa2-D_{m1}* mutant was found to be lethal in this shuffle out assay but when more candidates were tested it was recovered (data not shown).
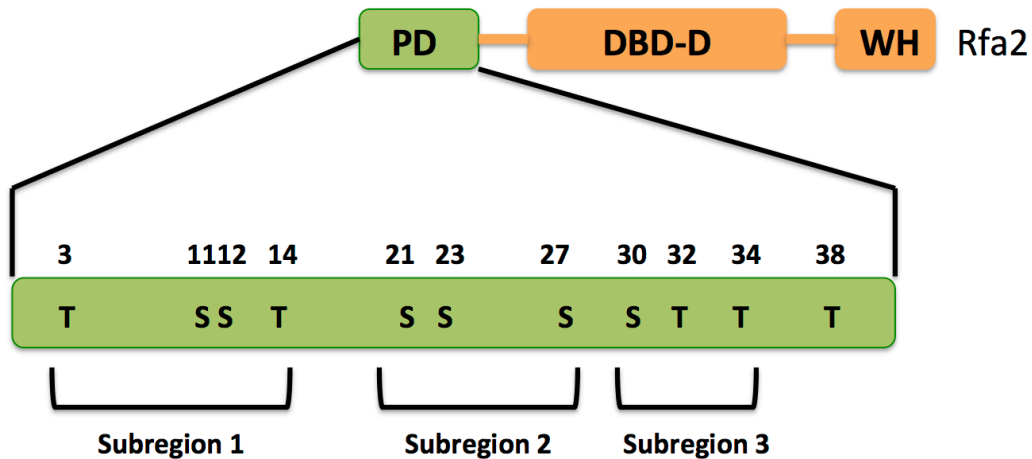
Figure 3.10: Diagrammatic representation of Rfa2 multi-mutants showing different clusters of phosphorylation site mutated together to form multimutants. S= Serine, T=Threonine, D_m = multiple sites mutated to Aspartic acid, A_m = multiple sites mutated to Alanine and ΔN= a portion of amino acid region deleted.

88

A.
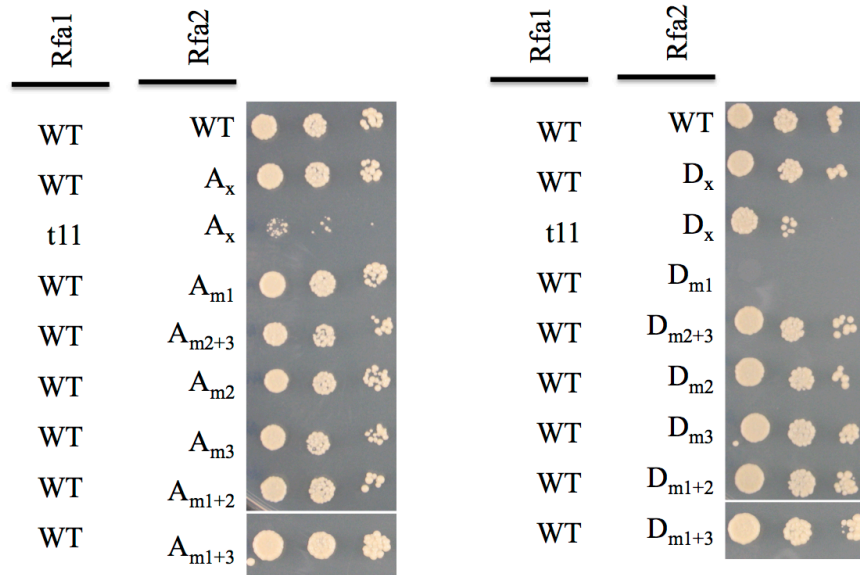
B.
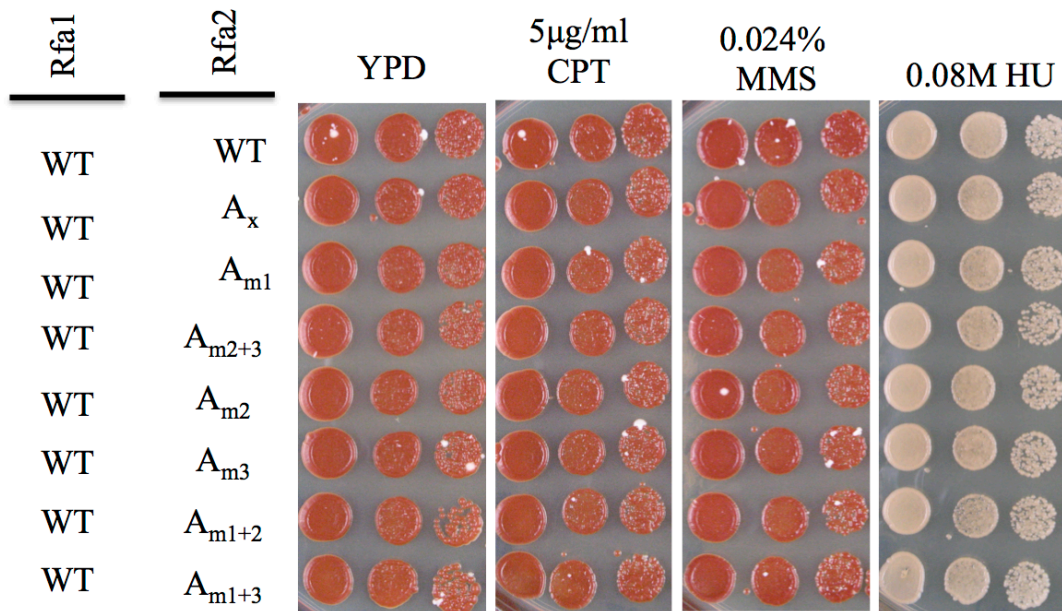
Figure 3.11: The cell viability and DNA damage assay of rfa2 NT multimutants. A) Shuffle out assay of Rfa2 multi-mutants showing successful shuffle out of all multi-mutants in RMY122A cells. B) DNA damage assay of all Am multi-mutants shows that all the alanine multi-mutants are resistant to DNA damage.

C.
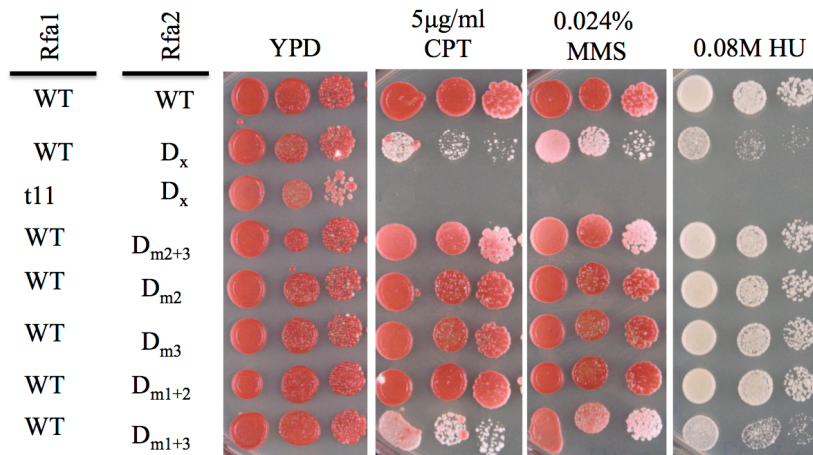
Figure 3.11: The cell viability and DNA damage assay of rfa2 NT multimutants (continued). C) DNA damage assay of all D_m multi-mutants showing that D_{m1+3} is sensitive to DNA damage.
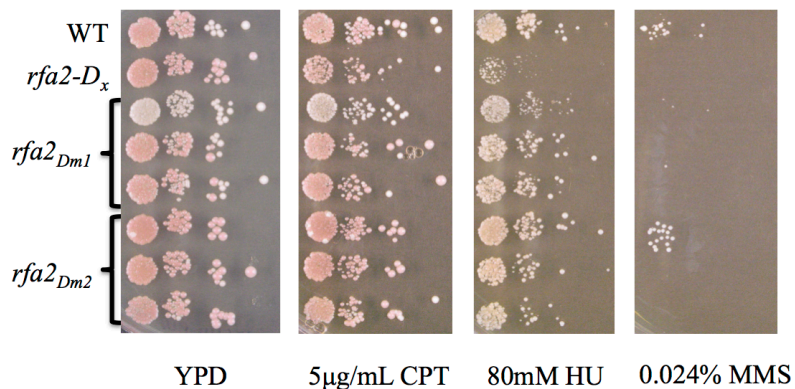
Figure 3.12: The DNA damage assay of three independent candidates showing *rfa2-D_{m1}*. The *rfa2-D_{m1}* is resistant to DNA damage. The *rfa2-D_{m2+3}* was used as control. The above DNA damage concentrations were picked so that they are not toxic but can show even subtle sensitivity differences.

When the *rfa2-D_m* and *rfa2-A_m* multi-mutants were subjected to DNA damage assay we found that all the A_m multi-mutants were resistant to DNA damage Figure 3.11 B. This was expected, as the *rfa2-A_x* mutant is resistant to DNA damage. When the *rfa2-Rfa2* multi-mutants were tested the *rfa2-D_{m1+3}* mutant was found to be slightly sensitive.

The *rfa2-D$_{m1}$* and *rfa2-D$_{m3}$* mutant however was found to be resistant to DNA damage (Figure 3.12 and 3.11 respectively). It is noteworthy, that *rfa2D$_{m1+3}$* sensitivity was similar to *rfa2-D$_x$* and *rfa1-t11 rfa2-D$_x$* double mutant is synthetically lethal. Hence, we can conclude that the region 1 and region 3 together contribute the damage sensitivity to the *rfa2-D$_x$* mutant.

A)
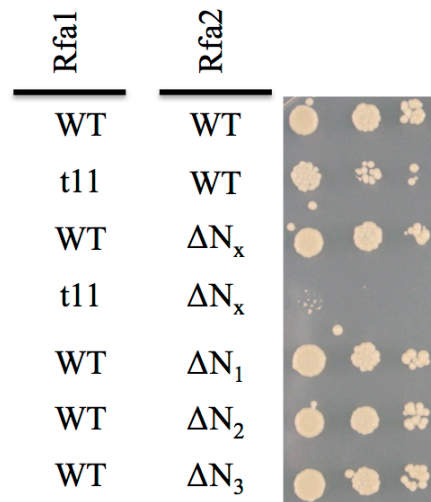
B)
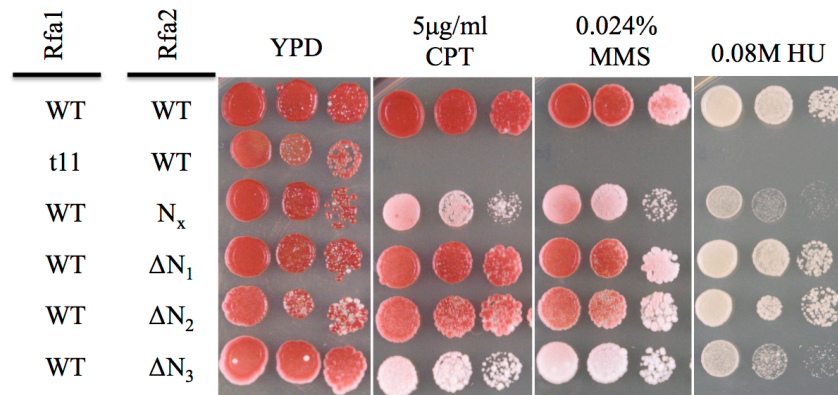
Figure 3.13: The cell viability and DNA damage assay of rfa2-ΔNm multimutants.
A) shuffle out assay of rfa2-Nm mutants in RMY122A background. All the multi-mutants shuffle out similar to WT cells. B) DNA damage assay of *rfa2-ΔN$_m$* mutants in RMY122A background. The *rfa2-ΔN$_{m3}$* mutant shows damage sensitivity similar to *rfa2-ΔN$_x$*.

91

The *rfa2-ΔN_m* multi-mutants were all able to shuffle out with similar frequency to that of WT and *rfa2-ΔN_x* cells. It is noteworthy that *rfa1-t11 rfa2-D_x* double mutant was found to be synthetically lethal 3.13 A. The cells were recovered and subjected to DNA damage assays. The *rfa1-t11* served as a negative control in this assay. The *rfa2-ΔN_3* mutant is showing a damage sensitive phenotype similar to *rfa2-ΔN_x* mutant Figure 3.13 B. Hence, we can conclude that the 3rd region of Rfa2- N-terminus that is S_{30}, T_{32} and T_{34} are important for DNA damage response.

The phosphorylation state of Rfa2-N-terminus affects RFA-Mre11 inter-complex interaction

In previous study, it is shown that the human Rpa1 N-terminus and Rpa2 phosphorylation are key components that regulate RPA and MRN complex interactions (OAKLEY *et al.* 2009). Also the component of MRN complex; NBS1 is shown to trigger Rpa2 phosphorylation by ATR (MANTHEY *et al.* 2007). Hence we chose to study this interaction further. In this study, we found that in *mre11Δ rfa2-D_x* double mutant is not viable (3.14B). To locate the key site responsible for this phenotype and in extension responsible for regulating RFA-MRX complex interaction, we tested the *rfa2-D_i* mutants in *mre11Δ* background.

All the individual mutants were able to support cells as sole copy of Rfa2 that is viable on 5-FOA (data not shown). The *mre11Δ* cells by themselves are sensitive to DNA damage. Therefore as shown in Figure 3.14A, all the individual mutants are sensitive to DNA damage and we cannot attribute this phenotype to any particular site. We further tested the *rfa2-D_m* mutants and as shown in Figure 3.14B the *rfa2-D_{m2+3}* and *rfa2-D_{m3}* mutants are sensitive in terms of cell viability on 5-FOA plates. However, they are not as

sensitive as $rfa2\text{-}D_x$. The $rfa2\text{-}D_{m1+3}$ mutant is as sensitive as $rfa2\text{-}D_x$, it is noteworthy

that $rfa2\text{-}D_{m3}$ itself survives slightly better. Hence the combination of region 1 and 3

leads to DNA damage sensitivity. This implies that phosphorylation of S3, S11, S12, S30,

T32 and T34 interferes with cell cycle regulation in absence of functional Mre11 protein.

We also tested all the $rfa2\text{-}A_m$ multi-mutants for viability on 5-FOA and all can survive as

expected (data not shown). However, DNA damage assay was not performed since

$mre11\Delta$ itself leads to sensitivity to DNA damage. In this study we used $rfa1\text{-}t11$ as a

control and we observed that in $mre11\Delta$ $rfa1\text{-}t11$ double mutant is lethal for cells. This

data imitates the data found in human cells, whereby both Rpa1 and Rpa2 N-terminus

regions were important for interaction with MRN complex (OAKLEY *et al.* 2009).

The phosphorylation state of Rfa2 N-terminus affects intra-complex interactions in

RFA complex

The Rfa1 subunit of RFA is thought to be the majorly involved in DNA-binding

activity, through DBD-A and DBD-B. The DBD-F of this subunit is thought to be

responsible in protein-protein interaction. A well-studied mutation in this domain is *rfa1-*

t11 (K45E). Interestingly this mutant is; replication proficient but recombination deficient

mutant (WANG and HABER 2004). It triggers checkpoint response after DNA damage,

however it interferes in the homologous recombination repair (VANOLI *et al.* 2010). In

our studies, we found that in cell viability assay $rfa1\text{-}t11$ $rfa2\text{-}D_x$ double mutant is viable

but rfa1-t11 $rfa2\text{-}\Delta N_x$ mutant is lethal for cells. This was a very interesting observation

for us since DBD-F and Rfa2 N-terminus asp peptide were shown to interact by NMR.

We further wanted to locate the region responsible for this phenotype and therefore we

co-transformed the *rfa1-t11* mutant plasmid with $rfa2\text{-}\Delta N_m$ multi-mutants. After that we

tested for their recovery on 5-FOA plates as described in methods. We found that the

rfa1-t11 rfa2-ΔN$_{m1}$ and *rfa1-t11 rfa2-ΔN$_{m3}$* were sensitive on 5-FOA (Figure 3.15). Hence

we can conclude that region 1 (S3, S11and S12) and region 3 (S30, T32 and T34),

contribute to the lethality of *rfa1-t11 rfa2-ΔN$_x$* double mutant.

A)
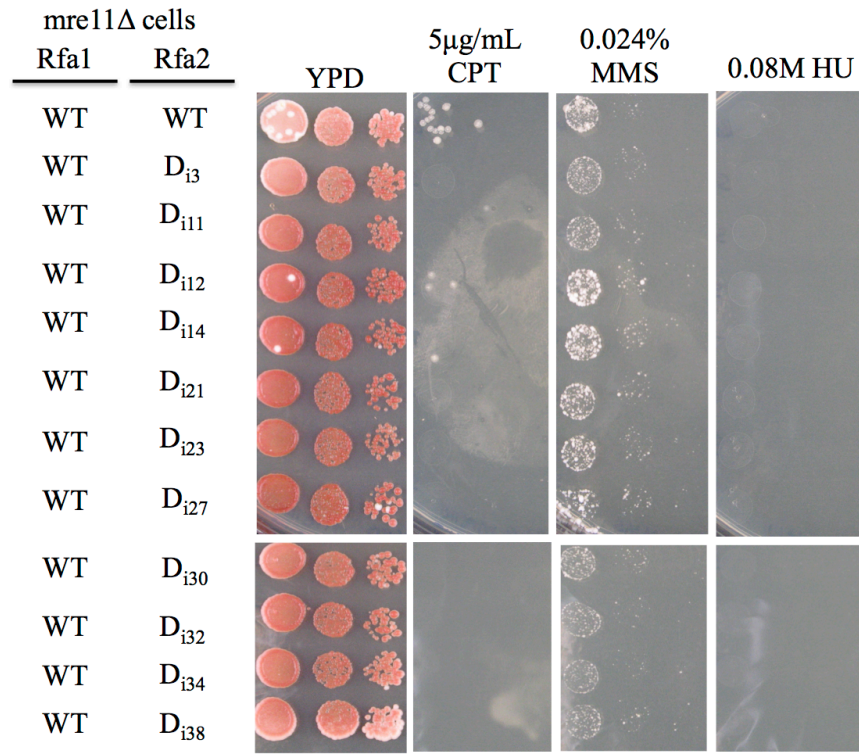
Figure 3.14: The DNA damage and cell viability assay of *rfa2-D$_i$* and *rfa2-D$_m$* mutants in mre11Δ background. A) A DNA damage assay showing that the rfa2-D$_i$ mutants can survive in unstressed condition in mre11Δ background but are sensitive to DNA damage.
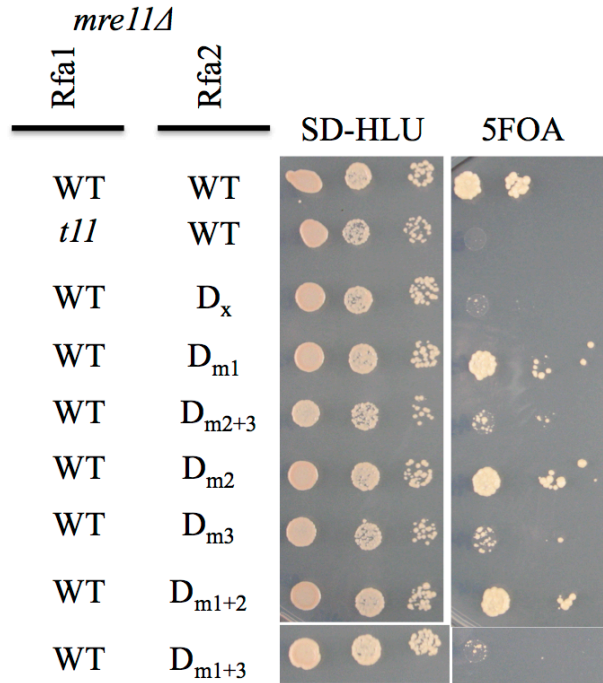
B)

	mre11Δ			
Rfa1	**Rfa2**		SD-HLU	5FOA
WT	WT			
t11	WT			
WT	D_x			
WT	D_{m1}			
WT	D_{m2+3}			
WT	D_{m2}			
WT	D_{m3}			
WT	D_{m1+2}			
WT	D_{m1+3}			

Figure 3.14: The DNA damage and cell viability assay of *rfa2-D$_i$* and *rfa2-D$_m$* mutants in mre11Δ background (continued). B) A shuffle out assay of *rfa2-D$_m$* multi-mutants showing *mre11 Δ rfa2-D$_x$* double mutant is lethal and that region 1 and 3 are responsible for this phenotype.

Rfa1	Rfa2	SD-HLU	5-FOA
WT	WT		
t11	WT		
WT	ΔN_x		
t11	ΔN_x		
t11	N_{m1}		
t11	N_{m2}		
t11	N_{m3}		
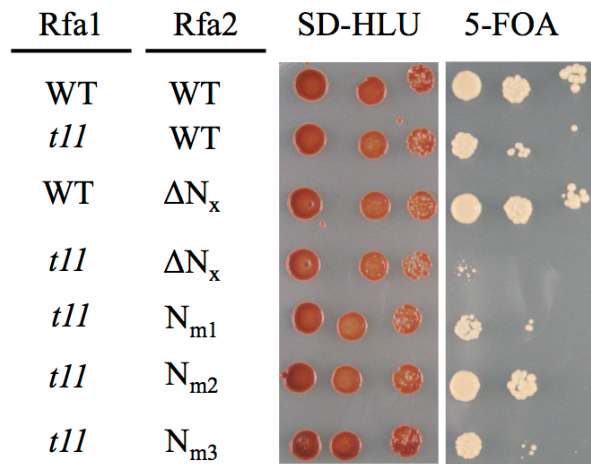
Figure 3.15: The shuffle out assay showing viability of *rfa1-t11 rfa2-ΔN$_m$* double mutants. The cell viability is determined on the basis of ability to grow on 5-FOA plates. The *rfa1-t11 rfa2-ΔN$_x$* mutant is lethal while Nm1 and Nm3 region with *rfa1-t11* are showing reduced growth on 5-FOA plates.

Deletion of Rfa2 N-terminus rescues the synthetic lethality caused by Rfa1 aromatic mutants

RFA is a heterotrimeric complex with multiple roles in DNA metabolism. It is well known that, proteins forming a single complex affect each other's function. The N-terminus of Rfa1 and Rfa2 are hypothesized to affect the function of RFA complex by interacting with other subunits. In humans, an NMR study showed direct interaction between the N-terminus of Rpa1 and Rpa2 (BINZ *et al.* 2003b). This interaction is thought to play role in regulating protein-protein interactions. We have previously shown that the *rfa2-ΔN$_x$ rfa1-t11* double mutant is not viable indicating that these mutants are not involved in the same pathway. In this study, we use mutagenesis to examine if the phosphorylation status of Rfa2 N-terminus affects function of Rfa1 with the help of *rfa1-Aro* mutants (Figure 3.16A). These aromatic residues are important for ssDNA-binding activity of Rfa1 and these residues are conserved in humans, mouse, drosophila and yeast (HASS *et al.* 2012).

A)

B)
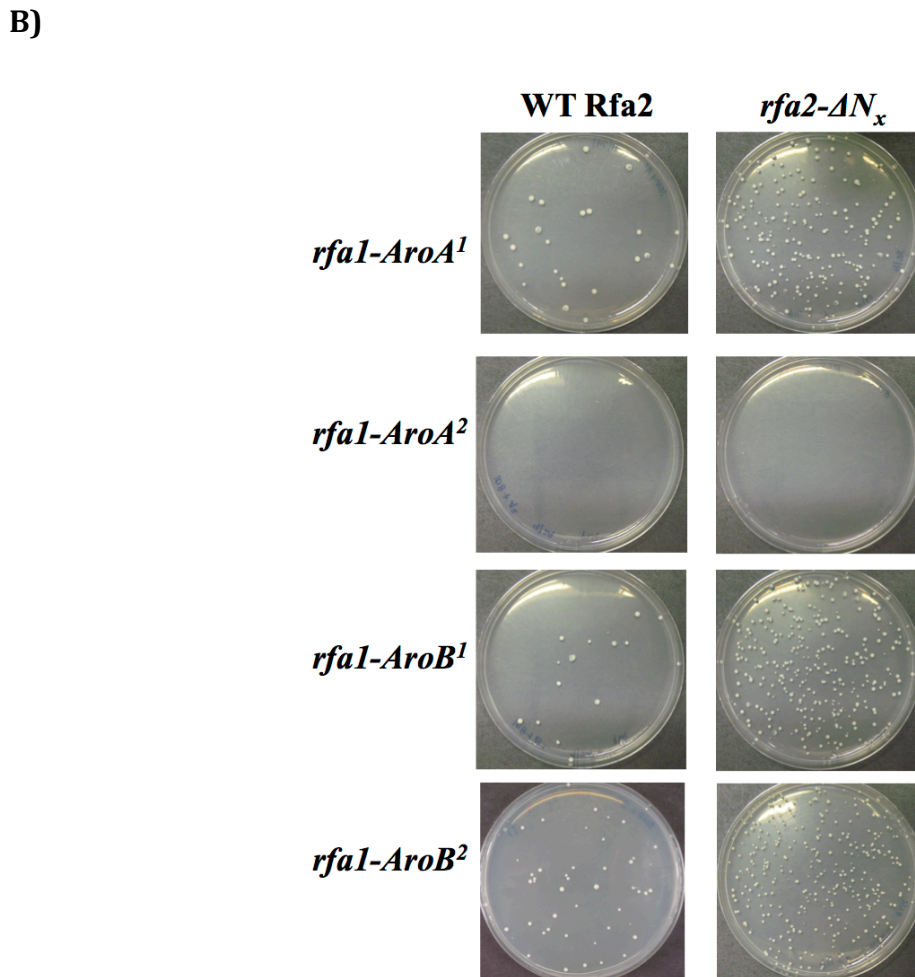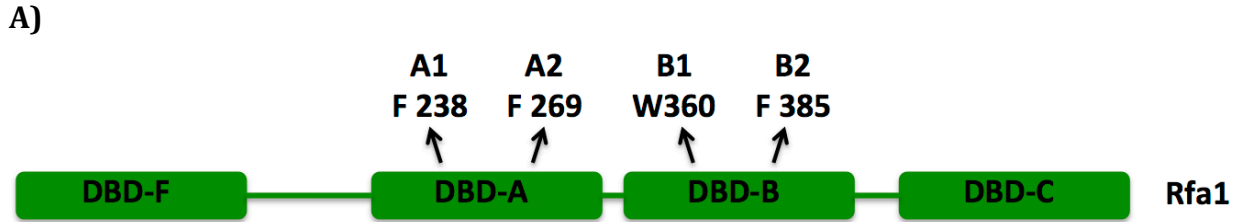
Figure 3.16: A diagram representing the *rfa1-Aro* mutants sites in Rfa1 and the shuffle out assay of *rfa1-Aro* single mutants. A) A diagrammatic representation of *rfa1-Aro* mutants showing location of aromatic amino acids essential for ssDNA-binding in DBD-A and DBD-B. These are renamed as A^1, A^2, B^1 and B^2 as shown here. B) All single *rfa1-Aro* single mutants expressed with WT copy of Rfa2 are shown n the left side while with *rfa2-ΔN$_x$* mutant are shown on the right.
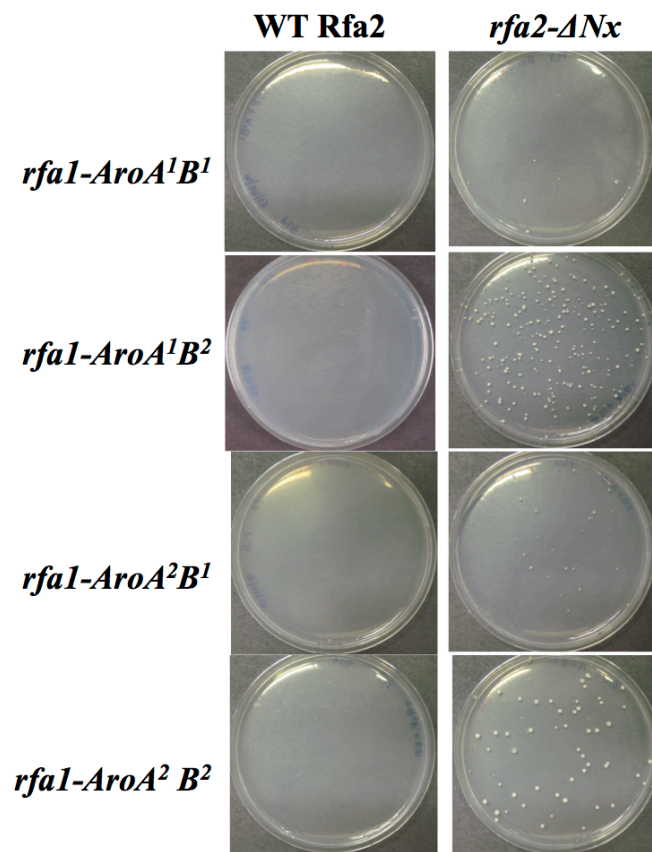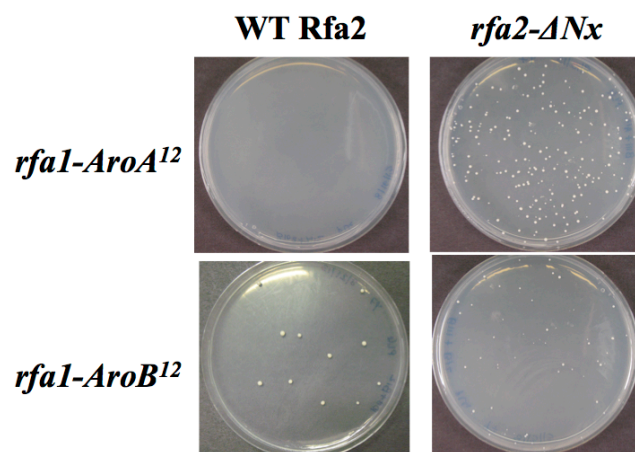
Figure 3.17: Shuffle out assay of *rfa1-Aro* double mutants. A) All the *rfa1-Aro* double mutant combinations located in different DBD are shown here, these are not viable in presence of WT Rfa2 but are rescued in presence of *rfa2-Nx*. B) All double mutants located in the same DBD are shown here this data indicates that aromatic a.a. in DBD-A are more important for cell survival as compared to DBD-B.

The *rfa1-Aro* mutant plasmids were co-transformed with WT Rfa2. All the triple

and quadruple *rfa1-Aro* mutants were lethal and we did not recover any colonies on 5-

FOA. All single mutants were viable except the *rfa1-AroA^2* mutant; in all three

independent experiments we did not recover any colonies on 5-FOA. This was

contradictory to previous study carried out by Philipova et al, which showed that *rfa1-*

AroA^1 (Phe 238) is lethal for cells (PHILIPOVA *et al.* 1996). In humans, it was shown that

the individual aromatic residue mutated to alanine reduces the affinity towards DNA only

moderately (WALTHER *et al.* 1999). Hence the recovery of *rfa1-A^1*, B^1 and B^2 is not

surprising and shows that in yeast it follows a similar pattern Figure 3.16 B.

It was previously shown that the ssDNA-binding activity of the RPA complex

requires both DBD-A and DBD-B. In fact the A and B domains by themselves bind DNA

weakly and stable ssDNA interactions is achieved only when both the domains are

present (BRILL and BASTIN-SHANOWER 1998; ARUNKUMAR *et al.* 2003). When we

studied combination double mutants whereby both domains have one mutation, we did

not recover cells. This is because function of both the domains was hampered. It was

shown that when we both the sites in the same domain are mutated it reduces the binding

of that domain (BASTIN-SHANOWER and BRILL 2001). These double mutants were

interesting to study, as we did not recover *rfa1-AroA^{12}* and *rfa1-AroB^{12}* was viable (Figure

3.17B). This suggests that the DBD-B has a dispensable function in ssDNA-binding,

which is also suggested by single mutant data where both DBD-B single mutants were

viable.

In order to study the effect of phosphorylation state of Rfa2 N-terminus on Rfa1

function, we studied *rfa1-Aro rfa2-ΔN$_x$* double mutants. As shown in Figure 3.16, Figure

3.17 and Table 3.4 surprisingly *rfa2-ΔN_x* rescues the *rfa1-Aro* single and double mutants. The only mutant that remains unaffected is *rfa1-AroA²*. All the DBD-B single and double mutants were found viable in presence of WT Rfa2, and we recovered more colonies in presence of *rfa2-ΔN_x*. This indicates that the absence of Rfa2 N-terminus enhances the growth. It is noteworthy that the *rfa1-AroA²* mutant shows a dominant negative phenotype. All the triple and quadruple mutants are lethal for the cells and were not recovered by *rfa2-ΔN_x* expression.

The Rfa2 extreme mutants do not interfere with DNA damage response and induce phosphorylation of Rfa2 in a region other than N-terminus

When subjected to DNA damage the cells undergo two processes, one is halt cell cycle progression and the second is to recognize and fix damage. The halting occurs via the DDR pathway, a complex signal transduction network, which regulates the response. The phosphorylation of checkpoint kinase Rad53 is considered as an ultimate mark of activation of the DDR pathway (MA *et al.* 2006) (JANKE *et al.* 2010). Another important feature of DDR pathway is the phosphorylation of Rfa2 in response to DNA damage by Mec1 (BRUSH *et al.* 1996) (JANKE *et al.* 2010). In this pathway the Rfa2 and Rad53 phosphorylation events are important and we hypothesize that they affect each other.

In this study we tested the phosphorylation of Rfa2 and Rad53 in cells, which contain the Rfa2 extreme mutants as the sole copy of Rfa2.

As shown in Figure 3.18 (A) when induced with 0.03% MMS we can see increased expression of Rfa2 as compared to unstressed samples in blot without Phos-Tag (upper blot). This increased expression is not unusual, it was previously demonstrated that RFA expression increases in response to DNA damage (TKACH *et al.* 2012). When

we add 50μM Phos-Tag we can see a doublet all across (lower blot). Again, the Rfa2 is known to be phosphorylated during G1/S transition and M phase of cell cycle (DIN *et al.* 1990) (FANG and NEWPORT 1993). However the distinct observation in this assay is that in *rfa2-D$_x$* sample the phosphorylated species is slightly higher in MMS induced sample as compared to WT and *rfa1-t11* cells. Also the phosphorylated species in the MMS induced *rfa2-ΔN$_x$* cells is higher and defined species. We lowered the concentration of Phos-Tag to 25 μM and we see doublet in MMS induced *rfa2-D$_x$* and *rfa2-ΔN$_x$* samples only validating the results we saw with 50μM Phos-Tag. This indicates that although Rfa2 gets phosphorylated during cell cycle, the *rfa2-ΔN$_x$* and *rfa2-D$_x$* mutants induce a different response (or condition), which triggers phosphorylation of Rfa2 at a site other than N-terminus.

We further tested if the Rfa2 extreme mutants cause any defect in DDR pathway and hence trigger phosphorylation of Rfa2. The Rad53 phosphorylation event is considered a hallmark of functional DDR pathway. As shown in Figure 3.18 C that Rad53 phosphorylation remains unaffected by all the Rfa2 extreme mutants. This indicates that in this experiment the DNA damage was successfully induced by MMS treatment and that Rfa2 mutants do not affect the DDR pathway.

A)

SDS-PAGE with 50μM Phos Tag
followed by αRfa2 Western Blot.

B)

SDS-PAGE with 25μM Phos Tag
followed by αRfa2 Western Blot.

C)
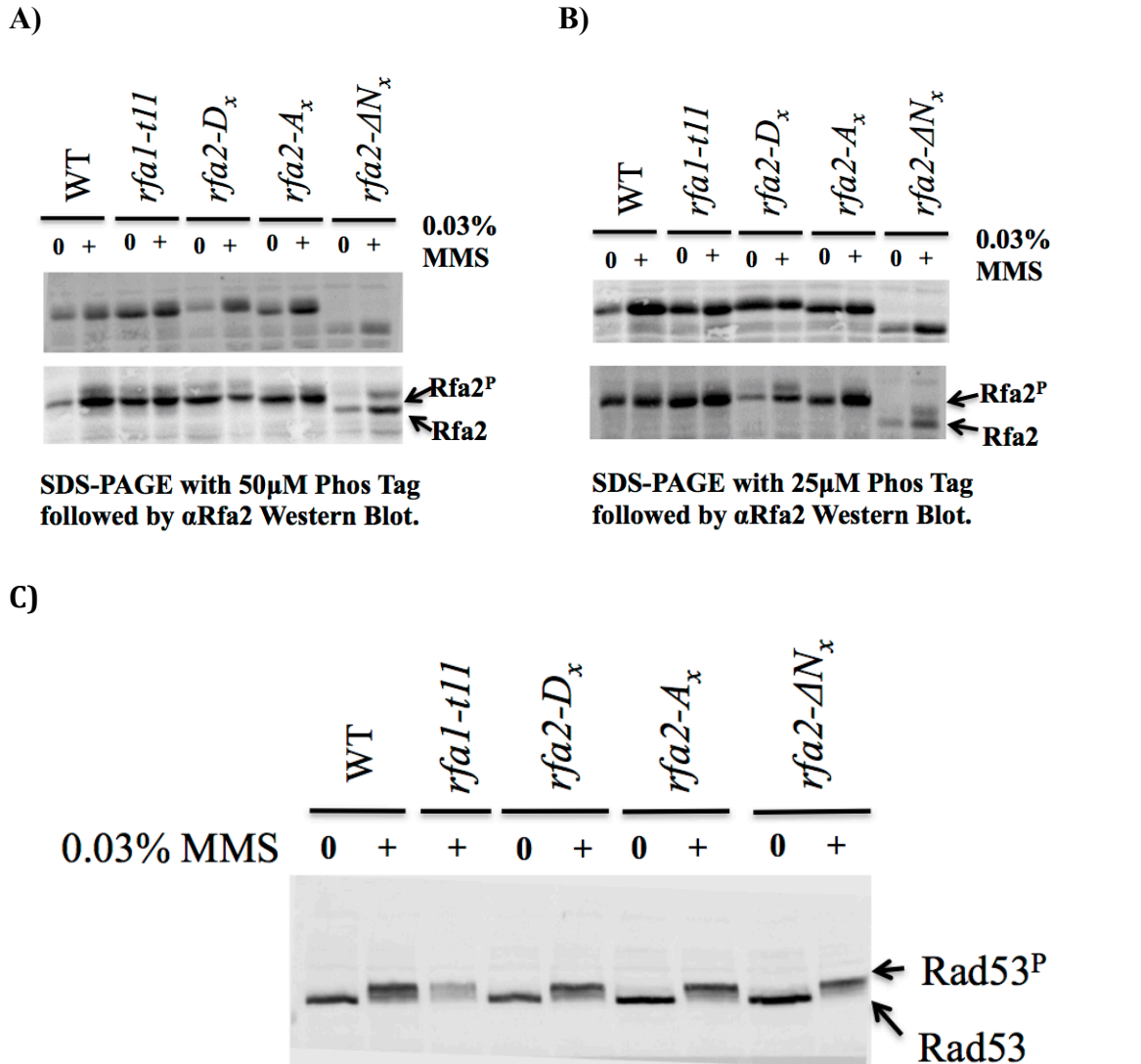
Figure 3.18: Western blots of rfa2 NT extreme mutants for detection of Rfa2 and Rad53 phoshprylation. A) The WB of Rfa2 extreme mutants unstressed (0) and MMS induced samples (+) with α-Rfa2 antibody. The upper blot is developed from 12% SDS-PAGE without Phos-Tag while lower one contains 50μM Phos-Tag. B) The WB of rfa2 extreme mutants unstressed (0) and MMS induced (+) samples. The upper blot is developed from 12% SDS-PAGE gel without Phos-Tag while lower one contains 25μM Phos Tag. C) WB of Rfa2 extreme mutants in unstressed (0) and MMS induced (+) samples developed with α Rad53 antibody showing Rad53 phosphorylation in all samples stressed with MMS.

Discussion

The Rfa2 N-terminus is essential but its phosphorylation is dispensable in yeast

It is well established in human cells, that the Rpa2 N-terminus is hyper-phosphorylated in response to DNA damage. In this study we established that in *S. Cereviciae*, the Rfa2 N-terminus phosphorylation is dispensable. There are 11 potential Ser and Thr sites located in this region and are similar in sequence as compared to their human counterpart. The *rfa2-D$_x$* mimics hyper-phosphorylated for of Rfa2 and supports cell viability. This mutant leads to a sensitive phenotype in presence of DNA damage. However, *rfa2-A$_x$*; a mutant incapable of getting phosphorylated in the Rfa2 N-terminus survives like WT cells in presence of damage. In fact its growth rate is better than wild type cells. This implies that in yeast cells phosphorylation at Rfa2 N-terminus can be avoided in presence of DNA damage. Despite the data shown by *rfa2-A$_x$* mutant we still see *rfa2-D$_x$* cells being sensitive to DNA damage.

This can be explained by considering three different points. Firstly, the *rfa2-D$_x$* represents continual phosphorylation that is an irreversible event, this indicates that de-phosphorylation of this domain would be important. Secondly, the phosphorylation mimicking state might trigger a cell cycle checkpoint. And lastly, the mutation affects the stability of the domain. We addressed this in some other studies carried out in our lab, which are explained further (see section on adaptation Figure 3.21).

Another important observation in this study shows that the *rfa2-ΔN$_x$* is able to support cell viability. This mutant represents absence of the Rfa2 NT (amino acids 1-38). However, in presence of DNA damage this mutant also shows sensitive phenotype. The *rfa2-A$_x$ and rfa2-ΔN$_x$* both mutants are incapable of getting phosphorylated, the

103

difference is physical absence of the domain in the later. Hence, the cells require Rfa2 N-terminus to recover from DNA damage in yeast irrespective of its phosphorylation state. This was also supported by the western blot data, when treated with MMS all the *rfa2* extreme mutants show phospho-species (Figure 3.18A).

Region 1 and region 3 of Rfa2 NT are accountable for DNA damage sensitivity in yeast

In humans, the phosphorylation of Rpa2 N-terminus occurs sequentially. This pattern is seen because different kinases (CDK or PIKK) have different sites as substrate. And phosphorylation at one site by a particular kinase known as priming, leads to phosphorylation at other sites (LIU *et al.* 2012). In order to decipher if similar priming reaction occurs in *S. Cerevisiae* we developed *rfa2* single mutants. If this priming reaction occurred the *rfa2-A_i* mutants would block further phosphorylation of Rfa2. However all the *rfa2-Ai* and *rfa2-Di* mutants showed robust growth and were resistant to DNA damage.

We further developed Rfa2 N-terminus multi-mutants to test if the priming requires more than one site and if any particular group was responsible for the DNA damage phenotypes shown by *rfa2-D_x* and *rfa2-ΔN_x*. We divided Rfa2 N-terminus in to group1 (S3, S11, S12 and T14), group2 (S21, S23 and S27) and group3 (S30, T32 and T34) as shown in Figure 3.9. The *rfa2-ΔN_x* mutant is sensitive to DNA damage and when we divided it to sub regions, *rfa2-ΔN_{m1}* and *rfa2-ΔN_{m3}* were found sensitive. Similarly, the *rfa1-t11 rfa2-ΔN_x* double mutant is lethal and this phenotype can also be attributed to region1 and 3. The *rfa2-D_x* mutant is sensitive to DNA damage and although *rfa2-D_{m1}* is not sensitive as *rfa2-D_{m3}* when combined as *Rfa2-D_{m1+3}* these two regions show the most

sensitive phenotype. Hence overall a combination of these two regions was found most sensitive. We conclude that all phenotypes observed with *rfa2-D$_x$* and *rfa2-ΔN$_x$* can be accounted to combination of region1 and region3. It is noteworthy that the similar region was found important in humans, to activate DNA damage response (L$_{IU}$ *et al.* 2012).

The Rfa2 is phosphorylated to a site located in unknown region of Rfa2 in *rfa2-D$_x$* and *rfa2-ΔN$_x$* mutants

Since rfa2-A$_x$ and *rfa2-ΔN$_x$* cannot be phosphorylated at the N-terminus it is evident that phosphorylation is occurring at some other site on Rfa2. A well-known site is S122 a substrate for Mec1 and the only known site phosphorylated in response to DNA damage. Other sites include T38, S115, S116, Y120, S122 and S189, which were suggested by mass spec analysis of phospho-peptides, but the conditions were not stated (www.phosphogrid.org). The western blot analysis of Rfa2 extreme mutants showed a phospho species in response to MMS induced DNA damage. Since all the three *rfa2* extreme mutants were incapable of getting phosphorylated at N-terminus, this species is generated due to phosphorylation at an unknown site. However, it is noteworthy that these distinct phospho-species are generated only in *rfa2-D$_x$* and *rfa2-ΔN$_x$* samples. It is possible that these mutants trigger Rfa2 phosphorylation for a cellular event apart from DDR.

It is clear from the Rad53 blots that all *rfa2* extreme mutants have a functional DDR pathway. And hence we can say that the N terminal phosphorylation does not affect DDR signaling pathway. In the future it will be interesting to study which sites are phosphorylated in the *rfa2-D$_x$* and *rfa2-ΔN$_x$* mutants using mass spectrometry.

The genetic interaction studies reveal *rfa2-D$_x$* and *rfa2-ΔN$_x$* show similar phenotype but have different mode of action

In this study, we found that *rfa2-D$_x$* and *rfa2-ΔN$_x$* are sensitive to DNA damage. The *rfa2-D$_x$* represents the hyper-phosphorylated state of the N-terminus or continual phosphorylation. While the *rfa2-ΔN$_x$* cannot be phosphorylated and might cause loss of interaction with other repair proteins. We found that both these mutants have different mode of action, through genetic interaction studies.
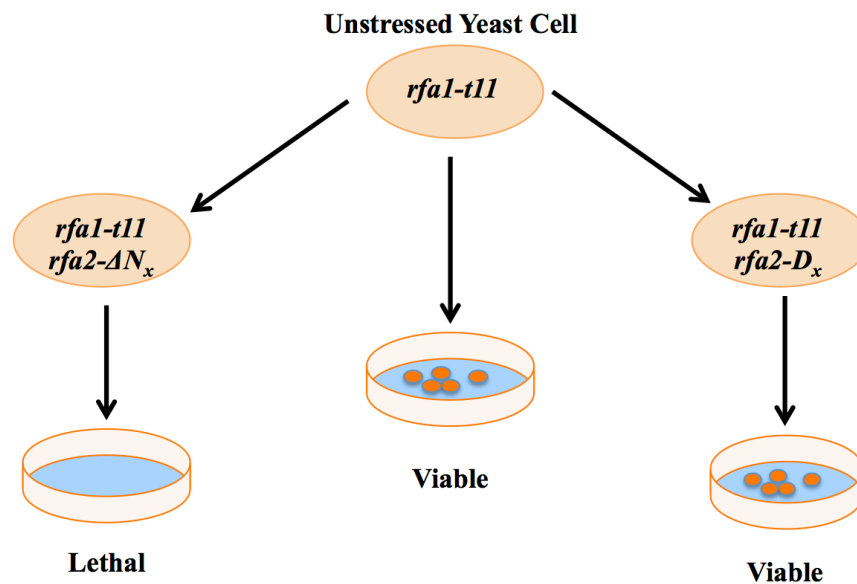
Figure 3.19: A model explaining the differences in mode of action of *rfa2-D$_x$* and *rfa2-N$_x$* mutants with the help of genetic interaction studies with *rfa1-t11*. This study indicates that *rfa1-t11* and *rfa2-D$_x$* have synergistic effect on cell survival.

The MRN complex has a role in DNA replication, DNA damage recognition, cell cycle regulation and repair (LAVIN 2007). In humans, the N-terminus of Rpa1 interacts with Mre11. When phosphorylated, Rpa2 disrupts tis interaction by binding to the Rpa1 N-terminus (OAKLEY *et al.* 2009). In yeast cells, we found that both *rfa1-t11, rfa2-D$_x$* and *mre11Δ* mutants are sensitive to DNA damage, but are viable in unstressed condition.

This indicates that individually they affect the DNA repair process. However, the $rfa2$-D_x $mre11\Delta$ double mutant is not viable in unstressed cells, showing additive effect of these mutants. We saw a similar phenotype with $rfa1$-$t11$ $mre11\Delta$ double mutant. This implies that $rfa2$-D_x affects cellular processes that are independent of Mre11.
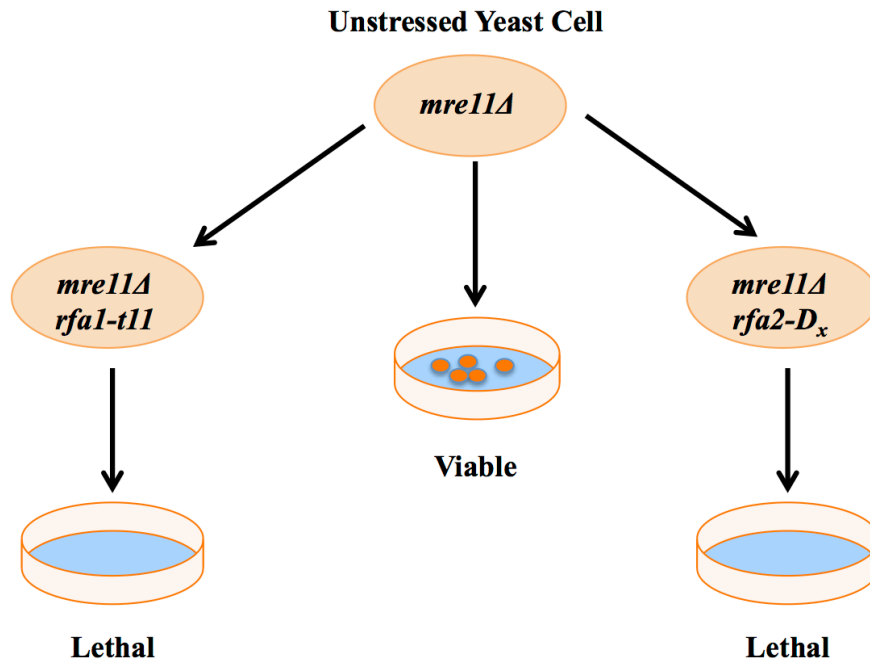
Figure 3.20: A model explaining the synergistic effect of *rfa1-t11* and *rfa2-D$_x$* mutants utilizing genetic interaction studies with Mre11. Both *rfa1-t11* and *rfa2-D$_x$* show non-viability in presence of *mre11Δ*.

As described previously, the proteins involved in the same complex regulate each other's function. We chose to study the $rfa1$-$t11$ mutant because it lies in the same complex but different protein and it is a recombination deficient mutant. The $rfa1$-$t11$ mutant itself in unstressed cells is slightly sensitive while $rfa2$-ΔN_x is not. When combined together, the $rfa1$-$t11$ $rfa2$-ΔN_x double mutant is lethal for cells in unstressed condition. Again, this indicates additive effect of the two mutants. It is noteworthy that the $rfa1$-$t11$ $rfa2$-D_x mutant is sensitive but not lethal for cells. Overall this indicates that

rfa2-D$_x$ and *rfa2-ΔN$_x$* have different effects on cellular growth and DNA damage (Figure 3.19). Also, the *rfa1-t11* and *rfa2-D$_x$* have synergistic effect (Figure 3.20).

Rfa2 N-terminus has a unique role in yeast cells and supports the data in this study

The role of Rfa2 N-terminus phosphorylation is dispensable in response to DNA damage, but it has a unique role in checkpoint adaptation. Data produced by my colleague Mr. Timothy Wilson shows that *rfa2* extreme mutants have differences in checkpoint adaptation and can possibly explain the differences in phenotypes of these mutants. Checkpoint adaptation is the ability of cells to override the G2/M checkpoint even in presence of DNA damage. The adaptation phenotype is assessed with the help of Rad53 phosphorylation. As the cells adapt in presence of broken DNA, the rad53 is dephosphorylated with time. While in adaptation deficient cells, the Rad53 is continually phosphorylated (PELLICIOLI *et al.* 2001). It was shown that the *rfa1-t11* mutation induces adaptation in *ku70Δ* cells and *tid1Δ* cells, which are otherwise adaptation deficient (LEE *et al.* 1998) (LEE *et al.* 2001). Similarly, in our studies we were able to show that *rfa2-D$_x$* induces adaptation in *ku70Δ* cells (GHOSPURKAR *et al.* 2015) see Figure 3.21. Again, this data shows that *rfa1-t11* and *rfa2-D$_x$* have a synergistic effect. This also explains the DNA damage sensitive phenotype of *rfa2-D$_x$*. On plates, the cells are undergoing continuous DNA damage (for around 48-50 hrs). The adaptation proficient cells have more genomic instability and hence show sensitivity. In all the assays the *rfa2-A$_x$* mutant phenotype found similar to WT cells. In this assay as well we found that the *rfa2-A$_x$ ku70Δ* double mutant was adaptation deficient like the WT cells. We hypothesize that the *rfa2-ΔN$_x$* will be adaptation deficient since it has been showing a phenotype different from *rfa2-D$_x$*.

Time (Hr) after HO Induction

	0	2	4	6	8	10	12	15	18

yku70Δ

yku70Δ rfa1-t11

yku70Δ rfa2-D$_x$

yku70Δ rfa2-A$_x$
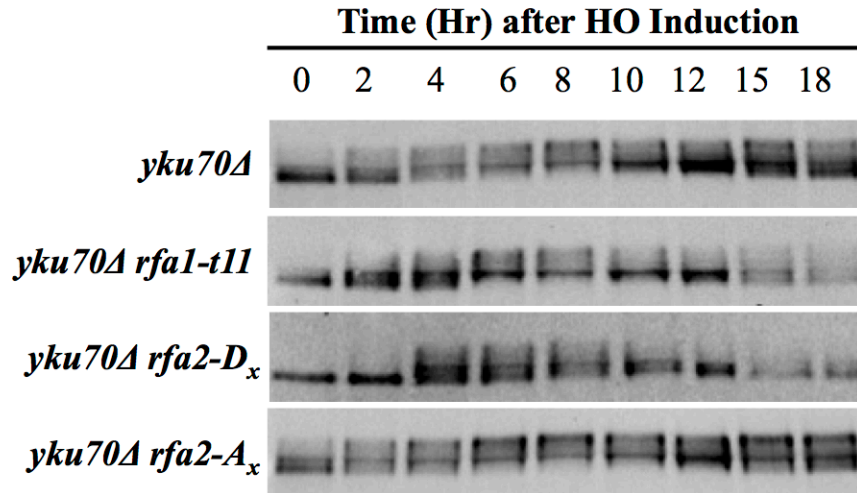
Figure 3.21: Western blot assay to detect Rad53 phosphorylation over the course of time depicting adaptation. This experiment analyses rad53 phosphorylation in response to DNA damage induced by HO endonuclease. The *yku70Δ* cells are adaptation deficient, and they can adapt only in presence of *rfa1-t11* and *rfa2-D$_x$*.

Rfa2 N-terminus deletion rescues the effect of Rfa1 aromatic mutants

Some of the *rfa1-Aro* single and double mutants display a 'no growth' phenotype in unstressed condition. However all of them except *rfa1-AroA^2* recover when *rfa2-ΔN$_x$* is present in cells Figure 3.22. The no growth phenotype can mean two things one, the cells are dead or two the cells are arrested i.e. the growth is temporarily inhibited. The ability to overcome the 'no growth' phenotype in presence of *rfa2-ΔN$_x$* indicates that the *rfa1-Aro* single or double mutants are inducing cell cycle arrest. And the presence of Rfa2 N-terminus is important for the inducing this checkpoint. In human cells, the *rpa1-AroA^{12}* and *rpa1-AroB^{12}* mutants caused cells to accumulate in G2/M phase of cell cycle and they do not localize to sites of DNA repair (HARING *et al.* 2008).

This data shows that the DBD-A aromatic residues are more important than the DBD-B residues. Because all the *rfa1-AroB* single mutants and *rfa1-AroB^{12}* mutant survive in unstressed condition. However, they show better recovery in presence of *rfa2-*

109

ΔN_x indicating that they also contribute probable DNA lesions but not to the point of inducing checkpoint.

The triple and quadruple mutant phenotypes are expected since 75% to 100% of the sites are mutated the RPA cannot bind ssDNA at all and therefore cells die. This was also shown with the help of in vitro data, whereby $rfa1\text{-}AroA^{12}B^{12}$ mutant did not bing ssDNA at all (BASTIN-SHANOWER and BRILL 2001). If we consider that, like humans, $rfa1\text{-}Aro$ single and double mutants are inducing G2/M checkpoint it indicates that irrespective of their ability to bind ssDNA the cells are able to go through replication. This can be explained using a model developed from previous work (BASTIN-SHANOWER and BRILL 2001) (BOCHKAREVA $et\ al.$ 2001).

The model here explains that the ssDNA-binding is initiated by DBD-A followed by binding of DBD-B. These two domains are essential in 8-10 nucleotide ssDNA-binding. As the length of ssDNA increases, the other DBDs of Rfa1 and Rfa2 are involved. Now, the longer ssDNA molecules are generated mostly during replication. Hence, this explains why the $rfa1\text{-}Aro$ mutants are able to replicate but not able to repair. The odd one out of these mutants is $rfa1\text{-}AroA^2$ which despite being a single mutant does not recover in presence of $rfa2\text{-}\Delta N_x$. This indicates that even this single mutation does not allow RFA binding to DNA and cells die.

One interesting observation is that in humans, these cells are shown to accumulate in G2/M, which is rescued by $rfa2\text{-}\Delta N_x$. However, the $rfa2\text{-}\Delta N_x$ does not help cells override the G2/M checkpoint induced by DSB (see section on adaptation). This indicates that the $rfa1\text{-}Aro$ mutants induce checkpoint using a pathway dissimilar to that used in DSB induced repair.
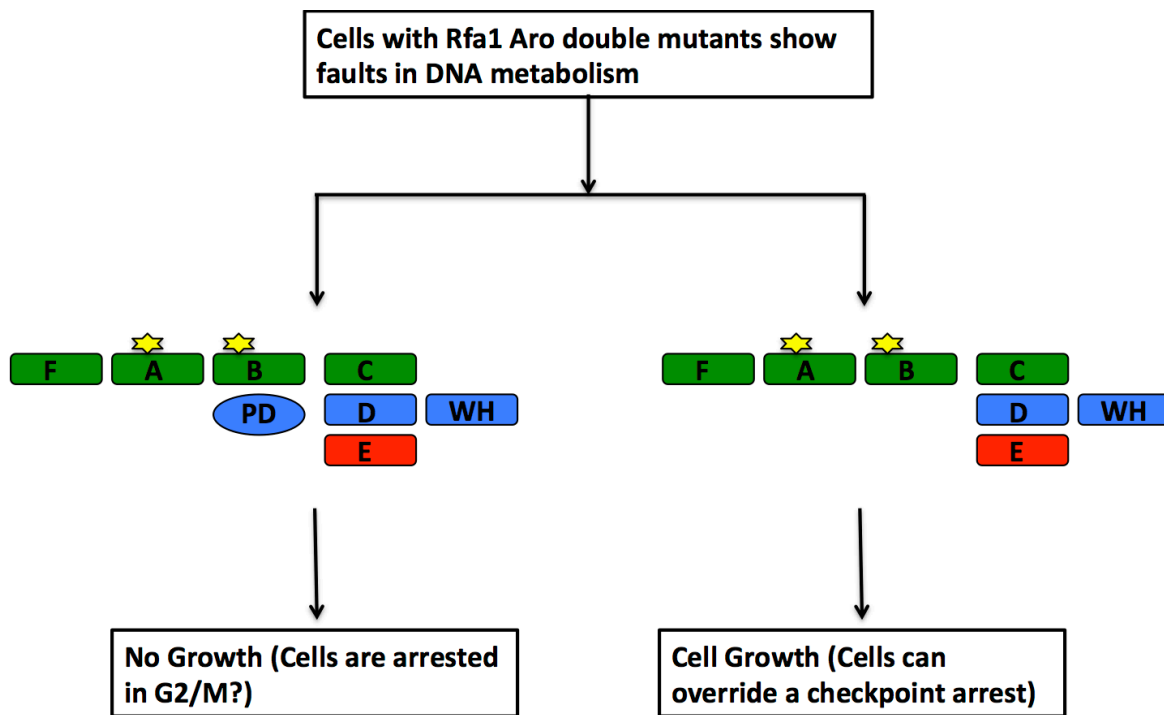
Figure 3.22: A model summarizing the results of *rfa1-Aro* mutants phenotype and their rescue in presence of *rfa2-ΔN$_x$*.

The potential molecular mechanism of Rfa2 NT in checkpoint activation

The genetic interaction data and the adaptation data indicated that the *rfa2-Dx* and *rfa2-ΔN$_x$* mutants have different molecular mechanism. As shown in Figure 3.23 we propose a model explaining the molecular mechanism through which these mutants may differ in their function. DBD-F of Rpa1 and the hyper-phosphomimetic Rfa2 NT peptides were shown to interact with each other in solution with NMR. The negatively charged Rpa2 NT peptide was proposed to act like a DNA molecule, which now can interact with the DBD-F, which has ssDNA-binding properties (OB fold). DBD-F of Rfa1 is also known to interact with a number of proteins. Thus we propose that the *rfa2-D$_x$* mutant is a phosphomimetic mutant and can interact with DBD-F (because of negative charges of

111

Aspartic acid). This then sequesters DBD-F from interacting with its potential protein partner (represented in model by 'X'): an interaction that activates checkpoint arrest. Therefore the *rfa2-D$_x$* causes loss of DBD-F interactions leading to checkpoint adaptation. The *rfa2-ΔN$_x$* mutant on the other hand does not have N- terminus thus it cannot sequester DBD-F from interacting with other proteins. Therefore the proper checkpoint arrest is activated.
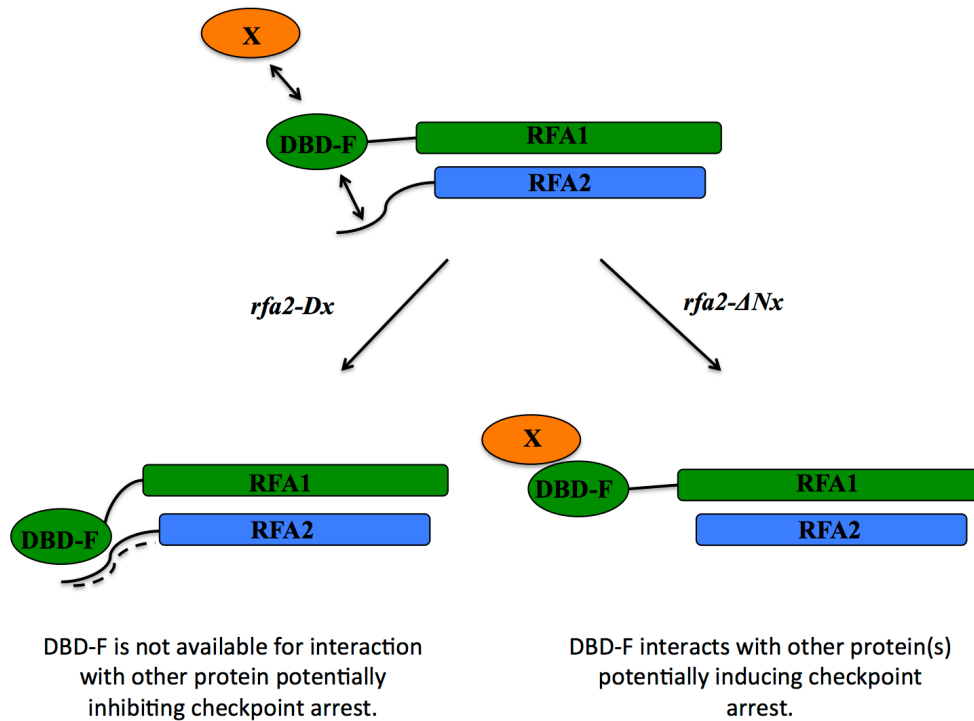
Figure 3.23: A molecular mechanism of Rfa2 N terminus extreme mutants. In the wild type cells DBD-F of Rfa1 has potential to interact with both Rfa2 NT and other protein partners. In *rfa2-D$_x$* cells the DBD-F is sequestered away from potential protein partners. This may play a role in overcoming checkpoint arrest.

<u>Future directions</u>

1.   It will be interesting to locate the sites of Rfa2 phosphorylation in *rfa2-D$_x$* and *rfa2-ΔN$_x$* mutants by mass spectrometry. Also, if those are sites known to be phosphorylated by a kinase or not.

2.　　The *rfa1-Aro* mutants arrested in G2/M checkpoint in humans, and we hypothesized that the in yeast similar checkpoint is induced. To prove this hypothesis we have developed degron system. Unfortunately at this point the degron fails to induce degradation at 37°C and needs to be retested.

3.　　We also want to utilize flow-cytometry to check if the *rfa1-Aro* mutants accumulate in G2/M like their human counterpart.

4.　　We have already tested that during adaptation the Rfa2 N-terminus gets phosphorylated (data not shown), in an experiment carried by my colleague Mr. Timothy Wilson. It would be interesting to see if region 1 and region 3 are phosphorylated in this assay. Also with the help of *rfa2-A$_i$* mutants we will be able to locate important residues and priming if at all it occurs.

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114

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CHAPTER 4. THE MCM COMPLEX INTERACTS WITH RFA1 THROUGH MCM5, A NOVEL INTERACTION DISCOVERED THROUGH THE YEAST TWO-HYBRID SYSTEM

Introduction

Proper genome duplication and maintenance are essential for cell survival. Replication, repair, and cell cycle regulation are interdependent processes that are coordinated to maintain genome stability. An error in any one of these processes can influence or affect the other process and have potentially negative consequences for the cell. The coordination of these processes is occurs through a complex network of proteins and protein modifications. In short, the protein-protein interactions serve to transduce "signals" that can regulate functions of a cell. Therefore, studying protein-protein interaction is an important aspect of understanding these processes.

A common intermediate to all DNA metabolic processes is ssDNA. Replication Protein A is ssDNA-binding protein and thus acts as a "sensor" of the intermediate to coordinate factors important in all of these processes. When ssDNA is generated, it is almost immediately bound by RPA. Thus whether the ssDNA needs to be processed by replication machinery or repair machinery is somewhat dependent on RPA. It is presumed that different ssDNA structures and how RPA interacts with these structures may be important for proper recruitment of downstream processing factors; however, it is currently unclear how RPA directs the cell to perform a particular process?

We and others have hypothesized that RPA regulates the fate of ssDNA generated through coordination of protein interactions, and that some of these protein interactions may be regulated by the post-translational modification of RPA. In this chapter, we

employ yeast two-hybrid assay to discover novel protein interactions of RPA. These assays were aimed to study two specific aspects of RPA: (1) whether differences in protein interactions between Rpa2 and Rpa4 might influence their differing functions, and (2) whether hyper-phosphorylation of the of Rfa2 NT affects the protein-protein interactions of the RFA complex.

Exploring protein-protein interactions through the yeast two-hybrid assay

Proteins are essential for carrying out the many cellular processes necessary for living cells. One can gain a lot of information about protein function by exploring protein sequence and structure, cellular localization, post-translational modifications, and protein-protein interactions These properties define the function of a particular protein(s) in the cell. Furthermore, protein-protein interactions are important because they are useful for characterization of function and identification of the molecular mechanism(s) by which a protein functions in the context of other proteins.

There are number of biochemical and biophysical methods available for studying protein interactions (Table 4.1). Most of the biophysical (e.g. spectrophotometric) assays require the proteins to be isolated from cells with their substrate or as a crystal. These assays are very informative and look closely into enzyme active site, mechanism of binding, and can lead to the identification of the essential binding regions on the basis of structure. One potential drawback of using these techniques is that the proteins are not in their native/natural biological state (*i.e.*, they are not in the context of other cellular proteins).

Table 4.1: List of methods used to study protein-protein interactions

Type of method	Method
Biophysical/ Spectroscopic	Mass Spectrometry, X-ray Diffraction, Surface Plasma Resonance, Fluorescence resonance energy transfer (FRET), and NMR.
Biochemical	Affinity Chromatography, Coimmunoprecipitation
Bioinformatics	Sequence based predictions
Molecular Biology/ Genetics	Yeast two-hybrid Assay, Mutagenesis

Although performed *in vitro,* the biochemical assays provide insight into these interactions. Mostly these are performed in an environment that mimics cellular conditions. These are generally performed with cell extracts or purified proteins. The success of detection of protein interaction in these assays depends on the (a) abundance/ amount of protein (PHIZICKY and FIELDS 1995), (b) whether the interaction is strong and direct, and (c) availability of antibody without cross reactivity. The challenges in this assay are purification of protein and stability of tag. Affinity purification followed by mass spectrometry detection is a very widely used technique. Potential obstacles to this type of assay include cost and accessibility to equipment.

One way to look at protein interactions in the cell is through the use of the yeast two-hybrid assay. When considering this methodology, one must keep in mind the advantages and disadvantages of this system. Some disadvantages include: (1) false-positives (VAN CRIEKINGE and BEYAERT 1999), (2) the fact that the assay uses *S. cerevisiae* as the host organism (lack of organism's other protein or improper protein

expression/folding/modification) (OSBORNE *et al.* 1995), (3) the potential toxicity of proteins, and (4) artifact/biologically irrelevant interactions (due to protein fragments and/or mislocalization. However, this system also has many advantageous features, including: (1) it is *in vivo* assay, (2) does not require purification or high quantity of protein (generally reduces cost, time, and effort), (3) can detect a wide range of strengths of interactions, (4) can provide relative quantitation of interaction, (5) can be used to screen a library of potential interactors, (6) since done in yeast, often provides for extrapolation to higher organisms, and (7) provides a simple system to detect interactions and map interacting regions.

Our aim was to screen through a library of proteins to find novel interactions with RPA subunits. We chose the yeast two-hybrid assay because it would allow us to screen through numerous potential interactors, allow for recovery of the clone expressing the interacting region, and allow for relatively straightforward mapping of the interacting region. The MCF7 cDNA library was screened with human Rpa1 and human Rpa4 as bait. The success of this screening was limited (as discussed in this chapter); however, my colleague, Mr. Gunjan Piya, used this same system to successfully screen a yeast genomic library with Rfa1-FLAB as a bait (PIYA *et al.* 2015). Also described in this chapter is work examining a novel interaction found in this screen between Mcm5 and Rfa1.

Why the Mcm5-Rfa1 interaction is of interest

Minichromosome Maintenance Complex (MCM complex) is a hetero-hexameric complex (*i.e.* it is made up of six closely-related proteins). These are named as Mcm2-7, and Mcm5 is a part of this complex. The MCM complex is a helicase important in

initiation and elongation of DNA replication (HESKETH *et al.* 2015). Two MCM hexamers are loaded on the dsDNA by the Origin Recognition Complex (ORC) along with Cdc6 and Cdt1 (SAMEL *et al.* 2014a). The six proteins form a closed ring structure around dsDNA, which passes through a gap between Mcm2 and Mcm5 (SAMEL *et al.* 2014b). In fact, this coordination between Mcm2 and Mcm5 is important in helicase loading and serves as the DNA entry gate (SAMEL *et al.* 2014a; BRUCK and KAPLAN 2015). The two hexamers then move in opposite directions to unwind dsDNA.

It is evident that Mcm5 and Rfa1 work in same pathway (*i.e.* replication). The RPA and MCM interaction has been suggested in literature a few times (OAKLEY and PATRICK 2010; LEVIDOU *et al.* 2012a). This is because the product of helicase activity of MCM complex (*i.e.* ssDNA) is a substrate for RPA. In fact, when human RPA was expressed as a complex in insect cell line it interacted with Mcm3-7 (NAKAYA *et al.* 2010). In mouse, Rpa1 and Rpa2 were reported to interact with Mcm2 and Mcm4 while Mcm6 interacted with Rpa1 only (KNEISSL *et al.* 2003). These studies have not investigated these interactions any further, and the identification of an interaction between Rfa1 and a fragment of Mcm5 provided for an opportunity to map this interaction further. Interestingly, in the previous studies, Mcm5 was not identified as in interactor.

Mcm5 overexpression is correlated with certain cancers (KELLY *et al.* 2012; DAS *et al.* 2013; YU *et al.* 2014). Also, interrelation of Rpa2 and Mcm5 expression was considered as prognostic significance in ovarian cancer (LEVIDOU *et al.* 2012b). A study carried out in our lab showed that in *S. cerevisiae* Mcm5 interacts with Rfa1, and that this interaction is slightly affected by the phospho-state of the Rfa2 N-terminus. These data

122

indicate the importance of Rfa1-Mcm5 interaction and in this study we aimed to further characterize this interaction.

Methods

Strains and plasmids

The yeast two-hybrid kit was ordered from Origene (DKT100). In this kit, four different yeast strains (Appendix A) were provided with reporter genes developed to detect different sensitivity levels of interaction. These four strains were EGY48, EGY194, EGY188, and EGY40, which contain a *LEU2* reporter gene regulated by decreasing numbers of *lexA* operator (O_{lexA}) sequences. EGY48 cells contain 6xO_{lexA}, providing for the most sensitivity for potentially weak interactions, while EGY40 cells containing no *lexA* operator sequences can serve as a negative control. All the tests were carried out in EGY48 cells, unless otherwise noted.

All plasmids used are described in Appendix B. The bait plasmid used in this study was pEG202 (*HIS3*, 2μ, *amp*^r^) which contains the constitutive P_{ADH1} promoter to drive gene expression of the lexA DNA-binding domain (BD)-fused bait protein (*i.e.*, RpaX, where X could be any of the subunits). pSH18-34 served as an additional reporter plasmid (*URA3*, 2μ, *amp*^r^, 8x O_{lexA}-*lacZ*). Prey plasmids were constructed in the vector pJG4-5 (*TRP1*, 2μ, *amp*^r^), which contains an inducible P_{GAL1} promoter driving expression of the B42 transcription activation domain (AD)-fused prey protein. For the yeast two-hybrid screening we ordered MCF7 cDNA library from Origene (DLH117). All bait plasmids containing *rfa1* gene fragments that encode for the individual domains of Rfa1 (Figure X) are derivatives of pGAL-lexA and were kindly provided by Dr. Susan Gasser (Friedrich Miescher Institute for Biomedical Research, Switzerland).

123

The various bait and prey vectors were generated using an *in vivo* homologous recombination cloning method. First, an RPA/RFA subunit was amplified by PCR using the appropriate pEGFP-RPA plasmid as a template and primers 260-269 (Appendix C). The primers used had homologous ends to pEG202 (bait). My colleague Mrs. Erica Mueller generated the prey RPA/RFA vectors.For generating the MCM prey vectors, genomic DNA isolated from RMY122-A cells was used as template. The various MCM subunits were amplified using primers 596-605. The genomic DNA from RMY122A cells was used as a template to amplify the 3 subregions of the *MCM5* gene. The primer sets (520-533) used to amplify these sub-regions are listed in appendix C. The pJG4-5-Mcm5$_{108-359}$ isolated from the yeast two-hybrid screen was used as template to generate the 50aa deletion plasmids. The 50 amino acid regions were deleted using mutagenic primers 509-513. The pEG202 vector was linearized utilizing the *Nco*I site, while pJG4-5 was linearized by a *Xho*I-*EcoR*I double digest. The linearized plasmid and PCR were co-transformed into EGY48 cells. Plasmid DNA were recovered from these cells and further confirmed by both diagnostic restriction digestion and DNA sequencing (Eton Biosciences). All generated plasmid constructs are listed in Appendix B.

Media used for two-hybrid assays

The viability of cells was tested on YPD plates (2% dextrose, 1% yeast extract and 2% peptone), and to detect any defects in mitochondria that might affect cell growth, cells were grown on YPG plates. The YPG media was similar to YPD except the carbon source was 3% glycerol. SD-HTU plates were used to recover cells with bait, prey, and reporter vectors in each cell. The effect of galactose induction on cells was tested on SG-HTU plates. Auto-activation was examined using SD-HLTU plates, and potential protein-

protein interactions were detected on SG-HLTU plates. The synthetic complete media was made with 2% dextrose (or 2% galactose), 0.5% ammonium sulfate, and 0.17% yeast nitrogen base without amino acid, and an appropriate amount of amino acid dropout mixture was added to this media. For the detection of expression of the *lacZ* reporter gene (encoding for β-galactosidase), 40 μg/mL of 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (X-gal) was added to SG-HTU media.

Yeast two-hybrid screen

Once the bait constructs were tested for auto-activation, the plasmid pPLG? (BD-Rpa1) was co-transformed with pSH18-34 ($8xO_{lexA}$-*lacZ* reporter) into EGY48 cells, and transformants were selected for by plating onto SD-HU (lacking histidine and uracil) media. One colony was used to make competent cells, the transformation efficiency of the cells was measured, and the MCF7 cDNA library was transformed into these cells and plated onto SD-HTU (lacking histidine, tryptophan, and uracil) media (40 independent transformation reactions). The resulting $1.68x10^6$ independent transformants were collected, titered, and $1.68x10^7$ cells (10-fold excess of original number of transformants) were plated onto diagnostic SG-HLTU (containing 2% galactose and lacking histidine, leucine, tryptophan, and uracil) media. Plates were incubated at 30°C, and starting on day four post-transformation, larger colonies were patched onto SD-HTU plates through day fifteen for Rpa4 and day ten for Rpa1. These initial patch master plates were then replica plated onto both SD-HLTU (negative growth control) and SG-HLTU; retest) and grown at 30°C for 3-4 days. Patches that only grew on the SG-HLTU plates were re-picked onto SD-HTU plates as secondary master plates and maintained for further characterization.

Isolation and analysis of prey plasmids (*TRP1* plasmids)

In our assay with BD-Rpa4 as bait, we had a system whereby all the three plasmids (bait, prey and reporter) contained an ampicillin-resistance (*amp^r^*) marker. First, the plasmids were isolated as a part of genomic DNA prep from each individual colony. These plasmids were transformed into DH10B bacterial cells through electroporation. Then individual bacterial colonies recovered on the LB+Amp plates were then tested with colony cracking followed by restriction enzyme digestion. This was to locate the individual bacterial colony with prey plasmid from a particular yeast candidate recovered in yeast two-hybrid. This process was performed until we found the prey plasmid for that particular candidate.

To overcome this tedious process for the BD-Rpa1 screen, we switched the *amp^r^* marker on bait (pEG202) and reporter (pSH18-34) plasmids to kanamycin-resistance (*kan^r^*). This allowed us to directly recover prey plasmid on LB+Kan plates after electroporation. These isolated candidates were tested by restriction enzyme digest to avoid any empty prey vectors.

After the prey plasmids were isolated they were sent for DNA sequencing (Eton Biosciences). The resulting sequences were then subjected to Nucleotide BLAST analysis to find the corresponding gene in the database.

Replica plating assays

About 200 ng of bait, prey, and reporter plasmid were co-transformed into EGY48 cells and isolated on SD-HTU plates. Individual colonies were picked as patches on SD-HTU media. These plates were then replica plated on YPD, YPG, SD-HTU, SG-HTU, SD-HLTU, SG-HLTU and SG-HTU+X-gal plates. The protein interaction was

126

determined by growth on SG-HLTU plates after replica plating. The development of blue color on SG-HTU+X-gal was considered as indicator of strong growth.

Results

Testing auto-activation for RPA subunit candidates

The canonical and alternative RPA complexes function differently with respect to DNA replication; the canonical RPA supports DNA replication while the alternative RPA does not (HARING *et al.* 2010). We hypothesize that Rpa2 and Rpa4 interact with different proteins, potentially leading to differences in function. To test this hypothesis we developed baits of all the four subunits. Before proceeding to the yeast two-hybrid assay, it was important to test auto-activation. If a bait protein can activate the expression of the reporter gene in the absence of an interacting prey partner, this is termed as auto-activation and indicates a false-positive interaction. In this case, the ability of cells to grow on SD-HLU plates indicates auto-activation. The lack of His and Ura in the media ensures that the cells retain bait and reporter plasmid respectively. The lack of leucine in the media tests the expression of reporter. To test auto-activation we transformed the various bait (pEG-202-Rpa1 through Rpa4) constructs with the reporter plasmid (pSH-18-34) and recovered them on SD-HU plates. The colonies were picked on a fresh plates as patches and grown at 30°C for 2 days. These were then replica plated to YPD, YPG, SD-HLU and SD-HU plates. We found that both yeast and human Rpa2 auto-activate (Figure 4.1). The auto-activation of human BD-Rpa2 was reported in a previous study (KESHAV *et al.* 1995). A slight auto-activation was observed for BD-Rpa4 for 4 independent isolates.

127
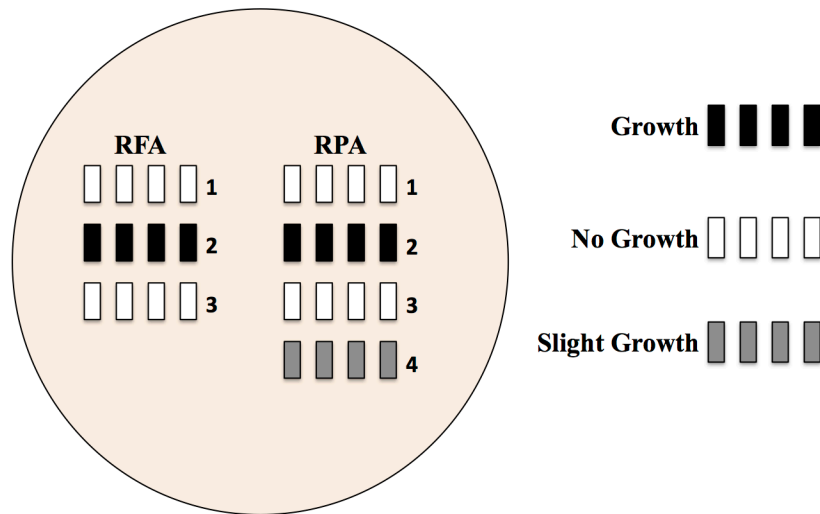
Figure 4.1: Testing auto-activation of RPA and RFA subunits. A schematic representation of RPA and RFA subunits shows both human and yeast Rpa2 auto-activates. The Rpa4-bait shows slight auto-activation.

Since the initial aim of the study was to compare the protein interactions between Rpa2 and Rpa4, the observation of auto-activation, especially for BD-Rpa2, made this a difficult task. Since BD-Rpa2 showed auto-activation we attempted to overcome this by lowering the sensitivity of detection of interaction by using a strain with a reduced number of *lexA* operators (Figure 4.2A). This would lead to detection of only strong bait-prey interaction and may reduce/eliminate the auto-activation observed. Unfortunately, a reduction in *lexA* operators did not reduce the observed auto-activation.

Because auto-activation was still observed for BD-Rpa2, we attempted to map the region causing transcriptional activation by using Rpa2-Rpa4 hybrid protein expressing constructs. Since the Rpa2 homolog Rpa4 displayed only a slight auto-activation, Rpa2-Rpa4 hybrid constructs (Figure 4.2B and C) were generated similar to Haring *et al*. The aim here was to replace the Rpa2 region responsible for auto-activation with the equivalent region of Rpa4; however, all of the hybrid proteins displayed auto-activation.

We would later determine that a full-length BD-Rpa4 also displays transcription

activation activity.

A)
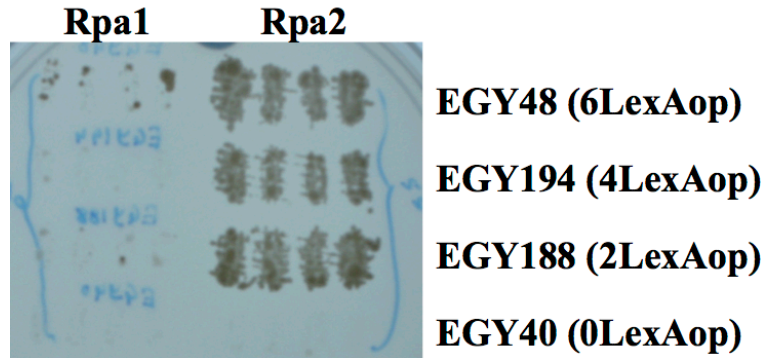
B)
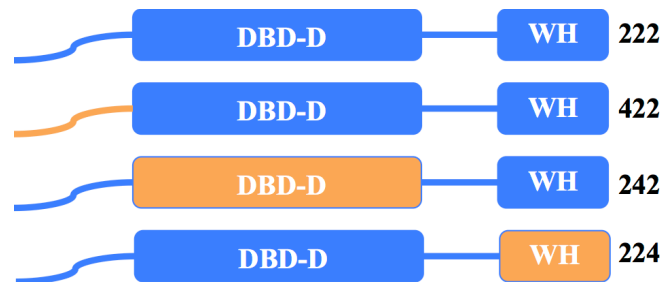
C)
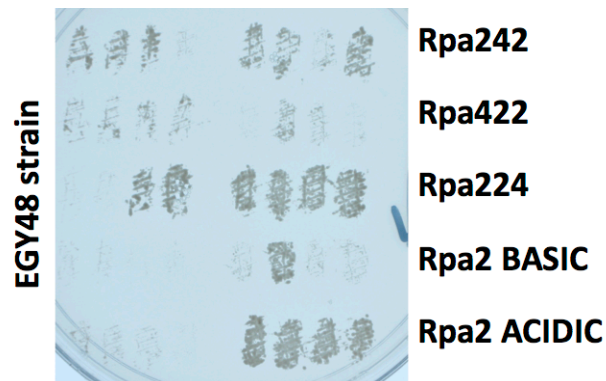
Figure 4.2: Overcoming LexA-Rpa2 auto-activation. A) Strains with different number of LexA operators do not reduce LexA-Rpa2 auto-activation. LexA-Rpa1 served as a positive control. B) A schematic diagram of Rpa2-Rpa4 hybrid constructs. The Blue color regions represent Rpa2 regions while the orange color regions represent Rpa4 regions. C) Eight different candidates were tested for each Rpa2/4 hybrid constructs on SD-HLU plates. Except Rpa422 we saw auto-activation for all constructs. However this construct was not chosen as slight auto-activation was observed.

Table 4.2: List of selected candidates discovered in yeast two-hybrid assay with Rpa4 as bait

Candidate (Gene Symbol)	Number of Hits	Description
Rpa2	2	Homologue of Rpa4
YY1	1	Transcription factor/ transcription repressor
INO80	1	Chromatin remodeling complex
RuvB like 2	1	Helicase
E4F1	2	Transcription factor, ubiquitin ligase, cellular signaling
MAPKKK14	4	Cellular Signaling, involved in CFC syndrome
HAX1	5	Promotes cell survival
RNA pol II (RBP II a)	1	RNA synthesis

Yeast two-hybrid assay with BD-Rpa4

BD-Rpa4 was chosen as the 'bait' vector to screen for novel protein interactions of alternative RPA complex, as it showed little auto-activation. The MCF7 cDNA library was chosen as the prey library. Colonies started appearing on SG-HLTU plates after 5 days and were picked over the next 10 days (15 day period post-transformation). About 150 candidates were picked, and replica plated on various diagnostic media plates as described in the methods. About 100 strong interactions were chosen on the basis of growth on SG-HLTU and development of blue color on SG-HTU+X-gal. The prey plasmids were first diagnosed using a colony cracking method, and then isolated by plasmid miniprep kit (Omega E.Z.N.A. Plasmid mini kit D6942-02). Isolated plasmids

were sent for sequencing to Eton Biosciences. The selected candidates are listed in Table 4.2. The INO80, YY1, and RuvB-like 2 form a complex, which functions in the same pathway (*i.e.*, homologous recombination repair) (Wu *et al.* 2007). It is known that Rpa4 supports homologous recombination and hence isolating these proteins in the screen was supportive of possible novel interaction discovery (KEMP *et al.* 2010). However, we identified that the pEG202-Rpa4 had a truncation (stop codon) after 34 amino acids, thus the assay was not performed with full-length protein. Therefore, the interactions recovered in this assay were not pursued further.

Testing auto-activation of full-length Rpa1 as bait
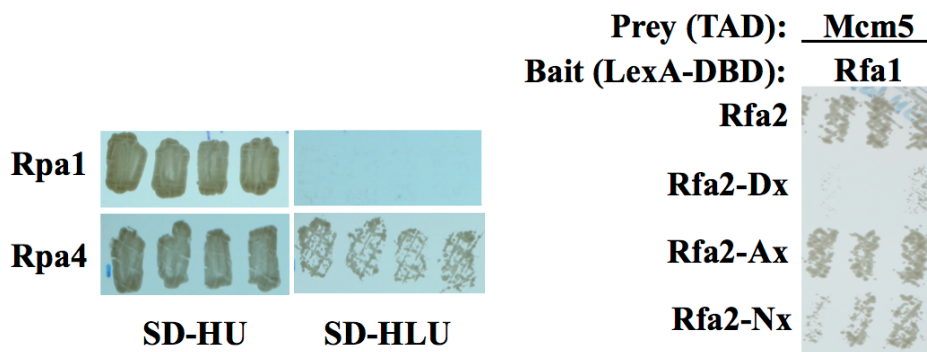
Figure 4.3: Preliminary data for yeast two-hybrid assays. Left panel Auto-activation of full-length BD-Rpa1 and BD-Rpa4 shows that the BD-Rpa1 does not activate and was used further for two-hybrid screen. Right panel In a screen performed by Mr. Gunjan Piya a novel interaction between Mcm5-Rfa1 was discovered this interaction is altered by *rfa2-Dx*.

The previous constructs were made with *Taq* DNA polymerase, which had introduced undesired mutations. We developed new constructs using *Phusion* DNA polymerase (New England BioLabs); these constructs were sequenced and carefully analyzed for mutations before use. Next, the constructs were tested for auto-activation. We found that like its homolog Rpa2, full-length Rpa4 displays auto-activation (Figure

4.3A). The BD-Rpa1 construct does not auto-activate, and a two-hybrid assay was

performed using BD-Rpa1 as bait.

Yeast two-hybrid assay with Rpa1

Table 4.3: A list of candidates discovered during yeast two-hybrid assay with Rpa1 as bait.

Candidate (Gene Symbol)	Number of Hits	Fusion Junction (Base number)	Description
Rpa2	100+	Not calculated	Replication protein A subunit (Known Rpa1 interaction)
HAX1	2	Not found	Promotes cell survival
E4F1	5	27	Transcription factor
HCFC1 (Host cell factor C1)	3	-70	Cell cycle and transcriptional regulation during herpes simplex virus infection.
HSPA8	3	542	Heat shock protein 8
DEAF1	1	27	Transcription factor
ZMYND19	9	-70	Binds to the C terminus of melanin-concentrating hormone receptor-1
TCEB2	16	-15	Transcription elongation
DNAJB6	2	90	DNAJ (Hsp40) homologue
DNAJA2	2	183	DNAJ (Hsp40) homologue

We recovered around 300 candidates in the yeast two-hybrid assay with Rpa1 as

bait. Candidates chosen were also selected on the basis of blue color developed on SG-

HTU+Xgal in addition to growth on SG-HLTU. Prey plasmid DNA were isolated from

100 candidates and sent for sequencing. The sequences were analyzed with BLAST tool

to identify the gene and the protein it encodes. Many of these candidates were Rpa2, which is a known interaction of Rpa1. This confirmed the authenticity of assay; however, it was also an unfortunate drawback for this assay. The Rpa1-Rpa2 interaction dominated the screen; and we could not recover many other novel candidates unless we screened through 1000's of additional candidates. Some of the interactions recovered in this assay (besides Rpa2) are listed in Table 4.3.

Rfa1 interacts with Mcm5 in the 108-359 amino acid region

In a yeast two-hybrid assay with Rfa1-FLAB (lacking DBD-C) as bait, a novel interaction with Mcm5 was discovered (PIYA *et al.* 2015). This interaction appears to be negatively affected by the state of the Rfa2 N-terminus (GP, unpublished data; Figure 4.3 B). The prey plasmid was identified to encode an Mcm5 fragment containing amino acids 108-359. We further characterized this interaction using different regions of the *MCM5* gene encoding for different regions of Mcm5 as prey and full-length Rfa1 as bait. Mcm5 was divided into three regions: 1-107 ($Mcm5_{1-107}$), 108-359 ($Mcm5_{108-359}$), and 360-end ($Mcm5_{360-775}$). We observed that $Mcm5_{108-359}$ shows strongest interaction with Rfa1 (Figure 4.4). We also tested full length Mcm5 as prey; however, detectable interaction with Rfa1 was not observed (data not shown; see discussion). The interaction observed with all three Mcm5 fragments indicates that the Rfa1-Mcm5 protein may not be limited to the $Mcm5_{108-359}$ region.
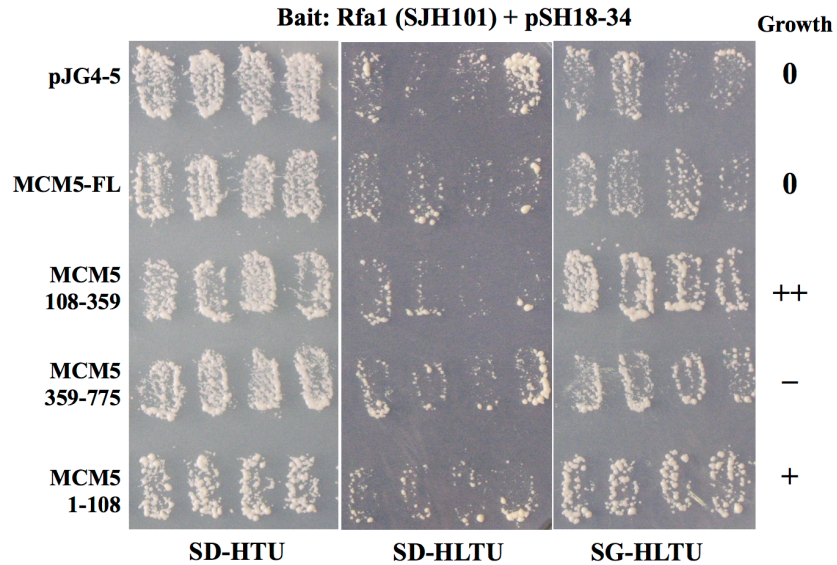
Figure 4.4: Replica plating assay-testing interaction of Mcm5 fragments with BD-Rfa1. In the BD-Rfa1-FLAB yeast two-hybrid screen we isolated the middle region of Mcm5 (108-359 aa). To analyze further points of interaction the other two regions 1-108 aa and 359-775 aa were cloned in pJG4-5 plasmid. Here we observe maximum growth with $Mcm5_{108-359}$. Growth Scale: $0 < - < + < ++$

A 50 amino acid subregion in Mcm5 appears important for interaction with Rfa1

To characterize the $Mcm5_{108-359}$ interaction with Rfa1, we generated 50 amino acid deletion mutants of the $Mcm5_{108-359}$ region. Five different deletion mutants were created as shown in Figure 4.5A. We tested interaction of these deletion mutants with Rfa1. If a region is important for interaction with Rfa1, its deletion will lead to reduced/lack of growth on SD-HLTU plates. We found that the 208-257 amino acid fragment of Mcm5 leads to reduced growth (Figure 4.5B).

A)

B)
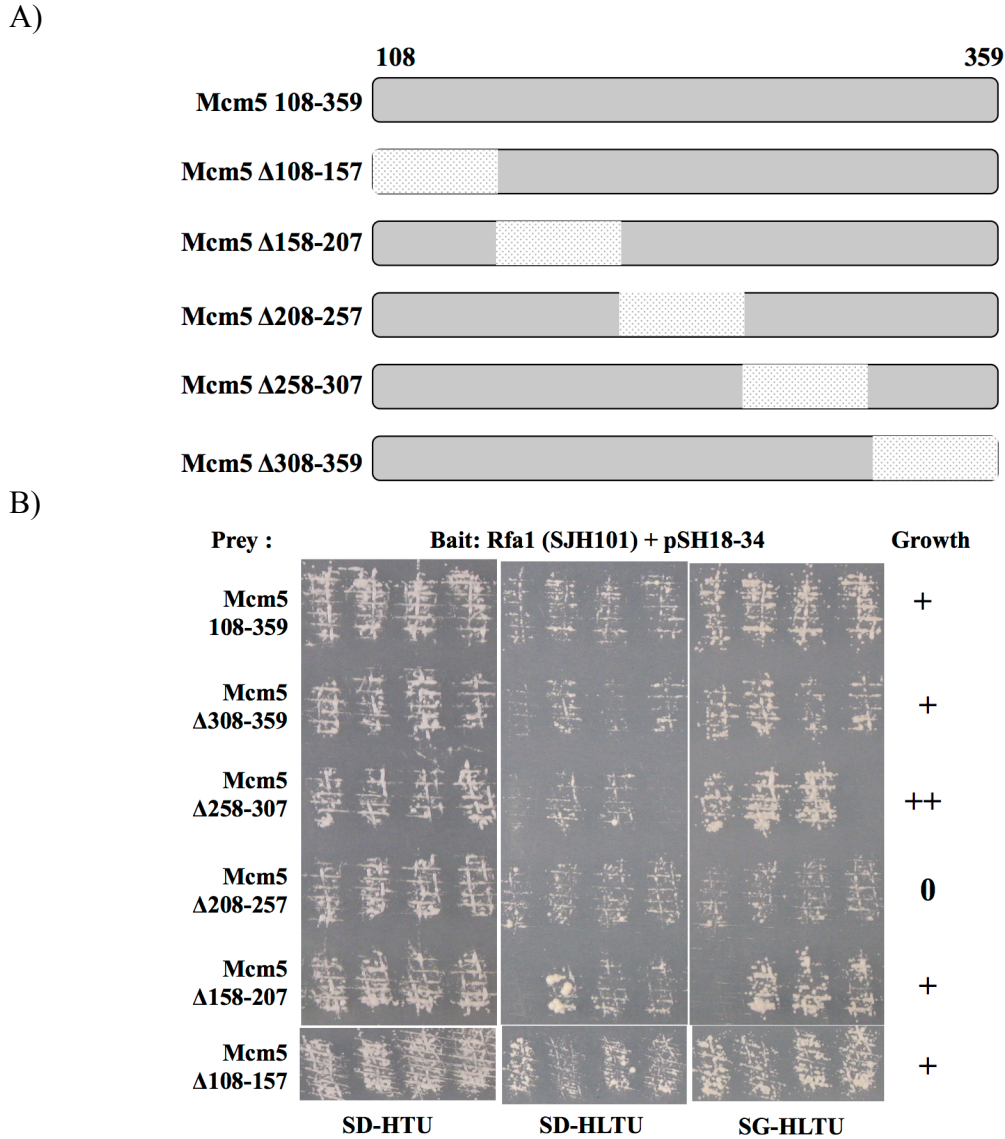
Figure 4.5: A 50 amino acid region is important for Mcm5-Rfa1 interaction. A) The cartoon representation of 50 amino acid deletion mutants created to locate important region in the Mcm5$_{108-359}$ fragment. B) A deletion of amino acids 208-257 in Mcm5$_{108-359}$ fragment lead to disruption of interaction with BD-Rfa1 indicating that this region is important for the Rfa1-Mcm5 interaction.

Rfa1 DBD-B is important for interaction with Mcm5

To further characterize the Rfa1-Mcm5 interaction, we tested different regions of Rfa1. The Rfa1 bait vectors, which encode for various regions of Rfa1, were kindly provided by Dr. Susan Gasser (Figure 4.6). The full length Rfa1 and Rfa2 served as a

135

control in these reactions while the vector 965 was empty backbone of bait vectors. In this study, we found that the DBD-B is important for interaction with Mcm5 (Figure 4.7A), as this combination provided for growth on SG-HLTU. All the controls show no growth with empty bait vector pGAL-lexA (965) or with prey vector pJG4-5 (see Figure 4.7C). We did not see development of blue color on SG-HTU+Xgal plates in this assay (data not shown). This lack of blue indicates that the Mcm5-Rfa1 interaction is a weak or transient interaction.
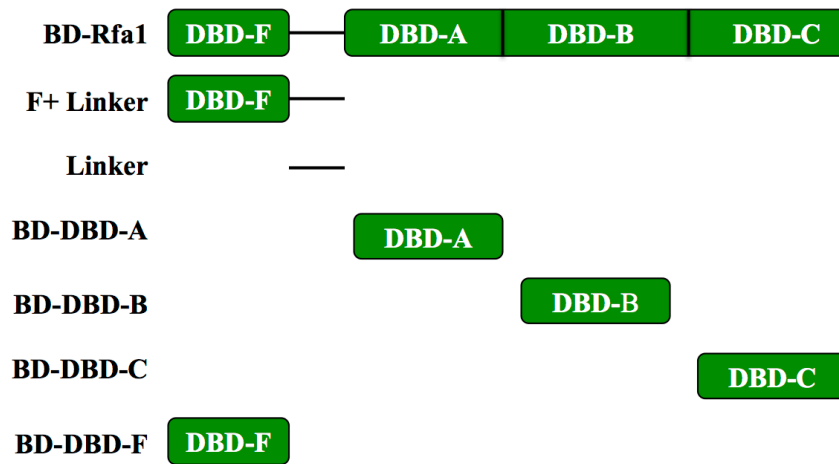
Figure 4.6: A diagrammatic representation of various BD-Rfa1 constructs provided by Dr. Susan Gasser. The Rfa1 protein is divided on the basis of its DNA-binding domains.

Rfa1 DBD-B is important for interaction with MCM complex

Although we located the 50 amino acid region in Mcm5 important for interaction with Rfa1, we cannot overlook that Rfa1 also interacted with $Mcm5_{1-108}$ and $Mcm5_{360-775}$ fragments. This indicated that other regions of Mcm5 could contribute to the interaction with Rfa1. The MCM complex proteins Mcm2-7 are all a closely related group of proteins and share sequence and structure similarities (FORSBURG 2004).

A)
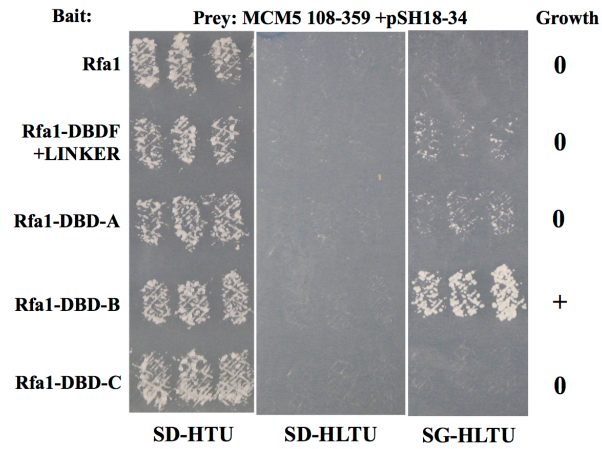
B)
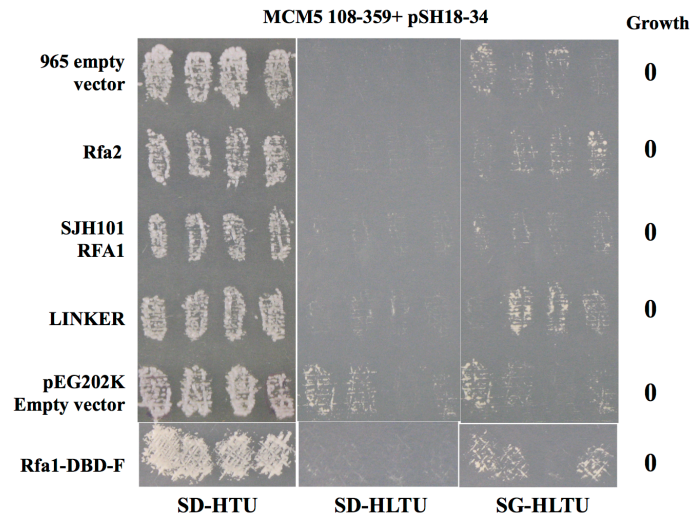
C)
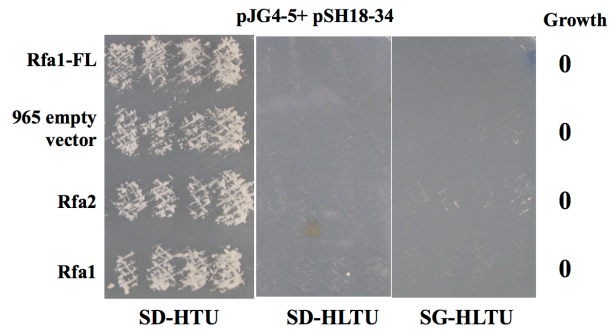
Figure 4.7: Replica plating assay to determine important region of interaction in Rfa1: A) Only DBD-B of Rfa1 interacts with $Mcm5_{108-359}$. B) Replica plating assay indicates Mcm5108-359 does not show interaction with full length Rfa1 and Rfa2. C) Controls for replica plating assay of $Mcm5_{108-359}$ with Rfa1 constructs provided by Dr. Susan Gasser.

Hence, it is possible that Rfa1 interacts with multiple subunits of MCM complex and indirectly with these other Mcm5 fragments. To test this possibility we developed prey vectors with different MCM subunits. These constructs were tested with Rfa1 bait vectors provided by Dr. Susan Gasser. We found in this assay that all the MCM subunits were able to interact with Rfa1 DBD-B (Figure 4.8) to various extents, although interaction with Mcm4 and Mcm5 appeared to be the strongest as indicated by growth on SG-HLTU.
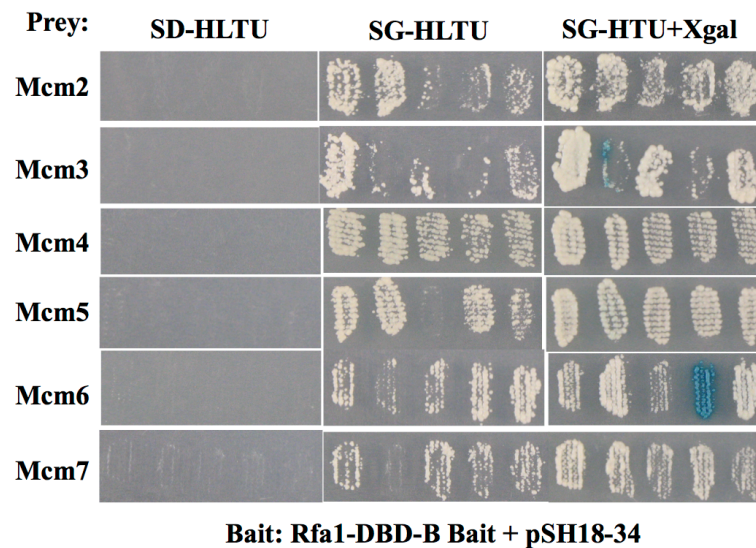
Figure 4.8: Replica plating assay to determine if Rfa1 interacts with other MCM subunits. The picture here indicates that all MCM subunits interact with Rfa1 through DBD-B (growth on SG-HLTU plates).

A model based on *Sulfolobus solfataricus* hints at function of Rfa1-Mcm5 interaction

The known full crystal structure of MCM complex is based on the MCM complexes from Sulfolobus solfataricus, Methanothermobacter thermautotrophicus, and Pyrococcus furiosus. We compared the 50 amino acid sequence of Mcm5 important for Rfa1 interaction with the MCM sequence of S. solfataricus. We found that this region is similar, and have some conserved residues (Figure 4.9).

138

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Sso            ITIDGILVKVTPVKERIYKATYKHIHPDCMQEFEWPEDEEMPEVLEMPTICPKCGKPGQF 180
mcm5           -----------------------PRSCLSTIESESSMANESNIGDESTKKNCG-PDPY 34
                                       .*:. :*  ..    . :   :   :** *. :

Sso            RLIPEKTKLIDWQKAVIQERPEEVPSGQLPRQLEIILEDDLVDSARPGDRVKVTGILDIK 240
mcm5           IIIHESSKFIDQQFLKL------------------------------------------ 51
               :* *.:*:** *   :
```

Figure 4.9: Sequence alignment of *Saccharomyces cerevisiae* Mcm5$_{208-257}$ and *Sulfolobus solfataricus* Mcm. The 50 amino acid region important for interaction between Rfa1 and Mcm5 is found to have similar sequence in *Sulfolobus solfataricus* Mcm. We utilized the crystal structure of Mcm from *S. solfactaricus* to gain insight on importance of this region in Rfa1-Mcm5 interaction.

The C terminus MCM structure of *S. solfataricus* was aligned with the near full length MCM structure from *S. solfataricus* and created a full MCM hexameric complex mimicking *S. cerevisiae* MCM with the help of PyMOL (The model was made by Ms. Jaime Jensen courtesy Colbert Lab). This was developed using the crystal structures available PDB ID 2VL6 (*Sso* N-terminal domain) and 3F9V (near full length crystal structure of *S. solfataricus* MCM). The 50 amino acid region similar in both species is highlighted in cyan blue (Figure 4.10 and 4.11). The model here illustrates the potential orientation of both the complexes on DNA. Based on this model the ssDNA generated due to helicase activity of archaeal MCM complex emerges from the N-terminus end. The Mcm5$_{208-257}$ region important for Mcm5-Rfa1 interaction is predicted to be located exposed and available for interaction on the N-terminal side. Thus supporting the hypothesis that after helicase activity of the MCM complex, the RFA complex would be in a position to readily bind ssDNA.
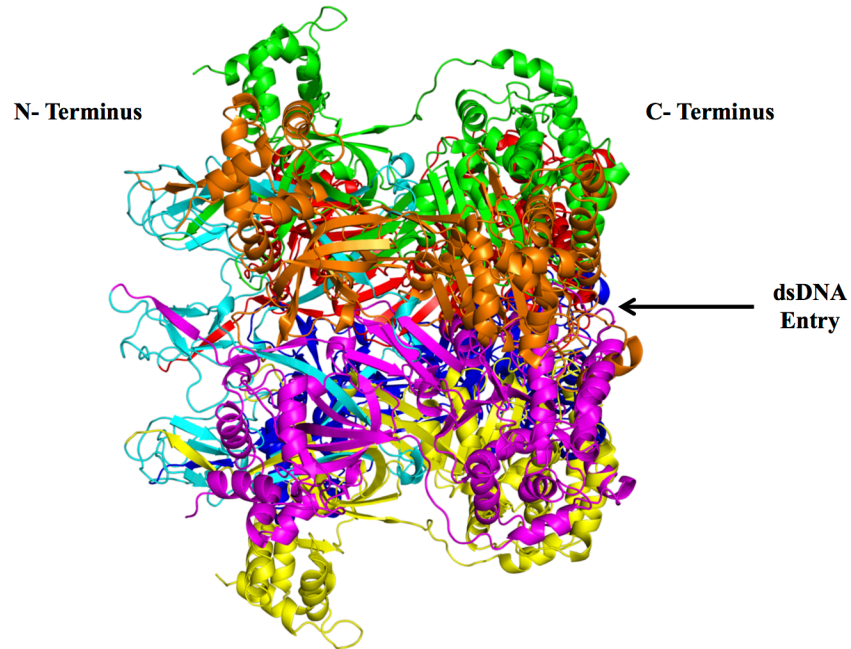
Figure 4.10: A ribbon structure of side view of MCM complex from *S. solfactaricus*
The C terminal region of the complex binds the dsDNA, the middle region is responsible for the helicase activity and N terminus is where ssDNA binds. The region similar to *S. cerevisiae* $Mcm5_{208-257}$ is indicated in cyan (developed using PyMol).
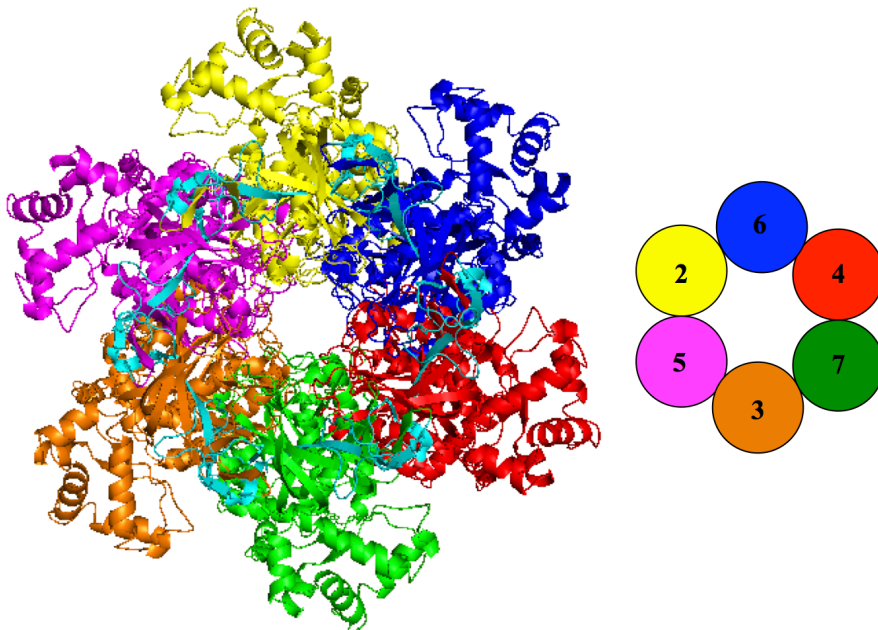
Figure 4.11: A ribbon structure of top view of *S. solfactaricus* MCM complex from N terminal side. An accompanying cartoon shows the arrangement of MCM subunits forming MCM complex in *S. cerevisiae*.

Discussion

The Rpa1 construct should be modified for successful yeast two-hybrid assay

In our yeast two-hybrid assay we found that the full-length Rpa1 does not auto-activate. Therefore, we performed our screen utilizing the BD-Rpa1 construct. However, since this construct had the ability to form functional RPA complex and the affinity amongst the subunits is very high (WOLD 1997), all the strong interactions recovered were Rpa2. Thus having full-length Rpa1 containing DBD-C (the region necessary to interact with other RPA subunits) while screening limited our ability to "fish-out" other potential protein candidates. Hence, for a successful screen we need to develop Rpa1-FLAB construct to limit its interaction with Rpa2. The downside of doing this would be an Rpa1 not present in the context of a full RPA complex, and this might influence which proteins are recovered.

The Mcm5$_{208-257}$ region appears important for Mcm5-Rfa1 interaction

In the screen performed with BD-Rfa1-FLAB as the bait, Mcm5$_{108-359}$ fragment was identified. This interaction was of interest, because it appeared to be affected by the phospho-mimetic state of yeast Rfa2 and these two protein complexes are involved in DNA replication (PIYA *et al.* 2015). Further mapping revealed that this interaction probably involves a 50 amino acid subregion in Mcm5 (*i.e.* Mcm5$_{208-257}$). However, this interaction was not tested with the deletion of 208-257 amino acid region in full length Mcm5. In fact, we did not see interaction of Rfa1 with full-length Mcm5. Sometimes over-expression or epitope-tagged versions of certain protein can be toxic to yeast cells. Alternatively, it is possible that full-length Mcm5 and full-length Rfa1 do not show interaction due to steric hindrance (full MCM and RPA complex) and/or that full-length

141

Mcm5 is recruited away from the two-hybrid reporter by its normal function in the cell. The lack of interaction between Rfa1 and Mcm5 might be attributed to one or more of these phenomenons, and this was not determined. The Mcm5$_{108-359}$ does not show this effect due to lack of functional full-length protein.

The potential importance of a putative interaction with this region of Mcm5 is indicated by a model developed on the basis of *S. solfataricus* MCM crystal structure (Figure 4.8). The positioning of this region is on the ssDNA exit side of the complex. Also, this region is not important for the *Sso* MCM complex formation. A P62L mutation in Mcm5, also known as the *mcm5-bob1* mutation, bypasses a kinase required for replication initiation and affects helicase activity of the complex (FLETCHER and CHEN 2006). This mutation is in the N-terminus of Mcm5 and indicates a regulatory role of N terminus via protein binding. This region of Mcm5 might facilitate interaction with downstream factors in replication, such as RFA.

Rfa1 DBD-B is important for interaction with MCM complex

The N-terminal domain (*i.e.*, DBD-F) is thought to mediate many of the Rfa1 protein-protein interactions. Our lab has demonstrated that DBD-F facilitates interactions with DDR proteins, and these interactions appear to be the strongest. On the other hand, the major contribution to RPA function of DBD-A and DBD-B is through ssDNA-binding activity. In our assay, DBD-B shows interaction with MCM subunits, which is somewhat novel. Also in our lab, many of the interactions we identified to occur with domains other than DBD-F are with proteins not specific to the DDR, and these interactions are typically weaker. The DBD-B binding to the Mcm5 in this case, seems more conveniently explained in the model diagrammed in Figure 4.12.
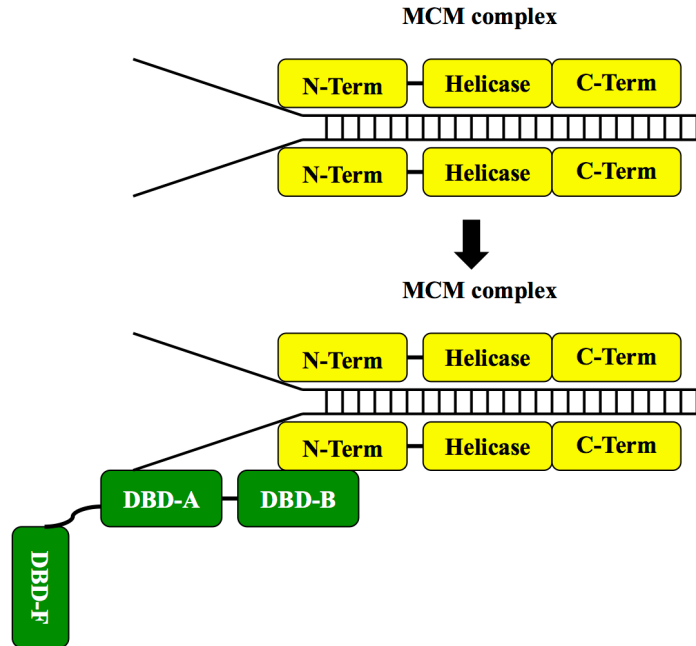
Figure 4.12: A model demonstrating the possible orientation of MCM and RFA complex (only Rfa1-FLAB) to DNA. The dsDNA enters the C-terminus side of the MCM complex, unwinds due to helicase activity located in the middle region and exits the complex from N-terminal side. At the N-terminal end the ssDNA is exposed to Rfa1, which can facilitate loading onto one of the ssDNA strands in the proper orientation.

The activity of the MCM complex is distributed in three regions; the C-terminus (aa 600-700) binds dsDNA and is important for heterohexameric formation. The central region (approximately aa 340-575) has the helicase activity and the N-terminus (aa 1-320) is where the ssDNA exits. The binding of Mcm5 to DBD-B might enhance two things: (1) positioning DBD-A and DBD-B closer and in proper orientation with one strand of the ssDNA, since RPA binds ssDNA with polarity, and (2) may assist in MCM movement by acting as a recipient for the newly unwound DNA. MCM helicase activity occurs *in vitro*; however, it has not been measured whether or not RPA enhances this helicase activity. It might not be surprising if it did, since RPA is known to interact with other helicases.

Future Directions

In most of our replica plating assays with various Mcm5 and Rfa1 mutants we did not find any substantial β-galactosidase activity (development of blue color on SG-HTU+Xgal plate). This was an indication of a transient or weak interaction between Rfa1 and Mcm5. We also, utilized liquid β-galactosidase assays and spot dilution assays (data not shown), which failed to show any reliable differences in interaction strength or allow for the observations of subtle differences in growth. The assays also showed a lack of consistency in results. To study the relevance of this particular interaction, it will be important to utilize or develop new methods. Some future directions to further improve or retest the assays discussed in this chapter are outlined below:

1. To test the novel interaction of human RPA subunits we should generate a BD-Rpa1-FLAB construct to limit the interaction with Rpa2.

2. The INO80/YY1/RuvB-like2 proteins act in the same pathway. In an experiment we found that all these proteins were able to interact with Rpa1. We further found that exogenous expression of Rpa4 interferes with this interaction and the Rpa1 DBD-B is important for this interaction (data not shown). However, since use of *Taq* DNA polymerase introduced random mutations in these constructs the data is not reliable. However, it will still be interesting to retest these interactions with a full-length non-mutated BD-Rpa1.

3. The Rfa1-Mcm5 interactions appear to be weak. Biochemical techniques like co-immunoprecipitation or testing *in vitro* interaction of purified RPA and MCM5 complex would be informative about the biological relevance of this interaction.

4. To determine the importance of the Mcm5 aa 208-257 region, a mutant of Mcm5 with the deletion of this mutant should be generated. Further, it should be tested if this mutant can support cellular replication (*i.e.*, test physiological relevance).

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146

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CHAPTER 5. FINAL REMARKS AND FUTURE DIRECTION

<u>Introduction</u>

DNA damage poses a daunting challenge to the cell. The cell must recognize and fix DNA damage in order to maintain the integrity of the genome and ensure proper cell function. Two things occur in response to DNA damage; along with DNA repair, the cells arrest the progression of cell cycle. RPA is a key player in replication, repair, and cell cycle regulation (FANNING *et al.* 2006). The primary biochemical function of RPA is to bind and protect ssDNA from nucleases and prevent secondary structure formation. Proteins necessary to process this ssDNA intermediate into dsDNA are presumably coordinated and loaded on to ssDNA through RPA. Subsequent displacement of RPA would then be required to completing the DNA processing. Therefore, the distinction as to which proteins will process the ssDNA is likely made through interaction with RPA. Coordination of RPA function is likely regulated by two factors: (1) differences in protein interactions at different stages of cell cycle, and (2) post-translational modifications of RPA and/or interacting proteins.

An extensively studied post-translational modification that RPA complex undergoes is phosphorylation. Of particular interest is the phosphorylation of Rpa2 N-terminus. The RPA complex is phosphorylated in a cell cycle dependent manner by cyclin-dependent kinases (CDK) (DIN *et al.* 1990; FANG and NEWPORT 1993), although the importance of this post-translational modification remains unclear. However, in human cells, the Rpa2 N-terminus is hyper-phosphorylated in response to DNA damage (LIU and WEAVER 1993; CARTY *et al.* 1994a). The Rpa2 hyper-phosphorylation has been studied predominantly in human cells, and only peripherally or recently in other

organisms such as *D. melanogaster*, *X. laevis*, and *C. albicans* (FANG and NEWPORT 1993; MITSIS 1995; WANG *et al.* 2013; GAO *et al.* 2014). The work presented in this thesis aims to elucidate the role of the Rpa2 N-terminus in response to DNA damage utilizing *S. cerevisiae* as a model organism. In order to understand the role of the Rpa2 N-terminus, we focused on answering the following specific questions: (1) Is the Rfa2 N-terminus hyper-phosphorylated in response to DNA damage? (2) What regions or amino acids of Rfa2 N-terminus are important for the DNA damage response, (3) Does Rfa2 hyper-phosphorylation affect protein interaction pattern of RFA complex? and (4) Can *S. cerevisiae* serve as a model to study other eukaryotic Rpa2 hyper-phosphorylation? The overarching goal of all of these studies was to use a simple organism with unique tools to study the importance of the Rpa2 N-terminus and gain insight into its function.

Discussion and future directions

Rpa2 NT phosphorylation has different implication in *H. sapiens* and *S. cerevisiae*

Rpa2 NT is phosphorylated in cell cycle dependent manner and in response to DNA damage in humans. The Rpa2 NT phosphorylation has important role in DNA repair while the role of Rfa2 NT phosphorylation remains elusive; based on following data discussed.

The human Rpa2 NT has 9 different S/T phosphorylation sites in the unstructured Rpa2 N terminus. These sites are phosphorylated by PI3 family of kinases ATM, ATR and DNA-PK in response to DNA damage. The cellular extracts from adozelesin treated 293 and HeLa cells were unable to support SV40 DNA replication in vitro, the replication resumed when purified non-phosphorylated RPA was added (CARTY *et al.* 1994b; LIU *et al.* 2000). The adozelsin (a drug used to teat cancer alkylates DNA)

treatment causes hyper-phosphorylation of the Rpa2 NT and thus indicates that the hyper-phosphorylated Rpa2 does not support replication. In human cells the Rpa2$_D$ mutant (8 sites mutated to D except S4) did not localize to the replication centers in vivo instead showed competent binding to DNA damage foci (VASSIN *et al.* 2004a). Rad51 failed to localize to HR induced damaged sites when Rpa2$_A$ mutant (7 sites mutated) is expressed exogenously, indicating that the Rpa2 phosphorylation is important for Rad51 recruitment (SHI *et al.* 2010). Rad51 is essential in homologous recombination repair. Rpa2A mutant (S26, S31 and T35) in *Xenopus laevis* stimulated S phase dependent checkpoint signaling, while the Rpa2D could not. This data indicated that the Rpa2 phosphorylation is not involved in checkpoint activation but maybe involved in repair or restart arrested fork. In fact, phosphorylated Rpa2 recruits PALB2 at stalled replication forks to maintain its integrity during replication stress (MURPHY *et al.* 2014).

The budding yeast Rpa2 NT has 11 different S/T potential phosphorylation sites. However the only known phosphorylation in this region occurs at S27 by Ime2 kinase, and this event is important in initiation of meiosis. Apart from this the Mec1 kinase phosphorylate both Rfa1 (at 178) and Rfa2 (at 122) subunits of the RFA complex; and the Rfa2 site lies outside of Rfa2 NT. However, when these sites were mutated to ALA they did not show any DNA damage sensitivity (GHOSPURKAR *et al.* 2015a). Therefore, we tested the *rfa2* NT extreme mutants and found that all these mutants are viable in unstressed condition (5-FOA). Although it is not a direct measurement of replication activity, the ability of these mutants as a sole Rfa2 copy to grow in unstressed condition indicates that these mutants can support DNA replication. This indicates that *rfa2-D$_x$* unlike its human counterpart can support DNA replication.

150

The *rfa2-D$_x$* and *rfa2-ΔN$_x$* mutants are sensitive to DNA damage irrespective of the type of DNA damaging agent and strain. The Rad53 phosphorylation data indicated that none of the *rfa2* extreme mutants cause defect in inducing DDR in presence of MMS. The ability of *rfa2-A$_x$* to act like WT Rfa2 indicates that for proper cellular replication and repair a 'normal' Rfa2 NT is required. The 'normal' here indicates that the Rfa2 NT must be present and should be devoid of any additional negative charges. Collectively, this data indicated that the Rfa2 NT is required for DDR but its phosphorylation is dispensable in yeast.

The *rfa2-ΔN$_x$* mutant was known to be viable as demonstrated in previous study (PHILIPOVA *et al.* 1996). However, we are the first group to show its DNA damage sensitivity in *S. cerevisiae.*

The human and yeast Rpa2 mechanisms differ in other two ways (1) The Rfa2 was shown to be phosphorylated even after deletion of 1-40 aa, this required a fully functional Mec1 kinase (SCHRAMKE *et al.* 2001). On contrary the deletion of N terminal 33 amino acids of Rfa2 eliminated Rpa2 phosphorylation in humans (HENRICKSEN *et al.* 1996). (2) The human Rpa2 NT phosphorylation is further intricately regulated by several kinases using single site priming mechanism discussed below.

The human Rpa2 NT is phosphorylated by several kinases and their target sites are somewhat overlapping, suggesting interplay of DDR regulation through Rpa2 NT phosphorylation. When *RPA2* S33 was mutated to alanine, this mutation suppressed the phosphorylation at the S29 and S4/8 sites of human Rpa2 NT (ANANTHA *et al.* 2007). Similarly, *RPA2* T21 and S33 when mutated to ALA showed an S phase checkpoint after UV induced damage, indicating that T21 is also important in establishing the primary

response in DNA damage (OLSON *et al.* 2006; ANANTHA *et al.* 2007). The *RPA2* S4/ S8 when mutated to ALA showed to block phosphorylation of S23 and S12 (LIU *et al.* 2012). All these data indicate that the phosphorylation at one site can regulate the phosphorylation of other, known as priming. The data is summarized in Figure 1.3.

All our *rfa2* extreme mutants showed phosphorylation of Rfa2 in response to DNA damage, supporting the 40 amino acid deletion data and this phosphorylation was thought to occur at the Mec1 site. Interestingly, both our damage sensitive mutants *rfa2-D_x* and *rfa2-$ΔN_x$* showed a slower migrating Rfa2 species compared to WT and *rfa2-A_x*. This implies that there is a different phosphorylation site(s) in these mutants apart from the known Mec1 site. Now, as both these mutants are damage sensitive it is possible that this phosphorylation is required for a process that is not addressed in this thesis (see section on adaptation below).

Since the WT Rfa2 phosphorylation is not detectable without PhosTag, and is thought to be subtle the detection of any priming reaction was impossible to detect. But the *rfa2-D_x* and *rfa2-$ΔN_x$* show DNA damage sensitivity so we tested if this phenotype can be attributed to a single phosphorylation site. We developed *rfa2* NT single mutants *rfa2-A_i* and *rfa2-D_i* and none of these mutants show any DNA damage sensitivity. Therefore we mutated multiple sites at a geographically convenient region and tested DNA damage sensitivity. The DNA damage phenotype can be attributed to the rfa2 region 1 and region 3, *i.e.,* Serine residues at position 3,11,12 and 30 and Threonine residues at 14, 32 and 34. Although, region 1 and region 2 of Rfa2 NT are shown to contribute to damage sensitivity, the fact that the extreme mutants show Rfa2 phosphorylation supports that, the phosphorylation of Rfa2 NT does not influence the

overall Rfa2 phosphorylation in yeast cells. Interestingly these regions coordinate that

target regions of ATM and ATR in humans. The homologues of these kinases are present

in yeast (Tel1 and Mec1). It is possible that these may be the potential kinases that

phosphorylate this region under suitable conditions. However, the data in this thesis is

just suggestive and there is no strong evidence (Figure 5.1).

A)

| | 4 | 8 | 12 | | 21 | 23 | | 29 | | 33 | |

Rpa2 NT MWNSGFESYGSSSYGGAGGYTQSPGGFGSPAPSQAEKKSR

ATM   DNA-PK   CDK   ATR

B)

| | 3 | | 11 | 12 | 14 | | 21 | 23 | | 27 | | 30 | 32 | 34 | |

Rfa2 NT MATYQPYNEYSSVTGGGFENSESRPGSGESETNTRVNTLT

TEL1 ?   DNA-PK ??   CDK ?   MEC1 ?
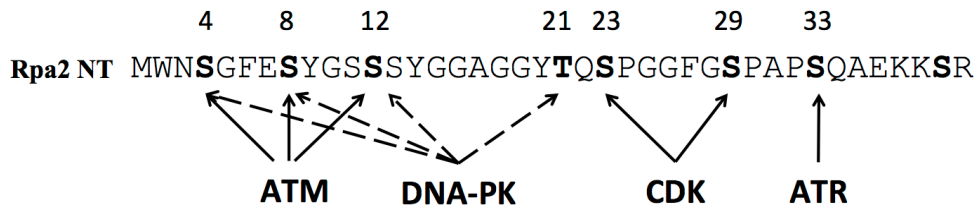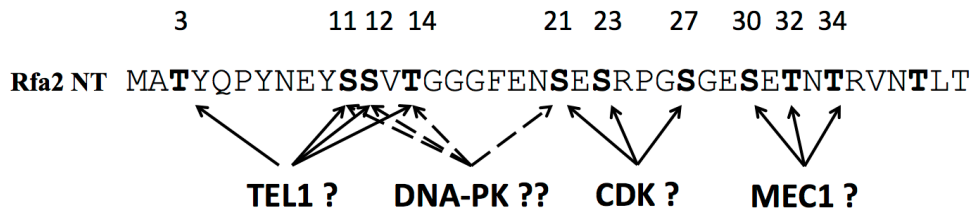
Figure 5.1: The phosphorylation pattern of Rpa2 NT in *H. sapiens* and *S. cerevisiae*
A) Phosphorylation of different Rpa2 NT sites by various kinases in H. sapiens (courtesy: Liu et al 2012) B) A phosphorylation pattern predicted on the basis of Liu et al in *S. cerevisiae*. The DNA-PK is questionable as there is no known homolog in yeast.

In future to further identify the sites of phosphorylation (observed in *rfa2-D$_x$* and

rfa2-ΔN$_x$) and importance of their function, I suggest that the following experiments can

be conducted (1) To locate the site the protein should be purified and sent for mass

spectrometry analysis. (2) To test whether the Mec1 phosphorylation site is involved, we

can combine the *rfa2-D$_x$* mutation with *rfa2-122A* should be combined and tested for

Rad53 and Rfa2 phosphorylation. (3) To locate the kinase responsible for the

phosphorylation of *rfa2-D$_x$* and *rfa2-ΔN$_x$* we can combine these mutants with kinase deletion strains and test effect on Rfa2 phosphorylation.

Phosphorylation state of Rfa2 NT affects protein-protein interactions

The post-translational modifications of proteins can result in changes in their structure and function. The protein-protein interactions are often affected/regulated by post-translational modification. Since, human Rpa2 NT phosphorylation occurs in response to DNA damage, it is proposed to alter the interaction of RPA with DNA and the replication/repair proteins (GEORGAKI and HÜBSCHER 1993; OAKLEY and PATRICK 2010). An Rpa2 NT mimicking peptide was shown to interact with DBD-F of Rfa1 (BINZ *et al.* 2003). The Rpa2 phosphorylation is also known to affect the interaction of RPA with Mre11, Nbs1, Rad51, and Rad52 (JACKSON *et al.* 2002; OAKLEY *et al.* 2009). All these proteins have repair specific function. The hyper-phosphorylated RPA is recruited away from the replication centers to the repair foci (VASSIN *et al.* 2004b). This can be implying towards changes in RPA-DNA as well as the protein interactions.

The projects discussed in this thesis had utilized two molecular biology methods to study protein-protein interaction: (1) through genetic interactions (*i.e.* double-mutant analysis) and (2) through yeast two-hybrid. The known Rpa2 NT interactions with Mre11 and Rpa1 were utilized in the genetic interaction analysis.

The genetic interaction data demonstrated that the *rfa2-ΔN$_x$* when combined with *rfa1-t11* is lethal, and when combined with *mre11Δ* survives. On the contrary the *rfa2-D$_x$* survives when combined with the *rfa1-t11* and both *rfa1-t11 mre11Δ* and *rfa2-D$_x$ mre11Δ* double mutants are lethal. This data indicated that the *rfa2-D$_x$* and *rfa1-t11* are involved in same pathway and *rfa2-ΔN$_x$* and *mre11Δ* are involved in same pathway (Figure 5.2).

154

The model is based on the type of damage and the response it generates. During

replication stress the RPA is already present at the replication site and does not follow

Mre11 activity while the general two stranded breaks (caused by agents like MMS and

UV) first require recognition of break followed by Mre11 activity. The resected DNA is

then bound by RPA.
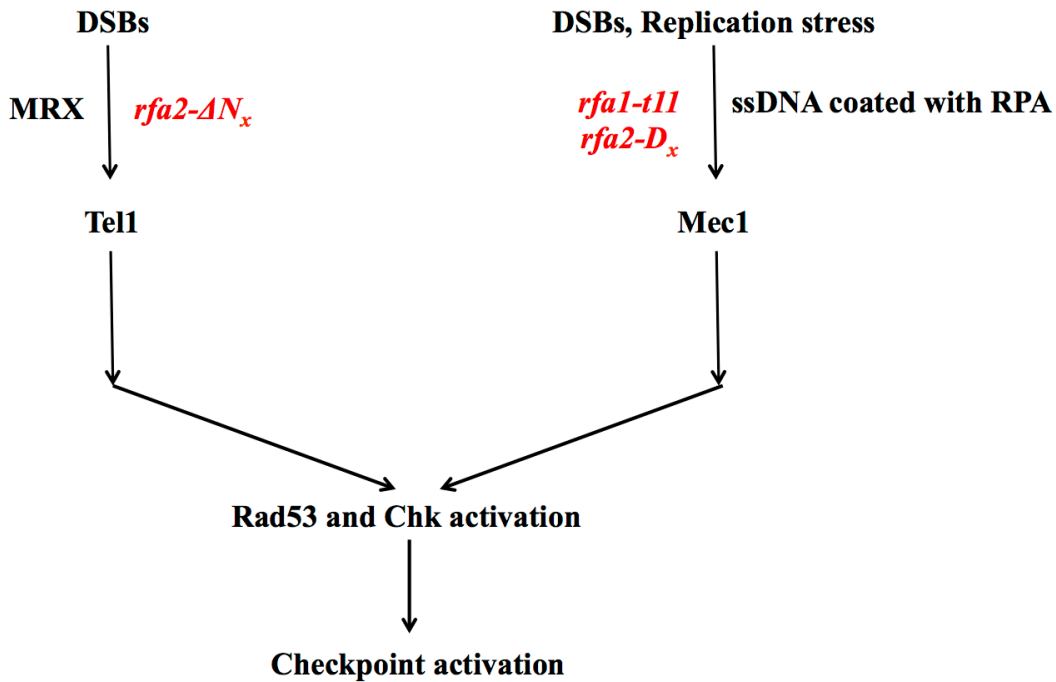
Figure 5.2: A model depicting the role of Rfa2 NT in DDR and checkpoint adaptation. A model based on the genetic interaction data between Rfa1, Rfa2 NT and Mre11. The model shows the placement of Rfa2 NT mutants according to agreement with genetic interactions. The model also suggests role of Rfa2 NT phosphorylation in Mec1/ Tel1 interplay.

We also utilized the *rfa1-aro* mutants to study the effect of Rfa2 phosphorylation

on complex interaction and DNA-binding. In humans, the *rfa1-aro* mutations lead to

accumulation of cells in G2/M phase (HARING *et al.* 2008; HASS *et al.* 2012). In our

study, we developed single through quadruple *rfa1-aro* mutants. We also combined the

rfa2-ΔNx with *rfa1-aro* mutants. The *rfa2-ΔNx* is found to rescue the absence or reduced

growth induced by *rfa1-aro* single and double mutants. This was another interesting observation. The ability of cells to be rescued by *rfa2-ΔN$_x$* indicates that the cells can go through replication. Whether this replication is defective or leads to any replication stress is unknown at this point. However, since the cells survive in presence of *rfa2-ΔN$_x$* it indicates the *rfa1-aro* mutants activated checkpoint (this hypothesis is discussed in the last section).

We utilized yeast two-hybrid assay as a direct approach to detect protein-protein interaction. The yeast two-hybrid screen with Rpa1 and Rpa4 were not successful. The screen conducted by my colleague Mr. Gunjan Piya clearly found new interactions of Rfa1. The work further elucidated that some of the Rfa1 protein interactions are influenced by Rfa2 NT phosphorylation state (PIYA *et al*. 2015). One such interaction between Rfa1 and Mcm5 is investigated in detail in this thesis.

The Mcm5 interaction with Rfa1 is novel, as it is not observed in human or mouse. A 50 amino acid region in Mcm5, 208-257 was found important for this interaction. However, our data indicates that this is a transient or weak interaction and needs to be investigated using other biochemical assays.

The *S. cerevisiae* can serve as a model system to study Rfa2 NT function

The Rpa2 NT phosphorylation is extensively studied in humans. However, there is still lack of clear understanding when it comes to function of this event. This is because the Rpa2 NT phosphorylation affects more than one aspect of DDR. The human cell line system although ideal, is not always convenient to handle and manipulate. Furthermore, experiments affecting essential genes often can only be tracked for 1-2 generations. While providing a snapshot into what is affected, it does not provide for determination of

156

whether effects have physiological consequences. Therefore, a simplistic model is would be greatly beneficial to study the function of Rpa2 NT phosphorylation.

S. cerevisiae is a single celled eukaryote, which executes all DNA metabolic processes similar to humans. These are easy to handle and more importantly are easy to manipulate genetically, which gives them great advantage over human cell lines. Therefore we first attempted to develop a 'humanized yeast' system to study RPA. In that, we aimed to develop yeast cells with human RPA as a sole copy of RPA.

It was important to understand the complex formation between in inter-species and intra-species subunits of RPA from human and budding yeast. To understand this we utilized yeast-two hybrid assay. This assay demonstrated that the Rpa1 /Rpa3 interaction is mediated by Rpa2 or Rpa4. Previous data have shown that the human RPA can form a stable complex with all three genes or a sub-complex of Rpa2-Rpa3 (HENRICKSEN *et al.* 1994). A direct interaction between Rpa1 and Rpa3 is not known. In our study, we found that the RFA1 does not interact with the RPA subunits and vice a versa. This can explain why previous attempts to replace Rfa1 with Rpa1 and Rfa2 with Rpa2 have failed. Therefore in this study we attempted to replace the full RFA complex with the RPA complex. However this attempt was not successful, as we did not achieve required level of RPA subunits expression.

Meanwhile, a study carried out by my colleague Mr. Timothy Wilson showed that, a hybrid protein whereby Rpa2 NT replaces the Rfa2 NT could survive in the yeast cells (GHOSPURKAR *et al.* 2015b). Now, since our data showed that the Rfa2 NT phosphorylation was dispensable in yeast, we further investigated if yeast cells have capability to phosphorylate the Rpa2 NT. We did observe hyper-phosphorylation of Rpa2

157

NT in response to DNA damage in yeast cells. This data indicated that the yeast cells

conserve the capability of Rpa2 NT hyper-phosphorylation, even if the event itself did

not appear necessary for DDR in yeast (Table5.1). Surprisingly, the phosphorylation of

the human Rpa2 NT occurred very similarly to that observed in human cells.

Table 5.1: A summary of Rpa2 NT phosphorylation observed in various hybrid proteins

Species	Phosphorylation	Species	Phosphorylation
S. cerevisiae	0	O. sativa (1)	+
S. pombe	+	O. sativa (2)	0
C. albicans	+	X. laevis	+
A. thaliana (A)	+	M. musculus	+
A. thaliana (B)	+	H. sapiens	+

Scale: 0 = no phosphorylation and + = phosphorylation

This data indicate that the role of Rpa2 NT phosphorylation as a regulatory

mechanism may have arisen during evolution. Further, the capability of yeast cells to

phosphorylate Rpa2 NT made them ideal to study human Rpa2 NT. In this study we

further investigated the evolutionary aspect of Rpa2 phosphorylation.

We developed mutants that encode the hybrid 2NT proteins from around six

different species apart from humans. The various species are listed in the Table 5.1. The

phylogenetic tree developed utilizing the Rpa2 NT sequences from all these organisms is

shown in Figure 5.3.

```
                                                    AtRpa2A 0.07375
                                                    AtRpa2B 0.08625
                                                    OsRpa32-2 0.09775
                                                    OsRpa32-1 0.13458
                                                    HsRpa2 0.01813
                                                    MmRpa2 0.02187
                                                    XlRpa2 0.07964
                                                    CaRfa2 0.13844
                                                    ScRfa2 0.12156
                                                    SpSsb2 0.12922
```
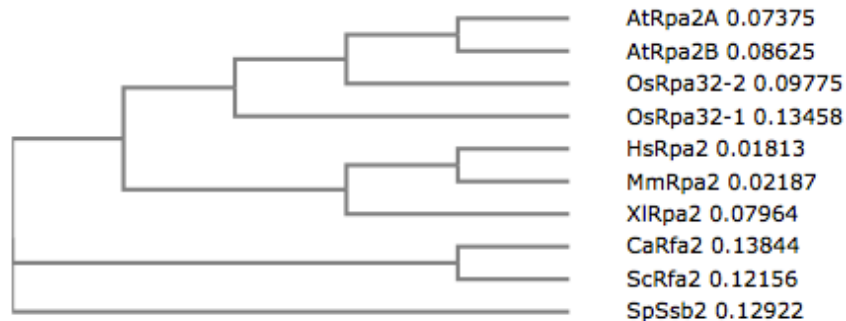
Figure 5.3: A phylogenetic tree developed from Rpa2 NT sequences from organisms in this study. Plant species, fungi and animal species studied in this dissertation club together. The *S. pombe* is closer to humans (1 branch) as compared to *S. cerevisiae.* The *O. sativa* homolog 2 is not phosphorylated although is closer to *A. thaliana* species which both get phosphorylated (1 branch).

The plants, animals and fungi are at the end of the phylogenetic tree. The fungi are closer to animals than plants. In this study we have selected organisms from each branch to understand the evolution of Rpa2 NT. First, the Rpa2 NT is an unstructured domain, as in the tree these sequences club the plant, fungi and animal species together indicated that the sequence is conserved. This is surprising because generally unstructured domain show less sequence conservation. Secondly the *S. pombe* is closer to humans as compared to any other fungi studied. A definite statement about how the evolution of this domain may have occurred is difficult to state with just eight different samples.

However, a phosphorylation pattern emerges when we take a closer look at the Rpa2 NT sequences from these species (Figure 5.4). The kinases recognize the SQ/TQ motif in the Rpa2 NT. The S. cerevisiae is the only species in this study, which lacks the SQ/TQ motif in the sequence. And this is the only species, which does not show any detectable phosphorylation in the Rpa2 NT. The Os2NT-2 sample did not show

phosphorylation even though SQ/TQ motif is present, this can be attributed to differences in functional compartmentalization of these proteins in plants.

```
ScRfa2    MATYQPYNEYSSVTGGGFENSESRPGSGESETNTRVNT......................LTPVTIKQ
CaRfa2    MSDFEYDNNNTGAFGNISQGGFNTEHAGSSQRQTTTQVRQS...................LTPVTIKQ
SpSsb2    MAYDAFGKPGYGPDFNSAFSPGMGGGAGFNEYDQSSQPSVDRQQGAGNK...........LRPVTIKQ
HsRpa2    MWNSGFESYGSSSYGGAGGYTQSPGGFGSPAPSQAEKKSRARAQH...............IVPCTISQ
MmRpa2    MWNSGFESFSSSTYGGAGGYTQSPGGFGSPTPSQAEKKSRVRAQH...............IVPCTISQ
XlRpa2    MWNNHGGFDGGYGGSGMGGGGGGYMQSPGGFGSPAPTQGEKKSRSRSQQ............IVPCTVSQ
AtRpa2A   MFSSSQFEPNSGFSGGGFMSSQPSQAYESSSSTAKNRDFQG...................LVPVTVKQ
AtRpa2B   MYGGDFDGNAAFAGGGFMPSQATTQAHESSSSLKVYVSDSRRFSGISAAKSSIRNRDVRTLLPLTLKQ
OsRpa32-1 MMSFSQPDAFSPSQFTSSQNAAADSTTPSKSRGASS.......................TMPLTVKQ
OsRpa32-2 MYGVGVGGGGGGNYDGGGGNASSLFGGGGFMPSQATNAAEGTSGGGGGFPKSRNAQA...LLPLTVKQ
```

Figure 5.4: A sequence alignment of Rpa2 NT from different species. The *S. cerevisiae* Rpa2 NT lacks the SQ/ TQ motif in its Rpa2 N terminus. The other species studied in this dissertation show the SQ/TQ motif conserved (denoted in Red).

So the convergent evolution (?) of the Rpa2 NT can be explained as follows the plant species have both SQ/TQ in the Rpa2 NT sequence and the kinases with ability to recognize them. The *S. cerevisiae* have lost the SQ/ TQ motif while diverging from the other fungi but retained the SQ/TQ motif recognizing kinases. The *S. pombe* have evolved differently from other fungi it retains both the parameters. The human cells not only retain both parameters but also have more intricate regulation of the Rpa2 phosphorylation event.

The Rfa2 NT phosphorylation has a unique role in *S. cerevisiae*

The data presented in this thesis demonstrates that the Rfa2 NT phosphorylation does not have a significant role in DNA damage response in *S. cerevisiae*. However, the *rfa2-D$_x$* and *rfa2-ΔN$_x$* are sensitive to DNA damage. A study by our group shows that the Rpa2 NT phosphorylation has a role in checkpoint adaptation. The experiments performed by my advisor Dr. Stuart Haring and Colleague Mr. Timothy Wilson indicated that the *rfa2-D$_x$* mutant is adaptation proficient while the *rfa2-A$_x$* is adaptation deficient

(GHOSPURKAR *et al.* 2015b). The *rfa2-ΔN_x* also shows adaptation deficient phenotype (unpublished SJH). Now the question is why these mutants with antagonistic function show similar phenotype.

In response to DNA damage the cells can lead to three distinct pathways. Firstly, they can successfully fix the damage and continue to progress through cell division. Secondly, the cells will not be able to fix damage and die i.e. apoptosis. Thirdly, the cells can cease the progression through cell cycle until they fix the damage. Curiously, the phenotypes of the (a) cells arrested in cell cycle and (b) cells that die are very similar, *i.e.* there is no substantial colony growth seen on the plates. We think, that the *rfa2-D_x* and *rfa2-ΔN_x* are similar in phenotype but different in mechanism as discussed above Figure 5.5.

Also, the *rfa2-ΔN_x* adaptation poses a question about the hypothesis we made in section on *rfa1-Aro* mutants. The hypothesis was, the *rfa1-Aro* mutants trigger a checkpoint leading to arrest of cells and expression of *rfa2-ΔN_x* leads to adaptation caused due to these mutations. In human cells, the *rpa1-Aro* mutants are shown to trigger a G2/M checkpoint arrest (HARING *et al.* 2008). Now the phosphorylated N terminus is proposed to bind the DBD-F of Rpa1 (BINZ *et al.* 2003). However, in study carried by our group have shown that the phosphorylated Rpa2 N terminus also interferes with DBD-B (Mueller and Haring unpublished). In this thesis, we have seen that the Rfa1-Mcm5 interaction is affected by Rfa2 phosphorylation and all the interactions are located in DBD-B of the Rfa2.

It is possible that the Rfa2 N terminus interaction (or phosphorylated N termius) is important to activate and establish checkpoint caused by *rfa1-Aro* mutants and since *rfa2-ΔN$_x$* lacks the ability to interact with DBD-B the checkpoint is override in these conditions.
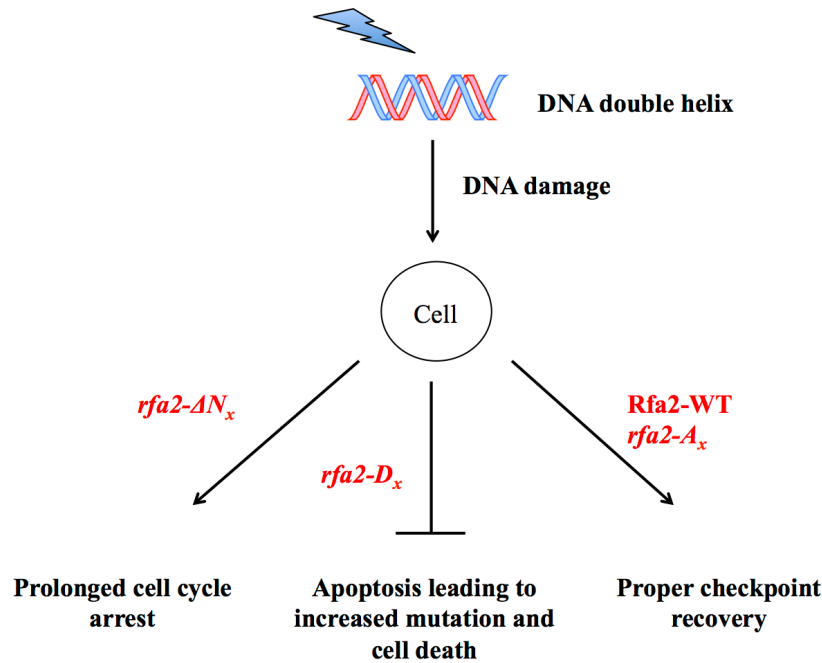
Figure 5.5: A model depicting various pathways that cells can undergo after damage. After exposed to DNA damage the cells can have different fate depending upon the type of DNA damage, whether it is fixed or whether the cells have suitable environment to survive. The model here represents the fate of cells depending upon the Rfa2 NT mutants present in the cells.

Summary

Overall, this study contributed four major things to previously unknown details about Rpa2 NT phosphorylation and are listed below

    I.   The Rfa2 NT is required for DDR in *S. cerevisiae* but its phosphorylation is dispensable. However Rfa2 NT phosphorylation plays a role in checkpoint adaptation.

II.   The *rfa2-ΔN$_x$* and *rfa2-D$_x$* have similar observed damage sensitivity but have different modes of action. Their action might lie in the ability of cells to release from a checkpoint properly. The genetic interaction study indicated that the phosphorylation state of Rfa2 NT directs the protein interaction pathway.

III.   The phosphorylation of Rfa2 NT influences the protein binding activity of RFA complex (Mcm5 interaction).

IV.   The *S. cerevisiae* do not require phosphorylation of Rfa2 NT in DDR, however the machinery for phosphorylation is conserved. This provided evidence for the potential of *S. cerevisiae* to be used as a model system to study eukaryotic Rpa2 NT phosphorylation.

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APPENDIX A. STRAINS

STRAIN	GENOTYPE
RMY122A	MATα leu2-3,112 trp1-1 can1-100 ura3-1 his3-11,15 rad5-535 rfa1Δ::TRP1 rfa2Δ::TRP1; this strain contains pJM132
RMY122A-mre11Δ	mre11Δ::kanMX derivative of RMY122
403 (K264-10D)	MATa, lys2-2, tyr1-2, his7-1, ura3-1, met13-c, cyh^r, trp5-c, leu1-c, ade2-1
163 (RM26-26C)	MATα, lys2-1, can^r, ura3-1, his1, ade5, CYH^s, trp5-2, leu1-12, ade2-1
EGY48	**MAT**α *trp1 his3 ura3 leu2*::6 LexAop-Leu2
EGY194	**MAT**a *trp1 his3 ura3 leu2*::4 LexAop-Leu2
EGY188	**MAT**a *trp1 his3 ura3 leu2*::2 LexAop-Leu2
EGY40	**MAT**α *trp1 his3 ura3 leu2*::0 LexAop-Leu2

APPENDIX B. PRIMERS

Primer Number	Primer Name	Sequence	Function	Chapter
5	Rpa1intoRfa1up	ATTACGGTAAAGGCGAAAC CAGCAAGAAGACCAGATTA TACTTACAAGAGATGGTCGG CCAACTGAGCGAGGGGG	Cloning	2
6	Rpa1intoRfa1down	ACATTCTCATATGTTACAT AGATTAAATAGTACTTGATT ATTTGATACATCACATCAAT GCACTTCTCCTGATG	Cloning	2
7	Rpa2intoRfa2up	GCAAAATCGATAGCGACTA TCTAGAACAGGCTAGTTTAA GCATATACATAATGTGGAAC AGTGGATTCGAAAGCT	Cloning	2
8	Rpa2intoRfa2down	AAATACACAAACGAATACT AAGAAATGCTAAAAAAATA ATCTATATATTTTTATTCTGC ATCTGTGGATTTAAAA	Cloning	2
9	Rpa3intoRfa3up	CACCCTTCACCATTCTTGTA GAACATCGTCTACCAGTAAC ACAAGTAAAAATGGTGGAC ATGATGGACTTGCCCA	Cloning	2
10	Rpa3intoRfa3down	AGACATATAGAGGCAACAG TACATAAAGGTAAGAATAA AAGCGATTTTAGTCAATCAT GTTGCACAATCCCTAAA	Cloning	2
11	Rpa4intoRfa2up	GCAAAATCGATAGCGACTA TCTAGAACAGGCTAGTTTAA GCATATACATAATGAGTAA GAGTGGGTTTGGGAGCT	Cloning	2
12	Rpa4intoRfa2down	AAATACACAAACGAATACT AAGAAATGCTAAAAAAATA ATCTATATATTTTCAATCAG CAGACTTAAAATGCTCC	Cloning	2
81	ura3-G418 newFOR	TGCCCAGTATTCTTAACCCA ACTGCACAGAACAAAAACC TCGTACGCTGCAGGTCGACG G	Cloning	2

Primer Number	Primer Name	Sequence	Function	Chapter
82	ura3-G418 newREV	TAATAACTGATATAATTAAA TTGAAGCTCTAATTTGTGAG ATCGATGAATTCGAGCTCGT	Cloning	2
101	shRPA1F	GATCCGGAATTATGTCGTAA GTCATTCAAGAGATGACTTA CGACATAATTCCTTTTTTAC GCGTG	Gene Silencing	2
102	shRPA1R	AATTCACGCGTAAAAAAGG AATTATGTCGTAAGTCATCT CTTGAATGACTTACGACATA ATTC	Gene Silencing	2
103	shRPA2F	GATCCGCCTAGTTTCACAAT CTGTTTTCAAGAGAAACAGA TTGTGAAACTAGGTTTTTTA CGCGTG	Gene Silencing	2
104	shRPA2R	AATTCACGCGTAAAAAACCT AGTTTCACAATCTGTTTCTC TTGAAAACAGATTGTGAAA CTAGGCG	Gene Silencing	2
105	shRPA3F	GATCCGATTGTAAATGAGCT ATATTTCAAGAGAATATAGC TCATTTACAATCTTTTTTACG CGTG	Gene Silencing	2
106	shRPA3R	AATTCACGCGTAAAAAAGA TTGTAAATGAGCTATATTCT CTTGAAATATAGCTCATTTA CAATCG	Gene Silencing	2
107	shRPA4F-1	GATCCGCACACATGATGCTG GATAATTCAAGAGATTATCC AGCATCATGTGTGTTTTTTA CGCGTG	Gene Silencing	2
108	shRPA4R-1	AATTCACGCGTAAAAAACA CACATGATGCTGGATAATCT CTTGAATTATCCAGCATCAT GTGTGCG	Gene Silencing	2
109	shRPA4F-2	GATCCGAGAGAAAGTCAAG CAGGTTTCAAGAGAACCTGC TTGACTTTCTCTCTTTTTTAC GAGTG	Gene Silencing	2
110	shRPA4R-2	AATTCACGCGTAAAAAAGA GAGAAAGTCAAGCAGGTTC TCTTGAAACCTGCTTGACTT TCTCTCG	Gene Silencing	2

Primer Number	Primer Name	Sequence	Function	Chapter
111	shRPA4F-3	GATCCGCCGACAGTGGTTTG GTAGATTCAAGAGATCTACC AAACCACTGTAGGTTTTTTA CGCGTG	Gene Silencing	2
112	shRPA4R-3	AATTCACGCGTAAAAAACC GACAGTGGTTTGGTAGATCT CTTGAATCTACCAAACCACT GTCGGCG	Gene Silencing	2
113	shRPA4F-5	GATCCGCATCCAGCTGTGAG TAATTTTCAAGAGAAATTAC TCACAGCTGGATGTTTTTTA CGCGTG	Gene Silencing	2
114	shRPA4R-5	AATTCACGCGTAAAAAACA TCCAGCTGTGAGTAATTTCT CTTGAAAATTACTCACAGCT GGATGCG	Gene Silencing	2
260	pEG202-Rpa1-FOR	TATTCGCAACGGCGACTGGC TGGAATTCCCGGGGATCCTT ATGGTCGGCCAGCTGAGCG A	Cloning	2/4
261	pEG202-Rpa1-REV	AAATTCGCCCGGAATTAGCT TGGCTGCAGGTCGACTCGAG TCACATCAATGCACTTCTCC	Cloning	2/4
262	pEG202-Rpa2-FOR	TATTCGCAACGGCGACTGGC TGGAATTCCCGGGGATCCTT ATGTGGAACAGTGGATTCA	Cloning	2/4
263	pEG202-Rpa2-REV	AAATTCGCCCGGAATTAGCT TGGCTGCAGGTCGACTCGAG TTATTCTGCATCTGTGGATT	Cloning	2/4
264	pEG202-Rpa3-FOR	TATTCGCAACGGCGACTGGC TGGAATTCCCGGGGATCCTT ATGGTGGACATGATGGACTT	Cloning	2
265	pEG202-Rpa3-REV	AAATTCGCCCGGAATTAGCT TGGCTGCAGGTCGACTCGAG TCAATCATGTTGCACAATCC	Cloning	2
266	pEG202-Rpa4-FOR	TATTCGCAACGGCGACTGGC TGGAATTCCCGGGGATCCTT ATGAGTAAGAGTGGGTTTG G	Cloning	2/4
267	pEG202-Rpa4-REV	AAATTCGCCCGGAATTAGCT TGGCTGCAGGTCGACTCGAG TCAATCAGCAGACTTAAAAT	Cloning	2/4

Primer Number	Primer Name	Sequence	Function	Chapter
268	pEG202-RFA1-FOR	TATTCGCAACGGCGACTGGCTGGAATTCCCGGGGATCCTTATGAGCAGTGTTCAACTTT	Cloning	2/4
269	pEG202-RFA1-REV	AAATTCGCCCGGAATTAGCTTGGCTGCAGGTCGACTCGAGTTAAGCTAACAAAGCCTTGG	Cloning	2/4
369	RPA1mRNA FOR-RT	CTAAGGCTTATGGTGCTTCAAAGAC	q-PCR	2
370	RPA1mRNA FOR-RT	CTAAGGCTTATGGTGCTTCAAAGAC	q-PCR	2
371	RPA2mRNA FOR-RT	GCACCTTCTCAAGCCGAAAA	q-PCR	2
372	RPA2mRNA REV-RT	CAGCTGAGATATAGTACAGGGCACAA	q-PCR	2
373	RPA3mRNA FOR-RT	GGAACCCCTTGATGAAGAAATCT	q-PCR	2
374	RPA3mRNA REV-RT	TGGCCTTGGCGGTTACTC	q-PCR	2
375	RPA4mRNA FOR-RT	GCAGAGAAGGCTTCAAATCACA	q-PCR	2
376	RPA4mRNA REV-RT	CGGGCCTCGATTGGTTT	q-PCR	2
658	Rfa1-cDNA FWD Set1	CTTCAAGGGCTGGGAAGAAA	q-PCR	2
659	Rfa1-cDNA REV Set1	TCCATAGGCCAACAGAGATAG	q-PCR	2
660	Rfa2-cDNA FWD Set1	GTGAGGGCAAAGACGCTAAT	q-PCR	2
661	Rfa2-cDNA REV Set1	GTCAAGGTCGTACAGCAGTTT	q-PCR	2
662	Rfa3-cDNA FWD Set2	CGACTCGTGGTATGAGTTTGT	q-PCR	2
663	Rfa3-cDNA REV Set2	AAAGCAACCACACCGTTTAAG	q-PCR	2
256	rfa2-S3D-remake	TTAAGCATATACAAAATGGCAGATTATCAACCATATAAC	Mutagenesis	3
152	rfa2-S11D FOR	CATATAACGAATATGATTCAGTAACGGGCG	Mutagenesis	3

Primer Number	Primer Name	Sequence	Function	Chapter
154	rfa2-S12D-FOR	CCATATAACGAATATAGTGATGTAACGGGCGGTG	Mutagenesis	3
156	rfa2-T14D-FOR	GAATATTCATCAGTAGATGGCGGTGGCTTTGAG	Mutagenesis	3
158	rfa2-S21D-FOR	GGCTTTGAGAACGACGAGAGTCGCCCAGGTAGTG	Mutagenesis	3
257	rfa2-S23D-remake	GTGGCTTTGAGAACTCAGAGGATCGCCCAGGTAGTG	Mutagenesis	3
162	rfa2-S27D-FOR	GTCCCGCCCAGGTCGTGGGGAGTCGGAAAC	Mutagenesis	3
164	rfa2-S30D-FOR	GTCCCCGCCCAGGATCCGGGGAGGATGAAACTAACACTAG	Mutagenesis	3
258	rfa2-T32D-remake	GGGGAGTCGGAAGATAACACTCGAGTTAACACCTTG	Mutagenesis	3
168	rfa2-T34D-FOR	GTCGGAAACTAACGATAGAGTAAACACCTTGACAC	Mutagenesis	3
170	rfa2-T38D-FOR	GAAACTAACACTAGAGTCGACACCTTGACACCTGTG	Mutagenesis	3
245	rfa2-S3A	TTAAGCATATACAAAATGGCAGCTTATCAACCATATAAC	Mutagenesis	3
246	rfa2-S11A	CATATAACGAATATGCTTCAGTAACGGGCG	Mutagenesis	3
247	rfa2-S12A	CCATATAACGAATATTCAGCTGTAACGGGCGGTG	Mutagenesis	3
248	rfa2-T14A	GAATATTCATCAGTAGCCGGCGGTGGCTTTGAG	Mutagenesis	3
249	rfa2-S21A	GGCTTTGAGAACGCTGAGAGTCGACCAGGTAGTGGGGAG	Mutagenesis	3
250	rfa2-S23A	GTGGCTTTCAGAACTCAGAGGCCCGCCCAGGTAGTG	Mutagenesis	3
251	rfa2-S27A	GTCCCGCCCAGGTGCCGGCGAGTCGGAAACTAAC	Mutagenesis	3
472	rfa2-S30A	GTCCCGCCCAGGATCCGGGGAGGCTGAAACTAACACTAG	Mutagenesis	3
253	rfa2-T32A	GGGGAGTCGGAAGCTAACACTCGAGTTAACACCTTG	Mutagenesis	3
254	rfa2-T34A	GTCGGAAACTAACGCTAGAGTAAACACCTTGACAC	Mutagenesis	3

Primer Number	Primer Name	Sequence	Function	Chapter
255	rfa2-T38A	GAAACTAACACTCGAGTTAACGCGTTGACACCTGTGACG	Mutagenesis	3
180	rfa1-F238A-BglII-FOR	CCGAGCCACGGCGGCTAATGATTTTGCTAC	Mutagenesis	3
182	rfa1-F269A-BlpI-FOR	CTCCAACCAGCTAAACCCCAAGCTACTAATCTAACAC	Mutagenesis	3
184	rfa1-W360A-EcoRV-FOR	CTCTGGGTTTTCGATATCTGTTGGCCTAGCGAATCAGCAAGCC	Mutagenesis	3
186	rfa1-F385A-NaeI-FOR	GTTCGTGTGACGGATGCCGGCGGCAAATCTTTGTC	Mutagenesis	3
327	rfa2-Asp(3,11,12,14) or rfa2-Dm1	ATATACACCATGGCAGATTATCAACCATATAACGAATATGATGATGTAGATGGCGGTGGC	Mutagenesis	3
328	rfa2-Asp(21,23,27,30,32,34) or rfa2-Dm2+3	TTTGAGAACGACGAGGATCGCCCAGGTGATGGGGAGGATGAAGATAACGATAGAGTTAAC	Mutagenesis	3
329	rfa2-Asp(21,23,27) or rfa2-Dm2	TTTGAGAACGACGAGGATCGCCCAGGTGATGGGGAGTCGGAAACTAAC	Mutagenesis	3
330	rfa2-Asp(30,32,34) or rfa2-Dm3	CGCCCAGGTAGTGGGGAGGATGAAGATAACGATAGAGTTAACACCTTGACA	Mutagenesis	3
331	rfa2-Ala(3,11,12,14) or rfa2-Am1	ATATACACCATGGCAGCTTATCAACCATATAACGAATATGCTGCTGTAGCCGGCGGTGGC	Mutagenesis	3
332	rfa2-Ala(21,23,27,30,32,34) or rfa2-Am2+3	TTTGAGAACGCTGAGGCCCGCCCAGGTGCCGGGGAGGCTGAAGCTAACGCTAGAGTTAAC	Mutagenesis	3
333	rfa2-Ala(21,23,27) or rfa2-Am2	TTTGAGAACGCTGAGGCCCGCCCAGGTGCCGGGGAGTCGGAAACTAAC	Mutagenesis	3
334	rfa2-Ala(30,32,34) or rfa2Am3	CGCCCAGGTAGTGGGGAGGCTGAAGCTAACGCTAGAGTTAACACCTTGACA	Mutagenesis	3

Primer Number	Primer Name	Sequence	Function	Chapter
338	RFA2-UP-NEW	TAGCAATTCCTTTGGCCTCG ATGAGCTTCC	Sequencing	3
339	RFA2-DOWN-NEW	GATAAAACCCTGGTCAGTCA AGGTCGTAC	Sequencing	3
340	pJG4-5-UP-Sequence	GATCCAGCCTGACTGGCTGA AATCGAATGG	Sequencing	4
341	pJG4-5-UP-Recover	TGGCGGATCAGGCGATTAA CGTGGTGCCGG	Sequencing	4
420	pEG202-LexA-SEQ-FOR	GGGCAATAAAGTCGAACTG TTGCCAG	Sequencing	4
421	pEG202-LexA-SEQ-REV	CCTGACCTACAGGAAAGAG TTACTCAAGAACAAGAATTT TCG	Sequencing	4
425	Rpa1-MID-FOR-SEQ	CAGCCTGTCACACACTTCTG GGGG	Sequencing	4
509	pJG4-5-mcm5-Delta308-359	TATTCCATCTATAATTCTAA AAATGGTGCCCGGAATTCG GCCGACTCGAGAAGCTTTGG A	Mutagenic-deletion	4
510	mcm5-Delta258-307	TCAAAGTTTATTGATCAACA GTTTTTAAAAGGATCCGGAA GGAGCGGGGGTGGAAAT	Mutagenic-deletion	4
511	mcm5-Delta208-257	AATTCTATCACAGGCAATAC CGTCAGTTTATTACAGGAAA TCCCAGAACTGGTTCCAGTA	Mutagenic-deletion	4
512	mcm5-Delta158-207	TTGAGAGATTTGGATTCTGA ACACGTCTCCCCACGTTCTT GCTTATCTACGATTGAGAGT	Mutagenic-deletion	4
513	pJG4-5mcm5-Delta108-157	GTGCCAGATTATGCCTCTCC CGAATTCCCTAAGATTGTCC GTTTATCAGGTATTATAATA	Mutagenic-deletion	4
520	NEW-pJG4-5-MCM5-FOR	CTACCTTATGATGTGCCAGA TTATGCCTCTCCCGAATTCA TGTCATTTGATAGACCGGA	Cloning	4
521	pJG4-5-mcm5(1-107)-REV	TTGACCAAACCTCTGGCGAA GAAGTCCAAAGCTTCTGCAG TCAAGCTCTGCTTAGAATAC	Cloning	4

Primer Number	Primer Name	Sequence	Function	Chapter
522	pJG4-5-mcm5(360-775)-FOR	CTACCCTTATGATGTGCCAGATTATGCCTCTCCCGAATTCCTAAGTAGAAACCCGAAGCT	Cloning	4
523	NEW-pJG4-5-MCM5-REV	TTGACCAAACCTCTGGCGAAGAAGTCCAAAGCTTCTCGAGTCATACACCACTTCTGTAAA	Cloning	4
532	pJG4-5-MCM5-FOR (108-359)	GCAACGGCGACTGGCTGGAATTCCCGGGGATCCGTCGACATGTCATTTGATAGACCGGAA	Cloning	4
533	pJG4-5-MCM5-REV (108-359)	AATTAGCTTGGCTGCAGGTCGACTCGAGCGGCCGCCATGTCATACACCACTTCTGTAAAT	Cloning	4
596	MCM2FL-pJG4-5-FOR	TCACCTTATGATGTGCCAGATTATGCCTCTCCCGAATTCATGTCTGATAATAGAAGACGT	Cloning	4
597	MCM2FL-pJG4-5-REV	TTGACCAAACCTCTGGCGAAGAAGTCCAAAGCTTCTCGAGTTAGTGACCCAAGGTATAAA	Cloning	4
598	MCM3FL-pJG4-5-FOR	TCACCTTATGATGTGCCAGATTATGCCTCTCCCGAATTCATGGAAGGCTCAACGGGATTT	Cloning	4
599	MCM3FL-pJG4-5-REV	TTGACCAAACCTCTGGCGAAGAAGTCCAAAGCTTCTCGAGTCAGACTCTCCAAACTTTAT	Cloning	4
600	MCM4-pJG4-5-FOR	TCACCTTATGATGTGCCAGATTATGCCTCTCCCGAATTCATGTCTCAACAGTCTAGCTCT	Cloning	4
601	MCM4-pJG4-5-REV	TTGACCAAACCTCTGGCGAGAAGTCCAAAGCTTCTCGAGTCAGACACGGTTATTCAGGC	Cloning	4
602	MCM6-pJG4-5-FOR	TCACCTTATGATGTGCCAGATTATGCCTCTCCCGAATTCATGTCATCCCCTTTTCCAGCT	Cloning	4
603	MCM6-pJG4-5-REV	TTGACCAAACCTCTGGCGAAGAAGTCCAAAGCTTCTCGAGTTAGCTGGAATCCTGTGGTT	Cloning	4
604	MCM7-pJG4-5-FOR	TCACCTTATGATGTGCCAGATTATGCCTCTCCCGAATTCATGAGTGCGGCACTTCCATCA	Cloning	4

Primer Number	Primer Name	Sequence	Function	Chapter
605	MCM7-pJG4-5-REV	TTGACCAAACCTCTGGCGAA GAAGTCCAAAGCTTCTCGAG TCAAGCGTCTTGTAGATCGA	Cloning	4

APPENDIX C. PLASMIDS[3]

Plasmid Name	Mutation	Backbone	Perm Number
pPLG1	pRS315-rfa2-S3D	pRS315-Rfa2	B-696
pPLG2	pRS315-rfa2-S11D	pRS315-Rfa2	B-697
pPLG3	pRS315-rfa2-S12D	pRS315-Rfa2	B-698
pPLG4	pRS315-rfa2-T14D	pRS315-Rfa2	B-699
pPLG5	pRS315-rfa2-S21D	pRS315-Rfa2	B-700
pPLG6	pRS315-rfa2-S23D	pRS315-Rfa2	B-701
pPLG7	pRS315-rfa2-S27D	pRS315-Rfa2	B-702
pPLG8	pRS315-rfa2-S30D	pRS315-Rfa2	B-703
pPLG9	pRS315-rfa2-T32D	pRS315-Rfa2	B-704
pPLG10	pRS315-rfa2-T34D	pRS315-Rfa2	B-705
pPLG11	pRS315-rfa2-T38D	pRS315-Rfa2	B-706
pPLG12	pRS315-rfa2-S3A	pRS315-Rfa2	B-707
pPLG13	pRS315-rfa2-S11A	pRS315-Rfa2	B-708
pPLG14	pRS315-rfa2-S12A	pRS315-Rfa2	B-709
pPLG15	pRS315-rfa2-T14A	pRS315-Rfa2	B-710
pPLG16	pRS315-rfa2-S21A	pRS315-Rfa2	B-711
pPLG17	pRS315-rfa2-S23A	pRS315-Rfa2	B-712
pPLG18	pRS315-rfa2-S27A	pRS315-Rfa2	B-713
pPLG19	pRS315-rfa2-S30A	pRS315-Rfa2	B-714
pPLG20	pRS315-rfa2-T32A	pRS315-Rfa2	B-715
pPLG21	pRS315-rfa2-T34A	pRS315-Rfa2	B-716
pPLG22	pRS315-rfa2-T38A	pRS315-Rfa2	B-717
pPLG23	pRS315-rfa2-Am1	pRS315-Rfa2	B-718
pPLG24	pRS315-rfa2-Am2	pRS315-Rfa2	B-719
pPLG25	pRS315-rfa2-Am3	pRS315-Rfa2	B-720
pPLG26	pRS315-rfa2-Am4	pRS315-Rfa2	B-721
pPLG27	pRS315-rfa2-Am1+3	pRS315-Rfa2	B-722
pPLG28	pRS315-rfa2-Am1+4	pRS315-Rfa2	B-723
pPLG29	pRS315-rfa2-Dm1	pRS315-Rfa2	B-724
pPLG30	pRS315-rfa2-Dm2	pRS315-Rfa2	B-725
pPLG31	pRS315-rfa2-Dm3	pRS315-Rfa2	B-726
pPLG32	pRS315-rfa2-Dm4	pRS315-Rfa2	B-727
pPLG33	pRS315-rfa2-Dm1+3	pRS315-Rfa2	B-728
pPLG34	pRS315-rfa2-Dm1+4	pRS315-Rfa2	B-729
pALS8	pRS315-rfa2-DeltaN1	pRS315-Rfa2	B-730
pALS9	pRS315-rfa2-DeltaN2	pRS315-Rfa2	B-731
pALS10	pRS315-rfa2-DeltaN3	pRS315-Rfa2	B-732
pENM10	pJG4-5-B42-HA-RFA1	pJG4-5	B-848

[3] **Note:** NA in perm number column indicates the plasmids not stored in perms.

Plasmid Name	Mutation	Backbone	Perm Number
pENM11	pJG4-5-B42-HA-RFA2	pJG4-5	B-849
pENM12	pJG4-5-B42-HA-RFA3	pJG4-5	B-850
pENM13	pJG4-5-B42-HA-Rpa1	pJG4-5	B-851
pENM14	pJG4-5-B42-HA-Rpa2	pJG4-5	B-852
pENM15	pJG4-5-B42-HA-Rpa3	pJG4-5	B-853
pENM16	pJG4-5-B42-HA-Rpa4	pJG4-5	B-854
pGP1	pEG202-lexA-rfa1-FLAB	pEG202	B-856
pSJH101	pEG202K-lexA-RFA1	pEG202	B-863
pPLG63	pJM132-Rpa2-Rpa3-Rpa1-kanMX (22G2)	pJM132	B-894
pPLG64	pJM132-Rpa4-Rpa3-Rpa1-kanMX-1 (3G1)	pJM132	B-895
pPLG65	pJM132-Rpa4-Rpa3-Rpa1-kanMX-2 (3G2)	pJM132	B-896
pPLG35	pRS313-Rfa1-A1	pRS313	B-912
pPLG36	pRS313-Rfa1-A2	pRS313	B-913
pPLG37	pRS313-Rfa1-B1	pRS313	B-914
pPLG38	pRS313-Rfa1-B2	pRS313	B-915
pPLG39	pRS313-Rfa1-A12	pRS313	B-916
pPLG40	pRS313-Rfa1-B12	pRS313	B-917
pPLG41	pRS313-Rfa1-A1B1	pRS313	B-918
pPLG42	pRS313-Rfa1-A1B2	pRS313	B-919
pPLG43	pRS313-Rfa1-A2B1	pRS313	B-920
pPLG44	pRS313-Rfa1-A2B2	pRS313	B-921
pPLG45	pRS313-Rfa1-A12B1	pRS313	B-922
pPLG46	pRS313-Rfa1-A12B2	pRS313	B-923
pPLG47	pRS313-Rfa1-A1B12	pRS313	B-924
pPLG48	pRS313-Rfa1-A2B12	pRS313	B-925
pPLG49	pRS313-Rfa1-A12B12	pRS313	B-926
pPLG52	pJG4-5-MCM5 FL	pJG4-5	B-929
pPLG53	pJG4-5-MCM5 1-108	pJG4-5	B-930
pPLG54	pJG4-5-MCM5 108-359	pJG4-5	B-931
pPLG55	pJG4-5-MCM5 359-775	pJG4-5	B-932
pPLG56	pJM132-Rpa4	pJM132	B-933
pPLG57	pJM132-Rpa2	pJM132	B-934
pPLG58	pJM132-Rpa4+3	pJM132	B-935
pPLG59	pJM132-Rpa2+3	pJM132	B-936
pPLG60	pEG202K-Rpa1-2	pEG202	B-937
pPLG61	pEG202K	pEG202	B-938
pPLG62	pSH18-34K	pSH18-34	B-939
pPLG63	pJG4-5-MCM2 FL	pJG4-5	NA
pPLG64	pJG4-5-MCM3 FL	pJG4-5	NA

Plasmid Name	Mutation	Backbone	Perm Number
pPLG65	pJG4-5-MCM4 FL	pJG4-5	NA
pPLG66	pJG4-5-MCM6 FL	pJG4-5	NA
pPLG67	pJG4-5-MCM7 FL	pJG4-5	NA
pPLG68	pJG4-5-MCM5 Δ108-157	pJG4-5	NA
pPLG69	pJG4-5-MCM5 Δ158-207	pJG4-5	NA
pPLG70	pJG4-5-MCM5 Δ208-257	pJG4-5	NA
pPLG71	pJG4-5-MCM5 Δ258-307	pJG4-5	NA
pPLG72	pJG4-5-MCM5 Δ308-359	pJG4-5	NA
pPLG73	pLVX-shRNA2-shRPA1	pLVX-shRNA2 (ZsGreen)	NA
pPLG74	pLVX-shRNA2-shRPA2	pLVX-shRNA2 (ZsGreen)	NA
pPLG75	pLVX-shRNA2-shRPA3	pLVX-shRNA2 (ZsGreen)	NA
pPLG76	pLVX-shRNA2-shRPA4-1	pLVX-shRNA2 (ZsGreen)	NA
pPLG77	pLVX-shRNA2-shRPA4-2	pLVX-shRNA2 (ZsGreen)	NA
pPLG78	pLVX-shRNA2-shRPA4-3	pLVX-shRNA2 (ZsGreen)	NA
pPLG79	pLVX-shRNA2-shRPA4-5	pLVX-shRNA2 (ZsGreen)	NA