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INVESTIGATING THE ROLE OF FUN30, A CHROMATIN REMODELER, IN DNA REPAIR

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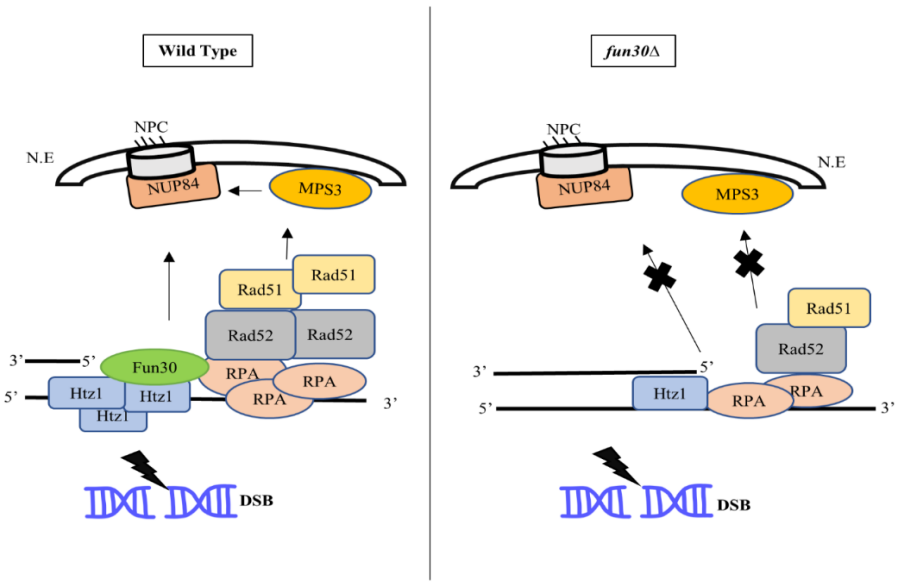

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United Arab Emirates University

College of Medicine and Health Sciences

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Mehwish Iqbal

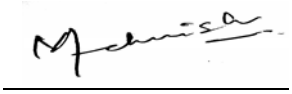
This dissertation is submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Biomedical Sciences

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

April 2022

Declaration of Original Work

I, Mehwish Iqbal, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this dissertation entitled "*Investigating the Role of Fun30, a Chromatin Remodeler, in DNA Repair*", 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 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 Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my dissertation have been properly cited and acknowledged in accordance with appropriate academic conventions.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.

Student's Signature:  Date: 14-7-2022

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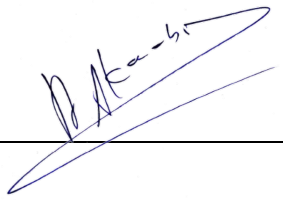
Signature  Date 01 August 2022

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Abstract

The repair of DNA double-strand breaks (DSBs) is crucial for maintaining genome stability. DSB repair needs to take place within the complex organization of the chromatin, and this requires changes in the chromatin structure adjacent to DSB sites. These changes occur through covalent histone modifications that alter histone-DNA contacts as well as by the action of ATP-dependent chromatin remodelers. Many chromatin remodelers, including Fun30, are involved in DSB repair. Fun30 facilitates DNA end resection at DSB site during the homologous recombination repair pathway. Apart from its role in DNA repair, Fun30 promotes gene silencing at heterochromatic loci such as telomeres, rDNA regions, and the mating-type locus *HML*α and *HMR*a, which are known to be clustered at the nuclear periphery. In this study, using mass spectrometry analysis of pulled down TAP-tagged Fun30, we observed co-purification of Fun30 with several nuclear pore proteins. Moreover, we also observed a reduced level of Mps3 and Nup84 at a single irreparable DSB in *fun30*Δ mutant suggesting that Fun30 helps to translocate irreparable DSBs towards the nuclear periphery. In addition, we observed that Fun30 supports histone H2A variant Htz1 recruitment at DSB. Thus, Fun30 favors the relocation of DSB by controlling the level of histone variant Htz1 and by favoring DNA end resection at the DSB site.

Keywords: DNA Repair, Chromatin Remodeling, Nuclear Envelope, Fun30, Resection.

Title and Abstract (in Arabic)

التحقيق في دور جهاز إعادة تشكيل الكروماتين Fun30 في إصلاح الحمض النووي

الملخص

يعد ترميم انقطاعات الحمض النووي المزدوجة (DSBs) أمرًا ضروريًا للحفاظ على استقرار الجينوم. يجب أن يتم ترميم ال DSB ضمن التنظيم المعقد للكروماتين، وهذا يتطلب تغييرات في بنية الكروماتين المجاورة لمواقع ال DSB. تحدث هذه التغييرات من خلال تعديلات هيستون التساهمية التي تغير تواصل الحمض النووي للهيستون وكذلك من خلال إجراءات إعادة تشكيل الكروماتين المعتمدة على ال ATP. تشارك العديد من أجهزة إعادة تشكيل الكروماتين، بما في ذلك ال Fun30، في ترميم ال DSB. يتم تحسين استئصال نهاية الحمض النووي في مواقع ال DSB بوجود ال Fun30 لتفضيل مسار ترميم إعادة ترتيب الجينات المتشابه. أثناء مسار إصلاح إعادة التركيب المتماثل، يفضل Fun30 استئصال نهاية الحمض النووي في مواقع DSB. بصرف النظر عن دوره في ترميم الحمض النووي، يشجع ال Fun30 إسكات الجينات في المواقع غير المتجانسة مثل التيلوميرات ومناطق ال rDNA وموضع التزاوج ال HMLα وال HMRa، والمعروف أنهما متجمعان في المحيط النووي. في هذه الدراسة، باستخدام تحليل قياس الطيف الكتلي لـ Fun30 الموسومة بعلامة ال TAP، نلاحظ التنقية المشتركة لـ Fun30 مع العديد من بروتينات المسام النووية. علاوة على ذلك، لاحظنا أيضًا انخفاضًا في مستوى Mps3 وNup84 عند ال DSB واحد لا يمكن ترميمه بغياب ال Fun30مما يشير إلى أن ال Fun30 يساعد على نقل ال DSBs غير القابلة للترميم نحو المحيط النووي. بالإضافة إلى ذلك، لاحظنا أن ال Fun30 يدعم توظيف ال Htz1 متغير الهيستون H2A عند ال DSB. وبالتالي، تفضل ال Fun30 نقل ال DSB من خلال التحكم في مستويات متغير الهيستون Htz1 وتفضيل استئصال نهاية الحمض النووي في موقع ال DSB.

مفاهيم البحث الرئيسية: ترميم الحمض النووي، إعادة تشكيل الكروماتين، دورة الخلية، الغلاف النووي، Fun30.

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Dedication

To my beloved children, Hoorain and Muhammad Yahya

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List of Abbreviations

BIR	Break-induced Replication
BP	Base Pair
CUE	Coupling of Ubiquitin Conjugated to ER Degradation
DDC	DNA Damage Checkpoint
DDR	DNA Damage Response
DNA	Deoxyribo Nucleic Acid
ChIP	Chromatin Immunoprecipitation
CPT	Camptothecin
DSB	Double-Strand Break
GFP	Green Fluorescent Protein
HU	Hydroxyurea
HR	Homologous Recombination
HO	Homothallic Switching
MMS	Methyl Methanesulfonate
MMEJ	Microhomology-Mediated End Joining
NHEJ	Non-Homologous End Joining
NER	Nucleotide Excision Repair
NPC	Nuclear Pore Complex
PCR	Polymerase Chain Reaction
RNAPII	RNA Polymerase II
RPA	Replication Protein A
SSA	Single Strand Annealing

SsDNA	Single-Stranded DNA
TCA	Trichloroacetic Acid
TAP	Tandem Affinity Purification

Chapter 1: Introduction

1.1 Overview

DNA encodes the genetic information that is required for development, growth and functioning of an organism. Nucleosomes are the basic building blocks of chromatin, consisting of approximately 146 base pairs (bp) of DNA wrapped around histone octamer. Nucleosomes are packaged in turn to folded structures that inhibit the interaction of DNA with any proteins. Access to chromatin is regulated by covalent posttranslational modification of histone proteins, and by the action of ATP-dependent remodeling enzymes. DNA is under continuous attack from both endogenous and exogenous damaging agents that could cause breaks in DNA. Double-strand breaks (DSBs) are among cytotoxic kind of DNA lesions. Genomic instability is a hallmark of cancerous cells. Cells have evolved different pathways to guarantee accurate transfer of the genetic information. Mutations in any of these mechanisms can result in genomic changes and lead to cells with altered growth and ultimately to cell death. Thus, the repair of DNA breaks is crucial for maintaining genome stability. Cellular response to damage includes mechanisms to halt the progression of the cell cycle and to restore the structure of the broken chromosome. Repair needs to take place within the complex organization of the chromatin, and this requires changes in chromatin adjacent to it. Unrepaired or persistent DNA breaks in genome is translocated towards nuclear periphery in order to prevent gross chromosomal rearrangement within the active euchromatic regions. The section below will demonstrate different kinds of DNA breaks that can result in genomic inaccuracy if not fixed with a special focus on DNA double-strand lesions that

become cytotoxic if not accurately repaired. Thus, an overview on the role of chromatin remodelers in DNA repair and the conditions in which DNA breaks moves to nuclear periphery will be presented.

1.2 Statement of the Problem

Understanding the DNA repair pathways that are undergoing in a eukaryotic cell is crucial. Fun30 (Function unknown now 30) is a chromatin remodeler with versatile properties. It is involved in maintaining silenced chromatin at heterochromatic regions and favoring homologous recombination (HR) pathway by assisting extensive DNA end resection. The budding yeast is a simple eukaryotic organism, and, like human's, yeast genome is also packaged into chromosomes that can be easily manipulated genetically. *Saccharomyces cerevisiae* has approximately 6,000 genes. Many of human orthologous genes are there in, yeast genome. For example, SMARCAD1, an orthologue of Fun30 in humans, shows, many properties like Fun30. Identification, characterization, and investigations of action of a chromatin remodeling complex and their role in DNA repair is very exciting and could lead to the development of potential drugs to cure human diseases, specifically cancer in the future. Cancer is now the second leading cause of death worldwide, third in the UAE and is recognized as a major healthcare problem (Al-Shamsi et al., 2022). The control of this disease and the development of novel therapies can benefit enormously from studying the basic mechanisms of transcriptional regulation and chromatin remodeling in yeast that can later be extended to the mammalian system.

1.3 Sources of DNA Damage

Living organisms are under continuous exposure to several DNA damaging substances that have impact on health and modulate disease-states. Maintaining correct genomic sequence information in an organism is important for the preservation of life. Genomic mutations play a vital role in its maintenance and evolution, while also lead to aging, many human diseases and cancer. DNA, the basic unit of inheritance, an inherently reactive substance, vulnerable to chemical distortions by different endogenous and exogenous sources. A majority of the endogenous DNA damage result because of the action of reactive oxygen species that are generated within the cell on the DNA (Chalissery et al., 2017). Exogenous DNA distortions can develop because of environmental, physical, and chemical sources such as alkylating agents, UV, and ionizing radiation (Chatterjee & Walker, 2017).

1.4 DNA Damage Responses

The capability of an organism to deal productively with DNA damage is important for genome stability and cell survival. Malfunctioning in these mechanisms can cause chromosomal abnormalities, deleterious mutations, and cancer. As part of the DNA damage response (DDR), cells have emerged with surveillance pathways, called DNA damage checkpoints to monitor the successful completion of cell cycle events and initiate a coordinated cellular response when damage is detected (Bartek & Lukas, 2007). Activation of the DNA damage checkpoints (DDC) results in cell cycle arrest, activation of transcriptional programs from genes like ribonucleotide reductase gene and thus the start of DNA repair pathways. If the DNA damage is extreme, cell enters cellular senescence or

programmed cell death. These pathways work to conserve genome integrity. The DDC responses are downregulated once the repair is done, and cells re-enter the cell cycle in a process called ‘recovery’. But if the DNA breaks are persistent or irreparable, cells can undergo adaptation and may re-enter the cell cycle with the damaged DNA (Clemenson & Marsolier-Kergoat, 2009).

One of the essential aspects of DDR is the repair of damaged lesions. The choice of a living cell with broken DNA is to either fix the damage, adapt to it, or to undergo programmed cell death and the decision depends on the extent of DNA damage. Cells have different pathways to detect DNA damage and ultimately repair it. The phase of cell cycle and kind of DNA damage decide the DNA damage repair mechanism that will be activated.

DNA lesions are mostly repaired by the following pathways:

Base Excision Repair (BER): Most the DNA modifications caused by oxidation, deamination and alkylation are repaired by BER (Krokan & Bjørås, 2013) and this pathway acts as a first line of defense upon DNA damage. The mechanism is as follow: firstly, the damaged base is excised from the break site with the help of enzymes that are called DNA N-glycosylases (e.g., Ntg1, Ntg2, Mag1, Ung1, Ogg1). This generates an abasic apurinic/apyrimidinic (AP) site that is sensed by AP endonucleases to produce 3’-substrate for the enzyme DNA polymerase δ to repair the damage and DNA ligase seal the break (Krokan & Bjørås, 2013).

Nucleotide Excision Repair (NER): Bulkier DNA lesions that can lead to DNA helix distortion are usually repaired by NER. At first different repair factors like Rad4-Rad23 and Rad14 recognize the damage, followed by excision of 24-27

nucleotide long region around the broken DNA with the help of Rad2 and Rad1-Rad10 endonucleases. The damaged nucleotides are removed by helicase Rad3 and transcription factor TFIIH, along with Replication protein A (RPA) and Mms19. The identification of broken DNA lesions in NER is divided in two pathways: global genome repair (GGR) and transcription coupled repair (TCR). GGR has the ability to fix broken DNA with the help of Rad16-Rad7 heterodimer. Whereas, in TCR damaged DNA lesions are approached by RNA Polymerase II (RNAPII), and repair usually happens on the template strand. To detect DNA damage, TCR needs a subunit of RNAPII, Rad26 or the Rpb9 (Rechkunova et al., 2021).

Mismatch Repair: It is the pathway of choice when replication errors or damaged lesions cause mispairing between bases. MUTSα and MUTSβ complexes recognize the mismatch and along with MUTLα translocate on the DNA to the nearest nick site to differentiate the mismatched daughter strand from the parent strand. Then exonuclease Exo1, DNA polymerase δ and ε, and DNA ligase Cdc9 are recruited to the damage site to fix the broken lesions (Fukui, 2010).

Post-Replication Repair: This pathway is further divided into two sub-pathways: 1) translesion synthesis (TLS) 2) template switching (TS). TLS is error-prone, whereas TS is error free. Both pathways of repair are referred as DNA damage tolerance mechanisms where replication machinery ignores the broken lesions and repair happens at later stages (Gao et al., 2017).

Double Strand Breaks (DSBs): DSBs are considered as the most deleterious DNA breaks in which both the strands are nicked at the same time, leading to cytotoxic genetic alterations like insertions/deletions or translocations. DSBs are recognized by proteins that are recruited at the damage site and lead to check point

activation. To sense DSBs, the checkpoint machinery also loads proteins that cause cell cycle arrest to fix the damage (Finn et al., 2012). Apart from DDR process, chromatin remodeling also occurs at the damage site. Remodeling results in the access of the damaged lesions to the repair pathways. The remodeling step is triggered by histone post-translational modifications (PTM) and by the help of specific remodeling complexes (Jeggo & Downs, 2014; Van & Santos, 2018). For example, H2A becomes phosphorylated at serine 129 in yeast (Lee et al., 2014; Redon et al., 2003). This modification expands at damage site (Lee et al., 2014; Renkawitz et al., 2013). Phosphorylated H2AX (γH2AX) acts as a docking site for remodeling enzymes like INO80 or SWR1. These remodelers trigger downstream different DDR factors (van Attikum et al., 2004). DSBs are repaired by several different mechanisms, two of them are:

Non-Homologous End Joining (NHEJ): NHEJ involves the simple re-ligation of broken ends of a DSB, and it occurs mostly during G1 phase of cell cycle (Barlow et al., 2008). NHEJ is error prone because nucleotides loss at the damage site could results in mutations. MRX complex recognizes the DNA lesions (Figure 1.1). The Yku70-Yku80 are recruited to the damage site and ligation is performed by DNA Ligase.

Homologous Recombination (HR): Another pathway to repair DSBs is HR, DNA end resection is critical to repair DSB through HR. Resection is highly regulated during different phases of cell cycle: little resection occurs in the G1 phase, therefore, NHEJ pathway is favored. Whereas in S, G2 and M phases of the cell cycle, resection becomes more prominent, and HR overcomes NHEJ (Cejka, 2015; Ira et al., 2004; Symington, 2014; Symington & Gautier, 2011). The first step in HR

pathway is 5'-3' resection of DNA ends to generate 3' overhangs that are required to load checkpoint and recombination proteins. Initial resection is initiated by Mre11-Rad50-Xrs2 and Sae2, whereas extensive resection is performed upon recruitment of Exo1 exonuclease or by Sgs1-Top3-Rmi1 along with Dna2 endonuclease (Cejka et al., 2010; Mimitou & Symington, 2008; Nicolette et al., 2010; Zhu et al., 2008). This leads to the formation of long stretches of single-stranded DNA (ssDNA) that are bound with RPA, a ssDNA binding protein. Then a nucleoprotein filament is formed by displacing RPA and recruiting Rad51, which along with Rad52 leads to formation of D-loop that invades the sister chromatids and search for homology. DNA polymerases Polδ or Polε extends the 3' end of the invading strand. The second end of the DSB forms a double Holliday junction, where their resolution could result in crossovers (Figure 1.1). Recently, the role of histone acetyl transferase NuA4 in strand invasion and D-loop formation is reported. NuA4 is recruited to donor sequences during recombination event and co-operates with other histone acetyl transferase, the SAGA complex, to favor DNA end resection and to recruit ATP dependent chromatin remodelers at the damage site (Cheng et al., 2021).

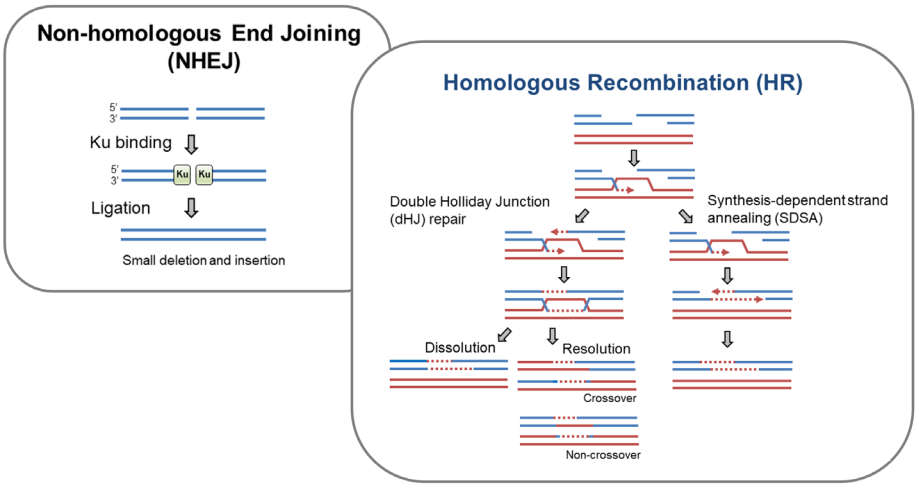

Figure 1.1: Non-homologous and homologous recombination pathways for DSB repair

DSBs can be repaired by different pathways. Non-homologous end joining (NHEJ) and homologous recombination (HR) are two major pathways to repair DSBs. NHEJ is Ku-dependent and ligates DSB ends without using a template. HR uses homologous sequences as repair templates and is considered as the most conserved repair mechanism (scripps.edu/wu/research.htm).

1.5 ATP-Dependent Chromatin Remodelers

Nucleosomes are the building blocks of chromatin. They consist of 146 bp of DNA, wrapped around a histone octamer. The core particle of nucleosome is made up of two H2A-H2B dimers and a H3-H4 tetramer (Pogna et al., 2010). Nucleosomes are further packaged to folded structures that limit the interaction of DNA with other proteins. Chromatin remolding alters the DNA configuration in such a way to allows physical interaction between DNA and different proteins. This is achieved by two broad classes of proteins (Eberharter et al., 2005). The first class comprises enzymes that play a role in catalyzing a variety of post-translational modifications, including, methylation, acetylation, ubiquitination, and phosphorylation of residues on the core region and N-terminus histone tails. These modifications act as a signal towards the downstream regulatory factors to relax the chromatin (Jenuwein & Allis, 2001). The

second class are the ATP-dependent chromatin remodelers that uses energy from ATP hydrolysis to disrupt histone-DNA interactions. This results in the eviction or sliding of nucleosomes from the DNA. These remodelers solely, or in combination with other chromatin remodelers, alter the chromatin structure to allow access of DNA by different proteins involved in replication, transcription and DNA repair (Hassan et al., 2001). Many of the chromatin remodelers have been shown to play a regulatory function in diverse processes. This illustrates that a specific set of signals is responsible for directing correct chromatin remodelers to their target site. There is a concept of "access repair restore" to fix damaged lesions in the context of chromatin (Green & Almouzni, 2002). According to this model, chromatin structure is altered at the DNA damage sites to allow the access of repair machinery to DNA and once repair has been performed, chromatin is restored in its original form. Chromatin remodelers play different roles in different biological processes. Phylogenetic classification of ATP-dependent chromatin remodelers separates them into several major groups:

The SWI/SNF Complex: In yeast, the first identified ATP-dependent chromatin remodeler was the SWI/SNF complex. It was identified through genetic screens for genes that have a role in yeast mating type switching (SWI), and for SUC2 gene expression, which encodes the enzyme invertase important for utilizing sucrose - sucrose non fermenting (Laurent et al., 1993). The SWI/SNF proteins form a large multi-subunit complex of 12 different proteins, with a core catalytic subunit, the SWI2/SNF2. This subunit contains a helicase-like ATPase domain having two tandem RecA-like folds and seven conserved helicase-related sequence motifs that classify it as part of the superfamily 2 grouping of helicase-like proteins (Flaus et al., 2006).

Mutants of this complex are sensitive to DNA distorting agents. SWI/SNF physically associate with damage site (Chai et al., 2005). SWI/SNF plays an important role in chromatin remodeling and exposes DNA for strand invasion and synapsis during HR pathway of DNA repair (Chai et al., 2005). SWI/SNF complex also removes Sir3, allowing promotion of recombination in heterochromatic regions (Sinha et al., 2009).

Many other proteins have been identified that resemble the Snf2 family. The Snf2 family is divided into 24 subfamilies depending upon similarities within these Snf2-specific motifs. Four of these families are well characterized chromatin remodelers, SWI/SNF, ISWI, CHD and INO80 subfamilies (Flaus et al., 2006). All four remodelers utilize energy from ATP to alter histone-DNA architecture.

The SWI/SNF subfamily remodelers: This family comprise of 8 to 14 subunits. Most of the eukaryotes have two SWI/SNF members, the SWI/SNF and the RSC complexes, with two catalytic subunits, the SNF2/SWI2 and the STH1 subunits, respectively. The catalytic ATPase subunit contains a helicase-SANT associated HSAdomain, which binds with bromodomain that identifies the acetylated lysine residues on histone N-terminal tails. SWI/SNF subfamily performs crucial tasks such as removing nucleosomes and facilitating processes like DNA replication initiation (Flanagan & Peterson, 1999), DNA repair (Chai et al., 2005) and transcription (Laurent et al., 1993).

The ISWI (imitation switch) subfamily remodelers: This family contains 2-4 subunits. In yeast, Isw1 and Isw2 are the catalytic subunits with specialized associated proteins. Towards the C-terminus of ISWI family of ATPases, a characteristic set of domains reside, including a SANT domain that interacts with

unmodified histone tails (Boyer et al., 2004). They also have HAND, SLIDE and SANT domains, that play a role in DNA binding. Some members of ISWI family like ACF and CHRAC maintain chromatin architecture and repress replication (Längst et al., 1999; Yang et al., 2006).

The CHD (chromodomain, helicase, DNA binding) subfamily of remodelers: The members of this family was first purified from *Xenopus laevis* and it contains 1 to 10 subunits. In budding yeast, the member of subfamily include Chd1. CHD1 has role in transcription elongation and the formation of regularly spaced nucleosomal arrays. The Mi-2/CHD complex can also deacetylate chromatin structures, repress transcription, and regulate development and promote nucleosome sliding (Bowen et al., 2004; Lusser et al., 2005). Towards the N-terminus of the catalytic subunit, this family contains two tandemly arranged chromodomains that allow it to bind with methylated lysines on the histone terminal tails. This family also contains DNA-binding domains and SANT domains.

The INO80 (inositol requiring 80) subfamily of remodelers: INO80 remodelers show an insert in the middle region of ATPase domain and have a RuvB like DNA helicase (Clapier & Cairns, 2009). This family composed of 10 subunits and contain the SWR1-related complexes that were isolated from budding yeast. SWR1 helps in the incorporation of Htz1 at the DSB site and this incorporation of Htz1 is important for the repair of persistent DSBs. The major characteristic of the INO80 subfamily is a ‘split’ ATPase domain. INO80 plays roles in many pathways, including transcription and repair of broken DNA lesions and helps in the restart of paused replication forks (Morrison et al., 2004).

1.6 Fun30, Chromatin Remodeler

Chromatin remodeler, Fun30 belongs to the SNF2 family. Fun30 regulates several cellular activities, including DNA repair, gene silencing and maintenance of chromatin structure (Dürr et al., 2006). Bioinformatics analysis revealed Fun30 as a member of a distinct subfamily that comprises of SMARCAD1 in humans, Fft1, 2 and 3 in *Schizosaccharomyces pombe* and ETL1 in mouse (Adra et al., 2000; Schoor et al., 1993). Fft3 and Fun30 share a high degree of similarity in their ATPase domains (Neves-Costa et al., 2009). Fun30, a 128 kDa protein, elute in fractions belonging to molecular mass of 250 kDa, suggesting that it exist as a dimer. Co-expression of TAP-tagged and HA-tagged Fun30 showed that HA-Fun30 could co-immunoprecipitate with TAP antibody (Awad et al., 2010). This indicates that Fun30 has ability to directly interact with another molecule of Fun30 and exists as a homodimer of ~250 kDa (Awad et al., 2010).

Like SWI/SNF, the ATPase activity of Fun30 is stimulated by binding to DNA or histone proteins (Awad et al., 2010). Like other remodeling enzymes, Fun30 also binds to DNA and chromatin fragments to perform remodeling activity. A previous study showed that Fun30 as a dimer can bind to single nucleosome with high preference (Awad et al., 2010). This binding interaction was based on the association of Fun30 with DNA flanking the nucleosome core. Like several other Snf2 family members, Fun30 also exhibits ATP-dependent chromatin remodeling activity (Awad et al., 2010). Apart from its remodeling activity, Fun30 can exchange histone dimers (H2A/H2B) better than repositioning nucleosomes, and to some extent it can catalyze transfer of histone octamers (Awad et al., 2010).

The ATPase domain of Snf2 family is divided into two parts: ATPase-N and ATPase-C that are connected by a linker. ATPase-N has the ATP-binding site and ATPase-C participates in tracking along the DNA (Durr et al., 2005). In *Saccharomyces cerevisiae,* structural features of Fun30 ATPase-C domain show major differences in its insertion regions. In Fun30, insertion I is composed of three helices, other remodeling enzymes like Chd1, Rad54, Snf2 exhibit two helices linked by loop. The main difference lies in insertion II region that in Fun30 is much larger and complex than other proteins. Insertion II is highly conserved and seems like hallmark of Fun30 subfamily (Liu & Jiang, 2017). Crystal structure of ATPase-C domain at 1.95Å resolution showed that Fun30 ATPase-C domain has same conserved motifs and structural folds as its homologues Fft3, Etl1, SMARCAD1 but Fun30 exhibit a helix-bundle insertion that is unique to Fun30 subfamily. Fun30 ATPase-C domain is monomeric in solution but dimerizes upon crystallization with molecular weight 34 kDa (Liu & Jiang, 2017). ATPase-C domain of Fun30 also has little affinity for dsDNA (Liu & Jiang, 2017). Fun30 uses ATPase-N domain to interact with dsDNA and form a super shift on native PAGE (Liu & Jiang, 2017), showing that Fun30 has affinity for dsDNA (Awad et al., 2010). Neves-costa et al. (2009) identified the putative CUE (coupling of ubiquitin conjugated to ER degradation) domain towards the N-terminal of Fun30 (Neves-Costa et al., 2009). The motif assists Fun30 to associate with ubiquitin. This motif stretches along 35 amino acids and is characterized by FP/LP (Phenylalanine or Leucine-proline) (Ponting, 2000). This conserved CUE motif is known to require for ubiquitin binding and different cellular functions. CUE domain is a conserved key characteristic of ETL1/Fun30 subfamily. Fft3 and Fft1 also contain a CUE motif towards N-terminus whereas in Fft2 this motif is located downstream of ATPase domain. This putative

CUE motif is evolutionary conserved from yeast to humans as mouse and human homologs ETL1, SMARCAD1 respectively, also contain CUE motif at N-terminal to ATPase domain (Neves-Costa et al., 2009). In budding yeast, CUE motif is necessary for complete functioning of protein as the over expression of CUE deletion and CUE point mutation result in diminished growth (Neves-Costa et al., 2009). This putative CUE motif in many other proteins is known to interact with ubiquitin (Shih et al., 2003).

1.6.1 Role of Fun30 in DNA Repair

1.6.1.1 Fun30 Promotes DNA End Resection

Fun30 is known to play role in DNA repair. It physically interacts with DNA near DSB end as it shows recruitment to about 2 kb on each side of DSB after 1h of DSB induction (Eapen et al., 2012). Fun30 promotes Sgs1 and Exo1 dependent DNA end resection (Chen et al., 2012) by utilizing its ATPase activity (Costelloe et al., 2012) and by altering nucleosome architecture (Eapen et al., 2012). But Sgs1 pathway depends more on Fun30 (Costelloe et al., 2012). Previous studies showed that *FUN30* deletion mutants exhibit the similar phenotype as the resection mutant *sgs1*Δ and *exo1*Δ. This indicates that Fun30 promotes DNA end processing and long-range resection as in absence of Fun30 the formation of longer ssDNA intermediates is abolished or delayed. Absence of Fun30 reduced the resection rate to approximately 1/3 to that of wildtype that is from 4kb/h to 1.2kb/h (Eapen et al., 2012). In *FUN30* deletion mutants resection is severely delayed at regions 5, 10 and 27-28 kb away from DSB site (Chen et al., 2012).

Single strand annealing (SSA) can be used as a marker for resection, in which DSB repair between two directs repeats require extensive resection to repair damage,

FUN30 deletion failed to repair through SSA (Chen et al., 2012). Deletion of *FUN30* allows only 40% of cells to repair damage by SSA and ATPase activity is necessary for this function (Eapen et al., 2012). It is known that cells lacking Fun30 are hypersensitive to topoisomerase I inhibitor camptothecin (CPT) (Neves-Costa et al., 2009) and expression of wildtype but not ATPase deficient (Fun30 K603R) mutant restore CPT sensitivity (Costelloe et al., 2012). Studies showed that resection related function of Fun30 is responsible to repair CPT induced DNA damage as expression of wildtype Exo1 but not ectopically expressed Exo1 (D173A) nuclease deficient mutant, suppresses both CPT hypersensitivity and resection defects (Costelloe et al., 2012). On the other hand, mutants in other chromatin remodelers like INO80, SWR1 or RSC are efficient in repair by SSA (Kalocsay et al., 2009; Shim et al., 2007; van Attikum et al., 2004). This shows that Fun30 has a role in extensive DNA end resection. Co-immunoprecipitation showed that Fun30 interacts with RPA, Exo1 and Dna2, showing Fun30 can directly control resection. As well as in *FUN30* deletion mutant resection enzymes are recruited to DSB site, but they fail to spread farther to the damage site (Chen et al., 2012). It was shown previously that Fun30 has no significant role in later steps of HR and strand invasion processes (Eapen et al., 2012). Overall, chromatin remodeling and DNA end resection mediated by Fun30 come in coupled manner and it is important for the repair of damaged DNA lesions.

Fun30 remodel and perform resection in Rad9 bound chromatin (Chen et al., 2012). Rad9 is negative regulator of resection and present an additional barrier to resection. Rad9 is recruited to histone H2A phosphorylated at Ser129 and histone H3 methylated at Lys79 by Dot1 (Wysocki et al., 2005). Among *rad9*Δ cells, resection rate is markedly increase and the double mutant *fun30*Δ *rad9*Δ are far better in resection compared to *fun30*Δ, this indicates that Fun30 becomes partially

dispensable in absence of γH2A.X and Dot1. Resection barrier generated by Rad9 may be overcome by Fun30 as more Rad9 was seen to be accumulated at DSB ends in *fun30*Δ compared to wildtype cells (Chen et al., 2012). Similarly, SMARCAD1, human homolog of Fun30, knockdown results in reduced accumulation of RPA in to ionizing radiation induced foci, as well as SMARCAD1 knockdown also cause reduction in ssDNA formation. Upon DNA damage SMARCAD1 co-localize with γH2A.X at DNA breaks, which show that SMARCAD1, like Fun30 is recruited to DSB and has a direct role in DNA repair (Costelloe et al., 2012). Overall, this indicates that, the role of Fun30 in favoring DNA end resection is conserved in its human homolog, SMARCAD1 (Costelloe et al., 2012).

1.6.1.2 Mechanism of Fun30 Recruitment to DSB and its Role in Checkpoint Adaptation

Fun30 is necessary for adaptation in response to DSB, and the defect in adaptation is linked to a functional consequence on checkpoint activation (Eapen et al., 2012). Cdk1 (cyclin dependent kinase 1) and cyclin functions together, so it is normal to expect an increase in cyclins at DSB when an increase in Cdk1 level is observed. Studies indicated that cyclins like Cln2, Clb2 and Clb5 show highest enrichment whereas some traces of Clb4, Clb6 and Clb3 were also observed (Chen et al., 2016). Cdk1 and cyclins share same genetic requirement for their recruitment to DSB, so it is possible that these proteins delivered as a complex. Clb2 and Clb5 play main role in Cdk1 recruitment to DSB as well as they stimulate Fun30 phosphorylation by Cdk1 (Chen et al., 2016). Clb5 plays role in extensive DNA resection and linked to both Sgs1 and Exo1 pathways of resection (Chen et al., 2016). Cdk1 activity plays key role in DNA damage response as Cdk1 recruitment decline dramatically in checkpoint deficient mutant *mec1*Δ *tel1*Δ *sml1*Δ, and its level

drops further in initial or extensive resection mutants *mre11*Δ *or sgs1*Δ *exo1*Δ (Chen et al., 2016). Cdk1 recruitment require formation of ssDNA, damage checkpoint activation, and kinase activity of Cdk1. Fun30 is phosphorylated by Cdk1 at S28 residue, this is necessary for its recruitment to DSB site (Chen et al., 2016). Together, it indicates that Fun30 phosphorylation by Cdk1 is important for its recruitment to damage site.

By yeast two hybrid system, it was reported that Fun30 interact with Dpb11, a critical regulator of genome maintenance in *S. cerevisiae* (Byeon et al., 2013; Gritenaite et al., 2014; Ohouo et al., 2010; Ohouo et al., 2013; Pfander & Diffley, 2011). Dpb11 has two BRCT domains that play important role in phospho protein binding (Leung & Glover, 2011). Co-immunoprecipitation experiments show interaction between Fun30 and BRCT1+2 domain of Dpb11, this interaction occurs in S or M phases of cell cycle but not during G1 phase and its regardless of damage (Bantele et al., 2017). Fun30 phosphorylation by Cdk1 (Chen et al., 2012; Chen et al., 2016) is necessary for its binding to Dpb11 as by inhibiting Cdk activity binding of Fun30 with Dpb11 is also strongly reduced (Bantele et al., 2017). Towards the N-terminus of Fun30, S20 and S28 are critical residue for phosphorylation of Fun30 and Fun30-Dbp11 interaction (Bantele et al., 2017; Chen et al., 2016).

In single standard annealing assay, a Dpb11 mutant that is deficient in Fun30 binding is deficient in SSA mediated survival and this defect is completely restored in Fun30-Dpb11 covalent fusion (Bantele et al., 2017). This indicates that Cdk-mediated interaction between Dpb11 and Fun30 is not only important for long-range DNA end resection but also for resection-coupled DNA repair. Mutants that are deficient in DNA end resection like *exo1*Δ *sgs1*Δ*, sae2*Δ*,* or *fun30*Δ are

hypersensitive towards the TOP1 inhibitor CPT (Chen et al., 2012; Costelloe et al., 2012; Eapen et al., 2012; Neves-Costa et al., 2009). The CPT sensitivity of Fun30 SSAA (S20A,S28A) is rescued by the expression of covalent Fun30-Dpb11 fusion (Bantele et al., 2017). Upon *RAD9* deletion, the CPT sensitivity of resection-deficient Fun30-SSAA mutant was further suppressed indicating that Fun30-Dpb11 antagonize the Rad9 (Bantele et al., 2017). Rad9 show interaction with Dpb11 and both share similar position on Dpb11 for binding (Pfander & Diffley, 2011). It was previously shown that 9-1-1 complex helps in loading of Dpb11 to DSB. This complex also show interaction with BRCT3+4 of Dpb11 (Wang & Elledge, 2002). Together, Fun30, Dpb11 and 9-1-1 forms a ternary complex that is cell cycle regulated. If there is any interruption in Dpb11-9-1-1 interaction or Fun30-Dpb11 interaction, Fun30 recruitment to DSB will be reduced (Bantele et al., 2017), this indicates that 9-1-1 complex favors Fun30 targeting to DSB.

Usually, chromatin is a hurdle for DNA end resection and the establishment of Dpb11–Fun30 complex needs to be activated in recombination-permissive stage of cell cycle to overcome the chromatin barrier. TOPB1, a human ortholog of Dpb11 interacts to SMARCAD1, and this binding is dependent on the phospho-protein binding site of TOPB1- BRCT1+2 (Bantele et al., 2017). Surprisingly, despite of low sequence conservation human SMARCAD1 also interacts with yeast Dpb11 which means SMARCAD1 and Fun30 can complement each other functionally. Fun30-SMARCAD1 chimera lacking the Dpb11 interacting region but having TOPB1-binding site of SMARCAD1 is able to rescue the effect of CPT of *fun30*Δ mutant (Bantele et al., 2017). Overall, SMARCAD1/Fun30 interaction with TOPBP1/Dpb11 is regulated through CDK phosphorylation and are conserved over billion years of eukaryotic evolution.

1.6.1.3 Fun30 and Cellular Response to Genotoxins

Fun30 has a potential to regulate cellular response positively or negatively depending on the genetic background of cell. Fun30 plays an important role in cellular tolerance to CPT, methyl methanesulfonate (MMS) and Hydroxyurea (HU). CPT can induce DSB by trapping topoisomerase when it covalently links nicked DNA. MMS methylates the nucleotides leading to DNA replication arrest and ultimately release of polymerase and helicase from replication fork and formation of single-stranded gaps (Byun et al., 2005; Jossen & Bermejo, 2013; Sogo et al., 2002). Arrested replication forks are repaired by the damage tolerance pathway, if not repaired can leads to DSB. Previous study showed that *FUN30* deletion differentially affects the cellular resistance in response to MMS, HU, and CPT (Bi et al., 2015). For the cellular tolerance in response to CPT, Fun30 is required. As its deletion makes cell sensitive to CPT (Eapen et al., 2012). But it has inhibitory role on tolerance to MMS and HU in cells lacking Rad5/Mms2/Ubcl3-mediated DNA damage template switching pathway (Bi et al., 2015). *fun30*Δ partially established the resistance of *rad5*Δ *mms*2Δ and *ubcl3*Δ mutants to MMS and HU (Bi et al., 2015). *FUN30* deletion reduces cellular resistance to MMS and HU at relatively high concentrations. Overexpression of Exo1 compensate for the deletion of *FUN30* in CPT resistance, indicating that Fun30 aid in repairing of CPT induced DSB by favoring DSB end resection (Bi et al., 2015). As previously mentioned, Fun30 directly associates with chromatin at DSB (Eapen et al., 2012). Expression of wildtype but not ATPase mutant recovers the cell from damage caused by CPT which indicates that resection mediated by Fun30 ATPase activity guard the cells from CPT induced DNA damage (Costelloe et al., 2012). However, deletion of *FUN30* leads to 5-fold increase in NHEJ (Eapen et al., 2012), suggesting that it

might act early in resection and control the rate of extensive resection. Upon *fun30*Δ, cells show segregation defects and remain arrested as a large, budded cell (Eapen et al., 2012). On the other hand, among *FUN30* deletion mutants, over expression of Exo1 reduces the MMS sensitivity (Bi et al., 2015), also pointing towards the role of Fun30 in DNA end resection in repair of MMS induced damage. In case of HU, sensitivity is not suppressed by overexpression of Exo1 in *fun30*Δ cells (Bi et al., 2015). Fun30 negatively regulates the HR-linked repair of ssDNA gaps generated by MMS (Bi et al., 2015). It also shows inhibitory role to recover from MMS induced G2/M arrest of *rad5*Δ cells (Bi et al., 2015). *In vivo*, on cellular level CPT leads to a rapid uplift in Fun30 level where as MMS leads to a gradual decline (Siler et al., 2017). There is a difference in pathways activated by CPT and MMS, as CPT arrest cell cycle at G2/M phases (Paulovich & Hartwell, 1995) while MMS hinders S phase progression (Redon et al., 2003). CPT induces a significant increase in Fun30 levels whereas MMS leads to a decrease in Fun30 levels. This can be explained by G2/M and S phase arrest by CPT and MMS, respectively. This indicates that Fun30 level fluctuates during cell cycle being highest at G2/M phase and lowest in early S phase (Siler et al., 2017).

Recent study shows that Fun30 phosphorylation increases upon treatment of DNA damage inducing agents such as MMS, and phleomycin, but it remains largely unaffected by treatment with HU (Chen et al., 2016). Cells arrested at G0, G2/M and S phase by HU shows that Fun30 abundance was too low in G0 cells, moderately high in S phase and much higher in G2/M phase (Siler et al., 2017). Under non-damaging conditions Fun30 is regulated in a cell cycle dependent manner. Fun30 level is low initially when cell enter in S-phase (30 min), and then significantly increases at G2-phase (90 min) (Siler et al., 2017). It was also shown that genotoxin-

resistance by Fun30 is mainly required in G2/M phase of cell cycle, during which Fun30 levels are highest. Recently work from our lab showed that chromatin remodeling by Fun30 is important in dealing with torsional stress and CPT-induced DNA damage (Al-Natour et al., 2021).

In response to CPT, Fun30 promotes the DDC activation. Rad53 phosphorylation is an indicator of checkpoint activation (Hustedt et al., 2013). CPT leads to enhanced phosphorylation of Rad53 in *fun30*Δ compared to wildtype cells . Whereas the DDC induced by CPT is dependent on Rad9 (Hustedt et al., 2013), a decrease in phosphorylation of Rad53 was observed in *rad9*Δ cells upon *FUN30* deletion as well as in WT cells . This shows that Fun30 negatively regulates the Rad9 dependent DDC activation upon treatment with CPT. Fun30 also act to dampen the MMS induced Rad9-dependent DDC activation but Fun30 has no control over Rad9-independent DDC activation by MMS . In case of HU, Fun30 has little or no role on effect on phosphorylation of Rad53 . CUE motif of Fun30 is not required for cellular resistance to CPT, MMS and HU but interestingly, mutation in CUE motif of Fun30 make *rad5*Δ cells more sensitive to MMS which means that CUE motif restricts the Fun30 to inhibit a putative mechanism for countering MMS induced replicative stress (Bi et al., 2015). Overall, it was shown that Fun30 plays an important role in DDC activation and in regulating cellular response to genotoxins.

1.6.2 Role of Fun30 in DNA Silencing

DNA in eukaryotes is packed into the chromatin. Heterochromatin generally made up of orderly arranged nucleosomes with specific histone marks that facilitate higher order packaging of chromatin. Heterochromatin in budding yeast is present at *HML*, *HMR* loci and also at telomeres (Rusche et al., 2003). These regions bear

characteristic histone modifications including hypoacetylation and hypomethylation (Ng et al., 2003; Rusche et al., 2003; Santos-Rosa et al., 2004). Sir proteins are evolutionary conserved and one of key player in formation of heterochromatin, Sir complex is composed of Sir2, Sir3 and Sir4 and they bind to nucleosomes (Moazed, 2001). Heterochromatin formation starts when Sir complex recruit to silencer regions near the *HML*, *HMR* loci or telomeric regions (Rusche et al., 2003). Sir complex deacetylates histone in adjacent nucleosomes upon its recruitment to silencer or telomeres (Moazed, 2001). Deacetylated nucleosomes have ability to bind additional Sir molecules because they can self-interact and bind hypoacetylated histones (Carmen et al., 2002; Hecht et al., 1996; Liou et al., 2005; Rudner et al., 2005). Through the repeated cycles of histone deacetylation and Sir complex recruitment, heterochromatin is formed (Rusche et al., 2003). Preexisting nucleosomes may be repositioned by chromatin remodeling factors but none of Sir proteins have chromatin remodeling activities. There are some chromatin remodelers like Isw1, Snf2 and Fun30 that have role in maintaining silencing at heterochromatic regions (Cuperus & Shore, 2002; Dror & Winston, 2004; Neves-Costa et al., 2009).

1.6.2.1 Fun30 is Involved in Silencing of Heterochromatic Locus HMR, Telomeres and at rDNA Repeats

In budding yeast, ATP dependent chromatin remodeling factors like ISWI1 and ISWI2 are linked to silencing of *HMR*, rDNA repeats but not telomeres, whereas SWI/SNF complex is involved in silencing of telomeres and rDNA repeats but not at *HMR* or *HML* locus (Cuperus & Shore, 2002; Dror & Winston, 2004; Mueller & Bryk, 2007; Mueller et al., 2007). *HMR* is one of the well characterized locus where heterochromatin structures get assembled (Rusche et al., 2003). Many *cis* acting sequences called silencers and *trans* acting factors are involved in maintaining

silencing at these regions. The *HMR-E* silencer is known to maintain repression and comprised of three sites including A, E, and B, that allow binding of origin recognition complex (ORC) and Rap1 (previously reviewed in (Rusche et al., 2003). Deletion of *FUN30* causes the reduction in *HMR-E* silencing (Neves-Costa et al., 2009). ATPase activity is essential for silencing functions as by mutating lysine residue in ATPase domain cancel its functioning. Fun30 and Isw1 play overlapping role in *HML* silencing because deletion of *FUN30* causes extensive changes in primary architecture of nucleosomes pertaining at *HML* (Yu et al., 2011). These changes are different to those caused by *sir2*Δ that completely abolishes heterochromatin. This suggests that in the absence of Fun30 silencing at *HML* loci is partially maintained that is different from fully silent state and fully derepressed form of chromatin. This intermediate state is characterized by hypoacetylation and hypomethylation of histones (Yu et al., 2011). *fun30*Δ generates relatively large breaks/gaps between nucleosomes and are disruptive to heterochromatin functioning (Yu et al., 2011). So, it is possible that Fun30 may remove the large gaps between nucleosomes and contribute to efficient silencing at heterochromatic loci.

Fun30 also interacts with ORC5 and plays role in cell cycle progression as FACS analysis indicates that double mutant of *orc5-1 fun30*Δ shows poor growth and mutant cells having genome content between 1n and 2n. Also, *orc5-1fun30*Δ double mutant has 2-fold reduction in bud formation causing defects in cell cycle (Neves-Costa et al., 2009). This indicates that an epistatic relation exists between ORC5 and Fun30 that needs to be explored further.

During eukaryotic DNA replication, negative supercoil is generated (Simpson et al., 1985). DNA at *HMR* or *HML* is negatively supercoiled when the region is

silent rather than when it is in transcriptionally active state (Bi & Broach, 1997, 1999; Cheng et al., 1998). *FUN30* deletion reduced negative supercoiling, whereas *SIR3* deletion completely disrupt the heterochromatin structure and reduce the negative supercoiling by linking number of ~9. It is obvious that *FUN30* deletion results in change in *HML* topology (Yu et al., 2011). *FUN30* deletion also change the positioning pattern of nucleosomes at *HML* locus, at least 12 nucleosome within *HML* locus and 4 nucleosomes outside of *HML* region (Yu et al., 2011). How Fun30 is directed towards heterochromatin is still not clear. There is possibility that CUE motif of Fun30 assist its directionality towards heterochromatin by recognizing ubiquitin component of heterochromatin. And the fact that partially silent chromatin structure form upon deletion of *FUN30* indicates the involvement of other chromatin remodelers, there is possibility that these factors play redundant role in heterochromatin.

rDNA locus consist of tandem array of 100-200 repeats of 9.1 kb units on chromosomes XII. Fun30 was shown to be involved in silencing of rDNA repeats (Neves-Costa et al., 2009). Fun30 occupancy is also high close to telomeres and drops towards centromeres (Durand-Dubief et al., 2012). This indicates that Fun30 play a role in maintaining silencing at these heterochromatic regions.

1.6.2.2 Fun30 in Supporting Point Centromere Functioning

Different factors contribute to the functional state of chromatin including chromatin remodelers, histone modification, histone variants and non-histone proteins. Histone variants are fundamental part in order to organize chromatin to delineate specific domains (Sarma & Reinberg, 2005). Histone H3 variant, CENP-A (CENH3) is known to characterized centromeric regions (Torras-Llort et al., 2009).

Centromeres serve as attachment point for kinetochore proteins that in turn interact with microtubules of the mitotic spindle. Eukaryotic centromere are enriched with histone H3 variant CENPA (called Cse4 in budding yeast) where they serve chromosome segregation and kinetochore assembly (Ekwall, 2007).

In budding yeast, centromeres are well positioned and composed of Cse4 containing variant nucleosomes, approximately 125 base pair comprising 3 regions (CDEI, CDEII, CDEIII) (Chen et al., 2000; Collins et al., 2005; Ekwall, 2007; Fitzgerald-Hayes et al., 1982; Furuyama & Biggins, 2007; Keith et al., 1999; Ortiz et al., 1999). A Cse4 specific histone chaperone, Scm3, promotes the recruitment of Cse4 (Camahort et al., 2007; Mizuguchi et al., 2007). Each single point centromere is important for viability. ChIP-Seq results for the whole genome wide binding profile for Fun30 shows that Fun30 is enriched at the intergenic regions. Within Open Reading Frame (ORF), Fun30 is depleted, whereas peaked at the 3' end of genes compared to the 5' start site (Durand-Dubief et al., 2012). Fun30 also show enrichment at the tRNA genes, small nuclear RNA, long terminal repeats, small nucleolar RNA and autonomous replicating sequences (ARS) (Durand-Dubief et al., 2012). Although telomeric repeats are enriched for Fun30 but subtelomeric elements are not. Co-immunoprecipitation experiments also confirmed the recruitment of Fun30 at centromeres (Durand-Dubief et al., 2012). Fun30 affects not only nucleosome positioning but also histone variant, Htz1 levels at centromeres (Durand-Dubief et al., 2012).

Fun30 is also involved in chromosomes segregation. Expression profiling of mRNA using RNA-seq in WT and *FUN30* deleted cells showed that Fun30 activity is mostly required to silence genes. Among the *FUN30* deletion affected genes, 573

genes were upregulated whereas 255 genes show downregulation. Gene ontology analysis confirmed that the upregulated genes group belongs to genes involved in meiosis and chromosomes segregation (Durand-Dubief et al., 2012). *FUN30* deletion causes the upregulation of genes involved in anaphase promoting complex, a component of kinetochore (Durand-Dubief et al., 2012). Together, this indicates the important role of Fun30 in chromosome segregation.

Transcription through centromeres is inhibited to establish and maintain centromere function (Grewal, 2010; Lacefield et al., 2009; Mythreye & Bloom, 2003). Whereas forced transcription through centromeres can disrupt its functioning (Doheny et al., 1993; Hill & Bloom, 1987; Ohkuni & Kitagawa, 2011). Upon introducing the transcription through CEN3 by using a centromere proximal GAL1 promoter by galactose addition and monitoring the segregation by live cell marking reveals that in wildtype cells, in the absence of transcription through CEN3 approximately 1% shows some segregation defects. Whereas, cells lacking Fun30 showed 3-fold increase in segregation defects compared to control cells. Transcription induction through CEN3 makes the situation worse and persistent transcription leads to loss of cell viability (Durand-Dubief et al., 2012). This indicates that Fun30 affects processes downstream of transcription i.e., reestablishment of chromatin.

Minichoromosomes loss assay depicts that Fun30 is important to maintain minichromosome through several generations. *FUN30* deletion causes the change in centromere silencing as deletion of *FUN30* increase the production of cryptic unstable transcript over the centromere compared to wildtype (Durand-Dubief et al.,

2012). This clearly shows that Fun30 promotes faithful chromosome segregation when centromere structure faces a challenge.

Fun30 plays a critical role to maintain normal CEN chromatin structure because deleting *FUN30* causes alteration in CEN flanking nucleosome positioning and MNase accessibility of nucleosome core particle (Durand-Dubief et al., 2012). So, Fun30 not only binds at centromeres but also play role in maintaining their structure.

Three-dimensional positioning of nuclear components and chromosomal loci is necessary for organization of genome processes like replication, transcription and translation and DNA repair. Interaction between nuclear periphery and chromatin is also important. Mostly nuclear envelope show reduces expression and repressive chromatin marks. In fission yeast, the inner nuclear membrane protein Man1, associates with a third of genome (Steglich et al., 2012). Fun30 homolog, Fft3 binds to subtelomeric gene and in cells lacking Fft3 the expression of subtelomeric genes get upregulated (Stralfors et al., 2011). Genome wide ChIP-chip data for RNA polymerase II, Histone variant H2A.Z and histone modification H4K12Ac showed enrichment over the subtelomeric regions in *fft3*Δ cells as compared to WT (Steglich et al., 2015). Subtelomeres also show enrichment for the Man1 (Stralfors et al., 2011) and this interaction is strongly reduced in cells lacking *fft3*Δ. Fft3 even though not enriched at the subtelomeres, affects gene expression in these domains by acting on their borders. Man1 and Fft3 play important role in tethering subtelomeres to nuclear envelope. In wild type cells, approximately 70% of telomeres signals lie in the outermost zone, close to nuclear envelope. Bqt4 is a known protein that helps the anchoring of telomeres to nuclear envelope (Chikashige et al., 2009). Among *bqt4*Δ

*fft3*Δ cells result in loss of peripheral localization of telomeres among more than 50% of cells (Steglich et al., 2015). Fun30 homolog, Fft3 also interacts with the LTRs genome wide and affects their nucleosome occupancy and peripheral localization. Fft3 also show a significant enrichment at tRNA genes (Steglich et al., 2015). Together, this indicates that Fun30 and its homolog, Fft3 play an important role in centromere functioning and telomere anchoring at the nuclear periphery, respectively.

1.6.3 Fun30 and Transcription

Evolutionary conserved ATP-dependent chromatin enzyme Fun30 has been linked with transcription, apart from its role in silencing and DNA end resection. ATP dependent chromatin remodeling enzymes are capable of changing nucleosome occupancy and position by evicting or sliding histone octamer, replacement of H2A-H2B with their variants or evicting H2A-H2B dimer (Hota & Bartholomew, 2011; Narlikar, 2010). Nucleosomes can impose a serious restriction to elongating RNAPII during transcription. So, RNAPII must prevail over the nucleosome burden upstream of transcribing gene. The mechanism by which RNAPII transcribes through nucleosome enriched regions is not defined well. One of the histone chaperones, FACT, is best described factor that modulates nucleosome barrier during RNAPII elongation. *In vitro*, through chromatin templates (Orphanides et al., 1998) and *in vivo*, by co-localizing with RNAPII, (Mason & Struhl, 2003; Saunders et al., 2003) FACT promotes RNA polymerase transcription. Few years back, in a report it was purposed that FACT reduces nucleosome fence by disrupting nucleosome-histone interaction without causing dimer removal (Hsieh et al., 2013). In *Saccharomyces*

cerevisiae, FACT also plays a part in reassembly of nucleosomes after transit of RNAPII (Belotserkovskaya et al., 2003; Jamai et al., 2009).

Fun30 is involved in repressing the expression of certain genes that are known to play a role in metabolism of amino acid and carbohydrates synthesis, as well as the stress response and meiosis related genes. In a synthetic lethal genetic screen, it is found that Fun30 interacts genetically with subunits of SWR1complex and histone H2A.Z (Krogan et al., 2003), meaning sickness can occur between the genes acting between parallel pathways. In *fun30*Δ *htz1*Δ mutants (in yeast, Htz1 is H2A.Z) exhibit sick phenotype indicating that Fun30 and H2A.Z are somehow functionally connected. By using two color gene expression microarray hybridization and data analysis 275 genes are identified as Fun30-dependent genes (Byeon et al., 2013). Among these genes 60% are involved in various biological processes. Approximately 15% of these genes belong to telomeric and subtelomeric regions, and the majority is located widespread along chromosomes (Byeon et al., 2013). This indicates that Fun30 is important in repression of a subset of genes. Fun30 is also involved in remodeling nucleosomes at 5' end of genes because in cells lacking Fun30 the nucleosome free region length distribution is significantly altered. Fun30 also alters the nucleosome positioning at the 5' end of its target genes mostly -1, +2 and +3 nucleosomes, nucleosomes that are around the +1 H2A.Z containing nucleosomes (Byeon et al., 2013). Role of Fun30 in transcription repression comes to front, as Fun30 changes the level of histone acetylation, methylation and ubiquitination at 5' end of its target genes. Among Fun30 repressed genes a substantial reduction in the level of H3K4me3, H3K14ac and H3K4ac is observed at the promoter region. In open reading frame of its target genes an increased in H2BK123 ubiquitination is noticed (Byeon et al., 2013).

In vivo, Fun30 is capable of sliding nucleosomes by using energy from ATP hydrolysis. Fun30 is required for the organization of nucleosomes at specific loci (Byeon et al., 2013) rather than global nucleosome organization like other chromatin remodelers, Isw1 and Chd1 (Gkikopoulos et al., 2011). Fun30 exhibits histone dimer exchange activity *in vitro* (Awad et al., 2010), there can be several possibilities by which Fun30 cause nucleosomes repositioning at 5' ends including: Fun30 can be directly involved in displacement or deposition of nucleosome, it can slide nucleosomes to specific loci or Fun30 slide flanking nucleosomes to allow nucleosome loss or gain by other nucleosome chaperone or by chromatin remodelers (Durand-Dubief et al., 2012; Stralfors et al., 2011).

Homolog of Fun30 in fission yeast, Fft3 shows prompt nucleosome disassociation to favor elongating RNAPII. Fft3 interacts with the RNAPII and in transcription dependent manner it facilitates the nucleosome disassembly (Lee et al., 2017). Fft3 shows a higher enrichment over transcribing region of gene compared to promoters and terminators (Lee et al., 2017). During RNAPII transcription, Fft3 helps to dissociate nucleosome at transcribing regions (Lee et al., 2017). FACT is one of main player in this process, suggesting both interact genetically. Both Spt16 and Fft3 may interacts to reduce nucleosomal barrier ahead of elongating RNAPII (Lee et al., 2017).

Fun30 homolog, Fft3 controls nucleosome occupation at transcribing regions in a way that depends on FACT. But Fft3 is not required for reassembly of nucleosomes as defects in FACT but not in Fft3 result in loss of nucleosomes at transcribing regions (Lee et al., 2017) In co-immunoprecipitation assay, Fft3 immunoprecipitated with RNAPII (either with phosphorylated CTD or

unphophorylated CTD). TAP purified Fft3 show interaction with RNAPII subunits and with Spt16 (Lee et al., 2017). The Ino80 and RSC complexes also affect RNAPII occupancy at promoter regions where as Fft3 has a distinguished feature that it recruits to transcribing regions.

The effect of *fun30*Δ on RNAPII is marginal because of functional redundancy among Fun30 and other remodelers. Previous studies shows that RSC remodeler complex enhance transcription *in vitro* (Carey et al., 2006), by localizing to coding regions and regulating histone occupancy (Ginsburg et al., 2009); (Spain et al., 2014). It will be interesting to get insight whether Fun30 plays a part with RSC complex in modulating RNAPII transcription at coding regions. SMARCAD1, human homolog of Fun30, was also found to localize replicating heterochromatic regions and have a role in maintaining silent heterochromatin (Doiguchi et al., 2016). SAMARCAD1 also associates with human FACT complex SUPT16H, SSRP1 (Doiguchi et al., 2016).

In another study, role of Fun30 chromatin family member Fft3 is shown to involve in suppressing the transcription of long terminal repeats. In budding yeast, the mobility of Ty1 retrotransposons is constricted by the activity of host genes including transcription and chromatin factors. In fission yeast, Tf2 retrotransposons level get upregulated dramatically among *fft2*Δ cells. Although the transcript of retrotransposons is there in wild type cells, but these transcripts are not mature enough to support reverse transcription and to integrate in host genome. Cells lacking Fft3 and Fft2 the transcripts can prime reverse transcription for themselves (Persson et al., 2016). Fft3, like Fun30 is known to bind with LTRs (Stralfors et al., 2011), ChIP-chip experiments suggest that Fft2 is also localized to Tf2 coding regions and

LTR elements (Persson et al., 2016). Fft2 and Fft3 both can substitute each other to some extent as LTR and Tf2 binding properties of Fft2 increases in *fft3*Δ (Persson et al., 2016). By sequencing the mononucleosomal DNA digested with micrococcal nuclease, a 50% decrease in the nucleosome occupancy is observed at the LTR U3 region in *fft2*Δ *fft3*Δ double mutant (Persson et al., 2016). It is also reported that LTR chromatin state in *fft2*Δ *fft3*Δ is the replica of stressed cells. In a recent article a model for transcription at Tf2 retrotransposons is proposed. In wild type cells retrotransposons are flanked by LTRs that contain U3, R and U5 sequences, Fft2 and Fft3 positioned a nucleosome over U3 region as an outcome transcription will initiates downstream of LTR region just upstream of protein coding region. The mRNA produced as a result is not able to perform reverse transcription that is a key step in retrotransposons life. Whereas in cells lacking Fft3 and Fft2 or both, LTR region is fully exposed transcription will start from the promoter and the resulting mRNA will be capable to undergo reverse transcription, by self-priming to the primer binding site in U5. The Tf2 encoding reverse transcriptase cleaves the self-primer from the rest of mRNA and cDNA will be generated. Reverse transcriptase and short cDNA transcript will hybridize to 3' end of mRNA producing double stranded cDNA that can integrate in the genome (Persson et al., 2016). Further studies will determine how well these pathways are phylogenetically conserved among other Fun30 chromatin remodeler family members.

1.6.4 Fun30 and mRNA Splicing

Fun30 have role in mRNA splicing as efficiency of pre-mRNA splicing is severely impaired and the loading of spliceosome machinery is compromised in Fun30-depleted cells. Splicing efficiency decreases from 1.3–1.9-fold at individual

loci in the absence of FUN30. In addition, Fun30 is recruited to the gene body of individual intron-containing genes. Pre-mRNA splicing efficiency is also dependent on the chromatin remodeling activity of Fun30. SMARCAD1, the human homolog of Fun30, also regulates alternative splicing (Niu et al., 2020).

1.7 Chromatin Dynamics as a Part of the DNA Damage Response

To conserve genome stability and to fix cytotoxic DNA breaks, cells evolved a damage response, which involves pathways to identify damage, transmit signal, and to repair damage, as explained in previous sections. Apart from checkpoint activation and chromatin reassembly, DNA repair leads to increase in diffusion of damaged chromatin. This response comprised different mechanisms that favor the encounter of damaged DNA and homology search, leading to enhance genomic stability and cell survival. However, under some conditions, unrestricted DSB mobility is distorting to cell, such as great number of broken lesions or DSBs occurring at TG-rich regions can cause misrepair and chromosomal rearrangements (Marcomini et al., 2018). Apart from the movement of damaged lesions, chromatin architecture away from DSBs is also changed, but the function of this response remains poorly uncharacterized (Cheblal et al., 2020; Mine-Hattab & Rothstein, 2012; Seeber, Dion, et al., 2013).

In last few years, many studies have characterized the nature of increased chromatin mobility after induction of DNA damage. For example, Homothallic switching (*HO*) endonuclease system in budding yeast, induce DSBs at the desired locus. This allows understanding the local movement of chromatin around the break site and the overall mobility of undamaged chromatin.

Local Mobility of Chromatin after DSB: A single and random induced DSB will cause increased in local movements. For instance, in budding yeast, a tagged locus that is several kb away from *HO* endonuclease cutting site, showed enhanced chromatin movement after the DSB induction (Kalocsay et al., 2009; Nagai et al., 2008; Oza et al., 2009). Similarly, formation of Rad52 repair foci after zeocin treatment resulted in increased mobility of chromatin (Dion et al., 2012). The increased chromatin mobility is directly associated with the exploration of nuclear area investigated by distorted loci, possibly favoring homology search (Dion et al., 2012). Whereas, enhanced chromatin dynamics are not useful when homologous template is available for repair (Dion et al., 2013).

Global Mobility of Chromatin after DSB: The term global dynamics first emerged after observing the chromatin mobility of an undamaged chromosome when a single DSB was induced in its homologous counterpart (Mine-Hattab & Rothstein, 2012). For example, a break induced at chromosome IV resulted in enhanced chromatin movement of a tagged locus on chromosome V (Schwartz, 2016). Similarly, a DSB that was introduced at the *MAT* locus on yeast chromosome III enhanced the movement of chromosome VI (Cheblal et al., 2020).

1.7.1 Alterations of the Chromatin Fiber after DSB Induction

Soon after the DSB formation in yeast, the phosphorylation of histone H2A occurs at the damage site, referred to as γH2A.X. This histone modification spreads bidirectionally over large domains surrounding the break site, spanning around 200–300 kb on yeast genome (Lee et al., 2014), and generating ‘DNA repair foci’. The reasons why such large chromosomal domains undergo modification remain mysterious. Recently, a new DNA damage induced chromatin modification at serine

15 of Histone H2A was reported, and this modification only happens when DNA end resection is engaged (Ahmad et al., 2021). Remodeling machinery removes the histones from the damaged site and studies showed that linker Histone H1 and core histone proteins were removed from the damage site (Challa et al., 2021). Remodeling complexes, INO80 and SWR1 are known to play role in enhanced chromatin dynamics after break induction. The large scale spreading of γH2A enhances chromatin mobility, as shown in yeast (Herbert et al., 2017). This indicates that chromatin remodeling as a response to DNA break plays vital role in enhanced chromatin dynamics.

1.7.2 From Chromatin Structure to DSB mobility

During the last decade, mobility of DSBs has been massively investigated. For many years, the issue of DSB mobility has been controversial in higher eukaryotes. However, in *S. cerevisiae*, studies have reported that DSB induction enhances the mobility of DSB itself as well as of undamaged chromosomes (Dion et al., 2012; Mine-Hattab & Rothstein, 2012; Seeber, Dion, et al., 2013). The place on the genome where DSB occurs usually determine the immobility, small scale movements or large-scale mobility and it also depends on the repair kinetic, and mechanisms used at that specific locus (Cho et al., 2014; Gandhi et al., 2012).

Function of this enhanced mobility of DSBs is usually to look for sequence homology (Dion et al., 2012; Mine-Hattab & Rothstein, 2012). However, enhance mobility of DSB may also be required to promote DSB clustering and tethering to subnuclear compartment, that will be discussed in the sections below.

1.7.3 DSB Clustering: An Outcome of DSB Mobility

An outcome of DSB motion is ‘clustering’ i.e., the capability of multiple DSBs to cluster together. Previous studies showed that two DSBs induced in yeast can lead to generation of a single Rad52 repair focus, which means several DSBs can unite in a ‘repair focus’ (Lisby et al., 2003). DSBs clustering is highly controlled mechanism, as it could also leads to enhanced rate of chromosomal translocations (Roukos et al., 2013), but its function remains mysterious. Clustered DSB foci also indicate ‘sequestration bodies’ where DSBs that are persistent or are undergoing slow repair can tether together to remain separated from rest of genome and wait for an adapted molecular and cellular response.

1.7.4 Structure of Yeast Nuclear Envelope

Nuclear periphery, the borderline between cytoplasm and nucleus, is composed of nuclear envelope and nuclear pores. Nuclear envelope is a bilayer membrane made of outer and inner nuclear membranes. Inner nuclear membrane, in yeast is composed of 10 proteins that also include Mps3, an important SUN domain protein (Figure 1.2). Mps3 has a role in duplication of spindle pole body and help to anchor telomeres at the periphery (Jaspersen et al., 2002). The N-terminal domain of Mps3 is not essential for its integration at nuclear envelope and for spindle pole body but required for DSB positioning and telomere tethering (Bupp et al., 2007; Oza et al., 2009). To provide a channel between nucleus and cytoplasm, about 100-200 nuclear pores per cell are there in yeast. Around 30 nucleoporins assemble to make nuclear pore complex (NPC), different porins further assembled to make sub-complexes that further make nuclear pores (Schwartz, 2016). This nuclear pore structure is conserved between yeast and higher organisms. The huge nuclear pore

sub-complex is Y-shaped Nup84, which comprise six core conserved members (Schwartz, 2016). Many other sub-complexes are also present at the ring structure (Figure 1.2). Nuclear baskets protrude into the nucleoplasm and interact with pore ring. Nuclear basket is composed of Mlp1, Mlp2, Nup1, Nup2 and Nup60 and it is important in DNA repair, transcriptional regulation, gene gating and RNA export (Sood & Brickner, 2014). Y-complex also plays an important role to fix broken DNA lesions, since mutants of this complex can cause hypersensitivity to DNA distorting agents (Nagai et al., 2008).

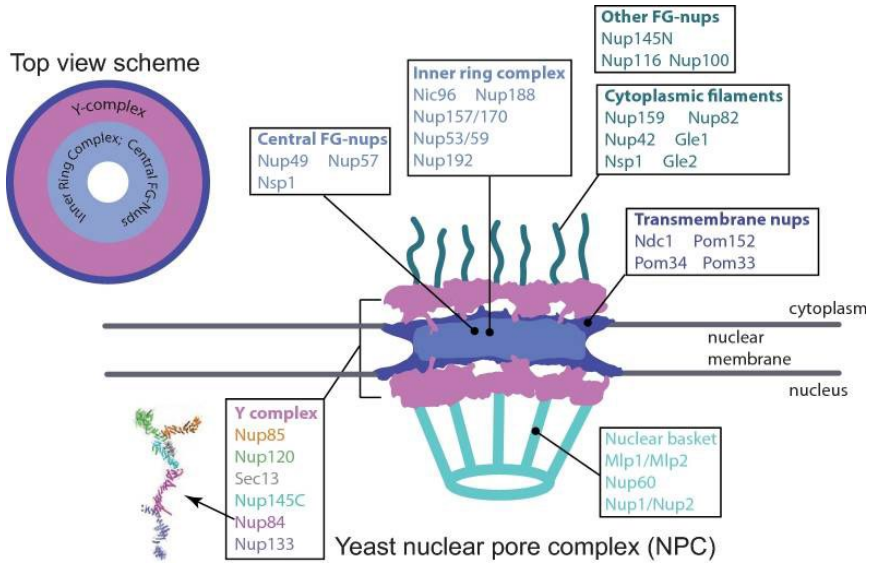

Figure 1.2: Yeast Nuclear Pore complex structure (Freudenreich & Su, 2016)

1.8 DSB Translocation to Subnuclear Compartments

DNA repairs occur in context of heavily packed nucleus. During HR repair of DSB there is an added task of correct homologous donor template. There are many proteins that favor this searching mechanism including Rad51 that allows synapsis (Symington, 2014). During S and G2 phases of cell cycle sister chromatids provide perfect choice for repair. Spontaneous damage that occurs during S-phase of cell

cycle usually repair very quickly. However, some type of DNA breaks appears to be more difficult for repair and can persist for hours, can lead to cell cycle arrest. During past few years, it has been shown that 'persistent' or 'difficult to repair' lesion translocate towards the periphery allowing alternative repair processes to complete the repair (Nagai et al., 2008; Oza & Peterson, 2010).

Movement of damaged DNA inside the nucleus has been noticed under different conditions. DSB induced in yeast by *HO* endonuclease does not result in relocation to the nuclear periphery, under normal conditions. However, when homologous sequences that are used for repair, were removed, or moved to some other chromosomal regions, then broken DNA translocate to nuclear periphery (Nagai et al., 2008; Oza et al., 2009). DSBs that required only 5 kb of resection and were repaired by SSA and did not translocate but those that required long-range resection around 30kb, moved to nuclear periphery (Oza et al., 2009). At nuclear periphery there are two major players to receive these 'persistent' DSBs: an inner nuclear membrane protein, Mps3 and Y-shaped Nup84 complex. Relocation depends on several factors including cell cycle stage: DSB-pore interaction can occur in any cell cycle phase, but DSB-Mps3 interaction occurs only in S and G2 phases (Horigome et al., 2014). DNA structure can also be one of factor as induced subtelomeric DSBs and eroded telomers associate with Nup84 complex, but intact telomers usually tethers at nuclear periphery by Mps3 (Bupp et al., 2007).

Apart from eroded telomeres and DSBs, collapsed replication forks can also translocate to nuclear pores (Nagai et al., 2008). HU stalled replication forks do not translocate to nuclear envelope (Nagai et al., 2008), whereas prolonged treatment with DNA alkylating agents, MMS prior to release in HU, triggers movement

towards nuclear periphery (Nagai et al., 2008). Overall, in yeast, it is well reported that persistent DSBs relocate towards nuclear periphery.

1.8.1 Mechanisms of DNA Breaks Relocation

Persistent DSBs are triggered towards nuclear periphery. Several evidences show that sumoylation pathway plays an important role in this process (Nagai et al., 2008). SUMO (small ubiquitin like modifier) modifies lysine residue of its target proteins. SUMO is added by SUMO ligases, E2 in yeast is Ubc9 and there are three E3 ligases Siz1, Siz2 and Mms21 (Sarangi & Zhao, 2015). Whereas SUMO is removed by Ulp1 and Ulp2. Ulp1 is located at the nuclear pores and shown to interact with Nup84 (Nagai et al., 2008). Many proteins are known that are sumolyated under DNA damaging conditions including many DNA repair proteins like Rad52, Mre11, Cdc13, RPA, sgs1, Smc5/6 and Srs2 (Sarangi & Zhao, 2015). Slx5/8 complex is SUMO-targeted ubiquitin ligases and it contains many SUMO-interacting motifs. Slx5/8 provides linking sites between onsite sumolyation at DNA lesions and translocation to NPC by physically interacting with Nup84 (Nagai et al., 2008). Both proteasome and Ulp1 are located at nuclear pores in order to facilitate degradation of sumolyated proteins that are translocated there (Géli & Lisby, 2015).

One model for Slx5/8 dependent translocation is that, upon DNA damage, at the break site sumolyated proteins are accumulated that can bind Slx5/8. Slx5/8 then mediates interaction with nuclear pores by its capability to bind Nup84 (Nagai et al., 2008). Recent studies shows that monosumolyation by Mms21is sufficient to shift a damaged DNA to Mps3 but relocation to Nup84 complex requires polysumolyation by Siz2 (Su et al., 2015). Slx5/8 is also important for CAG tract relocation (Su et al., 2015). Slx5/8 is only partially important for DSB-Nup84 interaction during S-phase

of cell cycle, but it fully required during G1-phase of cell cycle (Horigome et al., 2016). Nup84 also stabilize Ulp1 and allows its interaction with NPC (Palancade et al., 2007).

Many other proteins are known to involve in relocation of persistent DNA lesion to nuclear envelope. As deletion of Mec1/tel1, Swr1 and Rad9 protein leads to disruption in NPC localization. These proteins are crucial for DSB movement (Horigome et al., 2014). Another protein complex Smc5/6 has role in NPC tethering, Nse5 a subunit of Smc5/6, facilitates its recruitment to sites of replication stress and allow its direct interaction with Slx5. Abolishing this interaction results in decrease association with Mps3 (Horigome et al., 2016). Telomere binding protein Cdc13 has also been shown in DSB translocation to Mps3 (Horigome et al., 2016; Horigome et al., 2014). Recently, it was shown that DSB occurring in subtelomeric region relocate from nuclear envelope location, that is normally occupied by telomeres, to NPC by the help of cohibin, a telomere tethering complex, kinesin 14 a motor protein and α tubulin (Chung et al., 2015). Chung et al. (2015) proposed a model in which an active microtubule motor process moves damage between the NPC and Mps3. Thus, cell has evolved several overlapping complicated mechanisms to relocate DNA breaks to nuclear periphery.

1.8.2 Role of Chromatin Remodelers in Translocation of Persistent Breaks to Nuclear Periphery

Chromatin remodelers like RSC, SWI/SNF, SWR1 and INO80 are previously shown to play role in relocation of persistent DNA breaks to nuclear periphery (Chai et al., 2005; Kobor et al., 2004; Shen et al., 2000; Shim et al., 2005; van Attikum et al., 2004). All four above mentioned complexes have ability to change nucleosome positioning. Snf2, Sth1 and Ino80 ATPases can directly associate with DSB at the

yeast *MAT* locus (Chai et al., 2005; Downs et al., 2004; Morrison et al., 2004; Shim et al., 2005; van Attikum et al., 2004).

Many studies have revealed how chromatin remodeling shapes the nucleosome architecture around the DSB site. Mutations in RSC or INO80 complex result in impairment of core histone loss at DSB (Shim et al., 2007; Tsukuda et al., 2005). Single-stranded DNA formation is also delayed in these mutants (Shim et al., 2007; van Attikum et al., 2004). This means nucleosome remodeling by INO80 and RSC, allows access of DNA ends, to the enzymes that play role in end-processing and DNA repair. Despite of shared similarities between INO80 and SWR1 remodeling complexes, they show different binding affinities for histone H2A variants: INO80 binds γH2AX, while SWR1 binds both γH2AX and Htz1, with a preference to Htz1 (Morrison et al., 2004). Swr1 also recruit Htz1 proximal to DSB site (van Attikum et al., 2007).

In budding yeast, INO80, SWR-C, RSC complex and Fun30 are recruited at DSB on the other hand in mammalian cell, SWI/SNF homolog, INO80 and SRCAP are implicated in repair pathway choice (Chen et al., 2012; Costelloe et al., 2012; Peterson & Almouzni, 2013; Price & D'Andrea, 2013; Seeber, Hauer, et al., 2013; Smeenk & van Attikum, 2013). INO80 favors short-range DNA end resection and allows Rad51 binding (Papamichos-Chronakis et al., 2011; Tsukuda et al., 2005; van Attikum et al., 2007). Fun30 assist in long-range resection and it has ability to exchange histone dimers H2A-H2B (Awad et al., 2010; Chen et al., 2012; Costelloe et al., 2012). SWR-C exchange H2A-H2B dimers for Htz1-H2B (van Attikum et al., 2007). DSB-pore interaction does not need chromatin remodeler INO80 nor RAD51 recombinase activity, on the other hand, association of DSB with Mps3 require both

INO80 and SWR-C complex (Horigome et al., 2014). It was previously shown that, loss of Htz1 result in reduced recruitment of DSB to Mps3 in S and G2 phases of cell cycle (Kalocsay et al., 2009). Htz1 is also essential for proper insertion of Mps3 at nuclear periphery as it physically interact with Mps3 (Gardner et al., 2011). Htz1 incorporation at DSB is required for DSB translocation to both the sites: Nup84 complex and Mps3 (Horigome et al., 2014). Role of Htz1 and SWR-C in DSB repair appears to be conserved in mammals, as Swr1 homolog p400 ATPase and H2A.Z plays important part in Rad51-mediated repair (Costelloe et al., 2012; Courilleau et al., 2012; Xu et al., 2012). Taken together, many chromatin remodelers contribute to favor relocation of persistent DNA breaks to the nuclear periphery.

1.8.3 Potential Functions of DSB Relocation

The role of increased chromatin mobility after DSB formation could have different functional outcomes. The different parts of genome behave differently in DSB repair and repair efficiency depends on the position of the breaks. Different chromatin domains are referred as repair-repressive or repair-prone domains. Nuclear periphery and NPC are documented as repair-prone domains (Horigome et al., 2014; Kalocsay et al., 2009; Nagai et al., 2008), the repair-repressive regions comprised of heterochromatin, ribosomal DNA, and transcribed regions (Torres-Rosell et al., 2007).

DSBs in the repair-repressive regions should relocate to repair friendly regions. In budding yeast, increased chromatin mobility of a DSB induced at rDNA locus allowed its exclusion from the nucleolus (Torres-Rosell et al., 2007). Similarly, the enhanced local DSB mobility is observed after induction of DSB at *MAT* locus resulting in the relocalization of break to the nuclear periphery (Kalocsay et al.,

2009; Oza et al., 2009). Altered chromatin mobility enhances the chromosomal rearrangements. Persistent DSBs relocalize because of unavailability of donor sequences or the DSBs are irreparable. In case of irreparable DSBs, Rad51 remains bound to the damaged DNA, showing persistent homology search, resulting in relocation of breaks to the nuclear periphery (Kalocsay et al., 2009). Persistent DSB induced in yeast leads to their translocation towards NPC or Mps3 (Kalocsay et al., 2009; Oza et al., 2009).

To prevent gross chromosomal rearrangements or cell death, persistent DNA breaks relocalize to nuclear periphery. Mps3 usually suppresses HR and Nup84 favors HR which means Mps3 is repair repressive environment compared to Nup84 complex (Kalocsay et al., 2009; Oza et al., 2009). The usual functions of tethering DSB at the nuclear periphery by Mps3 are to delay HR repair, repress unequal sister chromatid recombination and repress HR with an ectopic donor (when donor locus present at the ectopic location). Whereas, NPC appears to be more permissive for different kinds of repair processes to happen (Kalocsay et al., 2009; Oza et al., 2009). Under different circumstances repair can be promoted or suppressed at nuclear periphery depending upon damage type and cell cycle phase. A persistent DNA break that has failed to be repaired by a conservative process, relocate to NPC that will allow some alternative repair mechanisms to promote survival at the expense of genome stability.

Alternate repair pathways, could be mutagenic, as repair from an ectopic donor could end in loss of heterozygosity, or micro homology mediated end joining (MMEJ) pathway that often cause insertions and deletions (Sfeir & Symington, 2015). Upon relocation of persistent DSB to the nuclear pores, MMEJ and ectopic

break-induced replication (BIR) act as an alternative repair mechanism (Horigome et al., 2016; Nagai et al., 2008). In case of subtelomeric DSB, survival usually depends upon BIR and BIR levels are decreased in mutants that are impaired in localization to NPC (Chung et al., 2015). Eroded telomeres can be fixed by type I or type II recombination, with type II more common (Churikov et al., 2016). Recent studies show that Nup60 undergoes monoubiquitylation and sumolyation. Ubiquitylated Nup60 was shown to interact with Nup84 complex to tether it to NPC and contributes to DNA repair (Niño et al., 2016). Overall, to prevent erroneous recombination and cell death DSBs are relocated towards the nuclear periphery.

1.9 Aims and Objectives

Chromatin remodeler Fun30 has been shown to facilitate DNA end resection at the DSB site during the HR pathway. To get further insight about the role of Fun30 in the repair of persistent DSBs we have designed the following aims:

1. Investigate the interacting partners of Fun30 protein.
2. Investigate the role of Fun30 in relocating persistent DSBs towards the nuclear periphery.
3. Determine the importance of Fun30-mediated DNA end resection in relocation of DSBs towards nuclear periphery.
4. Investigating the role of Fun30 in maintaining Htz1 level at DSB site.

To achieve this, we tested the effects of *FUN30* deletion in relocating persistent DSBs towards nuclear periphery. Results obtained from these studies will enhance our understanding regarding DNA repair and genome stability.

Chapter 2: Methods

2.1 Yeast Strain Construction

All the yeast strains used in this study were made by one step polymerase chain reaction (PCR)-mediated gene deletion or tagging (Longtine et al., 1998). Using this method, a null mutant of a gene can be generated, or proteins can be tagged at their C-terminus. Briefly, for gene deletion, the whole gene is replaced with a DNA cassette containing a selection marker using the HR machinery of the cell. Depending upon 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 Trp1 gene cassettes were amplified by PCR from pFA6a-kanMX6, pFA6a-His3MX6 plasmids or pFA6a-TRP1 plasmids, respectively (generous gifts from Professor Danesh Moazed, HMS, USA). The primers used for the amplifications were designed in a special way that would allow 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 (Figure 2.1). 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 (Figure 2.1). 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. DNA inserts containing the 13Myc tag were amplified from pFA6a-His3MX6 and pFA6a-13Myc-kanMX6 plasmid (Addgene).

All yeast strains were constructed using the following strategy. Briefly, PCR amplification (using Taq DNA Polymerase with ThermoPol buffer for amplifying cassettes for the deletions and Phusion HF polymerase (New England Biolabs) for amplifying cassettes for tagging. The size of the PCR product was confirmed on an agarose gel and the inserts were ethanol precipitated and dissolved in 20 µL of distilled water. Next, 10 µL of the insert was transformed into desired yeast cells. For the yeast transformation, cells were grown in 50 ml of YPD (1% yeast extract, 2% Bacto-peptone, and 2% glucose) media till mid-log phase OD_{600} of 0.5. Cells were then centrifuged, washed with water, and resuspended with 1 ml of buffer containing 100 mM Lithium Acetate and 0.5XTE for 10 minutes. 100 µL of cells were then mixed with 10 µL of 10 mg/ml of salmon sperm DNA, 10 µL of PCR product and 700 µL of a mix of 100 mM lithium acetate, 1X TE, and 40% polyethylene glycol. Cells were then incubated at 30ºC for 30 minutes, after that 80 µL of DMSO was added and heat shocked for 15 minutes at 42ºC. Following heat shock, cells were immediately kept on ice for 2 minutes, centrifuged, resuspended in 1 ml 1X TE buffer and plated directly on desired selection media. For antibiotic resistance, cells were resuspended in 1 ml YPD media, grown overnight at 30ºC and plated on next day on YPD-Geneticin (YPD containing 0.03% Geneticin) plates. Plates were grown for three days; colonies were re-streaked and allowed to grow for another three days at 30ºC and then screened for proper cassette integration by PCR with primers specific to the region of interest.

Plasmid pEAM67 (*EXO1, LEU2, 2*µ) and pEAM71 (*exo1-D173A, LEU2, 2*µ) were used for overexpression of Exo1 and Exo1 D173A mutant, respectively (Sokolsky & Alani, 2000). The yeast strains constructed during the study are listed in Table 1. Primers used in the study are mentioned in Table 2.

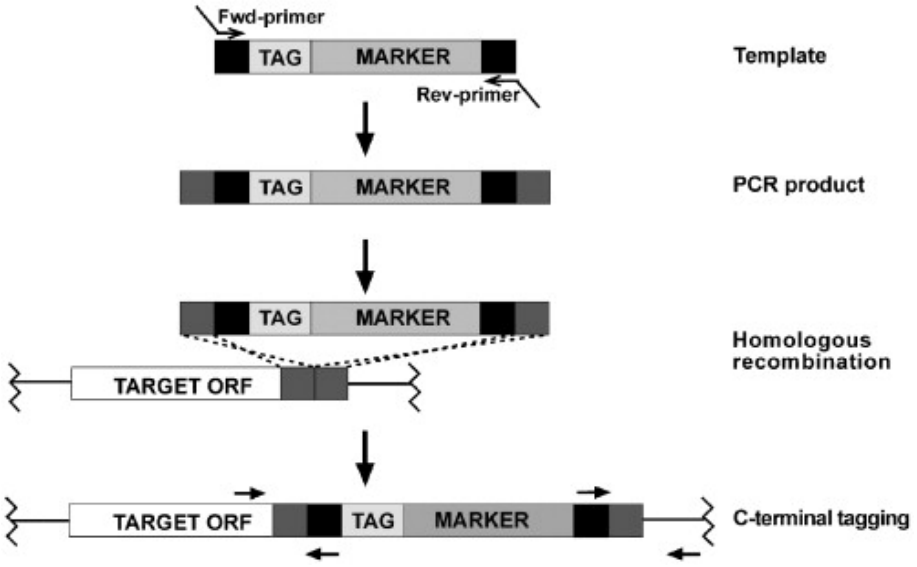

Figure 2.1: Overview of one step PCR-mediated gene tagging

Diagram illustrating C-terminal tagging using one-step PCR amplified cassette.

2.2 Split GFP Assay

Superfolder green fluorescent protein (GFP) was spilt into two parts, GFP1-10 (24 kDa) and GFP11 (3 kDa) (Cabantous et al., 2005). These do not fluoresce individually, but can reconstitute a working GFP, when both are expressed in the same cellular compartment. Plasmid PSJ1321 (pRS315-NOP1pr-GFP11-mCherry-Pus1 was purchased from Addgene. Yeast codon-optimized GFP11 and mCherry fused into the coding sequence of Pus1 was expressed under the NOP1 promoter. This resulting plasmid was then transformed in strain where Fun30 was tagged with GFP1-10. Localization of Pus1 and Fun30 was viewed under Nikon Eclipse 80i confocal microscope at 100X.

Mps3 was cloned at *NheI*/*SalI* sites by replacing *PUS1* from PSJ1321 (pRS315-NOP1pr-GFP11-mCherry-Pus1).

2.3 Tandem Affinity Purification (TAP) of Fun30

C-terminally TAP-tagged Fun30 was pull-down as described (Puig et al., 2001). Pull-down was done at high salt concentration (350 mM) as well as at low salt concentration (150 mM). Briefly, cells were grown in 6 L of YPD media until mid-log phase OD_{600} of 2-3. Cells were pelleted at 6000 RPM and resuspended in equal volume of TAP extraction buffer (25 mM Tris-HCL pH 8, 10% glycerol, 150 mM NaCl, 0.1% Tween-20, supplemented with protease inhibitors (1 µg/ml pepstatin, 2 µg/ml leupeptin, 2 µg/ml aprotinin, and 1 mM PMSF and 1 mM DTT). Cells were then lysed using bead beating (Hamilton Bead-beater), centrifuged at high speed 13,000 RPM for 30 minutes to remove debris, followed by another centrifugation step for supernatant using ultracentrifuge at 40,000 RPM for 20 minutes. The final concentration of NaCl in the extraction buffer was adjusted to 350 mM for pull-down at high salt condition. 500 µL of IgG sepharose fast flow beads (GE healthcare) were added and kept at 4ºC for 3 hours. The lysate was then allowed to drain by gravity flow in a 10 ml Poly-Prep chromatography column (Bio-Rad). Beads were washed thrice with TAP extraction buffer and once with TEV cleavage buffer (20 mM HEPES pH 7.4, 10% glycerol, 100 mM NaCl, 0.1% Tween-20, 1 mM DTT) supplemented with protease inhibitors (1 µg/ml pepstatin, 2 µg/ml leupeptin, 2 µg/ml aprotinin, and 1 mM PMSF). Fun30 was then eluted from IgG resin in 1 ml of the same buffer containing 300 units of TEV Protease at 4ºC overnight. Next day, the flow through was collected and washed with calmodulin binding buffer (10 mM Tris HCl pH 8, 300 mM NaCl, 2 mM $CaCl_2$, 1 mM Mg acetate, 1 mM Imidazole, 0.1% NP-40, 10% Glycerol, 0.5 mM DTT) supplemented with protease inhibitors (1 µg/ml pepstatin, 2 µg/ml leupeptin, 2 µg/ml aprotinin, and 1 mM PMSF), and added to 500

μL of Calmodulin affinity resin (Stratagene) and rotated for 2 hours at 4°C. Beads were collected and washed with calmodulin binding buffer. The bound Fun30 was eluted in 250 μL of calmodulin elution buffer (10 mM Tris-HCl pH 8, 150 mM NaCl, 1 mM Mg acetate, 1 mM imidazole, 2 mM EGTA, 0.1% NP-40, 10% Glycerol, 0.5 mM DTT) supplemented with protease inhibitors (1 μg/ml pepstatin, 2 μg/ml leupeptin, 2 μg/ml aprotinin, and 1 mM PMSF). Eluted Fun30 was pooled together and concentrated using Amicon Ultra centrifugal filter units with a 30 kDa cutoff value. Protein purity and integrity was monitored by western blotting using an anti-TAP antibody (Thermo scientific) and by silver staining. Eluted Fun30 was used for mass spectrometry analysis. The steps of the TAP purification method are illustrated below in Figure 2.2.

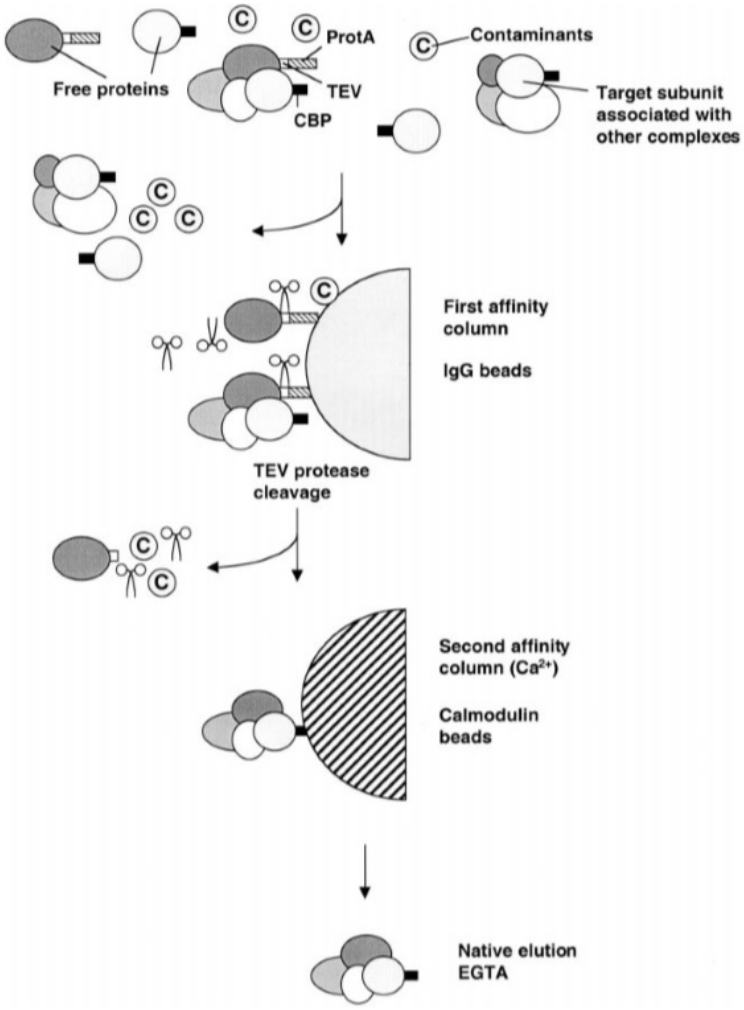

Figure 2.2: Illustration of Tandem Affinity Purification (TAP) method

Diagram of TAP method adapted from Puig, 2001 (Puig et al., 2001). The TAP tag consists of Calmodulin binding peptide (CBP) followed by a TEV cleavage site, then Protein A region. The first step of TAP purification involves incubating the cell lystaes to IgG beads, to allow binding of the protein of interest with the beads using Protein A region. Washing removes contaminant proteins in the lysate. The bound protein is then eluted from IgG beads by cleaving with TEV protease. The eluted protein is bound to a second affinity column, calmodulin beads, which interacts with the second tag on the protein, the CBP. Finally, the bound protein is eluted with EGTA which chelates Ca^{+}, thus releasing the interaction between CBP and calmodulin.

2.4 Co-Immunoprecipitation Assays

Mid-log phase OD_{600} 100 of exponentially growing unfixed cells were pelleted. Cells were lysed using 1X lysis buffer (100 mM Na-HEPES, pH 7.5, 130 mM NaOAc, pH 7.5, 2 mM EDTA, 2 mM EGTA, 10 mM MgOAc, 10% glycerol,

0.25% NP40, 3 mM DTT, supplemented with Protease Inhibitor (1 µg/ml pepstatin, 2 µg/ml leupeptin, 2 µg/ml aprotinin, and 1 mM PMSF)). Cell lysates were then quantified by Bradford (Biorad). 10-15 mg lysates were immunoprecipitated with 6 µg anti-Myc antibody (Sigma) and 30 µl Protein G Dynabeads were added and rotated for 3 hours at 4°C. For TAP tagged protein 30 µL IgG Sepharose beads (GE healthcare) were used for pull-down. Beads were washed with wash buffer, resuspended in 50 µl 2X SDS loading dye, denatured at 95°C for 5 minutes and loaded on SDS-PAGE followed by western blotting and probing with anti-Myc (Sigma), anti-Nsp1 (Thermo scientific) and anti-TAP (Thermo scientific) antibodies.

2.5 Chromatin Immunoprecipitation (ChIP) at *MAT* locus

Cells were grown YEP lactate (1% yeast extract, 2% Bacto-peptone, 3% glycerol, 2% lactic acid, and 0.05% glucose, pH 6.6), or SD-LEU (for all Exo1 plasmid carrying strains) and grown to mid-log phase OD_{600} of 0.3-0.4. At this stage, galactose was added to induce cut at the mating-type (MAT) locus by *HO* endonuclease. In a small fraction of cells, glucose was added to repress the expression of *HO* endonuclease (representing uncut ChIP samples). Total OD_{600} of 20 were collected after 0, 2, 4, and 6 hours of galactose addition, fixed with 1% formaldehyde for 10 minutes, followed by the addition of 0.125 M glycine for 5 minutes to quench the crosslinking. Cells were then centrifuged and washed with TBS buffer (50 mM Tris-HCl pH 7.5 and 150 mM NaCl). 1 ml of FA lysis buffer (50 mM HEPES pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.5 mM DTT supplemented with protease inhibitors). Glass beads were then added to lyse the cells and bead beating was done for 5 times for 1 minute each time. The lysate was collected by centrifugation, 400 ml of FA lysis buffer was

added to make the final volume to 1.4 ml. DNA was sheared using a chilled water bath Diagenode sonicator at high power for 7 minutes (30 seconds on, 30 seconds off). After that, lysate was centrifuged at 14,000 RPM for 10 minutes, and the whole-cell lysate was quantified using Bradford reagent. 0.5 mg lysate was diluted to 500 µl by adding FA lysis buffer. For immunoprecipitation, 2 µg anti-Myc antibody (Sigma) or 4 µg of anti-Histone H2A.Z antibody (Ab4174) was added and kept at 4°C for 3 hours with rotation followed by 30 µl washed Protein G Dynabeads for 2 hours at 4°C. An aliquot of each extract was saved and served as the input. Beads were washed with 1 ml FA lysis buffer, followed by 1 ml FA lysis buffer with 500 mM NaCl and 1 ml Wash buffer (50 mM Tris-HCl pH 7.5, 250 mM LiCl, 1 mM EDTA, 0.1% NP-40, 0.1% Na deoxycholate). This was followed by a final wash with TE Buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA). DNA was then eluted from beads with 100 µl of elution buffer containing (50 mM Tris-HCl pH 7.5, 1 mM EDTA, 1% SDS) while incubating at 65°C for 10 minutes with shaking at 12000 RPM. The elution was repeated, and eluates were pooled and decrosslinked together with the inputs overnight at 65°C. RNaseA/T1 was then added and incubated at 37°C for 30 minutes. DNA was then purified using a MinElute reaction cleanup kit (Qiagen). The immunoprecipitated and input DNA was analysed by quantitative realtime PCR with QuantStudio 7 Flex (Applied Biosystems) using SYBR™ green PCR master mix (Applied Biosystems). ChIP analysis was performed as described previously (Bennett et al., 2013; Ferrari et al., 2015; Marcomini et al., 2018; van Attikum et al., 2007). Briefly, for each time point, the signal from a site near the *HO* DSB at the *MAT* locus was normalized to that from the non-cleaved ACT1 locus in ChIP and input DNA samples. For each time point and site, the normalized ChIP signals were again normalized to the normalized input DNA signals, because end

resection can reduce the available DNA template. Finally, relative-fold enrichment was calculated by dividing the absolute-fold enrichment from induced cells to that of uninduced cells. Quantitation of DNA end resection by qPCR was calculated as described in (Eapen et al., 2012). Briefly, the PCR signal of input sample from each site near the DSB was normalized to the non-cleaved ACT1 site. The value for each time point was further normalized to the value for time '0' and plotted as a line graph against each time point.

2.6 Silver Staining

For silver staining, the SDS-PAGE 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 thrice (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 Fun30 band were visible the gel was fixed by adding 6% acetic acid and scanned.

2.7 Western Blotting

Western blot analysis was performed by running proteins, purified or whole cell lysate, on 6-12% SDS-PAGE 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 30 minutes. The membranes were then incubated overnight at 4ºC with the primary antibody. Next morning, membrane was washed thrice for 10 min each with PBST, 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), α-Myc Antibody (Sigma), α-Nsp1 (Thermo scientific) antibody.

2.8 Extraction of Total Cellular Protein by Trichloroacetic Acid (TCA)

Growing yeast cells to mid-log phase OD_{600} of 2.5-5 was collected by centrifugation, washed with 1 ml sterile water. Cells were then resuspended in cold (0.25 M NaOH/ 1% betamercaptoethanol) and incubated on ice for 10 minutes. 160 µl of 50% TCA was then added and incubated for another 10 minutes on ice. Precipitated proteins were centrifuged at 14,000 rpm for 10 minutes, washed with cold acetone and air-dried for 10 minutes. Proteins were resuspended in 100 µls 2X SDS PAGE loading buffer, boiled for 5 minutes at 95°C, and centrifuged at 13k rpm for 5 minutes. 10-15 µls of supernatant are loaded on SDS-PAGE gel followed by Western blotting.

2.9 Mass Spectrometry Analysis

TAP purified Fun30 was treated with 0.1U Benzonase (Sigma) for 30 minutes at 37°C, to remove any co-purified DNA. The solution was brought to 400 µls with 100 mM Tris-HCl pH 8.5 and chilled. 100 µL of 100% TCA was added and kept overnight at 4°C. Proteins were precipitated by centrifugation at 14000 rpm for 30 minutes at 4°C and washed twice with 500 µL ice-cold acetone then air-dried.

Mass spectrometry analysis for TAP-Fun30 was done at Taplin Mass Spectrometry Facility at Harvard Medical School.

2.10 Rapid Total Cellular Protein Extraction

Total cellular protein was extracted using a method adapted from (Neves-Costa et al., 2009) where yeast cellular proteins are rapidly extracted without mechanical disruptions. This was used to confirm successful tagging of proteins 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.

Table 1: List of yeast strains used in study

Strain	**Description**	**Source**
BY4741	MATa; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*	Euroscarf
JKM179	MAT*α ho*Δ *hml*Δ::ADE1 *hmr*Δ::*ADE1 ade1-100 leu2-3,112 lys5 trp1*::*hisG ura3-52 ade3*::*GAL*::*HO*	James E. Haber
AJC49	JKM179; Mps3-13 Myc: kanMX6	This Study
AZN3	BY4741; FUN30-13myc: kanMX6	This Study
AMI1	BY4741; Fun30 C-TAP: URA3	This Study
AMI2	BY4741; Fun30 C-GFP1-10: URA3	This Study
AMI5	AJC49; *fun30*Δ::TRP1	This Study
AMI6	BY4741; GFP11-mCherry-PUS1: LEU2	This Study
AMI7	AMI2; GFP11-mCherry-PUS1: LEU2	This Study
AMI13	AMI2; GFP11-mCherry-Mps3: LEU2	This Study
AMI14	BY4741; GFP11-mCherry-Mps3: LEU2	This Study
AMI22	BY4741; Mps3-13 Myc: His3MX6	This Study
AMI23	AMI1; Mps3-13 Myc: His3MX6	This Study
AMI24	AJC49; Fun30 C-TAP: TRP1	This Study
AMI25	JKM179; Nup84-13 Myc: kanMX6	This Study
AMI26	AMI25; *fun30*Δ::TRP1	This Study
AMI27	AMI25; Fun30 C-TAP: TRP1	This Study
AMI31	JKM179; *fun30*Δ::KAN	This Study
AMI34	AMI5; pEAM67, Exo1: LEU2	This Study
AMI35	AMI5; pEAM71, Exo1 D173A: LEU2	This Study
AMI36	AMI26; pEAM67, Exo1: LEU2	This Study

Table 2: List of primers used in the study

Name	Sequence
0.18 HO R FP	CCTGGTTTTGGTTTTGTAGAGTGG
0.18 HO R RP	GAGCAAGACGATGGGGAGTTTC
2.1 HO R FP	GCCTCTATGTCCCCATCTTGTCTC
2.1 HO R RP	GTGTTCCCGATTCAGTTTGACG
ACT1 FP	TGTCACCAACTGGGACGATA
ACT1 RP	GGCTTGGATGGAAACGTAGA
Myc check RP	TTAATTAACCCGGGGATCCG
Fun30 upstream 500 F.P	CATCCTACCAGATTCCCG
MI1 Fun30 GFP1-10 FP	TGGAGGATATAATTTATGATGAA AACTCGAAACCGAAGGGAACCAA AGAAGGTGACGGTGCTGGTTTA
MI2 Fun30 GFP1-10 RP	TGGTTTATTTTCTGCTTATCTATTT ACTTTTTTACTATATTTTTATTTAT TCGATGAATTCGAGCTCG
MI3 Mps3 Myc FP	TTCATCCCGCTTCTAACGTCCCAT CATTTGGCCAAGATGAGCTAGAT CAACGGATCCCCGGGTTAATTAA
MI4 Mps3 Myc RP	GCGATTTTCTGGGGGCCAGGGGG TTAGAACGTTTAATTTTTTATTGT CGTGAATTCGAGCTCGTTTAAAC
MI5 Mps3 check FP	CTAATAACCTACATATAATG
MI6 Fun30 check RP	CTTCACGAAAGTTTTACG
FP FUN30 c-ter tag check	GAAAAGATTCATCAACTGGC
Fun30 del FP	GAACGTAAACAAGAAAAAGAGA GAAAATACGCTATAGTTGAAAAC CGGATCCCCGGGTTAATTAA
Fun30 del RP	TATTTTCTGCTTATCTATTTACTTT TTTACTATATTTTTATTTATGAATT CGAGCTCGTTTAAAC
Fun30 del check	GCTCTATATTCGAGTTTGTTGC

Table 2: List of primers used in the study (Continued)

Name	Sequence
MI19 Mps3 Nhe1 FP	ACGATCGCTAGCAAAAAAATGTC TATGAATAACTCAAATGAGCA
MI20 Mps3 Sal1 RP	ACGATCGTCGACTTATTGATCTA GCTCATCTT
MI31 Myc-Nup84 F.P	AGTTAAAAGAGTATCTGGATCTC GTTGCTCGCACAGCAACCCTTTC GAACCGGATCCCCGGGTTAATTA A
MI32 Myc-Nup84 R.P	TTATTGCTGTTTACTTAAAATATA AACTTATTCTGCAATACATTAAT TGAGAATTCGAGCTCGTTTAAAC
MI33 Nup84 Check F.P	AGGGAAGCTTTATGTGGACGC
Fun30 c-myc F.P	ATATAATTTATGATGAAAACTCG AAACCGAAGGGAACCAAAGAAC GGATCCCCGGGTTAATTAA
Fun30 c-myc R.P	TTCTGCTTATCTATTTACTTTTTT ACTATATTTTTATTTATTTAGAAT TCGAGCTCGTTTAAAC
JC52 Fun30 C-TAP FP	GGATATAATTTATGATGAAAACT CGAAACCGAAGGGAACCAAAGA ATCCATGGAAAAGAGAAG
JC53 Fun30 C-TAP RP	GGTTTATTTTCTGCTTATCTATTT ACTTTTTTACTATATTTTTATTTA TTACGACTCACTATAGGG

Chapter 3: Results

3.1 Pull-Down of Fun30 Protein at Different Salt Concentrations

To gain insight about different functions of Fun30 protein, it was pulled down at two different salt concentration, 150 mM NaCl and 350 mM NaCl using tandem affinity purification (TAP) technique (Puig et al., 2001). The purpose to perform TAP at two different salt concentrations was to reveal difference between strong and weakly interacting partners that pull-down along with TAP tagged Fun30. Fun30 is a 128 kDa protein, shown as double bands (could be modified form of Fun30) that are usually noticed with purified Fun30 (Figure 3.1).

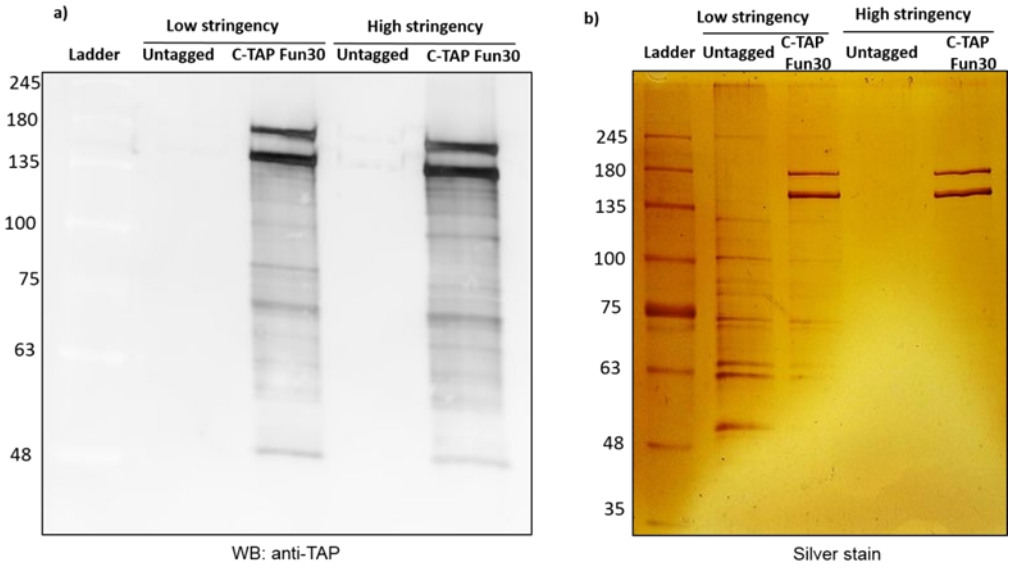

Figure 3.1: Pull-down of Fun30 by tandem affinity purification method

a) Western blot of low and high stringency pull-down of untagged and C terminal-TAP tagged Fun30. b) Silver staining of low and high stringency pull-down of untagged and C terminal-TAP tagged Fun30.

Pulled-down Fun30 was TCA-precipitated and sent for the mass spectrometry analysis, which indicate several binding partner including proteins belonging to the nuclear periphery (Table 3). Previously, our group has reported that Fun30 has

chromatin remodeling activity, and it can exchange histone dimer H2A-H2B (Awad et al., 2010). Later, it was shown that Fun30 has a role in DNA repair by favoring DNA end resection during HR (Chen et al., 2012; Costelloe et al., 2012). Pull-down of several proteins belonging to the nuclear periphery indicates that Fun30 might physically associate with periphery where it deals with heterochromatic regions and maintain them in their preferred silent state. ChIP-seq data of Fun30 showed its localization to several regions in the genome including centromeres, telomeric repeats, etc. (Durand-Dubief et al., 2012). Fun30 might also have a role in the repair of persistent DSBs that are targeted towards the nuclear periphery. Overall, our data shows that several proteins belonging to nuclear membrane pull-down with Fun30 at high salt stringency.

Table 3: Mass spectrometric hits of potential Fun30 interacting proteins (at high salt strigency) belonging to nuclear periphery

Gene Symbol	**No. of Unique Peptides in**	
	Pull-down	**Control**
FUN30	128	0
NSP1	30	3
NUP60	10	0
NUP2	10	0
NUP57	8	0
NUP116	4	0
NUP145	2	0
NUP49	2	0
NUP1	1	0

3.2 Fun30 Physically Interacts with Nsp1

The physical interaction between Fun30 and one of the top hits that was obtained from mass spectrometry, Nsp1, was tested by co-immunoprecipitation. To this end, Fun30 was tagged at the C-terminus with 13Myc-tag and

immunoprecipitation was done with anti-Myc antibody followed by western blot analysis for probing Nsp1 by anti-Nsp1 antibody (Figure 3.2). Results show that Fun30 interacts with Nsp1.

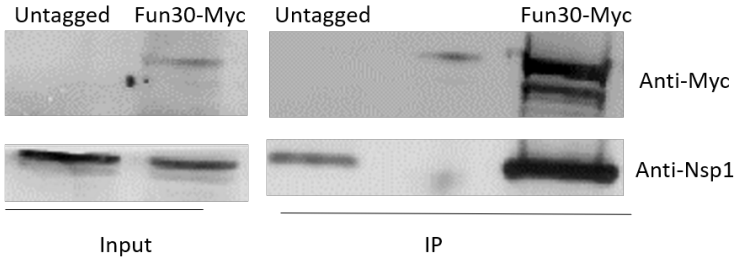

Figure 3.2: Fun30 physically interacts with Nsp1

Co-immunoprecipitation assay shows an interaction of Nsp1 with 13-Myc tagged Fun30. IP samples along with inputs were loaded on SDS-PAGE and analyzed by western blotting. Upper panel shows western blot probed with anti-Myc antibody to detect Fun30. Lower panel shows the blot probed with anti-Nsp1 antibody to detect the immunoprecipitated Nsp1.

3.3 Fun30 is a Nuclear Protein

To examine the localization of Fun30 in cell, we utilized a split GFP assay. In this assay, the superfolder GFP is split into two parts [GFP1-10 (24 kDa) and GFP11 (3 kDa)] asymmetrically where these do not fluoresce separately but can reconstitute a working GFP when expressed in the same cellular compartment (Cabantous et al., 2005; Cabantous & Waldo, 2006; Smoyer et al., 2016) (Figure 3.3a). Yeast codon-optimized GFP11 and mCherry were fused with the coding sequence of Pus1, a yeast nuclear protein, at the N-terminus and was expressed under the *NOP1* promoter. When *NOP1*pr-GFP11-*mCherry*-Pus1 was expressed alone, no green fluorescence was detected in the green channel, but fluorescence in the red channel showed the actual positioning of Pus1, i.e., nucleus (Figure 3.3b). Fun30 was tagged at the C-terminus with yeast codon-optimized GFP1-10 and was expressed under endogenous promoter. GFP1-10-Fun30 showed no fluorescence in the green or red channel. But

when *NOP1*pr-GFP11-*mCherry*-Pus1 and GFP1-10-Fun30 were co-expressed in the same cells, fluorescence at 488 nm was observed in both green channel and red channel, indicating the co-localization of Fun30 with Pus1 in the nucleus (Figure 3.3b).

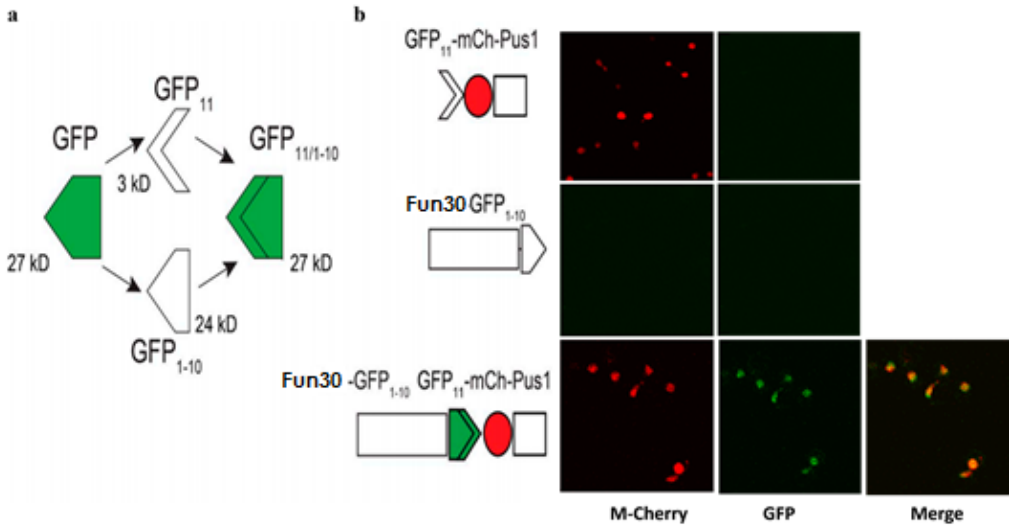

Figure 3.3: Fun30 is a nuclear protein

a) Schematic diagram showing functional GFP split into two parts: GFP1-10 and GFP11. b) Yeast codon-optimized GFP11 and mCherry fused into the coding sequence of Pus1 and was expressed under the NOP1 promoter. Fun30 was fused with GFP1-10 and was expressed under the endogenous promoter of Fun30. When *NOP1*pr-GFP11-*mCherry*-Pus1 was expressed alone, no fluorescence in the green channel was observed, but fluorescence in the red channel indicated the actual positioning of Pus1. When GFP1-10 was fused with Fun30 in *NOP1*pr-GFP11-mCherry-Pus1 expressing cells, GFP fluorescence was observed in the green channel at 488 nm indicating the co-localization of Fun30 and Pus1 in nucleus.

3.4 Investigating the Co-localization of Fun30 with Mps3 at the Nuclear Periphery

As mentioned in the introduction section that Fun30 has a role in silencing of heterochromatic regions that are towards the periphery of the nucleus, Fun30 like other chromatin remodelers might have role in relocating persistent DSBs towards the nuclear periphery. Therefore, Fun30 by physically interacting with the nuclear membrane proteins may help in the processing of persistent DSBs that are translocated towards nuclear periphery. For that reason, we wanted to investigate the

presence of Fun30 near the nuclear periphery. Mps3, an inner nuclear membrane protein, is important to tether persistent DSBs towards the nuclear periphery. To examine the co-localization of Fun30 with Mps3 near inner nuclear membrane, we utilized the split GFP assay as mentioned in material and methods (Figure 3.4a). Fun30 was tagged at the C-terminus with yeast codon-optimized GFP1-10 and was expressed under endogenous promoter. When *NOP1*pr-GFP11-*mCherry*-Mps3 and GFP1-10-Fun30 were co-expressed in the same cells, fluorescence at 488 nm was observed in both green channel and red channel. However, the long tagging of Mps3 in plasmid PSJ1321 (*NOP1*pr-GFP11-*mCherry*-Pus1) to replace Pus1 with Mps3, led to some mutation in the construct since we observed the expression of Mps3 throughout the cell, not only at the nuclear periphery (Figure 3.4b).

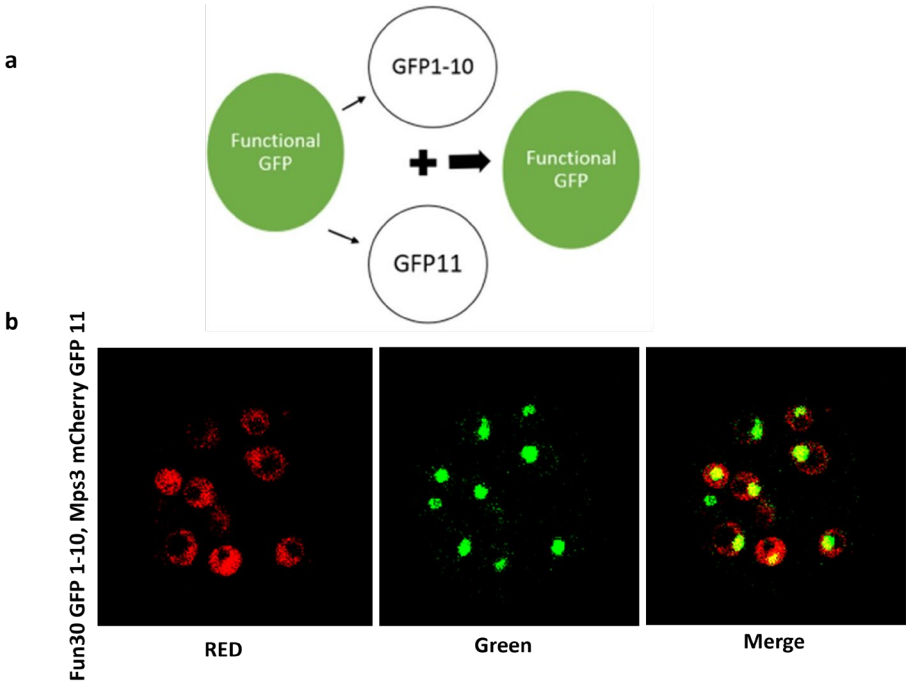

Figure 3.4: Checking the co-localization of Fun30 with Mps3

a) Schematic diagram showing functional GFP split into two parts: GFP1-10 and GFP11. b) GFP1-10 was fused with Fun30 in *NOP1*pr-GFP11-mCherry-Mps3 expressing cells.

3.5 Investigating the Physical Interaction between Fun30 and Mps3

As mentioned above, we were not able to visualize the co-localization of Mps3 and Fun30 at nuclear periphery using split-GFP assay. So, we sought to investigate the physical relationship between Fun30 and Mps3 using co-immunoprecipitation experiments, by tagging Mps3 with 13Myc-tag in a strain where Fun30 was tagged by TAP-tag. Since the TAP-tag constructs include a protein A component, therefore, IP can only be conducted in one direction (with IgG Sepharose beads). For example, when we are looking for an interaction between a TAP-tagged protein and a Myc-tagged protein can only be done by immunoprecipitating the TAP-tagged protein with IgG. In the other direction, the addition of the anti-Myc antibody will result in the immunoprecipitation of the TAP-tagged protein regardless of its binding to the Myc-tagged protein (IgG binding to protein A). For that reason, immunoprecipitation was done with IgG Sepharose beads (GE healthcare) followed by western blot analysis using anti-TAP and anti-Myc antibodies (Figure 3.5a). Results showed that no physical interaction was observed between Fun30 and Mps3 in our co-immunoprecipitation experiment.

We sought to investigate the physical interaction between Fun30 and Mps3 after inducing DNA damage. Towards this end, galactose-induced expression of *HO* endonuclease induces an irreparable DSB at the *MAT* locus on chromosome III in a donorless *hml*Δ*hmr*Δ strain. Immunoprecipitation was done using IgG Sepharose beads (GE healthcare). Results indicate that even after DSB induction no physical interaction was observed between Fun30 and Mps3 (Figure 3.5b). Surprisingly, we observed reduced binding of Fun30 after 4 hours of damage compared to uncut (Figure 3.5b). Together, we were not able to identify physical interaction between

Fun30 and Mps3 using Co-IP. But there could be a functional interaction between both proteins that will explore by ChIP and is discussed in section below.

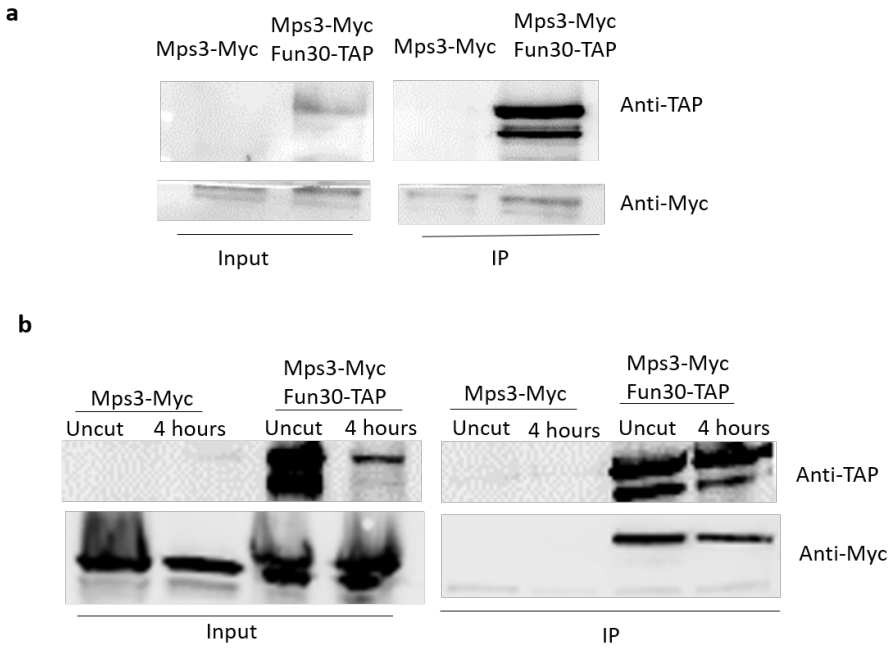

Figure 3.5: Investigating the physical interaction between Fun30 and Mps3

a) Co-immunoprecipitation assay to identify the physical interaction between TAP-Fun30 and 13-Myc tagged Mps3. IP samples along with inputs were loaded on SDS-PAGE and analyzed by western blotting. Upper panel shows western blot probed with anti-TAP antibody to detect Fun30. Lower panel shows the blot probed with anti-Myc antibody to detect Mps3. b) Co-IP assay after inducing a single irreparable DSB at the *MAT* locus. Samples were collected before the DSB induction and after the 4 hours of DSB induction. Fun30 was tagged with TAP-tag and Mps3 was tagged with 13-Myc tag.

3.6 Fun30 Assists Translocation of Persistent DSBs Towards Mps3

Chromatin remodelers like INO80 and SWR-C have a role in translocation of the DSB towards the nuclear periphery. DSB relocation to the nuclear periphery has been shown to be completely abolished upon loss of Swr1 or SWR-C component Arp6 (Horigome et al., 2014). Similarly, Htz1 is also shown to be important to shift damaged DNA to both Mps3 and nuclear pores (Horigome et al., 2014). In another

study, INO80 was shown to favor resection at the damage site by removing nucleosomes (Chen et al., 2012).

Extensive DNA end resection that generates long 3' single-stranded DNA is crucial for the translocation of DSB to the nuclear periphery. Based on the role of Fun30 in long-range resection during DSB repair and its sequence homology with the chromatin remodelers, Ino80 and Swr1 of the Snf2 remodeler family (Flaus et al., 2006), we hypothesized that Fun30 might also be involved in the translocation of persistent DSBs to the nuclear periphery. To test this, we have utilized a well-standardized system for monitoring the dynamics of recruitment of repair factors at a specific DSB by ChIP analysis (Bennett et al., 2013; van Attikum et al., 2007). In this system, galactose-induced expression of *HO* endonuclease induces an irreparable DSB at the *MAT* locus on chromosome III in a donorless *hml*Δ*hmr*Δ strain (Figure 3.6a). The association of Mps3 with the persistent DSB was monitored by ChIP with anti-Myc antibody followed by real-time PCR using primers specific to regions 0.18 kb and 2.1 kb to the right of DSB at 0, 2, 4, and 6 hours of induction of DSB in the presence or absence of Fun30 (0-hour time point refers to uncut). Kalocsay et al. (2009) found that Mps3 associate with the break only after few hours of DSB induction. We also observed that after break induction, Mps3 shows interaction with the DSB, reflecting the relocation of the DSB to the nuclear periphery (Figure 3.6b). This indicates that persistent DNA breaks translocate towards the nuclear periphery after DSB induction. At all the time points and both regions (0.18R and 2.1R) we have tested, *fun30*Δ mutants displayed 2-3 folds reduced Mps3 enrichment at DSB compared to the WT control (Figure 3.6b) suggesting that Fun30 plays an important role in the repair of persistent DSBs by favoring their translocation towards Mps3. To monitor the progression of DSB processing, we measured the loss in the PCR

signal using qPCR with primers around DSB sites (Figure 3.6c). We have also observed a slight delay in the resection rate upon the loss of Fun30 at 0.18R and 2.1R regions, indicating that apart from its role in long-range resection, Fun30 is also important for short-range resection, even close to the DSB ends (Figure 3.6c).

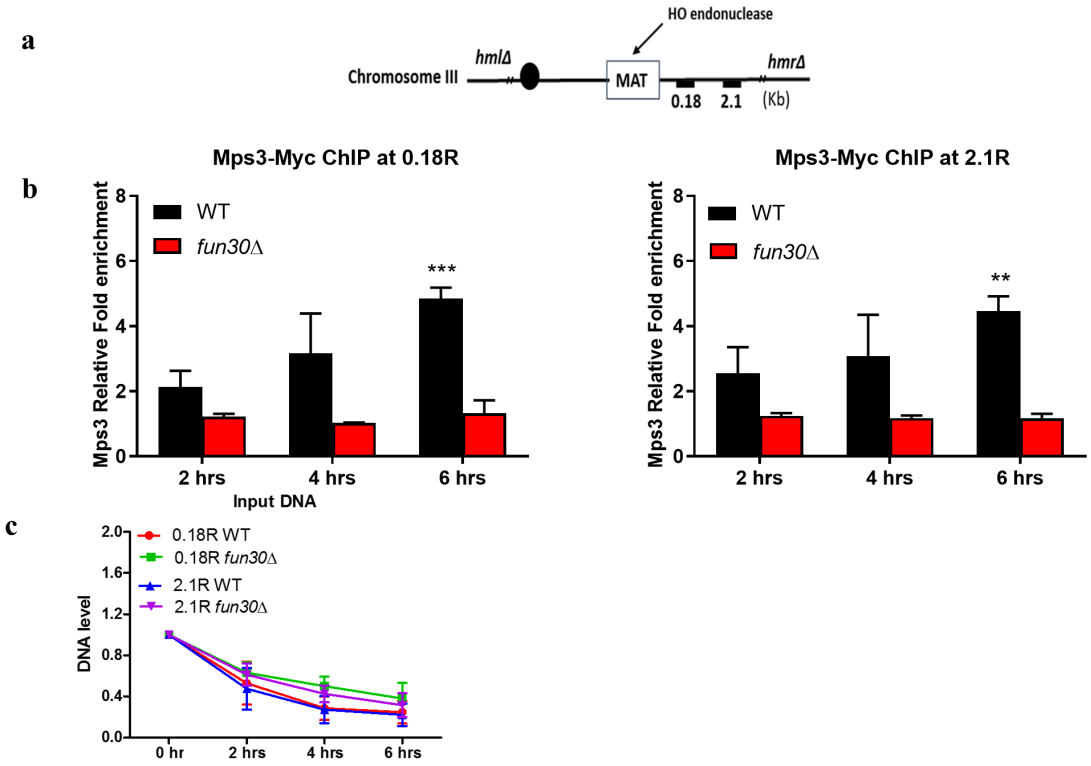

Figure 3.6: Fun30 facilitates relocation of persistent DSBs to Mps3

a) Schematic diagram of chromosome III of a donorless yeast strain harboring a galactose inducible *HO*-endonuclease. Primers used during ChIP analyses are indicated according to their distance from the DSB. b) ChIP for 13Myc-Mps3 using anti-Myc antibody immobilized on proteinG Dynabeads in the presence or absence of Fun30 showing reduced binding of Mps3 at the DSB in *fun30*Δ by real-time PCR for both the regions (0.18 kb and 2.1 kb) and all the time points tested. c) Quantitation of DNA end resection measured by real-time PCR using primers specific to the region 0.18R or 2.1R normalized to the non-cleaved ACT1 site. Values are presented as Mean ± SEM form 3-4 independent experiments. Two asterisks show *p*value <0.01 and three asterisks show *p*value <0.005 compared to its corresponding FUN30 deletion mutant.

3.7 Investigating the Physical Interaction between Fun30 and Nup84

In our ChIP experiments, we have shown that Fun30 translocates persistent DSB towards the Mps3. Next, we sought to investigate physical relationship between Fun30 and Nup84 (another nuclear protein that interacts with persistent DSBs). For that reason, a co-immunoprecipitation experiment was performed by tagging Nup84 with 13Myc-tag in a strain where Fun30 is tagged by TAP-tag. Pull-down was done using IgG Sepharose beads (GE healthcare) (Figure 3.7a). We observed nonspecific binding of Nup84 in our co-immunoprecipitation experiment (Figure 3.7a).

Next, we performed co-immunoprecipitation after inducing DNA damage at the *MAT* locus in a strain where Nup84 was tagged with 13Myc tag and Fun30 was tagged with TAP-tag. Immunoprecipitation was done using IgG Sepharose beads (GE healthcare). Results indicates that even after DSB induction, no physical interaction was observed between Fun30 and Nup84 (Figure 3.7b). Together, these data suggest that no physical interaction between Fun30 and Nup84 was seen in our co-IP experiment but there could be functional interaction that will be discussed in section below.

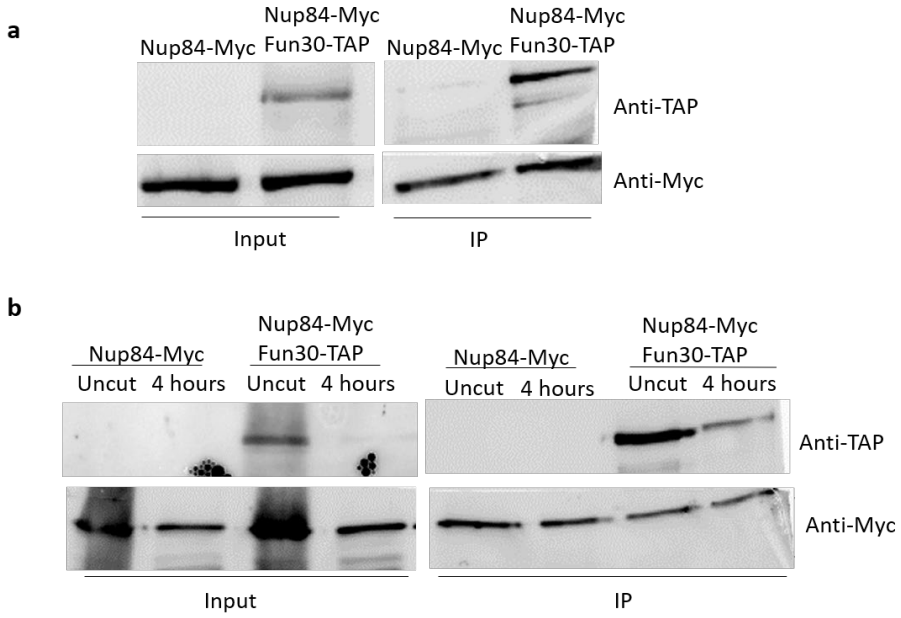

Figure 3.7: Investigate the physical interaction between Fun30 and Nup84

a) Co-immunoprecipitation assay shows an interaction of TAP-Fun30 with 13-Myc tagged Nup84. IP samples along with inputs were loaded on SDS-PAGE and analyzed by western blotting. Upper panel shows western blot probed with anti-TAP antibody to detect immunoprecipitated Fun30. Lower panel shows the blot probed with anti-Myc antibody to detect Nup84. b) Co-IP assay after inducing a single irreparable DSB at the *MAT* locus. Samples were collected before the DSB induction and after the 4 hours of DSB induction. Fun30 was tagged with TAP-tag and Nup84 was tagged with 13-Myc tag.

3.8 Fun30 Assists Translocation of Irreparable DSBs towards Nup84

At the nuclear periphery, Nup84 complex acts as one of the main players to receive 'persistent' or 'difficult to repair' DSBs. The NPC appears to be more permissive for repair since it favors several types of alternative repair pathways to fix the broken lesions. To study the role of Fun30 in translocation of DSB to Nup84 complex, the association of *HO*-induced DSBs with Myc-tagged Nup84 was monitored in the presence or absence of Fun30. An irreparable DSB was introduced by adding galactose and cells were collected after 0, 2, 4, and 6 hours post DSB induction as explained in previous section. At both regions (0.18R and 2.1R), Nup84 enrichment was decreased in *fun30*Δ mutant, compared to the WT control (Figure 3.8a). The relative fold enrichment of Nup84 at 0.18R and 2.1R peaked about 5 folds

at 6 hours post DSB induction, whereas in *fun30*Δ it decreases after DSB induction at both the regions tested (Figure 3.8a). At all the time points and both regions tested, *fun30*Δ mutant shows reduced Nup84 enrichment compared to the WT control. We have also observed a slight delay in the resection rate upon the loss of Fun30 at 0.18R and 2.1R, indicating that Fun30 is important for short-range resection close to the DSB ends (Figure 3.8b). These data together demonstrate a novel function of Fun30 in DNA repair, the translocation of persistent DSB to Nup84 complex.

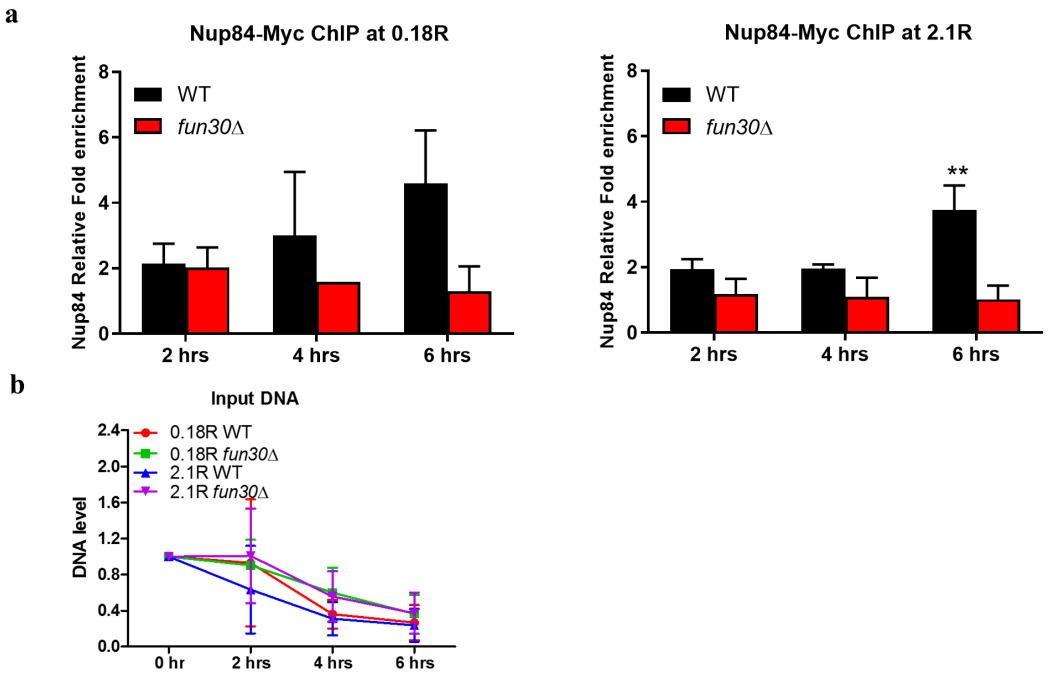

Figure 3.8: Fun30 facilitate relocation of persistent DSBs to Nup84 complex

a) ChIP for Myc-Nup84 using anti-Myc antibody immobilized on protein G Dynabeads, in the presence or absence of Fun30 showing reduced binding of Nup84 at the DSB in *fun30*Δ by real-time PCR for both the regions (0.18 kb and 2.1 kb). b) Quantitation of DNA end resection measured by real-time PCR using primers specific to the region 0.18R or 2.1R normalized to the non-cleaved ACT1 site. Values are presented as Mean ± SEM form 3-4 independent experiments. Two asterisks show *p*value <0.01 compared to its corresponding FUN30 deletion mutant.

3.9 Role of Fun30-assisted DNA End Resection in Relocation of Persistent DSBs towards Nuclear Periphery

In budding yeast, extensive DNA end resection at DSB is performed by Exo1. INO80 and Fun30 favor this process. Resection allows the binding of Rad52, which along with Rad51 has been shown to be necessary for shifting the persistent DSBs to Mps3 (Kalocsay et al., 2009; Oza et al., 2009). Fun30 also weaken the interaction between histones and DNA, facilitating Exo1 to perform the DNA end resection (Costelloe et al., 2012). Based on our observations, that *fun30*Δ results in a significant reduction in the translocation of DSB to the nuclear periphery, we hypothesized that Fun30-assisted DNA end resection might be playing a role in the relocation of DSB to the nuclear periphery. To test this, Exo1 or Exo1 D173A mutant was overexpressed in the Myc-tagged Mps3 in the presence or absence of Fun30.

ChIP for Myc-tagged Mps3 upon overexpression of Exo1 in the presence of Fun30 indicates that plasmid-based overexpression of Exo1 favors the translocation of DSB towards Mps3 to some extent (Figure 3.9a). Whereas, upon overexpressing Exo1-D173A, there is a slight reduction in Mps3 enrichment at the DSB site at both regions (0.18R and 2.1R) tested (Figure 3.9a). Overexpression of Exo1 in the Myc-tagged Mps3 strain in the absence of Fun30 did not significantly improve the translocation of persistent DSBs to Mps3 when compared to Exo1 D173A mutant (Figure 3.9b). This means that resection mediated by Fun30 is not fully compensated by the overexpression of Exo1.

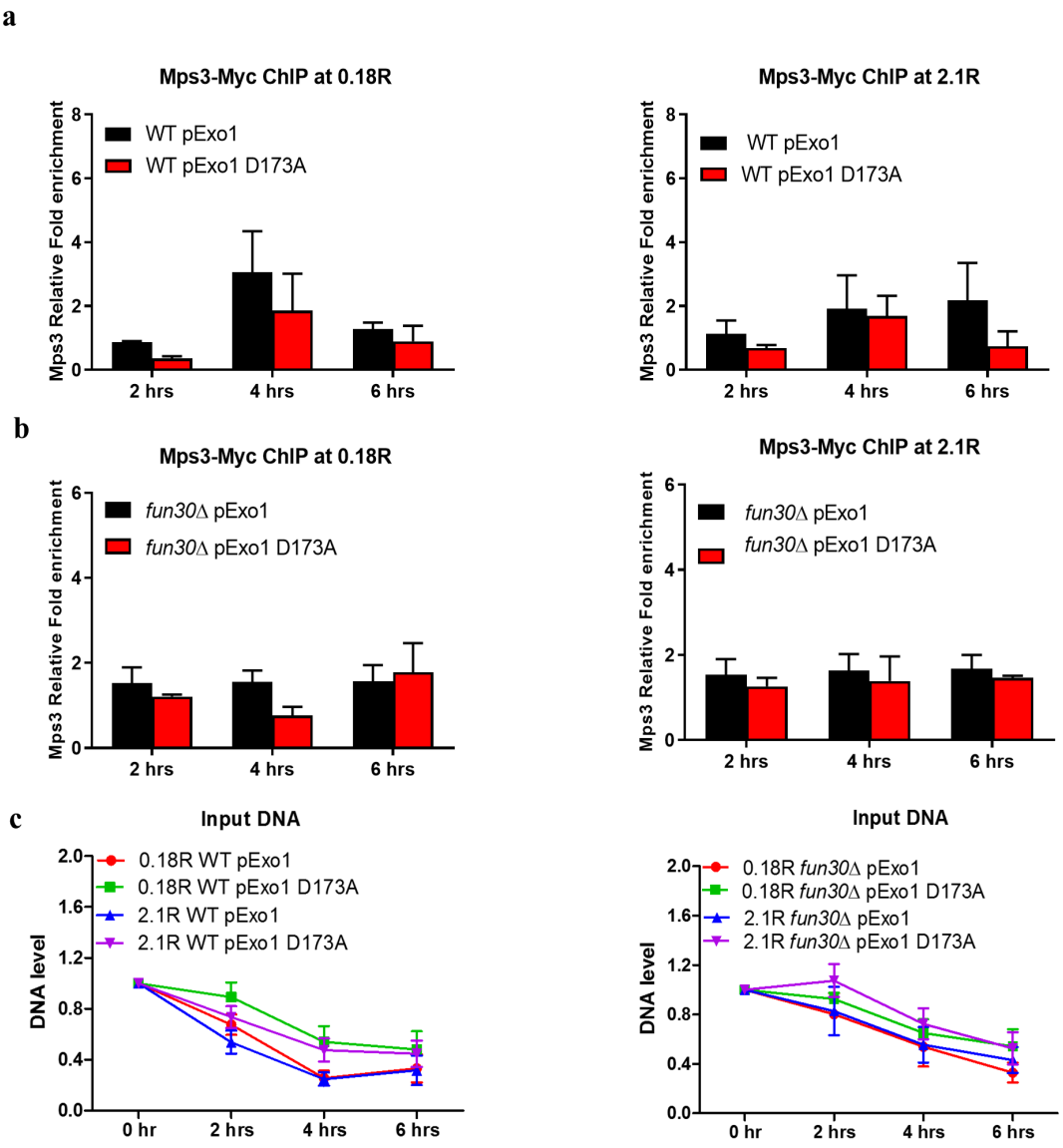

Figure 3.9: DNA end resection assisted by Fun30 did not significantly favors relocation of DSB towards Mps3

a), b) ChIP for Mps3-Myc using anti-Myc antibody immobilized on proteinG Dynabeads, upon plasmid-based overexpression of Exo1 or its mutant Exo1 D173A in the presence or absence of Fun30. c) Quantitation of DNA end resection measured by real-time PCR using primers specific to the region 0.18R or 2.1R normalized to the non-cleaved ACT1 site. Values are presented as Mean ± SEM form 3-4 independent experiments.

In the experiments above, we have shown that Fun30 favors the relocation of persistent DSBs towards the Nup84 pore complex. To get an insight into the role of Fun30-assisted DNA end resection in this translocation, Exo1 or Exo1 D173A

mutant was overexpressed in the Myc-tagged Nup84 strain in the absence or presence of Fun30. ChIP using anti-Myc antibody at 0, 2, 4, and 6 hours post DSB induction was performed. Results indicate that upon overexpression of Exo1 in the presence of Fun30 in Myc-tagged Nup84 yeast strain improved the relocation of DSB towards Nup84 (Figure 3.10a). Whereas, overexpression of Exo1 in Myc-tagged Nup84 strain in the absence of Fun30 partially compensated the loss of Fun30 at both 0.18R and 2.1R regions tested (Figure 3.10b). These data altogether indicate that DNA end resection favored by Fun30 helps to translocate DSB to Nup84 complex.

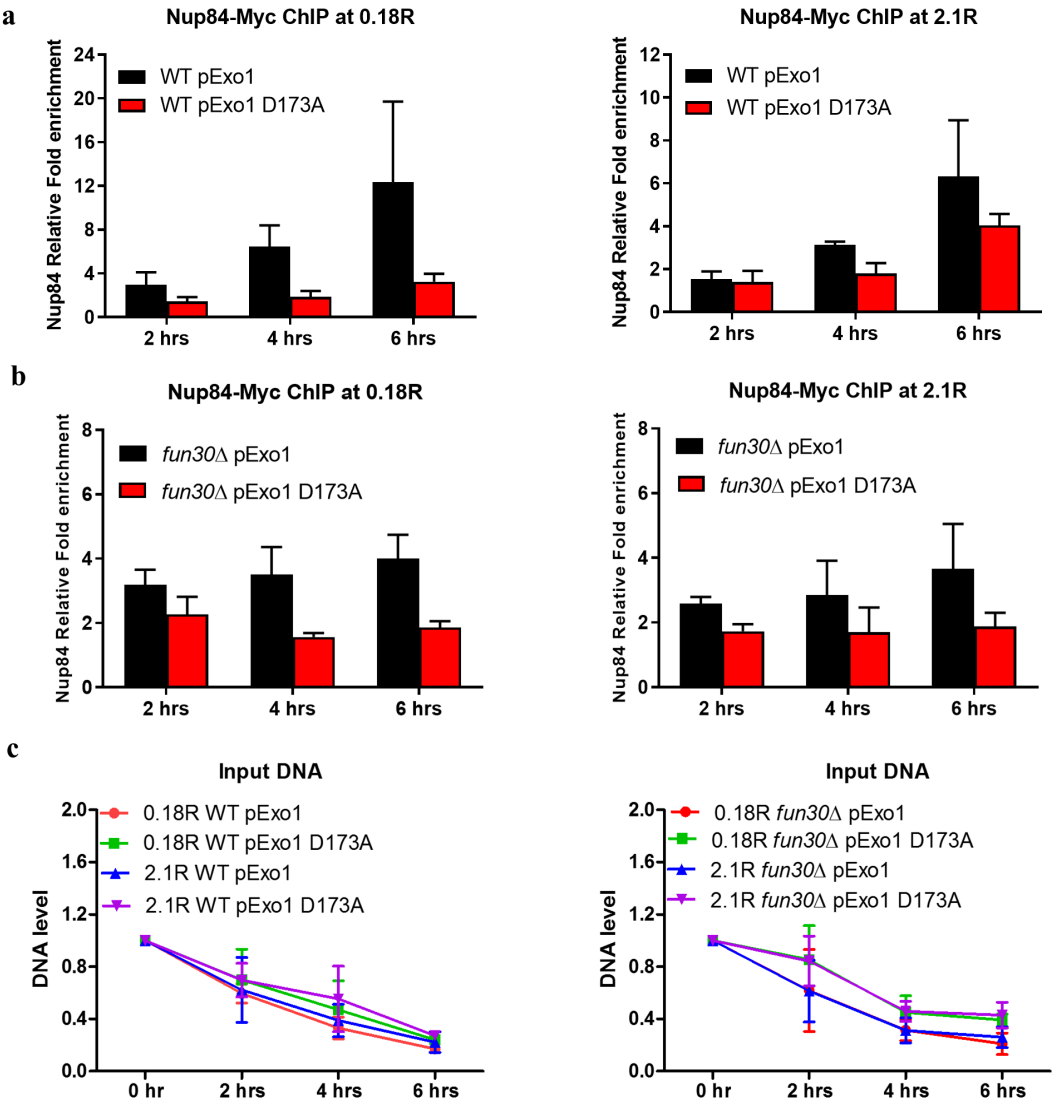

Figure 3.10: Fun30-mediated DNA end resection favors the translocation of persistent DSBs towards Nup84

a,b) ChIP for Nup84-Myc using anti-Myc antibody immobilized on proteinG Dynabeads in the presence or absence of Fun30. c) Quantitation of DNA end resection measured by real-time PCR using primers specific to the region 0.18R or 2.1R normalized to the non-cleaved ACT1 site. Values are presented as Mean ± SEM form 3-4 independent experiments.

3.10 Fun30 Supports Htz1 Levels at the DSB site

Histone variant Htz1 is deposited at DSB by SWR-C (Kalocsay et al., 2009). The translocation of persistent DSBs to Mps3 or nuclear pores decreased severely

upon deletion of SWR-C component *swr1* indicating the importance of SWR-C in shifting the DSB to both Mps3 and nuclear pores (Horigome et al., 2014). In another study, it was shown that Mps3 and Htz1 interact physically (Gardner et al., 2011) and the loss of Htz1 interaction with Mps3 due to mutation in Mps3 causes failure in its peripheral localization (Gardner et al., 2011).

To determine whether Fun30 influences the Htz1 enrichment at DSB, ChIP using anti-H2A.Z antibody at 0, 2, 4, and 6 hours after induction of DSB in the presence or absence of Fun30 was carried out. Results indicate that in the absence of Fun30, there is a reduction in the levels of Htz1 at the DSB site (Figure 3.11a). Previously, it was shown that Htz1 is enriched at DSB after 15 minutes of DSB induction and then its levels decreases with time (Kalocsay et al., 2009). Contradictory to Kalocsay et al. (2009), we have observed that the relative fold enrichment of Htz1 at 0.18R peaked around 4 folds at 6 hours post DSB induction, whereas in *fun30*Δ it decreases after DSB induction at both the 0.18R 2.1R regions tested. The reduced levels of Htz1 at an induced irreparable DSB account for the decreased translocation of DSBs to Mps3 and nuclear pores as it has been previously reported that H2A.Z incorporation is important to relocate DNA breaks to the nuclear periphery (Horigome et al., 2014). Quantitation of DNA end resection measured by real-time PCR using primers specific to the region 0.18R or 2.1R is indicated in (Figure 3.11b). Results suggest that by controlling Htz1 levels at DSB, Fun30 plays an important role in the relocation of irreparable DSB towards the nuclear periphery.

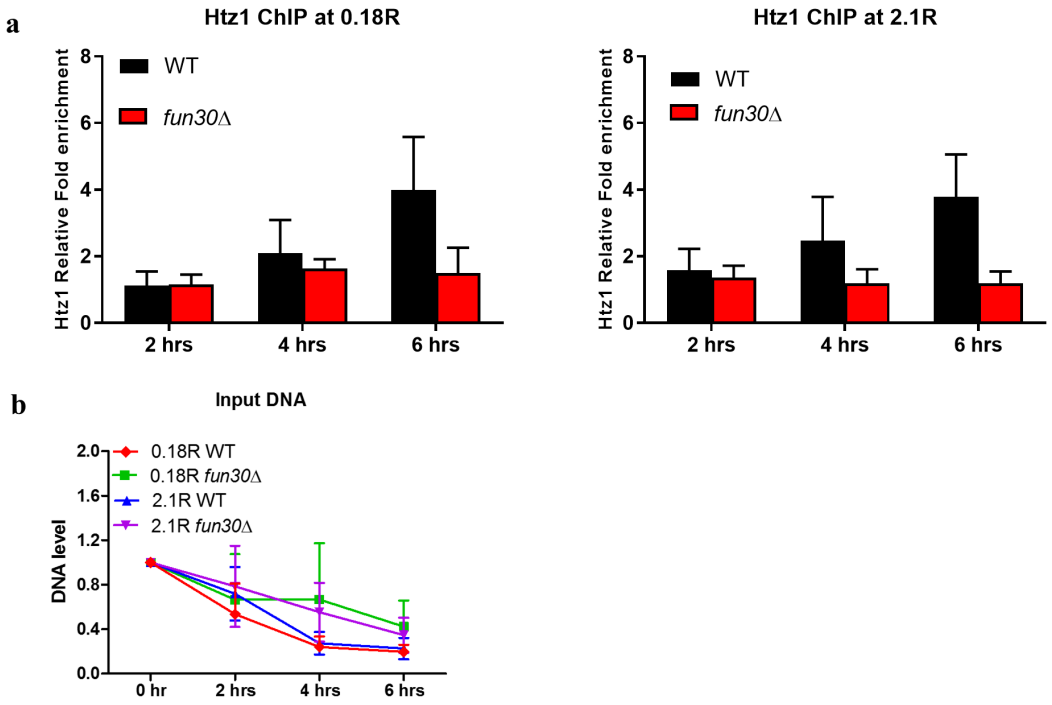

Figure 3.11: Fun30 controls Htz1 levels at DSB

a) ChIP for Htz1 using anti-H2A.Z antibody immobilized on proteinG Dynabeads in the presence or absence of Fun30 showing reduced binding of Htz1 at the DSB in *fun30*Δ by real-time PCR for both the regions (0.18 kb and 2.1 kb) tested. b) Quantitation of DNA end resection measured by real-time PCR using primers specific to the region 0.18R or 2.1R normalized to the non-cleaved ACT1 site. Values are presented as Mean ± SEM form 3-4 independent experiments.

3.11 Fun30-Facilitated DNA Resection Did not Support Htz1 at DSB

Above we have shown that Fun30 by controlling Htz1 levels at DSB, plays an important role in the relocation of persistent DSBs towards the nuclear periphery. To test the role of DNA end resection, facilitated by Fun30, in the recruitment of Htz1 at DSB, we have overexpressed Exo1 in the presence or absence of Fun30. ChIP using anti-H2A.Z antibody at 0, 2, 4, and 6 hours after DSB induction was carried out. Results indicate that Htz1 recruitment did not increase at the DSB upon Exo1 overexpression in the absence or presence of Fun30 at both the regions we have tested when compared to overexpression for nuclease defective mutant Exo1

D173A (Figure 3.12a and b). This means, Fun30-mediated DNA ends resection at DSB did not facilitate the Htz1 recruitment at the DSB. Previous studies showed that mutants deficient in H2A.Z show defects in DNA resection at the region flanking the DSB (Kalocsay et al., 2009). This indicates that Htz1 levels at the DSB affect the DNA end resection. It could be potentially Fun30 chromatin remodeling activity by which Fun30 supports Htz1 at DSB site.

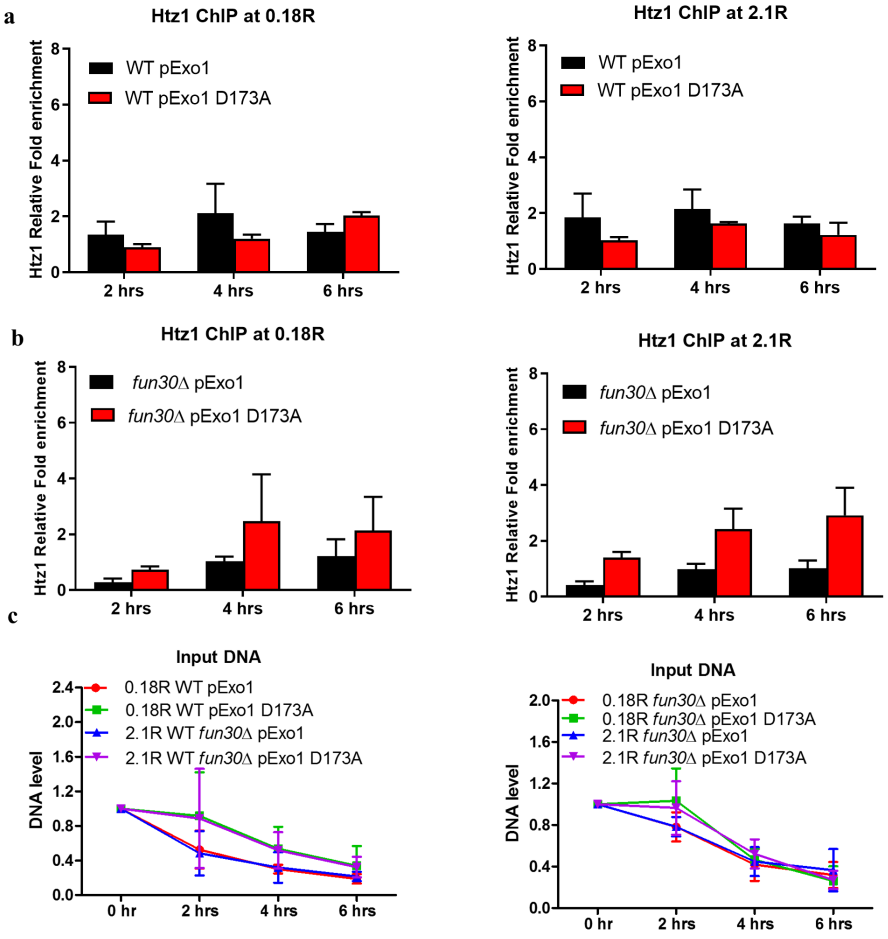

Figure 3.12: DNA end resection assisted by Fun30 did not help Htz1 recruitment at DSB

a,b) ChIP for Htz1 using anti-H2A.Z antibody immobilized on proteinG Dynabeads did not result in enhanced binding of Htz1 at the DSB upon overexpression of Exo1 and its mutant in *fun30*Δ and upon the presence or absence of Fun30. c) Quantitation of DNA end resection measured by real-time PCR using primers specific to the region 0.18R or 2.1R normalized to the non-cleaved ACT1 site. Values are presented as Mean ± SEM form 3-4 independent experiments.

Chapter 4: Discussion

Our mass spectrometry analysis of TAP purified Fun30 protein identified several nuclear pore proteins pulled down along with the Fun30. One of these pull-down proteins along with Fun30 was Nup60. Mutations in the outer ring of Nup84 sub-complex and the nucleoporin Nup60 have previously been shown to cause accumulation of DSB and these mutant cells were defective in DSB repair by NHEJ due to de-localization of Ulp1, a SUMO specific protease, from nuclear pore complex (Palancade et al., 2007). Nsp1, another yeast nuclear envelope protein, was also among the interacting partners pulled down along with Fun30. There are many other nucleoporin proteins that were pulled down with Fun30, including Nup2, Nup57, Nup116, Nup145, Nup49, and Nup1. Therefore, co-purification of Fun30 with several nuclear pore proteins indicates that Fun30 may perform different roles in the processing of persistent DNA lesions that translocate towards the nuclear periphery. Our co-immunoprecipitation confirms the physical interaction of Fun30 with Nsp1. This means that Fun30 by physically associating with the NPC might be playing an important role in the repair of persistent DSBs that relocate towards the nuclear periphery, an observation that needs further investigations.

Fun30 is known to interact with heterochromatic regions like telomeres and centromeres (Durand-Dubief et al., 2012). Cells strategically keep these heterochromatic regions towards the nuclear periphery as these regions are scarcely expressed. We hypothesized that Fun30 associates with the nuclear membrane proteins where it deals with heterochromatic regions and maintains them in their preferred silent state (Durand-Dubief et al., 2012). The fission yeast homolog of Fun30, Fft3, has been shown to be essential for maintaining proper chromatin

structure at the centromeres and subtelomeres (Steglich et al., 2015). Fft3 is also known to play role in maintaining nucleosome structure of insulator regions at the subtelomeric borders (Steglich et al., 2015). It was also reported that subtelomeres and insulator region move inside of the nucleus upon deletion of *FFT3* (Steglich et al., 2015). In the absence of Fun30 homolog Fft3, enhanced transcription and active histone marks were observed, and telomeric domain is released from the nuclear envelope (Steglich et al., 2015). When Bqt4, a protein that helps in the anchorage of telomeres to the nuclear envelope, is deleted in addition to Fft3 the entire telomere moves away from the nuclear envelope towards the interior of nucleus. In *fft3*Δ cells, tRNA clusters at the nuclear envelope also dissociate from the nuclear periphery (Steglich et al., 2015). In this study, we have shown that Fun30 physically associates with proteins belonging to the nuclear periphery. Histone H2A.Z variant, Htz1, was also pulled-down along with Fun30 in our mass spectrometry analysis (See Appendix). Previous studies have shown that Htz1 has a role in integrating Mps3 at the nuclear periphery and in the absence of Htz1, Mps3 loses its peripheral positioning and is diffused into the nucleus. Htz1 also physically interacts with Mps3 at the nuclear periphery. Future studies are needed to determine whether the interaction of Fun30 with Htz1 might also be important for the proper insertion of Mps3 at the nuclear periphery.

DNA damage can lead to mutations and genomic instability that can be lethal for cell survival, if remain unrepaired. Many nuclear events including DNA repair are known to relocalize to different nuclear sub compartments. Persistent DNA lesions or DNA breaks that take longer time than usual for repair shift towards the nuclear periphery. Mps3 and Nup84 act as two distinct DSB binding proteins on the nuclear membrane. It has been shown that DSB bind to Nup84 in any cell cycle

phase, but binding of DSB to Mps3 is limited to the S and G2 phases of the cell cycle (Horigome et al., 2014). Furthermore, DSB translocation towards nuclear periphery in *mps3*Δ*65-145* strain is abolished, and this defect is observed in G1 and S phases also, although DSBs did not relocate to Mps3 in the G1 phase (Horigome et al., 2014). Loss of Mps3 N-terminal also results in reducing DSB association with nuclear pores from 5-folds to 2-folds at 4 hours after DSB induction (Horigome et al., 2014). DSBs associate with Mps3 before being ultimately delivered to the Nup84 complex in a process that depends on the activity of motor protein Kinesin-14 (Chung et al., 2015). In this study, we have observed a gradual increase in the association of DSB with Mps3 and Nup84 from 2 to 6 hours after the DSB induction (Figure 3.6 and 3.8). Our results show a novel role of Fun30 in favoring the relocation of persistent DSB towards both Mps3 and Nup84.

When DSB occurs in a cell, many proteins, including several chromatin remodelers are recruited to the DSB. Translocation of the persistent DSBs to the pores during the G1 phase of cell cycle depends on the SWR-C chromatin remodeler because of the unavailability of resection (Horigome et al., 2014). SWR-C also assists in the deposition of Htz1 at DSB, critical in generating an open chromatin conformation at DSB that eventually helps in the loading of RPA, Rad52, and Rad51 at the DSB (Horigome et al., 2014). The deposition of H2A.Z nucleosomes on either side of nucleosome-free regions at the DSB generates a structure that resembles the transcriptional start site of genes (Price & D'Andrea, 2013). Therefore, by depositing H2A.Z on either side of the DSB, the cell may define the extent of the nucleosome-free region and generate a template that can limit DNA end resection. Chromatin remodeling at DSBs through the exchange of H2A.Z and H4 acetylation is therefore important for allowing further processing and repair of DSB (Price & D'Andrea,

2013). Similarly, presence of histone H3 and H2B on resected DNA has previously been reported (Costelloe et al., 2012). While, SWR-C was reported to deposit Htz1 at DSB site within 30 minutes of break formation but later it decreased (Kalocsay et al., 2009). This Htz1 incorporation is essential throughout the cell cycle to shift persistent DSBs towards the nuclear periphery (Horigome et al., 2014). Fun30 has also been shown to affect Htz1 levels genome-wide (Durand-Dubief et al., 2012). Our ChIP experiments show that Fun30 is important in maintaining Htz1 levels at DSB (Figure 3.11). We show that in the absence of Fun30, Htz1 levels drop at the single, induced, irreparable DSB, and this should ultimately affect the translocation of DSB to the nuclear periphery.

During the last few years, many studies have shown a role for Fun30 in long-range resection of 5' ends, an initial step in HR. This resection allows the recruitment of RPA proteins that along with Rad51 and Rad52 are required to shift DSBs to the nuclear periphery (Horigome et al., 2014). DNA breaks that are repaired by SSA with only 5 kb of resection do not translocate towards the nuclear periphery, but those that need 30 kb of resection are translocated to the nuclear periphery (Oza et al., 2009). In budding yeast, DSBs are initially sensed by both Ku and the Mre11-Rad50-Xrs2 (MRX) complex; MRX, in turn, recruits Tel1 kinase. If end ligation is not immediate, MRX makes a single-strand nick distal to the break, to initiate short-range resection (Cejka et al., 2010). The ssDNA is then extended by a second set of partially redundant enzymes, namely, Exo1, Dna2, and Sgs1 (Nicolette et al., 2010). DNA end resection is more efficient in S phase than in the G1 phase, and robust resection favors repair by HR over NHEJ, as long as a homologous template is available (Symington & Gautier, 2011). Fun30 being a chromatin remodeler weakens the interaction between DNA and histones, allowing Exo1 to perform the resection at

the DSB (Costelloe et al., 2012). Previous studies show that plasmid-based overexpression of Exo1 bypasses the need for Fun30 chromatin remodeler for DNA end resection (Costelloe et al., 2012). In this study we have shown that the DNA end resection facilitated by Fun30 partially facilitates the translocation of persistent DSB to the Nup84 complex (Figure 3.10). In the absence of Fun30, persistent DSB move better to the nuclear periphery upon overexpression of Exo1 compared to Exo1 D173A mutant. This indicates the importance of Fun30-mediated DNA end resection in the repair of persistent DNA lesions. Moreover, we have also observed a slight delay in the resection measured by qPCR upon the loss of Fun30 at 0.18R and 2.1R, indicating that Fun30 is also important for short-range resection close to the DSB ends.

In conclusion, we have uncovered a novel role of Fun30 in relocating persistent DSBs towards the nuclear periphery. Fun30, through different interdependent pathways, shifts these persistent DSBs to the nuclear periphery: one way is to facilitate DNA end resection at the DSB by helping the deposition of Htz1 at DSB (Figure 4.1). This translocation may provide DSB survivors with an advantage or disadvantage that may in turn be beneficial or deleterious to an organism, depending upon cellular and environmental conditions. Overall, the study of the relocation of persistent DSBs towards the nuclear periphery helps to improve our understanding of how the spatial segregation of repair in the highly compartmentalized nucleus contributes to maintaining genome stability and cell survival. Many secondary tumors or cancers emerge because of genomic rearrangements. It would be interesting to investigate whether the error-prone DNA repair process at the nuclear periphery on persistent DSB have any role in secondary cancers.

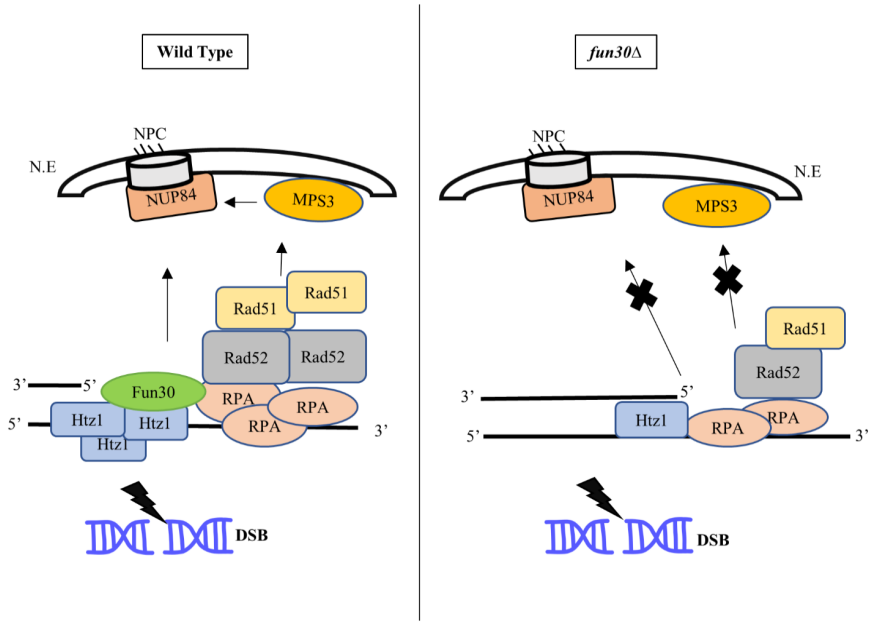

Figure 4.1: Role of Fun30 in relocation of persistent DSBs to nuclear periphery

Fun30's influence on DSB relocation to both Mps3 and Nup84 nuclear pore complex. Fun30-mediated DNA end resection favors the recruitment of Htz1, RPA, Rad52 and Rad51 proteins to the DSB site that allows its relocation towards Mps3. In the absence of Fun30, their recruitment is severely reduced. Fun30 also helps the Htz1 deposition around DSB that is critical in shifting DSB towards Mps3 and Nup84. In the absence of Fun30, Htz1 levels are reduced at the DSB site that ultimately affect DSB relocation to the nuclear periphery. (N.E: Nuclear Envelope, NPC: Nuclear Pore Complex, arrows indicate the direction of DSB movement).

Chapter 5: Conclusion

DNA double-strand break can result from intrinsic or extrinsic factors and is one of the most harmful forms of DNA damage. Unrepaired DSBs can lead to loss of genetic information and can cause genomic rearrangements. Chromatin remodeling helps to alter the configuration of chromatin in a way to allow the interaction between DNA and repair proteins. Fun30 is a chromatin remodeler that has sequence similarity with SWI/SNF and belongs to the Snf2 family of ATP-dependent chromatin remodelers. Previously, Fun30 has been shown to physically interact with DNA around DSB ends and it facilitates DNA end resection during homologous repair pathway. Here, we have shown that Fun30 helps to relocate persistent DSBs towards Mps3 and Nup84 complex at the nuclear periphery. *fun30*Δ mutants showed reduction in the relative enrichment of Mps3 and Nup84 compared to the wild type at all-time points and both the regions (0.18R and 2.1R) tested. Moreover, Fun30 supports histone variant Htz1 enrichment at DSB sites. This means that by controlling Htz1 levels at DSB, Fun30 plays an important role in the relocation of irreparable DSB towards the nuclear periphery. Our results support a model in which Fun30 by favoring the DNA end resection and by supporting Htz1 deposition at the DSB, helps to translocate DSB towards the nuclear periphery. In this study, we have uncovered a novel role of Fun30 in relocating persistent DSBs towards the nuclear periphery. We, therefore, believe this study expands our understanding of the basic mechanisms of DNA damage repair and cell survival.

5.1 Research Implications

The overall aim of research was to gain insight into the mechanisms of actions of chromatin-modifying complex, Fun30 and its role in relocating persistent

DSBs towards the nuclear periphery in the yeast *Saccharomyces cerevisiae*. The research will help us in understanding the molecular pathways that are undergoing in a eukaryotic cell. DNA damage can be deleterious to cell and could result in cell cycle arrest and eventually leads to cell death if it remains unrepaired. Cell has evolved different pathways to repair DSBs, HR is one of them. Fun30 is one of chromatin remodeler playing its role in HR pathway by facilitating long-range DNA end resection to generate 3'overhangs. These overhangs then invade the sister chromatid to search for homologous regions. We are focusing on yeast because it is a simple eukaryotic organism and like human DNA, it is also packaged in chromosomes and easy to manipulate genetically.

Cancer is a disease caused when cells divide uncontrollably and incidence rate for cancer is increasing day by day. Leading cancers in UAE population are breast, thyroid, colorectal, lung and cancer of cervix, and colorectal cancer is a second major cause of death in Emirati population. DNA damage, genetic instability, chromosomes abnormalities are players for cancer, if the damage exceed than normal capacity cell become cancerous. Studying DNA damage and silencing of heterochromatin will play a crucial role in cancer research and towards some potential solution of this catastrophic disease. The beauty of epigenetic modifications is that they can be reversed back and restored. Many tumor suppressor genes like Rbl2 downregulate and similarly many oncogenes erbB-2 are upregulated in carcinomas. The potential applications for the research are if we design epigenetic markers and products that can reverse back modification pattern in case of carcinomas. It is likely that errors in the function of these complexes can result in alterations in the life cycle of the cell that would lead to the development of cancer.

Thus, we are also interested in studying how chromatin misregulation contributes to cancer.

5.2 Potential Contributions and Limitations of the Study

In a eukaryotic cell, DNA damage can be caused by several factors such as ionizing radiations, UV light, and replicative stress. Cell has evolved different pathways to repair damage, HR is one of them. Double-stranded breaks are one of the most cytotoxic forms of DNA damage are mostly repaired by these mechanisms. Irreparable or persistent DSBs are relocated towards the nuclear periphery to avoid gross chromosomal rearrangements. The study of the relocation of persistent DSBs towards the nuclear periphery helps to improve our understanding of how the spatial segregation of repair in the highly compartmentalized nucleus contributes to maintaining genome stability and cell survival.

The following points will describe the limitations of study:

1) To test the co-localization of Fun30 with inner nuclear membrane protein, Mps3, it was cloned in the plasmid pSJ1321 purchased from Addgene. Plasmid pSJ1256 from Addgene was used for tagging Fun30 with GFP1-10. Surprisingly we observed that Mps3-mcherry was giving red signal throughout the yeast cell instead of localizing to spindle poles and nuclear periphery. Similarly, we were unable to show co-localization of Fun30 with our mass-spectrometry pull-down proteins i.e., Nsp1. Unsuccessful cloning of these proteins was one of limitation in our study.
2) The role of chromatin remodeling complexes in DSB repair has been studied using the *HO* system in *S. cerevisiae*. To investigate the role of Fun30 in relocation of persistent DSBs to nuclear periphery, we wanted to

delete *FUN30* gene in a donorless yeast strain harboring integrated arrays of lac operators ($LacO_R$) adjacent to the *HO* recognition site at the *MAT* locus. LacI-GFP fusion protein, which specifically binds the $LacO_R$ regions, will allow the visualization of the *MAT* locus position relative to the nuclear periphery, which will be identified by the nucleoporin protein Nup49-GFP fusion. Our aim was to track the movement of persistent DSB towards nuclear periphery upon *FUN30* deletion using confocal microscope. However, due to technical limitations we were not able to perform this experiment.

5.3 Future Prospects

In this study, we have identified the role of Fun30 in relocating persistent DSBs towards Mps3 and Nup84 complex. It would be interesting to find out through mutational analysis, if a particular motif within Fun30 is responsible for this translocation. Furthermore, the role of Fun30 in alternative repair pathways at the nuclear pores can be explored. Future studies will uncover the role of Fun30 at the nuclear periphery in the repair of broken lesions. Finding additional possible genetic interactions with other nuclear proteins and proteins involved in relocation and repair of persistent DSBs can help us in better understanding the importance of Fun30 in DNA repair and genomic stability. Human homolog of Fun30, SMARCAD1, has been found to play an important function in cancer development and progression and since we now know that cells lacking SMARCAD1 cannot efficiently repair DNA damage, perhaps future studies can be better designed in developing specific drugs that target DNA damage.

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List of Publications

Jalal, D., Chalissery, J., Iqbal, M., & Hassan, A.H. (2021). The ATPase Irc20 facilitates Rad51 nucleofilament formation during homologous recombination in yeast *Saccharomyces cerevisiae*. *DNA Repair*, 97, 103019. https://doi.org/10.1016/j.dnarep.2020.103019

Appendix

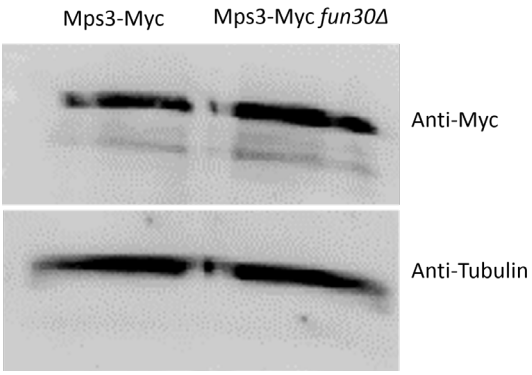

Figure 5.1: Western blot showing similar levels of Mps3 protein among Mps3-Myc and Mps3-Myc *fun30*Δ strains.

Table 4: Top hits for mass spectrometry for Fun30-TAP under low salt concentration

*Signal Intensity refers to the sum of intensity measurements for total peptides detected for the protein.

Gene Symbol	**Signal Intensity***		**Description**
	Fun30-TAP tagged **(Low salt, 150 mM)**	**Untagged Control**	
FUN30	1200000000	0	Snf2p family member with ATP-dependent chromatin remodeling activity
CCT5	2900000	0	Subunit of the cytosolic chaperonin Cct ring complex
CMK1	700000	0	Calmodulin-dependent protein kinase
ARC15	460000	0	Subunit of the ARP2/3

			complex;
CMK2	460000	0	Calmodulin-dependent protein kinase
CK2	460000	0	CK2 is a Ser/Thr protein kinase with roles in cell growth and proliferation
RIM1	320000	0	ssDNA-binding protein essential for mitochondrial genome maintenance
YGL041W-A	330000	0	Putative mitochondrial protein of unknown function
GSY2	150000	0	Glycogen synthase
RPN8	160000	0	Essential non-ATPase regulatory subunit of the 26S proteasome
GDI1	180000	0	Regulates vesicle traffic in secretory pathways by regulating the dissociation of GDP from the Sec4/Ypt/rab family of GTP binding proteins
YNR034W-A	230000	0	Protein of unknown function; expressed during diauxic shift and stationary phase
SEC1	160000	0	Sm-like protein involved in docking and fusion of exocytic vesicles
CKA1	210000	0	Cyclin-dependent kinase-activating kinase; required for passage through the cell cycle
GPP2	370000	0	DL-glycerol-3-phosphate phosphatase involved in glycerol

			biosynthesis
DCS1	160000	0	Non-essential hydrolase involved in mRNA decapping
OM45	150000	0	Mitochondrial outer membrane protein of unknown function
VAC8	130000	0	Vacuole-specific Myo2p receptor
BRE1	100000	0	E3 ubiquitin ligase
SMT3	400000	0	Translational repressor of the mitochondrial ATP6/8 Mrna
TAF12	100000	0	Subunit (61/68 kDa) of TFIID and SAGA complexes
PRE2	110000	0	Beta 5 subunit of the 20S proteasome
FIS1	90000	0	Protein involved in mitochondrial fission and peroxisome abundance
TMA20	130000	0	Protein of unknown function that associates with ribosomes
EMI2	65000	0	Hexokinase expressed during growth with low glucose levels
EHT1	770000	0	Octanoyl-CoA:ethanol acyltransferase
URA1	260000	0	Dihydroorotate dehydrogenase
RPS29B	320000	0	Protein component of the small (40S) ribosomal subunit
RPS29A	830000	0	Protein component of the small (40S) ribosomal subunit

DJP1	75000	0	ER-associated chaperone involved in protein targeting
SLA2	50000	0	Adaptor protein that links actin to clathrin and endocytosis
TIM9	380000	0	Essential protein of the mitochondrial intermembrane space
ATP7	210000	0	Subunit d of the stator stalk of mitochondrial F1F0 ATP synthase
PRE3	120000	0	Beta 1 subunit of the 20S proteasome
PRE7	150000	0	Beta 6 subunit of the 20S proteasome
SNC2	89000	0	Vesicle membrane receptor protein (v-SNARE)
CSR1	39000	0	Phosphatidylinositol transfer protein
TFB1	92000	0	Subunit of TFIIH and nucleotide excision repair factor 3 complexes
LSM6	58000	0	Lsm (Like Sm) protein
AHA1	48000	0	Co-chaperone that binds Hsp82p and activates its ATPase activity
CAR2	110000	0	L-ornithine transaminase (OTAse)
HEM15	110000	0	Ferrochelatase; a mitochondrial inner membrane protein
LYS1	120000	0	Saccharopine dehydrogenase (NAD+, L-lysine-forming)

UBP13	78000	0	Ubiquitin-specific protease that cleaves Ub-protein fusions
HYR1	100000	0	Glutathione peroxidase
BUR6	200000	0	Subunit of a heterodimeric NC2 transcription regulator complex
SLO1	80000	0	Protein interacting with Arl3p
DST1	170000	0	General transcription elongation factor TFIIS
YNL134C	130000	0	NADH-dependent aldehyde reductase
STE12	23000	0	Transcription factor that is activated by a MAPK signaling cascade
PUB1	130000	0	Poly (A)+ RNA-binding protein
YMR027W	39000	0	A metal-dependent phosphatase, part of the DUF89 protein family
NFU1	70000	0	Protein involved in Fe-S cluster transfer to mitochondrial clients
PRO1	51000	0	Gamma-glutamyl kinase
GLC3	48000	0	Glycogen branching enzyme, involved in glycogen accumulation
THR1	35000	0	Homoserine kinase
YNL208W	150000	0	Protein of unknown function; may interact with ribosomes
CYC1	52000	0	Cytochrome c, isoform 1; also known as iso-1-cytochrome c

NUM1	820000	0	Protein required for nuclear migration
CDS1	320000	0	Phosphatidate cytidylyltransferase
HHT1	160000	0	Histone H3; core histone protein required for chromatin assembly
WHI2	20000	0	Negative regulator of TORC1 in response to limiting leucine
RIB3	63000	0	3,4-dihydroxy-2-butanone-4-phosphate synthase (DHBP synthase)
RPB10	67000	0	RNA polymerase subunit ABC10-beta
RPC10	47000	0	RNA polymerase subunit ABC10-alpha
NIF3	34000	0	Protein of unknown function; similar to Listeria monocytogenes major sigma factor
GGA2	17000	0	Protein that regulates Arf1p, Arf2p to facilitate Golgi trafficking;
RTT103	50000	0	Protein involved in transcription termination by RNA polymerase II
CIR1	28000	0	Mitochondrial protein that interacts with frataxin
MCR1	30000	0	Mitochondrial NADH-cytochrome b5 reductase initiation of RNA
VMA6	27000	0	Subunit d of the V0 integral membrane

			domain of V-ATPase
JSN1	60000	0	Member of the Puf family of RNA-binding proteins
ECM1	35000	0	Pre-ribosomal factor involved in 60S ribosomal protein subunit export
ATP4	19000	0	Subunit b of the stator stalk of mitochondrial F1F0 ATP synthase
RTG1	120000	0	Transcription factor (bHLH) involved in interorganelle communication
STE5	120000	0	Pheromone-responsive MAPK scaffold protein
SWI3	20000	0	T Subunit of the SWI/SNF chromatin remodeling complex;
RPN6	55000	0	Essential, non-ATPase regulatory subunit of the 26S proteasome lid
CRM1	30000	0	Nuclear export factor, exportin
CCS1	27000	0	Copper chaperone for superoxide dismutase Sod1p
CBK1	28000	0	Serine/threonine protein kinase of the the RAM signaling network
NOP58	15000	0	Protein involved in producing mature rRNAs and snoRNAs
CKB1	55000	0	Beta regulatory subunit of casein kinase 2 (CK2)
ETT1	33000	0	Nuclear protein that inhibits replication of

			Brome mosaic virus
YDL121C	15000	0	A cargo receptor protein for Pma1p
YPT7	45000	0	Rab family GTPase; GTP-binding protein of the rab family
YER156C	49000	0	Protein involved in mitochondrial function
HMO1	44000	0	Chromatin associated high mobility group (HMG) family member
TMA46	36000	0	Protein of unknown function that associates with translating ribosomes
RTC3	65000	0	Protein of unknown function involved in RNA metabolism
BUD3	10000	0	Guanine nucleotide exchange factor (GEF) for Cdc42p
SSP120	49000	0	Protein packaged into COPII vesicles for ER to Golgi trafficking
MYO2	27000	0	Type V myosin motor involved in actin-based transport of cargos
YOP1	150000	0	Reticulon-interacting protein
PRE1	56000	0	Beta 4 subunit of the 20S proteasome
ISD11	28000	0	Cysteine desulfurase (Nfs1p) activator
GIP2	14000	0	Putative regulatory subunit of protein phosphatase Glc7p
LGE1	8100	0	Protein involved in histone H2B

			ubiquitination
ARI1	22000	0	NADPH-dependent aldehyde reductase
MHP1	17000	0	Microtubule-associated protein involved in microtubule organization
ATP5	75000	0	Subunit 5 of the stator stalk of mitochondrial F1F0 ATP synthase
SEC9	14000	0	t-SNARE protein required for secretory vesicle-plasma membrane fusion
GLC7	79000	0	Type 1 S/T protein phosphatase (PP1) catalytic subunit
UTP8	15000	0	Nucleolar protein required for export of tRNAs from the nucleus
RNQ1	24000	0	[PIN(+)] prion; an infectious protein conformation that is generally an ordered protein aggregate
DUO1	4600	0	Essential subunit of the Dam1 complex (aka DASH complex)
RPC40	40000	0	RNA polymerase subunit AC40
RHO1	62000	0	GTP-binding protein of the rho subfamily of Ras-like proteins
HTZ1	120000	0	Histone variant H2A.Z
YER010C	24000	0	Bifunctional HMG aldolase/oxaloacetate decarboxylase
CYT1	31000	0	Cytochrome c1;

			component of the mitochondrial respiratory chain
GCV2	35000	0	P subunit of the mitochondrial glycine decarboxylase complex
PCK1	230000	0	Phosphoenolpyruvate carboxykinase
STE24	18000	0	Highly conserved zinc metalloprotease
SBH2	53000	0	Ssh1p-Sss1p-Sbh2p complex component
SNA2	33000	0	Protein of unknown function; has similarity to Pmp3p
AIM29	35000	0	Protein of unknown function; epitope-tagged protein localizes to the cytoplasm
RPL29	280000	0	Ribosomal 60S subunit protein L25
COP1	45000	0	Alpha subunit of COPI vesicle coatomer complex
NCB2	18000	0	Subunit of a heterodimeric NC2 transcription regulator complex;
YME2	36000	0	Integral inner mitochondrial membrane protein
NCE102	75000	0	Protein of unknown function; contains transmembrane domains
FMP52	39000	0	Protein of unknown function; localized to the mitochondrial outer membrane

AWRI1631_41380	18000	0	Haploid derivative of South African commercial wine strain N96
COX4	24000	0	Subunit IV of cytochrome c oxidase
GIM4	21000	0	Subunit of the heterohexameric cochaperone prefoldin complex
ALD2	200000	0	Cytoplasmic aldehyde dehydrogenase
PFD1	28000	0	Subunit of heterohexameric prefoldin
DYN1	210000	0	Cytoplasmic heavy chain dynein

Table 5: Top hits for mass spectrometry for Fun30-TAP under high salt concentration

*Signal Intensity refers to the sum of intensity measurements for total peptides detected for the protein.

Gene Symbol	**Signal Intensity***		**Description**
	Fun30-TAP tagged **(High salt, 350 mM)**	**Untagged Control**	
FUN30	6200000000	0	Snf2p family member with ATP-dependent chromatin remodeling activity
VMA1	1100000	0	Subunit A of the V1 peripheral membrane domain of V-ATPase

KAR2	1600000	0	ATPase involved in protein import into the ER
CCT3	1400000	0	Subunit of the cytosolic chaperonin Cct ring complex
TIM44	1100000	0	Essential component of the TIM23 complex; tethers the import motor and regulatory factors
CCT8	1500000	0	Subunit of the cytosolic chaperonin Cct ring complex
CCT4	7500000	0	Subunit of the cytosolic chaperonin Cct ring complex
CCT6	1100000	0	Subunit of the cytosolic chaperonin Cct ring complex
CCT2	1800000	0	Subunit beta of the cytosolic chaperonin Cct ring complex
BRE5	1800000	0	Ubiquitin protease cofactor
CCT7	1200000	0	Subunit of the cytosolic chaperonin Cct ring complex
CST6	620000	0	Basic leucine zipper (bZIP) transcription factor from ATF/CREB family involved in stress-responsive regulatory network
TCP1	940000	0	Alpha subunit of chaperonin-containing T-complex;
CMK2	740000	0	Calmodulin-dependent protein kinase
TY1A	1800000	0	Beta subunit of fatty acid synthetase
NUP60	540000	0	FG-nucleoporin component of central core of the NPC
HSP104	770000	0	Disaggregase; heat shock protein that cooperates with Ydj1p (Hsp40) and Ssa1p (Hsp70)
SSD1	400000	0	Translational repressor; involved in polar growth

PFK1	370000	0	Alpha subunit of heterooctameric phosphofructokinase
NUP2	500000	0	Nucleoporin involved in nucleocytoplasmic transport
TRM1	600000	0	tRNA methyltransferase
FAS1	380000	0	Beta subunit of fatty acid synthetase
CCT5	730000	0	Subunit of the cytosolic chaperonin Cct ring complex
SYP1	410000	0	Negative regulator of WASP-Arp23 complex
NUP57	560000	0	FG-nucleoporin component of central core of the NPC
RVB1	390000	0	ATP-dependent DNA helicase, also known as pontin
SSZ1	330000	0	Hsp70 protein that interacts with Zuo1p (a DnaJ homolog)
ATP1	550000	0	Targeting subunit for Glc7p protein phosphatase
URA2	300000	0	Bifunctional carbamoylphosphate synthetase/aspartate transcarbamylase
RVS167	310000	0	Calmodulin-binding actin-associated protein
FAS2	390000	0	Alpha subunit of fatty acid synthetase
PDI1	470000	0	Protein disulfide isomerase; multifunctional oxidoreductase of the ER lumen,
CMK1	520000	0	Calmodulin-dependent protein kinase
RPS2	890000	0	Protein component of the small (40S) subunit
YBR139W	420000	0	Vacuolar serine-type

			carboxypeptidase
CLC1	150000	0	Clathrin light chain; subunit of the major coat protein involved in intracellular protein transport and endocytosis
SAN1	450000	0	Ubiquitin-protein ligase; involved in proteasome-dependent degradation of aberrant nuclear proteins
EFB1	960000	0	Translation elongation factor 1 beta
RAT1	260000	0	Nuclear 5' to 3' single-stranded RNA exonuclease
CDC48	240000	0	AAA ATPase with protein-unfoldase activity
ADE5,7	220000	0	Bifunctional enzyme of 'de novo' purine nucleotide pathway
KAP123	370000	0	Karyopherin beta
TIF4631	140000	0	Translation initiation factor eIF4G and scaffold protein
THR4	230000	0	Threonine synthase
PBP4	1400000	0	Pbp1p binding protein
BBC1	4400000	0	Protein possibly involved in assembly of actin patches
PEP4	520000	0	Vacuolar aspartyl protease (proteinase A)
RPS20	630000	0	Protein component of the small (40S) ribosomal subunit
YJU3	300000	0	Monoglyceride lipase (MGL) that hydrolyzes fatty acid ethyl esters
SAM1	350000	0	S-adenosylmethionine synthetase
RDL1	410000	0	Thiosulfate sulfurtransferase
ACO1	220000	0	Aconitase; required for the tricarboxylic acid (TCA) cycle

STI1	170000	0	Evolutionarily-conserved Hsp90 cochaperone
GLK1	250000	0	Glucokinase; catalyzes the phosphorylation of glucose
MET6	330000	0	Cobalamin-independent methionine synthase
RAD23	430000	0	Protein with ubiquitin-like N terminus
BUD3	160000	0	Guanine nucleotide exchange factor (GEF) for Cdc42p
PSP1	2000000	0	Asn and gln rich protein of unknown function
VMA8	120000	0	Subunit D of the V1 peripheral membrane domain of V-ATPase
OSH2	210000	0	Member of an oxysterol-binding protein family with seven members
ILV2	130000	0	Acetolactate synthase
HSP12	330000	0	Plasma membrane protein involved in maintaining membrane organization
SRV2	170000	0	CAP (cyclase-associated protein)
YGR250C	180000	0	RNA binding protein and negative regulator of START
RVB2	220000	0	ATP-dependent DNA helicase
SRP1	240000	0	Karyopherin alpha homolog
CMD1	230000	0	Calmodulin; Ca2+ binding protein
ABP1	230000	0	Actin-binding protein of the cortical actin cytoskeleton
TUB2	390000	0	Alpha-tubulin; associates with beta-tubulin
GRS1	110000	0	Cytoplasmic and mitochondrial glycyl-tRNA synthase

DUG1	220000	0	Cys-Gly metallo-di-peptidase
KRE2	1300000	0	Alpha1,2-mannosyltransferase of the Golgi
HYP2	820000	0	Translation elongation factor eIF-5A;
RRP15	180000	0	Nucleolar protein; constituent of pre-60S ribosomal particles
LAT1	210000	0	Dihydrolipoamide acetyltransferase component (E2) of the PDC;
EGD2	670000	0	Alpha subunit of the nascent polypeptide-associated complex
IDH2	260000	0	Subunit of mitochondrial NAD(+)-dependent isocitrate dehydrogenase
NUP116	160000	0	FG-nucleoporin component of central core of the NPC
GLN1	260000	0	Glutamine synthetase (GS)
LSB3	170000	0	Protein containing a C-terminal SH3 domain
LSP1	150000	0	Eisosome core component
SCP160	89000	0	Essential RNA-binding G protein effector of mating response pathway
SEC16	130000	0	COPII vesicle coat protein required for ER transport vesicle budding
ADE1	250000	0	N-succinyl-5-aminoimidazole-4-carboxamide ribotide synthetase
WTM1	430000	0	Transcriptional modulator; involved in regulation of meiosis
NRP1	210000	0	Putative RNA binding protein of unknown function
HRP1	230000	0	Subunit of cleavage factor I
RPL26B	730000	0	Ribosomal 60S subunit protein L26B

CBK1	160000	0	Serine/threonine protein kinase of the the RAM signaling network
RIM1	67000	0	ssDNA-binding protein essential for mitochondrial genome maintenance
RPL22A	320000	0	Ribosomal 60S subunit protein L22A
ILV3	90000	0	Dihydroxyacid dehydratase; putative 2Fe-2S protein
GND1	270000	0	6-phosphogluconate dehydrogenase (decarboxylating)
TPM1	200000	0	Major isoform of tropomyosin
VMA5	140000	0	Subunit C of the V1 peripheral membrane domain of V-ATPase
SEC53	970000	0	Phosphomannomutase
SAR1	190000	0	ARF family GTPase; component of the COPII vesicle coat
ZDS1	85000	0	Protein with a role in regulating Swe1p-dependent polarized growth
FIP1	110000	0	Subunit of cleavage polyadenylation factor (CPF)
PAN1	93000	0	Part of actin cytoskeleton-regulatory complex Pan1p-Sla1p-End3p
SER1	120000	0	3-phosphoserine aminotransferase
TRX1	5800000	0	Cytoplasmic thioredoxin isoenzyme
YLR407W	120000	0	Putative protein of unknown function
ERG13	98000	0	3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase
KAP95	87000	0	Karyopherin beta; forms a

			complex with Srp1p/Kap60p
BGL2	120000	0	Endo-beta-1,3-glucanase
SBP1	320000	0	Protein that binds eIF4G and has a role in repression of translation
IMD3	140000	0	Inosine monophosphate dehydrogenase
LYS12	170000	0	Homo-isocitrate dehydrogenase
PDB1	250000	0	E1 beta subunit of the pyruvate dehydrogenase (PDH) complex
HEM15	160000	0	Ferrochelatase; a mitochondrial inner membrane protein
URA7	87000	0	Major CTP synthase isozyme (see also URA8)
MHP1	91000	0	Microtubule-associated protein involved in microtubule organization
MCR1	180000	0	Mitochondrial NADH-cytochrome b5 reductase; involved in ergosterol biosynthesis
DBP5	2500000	0	Cytoplasmic ATP-dependent RNA helicase of the DEAD-box family
SPT4	370000	0	Spt4p/5p (DSIF) transcription elongation factor complex subunit
AHA1	96000	0	Co-chaperone that binds Hsp82p and activates its ATPase activity
PSP2	130000	0	Asn rich cytoplasmic protein that contains RGG motifs
FMP52	130000	0	Protein of unknown function; localized to the mitochondrial outer membrane
TDA11	85000	0	Putative protein of unknown function
ERG20	70000	0	Farnesyl pyrophosphate

			synthetase;
ADE13	140000	0	Adenylosuccinate lyase; catalyzes two steps in the 'de novo' purine nucleotide biosynthetic pathway
RCO1	65000	0	Essential component of the Rpd3S histone deacetylase complex; interacts with Eaf3p
TSL1	92000	0	Large subunit of trehalose 6-phosphate synthase/phosphatase complex
UBA1	81000	0	Ubiquitin activating enzyme (E1)
GSP2	310000	0	GTP binding protein (mammalian Ranp homolog)
IDH1	110000	0	Subunit of mitochondrial NAD(+)-dependent isocitrate dehydrogenase
SEC31	84000	0	Guanine nucleotide exchange factor (GEF)
ACS2	29000000	0	Acetyl-coA synthetase isoform

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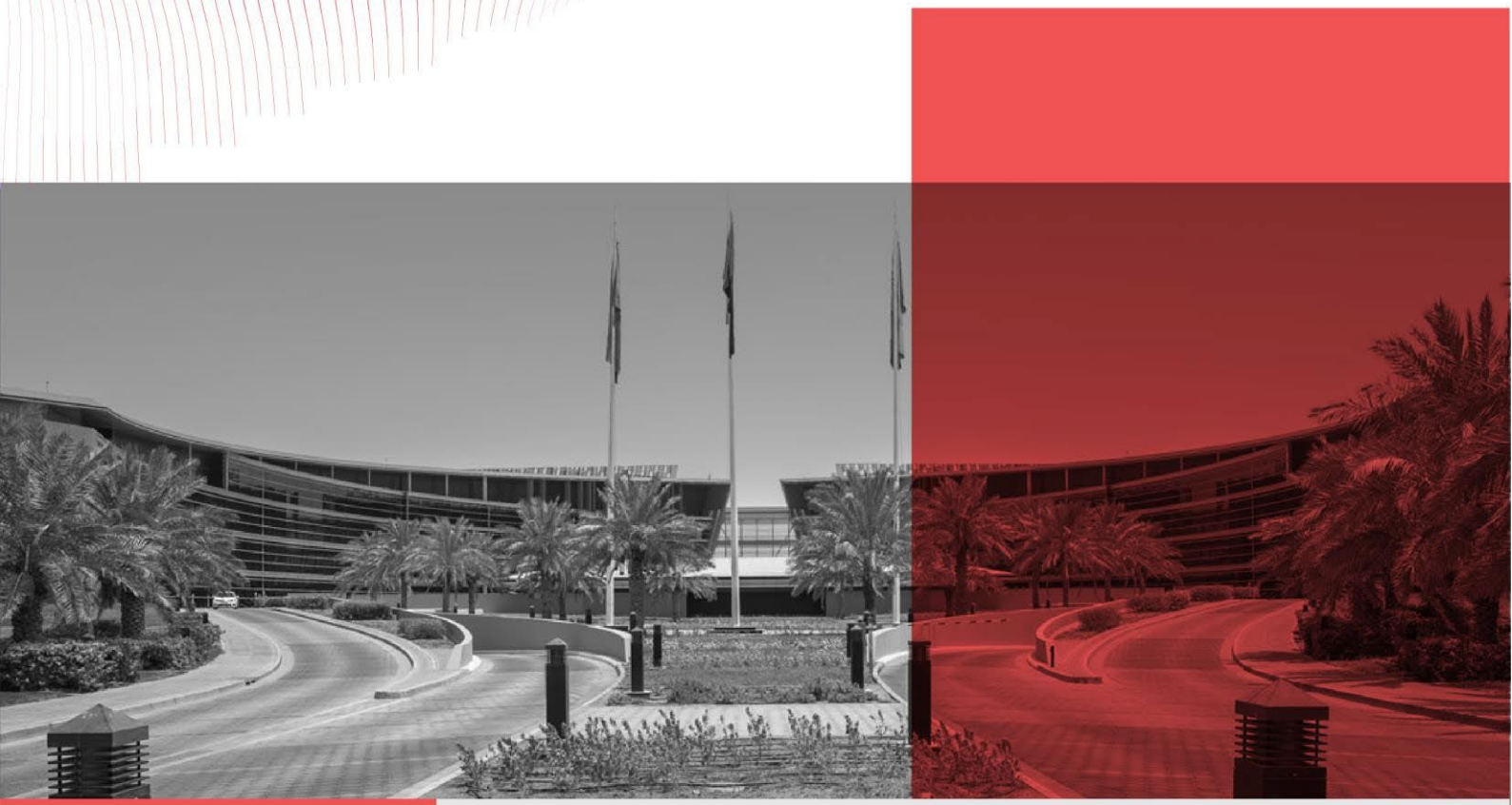

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Chromatin remodeler, Fun30, has a diverse function including its role in facilitating DNA end resection at the DNA double-strand break (DSB) site. In this study, we have uncovered a novel role of Fun30 in relocating persistent DSBs towards the nuclear periphery. Fun30 favors this relocation by controlling histone variant, Htz1, levels at the DSB site and by favoring DNA end resection.

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