Identification and characterization of novel genes involved in DNA double strand break repair process in the yeast *Saccharomyces cerevisiae*

by

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Abstract

DNA Double Strand Break (DSB) is the most severe form of DNA lesion. Unrepaired DSBs and/or mutations in key DSB repair genes can lead to genomic instability, cancer or even cell death. DSBs can be repaired by two independent pathways; Homologous Recombination (HR) and Non-Homologous End Joining (NHEJ). Both pathways are highly conserved from yeast to human. HR requires a homologous region to repair the damage but in NHEJ, the break ends are ligated directly. In *Saccharomyces cerevisiae,* the key protein complexes of the NHEJ pathway are: Yku70p/Yku80p (YKU), which binds to DNA and initiates the process and stabilizes the broken ends, Mre11p/Rad50p/Xrs2p (MRX), which brings broken ends close together and Dnl4p/Lif1p/Nej1p, which have ligase activities.

In this thesis, we aimed to identify novel proteins that influence the NHEJ process in yeast. To this end we performed a combination of high-throughput as well as low-throughput assays, and followed-up by verifying some of the findings. The initial large-scale plasmid repair screening that we performed revealed approximately 60 novel genes that might involve in the NHEJ process. Among these genes, *PPH3/PSY2*, *HUR1*, *BUB1/BUB2*, and *SBP1* were selected for follow up experiments. Small-scale plasmid repair and chromosomal break repair assays were used to verify the involvement of these selected candidates in the efficiency of NHEJ. Deletion of any one of the above selected genes showed a reduction in efficiency of both plasmid repair and chromosomal repair assays. Deletion of those genes also showed increased sensitivity to DNA damaging agents Hydroxyurea (HU) and bleomycin. Our follow-up genetic studies suggested a role

for *PPH3/PSY2* phosphatase complex in the NHEJ process through interaction with checkpoint kinases Rad53 and Chk1. We also propose a novel role for yeast uncharacterized ORF *HUR1,* in NHEJ pathway through its association with Nej1. We also report that *BUB1/BUB2* may affect the efficiency of NHEJ by limiting cell cycle progression. We suggest their joint participation with Apc9p (APC), Clb2p (cyclin), and Swi4p (SBF) proteins in the NHEJ repair process. Additionally, we hypothesize a role for the translational repressor *SBP1* in NHEJ pathway, connecting translation control to NHEJ.

DEDICATION

To my beloved husband and parents for their love, support and encouragement.

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Statement of Contribution

The thesis, "Identification and characterization of novel genes involved in DNA double strand break repair process in the yeast *Saccharomyces cerevisiae* " is composed of four studies. Currently, chapters 2 (*PLoS One,* 2014) and 4 (*MCB,* 2015) are published, and chapter 3 (*Gene Journal*, 2017) is submitted for publication.

Chapter 2, "Phosphatase complex Pph3/Psy2 is involved in regulation of efficient non-homologous end-joining pathway in the yeast *Saccharomyces cerevisiae*". Formulation of hypothesis, experimental design, development of the project, and data analysis were done by me. The manuscript was prepared by me. Computational work was done by Dr. Dehne and Dr. Green's group (Dr. Sylvain Pitre and Dr. Andrew Schoenrock), from Computer Sciences and Engineering Department, Carleton University. Dr. Bahram Samanfar contributed to Synthetic Genetic Array experiment. Dr. Matthew Jessulat and Daniel Burnside were involved in the analysis of the interaction data. Megan Sanders and Dr. Mohsen Hooshyar contributed to plasmid repair assay.

Chapter 3, "Uncharacterized ORF *HUR1* influences the efficiency of non-homologous end-joining repair in *Saccharomyces cerevisiae*". I came up with the hypothesis, experimental design and development of the project. I analyzed the data and wrote the manuscript. Experiments in yeast cells were carried out by me. Dr. Matthew Jessulat, Mary Daniel and Maryam Hajikarimlou contributed to plasmid repair experiments.

Computational work was done by Dr. Andrew Schoenrock. Dr. Mohsen Hooshyar and Daniel Burnside, contributed to drug sensitivity analysis.

Chapter 4, "Spindle Checkpoint Factors, Bub1 and Bub2, of *Saccharomyces cerevisiae* are required for non-homologous end joining double strand break repair". Wet lab experiments for this work were performed by me and Dr. Matthew Jessulat in Dr. Babu's lab from University of Regina. I participate in analyzing the plasmid, chromosomal repair assay, genetic interaction data and drug sensitivity spot test.

Chapter 5, "Translational repressor *SBP1* regulates the efficiency of non-homologous end joining repair in *Saccharomyces cerevisiae*". I worked out the hypothesis, experimental design and development of the project. Dr. Bahram Samanfar and Daniel Burnside were involved in the analysis of the data obtained from protein-protein interaction prediction and Pourya Honarvar helped with plasmid repair assay experiments.

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List of Abbreviations

$^1O_2$	Singlet oxygen
3-D	3-dimensional
6-4 PP	pyrimidine-pyrimidone photoproducts
AD	Activation domain
Alt-NHEJ	Alternative non-homologous end joining
AMPr	Ampicillin resistance
APC	Anaphase promoting complex
BD	Binding domain
c-NHEJ	classical- Non-homologous end joining
CBP	Calmodulin binding site
CPD	Cyclobutane pyrimidine dimers
DD	DNA damage
DDA	DNA damage array
DDR	DNA damage response
DNA	Deoxyribose nucleic acids
DSB	Double strand break
eIF4G	Eukaryotic initiation factor 4G
GAL	Galactose
GI	Genetic interaction
H_2O_2	Hydrogen peroxide

HO	Homothallic switching
HR	Homologous recombination
HU	Hydroxyurea
IR	Ionizing radiation
IRES	Internal ribosome entry site
kanMX4	Kanamycin resistance marker
Leu	Leucine
MMEJ	Micro-homology mediated end joining
MMS	Methyl methane sulfonate
MS	Mass spectroscopy
mSGA	Miniaturized SGA
Nat-R	Nourseothricin resistance
NHEJ	Non-homologous end joining
O_2–·	Superoxide
OH·	Hydroxyl radicals
ORF	Open reading frame
PIPE	Protein-protein interaction prediction engine
PPI	Protein-protein interaction
PSA	Phenotypic suppression analysis
RFC	Replication factor C
ROS	Reactive oxygen species
SAC	Spindle assembly checkpoint
SAM	S-adenosylmethionine

SDL	Synthetic dosage lethality
SGA	Synthetic genetic analysis
shRNA	Short hairpin RNA
TAP	Tandem affinity purification
TF	Transcription factor
UAS	Upstream activating sequence
Ura	Uracil
WT	Wild type
Y2H	Yeast 2 hybrid
YPD	Yeast extracts peptone dextrose

1 Chapter: Introduction

1.1 DNA damage and repair

The genetic information of every living organism, with the exception of certain viruses, is embedded within its sequence of deoxyribose nucleic acids (DNA). This sequence contains the "genetic code" which is the heritable information needed to describe an organism as an entity. Any small changes in the DNA could be harmful for the biology of a cell and, in theory, could lead to genetic instability. DNA damage is a constant threat to a cell's survival and it can have many causes and forms. In the simplest case, during DNA replication process, a nucleotide can be mis-inserted, deleted, or changed. DNA damage can constantly occur by exogenous and endogenous chemical agents. The main exogenous sources include certain environmental factors, UV radiation, and ionizing radiation, whereas endogenous causes of DNA damage include internal factors such as replication stress, and certain biochemical reaction products such as reactive oxygen species (ROS) (Friedberg EC et al. 2004, Zeman and Cimprich 2014 and Ciccia and Elledge 2010).

UV light is a common cause of DNA damage and could lead to mutations. It produces cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6-4) pyrimidone photoproducts (64PPs) lesions. The production of UV lesions is depended on its wavelength and energy absorption by DNA nucleotides.

DNA damage could also occur by ionizing radiation through formation of free-radicals. Ionizing damage could create the DSBs, which are the most severe forms of DNA damage (Mahaney et al. 2009).

ROS is one of the main sources of endogenous damage. ROS are produced as bi-product of cellular metabolism. In living organisms ROS are formed as superoxide ($O_2^{-\cdot}$), hydroxyl radicals ($OH^{\cdot}$), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$). $O_2^{\cdot -}$ is the most critical form of ROS as it produces several other forms of reactive oxygen (Bhattacharyya et al. 2014). Hydroxyl radical (•OH) is the most reactive of ROS and can cause damage to proteins and DNA (Birben et al. 2012).

Living cells contain other small reactive molecules that could also lead to DNA damage. *S*- adenosylmethionine (SAM) is an example of such molecules. It is a reactive methyl group donor that is involved in physiological enzymatic DNA methylation, which plays a role in regulation of gene expression (Holliday and Ho, 1998).

If it is not repaired, DNA damage can cause mutations, loss of genetic information or in some cases can be lethal (Burma et al. 2006, Zha et al. 2007, De Bont and van Labrebeke 2004). DNA replication and repair are essential components for the long-term survival of any organism. DNA damage can be repaired through different repair pathways. Many DNA repair proteins are conserved, from fungi to mammals, highlighting the importance repair pathways. In the case of Double Strand DNA Break (DSB), which is the most severe form of DNA damage, the cell responds to the break occur, through two main repair pathways, Non-Homologous End Joining (NHEJ) and Homologous Recombination (HR) (Chapman et al. 2012).

1.1.1 DNA double strand break repair

One of the most toxic forms of DNA lesions is a DSB, where both strands of DNA are cut at or near the same region (Czornak et al. 2008). DSBs can be caused by

different factors. It can be induced by replication stress/errors such as stalled replication fork, metabolic oxidative products as well as different natural and synthetic chemicals (Aylon et al. 2004). It can cause genetic instability, chromosomal abnormality, mutations and even cell lethality. Mutations in key DSB repair genes have been linked to cancer development and susceptibility in humans (Shrivastav et al. 2008). As indicated, DSBs can be repaired by two main mechanisms; Non-Homologous End Joining (NHEJ) and Homologous Recombination (HR). These repair pathways are highly conserved from single cell organisms such as *Saccharomyces cerevisiae* to more complex mammals such as human.

The cell may repair the damage through HR if the lesion happens within a sequence that has a homologous region somewhere else in the genome. The HR pathway is preferred in case of stalled replication forks because of availability of a homologous template (Shrivastav et al. 2008). Otherwise, NHEJ can be used to repair any DSBs. It is thought that these two pathways work independent of each other. HR is considered to be an error free repair pathway (Dudas et al. 2004). A template used during the repair process is often obtained from sister chromatids or homologous chromosomes and is used as an anchor to connect the broken sites of DSBs (Ataian and Kerbs 2006).

In NHEJ, the break ends can also be ligated directly or by use of micro-homologs. In contrast to HR, it does not require a homologous template. Although NHEJ has a higher risk of mistakes and mutagenesis (Pierce et al. 2001), in mammalian and higher eukaryotes NHEJ is the main pathway to repair DSBs (Saleh-Gohari et al. 2005).

Signaling proteins, histone modifiers and chromatin remodeling facilitate recruitment of DNA damage repair proteins to the site of the break (Ataian and Kerbs

2006). DNA damage checkpoints sense the damage and preparing the cell for repair through changes in cell cycle progression (Davidson and Brown 2008).

1.1.2 Cell cycle regulation and DNA damage response

In the presence of constant threat of damage to DNA, cells have evolved different mechanisms for maintenance of the genome. Before committing to mitosis cells pass through different cell cycle checkpoints. These checkpoints are activated in response to DNA damage, incomplete DNA replication and damaged replication complexes. By recognizing DNA damage and regulating cell cycle, they arrest or delay cell cycle progression to provide needed time for the cell to repair its DNA. Such checkpoints function as control mechanisms to stop or slow down the cell cycle process, creating stable conditions for damage to be repaired; any defect in checkpoint can lead to cellular damage and genome instability (Chen et al. 2008). Three general checkpoints are present in *S. cerevisiae*, the G1/S, Intra-S, and G2/M checkpoints. Cdc20/ Cdc28 (*CDK1)* (cell division cycle) were some of the first genes to be identified as important regulators of the cell cycle in yeast (Nasmyth et al. 2004). In *S. cerevisiae*, CDK1 is activated in G1 at the point of S phase entry (Toone et al. 1997). CDK1 is required for DSB-induced HR at different cell cycle steps and for efficient 5′ to 3′ resection of DSB ends (Ira et al. 2004). During G1, yeast cells can repair DSBs by NHEJ. During S and G2 phases, due to the presence of homologous template, HR is enhanced. Nonetheless, NHEJ remains active during those phases and competes with HR for DSB repair.

Key proteins of the DNA damage response pathway in *S. cerevisiae* are Tel1p and Mec1p, which are homologs of human ATM and ATR (Putman et al. 2009). DNA

damage detection begins with the *MEC1*-dependent DNA damage checkpoint in budding yeast. It is a sensor of ssDNA in the cell (Harrison and Haber, 2006). In case of DNA damage, Mec1p activation is dependent on Ddc2p and RPA (Zou and Elledge 2003) and Tel1p is activated by MRX complex (Mre11/Rad50/Xrs2) (Nakada et al. 2003). Tel1p also responds primarily to DSBs in parallel pathway with Mec1p, which is involved in a DNA damage checkpoint pathway. Rad9p phosphorylation and interaction with Rad53p and Chk1p is dependent on activation of Mec1p and Tel1p (D'Amours and Jackson 2001, Grenon et al. 2001, Usui et al. 1998, Naiki et al. 2004, Emili 1998, Blankley et al. 2004, Ma et al. 2006, Sweeney et al. 2005).

In *S.cerevisiae*, Rad9p, Rad17p, Rad24p, Mec3p, and Ddc1p checkpoint proteins are required for efficient recognition of damaged DNA (Majka et al. 2003). Replication factor C (RFC) forms a complex with Rad24p, and recruits Rad17p, Ddc1p, and Mec3p complex to the site of damage (Branzei and Foiani 2006). This in turn upregulates repair genes, downregulates cyclins and delays cell cycle progression. Rad53p plays an important role in S/G2 arrest. Rad53p has several targets; transcriptional regulator Swi6p, Dun1p and Dbf4p. Activation of these targets by Rad53p leads to limiting the replication of the genome and prevention of late starting of DNA replication origins (Lee et al. 2003, Duncker et al. 2002). Chk1p has an importance role in G2/M arrest of cell cycle as it arrests cell cycle at metaphase and inhibits the cell from entering anaphase. (Branzei D and Foiani M 2006). Chk1p also regulating phosphorylation of Pds1p in response to DNA damage, which is an important factor of the metaphase to anaphase transition. Delay or arrest in cell cycle provides the cell with more time to repair the damaged DNA.

Mec1p and Tel1p, phosphorylate H2A to form γH2A. γH2A is a key histone modification, which is activated in early stage of repair and serves as an indicator of a DSB damage (Fillingham et al. 2006). γH2A recruits several other repair proteins to the site of the DSB. INO80 is one of the complexes that is recruited by γH2A. INO80 is believed to facilitate the recruitment of MRX complex to the site of damage. Before repair is completed, γH2A is dephosphorylated and released from the site of the damage (Figure 1.1). It is not clear which proteins and signals are involve in this process.

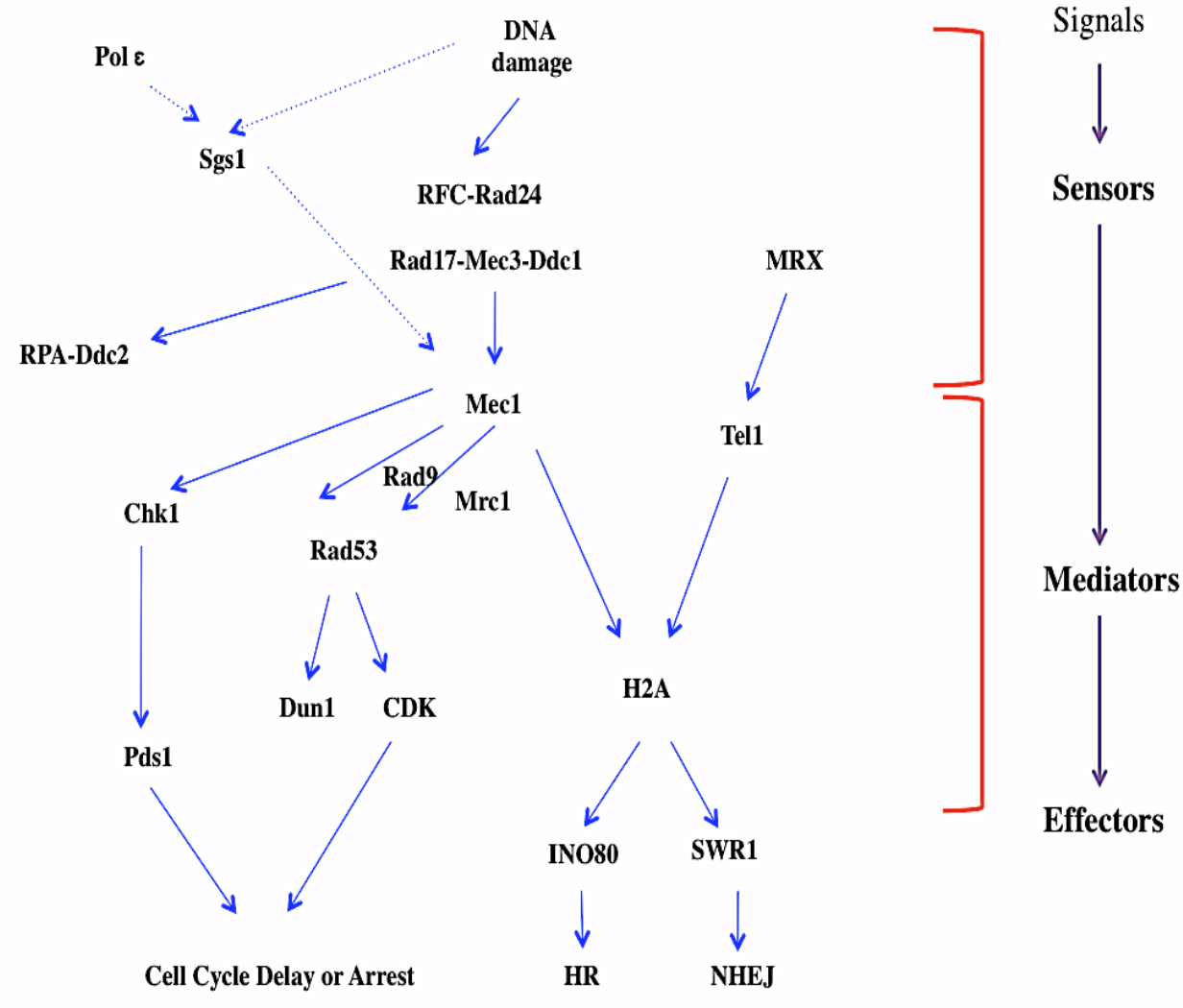


Figure 1.1: A diagram of DNA damage sensing and activation in yeast. It controlled by signal transduction pathways including sensors, transducers and effectors. Mec1, Tel1, Rad53 and Dun1 together regulate the DNA damage-induced process.

1.1.3 Homologous recombination repair

HR plays a fundamental role in DNA damage repair pathway to maintain genome stability. It facilitates the error-free repair pathway primarily during the S and G2 phases of the cell cycle (Mathiasen and Lisby 2014). HR pathway is the primary DSB repair system in most fungi including *S. cerevisiae*. Its activity is directly linked to the availability of a homologous DNA region. A cell can repair DNA damage through HR if the lesion happens within a sequence that has a homologous site elsewhere in the genome. HR utilizes an undamaged homologous template, preferably the sister chromatid or homologous chromosomes, to repair the broken sites of DSBs (Ataian et al. 2006, Kass et al. 2010), and it is considered to be an error free repair pathway (Dudas et al. 2004).

Repair of DSBs by HR is initiated by the 5′ to 3′ resection of DSB ends, which commits repair to this pathway. The 3′ Overhangs of DNA links with Rad52p and polymerized Rad51p, which cover up the ssDNA overhang. In yeast, the MRX complex, which is composed of Mre11p, Rad50p and Xrs2p binds to DNA and forms the basis of HR repair. Mre11p possesses a DNA binding activity (Usui et al. 1998, Furuse et al. 1998) that allows the recruitment of this complex to the DNA. The exact function of Xrs2p is not known and is thought to be involved in structure specific DNA binding and is reported to mediate the activity of Mre11p (Trujillo and Sung 2001). Rad51p binds DNA in an ATP-dependent manner and can mediate the identification of homologous sequences of DNA (Sung 1994). Then RPA binds to the ssDNA overhangs, recruiting Rad54p, Rad52p and Rad57/Rad55p to the DNA lesion. Rad54p interacts with Rad51p, ssDNA, and chromatin to stimulate pairing with homologous DNA (Dudáš et al. 2003).

The homologous region will be used as a template for replication by DNA polymerases (Shrivastav et al. 2008) (Figure 1.2A).

1.1.4 Non-homologous end joining repair

A more flexible alternative for HR repair system is NHEJ (Figure 1.2B) (Dudášová et al. 2004, Daley 2005). In NHEJ, the two broken strands of DNA can be ligated directly (classic pathway) or by use of micro-homologs (alternative-NHEJ or alt-NHEJ pathway). Since NHEJ does not use a homologous sequence, there is a higher risk of mistakes in repair that can lead to mutations (Pierce et al. 2001). Consequently, it is consider to be an error-prone pathway. Generally yeast cells repair DSBs by NHEJ in G1, while HR happens specifically in late S and G2 phases because of template accessibility. However, NHEJ still remains active during other phases of cell cycle and competes for DSBs.

In mammalian, NHEJ is the main pathway to repair DSBs; in comparison to yeast where it plays a secondary role (Saleh-Gohari et al. 2005). NHEJ pathway is highly conserved from yeast to mammals; with the exception of DNA-PKCs and Artemis (please see below).

The first step in DSBs repair pathway through NHEJ is the recognition of the broken ends of DNA. The formation of a bridge to bring the broken ends of DNA together is considered the second step. In the last step of the repair, ligation and repair of the break takes place (Reviewed by Chiruvella et al. 2013 and Shrivastav et al. 2008 and Daley et al. 2005). In *S. cerevisiae,* the key protein complexes of the NHEJ pathway are

the MRX (Mre11p, Rad50p, and Xrs2p), YKU (Yku70p/Yku80p) and Lif1p/Dnl4p (Daley et al. 2005).

The first complex to bind to the site of damage is Yku70p/Yku80p heterodimer (Yku complex), which binds to DNA double strand break ends; it forms a ring which is required as a factor for protecting and stabilizing the broken ends of DNA from degradation. Once the YKU complex is bound to the site of damage, it recruits other factors to the site of break. It should be noted that MRX complex recruitment to the site of damage is reported to be independent of YKU, but its binding to the site of damage is reduced in the absence of YKU (Wu et al. 2008 and Zhang et al. 2007).

MRX is the second complex that is recruited to the site of damage. It is composed of two subunits of Rad50p and Mre11p and one subunit of Xrs2p. The contribution of MRX complex in NHEJ repair pathway is not well understood (D'Amours and Jackson, 2002). MRX complex controls end bridging of the two broken ends of DNA and forms a bridge, which brings the broken ends closer to each other and prepares them for ligation. Rad50p is the largest protein of MRX complex and contains coil-coil regions that separate its two ATPase domains. These domains are placed on either side of the damage that is connected by an Mre11 dimer, which also mediates interactions between Rad50p and Xrs2p (Hopfner et al. 2000). It is thought that Xrs2p is one of the key proteins for targeting of the MRX complex to the damage site, although both the complex and every individual member of the complex can bind to DNA directly (Furuse et al. 1998, Trujillo and Sung 2001).

The third complex is Dnl4p/Lif1p, which has ligase activities. DNA ligation is a fundamental requirement for DSB repair. Lif1p interacts with N-terminus of Xrs2p and

Dnl4p, and performs ligation of DNA ends (Best et al. 2009, Hefferina et al. 2005). Lif1p recruits Dnl4p to the site of damage and promotes its stability and ligase activity at the site of damage (Wu et al. 2008, Teo S, Jackson 2000). Dnl4p/Lif1p recruitment to damage site is depended on the YKU (Zhang et al. 2007). The interaction between Yku80p C-terminus and Dnl4p is critical to Dnl4p activity (Palmbos et al. 2008). Nej1p binds to Dnl4p/Lif1p complex through an interaction with Lif1p, although its role is still unclear (Deshpande and Wilson 2007, Ooi et al. 2001). The interaction is mediated between C-terminus Nej1p and N-terminus of Lif1p (Ooi et al. 2001, Mahaney et al. 2014). Recent studies suggest that Nej1p is recruited to the break site, interacts with DNA at the site of damage, and may play a regulatory role in NHEJ (Brandi et al. 2014).

Three main complexes involved in NHEJ in *S. cerevisiae* have functional homologues in human; Yku70 and Yku80 are *S. cerevisiae* homologues of Ku70 and Ku80, respectively, Rad50 and Mre11 of MRX are both present in humans. Yeast Xrs2 has human Nbs1 homolog, and both human and *S. cerevisiae* have lig4 (Hefferina et al. 2005). Consequently, lower organisms such as *S. cerevisiae* can serve as good model organisms to study NHEJ and help to understand the underlying principles of DSB repair in humans and certain diseases that are linked to NHEJ (Aylon and Kupiec 2004, Emerson et al. 2016).

Several other factors have been shown to be involved in the NHEJ process. End processing at the DNA breaks is one of the steps that is not clearly understood because of involvement of different overlapping pathways. For example, Pol4p, a Pol X family polymerase, and Rad27p, a 5’ nuclease, are associated with gap filling and end processing, respectively (Bebenek et al. 2005, Daley and Wilson et al. 2008). Recent

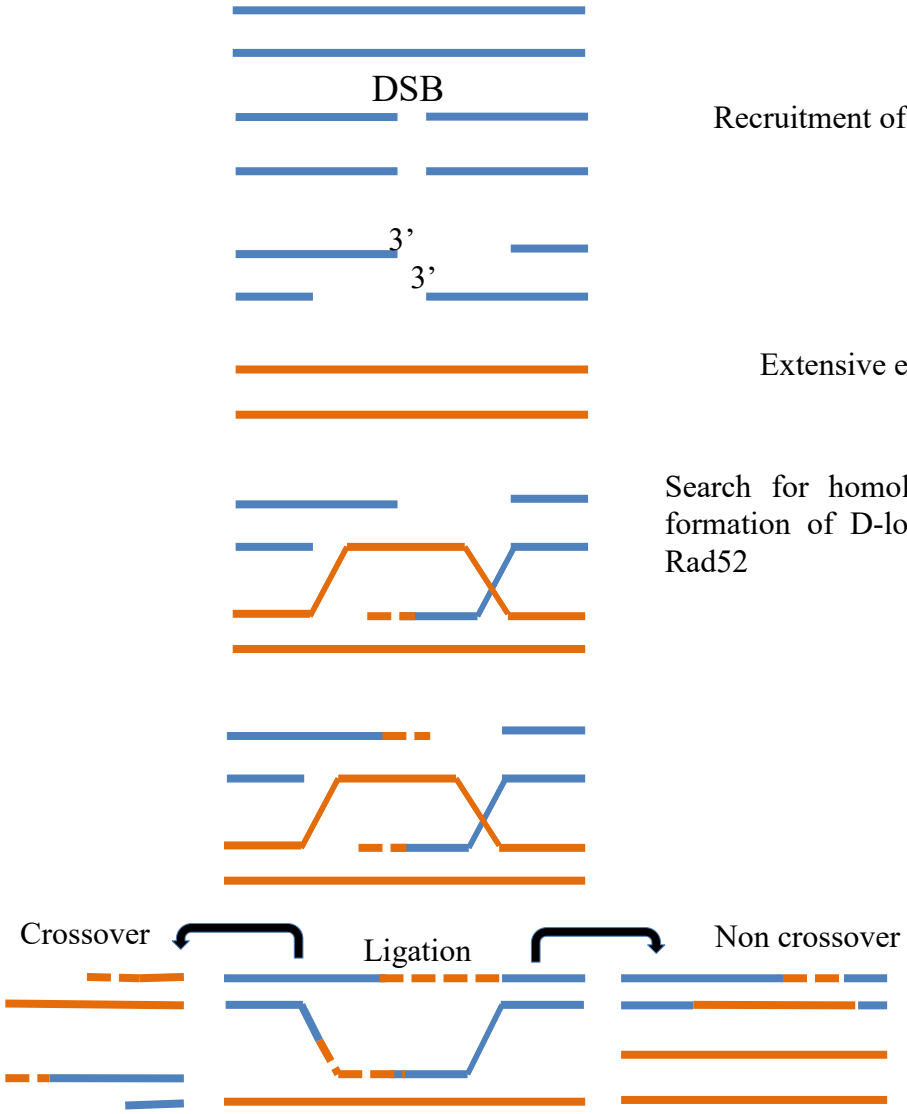
paper reports the interaction of Nej1p with Pol4p and Rad27p, and modulating their DNA synthesis and nuclease activities by Nej1p (Yang et al. 2015). Pol4p activity is depended on the interaction of Pol4p with Dnl4/Lif1 complex. Pol4p activation in NHEJ pathway is regulated by Tel1p (Ruiz et al. 2013). Also other factors such as Pol2p, Pol3p, Exo1p and Tdp1p have been reported to be involved in NHEJ end processing in *S. cerevisiae* (Galli et al. 2015).

In the past few years a number of additional genes have been linked to the efficiency of NHEJ pathway. These include, Rtt109p and Vps75p, a histone acetyl transferase, Sub1p, a transcriptional regulator and Tpk1p, protein kinase. This suggests that there may remain a number of other uncharacterized genes in NHEJ pathway that need to be further investigated.

Although the basic principles of classic NHEJ pathway have been well elucidated, different studies show that deletion of the key NHEJ genes does not fully deactivate the repair process. This suggests the presence of an additional pathway and eventually led to the discovery of alt-NHEJ pathway, also known as micro-homology mediated end joining (5), which may serve as an alternative or backup to NHEJ. The classic NHEJ pathway is dependent on the key factors YKU and Dnl4p, however, the alt-NHEJ seems to only require the MRX complex (Emerson et al. 2016, Kostyrko and Mermod 2016). Classic NHEJ can cause insertions or deletions of less than 5 bp while nucleotide changes up to 25 bp can take place in alt-NHEJ (Lee and Lee 2007). The control and mechanistic details of alt-NHEJ are less clear; different studies have reported that this alternative pathway could be an extension of classic NHEJ, or substitutions of some genes for the

loss of others (Chiruvella et al. 2014). It is still unclear if alt-NHEJ pathway consists of one or several separate pathways.

A

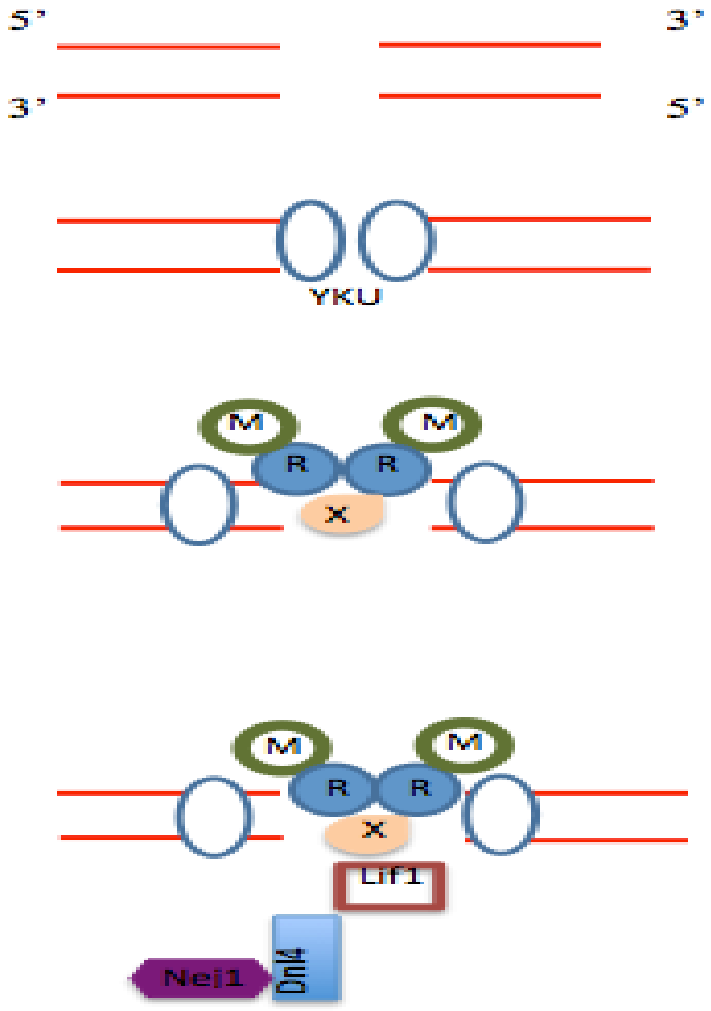


Recruitment of MRX and resection

Extensive end resection

Search for homologous sequence and formation of D-loop PRA, Rad51 and Rad52

B



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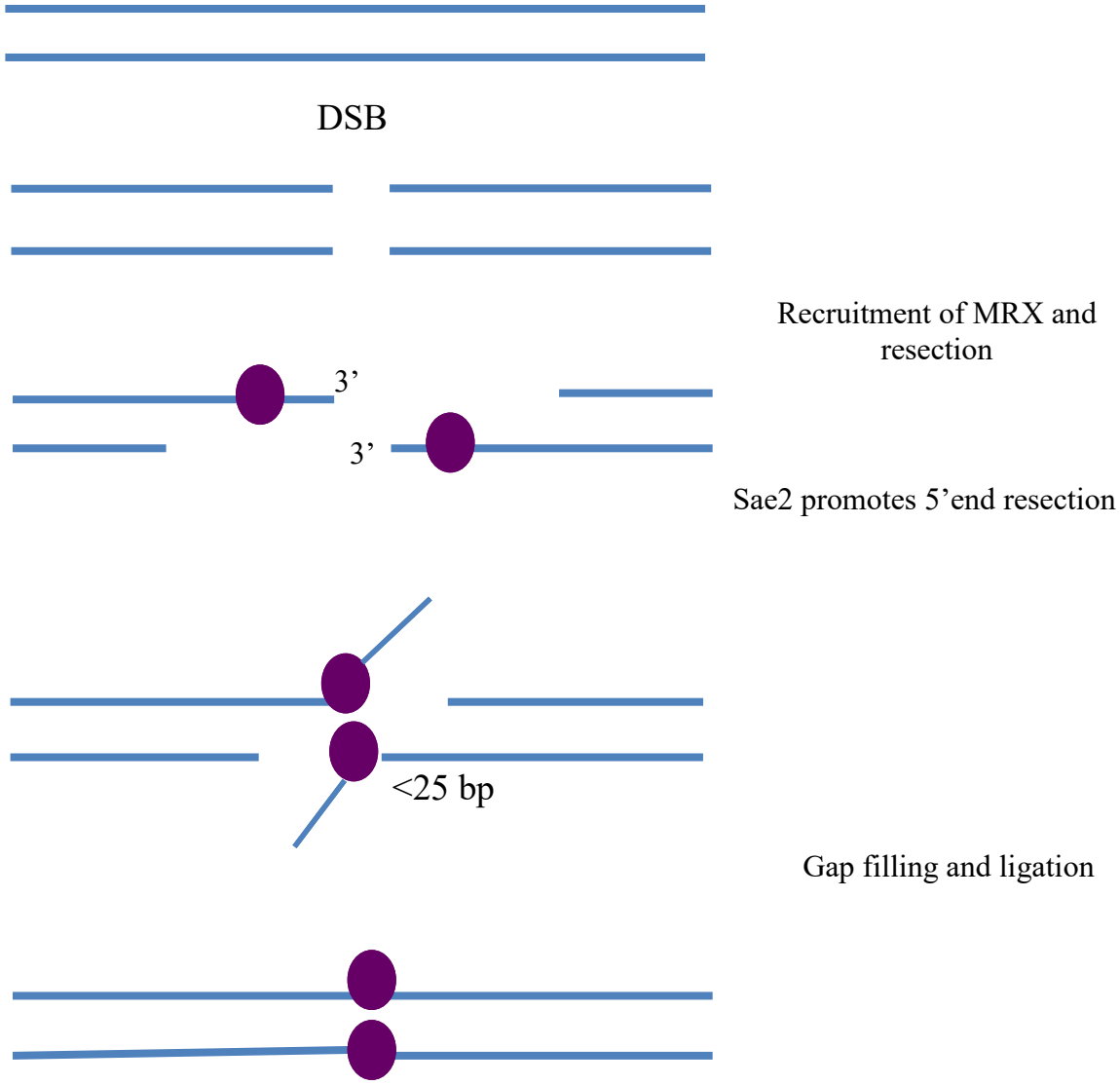


Figure 1.2: DSBs are repaired by HR, NHEJ and MMEJ. (A) HR uses a homologous region, (B) NHEJ repairs by direct re-joining. The key protein complexes of the NHEJ pathway are the MRX (Mre11p (M), Rad50p (R), and Xrs2p (X)), YKU (Yku70p/Yku80p) and Lif1p/Dnl4p, (C) MMEJ YKU independent pathway.

1.2 Systems biology

For many years, in molecular biology, the function of individual genes was investigated one at the time using mostly model organisms such as *S. cerevisiae*, *Escherichia coli, Drosophila melanogaster*, etc. A combination of all the different individual studies helps to understand the basic of different cellular pathways. In this approach, however, there are often many gaps and the relationship between genes in various contexts may not be studied in detail.

In recent years, with the advancement of new technologies, we now have the means to investigate the biology of a gene in a large-scale and a systematic manner, providing a better context to elucidate their functions (Schoenrock et al. 2014, Babu et al. 2011, Guénolé et al. 2013, Hu et al. 2009, Parson et al. 2004 and Hillenmeyer et al. 2008). Systems molecular biology focuses on examining the network of molecular components within a biological system, and attempts to increase understanding of cellular factors in association with other factors in the cell. It is thought that it could be used to provide deeper understanding of functional relationships between genes, pathways and other cellular components from a sub-cellular to an organism level as a whole system (Aggarwal and Lee 2003, Castrillo and Oliver 2004, Aloy and Russell 2006).

In the context of high-throughput studies, the budding yeast, *S. cerevisiae*, has emerged as a model organism of choice to examine the biology of eukaryotic cells at a systems level (Samanfar et al. 2013, Makhnevych et al. 2009). Early availability of the genome-wide DNA sequence of *S. cerevisiae,* non-essential gene deletion libraries, over expression libraries, conditional essential gene-knockdown arrays, etc. made yeast an

ideal model organism in systems biology. It has been utilized in different large-scale systematic studies such as genetic interaction mapping (Krogan et al. 2006, Alamgir et al. 2008, Costanzo et al. 2011, Samanfar et al. 2013, Samanfar et al. 2014, Omidi et al. 2014), protein interaction studies (Pitre et al. 2006, Schoenrock et al. 2014), gene expression analyses (Jessulat et al. 2010), etc.

1.2.1 Functional genomics

In simple terms, functional genomics refers to investigation of functions for genes in a high-throughput (genomic-scale) manner. During the past two decades, comprehensive systematic approaches, such as genome sequence analysis, gene expression studies, Genetic Interaction (GI) arrays and Protein-Protein Interaction (PPIs) networks, have been used to study and understand the function of genes.

The yeast *S. cerevisiae* has been used as a first model eukaryote organism to study large-scale gene functions (Giaever and Nislow 2014, Samanfar et al. 2013, Suter et al. 2006 and Sharifipoor et al. 2012). Yeast became a powerful genetic model organism for systems molecular biology and functional genomics investigations because of early availability of fully sequenced genome, availability of non-essential and essential deletion mutants strain library (Winzeler et al. 1999) and yeast gene overexpressed arrays (Zhu et al. 2001, Sopko et al. 2006) in addition to simple DNA transformation methodology with high efficiency and simple genetics. In the deletion mutant strain collection, each of the open reading frames (ORFs) is deleted and replaced by kanamycin resistance marker (kanMX4) using homologous recombination, which works very efficiently in yeast. Synthetic Genetic Array (SGA), Synthetic Dosage Lethality (SDL)

and Phenotypic Suppression Array (PSA) techniques have been useful in the identification of genes involved in different pathways and global mapping networks of functional interactions between genes (Tong et al. 2001, Sopko et al. 2006, Alamgir et al. 2008, Omidi et al. 2014, Samanfar et al. 2013). Combining genetic interaction analysis with chemical-genomics has also proven to be a good tool to study novel gene functions (Alamgir et al. 2008, Alamgir et al. 2010). Despite major advances in functional genomics tools, and availability of comprehensive interaction data, there remains numerous unknown and uncharacterized genes in yeast that require further investigation.

1.2.2 Genetic interaction analysis

In a general sense, Genetic Interactions (GI) refer to a functional association between a group of genes or genetic pathways (Boucher and Jenna 2013). It point to a higher level of association and a complex network, relationship, between gene functions. GI is defined by an unpredictable phenotype caused by overexpression or deletion of two or more genes together that is not easily explained by investigating the phenotype for changes in expression of each gene alone (Dixon et al. 2009). Generally, GI is detected by comparing and analyzing the phenotype of the individual single mutant strain to the double mutant strains phenotype. In yeast, the growth phenotype of the strains is often used as fitness measure by detecting colony size within the array of single and double mutant strains. In this case the phenotype (colony size) of a single mutant is measured, compared to surrounding colonies and then compared to the phenotype of the double mutant strain, which is normalized to the surrounding double mutants. If the phenotype is different than expected, the two genes are said to be interacting. For example, if deletion

of gene 1 or 2 alone results in no reduction in growth but deletion of both genes together causes a reduction in growth, it is then said that gene 1 and gene 2 are genetically interacting. When two genes are interacting, it is generally accepted that they are functionally related. The functional relationship might be explained by their activity in overlapping pathways, similar pathways, or signaling (Ui et al. 2005). GIs have been systematically studied in different organisms such as *S. cerevisiae, E. coli*, *C. elegans* and human (Jessulat et al. 2008, Omidi et al. 2014, Babu et al. 2011, Sugimoto 2004, Schoenrock et al. 2014) to identify functional relationship or correlation between different genes or their related products in different pathways.

GIs are divided in two broad categories; negative and positive interactions. Negative interactions describe the double mutants or overexpress/deletion phenotype when the phenotype is worse than expected compare to single deletion mutants. This includes synthetic lethality, where combined mutations are lethal, or synthetic sick where double mutants have a reduced growth rate. Negative interaction is thought to represent overlapping roles for the two deleted genes in parallel biological pathways. Deleting one gene (Gene A) alone does not have any effects on normal viability, meaning that the other gene (Gene B) with overlapping function in a parallel pathway may compensate for the lack of gene A. Removing both genes will result in a very sick or non-viable colony because the compensatory pathway is no longer functional. Positive interactions also suggest overlapping function. For example, if the function of one pathway is interrupted by deletion of one gene (Gene A), deletion of second gene (Gene B) prevents impairment of viability. Deletion A or B, which function in the same pathway, causes defects in fitness; however, double mutants completely shut down the pathway and trigger another

pathway that compensates for the absence of both genes (Figure 1.3) (Bryshnikova et al. 2013).

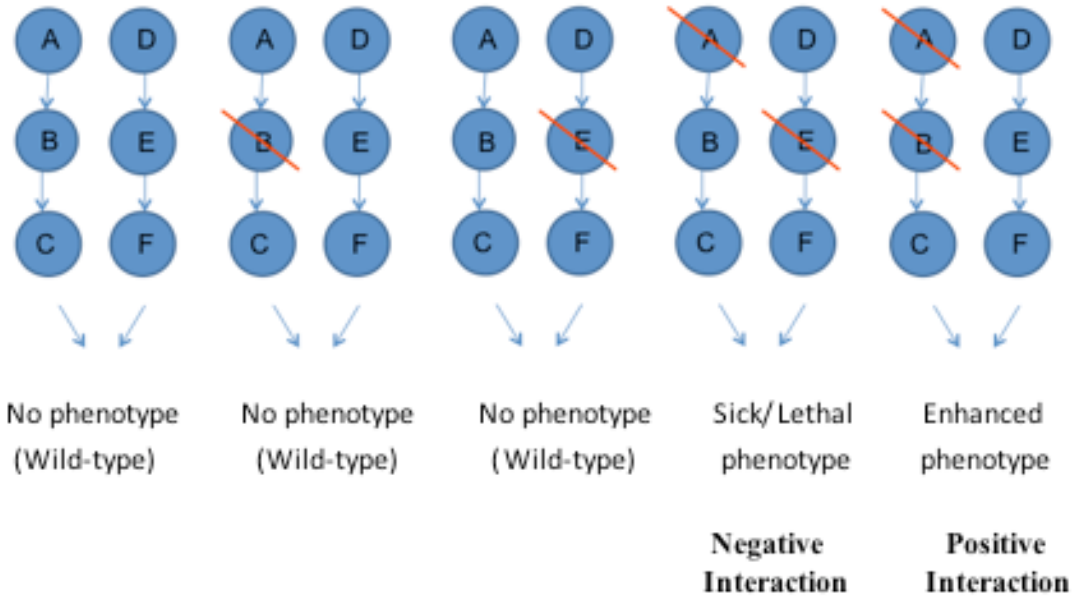


Figure 1.3: Genetic interaction analysis. Deletion of B or E genes individually in parallel pathways result in no phenotypic consequence. Deletion of both genes A and E together, that function in parallel pathways result in sick or lethal phenotype. This is an example of a negative interaction. Positive interaction refers to enhanced phenotype resulting from deletion of two genes A and B in the same pathway.

The availability of gene knockout collections in yeast, along with simple mating system and genetics made *S. cerevisiae* an ideal model organism for GI analysis. SGA a method developed in yeast by which through mating and a series of selections, double gene deletion mutants are generated in a high throughput manner (Figure 1.4) (Tong et al. 2001, Hillenmeyer et al. 2008). In this way an array of single gene knockout strains carrying the *kan* resistance marker in "a" mating type, MATa, is crossed with a single gene knockout of "α " mating type, MATα. Through a series of selections, an array of double mutants of "a" mating type is generated. The growth of these newly generated colonies is compared to the growth of their single mutants.

Synthetic Dosage Lethality (SDL) is a modified SGA technique designed to generate strains in which one gene is deleted and the other is overexpressed (Figure 1.5) (Sopko et al. 2006). Plasmid-based gene over expression strains of mating type α, are crossed to the series of single deletion mutants. After a series of selections, strains carrying a gene deletion and a gene overexpression are selected. Synthetic sick or recovery phenotypes are measured on the basis of the relative size of the colonies in each plate (Costanzo et al. 2011). Phenotypic suppression array (PSA) analysis is a modification of SDL. In PSA, compensation of phenotype in the presence of an inhibitory drug with a known mode of activity is studied (Alamgir et al. 2008, Alamgir et al. 2010). If overexpression of a gene "A" compensates for the fitness reduction (in the presence of a drug) caused by deletion of gene "B", then a functional relationship between the two genes can be hypothesized.

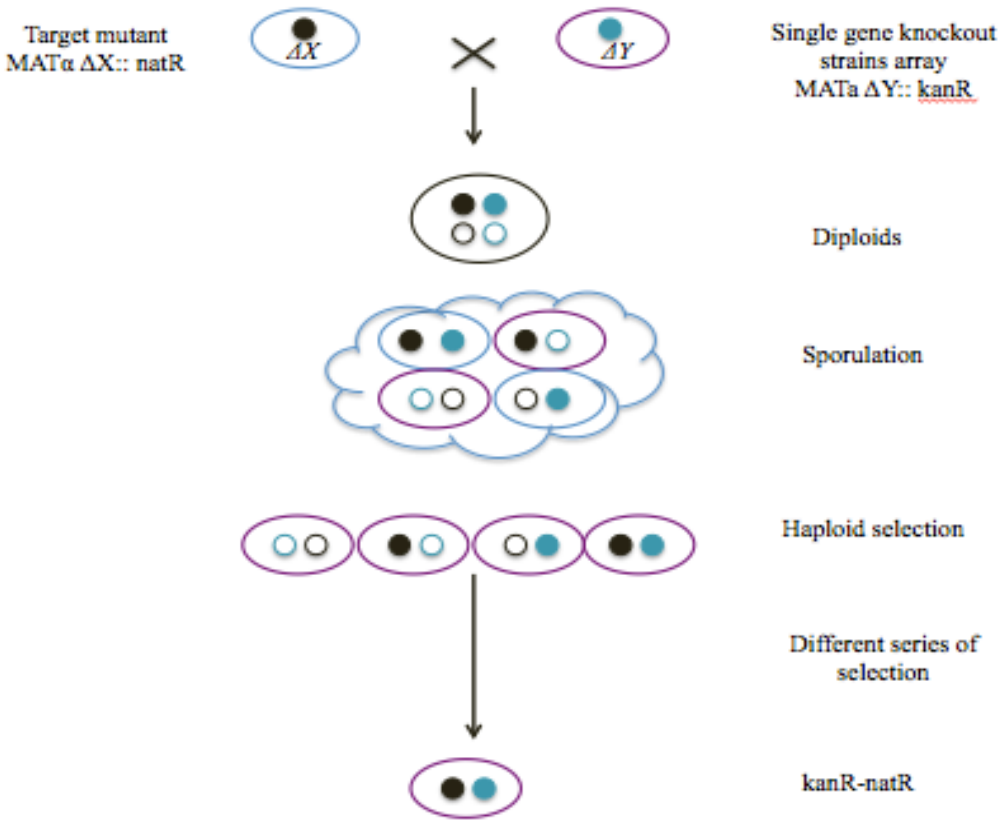


Figure 1.4: Synthetic Genetic interaction (SGA) methodology. An array of single gene knockout strains carrying *kan* resistance marker in MATa, is crossed with a single gene deletion of MATα. A double mutant of "a" mating type is generated after series of selections.

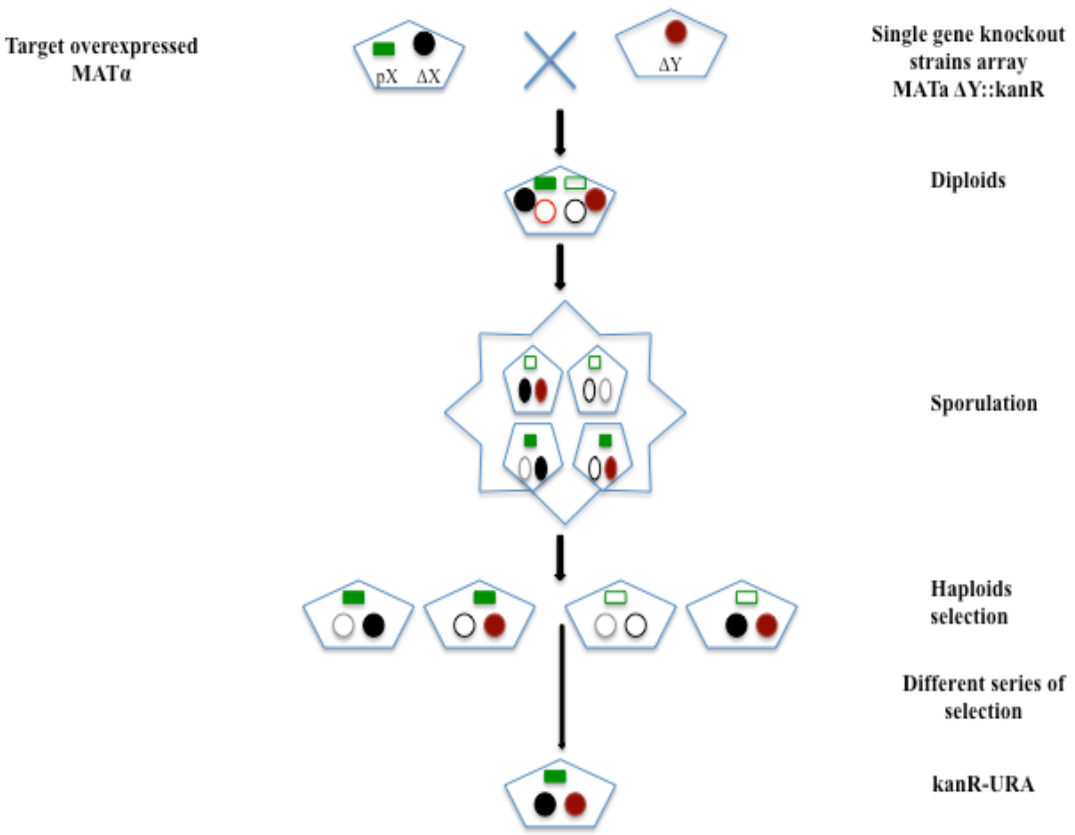


Figure 1.5: Method of Synthetic Dosage Lethality (SDL). An array of single gene knockout strains carrying *kan* resistance marker in MATa, is crossed with an overexpression gene in MATα. A gene deletion and a gene overexpression in MATa is selected after series of selections.

1.2.3 Protein-protein interactions

Proteins are key biomolecules that interact with each other to carry out particular cellular functions. The interaction between proteins is called Protein-Protein Interaction (PPIs). These interactions are important for the activity of proteins and hence essential to cell survival. Cellular pathways such as DNA replication, transcription, translation and signal transduction all require PPIs (Krogan et al. 2006). PPIs within a complex can be classified as (a) permanent, in which proteins of the complex have higher affinity for each other and interact longer, or (b) transient interactions that are temporary and often happen under specific cellular and physiological conditions (Criekinge and Beyaert 1999, Nooren and Thornton 2003, Aylon and Kupiec 2004).

During the past decades, different wet lab techniques and methods have been successfully applied to global large-scale PPIs detection in yeast. These methods include Yeast two-hybrid (Y2H) (Fields and Song, 1989) and Tandem Affinity Purification (TAP) tag coupled with mass spectroscopy (MS) (Rigaut et al. 1999).

In Y2H, transcription factor (TF) proteins are used to detect PPIs (Fields and Song 1989). Y2H is based on the activity of TFs that contain two protein domains with specific functions, a DNA binding domain (BD) and activation domain (AD) (Brückner et al. 2009, Criekinge et al. 1999). The binding domain is responsible for binding to the upstream activating sequence (UAS) and the activation domain is responsible for activating of transcription process (Young, 1998). The activity of this TF is dependent on the physical distance between the two domains. In Y2H analysis binding domain is fused to one of the proteins of interest that is called the bait protein, or the bait, and activation domain is fused to another protein of interest called the prey protein or the prey. One of

the initial pair of TF domains used in Y2H is AD and BD of GAL4 gene. This TF can bind to the Gal4 promoter of which is often placed upstream of a β-galactosidase reporter gene (Suter et al. 2006). This LacZ reporter system is activated when the UAS-BD-AD form a complex and hence produced β-galactosidase.

The TAP method was developed as a method of choice for the native purification of protein complexes (Puig et al. 2001, Li 2010). It is a fast and efficient affinity based purification method that results in high yield of protein complexes under physiological conditions. In TAP process, the target protein is fused to a C- or N-terminus of a double tag. The complexes are isolated in two rounds of affinity purification reducing the presence of co-purified contaminants (Jessulat et al. 2010). The original TAP tag was made up of an IgG binding domain of *Staphylococcus aureus* protein A and a calmodulin binding protein (CBP), separated by a TEV protease cleavage site (Puig O et al. 2001). It has shown that TAP is more sensitive than Y2H method in detecting PPIs. It also has lower error rates resulting in less false positives (Sun et al. 2013). Contrary to Y2H which is used to study binary interactions, TAP method has the ability to disclose multi-component interactions (Li 2010).

Although, Y2H and TAP method have been successfully applied in yeast genomics providing scientist with a wealth of PPI data, these methods possess inherent disadvantages. For example, they cannot be applied to all proteins and the high rate of false positive and negatives associated with these methods has led to little overlap between various global PPI studies (De Las Rivas and Fontanillo 2010). There are some disadvantage for those techniques such as time consuming and long time and expensive procedure, high rate of false positives and negatives, etc. (Skrabanek et al. 2008 and

Brückner et al. 2009). Also, it is difficult to use these techniques for all types of proteins (Berggård et al. 2007).

Recently, computational models and bioinformatics tools have been established to detect PPI networks. Using such computational tools provides the opportunity to discover additional PPIs in a faster and cost efficient manner. Some of these methods predict PPIs based on 3-dimensional (3-D) structure of proteins (Ohue et al. 2013 and Keskin et al. 2008). Others are based on co-conservation of proteins during the course of evolution (Skrabanek et al. 2008 and Muley and Ranjan 2013).

There are also other tools that use primary structure signals as a mean to predict interactions (Gomez et al. 2003, Pitre et al. 2008, and You et al. 2014). In one of these methods, co-occurrence of short polypeptide regions was used to detect novel PPIs. This method has been used to map interaction networks in *S. cerevisiae*, *Schizosaccharomyces pombe, Caenorhabditis elegans, Arabidopsis thaliana*, and humans (Pitre et al. 2006, 2008, 2012 and Schoenrock 2014). An advantage of this method is that the site of interactions between a pair of proteins can also be predicted.

1.3 Focus

As mentioned, DSBs are the most severe form of DNA damage. They are caused by both endogenous and exogenous DNA damaging factors (Czornak et al. 2008). There are two mechanisms for DSB repair, HR and NHEJ. In NHEJ, the two broken ends of DNA are ligated back together directly without the use of a homologous region. Mutations in key DSB repair genes have been linked to the onset of cancer and susceptibility to other diseases in humans including certain neurodegenerative diseases

and immunodeficiency syndromes due to inability of repair systems to maintain genetic materials (Durant and Nickoloff 2005, Frappart and McKinnon 2008, Gennery 2006). The NHEJ pathway is highly conserved from yeast to human, making yeast a suitable tool for studying NHEJ. The availability of yeast gene knockout and overexpression collections, its simple life cycle and genetics, and advanced tools for genome manipulation has made yeast an ideal model organism for studying gene function. Thus, *S. cerevisiae*, is an ideal choice for large-scale GI assays. In addition, the availability of various genomic resources and advanced DNA transformation/manipulation methodologies has made yeast an ideal "tool" for performing genome-wide analysis.

Over the past few decades the process of DNA damage repair has been extensively studied. Consequently, much has been learned about its mechanisms as well as the key factors involved in it. Subsequent studies showed that deletion of the key NHEJ genes did not deactivate and arrest the repair process. This suggests the presence of alternative repair pathways and genes that can mediate this process in the absence of the known key factors. A growing number of recent studies have revealed a number of additional genes to influence the efficiency and/or accuracy of NHEJ (Ruiz et al. 2013, Omidi et al. 2014, Jessulat et al. 2015, Heo et al. 2015, Kostyrko and Mermod, 2016) suggesting the presence of other genes that might also influence NHEJ. The objective of this thesis is to identify and study novel genes involved in the NHEJ pathway in yeast *S. cerevisiae.*

Previous work demonstrated that a series of other cellular processes, including cell cycle, histone and chromatin modification, transcription, etc. can also influence the efficiency of NHEJ. To this end, and to further study DNA damage repair, we previously

generated a DNA damage deletion mutant array, along with its corresponding overexpressed array forming a single user-friendly 384-spot array plate. Each colony represents a specific gene deletion strain in which a particular gene has been replaced by a kanamycin-resistance marker conferring resistance to antibiotic geneticin (G418). This plate was used to uncover new genes that could influence the efficiency of NHEJ. This was accomplished by a large-scale *in vitro* plasmid-based NHEJ DNA repair screening subjecting each strain to one round of *in vitro* plasmid repair screening. Deletion strains in this array included genes known to participate in NHEJ (10), other DNA repair pathways (57), response to stress or DNA damage (20), DNA replication (18), cell cycle or division (64), histone or chromatin modification (91), cell maintenance and structure (24), as well as genes with pleiotropic or unclear (37) functions. All of these candidates are known and potential DNA repair-related genes that are localized to nucleus and chosen from the literatures and public databases.

The initial large-scale screening suggested possible novel roles for approximately 60 genes in NHEJ. It should be noted that due to the large-scale nature of this screening many of these genes might be false positives. Similarly, there may be other positives that were not detected as a result of a high rate of false-negatives associated with such screens. Among the newly identified genes with possible association with NHEJ efficiency, we selected a few for follow-up studies. These 6 genes are localized to nucleus and may express in response to DNA damage. These candidates were not previously reported to have direct function within DSB repair.

At the time, the *PPH3*/*PSY2* phosphatase complex was known to be involved in cell cycle regulating recovery from the Rad53p DNA-damage checkpoint. Activation of

the Rad53 is a critical response to DNA damage, which maintain genome stability at the replication forks (O'Neill et al. 2007); *HUR1* was an uncharacterized gene, which characterizing it may offer new insights towards the elucidation of new pathways; Bub1p and Bub2p function as initial sensors of DNA damage during mitosis, and previous biochemical and genetic investigations have indicated a strong functional coupling between the SAC and the DDR (Kim et al. 2008). *BUB1*/*BUB2* were two interacting proteins with known function in limiting cell cycle progression by averting the activation of the Anaphase Promoting Complex (APC) (Musacchio et al. 2007). This complex functions as an initial sensor of DNA damage during mitosis (Kim and Bruke, 2008). *SSB1* was directly bind eIF4G and has a role in repression of translation (Rajyaguru et al. 2012). Inclusion of these genes in a list of candidates for additional investigations represented a combination of low (*PPH3*/*PSY2*), medium (*BUB1*/*BUB2*) and high risk (*HUR1* and *SSB1*) projects (appendix A.3).

In this thesis, to better understand the activity of the selected genes, small-scale plasmid repair assays were used to verify the involvement of the target genes in the efficiency of NHEJ. To verify the results of the small-scale plasmid repair assay in a chromosomal context, chromosomal DSB repair assays were performed. To support a role of target genes in DNA repair, drug sensitivity assays was carried out in the presence of DNA damage agents. To mechanistically study the activity of genes of interest, plasmid repair assay was repeated on targeted double gene deletions. Large-scale GI between the candidate genes and genes with known function in DNA repair was investigated to better understand the activity of the target genes in NHEJ. A PPI

prediction analysis was performed to study the physical interaction networks between genes that may have similar function in NHEJ pathway.

While the HR is the prevalent pathway in the unicellular budding yeast *Saccharomyces cerevisiae* (Dudas et al. 2003), the NHEJ is more prevalent in mammalian cells, especially those that are quiescent (Yano et al. 2009), and can repair DNA lesions even if there is no homologous strand (Lisby et al. 2009). Notably, the impairment of NHEJ in mammalian cells is frequently linked to genomic instability, cancer, and lymphoid V(D)J (i.e., variable, diversity, and joining gene segments) recombination defects. Therefore, a detailed molecular understanding of this pathway would provide critical insight into the genetic risk factors related to carcinogenesis or immunological disorders (Van Gent et al. 2001). For example, recent observations in mammalian cells have indicated that Bub1 functions in the DDR signaling pathway (Yang et al. 2012) but its role in supporting NHEJ efficiency remains unclear. Pph3/Psy2' homolog in higher eukaryotes such as human are identified and their conserved roles are indicating functional conservation through evolution in yeast to human (O'Neill et al. 2007). So, further investigation on these selected candidates in human will help to understand the mammalian function.

2 Chapter: Phosphatase complex Pph3/Psy2 is involved in regulation of efficient non-homologous end-joining pathway in the yeast *Saccharomyces cerevisiae*

Omidi K, Hooshyar M, Jessulat M, Samanfar B, Sanders M, Burnside D, Pitre S, Schoenrock A, Xu J, Babu M and Golshani A.

Contribution: Formulation of hypothesis, experimental design, development of the project, and data analysis were done by me. The manuscript was prepared by me. Computational work was done by Dr. Dehne and Dr. Green's group (Dr. Sylvain Pitre and Dr. Andrew Schoenrock), from Computer Sciences and Engineering Department, Carleton University. Dr. Bahram Samanfar contributed to Synthetic Genetic Array experiment. Dr. Matthew Jessulat and Daniel Burnside were involved in the analysis of the interaction data. Megan Sanders and Dr. Mohsen Hooshyar contributed to plasmid repair assay.

2.1 Abstract

One of the main mechanisms for double strand DNA break (DSB) repair is the non-homologous end-joining (NHEJ) pathway. Using plasmid and chromosomal repair assays, we showed that deletion mutant strains for interacting proteins Pph3p and Psy2p had reduced efficiencies in NHEJ. We further observed that this activity of Pph3p and Psy2p appeared linked to cell cycle Rad53p and Chk1p checkpoint proteins. Pph3/Psy2 is a phosphatase complex that regulates recovery from the Rad53p DNA damage checkpoint. Overexpression of Chk1p checkpoint protein in a parallel pathway to Rad53p

compensated for the deletion of *PPH3* or *PSY2* in a chromosomal repair assay. Double mutant strains *Δpph3/Δchk1* and *Δpsy2/Δchk1* showed additional reductions in the efficiency of plasmid repair, compared to both single deletions, which is in agreement with the activity of Pph3p and Psy2p in a parallel pathway to Chk1p. Genetic interaction analyses also supported a role for Pph3p and Psy2p in DNA damage repair, the NHEJ pathway, as well as cell cycle progression. Collectively, we report that the activity of Pph3p and Psy2p further connects NHEJ repair to cell cycle progression.

2.2 Introduction

Among DNA lesions, double strand DNA breaks (DSBs) are regarded as the most severe form of DNA damage. The mechanisms for DSB repair are divided in two independent pathways, Homologous Recombination (HR), and Non-Homologous End Joining (NHEJ). HR utilizes an undamaged homologous template, preferably the sister chromatid or homologous chromosomes, to repair the broken sites of DSBs (Ataian and Kerbs 2006, Kass and Jasin 2010), and is considered to be an error free repair pathway (Dudas and Chovanec 2004). A more flexible alternative to the HR repair system is NHEJ (Dudasova et al. 2004, Daley et al. 2005). In NHEJ, the two broken strands of DNA can be ligated directly. Because NHEJ does not use a homologous template, there is a higher risk of errors in repair, which can result in mutations (Pierce et al. 2011). NHEJ is the main pathway to repair DSBs in mammals (Saleh-Gohari at al. 2005).

The NHEJ pathway is highly conserved from yeast to human. Yku70p and Yku80p are *S. cerevisiae* homologs of Ku70p and Ku80p, respectively, which bind to DSB ends; they form a ring that is required as a factor for protecting and stabilizing the

broken ends of DNA from degradation. The MRX (Mre11p, Rad50p, Xrs2p) complex in yeast is homologous to MRN (Mre11p, Rad50p, Nbs1p) in mammalian cells. It forms a bridge between the two broken ends of DNA and brings the broken ends closer to each other preparing them for ligation. The MRX complex is recruited by Yku70/Yku80 to the site of a DNA break. It is thought that Xrs2p is one the key proteins for targeting of the MRX complex to the damage site, although both the complex and all individual members of the complex can bind to DNA directly (Furuse et al.1998, Trujillo and Sung 2001). Recent evidence suggests that MRN may function in multiple steps of NHEJ in mammalian cells (Zha et al. 2009). The Dnl4/Lif1 complex is the homolog of mammalian DNA ligase XRCC4, which has ligase activities. Lif1p interacts with Xrs2p and Dnl4p, and Dnl4p performs the ligation of DNA (Hefferina and Tomkinson 2005, Deshpande and Wilson 2007, Chen et al. 2010). Nej1p binds to the Dnl4/Lif1 complex through an interaction with Lif1p. Although its exact role is still unclear, recent investigations suggest it is recruited to the site of break, interacts with DNA and participates in the final steps of ligation (Chen and Tomkinson 2011) Plasmid repair analyses have demonstrated that NEJ1p is required for NHEJ to function at high efficiency (Chen and Tomkinson 2011).

The efficiency of NHEJ depends on a number of factors. For example, different histone acetyltransferases are required for NHEJ efficiency (Jessulat at al. 2008, Oike et al. 2012). Another study reported that NHEJ is dependent on different stages of the cell cycle; NHEJ activity is higher in G1 compared to G2/M (Zhang et al. 2009). In a recent study, methylation of histone H3 lysine 36 was shown to enhance the efficiency of NHEJ (Fnu et al. 2001).

Before committing to mitosis, cells pass through different cell cycle checkpoints. Checkpoints can be activated in response to DNA damage, incomplete DNA replication and damaged replication complexes. By recognizing DNA damage and regulating cell cycle arrest, they delay cell cycle progression to provide additional opportunity for DNA repair. Defects in checkpoint function can cause genomic instability (Myung and Kolodner 2002). Temporal association between the cell cycle and DNA damage is thought to begin with Mec1p, a DNA damage dependent checkpoint gene (Harrison and Haber 2006). Mec1p phosphorylates Rad9p (Naiki et al. 2004, Emili 1998). Phosphorylation of Rad9p further stimulates the activity of Mec1p to trigger several kinases including Rad53p and Chk1p (Blankley and Lydall 2004, Ma et al. 2006, Sweeney et al. 2005). The checkpoint Rad53p is a key protein in response to DNA damage. Activation of Rad53p up-regulates repair genes, down-regulates cyclins and delays cell cycle progression. The phosphatase complex Pph3/Psy2 negatively regulates Rad53p activity by dephosphorylating it and allowing cell cycle progression to continue (O'Neill et al. 2007). Recently, it was shown that the deletion of *PPH3* reduced the ability of cells to complete DSB repair via HR (Kim et al. 2011). *PPH3* (YDR075W) and *PSY2* (YNL201C) are located in the nucleus on chromosome IV and chromosome XIV, respectively. Pph3p and Psy2p localize to foci on meiotic chromosomes. The abundance of PPH3 proteins is 2840 molecules/cell and the protein domain of 1-308 is serine/threonine protein phosphatase.

Here, we posit that in addition to the previously known components of the NHEJ pathway, there are also other genes that may affect the efficiency of NHEJ. Here, we identified Pph3 and Psy2 from a comprehensive plasmid-based DNA repair screening.

we report that the deletion of *Pph3* and *Psy2* reduces the efficiency of NHEJ in *S. cerevisiae*. We further illustrate that this activity appears connected to cell cycle regulation.

2.3 Materials and Methods

2.3.1 Yeast strains and plasmid

The yeast strains are gene deletion variants of S288C *(*MAT *a* orfΔ::kanMX4 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0), described in (Winzeler et al. 19990. JKM139 (MATa hmrΔ::ADE1 hmlΔ::ADE1 ade1-100 leu2-3,112 lys5 trp1::hisG ura3-52 ade3::GAL-HO) strain is described in Moore and Haber 1996, and Lee et al. 1998. Yeast mating type alpha strain Y7092 (MATa can1Δ::STE2pr-HIS3 lyp11Δ ura31Δ 0 leu21Δ0 his31Δ1 met151Δ0) was used for mating experiments (Tong and Boone 2007). For plasmid repair assay a derivation of plasmid p416 with a LacZ insert following the GAL promoter region (Alamgir et al. 2008) was used. Gene knockouts are produced by transformation with a PCR product containing a NAT selection gene as described in (Winzeler et al. 1999). The DNA damage (DD) array was generated on the basis of GO term by arraying gene deletion mutants for 384 genes with known or potential involvement in DNA damage response, DNA replication, cell cycle progression or localization in nucleus. Overexpression plasmids which used are 2-micron plasmid as explained in Sopko et al. 2006.

2.3.2 Plasmid repair assay

A unique XbaI restriction site was used for plasmid (p416) linearization and the repair assay was performed as in (Jessulat at al. 2008). Each experiment was repeated at least five times.

2.3.3 Chromosomal repair assay

Chromosomal DSBs assay was performed using JKM139 strain. Serial dilutions of cells (10^{-3}–10^{-5}) were exposed to galactose for 90 minutes to induce HO endonuclease and compared to those before exposure. Number of colonies formed before and after induction of HO endonuclease was used as a measure of survival and the efficiency of the cell to repair induced DSBs. Each experiment was repeated at least five times. For compensation experiments, gene overexpression in a single mutant background was generated by transforming the JKM139-based gene deletion strains using a corresponding plasmid carrying the target gene (Sopko et al. 2006), or an empty vector as a control. To study NHEJ efficiency in different phases of the cell cycle, cells were synchronized in G1, S, and G2/M phases using drug treatment with 10 µg/ml alpha-factor, 0.2 M hydroxyurea, and 15 µg/ml nocodazole, for 2.5 hours, before exposure to galactose.

2.3.4 Drug sensitivity spot test

A series of single and double deletion mutants grown to mid-log were diluted (10^{-2}–10^{-5}) and spots of 15 µl of each dilution were placed on YPD plates containing 60 mM hydroxyurea (HU), 4 µg/ml bleomycin, or no drug as a control. Reduced colony size and numbers represented increased sensitivity.

2.3.5 Genetic interaction analysis

Genetic interaction between target genes and DNA damage array (DDA) genes was examined using a miniaturized version of Synthetic Genetic Array (SGA) analysis (Tong et al. 2001). In the miniaturized SGA (mSGA), a target gene is deleted or overexpressed (plasmid-based), in an alpha mating type strain and crossed to two arrays of 384 gene deletion strains, one for target genes (DD array) and the other random (as a control) (Alamgir et al. 2008). Double mutant strains were scored for fitness as in (Memarian et al. 2007, Samanfar et al. 2013) with some modifications. In brief, average colony size (S_{ave}) was calculated by summing the size of all colonies on a plate and dividing by the total number (384). S_{ave} was subtracted from each colony to derive a relative size for individual colonies. Each experiment was repeated three times and those colonies that had a reduction of 30% or more in two of the three repeats were deemed "positive". Synthetic sick interactions (positives) were categorized as follows: moderate (30–49% reduction), strong (50–69%), and very strong (70–99%), as in (Alamgir et al. 2010). For conditional interactions, the above analysis was repeated in the presence of low (sub-inhibitory) concentrations of DNA damage-inducing drugs. Hits were confirmed by random sporulation. Synthetic dosage lethality (SDL) analysis was performed as above with the exception that overexpression plasmids were transformed into the above deletion arrays as in (Alamgir et al. 2010). Gene classification on the basis of cellular process and function was performed by Yeast Features (http://software.dumontierlab.com/yeastfeatur es/), Yeast Genome Database (http://www.yeastgenome.org/) and GeneMANIA (http:// www. genemania.org/).

2.3.6 Protein-protein interaction prediction

Protein-Protein Interactions (PPIs) were predicted on the basis of co-occurring polypeptide regions as in (Pitre et al. 2008). An updated high confidence PPI database (approximately 55,000 interactions) was generated from published data (BioGRID: www.thebiogrid.org and DIP: www.dip.doe-mbi.ucla.edu). The analysis was performed at 99.95% specificity (a measure for false positive prediction), generating a sensitivity (percentage of interactions that can be identified from the total interactions that a protein makes) of 28% in comparison to the sensitivity of 14.6% in (Pitre et al. 2008) estimated by leave-one-out analysis. The local regions that mediate PPIs were predicted using PIPE-site algorithm (Amos-Binks et al. 2011).

2.4 Results and Discussion

2.4.1 Deletions of *PPH3* and *PSY2* reduced the efficiency of NHEJ in a plasmid based repair assay

To evaluate the activity of Pph3/Psy2 complex on the efficiency of NHEJ, a plasmid repair assay was utilized (Shim et al. 2005, Boulton and Jackson, 1998). Equal amounts of circular and linearized plasmids were transformed separately to both wild-type and deletion mutants for *PPH3* and *PSY2*. Transformed cells were plated on a selective media in a way that only cells receiving an intact plasmid or cells capable of repairing a received digested plasmid would form a colony. In this case, DNA repair is limited to NHEJ because the break site has no homologous region within the genome of *S. cerevisiae.* The number of colonies formed from linearized plasmids is related to

colonies formed from intact plasmids, and this ratio reflects the proportion of successful NHEJ events that have occurred. Previously, using this assay, deletion effects for the *RSC2*, a member of the RSC, chromatin remodelling complex (Shim et al. 2005), *SIR2*, *SIR3*, *SIR4* proteins involved in telomere maintenance (Boulton and Jackson, 1998), and yeast histone acetyltransferase *RTT109* have been evaluated (Jessulat et al. 2008). Deletion of *YKU70* or *YKU80* reduced NHEJ efficiency to approximately 6% and has been used as a positive control (Shim et al. 2005, Jessulat et al. 2008).

It was observed that efficiency of NHEJ for individual deletions of *PPH3* and *PSY2* was approximately 24% and 28%, respectively (Figure 2.1). Deletion of both *PPH3* and *PSY2* had a NHEJ efficiency of approximately 25%. These data are in agreement with the involvement of Pph3/Psy2 phosphatase complex in efficient NHEJ of a plasmid DNA.

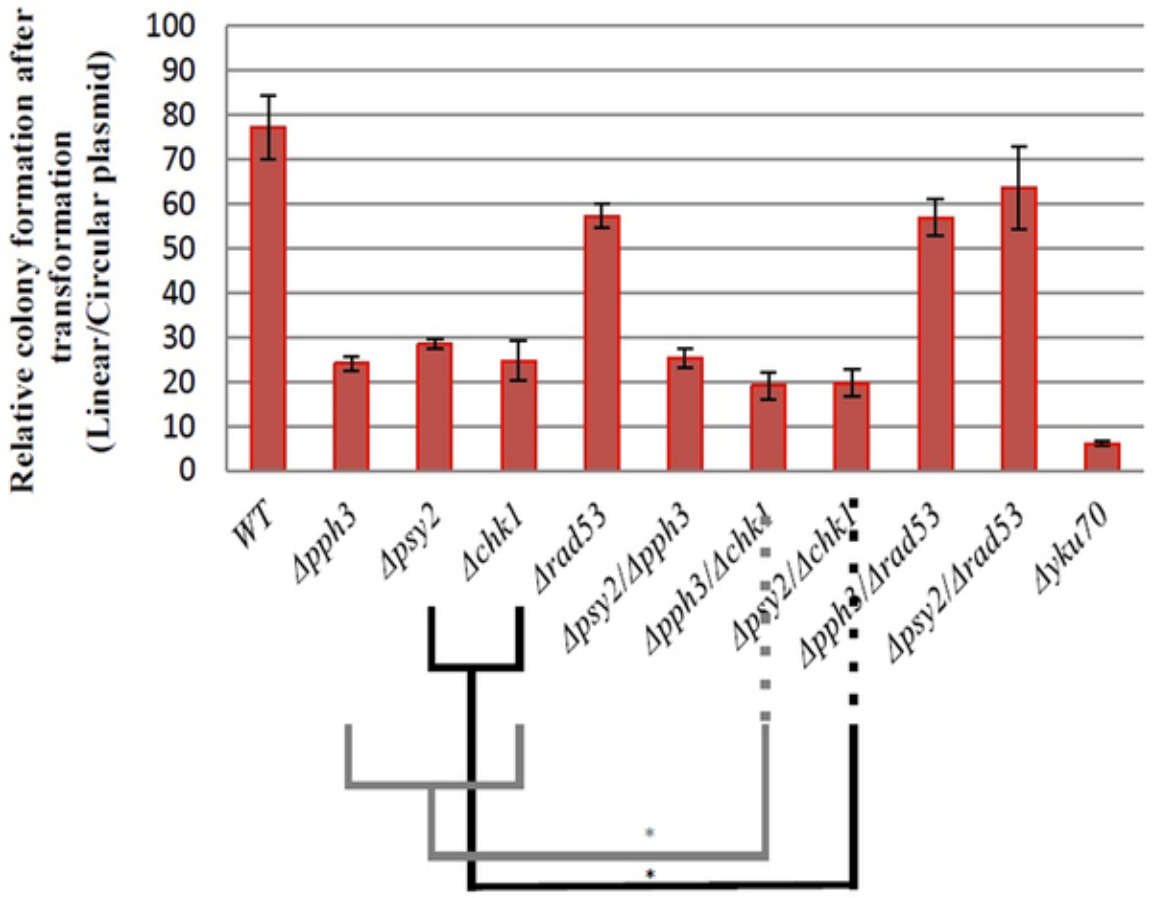


Figure 2.1: Plasmid repair efficiency for different yeast strains. Repair efficiency of breaks with overhangs in *Δpph3* and *Δpsy2* strains have reduced efficiencies in repair. *Δpph3/Δchk1* and *Δpsy2/Δchk1* have reduced efficiencies comparable to those for *Δpph3* and *Δpsy2*. The rate of plasmid repair in WT for breaks with cohesive ends is approximately 78%. Error bars represent standard deviation. Each experiment was repeated at least five times. *Indicates P value of <0.05. Δyku70 was used as a positive control.

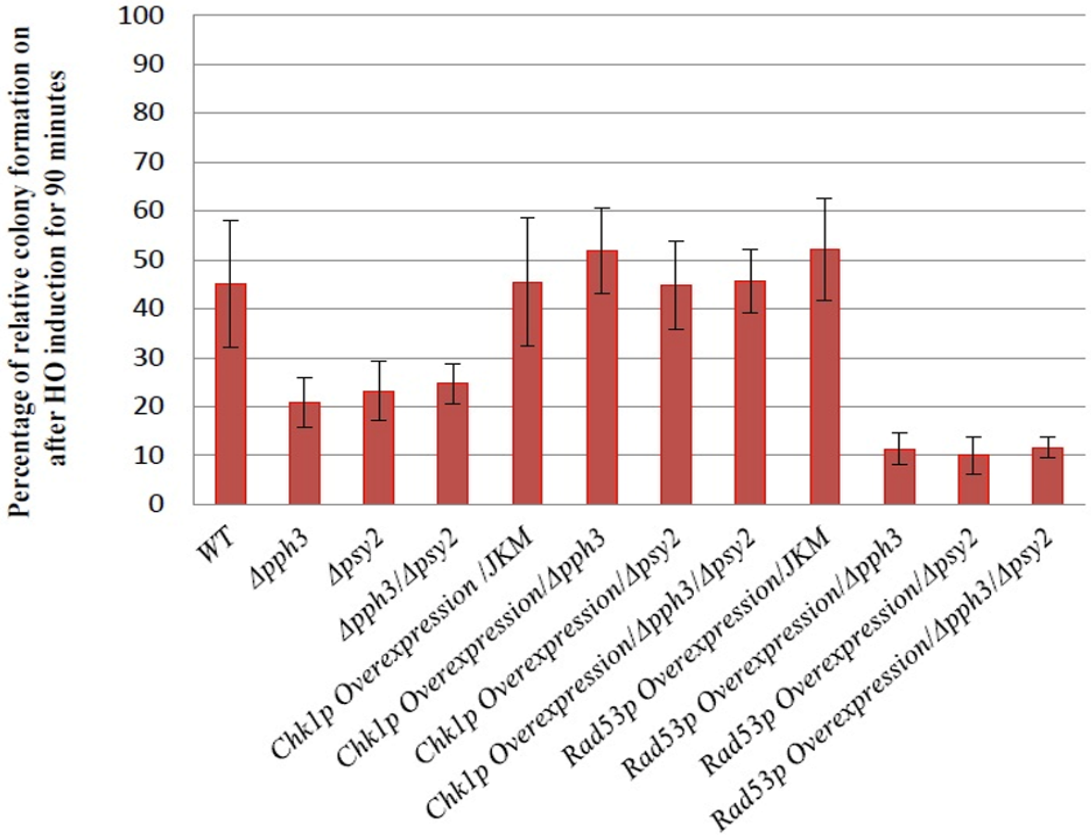
2.4.2 The effect of Pph3p and Psy2p on efficient NHEJ is relevant in a chromosomal context

We subsequently sought to verify the involvements of Pph3p and Psy2p in efficient NHEJ in a chromosomal context, using a JKM139 strain-based chromosomal break assay (Moore and Haber, 1996). In this assay, the target genes are knocked-out in a JKM139 strain background and the viability of target gene deletion mutants are evaluated after exposure to galactose. JKM139 strain carries a GAL promoter in front of an endonuclease specific to the HO site. The presence of galactose induces the production of this endonuclease and consequently results in chromosomal breakage at the HO sites. Wild-type, *Δpph3,* and *Δpsy2* cells (JKM139 background) were exposed to DSB inducing conditions for 90 minutes and allowed to form colonies (Figure 2.2A). Fractions of colonies formed before and after exposure to galactose were used as a measure of survival and were related to the ability of the cell to repair induced DSBs (Figure 2.2A). As expected, *Δpph3* and *Δpsy2* strains had a reduced ability to survive when DSBs were induced compared to wild-type, further supporting the involvement of Pph3p and Psy2p in the efficiency of NHEJ.

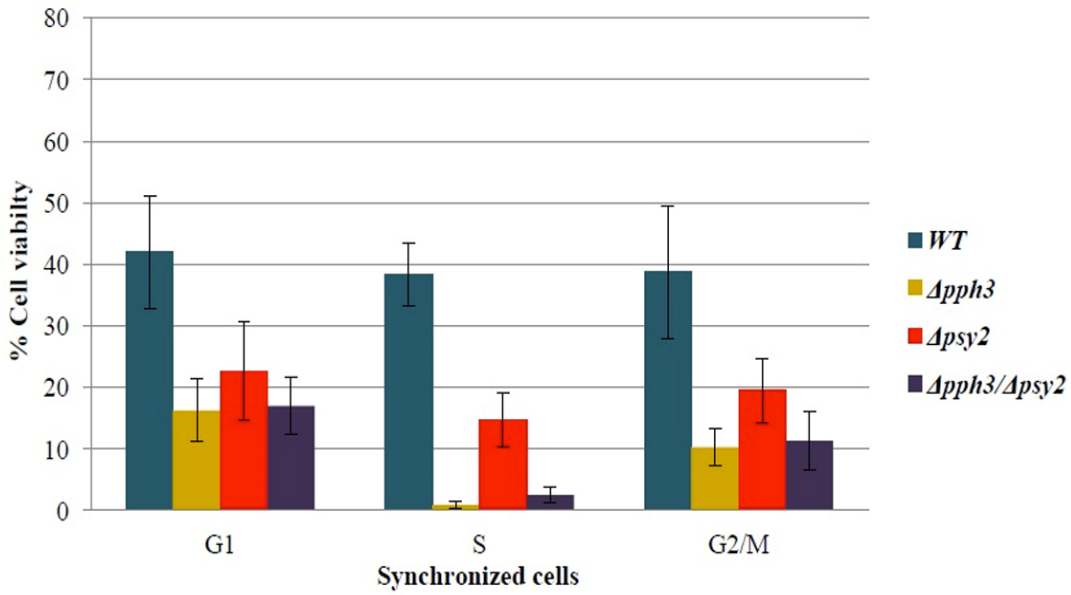
Cell cycle dependency for *Δpph3* and *Δpsy2* strains was investigated by synchronizing the cells in G1, S, and G2/M phases by treating the cells with alpha-factor, hydroxyurea, and nocodazole, respectively, before HO endonuclease induction. Alpha-factor causes cell cycle arrest and inhibits mating. HU inhibits DNA synthesis by limits the dNTPs at the replication forks (Rittberg and Wright 1989), and nocodazole inhibits the microtubule polymerization (Blajeski et al. 2002). It was observed (Figure 2.2B) that *Δpph3* and *Δpsy2* strains had their lowest NHEJ efficiencies in S phase (1% and 14%,

respectively). The significant reduction in the efficiency of NHEJ for *Δpph3* strain appears to separate the activity of *PPH3* from *PSY2* during S phase. A possible explanation is that in S phase, in addition to its *PSY2*-dependent activity, *PPH3* might also affect NHEJ efficiency through an additional pathway, which is independent of *PSY2*.

A


Percentage of relative colony formation on after HO induction for 90 minutes
100
90
80
70
60
50
40
30
20
10
0
WT
Δpph3
Δpsy2
Δpph3/Δpsy2
Chk1p Overexpression /JKM
Chk1p Overexpression/Δpph3
Chk1p Overexpression/Δpsy2
Chk1p Overexpression/Δpph3/Δpsy2
Rad53p Overexpression/JKM
Rad53p Overexpression/Δpph3
Rad53p Overexpression/Δpsy2
Rad53p Overexpression/Δpph3/Δpsy2

B


80
70
60
50
40
30
20
10
0
% Cell viability
G1
S
G2/M
Synchronized cells
WT
Δpph3
Δpsy2
Δpph3/Δpsy2

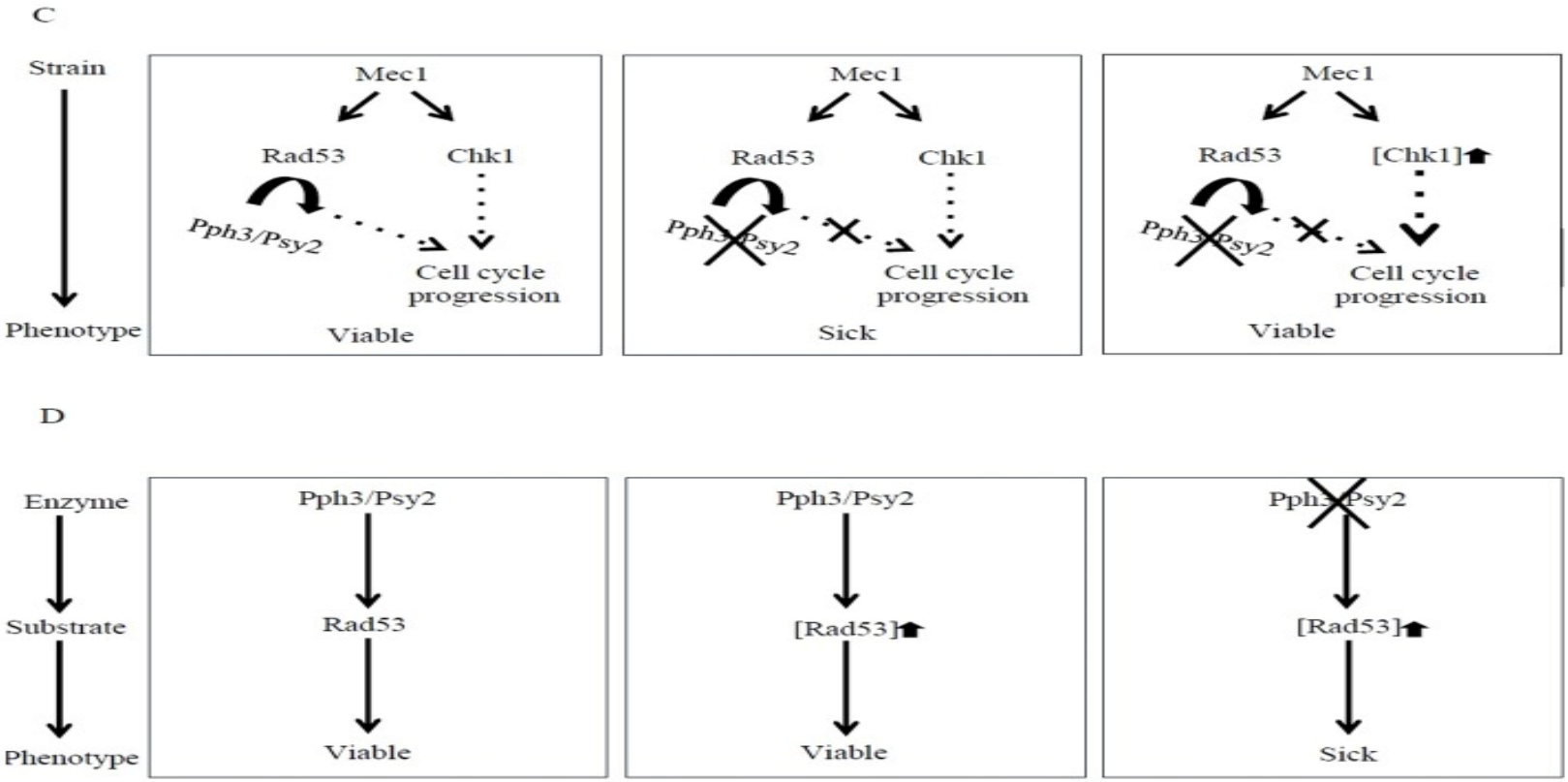


Figure 2.2: Phenotypic analysis of JKM139-based strains. (A) Fraction of the colonies that grew after HO endonuclease induction. Deletion mutant for *PPH3* or *PSY2* had reduced survival and recovered when *CHK1* was overexpressed. Overexpression of *RAD53* reduced survival when *PPH3* or *PSY2* were deleted. (B) Fraction of the colonies that grew after HO endonuclease induction when cells were synchronized in G1, S or G2/M phases. (C) Illustration of conceptual basis for the observed activity of Pph3/Psy2 complex and Chk1p in parallel pathways. (D) Illustration of conceptual basis for the observed activity Pph3/Psy2 complex (enzyme) in relationship to Rad53p (substrate). Overexpression of the substrate in the absence of the enzyme can result in a very sick phenotype. [X]↑ refers to overexpression of gene X.

2.4.3 The Pph3/Psy2 complex functions in association with components of cell cycle

The *PPH3/PSY2* complex is associated with a cell cycle checkpoint through dephosphorylation of the checkpoint protein Rad53p (O'Neill et al. 2007, Kim et al. 2011). Above, we showed that deletion of individual and both members of this complex reduced efficiency in NHEJ as measured by plasmid repair analysis. To determine if these results are in fact associated with checkpoint activity, the activity of other related checkpoint proteins was investigated for their effect on NHEJ using plasmid repair assay. We observed that NHEJ efficiency for deletion of *CHK1* was 25%. Deletion of *RAD53* (Taylor et al. 2005, Chen et al. 2007), which works in parallel with *CHK1*, reduced NHEJ efficiency to 57% (Figure 2.1).

Double mutant strains *Δpph3/Δchk1* and *Δpsy2/Δchk1* showed NHEJ efficiency of 19% and 20%, respectively. NHEJ efficiency for *Δpph3/Δrad53* and *Δpsy2/Δrad53* double mutant strains were 57% and 64%, respectively, which were similar to that for *Δrad53* (57%) single mutant suggesting that the effect of these two genes on NHEJ is likely within the same pathway as Rad53p. In this context, Rad53p appears to be upstream of Pph3p and Psy2p that the activity of these two proteins is dependent on the presence of Rad53p. A possible explanation is that deletion of *RAD53* triggers a second parallel pathway, for example Chk1p-dependent pathway, which works independent of Pph3p and Psy2p. This second parallel pathway is not triggered when *RAD53* is intact.

2.4.4 Overexpression of Chk1p can recover DNA damage sensitivity phenotypes in *Δpph3* and *Δpsy2* mutants in JKM139

We also used the JKM139 strain to detect phenotypic compensation in a chromosomal assay. Overexpression of genes in the DNA damage repair pathways was evaluated for its ability to compensate for a phenotype caused by deletion of *PPH3* and *PSY2*. In this way, genes that have compensating functions can be identified.

It was observed that overexpression of *CHK1* compensated for the absence of either *PPH3* or *PSY2* in a JKM139 assay (Figure 2.2A). Such a recovery provides strong support for a functional association for *CHK1* with *PPH3* and *PSY2.* This is explained by the activity of Chk1p being in a parallel pathway that is compensatory to that of Pph3p and Psy2p, in response to activation of Mec1 (Figure 2.2C). Of interest, overexpression of *RAD53* had a compounding effect on phenotypes of *PPH3* and *PSY2* deletions (Figure 2.2A and 2.2D). Deletion strains for *PPH3* and *PSY2* grew very poorly (sick phenotype) if *RAD53* was overexpressed when DSB was induced. This observation is in accordance with the assumption that a certain equilibrium between "enzyme and substrate" can be important for cell viability (Sopko et al. 2006) (Figure 2.2D). Rad53p (substrate) is known to be dephosphorylated by the Pph3/Psy2 complex (enzyme). In this context, overexpression of the substrate in the absence of the enzyme caused a conditional sick phenotype. Overexpression of *CHK1* or *RAD53* alone did not affect the phenotype of a wild-type JKM139 strain.

2.4.5 Drug sensitivity analysis

It is expected that deletion of genes involved in a DNA repair pathway might change (usually elevate) the sensitivity of their deletion strains to DNA damage-inducing drugs. We used drug sensitivity to bleomycin and hydroxyurea (HU), to further study the activity of Pph3p and Psy2p. Bleomycin causes DSBs via a free-radical mechanism, and HU generates DNA replication errors that can lead to DSB (Bradley and Kohn 1979, Rittberg and Wright 1989). The *Δrad53* strain showed sensitivity to HU (Appendix B.1) and *Δpph3, Δpsy2, Δchk1* and *Δrad53* strains all showed increased sensitivity to bleomycin (Figure 2.3), confirming previous observations (Keogh et al. 2006, O'Neill et al. 2007, Kim et al. 2011). Double mutant strains *Δpph3Δchk1* and *Δpsy2Δchk1* had elevated sensitivity in comparison with single mutants. This is in agreement with the activity of Chk1p in a parallel pathway to Pph3p and Psy2p. Double deletion mutants *Δpph3Δrad5*3 and *Δpsy2Δrad53* showed similar sensitivity to bleomycin as *Δrad53*. This can be explained by the activity of Pph3p and Psy2p, which is dependent on the presence of Rad53p, as above.

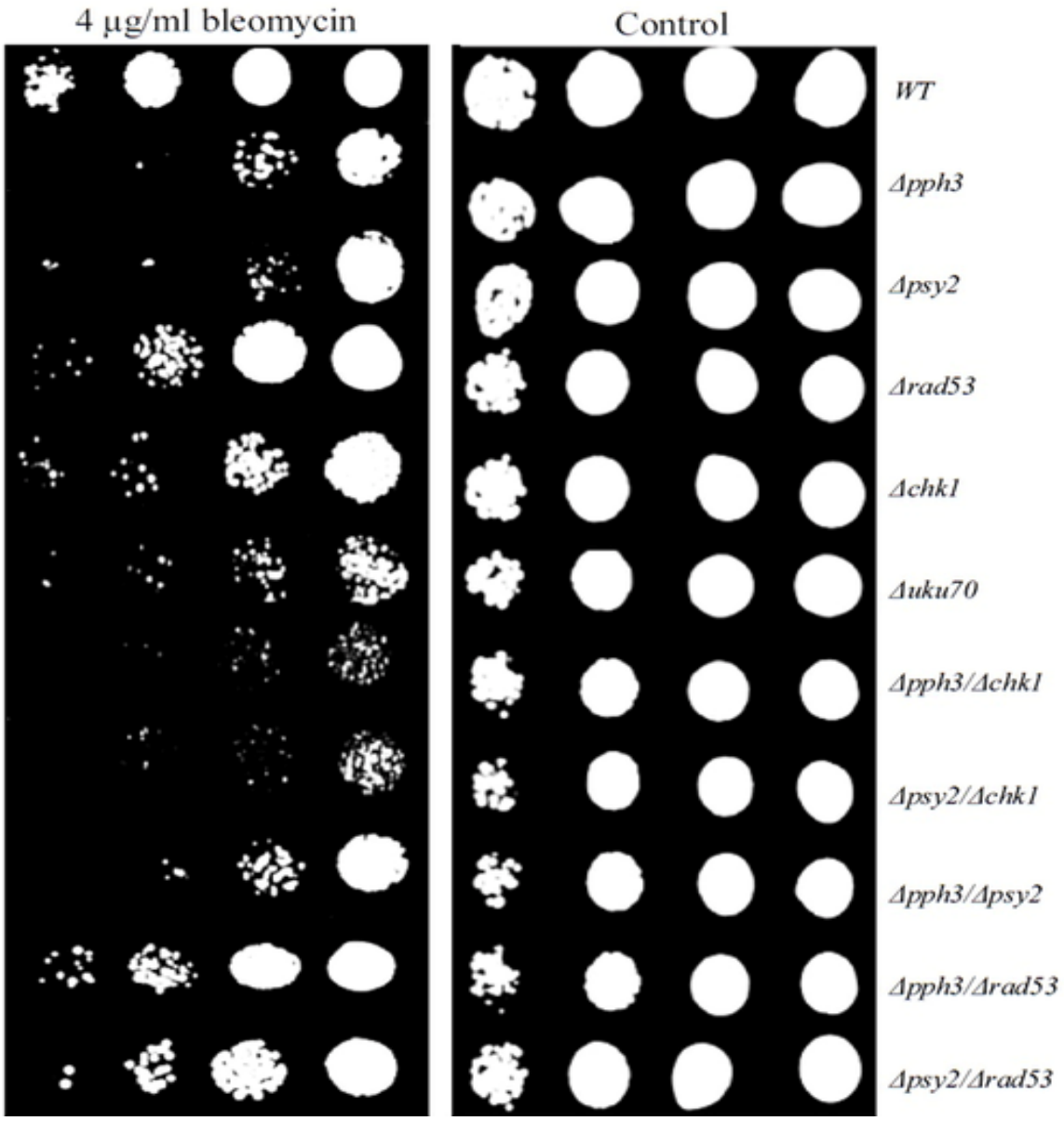


Figure 2.3: Strain sensitivity analysis to bleomycin. Single deletion mutants for *PPH3* or *PSY2* showed increased sensitivity to DSB inducing agent bleomycin. Double deletion mutant strains *Δpph3Δchk1* and *Δpsy2Δchk1* had elevated sensitivity in comparison to single deletion mutants *Δpph3* and *Δpsy2*. In contrast double deletion mutant strains *Δpph3Δrad53* and *Δpsy2Δrad53* had reduced sensitivity in comparison to single deletion mutants *Δpsy2* and *Δpph3*.

2.4.6 Genetic interactions analysis for *PPH3* and *PSY2*

A genetic interaction refers to phenotypes of overexpression/deletion of two genes together that are not easily explained by the investigation of two single genes alone (Dixon et al. 2007). It reveals a higher order pathway association between genes and their functions. Since functionally related genes often genetically interact with one another (Boon eta l. 2007), one way that the function(s) of a gene is studied is through the genetic interactions that it makes with other genes with known functions. In this context, genetic interactions are divided into two groups of negative and positive interactions. A more extreme phenotype for a double mutant than expected infers a negative or aggravating interaction, whereas in positive or alleviating interaction the phenotype of the double mutant is less severe. A negative genetic interaction is often observed when two genes interact through parallel pathways (Baryshnikova et al. 2010). To further study the activity of *PPH3* and *PSY2* we examined their negative genetic interactions under standard laboratory growth condition and in the presence of sub-inhibitory concentrations of DNA damaging agents bleomycin and HU. In this way, conditional genetic interactions were investigated when DNA damage was induced. We used the method of synthetic genetic array (SGA) analysis (Tong et al. 2001) to examine sick phenotypes (negative interactions) for two mini-arrays, one for DNA damage (DD) that is a collection of 384 deletion strains for genes associated with DNA damage response, DNA replication, cell cycle progression and other interesting genes whose products are localized to nucleus, and a second array that contains 384 random deletion strains, used as a control. Using a DD array, 25 and 12 synthetic sick interactions were observed for *PPH3* and *PSY2* respectively (Figure 2.4) in comparison to 4 and 3 in a random array.

Illustrated in Figure 2.4 on the basis of their cellular process, the interacting genes can be grouped into two categories of cell cycle progression or DNA repair (or both) connecting the activity of *PPH3* and *PSY2* to both cell cycle progression and DNA repair with P values of 2.65×10^{-11} and 2.95×10^{-27} for *PPH3* and 8×10^{-13} and 5.98×10^{-10} for *PSY2*, respectively, with the assumption that random array represents the global distribution of negative interactions. This is in agreement with the enrichment of negative interactions previously reported for *PPH3*, 6.78×10^{-9} and 5.73×10^{-6}, and *PSY2*, 5.73×10^{-6} and 2.3×10^{-4}, for response to DD and cell cycle progression, respectively (Costanzo et al. 2010, Collins et al. 2007). Differences in the genetic interaction profiles for *PPH3* and *PSY2* may underscore their additional functions within the cell that are independent of each other. For example, unlike *PSY2*, *PPH3* does not form negative genetic interactions with HR genes, suggesting that a previously reported role for *PPH3* in HR (Kim et al. 2011) is independent of *PSY2*.

Presence of sub-inhibitory concentrations of bleomycin (3 μg/ml; MIC=7.5 μg/ml) or HU (45 mM; MIC=150 mM) generated a number of previously unreported conditional negative interactions (Figure 2.4). As expected, majority of these new interactions are linked to the DD response. For example, *MAG1* encodes a 3-methyl-adenine DNA glycosylase that initiates base excision repair, *PMS1* encodes a mismatch repair protein, and *XRS2* is a DSB repair protein, among others. Of interest, *CHK1* formed a conditional negative genetic interaction with both *PPH3* and *PSY2* in the presence of bleomycin. In the presence of HU, *CHK1* also interacted with both *PPH3* and *PSY2*. These conditional interactions are in agreement with a DD dependent functional association for Pph3p and Psy2p with Chk1p. The slightly different between degree of

sickness using bleomycin vs. HU was observed, which could be related to the different mode of actions of these drugs. Bleomycin chelates metal ions that produce free radicals which results in DSBs (Cannan and Pederson 2016, Della Latta et al. 2015, Peral et al. 2015), and HU induces DNA replication errors by nucleotide depletion, which may lead to DSBs (Rittberg et al 1989, Koç A et al. 2004). It should be noted that due to the large-scale nature of the GI screening some of these genes might be false positives.

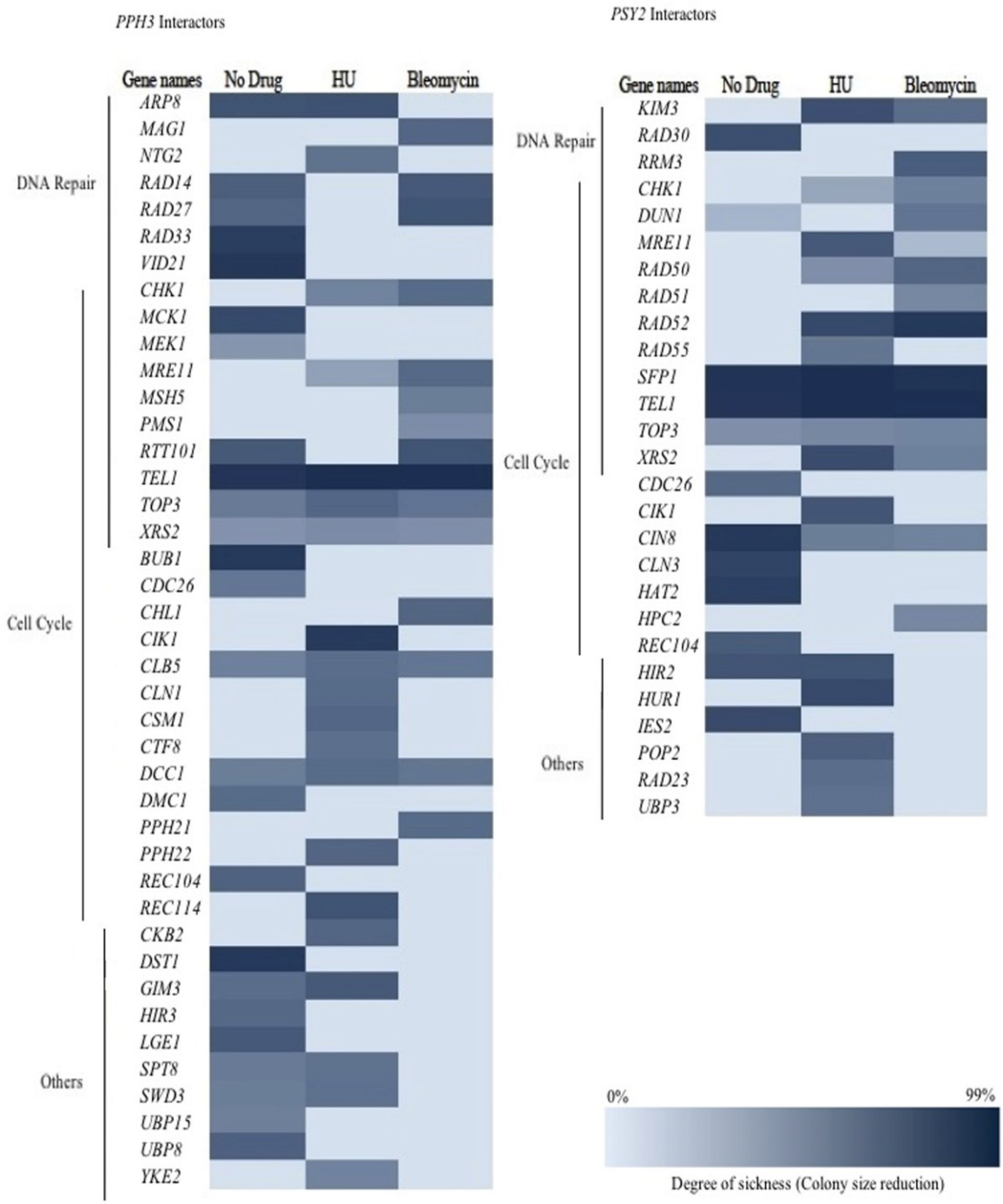


Figure 2.4: Analysis of the synthetic sick interactions for *PPH3* (A) and *PSY2* (B). Most of the interactors are involved in cell cycle progression and DNA repair with P values of 2.65×10^{-11} and 2.95×10^{-27} for *PPH3* and 8×10^{-13} and 5.98×10^{-10} for *PSY2*, respectively. Conditional interactions were identified in the presence of sub-inhibitory concentrations of HU (45 mM) or bleomycin (3 µg/ml).

Overexpression of certain genes may have no phenotypic consequence for a wild-type strain; however, when a second gene is deleted, the same overexpression may result in an unexpected phenotype such as sickness or lethality. This type of interaction is termed Synthetic Dosage Lethality (SDL) (Kroll et al. 1996, Magtanong et al. 2011) and is often used to study the relationship between regulator and substrate where the overexpression of the substrate in the absence of the regulator often causes a severe phenotype (Douglas et al. 2012, Sharifpoor et al. 2012). To study potential regulators for the Pph3/Psy2 complex, we examined the overexpression phenotypes for *PPH3* and *PSY2* on DD array in the presence and absence of sub-inhibitory concentration of DNA damage drugs bleomycin and HU as above. In the absence of DNA damage, overexpression of *PPH3* or *PSY2* did not form any SDL interactions. However, when DNA damage was induced, overexpression of either *PPH3* or *PSY2* formed SDL interactions with gene deletion strains for each of the three members of MRX complex *MRE11*, *RAD50* and *XRS2* (P value of 5.43×10^{-16}) (Figure 2.5). These data are in agreement with DNA damage dependent regulation of Pph3/Psy2 by MRX complex. MRX is an evolutionarily conserved complex that recognizes and binds DSBs and regulates the activity of the major DSB response kinases Mec1p and Tel1p. Since the Pph3/Psy2 complex dephosphorylates activated Rad53, regulation of Pph3p and Psy2p by the MRX complex may explain previous finding that the activity of Mre11p is linked to accumulation of phosphorylated Rad53p (Clerici et al. 2006, Limbo et al. 2011). In light of our finding here, a plausible model is that MRX complex might promote the activity of Pph3p and Psy2p to dephosphorylate activated Rad53p and hence regulate cell cycle progression during DD. This model merits further investigation. Of interest, *CHK1*

was also found as a conditional interacting partner when either *PPH3* or *PSY2* was overexpressed. These SDL interactions further connect the activity of Chk1p with Pph3p and Psy2p during DNA damage and suggest that the Pph3/Psy2 complex might also be under the conditional regulation of Chk1p kinase. Other genes that formed conditional SDL interactions with *PPH3* and *PSY2* are *SAW1* and *NEJ1*. Saw1p facilitates the binding of the Rad1/Rad10 complex to the site of DNA damage during HR (Li et al. 2013) and Nej1p is a regulator of NHEJ (Valencia et al. 2001).

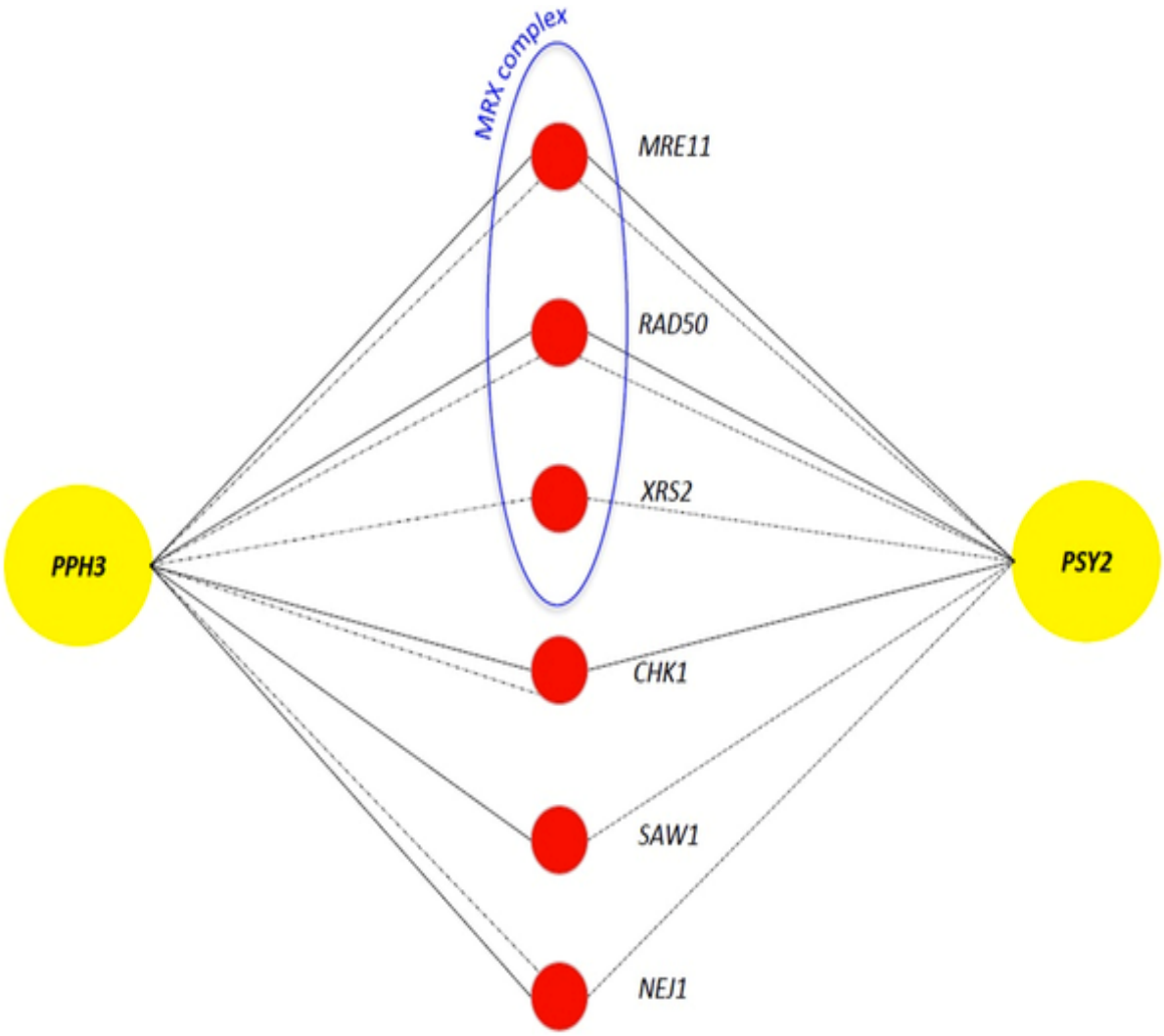


Figure 2.5: Synthetic dosage lethality (SDL) analysis. Overexpression of *PPH3* and *PSY2* formed conditional SDL interactions with members of MRX complex, in addition to *CHK1*, *SAW1* and *NEJ1*. Solid and dashed lines represent interactions found in the presence of bleomycin (3 µg/ml) and HU (45 mM), respectively.

2.4.7 Protein-protein interaction prediction

Proteins often realize their function through interactions with one another. The overall profiles of such interactions can reveal information about the function as well as the cellular process in which proteins participate. Some PPIs are mediated by a finite number of short interaction motifs (Neduva and Russell 2006, Jessulat et al. 2011). Such interactions can be studied by examining the co-occurrence of small polypeptide regions that are significantly enriched in the dataset of high confidence interacting proteins (Piter et al. 2008, 2012). One advantage of this method is that the polypeptide regions that are responsible for a physical interaction between proteins can be identified (Amos-Binks et al. 2011). Here, we examined the possible proteome-wide interactions that Pph3p and Psy2p make on the basis of short interacting motifs. Their predicted interaction partners, along with their proposed site of interactions, are represented in Table 2.1. A potential interaction between amino acids 16–231 for Pph3p and 317–337 for Psy2p was identified. Previously, Pph3p was reported to interact with Psy2p (O'Neill et al. 2007); however, the region responsible for this physical association remained unclear. Similarly, interactions between Pph3p (amino acids 298–336) and Rad53p (amino acids 266–268), as well as Psy2p (amino acids 587–608) and Rad53p (amino acids 298–336) were proposed. A number of these interactions appear to be competing for the same binding site on Pph3p and Psy2p. Such competing interactions may function as regulators of activity. For example, Rrd1p, a cell cycle regulator that activates PP2A phosphatase, competes for the same region of Pph3p (amino acids 213–286) as Rad53p. Rrd1p abundance is reported to increase in response to DD (Tkach et al. 2012) and can potentially outcompete the interaction of Pph3p with Rad53p, preventing the

dephosphorylation of Rad53p by Pph3p in response to DNA damage. Further investigations are needed to examine the validity of this model of regulation for Pph3/Psy2 activity. The interacting partners of Pph3p and Psy2p can be grouped into two general categories of DNA damage response and cell cycle progression. These PPI profiles are in agreement with the activity of Pph3/Psy2 in regulating the cell cycle in response to DNA damage.

Table 2.1: Protein-Protein interaction prediction for Pph3p and Psy2p. Of the 24 proteins that interact with Pph3p, 6 have a role in DNA repair, 8 in both DNA repair and regulation of cell cycle, and 10 in "other" cellular processes.

Gene Names	Site of Interaction	Description
Pph3 Interacting Partners		
*HTA1**	213–286	Core histone protein; DNA damage-dependent phosphorylation by Mec1p facilitates DNA repair
*HTA2**	213–286	Core histone protein; DNA damage-dependent phosphorylation by Mec1p facilitates DNA repair
*SPT4**	201–248	Regulation of transcription elongation; transcription-coupled DNA repair
*SPT5**	56–136	Component of the universally conserved Spt4/5 complex; has a role in transcription-coupled DNA repair
*TIP41**	28–74	Regulator of PP2A pathway; protein abundance increases in response to DNA replication stress
TDH2	56–136	Glyceraldehyde-3-phosphate dehydrogenase; protein abundance increases in response to DNA replication stress
*HTB1**	56–253	Core histone protein required for chromatin assembly; regulates meiotic DSB formation
*HTB2**	56–253	Core histone protein required for chromatin assembly; regulates meiotic DSB formation
PSY2	16–231	Subunit of protein phosphatase PP4 complex; regulates recovery from the DNA damage checkpoint
*PSY4**	213–248	Regulatory subunit of protein phosphatase PP4; recovery from the DNA damage checkpoint
*RAD53**	266–286	Protein kinase required for cell-cycle arrest in response to DNA damage
*RRD1**	213–286	Peptidyl-prolyl cis/trans-isomerase involved in G1 phase progression, and DNA repair
SRS2	60–136	DNA helicase and DNA-dependent ATPase involved in DNA repair and checkpoint recovery
TDH1	56–136	Glyceraldehyde-3-phosphate dehydrogenase; protein abundance increases in response to DNA replication stress
CCT2	56–156	Subunit beta of the cytosolic chaperonin Cct ring complex, related to Tcp1p/required for the assembly of actin and tubulins in vivo
CCT3	56–253	Subunit of the cytosolic chaperonin Cct ring complex, related to Tcp1p/required for the assembly of actin and tubulins in vivo
DIA4	31–73	Probable mitochondrial seryl-tRNA synthetase
PRO1	26–46	Gamma-glutamyl kinase; catalyzes the first step in proline biosynthesis
*SSD1**	215–242	Translational repressor with a role in polar growth and cell wall integrity
STE12	56–136	Transcription factor that is activated by a MAP kinase signaling cascade
TAP42	56–138	Essential protein involved in the TOR signaling pathway
TCP1	56–253	Alpha subunit of chaperonin-containing T-complex, which mediates protein folding in the cytosol
TDH3	77–97	Glyceraldehyde-3-phosphate dehydrogenase involved in glycolysis and gluconeogenesis
YHR033W	213–243	Protein of unknown function

Psy2p Interacting Partners		
*HTA1**	121–152	Core histone protein; DNA damage-dependent phosphorylation by Mec1p facilitates DNA repair
*HTA2**	121–152	Core histone protein; DNA damage-dependent phosphorylation by Mec1p facilitates DNA repair
KSP1	587–608	Serine/threonine protein kinase; protein abundance increases in response to DNA replication stress
MCK1	587–608	Dual-specificity serine/threonine and tyrosine protein kinase; Involved in control of chromosome segregation and in regulating entry into meiosis
*SPT4**	405–430	Regulation of transcription elongation; transcription-coupled DNA repair
*SPT5**	590–612	Component of the universally conserved Spt4/5 complex; has a role in transcription-coupled DNA repair
*TIP41**	543–565	Regulator of PP2A pathway; protein abundance increases in response to DNA replication stress
WSS1	145–179	Protein of unknown function; has a suggested role in the DNA damage response
*HTB1**	405–428	Core histone protein required for chromatin assembly; regulates meiotic DSB formation
*HTB2**	405–428	Core histone protein required for chromatin assembly; regulates meiotic DSB formation
PHO85	587–608	Cyclin-dependent kinase; involved in regulating the cellular response to nutrient levels and environmental conditions and progression through the cell cycle
PPH3	317–337	Catalytic subunit of protein phosphatase PP4 complex; regulates recovery from the DNA damage checkpoint
*PSY4**	815–836	Regulatory subunit of protein phosphatase PP4; recovery from the DNA damage checkpoint
*RAD53**	587–608	Protein kinase required for cell-cycle arrest in response to DNA damage
*RRD1**	422–442	Peptidyl-prolyl cis/trans-isomerase involved in G1 phase progression, and DNA repair
AAD6	590–614	Putative aryl-alcohol dehydrogenase involved in oxidative stress response
ARG82	590–612	Inositol polyphosphate multikinase; diphosphoinositol polyphosphate synthase activity
BEM2	143–170	Rho GTPase activating protein (RhoGAP) involved in the control of cytoskeleton organization and cellular morphogenesis
GDS1	568–588	Protein of unknown function
HEF3	154–177	Translational elongation factor EF-3; stimulates EF-1 alpha-dependent binding of aminoacyl-tRNA by the ribosome
PGA2	143–179	Essential protein required for maturation of Gas1p and Pho8p; involved in protein trafficking
PGC1	467–487	Phosphatidylglycerolphosphate synthase; catalyzes the synthesis of phosphatidylglycerolphosphate from CDP-diacylglycerol and sn-glycerol 3-phosphate
RPC25	12–36	RNA polymerase III subunit C25; required for transcription initiation
*SSD1**	155–179	Translational repressor with a role in polar growth and cell wall integrity
STD1	154–182	Protein involved in control of glucose-regulated gene expression
YEF3	154–177	Gamma subunit of translational elongation factor eEF1B; stimulates the binding of aminoacyl-tRNA (AA-tRNA) to ribosomes

2.5 Concluding Remarks

In this study, we show that interacting proteins Pph3p and Psy2p affect the efficiency of NHEJ in the unicellular budding yeast *S. cerevisiae*. Deletion of either *PPH3* or *PSY2* genes reduced NHEJ efficiency both in the context of chromosomal and plasmid DSB repair. Pph3p and Psy2p form a phosphatase complex, which dephosphorylates Rad53 checkpoint kinase (O'Neill et al. 2007). Our analyses using a plasmid repair assay suggested a functional connection between the activity of Pph3p and Psy2p on NHEJ through checkpoint protein Rad53. Similarly, phenotypic suppression analysis revealed that overexpression of Chk1p, another checkpoint kinase that works in parallel to Rad53p, compensated for the absence of either *PPH3* or/and *PSY2* genes in a chromosomal based repair assay. Double deletion mutant strains for either *PPH3* or *PSY2* with CHK1 showed additional reduction in the efficiency of plasmid repair through NHEJ than single mutant. Our genetic interaction analyses revealed synthetic sick phenotypes for both *PPH3* and *PSY2* with DNA damage response genes that function in regulation and upstream to DNA damage repair pathway, in addition to genes involved in cell cycle progression. This observation is in clear agreement with the activity of Pph3/Psy2 in cell cycle progression and further indicates that their effect on NHEJ is not at the mechanistic but rather at the regulatory level. This is consistent with previously reported activity of Pph3p in the HR pathway (Kim et al. 2011). In support of a role for Pph3/Psy2 in regulation of DNA damage response via cell cycle, the PPI analysis

reported here suggested that both Pph3p and Psy2p interact with both DNA damage response and cell cycle progression proteins.

Dephosphorylation of Rad53p by Pph3/Psy2 releases cell cycle arrest. Pph3p also dephosphorylates γH2AX, which regulates DD checkpoint protein activity. This regulation is through chromatin modification (Keogh et al. 2006). A recent study by Kim et al. reported a role for Pph3p in DSB repair through HR (Kim et al. 2011). Here, we show that Pph3p and Psy2p also affect the efficiency of NHEJ. We also present genetic evidence for conditional cross-talk and functional associations between Pph3p and Psy2p with checkpoint kinases Rad53p and Chk1p. These associations can be triggered by bleomycin, HU and HO endonuclease.

3 Chapter: Uncharacterized ORF *HUR1* influences the efficiency of non-homologous end-joining repair in *Saccharomyces cerevisiae*

Omidi K, Jessulat M, Hooshyar M, Burnside D, Kazmirchuk T, Schoenrock A, Haji karimlou M, Daniel M, Babu M, Ramotar D, Samanfar B, Dehne F, Golshani A.

Contribution: I came up with the hypothesis, experimental design and development of the project. I analyzed the data and wrote the manuscript. Experiments in yeast cells were carried out by me. Dr. Matthew Jessulat, Mary Daniel and Maryam Hajikarimlou contributed to plasmid repair experiments. Computational work was done by Dr. Andrew Schoenrock. Dr. Mohsen Hooshyar and Daniel Burnside, contributed to drug sensitivity analysis.

3.1 Abstract

Non-Homologous End Joining (NHEJ) is a highly conserved pathway that repairs Double-Strand Breaks (DSBs) within DNA. Here we show that the deletion of yeast uncharacterized ORF *HUR1,* Hydroxyurea Resistance1 affects the efficiency of NHEJ. Our findings are supported by Protein-Protein Interaction (PPI), genetic interaction and drug sensitivity analyses. To assess the activity of *HUR1* in DSB repair, we deleted its non-overlapping region with *PMR1*, referred to as *HUR1-A.* We observed that similar to deletion of *TPK1* and *NEJ1*, and unlike *YKU70* (important for NHEJ of DNA with overhang and not blunt end), deletion of *HUR1-A* reduced the efficiency of NHEJ in both overhang and blunt end plasmid repair assays. Similarly, a chromosomal repair assay showed a reduction for repair efficiency when *HUR1-A* was deleted. In agreement with a functional connection for Hur1p with Tpk1p and NEJ1p, double mutant strains *Δhur1-A/*

Δtpk1, and *Δhur1-A/Δnej1* showed the same reduction in the efficiency of plasmid repair, compared to both single deletion strains.

3.2 Introduction

DNA Double-Strand Breaks (DSBs) are the most severe form of DNA damage. In the event of DSBs, there are two independent repair pathways: Homologous Recombination (HR), and Non-Homologous End Joining (NHEJ). When the break occurs within a sequence that has a homologous region elsewhere in the genome, the cell may repair the damage through HR. HR uses a break region's homology as a template to coordinate efficient repair (reviewed in Dudáš and Chovanec 2004). HR is the primary DSB repair system in baker's yeast *Saccharomyces cerevisiae*, and is considered to be less error-prone than its alternative.

A more versatile alternative to HR is classical Non-Homologous End joining (c-NHEJ) in *S. cerevisiae* (Dudášová et al. 2004, Daley et al. 2005). NHEJ is used to repair any DSB regardless of sequence homolog availability. Initial recognition of the DNA lesion in NHEJ is through the Yku70p-Yku80p heterodimer (YKU complex), which binds to DNA ends (Milne et al. 1996, Siede et al. 1996). Recognition of the DSB and recruitment of the YKU complex to the site of damage involves a cascade of DNA damage checkpoints in which Tel1p, Mec1p and Rad53p play central roles (Ataian and Krebs 2006). The proteins Mre11p, Rad50p, Xrs2p form a complex termed MRX, which is then recruited to the broken ends of DNA. The exact contribution of MRX in the NHEJ pathway is not fully understood, because of the involvement of this complex in other cellular pathways (Emerson et al. 2016). The MRX complex strongly stimulates DNA

ligation. Ligase activity in NHEJ is the result of the Dnl4p/Lif1p complex (Chen et al. 2001). Nej1p binds to the Dnl4/Lif1 complex through an interaction with Lif1, but Nej1 is not critical for Dnl4/Lif1 complex formation (Ooi et al. 2001, Emerson et al. 2016). Although details of Nej1p's role is still unclear, recent papers suggest it recruits to the site of the break, interacts with DNA, and may function as a regulatory protein (Brandi et al. 2014). Plasmid repair assays have shown a role of Nej1p in NHEJ (Deshpande et al. 2007, Emerson et al. 2016). Nej1p also affects NHEJ via YKU independent pathways such as micro-homology mediated end joining (MMEJ), which is a subset of alternative NHEJ (alt-NHEJ) (Lee and Lee 2007, McVey and Lee 2008). MMEJ, which requires Nej1, plays a significant role in the repair of DNA breaks with blunt ends (Lee and Lee 2008). The process of alt-NHEJ is not fully clear, but there is agreement that the MRX complex is necessary for that process (Lee and Lee 2007, Emerson et al. 2016).

Recent literature as well as studies in our laboratory have identified a growing number of genes with novel roles in the process of DNA damage repair and NHEJ, such as Rtt109p, Sub1p, Pph3p, Psy2p, Tpk1p etc., which suggests the existence of other uncharacterized proteins in the NHEJ pathway (Shim et al. 2005, Jessulat et al. 2008, Yu et al. 2013, Omidi et al. 2014, Jessulat et al. 2015, Hooshyar et al. 2016). For example, Jessulat et al. proposed a novel function for the Rtt109p-Vps75p involved in the efficiency of NHEJ in yeast *S. cerevisiae* (Jessulat et al. 2008). *SUB1* was found to be required for NHEJ repair of DSBs in plasmid DNA, but not in chromosomal DNA. It also may play a role in the fidelity of NHEJ (Yu et al. 2013). Deletion of *PPH3* and *PSY2* was shown to reduce the efficiency of NHEJ in yeast through cell cycle regulation (Omidi et al. 2014).

Here, we are interested in studying the uncharacterized ORF *HUR1*. *HUR1*, Hydroxyurea Resistance1, is a functionally uncharacterized ORF that is reported in the Saccharomyces Genome Database (http://www.yeastgenome.org) as a protein of unknown function with no known biological activity. Its expression is reported to be unregulated under different DNA damage conditions (Fry et al. 2003, Takagi et al. 2005, Borde et al. 2009). *HUR1* (YGL168W) is located in the nucleus on chromosome VII. The protein domain of 1-31 is predicted signal peptide and domain 34-110 is region of a membrane-bound protein predicted to be outside the membrane, in the cytoplasm.

Its locus partially overlaps with *PMR1*, a calcium import gene associated with Golgi. In this report, we delete the first 70 amino acids of *HUR1* (*HUR1-A* ORF*)* without affecting its overlap with *PMR1* and report that *HUR1* influences the efficiency of NHEJ in yeast.

3.3 Materials and Methods

3.3.1 Yeast strains and plasmid

The yeast strain *S. cerevisiae* deletion library in BY4741 background (MATa orfΔ::KanMX4 *his3Δ leu2Δ met15Δ ura3Δ*) (Winzler et al. 1999) was utilized unless stated otherwise. Since *HUR1* partially overlaps with *PMR1*, the first 70 amino acids of *HUR1* were deleted (*Hur1-A)* without affecting its overlap with PMR1 in BY4741 and Y7092 (MATa can1Δ::STE2pr-HIS3 lyp11Δ ura31Δ leu21Δ his31Δ met151Δ) background strains by PCR transformation containing NAT selection gene (Tong and Boone 2007).

The yeast gene deletion strains and plasmids were used to perform the experiments as in (Omidi et al. 2014). Plasmid p416 that carries a URA3 marker and Ampr gene (Jessulat et al. 2008), and Ycplac111 with Leu2 marker, and Ampr, were used for plasmid repair assays for overhang and blunt end repairs, respectively.

3.3.2 Protein-protein interaction prediction

Prediction of Protein-Protein Interactions (PPIs) were based on the co-occurring polypeptide regions (Pitre et al. 2008). An updated list of high confidence PPIs was used from published data (BioGRID: www.thebiogrid.org and DIP: www.dip.doe mbi. ucla.edu). The sensitivity to measure false positive prediction was set at 99.55%. The regions that mediate PPIs were predicted using PIPE-site algorithm (Amos-Binks et al. 2011).

3.3.3 Genetic interaction analysis

The Genetic Interaction (GI) analyses with the DNA damage array (DDA) was performed using the principles of synthetic genetic array (SGA) analysis (Tong et al. 2001) as in (Alamgir et al 2008). Conditional GI analyses were performed in the presence of mild DNA damage conditions induced by low concentrations of bleomycin and HU. Fitness was scored by colony size measurement as in (Memarian et al. 2007, Jessulat et al. 2008, Samanfar et al. 2014). Phenotypic rescue analysis was performed using overexpression plasmids were transformed into the above deletion arrays as in (Alamgir et al. 2010). Each experiment was repeated three times. Double mutant strains with

relative reduced fitness of 20% or more in at least two experiments were considered positive hits and were further confirmed using spot test analysis.

3.3.4 Drug sensitivity spot test

Yeast strains deletions and wild-type were grown in YPD at 30^{o}C to saturation then 15 µl of each spotted on the YPD media containing 60 mM hydroxyurea (HU) or 4 µg/ml bleomycin and drug free media. Series of cell dilutions 10^{-2} -10^{-5} were used as explained in (Jessulat et al. 2008, Hooshyar et al. 2016).

3.3.5 Plasmid repair assay

p416 and Ycplac111 were digested at their unique XbaI and SmaI restriction sites within regions with no homology to yeast chromosomes, respectively. The assay was performed in this study as in (Jessulat et al. 2008, Omidi et al. 2014). Each experiment was repeated at least five times. At least 250 colonies were counted for strains with low NHEJ efficiency. To calculate the p-values two-tailed distribution t-test was used.

3.3.6 Chromosomal repair assay

A JKM139-based chromosomal DSB repair was done by knocking-out target genes in JKM139 strain (Moore and Haber 1996, Omidi et al. 2014). This strain carries a GAL promoter in front of an endonuclease specific to HO site. Different serial dilutions between 10^{-2} -10^{-5} for mutant and wild type strains grown to OD 1, were plated on media containing galactose or glucose (as a control). Colony growth differences were used as a measure of survival and related to the ability of the cell to repair induced DSBs. For

phenotype compensation experiments, gene overexpression in an individual mutant background was used as in (Jessulat et al. 2008). Each experiment was repeated five times. To calculate the p-values two-tailed distribution t-test was used.

3.4 Results and Discussion

3.4.1 *HUR1*: a candidate gene involved in DNA damage repair

3.4.1.1 Protein-protein interaction analysis

PPIs display overall profiles and essential aspects of all biological pathways and mechanisms within a cell (Dittrich et al. 2008). As a general rule, proteins with similar functions physically interact with one another within a cell. Consequently, PPIs have been used as a mean to study protein functions (Butland et al. 2007, Wood et al. 2003) and to uncover novel activities for proteins in different organisms (Hu et al. 2009, Krogan et al. 2003, Jin et al. 2007). For example, if protein X is found to physically interact with proteins involved in the process of transcription, it is generally thought that protein X may also play a role in this process. PPIs can be studied using various biochemical and computational approaches with each having inherent advantages and disadvantages. One such computational tool predicts PPIs on the basis of co-occurring short polypeptide regions (Pitre et al. 2008, Pitre et al. 2012). It has one of the highest specificities (low false positives) and sensitivities (low false negatives) among the computational tools and has been used to study novel protein functions for different yeast proteins (Pitre et al. 2012 Schoenrock et al. 2014). To study Hur1p function, we used the principles of co-occurring peptides as a mean of interactions (Jessulat et al. 2011) to study its proteome

wide PPI map (Appendix B.2). In this way, the updated database of high confidence PPIs were searched for pairs of small overlapping windows of 20 amino acids long that co-occur within interacting partners only. To this end, 3 high confidence interactions were found for Hur1p protein with Tpk1p, Tpk2p and Tpk3p proteins (Figure 3.1A). TPK family of proteins constitutes cAMP-dependent protein kinases that promote vegetative growth in response to nutrients. We have recently shown that their deletion reduces the efficiency of NHEJ and that this activity for *TPK1* is connected to *NEJ1* function in a YKU independent manner. In agreement with this data, a physical interaction between *HUR1* and *TPK1* has been previously reported (Ptacek et al. 2005). Besides this one interaction for Hur1 no other PPIs are reported in literature.

3.4.1.2 Genetic interaction analysis

Genetic Interaction (GI) analysis often shows functional redundancy of genes in different pathways and higher order pathway association (Bandyopadhyay et al. 2010, Costanzo et al. 2011). They are determined by comparison of a double mutant's phenotype(s) to individual phenotypes of single mutants, by the deletion of an individual gene with deletion or overexpression of a second gene. A GI is formed if the presented phenotype cannot be explained by observing the phenotype of the single gene deletion or overexpression alone (Boone et al. 2007). Negative interactions or synthetic sickness refer to the double mutant phenotypes, which is more intense (worse) than expected compared to single deletion mutants. Positive or alleviating interaction describes the interactions where a second mutation compensates for the deletion of the first gene, so the phenotype of the double mutant is less severe (Baryshnikova et al. 2010).

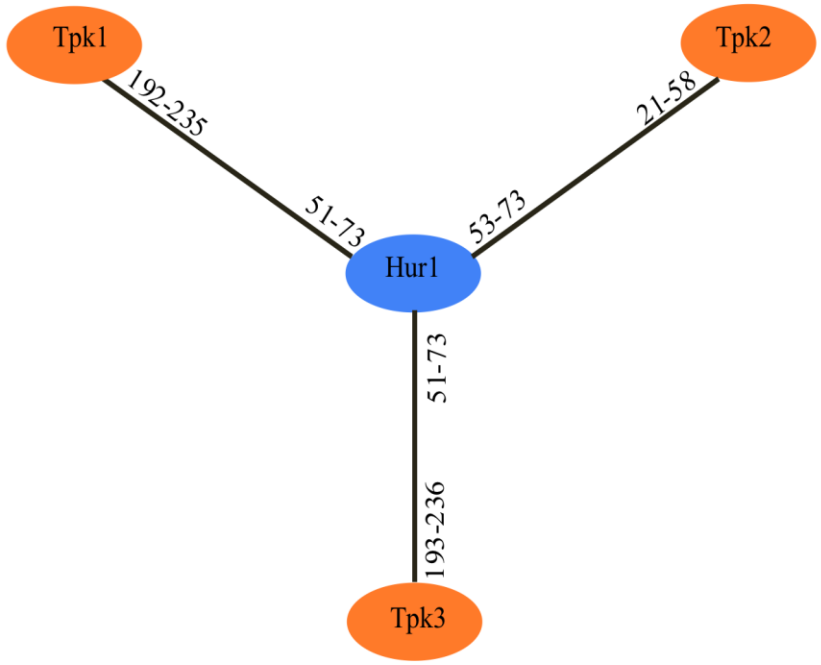
To investigate the activity of Hur1p, the part of *HUR1* (amino acid 1-70) that does not overlap with *PMR1* was deleted, forming a partial deletion for the *HUR1* gene (*HUR1-A*). We used a modified Synthetic Genetic Array (SGA) method to explore GIs and networking for *HUR1-A* (Tong et al 2001, Alamgir et al. 2008). In this way, SGA analysis was performed for *HUR1-A* with two sets of 384 gene deletion strains. One of these arrays contains 384 deletion strains that play a role in DNA damage response, cell cycle progression, checkpoints, DNA replication, and chromatin modifications. The second set contains a collection of 384 random deletion strains, used as a control.

Illustrated in Figure 3.1B, we observed that *HUR1-A* deletion negatively interacts with a number of DNA damage repair and cell cycle progression genes including *RAD52, RAD18, RAD4,* and *BUB1* (Figure 3.1B), *RAD52* is a part of HR pathway; *RAD18* is involved in post-replication repair; *RAD4* protein product binds to damaged DNA during nucleotide excision repair; and *BUB1* codes for a checkpoint protein that affects NHEJ. *HUR1-A* did not form negative interactions with key NHEJ genes. This pattern of negative interactions is very similar to those for well-defined NHEJ genes such as *YKU70*, *YKU80*, *DNL4* and *TPK1* (Koh et al. 2010). Negative GIs are often formed between genes involved in parallel pathways (Boone et al. 2007). When DNA damage was induced in the presence of sub-inhibitory concentrations of HU (45 mM) and bleomycin (3 μg/ml) a few additional negative GIs were formed. For example, *RTT107* is involved in recruitment of DNA repair complex SMC5/6 to DSBs and *RAD16* participates in nucleotide excision repair pathway.

Another type of genetic interaction is dosage suppression where overexpression of a target gene compensates for a phenotype caused by the deletion of a second gene. This

type of phenotypic compensation is termed dosage suppression (Magtanong et al. 2011). Unlike negative GIs explained above, this type of interaction often happens between genes within the same pathway. In the absence of DNA damage drugs, overexpression of *HUR1* did not form any GIs. However, overexpression of *HUR1* compensated for deletion of several key NHEJ genes in the presence of HU or bleomycin. Overexpression of *HUR1* formed GIs with *RAD50* and *XRS2* in the presence of both HU and bleomycin. It also compensates for lack of other NHEJ genes such as *YKU80*, *DNL4* and *LIF1* in the presence of HU (Figure 3.1C). These phenotypic suppression interactions are in the agreement with an involvement for *HUR1* in the NHEJ pathway.

A



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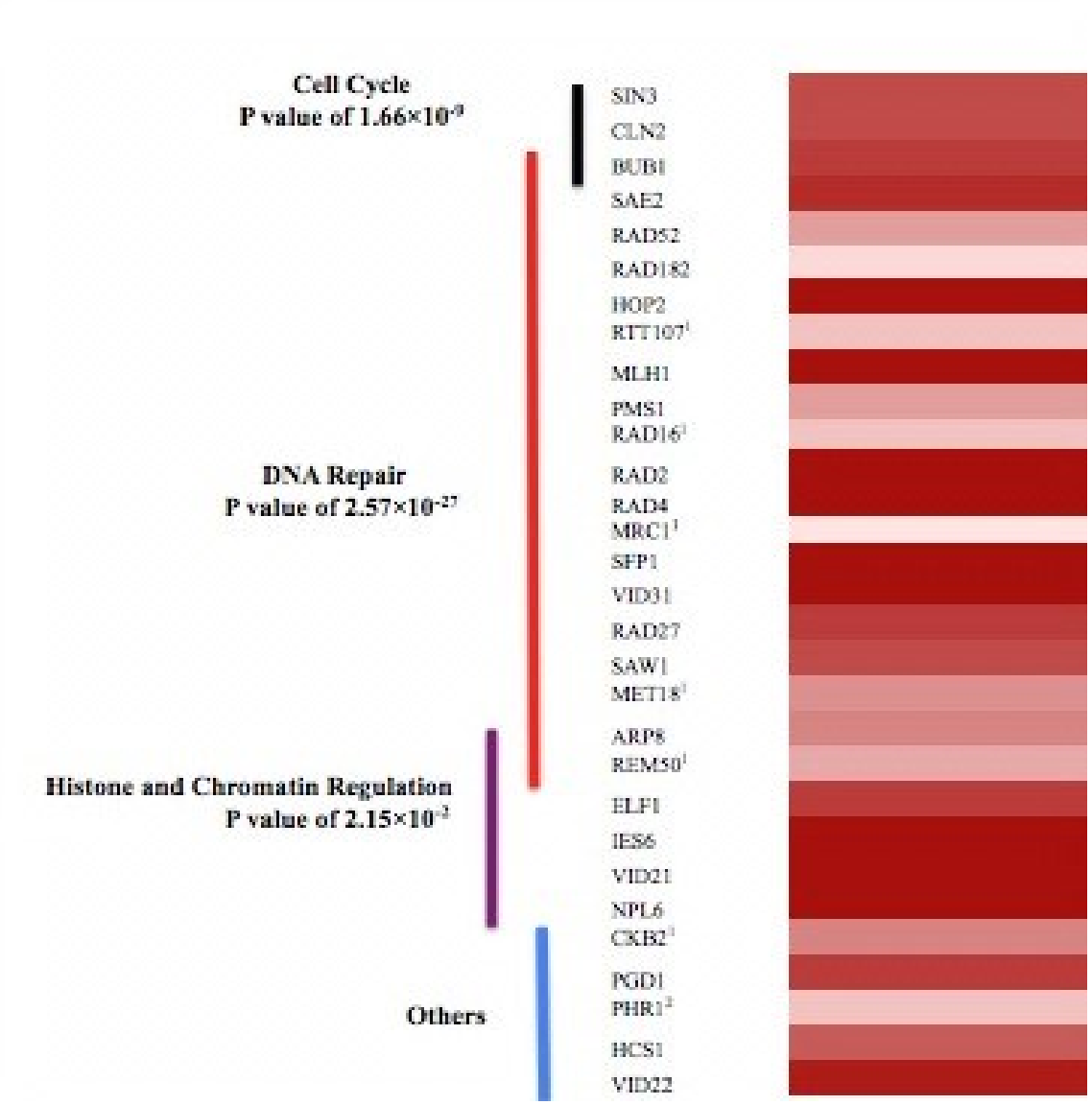
Cell Cycle
P value of 1.66×10-9
DNA Repair
P value of 2.57×10-27
Histone and Chromatin Regulation
P value of 2.15×10-2
Others
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CLN2
BUB1
SAE2
RAD52
RAD182
HOP2
RTT107
MLH1
PMS1
RAD16
RAD2
RAD4
MRC1
SFP1
VID31
RAD27
SAW1
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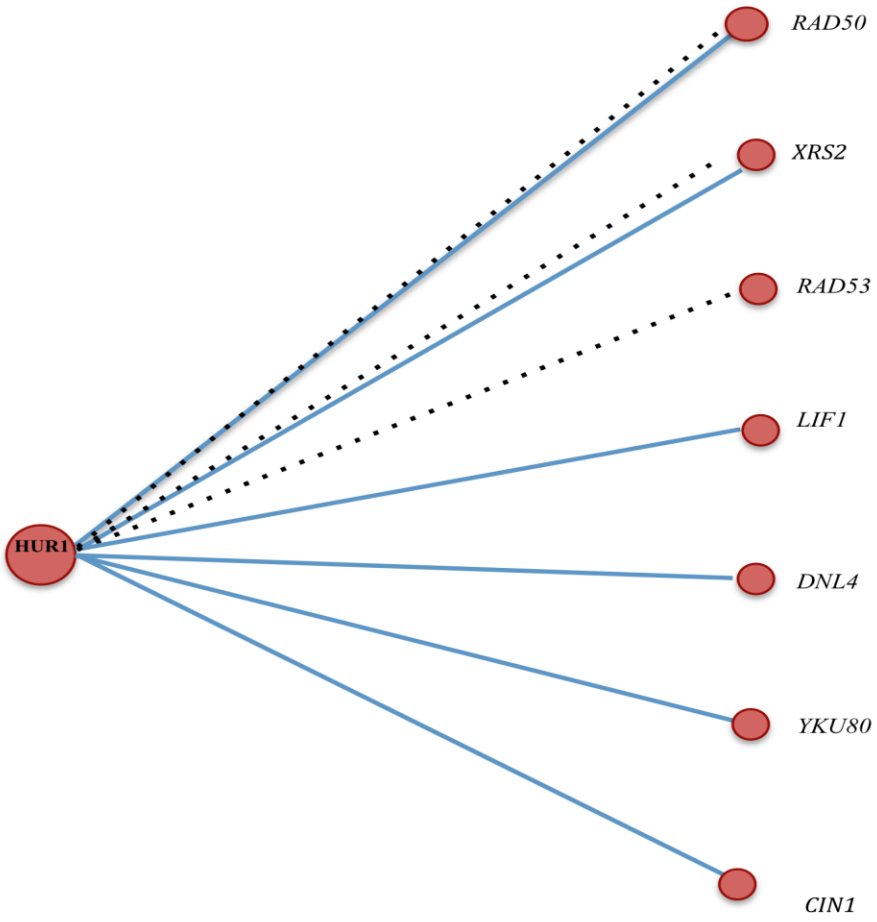

Figure 3.1: Analysis of the protein-protein interactions prediction and genetic interactions for *HUR1-A.* A) 3 interactions were predicted for Hur1p protein with Tpk1p, Tpk2p and Tpk3p proteins. The predicted sites of interaction for interacting proteins are indicated. B) *HUR1-A* formed negative genetic interactions with a number of genes involved in DNA damage repair genes. Conditional interactions were selected in the presence of 60 mM HU (1) and 4 µg/ml bleomycin (2). C) Overexpression of *HUR1* compensated for key NHEJ in the presence of 60 mM HU (Solid lines) and 4 µg/ml bleomycin (Dash lines).

3.4.2 Drug sensitivity analysis

It is expected that deletion of genes involved in DNA repair pathways might change the sensitivity of yeast to different DNA damage-inducing drugs (Birrell et al. 2001). To this end, we used bleomycin and HU to study drug sensitivity. Bleomycin uses a free-radical-based mechanism resulting in DSBs, and HU generates DNA replication errors through the depletion of dNTPs that can lead to DSB (Bradley and Kohn 1979, Rittberg and Wright 1989, Koç et al. 2004). We observed a mild sensitivity for *Δhur1-A* strain in the presence 4 µg/ml bleomycin of and a more pronounced sensitivity to 60 mM HU (Figure 3.2). This is in contrast to the previous report where the sensitivity of full length *HUR1* deletion strain was attributed to its overlap with *PMR1* gene (Jordan et al. 2007). We believe that the strong phenotypes of the *PMR1* null mutants could overshadow those displayed by the *HUR1* mutants. To investigate if reintroduction of *HUR1* into its deletion mutant strain would reverse the observed sensitivity, a *HUR1* overexpression plasmid was used. Introduction of plasmid-born Hur1p into the *Δhur1-A* deletion mutant reversed the drug sensitivities, suggesting that the observed phenotypes was as a result of deletion of *HUR1-A*.

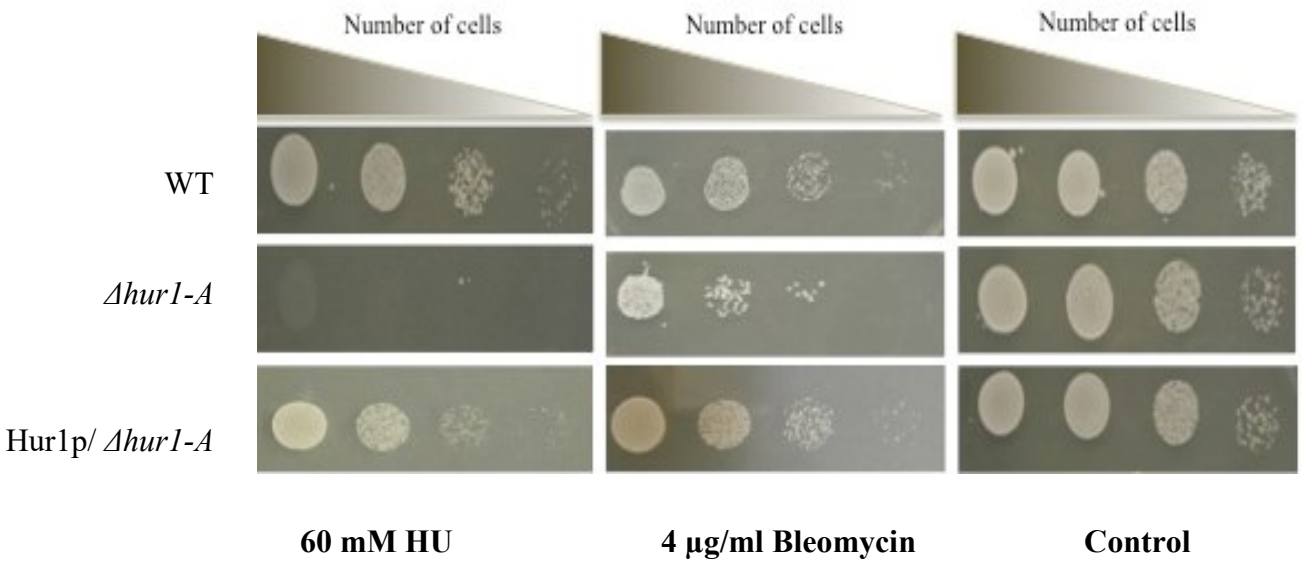

Figure 3.2: *HUR1-A* sensitivity to bleomycin and HU. Single mutant of *HUR1-A* showed increased sensitivity in presence of bleomycin (4 µg/ml) and HU (60 mM). Overexpression of Hur1p compensated for *Δhur1-A.*

3.4.3 *HUR1* deletion reduced the efficiency of NHEJ in a plasmid based repair assay independent of its overlap with *PMR1*

To investigate the activity of Hur1p on the efficiency of NHEJ, a plasmid repair assay was utilized (Boulton and Jackson 1998). This assay has been used to identify novel genes involve in NHEJ pathway (Shim et al. 2005, Jessulat et al. 2008, Yu and Volkert 2013). Equal amounts of intact and linearized plasmids with overhangs were used to separately transform the wild-type, Δ*hur1-A,* Δ*pmr1-A* and Δ*yku70* strains. Δ*hur1-A* and Δ*pmr1-A* represent partial gene deletions for *HUR1* and *PMR1*, respectively, without compromising the overlap region between the two genes. In this way, the influence of each gene on NHEJ can be evaluated independent of the other. In this assay, only the cells that contain circular (repaired) plasmids would form a colony. DNA repair is limited to NHEJ due to the lack of homology between the break site on the plasmid and *S. cerevisiae* genome. The number of colonies formed from transformation with linearized plasmids is related to colonies formed from circular plasmids, and the ratio represents the efficiency of plasmid repair that have occurred. It was observed that Δ*hur1-A* showed approximately 86% reduction in plasmid repair efficiency. In contrast, the efficiency of NHEJ was reduced by approximately 18% for Δ*pmr1-A*. The efficiency of NHEJ in Δ*hur1* was similar to Δ*hur1-A* (Figure 3.3). This observation suggests that *HUR1* influences the efficiency of NHEJ of plasmid DNA and that this activity is independent of its overlap with *PMR1* gene.

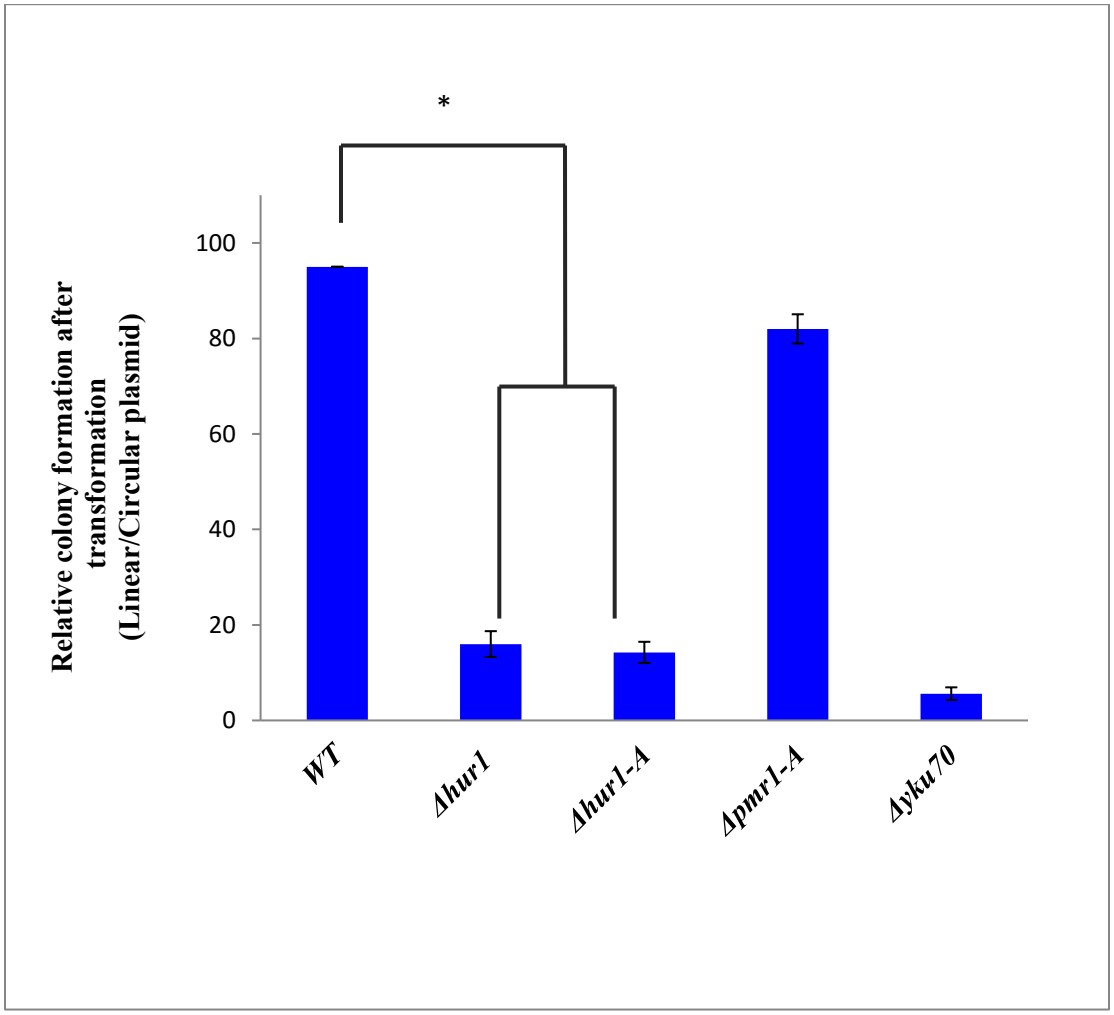

Figure 3.3: Plasmid repair assay for Hurl and Pmr1 deletion strains. Deletion of *HUR1* reduces the efficiency of repair for plasmids with overhangs. *Δhur1-A* reduced the efficiency of NHEJ similar to *Δhur1* but *Δpmr1-A* did not affect the efficiency of NHEJ. Error bars represent standard deviation. Wild type values are normalized to 100% and other values are related to this value. Experiments were repeated at least five times; *p*-value ≤ 0.05 calculated using Student's *t*-test.

Next we investigated the repair efficiency of Δ*hur1-A* for DSBs with blunt ends. For this, we tested the transformation efficiency using YCplac111 plasmid (Bahmed K et al. 2010) cut with Sma1 (leaving blunt ends). As before, equal amounts of intact and linearized plasmids with blunt ends were used to transform wild-type, Δ*hur1-A,* and Δ*yku70*. The Δ*hur1-A* strain showed 84% reduction in plasmid repair efficiency for blunt end repair suggesting a role for *HUR1* in blunt end DNA repair (Figure 3.4A). In agreement with previous observations (Boulton and Jackson 1996) deletion of *YKU70* did not show a reduction in blunt end repair; the reason for this observation is still unclear. Repair of the blunt end DSBs is generally accepted to be independent of YKU activity (Yu and Volkert 2013). In this way, it appears that the influence of *HUR1* on NHEJ might be different from *YKU70*'s activity.

3.4.4 Plasmid repair analysis of double mutant strains suggest that *HUR1-A, TPK1* and *NEJ1* function in the same pathway and parallel to *YKU70*

Single gene deletion mutant strains for *HUR1-A*, *YKU70*, *NEJ1*, and *TPK1* showed significant reduction in NHEJ compare to the WT. To further investigate the functional relationship between these genes, a plasmid repair assay for double gene deletion strains was carried out. Deletion of two genes in the same pathway within a cellular process generally causes phenotypes similar to deletion of a single gene alone. However, an aggravating change in the phenotype is expected if the two deleted genes work in parallel pathways that can compensate one another. It was observed that strains carrying gene deletions for *HUR1-A* along with *TPK1* (*Δhur1-A/*Δ*tpk1)* or *NEJ1* (*Δhur1-A/*Δ*nej1)* have the same reduced repair efficiency as their corresponding single gene

deletion mutants for *HUR1*, *TPK1* and *NEJ1*. Double mutant strains *Δhur1-A*/*Δtpk1,* and *Δhur1-A*/*Δnej1* showed NHEJ efficiency of 12.5 % and 13.75%, respectively for overhang end repairs (Figure 3.4B) suggesting that *HUR1* functions in the same pathway as *TPK1* and *NEJ1*. In contrast, the double mutant strain for *HUR1-A* and *YKU70* (Δ*hur1-A*/Δ*yku70*), showed an increase in reduction of repair efficiency compared to single mutants (P value < 0.5). *Δhur1-A*/*Δyku70* showed NHEJ efficiency of approximately 2.75% for overhang ends, which is lower than NHEJ efficiency of either *HUR1-A* or *YKU70* single mutants alone (Figure 3.4B). As indicated, unlike *TPK1* and *NEJ1* (Hooshyar et al. 2016), DSBs with blunt ends are repaired independently of the YKU complex (Boulton and Jackson 1996, Yu and Volkert 2013). We observed that Δ*yku70* showed less than 5% reduction in NHEJ efficiency of blunt ends (Figure 3.4A). However, similar to above, *Δhur1-A*/*Δyku70* had a decrease in the repair efficiency by approximately 81%, equivalent to that for *Δhur1*-A (Figure 3.4A). Consequently, it appears that *HUR1-A* influences blunt end repair, and that this activity, like that for *TPK1* and *NEJ1* (Hooshyar et al. 2016) seems to be independent of YKU complex.

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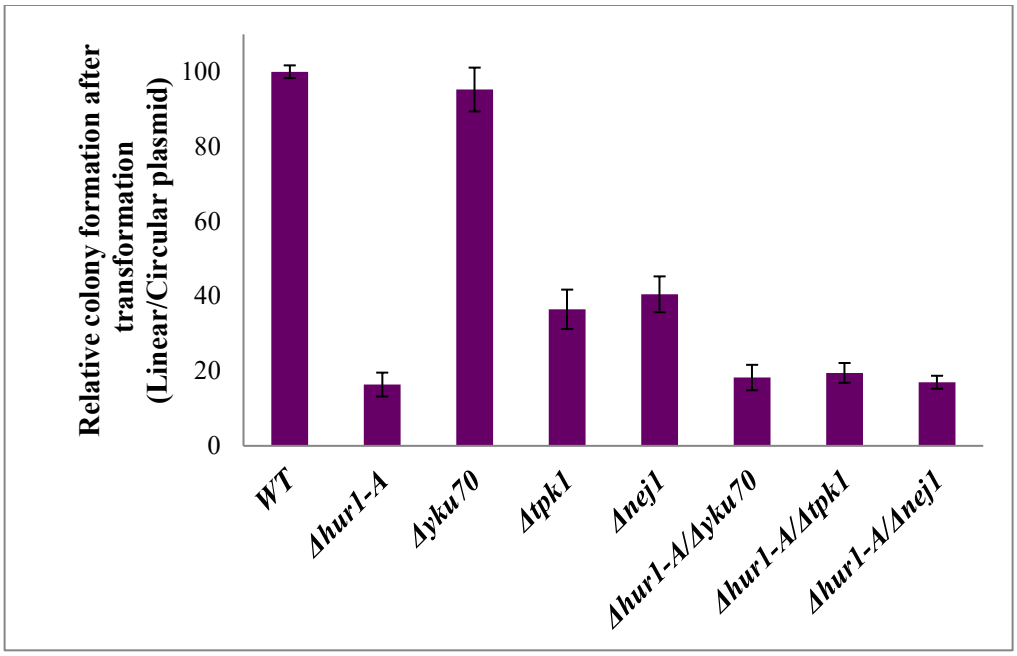

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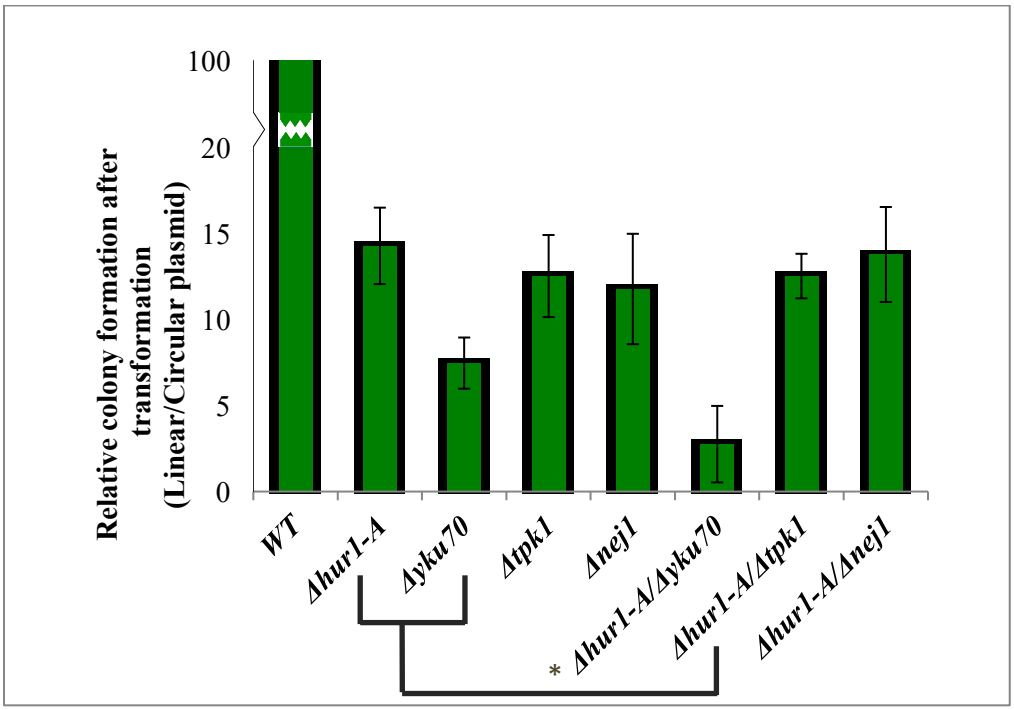

Figure 3.4: Plasmid repair efficiency for different yeast strains. Δhur1-A shows reduction in NHEJ efficiency for both plasmid repair assay with blunt ends (A) and overhangs (B). Deletion of double mutants of Hur1-A/Yku70 shows significant reduction compare to Δhur1-A alone. Deletion of double mutants of Hur1-A/Tpk1 and Hur1-A/Nej1 similar reduction compare to single mutant. Error bars represent standard deviation. Wild type values are normalized to 100% and other values are related to this value. Experiments were repeated at least five times; p-value ≤ 0.05 calculated using Student's t-test.

3.4.5 The effect of Hur1p half on efficient NHEJ is relevant in a chromosomal context

We used the JKM139 strain to further investigate and confirm the effect of *Hur1-A* on the efficiency of NHEJ to repair chromosomal DSBs (Moore and Haber 1996). In this assay, the target gene is deleted in a JKM139 strain background and the viability of target gene deletion mutant is evaluated after DSB induction by exposure to galactose. JKM139 strain carries a GAL promoter in front of an endonuclease specific to the HO site. The presence of galactose induces the production of this endonuclease and consequently results in chromosomal breakage at the HO sites. There is no homologous region to HO site in this strain limiting the repair to NHEJ. Equal amount of cells for WT, and *Δhur1-A* (JKM139 background) were serially diluted and plated on both galactose and glucose (control) medium to form the colonies. The ratio of comparing the number of colonies in glucose and galactose were used as a measure of survival and were related to the ability of the cell to repair induced DSB (Figure 3.5). As expected, *Δhur1-A* had a reduced ability to survive when DSB was induced. This observation verify the results of the plasmid repair assay in a chromosomal context for *Δhur1-A* gene. To further investigate if the observed inability of *Δhur1-A* is in fact a result of the deletion of *HUR1-A* and not a secondary mutation within the genome, the *HUR1* expression plasmid was reintroduced into *Δhur1-A*. The introduction of this plasmid compensated for the deletion of *HUR1-A* in chromosomal break assay confirming that the observed phenotype was a consequence of *HUR1-A* deletion and not a secondary unwanted mutation.

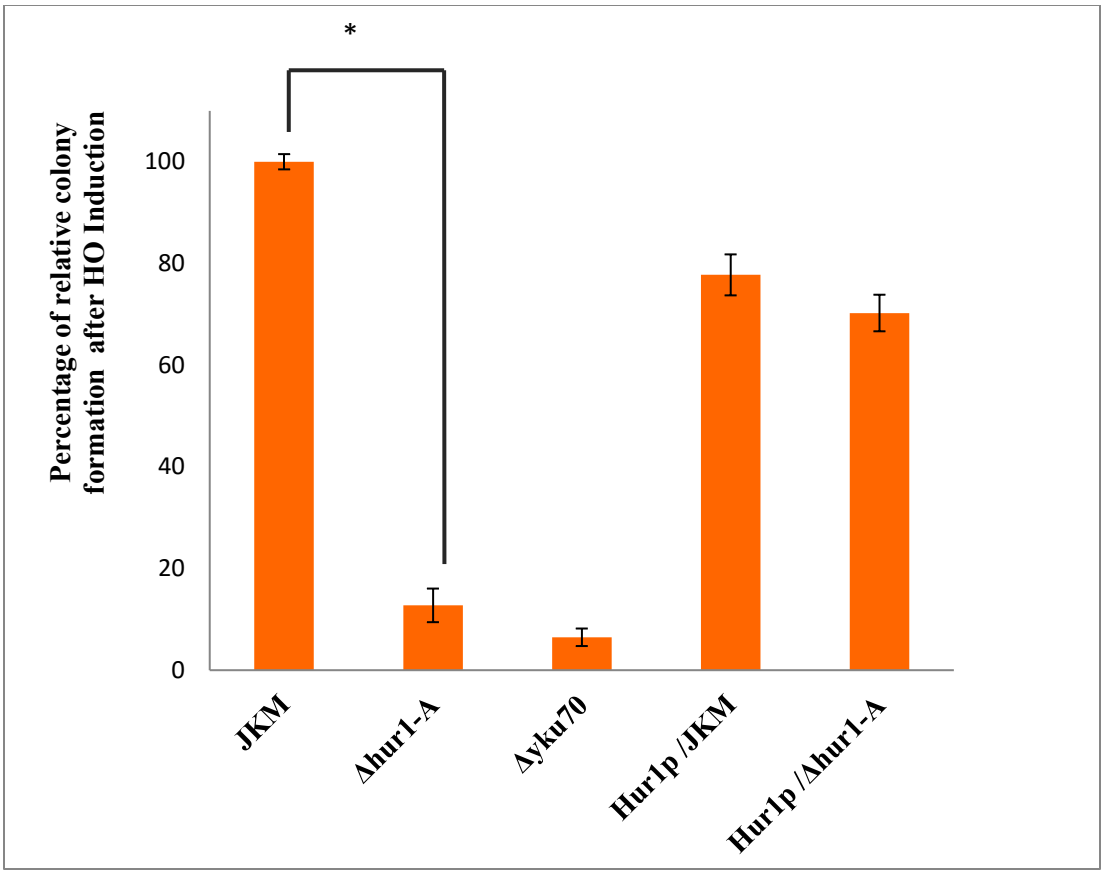

Figure 3.5: Repair efficiency of chromosomally induced breaks. Comparing colony formation after DSB induction (in the presence of galactose). Deletion mutant for *HUR1* had a reduced relative colony survival (87.25% reduction). Wild type values are normalized to 100% and other values are related to this value. Experiments were repeated at least five times; p-value ≤ 0.05 calculated using Student's t-test.

3.5 Concluding Remarks

In this study, we use genetic evidence to report a role for functionally uncharacterized ORF *HUR1* in the process of NHEJ in *S. cerevisiae*. We provide evidence-linking *HUR1* to NHEJ through its interaction with *TPK1*. Our genetic interaction analyses revealed negative interaction between *Hur1-A* and genes involved in DNA damage repair pathways. Deletion of the region of *HUR1* that has no overlap with *PMR1* (*HUR1-A*) reduced NHEJ efficiency in both chromosomal and plasmid repair assays suggesting that the NHEJ activity observed is related to *HUR1* and is independent of *PMR1*. Similar to *TPK1* and *NEJ1*, and unlike YKU complex, Hur1p appears to be involved in blunt end DSB repair as well as repair of overhangs. In agreement with this, double gene deletion mutant strains Δ*hur1/*Δ*tpk1,* and Δ*hur1/*Δ*nej1* show similar reduction compared to single gene deletion mutant strains. Additional investigations of *HUR1* in the context of DNA repair would help us further understand the activity of this protein in yeast.

4 Chapter: Spindle checkpoint Bub1 and Bub2, of *Saccharomyces cerevisiae* are required for non-homologous end joining double strand break repair

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Contribution: Wet lab experiments for this work were performed by me and Dr. Matthew Jessulat in Dr. Babu's lab from University of Regina. I participate in analyzing the plasmid, chromosomal repair assay, genetic interaction data and drug sensitivity spot test.

4.1 Abstract

The core components of the Non-Homologous End Joining (NHEJ) pathway for the efficient repair of double-strand break (DSB) DNA lesions have been extensively studied, yet it is likely that this pathway involves the concerted actions of many unidentified DNA-repair related factors and cellular processes. While large-scale phenomics screens have identified genes required for resistance to DNA damaging agents, the progress towards uncovering new factors governing the underlying mechanism of NHEJ repair has so far been limited. Here, we present a systematic and unbiased plasmid-based DNA-repair screen specific to NHEJ in the yeast *Saccharomyces cerevisiae*, encompassing a selected set of 371 known and predicted non-essential DNA-repair related components. Among the newly identified NHEJ factors, the evolutionarily conserved spindle assembly checkpoint (SAC) genes, Bub1 and Bub2, and the non-

essential components of the anaphase promoting complex (APC), cyclins, and SBF transcription complex (Swi4-Swi6) were critical for efficient NHEJ. A targeted yeast genetic screen showed *bub1* and *bub2* to interact genetically with components of DNA-repair related processes. Consistent with this finding, the *bub1* and *bub2* mutant strains either alone or in combination with *apc9* (APC), *clb2* (cyclin), and *swi4* (SBF) showed increased sensitivity to DNA damaging agents. Thus, our data support a dual functional role for Bub1 and Bub2 in mitotic exit and NHEJ, and suggest their joint participation with the components of Apc9, Clb2, and Swi4 in the NHEJ repair process.

4.2 Introduction

The repair of DNA double strand breaks (DSBs) is an essential process for the preservation of genome integrity and the normal functioning of the cell (Wyman et al. 2006). These cytotoxic lesions are repaired by major DSB repair pathways including the homologous recombination (HR) (Wyman et al. 2006, Li et al.2008) and non-homologous end joining (NHEJ) systems (Dudasova et al. 2004, Daley et al. 2005, Wyman et al. 2006, Lieber et al. 2010). While the former is a prevalent pathway in the unicellular budding yeast *Saccharomyces cerevisiae* (Dudas et al. 2004), the latter pathway is predominant in mammalian cells, especially in quiescent cells (Yano et al. 2009), and can repair DNA lesions even if there is no homologous strand (Moore and Haber 1996, Takata et al. 1998, Mao et al.2008, Lisby et al. 2009). Most notably, the impairment of NHEJ in mammalian cells has been frequently linked to genomic instability, cancer, and lymphoid V (D) J (i.e. variable, diversity, and joining gene segments) recombination defects, and therefore a detailed molecular understanding of

this pathway will provide critical insights into the genetic risk factors for carcinogenesis or reduced immune system function (O'Driscoll et al. 2002, Burma at al. 2006, de Villartay et al. 2009).

As in mammalian systems, the analogous core components of the classical NHEJ pathway in *S. cerevisiae* are dependent on three major complexes (i.e. YKu, MRX, and DNL4), which are recruited rapidly to DSBs (Daley et al. 2005). First, the yeast Ku heterodimer (yku70-yku80) binds to each broken end of a DSB, and serves as an anchor for protein complexes involved in securing and annealing the break, and also suppresses the competing HR pathway (Taccioli et al. 1994, Sonoda et al. 2006). Second, the DSB processing complex MRX (Mre11-Rad50-Xrs2) spans the lesions and acts at an early stage of both NHEJ and HR repair pathways (Usui et al. 1998, Chen et al. 2001). Third, the Dnl4 complex (i.e. Dnl4-Lif1-Nej1) functions as a DNA ligase in the rejoining step of the DSB ends (Teo et al.1997, Wilson et al.1997). While these core protein complexes have long been elucidated, efficient repair by the NHEJ depends on a wide array of other coordinated cellular processes (McKinney et al. 2013). For example, the yeast chromatin structure-remodeling (Shim et al. 2005) and histone acetyltransferase complexes (Collins et al. 2007, Driscoll et al. 2007, Han et al. 2007, Tsubota et al. 2007, Jessulat et al. 2008) and the phosphorylation of histone H2A (also known as γ-H2A) in yeast have been shown to be essential for efficient NHEJ activity (Downs et al. 2000, Fernandez-Capetillo et al. 2004, Van Attikum et al. 2004). As well, the effective repair of DSBs depends on the DNA damage response (DDR) pathway, which detects the DNA lesions via recognition factors of the MRX complex (Lee et al. 2007), and controls the activity of protein kinases including Rad9, Rad53, and the checkpoint effector protein, Chk1 (Emili

et al. 1998, Vialard et al. 1998, Stracker et al. 2007). These kinases induce cell cycle arrest and the up-regulation of DNA-repair systems, leading to profound alterations in chromatin structure, dynamics, and gene expression (Vialard et al. 1998, Chen et al. 2004, Stracker et al. 2007).

Aside from these processes, DNA damage has also been shown to trigger the spindle assembly checkpoint (SAC) pathway, and thus prevent cell cycle progression in the presence of DNA damage prior to cell commitment to anaphase (Kim et al. 2008). In particular, the SAC kinase protein, Bub1, in conjunction with other kinetochore factors essential for the SAC pathway (e.g., Mad1, Mad2, Mad3, and Bub3) mediates cell cycle arrest in the event of spindle fiber damage (Lew et al. 2003, May et al. 2006, Musacchio et al. 2007, Burke et al. 2008). Another, SAC pathway-associated component, Bub2, that localizes to the spindle pole body, forms an association with its GTPase-activating co-complex member, Bfa1, to trigger a delay in anaphase progression or mitotic exit (Hu et al. 2001, Molk et al. 2004). Both the Bub1 and Bub2 branches of the SAC pathway also limit cell cycle progression by preventing the activation of the anaphase promoting complex (APC), an essential, multi-subunit, E3 ubiquitin-protein ligase that depends on the co-activators Cdc20 (Musacchio et al. 2007) and Cdh1 (Buschhorn et al. 2011) to target anaphase-inhibitory substrates such as B-type cyclins. These B-type cyclins act as key regulators of cell cycle proteins (e.g., activation of cyclin-dependent protein kinase, Cdc28 by the mitotic cyclin Clb2) (Enserink et al. 2010), and are tightly regulated by major transcriptional factors such as the components of the SBF complex (i.e. Swi4-Swi6 cell cycle box binding factor), which regulates proteins involved in budding, spindle pole formation, cell wall biogenesis, and DNA synthesis (Iyer et al. 2001). Notably, genome-

wide pooled short hairpin RNA (shRNA) screens in different cancer cell lines (Marcotte et al. 2012) identified components of the SAC, APC, and cyclins to be essential for proliferation in certain cancer-specific cells (e.g., breast and pancreatic cancers), suggesting an intricate relationship of this mitotic checkpoint abnormality in tumor progression beyond DSB repair (Schvartzman et al. 2010).

While Bub1 and Bub2 activate the mitotic checkpoint via distinct pathways (Fraschini et al. 1999) they work in concert with the major target components of the APC, cyclins, and SBFs in the checkpoint and cell cycle control mechanism (Siegmund et al. 1996, Visintin et al. 1997, Zich et al. 2010). Moreover, the ability of these BUB proteins to interchangeably act as DNA damage checkpoint kinase (e.g., Rad9 and Rad53) substrates suggests that the BUB pathways are efficient at responding to disruptions of the kinetochores (Kim et al. 2008), but also implies a likely role for these proteins in the response to mitotic DSBs. Consistent with the later notion, recent evidence in mammalian cells has indicated that Bub1 functions in the DDR signalling pathway (Yang et al. 2012), but its role in NHEJ remains unclear. *BUB1* (YGR188C) and *BUB2* (YMR055C) are located in the nucleus on chromosome VII and chromosome XIII, respectively. The protein abundance for Bub1 and Bub2 are 414 molecules/cell and 457 molecules/cell, respectively. The protein domain of 26-1019 is bub1 homologue-related and Bub2 domain 1-305 is tbc1 domain family member gtpase-activating protein.

In this study, we posit that in addition to the known components of the NHEJ pathway, there are yet several unidentified proteins that are required for efficient NHEJ, and that many of these new targets can be found within the spindle regulation, cell cycle, histone and chromatin modification, and other cellular pathways. Accordingly, a

comprehensive plasmid-based DNA-repair screening approach, specific to NHEJ in *S. cerevisiae* was employed in our laboratory. Among the newly identified NHEJ factors, Bub1 and Bub2, and several other non-essential components of the APC, as well as cyclins that are regulated by the APC, and SBF were found. Synthetic genetic interaction screens of *bub1* and *bub2* with the selected target set of DNA-repair related genes also showed a relationship with components of the DNA damage, repair, and epigenetic regulation pathways. Our findings reveal that the Bub1 and Bub2-mediated branches of the SAC orchestrates with Apc9 (APC), Clb2 (cyclin), and Swi4 (SBF) to alter NHEJ efficiency, which overlaps with DDR that utilizes Rad53 kinase and the phosphorylated H2A or H2AX from the DNA break.

4.3 Materials and Methods

4.3.1 Yeast strains and media

Mutant strains in JKM139 strain background were generated by lithium acetate transformation procedure (Winzeler et al. 1999) with a PCR product containing the resistance *NATMX6* (nourseothricin resistance) cassette flanked by homologous regions to the gene of interest. Non-essential haploid *MATa* Kan-marked deletion mutant strains were obtained from the yeast deletion collection (Costanzo et al. 2010). Wild-type and mutant strains were cultured in standard yeast media growth conditions, except as noted.

4.3.2 Compilation of DNA-repair related target set

An exhaustive review of literature studies, GO annotations, and public database surveys (Cherry et al. 2012, Chatr-Aryamontri et al. 2013) were used to manually curate genes that are of relevance to DNA-repair processes. In certain cases, gene candidates were included based on their epistatic associations with known NHEJ factors (Costanzo et al. 2010, Cherry et al. 2012) or when displayed hypersensitivity to DNA damage-inducing agents targeting NHEJ pathway, as assessed in large-scale chemical-genetic screens (Hillenmeyer et al. 2008). The target index also includes some known key NHEJ factors as positive controls, to assess the reliability of our assay and data quality.

4.3.3 Other methods

NHEJ plasmid-based and chromosomal DSB repair assays and SGA analysis were performed as discribed in chapter 2 and 3.

4.4 Results

4.4.1 Large-scale plasmid-based DSB screening reveals new NHEJ repair targets

Prior to the systematic screening for identifying genes with reduced NHEJ efficiency, a previous study performed an exhaustive survey on the literature and public databases (Cherry et al. 2012) and compiled 510 known and putative DNA-repair-related functional assignments. Then a selected subset of 371 non-essential DNA-repair related genes, which were manually curated into 11 broadly representative bioprocesses and 30 subclasses were selected (Figure 4.1). These included genes known to participate in

NHEJ (Moore and Haber 1996), DNA related repair (Sulek et al. 2007), response to stress or DNA damage (Wilson et al. 1997), DNA replication (Usui et al. 1998), cell cycle or division (Hegde et al. 2000), histone or chromatin modification (Truman et al. 2009), cell maintenance and structure (Driscoll et al. 2007), as well as genes with pleiotropic or unclear (Burke et al. 2008) functions (Figure 4.1).

To identify the gene targets of the NHEJ pathway, we exploited our established *in vitro* plasmid DSB repair assay (Jessulat et al. 2008) by introducing the linearized plasmid p416 containing the URA3 selection marker into an array of target single gene deletion mutants with the plasmid cleavage site (i.e. an induced DSB) in a region with no homologous sequence available in the genome to repair. In parallel, an equal volume of undigested plasmid p416 was also transformed as a control (i.e. non-linearized or circular plasmid) for quantifying NHEJ repair efficiency. After outgrowth for 48-72 hrs at 30 °C, the colony growth of the single gene deletion mutants on media lacking uracil was quantified to uncover NHEJ targets. Mutant strains derived from the linearized plasmid that display less colony growth relative to those from the circularized control plasmid grown under uracil limiting medium indicates that the linearized plasmid is not efficiently re-circularized for promoting replication and growth in the absence of uracil.

For example, defects in the components of the Yeast "Ku" complex (Siede et al. 1996, Milne et al. 1996) strains depleted for the subunits of the DNL4 complex (Herrmann et al. 1998, Teo et al. 2000, Valencia et al. 2001, Sulek et al. 2007) strains lacking the NHEJ-related end-processing factors, *pol4* and *rad27* (Wilson and Lieber 1999, Wu et al. 1999) and mutant strain, *mre11*, defective for DSB repair (Symington 2002) showed a significant reduction in NHEJ efficiency. Loss of chromatin assembly

and silencing factors, *sir2*, *sir3*, and *sir4* also showed reduced NHEJ efficiency, emphasizing the functional role of these components in the NHEJ repair pathway (Critchlow et al.1998, Hegde et al. 2000).

Together with the identification of well-studied NHEJ targets, our assay also uncovered several new candidates that are relevant for NHEJ (Appendix B.3). These include two SAC encoding genes, Bub1 and Bub2, whose corresponding mutants displayed marked reductions in NHEJ efficiency. Both Bub1 and Bub2 function as initial sensors of DNA damage during cellular division (Burke et al. 2008), and previous biochemical and genetic investigations have indicated a strong functional coupling between the SAC and DDR, with DNA lesions being capable of causing cell cycle arrest through a SAC-dependent mechanism (Kim et al. 2008, Garber et al. 2002) Consistent with this notion, in contrast to known SAC factors (*bfa1*, *bub3*, *mad1*, and *mad2*), the reduced colony formation of yeast cells due to the loss of *bub1* and *bub2*, suggests a more likely role of these components in NHEJ DNA-repair, and hence we chose to investigate the activity of these two candidates in more detail.

The plasmid repair assay in small-scale was repeated to support the involvement of Bub1 and Bub2 in NHEJ process. It was observed that Δ*bub1* and Δ*bub2* showed 81% and 78.4% reduction in plasmid repair efficiency, respectively (Figure 4.2A). This supports the large-scale plasmid repair assay, which suggests the role of these components in NHEJ pathway.

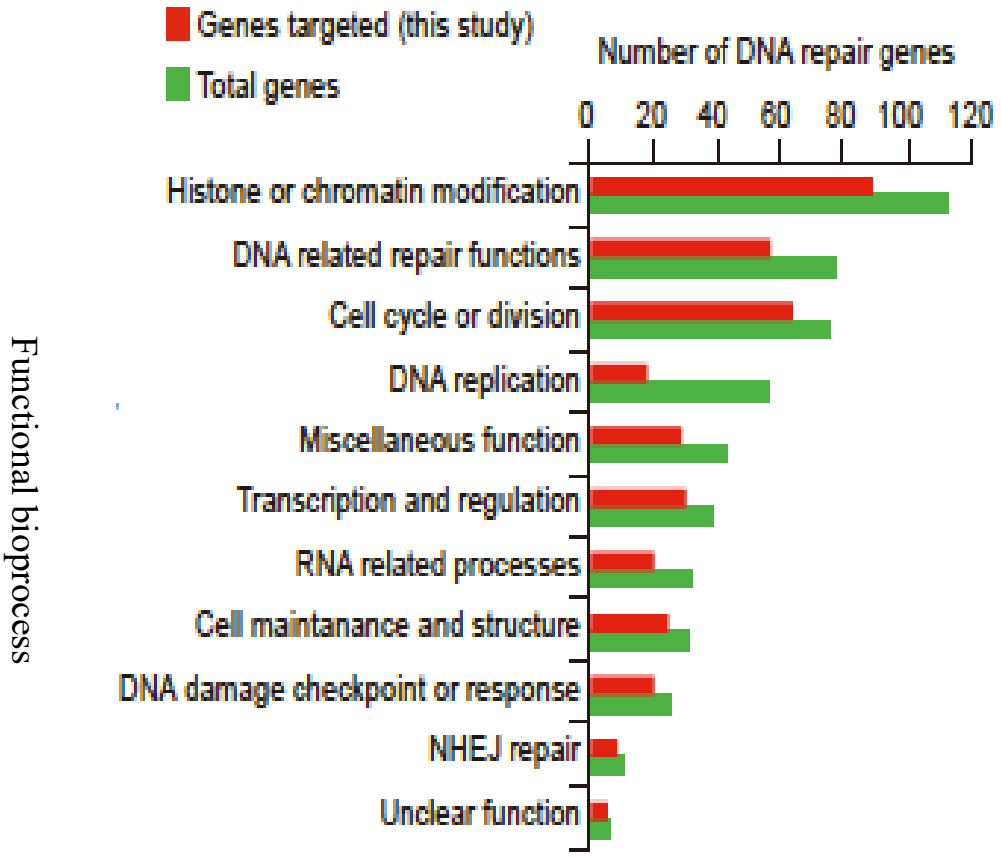
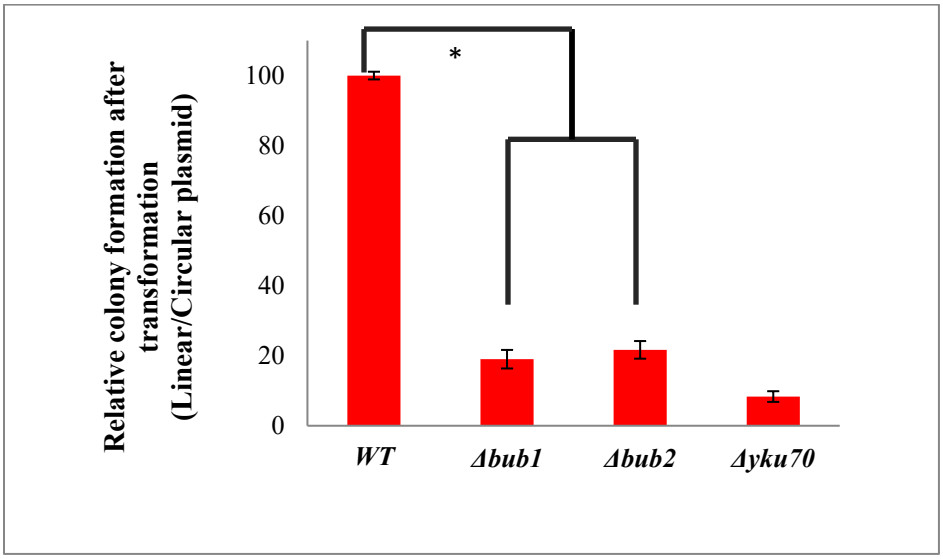


Figure 4.1: NHEJ targets. Functional distribution of known and putative DNA-repair related target genes grouped into 11 broadly representative bioprocesses.

4.4.2 Bub1 and Bub2 are required for chromosomal DSB repair by NHEJ

Since plasmid based DSBs, as employed in this study for assessing NHEJ repair, may be functionally distinct from chromosomal DSB's (Vigasova et al. 2013, Yu and Volkert et al. 2013), we re-examined the SAC *bub1* and *bub2* mutant strains that displayed reduced NHEJ by a plasmid based rejoining assay to verify if Bub1 and Bub2 influence the efficiency of chromosomal DSB repair by NHEJ (Figure 4.2B). Consequently, we constructed the *bub1* and *bub2* mutants in JKM139 yeast background strains expressing site-specific HO (i.e. Homothallic switching) endonuclease on media containing galactose as a carbon source, resulting in cleavage of a restriction site at the *MAT* locus to produce an *in vivo* chromosomal DSB (Moore and Haber 1996). Notably, the parental JKM139 strain that we used lack the two silent copies of the *MAT* locus (i.e. the HML and HMR loci located at the left and right end of the chromosome III, respectively), required for the repair of Homothallic switching (HO)-induced DSBs by HR (Figure 4.2B). Efficient repairing of this break will depend critically on the NHEJ process to accomplish the repair required for cell's survival (Van et al. 2004). As expected, compared to wild-type cells, both the isogenic *bub1* and *bub2* mutant strains displayed significantly reduced (p-value < 0.05) colony formation at 48-72 h after cleavage was induced by treatment with galactose (Figure 4.2B).

A



B

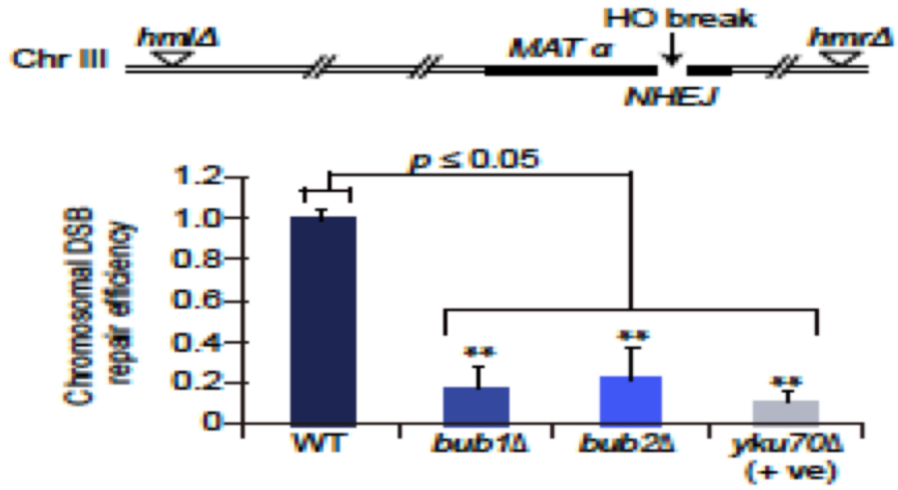


Figure 4.2: Identification of potential NHEJ targets from plasmid-based and chromosome DSB repair screens. (A) *Δbub1* and *Δbub2* show reduction in NHEJ efficiency for plasmid repair assay with overhangs. (B) Schematic illustration (top) of the haploid yeast JKM139 strain on chromosome III expressing galactose-inducible HO endonuclease at *MAT* locus, bearing the deletions of HML and HMR donor loci. Repair efficiency of HO-induced chromosomal DSBs (bottom) in the indicated mutant and WT strains. Efficiency was determined by counting the number of surviving colonies on galactose versus glucose medium. Values indicate mean ± SEM of three independent experiments; p-value ≤ 0.05 calculated using Student's t-test.

4.4.3 Bub1 and Bub2 interact genetically with NHEJ and other DNA-repair related processes

To gain additional insight into the *in vivo* functions of Bub1 and Bub2, and their potential role in DNA-repair pathways, we adapted the SGA (synthetic genetic array) approach (Tong et al. 2001, Costanzo et al. 2010) to identify genes interacting genetically with *bub1* and *bub2* mutants. Double mutants were generated by mating the *bub1* and *bub2* "query" deletion mutants against the selected target subset of 371 "array" non-essential single gene mutant strains. Functional relationships between genes were assessed based on the growth fitness of the inviable or slow-growing double mutant meiotic progenies. Negative or positive genetic interactions suggests that the protein products of the two genes are functionally related and are usually interpreted in terms of redundancy (parallel pathways) or similarity (same pathway) of function (Tong et al. 2001, Costanzo et al. 2010, Beltrao et al. 2010).

In agreement with the plasmid based repair assay, we were able to identify synthetic sick or lethal interactions of *bub1* and *bub2* with ~52% (194 of 371) of the DNA-repair related genes. Of these, our screens correctly captured ~87% (116 of 134) of the genes that were reported previously to interact negatively with *bub1* or *bub2* (Figure 4.3A). For example, we captured the synthetic sick or lethal phenotype between *bub1* and the NHEJ factor *mre11* (Collins et al. 2007). Likewise, the Bub1 or Bub2 network confirmed negative interactions with genes known to be involved in histone modification (e.g., *rtt109*), the cell cycle (e.g., *clb3*), transcriptional regulation (e.g., *swi4*), and other processes (Figure 4.3A). Although previous genetic evidence indicates that Bub1 and Bub2 function in distinct branches of the SAC pathway (Hardwick et al. 1998, Fraschini

et al.1999) about 9% (33 of 371) of the DNA-repair related targets displayed synthetic sick or lethal relationships with both of these genes (e.g., *xrs2* and *mre11*), suggesting that Bub1 and Bub2 coordinate their function with these genes to mediate DSB repair.

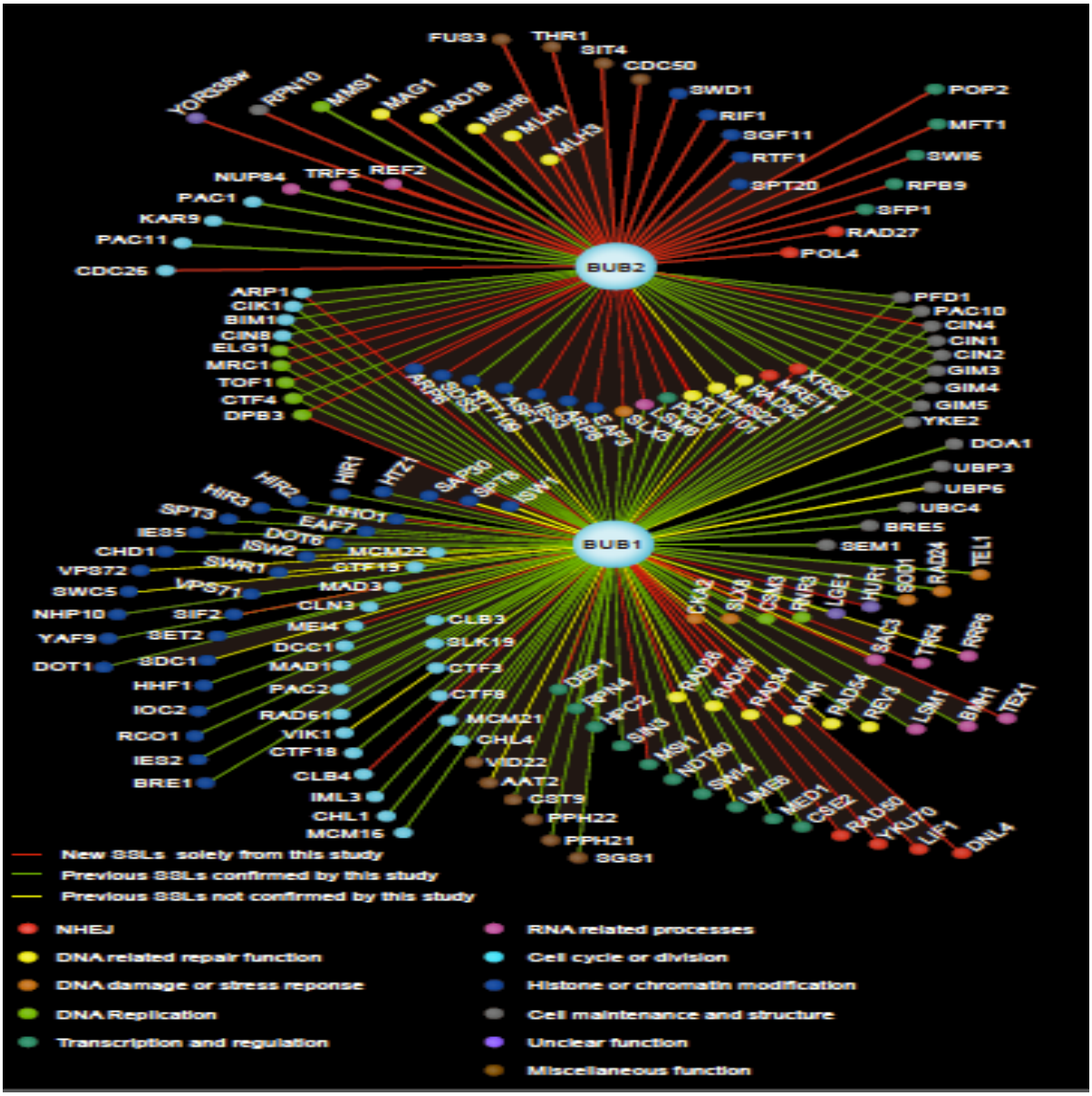


Figure 4.3: Synthetic lethal screens of Bub1 and Bub2 on DNA-repair related targets. Network showing synthetic sick and lethal (aggravating) interactions (colored edges) between Bub1 and Bub2 and the components of known and putative DNA-repair related processes. Genes belonging to specific DNA-repair processes are shown with similar color coded nodes. In each process, interactions that were reported only from previous studies (yellow), novel (red), and previously reported and validated by our screens (green) are displayed.

Overexpression-based Phenotypic suppression array (PSA) analysis for *BUB1*, and *BUB2* were performed as in (Alamgir et al. 2010). The suppression analyses were carried out in the presence of DNA damage agents hydroxyurea (HU) and bleomycin. Those gene deletions whose sensitivity to drug treatment were compensated by the overexpression of target genes were selected. These genes may represent overlapping/related functions to the target genes. Comparing colony growth size of overexpressed-deletion strains to deletion set suggest that overexpression of *BUB1* and *BUB2* compensated for deletion of several key genes involved in NHEJ **(**Figure 4.4). These data further support a role for *BUB1* and *BUB2* in DSB repair.

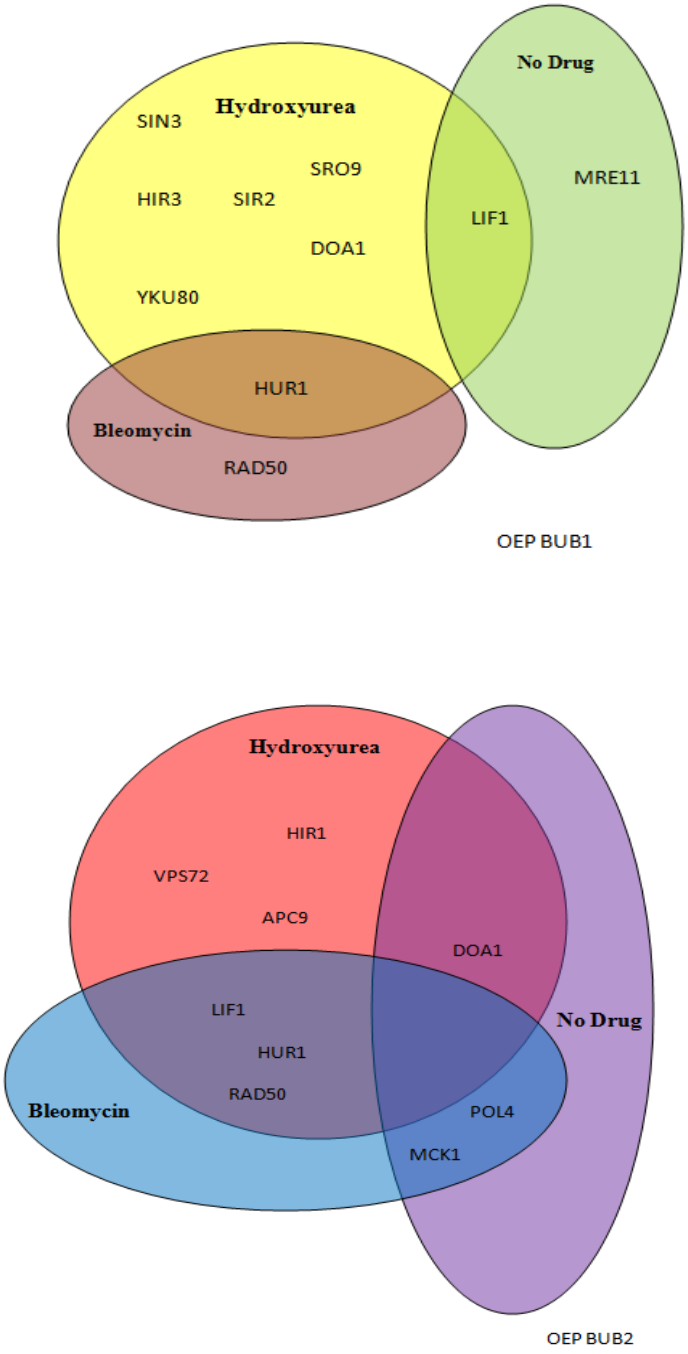


Figure 4.4: Venn diagrams of overexpression (OEP) of *BUB1* and *BUB2*. (A) Venn diagram showing that the overexpression of *BUB1* compensated for deletion of several key genes involved in NHEJ process in the presence of DNA damage agents bleomycin and HU. (B) Venn diagram showing that the overexpression of *BUB2* compensated for deletion of several key genes involved in NHEJ process in the presence of both bleomycin and HU.

4.4.4 Bub1 and Bub2 participates with Apc9, Clb2, and Swi4 factors in NHEJ repair

Cell cycle arrests by the SAC in response to DNA damage hinders the activation of the APC (Brito et al. 2006), which is involved in the regulation of cyclin-dependent kinases such as Cdc28 (Enserink et al. 2010) and SBF transcription factors (Costanzo et al. 2004). While several APC (e.g., *apc9*, *cdc26*, *swm1*), cyclins (e.g., *clb2, clb5*), and SBF (e.g., *swi4*, *swi6*) components that are not essential for viability exhibited reduction in NHEJ, we choose *apc9*, *clb2*, and *swi4* for further investigations, as loss-of-function of these alleles from our *in vitro* plasmid-based DSB repair screens showed a roughly 15 to 40% reduction in NHEJ repair compared to others (Appendix B.3).

Furthermore, these cell cycle regulatory factors are particularly crucial for cell survival as loss of *apc9* leads to reorganization of the APC and degradation of mitotic APC substrates (Zachariae et al.1998, Page et al.2005), whereas the mitotic cyclin, Clb2 is primarily involved in the maintenance and assembly of spindle during the G2/M phase of the cell cycle, where SAC is expected to be active (Grandin and Reed 1993, Schwob and Nasmyth 1993). As well, previous biochemical evidence suggests that the binding of Clb2 with the ankyrin repeats of the DNA-binding transcription factor, Swi4 during the G2/M phase dissociates SBF complex by disrupting Swi4-Swi6 interaction (Siegmund and Nasmyth 1996, Baetz and Andrews 1999). This previous knowledge on the biological interplay among these individual components enabled us to examine further the joint influence of Bub1 and Bub2 checkpoint proteins on Apc9, Clb2, and Swi4 for NHEJ activity.

Indeed, we found that double deletion combinations of *bub1* and *bub2* with *apc9*, *clb2*, and *swi4* showed no further decrease in NHEJ repair efficiency to those of the single mutants (Figure 4.5A). Consistent with this, HO-induced chromosomal DSBs by the addition of galactose in JKM139 strains lacking both *bub1*/*bub2* and *apc9*/*clb2*/*swi4* double mutants also showed reduced (p-value < 0.05) colony formation to the extent similar to the corresponding single mutants (Figure 4.5B), suggesting an intimate coupling of Bub1 and Bub2 with Apc9, Clb2, and Swi4 in NHEJ repair.

4.4.5 Apc9, Clb2, and Swi4, like Bub1 and Bub2 are required for resistance to DNA damage

As genotoxic agents induce single-and double-strand breaks (Tam et al. 2007), we compared the sensitivity of the yeast *bub1* and *bub2* mutant strains to DNA damage induced by the exogenous genotoxic agent, HU that is known to arrest DNA replication and synthesis by inhibiting ribonucleotide reductase and limiting 2′-deoxynucleoside 5′-triphosphate pools, which initiates subsequent chromosomal breaks (Koc et al. 2004). Strains deleted individually for Swi4 subunit of the SBF complex are known to be hypersensitive to HU-induced DNA damage (Queralt and Igual 2005). Consistent with this, like the positive NHEJ repair mutant strain *yku70*, loss of *bub1* and *bub2* increased the sensitivity of the yeast cells to HU to the same extent as *apc9*, *clb2*, and *swi4* single gene deletion mutations (Figure 4.5C). The observed phenotypic defect of the single gene deletion mutants was further reconfirmed by restoring the HU resistance of the respective null mutant strains to wild-type levels through ectopic expression of these factors in *trans*

(Figure 4.5C; representative *bub1* and *bub2* phenotypic complementation based on plasmid rescue shown).

Furthermore, double deletion combinations of yeast *bub1* and *bub2*, respectively with *apc9*, *clb2*, and *swi4* displayed similar levels of sensitivity to HU as those of the single mutants (Figure 4.5C). Collectively, the lack of synergism in the *bub1*- or *bub2*-*apc9*/*clb2*/*swi4* double knock-out strains suggests that Bub1 and Bub2 are likely to function with Apc9, Clb2, and Swi4 in the same DNA-repair pathway for maintaining genome integrity, consistent with the plasmid-based NHEJ repair and GI screening.

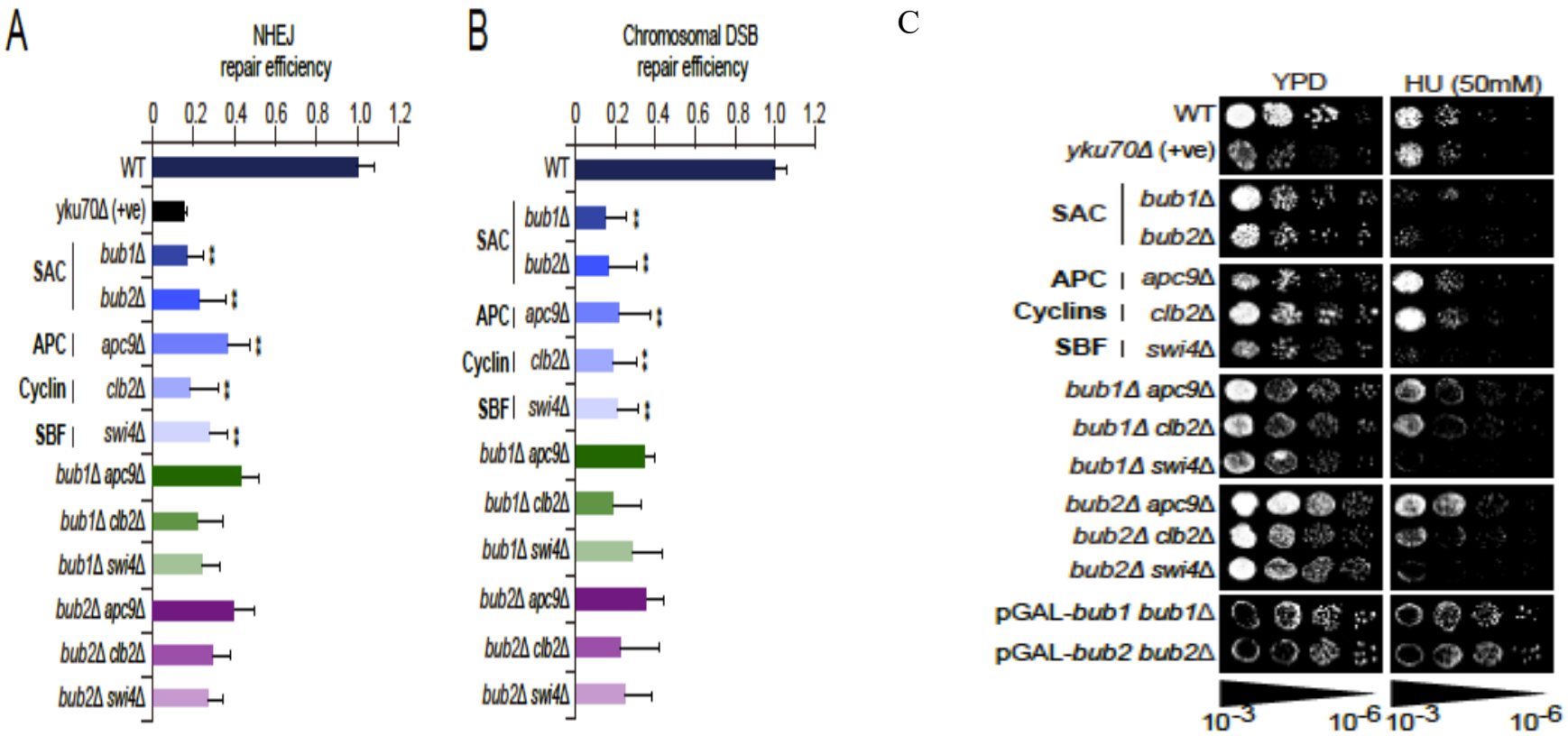


Figure 4.5: Bub1 and Bub2 functions jointly with APC, cyclins and SBF to promote NHEJ. NHEJ (A) and chromosomal DSB (B) repair efficiency of the indicated *bub1* or *bub2* double mutants and their corresponding single mutants. Highlights indicate genes tested in APC, cyclins, and SBF. (C) Serial cell dilution assay showing the growth inhibition of the indicated strains exposed to hydroxyurea (HU; 50mM). Phenotypic complementation of *bub1* and *bub2* is shown using a pGAL vector expressing wild-type copies of *bub1* and *bub2* in their respective mutant background on galactose-containing medium treated with and without HU. Data (A, B) are represented as the mean ± SEM (n = 3); asterisk indicate significant (Student's *t*-test; *p*-value ≤0.05) difference between mutant and WT.

4.4.6 Overexpression of *BUB1* and *BUB2* can recover DNA damage sensitivity phenotypes in *Δrad50* deletion strain mutant in spot test sensitivity analysis

To confirm some of the observations made by large-scale PSA analyses above, serial dilution drug sensitivity analysis was performed with DNA damage inducing agents bleomycin and HU. As explained earlier, if a gene of interest is involved in DNA repair, it may be expected that its deletions cause increased sensitivity to different DNA damaging agents. Serial dilution of a set of key NHEJ deficient deletion strains, the same set of deletions strains carrying an overexpression of a target gene (*BUB1* or *BUB2*), and wild type (control) were plated on media containing DNA damaging agents. Our analysis verified a number of observations we made above. For example, *Δrad50* showed sensitivity to bleomycin and overexpression *BUB1* compensated for this sensitivity of *Δrad50* suggesting a possible functional overlap for *BUB1* and *RAD50* (Figure 4.6). These observations confirm that the above large-scale analysis can be successfully used to identify compensation between genes.

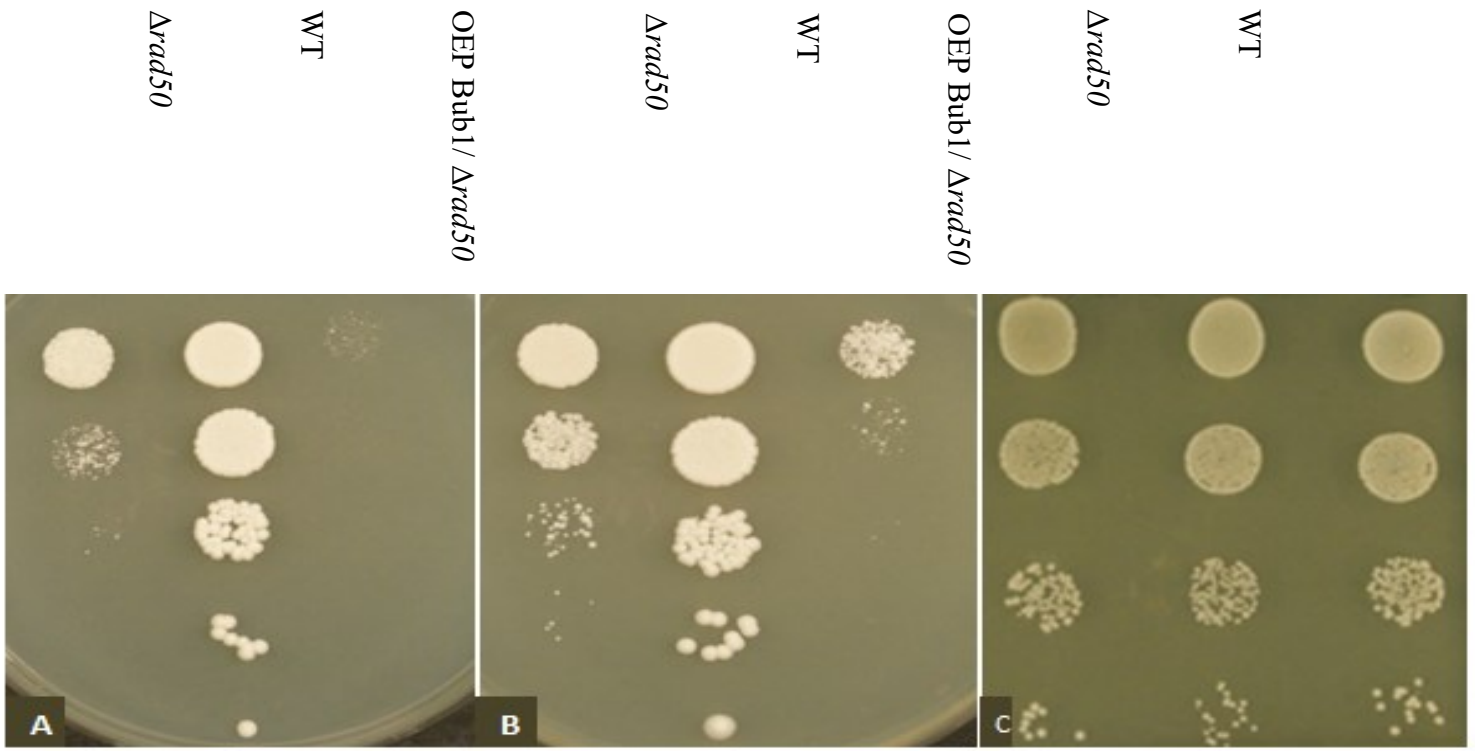


Figure 4.6: Serial cell dilution assay. (A) 1 µg/ml bleomycin, (B) 0.5 µg/ml bleomycin, (C) Control. Deletion of *RAD50* makes the cells sensitive in the presence of different concentration of bleomycin (0.5-1 µg/ml). Overexpression (OEP) of Bub1 compensated for deletion of *RAD50*.

4.5 Discussion

Understanding how the cell manages DNA damage through DSB repair pathways is of fundamental importance, as incorrect repair of the DSBs by NHEJ can lead to genomic instability, cancers, and cell death (Saberi et al. 2007, Shrivastav et al. 2008). Genetic investigations have identified gene products (e.g., YKu70, Mre11-Rad50-Xrs2) operating in NHEJ, a process that has long been appreciated for its primary role in recognizing and repairing DSBs to maintain genome integrity (Shrivastav et al. 2008). However, the identity, mechanism, and regulation of many factors that exist in the canonical NHEJ pathway still remains unknown. In this study, using a comprehensive plasmid-based DSB repair screening approach (Han et al. 2007), we provide evidence that there are several new targets of *S. cerevisiae* that operate in NHEJ, and we characterized in further detail two components of the SAC pathway, Bub1 and Bub2.

The growing body of literature evidence suggests that DSBs caused by DNA alkylating agents such as methyl methane sulfonate (MMS) and HU activate the SAC, independent of the kinetochore (Stracker et al. 2009), and that activation involves many of the signaling proteins that are regulated by DDR. For example, the key mitotic regulator Pds1 (also known as "securin" in humans), a downstream effector of the SAC, is associated with DNA damage checkpoints as well as with the subunit of a DNA-dependent protein kinase involved in NHEJ repair, underscoring the intricate relationship between the SAC and DDR surveillance pathways (Cohen-Fix and Koshland 1997, Featherstone and Jackson 1999, Wang et al. 2001). Activation of these pathways in response to stress or DNA damage triggers phosphorylation changes of the checkpoint

kinase Rad53 and γ-H2A, which recruit key substrate proteins involved in DSB repair (e.g., transcription cofactors such as Swi6), and serve as molecular signatures for DSB formation (Rogakou et al. 1998, Lee et al. 2003). This rapid activation by Rad53 slows down the checkpoint proteins by delaying the cell cycle progression and nuclear division, which in turn leads to the accumulation of large budded cells (Lee et al. 2003).

While we may have missed some epistatic interactions, our genetic screening indicates that Bub1 and Bub2 are aggravating with genes operating in DNA-repair-related pathways, including the subset of well known DSB genes in NHEJ. Previous observations have indicated that the SAC operates through the regulation of the APC, which, in association with Cdc20, ubiquitinates and targets mitotic cyclins for degradation under the tight regulation of SBF transcription factors (Brito and Rieder 2006, Kim et al. 2008). Decreased regulation of mitotic cyclin (Clb2) levels in response to SAC (Bub1 and Bub2) activation, as well as the NHEJ defects caused by the SAC (*bub1* or *bub2*), APC (*apc9*), cyclin (*clb2*), and SBF (*swi4*) had led to the notion that the components involved in these processes are likely to function jointly on a common biochemical pathway. Our findings indeed indicate that the double-mutants of *bub1* or *bub2* with *apc9*, *clb2*, or *swi4* genes had no additional loss of NHEJ efficiency as compared to their respective single mutants. Likewise, the cellular sensitivity of *bub1* or *bub2* with *apc9*, *clb2*, or *swi4* double mutants in HU or BLM were no more sensitive than their corresponding single gene deletion mutant strains, strongly suggesting that Bub1 or Bub2 likely act in concert with Apc9, Clb2, or Swi4 in the NHEJ pathway.

While the results presented in this study primarily emphasize the digenic combination of *bub* with *apc9*, *clb2*, and *swi4* in NHEJ repair, additional relationships

may have been overlooked, as we did not test the role of Bub proteins with all of the components of the APC and all cyclins. Conversely, there were few non-essential components of the APC, and cyclins that we tested failed to show reduction in NHEJ, suggesting that only certain components of the APC and cyclins tend to function specifically in NHEJ by cooperating with Bub1 and Bub2, while others may either have a role in an unrelated process or an opposing (i.e. antagonistic) function with Bub1 and Bub2. The latter notion is indeed supported by the findings from the quantitative *bub1* and *bub2* genetic screens in distinct types of DNA damaging conditions (Guenole et al. 2013), which showed positively (i.e. shared relationship) and negatively (inhibitory or antagonistic relationship) correlated genetic interaction profiles with certain components of APC, indicating specialized functionalities.

Altogether, our results support a model where Bub1 and Bub2 proteins act as DNA damage sensors, triggering an enhancement of NHEJ activity in response to DNA damage. This is consistent with the synergistic interactions previously observed between DSBs and SAC genes (Dotiwala et al. 2010), as well as with the role of the Bub1 kinetochore protein in the DDR signaling pathway (Yang et al. 2012). Moreover, our model suggests that besides the functionally distinct role played by Bub1 and Bub2 in activating the mitotic checkpoint (Fraschini et al. 1999), they function in cooperation with a suite of APC, cyclin, and SBF factors to promote the repair of DNA breaks in NHEJ. While further mechanistic work is required to understand how these various components contribute to the canonical NHEJ, the results presented here elucidate a fundamental role for Bub1 and Bub2 in the cellular response to DSBs mediated by the NHEJ repair pathway.

In summary, we have generated a valuable resource by identifying a comprehensive set of potential candidates governing the NHEJ repair pathway. Conservation of these gene candidates in human and its relationship to cancerogenesis (Marcotte et al. 2012) (i.e. breast, pancreatic) further indicates a broad applicability of these findings to inform future study aimed at characterizing the mechanistic function for some of these variants in human tumors and in the development of target- and mechanism-based therapeutics.

5 Chapter: Translational repressor *SBP1* regulates the efficiency of non-homologous end joining repair in *Saccharomyces cerevisiae*

Contribution: I worked out the hypothesis, experimental design and development of the project. Dr. Bahram Samanfar and Daniel Burnside were involved in the analysis of the data obtained from protein-protein interaction prediction and Pourya Honarvar helped with plasmid repair assay experiments.

5.1 Abstract

DNA double strand breaks (DSBs) can be repaired through two independent repair pathways; non-homologous end joining (NHEJ) and homologous recombination (HR). The NHEJ pathway is highly conserved from yeast to human and directly ligates two broken strands of DNA. Considering the cell as a highly interconnected network of genes and pathways; NHEJ could occur through association with other cellular processes. Here we report a novel role for the member of translation regulatory pathway, Sbp1p, in DSB repair via NHEJ. Sbp1p binds eIF4G and has a role in repression of translation. Previously known as Ssb1, it has been reported as a major single strand RNA binding protein. Deletion of *SBP1* reduces repair efficiency of DNA breaks with cohesive ends but not blunt ends in plasmid repair assays. Deletion of *SBP1* also showed reduction in a chromosomal repair assay. Additionally, deletions of *SBP1* make the cells sensitive to the DNA damaging agents bleomycin and hydroxyurea (HU). Our genetic interaction (GIs) analysis also supported a role for *SBP1* in DNA damage repair pathway. *SBP1* genetically interacts with the NHEJ genes *MRE11*and *VPS75*, as well as the HR gene

RAD52. We hypothesize that *SBP1* binding protein is involved in NHEJ through negative regulation of translation in response to DNA damage.

5.2 Introduction

During the cell's lifecycle, the cellular DNA is constantly exposed to different types of reagents that result from the byproducts of endogenous processes or exogenous sources such as chemical agents or ionizing radiation (IR) that induce DNA damage (Friedberg et al. 2004, Zeman and Cimprich 2014 and Ciccia and Elledge 2010). Among different types of DNA damage, double strand breaks (DSBs) are one of the most severe forms of DNA damage. DSBs can affect DNA replication and gene expression that lead to the loss of chromosome fragments, genetic instability or even death (Burma et al. 2006, Zha et al. 2007). In response to DSBs, the cell initiates different mechanisms to repair the breaks. The two primary independent mechanisms responsible for repair of DSBs are Homologous Recombination (HR) and Non-Homologous End-Joining (NHEJ) pathways. These two pathways compete independently of each other for repair of DSBs. The choice of the pathway depends mainly on the form of the DSBs, presence of homologous sequence and the phase of the cell cycle. HR is a prominent repair mechanism in the yeast *Saccharomyces cerevisiae*, and is mainly activated in the late S and G2 phase of the cell cycle. HR is considered to be an error-free pathway (Mao et al. 2008). NHEJ is the error-prone pathway, and is dominant in higher eukaryotes (Mao et al. 2008). Although NHEJ is mainly active in G1 stage, cell' can initiate repair through NHEJ process during all phases of the cell cycle.

In *S. cerevisiae*, NHEJ is initiated via binding of YKU complex (Yku70-Yku80

heterodimer) to the broken DNA ends. YKU complex recruits other NHEJ repair members such as Dnl4/Lif1 and Nej1p to the site of damage (Milne et al. 1996, Chen and Tomkinson 2011 and Yano et al. 2008). The second complex that binds to the broken ends of DNA is the MRX complex, comprised of Mre11, Rad50 and Xrs2 (Emerson et al. 2016). The MRX complex stimulates and initiates the ligation (Chen et al. 2001, Yang et al. 2015). Nej1p binds to the ligation complex (Dnl4/Lif1) via an interaction with Lif1p (Ooi et al. 2001, Emerson et al. 2016). Nej1 also plays a role in a third pathway of DSB repair called alternative-NHEJ (alt-NHEJ), or microhomology-mediated end-joining (MMEJ) (Lee and Lee 2007, Bennardo et al. 2008, Paul et al. 2013). The MMEJ pathway plays an important role in the repair of DNA breaks with blunt ends (Lee and Lee 2007). Although these core protein complexes are the key factors in DSB repair via NHEJ, efficient repair by this process is also depended on a variety of other cellular processes and pathways such as chromatin structure-remodeling (Shim et al. 2005, Bonetti et al. 2013), histone acetyltransferase activity, cell cycle proteins, etc. (Collins et al. 2007, Badura et al. 2012, Mckinney et al. 2013, Braunstein et al. 2007, Silvera et al. 2010).

We believe that in addition to the known factors and pathways involved in NHEJ processes, there is other unidentified proteins that can affect efficient NHEJ repair. In this study, *SBP1*, previously known as *SSB1* for single strand RNA binding protein 1 (Jong et al. 1987) has been identified as a factor playing a role in efficient DSB repair. *SBP1* (YHL034C) is located in the nucleus on chromosome VIII. The protein abundance is 40364 molecules/cell. The protein domain of 28-286 is single-stranded nucleic acid-binding protein. *SBP1* is the eukaryotic initiation factor 4G (eIF4G) binding protein that negatively regulates translation in response to stress and damage. In the case of DNA

damage, it is reported that cells respond by downregulating general protein synthesis (Braunstein et al. 2009). Although translational regulation in response to DNA damage is poorly understood, recent studies show that the level of expression of eIF4G is increased in presence of DNA damage such as human breast cancer (Braunstein et al. 2007, Silvera et al. 2010). This elevation, which is correlated with the translation initiation stage, makes a critical connection between the DNA damage response and expression of DNA repair genes. Due to the possible interaction between translation and expression of DNA damage repair genes, we hypothesize the presence of a cross-communication pathway that can influence both processes of translation and DNA repair. Here, we show that the deletion of the translation regulator gene *SBP1* reduces the efficiency of NHEJ in *S. cerevisiae*. This activity may verify the connection of DNA repair to translation regulation.

5.3 Materials and Methods

Please refer to chapter 3 for yeast and plasmid strains information. NHEJ plasmid and chromosomal DSB repair assays; drug sensitivity and SGA analysis were performed as described in chapter 2 and 3.

5.4 Results and Discussion

5.4.1 Sbp1 physically interacts with a number of DNA damage repair proteins

Generally speaking, interacting proteins are functionally related as proteins often physically interact with one another to perform their functions. Consequently, the

unknown function of proteins can be studied by elucidating the activity of the proteins with which it interacts (Krogan et al. 2006 and Butland et al. 2005). Protein-protein interactions can be detected using various approaches including different computational tools (Pitre et al. 2008 and Muley and Ranjan 2013). To this end, we used a computational method that works based on the co-occurring short polypeptide regions to predict PPIs (Pitre et al. 2008, Pitre et al. 2012). Previously we used that same approach to predict a comprehensive all against all PPI map for yeast (Pitre et al. 2008). Using the same principle, with an updated database of known interacting proteins, here we predicted that yeast protein Sbp1 interacts not only with several translation regulation proteins which has been reported previously (Segal et al. 2006, Mitchell et al. 2013), but also it interacts with several genes involve in DNA damage response. These novel interacting partners include Bre1p, Fun30p, Rtt107p, and Pol32p (Figure 5.1). Sbp1p also interacted with NHEJ proteins Sub1p and Psy2p, and a few chromatin and histone modification proteins, namely Hta1p, Hta2p, Hhf1p and Hhf2p (Figure 5.1). Based on these interactions, we hypothesize that Sbp1p might also play a role in DNA damage repair.

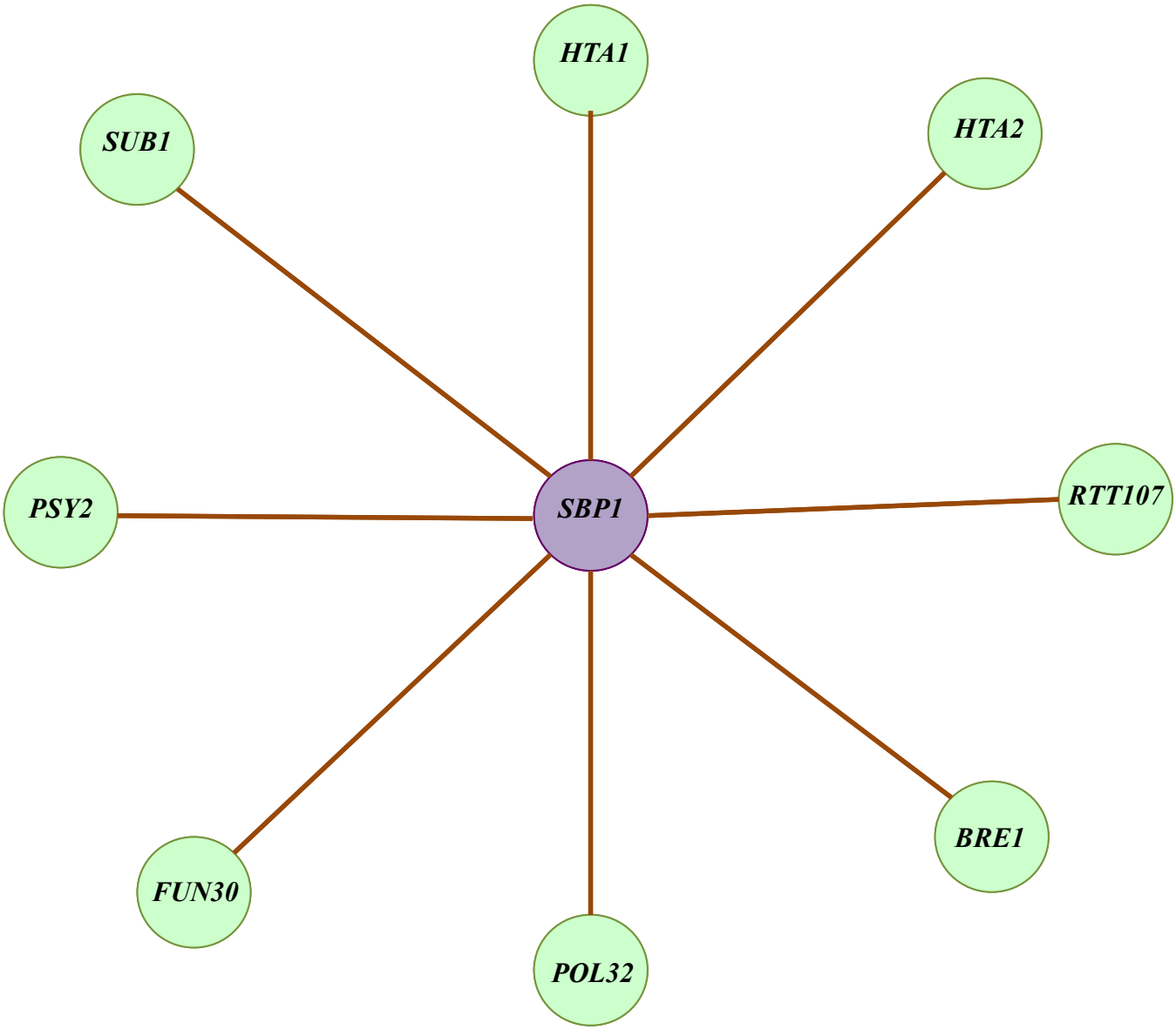
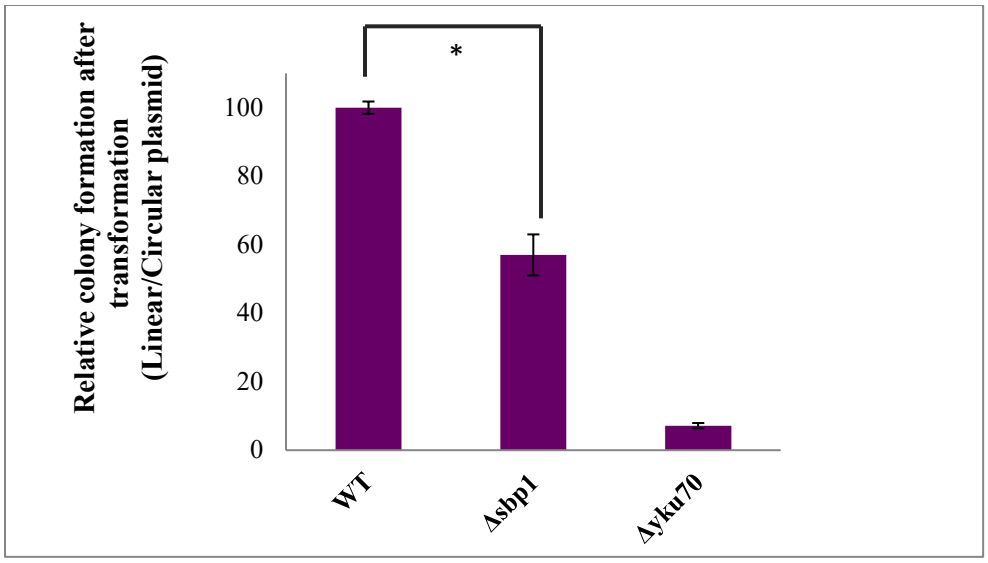


Figure 5.1: PPI prediction for Sbp1p. Sbp1p is predicted to interact with several proteins involve in DNA damage repair process including Pol32p, and Bre1p. It is also predicted to interact with two NHEJ proteins Psy2p and Sub1p. The analysis was performed at 99.95% specificity (a measure for false positive prediction).

5.4.2 Plasmid repair analysis suggest a role for *SBP1* in NHEJ

Plasmid-based repair assays have been utilized to evaluate activity of novel genes involved in NHEJ repair pathway (Shim et al 2005, Jessulat et al 2008, Omidi et al 2014). For this assay, p416 and YCplac111 plasmids were digested with XbaI and SmaI restriction enzymes at corresponding restriction sites within a regions that has no homology to yeast chromosomes. This allows investigating the role of genes in repair of overhangs and blunt ends through NHEJ, respectively. In this study, deletion of *SBP1* was used to examine its involvement in overhangs and blunt ended breaks in NHEJ repair. Equal amount of circular and linearized plasmids were transformed into Δ*sbp1*, WT and Δ*yku70* (as apositive contorl) strains. The ratio of repaired linearized plasmid to circular plasmid of mutant were calculated and normalized to the WT ratio. It was observed that the efficiency of repair in *Δsbp1* with overhangs and blunt end were 57% and 96.33%, respectively compared to the WT (Figure 5.2). This suggests that deletion of *SBP1* reduces the repair efficiency in overhangs ends but not blunt end (p-value < 0.05) compared to the WT. Our data indicate that *YKU70* mutant strains (*Δyku70)* were also deficient in repair of broken end with overhangs but not blunt ends. This is in agreement with previous studies indicating that the repair of DSBs with blunt ends through NHEJ acts independent of YKU complex (Boulton and Jackson 1996, Yu and Volkert 2013). As presented in figure 5.2 our results suggest the potential involvement of *SBP1* in repair efficiency of overhangs through NHEJ.

A.



B.

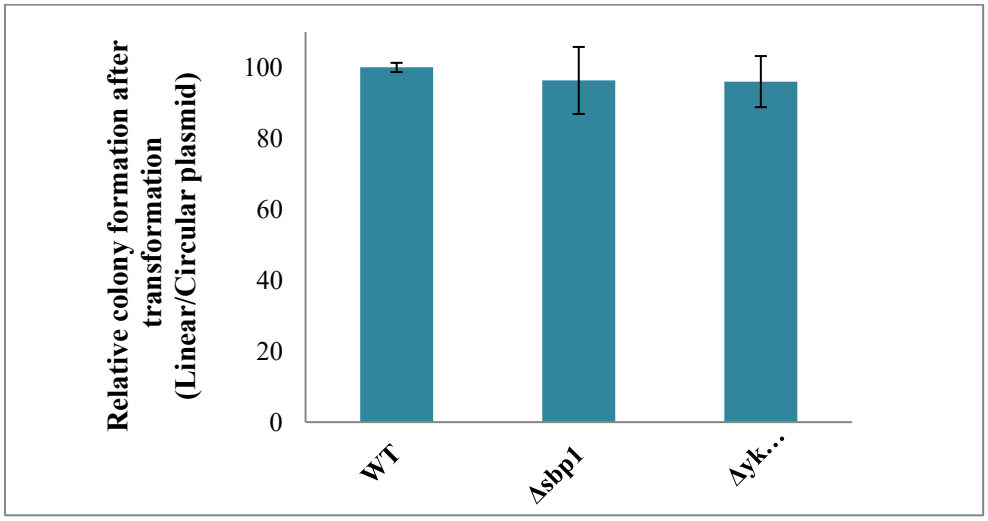


Figure 5.2: Plasmid repair efficiency. Deletion of *SBP1* reduced repair efficiency of breaks with cohesive ends (A) but not blunt ends (B) in a plasmid repair assay. Wild type values are normalized to 100% and other values are related to this value. Experiments were repeated at least five times; p-value ≤ 0.05 calculated using Student's t-test.

5.4.3 The effect of Sbp1p on the efficiency of NHEJ in a chromosomal context

The plasmid repair assay has shown reduction in repair deficiency of *Δsbp1* in NHEJ. Next, we tested the involvements of Sbp1p in NHEJ efficiency in a chromosomal context. In this assay, *SBP1* was deleted in a JKM139 strain. The JKM139 strain has a GAL-promoter linked to HO-endonuclease, which is expressed in the presence of galactose. This results in chromosomal breakage at the HO specific sites, which have no homology to other sequences in the *S. cerevisiae* genome (Moore and Haber 1996). Deletion strains of *SBP1*, WT and *YKU70* (as a positive control) were grown to saturation and transferred onto solid media containing glucose or galactose. The number of colonies formed in deletions strains was used to measure the repair efficiency in comparison to WT. As expected, *Δsbp1* showed reduction in the efficiency of repair compared to WT in JKM139 background (Figure 5.3**).** These data are in agreement with the plasmid repair assay, further supporting the involvement of Sbp1p in the efficiency of NHEJ.

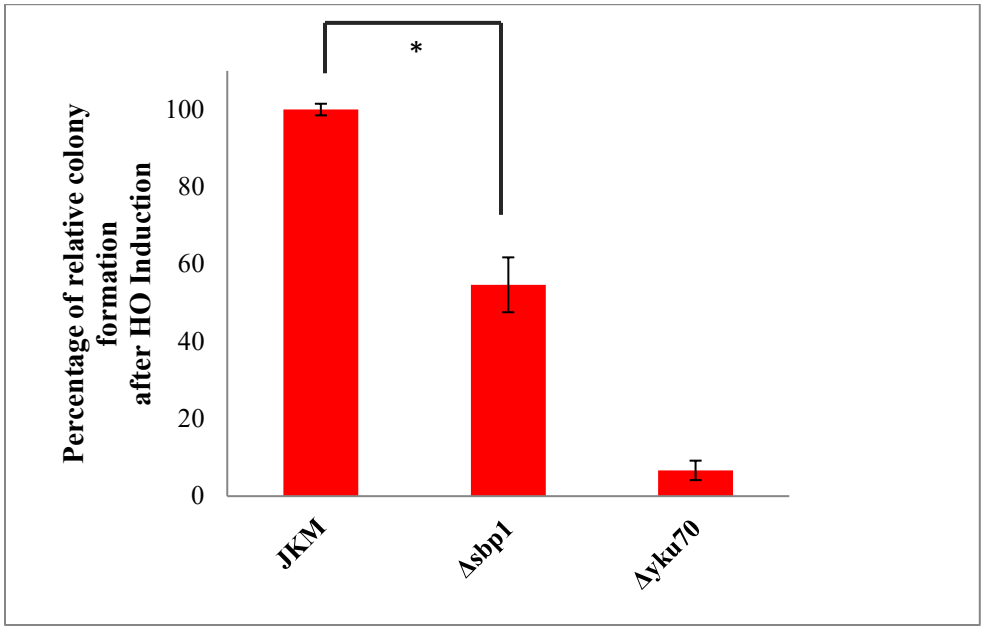


Figure 5.3: Repair efficiency of chromosomally induced breaks by HO-endonuclease in JKM139 strains. Chromosomal breaks are induced by HO-endonuclease in JKM139 strains. Deletion of *SBP1* reduced viability under DSB induction. JKM (WT) was normalized to 100% and other values were normalized to this value. Experiments were repeated at least 5 times each. *P*-value ≤ 0.05 calculated using Student's *t*-test. Δyku70 was used as a positive control.

5.4.4 Drug sensitivity analysis of *SBP1*

It has been well studied that the deletion of genes involved in DNA damage repair will likely cause an increase in the sensitivity of the mutant strain to DNA damaging agents (Birrell et al. 2001, Jelinsky et al. 2000 and Chang et al. 2002). In this study, we used DNA damaging drugs bleomycin and HU to examine sensitivity of the *Δsbp1* strain. Bleomycin promotes the production of free radicals which can lead to DSBs (Cannan and Pederson 2016, Della Latta et al. 2015, Peral et al. 2015), and HU induces DNA replication errors by nucleotide depletion, which results in DSBs (Rittberg et al 1989, Koç A et al. 2004). In this study, *Δsbp1* mutants show sensitivity to both HU and bleomycin compared to WT (Figure 5.4). These results further support an involvement for *SBP1* in DNA damage repair.

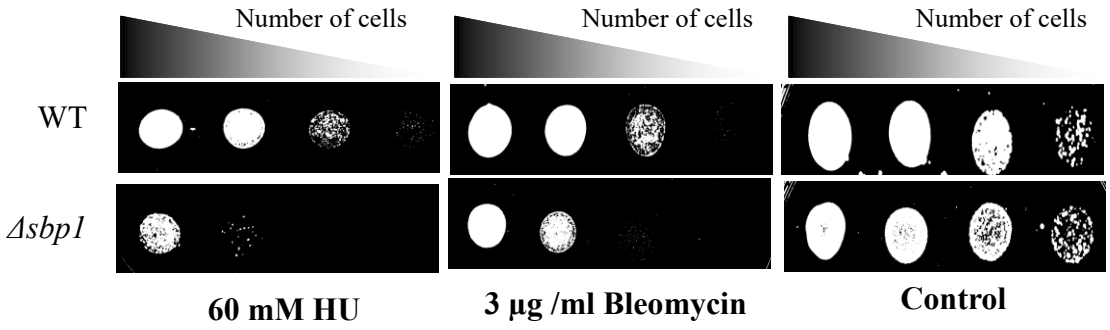


Figure 5.4: *SBP1* sensitivity to DNA damage drugs. Deletion of *SBP1* showed sensitivity to HU (60 mM) and bleomycin (3 µg/ml). Cell survival is represented as spot tests. Experiments were repeated 5 times with similar results.

5.4.5 Genetic interaction analysis supports a role for *SBP1* in DNA damage repair

Genetic interaction (GI) analysis is one way to study the functional relationships of different genes. It is often formed amongst genes with similar functions. It can be explained through overlapping pathways. In simple terms, when deletion of two genes together result in a phenotype that significantly differs from deletion of each gene alone it is said that the two genes are genetically interacting. GIs are divided into negative and positive interactions. In negative interactions, deletion of two genes resulting in more extreme phenotype (or cell lethality), whereas in positive interaction the double mutant fitness is less severe or compensated (Baryshnikova et al. 2010) in comparison to single mutants. To investigate the activity of the target gene, the GIs between target gene *SBP1* and the set of DNA damage repair genes were carried out. The DNA damage array (DDA) contains 384 mutant strains for genes functioning in DNA repair, cell cycle chromatin modifications, checkpoints and related processes. We used the principles of Synthetic Genetic Array (SGA) analysis to evaluate GIs between genes (Tong et al. 2001). In this assay, the target gene, *SBP1* was crossed with the DDA, to investigate interacting partners through overlapping pathways. As a control, the target gene was crossed with a second array of random gene deletions with no reported function related to DNA repair process. The double deletion mutants were scored for fitness by comparing the size of the colonies (Tong et al. 2001, Memarian et al. 2007). SGA analysis indicated that *SBP1* genetically interacts with key member of genes involved in NHEJ such as *MRE11*, *VPS75* as well as HR gene *RAD52.* It also interacts with several histones related proteins such as *HTA1*, *HTB1*, and *HTZ1*. These interactions support a role of *SBP1* gene in DNA damage repair with P values of 2.53×10^{-14} (Figure 5.5).

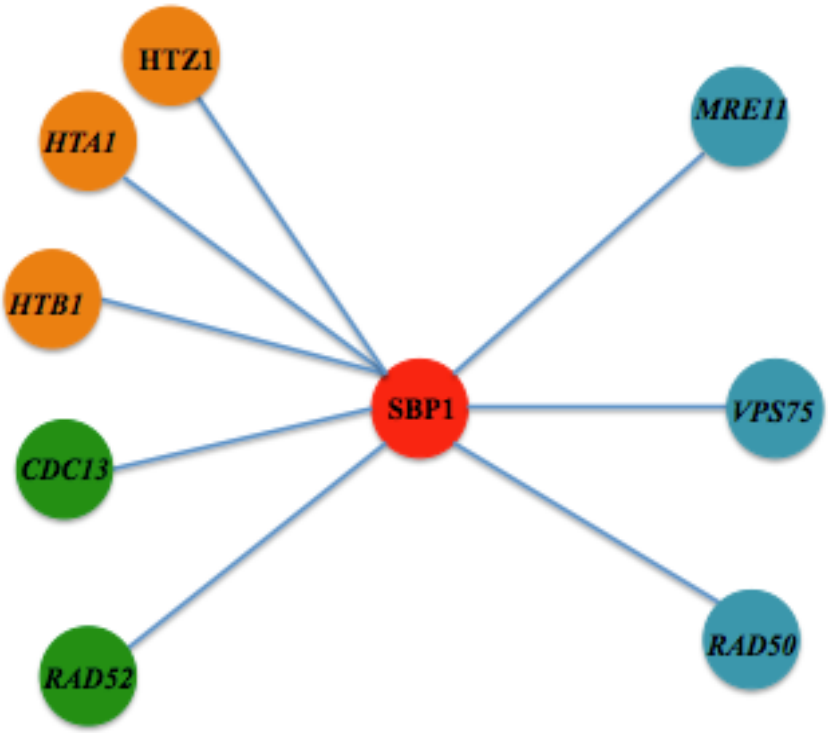


Figure 5.5: Genetic interaction of *SBP1* showed several interactions with NHEJ genes (blue) with P values of 6.21×10^{-4}, HR genes (green) with P values of 1.7×10^{-5}, and histone related genes (orange) with P values of 4.89×10^{-9}.

5.5 Concluding Remarks and Future Directions

SBP1, previously known as Ssb1p has been reported as a single strand RNA binding protein (Jong et al. 1987). As mentioned above there may be a correlation between translation initiation and DNA damage responses. It has been reported that Sbp1 functions in the repression of translation by conversion of mRNAs from translation heading for de-capping and mRNA degradation (Segal et al. 2006). Recent studies showed a relationship between translation and mRNA de-capping (Sheth and parker 2003, Cougot et al. 2004). In yeast, de-capping is one of the dominant processes that represses translation. Generally speaking, de-capped RNA will not translate or will translate at a lower efficiency by other initiation methods such as IRES (Internal Ribosome Entry Site) (Komar et al. 2003). Sbp1p protein also binds to eIF4G, one of the key elements in translation initiation, inactivating this important initiation factor, and in this manner can repress translation also. In this work, we propose a novel function for Sbp1p in efficiency of DNA damage repair process via NHEJ through connection with translation regulation in yeast *S. cerevisiae.*

Our analysis showed that NHEJ efficiency of cohesive breaks is reduced in deletion mutants for *SBP1* but not in blunt ends in plasmid repair assay. Deletion of *SBP1* also showed reduction in chromosomal DSB breaks. Furthermore, *SBP1* shows GIs with genes involved in DNA damage repair. Future investigation of the relationship between *SBP1* and key NHEJ genes (for example by overexpression of *SBP1* with DNA damage genes) will help us better understand the involvement of this gene in NHEJ pathway. Investigating the activity of *SBP1* in the context of NHEJ can further help us elucidate the communication and cross-talk between translation and NHEJ.

6 Chapter: Conclusion and future directions

6.1 Final Thoughts and Concluding Remarks

During the cells' lifecycle, cellular DNA is constantly exposed to different types of DNA damage. Among different types of damages DNA double strand break (DSB) is one of the most severe forms as it could directly affect the cells sustainability. It could lead to chromosomal rearrangement, gene mutations, and cell death (Aparicio et al. 2014). A study performed by Frappart et al. 2008, demonstrated that the inability for cells to repair DSBs may cause neurodegenerative diseases including the formation of brain tumors (Frappart et al. 2008). Primary immunodeficiency syndromes are also linked to defects in DSB repair (Gennery et al. 2006). Mutations in key DSB repair genes within the Homologous Recombination (HR) and Non-Homologous End Joining (NHEJ) pathways have been involved in the onset of cancer (Shrivastav et al. 2008). Mutations in *RAD51*, *BRCA1*, *BRCA2* and DNA PK genes have been linked to different types of cancer and cancer susceptibility (Dietlein et al. 2014, Aparicio et al. 2014, Wiegmans et al. 2014). Patients with immunodeficiency syndromes have mutations in NHEJ genes DNA PK, Lig4 and XLF, which is linking NHEJ with immune disease (Woodbine et al. 2014). Consequently, understanding the comprehensive mechanisms of how the cell governs DNA damage via DSB repair pathways is important.

The NHEJ pathway is highly conserved between yeast and human. As a result, the availability of gene knockout (~5000 genes) and overexpression arrays and simple genetic and sexual life cycle of *S. cerevisiae* made this organism an ideal choice for studying functional genomics. In addition, genetic manipulation in yeast is common as

various methodologies and advanced tools for genome manipulation exist. Consequently, we feel that *S. cerevisiae* is an ideal eukaryotic model organism for functional genomic screens to study DSB repair.

There are a series of cellular processes including cell cycle, histone and chromatin modification, transcription etc. that may affect the efficiency of NHEJ. Recent studies in our lab as well as those of others have shown the new involvement of several genes in the NHEJ process (Jessulat et al. 2008, Ruiz et al. 2013, Jessulat et al. 2015, Heo et al. 2015), suggesting the presence of other novel genes that might affect this repair pathway. It was observed that these newly identified genes are involved in various processes that directly or indirectly influence the efficiency of NHEJ pathway. For example, *SUB1* plays a role in the fidelity of NHEJ (Yu et al. 2013). *TBF1/VID22* affect chromatin structure in the presence of DSBs (Bonetti et al. 2013). *TDP1* regulates the processing of DNA end and the fidelity of NHEJ (Bahmed et al. 2010).

In this thesis, we hypothesized that there are a number of previously characterized or uncharacterized genes that affect NHEJ and genetic regulation of NHEJ. Some of these genes may function through an overlap with other pathways and cellular processes in the cell. Others may play regulatory roles in NHEJ pathway, which is dependent or independent of other DNA repair pathways.

Previous high throughput investigations using large-scale Genetic Interactions (GI), Protein-Protein Interactions (PPIs), gene expression profiles, and RNA sequencing (Brown et al. 2006, Chang et al. 2002, Hendry et al. 2015, Bandyopadhyay et al. 2010, Giaever and Nislow 2014) has confirmed the complex dynamics of network that can govern DSB repair. In addition, bioinformatic tools have also helped in discovering new

elements that can influence DSB repair (Pitre et al. 2008). Much has been learned from these foundational studies promoting us to continue our search for additional genetic factors that can influence DSB repair.

As a part of this thesis we have identified approximately 60 novel genes, which might affect the efficiency of NHEJ. Among these genes, we have selected *PPH3, PSY2*, *HUR1*, *BUB1, BUB2*, and *SBP1* (*SSB1*), for further investigation. These genes were not previously reported to have direct function within DSB repair (see below). At the time, these genes represented a balanced combination of high risk (*HUR1, SBP1*), low risk (*PPH3, PSY2)* and intermediate (*BUB1, BUB2*) research goals.

At the initial stages of this thesis, *PPH3/PSY2* protein phosphatase complex was known to be necessary for Rad53 dephosphorylation and efficient recovery from the checkpoint (Vazquez-martin et al. 2008, O'Neill et al. 2007). *HUR1* was an ORF with uncharacterized function. *HUR1* was found to have an overlap with the *PMR1* gene ,which confers resistance to hydroxyurea (Jordan et al. 2007). *BUB1* and *BUB2* were known as non-essential protein kinases involved in the spindle checkpoint in cell cycle. Bub1p interacted with kinetochore, while Bub2p bond to the spindle (Hardwick, 1998). *SBP1* previously known as *SSB1* was identified as a eukaryotic initiation factor 4G (eIF4G) binding protein which negatively regulated translation in response to exogenous agents (such as UV damage) (Rajyaguru et al. 2012).

We successfully illustrated that all of the selected genes in this thesis do carry a novel function in influencing NHEJ repair process (Figure 6.1). Small-scale plasmid repair assays demonstrated the involvement of all 6 candidates in affecting the efficiency of NHEJ. We also found the involvement of these 6 target genes to be relevant in a

chromosomal context. These findings were supported by drug sensitivity assays showing an increased sensitivity for their deletion mutants to DNA damage drug agents, hydroxyurea (HU), and/or bleomycin. Next, we investigated the large-scale GI (synthetic genetic array or synthetic dosage lethality) between our 6 genes of interest and genes with known function in DNA repair, which showed the interactions between those candidate genes and other genes in related pathway.

We have shown that the activity of Pph3p and Psy2p connect NHEJ repair to cell cycle progression. *PPH3/PSY2* form a phosphatase complex regulates NHEJ through cross-talk and functional connection with checkpoint kinases Rad53p and Chk1p. previous studies showed that Pph3/Psy2 regulates recovery from the Rad53 DNA damage checkpoint. In the absence of Pph3/Psy2, Rad53p dephosphorylation is not accrued and the DNA synthesis is delayed during recovery from DNA damage (O'Neill et al. 2007). Checkpoints can be activated in response to DNA damage before cells enters to mitosis. Checkpoint proteins Tel1p, Mec1p and Rad53p play critical roles in regulation of key proteins of the DNA repair pathway (Ataian and Kerbs 2006). Any errors in the checkpoint mechanisms may cause genomic instability (Myung and Kolodner 2002). So, this is an important finding which may help to better understand the NHEJ process and the connection between different pathways in the cell.

We report a role for functionally uncharacterized ORF *HUR1* as a novel NHEJ-related gene that reduces the efficiency of NHEJ in both plasmid repair and chromosomal repair assays in *S. cerevisiae.* Our predicted PPIs data demonstrate the physical interaction between *HUR1* and *TPK1.* We provide evidence connecting the involvement

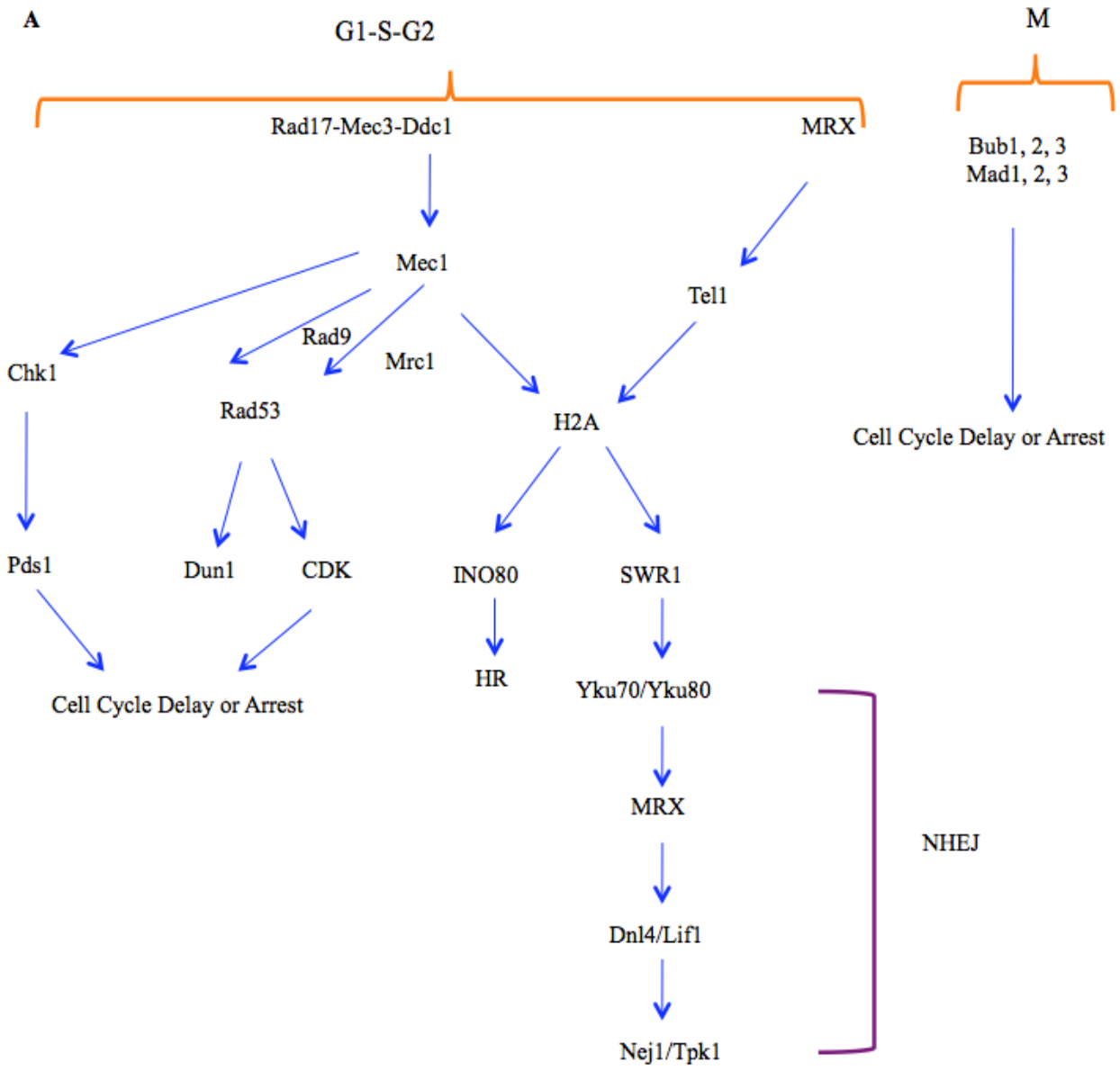
of Hur1p in NHEJ through association with Tpk1p and Nej1p. Characterizing the role for *HUR1* may help understanding the elucidation of new pathways.

In this study, we also have shown, Bub1/Bub2 are involved in mitotic exit as well as NHEJ process, which we have shown the presence of connection with Apc9p (APC), Clb2p (cyclin), and Swi4p (SBF) proteins in the NHEJ repair pathway. It was also shown that either single mutants *bub1* and *bub2* or their double mutants with *apc9*, *clb2* and *swi4* showed sensitivity to DNA damaging drugs. Previous study has been shown; DNA damage triggers the Spindle Assembly Checkpoint (SAC) pathway in yeast, preventing cell cycle progression prior to cell commitment to anaphase (Bloom and Cross 2007). Both the Bub1 and Bub2 (herein referred as "Bub") branches of the SAC pathway also limit cell cycle progression by preventing the activation of the Anaphase Promoting Complex (APC).

SBP1 is the eukaryotic initiation factor 4G (eIF4G) binding protein, which we have shown its sensitivity to both HU and bleomycin DNA damage agents. We also reported its involvement in NHEJ pathway through its possible connection with translation regulation genes that must be studied further to better understand the functional association between pathways.

The important observations we made here provide evidence for existence of possibly other novel functions for different genes in the context of NHEJ. The screens that we have used in this thesis are by no means exhaustive. Like all screens, they suffer from high rates of false negatives. There is also the possibility that a gene may only gain a new function under specific conditions, for example under DNA damage conditions such as exposure to UV. In this case it would be very difficult to predict these functions

under standard laboratory conditions. An important message that this thesis carries is that our knowledge of functional genomics is by no means complete. There is a need for additional screens and methodologies that can be used to investigate novel gene functions in a more comprehensive manner.


A
G1-S-G2
M
Rad17-Mec3-Ddc1
MRX
Bub1, 2, 3
Mad1, 2, 3
Mec1
Tel1
Chk1
Rad9
Mrc1
Rad53
H2A
Cell Cycle Delay or Arrest
Pds1
Dun1
CDK
INO80
SWR1
HR
Yku70/Yku80
Cell Cycle Delay or Arrest
MRX
NHEJ
Dnl4/Lif1
Nej1/Tpk1

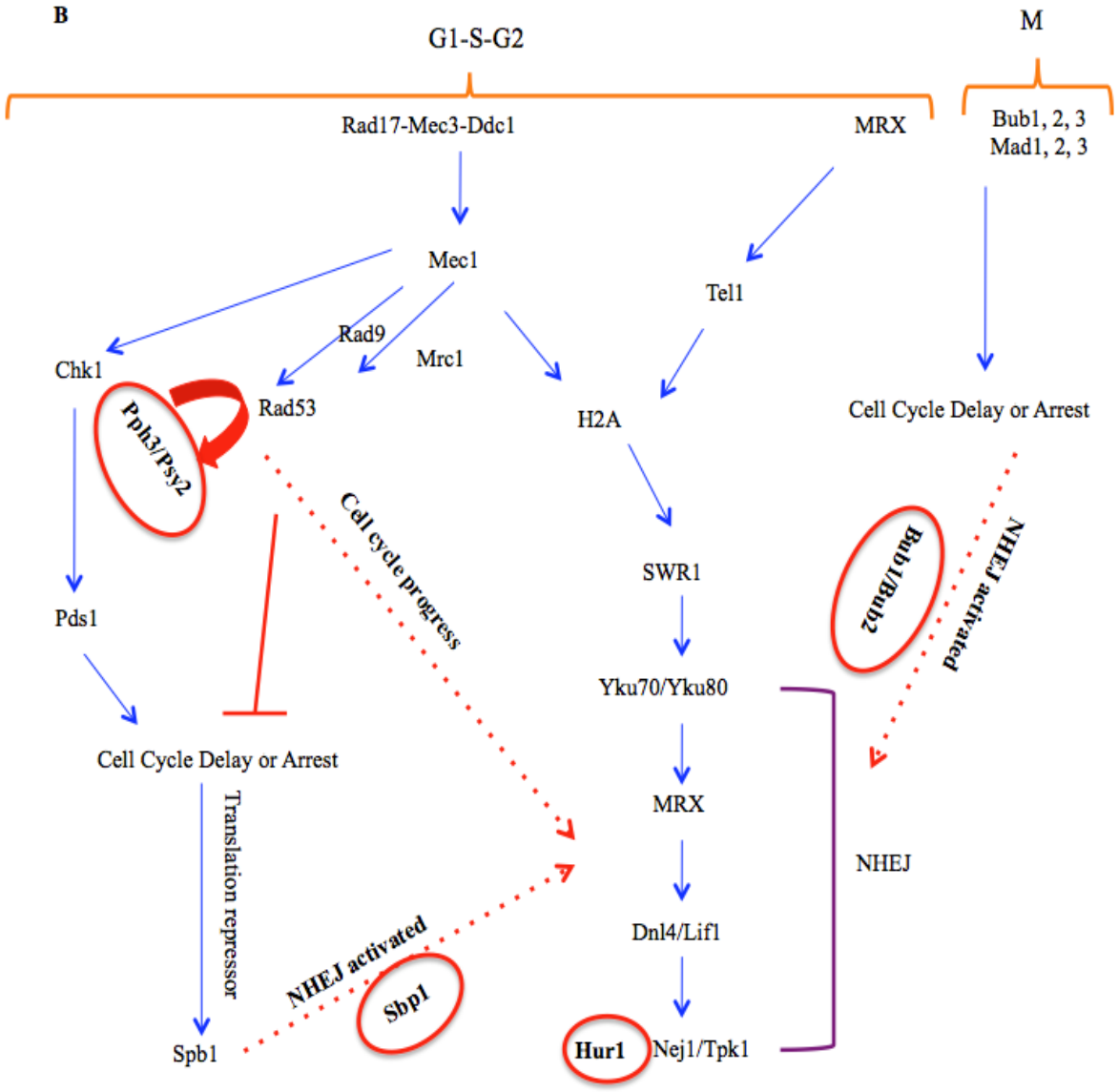


Figure 6.1: DNA repair genes and pathways in *S. cerevisiae.* The orange horizontal lines indicate the cell cycle. (A) The genes previously known to play a role under DNA damage conditions are listed below each phase of the cell cycle. The central signal transduction pathway involving *MEC1*, *RAD53*, *CHK1*, *PDS1*, and *DUN1,* which activated and recruits DNA repair genes in response to DNA damage. (B) The red circles or lines are related to involvement of six novel identified candidates in NHEJ process. All of the selected genes in this thesis do carry a novel function in influencing NHEJ repair process.

6.2 Future Directions

Identification and characterization of novel NHEJ repair genes will lead to a better understanding of the fundamental process of DNA repair. Due to the primary importance of DNA damage in the onset of numerous genetic diseases such as cancer, neurodegenerative disorders and immunodeficiency syndromes, the results produced by this research combined with future studies may be consequential to human health.

In this thesis, we have produced a valuable resource by identifying several novel candidate genes influencing the NHEJ repair pathway. *BUB1*'s NHEJ-linked function appears to be conserved in mammalian cells (Jessulat et al, 2015). Future studies could focus on the conserved homologs of the remaining genes identified in this study (~ 60 of them). Different tools such as advanced PPI prediction tools may also be used to predict additional human PPIs that in turn could be used to predict novel gene functions. Improved GI detection analysis can also be utilized to develop a list of novel genes which influence NHEJ pathway. Combining these two datasets may be useful in the identification of novel gene candidates involved in different repair pathways. Conservation of these genes in humans and their relationship to tumorigenesis (Marcotte et al. 2012) and other diseases further illustrates the importance of understanding of the genes related to different diseases, which may lead to development of new therapies.

In this study we utilized our PPI prediction tool to identify potential interactions between our candidates and the genes, which may play a role in the NHEJ pathway

Pull down assays could further be utilized to indicate the recruitment of our novel candidates to the site of a DNA break. The dependency of recruitment of the target genes

via the key NHEJ genes to the DSBs site could also be investigated. It helps to understand other key genes and overlapping pathways, which may influence NHEJ.

The role of *SBP1* in NHEJ is not fully understood. Additional experiments are needed to investigate the role of *SBP1* in the repair damage process. GI analysis using overexpression of Sbp1p and set of DNA damage mutants could help to investigate relations between genes in overlapping pathways.

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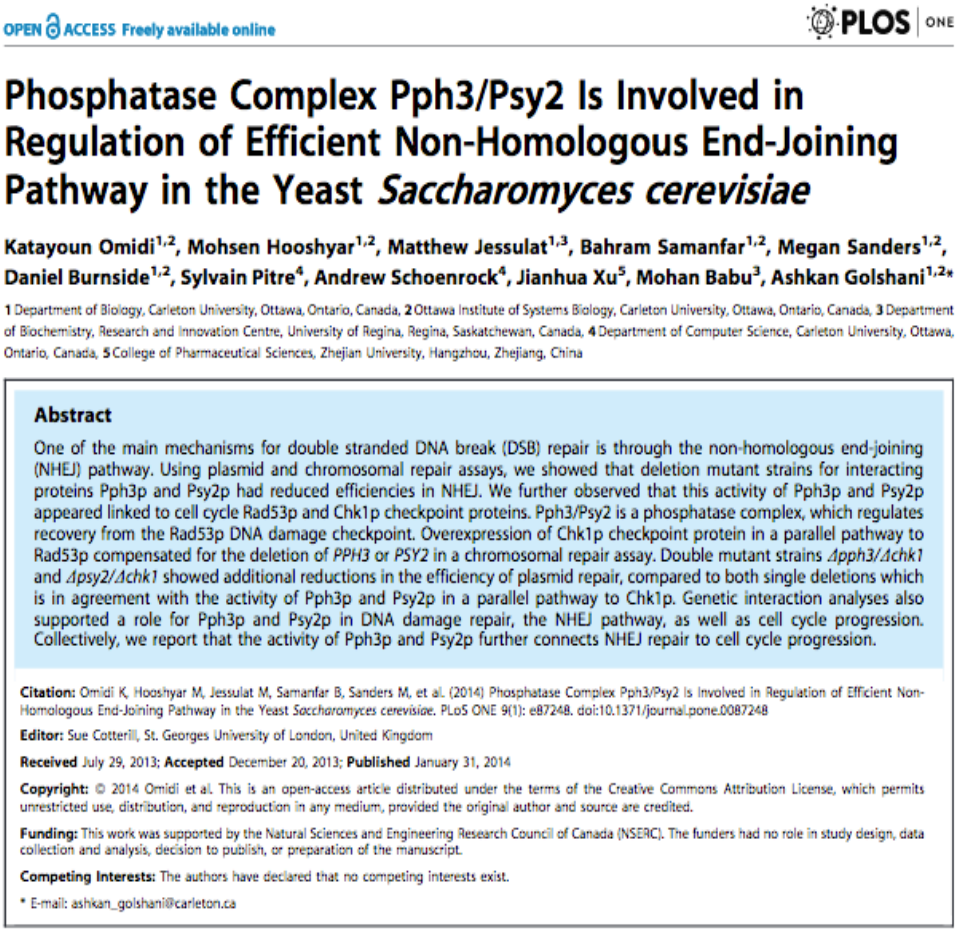
Appendix A

Appendix A.1 List of publications

- **Omidi K**, Hooshyar M, Jessulat M, Samanfar B, Sanders M, Burnside D, Pitre S, Schoenrock A, Xu J, Babu M and Golshani A: Phosphatase Complex Pph3/Psy2 is Involved in regulation of efficient Non-Homologous End-Joining pathway in the yeast *Saccharomyces cerevisiae*. *PLoS One*. 2014;9(1):e87248.



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PLOS ONE

Phosphatase Complex Pph3/Psy2 Is Involved in Regulation of Efficient Non-Homologous End-Joining Pathway in the Yeast *Saccharomyces cerevisiae*

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Abstract

One of the main mechanisms for double stranded DNA break (DSB) repair is through the non-homologous end-joining (NHEJ) pathway. Using plasmid and chromosomal repair assays, we showed that deletion mutant strains for interacting proteins Pph3p and Psy2p had reduced efficiencies in NHEJ. We further observed that this activity of Pph3p and Psy2p appeared linked to cell cycle Rad53p and Chk1p checkpoint proteins. Pph3/Psy2 is a phosphatase complex, which regulates recovery from the Rad53p DNA damage checkpoint. Overexpression of Chk1p checkpoint protein in a parallel pathway to Rad53p compensated for the deletion of *PPH3* or *PSY2* in a chromosomal repair assay. Double mutant strains *Δpph3/Δchk1* and *Δpsy2/Δchk1* showed additional reductions in the efficiency of plasmid repair, compared to both single deletions which is in agreement with the activity of Pph3p and Psy2p in a parallel pathway to Chk1p. Genetic interaction analyses also supported a role for Pph3p and Psy2p in DNA damage repair, the NHEJ pathway, as well as cell cycle progression. Collectively, we report that the activity of Pph3p and Psy2p further connects NHEJ repair to cell cycle progression.

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- Jessulat M, Malty R, Nguyen-Tran D, Deineko V, Aoki H, Vlasblom J, **Omidi K**, Jin K, Minic Z, Hooshyar M, Burnside D, Samanfar B, Phanse S, Freywald T, Prasad B, Zhang Z, Vizeacouma F, Krogan N, Freywald A, Golshani A, and Babu M: Spindle checkpoint factors Bub1 and Bub2 promote DNA double strand break repair by Non-Homologous End Joining. *Molecular and Cellular Biology*. 2015;35(14):2448-2463.

Spindle Checkpoint Factors Bub1 and Bub2 Promote DNA Double-Strand Break Repair by Nonhomologous End Joining

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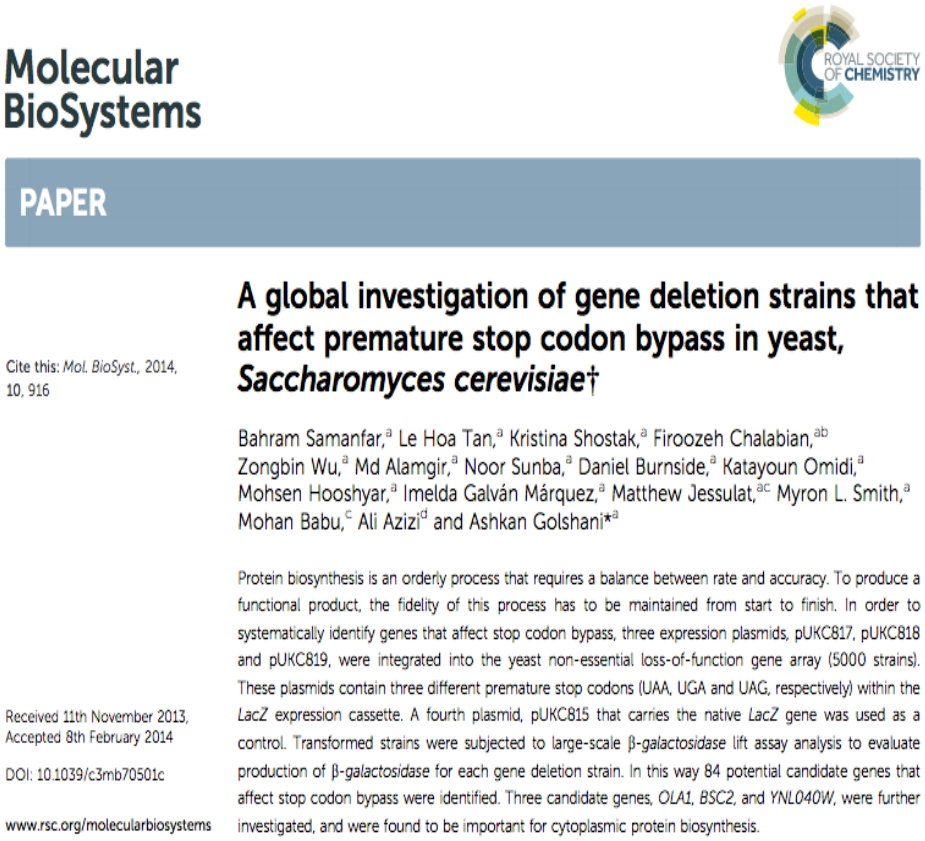
The nonhomologous end-joining (NHEJ) pathway is essential for the preservation of genome integrity, as it efficiently repairs DNA double-strand breaks (DSBs). Previous biochemical and genetic investigations have indicated that, despite the importance of this pathway, the entire complement of genes regulating NHEJ remains unknown. To address this, we employed a plasmid-based NHEJ DNA repair screen in budding yeast (*Saccharomyces cerevisiae*) using 369 putative nonessential DNA repair-related components as queries. Among the newly identified genes associated with NHEJ deficiency upon disruption are two spindle assembly checkpoint kinases, Bub1 and Bub2. Both observation of resulting phenotypes and chromatin immunoprecipitation demonstrated that Bub1 and -2, either alone or in combination with cell cycle regulators, are recruited near the DSB, where phosphorylated Rad53 or H2A accumulates. Large-scale proteomic analysis of Bub kinases phosphorylated in response to DNA damage identified previously unknown kinase substrates on Tel1 S/T-Q sites. Moreover, Bub1 NHEJ function appears to be conserved in mammalian cells. 53BP1, which influences DSB repair by NHEJ, colocalizes with human BUB1 and is recruited to the break sites. Thus, while Bub is not a core component of NHEJ machinery, our data support its dual role in mitotic exit and promotion of NHEJ repair in yeast and mammals.

- Samanfar B, Tan L.H, Shostak K, Chalabian F, Wu Z, Alamgir M.D, Sunba N, Burnside D, **Omidi K**, Hooshyar M, Galván Márquez I, Babu M and Golshani A: A Global investigation of gene deletion strains that affect premature stop codon bypass in yeast, *Saccharomyces cerevisiae. Molecular Biosystems.* 2014, 10(4):916-24.



Molecular BioSystems

PAPER

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A global investigation of gene deletion strains that affect premature stop codon bypass in yeast, *Saccharomyces cerevisiae*†

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Protein biosynthesis is an orderly process that requires a balance between rate and accuracy. To produce a functional product, the fidelity of this process has to be maintained from start to finish. In order to systematically identify genes that affect stop codon bypass, three expression plasmids, pUKC817, pUKC818 and pUKC819, were integrated into the yeast non-essential loss-of-function gene array (5000 strains). These plasmids contain three different premature stop codons (UAA, UGA and UAG, respectively) within the *LacZ* expression cassette. A fourth plasmid, pUKC815 that carries the native *LacZ* gene was used as a control. Transformed strains were subjected to large-scale β-*galactosidase* lift assay analysis to evaluate production of β-*galactosidase* for each gene deletion strain. In this way 84 potential candidate genes that affect stop codon bypass were identified. Three candidate genes, *OLA1*, *BSC2*, and *YNL040W*, were further investigated, and were found to be important for cytoplasmic protein biosynthesis.

- Schoenrock A, Samanfar B, Pitre S, Hooshyar M, Jin K, Philips C, Wang H, Phanse S, **Omidi K**, Gui Y, Alamgir Md, Wong A, Barrenas F, Babu M, Benson M, Langston M, Green JR, Dehne F, and Golshani A: Efficient prediction of human protein-protein interactions at a global scale. *BMC Bioinformatics*. 2014;15(1):383.

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BMC Bioinformatics

RESEARCH ARTICLE Open Access

Efficient prediction of human protein-protein interactions at a global scale

Andrew Schoenrock[1], Bahram Samanfar[2], Sylvain Pitre[1†], Mohsen Hooshyar[2†], Ke Jin[3], Charles A Phillips[4], Hui Wang[5,6], Sadhna Phanse[3], Katayoun Omidi[2], Yuan Gui[2], Md Alamgir[2], Alex Wong[2], Fredrik Barrenäs[5,6], Mohan Babu[7], Mikael Benson[5,6], Michael A Langston[4], James R Green[8], Frank Dehne[1] and Ashkan Golshani[2*]

Abstract

Background: Our knowledge of global protein-protein interaction (PPI) networks in complex organisms such as humans is hindered by technical limitations of current methods.

Results: On the basis of short co-occurring polypeptide regions, we developed a tool called MP-PIPE capable of predicting a global human PPI network within 3 months. With a recall of 23% at a precision of 82.1%, we predicted 172,132 putative PPIs. We demonstrate the usefulness of these predictions through a range of experiments.

Conclusions: The speed and accuracy associated with MP-PIPE can make this a potential tool to study individual human PPI networks (from genomic sequences alone) for personalized medicine.

Keywords: Protein-protein interactions, Computational prediction, Human proteome, Massively parallel computing, Personalized medicine, Interactome, Network analysis

- Samanfar B, **Omidi K**, Hooshyar M, Laliberte B, Alamgir M, Seal AJ, Ahmed-Muhsin E, Viteri DF, Said K, Chalabian F, Golshani A, Wainer G, Burnside D, Shostak K, Bugno M, Willmore WG, Smith ML, Golshani A: Large-scale investigation of oxygen response mutants in *Saccharomyces cerevisiae*, *Molecular bioSystems*. 2013, 9(6):1351-1359.

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Large-scale investigation of oxygen response mutants in *Saccharomyces cerevisiae*†

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A genome-wide screen of a yeast non-essential gene-deletion library was used to identify sick phenotypes due to oxygen deprivation. The screen provided a manageable list of 384 potentially novel as well as known oxygen responding (anoxia-survival) genes. The gene-deletion mutants were further assayed for sensitivity to ferrozine and cobalt to obtain a subset of 34 oxygen-responsive candidate genes including the known hypoxic gene activator, *MGA2*. With each mutant in this subset a plasmid based β-galactosidase assay was performed using the anoxic-inducible promoter from *OLE1* gene, and 17 gene deletions were identified that inhibit induction under anaerobic conditions. Genetic interaction analysis for one of these mutants, the RNase-encoding *POP2* gene, revealed synthetic sick interactions with a number of genes involved in oxygen sensing and response. Knockdown experiments for *CNOT8*, human homolog of *POP2*, reduced cell survival under low oxygen condition suggesting a similar function in human cells.

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- Jessulat M, Pitre S, Gui Y, Hooshyar M, **Omidi K**, Samanfar B, Tan LH, Alamgir MD, Green J, Dehne F, Golshani A. Recent advances in protein-protein interaction prediction: Experimental and computational methods. *Expert Opinion. Drug Discovery*. 2011, 6(9): 921-935.

Recent advances in protein-protein interaction prediction: experimental and computational methods

Jessulat M, Pitre S, Gui Y, Hooshyar M, Omidi K, Samanfar B, Tan LH, Alamgir M, Green J, Dehne F, Golshani A

Proteins within the cell act as part of complex networks, which allow pathways and processes to function. Therefore, understanding how proteins interact is a significant area of current research. Areas covered: This review aims to present an overview of key experimental techniques (yeast two-hybrid, tandem affinity purification and protein microarrays) used to discover protein-protein interactions (PPIs), as well as to briefly discuss certain computational methods for predicting protein interactions based on gene localization, phylogenetic information, 3D structural modeling or primary protein sequence data. Due to the large-scale applicability of primary sequence-based methods, the authors have chosen to focus on this strategy for our review. There is an emphasis on a recent algorithm called Protein Interaction Prediction Engine (PIPE) that can predict global PPIs. The readers will discover recent advances both in the practical determination of protein interaction and the strategies that are available to attempt to anticipate interactions without the time and costs of experimental work. Expert opinion: Global PPI maps can help understand the biology of complex diseases and facilitate the identification of novel drug target sites. This study describes different techniques used for PPI prediction that we believe will significantly impact the development of the field in a new future. We expect to see a growing number of similar techniques capable of large-scale PPI predictions.

- Ogunremi D, Nadin-Davis S, Dupras AA, Márquez IG, **Omidi K**, Pope L, Devenish J, Burke T, Allain R, Leclair D. Evaluation of a Multiplex PCR Assay for the Identification of Salmonella Serovars Enteritidis and Typhimurium Using Retail and Abattoir Samples. *Journal of Food Protection*. 2017, 80(2):295-301.

Evaluation of a multiplex pcr assay for the identification of Salmonella serovars enteritidis and typhimurium using retail and abattoir samples

Ogunremi, Dele; Nadin-Davis, Susan; Dupras, Andrée Ann; Márquez, Imelda Gálvan; Omidi, Katayoun; Pope, Louise; Devenish, John; Burke, Teresa; Allain, Ray; Leclair, Daniel

A multiplex PCR was developed to identify the two most common serovars of Salmonella causing foodbome illness in Canada, namely, serovars Enteritidis and Typhimurium. The PCR was designed to amplify DNA fragments from four Salmonella genes, namely, invA gene (211-bp fragment), iroB gene (309-bp fragment), Typhimurium STM 4497 (523-bp fragment), and Enteritidis SE147228 (612-bp fragment). In addition, a 1,026-bp ribosomal DNA (rDNA) fragment universally present in bacterial species was included in the assay as an internal control fragment. The detection rate of the PCR was 100% among Salmonella Enteritidis (n = 92) and Salmonella Typhimurium (n = 33) isolates. All tested Salmonella isolates (n = 194) were successfully identified based on the amplification of at least one Salmonella-specific DNA fragment. None of the four Salmonella DNA amplicons were detected in any of the non-Salmonella isolates (n = 126), indicating an exclusivity rate of 100%. When applied to crude extracts of 2,001 field isolates of Salmonella obtained during the course of a national microbiol ogical baseline study in broiler chickens and chicken products sampled from abattoir and retail outlets, 163 isolates, or 8.1%, tested positive for Salmonella Enteritidis and another 80 isolates, or 4.0%, tested as Salmonella Typhimurium. All isolates identified by serological testing as Salmonella Enteritidis in the microbiological study were also identified by using the multiplex PCR. The new test can be used to identify or confirm pure isolates of the two serovars and is also amenable for integration into existing culture procedures for accurate detection of Salmonella colonies.

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- **Omidi K**, Jessulat M, Hooshyar M, Burnside D, Kazmirchuk T, Schoenrock A, Hajikarimlou M, Daniel M, Babu M, Ramotar D, Samanfar B, Dehne F, Golshani A. uncharacterized ORF *HUR1* influences the efficiency of non-homologous end joining repair in *Saccharomyces cerevisiae*. *Gene Journal*. 2017, Submitted, GENE-D-17-00492.

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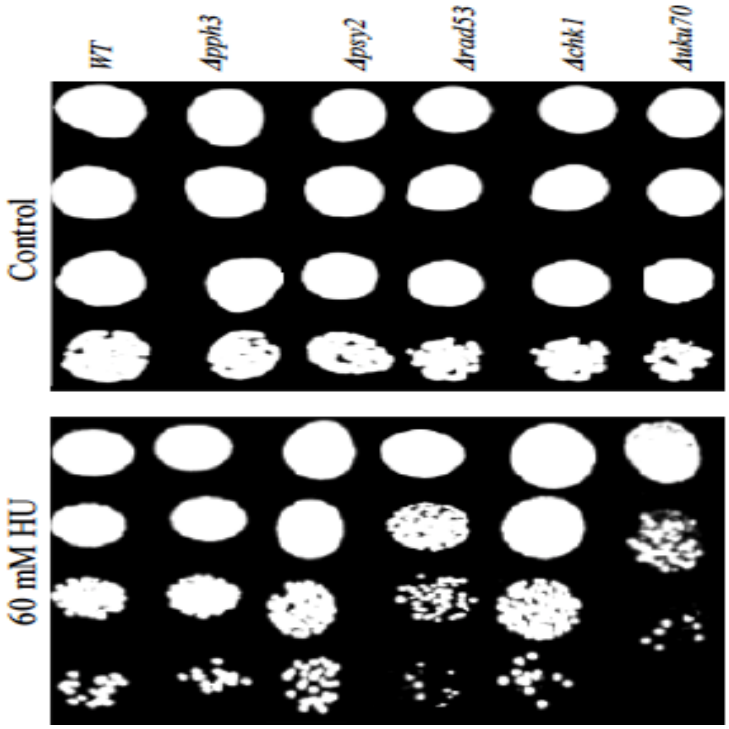
Appendix A.2 List of primers

Primer	Gene	Role	Sequence
KO-PPH3-F	**PPH3**	**Forward primer**	TTCTTTTGCAATTCAGGACACTTTAACTTGTAGTCACACGCTAG TCCACGTAGCAAAGTACACATACGATTTAGGTGACAC
KO-PPH3-R	**PPH3**	**Reverse primer**	TATTGCATTATGAAGAAAAAAAGAAAAATGCACTTGACAATTA GAGTGCCTGTTAAAAATAATACGACTCACTATAGGGAG
KO-PPH3-Con	**PPH3**	**Confirmation primer**	TATGACTAGACAGACATCTTAC
KO-PSY2-F	**PSY2**	**Forward primer**	GTCTGTGTTTGAAACAGAAATTCTAACTGAAAAGTTTAGGATTT ACGTATAGTAAGAGTACACATACGATTTAGGTGACAC
KO-PSY2-R	**PSY2**	**Reverse primer**	ACTTCTTACAACCATGACCGTTGTGCTAGCTTTTTATTCTTCTTT CCAACAAGGAAAAACAATACGACTCACTATAGGGAG
KO-PSY2-Con	**PSY2**	**Confirmation primer**	AGCCATGCAGCTCTCACATAAG
KO- HUR1-F	**HUR1**	**Forward primer**	GAAAAAGAAGGAAAGAATAAAATTTTATATTGCT TTGTATATA CGAAAAGTCGCCCATACCACATACGAT TTTAGG TGACAC
KO-HUR1-R	**HUR1**	**Reverse primer**	GTCAATCTTCGAAATCGGCTTTTTCACGAACAAAATGTTCACT ACACGCCGTTGGACTGAATACGACTCACTATAGG GAG
KO-HUR1-Con	**HUR1**		ATATTTGTCGAAGGTATAAATGAGGAAGG
KO- BUB1-F	**BUB1**	**Forward primer**	TTTCATCATGGAAAGATTGACGGTTCCTATTGTTTGAAT GTTAA CGCTGACCAGGAACACATACGATTTTAGGTGACAC
KO- BUB1-R	**BUB1**	**Reverse primer**	ACCTGCTAGTTTTATATTACCTTTTATATATTAATGTTACAATTG GCTCTACCTGAAGTTAATACGACTCACTATAGGGAG
KO- BUB1-Con	**BUB1**	**Confirmation primer**	CTCTACTAATTAATAGCTGG
KO- BUB2-F	**BUB2**	**Forward primer**	ATAATAGTAACGACGTAAAGGTAAAAGAAACAACAGACTTTT AAACTTGTTAACTTTTGCCACAIACGATTTTAGGT GACAC
KO- BUB2-R	**BUB2**	**Reverse primer**	GAAGGTGAAGGATATGCGTACGAGCCATTTTCATTTCG TGCAA TAATAGAATAATAACCTAATACGACTCACTATAG GGAG
KO- BUB2-Con	**BUB2**	**Confirmation primer**	ACCTGTCTTATGCTATAATGACACTGGAGG
KO- SBP1-F	**SBP1**	**Forward primer**	GTTTCTTGCTTTTGGATTTTCAGATGTCCCAAGATCATTACAGTA TTTTAATTGAACAAA
KO- SBP1-R	**SBP1**	**Reverse primer**	GAGAAAACAAAATTTATATACAATATAAGTAATATTCATATATA TGTGATGAATGCAGTC
KO- SBP1-Con	**SBP1**	**Confirmation primer**	CTAGATCATCTCATGCATGAT
KO-NAT-Con	**NAT**	**Confirmation primer**	TCCAGTGCCTCGATGGCCTCGGCG

Appendix A.3 List of selected candidates

Gene Names	Name Description	Functions
PPH3	Protein PHosphatase	PPH3 regulates recovery from the DNA damage checkpoint Rad53
PSY2	Platinum Sensitivity	PPH3 regulates recovery from the DNA damage checkpoint Rad53
HUR1	HydroxyUrea Resistance	Protein of unknown function
BUB1	Budding Uninhibited by Benzimidazole	Protein kinase that involves in the spindle assembly cell cycle check-point
BUB2	Budding Uninhibited by Benzimidazole	BUB2 arrests cell cycle progression before entering anaphase in response to spindle and kinetochore damage
SBP1 (SSB1)	_	SBP1 binds to eIF4G and regulates translation repression

Appendix B



Appendix B.1: Strain sensitivity analysis to Hydoxyurea (HU). Single deletion mutants for *PPH3* or *PSY2* showed similar sensitivity to DSB inducing agent HU similar to WT. In contrast deletion mutant strains *Δrad53* and *Δyku70* had reduced sensitivity in comparison to single deletion mutants *Δpsy2*, *Δpph3* and WT.

Appendix B.2 Ordered list of PPIs predictions for Hur1p

Protein B	Protein A	Score
YPL240C	YGL168W	99.965
YMR186W	YGL168W	99.965
YBL032W	YGL168W	99.835002
YLL039C	YGL168W	99.813002
YJL164C	YGL168W	99.809003
YPL203W	YGL168W	99.684
YKL166C	YGL168W	99.645001
YFR004W	YGL168W	99.641001
YBR010W	YGL168W	99.593002
YNL031C	YGL168W	99.593002
YNL030W	YGL168W	99.585003
YBR009C	YGL168W	99.585003
YNL255C	YGL168W	99.545997
YNL197C	YGL168W	99.431002
YDR139C	YGL168W	99.409997
YKR094C	YGL168W	99.356002
YIL148W	YGL168W	99.356002
YMR272W-A	YGL168W	99.158001
YLL024C	YGL168W	99.124002
YAL005C	YGL168W	99.106002
YLR167W	YGL168W	99.1
YDL229W	YGL168W	99.096
YNL209W	YGL168W	99.080002
YBL075C	YGL168W	99.075001
YER103W	YGL168W	99.045998
YLR096W	YGL168W	98.951
YHR027C	YGL168W	98.930001
YIL030C	YGL168W	98.751003
YJL034W	YGL168W	98.742002
YOR174W	YGL168W	98.697001
YHR020W	YGL168W	98.658001
YNR051C	YGL168W	98.645997
YOL133W	YGL168W	98.592001
YML058W	YGL168W	98.525
YCL037C	YGL168W	98.449999
YGR060W	YGL168W	98.418999
YER070W	YGL168W	98.277998

YNL189W	YGL168W	98.233998
YIL066C	YGL168W	98.203999
YBR106W	YGL168W	98.114002
YLR292C	YGL168W	98.114002
YOR326W	YGL168W	98.045999
YDL055C	YGL168W	98.013002
YBR200W	YGL168W	97.916001
YNL272C	YGL168W	97.898
YOR371C	YGL168W	97.894001
YKL029C	YGL168W	97.856998
YKL117W	YGL168W	97.834998
YMR047C	YGL168W	97.821999
YKL049C	YGL168W	97.801
YJR045C	YGL168W	97.798997
YGL059W	YGL168W	97.795999
YER008C	YGL168W	97.772998
YFL037W	YGL168W	97.684002
YKL068W	YGL168W	97.658998
YEL030W	YGL168W	97.624999
YKR016W	YGL168W	97.430998
YML085C	YGL168W	97.421998
YKL209C	YGL168W	97.390997
YHR016C	YGL168W	97.367001
YDR122W	YGL168W	97.277999
YML124C	YGL168W	97.228998
YDR477W	YGL168W	97.196001
YGR262C	YGL168W	97.136998
YFR024C-A	YGL168W	97.132999
YPL204W	YGL168W	97.060001
YLR369W	YGL168W	97.022003
YGR119C	YGL168W	96.952999
YDR510W	YGL168W	96.938002
YJR059W	YGL168W	96.899003
YLL060C	YGL168W	96.77
YKL145W	YGL168W	96.732998
YGL172W	YGL168W	96.714002
YGR037C	YGL168W	96.709001
YGR128C	YGL168W	96.696001
YGL097W	YGL168W	96.662003
YBL026W	YGL168W	96.616
YHR205W	YGL168W	96.565002

YGL092W	YGL168W	96.552002
YPL209C	YGL168W	96.456999
YHR019C	YGL168W	96.381998
YDR192C	YGL168W	96.346003
YOR117W	YGL168W	96.325999
YGL195W	YGL168W	96.270001
YHR200W	YGL168W	96.249002
YGR252W	YGL168W	96.135002
YER165W	YGL168W	96.114999
YDR515W	YGL168W	96.076
YKL053C-A	YGL168W	96.060997
YER151C	YGL168W	95.977998
YJL041W	YGL168W	95.973998
YHR007C	YGL168W	95.947999
YKL126W	YGL168W	95.915997
YOR098C	YGL168W	95.874
YDL224C	YGL168W	95.837998
YBR160W	YGL168W	95.828998
YDR205W	YGL168W	95.716
YDL156W	YGL168W	95.503998
YOR133W	YGL168W	95.497
YDR385W	YGL168W	95.497
YDR062W	YGL168W	95.454001
YBL007C	YGL168W	95.376998
YJL026W	YGL168W	95.359999
YDR229W	YGL168W	95.305002
YHR135C	YGL168W	95.283002
YDR381W	YGL168W	95.26
YIL068C	YGL168W	95.213997
YHR103W	YGL168W	95.200002
YER146W	YGL168W	95.182002
YHR165C	YGL168W	95.155001
YJL085W	YGL168W	95.137
YBL105C	YGL168W	95.118999
YHR170W	YGL168W	95.055997

Appendix B.3: Catalogue of DNA-repair related target mutants showing varying efficiency of NHEJ repair. The ability of target genes to repair DSBs by NHEJ is highlighted in red, whereas genes not involved in NHEJ are shown in green. Asterisk denotes genes previously-defined in NHEJ pathway. Efficiency of NHEJ repair is computed based on the number of stable transformants obtained from the linear over circular plasmid. Data is normalized based on WT ligation efficiency.