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Molecular Functions of the Chromatin Remodeler Fun30

Zeina Salim Al-Natour

United Arab Emirates University

College of Medicine and Health Sciences

MOLECULAR FUNCTIONS OF THE CHROMATIN REMODELER FUN30

Zeina Salim Al Natour

This dissertation is submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy

Under the Supervision of Dr. Ahmed H. Hassan Al Marzouqi

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Declaration of Original Work

I, Zeina Salim Al Natour, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this dissertation entitled "*Molecular Functions of the Chromatin Remodeler Fun30*", hereby, solemnly declare that this dissertation is my own original research work that has been done and prepared by me under the supervision of Dr. Ahmed H. Hassan Al Marzouqi in the College of Medicine and Health Sciences at UAEU. This work has not previously been published or formed the basis for the award of any academic degree, diploma or a similar title at this or any other university. Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my dissertation have been properly cited and acknowledged in accordance with appropriate academic conventions. I further declare that there is no potential conflict of interest with respect to the research, data collection, authorship, presentation and/or publication of this dissertation.

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Approval of the Doctorate Dissertation

This Doctorate Dissertation is approved by the following Examining Committee Members:

1) Advisor (Committee Chair): Dr. Ahmed H. Hassan Al Marzouqi
   Title: Associate Professor
   Department of Biochemistry and Molecular Biology
   College of Medicine and Health Sciences
   Signature _____ Date __January 4, 2017__

2) Member: Prof. Haider Raza
   Title: Professor
   Department of Biochemistry and Molecular Biology
   College of Medicine and Health Sciences
   Signature _____ Date __January 4, 2017__

3) Member: Prof. Tahir Rizvi
   Title: Professor
   Department of Microbiology and Immunology
   College of Medicine and Health Sciences
   Signature _____ Date __January 4, 2017__

4) Member: Prof. Samir Attoub
   Title: Professor
   Department of Pharmacology and Therapeutics
   College of Medicine and Health Sciences
   Signature _____ Date __January 4, 2017__

5) Member (External Examiner): Dr. Woojin An
   Title: Associate Professor
   Department of Biochemistry and Molecular Biology
   Institution: USC/Norris Comprehensive Cancer Center, USA

   Signature _____ Date __January 4, 2017__

This Doctorate Dissertation is accepted by:

Acting Dean of the College of Medicine and Health Sciences: Prof. Ruth Langer

Signature _____ Date 10/5/2017

Dean of the College of Graduate Studies: Prof. Nagi T. Wakim

Signature _____ Date 25/5/2017

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Advisory Committee

1) Advisor: Dr. Ahmed H. Hassan Al Marzouqi

Title: Associate Professor

Department of Biochemistry

College of Medicine and Health Sciences

2) Member: Prof. Haider Raza

Title: Professor

Department of Biochemistry

College of Medicine and Health Sciences

3) Member: Prof. Tahir Rizvi

Title: Professor

Department of Microbiology

College of Medicine and Health Sciences

4) Member: Prof. Samir Attoub

Title: Professor

Department of Pharmacology

College of Medicine and Health Sciences

Abstract

Many studies have identified conserved ATP-dependent chromatin remodeling complexes whose functions are to modulate DNA access by relieving chromatin-mediated repression. We have previously characterized Fun30 in *Saccharomyces cerevisiae* as a homodimer with ATP-dependent chromatin remodeling activity. Other studies have shown that Fun30 plays a role in maintaining the silenced state of subtelomeric and centromeric chromosomal regions. Fun30 has also been shown to play an important role in DNA damage repair by facilitating long range resection of DNA in Double Strand Breaks. This thesis was focuses on understanding the mechanisms by which Fun30 is involved in DNA damage repair. Results presented here show that Fun30 can anneal complementary strands of DNA that is facilitated by ATP hydrolysis and a helicase activity in the presence of trap DNA. In addition, Fun30 was found to be able to relax both negatively and positively supercoiled DNA in an ATP-independent manner and cleave a 3' overhang in a forked DNA duplex or a duplex that has a protruding 3'. Annealing and 3' flap endonuclease activities of Fun30 suggest a mechanism by which Fun30 can facilitates double strand break repair by the Single Strand Annealing pathway, while a potential helicase activity can facilitate Synthesis Depended Strand Annealing and as a result reduce the generation of recombination intermediates. Moreover, employing *in vivo* approaches, we show that Fun30 genetically interacts with the Mus81 nuclease upon chronic treatment with chemicals that stall the replication fork, suggesting that Fun30 deletion might lead to the accumulation of toxic recombination intermediates that are difficult to resolve in the absence of Mus81. We also found that Fun30 deletion affects the cell cycle progression of cells lacking TopI, without affecting the viability of the cells. This might explain a function for Fun30 in facilitating the progression of the cell cycle in the presence of torsional stress which can be induced by TopI deletion. Moreover, we found that Fun30 is not involved in removing camptothecin induced TopI/DNA complexes since no genetic interaction between Tdp1 and Fun30 was observed. Furthermore, we show that Fun30 genetically interacts with Asf1 under DNA damaging conditions, suggesting that Fun30 is required in the absence of Asf1. Finally, couple of models are proposed that explain how Fun30 annealing and nuclease activities may be important in the Single Strand Annealing pathway and how Fun30

helicase activity might be used to reduce the level of toxic recombination intermediates and thus maintain genomic stability, which if compromised could lead to cancer or other diseases.

Keywords: Fun30, Chromatin Remodeling, DNA Double Strand Breaks, DNA Damage Repair.

Title and Abstract (in Arabic)

الوظائف الجزيئية للبروتين المعدل لبنية الكروماتين الFun30

الملخص

بينت العديد من الدراسات دورمركبات بروتينية تستخدم الطاقة الناتجة من كسر جزيء الأدونيسين الثلاثي الفوسفات (ATP)، في التعديل من طبيعة الكروماتين المثبطة والمعيقة لوصول العديد من البروتينات للحمض النووي (DNA). لقد بينا في دراسة سابقة دور بروتين ال Fun30 المتواجد في خميرة الخباز, الموجود على شكل جزئ ثنائي متماثل في الحفاظ على طبيعة الكروماتين المكثفة والمثبطة في القسيم المركزي و التيلوميرات .كما وجدت دراسات مؤخرة دور لل Fun30 في تحفيز عملية تشكيل نهايات ال DNA عند اماكن القطع ثنائية الطرف وذلك عن طريق تحفيز تآكل سلسلة واحدة من كل طرف وهذه الخطوة تعد من الخطوات الأولية في عملية ترميم انقطاعات الDNA. تركز هذه الأطروحة على إيجاد آليات أخرى لفهم دور بروتين ال Fun30 في عملية ترميم ال DNA . تظهر نتائج البحث أن لل Fun30 قدرة على تحفيز الازدواج بين جزيئات ال DNA المكملة لبعضها. بالإضافة لذالك وجدنا أن لل Fun30 قدرة على فك هذا الازدواج ولكن فقط بوجود مصيدة من ال DNA وباستخدام الطاقة الناتجة من كسر ال ATP. كما بينا أن لل Fun30 قدرة على إحداث قطع في جزى ال DNA مما يساعد في إزالة الالتفافات في جزيئات ال DNA الدائرية وأيضا في إزالة أطراف ال ’3 من ال DNA ذا الفرعين أو فرع واحد. وجدنا في دراسات في الوسط الحيوي أن هناك تفاعل جيني بين بروتين ال Fun30 وبروتين ال Mus81 وذالك فقط في وجود مواد تؤثر على ال DNA ، وكون أن لل Mus81 دور في التقليل من دور الأثار السلبية لعمليات التهجين في ال DNA هذه النتيجة تؤكد أن لل Fun30 دور مماثل ولو بشكل مختلف. ايضا وجدنا أن ال غياب ال Fun30 في سلالة تفتقد لبروتين ال Top1 أثر على سير دورة الخلية من دون التأثير على حياتها. هذا قد يدل على أهمية ال Fun30 في غياب بروتين له دور في تقليل من صعوبات نسخ ال DNA بسبب إلتفافات الجزيء. عدم وجود تفاعل جيني بين بروتين ال Fun30 و بروتين ال Tdp1 يضحد فكرة أن لل F un30 دور مباشر في إزالة مركبات ال Top1/DNA والتي يتسبب بها مركب الCamptothecin. وهذا يدلل أن لل Fun30 آلية أخرى تختلف عن ال Tdp1. أيضا وجدنا ال Fun30 مهم في غياب بروتين ال Asf1 وذلك في وجود مواد تؤثر على طبيعة ال DNA أو عملية نسخه مما يدل على أهمية ال Fun30 في حال تعرض ال DNA لاي مواد

مسمة. نقدم في هذه الدراسة نموذجين لشرح آلية محتملة لل Fun30 في عملية ال SSA وهي نوع من علميات ترميم إنقطعات ال DNA وذالك عن طريق أستخدام ال Fun30 قدرته على تحفيز الازدواج بين جزيئات ال DNA وأيضا عن طريق إزالة الأطراف الزائدة وذلك للتمهيد لترميم القطع. بينما النموذج الثاني يقدم آلية محتملة لل Fun30 للتخفيف من الآثار الجانبية والضارة لعمليات تهجين ال DNA الزائدة وذالك عن طريق إزالة تركيبات ال DNA المتشكلة بواسطة استخدام قدرته على فك الاندماج بين جزيئات ال DNA . وبذالك قد يساهم ال التقليل من فرص عدم استقرار ال DNA و الذي يؤدي في حال حصوله إلى طفرات وتغييرات جينية تؤدي إلى حدوث السرطان وأمراض أخرى.

مفاهيم البحث الرئيسية: مركبات معدلة لبنية الكروماتين, بروتين ال Fun30 قطع ثنائي الطرف، ترميم الحمض النووي.

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Dedication

To my beloved parents and family

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List of Abbreviations

BIR	Break Induced Replication
CPT	Camptothecin
CY5	Cayanine5
DMSO	Dimethyl Sulfoxide
DTT	1,4-Dithiothreitol
Etl1	Enhance trap locus
FACS	Fluorescent Activated Cell Sorting
HU	Hydroxyurea
IgG	Immunoglobulin G
MMS	Methyl methanesulfonate
PCR	Polymerase Chain Reaction
SDSA	Synthesis Dependent Strand Annealing
Smarcad1	SWI/SNF-Related, Matrix-Associated Actin-Dependent Regulator Of Chromatin, Subfamily A, Containing DEAD/H Box 1
SSA	Single Strand Annealing
TAP	Tandem Affinity Purification
TCA	Trichloroacetic acid

Chapter 1: Introduction

1.1 Overview

All aspects of an organism life from structure to function are encoded in genes that are made of DNA, a complex chemical structure that is often referred to as the blueprint of life. Accurate and complete duplication followed by even transmission of the two copies of the genome to offspring cells is vital to maintain cell viability and functionality. Genomic instability, is a word used to describe increased tendency of genome alteration during cell life cycle (Shen 2011). These genomic alterations can be as simple as changing a single DNA base pair leading to silent, missense, and nonsense mutations, or the deletion or insertion of a single base pair. Gross DNA changes such as inversions, translocations, deletions or duplications of longer stretches of DNA can also happen. Additionally, more extreme form of instability can happen in the form of chromosomal loss or gain, or what is known as aneuploidy. Mutations are often perceived as the culprit behind many diseases; however, mutations can also cause variations that are needed for evolution. Knowing that many diseases have genetic bases has intrigued scientists for a long time as they try to explore how genetic instability is triggered and what mechanisms are utilized by cells to reduce its incidence. Interestingly, genomic instability is a hallmark of cancerous cells and heterogeneity of cancer cells' genetic background provides a strong evidence for this instability. The cells of all organisms have evolved several conserved mechanisms to ensure proper transmission of the genetic material. Malfunctioning of any of the machineries involved in this process can lead to genomic alteration and thus to either cell death or cells with altered growth that, in humans, can be the signal for the initiation of cancer. Homologous recombination is one of mechanisms the cell uses to

ensure genome stability. Interestingly if this mechanism goes uncontrolled, it can itself lead to genomic instability. The following sections will explain the types of DNA damage, which can lead to genomic instability if not repaired, with an emphasis on DNA double strand breaks that are considered deleterious to the cell, if not promptly and accurately repaired.

1.2 Endogenous and Exogenous Sources of DNA Damage

An organism's cell is continuously challenged by agents that threaten its DNA integrity. Endogenous or simultaneous damage happens during the regular life cycle and can be induced by chemicals that are released during normal cell metabolic activities. For example, reactive oxygen species generated during metabolism can lead to both base damage and DNA double strand breaks. Other form of damage is the simultaneous loss, deamination, or alkylation of DNA bases. In addition, modified bases located on template strand, if not repaired or tolerated, can lead to erroneous incorporation of nucleotides during replication. Exogenous sources of DNA damage can be either of physical or chemical nature. For example, ultraviolet radiation (UV) leads to pyrimidine dimer formation, gamma radiation leads to the formation of double strand breaks. Chemicals that damage DNA bases are like benzo(a)pyrene, aflatoxin, and nitrosamine are just a few examples of an arsenal of damage inducing chemicals (Boiteux and Jinks-Robertson 2013). To maintain genome stability, the cell has to accurately copy the DNA, which can only be done by a robust replication mechanism that ensures proper selection of nucleotides. This is done by the 3'-5' proof reading activity found in replicative polymerases. Moreover, fine tuning of replicative enzymes' functions is also aided by other interacting partners. Therefore, in addition to the previously mentioned sources of DNA damage, inactivation or suppression of

the proofreading function or regulatory proteins of the cells' replicative machinery can lead to genomic instability. Interestingly, this suppression can also be caused by mutations introduced into the proteins involved (Skoneczna, Kaniak, and Skoneczny 2015). It is interesting to know that cells have evolved several mechanisms to deal with different types of damage. Base Excision Repair (BER) pathway is used to remove damaged bases or to repair apyrimidinic/apurinic (AP) sites, while helix-distorting lesions that interfere with base pairing (such as those induced by UV damage or chemicals that cause bulky DNA adducts) are repaired by the Nucleotide Excision Repair (NER). Errors made during DNA replication, by inserting wrong nucleotides, will also lead to helical distortions that are sensed and repaired by the Mismatch Repair (MMR) pathway. The same pathway also acts when non-identical duplexes exchange strands during recombination (Boiteux and Jinks-Robertson 2013). Since double strand breaks are very toxic lesions and are a major source of genomic instability, the next sections will elaborate more on how they are generated and the mechanisms that are utilized by the cell to repair them.

1.3 DNA Double Strand Break (DSB)

DNA double-strand breaks (DSBs) are the most lethal forms of DNA damage, which if not repaired, can lead to either cell death or genomic instability. DSBs form upon simultaneous breaking of two complementary stands of the DNA double helix at sites that are very close to one another. These free DNA ends if not juxtaposed or repaired properly can haphazardly recombine or get joined with other regions in the genome leading to genome instability (Jackson 2002). DSBs are induced by many exogenous factors such as exposure to ionic radiation or treatment with radiomimetic drugs in which a break is induced by free radical mechanisms, DNA

replication inhibitors and topoisomerase poisons. Endogenous factors like oxidative damage can also lead to DSB formation (Povirk 2012). Interestingly, double-strand breaks can also be generated in normal cell cycle during replication. Although replication is highly controlled with all factors working synchronously, there are moments when the machinery faces obstacles that compromise its effectiveness. Replication stress is defined as the slowing or stalling of the progression of a replication fork. Beside exogenous chemicals that can cause replication stress, there are endogenous sources of replication stress inside the cell. Sources of replication stress can be: 1) Passing of replication fork over nicks that are generated during normal processes like DNA repair or as a result of DNA relaxing, which can later be converted to double-strand breaks, 2) When a replication forks encounters replicated DNA lesions that form due to exogenous or endogenous sources of DNA damage, 3) Misincorporation of ribonucleotides, 4) When replication forks encounter DNA secondary structures that are formed because of DNA sequences such as hairpins, triplexes and G-quadruplexes, 5) Collision between replication and transcription machinery at highly transcribed sites or improper processing of RNA transcripts, 6) Depletion of nucleotides due to firing of too many origins as a result of oncogene activation, 7) Passing through fragile sites, which lack origins of replications, given no backup mechanism to rescue a stalled replication fork with a converging fork, and finally 8) Replication in DNA regions that have high chromatin compaction (Zeman and Cimprich 2014).

Interestingly, although being lethal to the cell, generation of DSBs can be induced by the cell in a programmed way to accomplish particular tasks such as maturation of lymphocytes in human (Schatz and Swanson 2011), or during meiosis,

in which programmed induction of DSBs followed by homologous recombination promotes pairing interactions between homologous chromosomes, a step which is important for segregation of chromosomes during meiosis in both yeast and human (Borde and de Massy 2013). In budding yeast, mating type switching also requires the generation of double-strand breaks that are repaired by homologous recombination to achieve type switching (Haber 2012). Accurate repair of DNA double strand break is crucial to ensure genomic stability. Although the cell makes use of several mechanisms to repair a DSB, these mechanisms have different efficiency. This is because some mechanisms are error-prone and might introduce changes to the repaired DNA. However, the cells utilizes all the different pathways available to it while ensuring proper balance and regulation of their frequency as well as the temporal activation of some pathways, all of which reduces the side effects of less efficient repair. The different pathways for repairing DSBs include Non-Homologous End Joining (NHEJ) and Single Strand Annealing (SSA), which are considered error-prone, in addition to homologous recombination (HR), which is more accurate and is believed to be error-free, as well as synthesis dependent strand annealing (SDSA), which is a variant of homologous recombination. The following sections will give a brief description of some of these pathways.

1.3.1 Repair of DSBs by Non-Homologous End Joining (NHEJ)

The simplest solution of repairing a DNA DSB would be simply re-ligating the two ends, without seeking homologous region on sister chromatid, in order to make sure that ligation does not happen with a remote or unrelated DNA fragment on other non-homologous chromosomes. Otherwise, this can lead to deleterious genomic rearrangements. However, precision of this type of repair depends on the nature of the

free ends of the induced DSBs. Error free and perfect ligation happens when the two ends are of perfect complementarity or the structure of ends leads to no deletions and is simply repaired by gap filling as is the case in juxtaposing blunt ends with a protruding 3' or 5' ends. DNA ends with less complementarity can lead to imperfect ligation and deletions and thus is error-prone (Feldmann et al. 2000, Wilson and Lieber 1999). Non-homologous end joining is the favored pathway for repairing DSBs in human. While NHEJ is functional in budding yeast, HR is the dominant pathway (Boulton and Jackson 1998). Genes that are involved in this pathway can be categorized into four groups: yKu70/yKu80, Dnl4/Lif1, Sir2/Sir3/Sir4, and Mre11/Rad50/Xrs2 (MRX) complex. Ku70 and Ku80 proteins form a heterodimer that binds to the end of double-strand breaks (Milne et al. 1996), such as blunt ends, 5′, or 3′ overhangs (Boulton and Jackson 1998, Martin et al. 1999). Strong binding to DNA ends is believed to aid in bringing the two ends together for juxtaposing them (Pang et al. 1997). It has been proposed that Ku70/Ku80 heterodimer has a role in protecting the ends of DSBs and facilitates the ligation of the ends either directly or by recruiting ligase enzyme or nucleases that would work ahead of ligase enzyme (Lewis and Resnick 2000). Dnl4/Lif1 physically interacts with each other, with Dnl4 having an ATP-dependent ligase activity (Herrmann, Lindahl, and Schär 1998). Since not all DSB DNA ends are compatible with ligation, processing of these ends is a must, but such processing might cause alterations to the ends and so repair will be error prone. The MRX complex is believed to play a role in this end processing (Connelly and Leach 2002)**.** Finally, the Sir proteins have been shown to localize to DSB sites. Their actual role, however, at DSBs is not yet known, but it has been suggested that Sir proteins inhibit gene expression at DSB sites after repair (Martin et al. 1999).

1.3.2 Repair of DSBs by Seeking Homologous Regions (Error-Free)

Homology based repair might be the best mechanism evolved by the cell to avoid introducing errors while repairing double-strand breaks. Interestingly, this homology search might also be one way by which genomic instability is initiated. Many factors can affect the outcome of DSB repair, such as the nature of the break as being one-ended or double-ended DSB, or the presence of double-strand breaks in certain chromosomal features such as repetitive DNA sequences, or crossing over, especially between homologous chromosomes, can lead to genomic instability. The following sections will give a brief explanation of each possible means of homology-based repair.

1.3.2.1 Homologous Recombination (HR)

From its name, homologous recombination implies that this kind of repair depends on finding a homologous sequence, which can be either on a sister chromatid or on a homologous chromosome. Using homologous sequence as a template allows for error-free repair of double-strand breaks. In yeast, this pathway acts on both programed and un-programed DSBs. Moreover, homologous recombination allows the rescuing of stalled replication forks (Yeeles et al. 2013, Petermann et al. 2010). The key step in this pathway is the nuclease-mediated processing of the DNA ends at the DSB sites and is called 5'-3' DNA end resection resulting in DSBs with 3' overhangs or tails. Resection can proceed in two phases, with limited resection mediated by the MRX complex and Sae2, while subsequent extensive resection is mediated by the Exo1, a 5' to 3' exonuclease, and the Sgs1 helicase together with Dna2 nuclease (Mimitou and Symington 2008, Nicolette et al. 2010, Cejka, Cannavo, et al. 2010). Resection will help in forming DNA 3' tails that will seek homology; however,

this is not a task that can happen by simply annealing the 3’ overhang to a complementary DNA. Many factors and structures need to be formed to allow this search and synapsis. Once generated, the 3’ single-stranded DNA tails are bound by replication protein A (RPA). RPA binds with high affinity to the single strands. This binding allows the elimination of any secondary DNA structures that would form on the single stranded DNA (Chen, Lisby, and Symington 2013). Following resection, presynaptic filaments are formed by binding of Rad51 protein to single-stranded DNA, and are the structures that will lead to strand invasion (Shinohara, Ogawa, and Ogawa 1992, Sung 1994). Although RPA binding helps in eliminating secondary DNA structures, which is believed to assist in presynaptic filament formation, it was found that RPA itself, can cause an impediment to the binding of Rad51. This obstacle is overcome by the aid of recombination mediators such as Rad52 (Sung 1997), while Rad55-Rad57 complex aids in stabilizing the Rad51 filaments (Liu et al. 2011). The formation of the presynaptic filament is a critical step for strand invasion and homology searching on a sister chromatid or homologous chromosome. When a homologous region is found, a heteroduplex is formed where the invading strand displaces a DNA strand from a DNA duplex and anneals with its homologous region forming what is known as the D-loop. This is aided by Rad54, which has been shown to assist in D-loop formation in an ATP-dependent manner and by inducing topological changes to the target or donor DNA (Van Komen et al. 2000, Wright and Heyer 2014). After invasion, the 3’ invading strand acts as a primer for extension by a DNA polymerase. Interestingly, Rad54 helps this function by removing the Rad51 protein from the 3’ end in order to facilitate the access of a DNA polymerase (Li and Heyer 2009). Therefore, not only does Rad54 have a role in D-loop formation and extension, but also in disrupting it (Wright and Heyer 2014). There are three possible sub-

pathways of homologous recombination as described below, all of which share these steps initially (Figure 1.1).

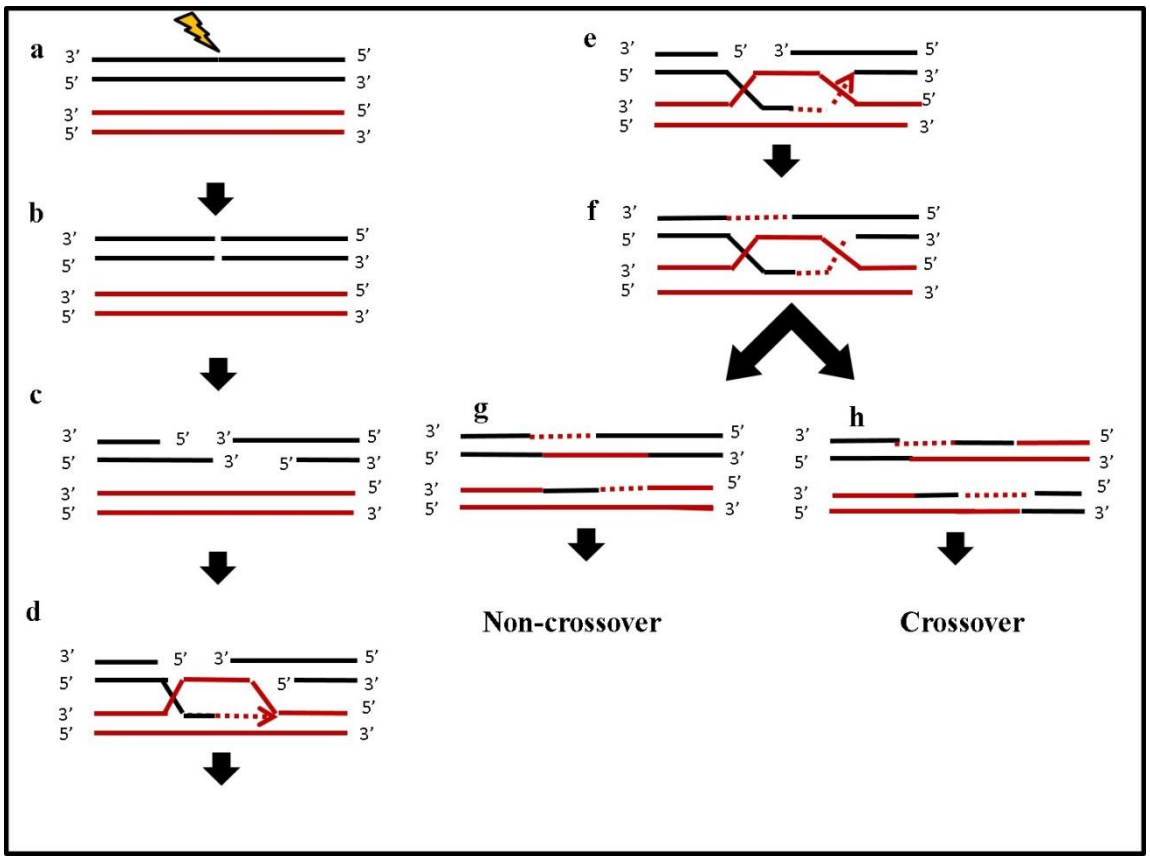


Figure 1.1: Double strand break repair by homologous recombination

Exposure to DNA damaging agents (a) causes the formation of Double Strand Breaks (b), followed by DNA resection (c), and strand invasion (d). This is followed by 3' primer extension (e), second end capture and the formation of double Holliday Junctions (dHJ) (f) leading to the resolution of dHJ into non-crossover (g) or crossover products (h).

1.3.2.2 Break Induced Replication (BIR)

While a two-ended DSB is the ideal structure for error free and safe repair, the cells can still perform HR in cases where the DSB is one-ended. One-ended DSB are formed in many ways; one way is through the collapse of the replication fork. Replication stress caused by endogenous or exogenous factors can lead to the collapse

of the replication fork and the generation of DSB with one end (Zeman and Cimprich 2014). Another source of one ended DSBs would be eroded telomeres (Lydeard et al. 2007). Although the initial steps of resection and invasion are similar, the only difference is that in the absence of a second end, the 3' end of the invading strand in the D-loop will be extended by DNA synthesis until it copies the rest of the donor chromosome. This process is termed Break Induced Replication (BIR) and it can restore the whole length of a broken chromosome. Hence, it is a way to rescue collapsed and broken replication forks. Moreover, BIR also works to maintain eroded telomeres (Lydeard et al. 2007). However, BIR can also lead to Loss of Heterozygosity (LOH). This happens if during BIR a homologous chromosome is used instead of a sister chromatid. Genomic instability can also be induced if BIR occurs at sites of internal repeated sequences (Llorente, Smith, and Symington 2008). BIR can be either Rad51-dependent or Rad51-independent. Rad51-dependent BIR makes use of some factors (such as resection proteins Rad52, Rad55-Rad57, and Rad54) that act in repairing two-sided DSBs, during homologous recombination. On the other hand, Rad51-independent BIR, is more mutagenic and it has been proposed that the ends of DSBs invade ectopic regions exposed during processes such as replication and transcription (Sakofsky, Ayyar, and Malkova 2012). A schematic representation of BIR pathway is shown below in Figure 1.2.

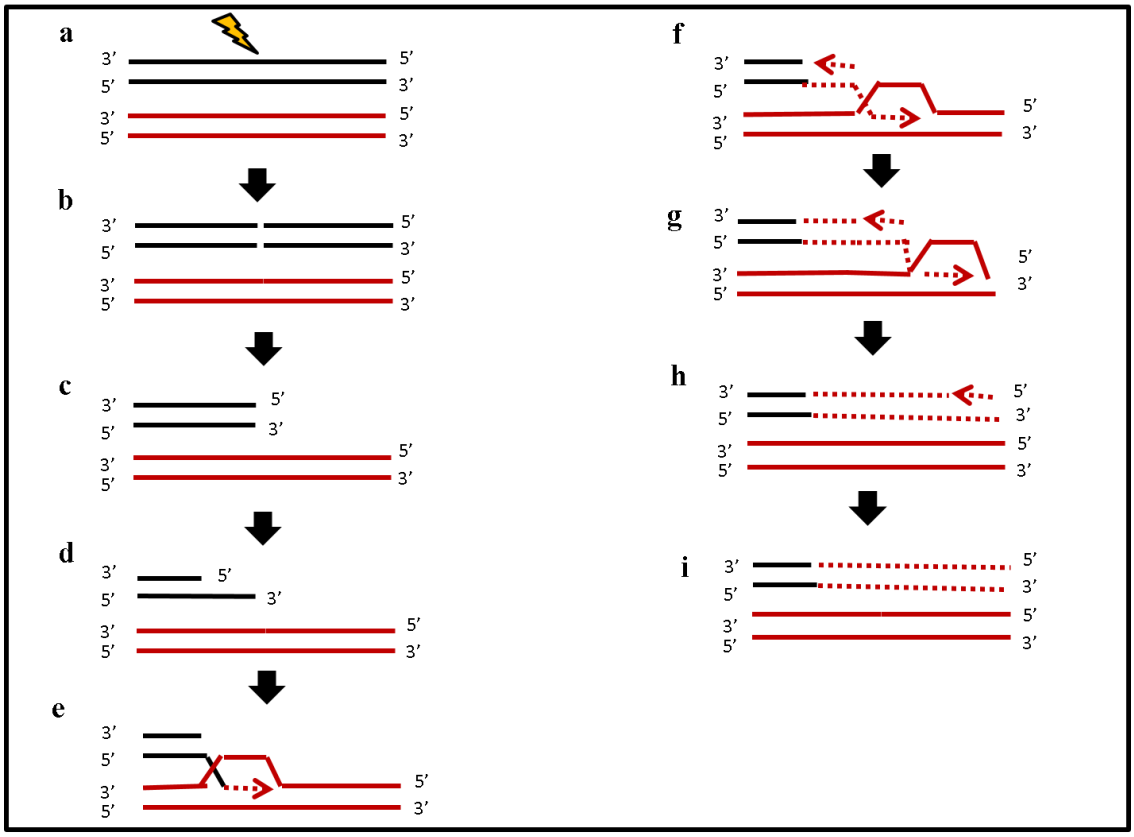


Figure 1.2: Double strand break repair by break induced replication

Exposure to DNA damaging agent (a) causes the generation of Doubles Strand Breaks (b), which lead to the loss of one side of the break leaving behind one end of the double strand break (c). This is followed by DNA resection (d), strand invasion and D-loop formation (e). Extension of D-loop (f) leads to its dissociation (h), and finally replication of the complementary strand (i).

1.3.2.3 Synthesis Dependent Strand Annealing (SDSA) and double Holliday Junctions (dHJ)

When a D-loop is formed in a two-ended DSB, the D-loop can proceed in two possible pathways. In one case, the D-loop is reversed and the 3’ end of the newly synthesized strand anneals to the second end of the DSB, followed by DNA synthesis and ligation. This pathway is known as Synthesis Dependent Strand Annealing (SDSA) and is the predominant pathway in somatic cells and helps the cell avoid unwanted genetic crossovers (McMahill, Sham, and Bishop 2007). Several proteins are involved in the D-loop disruption such as Srs2 (Ira et al. 2003) and Mph1 (Prakash

et al. 2009), which are both helicases, as well as Top3 (Fasching et al. 2015). In the other option, the D-loop is maintained and a second end capture takes place. This leads to the formation of double Holliday junctions (dHJ). Such a structure needs to be resolved, if not resolved, these dHJ can lead to genomic instability. Resolving of dHJ can be achieved in two different ways. One way makes use of structure specific nuclease such as Mus81-Mms4, Yen1, and Slx1-Slx4. These nucleases can have different substrate preference and the generation of Crossover (CO) or Non-Crossover (NCO) products depends on the orientation of the cut induced by these nucleases (Matos and West 2014). Another mechanism, which leads to NCO products, is known has Holliday junction dissolution. In this pathway, the Sgs1-Top3-Rmi1 protein complex aids in promoting the migration of two Holliday Junctions toward each other by the action of Sgs1 helicase. The resulting structure, which is formed from interlinked DNA molecule is then decatenated or detangled with the action of Top III topoisomerase (Cejka, Plank, et al. 2010). The SDSA pathway is illustrated in Figure 1.3.

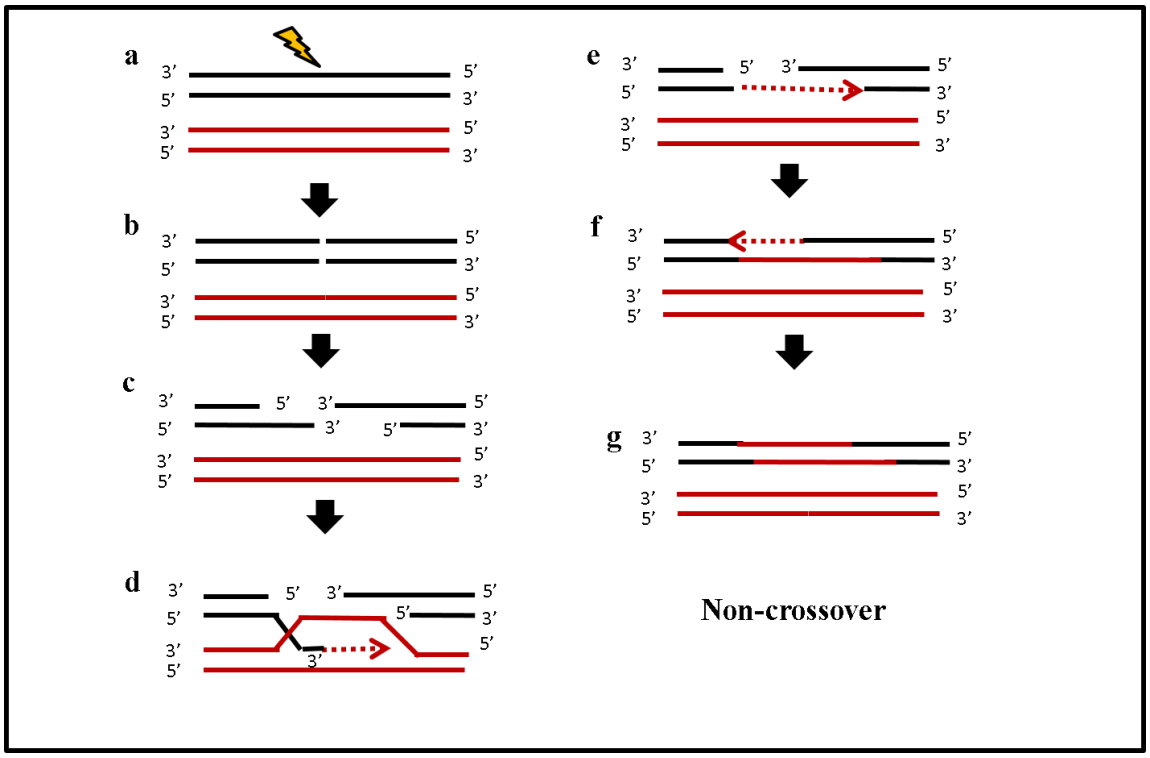


Figure 1.3: Double strand break repair by synthesis dependent strand annealing

Exposure to DNA damaging agent (a) causes the generation of Double Strand Breaks (b), followed by DNA end resections (c), strand invasion and extension (d). This is followed by strand displacement and annealing to other part of the break (e), gap filling (f) leading to non-crossover products (g).

1.3.2.4 Single Strand Annealing (SSA)

It is interesting to note that the position of a double strand break can affect the outcome of its repair. Single Strand Annealing pathway is the term usually used to describe a pathway of repair that deals with DSBs that exists in a region flanked by direct DNA repeats. Although SSA can help rescue broken chromosomes, this process leads to a loss of DNA between the repeats and a reduction of the repeats to a single copy, which can in turn lead to genomic rearrangements and thus is considered error prone. Similar to HR, SSA is initiated by 5' to 3' DNA resection. Extensive resection will unmask homologous single-stranded DNA on both sides of the break allowing them to anneal to each other (Bhargava, Onyango, and Stark 2016). Although SSA

requires Rad52, unlike HR it does not require other factors such as RAD51, RAD54, RAD55, and RAD57 (Ivanov et al. 1996). Since Rad52 has DNA annealing activity, it is believed that it aids in annealing the complementary strands together. During SSA, annealing of DNA can lead to branched structures having overhangs of non-homologous DNA or 3' flap, which should be removed to allow for completion of repair. Interestingly, mismatch proteins Msh2 and Msh3 are believed to recognize such structures, stabilize them, and facilitate the cleavage of these non-homologous overhangs by the Rad1/Rad10 nucleotide excision repair nuclease (Sugawara et al. 1997). Saw1 was identified in a screen for mutants defective in SSA and found to be required for the recruitment of Rad1/Rad10 endonuclease to 3' flaps during SSA (Li et al. 2008). More recently, it has been found that Saw1-mediated recruitment of Rad10 is only required for long 3' flaps that are formed in the G1 phase (Mardirosian et al. 2016). Repair of DSBs by Single Strand Annealing is illustrated in Figure 1.4.

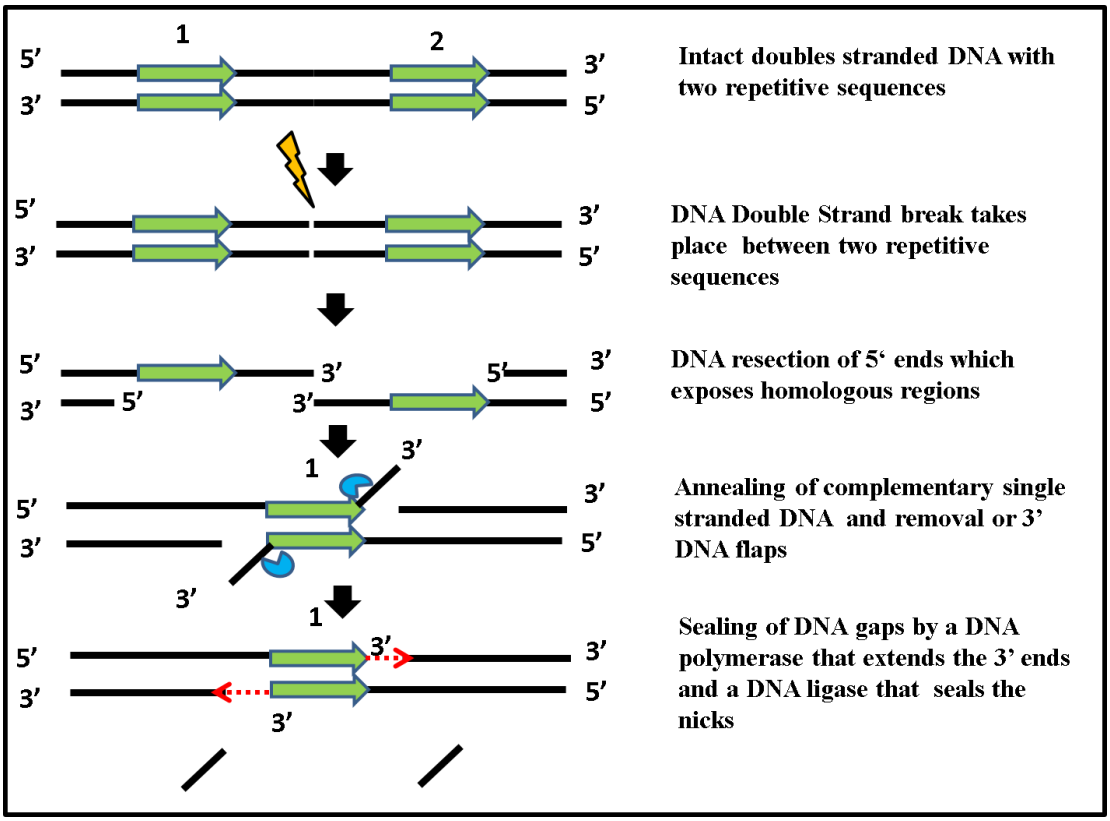


Figure 1.4: Double strand repair by single strand annealing

Exposure to DNA damaging agent causes the generation of Double Strand Breaks, followed by DNA end resections exposing homologous regions. Annealing of complementary single stranded DNA and the removal of 3’ DNA flaps is followed by DNA extension and sealing of the gaps.

1.4 The Chromatin Structure and ATP-Dependent Chromatin Remodeling

In eukaryotes, the DNA does not exist alone; but rather it interacts with several types of proteins. Some proteins bind transiently and represent binding factors or proteins that catalyze several DNA transactions such as DNA replication, transcription, DNA damage repair, and chromosomal segregation. On the other hand, other proteins have structural functions and their interaction with the DNA form a DNA-protein complex termed chromatin allowing the organization of DNA into higher order structures. The basic unit of chromatin is the nucleosome, which is made up of ~ 147 bp of DNA wrapped around an octameric complex of basic proteins called

histones. The histone octamer is composed of two dimers of histones H2A-H2B and a tetramer of H3-H4. Nucleosomal arrays on DNA form an 11 nm fiber or what is usually termed as the beads-on-a-string structure. Nucleosomal arrays further folds to form a 30 nm fiber (Li and Zhu 2015). Further condensation and compaction leads to the formation of the highly compacted mitotic chromosomes, however, the condensation mechanisms is still not clear and different models have been proposed to explain how this is accomplished (Antonin and Neumann 2016). DNA compaction can resolve the problem of DNA storage in the nucleus and make its movement and segregation possible with less tangling of the DNA molecule. Although nucleosomes are helpful in condensing the DNA, they can hinder other cellular processes as their protein machineries need to be able to access DNA to carry on their functions (Clapier and Cairns 2009). These DNA processes include DNA replication, transcription, and DNA damage repair, as mentioned above. For example, transcription can be negatively affected by blocking an enhancer or a promoter region by nucleosomes. Similarly, nucleosomes might present roadblocks to the elongation step of transcription. Initiation of replication requires the recognition of certain DNA elements that could be blocked by nucleosomes. In addition, replication elongation could also be hampered by nucleosomes that are ahead of the replication fork. Many steps during repair might also be affected by the presence of nucleosomes (Clapier and Cairns 2009).

In budding yeast, as in higher eukaryotes, the chromatin is organized into domains of transcriptionally active and transcriptionally repressed domains known as euchromatin and heterochromatin, respectively. These domains are usually found juxtaposed with one another. Heterochromatin regions have their signature histone modifications, which are hypoacetylation of different histones and hypomethylation

of lysine 4 and lysine 79 of histone H3. In yeast, they are found at regions such as in the mating type loci HML and HMR as well as in telomeres (Katan-Khaykovich and Struhl 2005). It is clear that establishing repressed chromatin is also required for the formation of functional genomic domains such as telomere and centromeres. This shows that relaxing or condensing the chromatin structure are two important competing activities during the cell cycle. Chromatin is a dynamic structure and cells have evolved mechanisms to overcome its inhibitory effects. Cells have also developed mechanisms to establish repressed domains. Mechanisms include posttranslational modification of the histone tails, which might loosen the contact between histones and DNA making a region more accessible or might create binding signals to recruit other proteins and allow further remodeling. Such modifications include acetylation, phosphorylation, methylation, ubiquitination, and sumoylation of histone tails. These mechanisms involve complexes that use energy of ATP hydrolysis, referred to as ATP-dependent chromatin remodeling, to modulate the chromatin structure. This involves physical movement of nucleosomes either by sliding them in cis or by evicting them in trans to expose the underlying DNA regions.

Other form of ATP-dependent remodeling involves the exchange of histone variants or evicting histone dimers. For more detail on various mechanism of chromatin remodeling complexes see reviews (Vignali et al. 2000, Henikoff 2016). Such physical effects seem to be triggered by the ATP-dependent translocation activity of these proteins that generate loops on the surface of the nucleosome, which can expose DNA on the nucleosome surface or in case of diffusion of the loop can lead to the repositioning or sliding of the nucleosome. ATP-dependent chromatin remodeling proteins belong to the helicase-like superfamily 2 (SF2) based on the presence of

several conserved helicase motifs in the ATPase domain. Most of the ATP-dependent remodeling proteins form protein complexes with other proteins that might confer regulatory effects on the catalytic subunit or provide accessory binding domains. This might explain the different activities that are catalyzed by remodeling complexes despite belonging to the same families. The catalytic subunit is usually the one with the ATPase domain (Flaus and Owen-Hughes 2011). Many ways can be used to classify proteins. Despite homology in the ATPase domain, some of these remodeling proteins were found to have extra domains giving each protein its unique function. Based on these extra domains, proteins were further classified onto into four major families. 1) The SWI/SNF family: beside the conserved ATPase domain, the members of this family have HSA domain on the N-terminus and a bromodomain on the C-terminus. A bromodomain allows binding to acetylated lysines on histone tails. The members of this family can both slide and eject nucleosomes. In yeast, SWI/SNF and RSC complexes belong to this family. 2) The ISWI family: in addition to the ATPase domain, they have SANT and SLIDE domains, which form a nucleosome recognition module. Most of the members of this family have been found to optimize nucleosome spacing. In yeast, ISW1a, ISW1b, and ISW2 complexes belong to this family. 3) The CHD family: They have two chromodomains at the N-terminus, which is believed to bind methylated histone tails. Proteins in this family can slide and eject nucleosomes and may also have repressive effect. In yeast, CHD1 which belongs to this family does not form a complex. 4) The INO80 family: They are known to have a split ATPase domain or a long insertion in the middle of the domain. Members of this family have been shown to facilitate transcription and replication and some, like SWR1, can replace H2A-H2B dimer with H2A.Z-H2B dimer. In yeast, the INO80 and SWR1 complexes belong to this family of remodelers (Clapier and Cairns 2009). In another

classification of the proteins that share sequence homology in their helicase related-regions, Rad54 was placed under the Rad54-like subfamily, while Fun30 was classified under the SWR1 subfamily (Flaus et al. 2006). Functions of some of these chromatin remodelers are discussed in more detail below. As mentioned above, DNA damage repair is one of many cellular mechanisms that are affected by the chromatin structure, and therefore, how ATP-dependent chromatin remolding complexes are involved in DNA damage repair, concentrating on DSB repair by HR, is also discussed below.

1.4.1 Heterochromatin and Chromatin Remodeling

Many lines of evidence indicate that heterochromatic regions are problematic for repair. For example, in humans, it has been found that mutations in many cancer genomes accumulate at high levels in repressed or heterochromatic domains (Schuster-Böckler and Lehner 2012). Moreover, mutations were found to locate to nucleosomal DNA, which is masked because of the presence of nucleosomes (Tolstorukov et al. 2011). In addition, heterochromatin domains have been found to have much slower rates of DNA repair (Goodarzi et al. 2008). All of these reflect a clear difficulty in either recognizing DNA damage or in processing/repairing it in the context of heterochromatin regions. Interestingly, this can also happen on a single nucleosome surface where access to DNA is inhibited by the nucleosome itself. Although chromatin itself can be repressive, but at the same time, it is the platform from which signaling for repair is triggered. The “access-repair-restore model” is a model that has been proposed to describe the steps required to repair DSB. This model signifies the importance of recognizing DNA damage in different chromatin structures, followed by remodeling chromatin, which will help in this recognition as well as in

allowing the access of repair proteins. Then, following repair, chromatin restoration takes place (Smerdon 1991). The chromatin remodeling is achieved by the action of ATP-dependent chromatin modifying complexes. In the following sections, how some ATP-dependent remodeling complexes are involved in facilitating the repair of DSB by HR will be discussed, in addition to how some ATP-dependent chromatin remodelers work to facilitate different stages of HR, such as the initial stages of resection, formation of synaptic nucleoprotein filaments, strand invasion, DNA synthesis, and check point activation and adaptation.

1.4.1.1 The SWI/SNF Complex

SWI/SNF is a multi-subunit complex, with the Swi2/Snf2 subunit as its catalytic subunit, which belongs to the SWI/SNF family. Similar to other remodelers, mutants of this complex was also shown to be sensitive to DSB inducing chemicals. Moreover, SWI/SNF has been shown to be recruited to sites of induced DSB (Chai et al. 2005). Interestingly, SWI/SNF has also been shown to associate with both the recipient locus, at which the double strand is induced, and the donor locus (Chai et al. 2005) . The recruitment of both Rad52 and Rad51 to donor site indicates synapsis event between the recipient and donor site. Interestingly, both Rad51 and Rad52 recruitment to DSB was reduced in the *Δsnf5* strain, and subsequently, DNA synthesis and ligation were inhibited. All of this point to a role for the SWI/SNF complex in remodeling and exposing donor DNA to allow invasion and synapsis steps during DSB repair by HR (Chai et al. 2005). Furthermore, *in vitro* assays have shown that the SWI/SNF complex is able to eject Sir3 that was reconstituted on mini-chromosomes facilitating Rad54 mediated D-loop formation. This suggests a possible role for the SWI/SNF complex in promoting recombination in heterochromatic regions, which are

hard to access due to their compacted and repressed nature (Sinha et al. 2009). The data on the SWI/SNF complex altogether reveal a role of SWI/SNF complex in facilitating DSB repair by remodeling chromatin at DSB sites making access of repair proteins possible.

1.4.1.2 The RSC Complex

RSC is a multi-subunit complex, with Sth1 as it catalytic subunit, and belongs to the SWI/SNF family. The RSC complex mutants have been found to be sensitive to DSB inducing chemicals and UV, implicating it in DSB repair (Liang et al. 2007). Genetic interaction between double mutant of RSC and Ku70 supports its implication in HR (Liang et al. 2007). The RSC complex was shown to affect very early and late stages of HR. The early recruitment of RSC to DSB supports its role in facilitating the initial steps of repair (Liang et al. 2007). For example, RSC has been shown to be required for the recruitment of Mec1 and Tel1 proteins, which are proteins that are involved in DNA damage check point and can facilitate Rad9 recruitment as well as Rad53 activation for check point activation (Liang et al. 2007). Deletion of Sth1, the catalytic subunit of RSC, was shown to reduce chromatin remodeling around DSB sites (Shim et al. 2007). Similarly, Sth1 deletion caused reduced recruitment of Mre11 suggesting a role for the RSC complex in remodeling the chromatin to allow access of repair proteins to the site of damage (Shim et al. 2007). Interestingly, unlike most ATP-dependent chromatin remodelers, RSC was not found to be recruited to γH2A.X, and instead γH2A.X levels were found to be dependent on RSC (Liang et al. 2007). Moreover, depletion of Sth1 was also found to reduce the recruitment of RPA, indicating reduced resection (Shim et al. 2007, Liang et al. 2007). RSC was also found to have a role in the latest step of HR, the ligation of the 3’ end of the invading strand

after synthesis, such a role has been explained by possibly dissociating the invading strand (Chai et al. 2005). All in all, these data point to the role of the RSC in facilitating DSB repair during HR by facilitating the recruitment of DNA repair factors by dissociating the invading strand to allow its ligation with the other end of the DSB.

1.4.1.3 The INO80 Complex

Ino80 is the catalytic subunit of the INO80 complex, which is one of the most intensively studied ATP-dependent chromatin remodelers, with numerous evidences implicating it in HR. An important role of Ino80 in HR was revealed when the deletion of the Ino80 subunit itself or the deletion of the ATPase stimulating subunits Arp8 or Arp5 were shown to make the strains sensitive to DSB inducing agents (van Attikum et al. 2004). A direct role in DSB repair was demonstrated from findings that show Ino80 localizes at and binds to induced DSB sites. This recruitment was found to be dependent on γH2A.X, a phosphorylated histone H2A at serine 129 that is induced upon DNA damage (van Attikum et al. 2004). The Ino80 recruitment to phosphorylated H2A has also been found to be dependent on the Nph10 subunit of the complex (Morrison et al. 2004). Data on the role of Ino80 on DNA resection, which is the first step in HR, are contradictory. While one study has found that resection was deficient in a strain lacking Arp8 (van Attikum et al. 2004), other studies have shown no effect on resection (Tsukuda et al. 2005, Papamichos-Chronakis, Krebs, and Peterson 2006). Interestingly, in one of these studies, where reduced eviction of nucleosomes at DSB in *Δarp8* was observed, resection was shown to proceed normally, but recruitment of Rad51 was delayed. This suggests a possible role for Ino80 in remodeling the chromatin in order to facilitate the recruitment of Rad51 (Tsukuda et al. 2005). Furthermore, work by Tsukuda et al. has highlighted the

difference in recruitment of Rad51 in haploid versus diploid cells. They have shown that recruitment of Rad51 was delayed in the *Δarp8* strain in haploid cells, which lack donor DNA, while its recruitment was normal in diploid cells, however, filament transfer to donor DNA is delayed in these cells. All of these results, in addition to the observed alteration in gene conversion tracts in Arp8 mutants, suggest a role for Ino80 at early and late stages of HR (Tsukuda et al. 2009). The sensitivity to DSB in *ino80* mutants was the motive for checking the efficiency of DNA damage repair by HR and NHEJ. Interestingly, *ino80* mutant were proficient at both repair pathways indicating that the sensitivity only occurs in persistent DSB damage. That was confirmed by the inability of the *ino80* mutants to achieve check point adaptation which was supported by the presence high Rad53p kinase activity. Inability of the *ino80* mutant to induce full phosphorylation of H2A was suggested to account for the inability of the strain to achieve adaptation after check point activation, however, observing that H2A phosphorylation site were not defective in adaptation excluded this possibility. Rather it was found that Ino80 inhibits Swr1-dependent exchange of γH2A.X with H2A.Z histone variant and thus this indicates that normal level of γH2A.X, which is maintained by Ino80, seems to be important for check point adaptation (Papamichos-Chronakis, Krebs, and Peterson 2006). Regarding DNA damage check point activation, it was shown that a mutant lacking Ino80 activity was proficient in activating G1 check point, excluding a role for Ino80 remodeling activity in activating G1 checkpoint (Javaheri et al. 2006). Another study has shown that the loss of γH2A.X, H2A.Z, and H3 around DSB was dependent on Ino80 and that its remodeling activity is important for Mre11 recruitment and thus for G2/M check point activation (van Attikum, Fritsch, and Gasser 2007). Yet, In another study, despite seeing a less significant role in repairing single induced DSB, Ino80 was found to be involved in

damage-induced sister chromatid recombination and interchromosomal recombination between hetero-alleles (Kawashima et al. 2007).

1.4.1.4 The SWR1 Complex

SWR1 is a multi-subunit complex with Swr1 as its catalytic subunit that belongs to the Ino80 family of chromatin remodelers. The Swr1 complex has been shown to exchange H2A-H2B dimers for H2A.Z-H2B in nucleosomal arrays, *in vitro*. H2A.Z, as an *in vivo* substrate of Swr1, was initially suggested following Swr1 purification with the H2A.Z-H2B dimer (Mizuguchi et al. 2004). A direct role of Swr1 in DSB repair is supported by its recruitment to DSB sites, which has been shown to be mediated by its interactions with γH2AX through the Arp4 subunit of the complex (Downs et al. 2004). Strains with deletion of Swr1 were also shown to be sensitive to the DNA damaging agent MMS (Mizuguchi et al. 2004). Despite its *in vitro* activity in exchanging histones dimers, SWR1 was not found to have a role in exchanging H2A-H2B for H2A.Z-H2B dimers at DBS *in vivo*, except when Ino80 was deleted, suggesting an antagonistic function between Ino80 and Swr1. This became clearer when a defect in checkpoint adaptation upon Ino80 deletion was shown to be rescued by deleting Swr1 (van Attikum, Fritsch, and Gasser 2007, Papamichos-Chronakis, Krebs, and Peterson 2006).

1.4.1.5 The Rad54 Protein

Rad54 belongs to the Rad54-like subfamily. Mutants of Rad54 are extremely sensitive to ionizing radiations, which are known to induce DSBs. This sensitivity reflects the inability of these mutants to repair DSBs. Rad54 protein has also been shown to have a role in strand invasion by facilitating the pairing between

Rad51 filaments and the homologous region, which is also supported by demonstrating both physical and functional interactions between the two proteins (Jiang et al. 1996, Petukhova, Stratton, and Sung 1998, Clever et al. 1997). This is not due to the increased formation of presynaptic filament, but rather due to interactions with the presynaptic Rad51 nucleoprotein filaments (Solinger et al. 2001). Besides being involved in D-loop formation, Rad45 has been shown to have another postsynaptic role by stimulating the extension of the D-loop in an ATP-dependent manner (Solinger et al. 2001). Heteroduplex DNA (hDNA) or the D-loop formation has been suggested to be facilitated by ATP-dependent conformational changes induced by the Rad54 in the duplex DNA that are believed to lead to transient opening of the DNA duplex and thus facilitating the joining of the presynaptic filament (Petukhova et al. 1999). Rad45 was also shown to be required for D-loop extension by DNA polymerase, which is facilitated by the removal of Rad51 blocking the 3' end of the invading strand (Li and Heyer 2009). Another study has shown that the ATP-dependent activity of Rad54 can facilitate the removal of Rad51 from presynaptic filaments which would facilitate D-loop formation and extension (Solinger, Kiianitsa, and Heyer 2002). Moreover, Rad54 has been shown to have remodeling activity on nucleosomal DNA by increasing its accessibility without disrupting nucleosomal positions, which implicate its remodeling activity in facilitating homologous pairing in the context of chromatin (Jaskelioff et al. 2003). In another study, it was shown that Mating type switching can be blocked in a strain lacking rad54, implicating its role in facilitating strand invasion into donor sequences embedded in heterochromatin (Sugawara et al. 1995). All of these findings on Rad54 highlight its role in several stages during HR beside a role in facilitating DSB that exist at compacted chromatin regions.

1.5 The Fun30 Protein

Fun30 is a yeast protein that has sequence homology with other ATP-dependent chromatin remodeling proteins such as the Swi2 subunit of the yeast SWI/SNF complex. Biochemical and functional characterization of Fun30 in our laboratory, has demonstrated its ATP-dependent remodeling function *in vitro* (Awad et al. 2010). In this study, TAP-tagged Fun30 was purified by the Tandem Affinity Purification method and it was found to form a homodimer. It was also shown that Fun30 has ATPase and histone dimer exchange activities, in addition to its chromatin remodeling activity (Awad et al. 2010). Later work by Byeon et al. reproduced some of these results (Byeon et al. 2013). Since this thesis is focused on Fun30, below I will provide a detailed literature review on the current understating of Fun30 functions.

1.5.1 Fun30 Remodels Chromatin

Many lines of evidence have been presented during the last few years showing a role for Fun30 or its orthologs in remodeling the chromatin structure *in vivo*. While most of the data support a role of Fun30 and its homologs in silencing by establishing heterochromatin at chromosomal features such as centromeres, telomeres, or mating type loci (in yeast). Work done by others has highlighted another possible role for Fun30 in establishing active chromatin as the case with the human ortholog of Fun30 called Smarcad1, suggesting that it might have a dual effect on chromatin. In the coming sections, I will elaborate more on the importance of establishing heterochromatin at certain chromosomal features and how Fun30 or its homologs/orthologs are involved in ensuring proper function. In addition, the role of Fun30 in remodeling promoters affecting transcription will be discussed in this section.

A possible role of Fun30 in gene silencing was first suggested by Neves-Costa et al. after observing that *fun30* was among genes identified in a synthetic lethality screen. That screen aimed at finding genes that interacted genetically with temperature sensitive mutants of *ocr2* and *ocr5*. Observing that *HST1, HST3,* and *SUM1* genes, which were identified in the same screen were involved in gene silencing, led the authors to test whether Fun30 also has a role in gene silencing (Neves-Costa et al. 2009). This was confirmed by Neves-Costa et al. and other groups, who showed that Fun30 is required for suppressing reporter genes that were inserted in heterochromatic loci (Neves-Costa et al. 2009, Strålfors et al. 2011, Durand-Dubief et al. 2012). I will discuss this further in later sections.

Many assays done using MNase to map nucleosomes borders and particular heterochromatin loci have shown that proper insertion and spacing of nucleosomes requires the activity of Fun30 (Yu, Zhang, and Bi 2011, Neves-Costa et al. 2009). In one study, in which genomic DNA extracted from both wild-type and Fun30 deletion strains (which were resolved on gel after MNase digestion) revealed no significant difference between the two strains in global chromatin structure (Neves-Costa et al. 2009). While a more sensitive assay, that can map the borders of nucleosomes employing sequencing of MNase digested DNA, showed a more clear difference between the Fun30 mutant and the wild-type strains (Byeon et al. 2013). In addition, with regards to histone modifications, a change in H2A.Z distribution was observed upon Fun30 deletion (Durand-Dubief et al. 2012).

Interestingly, in budding yeast, most of the information gained on the role of Fun30 in establishing heterochromatin is derived from work done on chromosomal

features made of repetitive sequences such as telomeres and centromeres, which are known to have no genes or code for non-coding RNA. Another important aspect of remodeling takes place at gene promoters where remodelers aid in turning genes on or off. RNA-Seq data by Druand-Dubief et al. showed that there is a weak correlation between gene expression and Fun30 recruitment to promoters, and that the changes which occurred in gene expression after deleting Fun30 were rather a response to other events affected by the absence of Fun30 (Durand-Dubief et al. 2012). By performing RNA expression analysis, utilizing hybridization microarrays in budding yeast, Byeon et al. showed that the expression of 275 genes was dependent on Fun30, 88% of which were upregulated in the absence of Fun30, indicating a role of Fun30 in repressing their expression. These results suggested that gene silencing was not due to their proximity to chromosomal features, which are known for their heterochromatic nature and maintained by Fun30. Moreover, they suggest that repression was not due to exclusive binding of Fun30 to the promoters since Fun30 binding was found to be throughout the open reading frame of the genes. However, Fun30 deletion was shown to affect the length of the Nucleosome Free Region (NFR) by affecting the positions of nucleosomes flanking these regions. It was found that nucleosomes at positions -1, +2 and +3 were shifted, while no change of the +1 nucleosome was observed. In addition, histone modifications that are hallmarks of active chromatin, such as H3K4me3, H3K14ac, and H4Kac were underrepresented at affected promoters. Moreover, higher levels of ubH2B at these genes were detected. However, it was suggested that repression by Fun30 is not dependent on transcription since not all genes which were targeted by Fun30 displayed altered transcription, and so it was suggested that Fun30 chromatin remodeling has function other than transcription at these genes (Byeon et al. 2013).

Other evidence on Fun30 requirement for proper positioning of nucleosomes came from work on retrotrasposons, whose transcription is highly regulated in wild-type cells under normal conditions. Such regulation of transcription was proposed by blocking the access of transcriptional machinery to the first transcription start site (TSS) and directing it to a downstream TSS, leading to truncated version of retrotrasposon transcript that fails to integrate into the genome (Persson et al. 2016). Interestingly, in this study, it was found that nucleosome positioning by Fun30 at the flanking long terminal repeat region will drive this preference for the downstream TSS, since Fun30 deletion led to less nucleosome occupancy and a higher transcription level of the intact transcript. This led the authors to suggest that Fun30 is important for regulating retrotrasposon expression in normally growing cells since its repression is alleviated under stress conditions (Persson et al. 2016). Remarkably, consistent with these finding, a screening study that aimed at identifing host factors that affect transposition Ty3 retrotrasposon in yeast, Fun30 deletion was shown to cause 40% reduction in transposition, which was explained by altered pattern of processing Gag3-p derived protein (IN) and reduced amount of cDNA (Aye et al. 2004). Although this study shows less expression of retrotrasposon upon *FUN30* deletion, this might have been due to differences in the design of the experiment.

Other evidence on the possible function of the protein on the chromatin structure came from the human Smarcad1. Proteomic analysis of Smarcad1, which was immunoprecipitated form mammalian cells, has shown that Smarad1 formed stable interaction with Kap1, a co-repressor protein, while it interacted with histone modifying complexes such as HDAC1, HDAC2, and the H3K9 methyltransferase

complex G9A/GLP in a sub-stoichiometrical fashion (Rowbotham et al. 2011). All of these proteins play a role in chromatin silencing by modifying histone tails. Moreover, stable and transient deletion of Smarcad1 from HeLa cells, has been shown to cause global upregulation of H3 and H4 histone acetylation accompanied by concomitant decrease in H3K9 di- and tri-methylation, all of which are hallmarks of euchromatin (Rowbotham et al. 2011). Observing a similar effect when Kap1 was deleted suggested that the two proteins work together in regulating heterochromatic histones modifications. In cells lacking Smarad1, only cells ectopically expressing wild type Smarcad1 were able to reverse these changes in histone modifications, while the ATPase mutant failed to do so, showing that this activity of Smarcad1 was ATP-dependent (Rowbotham et al. 2011). These findings led the authors to suggest that Smarcad1 is remodeling the chromatin structure at these sites, requiring ATP hydrolysis (Rowbotham et al. 2011). Contrary to these results, another study demonstrated that Smarcad1 induces open chromatin structure by facilitating histone acetylation (Doiguchi et al. 2016). In this study, Smarcad1 was identified as the factor required for ATP-dependent acetylation of H2A histones by CBP-p300 histone acetyltransferase in Drosophila nuclear extract. Although no tight complex was formed between the two proteins, Smarcad1 was required for acetylation of nucleosomal histones which are not the preferable substrates for acetylation compared to free histones. Authors have observed enhanced level of transcription of 12 genes *in vivo* upon overexpression of Smarcad1, moreover, Smarcad1 was shown to activate the transcription of DNA templates with promoters of some of these genes *in vitro*. Similarly, down regulation of some genes was observed upon knockdown of Smarcad1 (Doiguchi et al. 2016). Moreover, it was found that CBP was required for localizing of Smarcad1 to promoters (Doiguchi et al. 2016). All these data suggest multiple

possible roles for Fun30 including remodeling chromatin to achieve gene silencing or activation, in addition to its non-transcription related functions.

1.5.2 Silencing of Mating Type Loci HMR and HML in Yeast

Budding yeast cells can be either haploid with MATa or MATα types or they can be MATa/MATα diploids. The MAT locus is located in the middle of the right arm of chromosome III. Interestingly, the same chromosome also contains two different alleles of MAT locus, which are HMRa and HMLα and are proximal to telomeres. A programed induced DSB in the middle of MAT locus will induce its repair by homologous recombination by using any one of the two alleles as donor DNA, which will dictate the type of the cell it will become. This property of budding yeast allows the cells to be homothallic. In other words, mating-type switching allows the generation of haploid cells of opposite types in the same colony, which allows mating between different cell types and thus self-diploidization (Haber 2012). Acting as donors to allow mating-type switching implies that the genes at these allelic loci should be suppressed to maintain the newly acquired identity of the cell. This is accomplished by establishing silenced chromatin over these regions (Hickman, Froyd, and Rusche 2011).

Both Fun30 and Fft3 (Fun30 ortholog in Fission yeast) proteins have been implicated in the establishment of heterochromatin at these silenced mating type loci (Neves-Costa et al. 2009, Yu, Zhang, and Bi 2011). In one study, the authors showed that the deletion of Fun30 leads to the expression of an ADE2 reporter gene, inserted in the HMR locus, which is normally repressed in the wild-type strain. A direct role for Fun30 in establishing heterochromatin at HMR locus implies that it should be

physically located to chromatin to remodel it. Localization of Fun30 was confirmed by means of chromatin immunoprecipitation (ChIP), followed by quantitative PCR using primers spanning the boundaries of the HMR locus (Neves-Costa et al. 2009, Yu, Zhang, and Bi 2011). Fun30 was also shown to be localized to tRNA gene, which acts as a barrier gene separating heterochromatin from euchromatin, at HMR locus, while fun30 occupancy was found to be less in genes downstream of tRNA where euchromatin is present. Furthermore, the role of Fun30 in establishing heterochromatin was further demonstrated by showing that chromatin extracted from the Fun30 deletion strain had higher accessibility to micrococcal nuclease (MNase) when compared to chromatin of wild-type strain. However, localized sensitization to MNase digestion did not reflect a global change in chromatin structure in the absence in Fun30 (Neves-Costa et al. 2009). In addition, the Fun30 ATPase domain was also shown to be required for its role in silencing of the ADE2 reporter gene, embedded in the HMR locus, which supports its role as an ATP-dependent remodeler in heterochromatin establishment in budding yeast (Neves-Costa et al. 2009).

Similar to the Fun30 role at HMR, work done by Yu and co-workers have also revealed a role of Fun30 in silencing at the HML locus (Yu, Zhang, and Bi 2011). Here, Fun30 deletion strain was shown to reduce silencing of a URA3 gene that was inserted in the HML locus, which was confirmed by the presence of higher level of URA3 mRNA in the Fun30 deletion strain compared to the wild-type strain. As was in the case of the HMR locus, Fun30 was also found to be enriched at the HML locus. Moreover, a DNA topology based assay was utilized to confirm the role of Fun30 in establishing repressive chromatin structure at the HML locus. The assay was performed by resolving topoisomers of a circular minichromosome that was excised

from the HML locus, for both *Δfun30* and wild-type strains. The topoisomers isolated form the *Δfun30* strain displayed a pattern typical of more relaxed plasmid when compared to those from wild-type cells and the linking number was found to be reduced by 2. Reduced linking number indicates lower density of nucleosomes supporting a role of Fun30 in maintaining the heterochromatin structure. Since the change observed was not as severe as the change observed in DNA extracted from sir3 deletion strain, it was suggested that silencing the HML locus is not completely dependent on Fun30 and that it is required for full transcriptional silencing (Yu, Zhang, and Bi 2011). Furthermore, nucleosome mapping by MNase digestion followed by indirect labeling gave more insight into the nature of the chromatin changes, where mapping of the borders of nucleosomes reflected an action of nucleosome sliding. Since the chromatin pattern formed in absence of Fun30 was neither similar to chromatin formed in sir2 deletion strain nor in the wild-type strain, it was suggested that Fun30 is required to complete the establishment of heterochromatin from an intermediate state of chromatin which was suggested to be downstream of Sir2 action. This was concluded from the observation that Fun30 deletion did not affect chromatin structure in a Sir2 deletion strain. Moreover, the intermediate state chromatin that forms in the absence of Fun30 was shown to retain all hallmarks of heterochromatin such as hypoacetylation and hypomethylation, suggesting that Fun30 acts just downstream of sir2 protein and is more likely involved in establishing regular arrays and removing gaps between nucleosomes. Gapless chromatin is important for complete establishment of heterochromatin (Yu, Zhang, and Bi 2011).

1.5.3 Role of Fun30 at Silencing rDNA

In *Saccharomyces cerevisiae* ribosomal DNA (rDNA), which form 100-200 copies of tandem repeats (Petes and Botstein 1977), is another DNA region that needs to be silenced. This silencing provides a mechanism by which recombination is regulated at these regions and could lead to genomic instability and cell senescence, if goes uncontrolled (Huang et al. 2006). Similar to its role in maintaining silenced chromatin at telomeres, Fun30 has also been found to be important to maintain the repressed state of reporter genes that were inserted in rDNA (Neves-Costa et al. 2009). Although this silencing might suggest an important role for Fun30 in suppressing genomic instability, no information on whether Fun30 does affect recombination level at this locus is available.

1.5.4 Role Fun30 and its Homologs at Telomeres

Unlike bacterial DNA which is circular, eukaryotic DNA is linear with free ends. These ends of chromosomes, called telomeres, have a special nucleoprotein structure. In budding yeast, telomeres are mainly made of repetitive DNA at subtelomeric region, followed by repetitive sequences of $C_{1\text{-}3A}/TG_{1\text{-}3}$ forming stretches of double stranded DNA that extend to 300 ± 75 bp. These telomeric repeats are further extended by a G rich strand forming a 3’ overhang which range from 12 to 15 nucleotides in length (Wellinger and Zakian 2012). End-replication problem was a term used to describe the problem of telomeres shorting because of the inability of DNA polymerase to achieve complete replication of linear chromosomes. This happens because on the lagging strand, a gap on the 5’ end of the newly replicated strand will be left as a result of subsequent removal of the RNA primer (Lingner, Cooper, and Cech 1995). This process takes place upon each round of cell division and

therefore, the length of telomeres needs to be restored. Protection of chromosomes ends is crucial for cell viability and their continuous shortening or erosion leads to cells senescence and chromosomal loss. In budding yeast, telomeres length is mainly maintained by the telomerase enzyme. Telomerase has an RNA template that anneals to the telomeres single-stranded terminus and act as a template for elongating it using the reverse transcriptase activity of the enzyme (Lendvay et al. 1996). Since this section focuses on the role of Fun30 at supporting telomere heterochromatic structure, it will be useful to discuss the nature of chromatin structure at telomeres in budding yeast.

Chromatin at telomeres is observed at the double-stranded part with the TG telomeric repeats, which act as a platform for RAP1 protein binding that in turn recruits the Sir complex. The Sir complex then spreads to neighboring sub-telomeric regions to ensure the formation of repressive heterochromatin by inducing hypoacetylation of nucleosomes (Ottaviani, Gilson, and Magdinier 2008). The action of Sir complex takes place in two steps, Sir4 and Sir2 physically interact to form a dimer, which deacetylates lysine 16 in the histone H4 N-terminal tail and help in recruiting Sir3 (Oppikofer et al. 2011). In budding yeast, proteins involved in establishing chromatin structure at telomeres were shown to affect telomere length. For example, mutations in *sir3* and *sir4* genes of the sir complex slightly reduce telomere length (Palladino et al. 1993). In contrast, mutations in Rap1 have been shown to cause dramatic increase in the length of telomeres, which were unstable and eventually were deleted leading to chromosomal loss (Kyrion, Boakye, and Lustig 1992, Liu, Mao, and Lustig 1994). Similarly, mutations in two Rap1 interacting proteins or telomerase-repressing factors, Rif1 and Rif2 have been shown to result in telomeres elongation (Wotton and Shore

1997). Therefore, a balance should exist between activities of telomere elongation and reduction to ensure proper telomere lengths for its optimum function. Interestingly, Fun30 and its *Schizosaccharomyces pombe* homolog have recently been shown to have role in maintaining chromatin structure at telomeres, which suggest a possible role in maintaining telomeres stability and thus maintaining genomic stability, the following section will discuss these roles.

Two separate groups have shown that Fun30 is enriched at telomeric repeats compared to distances away from the telomeres (Neves-Costa et al. 2009, Durand-Dubief et al. 2012). Evidence for the role of Fun30 at telomeres is deduced from work on Fft3, the *S. pombe* homolog of Fun30. A possible role for Fft3 at telomeres was suggested after observing that more than 50% of genes that are up-regulated in a strain lacking Fft3 were mapped to sub-telomeric regions of the chromosomes, supporting the implication of Fft3 in silencing genes at subtelomeric regions. Direct role of Fft3 in silencing telomeres was demonstrated from ChIP-chip data, which showed that Fft3 is enriched at 100 kb from telomere ends, a chromosomal location that delineate transition between euchromatin and sub-telomeric chromatin (Strålfors et al. 2011). Deletion of Fft3 caused the spreading of euchromatin histone marks such as H4K12ac and H2A.Z to sub-telomeric heterochromatin, in addition to concomitant increase in Poll, which indicates higher level of transcription (Strålfors et al. 2011, Steglich et al. 2015). There are contradictions on the nature of the DNA region where Fft3 binds at telomere. Strålfors and co-workers by performing ChIP-chip on Fft3 has shown that the Fft3 peaks were sharply positioned at four tandem Long Terminal Repeats (LTRs), which are free of nucleosomes and it has been shown that Fft3 was important to keep these four LTRs free of nucleosomes, while not affecting the downstream genes. This

suggests a possible Fft3 remodeling activity needed to evict nucleosomes and ensure a nucleosome free LTRs, which may act as an insulator region. While work done by Steglich and co-workers, using MNase-Seq has shown that the LTR region was normally occupied with nucleosomes and that Fft3 was important to preserve the nucleosome occupancy at the LTRs (Steglich et al. 2015). These data suggest that Fft3 maintains proper heterochromatin structure at sub-telomeres by acting as a component of an insulator and functions by inhibiting the formation or spreading of euchromatin to the subtelomeric regions of chromosomes (Strålfors et al. 2011).

Not only does Fft3 affect the composition of chromatin at sub-telomeres, but it was also shown to affect nuclear organization of sub-telomeres. The importance of this fact comes from knowing that the binding of particular chromatin domain to the nuclear periphery is believed to help in finding an environment that provides silencing factors required for facilitating the formation of heterochromatin, which further emphasizes the role of Fft3 in silencing (Taddei and Gasser 2012). This role was demonstrated by observing a reduced binding of Man1, an inner nuclear membrane protein, to sub-telomeres in the absence of Fft3. Such a reduction would only be explained by reduced localization of sub-telomeres to the nuclear envelope. This result was confirmed by the reduced amount of Taz1 (which binds to telomeres) at the nuclear periphery. This reduction of subtelomeric region anchoring to nuclear envelope was exacerbated when both Fft3 and Bgt4, a protein required for anchoring telomeres to the nuclear envelope, were deleted. Such a change in the nuclear localization of subtelomeres suggests cooperation between the two proteins in maintaining subtelomeres and telomeres anchorage to the nuclear envelope. In addition, Fft3 localization at LTR was shown to be important for LTR anchoring to

the nuclear envelope since deletion of Fft3 has been shown to reduce association of the Man1 with the LRT region (Steglich et al. 2015). In humans, no information on the localization of Smarcad1 to telomere repeats is available, but the fact that it localizes to DNA repeat sequences at centromeres and pericentric regions as mentioned previously, it would suggest that it is possible to bind to other repeat elements in the genome as those found at telomeres. Indirect evidence on its role at telomeres is drawn from ChIP data which show increase in H3kac and H3K9me3 levels and decrease in HDAC1 and KAP1 levels at telomeric repeats when Smarcad1 was depleted, similar to effects seen on centromeres (Rowbotham et al. 2011).

Finally, a study aimed at finding out the free energy spent on the formation of nucleosomes on the DNA of telomeric repeats has shown nucleosomes reconstituted on telomeric DNA formed the least stable DNA nucleosome *in vitro* and nucleosomes needed less energy to get mobilized. This indicates that telomeric DNA can form highly mobile and unstably positioned nucleosomes (Filesi et al. 2000). All these findings on a role of Fun30 in silencing suggest a possible role in establishing stable nucleosomes which are hard to evict, therefore forming a more stable platform for heterochromatin establishment.

1.5.5 A Role of Fun30 and its Homologs at Centromeres

Centromeres are DNA loci on chromosomes that act as platforms for the assembly of a nucleoprotein complex known as the kinetochore. The kinetochore has several functions such as contributing to cohesion between sister chromatids, in the attachment of spindle tubules, which are key player in segregating chromosomes during mitosis and meiosis, and in activating cell cycle arrest in case of inappropriate

attachment of chromosomes to microtubules (Smith 2002). Centromeres also are known to have a unique variant of histone H3 known as Cse4 in budding yeast or CENP-A in higher eukaryotes, which plays a role in proper centromere assembly (Smith 2002). In budding yeast, the centromere is made up of 125 bp sequence with three DNA elements CDEI, CDEII and CDEIII, which all together define a centromere site and this site is where the single centromeric nucleosome containing Cse4 binds (Biggins 2013). Centromeres in higher eukaryotes are more complicated and don't have a strict sequence identity like in budding yeast, and are rather spread over longer stretches of AT-rich DNA (De Rop, Padeganeh, and Maddox 2012). In higher eukaryotes, heterochromatin, which is established at regions of centromeric chromatin or pericentric chromatin, has been shown to be important for both kinetochore assembly and cohesion between centromeres (Pidoux and Allshire 2005).

Fun30 and its orthologs have been shown to play a role in maintaining proper chromatin structure and centromeric regions. In budding yeast, ChIP-Seq data provided evidence for a direct role of Fun30 at centromeres (Durand-Dubief et al. 2012). The study has shown that Fun30 had the highest enrichment at centromeres when compared to other intergenic sites. The Fun30 occupancy was seen to be broad and it included the centromeres and the flanking pericentromic chromatin (Durand-Dubief et al. 2012). Similarly, in *Schizosaccharomyces pombe*, ChIP-chip data has shown that the Fun30 ortholog Fft3, was enriched at the central cores of the centromeres (Strålfors et al. 2011). Knowing that S. *Pombe* centromeres are mapped to larger chromatin region when compared to budding yeast centromeres (Clarke 1990), this might explain the unique details that are revealed about Fft3 at centromeres. Fft3 was shown to be enriched at the central core domain and high peaks were detected

at tRNA genes, which are located at the inner repeats (*imr)*, and act as insulators that separate the central core domain from the surrounding heterochromatin. The surrounding heterochromatin was found to be depleted of Fft3; however, Fft3 was enriched at the transition from the surrounding pericentric heterochromatin and the euchromatin at the inverted repeats (IRC), with no enrichment at euchromatin. All of these findings indicate a possible role of Fun30 in preserving the borders between these antagonistic chromatin domains by acting as a component of insulators, and thus in maintaining centromeres (Strålfors et al. 2011).

Deleting a protein that has a role in maintaining centromeres structure or function should have adverse effects on critical cellular process such as maintaining chromosome stability and segregation as well as cell viability. Interestingly, in support of this notion, RNA-Seq results obtained from wild type and *Δfun30* strains have shown that most of the genes that were upregulated in *Δfun30* are involved in chromosome segregation and meiosis such as the genes of anaphase promoting complex and genes that either codes for the components of the kinetochore or are required for its assembly (Strålfors et al. 2011). This change in genes expression in these categories of genes was found to be a cellular response to the absence of Fun30 rather than direct effect of Fun30 on their promoters since there was no strong correlation between Fun30 localization and the expression of those genes. Furthermore, it was observed that genes that have similar genetic interaction profile of Fun30 were mostly genes involved in chromosome segregation and meiosis (Strålfors et al. 2011, Durand-Dubief et al. 2012). In an another study, Fun30 was found among genes whose over expression led to genomic instability, however, a deletion of the gene did not cause chromosomal loss as was expected from the over dosage effect

(Ouspenski, Elledge, and Brinkley 1999). This observation was contradicted by other studies on both budding yeast and *S. pombe*, which showed that when wild type, *Δfun30,* or *Δfft*3 cells were transformed with a minichromosome, *Δfun30* and *Δfft*3cells were found to have higher frequency of chromosomal loss compared to the wild type cells (Durand-Dubief et al. 2012, Strålfors et al. 2011). Such a defect in *Δfft3* was further confirmed by staining of chromosomes with DAPI to monitor their segregation. These results suggest a role for Fun30 and its ortholog Fft3 in kinetochore function (Strålfors et al. 2011). Durand et al. has employed multiple approaches to study the role of Fun30 in centromere function, all of which aimed at compromising the function of the centromeres in budding yeast. In one approach, a conditional Cse4 mutant was used and it was shown that cells harboring cse4-1 mutant failed to form a proper centromere at semi permissive temperature. Fun30 was found to be important for cell viability in this strain and the phenotype was rescued by ectopically expressing wild type Fun30 but not the Fun30 ATPase domain mutant. In another approach, centromere function was disrupted by forcing transcription from a promoter that is placed close to CEN3. Chromosomes were not properly segregated under this condition even in wild type, but in the *Δfun3* segregation was more defective and even led to loss of cell viability after days of induced transcription. In the third approach, a strain harboring dicentric chromosome was used. In this assay, maintaining both centromeres leads to chromosomal loss and reduced cell viability, a phenotype of wild type strain since centromeres function is intact. Since fun30 deletion was found to suppress this phenotype it was taken as a further confirmation for its role supporting centromere function (Durand-Dubief et al. 2012). Taken together, it seems that fun30 has a direct role in supporting centromere function.

The requirement of Fun30 ATPase activity at centromeres and the increase in the RNA transcript of CEN3 region in the *Δfun30* compared to the wild-type strain suggest modification of the chromatin structure at centromeres which might help in maintaining a silent status of chromatin (Durand-Dubief et al. 2012). Such silencing would inhibit the deleterious effects of cryptic transcription on centromere function. Detecting any change in the nucleosome occupancy or even a change in histone variant occupancy will indicate a direct role of Fun30 on the chromatin structure. By employing ChIP, Fun30 deletion was found not to affect the level of Cse4 histone variant at CEN3, demonstrating no role for Fun30 in evicting canonical nucleosomes to facilitate the binding of the centromeric form. However, Fun30 deletion was found to affect the positions of nucleosomes over both the centromeres and the surrounding chromatin (Durand-Dubief et al. 2012). Histone H2A.Z is another histone variant that binds to gene promoters more than the Open Reading Frames (ORFs). Interestingly, Fun30 deletion was found to alter H2A.Z binding at promoters of genes in the vicinity of the majority of centromere (Durand-Dubief et al. 2012). In this study, a reduced binding of H2A.Z at promoters and an increased binding of H2A.Z in the ORFs was observed. Such a change might affect the silencing of genes at centromeres. These data highlight the importance of Fun30 for proper chromatin structure at centromeres (Durand-Dubief et al. 2012).

Additional support for the role of Fun30 in supporting the chromatin structure at centromeres comes from it is ortholog in *S. Pombe*. Besides being recruited to centromeric region Fft3, similar to Fun30, was shown to be required for maintaining the silencing status of the centromeric region (Strålfors et al. 2011). This was determined by rendering cells sensitive to FOA when the Ura4+ gene was placed

within the centromere. ChIP-chip data for H3 showed that chromatin at IRC and tRNA elements, which were free of H3, remained free of H3 even when Fft3 was deleted, ruling out a role of Fft3 in evicting H3 from these elements. Interestingly, Fft3 deletion caused a 3.5 fold increase of H3 at *imr3* region with a simultaneous decrease of Cnp1, a H3 variant that is present at centromeres, at the central core domain. Moreover, three distinct marks of euchromatin were found to increase at insulator regions. At the *imr* region, both H3K9ac and H4K12ac were increased, while H2A.Z was increased at both *imr* and IRC elements and at tRNA genes at the extremities of centromeres (Strålfors et al. 2011). Therefore, although the deletion of Fun30 did not cause a decrease in the Cse4 at centromeres, as was the case with Fft3 deletion, both proteins seems to act similarly in counteracting the spread of euchromatin at centromeres, which was evident by the spread of histone modifications that demarcate euchromatin. By employing such strategy, Fun30 as well as Fft3 are preserving the unique chromatin structure at centromeres and supporting their functions.

In human, Smarcad1 was also shown to have a similar role, with more details on the mechanism of silencing (Rowbotham et al. 2011). A direct role of Smarcad1 in silencing at centromeres was shown by its localization to pericentric chromatin during replication in synchronized and asynchronized NIH 3T3 mouse cells. ChIP experiments revealed that Smarcad1 knockdown cells had increased occupancy of H3Ac and decrease in occupancy of H3K9me3, with a simultaneous decrease in HDAC1 and KAP1 at both centric and pericentric repeats. An effect of Smarcad1 deletion on centromere function due to heterochromatin disruption at pericentric regions was demonstrated by showing increased frequency of misaligned chromosomes at metaphase and lagging chromosomes and DNA bridges during

anaphase and telophase. This reflects defects in chromosome segregation in the Smarcad1 deletion. The requirement of ATPase activity to suppress defects in histone modifications observed in absence of Smaracd1 shows that Smarcad1 mediates all its function through its remodeling activity which requires ATP hydrolysis (Rowbotham et al. 2011).

1.5.6 Role of Fun30 in Double-Strand Break Repair

1.5.6.1 A possible role in DNA repair

Yeast strain lacking *Fun30* gene doesn't show high sensitivity to many DNA damaging agents (Neves-Costa et al. 2009), and if this sensitivity was observed it was not as high as that observed in other strains lacking genes involved in cell cycle checkpoints or those implicated in DNA damage repair. However, cells lacking Fun30 are sensitive to high concentrations of Camptothecin (Neves-Costa et al. 2009), a topoisomerase II inhibitor. In humans, Smarcad1 deletion also confers sensitivity to camptothecin and poly (ADP-ribose) polymerase inhibitor (Costelloe et al. 2012). Lack of sensitivity to most DNA damaging agents doesn't rule out a role of Fun30 in DNA damage repair. Redundancy of genes having similar roles might explain the non-essential role of a gene during DNA damage. Recently, many studies have revealed that Fun30 plays an important role in repair of DNA double-strand breaks. However, this role was shown to be mainly confined to DNA resection, the first step in the double-strand break repair during homologous recombination. The motivation for searching for such a role was not because of high sensitivity of Fun30 mutants to DNA damaging agents as mentioned above, but rather, it was suggested after observing certain phenotypes of the *Δfun30* strain during high throughput screens. Moreover, the ATP-dependent remodeling activity of Fun30, was another reason to think of such a

role since chromatin remodeling is believed to play an important role in relieving any obstacles posed by the chromatin structure during DNA repair.

Finding out the possible role of a protein in DNA double-strand break repair can simply be done by testing the viability of a deletion strain under induced double-strand break or by studying the kinetics of repair of induced cut sites. Eapen et al. showed that the *Δfun30* strain was as efficient as the wild-type strain in the repair of an *HO*-induced cut in Matα locus by gene conversion (Eapen et al. 2012). This was shown by both strains having similar viability and gene conversion products after damage. In their study another pathway, Break Induced Replication (BIR), was also shown not to be affected in the absence of Fun30. Both of these results ruled out a role for Fun30 in strand invasion or subsequent events in homologous recombination. Interestingly, the viability of the Fun30 deletion strain was reduced to 40% when a cut was induced between two repeated sequences that were 25kb apart (Eapen et al. 2012). Such repair is accomplished through single-strand annealing (SSA) which requires extensive resection to allow for homology searching around the induced cut. A direct role for Fun30 in homologous recombination was confirmed in another study, in which a genetic screen was performed on 4,836 diploid yeast deletion mutants to identify genes that alter the integration rate of URA3 cassette at two separate loci (Chen et al. 2012a). An increase in the rate of gene integration in *Δfun30*, similar to the phenotype observed upon deletion of other genes involved in homologous recombination such as sgs1 and exo1, suggested a possible role for Fun30 in homologous recombination (Chen et al. 2012b). In a similar approach, Costelloe et al., using a genomic approach, also provided evidence that the Fun30 deletion mutant caused an increased efficiency in both break induced replication and gap repair. The two assays employed here relied

on transforming linearized fragments of DNA into a pool of mutants (Costelloe et al. 2012). These results contrast the findings of Eapen et al.; however, this can be due to differences in the assays used or the strains backgrounds. On the other hand, Smarcad1 deletion, unlike Fun30, was shown to affect DNA damage repair by gene conversion (Costelloe et al. 2012).

1.5.6.2 Fun30 Promotes Long Range Resection of DNA

Stabilization of the transformed linear DNA and slowing its degradation is the only explanation given to account for the increased rate of gene targeting, BIR, and gap repair when resection genes Sgs1 and Exo1 were deleted. Since Fun30 deletion showed similar phenotype, it was suggested that Fun30 might have a role in DNA resection (Costelloe et al. 2012, Chen et al. 2012b). A role for Fun30 in DNA resection was also suggested after observing less viability in strains that rely on SSA for repair when Fun30 was deleted since extensive resection is required in this pathway. Moreover, observing that *HO* cut DNA bands were maintained for longer time was explained by reduced resection of the cut DNA in the absence of Fun30 (Eapen et al. 2012). Many groups have used different approaches to assess the role of Fun30 in resection. Eapen et al. monitored resection progression by measuring the amount of DNA at different distances from the induced cut by PCR, where less PCR product reflected less template availability due to DNA degradation (Eapen et al. 2012). For this purpose, resection was measured in JKM179 strain that has *HO* cut site in Matα locus lacking a donor site. Resection rate was found to be reduced from 4.0 kb/h down to 1.2 kb/h when Fun30 was deleted (Eapen et al. 2012). On the other hand, Costelloe et al. analyzed the length of single stranded DNA generated by restriction enzyme digestion using a probe for *HO* cut after inducing the cut in the *HO* locus. Since

resection causes the loss of restriction enzyme sites, different length of DNA would be generated after induction of the cut. It was observed that short ssDNA is normally formed in the absence of Fun30, while long ones were abolished indicating that Fun30 played a role in long range resection (Costelloe et al. 2012). Similarly, Chen et al. found that Fun30 deletion delayed resection at regions which are 5, 10, and 28 kb pairs away from the *HO* cut, while resection close the *HO* cut was not affected (Chen et al. 2012b). This group used a different technique in which restriction site loss due to resection was monitored by probing different regions at and away from the cut site. Reduced resection at greater distances was observed with reduced recruitment of two single-stranded DNA binding proteins (Rad51 and RPA) at those regions, while their recruitment to DNA break proximal regions was not reduced significantly (Chen et al. 2012b, Costelloe et al. 2012). Genetic interaction assays helped further in finding a possible role for Fun30 in the two known major pathways of DNA resection, the Exo1 and the Sgs1 pathways. Single deletion of each of Sgs1 and Exo1 was shown to reduce resection, while the double deletion of these genes caused a complete inhibition of DNA resection. By deleting Fun30, along with these genes, it was found that resection was decreased in *Δsgs1Δfun30* and *Δexo1Δfun30* double mutants when compared to single mutants of each of *sgs1* and *exo1*. Interestingly, this reduction in resection was similar to that observed when Fun30 alone was deleted, suggesting that Fun30 can facilitate both resection pathways (Eapen et al. 2012). Similar data was observed by two other groups, while one group noticed that the double deletion *Δsgs1Δfun30* or *Δexo1Δfun30* had a more severe defect in resection, when compared to *Δfun30* alone. Furthermore, the resection reduction in the triple mutant (*Δsgs1Δexo1Δfun30)* was similar to that observed in the double mutants *Δsgs1Δexo1*, leading to the same conclusion (Chen et al. 2012b). These differences in observations might be due to

differences in the assays used to monitor resection and perhaps difference in the genetic backgrounds of strains utilized. Same observation was made by Costelloe et al., although they concluded that Fun30 contributes more to the Sgs1 pathway than to the Exo1 (Costelloe et al. 2012). Another piece of evidence that supports the role of Fun30 in the Sgs1 and Exo1 pathways is the reduced recruitment of both proteins to DNA regions that are away from the induced cut in the *Δfun30*, indicating reduced efficiency of resection at areas distant from the cut in the mutant. Moreover, reduced recruitment of single stranded DNA binding proteins such as RPA and Rad51 to a DNA region that is 5 kb away from the cut in *Δfun30* mutant supports this conclusion (Chen et al. 2012b). In human, a similar role for Smarcad1 in DNA resection was concluded when a reduced level of RPA on damage sites as well as less single-stranded DNA was observed in Smarcad1 knockout cells (Costelloe et al. 2012).

1.5.6.3 Fun30 Recruitment to DSB Sites

Fun30's role in long range resection can either be direct or indirect. A direct role implies that Fun30 must associate with DNA regions around the double strand breaks. To address this, using ChIP, Fun30 has been shown to be enriched at double strand break sites after one hour and to distant regions in both directions by a later time (Chen et al. 2012b, Costelloe et al. 2012, Eapen et al. 2012). Fun30 association with chromatin is not independent of other factors involved in this pathway since other proteins of DNA resection such as Dna2, Exo1 and RPA were co-immunoprecipitated with Fun30 upon damage (Chen et al. 2012b). An indirect role for Fun30 in long range DNA resection can be a possible way by which Fun30 acts, for example by altering the expression of DNA repair genes or by altering the general structure of chromatin. However, genome-wide gene expression analysis has revealed no effect of Fun30 deletion on expression of genes involved in DNA damage repair. Moreover, chromatin

analysis has shown no difference in the chromatin structure at the *HO* cut site in the wild-type or the *Δfun30* strains (Chen et al. 2012b). In addition, consist with the recruitment of Fun30 to DSB, Smarcad1 in humans was also found to be recruited to laser-induced DNA damage sites and nuclease induced double strand breaks, which was confirmed by co-localization with γH2AX. This recruitment was prior to RPA binding, but it was simultaneous with Exo1 recruitment (Costelloe et al. 2012).

1.5.6.4 Fun30 Remodels Chromatin at Sites of DNA Damage

Fun30 was first identified as an ATP-dependent chromatin remodeler in our lab (Awad et al. 2010) by showing that it has an ATPase activity with the ability to execute histone dimer exchange and to increase accessibility to chromatin, similar work was reproduced by other group (Awad et al. 2010, Byeon et al. 2013). Being recruited to DNA double strand break sites implies its direct action. It has been suggested that Fun30 activity in remodeling the structure of chromatin around a double strand break is the mechanism by which long range resection is facilitated. If chromatin remodeling is taking place, it was believed to be coupled to resection and not independent of it. That was explained by the similar kinetics of Fun30 recruitment and resection at DNA double strand break sites. Besides that, the two resection proteins Exo1 and Sgs1 failed to bind to distant regions in the absence of Fun30 (Chen et al. 2012b). However, all these studies do not demonstrate that Fun30 is involved in chromatin remodeling. To find out if Fun30 alters the accessibility of chromatin at a cut site, cleavage of an *HO* site in HMR was monitored (Eapen et al. 2012). In this locus, which is inaccessible due to a compact chromatin structure, no difference in the cleavage of the *HO* site between the wild-type and *Δfun30* was observed. Moreover, although resection was found to be reduced on the side of an *HO* cut site that was

proximal to an HMR locus in the wild-type strain, it was not possible to monitor resection in absence of Fun30 due to overall reduced level in resection in the *Δfun30* strain (Eapen et al. 2012). So, resection in the context of compact chromatin was not very informative. However, coupled chromatin remodeling and resection was further demonstrated by observing no change in H3 occupancy before resection in both the wild-type and the *Δfun30*, while longer occupancy in *Δfun30* was explained by reduced resection (Chen et al. 2012b). The coupled loss of H2B and H3 with resection around the *HO* induced cut was similar in the wild-type and the *Δfun30* cells, which was explained by Fun30 remodeling chromatin by altering its accessibility rather than affecting histone occupancy (Costelloe et al. 2012).

The nature of chromatin around a DNA double strand break is different from the surrounding chromatin because of the strong phosphorylation of H2AX leading to γH2AX, which marks the region for further signaling towards DNA damage repair. Fun30 dimer exchange activity was suggested to alter this chromatin mark, but similar kinetics of γH2AX formation and decrease was observed in both the wild-type and the *Δfun30* mutant cells, which suggests no role for Fun30 in γH2AX kinetics (Eapen et al. 2012). Moreover, using an *in vitro* binding assay, it was shown that Fun30 binds more strongly to un-phosphorylated H2A compared to γH2AX. When Fun30 was deleted in an H2A-S129A mutant strain, which is known to increase resection rate, the level of reduction in resection was comparable to that observed in the wild-type strain (Eapen et al. 2012). The check point protein Rad9 is known to inhibit DNA resection when it binds to chromatin. There is good evidence that Fun30 is required to overcome this inhibition by Rad9 since Fun30 becomes dispensable in the absence of Rad9 or the two histone modifications, H3K79 methylation and γH2A, which are both required

for its recruitment to double strand breaks (Chen et al. 2012b). Further support for this notion was obtained when the resection rate in *Δfun30* and *Δfun30 H2A-S129A* were compared. It was found that while the resection rate in the double mutant of *Δfun30 H2A-S129A* was lower than in the wild-type strain, it was still higher than in the *Δfun30* (Eapen et al. 2012). Similarly, Smarcad1 was also shown to function in the same way. It has been shown that H2A ubiquitination at DNA damage sites by the BRACA-BARD1 ubiquitin ligase activity signals for the recruitment of Smarcad1 through its ubiquitin-binding CUE domain, which in turn enables Smarcad1 to reposition 53BP1, the homologue of Rad9 in yeast, and relief its inhibitory effect in order to facilitate DNA resection (Densham et al. 2016).

1.5.6.5 Fun30 Plays a Role in Single Strand Annealing (SSA)

Since the efficiency of induced DSB was not affected in the absence of Fun30, it was logical to check other pathways such as the single strand annealing (SSA) pathway. To test whether Fun30 plays any role in this pathway, an established assay was used in which a break is induced between two repeated sequences where the repair process leads to the loss of the sequence in between them as the two sequences are complementary (Eapen et al. 2012). Using this assay, it was found that Fun30 deletion reduced the viability of cells down to 40% when the distance between the repeats was 25 kb, while cell viability was not affected when the distance was 5 kb. When Rad51 was deleted in the same strain to inhibit break induced replication, SSA product was completely abolished and cells lost their viability. These observations, indicate a potential role for Fun30 in SSA and since the *HO* cut product was not reduced, it further suggested that Fun30 must have a role in 5’ to 3’ resection (Eapen et al. 2012).

1.5.6.6 Remodeling Mediated Resection by Fun30

In a strain in which an induced cut is only repaired by SSA, reduced viability, upon induction of DNA cut in *Δfun30,* was rescued when a plasmid expressing wild-type Fun30 was transformed, while transforming with Fun30 having an ATPase defect failed to do the same. This data provides more direct support for an ATP-dependent activity (i.e. chromatin remodeling) of Fun30 in facilitating SSA (Eapen et al. 2012). Similarly, reduced resection level in *Δfun30* was only restored upon ectopic expression of the wild-type Fun30 but not its ATPase mutant (Costelloe et al. 2012). The Fun30 ATPase activity is also required to confer resistance to camptothecin, suggesting that the ATP-dependent activity of Fun30 that facilitate resection is also vital for this resistance. This was supported by rescuing this defect by ectopic expression of Exo1, which most probably does so by compensating the defects in resection (Costelloe et al. 2012). Similarly, ectopic expression of Exo1 was shown to suppress defects in check point adaptation that was observed after prolonged exposure of *Δfun30* to DNA damage. It is believed that these defects was mostly likely because of inefficient resection, which is believed to be rescued by Exo1 (Eapen et al. 2012). In humans, reduced level of RPA recruitment to damage DNA sites in Smarcad1 knockdown cells was shown to be partially rescued by overexpression of Exo1, and thus supporting its role in resection. If Fun30 can facilitate Exo1 and SGS1 resection pathways, this should be evident from the sensitivity of double deletion mutants to camptothecin, which should not be different from a single deletion of Fun30. However, this was not the case since it was shown that double deletion of Exo1 and Sgs1 was more sensitive to camptothecin compared to the single deletion of each

gene (Eapen et al. 2012, Costelloe et al. 2012). This suggests that Fun30 can perhaps mediate repair by other means beside resection.

1.5.7 Role of Fun30 and its Homologs in DNA Replication

Deletion of Fun30 did not affect cell cycle progression kinetics, which rules out a potential role for Fun30 in DNA replication. However, deletion of Fun30 in an orc5-1 mutant background rendered the cell less viable at different temperatures, caused the abnormal cell cycle progression with cells accumulating in G1 phase, and led to the reduced bud formation, all hinting at Fun30 functioning in cell cycle progression (Neves-Costa et al. 2009). This however, does not necessarily mean a role in the DNA replication process itself, but rather it might include any actions to achieve integrity of the DNA being replicated, or stabilizing the replication forks upon damage, or maintaining proper chromatin assembly during replication, etc. Fun30 is required to confer resistance to MMS, HU, and camptothecin. All of these are DNA damaging agents that are known to affect the progression of the replication fork leading to extended regions of single-stranded DNA as in the case with MMS and HU, or by inducing double-strand breaks and replication fork collapse as is the case with camptothecin (Neves-Costa et al. 2009, Bi et al. 2015). Recently, it has been shown that Fun30 negatively regulates resistance of *Δrad5* strain, which lacks an error free DNA damage tolerance mechanism, to MMS and HU (Bi et al. 2015). Moreover, it was found that the ectopic expression of Exo1 could only rescue sensitivity to MMS, but not to HU treatment. This negative regulation is mediated by inhibiting Rad51-dependent recombination which would be an alternative option available for cells to survive damage in case DNA damage tolerance pathways are not available. So this seems another mechanism, besides resection, by which Fun30 is believed to be

involved in the DNA damage process. A similar anti-recombinogenic role was also shown for Srs2, which was also shown to rescue *Rad5* mutant under damage, however Srs2 role was shown to be more important than Fun30 (Bi et al. 2015). In human, while Smarcad1 knockout cells did not perturb the cell cycle, a role for Smarcad1 in maintaining heterochromatin domain during replication was observed (Rowbotham et al. 2011). Smarcad1 was found to physically interact with PCNA, which is an important component of the replication machinery. This was supported by data that showed the enrichment of Smarcad1 at replication forks. It was shown that Smarcad1 through its interaction with Kap1 and HDAC1/2 aids in establishing heterochromatin by down-regulating acetylated H3 and H4 and facilitating H3K9 methylation, all of which ensure proper maintenance of heterochromatin structure during replication (Rowbotham et al. 2011).

1.5.8 Regulation of the Fun30 Activity

A genome-wide screen for possible targets for Cdk1 has identified Fun30 as a possible substrate (Ubersax et al. 2003). Further confirmation towards this is drawn from the fact that Fun30 has been shown to be phosphorylated *in vivo* and *in vitro* by Cdk1. The recruitment of Cdk1-cyclin complexes to DSB ends provide means for regulating the Fun30 activity that is involved in DNA damage repair. Fun30 phosphorylation was shown to be confined to the S and G2 phases of the cell cycle in normal conditions and was increased upon damage with MMS. Additionally, phosphorylation of Fun30 at serine 20 and 28 were found to be important for efficient double strand repair, SSA, and for conferring resistance to DNA damage induced by camptothecin and HU. However, while this phosphorylation of Fun30 by Cdk1 was shown not to be important for the initial recruitment of Fun30 to DBS, it was important for its spreading to distance regions. Furthermore, it was shown that cyclins Clb2 and

Clb5, which are the major cyclins needed for cdk1 recruitment to the DBS, were important for the facilitation of the *in situ* phosphorylation of Fun30 (Chen et al. 2016).

1.5.9 Smarcad1 and its Implication in Development and Cancer

From all the above, we can conclude that Fun30 and its orthologs play multiple, but similar roles in the cell. In yeast, the *fun30* gene is not essential, but its loss can lead to genomic instability. Implication in genomic instability can be deduced based on its various functions from supporting centromeres functions, to its roles in telomeres, DNA damage repair, and silencing. In simple unicellular organisms like yeast, this can lead to less viability with progressive cell division and finally cell death. However, in higher eukaryotes, genomic instability can lead to cell death or cancer development, compromising the life of an organism and if untreated it can lead to death. The development of cancer, however, might not be the only disease that affects an organism's life. Genes affecting the early stages of embryonic development or during growth can have serious effects on an organism's life as well. Some cancer cases are easily treated, whereas, many developmental disorders are hard to treat. Interestingly, the *smarcad1* gene was found to be implicated in both the development of the organism as well as cancer development. We will discuss both of these below.

1.5.9.1 The Role of Etl-1 and Smarcad1 in Development

The first hint on the role of the Smarcad1 in development came from work on mice (Soininen et al. 1992). The enhancer trap technique allows for tracing the expression pattern of a random gene. Using such an assay, the nature of the expression, and whether it is spatial, temporal, or ubiquitous with no specific pattern, can be elucidated. This relies on the random insertion of a *lacZ* reporter gene, which has a

promoter but lacks an enhancer making expression impossible. By random insertion, the *lacZ* insert will highjack the enhancer of a random gene and acquires the expression pattern of that gene (Springer 2000). In mice, this led to the identification of the Enhancer trap locus 1 (*Etl-1*) gene, the mouse homolog of Fun30 and Smarcad1. The expression of *Etl-1* was found to be widely spread during development, but with higher levels at both central nervous system and epithelial cells (Soininen et al. 1992). However, in adult mouse, *Elt-1* expression was found to be more spread in most tissues (Schoor, Schuster-Gossler, and Gossler 1993). Utilizing antibodies generated against Etl-1 allowed for better understanding of protein levels and localization at different stages before and after zygote formation and development. Etl-1, which was shown to be mainly nuclear, was found to have biphasic expression during early embryogenesis, and was suggested to be required for the onset of embryonic transcription (Schoor, Schuster-Gossler, and Gossler 1993). Further work supporting a role in cell differentiation was substantiated by analyzing the average expression of genes of mixed cell lines. Smarcad1 depletion by shRNA from embryonic stems cells was shown to affect pluripotency and self-renewal leading to differentiation. That was shown by a reduction of pluripotency markers and induction of differentiation markers suggesting a role of Smarcad1 in preserving the stemness of the cells and further in deciphering that Smaracad1 might act as a sequence specific transcription factor rather than a chromatin remodeler (Hong et al. 2009). A role of Smarcad1 in neurogenesis in mouse brain was also provided in a study that aimed at knowing which genes are involved in the generation of interneurons of the olfactory bulb (Lim et al. 2006). This study showed that Smarcad1 was expressed at moderate levels at all brain parts, but was more expressed in the sub-cortical zone and the olfactory bulb (Lim et al. 2006). Furthermore, it was shown that in adult mice, deleting *Etl1* was not lethal, but mice

lacking it suffered from retarded growth, peri- and post-natal lethality, less fertility, defects in the sternum and vertebral column, as well as respiratory failure (Schoor et al. 1999).

In humans, a link of Smarcad1 to fertility was also demonstrated after gene expression analysis was performed on sperms of fertile and infertile men, and interestingly, Smarcad1 was among genes that were upregulated in sperms of the asthenozoospermic group (reduced sperm motility) (Bansal et al. 2015). Smarcad1 was found to have two isoforms, and while the expression of the intact form was ubiquitous, the short isoform was expressed exclusively in the skin. Moreover, a mutation (c.378+1G>T, which is mapped to the first intron of the short isoform of Smarcad1), was identified in people suffering from adermatoglyphia (absence of finger prints) and reduced hand transpiration, which is also known as immigration disease. This mutated form of the short isoform, is believed to abolish a donor splice site and to decrease the stability of the Smarcad1 RNA (Nousbeck et al. 2011) and is also identified in people with Basan syndrome, which shares some symptoms with adermatoglyphia (Li et al. 2016).

1.5.9.2 The Role of Smarcad1 in Cancer

Higher expression of Smarcad1 leading to the activation of E1A viral transcriptional factor and thus allowing the expression of a silent reporter gene (Adra et al. 2000) can be explained in several ways. One possible explanation is that Samrcad1 can cause genetic rearrangement that would place the gene in a more active chromatin location. This suggests a role in genetic instability. Interestingly, Smarcad1 was mapped to chromosome 4q22-23, which is known to be a fragile site in which

deletions and mutations are frequent and are correlated with many diseases including cancers (Adra et al. 2000). Similarly, another study found that loss of heterozygosity in tissues of patients with head and neck cancers occurred frequently in chromosome region 4q22-35, where smarcad1 is located with some other genes. This suggests a possible tumor suppressor role for Smarcad1 (Cetin et al. 2008). Moreover, when gene expression profile was used to predict survival time for patients with bladder cancer, it was found that the expression of Smarcad1 was a predictor of increased survival time (Tapak et al. 2016). In another study, SNPs were identified as predictors for sensitivity to capecitabine, a drug which is widely used in the treatment of breast, colorectal, and gastric cancers. Performing genome-wide association studies on lymphoblastoid cell lines from individuals across the world, many SNPs in smarcad1 were identified, in addition to a missense variant rs11722476, in which a serine was changed to asparagine, suggesting that smarcad1 polymorphism may have a role in capecitabine sensitivity (O'Donnell et al. 2012).

A more recent study has shown that MDA-MB-231 cells, triple negative breast cancer cells, which are highly metastatic and invasive cancer cells, had a higher level of Smarcad1 expression when compared to the non-invasive cells (Al Kubaisy et al. 2016). In this study, the authors demonstrated that the Smarcad1 knockdown in this cell line caused an increase cell-cell adhesion, and a significant decrease in cell migration, invasion, and metastasis, when compared to the non-invasive breast cancer cells T47D. It was suggested that this is mediated at least in part by a strong inhibition of STAT3 phosphorylation (Al Kubaisy et al. 2016).

1.6 Aims and Objectives

Homologous recombination (HR) is a multi-step process that is regulated at different levels for efficient DSB repair and to avoid excessive levels of recombination. If HR is not properly regulated, the accumulated recombination intermediates can be toxic to the cells and could lead to increased levels of illegitimate recombination, which would affect the overall genomic stability. Given that Fun30 has been shown to facilitate long range DNA end resections implicating it in DSB repair, we hypothesized that Fun30 plays a direct role in various steps of homologous recombination. The main objectives of this study was to investigate the potential regulatory role of Fun30 during HR. Towards this, we initially studied the relevant *in vitro* enzymatic activates of Fun30 such as its ability to unwind double stranded DNA (i.e. helicase activity) as well as its other potential functions that are important for HR and DNA repair. Moreover, we investigated the *in vivo* roles of Fun30 and specifically how it functions compared to other proteins that are implicated in HR under DNA damaging conditions. To achieve this, we tested the effects of Fun30 deletions in strains that lack genes directly involved in DNA damage repair, homologous recombination, or replication.

Chapter 2: Materials and Methods

2.1 Construction of Yeast Strains

Yeast strains were made by using the one-step PCR-mediated gene deletion or tagging (Longtine et al. 1998). With this method, a null mutant of a gene can be generated or proteins can be tagged at either their N- or the C-terminus (Figure 2.1). In general, for gene deletion, the whole gene is replaced with a DNA cassette containing a selection marker by homologous recombination. Depending on the type of selection marker, a strain will either acquire resistance to antibiotic or it will be able to grow on synthetic media lacking a specific amino acid. For gene deletions, DNA inserts having either KanMX or His3 gene cassettes were amplified by PCR from pFA6a-kanMX6 and pFA6a-His3MX6 plasmids, respectively (generous gift from Professor Danesh Moazed, HMS, USA). For tagging Fun30 at its C-terminus with a Flag-tag, an insert that had 3XFLAG sequence upstream of the KanMX open reading frame was amplified from the p3FLAG-KanMX plasmid (generous gift from Professor Danesh Moazed, HMS, USA). The primers used for amplifications (see Table 2.2) were designed in a special way that would allow the proper integration of the insert DNA. For gene deletions, the forward primers had 40 to 45 bp complementary to the sequence upstream of the start codon of the gene of interest, followed by a sequence that acted as a forward primer for amplifying the cassette from the plasmid. The reverse primers had 40-45 bp complementary to the sequence downstream of the stop codon of the gene of interest, followed by a sequence that acted as a reverse primer for amplifying the cassette from the plasmid. The primers for tagging the protein of interest were designed similarly however, the forward primers for these constructs had 40 to 45 bp complementary to the sequence upstream of the stop codon of the gene of

interest, a diagram illustrating gene deletion and tagging by this one-step PCR-mediated replacement is shown in below in Figure 2.1A and B, respectively.

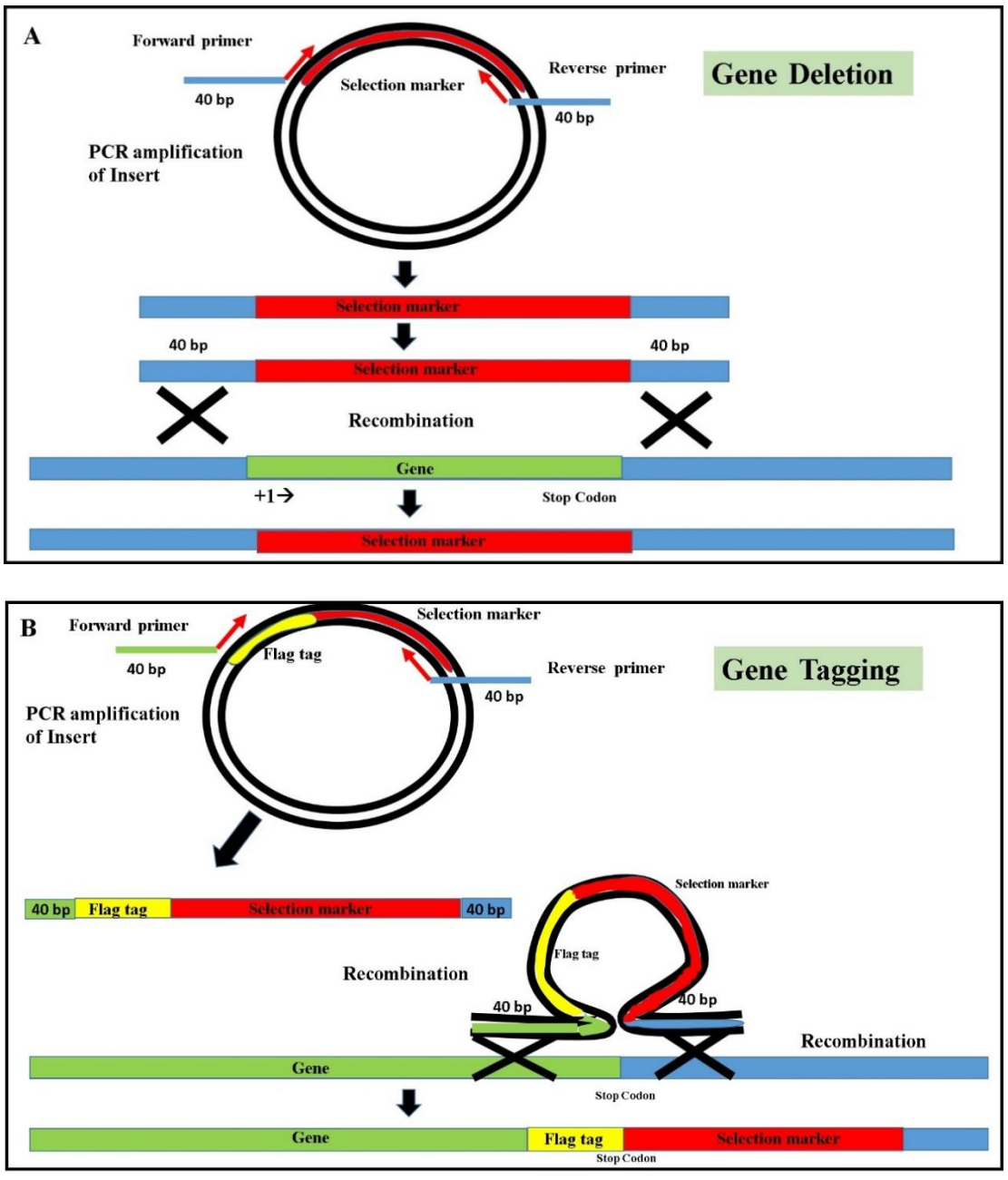


Figure 2.1: A diagram illustrating gene deletion (A) or tagging (B) by one-step PCR-mediated replacement

For all yeast strain constructions, the following procedure was followed. Briefly, following PCR amplification (using Taq DNA Polymerase/with thermoPol buffer for amplifying cassettes for the deletions and fusion HF polymerase (New

England Biolabs) for amplifying cassettes for tagging), of the appropriate cassette that would be inserted in the genome, and following confirmation of the size of the PCR product on an agarose gel, the inserts were ethanol precipitated, and dissolved in 15 µl of distilled water, and transformed into yeast cells. For yeast transformation, a single colony of wild type yeast BY4741strain was grown in 50 ml YPD media (1% yeast extract, 2% Bacto-peptone) until an OD_{600} of 0.5. Cells were then pelleted by centrifugation at 3,000 RPM for 3 minutes, washed with 25 ml of sterile distilled water, and resuspended and incubated in 2 ml of buffer containing 100 mM Lithium Acetate and 0.5X TE for 10 minutes at room temperature. 100 µl of the cells were then initially mixed with 10 µl of 10 mg/ml Salmon sperm DNA (Life technologies) and 15 µl of PCR product, followed by the addition of 700 µl of a mix of 100 mM Lithium Acetate, 1X TE, and 40% polyethylene glycol. Cells were mixed and incubated at 30 ^{o}C for 30 minutes with continuous shaking before 85 µl of DMSO were added to the cells and heat shocked by incubation at 42 ^{o}C for 7 minutes. Cells were then suspended in 1X TE buffer and pelleted, resuspended again in 2 ml YPD media and allowed to grow overnight. Next day, the cells were pelleted, washed with 0.5 TE, resuspended in 1 ml of 0.5X TE, and 25 to 100 µl plated on YPD plates (1% yeast extract, 2% Bacto-peptone, 2% Agar) containing 0.03% Geneticin for selection for Geneticin-resistance or on SD/-His plates for selection for His+ cells. After plates were grown for 3 days, single colonies were re-streaked on selective plates. All strains were constructed using the BY4741 wild-type strain as a background strain, except for Fun30-TAP strain which was purchased from Euroscarf. All constructed strains used in this thesis are listed below in Table 2.1.

Yeast Strain Name	Description
BY4741	MATa; *his3Δ1; leu2Δ0; met15Δ0; ura3Δ0* (Euroscarf, Germany)
Fun30-TAP (SC0012)	SC0000; MATa; *ura3-52; lei2-3,112; YALO19w::TAP-KIURA3* (Euroscarf, Germany)
AZN1	BY4741; *FUN30-3X Flag:kanmx*
AZN4	BY4741; *Δfun30::kanMX6*
AZN5	BY4741; *Δfun30::His3MX6*
AZN6	BY4741; *Δtdp1:: kanMX6*
AZN7	BY4741; *Δtdp1:: kanMX6, Δfun30:: His3MX6*
AZN8	BY4741; *Δmus81:: kanMX6*
AZN9	BY4741; *Δmus81:: kanMX6, Δfun30:: His3MX6*
AZN10	BY4741; *Δasf1:: kanMX6*
AZN11	BY4741; *Δasf1:: kanMX6, Δfun30:: His3MX6*
AZN13	BY4741; *ΔtopI::kanmx*
AZN16	BY4741; *ΔtopI::kanmx; Δfun30::His3MX6*

Table 2.1: Constructed yeast strains

Double deletions were constructed in a similar fashion by constructing one mutant and confirming it, followed by constructing the second mutant using the first mutant as a background. Since two consecutive transformations are done for these, the PCR inserts that are used in the transformations should have different selection markers to allow selection. Once strains were made, they were confirmed by primers that would amplify an accurate PCR product only when integrated in the right place,

since selection on plates can have a lot of false positives that result from faulty integration at other genomic loci. For this, single yeast colonies were grown in YDP media overnight. Cells were then pelleted, bead-beated in extraction buffer (150 mM NaCl, 50 mM Tris) to lyse the cells, and mixed with 0.1% Tween-20. Following, phenol chloroform extraction, DNA was ethanol precipitated and checked for accurate integration by PCR. PCR was done using appropriate primers and suitable cycling conditions. In general, to check for gene deletions, a forward primer was selected in a region of 500 to 1000 upstream of a start codon, and a reverse primer within the integrated cassette. For gene tagging confirmations, similar forward and revers primers were designed. All primers used in constructing and confirming the strains are listed below in Table 2.2.

Primer Name	**Sequence**	**Description**
FP Fun30 C-Flag	**ATATAATTTATGATGAAAA CTCGAAACCGAAGGGAAC CAAAGAA**AGGGAACAAAAG CTGGAG	Forward primer for making a Flag-tagged Fun30 strain at the C-terminus includes 44 bp complementary to the sequence upstream of Fun30 gene (in bold) + 20 bp acting as a forward primer for amplifying Flag- KanMX cassette from p3FLAG-KanMX.
RP Fun30 C-Flag	**TTCTGCTTATCTATTTACT TTTTTACTATATTTTTATTT ATTTA**CTATAGGGCGAATTG GGT	Reverse primer for making a Flag-tagged Fun30 strain at the C-terminus includes 44 bp complementary to the sequence downstream of Fun30 stop codon (in bold) + 20 bp acting as a

		reverse primer to amplify the KanMX cassette from p3FLAG-KanMX.
FP Fun30 C-Flag Check	GAAAAGATTCATCAACTGGC	Forward primer for confirmation of the Fun30 C-Flag tag is complementary to a 156 bp upstream of Fun30 stop codon.
RP Fun30 C-Flag Check	GACAATTCAACGCGTCTGTGAG	Reverse primer for confirmation of the Fun30 C-Flag tag is complementary to a sequence 271bp downstream of the Flag KanMX cassette from p3FLAG-KanMX.
FP *Δfun30*	**GAACGTAAACAAGAAAAAGAGAGAAAATACGCTATAGTTGAAAAC**CGGATCCCCGGGTTAATTAA	Forward primer for making the *Δfun30* strain that includes 45 bp complementary to the sequence upstream of the Fun30 gene (in bold) + 20 bp acting as a forward primer for amplifying the KanMX cassette from pFA6a-kanMX6.
RP *Δfun30*	**TATTTTCTGCTTATCTATTTACTTTTTTACTATATTTTTATTTAT**GAATTCGAGCTCGTTTAAAC	Reverse primer for making the *Δfun30* strain that includes 45 bp complementary to the sequence downstream of Fun30 stop codon (in bold) + 20 bp acting as a reverse primer for amplify KanMX cassette from pFA6a-kanMX6.

FP *Δfun30* Check	CATCCTACCAGATTCCCG	Forward primer for confirming the Fun30 deletion is complementary to a sequence 500 bp upstream of Fun30 start codon.
FP *ΔtopI*	**CTAAAGGGAGGGCAGAGC TCGAAACTTGAAACGCGTA AAA**CGGATCCCCGGGTTAAT TAA	Forward primer for making the *ΔtopI* strain includes 40 bp of complementary to the sequence upstream of the TopI gene (in bold) + 20 bp acting as a forward primer for amplifying KanMX cassette from pFA6a-kanMX6.
RP *ΔtopI*	**TGAATGTATTTGCTTCTCC CCTATGCTGCGTTTCTTTG CG**GAATTCGAGCTCGTTTAA AC	Reverse primer for making the *ΔtopI* strain includes 40 bp complementary to the sequence downstream of the TopI gene stop codon (in bold) + 20 bp acting as a reverse primer to amplify KanMX cassette from pFA6a-kanMX6.
FP*ΔtopI* Check	GATAATGCTGCTATCCGAG	Forward primer for confirming the TopI deletion is complementary to a sequence 1000 bp upstream of the start codon of the TopI gene.
FP *Δtdp1*	**CAGACAAGAATGATGATA ATGTGTTTTCAACCGATCA TTA**CGGATCCCCGGGTTAAT TAA	Forward primer for making the *Δtdp1* strain includes 40 bp of complementary to the sequence upstream of the Tdp1 gene (in bold) +

		20 bp acting as a forward primer for amplifying KanMX cassette from pFA6a-kanMX6.
RP *Δtdp1*	**AGTGATGCCTAAGTGGAC AGCAACATCGCGCTCTTAC TTG**GAATTCGAGCTCGTTTA AAC	Reverse primer for making the *Δtdp1* strain includes 40 bp complementary to the sequence downstream of the Tdp1 stop codon (in bold) + 20 bp acting as a reverse primer to amplify the KanMX cassette from pFA6a-kanMX6.
FP*Δtdp1* Check	ATGCAAATGTTGATTAAATT ATG	Forward primer for confirming the Tdp1 deletion is complementary to a sequence 1000 bp upstream of the start codon of the Tdp1 gene.
FP *Δmus81*	**TCAAAGGATTGATACGAAC ACACATTCCTAGCATGAAA GC**CGGATCCCCGGGTTAATT AA	Forward primer for making the *Δmus81* strain includes 40 bp complementary to the sequence upstream of the Mus81 gene (in bold) + 20 bp acting as a forward primer for amplifying KanMX cassette from pFA6a-kanMX6.
RP *Δmus81*	**TCTTTATAAAACCTTGCAG GGATGACTATATTTCAAAT TG**GAATTCGAGCTCGTTTAA AC	Reverse primer for making the *Δmus81* strain includes 40 bp complementary to the sequence downstream of the Mus81 stop codon (in bold) + 20 bp acting as a reverse

		primer to amplify the KanMX cassette from pFA6a-kanMX6.
FP*Δmus81* Check	ATGGCTGACGACTACGGT	Forward primer for confirming the Mus81 deletion is complementary to the sequence 1000 bp upstream of the start codon of the Mus81 gene.
FP *Δasf1*	**CTCTCCCTACCATCCAATTGAAACATAAGATATAGAAAAG**CGGATCCCCGGGTTAATTAA	Forward primer for making the *Δasf1* strain includes 40 bp complementary to the sequence upstream of the Asf1 gene (in bold) +20 bp acting as a forward primer for amplifying the KanMX cassette from pFA6a-kanMX6.
RP *Δasf1*	**CTCTCTTGCAGGTACCATTAATCTTATAACCCATAAATTC**GAATTCGAGCTCGTTTAAAC	Reverse primer for making the *Δasf1* strain includes 40 bp complementary to the sequence downstream of the Asf1 stop codon (in bold) + 20 bp acting as a reverse primer to amplify the KanMX cassette from pFA6a-kanMX6.
FP *Δasf1* Check	TGCTCGATCTTCTATCCT	Forward primer for confirming the Asf1 deletion is complementary to a sequence 1000 bp upstream of the start codon of the Asf1 gene.
RP *Δ* Check	TTAATTAACCCGGGGATCCG	Reverse primer for confirming any gene deletion is complementary to the

		KanMX and His cassette in pFA6a-kanMX6 and pFA6a-His3MX6, respectively

Table 2.2: List of primers used for gene deletions, tagging, and confirmations of the yeast strains made

2.2 Tandem Affinity Purification (TAP) of Fun30

For all the *in vitro* assays, purified Fun30 TAP-tagged at its C-terminus was used. Fun30 was purified, as described previously (Puig et al. 2001) from a Fun30-TAP strain of *Saccharomyces cerevisiae*, purchased from Euroscarf. The TAP method allows the purification of a protein over two affinity columns. The Fun30 TAP-tagged yeast strain was first streaked on a fresh YPD plate, allowed to grow for three days at 30 ^{o}C, followed by inoculation of a single yeast colony in YPD media until an OD_{600} of 2-3. Cells from 6 liters of culture were then pelleted at 6,000 RPM for 10 minutes, resuspended in an equal volume of TAP extraction buffer (25 mM Tris-HCL pH 8, 10% glycerol, 150 mM NaCl, 0.1% Tween-20, a complete tablet of protease inhibitors (Roche), 1 mM DTT, and 1 mM PMSF), and lysed by bead-beating (Hamilton Bead-beater). Breaking was done till 90% of cells were lysed as assessed by observing the cells under a light microscope. The supernatant was then centrifuged at 13,000 RPM for 30 minutes to remove cell debris. This was followed by another centrifugation step of the supernatant using an ultracentrifuge at 40,000 RPM for 2 hours. The whole cell extract from 6 liters of yeast cell, were then supplemented with NaCl to 350 mM final concentration (pH adjusted to 8 pH with NaOH) and added to 500 µl IgG Sepharose Fast

Flow beads (GE Healthcare) for 3 hours at 4 ^{o}C. The lysate was then allowed to drain by gravity flow in a 10 ml Poly-Prep chromatography column (BioRad). The beads were washed three times with TAP extraction buffer, and once with the same buffer supplemented with 1 mM DTT, 1 µg/ml pepstatin, 2 µg/ml leupeptin, and 1 mM PMSF, Fun30 was then eluted from the IgG resin in 1 ml of the same buffer containing 300 units of TEV Protease at 4 ^{o}C overnight. The flow-through containing Fun30 was then collected, washed with 3 ml of TAP extraction buffer, supplemented with NaCl to 300 mM final concentration and 2 mM $CaCl_2$, 0.5 mM DTT, and proteases inhibitors (1 µg/ml pepstatin, 2 µg/ml leupeptin and 1 mM PMSF), and added to 500 µl of Calmodulin affinity resin (Stratagene) for 3 hours at 4 ^{o}C. The beads were collected by centrifuging at 1,000 RPM, washed twice with 5 ml of the Calmodulin binding buffer, and twice with same buffer but containing 150 mM NaC instead of 300 mM. The bound Fun30 was then eluted from Calmodulin beads in 250 µl (10 times) of an elution buffer containing 10 mM Tris (pH 8), 150 mM NaCl, 2 mM $MgCl_2$, 1 mM Immidazole, 2 mM EGTA, 0.1 NP-40, 10% Glycerol, 1 mM DTT, 1 µg/ml pepstatin, 2 µg/ml leupeptin, and 1 mM PMSF. Elution done at room temperature for 5 minutes each time and the eluted Fun30 collected by centrifuging at 1,000 RPM. The eluted fractions were finally pooled and concentrated using Amicon Ultra centrifugal filter units with a 30 kDa cutoff value. Protein purification and integrity was monitored by western blotting using an anti-TAP antibody (Thermo scientific) and by silver staining. The concentration of Fun30 was calculated by western blotting, comparing Fun30 intensity with known amounts of recombinant Snf6 protein that had a C-terminal Calmodulin Binding Peptide tag using an anti-Calmodulin Binding Protein antibody (Millipore). The steps of the TAP purification method is illustrated below in Figure 2.2.

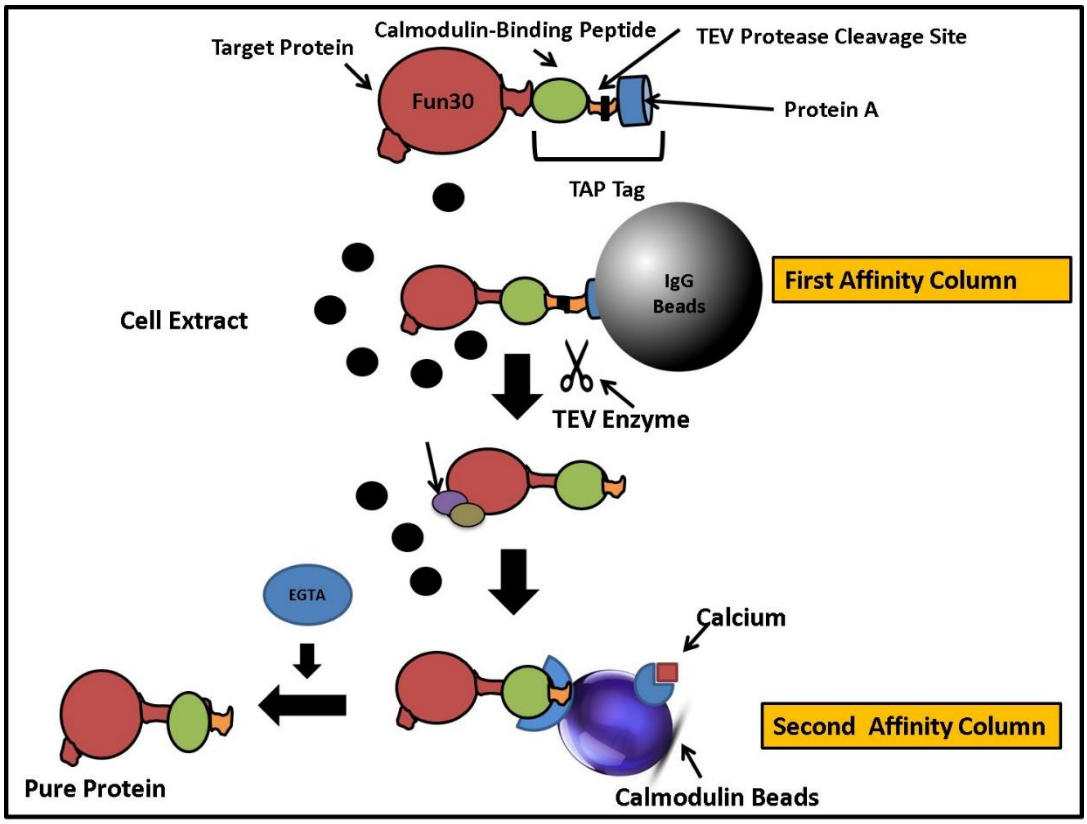


Figure 2.2: A schematic representation of the Tandem Affinity Purification (TAP) method

2.3 Western Blotting

The concentration of protein lysates were measured by Bradford assay using the Bio-Rad protein detection kit (Bio-Rad). Western blot analysis was performed by running proteins (either purified in the case of Fun30 or lysates) on 6-15% SDS gels, transferred to a nitrocellulose membrane at 100 V for 1.5 hour, and blocked in 50 ml of PBS–Tween 20 (144 mM NaCl, 2.6 mM KCl, 10 mM Na_2HPO, 1.7 mM KH_2P0_4., 0.05% Tween 20, pH 7.4) containing 5% Milk at 4 °C for 1 hour. The membranes were then incubated overnight at 4 ^oC with the proper dilution of the primary antibody, washed three times for 10 min each with PBS–0.05% Tween 20, and incubated for 1 hour with 1:10,000 dilutions of the corresponding secondary antibodies**.** Primary

antibodies used in this thesis were: α-TAP antibody (Thermo scientific) at 1:1,000 dilution, monoclonal anti-flag M2-Peroxidase (HRP) antibody produced in mouse (Sigma, A8592) at 1:1,000 dilution, anti-Rad53 antibody (Abcam, 104232) at 1:2,000 dilution, anti-histone H3 antibody (Abcam, ab1791) at 1:1,000 dilution, anti-(phospho S129) Histone H2A antibody at 1:1,000 dilution, and anti-β-tubulin monoclonal antibody (Sigma, T-4026) at 1:500 dilution.

2.4 Silver Staining

For silver staining, the SDS gel was first fixed with 50 ml fixation solution (50% ethanol, 10% acetic acid, and 40% distilled water) at room temperature overnight, followed by the addition of 50 ml of 30% ethanol for 15 minutes while shaking. The gel was then washed three times (for 5 minutes each) with distilled water, sensitized by adding 50 ml of 0.02% sodium thiosulphate for 1.5 minutes, washed again 3 times for 30 seconds each with distilled water, and incubated for 25 minutes in 50 ml of 0.2 % silver nitrate solution. The gel was finally washed 3 times again with distilled water and developed by adding 50 ml of developing solution (6% sodium carbonate supplemented with 1 ml of 0.02% sodium thiolsulphate buffer and 25 µl of formaldehyde). When band were visible the gel was fixed by adding 6% acetic acid and scanned.

2.5 DNA Substrates

All oligonucleotides used in this study were purchased from Metabion. Table 2.3 below lists all the oligonucleotides sequences used to prepare the DNA substrates in our *in vitro* experiments.

Name	**Sequence**
1T	5’-Cy5-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT TTTTTTTTTTTTGACGCTGCCGAATTCTGGCTTGCTAGGACAT-3’
2T	5’-ATGTCCTAGCAAGCCAGAATTCGGCAGCGTCTTTTTTTTTTTTT TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT-3’
1T trap	5’-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT TTTTTTTTTTTTGACGCTGCCGAATTCTGGCTTGCTAGG ACAT-3’
1N	5’-Cy5- GACGCTGCCGAATTCTGGCTTGCTAGGACAT-3’
2N	5’-ATGTCCTAGCAAGCCAGAATTCGGCAGCGTC-3’
HJ1	5’-GACGCTGCCGAATTCTGGCTTGCTATCATGTGGTAACTCATATTCT TACC-3’
HJ2	5’-GGTAAGAATATGAGTTACCACATGATAGCAATGAGTCCGTCGACT ACTCA-3’
HJ3	5’-TGAGTAGTCGACGGACTCATTGCTATCATGTCGTCGTAATATGAC GTCGG-3’
2E CY5	5’-Cy5-CCGACGTCATATTACGACGACATGATAGCAAGCCAGAAT TCGGCAGCGTC-3’
Reg1	5’-ACAGCACCAGATTCAGCAATTAAGCTCTAAGCCATCCGCAAAA ATGACCTCTTATCAAAAGGA-3’
Reg2	5’-Cy5-TCCTTTTGATAAGAGGTCATTTTTGCGGATGGCTTAGAGCTTA ATTGCTGAATCTGGTGCTGT-3’
Reg71	5’-CTTTAGCTGCATATTTACAACATGTTGACCTACAGCACCAGATTC AGCAATTAAGCTCTAAGCCATCCGCAAAAATGACCTCTTATCAAA AGGA-3’
Reg177	5’-CCTTTTGATAAGAGGTCATTTTTGCGGATGGCTTAGAGCTTAATT GCTAATCTGGTGCTGTAGGTCAACATGTTGTAAATATGCAGCTAAAG-3’

Table 2.3: A list of oligonucleotides that were used in reconstituting DNA substrates for the *in vitro* assays

1T,2T,1N, 2N, HJ1, HJ2, HJ3, and 2E CY5 were adapted from (Kaplan, Davey, and O'Donnell 2003), while Reg1, Reg2, Reg71, and Reg177 were adapted from (Bugreev, Rossi, and Mazin 2011).

In general, all DNA substrates were prepared by mixing the appropriate oligonucleotides in an annealing buffer (36 mM Tris-HCl, 17 mM magnesium acetate, 34% glycerol, 230 µM EDTA, 67 µg/ml BSA, 8.3 mM DTT (pH 7.5)) at 37 ºC overnight, as described previously (Kaplan and O'Donnell 2002). The labeled oligonucleotide was always added at a concentration of 100 nM and the unlabeled complementary strand at 150 nM. For the forked DNA duplex template, 1T was mixed with 2T; for the duplex template with 5' overhang, 1T was mixed with 2N; for the duplex template with 3' overhang, 1N was mixed with 2T; and for the blunt ended duplex template, 1N was mixed with 2N. The Holliday Junction substrate was reconstituted by incubating 100 nM 2E CY5, 150 nM HJ3, 225 nM HJ1, and 337 nM HJ2 in the annealing buffer at 37 ºC overnight. The success of substrates reconstitution was assessed by resolving them on 8% native PAGE gel in 0.5X TBE. Oligonucleotides for the regression assay were adapted from Bugreev et al. (Bugreev, Rossi, and Mazin 2011), however, we labeled the oligonucleotides with Cy5 instead. For nascent replication forks used in the regression assay, a Cy5-labeled tailed DNA Reg71/Reg2-Cy5 and a non-labeled tailed DNA Reg117/Reg1 were formed by mixing 1µM of Reg71 and Reg2-Cy5 or Reg117 and Reg1 in 100 µL 1X SSC buffer (15 mM Sodium citrate (pH 7), 150 mM NaCl), boiled for 3 minutes at 95 ºC, and annealing was allowed for 1 hour at hybridization temperature of 44 ºC.

This hybridization temperature was calculated by using the formula (Hybridization Temperature=1.24 × Tm-43.8), where Tm is the melting temperature of the double stranded part of the tailed substrate calculated using Promega website (www.promega.com/a/apps/biomath/index.html?calc=tm) as described previously (Rossi et al. 2010). Figure 2.3 below shows all the DNA substrates used in our *in vitro* assay in the thesis.

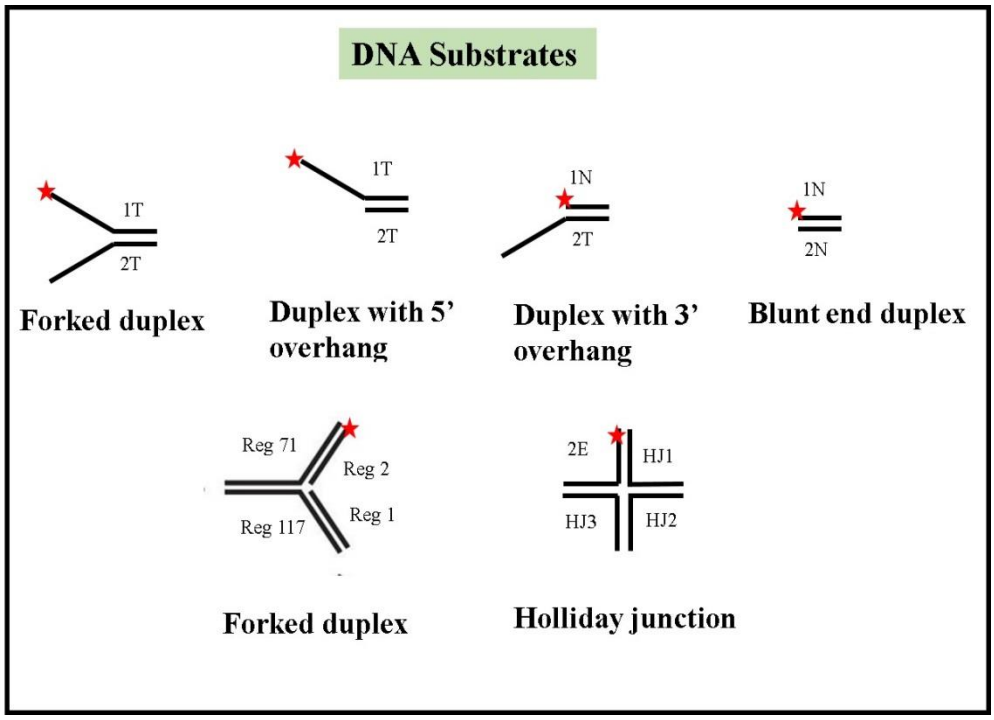


Figure 2.3: DNA Substrate used in the in vitro assays in this thesis
Red star shows the fluorescently-labeled oligonucleotide with Cy5.

2.6 Helicase Assay

For helicase assay, 1 nM of Cy5-labeled forked duplex template was incubated with indicated amounts of Fun30 in a 20 µl reactions in a Helicase Buffer H containing 50 mM Tris–HCl (pH 7.5), 80 mM NaCl, 4 mM $MgCl_2$, 4 mM ATP, mM DTT, and 0.1 mg/ml BSA) for 30 min at 30 °C in the absence or presence of different concentrations of trap DNA (30-90 nM). Where indicated in the figures, ATP was omitted from the reactions and $MgCl_2$ concentration was modified accordingly. Reactions were stopped and the samples de-proteinized by adding 5 µl of 5X Stop Buffer (100 mM EDTA, 2.5% SDS, and 1 mg/ml proteinase K) and incubating at 37 °C for 10 min. The reaction products were resolved on a native 8% polyacrylamide gel (acrylamide to bis-acrylamide ratios 29:1) run in 0.5X TBE at 120 V. The Cy-5 labeled DNA was visualized using a PhosphoImager Typhoon.

2.7 Strand Annealing Assays

Strand annealing assay was performed using partially complementary oligonucleotides 1T and 2T, as shown in Figure 2.3, to produce a forked duplex template. The two oligonucleotides were used at a concentration of 0.5 nM each, in a 20 µl reaction and were carried out in buffer H without ATP. Where indicated in the figures, ATP was added at the marked concentrations, $MgCl_2$ concentration was modified accordingly, or 22 nM SSB was added. Concentrations of Fun30 used are indicated in the figures. Reactions were incubated for 10 min at 30 °C, stopped by adding 5 µl of 5X Stop Buffer (100 mM EDTA, 2.5 % SDS, and 1 mg/ml of proteinase K) and incubating at 37 °C for 10 min. The reaction products were resolved on a native 8% polyacrylamide gel (acrylamide to bis-acrylamide ratios 29:1) run in 0.5X TBE at 120 V. The Cy-5 labeled DNA was visualized using a PhosphoImager Typhoon, as before.

2.8 Regression Assay

Regression assay was performed on nascent replication fork templates as described above. 2 nM of Cy5-labeled tailed DNA (Reg71/Re2-Cy5) was mixed with 3 nM of non-labeled tailed DNA (Reg117/Reg1) in 20 µl of Buffer H in the absence or presence of 4 mM ATP and incubated at 37 ^{o}C for 15 min. Then, different concentrations of Fun30 (16-48 nM) was added to the reactions and incubated for 30 min. at 30 ^{o}C. The reaction was then stopped, as before, by adding 5 µl of 5X Stop Buffer (100 mM EDTA, 2.5 % SDS, and 1 mg/ml of proteinase K) and incubating for 10 min at 37°C. The reaction products were resolved on a native 8% polyacrylamide

gel (acrylamide to bis-acrylamide ratios 29:1) run in 0.5X TBE at 120 V. The Cy-5 labeled DNA was visualized using a PhosphoImager Typhoon, as described earlier.

2.9 Branch Migration Assay

For the Branch migration assay, different concentration of Fun30 (15-30 nM) was added to 5 nM of Cy5-labeled Holliday Junction template in a 20 µl reactions in buffer H (50 mM Tris–HCl (pH 7.5), 80 mM NaCl, 4 mM $MgCl_2$, 8 mM ATP, mM DTT, and 0.1 mg/ml BSA) for 30 min at 30°C. Where indicated in the figures, ATP was omitted from the reactions. Reactions were stopped, as before, by adding 5 µl of 5X Stop Buffer (100 mM EDTA, 1% SDS, and 1 mg/ml proteinase K) and incubating at 37 °C for 10 min. The reaction products were resolved on a native 8% polyacrylamide gel (acrylamide to bis-acrylamide ratios 29:1) run in 0.5X TBE at 120 V. The Cy-5 labeled DNA was visualized using a PhosphoImager Typhoon, as before.

2.10 DNA Supercoiling/Relaxing Assay

For the Supercoiling/Relaxing assay, different concentrations of Fun30 was added to 50 ng of the pG5E4-5S plasmid in a 20 µl reaction containing the TopI Buffer (25 mM Tris-HCl (pH 7.5), 50 mM NaCl , 5 mM $MgCl_2$, 100 µg/ml BSA, and 0.5 mM DTT) and incubated at 30 ^oC for 1 hour. The reactions were then stopped by adding 5 µl of 5X Stop Buffer (100 mM EDTA, 2.5% SDS, and 0.2 mg/ml Proteinase K) and incubating at 37 ^oC for 10 minutes. The reactions products were resolved on a 0.7% agarose gel in 1X TAE (pH 8) at 100 V for 2 hours. The reaction products were run on gels prepared without or with 1.5 µg/ml chloroquine. The gel was stained with ethidium bromide and visualized under UV using the Typhoon.

Positively supercoiled plasmids were generated by adding 10 units of Topoisomerase I in the presence of 20 µM Netropsin (a chemical that intercalates into DNA causing over twisting of DNA) to 5 µg of the pG5E4-5S plasmid in a 500 µl reaction for four hours (Figure 3.7A) at 37 ^{o}C. The reactions were then stopped by adding 100 µl of 5X Stop Buffer (100 mM EDTA, 2.5% SDS, and 1 mg/ml Proteinase K), extracted twice with phenol/chloroform and once with chloroform to remove any remaining Netropsin and phenol. DNA was ethanol precipitated, air dried, and dissolved in 30 µl of water. The efficiency of generating positively supercoiled plasmid was assessed by resolving the plasmid on 0.7% agarose gels without and with 1.5 µg/ml chloroquine. For the supercoiling/relaxing assay using cellular extracts, cells were bead-beated in Extraction Buffer (100 mM HEPES (pH 8), 20 mM Mg $(Ac)_{2,}$ 150 mM NaCl, 10% Glycerol, and 0.1% Tween-20). The eluent containing total cell proteins were normalized to 2 mg/ml, serially diluted (1:2 ratio) using the Extraction Buffer, and 2 µl of each dilution used in the reactions containing 100 ng of pG5E4-5S plasmid in 20 µl reactions for 1 hour at 37 ^{o}C. Samples were then treated and resolved, as described previously.

2.11 Nuclease Assay

For the nuclease assay, different concentrations of Fun30 (15-20 nM) was added to 1 nM of various Cy5-labeled duplex substrates in 20 µl reactions containing Nuclease Buffer (50 mM Tris–HCl (pH 7.5), 80 mM NaCl, 4 mM $MgCl_2$, mM DTT, and 0.1 mg/ml BSA) and incubated for 1 hour at 30 °C. The reactions were stopped by the adding 5 µl of 5X Stop Buffer (100 mM EDTA, 2.5 % SDS, and 1 mg/ml proteinase K) by incubating at 37 °C for 10 min. The reaction products were then resolved on a native 8% polyacrylamide gel (acrylamide to bis-acrylamide ratios 29:1) run in 0.5X

TBE at 120 V. The Cy-5 labeled DNA was visualized using a PhosphoImager Typhoon.

2.12 Rapid Total Cellular Protein Extraction

Total cellular protein was extracted using a method adapted from costa et al. (Neves-Costa et al. 2009) where yeast cellular proteins are rapidly extracted without mechanical disruptions. This was used to confirm successful tagging of proteins (in this case Flag-tagged Fun30) as well as checking for protein expression under different conditions. For protein expression, 2 ml of YPD was inoculated with a single colony of the appropriate strain and grown overnight until saturation. Cells were then harvested, washed, resuspended in 500 µl distilled water and 500 µl 0.3 M NaOH, and incubated for 5 minutes at room temperature, followed by pelleting of the cells at 7,000 RPM. The cells were then resuspended in 100 µl 2X SDS dye, boiled for 5 minutes, and 10 µl of the supernatant was analyzed by Western blotting for protein expression.

2.13 Extraction of Total Cellular Protein by TCA Method

Pellets from the AZN1 yeast strain equivalent to an OD_{600} of 10 was collected from exponentially growing culture by centrifugation, washed with 20% trichloroacetic acid (TCA), and frozen. To extract total cellular proteins, following thawing, the pellets were resuspended in 250 µl 20% TCA, and bead-beated with 250 µl of glass beads at maximum speed for 3 pulses (each pulse lasting 1 minute, with 1 minute of rest on ice in between each pulse). Following this, bottom of the tubes were pierced with a hot needle, placed in another tube and centrifuged at 6,000 RPM for 3 minutes. Again, the beads were washed with 1 ml of 5% TCA and centrifuged again. The drained lysates were then centrifuged at 13,000 RPM for 10 minutes, and the

pellets were suspended in 750 µl of 100% Ethanol, sonicated for 5 seconds, and centrifuged again at 13,000 RPM for 5 minutes, followed by drying at 65 ^{o}C. Finally, 50 µl of 1 M Tris (pH 8) and 100 µl of 2X SDS PAGE Loading Buffer were added to the pellets. The samples were boiled, and protein expression was checked by running on 8% SDS-PAGE followed by Western blotting.

2.14 Preparation of Cytoplasmic and Nuclear Fractions

Cytoplasmic and nuclear protein fractions were prepared from the yeast Flag-tagged Fun30 strain cell culture equivalent to an OD_{600} of 10. The cell pellet (~ 9×10^8 cells) were collected, washed, and stored at -80 ^{0}C overnight. Next day, the pellet was thawed and resuspended in 200 µl of Sorbitol Buffer (1M Sorbitol, 100 mM EDTA, 14 mM 2-mercaptoethanol), and supplemented with 15 units of zymolyase-20T, mixed gently, and incubated for 30 minutes at 30 ^{o}C. The extent of spheroblast formation was assessed by measuring the OD_{600} of the cells where a value of less than 0.1 indicated complete spheroblasting. The spheroblasts were pelleted in pre-cooled microfuge tubes at 4,000 RPM for 2 minutes. The spheroblasts were then washed gently with 1 ml of chilled 1.2 M Sorbitol, pelleted at 4,000 RPM for 2 minutes, and resuspended in 400 µl Extraction Buffer (10 mM MgCl, 15 mM EGTA, 10 mM EDTA, 0.5% Triton, 400 mM sorbitol, 1 mM DTT, 1X protease inhibitors cocktail, and 1 mM PMSF), mixed gently, and incubated for 10 minutes on ice. The suspension was split into two tubes (200 µl in each tube); one as a measure for total cellular protein levels, and the other was further processed to isolate nuclear and cytoplasmic fractions. For this, the sample was centrifuged at 13,000 RPM for 10 minutes, supernatant saved, and the pellet was washed with 10 µl Lysis Buffer, and the supernatants (the cytoplasmic fraction) were pooled. The pellet (the nuclear fraction) was resuspended in 210 µl

Lysis Buffer by sonication for a few seconds. 35 µl 3% SDS sample loading buffer was added to each of the cytoplasmic and nuclear fractions, boiled for 5 minutes at 95 ^{o}C, and the protein levels assessed by resolving 50 µl of each sample on a 8% and 15% SDS-PAGE gels and analyzed by Western blotting for Fun30-Flag, tubulin as an internal control for the cytoplasmic fraction and histone H3 as an internal control for the nuclear fractions.

2.15 Chromatin Association Assay

The chromatin association assay was performed as before for the preparation of cytoplasmic and nuclear fractions except following spheroblasting, the pellet was resuspended in 400 µl of Extraction Buffer that contained 100 mM KCl, 50 mM HEPES-NaOH (pH 7.5), 2.5 mM $MgCl_2$, 0.5% Triton X-100, 1X protease inhibitors cocktail. To separate soluble and chromatin-bound fractions, following sample splitting and centrifugation as before, the supernatant (the soluble cytoplasmic and nuclear fraction) was separated from the pellet (the chromatin bound fraction). The pellet was washed and lysed, as described above. Samples were loaded on 8% and 15% SDS-PAGE gels and analyzed by Western blotting for Fun30-Flag, Tubulin and histone H3 as described earlier.

2.16 Growth Assays

For growth assay, cells growing at log phase were normalized to an OD_{600} of 0.3, serially diluted (1:10), and spotted, using a 48 Pin Multi-Blot Replicator (V&P Scientific, INC), on YPD plates or YPD plates containing Methyl Methane Sulphonate (MMS), 99% (Sigma -Aldrich), (S)-(+)-Camptothecin (Sigma),

Hyroxyurea (HU) (Sigma), at concentrations specified in the figures. Plates were allowed to grow for 3-5 days at 30 ^{o}C.

2.17 Cell Cycle Analysis by Flow Cytometer (FACS)

For synchronization of cells at the G1 phase, cells were grown in 50 ml flasks till they reached an OD_{600} of 0.2, when α-factor was added to a final concentration of 10 µg/ml followed by additional 2 hours of incubation and growth at 30 ^{o}C. Cells were then checked under the light microscope to confirm complete block at G1 by cells a an altered "shmoo" morphology, then extensive washing was done (3 times with 25 ml of chilled distilled water) to remove the α-factor. Cells were then resuspended in YPD and allowed to grow and progress through the cell cycle. Samples of 10 x 10^6 cells were taken from the culture before synchronization, zero time, and the different time intervals following the release. The cells were centrifuged and resuspended in 300 µl distilled water, and following the drop-wise addition of 100% ethanol and vortexing, they were stored at 4 ^{o}C for overnight fixation. Next day, cells were centrifuged, washed with 0.2 M Tris-HCl (pH 8), and 3 x 10^6 cells were then resuspended in 200 µl of Tris-HCl (pH 8) containing 0.2 mg/ml RNase A and incubated for 2 hours at 37 ^{o}C. Samples were then centrifuged and resuspended in 50 µl of 0.2 M Tris-HCl (pH 8) containing 1.5 mg/ml proteinase K and incubated for 2 hours at 37 ^{o}C. 1 ml of 5 µg/ml Propidium Iodide was then added to each samples stain the DNA content and incubated at 4 ^{o}C overnight, and sonicated for 5 seconds before they were analyzed using BD FACSCanto II Flow Cytometer. Cell profiles were analyzed using the FlowJo_V10 software. The same was followed for analyzing cell cycle of HU or camptothecin treated cells.

Chapter 3: Results -Biochemical Characterization of Fun30

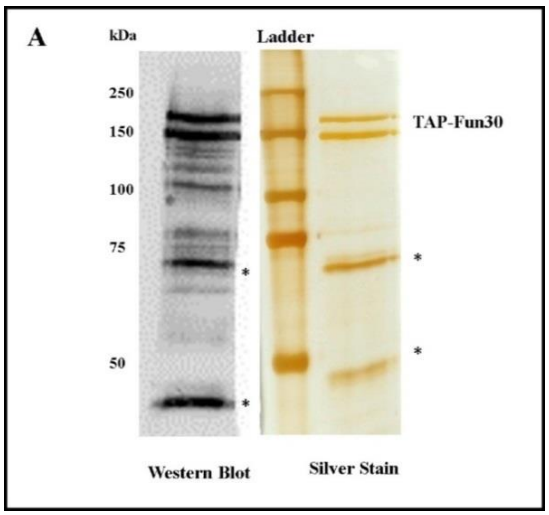
3.1 Overview

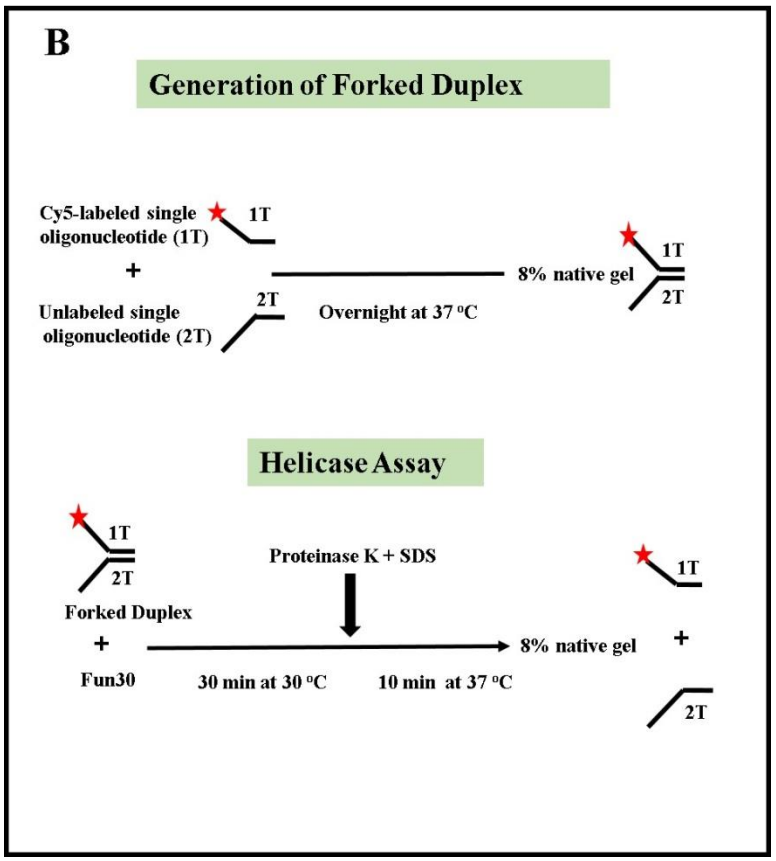
Many studies on Fun30 have focused on its role as an ATP-dependent chromatin remodeler. This has been based on the sequence homology between its ATPase domain and the ATPase domain of other ATP-dependent chromatin remodelers. From its name, a helicase domain is supposed to confer helicase activity onto the protein that harbors it, which allows the unwinding of duplex DNA. Interestingly, most of the chromatin remodelers lack this activity (Watanabe et al. 2015). The helicase activity of Fun30 has not been tested and therefore, one of our aims has been to investigate whether Fun30 has a helicase activity. Additionally, we investigated other potential activities of Fun30 *in vitro*.

3.2 Fun30 Can Anneal Complementary Strands of DNA

Fun30 has been shown to have a role in controlling HR, as mentioned in the introduction. Having a helicase domain raised the question of whether Fun30 has a helicase activity, which is a common activity for many proteins involved in recombination. Testing whether Fun30 has a helicase activity or other catalytic activities can be performed *in vitro* using purified proteins and reconstituted DNA substrates. In general, for all our *in vitro* biochemical assays, Fun30 was purified using a C-terminally TAP-tagged Fun30 yeast strain by tandem affinity purification (TAP) method (Puig et al. 2001). Purification was monitored by western blot using an anti-TAP antibody and confirmed by silver staining (Figure 3.1A). Fun30 is a 128 kDa protein that is shown as double bands, which are normally observed with purified Fun30. Other bands on both gels are likely degradation products.

To test a potential helicase activity of Fun30, increasing concentrations of Fun30 were added to a fluorescently (Cy5)-labelled forked duplex oligonucleotide template in the presence or absence of ATP. The reaction products of this helicase assay (Figure 3.1B) were resolved on native gels and visualized by measuring fluorescence using Typhoon^{Tm} FLA9500. Generation of single stranded templates would indicate that Fun30 had a helicase activity and was able to break the hydrogen bonds holding the two DNA strands together. However, under these conditions, with increasing concentrations of Fun30, we did not observe the generation of single strand oligonucleotides (products of helicase activity) either in the absence or the presence of ATP (Figure 3.1C, lanes 3-5 and lanes 7-9 in the absence or presence of ATP, respectively). Lane 1 shows the initial forked duplex denatured by heat to show where the single stranded oligonucleotide product generated in case of a helicase activity would run on the gel.





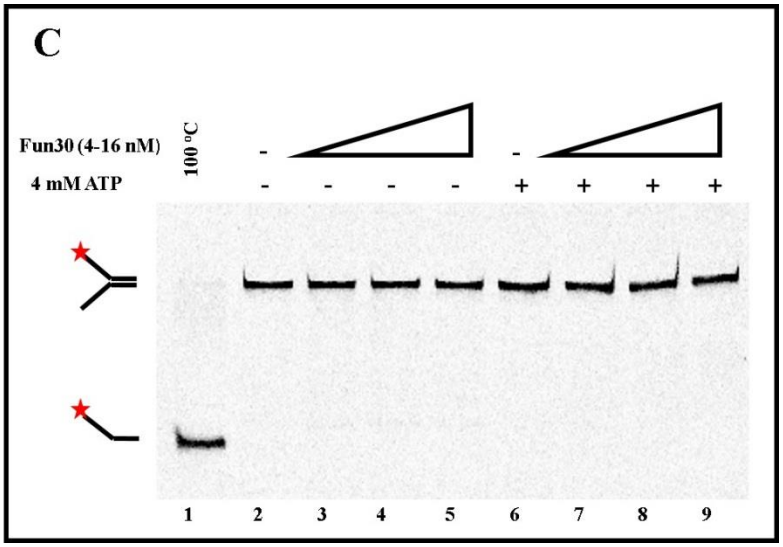


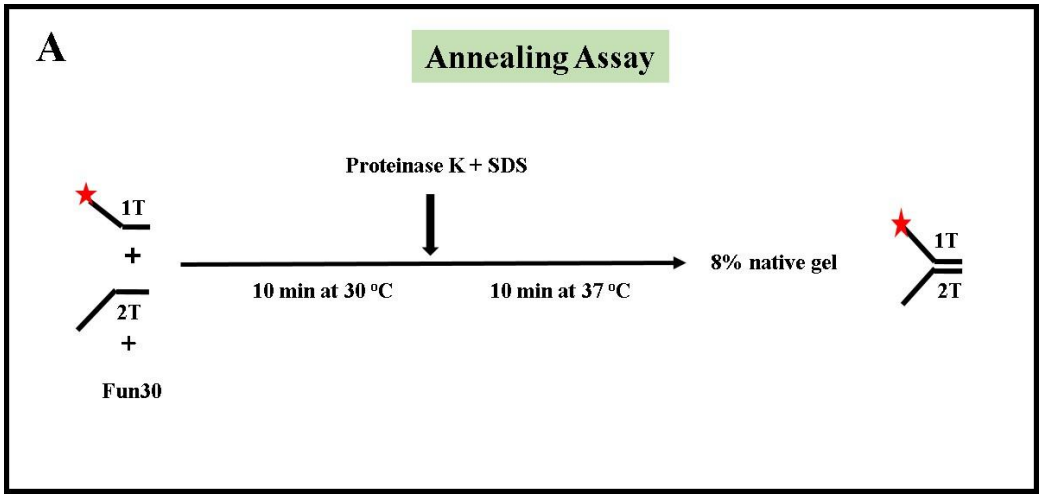
Figure 3.1: Fun30 does not have a helicase activity

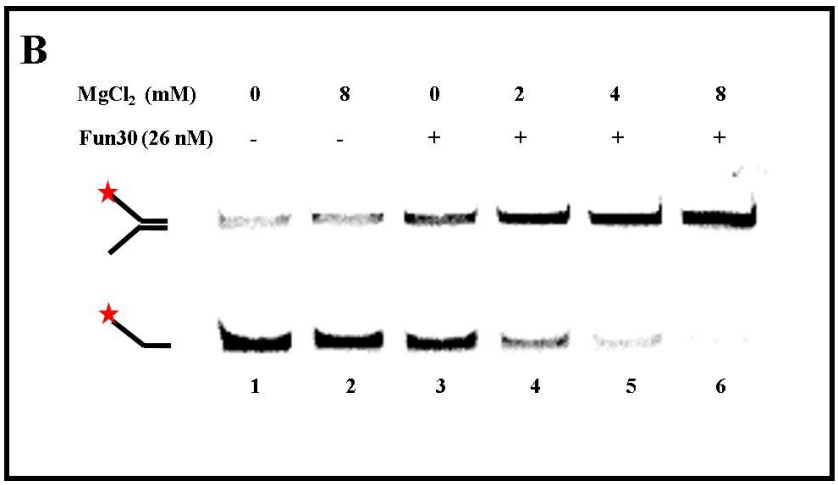
(A) Western blot analysis and silver staining of C-terminally TAP-tagged Fun30 yeast strain purified by tandem affinity purification (TAP) method. For monitoring Fun30 purification by western blotting, an anti-TAP antibody was used. Purified Fun30 on gels run as a double band. The asterisks show Fun30 degradation products. (B) Diagram showing the generation of the forked DNA duplex as a substrate for the helicase assay. Red star shows the fluorescently-labeled oligonucleotide with Cy5. (C) Helicase assay performed with increasing concentrations of Fun30 (4-16 nM) in 20 µl

reactions containing 1 nM forked DNA duplex that has one oligonucleotide fluorescently-labeled at its 5' end by Cy5, in the presence or absence of 4 mM ATP. The reactions were incubated for 10 minutes at 30 °C, stopped by adding 5 µl of stop buffer (containing 2.5% SDS and 1mg/mL proteinase K), resolved on 8% native gel, and scanned. Lane 1 shows forked duplex boiled at 100 °C as a control for completely opened duplex showing the size of the single labeled oligonucleotide. Lanes 2 and lane 6 show the forked duplex in the absence or presence of 4 mM ATP and do not contain Fun30. They show the stability of the DNA duplex under these conditions. Lanes 3-5, and 7-9 have increasing amounts of Fun30 in the absence or presence of ATP, respectively. The lack of single stranded oligonucleotides in the absence or presence of ATP, suggests that Fun30 does not have a helicase activity under these conditions.

Before concluding that Fun30 does not have a helicase activity, we thought to check if Fun30 might instead have an annealing activity that is masking a possible helicase function. To test this possibility, Fun30 was incubated with partially complementary single stranded oligonucleotides in the presence of increasing concentrations of $MgCl_2$ (Figure 3.2A). Here, only one of the partially complementary strands is fluorescently (Cy5)-labelled. In the event that the oligonucleotides are annealed together, a forked duplex will be formed, which migrates slower than a single stranded labeled oligonucleotide. The results (Figure 3.2B) show that Fun30 was able to achieve partial annealing of the oligonucleotides in the absence of $MgCl_2$ (compare lanes 1 and 3), while annealing levels increased in the presence of $MgCl_2$ (lanes 3-5), and was highest at a concentration of 8 mM $MgCl_2$ (lane 6; compare lanes 6 and 2). Next, we investigated how the hydrolysis of ATP by Fun30 would affect this annealing activity. If ATP hydrolysis was required for a potential helicase activity of Fun30, then we would expect to see inhibition of annealing in the presence of ATP. To test the effects of ATP on the helicase activity of Fun30, the annealing assay was repeated in the presence or absence of ATP as well as its non-hydrolysable form (ATP-γ-S) (Figure 3.2C). We observed that 4 mM ATP could partially inhibit the annealing

activity of Fun30 (compare lanes 2 and 4). Surprisingly, ATP-γ-S was able to inhibit the annealing activity of Fun30 even more (compare lane 4 and 6). This suggests that the inhibition of annealing activity may not be solely because of ATP hydrolysis or possibly antagonizing a potential helicase activity, but instead it may also be possible that ATP-γ-S binding has an allosteric effect that weakens the annealing activity of Fun30.





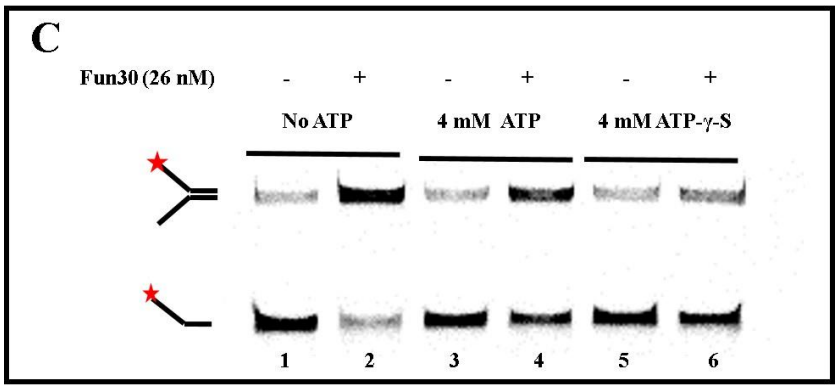


Figure 3.2: Fun30 can anneal complementary strands of DNA

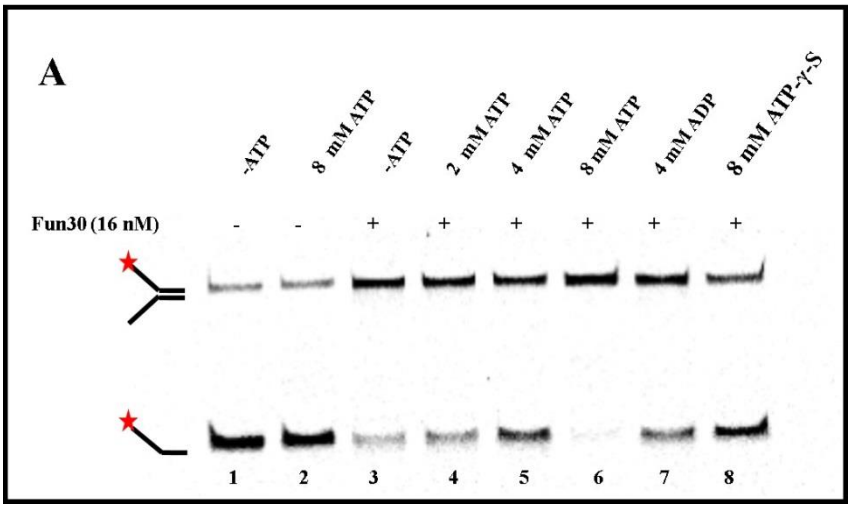
(A) Diagram of the annealing experiment. (B) Annealing assay performed by incubating 26 nM of Fun30 in 20 µl reactions containing 0.5 nM of each the two partially complementary single stranded oligonucleotides with one fluorescently-labeled at its 5' end cy Cy5 (Cy5-1T and 2T), in the presence of increasing amounts of $MgCl_2$ (0-8 mM). The reactions were incubated for 10 minutes at 30 ^oC, stopped, run on a native gel and scanned, as described before. Lanes 1 and 2 are control lanes in the absence of Fun30 to show that spontaneous annealing of these single stranded oligonucleotides does not take place under these conditions. Lanes 3-6 contain Fun30 with increasing concentrations of $MgCl_2$. Results show that Fun30 was able to achieve partial annealing of the oligonucleotides in the absence of $MgCl_2$ (compare lanes 1 and 3), while annealing levels improved with increasing $MgCl_2$ concentrations (C) Annealing assay to test the effects of ATP and its non-hydrolysable form ATP-γ-S on the annealing activity of Fun30. The assay was repeated with 26 nM of Fun30 in the presence or absence of 4 mM ATP or ATP-γ-S. The reactions were processed and analyzed as mentioned above. Lanes 1, 3, and 5 are control in the absence of Fun30 to show that no or little spontaneous annealing under these conditions. Lanes 2 shows enhanced annealing by Fun30 in the absence of ATP, which is inhibited by ATP and to a greater extent by ATP-γ-S (shown in lanes 4 and 6).

3.3 Fun30 Annealing Activity has a Biphasic Mode in the Presence of ATP

Inhibition of annealing activity by ATP and the non-hydrolysable form of ATP can be explained in different ways. It is possible that ATP is needed for the helicase activity of Fun30, which in turn can affect its annealing activity. Alternatively, another possibility is that ADP (the byproduct of ATP hydrolysis) can allosterically inhibit the annealing activity of Fun30. The inhibition of Fun30 annealing activity by ATP-γ-S, however, can only be explained through allosteric effects. To investigate the

effect of ATP binding and/or hydrolysis on the annealing activity of Fun30, we repeated the annealing assay in the presence of different concentrations of ATP (0-8 mM), 4 mM ADP or 8 mM ATP-γ-S (Figure 3.3A). The results show that there is an intrinsic annealing activity of Fun30 in the absence of ATP as previously observed in Figure 3.2 (Figure 3.3 lane 3), which was inhibited by ATP concentrations of up to 4 mM (Figure 3.3A, lanes 3-5). However, surprisingly, this inhibition did not hold true with higher ATP concentrations, where we observed efficient and complete annealing activity of the two strands by Fun30 at 8 mM ATP (Figure 3.3A, lane 6). 4 mM ADP was found to inhibit annealing by Fun30 to a similar extent as when 4 mM ATP was used in the reaction (compare lanes 5 and 7). Moreover, when we used 8 mM ATP-γ-S, we observed significant inhibition of annealing activity compared to when 8 mM ATP was used (Figure 3.3A, compare lanes 6 and 8). This is constituent with our results in Figure 3.2C when 4 mM ATP or ATP-γ-S was used in the reaction. These results show that ATP hydrolysis (and not binding per se) is required for efficient annealing activity of oligonucleotides by Fun30. To confirm these results, we repeated the experiments with increasing Fun30 concentrations. Under these conditions, the effects of increasing ATP concentrations (0, 4, and 8 mM) as well as 4 mM ADP on the annealing activity by Fun30 was tested (Figure 3.3B). Again, we observed that the presence of 4 mM ATP inhibited the annealing of the oligonucleotides by Fun30 (Figure 3.3B, compare first and second panels), whereas, 8 mM ATP led to more efficient annealing at lower Fun30 concentrations even compared to when ATP was absent (Figure 3B, compare the first 3 panels). Similar to Figure 3.3A, again we observed that 4 mM ADP also inhibited the annealing by Fun30 to a similar extent seen with 4 mM ATP (Figure 3.3B, compare second and fourth panels). These results show that while ATP at lower concentrations inhibits Fun30 annealing activity, at

higher concentrations it can promote this activity. This suggests that ATP can affect Fun30 annealing activity in a biphasic mode. In other words, Fun30 has an intrinsic annealing activity, which can be non-enzymatic and might be facilitated by molecular crowding or possibly because of the ability of Fun30 to bind to DNA, which might help in bringing DNA molecules closer together. Furthermore, the addition of ATP at lower concentration leads to a decreased annealing activity (similar to when the same concentration of ADP is used) possibly because of allosteric inhibitory effect. With increased ATP concentrations (to 8 mM), this allosteric inhibition could possibly overcome this inhibition and lead to enhanced annealing perhaps by competing with ADP or just as a result of increased ATP hydrolysis or increased Fun30 binding. The inhibition of annealing by the non-hydrolysable form of ATP might be explained by the requirement of ATP hydrolysis for annealing or alternatively an allosteric effect like with ADP. The reason for enhanced annealing activity of even lower concentrations of Fun30 at 8 mM ATP could possibly be due to increased affinity of Fun30 for DNA in higher concentrations of ATP.



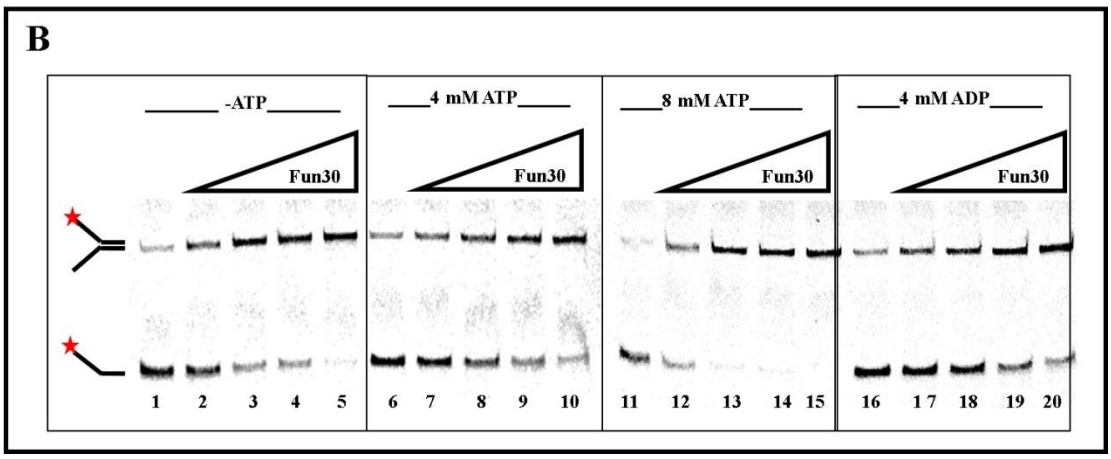


Figure 3.3: Fun30 annealing activity has a biphasic mode in the presence of ATP

(A) Annealing assay repeated with 16 nM of Fun30, the two single stranded oligonucleotides in the presence or absence of increasing concentrations of ATP (2-8 nM), ADP, and ATP-γ-S concentrations. The reactions were processed and analyzed as before. Lane 1 and 2 are control reactions with no or 8 mM ATP, respectively showing that annealing does not occur in the absence of protein. With the addition of Fun30 in lanes 3-6, annealing of the two strands is observed as indicated by the generation of the forked structure, which is initially inhibited by 4 mM ATP in the reaction and then improved upon further ATP addition (at 8 mM). Lanes 7 and 8 show the effects of ADP and ATP-γ-S on Fun30 annealing activity. (B) Annealing assay in the presence of increasing Fun30 (5-25 nM) and ATP (0-8 mM) concentrations or 4 mM ADP. Lanes 1, 6, 11, and 16 are control reactions without Fun30 that show the lack of spontaneous annealing under these conditions. The results again point to a biphasic mode of Fun30 annealing activity in the presence of increasing ATP concentrations.

3.4 Fun30 Annealing Activity is inhibited by Single Stranded DNA Binding Protein (SSB)

In vitro assays can provide clues on the possible activities of a protein, but despite the usefulness of these assays, they do not reflect the complexity that exists in the cell environment where many factors may be competing for the same substrate. Within the cell, DNA is not free and instead is bound by different proteins that recognize various DNA structures in order to perform different functions. Since Fun30 has an annealing activity, we investigated whether this annealing activity is affected

by the presence of proteins that bind to single-stranded DNA. Replication protein A (RPA) would is a good candidate since it is known to bind to single stranded DNA in yeast, however, since we were not successful in purifying a nuclease free form of this protein, instead we used SSB protein (a single stranded DNA binding protein from bacteria) for this assay (Figure 3.4). Here, we tested the Fun30 annealing activity on single stranded oligonucleotide templates that were already bound with SSB. We observed that SSB inhibited the Fun30 annealing activity under reaction conditions lacking ATP (Figure 3.4A, compare lanes 1-3 and 4-6). Since we previously showed that Fun30 annealing activity is enhance in the presence of 8 mM ATP (Figure 3.3), we tested whether 8 mM ATP caused Fun30 to overcome this inhibitory effect of SSB. The results show that when SSB was present in the reaction, Fun30 was unable to anneal the DNA oligonucleotides even in the presence of 8 mM ATP (Figure 3.4B, lanes 5-6). This shows that SSB inhibits Fun30 annealing activity even under conditions of higher ATP concentrations. This, however, could perhaps be due to a higher affinity of SSB to DNA compared to Fun30 competing with Fun30 on binding to the same substrates or possibly because SSB is not a native yeast protein that Fun30 would interact with in the cell. RPA would be a preferable competitor for substrate binding, especially since RPA has been shown to co-immunoprecipitate with Fun30 under DNA damaging conditions (Chen et al. 2012a).

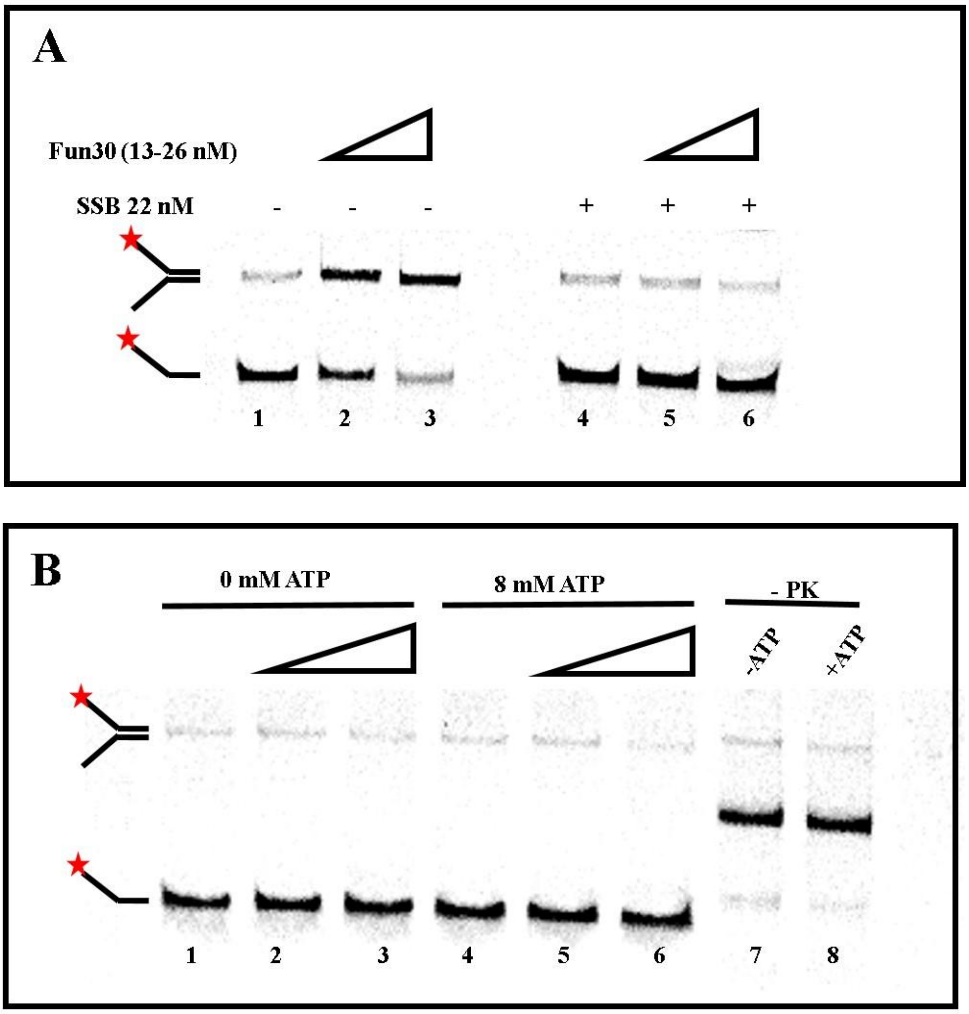


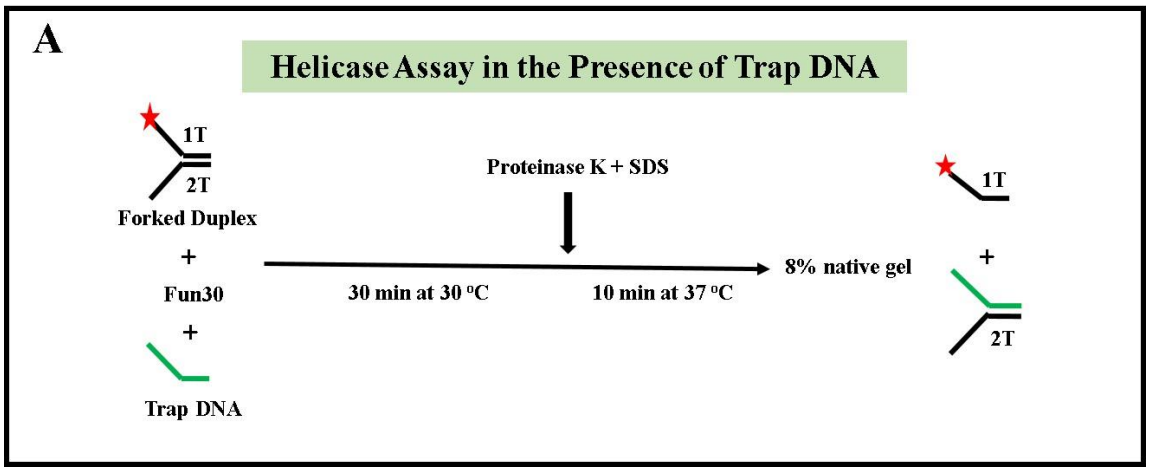
Figure 3.4: Fun30 Annealing Activity is inhibited by single stranded DNA binding protein (SSB)

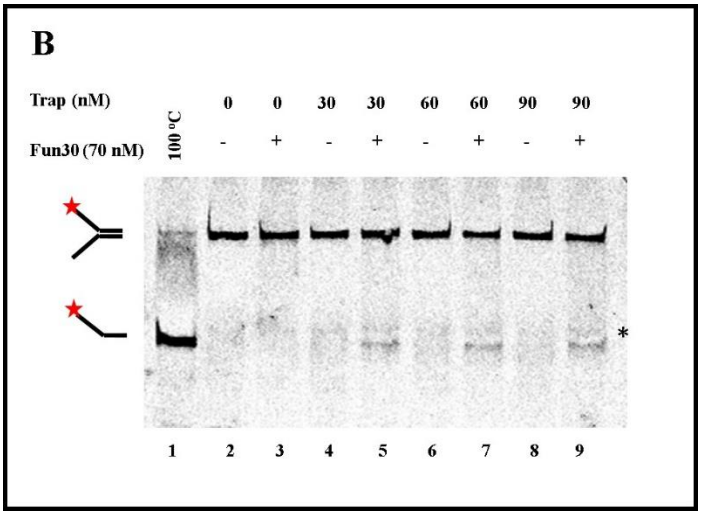
(A) Annealing assay in the presence of 22 nM SSB. Increasing concentrations of Fun30 (13 or 26 nM) were added to the single stranded oligonucleotides in the presence or absence of 22 nM SSB and the reactions were processed and analyzed as before. Lanes 1 and 4 are control reactions in the absence of Fun30 showing little self-annealing under these conditions. Lane 5 and 6 show reduced Fun30 annealing activity in the presence of SSB. (B) The same annealing assay as in (A) in the presence of 8 mM ATP. Lanes 5 and 6 show that even 8 mM ATP (which was able to increase Fun30 annealing activity in Figure 3.3) was unable to overcome SSB inhibition of Fun30 annealing activity. Lanes 7-8 are controls where the reaction were not digested by proteinase K and thus show the binding of SSB to the single stranded Cy5-labeled template causing a band shift.

3.5 Fun30 has a Helicase Activity in the Presence of Trap DNA

We could not observe any helicase activity for Fun30 at conditions seen with many potent helicases. However, the question of whether Fun30 has a helicase activity was raised since when we observed slight inhibition of annealing activity at 4 mM ATP concentrations. It could be possible that there is a balance between helicase and annealing activity or a weak helicase activity was being masked by a stronger annealing activity as mentioned earlier. To test this possibility, the conditions of the experiment were modified so that we could detect a possible week helicase activity of Fun30. For this the assay was performed by incubating Fun30 with forked duplex DNA under conditions at which annealing activity was not optimal (4mM $MgCl_2$, and 2 mM ATP) Moreover, the reactions were supplemented with unlabeled trap DNA, which was partially complementary to the unlabeled DNA strand of the forked template (Figure 3.5A). In this assay, where different concentrations (30-90 nM) of the trap DNA was used, the presence of the trap DNA would allow the capture of any released unlabeled oligonucleotide as a result of unwinding and thus generating a faster migrating single stranded labeled oligonucleotide. Under these conditions, the concentrations of the trap DNA was in excess molar ratio compared to the forked substrate and would thus compete with the unlabeled strand making the re-annealing of the released labeled strand less favorable and thus allow the detection of the freed labeled DNA (Figure 3.5A). Our results showed that Fun30 was unable to unwind forked duplex substrate in the absence of trap DNA (Figure 3.5B, lanes 2-3). However, adding 30 nM trap DNA enabled us to visualize this DNA unwinding/helicase activity of Fun30 (lanes 2-5). However, we did not observe enhanced Fun30 helicase activity with increased trap DNA concentrations (lanes 6-9). As this observed Fun30 helicase activity was quite weak, we tested the effects of different ATP concentrations in the

presence of 8 mM magnesium chloride and 30 nM trap DNA. Under these conditions, again, we observed that Fun30 could unwind the forked template (Figure 3.5C), however, no improvement in the unwinding activity of Fun30 was observed at different ATP concentrations (lanes 4-6). It is worth noting that we needed to use a much higher concentrations of Fun30 (3-5 folds higher) to detect its helicase activity compared to the amounts needed for observing its annealing activity.





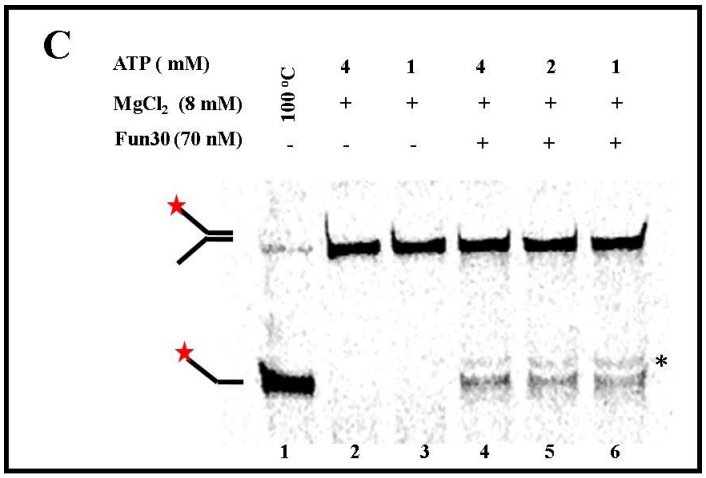


Figure 3.5: Fun30 has a helicase activity in the presence of trap DNA

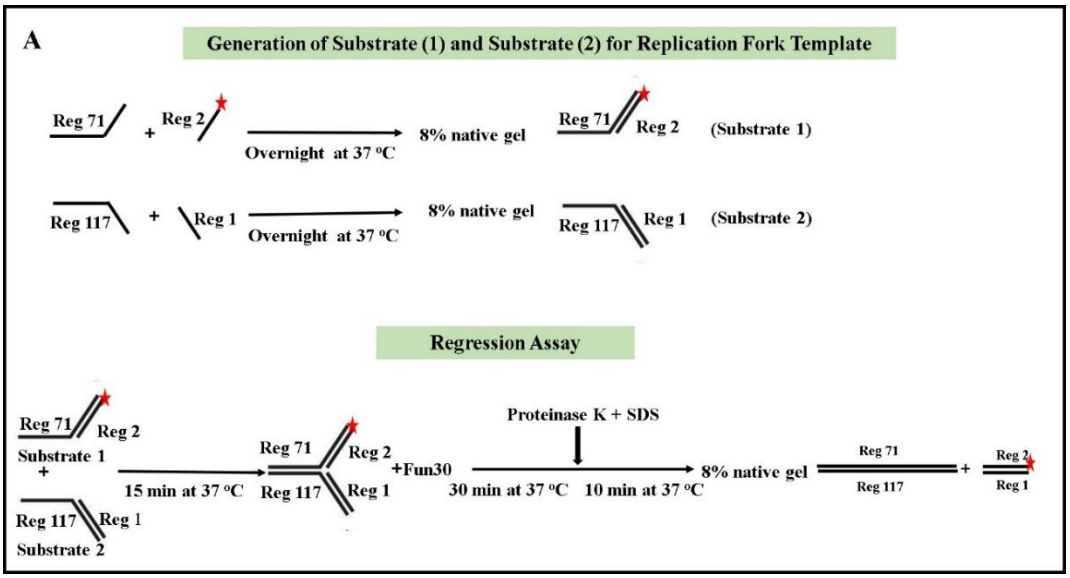
(A) Diagram outlining the helicase assay in the presence of trap DNA. The trap DNA is partially complementary to the unlabeled strand of the fork structure (or is the same as the labeled strand for the forked template). (B) Helicase assay performed in Figure 1 was repeated in the presence of different amount (30, 60, and 90 nM) of trap DNA. Here, 70 nM Fun30 was added to 20 µl reactions containing 1 nM Cy-5 labeled forked duplex template, 4 mM $MgCl_2$, and 4 mM ATP. The reactions were processed and analyzed as before. Lane 1 is the template boiled at 100 ^oC as a control to show the migration of a completely unwound/open duplex and lane 2 shows the position of the intact forked template. Results show a weak but consistent and reproducible unwinding/helicase activity by Fun30. (C) Helicase assay in (B) repeated with 30 nM trap DNA in the presence of increasing ATP concentrations. Here, 70 nM of Fun30 was added to 20 µl reactions containing 1 nM Cy-5 labeled forked duplex template, 8 mM $MgCl_2$, 30 nM trap DNA, and 1, 2 or 4 mM ATP. Results show no improvement in the helicase activity of Fun30 at different ATP concentrations. Asterisk point to the cleaved products (upper band) explained later in Figure 3.8.

3.6 Fun30 has a Week ATP-Independent Regression Activity and cannot Cause Holliday Junction Migrations

DNA unwinding/helicase and annealing activities are important mechanisms by which many proteins exert their effects during processes such as recombination or DNA damage repair. Since we had shown that Fun30 harbors both of these opposing activities, next we wanted to investigate whether Fun30 can utilize these activities to resolve DNA structures that are generated during the course of replication or during DNA damage repair. As mentioned previously, camptothecin induced damage can lead

to the buildup of torsional strain, in which case the replication fork might regress or collapse to allow for damage repair or recombination mediated repair. Because the Fun30 null and ATPase domain mutants are sensitive to camptothecin, we investigated whether Fun30 can cause the regression of replication forks. To test this, a DNA substrate that represents replication fork was constructed and a product of regression activity measured with increasing concentrations of Fun30 (Figure 3.6A). Lane 1 shows the size of the product formed if regression was complete. Our results showed a slight increase in formation of the regression product in the presence or absence of ATP with increasing Fun30 concentrations (Figure 3.6B, lanes 2-5 and 6-9 for 0 and 4 mM ATP, respectively). However, this increase was not very significant and since observed even in the absence of ATP, might not be important *in vivo*. This is because mutations in the Fun30 ATPase domain have also been implicated in sensitivity to DNA damage by camptothecin, as mentioned earlier. However, as we have tested other substrates such as those that have single stranded DNA gaps, we cannot make concrete conclusions about the regression activity of Fun30.

Furthermore, to test whether Fun30 can cause the migration of Holliday junctions, which is a byproduct of HR, a substrate that represents a single Holliday junction was constructed (Figure 3.6C). Lanes 1-3 show partial reconstitution of substrates as control. Furthermore, the results show that when Fun30, in the presence of ATP, was incubated with the Holliday junction substrate, no product of migration was observed (Figure 3.6D. lanes 4-7). In summary, we conclude that Fun30, under conditions we tested, is unable to regress replication forks or cause the migration of Holliday Junctions.


A
Generation of Substrate (1) and Substrate (2) for Replication Fork Template
Reg 71
Reg 2
Overnight at 37 °C
8% native gel
Reg 71
Reg 2
(Substrate 1)
Reg 117
Reg 1
Overnight at 37 °C
8% native gel
Reg 117
Reg 1
(Substrate 2)
Regression Assay
Reg 71
Reg 2
Substrate 1
+
Reg 117
Reg 1
Substrate 2
15 min at 37 °C
Reg 71
Reg 2
Reg 117
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Proteinase K + SDS
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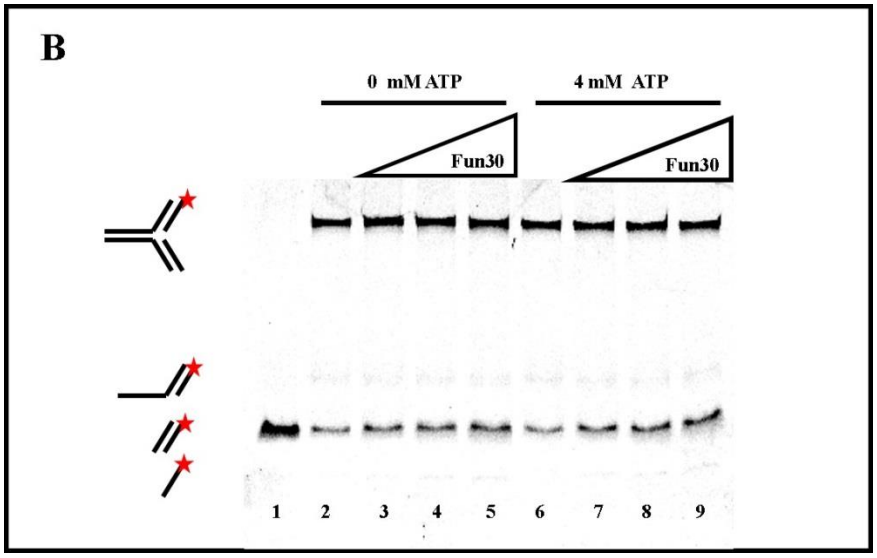
8% native gel
Reg 71
Reg 117
+
Reg 2
Reg 1


B
0 mM ATP
4 mM ATP
Fun30
Fun30
1
2
3
4
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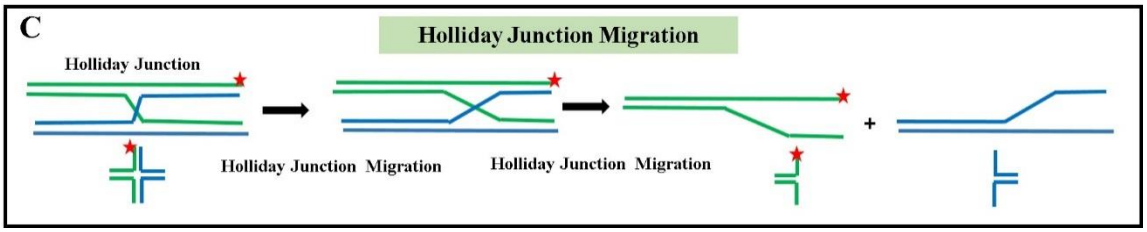
6
7
8
9


C
Holliday Junction Migration
Holliday Junction
Holliday Junction Migration
Holliday Junction Migration
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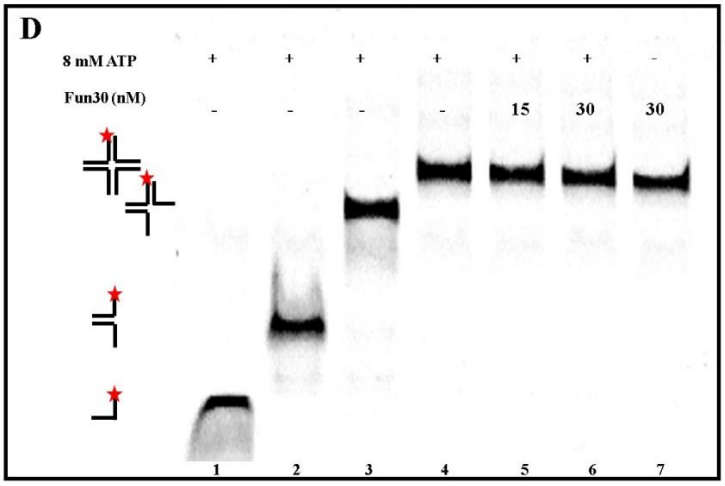


Figure 3.6: Fun30 has a weak ATP-independent replication fork regression activity and cannot cause holiday junction migrations

(A) Diagram illustrating the construction of the replication fork substrate and the expected fork regression products. Regression assay substrates 1 and 2 were prepared by mixing them in a reaction containing annealing buffer overnight, as described in the materials and methods. To generate the replication fork substrate 2 mM of substrate (1) was incubated with 3 nM of substrate (2) in 20 µl reactions for 15 min at 37 ºC. (B) Regression assay performed by incubating increasing concentrations of Fun30 (16, 32, and 48 nM) with the annealed regression template in the absence of presence of 4 mM ATP. The reactions incubated for 30 minutes at 30 ºC, stopped with proteinase K, and the products resolved on 8% native gel in 0.5X TBE buffer, and scanned by Typhoon. Lane 1 shows the size of the labeled product that would form if regression is completed and lanes 2 and 6 are controls showing the replication fork substrate in the absence of Fun30. Results show a weak regression activity with higher Fun30 concentrations after 30 minutes of incubation. (C) Diagram showing the substrate used in the Holliday Junction migration experiment. (D) Holliday Junction migration experiment performed by incubating Fun30 (15 or 30 nM) to 20 µl reactions containing 5 nM Holliday Junction substrate and 8 mM ATP. The reaction was incubated for 30 minutes and the samples were processed and analyzed as mentioned above in (B). Lanes 1-3 are control samples showing partial reconstitutions of the substrate, lane 4 is the fully reconstituted substrate in the absence of Fun30. Results in lanes 5-7 show that Fun30 does not cause Holiday Junction migration.

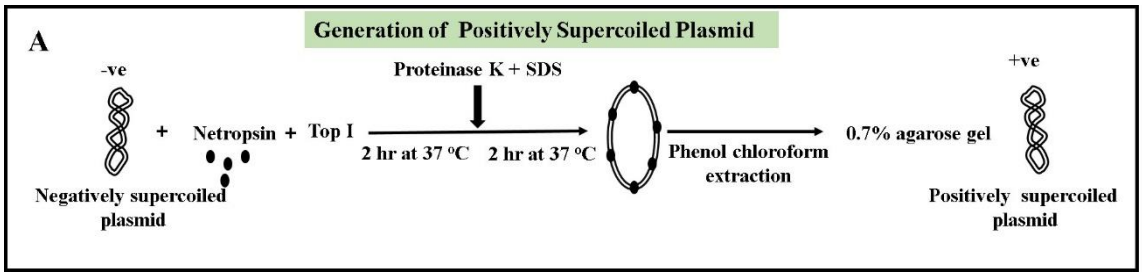
3.7 Fun30 Can Relax both Positively and Negatively Supercoiled DNA in an ATP-Independent Manner by Nicking DNA

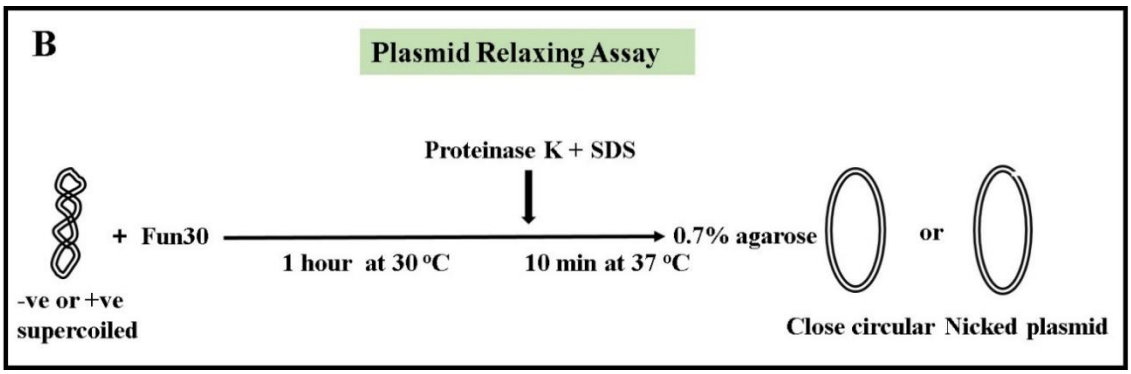
Cells lacking Fun30 were found to be sensitive compared to cells expressing a mutant form of Top1, which, as mentioned previously, stabilizes the Top1-DNA

complex leading to DNA damage similar to the damage induced by camptothecin, which *Δfun30* is also sensitive to (Neves-Costa et al. 2009). Here, we tested whether Fun30 is involved in relieving the torsional strain that builds up upon such kind of damage. Positively supercoiled plasmid DNA was formed by relaxing pG5E4-5S plasmid with Topoisomerase I enzyme in the presence of Netropsin (Figure 3.7A). To test whether Fun30 plays a role in relieving DNA with torsional strain, increasing concentrations of purified Fun30 were incubated with negatively and positively supercoiled pG5E4 plasmid and resolved on 0.7% agarose gels with or without chloroquine (Figure 3.7B and Figure 3.7C, right and left panels, respectively). Chloroquine helps in confirming the proper constitution of positively supercoiled DNA since negatively supercoiled DNA will migrate slower as a diffused smear in the presence of chloroquine, while positively supercoiled DNA will migrate faster and is more compact, compared to when chloroquine is not in the gel. In addition, chloroquine will help in differentiating between closed circular DNA and nicked circles, where a closed circular plasmid will migrate faster than a nicked circle, in presence of chloroquine. Our results show that Fun30 can relax negatively supercoiled DNA (Figure 3.7C, lanes 1-3, both panels), as observed by the reduce intensity of negatively supercoiled DNA band and increase relaxed circles. Furthermore, a similar result was observed with positively supercoiled DNA (Figure 37C, lanes 4-6, both panels). Lanes 7 and 8, in both panels, show Top1 relaxed plasmid and Kpn1 digested linear DNA as controls. Interestingly, this Fun30 relaxing activity does not seem to be typical of Topoisomerase I relaxing activity, but rather it was achieved by nicking the plasmid since the Fun30 relaxed products did not form a ladder of topoisomers (Figure 3.7C, compare lanes 2-3 and 5-6 to lane 7). Moreover, in the presence of chloroquine (Figure 3.7C, right panel) the bands migrated where nicked circles run and not where

closed circles migrate on the gel (right panel, compare lanes 5-6 to lane 7). Therefore, our results suggest that Fun30 can relax supercoiled plasmids by generating nicks.

To investigate the effect of ATP on the relaxing activity of Fun30, we repeated the relaxing assay above in the presence or absence of ATP with and without chloroquine (Figure 3.7D, right and left panels, respectively). For this, Fun30 was incubated with the negatively supercoiled 5 kb pG5E4-5S plasmid in the absence or presence of 4 mM ATP and the relaxing assay carried out as described above. The results showed that ATP did not significantly affect Fun30 relaxing activity (Figure 3.7D, compare lanes 2 and 4, both panels). In summary, here we show that Fun30 can relax both positively and negatively supercoiled plasmid DNA in an ATP-independent manner through DNA nicking.





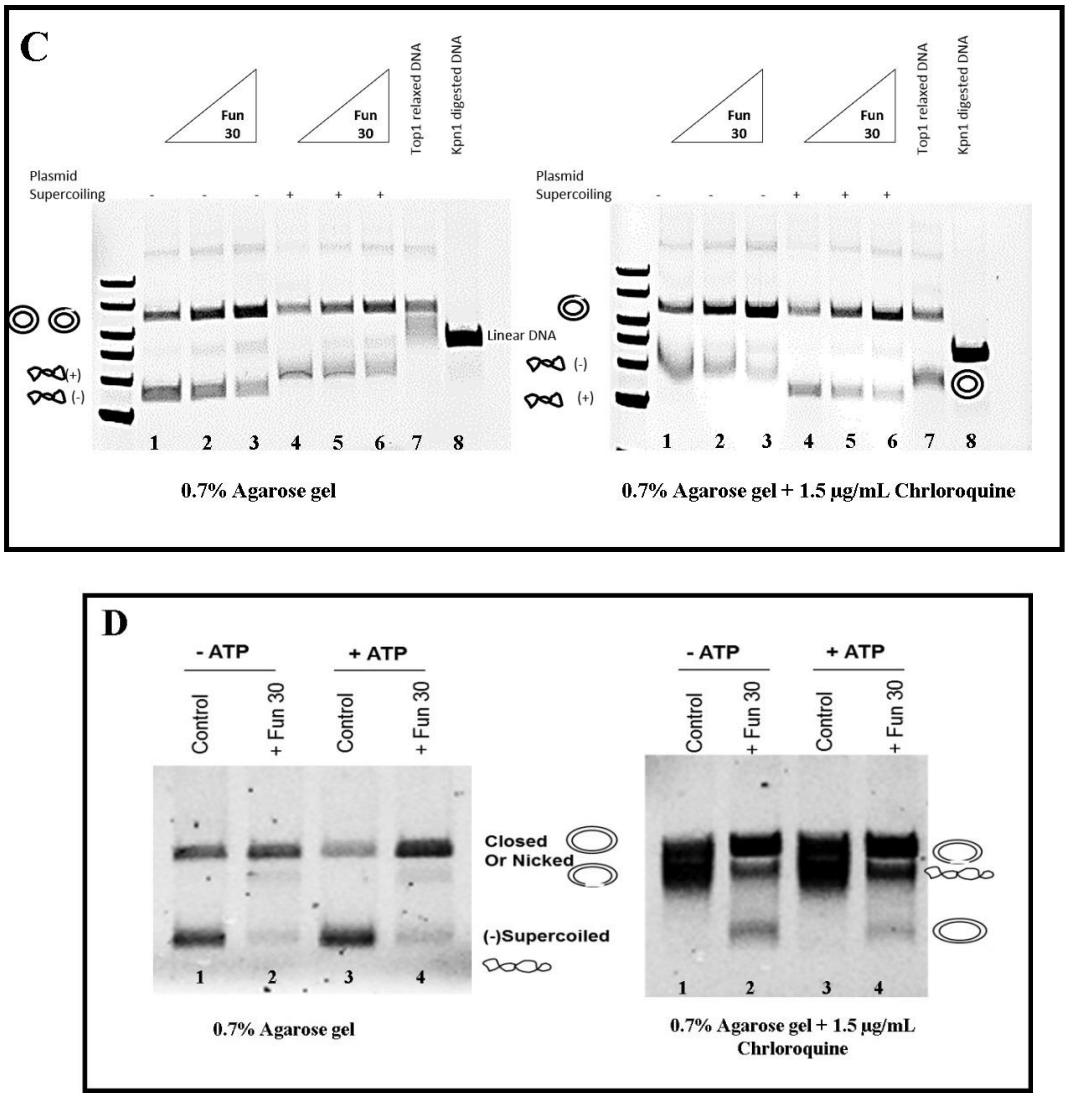


Figure 3.7: Fun30 can relax both negatively and positively supercoiled DNA in an ATP-independent manner by nicking DNA

(A) Diagram showing preparation of negatively and positively supercoiled DNA. Positively supercoiled pG5E4-5S plasmid was prepared by relaxing the plasmid by calf topoisomerase I in the presence of 20 µM netropsin, as described in materials and methods. (B) Diagram outlining the supercoiling/relaxation assay. (C) Supercoiling or relaxation assay performed by Fun30 (16 or 32 nM) in 20 µl reactions containing Top1 relaxing buffer, 50 ng of negatively or positively supercoiled pG5E4-5S plasmid. The reactions were incubated at 30 ^{o}C for 1 hour, stopped by proteinase K and SDS, resolved on 0.7% agarose gel with or without chloroquine, stained with ethidium bromide, and scanned by Typhoon. Lanes1 and 4, in both the left and the right panels, are controls showing the migration of negatively and positively supercoiled DNA, respectively. Negatively supercoiled DNA runs faster than positively supercoiled plasmid in gels lacking chloroquine (compare lanes 1 and 4 in left panel), while positively supercoiled plasmid runs faster in gels with chloroquine (compare lanes 1 and 4 right panel). Lanes 7, in both panels, are control samples showing TopI relaxed

plasmid and lane 8 show the plasmid linearized following Kpn1 digestion. Covalently closed relaxed plasmids and nicked plasmids run together in gels lacking chloroquine (lane 7, left panel), while covalently closed DNA migrates faster that nicked plasmid in chloroquine containing gels (lane 7, right panel). Results show that Fun30 can relax both negatively and positively supercoiled DNA. (D) Supercoiling or relaxation assay performed as above in Figure 3.7C in the presence of ATP. Here, 30 nM of Fun30 was incubated with either 50 ng of negatively supercoiled or positively supercoiled pG5E4-5S plasmid in the presence or absence of 4 mM ATP. The reaction was carried out and analyzed as above in the gels lacking or containing chloroquine shown in the left and the right panels, respectively. Results show that ATP did not significantly affect Fun30 relaxing activity.

3.8 Fun30 has a Nuclease Activity on 3' Overhangs

The observed nicking activity of Fun30 on supercoiled plasmids might indicate the presence of a nuclease activity on other DNA substrates. To test this, fluorescently labeled DNA substrates of structures such as forked duplex and DNA duplex with protruding 3' or 5' ends were prepared. In this assay, we tested whether Fun30 has a 3' or 5' nuclease activity. Our results show that Fun30 can only cleave 3' overhang (Figure 3.8A, lanes 3-4 and 7-8). Lane 1 shows the migration of the single stranded oligonucleotide as control and lanes 5 and 9 show where the products of the 3' overhang cleaved DNA for templates used in lanes 2-4 (forked duplex) and 6-8 (DNA with 3' overhang) run, respectively (Figure 3.8A). Arrows point to the products of the nuclease activity of Fun30 as a result of cleavage of 3' overhangs. These results show that Fun30 is able to cleave 3' overhangs in both the forked or 3' overhang substrates used in the reaction (Figure 3.8A, compare lanes 3-4 with lane 5 and lanes 7-8 with lane 9). In another experiment with additional substrates revealed the same results in that Fun30 nuclease activity was more efficient on substrates with protruding 3' ends (Figure 3.8B, compare lanes 2 and 4). Fun30 showed little or no activity on a substrate with protruding 5' end or blunt end DNA (Figure 3.8B, lanes 6 and 8,

respectively). From these results, we conclude that Fun30 preferentially cleaves substrates with 3’ overhangs.

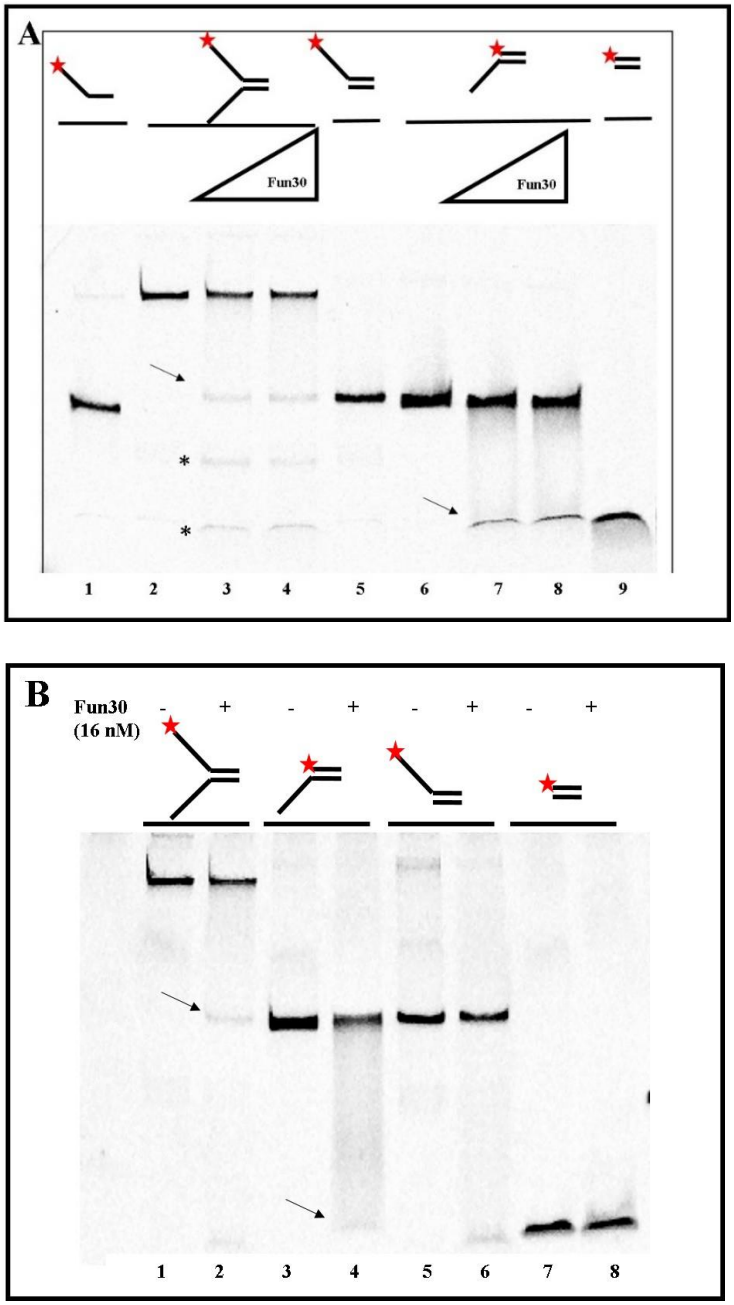


Figure 3.8: Fun30 can cleave 3’ overhangs in a forked duplex and in a DNA duplex with protruding 3’ ends in the absence of ATP

(A) Nuclease assay performed by adding Fun30 (15 or 20 nM) to 20 µl reactions containing 1 nM of Cy5-labeled forked duplex, or duplex labeled DNA with 3’ overhangs. The reactions were incubated for 30 minutes at 30 ^{o}C, stopped by proteinase K, resolved on 8% native acrylamide, and scanned using the Typhoon. Lane

1 is a control sample showing the migration location of the Cy5-labeled single strand oligonucleotide of the duplex DNA. Lane 5 shows the migration of a duplex DNA with 5’ overhang and lane 9 shows the location of blunt ended DNA duplex. The arrow in lanes 3 and 4 is pointing to the cleaved product of forked duplex template which migrate same as the DNA duplex with 5’ overhang in lane 5. The arrow in lanes 7 and 8 points to the cleaved product of the template with the 3’ overhang, which migrate the same as the blunt ended DNA duplex in lane 9. Asterisks show other possible cleaved products. (B) Nuclease assay repeated with either 1 nM each of labeled forked duplex, duplex with 3’ overhangs, duplex with 5’ overhangs, and blunt ended DNA duplex as described above in (A). Results show that Fun30 nuclease activity is more efficient on substrates with protruding 3’ ends.

Chapter 4: Results - The *In Vivo* Functions of Fun30

4.1 Role of Fun30 during Camptothecin Damage

4.1.1 Overview

DNA unwinding is an activity that takes place during several DNA processes such as DNA replication, transcription, and DNA damage repair. DNA unwinding would be easily accomplished if DNA was free to rotate. However, any rotation is hindered because of the constraints imposed by DNA binding proteins or due to DNA anchorage to sub-nuclear compartments. This will eventually lead to the accumulation of torsional strains, which is best explained by the generation of supercoiled DNA. Topoisomerases in general are enzymes that can relieve DNA topological strains (Postow et al. 2001, Bush, Evans-Roberts, and Maxwell 2015). Topoisomerase I (Top1) relaxes both negative and positive supercoils. Briefly, it acts by introducing a single strand nick that allows the DNA to relax, followed by re-ligation of the nicked DNA (Saleh-Gohari et al. 2005). In cancer therapy, many anticaner drugs act by targeting topoisomerases. Camptothecin, is a plant alkaloid that targets the Top1 enzyme to induce its toxicity (Nitiss and Wang 1988). During the cleavage step which is transient, a tyrosine in the active site of TopI attaches to the 3'-phosphate of the cleaved strand forming a transient covalent enzyme-DNA intermediate or a 3'-phosphtyrosyl and a 5'-OH end (Koster et al. 2007). Camptothecin acts by stabilizing this enzyme/DNA complex and thus delays the ligation step leading to single strand nicks (Megonigal, Fertala, and Bjornsti 1997). It is believed that such single strand nicks do not cause a problem per se, unless they are approached by a progressing replication fork, which could lead to replication fork collapse by converting these

single strand nicks into double strand breaks, ending in serious DNA damage, cell cycle arrest at G2 phase, and eventually cell death (Megonigal, Fertala, and Bjornsti 1997, Strumberg et al. 2000). Therefore, it is not affecting the activity of TopI that caused the damage observed with camptothecin but rather it is the stabilization of the enzyme/DNA complex that leads to DNA damage. This is supported by studies which show that Top1 is not an essential gene in yeast and that the deletion of Top1 confers resistance to camptothecin (Nitiss and Wang 1988, Megonigal, Fertala, and Bjornsti 1997). Moreover, Top1 mutants that lead to increased stabilization of enzyme/DNA complex were shown to kill cells in a mechanism similar to camptothecin, while a mutation that affects the tyrosine in the active site was shown to confer resistance to camptothecin toxicity (Megonigal, Fertala, and Bjornsti 1997). However, in a different study, another mechanism was proposed to explain the camptothecin toxicity (Koster et al. 2007). In this study, it was shown that Topotecan, a camptothecin analogue, causes reduced efficiency in relaxing positively supercoiled DNA as shown by the accumulation of a positively supercoiled 2 μm plasmid in treated cells. These positively supercoiled plasmids were also found not to be relaxed efficiently by Topoisomerase II (TopII). Based on their results, these authors suggest that this decreased ability to relax positively supercoiled DNA leads to the accumulation of toxic DNA lesions that could result in the stalling of the replication fork and its collapse generating toxic DSB (Koster et al. 2007). Camptothecin induced double-strand breaks are one-ended breaks, since they form when the replication machinery runs into single strand breaks. Such kind of one-ended break is repaired by homologous dependent-break induced replication (Saleh-Gohari et al. 2005). In support of this, DSBs induced by camptothecin were suggested to be repaired by HR as camptothecin treatment was shown to induce high levels of recombination. Such

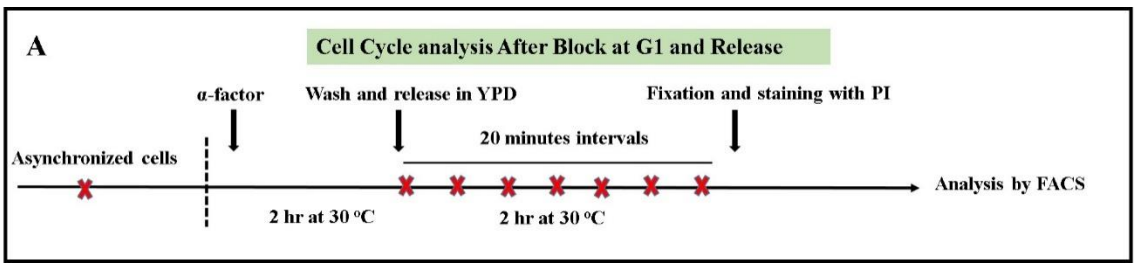
reliance of camptothecin-induced damage on HR was confirmed by showing that the sensitivity of yeast cells to camptothecin was enhanced when Rad52 was mutated (Nitiss and Wang 1988). This enhanced sensitivity after camptothecin treatment was believed to be from higher recombination levels rather than mutagenesis (Nitiss and Wang 1988). Although replication fork running into a TopI/DNA complex leading to DSB is a widely accepted mechanism, there also seems to be another source of mutagenicity that can result from the same TopI/DNA complex. This has been suggested to happen when a TopI/DNA linked to a nick is close to a downstream nick which is less than six base pairs away (Pommier et al. 1995). Release of this DNA fragment will lead to a gap and thus to the loss of the ligation partner. Such a structure will facilitate the strand invasion of even partially complementary DNA and its ligation to the 3' end leading to intermolecular illegitimate recombination (Pommier et al. 1995). In fact, in one study, it has been shown that illegitimate recombination was increased when TopI was overexpressed in yeast, which was explained by the ability of TopI to ligate non-homologous ends (Zhu and Schiestl 1996).

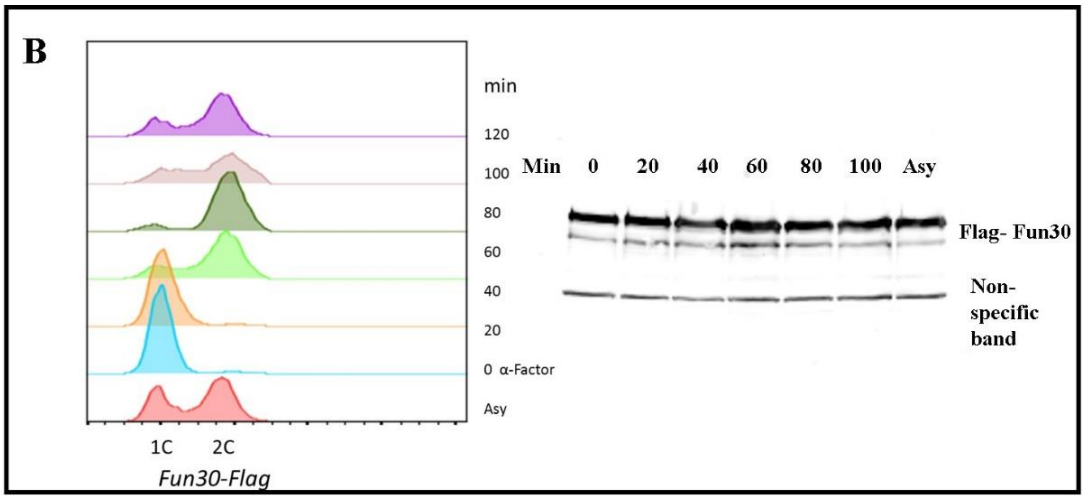
FUN30 is a non-essential gene, however, in the last decade some studies have pointed to its involvement in various cellular functions including HR, most of which have used Fun30 deletions to look for phenotypic difference. However, the fact that many non-essential genes have been found to at least be required in the absence of other pathways or genes, inspired many to look for such possible backup systems or redundant pathways in the case of Fun30 as well. Such redundant pathways can explain the non-essentiality of many genes as redundancies in the functions of certain genes can lead to no observable effect in a particular deletion. This chapter will focus on the roles of Fun30 in DNA repair and HR. For these *in vivo* experiments, we constructed

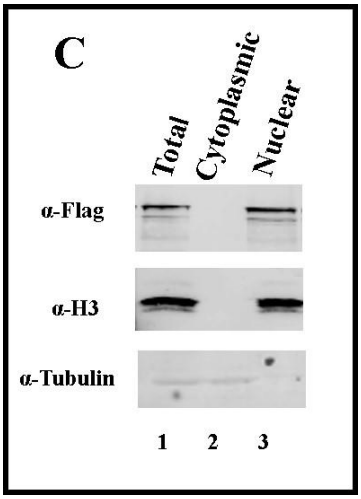
yeast strains lacking particular genes that are involved in DNA repair or HR in a wild-type or a Fun30 deletion backgrounds and screened for any potential genetic interactions between Fun30 and these genes. These experiments helped us shed light on any possible redundant pathways between Fun30 and genes involved in DNA repair and HR, in an attempt to better understand the *in vivo* functions of Fun30.

Among all the DNA damaging agents that have been tested on *Δfun30* strain, camptothecin had the highest toxicity. Such a response to camptothecin raises many questions such as: 1) Is Fun30 involved in resolving the lesions induced by this toxin? 2) Does Fun30 relieve constraints posed by this damage? And 3) Is Fun30 involved in resolving the intermediates that result from the repair of this kind of damage? To answer these questions, we compared *Δfun30* sensitivity to other null mutant strains that are known to repair such lesions or are known to be sensitive to them. Two genes (Tdp1 and Mus8) were chosen based on their role in camptothecin damage repair and TopI as it is implicated in this damage. However, first we checked the levels of Fun30 expression throughout the cell cycle. Regulation of protein activity can take place at different levels; one of those ways is regulation of its expression. Knowing whether the expression of a protein changes during the cell cycle can give us a clue on the importance of this protein at a particular phase of the cell cycle. To find out if Fun30 expression is altered during the cell cycle, a flag-tagged Fun30 strain was grown in YPD, synchronized with α-factor, and then released and grown for 2 additional hours in YPD. Samples were taken at different time intervals for both protein analysis and cell cycle progression (Figure 4.1A). FACS analysis performed at different times following release from G1 arrest with α-factor show progression of the flag-tagged Fun30 strain through the cell cycle (Figure 4.1B, left panel). The expression of Fun30

at the same time intervals (Figure 4.1B, right panel) shows no detectable change in protein expression at different stages of the cell cycle, suggesting that Fun30 is a ubiquitous protein. Since protein expression was not altered during the cell cycle, Fun30 localization in the cell was investigated. For this, we gently digested yeast cells expressing flag-tagged Fun30 with lysozyme and separated nuclear and cytoplasmic fractions following zymolyase digestion and spheroblast preparation. The expression of Fun30 in nuclear and cytoplasmic fractions was checked by western blot analysis (Figure 4.1C). Lane 1 is the total cellular protein levels. Our results show that Fun30 is a nuclear protein (see lanes 2 and 3). A nuclear protein can be either free or bound to chromatin constitutively or might get recruited to chromatin at certain phases of the cell cycle. To gain insight into the distribution of Fun30 in the nucleus between soluble and chromatin-bound fractions, flag-taggedFun30 cells were synchronized, released in YPD, and collected at different times following release as described above. Following separation of soluble and chromatin-bound fractions, where the soluble fraction included both cytoplasmic and nucleoplasmic proteins, western blot analysis was performed (Figure 4.1D). Our results show that Fun30 at G phase is mainly in the soluble fractions (lane 6), while it gets gradually recruited to chromatin during the S phase (compare lanes 9 and 12 to lane 6). Overall, these results show that Fun30 is a nuclear protein that is mostly in the soluble fraction that is recruited to chromatin as the cells progress through the S phase of the cell cycle.







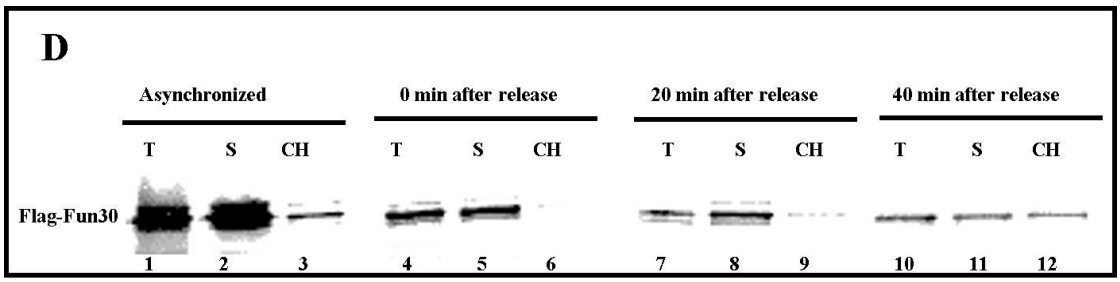


Figure 4.1: Fun30 is a ubiquitously expressed nuclear protein that is recruited to chromatin during the S phase of the cell cycle

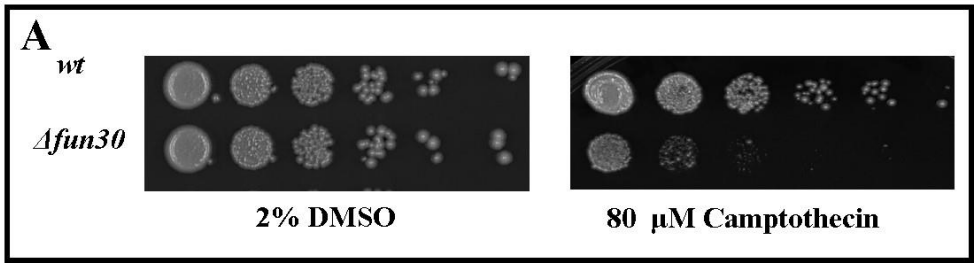
(A) Diagram outlining experiments where cells were cultured in YPD, synchronized with α-factor and released following this arrest, samples were taken at different time intervals (shown by x) for protein expression analysis and cell cycle progression. (B) Flag-tagged Fun30 strain progresses normally through the cell cycle and is

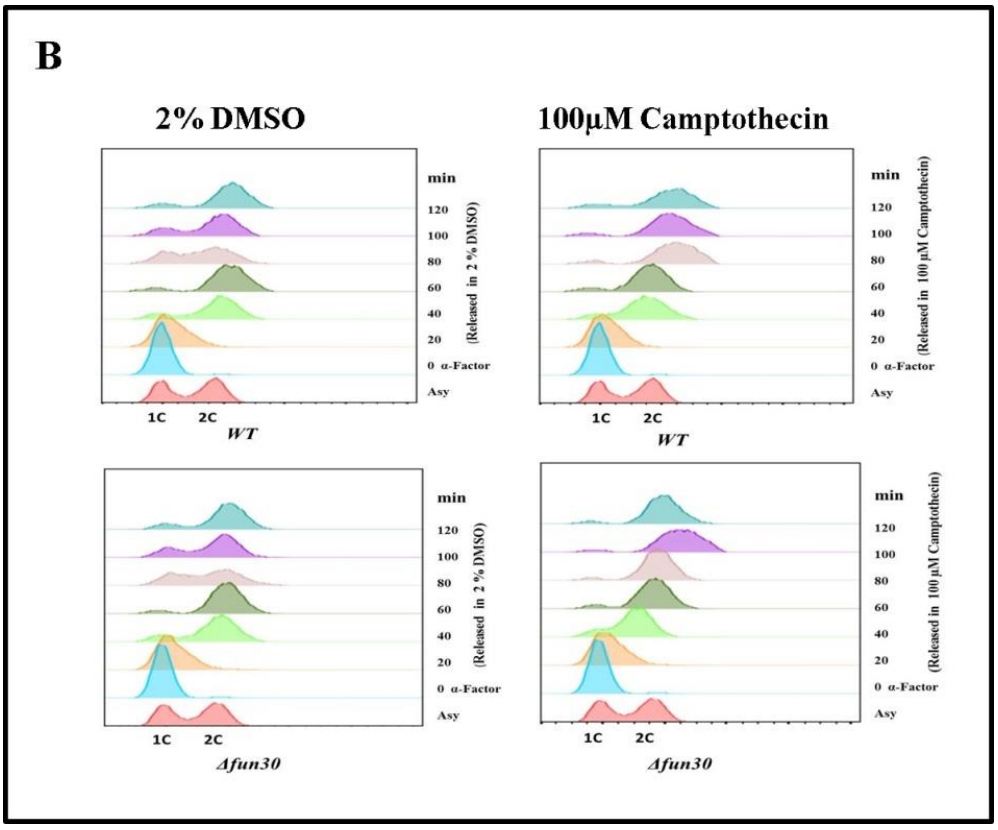
ubiquitously expressed. Flag-tagged Fun30 yeast cells were synchronized at the G1 phase by supplementing the media with 10 µg/ml of α-factor and incubating for 2 hours at 30 ^{o}C. Synchronization was confirmed under microscope, cells were subsequently washed extensively with water and released in YPD media and allowed to grow at 30 ^{o}C. Samples taken at release and every 20 minutes following release for up to 2 hours were fixed and processed for FACS analysis. Cells pellets equivalent to 2 OD at 600 nm were harvested at the same time intervals for protein expression analysis. For this, total cell protein was prepared with the rapid extraction method described in materials and methods, 30 µl of the protein were resolved on 6% SDS-PAGE gel and protein expression checked by western blot using anti-flag antibody. The left panel shows the cell cycle progression of the flag-tagged Fun30 strain following release. Results show normal progression of the strain through the cell cycle. The right panel shows ubiquitous expression of Fun30 during different phases of the cell cycle. (C) Fun30 localization in the cell. The distribution of Fun30 between the nucleus and the cytoplasmic fraction were measured in the flag-tagged Fun30 strain by growing these cells in a culture until an OD_{600} of 0.3. Cells equivalent to OD_{600} of 10 were then pelleted, and the cell walls gently digested with zymolyase to form spheroblasts. Spheroblasts were then pelleted and an extraction buffer with Triton x-100 and sorbitol was added to release the cytoplasmic protein, which were separated from the nuclear fraction by centrifugation at high speed, and resolved on an SDS-PAGE gel followed by Western blot analysis. Lane 1 shows total cell protein. Lane 2 and 3 show cytoplasmic and nuclear fractions, respectively. Results point to the Fun30 being a nuclear protein. (D) Chromatin association assay show the distribution of Fun30 in the nucleus between the soluble and chromatin-bound fractions. For this, flag-tagged Fun30 cells were synchronized with 10 α-factor and samples collected at different time intervals (0, 20, and 40 minutes) after release from G1, soluble and chromatin-bound fractions were separated and analysis by Western blotting. T is the total proteins, S is soluble and CH is chromatin-bound fractions. Results show that while Fun30 is mostly in the soluble nuclear fractions in asynchronized cells and in G1, it associates with chromatin during the S phase of the cell cycle.

4.1.2 Progression through the S Phase is Slightly Slower in the *Δfun30* Compared to the Wild-type in the Presence of Camptothecin

Deletion of Fun30 makes cells more sensitive to camptothecin. As mentioned previously, this chemical can stabilize TopI/DNA complexes leading to accumulated torsional strain, stall the replication fork, and potentially cause DSBs. Such conditions can result in the activation of the S phase cell cycle check point and can consequently

lead to a slower replication fork movement or even replication pause. The sensitivity of *Δfun30* to camptothecin led us to investigate whether this sensitivity was because of the inability of cells to deal with camptothecin damage and thus affect the progression of replication forks during the S phase. Towards this, wild-type and *Δfun30* cells were serially diluted and spotted on YPD plates in the presence of 80 µM camptothecin (Figure 4.2A). Camptothecin was dissolved in 2% DMSO and therefore growth on a 2% DMSO plate was the control. As in previous studies, *Δfun30* was found to be more sensitive to camptothecin compared to the wild-type cells (Figure 4.2A). To test if the progression of S phase was affected in *Δfun30*, both wild-type and *Δfun30* were synchronized at G1 with α-factor and then released, washed, and grown in YPD containing either 2% DMSO for control or 100 µM camptothecin. Progression through the cell cycle was monitored by taking cells at 20 minute intervals, fixing, and staining them with Propidium Iodide (PI), followed by FACS analysis (Figure 4.2B). The results show that both strains (*Δfun30* and *WT*) had similar kinetics of progression through the S phase in presence of 2% DMSO, while upon camptothecin treatment, *Δfun30* cells had slightly slower progression compared to wild-type cells. This was more evident when the cell profile of wild-type and *Δfun30* samples were taken after 40 minutes (Figure 4.2C) and 80 minutes (Figure 4.2C and D) were overlapped. These results show that cell cycle progression for *Δfun30* cells are somewhat delayed compared to the wild-type upon camptothecin damage.




B
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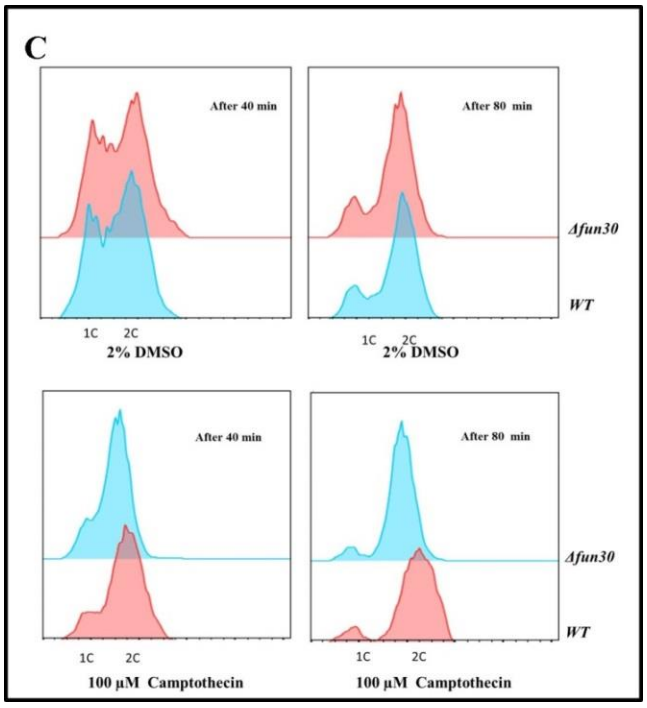
100µM Camptothecin
min
120
100
80
60
40
20
0 α-Factor
Asy
(Released in 2 % DMSO)
(Released in 100 µM Camptothecin)
1C
2C
WT
Δfun30


C
After 40 min
After 80 min
Δfun30
WT
1C
2C
2% DMSO
100 µM Camptothecin

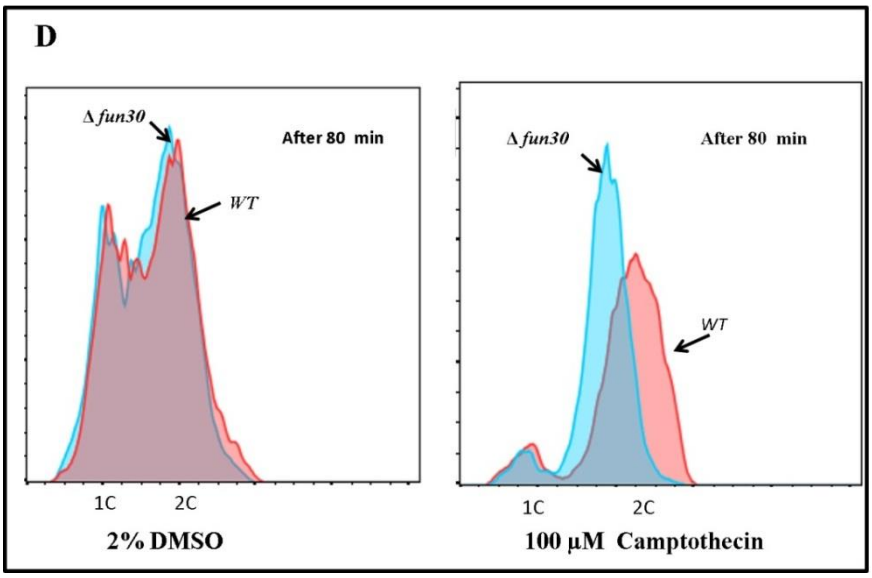


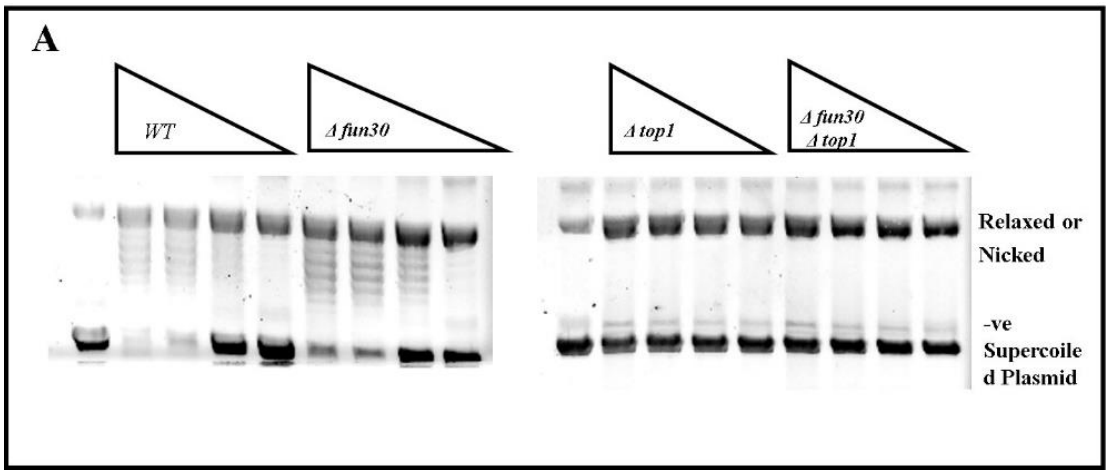
Figure 4.2: Fun30 deletion is sensitive to camptothecin and has a slower progression through the cell cycle compared to wild-type

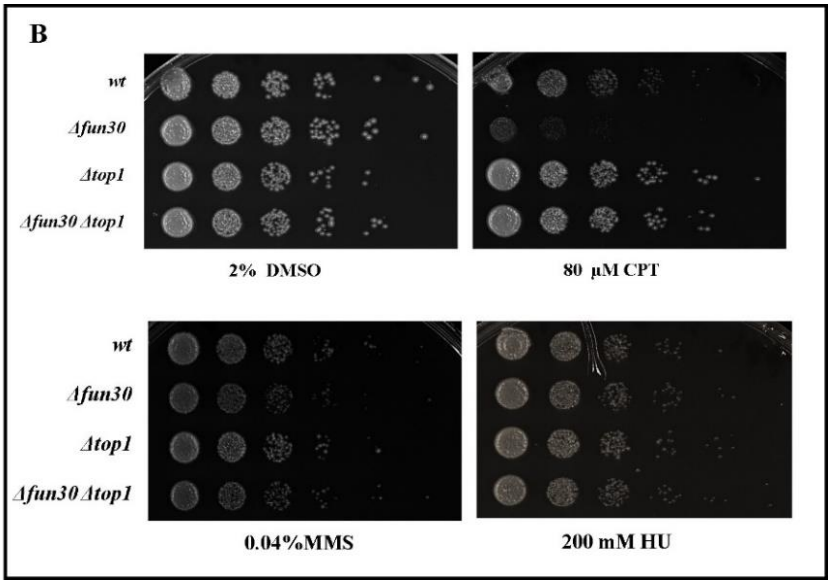
(A) Growth assay performed when wild-type and the *Δfun30* mutant were serially diluted and spotted on YPD plates containing either 2% DMSO as control or 80 µM camptothecin and allowed to grow for 2 days. In the presence of 80 µM camptothecin, cells lacking Fun30 grow much slower compared to the wild-type cells. (B) The progression of cell lacking Fun30 through the cell cycle is delayed. Wild-type and *Δfun30* mutant cells were synchronized at G1 phase with α-factor, washed and released from G1 by suspending them in YPD media containing either 2% DMSO or 100 µM camptothecin. Samples were taken at 20 minute time intervals, stained and analyzed for DNA content by FACS. (C) The overlay of FACS profile of wild-type and *Δfun30* samples taken after 40 or 80 minutes (left and right panels, respectively) in control media with 2% DMSO or 100 µM camptothecin (upper and lower panels, respectively). (D) The overlay of FACS profile of wild-type and *Δfun30* samples taken after 80 minutes in control media with 2% DMSO or 100 µM camptothecin (left and right panels, respectively) for better comparison.

4.1.3 The Sensitivity of *Δfun30* to Camptothecin is Specific to TopI Lesions

To test the specificity of Fun30 to Top1 lesions that are induced by camptothecin, we investigated the sensitivity of *Δfun30* to camptothecin in a strain that lacked Top1. First, we tested the effects of Top1 deletion on the relaxing activity in the cells. This was done by adding a serial dilution of cellular extracts of wild-type, *Δfun30, Δtop1,* and *Δfun30Δtop1* strains to a reaction buffer that had negatively

supercoiled pG5E4-5S plasmid. Our results show lack of relaxing activity in the extract of *Δtop1* and *Δfun30Δtop1* (Figure 4.3A, right panel). This is in contrast to the relaxing activity observed in the wild-type and *Δfun30* strains (Figure 4.3A, left panel). If the sensitivity to camptothecin that is conferred by Fun30 deletion is specific only to Top1 lesions, then this implies that deletion of TopI should reverse the sensitivity of *Δfun30* cells. To test this, wild-type, *Δfun30, Δtop1, Δfun30Δtop1* cells were serially diluted and spotted on YPD plates containing either 2% DMSO for control or 80 µM camptothecin, 0.04% MMS, or 200 mM HU (Figure 4.3B). Results shows that while *Δfun30* was found to be slightly sensitive to MMS and HU compared to the wild-type, it was more sensitive to camptothecin as shown before. More importantly, this camptothecin sensitivity was suppressed when TopI was deleted (i.e. in the double deletion strain (*Δfun30Δtop1*)). This was confirmed by FACS analysis, which shows that wild-type and *Δfun30* cells were blocked at G2 upon camptothecin addition, while *Δtop1* and *Δfun30Δtop1* mutants were able to proceed normally thought the cell cycle despite camptothecin treatment (Figure 4.3C). The absence of damage in *Δtop1, Δfun30Δtop1* strains was further confirmed by the absence of the damage signals such as Rad53 phosphorylation in cell extracts of these strains, following camptothecin treatment (Figure 4.3D, lanes 7-8). Under the same conditions, H2A phosphorylation was the same in all of these strains. These findings show that Fun30 deletion confers sensitivity to camptothecin and suggest that this sensitivity is specific to TopI lesions induced by camptothecin.


A
WT
Δ fun30
Δ top1
Δ fun30 Δ top1
Relaxed or Nicked
-ve Supercoiled Plasmid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wt
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Δtop1
Δfun30 Δtop1
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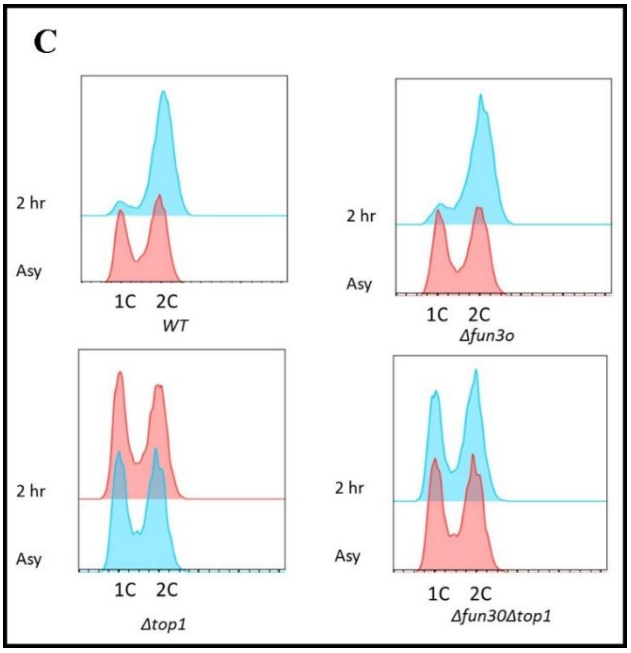
0.04%MMS
200 mM HU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Asy
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WT
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Δtop1
Δfun30Δtop1

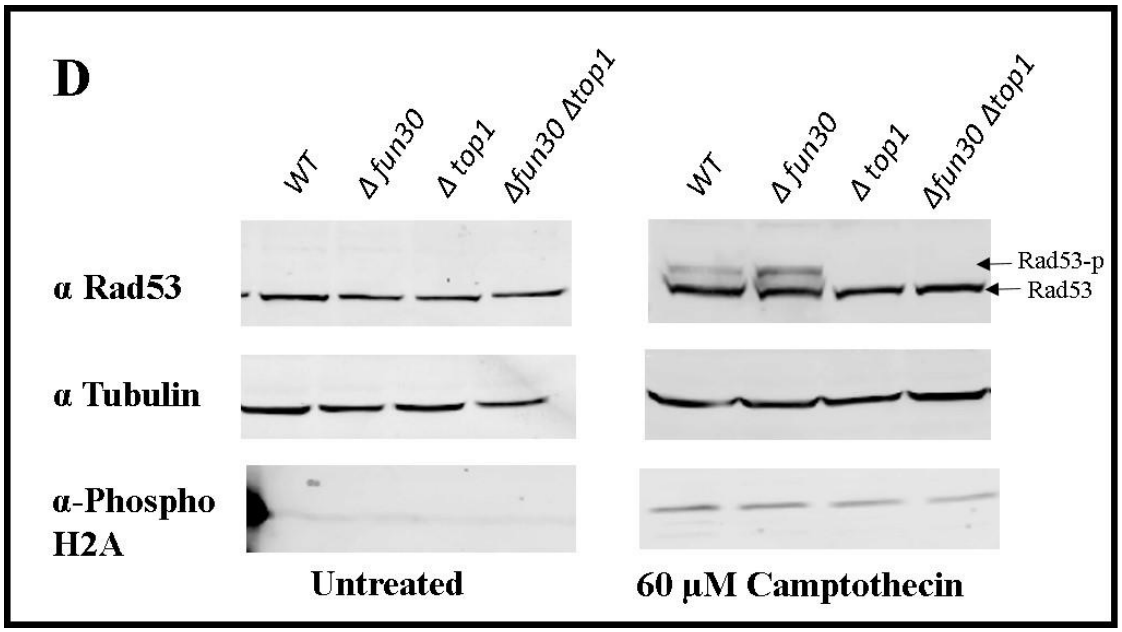


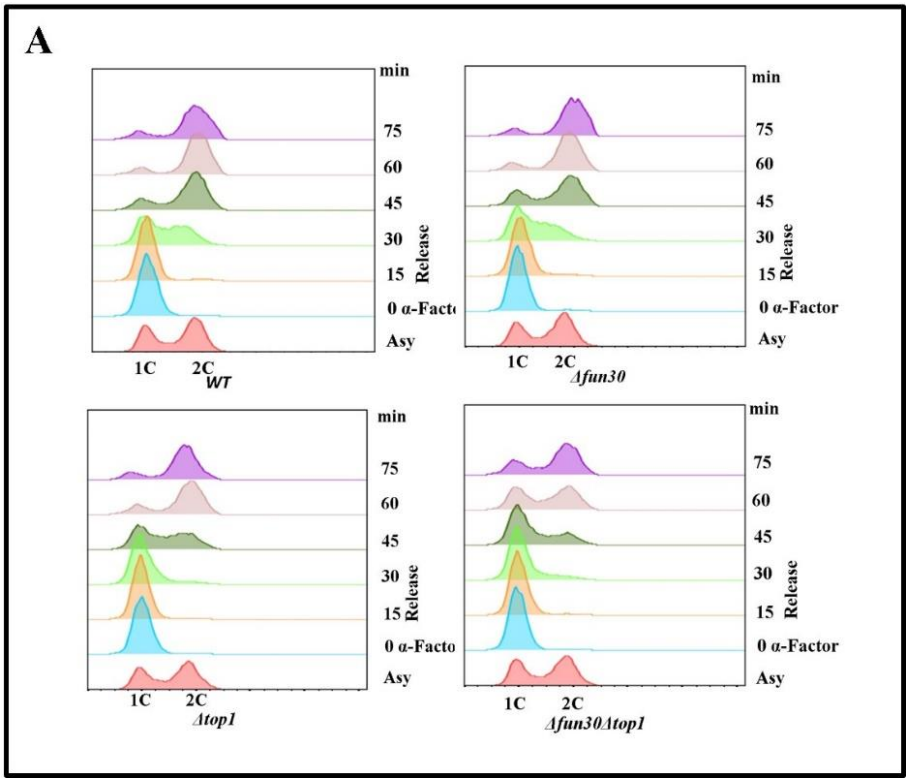
Figure 4.3: The sensitivity of *Δfun30* to camptothecin is specific to TopI lesions

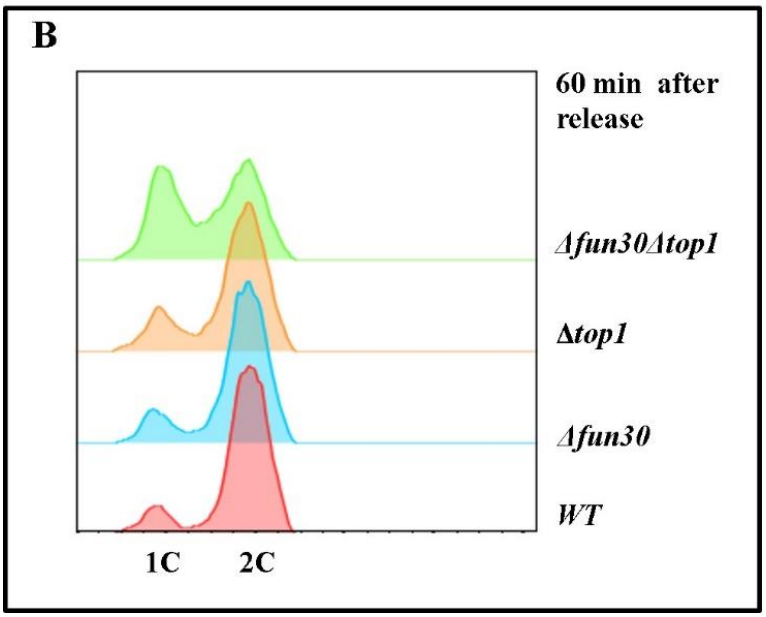
(A) DNA relaxing activity assessed in cells extracts prepared from wild-type, *Δfun30, ΔtopI,* and *Δfun30ΔtopI* strains. 100 ng of pG5E4-5S was incubated with 2 µl from each of the serial dilution of cellular extracts from each of these strains in a 20 µl reaction containing TopI buffer for 1 hour, stopped, the products resolved on 0.7% agarose gel, stained with ethidium bromide, and scanned using the Typhoon. The left gel shows extracts from the wild-type and *Δfun30* with intact relaxing activity as expected, and the right gel shows extracts from *ΔtopI* and *Δfun30ΔtopI* cells with no relaxing activity. (B) Growth assay of wild-type, *Δfun30, ΔtopI*, and *Δfun30ΔtopI* cells on YPD plates containing 80 µM camptothecin, 0.04% MMS, or and 200 mM HU. Results show that while *Δfun30* is slightly sensitive to MMS and HU compared to the wild-type, it is more sensitive to camptothecin, as shown before. More importantly, this camptothecin sensitivity is suppressed when TopI is also deleted (in the *Δfun30Δtop1*strain). (C) FACS profile of these strains following treatment with 60 µM camptothecin. Results show that wild-type and *Δfun30* cells are blocked at G2 upon camptothecin addition, while *Δtop1* and *Δfun30Δtop1* mutants are able to proceed normally thought the cell cycle despite camptothecin treatment. (D) Levels of Rad53 phosphorylation and γH2A in cell extracts of these strains following camptothecin treatment. Total cell lysates was extracted from these cells after two hours of treatment with 60 µM camptothecin by breaking the cells in the presence of TCA. The expression of Rad53, γH2A, and tubulin and was detected by Western blotting. The levels of RAD53 phosphorylation in *ΔtopI* and *Δfun30ΔtopI* mutants is greatly reduced in camptothecin-treated extracts, while γH2A levels are not significantly affected.

4.1.4 Fun30 is Required for Normal Progression Through the S Phase of the Cell Cycle in Cells Lacking TopI

Accumulation of positively supercoiled DNA and the inability of TopII to remove these torsional strains followed by replication fork collapse as a result of stalling have been suggested as a mechanism to explain the toxicity of camptothecin. However, it is also possible that the presence of camptothecin could hinder TopI activity, which in turn could lead to the accumulation of positively supercoiled DNA. Since Fun30 deletion leads to increased camptothecin sensitivity, we suggest that Fun30 might be a backup pathway to remove the torsional strains built as a result of the reduced activity of TopI. However, our results on the effects of Fun30 and TopI double deletion on cell viability showed that *Δfun30ΔtopI* grows normally in the absence or presence of camptothecin. Lack of any sensitivity in the double deletion strain might be due to the presence of other topoisomerases such as TopII that would relax any buildup of torsional strains. It is also reasonable to assume that any torsional strain would lead to some effect on the rate of replication. Therefore, we sought to test the effect of these deletions on cell cycle progression under non-damaging conditions. Towards this, wild-type, *Δfun30, ΔtopI*, and *Δfun30ΔtopI* cells were synchronized with α-factor at G1, released in YPD, and their progression through the S phase was monitored by FACS (Figure 4.4). The results show that while the wild-type and *Δfun30* strains progressed normally through the cell cycle, *ΔtopI* cells were slightly slower (Figure 4.4A). However, the progression of the double deletion (*fun30ΔtopI*) was significantly slower as evident by increased accumulation of cells at the G1 phase in *Δfun30ΔtopI* compared to the other cells tested (Figure 4.4A). This delay in progression through the cell cycle can be better observed when the cell profile of FACS samples taken after 60 minutes were overlapped (Figure 4.4B). The accumulation of

the double deletion cells at the G1 phase could be due to the inability of these cells to maintain the normal rate of replication because of the buildup of torsional strains. Alternatively, these cells could experience more difficulty in exiting the G1 phase. Measuring the doubling time of each strain might provide some answers towards this end, since a higher doubling time would indicate a slower replication. For this, we measured the doubling time of the different strains (Figure 4.4C). The results show no significant difference in their doubling time, although that *Δfun30Δtop1* grew slower. These data suggest that the *Δfun30Δtop1* cells have more difficulty in entering the S phase of the cell cycle; however, those that succeeded will proceed with normal kinetics (Figure 4.4C).





C

Strain	Doubling Time
Wild	75.84
Δ Fun30	78.6
Δ Top1	75
Δ Fun30 Δ Top1	77.4

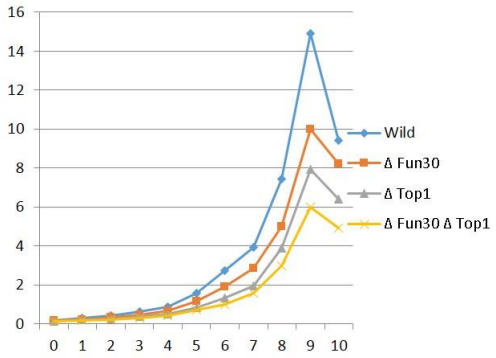


Figure 4.4: Fun30 deletion affects normal progression of cells that lack TopI

(A) FACS analysis of DNA content of wild-type, *Δfun30*, *ΔtopI*, and *Δfun30ΔtopI* cells under non-treated conditions. These cells were synchronized at the G1 phase with α-factor, washed and released from G1 by suspending them in YPD. Samples were taken at 20 minute time intervals, and analyzed for DNA content by FACS, as before. (B) An overlay of FACS profile of these strains after 60 minutes of release in YPD for better comparison. Results show while the wild-type and *Δfun30* strains progressed normally through the cell cycle, *Δfun30ΔtopI* cells were significantly slower. (C) Doubling time of the different strains measured in liquid YPD media. Results show no difference in their doubling time of the strains, although *Δfun30ΔtopI* cells grow slower.

4.1.5 Fun30 is not Redundant with Tdp1

Covalent complexes formed between TopI and DNA during the process of DNA relaxation should be removed before DNA is re-ligated. However, as mentioned above camptothecin can delay this step leading to dead-end complexes and consequently to cell death (Figure 4.1A). However, cells have developed mechanisms to resolve such DNA lesions. Tdp1 (tyrosyl-DNA phosphodiesterase 1) which is highly conserved in eukaryotes, was initially identified based on its ability to remove these dead-end complexes (Yang et al. 1996, Pouliot et al. 1999). In budding yeast, Tdp1 activity was mainly detected in yeast extracts using substrates with TopI/DNA complexes; however, *in vivo* toxicity to camptothecin has been reported to be not very significant (Pouliot et al. 1999, Liu, Pouliot, and Nash 2004). The presence of other pathways or mechanisms that work in parallel to Tdp1 could explain this as the sensitivity to camptothecin would only be observed when two or more pathways are deleted together (Pouliot et al. 1999, Liu, Pouliot, and Nash 2004). Interestingly, despite the lack of sensitivity on cell growth, deletion of Tdp1 causes increased spontaneous mutation rates, but with no gross chromosomal rearrangements (Liu, Pouliot, and Nash 2004). In addition, cells lacking Tdp1 treated with camptothecin were found to accumulate in mid-anaphase suggesting a role in genome stability (Pouliot et al. 1999, Liu, Pouliot, and Nash 2004). Other pathways that were found to be redundant with Tdp1 included the structure specific nucleases Rad1, Mus81, SLX4, and Sae2 (Deng et al. 2005, Liu, Pouliot, and Nash 2002, Vance and Wilson 2002). Interestingly, Tdp1 is only able to cleave TopI/DNA complexes that are at the ends of a DNA but not when they are located in the middle of a nicked duplex. This indicates that Tdp1 can work on TopI/DNA complexes only after DSBs are formed. This is in contrast to the idea that Tdp1 has a protective role in preventing DSB formation by

removing lesions before they develop into DSBs. However, such requirement for DSB was further confirmed when Rad52 was shown to be epistatic to Tdp1. The higher sensitivity of Rad52 deletion strain compared to Tdp1 deletion was explained by presence of other pathways that depend on Rad52 along with Tdp1 to repair this damage (Pouliot, Robertson, and Nash 2001).

Since Fun30 deletion sensitivity to camptothecin was found to be specific to TopI lesions, we sought to test whether Fun30 acts in a parallel or redundant pathway with Tdp1, a phosodiesterase which removes the TopI/DNA complexes. To accomplish this, wild-type, *Δtdp1, Δfun30* and the double mutant *Δtdp1Δfun30* cells were serially diluted and spotted on YPD plates containing either 40 or 80 µM camptothecin or 2% DMSO for control, as before (Figure 4.5B). If Fun30 acts in a parallel pathway or is redundant to Tdp1, then the double mutant should display increased sensitivity to camptothecin. All strains grew normally in 2% DMSO containing YPD plates, however, *Δfun30* and *Δtdp1* cells were both found to be more sensitive to 80 µM camptothecin compared to the wild-type cells. Moreover, *Δfun30* showed slightly higher sensitivity than the *Δtdp1* cells, under these conditions. The double deletion (*Δtdp1Δfun30*), was found to be as sensitive as *Δfun30* alone (Figure 4.5B). These results suggest that Fun30 is not likely to act in similar manner as Tdp1 in removing the TopI/DNA complexes, and the higher sensitivity of *Δfun30* compared to *Δtdp1* indicate that Fun30 may play a more important role than Tdp1 during camptothecin damage. The epispastic relation between these two genes suggests that Fun30 might be working downstream of Tdp1 (and other genes that may work redundantly with Tdp1) and is probably involved in resolving downstream products that result from repairing camptothecin damaged sites.

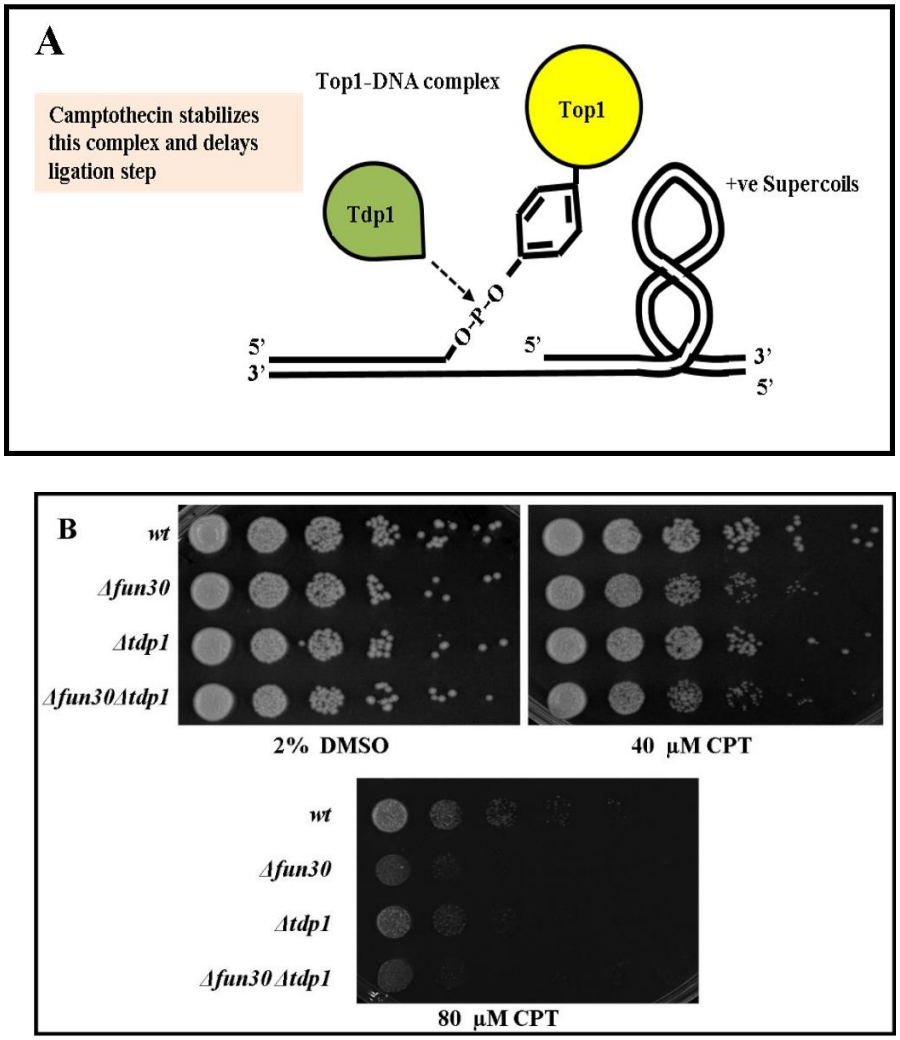


Figure 4.5: Fun30 is not redundant with Tdp1

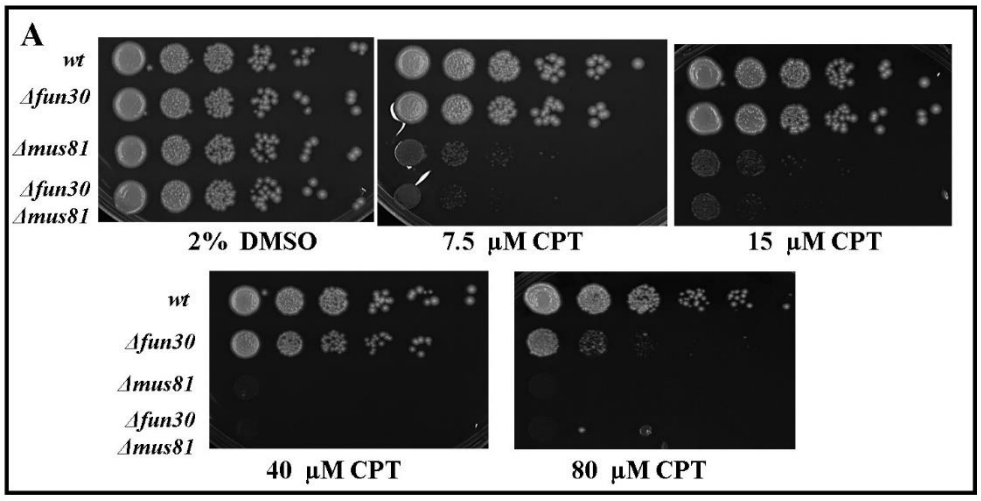
(A) A diagram that shows the substrate for Tdp1. (B) Growth assay of wild-type, *Δtdp1*, *Δfun30*, and *Δfun30Δtdp1* cells were serially diluted and spotted, as before, on YPD plates with either 2% DMSO as a control or 40 and 80 µM camptothecin. The *Δfun30Δtdp1* cells exhibits similar sensitivity to that of *Δfun30* ruling out a redundant role between Fun30 and Tdp1.

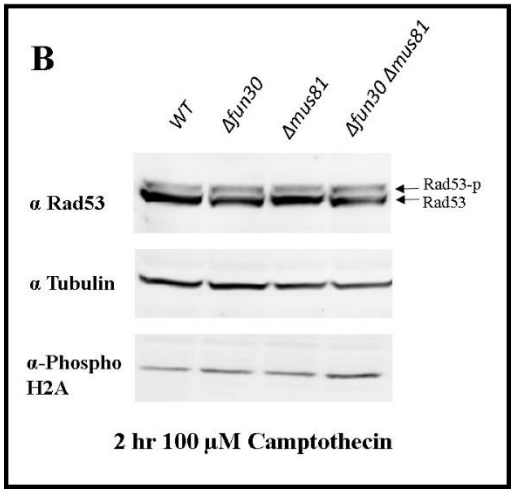
4.1.6 Fun30 Deletion is Less Sensitive to Camptothecin Induced Damage Compared to Mus81 Deletion and Fun30 Genetically Interact with Mus81 upon Camptothecin Induced Damage

Cells lacking Mms4-Mus81 are sensitive to camptothecin induced DNA damage, and as mentioned above Mus81 has been shown to be redundant with Tdp1.

This sensitivity could possibly be due to the role of Mms4-Mus81 in dealing with recombination products that form as result of the regression of a stalled replication forks. However, expression of RusA, a bacterial HJ resolvase, was shown to partially rescue the sensitivity of the mutant to camptothecin (Bastin-Shanower et al. 2003). This rescuing was not dependent on HR, ruling out a role in resolving HR structures into DSBs. It is believed that Mus81, which acts as a structure-specific nuclease, is most probably involved in the later steps of SDSA, where an invading 3' strand is over replicated and is displaced back and annealed to its parental molecule forming a 3' flap. This 3' flap is most probably the substrate for Mus81 and the removal of this flap would allow the completion of the repair (Bastin-Shanower et al. 2003).

The Mus81 endonuclease plays a role in resisting camptothecin induced damage. To explore if Fun30 act in a parallel pathway to Mus81 in repairing camptothecin induced damage, both single mutants of *Δfun30* and *Δmus81* and the double mutant strain *Δfun30Δmus81* were serially diluted and spotted on YPD plates containing increasing concentrations of camptothecin (7.5 to 80 µM) or 2% DMSO for control, as before (Figure 4.6A). All strains grew normally in 2% DMSO containing YPD plates. Unlike *Δfun30* cells which were sensitive to high concentrations (80 µM) of camptothecin compared to the wild-type, *Δmus81* cells were sensitive to much lower concentrations of camptothecin (7.5 µM) (Figure 4.6A). The double mutant (*Δfun30Δmus81*) was only slightly more sensitive to camptothecin at lower concentrations (7.5 or15 µM) compared to the single Mus81 deletion (*Δmus81*). These findings indicate that Mus81 has a more important role in repairing camptothecin induced damage than Fun30, while the slight increased sensitivity of the double mutant indicates that Fun30 acts in a parallel pathway with Mus81.


A
wt
Δfun30
Δmus81
Δfun30
Δmus81
2% DMSO
7.5 µM CPT
15 µM CPT
wt
Δfun30
Δmus81
Δfun30
Δmus81
40 µM CPT
80 µM CPT


B
WT
Δfun30
Δmus81
Δfun30 Δmus81
α Rad53
Rad53-p
Rad53
α Tubulin
α-Phospho H2A
2 hr 100 µM Camptothecin

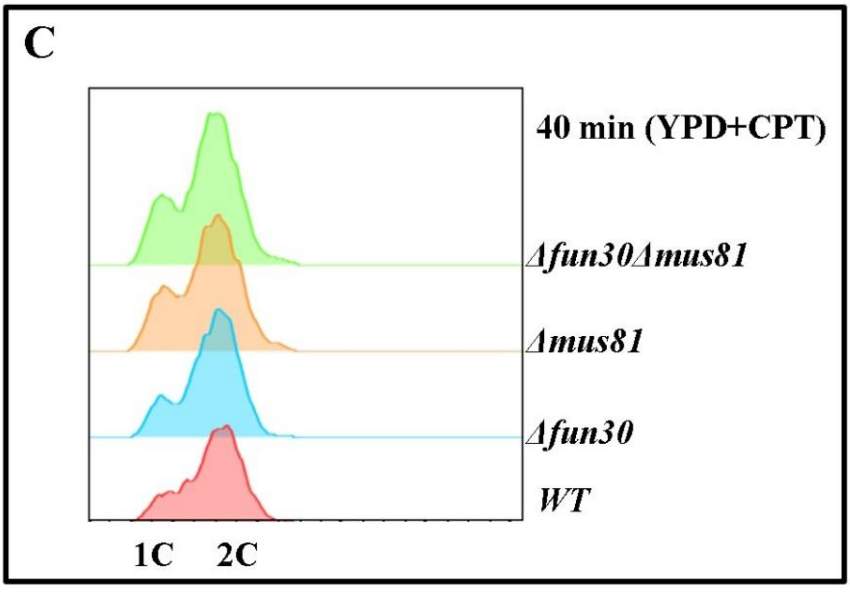

C
40 min (YPD+CPT)
Δfun30Δmus81
Δmus81
Δfun30
WT
1C
2C

Figure 4.6: Fun30 deletion is less sensitive to camptothecin induced damage compared to Mus81 deletion and Fun30 genetically interact with Mus81 upon camptothecin induced damage

(A) Growth assay comparing Fun30 and Mus81 deletions to the double deletion following camptothecin addition. Wild-type *Δfun30*, *ΔMus81*, and *Δfun30Δmus81* cells were serially diluted and spotted, as before, on YPD plates with either 2% DMSO as a control or increasing concentrations of camptothecin (7.5-80 µM). *Δmus81* mutant is far more sensitive than *Δfun30* indicating a more important role of Mus81 than Fun30 upon camptothecin damage. The slight increase in sensitivity in the double deletion (*Δfun30Δmus81*) indicates that Fun30 and Mus81 may act in independent pathways but genetically interact. (B) Higher sensitivity of *Δfun30Δmus81* is not due to defects in the cell cycle checkpoint. Total cell lysates was extracted from the cells after two hours of treatment with 100 µM camptothecin by breaking the cells, as described before in the presence of TCA. The expression of Rad53, γH2A, and tubulin and was detected by Western blotting. The levels of RAD53 phosphorylation in these deletions were not affected with camptothecin treatment suggesting that the higher sensitivity observed in the double deletion in (A) was not due to defects in cell cycle checkpoint activation. (C) Higher sensitivity of the *Δfun30Δmus81* to camptothecin is not due to replication defects. The wild-type, *Δfun30*, *Δmus81*, and *Δfun30Δmus81* cells were blocked at the G1 phase by α-factor and released into YPD media containing 100 µM camptothecin. Samples were taken at 20 minute time intervals and analyzed for DNA content by FACS. The figure shows an overlay of FACS profile of these strains after 40 minutes of release in YPD. As can be seen *Δfun30* progressed slower through the S phase in the presence of camptothecin compared to the wild-type. The *ΔMus81* cells progressed at an even slower rate, however the double deletion was not much slower than *ΔMus81* alone, suggesting that defects in replication is not likely the reason for the increased sensitivity observed in the double mutant.

4.1.7 Higher Sensitivity of *Δfun30Δmus81* is not due to a Defect in the Cell Cycle Checkpoint

In activation of cell cycle checkpoints can lead to serious cell damage. The higher sensitivity of the double deletion of Fun30 and Mus81 (*Δfun30Δmus81*) to camptothecin damage raised a question on whether this was due to defective checkpoint activation in the double mutant. To test this possibility, we checked the expression of phosphorylated Rad53 and γ-H2A in these strains following damage

(Figure 4.6B). The results show no observable difference in the expression of these phosphorylated protein suggesting that the higher sensitivity in the double deletion was not due to defective checkpoint activation. Another explanation for the higher sensitivity of the double mutant can be the inability of the mutant to progress through the S phase or to maintain proper rate of replication in the presence of camptothecin damage. To test this, all strains were again blocked at the G1 phase, as mentioned above, and were released in YPD with 100 μM camptothecin (Figure 4.6C). Samples were taken at time intervals of 20 minutes, and processed for FACS. An overlay of FACS profiles of the four strains after 40 minutes (Figure 4.6C) shows a slight cell cycle delay in the absence of Fun30 as observed with the growth assay in Figure 4.6A. This delay was more significant in the Mus81 deletion. However, the double deletion mutant did not show further delay in replication, suggesting the reason for higher sensitivity in the double mutant is not due to defects in replication.

4.2 Fun30 Genetically Interacts with Mus81 upon Treatment with other DNA Damaging Agents (HU and MMS)

4.2.1 Overview

Many lines of evidence point to the role of Fun30 in HR. The first evidence for the role of Fun30 in HR came when Fun30 was shown to facilitate long range DNA resection, which is an initial step in HR. However, defects in DNA resection is not a problem that would impede repair since cells are able to cope with this defect and efficiently repair DSBs. Therefore, it is possible that Fun30 plays additional roles other

than DNA resection since resection alone cannot explain the phenotypes observed in Fun30 deletion. Other studies show increased levels of recombination and crossover products in the absence of Fun30 (Chen et al. 2012a), as well as increased BIR (Costelloe et al. 2012), suggesting an anti-recombinogenic role of Fun30. Moreover, it has been shown that Fun30 deletion can reverse cell sensitivity to DNA damaging agents in the absence of Rad5, which is implicated in post replication repair pathway (Bi et al. 2015). In an another study, possible genetic interaction between Fun30 and Sgs1 (a DNA helicase involved in dissolution of double Holliday Junctions) in the presence of camptothecin damage was suggested since the double deletion was found to be more sensitive than each of the single deletions (Chen et al. 2012a). If the role of Fun30 was only to facilitate DNA resection through Sgs1 (i.e. they are in the same pathway), then this genetic interaction should not be additive. These results suggest that Fun30 and Sgs1 have redundant roles which can affect the viability of cells during DNA damage. Based on these observed phenotypes that support an anti-recombinogenic role for Fun30, we suggest that Sgs1, which has a role in resolving recombination intermediates that form during HR, may genetically interact with Fun30. In other words, if Fun30 deletion causes increased recombination, then there will be increased level of recombination intermediates that could add additional problems to the cell in the absence of Sgs1. These difficulties may be more apparent under DNA damaging conditions which rely on HR for repair. If repair is not efficient, then, this will result in the accumulation of toxic recombination products. Based on the genetic interaction between Fun30 and Sgs1, we hypothesized that Fun30 may also genetically interact with Mus81, a nuclease that is involved in resolving recombination products such as HJ similar to Sgs1. Below I briefly explain the role of Mus81 in recombination and other pathways.

Mms4-Mus81 complex was identified in a synthetic lethal screen that aimed at finding proteins that are required in the absence of Sgs1 (Mullen et al. 2001). Forming a heterodimer explains why single deletions of each of the subunits of the Mms4-Mus81 complex as well as the double deletion resulted in similar sensitivity to DNA damaging agents observed. Based on the observed synthetic lethality with Sgs1, it was proposed that the Mms4-Mus81 complex is involved in resolving recombination intermediates, which are generated during DNA damage or meiosis, and act as a backup pathway that rescues stalled replication forks in absence of Sgs1 (Mullen et al. 2001). Synthetic lethality between Mus81 and Sgs1 was found to be dependent on recombination since deleting recombination proteins was able to rescue these cells. Interestingly, it was found that these recombination events were not generated as a result of DSBs, but rather they were initiated following single-stranded DNA gaps (Fabre et al. 2002). In addition, during meiosis, Mus81 and Sgs1 were shown to be involved in resolving aberrant joint molecules that form during meiotic recombination, which explains the failure of chromosomal segregation in the absence of Mus81 and Sgs1 (Oh et al. 2008). Interestingly, diploid cells with Mus81 deletion were found to be more sensitive to MMS than haploid cells, which might suggest a possible role for Mus81 in resolving inter-homolog recombination intermediates. A more recent study confirmed a role for Mus81 in recombination by showing negative genetic interaction between Mus81 and Srs2, a helicase plays a role in recombination (Keyamura, Arai, and Hishida 2016). It was found that double mutants of Mus81 and Srs2 in diploid cells had less growth compared to haploid cells. This growth defect in the double mutant was also recombination dependent since deletion of rad52 and rad51 was able to rescue this defect (Keyamura, Arai, and Hishida 2016). Genetic interactions with nucleases involved in resolving Holliday junctions have been very helpful in

demonstrating the role of Mus81 in resolving joint molecules that result from HR. For example, Yen1, a nuclease that can cleave Holliday junctions, has been shown to interact genetically with Mus81 (Blanco et al. 2010). In this study, it was shown that double deletion of Mus81 and yen1 renders the cells very sensitive to DNA damaging agents, and that this sensitivity was believed to be as a result of the accumulation of toxic recombination intermediates that were not resolved. This was concluded based on the finding that the deletion of Rad52, which abrogates recombination, suppressed the sensitivity of the double deletion upon DNA damage (Blanco et al. 2010). Moreover, Yen1 and Mus81 were found to work redundantly, *in vivo*, to resolve Holliday junctions (Tay and Wu 2010). Nucleases that resolve the Holliday junctions can lead either to crossovers or to non-crossover products. Therefore, depletion of nucleases or their inactivation might affect the outcome of products. Interestingly, deletion of Mus81 was shown to lead to decreased crossovers and that Yen1 could be a redundant activity in the absence of Mus81 (Ho et al. 2010). In addition, it has also been found that the double deletion of Mus81 and Yen1 channels the DNA intermediates to break induced replication instead of leading to increased non-crossover products (Ho et al. 2010). However, in this case, Mus81 was found not to form a complex of two heterodimers, and thus was considered different from most nucleases that cleave Holliday junctions and are made of a tetramer. Moreover, the inability of Mus81 to cleave Holliday junction substrates *in vitro*, suggests that crossing over is not achieved by canonical ways of cleaving Holliday junctions, but rather it is accomplished by cleaving incomplete intermediates (Schwartz et al. 2012).

As a nuclease, Mms4-Mus81 has been shown to be a structure specific nuclease since Mus81 was found to act on a wide range of substrates (Kaliraman et al. 2001, Bastin-Shanower et al. 2003, Ehmsen and Heyer 2008). *In vitro*, the preference

of substrates was found to be for 3’ flapped structures, replication fork-like structures, nicked four-way Holliday junctions, three-way Holliday junctions with protruding 3’ or 5’ single-stranded DNA and displacement loop structure in a manner that is dependent on the enzyme concentration. At high concentrations it was found that the Mms4-Mus81 complex can cleave forked duplex and 5’ flap structures, but not intact Holliday junctions (Ehmsen and Heyer 2008). Additionally, in a different study, Mus81 was found to be able to cleave cruciforms that are formed at palindromic inverted repeats (Cote and Lewis 2008). Since cruciforms resemble four-way Holliday junctions, it was suggested that Mus81 might have a role in resolving intact Holiday junctions (Cote and Lewis 2008). Furthermore, Mus81 has also been implicated in the cleavage of branched structures independent of the DNA sequence, and instead the presence of a 5’ end or a gap of less than four nucleotides in length was important for recognition and cleavage (Bastin-Shanower et al. 2003).

Finally, Mus81 has also been implicated in genomic stability by studying the kinetics of replication fork at rDNA, which are rich in repetitive sequences (Ii, Ii, and Brill 2007). It was found that Mus81 deletion resulted in increased pausing of the replication fork at replication fork barriers and increased accumulation of x-shaped molecules. The amount of these x-shaped molecules was reduced in a Rad52 deletion indicating that these molecules are generated as a consequence of recombination (Bush, Evans-Roberts, and Maxwell 2015). Moreover, Mus81 deletion was also shown to lead to rDNA expansion. Therefore, it was proposed that Mus81 can resolve Holliday Junctions that form from DNA loops resulting from extra copies of DNA sequences that are generated during replication, by excising these extra repeats of DNA, Mus81 maintain the rDNA repeat sequence number (Ii, Ii, and Brill 2007).

4.2.2 Fun30 is Required in the Absence of Mus81 upon DNA Damage by MMS or HU

The notion that Fun30 is anti-recombinogenic led us to hyppthesize that in absence of Fun30, toxic recombination intermediates might accumulate as their resolving could be hindered in cells that lack other proteins involved in the resolution of recombination intermediates. To test this, mutants of each *Δmus81*, *Δfun30* and *Δfun30Δmus81* were constructed and cultures of each were serially diluted and spotted on YPD media having different concentration of MMS or HU (Figure 7 A and B, respectively). *Δmus81* cells were found to be more sensitive to MMS and HU compared to *Δfun30,* as was the case with camptothecin shown in Figure 4.6A, suggesting that Mus81 has a more vital role than Fun30 in resolving DNA damage caused by MMS and HU as well as camptothecin. The double deletion strain (*Δfun30Δmus81*) showed a slightly higher sensitivity to both MMS and HU than the Mus81 deletion alone (Figure 7 A and B, respectively). This suggests that Fun30 and Mus81 act in parallel pathways and that Fun30 is needed for the repair of damaged DNA in the absence of Mus81. These results also suggest that Fun30 genetically interacts with Mus81 upon treatment with DNA damaging agents such as HU and MMS. These data also support the increased production of recombination intermediates (upon DNA damage) in the Fun30 deletion strain and the inability of these cells to deal with them if they also lack Mus81.

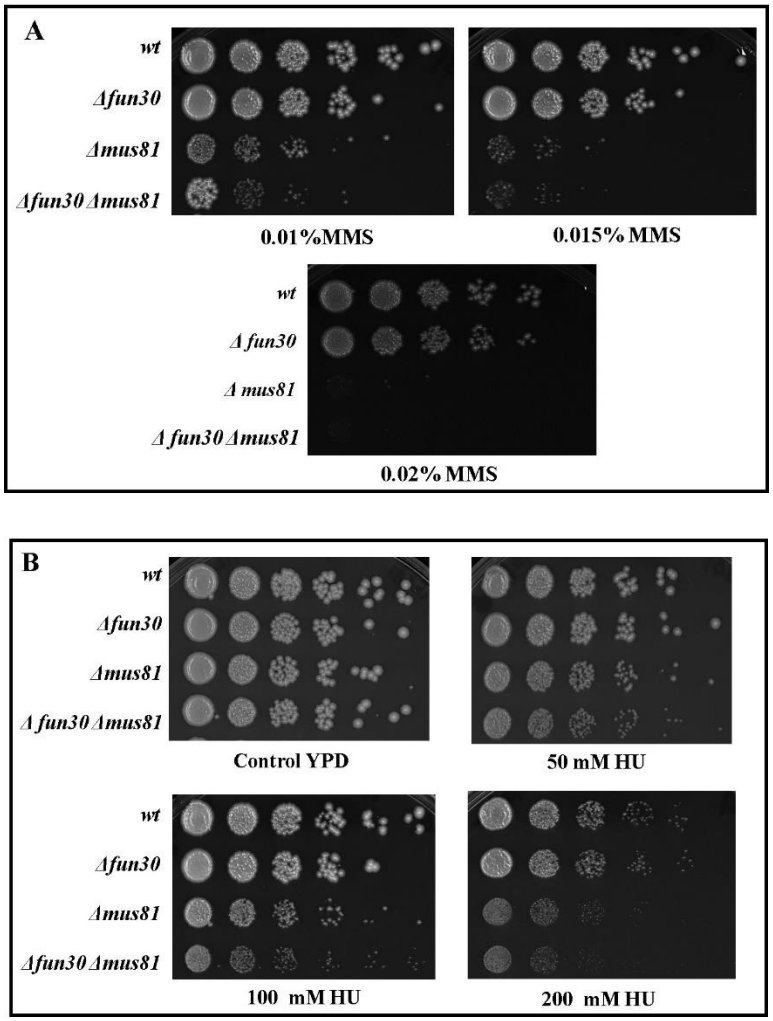


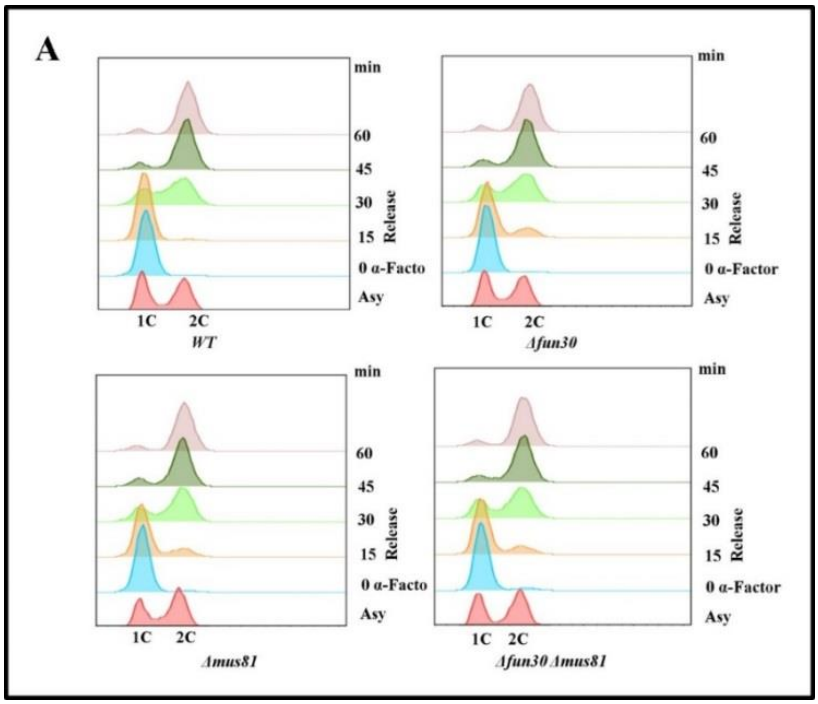
Figure 4.7: Fun30 is required in the *Δmus81* strain upon DNA damage by MMS or HU

(A) Growth assay comparing Fun30 and Mus81 deletions to the double deletion following addition of increasing concentrations of MMS (0.01%-0.02%). (B) Growth assay comparing Fun30 and Mus81 deletions to the double deletion following addition of increasing concentrations of HU (50-200 mM). *Δmus81* mutant is more sensitive than *Δfun30* in both MMS and HU. The increase in sensitivity in the double deletion (*Δfun30Δmus81*), upon DNA damage that stalls replications forks, indicates that Fun30 and Mus81 may act in independent pathways but genetically interact.

4.2.3 Higher Sensitivity of *Δfun30Δmus81* upon HU Treatment is not due to Delays in the Cell Cycle Progression

The increased sensitivity of the double deletion (*Δfun30Δmus81*) compared to the *Δmus81* alone to HU was perhaps a bit more significant than with MMS as

evident by the smaller size of colonies at higher HU dilutions (Figure 4.7, compare A and B). Therefore, we tested if *Δfun30Δmus81* cells suffered from any problems during replication either under normal growth conditions or after acute treatment with HU, which is known to act during replication by depleting the nucleotide pool leading to stalled replication forks. To check the effect of the deletions under normal conditions, wild-type, *Δfun30*, *Δmus81*, and *Δfun30Δmus81* cells were first blocked at the G1 phase with α-factor and then released in YPD, samples were taken at different time intervals and analyzed using FACS (Figure 4.8A). The results show no difference in the cell cycle profile of these strains under normal/untreated conditions (Figure 4.8A). This suggests that Fun30 is not required for bulk DNA replication in the absence of Mus81 when there is no DNA damage. To test the effects of HU on cell cycle progression of these mutants, the different strains were treated with 200 mM HU for 2 hours, washed and released in YPD, and samples were taken at different time intervals, and analyzed using FACS (Figure 4.8B). The results show that under these conditions, cell cycle progression was also not affected, indicating that bulk replication of DNA was not affected in *Δfun30Δmus81* after acute treatment with HU (Figure 4.8B) and that this double deletion strain was not defective in resuming stalled replication forks after acute HU treatment.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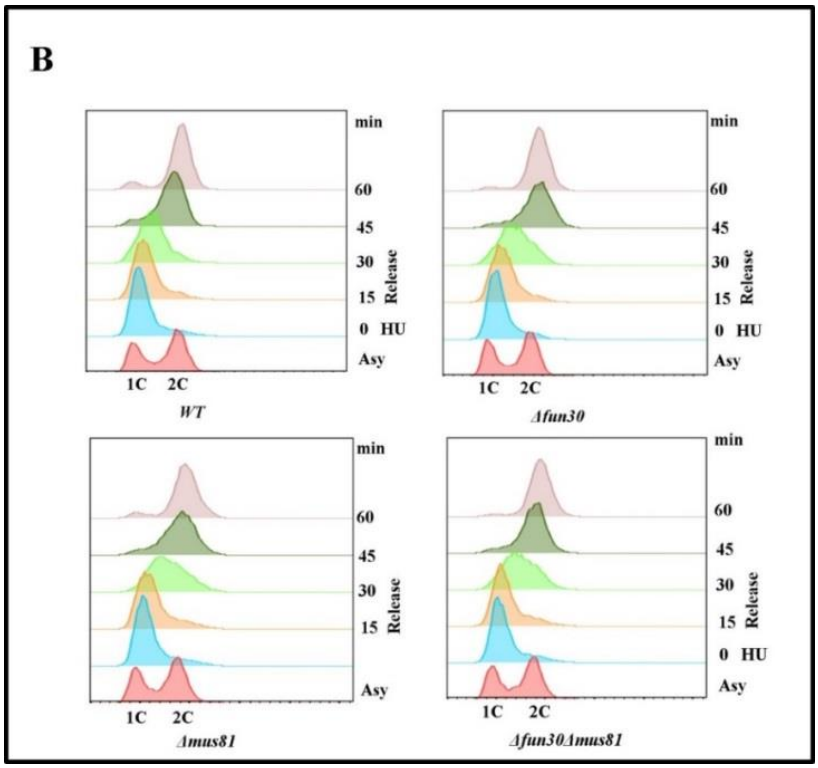
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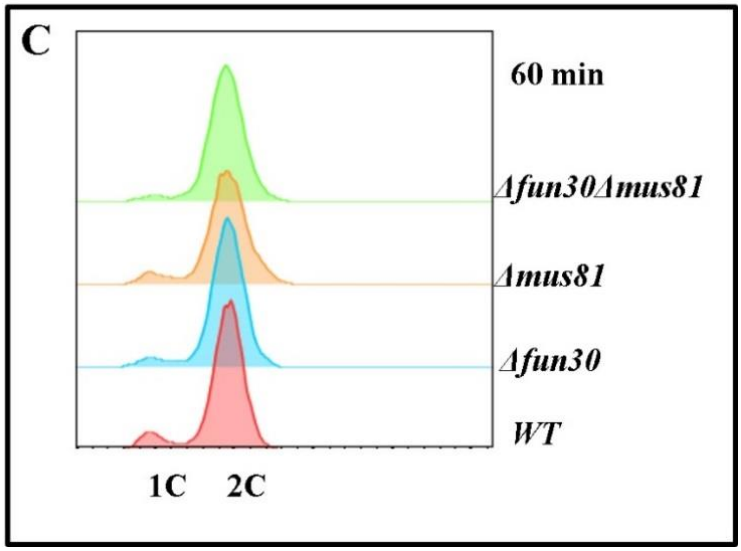


Figure 4.8: Higher sensitivity of *Δfun30Δmus81* upon HU treatment is not due to delays in the cell cycle progression

(A) FACS analysis of DNA content of wild-type, *Δfun30*, *Δmus81*, and *Δfun30Δmus81* under normal non-treated conditions. These cells were synchronized at the G1 phase with α-factor, washed and released from G1, as described before. Samples were taken at 15 minute time intervals and analyzed for DNA content by FACS. (B) FACS analysis of these strains after receiving an acute treatment of HU for 2 hours. (C) An overlay of FACS profile of samples upon HU treatment after 60 minutes of release in YPD for better comparison. Results show that cell cycle progression was also not affected under these conditions.

4.3 Fun30 Plays no Role in Replication in the Absence of Asf, but Genetically Interacts with Asf in the Presence of DNA Damage

4.3.1 Overview

As mentioned earlier in chapter 1, Fun30 has an ATP-dependent dimer exchange and nucleosome sliding activities in addition to its ability to affect the chromatin structure at certain DNA loci. These observations suggest that Fun30 may have a function similar to histone chaperones that act during replication. Experimentally, this is supported by showing that Fun30 is recruited to origins of replication and genetic interaction with ocr2 mutants (Neves-Costa et al. 2009).

Histones dimer deposition takes place during DNA replication to restore the chromatin structure following DNA replication. Several proteins or histone chaperones are involved in this process. Therefore, we decided to investigate whether Fun30 works redundantly with any of the proteins or chaperones that play a role in histone deposition in order to ensure proper chromatin assembly.

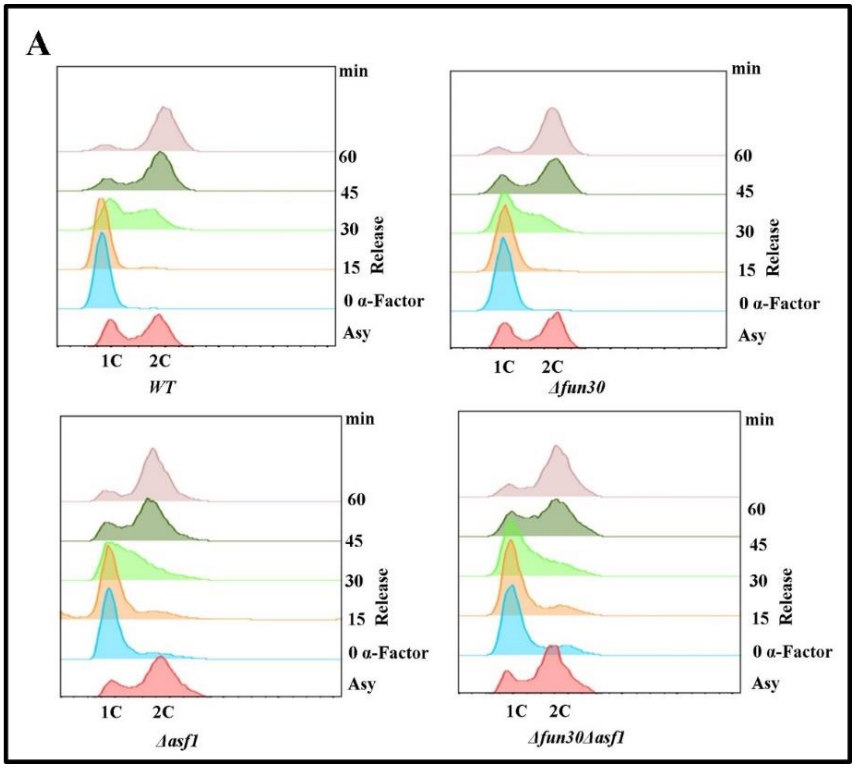
After DNA damage, the damage repair machinery has to access DNA, which is mainly hindered by the chromatin structure. Chromatin modifications are believed to relieve this chromatin mediated repression allowing the establishment of a more accessible environment where for example repair machinery can now access the underlying DNA. During replication, nucleosomes having histone H3 acetylated at lysine 56 (H3-K56) are deposited on the newly replicated DNA, which is then fully removed at the G2 phase of the cell cycle. However, during DNA damage, the removal of this H3-K56 acetylation is delayed in order to allow better access for repair factors. It has been shown that defects in acetylation of the histone H3 at K56 confers sensitivity to several DNA damaging agents (Masumoto et al. 2005). The deposition of histones on DNA is mediated by histone chaperones Asf1 and CAF1, both of which have been implicated in replication-dependent histone assembly (Recht et al. 2006). During replication, CAF1 acts by depositing H3 and H4 through its interactions with PCNA (Recht et al. 2006). The importance of Asf1 during replication was also demonstrated when cells lacking Asf1 were shown to be sensitive to DNA damaging agents that act on replicating DNA. This hypersensitivity of *Δasf1* cells to DNA damaging agents was found to be associated with the loss of H3-K56 acetylation, suggesting thatAsf1 may be important in maintaining genomic stability during replication (Recht et al. 2006). Moreover, Asf1 has been shown to facilitate the

acetyltransferase activity of Rtt109, which is the predominant histone acetyltransferase for histone H3-K56 (Driscoll, Hudson, and Jackson 2007). Acetylated H3-K56 also facilitates the deposition of H3 and H4 on DNA by enhancing the binding of these histones to CAF1 and Rtt106 which catalyze the depositioning of these histones (Yadav and Whitehouse 2016). *Δasf1* cells were found to have a distorted profile with cells accumulating at metaphase as a result of activation of cell cycle check points. However, this high level of damage was shown not to be due to defects in DSBs repair, but rather was due to excessive damage resulting from the altered structure of chromatin (Ramey et al. 2004).

4.3.2 Fun30 is not Required of Cell Cycle Progression in *Δasf1* under normal conditions

Despite that Fun30 deletion does not have any effect on cell cycle progression; we hypothesized that this may be due to possible redundancies of Fun30 with other genes the deletions of which can reveal such a role of Fun30. To test this, we checked the cell cycle progression in *Δasf1Δfun30* double deletion. Wild-type, *Δfun30*, *Δasf1*, *and Δfun30Δasf1* cells were synchronized with α-factor and then released in YPD, cells samples taken at different time intervals and analyzed by FACS (Figure 4.9). The results show that there is no difference in the cell cycle profile between the four strains (Figure 4.9A). By overlaying of the FACS profiles of samples analyzed 30 or 45 minutes following release one can appreciate these results better (Figure 4.9B). In other words, the deletion of Fun30 in an Asf1 deleted background did not show any effect on the cell cycle progression of these cells. As mentioned previously, cells lacking Asf1 will have some spontaneous damage, and therefore to test the levels of DNA damage and to see whether Fun30 deletion has an effect on this,

we checked the level of Rad53 phosphorylation in these strains (Figure 4.9C). Wild-type cells and *Δfun30* were found to lack any Rad53 phosphorylation, while *Δasf1* cells were found to have the expected level of Rad53 phosphorylation. Moreover, the double deletion (*Δfun30Δasf1*) did not result in an increased level Rad53 phosphorylation, suggesting no further damage is observed as a result of Fun30 deletion in cells lacking Asf1. These results are consistent with our FACS data that show no effect on the cell cycle progression in the deletions, even in the double mutant. This indicates that *Δasf1* cells can proceed normally though the cell cycle in the absence of Fun30 and that, under normal conditions, Fun30 does not seem to have a role similar to that of Asf1 in replication.



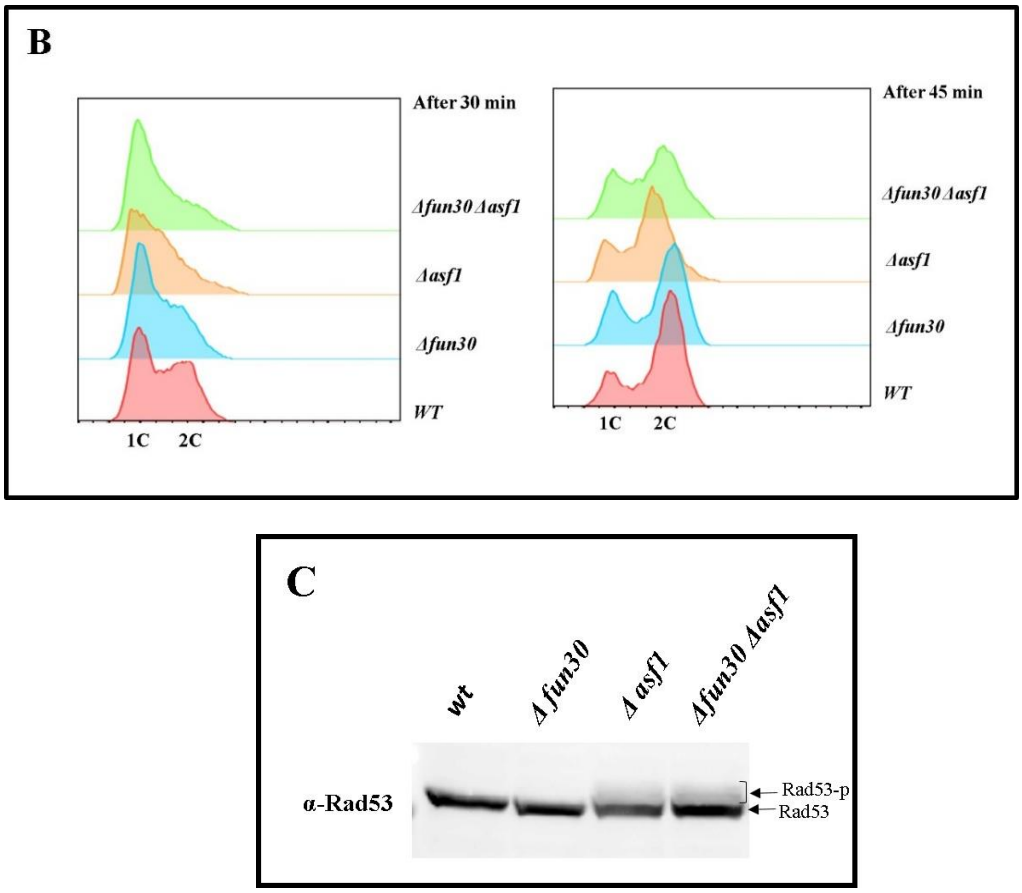


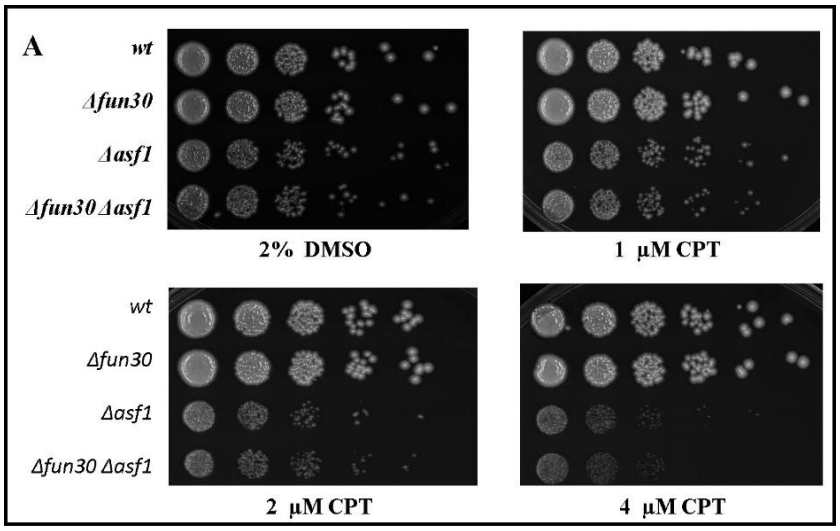
Figure 4.9: Fun30 is not required of cell cycle progression in *Δasf1* under normal conditions

(A) FACS analysis of DNA content of wild-type, *Δfun30*, *Δasf1*, and *Δfun30Δasf1* under normal non-treated conditions. These cells were synchronized at the G1 phase with α-factor, washed and released from G1, as described before. Samples were taken at 15 minute time intervals and analyzed for DNA content by FACS. (B) An overlay of FACS profile of samples after 30 or 45 minutes of release in YPD for better comparison. Results show no effect on the cell cycle progression in the deletions under normal conditions. (C) Total cell lysates extracts from the cells, as described before in the presence of TCA, show RAD53 phosphorylation is similar in the *Δasf1* and the double deletion (*Δfun30Δasf1*). These results indicate that *Δasf1* cells can proceed normally though the cell cycle in the absence of Fun30.

4.3.3 Fun30 Genetically Interacts with Asf1 in the Presence of DNA Damage

Since Asf1 has been shown to be important for resistance to DNA damage during replication, we tested whether Fun30 is required in cells lacking Asf1 (*Δasf1*)

upon DNA damage. For this, wild-type, *Δfun30, Δasf1*, *and Δfun30Δasf1* cells were serially diluted and spotted on YPD plates containing different concentrations of camptothecin, MMS, and HU (Figure 4.10 A, B, and C, respectively). As expected, *Δasf1* cells were found to be sensitive to all of these DNA damaging agent even at low concentrations compared to *Δfun30* cells, suggesting that Asf1 plays a more important role in the presence of DNA damage than Fun30. However, we observed a slight higher sensitivity to these chemicals (especially MMS and HU) in the *Δfun30Δasf1* cells compared to the *Δasf1* cells. This suggests that Fun30, under these DNA damaging conditions, might be involved in a redundant pathway with Asf1 in dealing with DNA damage. The sensitivity of *Δfun30Δasf1* to HU is possibly because of the inability of replication forks in these cells to resume after stalling due to acute HU treatment. To test whether Fun30 is required in cells lacking Asf1 to resume stalled replication forks, the different strains (wild-type, *Δfun30, Δasf1,* and *Δfun30Δasf1)* were treated with 200 mM HU for 2 hours, washed and released in YPD, samples were taken different time intervals, and analyzed using FACS (Figure 4.10D). The results show that under these conditions, cell cycle progression was also not affected in the double deletions (*Δfun30Δasf1*), suggesting that Fun30 is not required in the absence of Asf1 for resuming stalled replication forks after acute HU treatment. Moreover, the enhanced sensitivity observed with chronic HU treatment (on plates, Figure 4.10C) might have resulted from the differences in the level of DNA damage caused by chronic versus acute HU treatment. It is likely that acute HU treatment does not lead to extensive DNA damage, while chronic HU treatment might lead to replication fork collapse and increased DNA damage that requires the function of Fun30 in the absence of Asf1.


A
wt
Δfun30
Δasf1
Δfun30 Δasf1
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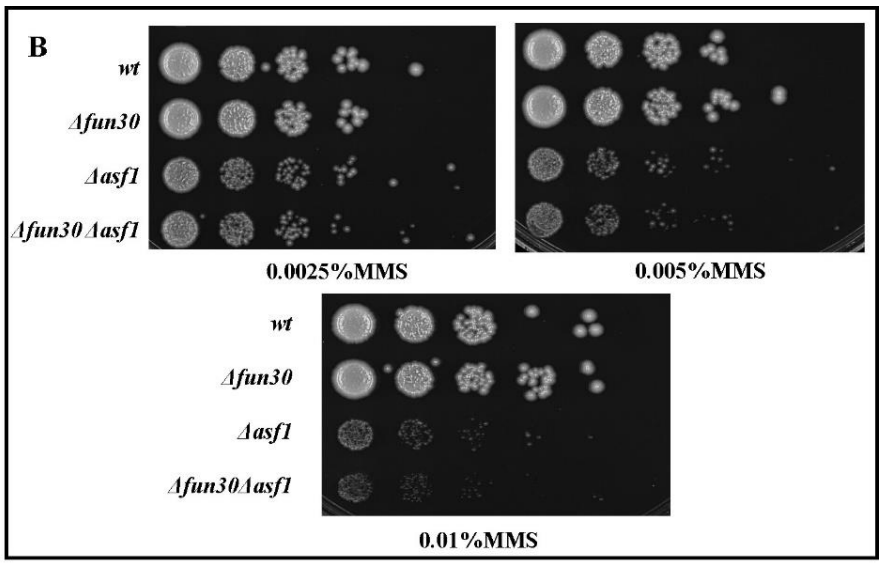
wt
Δfun30
Δasf1
Δfun30 Δasf1
2 µM CPT
4 µM CPT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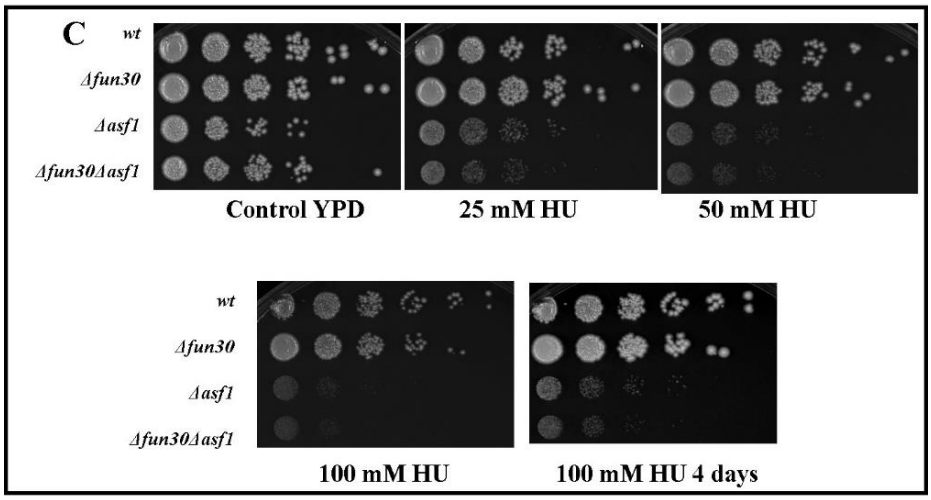
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100 mM HU
100 mM HU 4 days

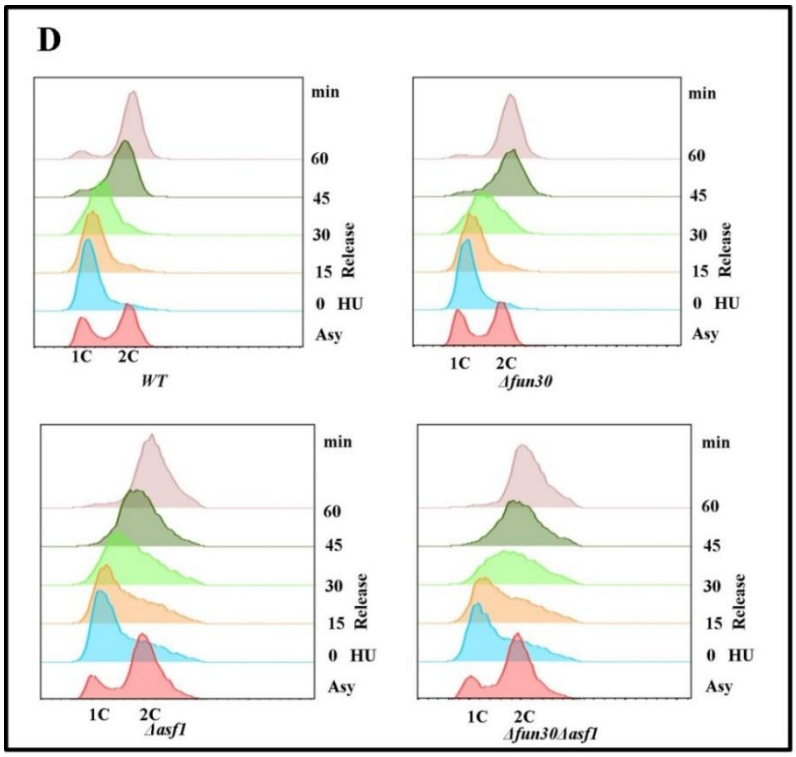

Figure 4.10: Fun30 deletion is less sensitive to DNA damage compared to Asf1 deletion and Fun30 genetically interact with Asf1 upon DNA damage

(A) Growth assay comparing Fun30 and Asf1 deletions to the double deletion following addition of low concentrations of camptothecin (1-4 µM). (B) Growth assay comparing Fun30 and Asf1 deletions to the double deletion following addition of increasing concentrations of MMS (0.0025%-0.01%). (C) Growth assay comparing Fun30 and Asf11 deletions to the double deletion following addition of increasing concentrations of HU (25-100 mM). *Δasf1* mutant is more sensitive than *Δfun30* to DNA damage induced by camptothecin, MMS, and HU. The increase in sensitivity in the double deletion (*Δfun30Δasf1*), upon DNA damage, indicates that Fun30 and Asf1 may act in independent pathways but genetically interact. (D) FACS analysis of these cells after receiving an acute treatment of HU for 2 hours. The cells were then washed and grown in YPD, as described before. Samples were taken at 15 minute time intervals and analyzed for DNA content by FACS. Results show that under these conditions, cell cycle progression was not affected in the double deletions (*Δfun30Δasf1*), suggesting that Fun30 is not required in the absence of Asf1 for resuming stalled replication forks after acute HU treatment.

Chapter 5: Discussions and Future Prospects

5.1 Discussions

5.1.1 The *In Vitro* Activities of Fun30

Our laboratory has previously shown that Fun30 is able to remodel the chromatin structure *in vitro* (Awad and Hassan 2008, Byeon et al. 2013). Furthermore, in *vivo*, Fun30 was found to be implicated in establishing silent chromatin at chromosomal domains such as centromeres and telomeres (Neves-Costa et al. 2009, Durand-Dubief et al. 2012). Interestingly, in the last few years, several studies suggested a role for Fun30 in the repair of DSBs, specifically, the long range resection of 5' ends of DSBs, which is one of the initial step in homologous recombination (Chen et al. 2012b, Costelloe et al. 2012, Eapen et al. 2012). However, even though resection was reduced in cells that lacked Fun30, no defects in cell viability were observed under DNA damaging conditions or when a single DSB was induced. This indicates that the repair efficiency was not affected significantly in these cells, even when the amount of resection was reduced. Moreover, gene conversion was shown not to be affected in *Δfun30*, indicating that the efficiency of DSB repair was not reduced in the absence of Fun30 (Eapen et al. 2012). In addition, deleting Fun30 was found to enhance the integration of DNA inserts into the genome, to increase the levels of Break Induced Replication (BIR) (Costelloe et al. 2012), and raise the levels of crossover products (Chen et al. 2012a), which suggest increased recombination in the absence of Fun30. In a later study, Fun30 was suggested to negatively regulate Rad51-dependent HR (Bi et al. 2015). All of these findings show that the cells undergo uncontrolled recombination in the absence of Fun30.

This thesis was focused on understanding the molecular functions of Fun30 that can better explain its role during homologous recombination. In *in vitro* assays, in which purified Fun30 and Cy5-labeled DNA substrates were used, we identified three distinct enzymatic activities for Fun30. First, Fun30 was found to be able to anneal partially complementary strands of DNA in the absence of ATP, which became more proficient at high ATP concentrations (8 mM). The inhibition of Fun30 annealing activity when a non-hydrolysable form of ATP was used suggested that ATP hydrolysis was required for Fun30 annealing activity. Fun30 annealing activity is important in various pathways such as in the Single Strand Annealing pathway of DSB repair. Previous studies have shown that Fun30 deletion caused a reduced efficiency of DSB repair by SSA (Eapen et al. 2012, Costelloe et al. 2012). Although deletion of Fun30 was found not to affect the viability of cells when a cut was induced between two closely positioned repetitive sequences, the viability reduced significantly in a strain where the two sequences were farther apart (Eapen et al. 2012). The distance effect was explained by the need for extensive long range resection which was shown to be facilitated by Fun30. Based on earlier studies it has been believed that Fun30 facilitated both Sgs1 and Exo1 resection pathways (Eapen et al. 2012, Costelloe et al. 2012). Fun30 deletion was also shown to decrease the rate of resection. By assuming that resection will reach normal levels after longer time in *Δfun30*, it is possible that SSA, rather than resection per se, maybe defective in a subsequent step in SSA. During SSA, partially complementary DNA stands that are exposed on both sides of a DSB as a result of resection could be annealed together by Fun30. Our results demonstrating *in vitro* annealing activity of Fun30 supports this possibility. The observed increased efficiency of annealing activity by Fun30 in the presence of 8 mM ATP (Figure 3.3B) to anneal a fraction of DNA molecules that was not annealed in the absence of ATP,

even if the time of the reaction was extended, suggests that Fun30 is capable of annealing complementary DNA oligonucleotides when they are present at very low concentrations. Low concentrations of partially complementary strands can be in theory similar to when tandem DNA sequences are located at greater distances from each other. This can possibly explain why a strain where a DSB was induced between distant repeats showed reduced viability in the absence of Fun30. Therefore, Fun30 can be dispensable for annealing of closely positioned repetitive sequences, probably because of redundancy with other proteins or as a result of spontaneous re-annealing due to the proximity of the DNA repeats. Furthermore, Fun30 annealing activity might also be important in stabilizing replication forks when they are stalled by chemicals such as camptothecin, MMS and HU. This can be achieved by preventing the peeling of nascent DNA strands from their template DNA and thus preventing them from invading ectopic or non-allelic regions of the genome leading to illegitimate recombination and genomic instability. Figure 5.1A represents a plausible model depicting Fun30 resection as well as annealing activities during SSA.

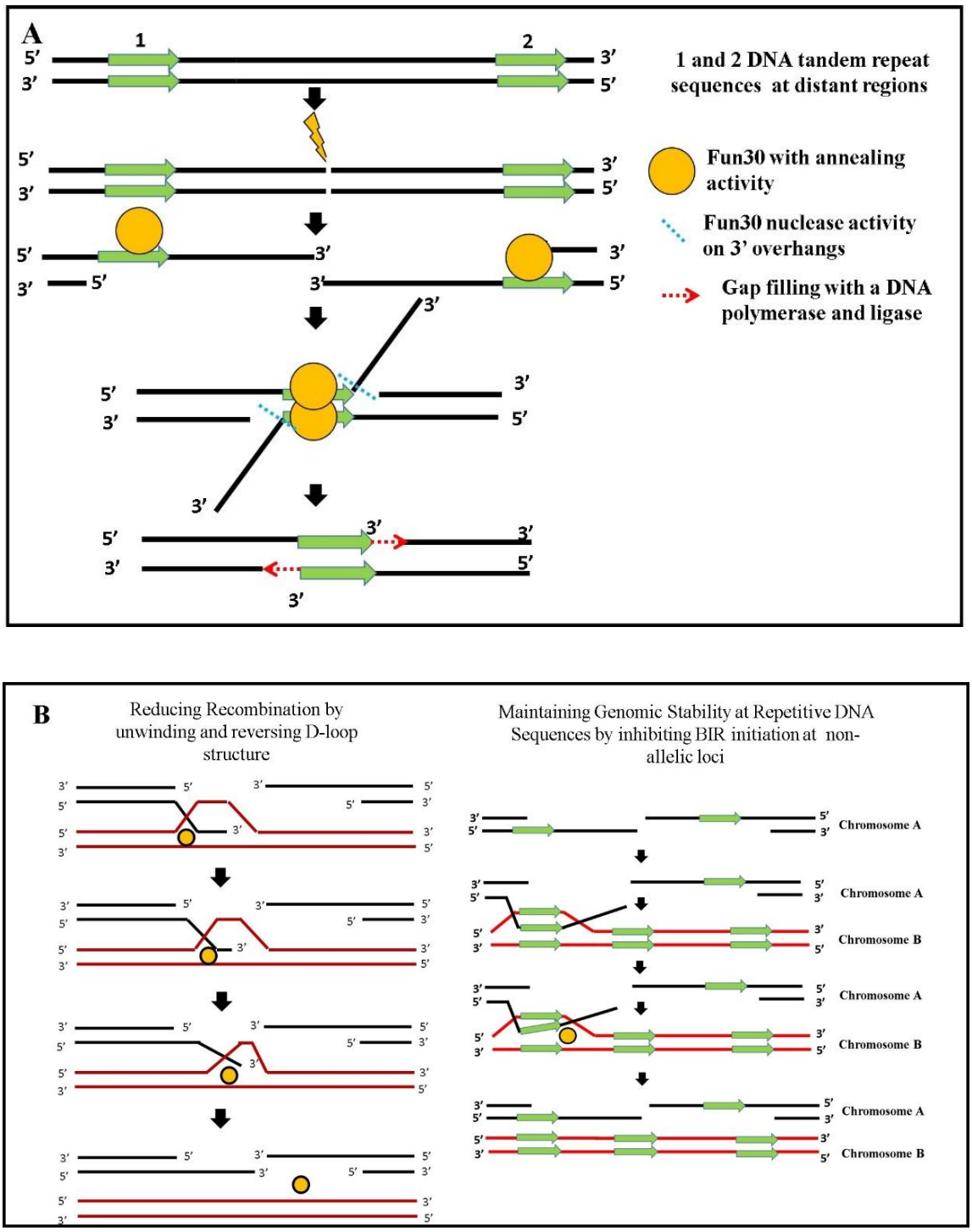

Figure 5.1: Models illustrating Fun30 functional activities

(A) Fun30 functions during SSA. (B) Fun30 role during HR and BIR by unwinding D-loops.

Another activity of Fun30 observed in our *in vitro* experiments is its ability to unwind a forked duplex indicating that Fun30 can act as a helicase. Since the Fun30 helicase function was only detected in the presence of trap DNA, it is likely that this Fun30 activity is masked by its strong annealing activity that opposes any helicase function. Because trap DNA is not present in cells, it is possible that interactions of single-stranded DNA with other proteins might aid Fun30 in unwinding its substrates. A good candidate for this is the Replication Protein A (RPA), which is the major protein that binds to single-stranded DNA in eukaryotic cells. Since deletion of Fun30 has been shown to increase the rate of recombination and BIR (Costelloe et al. 2012), we believe that Fun30 might utilize its helicase activity to unwind the D-loop or its extended form to reduce HR levels or channel repair to the SDSA pathway as suggested in the model (Figure 5.1B). Such control over the D-loop fate can help in reducing the levels of BIR or crossover products, which explains why their incidence is increased when Fun30 is deleted. Previously observed negative genetic interactions between Fun30 and Sgs1, which also plays a role in the dissolution of double Holliday junction (dHJs), suggests that Fun30 might act in a redundant pathway, where it might utilize both its annealing and helicase activities to facilitate the migration and the merging of the dHJs in a manner similar to Sgs1. This will lead to less dHJs, and thus fewer substrates for dHJs resolvases, whose end products can be either crossovers or non-crossovers. However, we could not detect branch migration by Fun30. We believe that this could be because the Holliday junction substrate used was not ideal since it contained regions of non-complementarity and thus could not efficiently be annealed together by Fun30.

A third functional activity of Fun30 observed in our *in vitro* experiments was a nuclease activity as shown by its ability to relax both positively and negatively

supercoiled DNA. This relaxing is achieved by DNA nicking suggesting a possible nuclease activity of Fun30, which was confirmed by using different DNA substrates. Fun30 was able to cleave the 3' end of both a forked substrate and a DNA duplex substrate with 3' overhang. Similar to annealing and helicase activities of Fun30, a potential nuclease activity, albeit a weak one, can be important in facilitating certain steps during HR or DNA damage repair. Cleaving 3' overhangs of a forked duplex DNA might be required during SSA where a non-complementary 3' overhang needs to be cleaved with a nuclease before the polymerase can fill the gap. In addition, since a forked duplex and a DNA duplex with 3' overhang both resemble a stalled replication, it is conceivable that Fun30, through its nuclease activity, might cleave stalled replication forks and help in resuming replication. Nicking supercoiled DNA raised a question as to whether Fun30 can relax supercoiled DNA that have accumulated during DNA replication or as a result of treatment with certain DNA damaging agents that lead to their accumulation. A nuclease activity can also be useful in separating the tangled DNA duplexes that results in the final steps of dHJs, similar to the roles that Sgs1 and Top III play there. Additional experiments are needed to decipher these possibilities.

5.1.2 The *In Vivo* Functions of Fun30

Fun30 deletion shows exceptional sensitivity to camptothecin-induced DNA damage, which is significantly reduced when these cells are treated with other DNA damaging agents such as MMS and HU (Neves-Costa et al. 2009, Costelloe et al. 2012, Bi et al. 2015). As mentioned previously, camptothecin induces damage mainly by stabilizing TopI/DNA adducts that develops into DSBs during replication. Interestingly, despite being generated at S-phase, the camptothecin induced damage is

invisible to the intra-S-phase checkpoint of the cell cycle, which allows the cells to progress normally through the S phase, and thus, the damage is can only be appreciated after bulk replication of DNA as a result of which the cells are blocked at G2 (Redon et al. 2003). To gain more insight into the role of Fun30 upon camptothecin induced damage, we studied the effects of camptothecin on cell cycle progression in Fun30 deletion cells and double deletions of Fun30 with genes involved in resistance to camptothecin. While bulk replication of DNA was not significantly affected in the presence of 100 µM camptothecin in *Δfun30*, a slower progression through the cell cycle in the deletion compared to wild-type cells upon camptothecin addition was observed. This suggests that Fun30 might play a role in stabilizing the replication forks in the presence of torsional strain imposed by camptothecin. One possible mechanism that Fun30 can achieve this is through utilizing its annealing activity to prevent fork reversal. In support of this, we found that Fun30 was not able to cause significant regression of replication forks, even in the presence of ATP. Since the observed delay in cell cycle in the Fun30 deletions was at later stages of the S phase, it is possible that these cells accumulate some forms of DNA intermediates or structures that affect the progress of DNA replication at the later stages of S phase. Suppression of Fun30 sensitivity to camptothecin in a TopI deletion background (i.e. in the double deletion *Δfun30ΔtopI*) confirmed that this may be due to TopI/DNA adducts rather than other possible effects of camptothecin. Because camptothecin can induce torsional strains, we postulated that Fun30 might be required in the absence of TopI; however, we did not observe growth defects in the double mutants compared to the *ΔtopI* cells, indicating that Fun30 does not play a significant role in the absence of TopI. Interestingly, under non-damaging conditions, the *Δfun30Δtop1* double deletion showed more cell accumulation in G1 compared to either of the single mutants.

However, since the doubling time was comparable between wild-type and the deletion strains, we believe that this altered profile is probably not due to a reduction in the overall rate of replication, but rather it might reflect difficulties in the initiation of replication. This is supported by previous findings where replication was shown to be affected when *fun30* was deleted in an *orc2* conditional mutant (Neves-Costa et al. 2009). All these finding demonstrate that the Fun30 sensitivity to camptothecin is not due to torsional stress but is rather because of TopI/DNA mediated damage.

To find out how Fun30 plays a role in other pathways that involve camptothecin damage, we looked for genetic interaction between Fun30 and Tdp1, a protein which is involved in removing TopI/DNA complexes utilizing its phosphodiesterase activity. We observed that Fun30 may play a more important role than Tdp1 during camptothecin damage and that Fun30 might be working downstream of Tdp1. In other words, Fun30's role in dealing with camptothecin damage may not involve direct repair or removal of the TopI/DNA lesion like Tdp1. Mus81 is a nuclease that has also been shown to play a role upon camptothecin damage. Therefore, we tested whether Fun30 is redundant with Mus81 or acts in a parallel pathway. Growth assays showed that *Δmus81* was more sensitive to camptothecin compared to *Δfun30* cells, which was slightly increased in the *Δfun30Δmus81* deletion indicating that Fun30 and Mus81 may act via independent pathways. While, bulk replication of DNA in *Δfun30Δmus81* cells was not altered in the presence of camptothecin, these double deletions experienced a slight delay at the later stages of the S phase, indicating that some events (or certain DNA structures that might accumulate) hinder replication in the late stages of S phase. As mentioned previously, camptothecin induced damage relies mainly on recombination for repair or

alternatively recombine randomly with remote DNA sequences leading to genomic instability. Observing no change in the levels of γH2A in these mutants following camptothecin damage indicates that neither Mus81 nor Fun30 are involved in inducing DSBs, and instead it is less likely that they are both involved in cleaving and in rescuing stalled replication forks. Moreover, the levels of RAD53 phosphorylation in these mutants was not very different indicating that higher sensitivity of the *Δfun30Δmus81* was not due to a defect in the G2 checkpoint. These finding together indicate that the action of Fun30 following camptothecin damage does not involve removing of the TopI/DNA adduct, a mechanism used by Tdp1.

Like Mus81, Fun30 seems to be involved in rescuing stalled replication forks. It is most likely that the observed enhanced sensitivity to camptothecin in Fun30 and Mus81 deletion strains are because of HR recombination intermediates that have accumulated at the end of S phase and are toxic if not resolved. Since Fun30 was suggested to negatively regulate recombination, we postulated that deleting Fun30 will lead to the accumulation of toxic recombination intermediates, which could be amplified by inhibiting one of the pathways involved in resolving these recombination intermediates, and hence would increase the sensitivity of the cells to DNA damaging agents that induce double strands breaks and initiate HR. Previous studies have shown that deleting both Sgs1 and Fun30 made the cells more sensitive to chronic treatment with camptothecin (Costelloe et al. 2012). This increase in sensitivity was believed to cause enhanced defects in DNA resection in the double deletion strains. But knowing that Sgs1 is also involved in resolving recombination intermediates indicates that resection is not the only mechanism that can account for this sensitivity, but rather, the accumulation of toxic recombination intermediates that are hard to resolve, might lead

to chromosomes intertwining and eventually hampering chromosomal segregation, leading to cell death. To test this, we examined how Fun30 and Mus81 deletion strains responded to different types of DNA damaging agents. Mus81 has a well-established role in resolving recombination intermediates by utilizing its nuclease activity. To induce DNA damage, HU, a ribonucleotide reductase inhibitor that is believed to stall replication forks by inhibiting the synthesis of dNTP and is known to caused replication forks breakage with chronic durations (Koc et al. 2004) or methylmethane sulphonate (MMS), a DNA alkylating agent that modifies guanine to 7-methylguanine and adenine to 3-methlyladenin leading to base mis-pairing and replication fork stalling (Lundin et al. 2005), were used. Similar to the results obtained with camptothecin induced damage, *Δmus81* was more sensitive to chronic treatments of HU and MMS than *Δfun30*, indicating the more important role of Mus81 in DNA damage compared to Fun30. However, the increased sensitivity in the *Δfun30Δmus81* double deletion upon DNA damage that stalls replications forks indicates that Fun30 and Mus81 may act independently in processing DNA damage. This higher sensitivity, however, was not due to delays in the cell cycle progression under normal cell conditions or after release from HU, indicating that bulk DNA replication was not affected. Moreover, previous work had shown that Mus81 is not required for resuming HU stalled replication forks (Saugar et al. 2013). Therefore, since the *Δfun30Δmus81* cells were more sensitive compared to the single deletion upon chronic treatment, it suggests that perhaps this is due to events that occur after bulk DNA replication. Since chronic treatment with HU will eventually lead to the generation of DSBs followed by repair through HR, it is likely that recombination intermediates that tend to accumulate as the cell progress through the S phase become more toxic when both *fun30* and *mus81* are absent. Similar to its enhanced sensitive with HU, the *Δfun30Δmus81* cells

were also more sensitive to chronic treatment with MMS when compared to single mutants, although to a lesser extent. This may be due to a less dependence of the repair of MMS-induced lesions on HR and the availability of other repair pathways, such as BER, that deal with this kind of damage. Further support for the role of Fun30 in reducing recombination intermediates comes from the fact that the nuclease activity of Mus81 in budding yeast is highly regulated and is only activated at the end of the S phase when bulk genome replication is completed (Gallo-Fernandez et al. 2012, Saugar et al. 2013). This Mus81 activity is mainly to resolve dHJ intermediates, and thus the genetic interaction between *FUN30* and *MUS81* could be attributed to this most prominent activity of Mus81. Similar to HU or MMS treatment, the sensitivity of *Δfun30Δmus81* to camptothecin can also be due to the accumulation of toxic recombination intermediates. The high sensitivity of *Δfun30* cells to camptothecin compared to HU and MMS could possibly be because of the higher levels of recombination that occur upon camptothecin damage.

Because Fun30 has both a remodeling activity and plays a role in replication, we investigated whether Fun30 is required in the absence of Asf1, a histone chaperone that plays a role in histone deposition during replication. We show that Fun30 genetically interacts with Asf1 upon DNA damage (as observed by enhanced sensitivity in the double mutant), although the Fun30 deletion alone is less sensitive than the Asf1 deletion upon chronic DNA damage. This suggests that Fun30 might not be involved directly in replication during damage, but rather it maybe more involved in suppressing downstream genomic instabilities that may result from repairing highly damaged DNA in the absence of Asf1. This was further supported when cell cycle progression was shown not to be affected in the double deletions (*Δfun30Δasf1*),

suggesting that Fun30 is not required in the absence of Asf1 for normal replication or for resuming stalled replication forks after acute HU treatment. The fact that Fun30 is recruited to DNA during replication with no significant effect on bulk DNA replication suggests that Fun30 may perhaps be required to deal with the HR intermediates that tend to accumulate as replication progress. Several observations on Fun30 or its homologs support its role in suppressing hyper-recombination and thus maintaining genomic stability. First, deletion of *fft3*, a *fun30* ortholog in *S. pombe*, or knocking down of *Smarcad1*, the human ortholog, were shown to affect chromosomal segregation (Rowbotham et al. 2011, Strålfors et al. 2011), which could be because of a possible failure in resolving the excessively produced dHJs that arise during the S phase of the cell cycle. Moreover, the recruitment of Fun30 to certain genomic regions such as centromeres, telomeres, and rDNA is quite interesting since they all have a common property of sharing repetitive DNA sequences. If a DNA break in such repeat-rich regions is not repaired efficiently by SSA or other means, the resected ends can undergo homologous recombination with the same chromosome, which may lead to loss of DNA and reduced cell viability. Alternatively, the resected ends can seek homology on non-allelic repetitive DNA sequences in the genome, initiating BIR that could lead to gross chromosomal changes and genomic instability. Therefore, SSA would be the best repair pathway in terms of genomic stability.

In summary, in addition to the resection activity that Fun30 facilitates, results presented in this thesis demonstrate that it might also play an important role in maintaining genomic stability, especially in highly repetitive DNA regions, by facilitating SSA and avoiding other more problematic repair pathways such as BIR at ectopic regions, or by utilizing its helicase activity to reduce the D-loop formation, or

by channeling recombination intermediates towards SDSA. All these actions will cause the reduction of crossover levels and thus loss of heterozygosity or the levels of BIR in order to avoid gross genomic changes. Fun30 annealing activity might also help in inhibiting illegitimate recombination by stabilizing nascent DNA molecules at stalled replication forks and preventing them from seeking homology or starting illegitimate recombination.

5.2 Future Prospects

In this study, we have identified three enzymatic activities for Fun30 annealing, helicase and nucleases activities, all of which may be important in maintaining genomic stability. It would of interest to find out, through mutational analysis, if a particular motif within Fun30 is responsible for these activities. Identifying such motifs might help shed light on the importance of each of these activities in maintaining genomic stability. Moreover, the helicase activity of Fun30 can be further studied using other DNA substrates in the presence and absence of RPA, which usually assists helicases by conferring a regulatory effect or by trapping released strands of DNA. Finding additional possible genetic interactions with other nucleases involved in the cleavage of 3' ends can help us in understanding the importance of Fun30 nuclease activity as to which repair pathway it may be most involved in. Smarcad1, the Fun30 ortholog in humans, has been found to play an important function in cancer development and progression and since we now know that cells lacking Fun30 cannot efficiently overcome excessive accumulation of recombination intermediates or DNA damage induced at repetitive DNA sequences, perhaps future studies can be better designed in developing specific drugs that target such damage or regions in target cells

or perhaps even effect recombination that could signal cell death due to their inability to segregate chromosomes.

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