Identification of Novel Mediators of Efficient Non-Homologous End Joining in the Yeast

Saccharomyces cerevisiae

by

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Abstract

Non-homologous end joining (NHEJ) is an important mechanism for DNA repair. In order to identify novel genes that affect this process we use a plasmid-based screen for gene deletion mutants (a subset of the yeast gene deletion array, ~380 genes) impaired for NHEJ efficiency in the model organism yeast, *Saccharomyces cerevisiae*. Our screen successfully identified known key NHEJ genes (such as MRE11), as well as factors previously linked to NHEJ (such as RSC2), supporting the usefulness of this screen. To confirm the influence of some of the identified candidate genes on NHEJ, drug sensitivity screens and chromosomal DNA damage screens based on the JKM139 yeast strain were used. Our results indicate NHEJ efficiency is impaired in deletion mutants lacking genes, including HUR1 (an uncharacterized gene), RTT109 and VPS75 (a complex which acetylates histones), BUB1, BUB2 and MAD3 (genes involved in the spindle assembly checkpoint), among others. BUB1 and BUB2 were found to influence the efficiency of NHEJ through an Anaphase-Promoting Complex mediated effect. This research can serve as the basis for a more complete understanding of the pathways and processes needed for reliable and efficient NHEJ in yeast.

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List of Abbreviations

A-NHEJ- Alternative non-homologous end joining

AP-Apurinic/aprimidinic site

APC- Anaphase promoting complex

ATP- Adenosine triphosphate

BER- Base excision repair

BSA- Bovine serum albumin

°C- Degrees celsius

DMA- Deletion mutant array

DNA- Deoxyribonucleic acid

DR- Direct repair

DSB- Double-strand break

GAL- Galactose

GG-NER- Global genome nucleotide excision repair

H3K56- Histone 3 lysine 56

HAT- Histone acetyltransferase

HU- Hydroxyurea

HR- Homologous recombination

IDL- Insertion/deletion loop

IR- Ionizing radiation

MALDI-TOF- Matrix-assisted laser desorption/ionization time of flight

MMR- Mismatch repair

MMS- Methyl methanesulfonate

MS- Mass spectrometry

NER- Nucleotide excision repair

NHEJ- Non-homologous end joining

OD- Optical density

PCR- Polymerase chain reaction

RNA- Ribonucleic acid

SDS-PAGE- Sodium dodecylsulfate polyacrilamide gel electrophoresis

SGA- Synthetic genetic array

ssDNA- Single-strand deoxyribonucleic acid

TAP- Tandem affinity purification

TC-NER- Transcription-coupled nucleotide excision repair

URA- Uracil

UV- Ultraviolet

V(D)J-Variable (diverse) joining

YPD- Yeast extract peptone dextrose media

YPGAL- Yeast extract peptone galactose media

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Chapter 1: Introduction

DNA Damage and repair

Within every organism is a polymer composed of a sequence of deoxyribose nucleic acids (DNA). This sequence contains the genetic code for each protein the organism creates, as well as regulatory regions, sequences for RNA essential for cellular processes, and structural and protective regions such as telomeres. While mutations to the DNA sequence of an organism are required for evolution and are an inevitable part of life, each mutation event carries significant risk to the cell or organism. Accurate DNA replication and reliable DNA repair is therefore essential to the long-term survival of any organism or species. As a consequence, the cell diverts considerable resources into the genome's integrity, responding to and correcting any challenges to the genome's faithful maintenance and replication of essential genetic sequences. Many repair pathways also have deep phylogenetic histories, as evidenced by the fact that many DNA repair proteins are largely conserved from fungi, such as *Saccharomyces cerevisiae*, to mammals, and many repair strategies are largely maintained across significantly diverse evolutionary lineages.

DNA damage can have many causes and take many forms. In the simplest case, a nucleotide may be mis-inserted during the process of DNA replication, or a nucleotide may be altered by an exogenous or endogenous chemical agent or by radiation. These types of DNA damage will cause minor, localized disruptions in the double-helix structure or in faithful base-pairing interactions, and as a consequence, may interfere with the transcription or translation process or the preservation of genetic information

across generations. These forms of DNA damage can be repaired by Direct Repair (DR), Base Excision Repair (BER), Nucleotide Excision Repair (NER), MisMatch Repair (MMR), or by a combination of these and other DNA repair pathways.

In a more severe case, both strands of DNA could be cut at or near the same location. In this event, a double-strand break (DSB) occurs. This form of DNA damage is regarded as perhaps the most severe type of damage. It can be caused by a variety of factors; chemical agents, ionizing radiation, stalled replication forks, the activity of restriction endonucleases, and other factors. The consequences of an unrepaired or misrepaired DNA DSB lesion can be dramatic, and at least two primary DSB repair pathways have evolved; Homologous Recombination (HR) and Non-Homologous End Joining (NHEJ).

Direct Repair (DR)

In the event of minor chemical modifications of nucleotides, the simplest form of DNA repair is Direct Repair (DR). DR utilizes single enzymes to directly reverse DNA damage before it can result in mutagenesis. However, the type of enzyme used must be specific to the damage repaired, making each DR pathway very limited in the type of DNA damage it can respond to. In *S. cerevisiae*, DR is very limited, though it is a more important pathway in *E. coli*. There are three broad categories of DR enzymes (reviewed in Dalhus *et al.* 2009): photolyases, methyl transferases, and oxidative demethylases, or dioxygenases. While both photolyases and dioxygenases are metabolically inexpensive and reliably restore the original sequence properly, methyl transferases can be more

costly, as they transfer the inappropriate methyl group to themselves, and then must be degraded.

Photolyases are a light-dependent group of DNA repair enzymes. This family includes the yeast protein Phr1p, which provides light-dependent repair of UV-induced DNA damage, specifically pyrimidine dimers (Sancar 1990). The protein uses photon energy from light of low wavelength (250-300nm) to excite an electron in FADH^- , which can then be transferred to the dimer to be repaired. The dimer is split by the charge, and the free electron is then transferred back to FADH^- , without decay of the protein (Dalhus *et al.* 2009). Interestingly, in yeast, this protein also stimulates the NER pathway (Sancar 1990), which can also deal with pyrimidine dimer DNA damage. The protein's expression level is upregulated in the presence of UV, but also in the presence of some chemical agents such as methyl methanesulfonate (Sebastian *et al.* 1990), which causes methylation damage to DNA (Chang *et al.* 2002). By contrast, it is repressed by factors Rph1p and Gis1p (Jang *et al.* 1999), with Rph1p being phosphorylated by Rad53p kinase in the event of DNA damage (Kim *et al.* 2002). In this way, Phr1p serves both as a DR protein specifically, and as a link in the broader DNA damage response, tying the DNA damage checkpoint to repair systems for pyrimidine dimers and other chemically-induced damage types.

Oxidative demethylases, or dioxygenases, repair DNA damage such as 1-methyl adenine, 3-methyl cytosine and similar lesions, and can function on single-stranded or double-stranded DNA, or even RNA (reviewed in Dalhus *et al.* 2009). Proteins such as the *E. coli* AlkB help the cell deal with alkylating agents (Chen *et al.* 1994), and function

by 'flipping' the compromised nucleotide into a hydrophobic pocket to scan for a positive charge (Dalhus *et al.* 2009). While human proteins satisfying this function have been identified (Kurowski *et al.* 2003), there are no currently known yeast proteins in this category.

DNA methyl transferases can also attack lesions caused by methylated nucleotides. The *E. coli* protein Ada contains two independent active regions; a C-terminal region which corrects O₆ methyl guanine and O₄ methyl thymine (Demple *et al.* 1985), and an N-terminal region which deals with methyl phosphotriester lesions on the DNA backbone (Sedgwick *et al.* 1998). In both cases, the methyl group is permanently transferred to a cysteine residue on the transferase itself. There is also the Ogt protein in *E. coli*, which has homologs from archaea to humans (Roberts *et al.* 2006, Daniels *et al.* 2000). However, again yeast does not appear to possess proteins in this category.

Base Excision Repair (BER)

The BER pathway (reviewed in Robertson *et al.* 2009, Memisoglu and Samson 2000) represents a significant damage repair pathway for minor disruptions to the helix structure. These distortions can include chemical modifications to bases such as alkylation, oxidative damage, deamination, and also missing bases (Robertson *et al.* 2009). The relevance of this pathway in cancer therapy is significant; many chemotherapy agents can cause DNA damage this pathway can correct (Kelley *et al.* 2003). Compromised BER has also been linked to cancer and age-related degenerative illnesses (Wilson and Bohr 2007). While the overall pathway exists from *E. coli* to yeasts

to humans, there is significant divergence in the structure and behaviour of the enzymes involved (Kelley *et al.* 2003). Because of this, the yeast BER pathway may serve as a useful initial model, but drug sensitivity results in yeast cannot necessarily be expanded to humans.

There are two types of BER; short patch (Kubota *et al.* 1996), which replaces a single damaged nucleotide, and long patch, which replaces a larger section of DNA after the damaged nucleotide (Frosina *et al.* 1996). The 'decision' between long and short patch repair is poorly understood, but two theories exist to explain the selection (Robertson *et al.* 2009). Short patch repair may be preferred if the local ATP concentration is high (Petermann *et al.* 2003). Alternatively, it may be preferred if the 5'-terminal deoxyribosephosphate is dealt with efficiently (Klungland and Lindahl 1997).

The process of BER depends on a minimum of 4 proteins: a glycosylase, an endonuclease or DNA lyase, a polymerase, and a ligase (Kubota *et al.* 1996). Together, these proteins identify the lesion, remove the affected nucleotide, fill the gap, and ligate the new DNA to the existing backbone.

Glycosylases are responsible for identifying the damaged base and cleaving the N-glycosidic bond (Lindahl 1974), creating an apurinic or apyrimidinic (AP) site. Some glycosylases also have AP lyase activity (O'Connor and Laval 1989). Each glycosylase is specific to a type of base modification. In *S. cerevisiae*, Ogg1p is a mitochondrial glycosylase that targets 8-oxoguanine (Nash *et al.* 1996). Ung1p is a uracil glycosylase, removing uracil residues (Burgers and Klein, 1986). Both Ntg1p and Ntg2p, a pair of glycosylases with similarity to *E. coli* endonuclease III, deal with oxidative damage (Eide

et al. 1996). And Mag1p compensates for 3-methyl-adenine residues, helping to deal with alkylation damage (Chen *et al.* 1989). Once a glycosylase has identified and removed a chemically-modified base, the resulting abasic site becomes the common substrate for subsequent reactions.

The primary AP endonuclease in yeast is Apn1p, with Apn2p playing a less significant role. Apn1p has been estimated to make up more than 97% of the endonuclease activity in this pathway (Popoff *et al.* 1990), with Apn2p showing little phenotypic effect *in vivo* unless in a double-mutant with Apn1p (Johnson *et al.* 1998). These enzymes nick the DNA at the 5' end of the AP site, processing it by removing the 5' deoxyribophosphate to prepare for fresh DNA synthesis. This creates a single nucleotide gap to be filled by polymerase III (the yeast polymerase δ), which depends on the Proliferating Cell Nuclear Antigen (PCNA) for loading in long-patch BER (Maga and Hübscher 2003). Ligation then proceeds by the action of Cdc9p (Wu *et al.* 1999).

Nucleotide Excision Repair (NER)

In the event of minor distortions in the DNA double helix or in some forms of improper base-pairing interactions, DNA repair primarily relies on NER (reviewed in Hoeijmakers 2001, Prakash and Prakash 2000). This pathway represents an important mechanism for dealing with disruptions caused by factors such as UV irradiation, some oxidative stresses, and intrastrand crosslinks, as well as interstrand crosslinks with the help of the HR pathway (Hoeijmakers 2001, Prakash and Prakash 2000, Lehoczky *et al.* 2007), both across the genome and at areas in the process of transcription. Failure or

impairment of this pathway is connected to a variety of human disorders, including susceptibility to skin cancer, sensitivity to sunlight, and neurodegenerative disorders (reviewed in Cleaver *et al.* 2009).

Global Genome NER (GG-NER) in yeast is a multi-step process that requires several protein complexes. Initially, the compromised section of DNA must be identified. The two strands of DNA must be unwound. The damaged strand must be identified from the undamaged strand. A section of DNA 25-30 nucleotides can then be removed through two incisions in the DNA strand. Finally, a new stretch of DNA can be assembled and ligated to restore the original DNA sequence.

Initial identification of the DNA disruption is done by a complex of yeast proteins Rad7p and Rad16p. This complex is an ATP-dependent DNA damage sensor (Guzder *et al.* 1997) that actively tracks along the double-helix structure until a change in local structure prevents it from proceeding (Guzder *et al.* 1998a). This complex is believed to be the first step in identifying the lesion, and subsequently recruits further proteins to the area in need of repair. However, if this complex is missing or non-functional, while the active search for DNA damage may not proceed, random encounters between other DNA damage recognition proteins and the compromised sequence still occur at a reduced rate (Hanawalt 2002).

Transcription-Coupled NER (TC-NER) is largely similar in the process of repair to GG-NER, however, these lesions are identified in a different manner which is linked to the site of active DNA transcription. When active transcription proteins meet a lesion they cannot pass, RNA polymerase II is disassembled (Malik *et al.* 2008) and

identification of the lesion appears to include recruitment of Rad26p (Malik *et al.* 2010). Beyond the identification, lesion repair is conducted in a similar manner (de Boer and Hoeijmakers 2000). Overall, the transcription-coupled lesion repair mechanism is considerably faster in producing a NER event.

Protein Rad7p can bind the Rad4p-Rad23p complex, which can also bind a region of DNA in a non-ATP dependent manner, and possesses a particular affinity for DNA damaged by UV light (Guzder *et al.* 1998B, Jansen *et al.* 1998). This complex further assists in identifying DNA that is damaged and acts as a recruiter and tether for other NER factors (Prakash and Prakash 2000). While dispensable for DNA incision, this complex enhances the activity of Rad7p-Rad16p and NER when bound (Guzder *et al.* 1999).

The 6 protein TFIIH complex (composed of Rad3p, Rad25p, Ssl1p, Tfb1p, Tfb2p and Tfb3p) serves as a helicase in NER. Specifically, proteins Rad3p and Rad25p provide helicase activity necessary for NER to function. Without Rad3p, Rad25p, or the remaining 4 subunit components of TFIIH (which can be purified as a single subunit of the complex), or with mutant variants of Rad3p or Rad25p lacking helicase activity, DNA damage induced by UV light is not excised by the NER pathway (Sung *et al.* 1996). The TFIIH complex also plays a role in transcription. Interestingly, the helicase activity of Rad3p appears dispensable for transcription, but the entire Rad3p protein is not (Sung *et al.* 1996). Proteins Rad3p and Rad25p translocate along single-stranded DNA in opposing directions around the lesion; Rad3p travels in a 5' to 3' direction, while Rad25p travels 3' to 5' (Sung *et al.* 1987, Guzder *et al.* 1994). In this manner, a space of single-

stranded DNA is created around the DNA damage to be excised, which is essential for the endonuclease activity of NER to occur.

Rad2p and a complex of proteins Rad1p and Rad10p provide the excision activity for NER. Both are ssDNA endonucleases, and both are responsible for cleavage at one side of the lesion. Rad2p cleaves at the 3' end, where the ssDNA ends and dsDNA begins (Habraken *et al.* 1995). Rad1p and Rad10p form a functional complex (Bailly *et al.* 1992), with Rad1p being the catalytic subunit (Aravind *et al.* 1999, Newman *et al.* 2005). This complex cuts at the 5' end of the lesion (Tomkinson *et al.* 1993, Sung *et al.* 1993), again where the ssDNA joins with duplex DNA (Bardwell *et al.* 1994). *In vivo*, this activity depends on the DNA damage recognition protein Rad14p, while *in vitro* this additional protein is unnecessary (Guzder *et al.* 2006). In this way, a short stretch of ~30 nucleotides of ssDNA is excised around the lesion, which can then be replaced. Once the damaged DNA is excised, the gap is filled by polymerase β , and is ligated into the existing DNA strand.

RPA, a heterotrimeric protein encoded by genes RFA1, RFA2 and RFA3, is a single-stranded DNA binding protein with a well-studied and significant role in DNA metabolism (Wold 1997). This protein is loaded to the break late in the process of NER (Prakash and Prakash 2000). The purpose of this protein appears twofold; it both assists in the efficient incision of UV-damaged DNA (Guzder *et al.* 1997) and directs the DNA damage response factor Rad24p-RFC, which in turn loads the Rad17p/Mec3p/Ddc1p checkpoint clamp protein onto the 5' end of the ssDNA (Majka *et al.* 2006). Important for this activity is phosphorylation by the Mec1p kinase (Kim and Brill 2003). The RPA

therefore serves as a link between the process of NER and the wider DNA damage response of the cell, which includes a temporary pause in cell division while the lesion is repaired.

While long known to be important in NER, the function of Mms19p (also referred to as Met18p) has only recently been determined. Mms19p was identified *in vivo* to function in both GG-NER and TC-NER, as well as in transcription (Queimado *et al.* 2001, Lombaerts *et al.* 1997, Lauder *et al.* 1996, Miller *et al.* 1982). However, *in vitro* reconstitution of this pathway found this protein unnecessary (Araujo *et al.* 2000). Ultimately, it was proposed that Mms19p stabilized the TFIIH complex, and therefore indirectly supported faithful NER (Hatfield *et al.* 2006). Further research revealed the Mms19p protein has a crucial role in regulating Rad3p concentration in the cell (Kou *et al.* 2008). Without a sufficient concentration of Rad3p, a key component of the TFIIH complex, NER efficiency was significantly impaired.

NER can effectively deal with intrastrand crosslinks, such as those caused by UV light, but interstrand crosslinks such as those caused by cisplatin or other anti-cancer compounds provide a more difficult challenge, as the damage is not limited to one DNA strand. In these cases, a combination of NER and other DNA repair pathways, such as HR, can be used. It is also known that in the process of correcting these lesions during transcription, a DSB may be formed, necessitating either HR or NHEJ. The complex interactions of these DNA repair pathways in response to an interstrand crosslink are reviewed in Lehoczký *et al.* (2007).

As early research in NER was frequently done using 'naked' DNA templates, the *in vivo* behaviour of this pathway, where chromatin structure and histones would play a significant role in NER regulation, were only elucidated later. The relevance of chromatin and histone modifications in DNA repair efficiency will be discussed in a subsequent section.

MisMatch Repair (MMR)

MMR serves as one of the cell's repair mechanism for mis-paired nucleotides, such as G/T mispairing or unpaired nucleotides, and also small insertion or deletion loops (IDLs) (reviewed in Marti *et al.* 2002, Lamers *et al.* 2000, Obmolova *et al.* 2002). However, BER and NER can also play a role in dealing with this type of damage. These mispairing events can be caused by errors in replication, chemical alteration of bases, or during crossovers. In the event of a compromised MMR pathway, yeast cells suffer an increase in post-meiotic segregation events (Williamson *et al.* 1985). In humans, impairment of the pathway can lead to an increased predisposition to colorectal cancers or sporadic tumors (Peltomaki and Vasen 1997, Jiricny and Nyström-Lahti 2000). While this pathway has historically been studied primarily in *E. coli*, where it is based on the MutS and MutL proteins, phylogenetic and molecular research has revealed MMR in yeast to be very similar, though also more complex.

In yeast, 2 complexes identify the lesion; the MutS α complex and MutS β complex. MutS α is composed of *E. coli* MutS homolog proteins Msh2p and Msh6p, and deals with base-base mismatches and single unpaired nucleotides (Alani 1996, Johnson

et al. 1996, Marsischky *et al.* 1996). MutS β is a heterodimer of Msh2p and Msh3p, and deals either with single or multiple unpaired nucleotides (Habracken *et al.* 1996, Johnson *et al.* 1996, Marsischky *et al.* 1996). In both cases, the Msh2p protein provides a structural scaffold, while the variable second protein provides the activity (Marsischky *et al.* 1996, Bowers *et al.* 1999, Lamers *et al.* 2000, Obmolova *et al.* 2000). DNA damage is identified by a phenylalanine residue, which can be inserted into the DNA at a region of weakened or absent binding (Obmolova *et al.* 2000).

Interestingly, there are other MutS homologs in yeast, including Msh1p (Marti *et al.* 2002). These other homologs do not appear to function in nuclear MMR, but Msh1p does seem to protect the mitochondria, which is lost in a few generations in $\Delta msh1$ yeast (Reenan and Kolodner 1992). It is believed that Msh1p may act as a homodimer in the mitochondria, similar to the *E. coli* MutS protein, which dimerizes so that one subunit can interact at the damage site while the other provides structural support (Marti *et al.* 2002).

There are also multiple MutL homologs in yeast: Pms1p, Mlh1p, Mlh2p and Mlh3p (Kramer *et al.* 1989, Prolla *et al.* 1994a, Flores-Rozas and Kolodner 1998). Yeast protein Mlh1p forms heterodimers with each of the other proteins (Wang *et al.* 1999), forming three complexes with distinct roles. Mlh1p and Pms1p form the primary MutL complex, MutL α , which can interact with both MutS α and MutS β to deal with all lesion types (Prolla *et al.* 1994b, Flores-Rozas and Kolodner 1998). Mlh1p and Mlh3p form the MutL β complex, which only interacts with MutS β (Flores-Rozas and Kolodner 1998). Less is known about the Mlh1p-Mlh2p complex, which is less important to efficient

MMR, but appears to suppress frameshift mutations with some efficiency (Harfe *et al.* 2000).

PCNA, encoded by the yeast gene POL30, interacts with the MutS complexes through binding sites on Msh3p and Msh6p (Flores-Rozas *et al.* 2000). This complex has a significant role in DNA replication and other processes, but in MMR, it disrupts the previously existing ternary complex between MutS and MutL subunits and DNA, identifies the lesion, increases MutS α binding affinity, and creates a 'sliding clamp' which moves along the DNA to find discrimination signals and excision factors, which will direct the subsequent excision of the damaged DNA strand (Flores-Rozas *et al.* 2000, Bowers *et al.* 2001)

The DNA lesion may be excised by one of several mechanisms. Yeast nuclease Exo1p is known to interact with Msh2p, Msh3p and Mlh1p (Tishkoff *et al.* 1997, Tran *et al.* 2001) and provides catalytic activity during MMR (Tran *et al.* 2002). However, DNA polymerase III (δ in mammals) can also function in removing the lesion (Tran *et al.* 1999), as well as provide synthesis of new DNA (Tran *et al.* 1999, Longley *et al.* 1997). Once the novel DNA is created, it can be ligated to complete the MMR process.

Double-strand break repair

The DSB can have a significant impact on the viability of the cell. If a DSB is not repaired efficiently, the result can be loss of genetic information, misalignment of chromosomes during cell replication, and even cell death, while if misrepaired, the lesion can lead to mutation, chromosomal rearrangement, and potentially

carcinogenesis in a multicellular organism. In fact, mutations in key DSB repair genes have been linked to cancer development and susceptibility in humans. Despite this risk, DSB's are also induced by the cell in a controlled fashion during V(D)J recombination in the development of the immune system, a process that requires efficient DSB repair to function. Impaired DNA repair function due to genetic mutation can therefore also lead to compromised immune system function (reviewed in de Villartay 2009).

In the event of a DSB, there are two primary methods to repair the lesion. If the lesion occurs within a sequence that has a homologous region elsewhere in the genome, the cell may repair the damage through Homologous Recombination (HR). Alternatively, Non-Homologous End Joining (NHEJ) can be used to repair any DSB regardless of sequence homolog availability.

The 'choice' of DSB repair pathway to initiate is not a simple one, depending on multiple factors, competitive elements, and also sharing some protein factors and regulators. However, each pathway is regarded as genetically and functionally distinct, despite some overlapping proteins and complexes.

Homologous Recombination

The primary DSB repair system in yeast (particularly in S-phase), and a pathway considered to be less error-prone than its alternative, homologous recombination is a DSB repair system that uses a break region's homology to a template region to coordinate efficient repair (reviewed in Dudáš and Chovanec 2003). While the repair of

a break using a homologous region as a template reduces the chances of a mutation arising, there is still the risk of loss of heterozygosity.

The MRX complex (composed of two units of Mre11p, two units of Rad50p and one unit of Xrs2p) forms the basis of this type of repair (though it also plays a significant role in NHEJ, see below). Mre11p possesses a DNA binding activity (Usui *et al.* 1998, Furuse *et al.* 1998) that allows the recruitment of this complex to the DNA. A Mn^{2+} -dependent 3'-5' exonuclease activity is also present (Usui *et al.* 1998, D'amours and Jackson 2002, Paull and Gellert 1998, Trujillo *et al.* 2001). This allows for the unwinding of a DNA duplex and the cleavage of hairpin structures (Paull and Gellert 1999), and also aids in allowing complementary sequences to anneal (de Jager *et al.* 2001). In the presence of ATP, Rad50p has the ability to degrade 3' overhangs at the DNA lesion (Trujillo *et al.* 2001). As the complex contains two of each of these subunits, both ends of the DSB can be bound and processed simultaneously. Together, this complex allows for the processing of broken DNA ends (which is essential, as much of the genetic material on either side of the lesion is degraded to prepare for fresh synthesis), controls sister chromatid interactions, and can initiate, stabilize and cause D-loop formation at the site of HR (Hopfner *et al.* 2002a, Hopfner *et al.* 2002b). At this point in the repair event, the lesion is processed on both sides to create a long stretch of single stranded DNA, capable of being used to search for homologous regions.

Rad51p binds DNA in an ATP-dependent manner and can catalyse the identification of homologous sequences of DNA, allowing a template to be found (Sung *et al.* 1994). This strand exchange is stimulated by replication protein A (Sugiyama *et al.*

1997, Namsaraev and Berg 1997) and is stabilized by the Rad55p-Rad57p heterodimer (Sung 1997). At this stage in the DNA repair process, single-stranded DNA, bound by RPA and Rad51p, invades intact DNA sequences in search of a template to copy for the repair. This strand invasion results in a D-loop, as the dsDNA containing a homologous sequence is unwound to allow the invading strand to anneal.

Rad54p interacts with Rad51p, ssDNA, and chromatin to stimulate pairing between homologous DNA sequences (Dudáš and Chovanec 2003). It has a chromatin remodelling activity, allowing it to slide nucleosomes along the DNA, creating an opening for the necessary repair interactions to occur.

Rad52p is considered an essential component of the HR pathway, so much so that its deletion is often used as a control for no HR activity. It functions by stimulating the Rad51p strand exchange (Sung *et al.* 1997), and also by forming rings around the DNA (Dudáš and Chovanec 2003), causing the annealing of complementary strands. This activity is assisted by Rad59p, which anneals complementary strands less well (Davis and Symington, 2001), and appears to help remove interference from RPA.

Repair by HR is complete when the Holliday junctions (structures of intersection between the two strands exchanging DNA during this repair) are resolved through nucleolytic cleavage and ligation. In yeast, this occurs via the Holliday junction resolvases Yen1p (Ip *et al.* 2008).

Non-Homologous End Joining

A more versatile alternative to HR is Non-Homologous End joining (NHEJ) (reviewed in Dudášová *et al.* 2004, Daley *et al.* 2005). In this pathway, the two broken strands of DNA are directly re-ligated by DNA Ligase 4 (LIG4 or DNL4), sometimes but not always after stages of end processing. While possible at any stage of the cell cycle, without a DNA template to ensure correct sequence, this pathway is regarded as more mutagenic than HR (Pierce *et al.* 2001).

Initial recognition of the DNA lesion is the result of binding by the Yku70p-Yku80p heterodimer, which binds to DNA ends in a sequence-independent manner (Milne *et al.* 1996, Siede *et al.* 1996). This heterodimer forms a ring, which encircles the broken DNA ends (Walker *et al.* 2001) to protect the DNA from degradation by other factors. This serves to identify the ends to be rejoined and recruit subsequent repair factors such as the MRX complex (Dudášová *et al.* 2004). Interestingly, the binding of this duplex seems to occur in only one orientation (Daley *et al.* 2005). Both Yku70p and Yku80p have identified homology to their human counterparts (Feldmann and Winnacker 1993, Feldmann *et al.* 1996).

The proteins Mre11p, Rad50p, Xrs2p form a complex termed MRX, which is then recruited to the broken ends of DNA. The complex is composed of 2 Rad50p subunits, 2 Mre11p subunits, and one Xrs2p subunit, with binding between Rad50p and Xrs2p depending on the presence of Mre11p (Chen *et al.* 2001, Dudášová *et al.* 2004). Both the complex and its individual components can bind DNA, but it is the Xrs2p protein that specifically targets the complex to the broken ends of DNA (Furuse *et al.* 1998, Usui *et*

al. 1998, Trujillo *et al.* 2001, Trujillo *et al.* 2003). In NHEJ, this complex plays a role as a bridging complex between the two broken DNA segments, and as a scaffold for other protein complexes to bind near the break (Chen *et al.* 2001). Specifically, Xrs2p shows affinity for Lif1p (a component of the NHEJ ligase complex), which is increased by Yku70p (Dudášová *et al.* 2004). The MRX complex is not limited to a function in NHEJ; it also plays a significant role in HR, as well as the DNA damage checkpoint (D'Amours and Jackson 2002, Grenon *et al.* 2001, van den Bosch *et al.* 2003). Very recently, the MRX complex was also linked to efficient polymerase activity in BER (Steininger *et al.* 2010).

Ligase activity in NHEJ is the result of the Lig4p/Lif1p/Nej1p complex. Lig4p is an ATP-dependent ligase, providing the activity necessary to rejoin the broken DNA ends (Schär *et al.* 1997). Lif1p targets the ligase to the site of repair (Teo and Jackson 2000) and interacts with physically with Xrs2p. Binding also occurs between Lig4p and the Yku80p protein (Palmbos *et al.* 2008). Both Lig4p and Lif1p form a stable heterodimer (Herrmann *et al.* 1998), with Nej1p additionally binding to both proteins. The role of Nej1p has previously been somewhat controversial. Initial results of plasmid repair assays demonstrated a role in NHEJ efficiency, and suggested a nuclear localization role for the protein based on Lif1p (Valencia *et al.* 2001). However, these localization results were not consistent with other groups (Kegel *et al.* 2001). Subsequently, a DNA-binding function for Nej1p was established, which suggests Nej1p and Lif1p both serve in recruiting the ligase to the site of DNA repair (Sulek *et al.* 2007).

In some cases, the broken DNA ends being rejoined are not blunt ends, and do not contain complementary DNA sequence overhangs. In these cases, or in the cases of

chemically-altered nucleotides being part of the overhang, a level of DNA end processing must be used to allow the joining to proceed properly. Pol4p, a polymerase whose deletion strains show mild sensitivity to Methyl Methanesulfonate (MMS) and ionizing radiation (IR) (Leem *et al.* 1994), interacts physically with, and is stimulated by, Lig4p (Tseng and Tomkinson 2002). The 5'-3' flap endonuclease Rad27p also shows sensitivity to MMS and IR, as well as UV and other DNA damaging agents (Hansen *et al.* 2000, Reagan *et al.* 1995, Symington 1998, Wu and Wang 1999). Both these factors appear to play a role in the subset of DSB's that require end processing prior to ligation (Wilson and Lieber 1999, Wu *et al.* 1999b)

While the above is the canonical NHEJ pathway, there has long been evidence of NHEJ activity even in the absence of key NHEJ factors such as Yku70p or Lig4p, albeit at a greatly reduced efficiency (Dudášová *et al.* 2004). This has led further research to identify an Alternate NHEJ (A-NHEJ), which functions in a limited capacity in the absence of all required factors for NHEJ. Rather than depend on Yku70p/Yku80p, A-NHEJ appears to use a short (only a few nucleotides) stretch of homology to rejoin the break (reviewed in Lieber 2010, Mcvey and Lee 2008), leading it to also be called Microhomology-Mediated End Joining. However, the need for a short overhang of complementary bases, and the absence of Yku70p/Yku80p to protect the ends of DNA from digestion, can lead to significant loss of sequence at the break site before a homologous stretch is found, making the A-NHEJ system not only less efficient, but also dramatically more mutagenic (McVey and Lee 2008, Bennardo *et al.* 2008).

Recent work has determined some prokaryotes possess a NHEJ pathway as well, one resembling the A-NHEJ system in that it requires short homologies to work.

Mycobacterium tuberculosis possesses a two-protein NHEJ system that relies on an Mt-Ku homodimer and the multifunctional Mt-Lig, as well as short homologies (Della *et al.* 2004). *E. coli*, despite having previously been used as a negative control for NHEJ, was recently determined to have a limited capacity for NHEJ, also relying heavily on microhomologies to coordinate the often mutagenic repair (Chayot *et al.* 2010).

Histone modification in DNA repair

Inside the cell, DNA is usually present within the context of some form of chromatin or histone packaging. This packaging has the potential to be a serious barrier to DNA repair systems, as a protein's physical access to a DNA lesion is required for repair to take place. As a result, various histone modifications are used by the cell to signal an unwinding of the nucleosome, facilitating DNA repair before the DNA is repackaged.

One key histone modification, termed γ H2A (or γ H2AX in mammals), marks a DSB lesion early in the repair process (Fillingham *et al.* 2006). γ H2A represents a serine phosphorylation on the carboxy-terminal region of histone 2A. This specific alteration in the histone occurs up to hundreds of kilobases up- and downstream of the lesion, but strangely, is reduced at the site of the break itself (Downs *et al.* 2000, Rogakou *et al.* 1998, Fillingham *et al.* 2006). It is known that this effect is mediated through Mec1p and Tel1p, both key signals in the broader context of DNA repair.

γ H2A is responsible for several recruitment events near the site of the DSB. In vitro, it has been demonstrated that NuA4 binds to this histone modification (Downs 2004). Recruitment of this complex results in the acetylation of histone H4 at the site of a DSB, which causes the chromatin to loosen and allow other factors access (Downs 2004, Bird 2002). Recruitment of INO80 also depends on γ H2A (van Attikum *et al.* 2004), as well as on Mre11p, and when recruited this chromatin remodelling complex removes the nucleosomes that otherwise bind the DNA (Tsukuda *et al.* 2005). Cohesin, a complex which holds sister chromatids together, binds along the same regions as γ H2A in a γ H2A-dependent manner (Glynn *et al.* 2004, Ünal *et al.* 2004). SWR-C may or may not be recruited by γ H2A (Downs *et al.* 2004), but there is a physical interaction between the two (Morrison *et al.* 2004), and this complex swaps H2AZ, a modified histone, for H2A along the lesion (Krogan *et al.* 2003, Kobor *et al.* 2004).

Before repair is complete, γ H2A is lost along the site of DNA repair. It is unclear what signal is responsible for the dephosphorylation and release of this histone variant, but it has been speculated that release must occur before repair can complete due to possible interference from cohesin (Fillingham *et al.* 2006).

H3K56 acetylation (of lysine 56 on histone 3) is another important histone modification in DSB repair. The major acetyltransferase responsible for this modification was not known until recently. Rtt109p has been shown to acetylate H3K56 (Han *et al.* 2007b, Driscoll *et al.* 2007), an activity that is Asf1-dependent (Downs 2008). This modified histone is incorporated into newly-synthesized DNA and is deacetylated as the cell proceeds through replication (Downs 2008). Cells which lack this histone

modification are sensitive to a variety of DNA damage inducing agents (Fillingham *et al.* 2008). Deacetylation occurs in human tissues by the activity of HDAC1 and HDAC2, which has recently been shown to occur following DNA damage at the damage site (Miller *et al.* 2010). Activity of these deacetylases appears to correspond with γ H2AX, and therefore areas of DNA damage, and repair by NHEJ appears unable to proceed when this activity is eliminated (Miller *et al.* 2010). While it is unclear how exactly this signal promotes DNA repair, it has been suggested that this may be a part of a larger 'histone code', and there has also been limited evidence suggesting a function in gene regulation at promoter sites (Downs 2008, Adkins *et al.* 2004, Rufiange *et al.* 2007) or suggestion of a role in DNA repair pathway choice or the blocking of transcription factor binding, which may interfere with repair (Miller *et al.* 2010).

The DNA damage checkpoint and cell cycle arrest

In the event of DNA damage, the cell responds by engaging the appropriate DNA repair pathway. In addition, the cell cycle delays to allow repair time to complete. The sequence of events leading to cell cycle arrest in response to DNA damage is the DNA damage checkpoint.

Detection of DNA damage begins with the protein Mec1p, which is a sensor of ssDNA in the cell (Harrison and Haber, 2006). Mec1p then phosphorylates Rad9p (Naiki *et al.* 2004, Emili *et al.* 1998), which stimulates Mec1p to further phosphorylate Chk1p and Rad53p (Blankley *et al.* 2004, Ma *et al.* 2006, Sweeney *et al.* 2005). This in turn upregulates repair genes, downregulates cyclins and delays cell cycle progression (Chen

and Sanchez 2004, Weinert 1998). A single break is sufficient to stall the cell cycle (Harrison and Haber 2006). Chk1p mediates the cell cycle by phosphorylation of Pds1p (Wang *et al.* 2001), which binds Esp1p and prevents cyclin destruction (Ciosk *et al.* 1998). Rad53p has multiple targets; Transcription factor Swi6p (Sidorova and Breeden, 1997), Dun1p, and Dbf4p (Lee *et al.* 2003, Duncker *et al.* 2002) are all affected, thereby reducing transcription of genes involved in the cell cycle and limiting the replication of the genome.

Objective

Given recent work has uncovered novel genes involved in NHEJ repair efficiency (Shim *et al.* 2005), and that there are a significant number of still-uncharacterized genes and unexplained genetic interactions, we hypothesized that there are likely genes which play a currently unknown function in efficient NHEJ repair. This research project aimed to identify and characterize some of these previously unknown NHEJ-related genes.

Because of the conservation of this pathway between *S. cerevisiae* and higher eukaryotes, we have chosen to use *S. cerevisiae* as our model organism, to take advantage of the ease of use and manipulation, significant previous genetic and proteomic research both within these pathways and across a wide range of cellular functions, and availability of resources such as the Saccharomyces Genome Database and yeast gene deletion arrays (Tong *et al.* 2001). However, this research is likely to have broad applicability across a wide variety of organisms.

Methodology

Using an available subset of gene deletion mutants in *S. cerevisiae*, we screen for genes defective in NHEJ using a plasmid repair assay. This assay would be supported by a screen for sensitivity to known DNA damaging agents. Any candidates identified in the initial plasmid repair screen would be confirmed by multiple replicates of the plasmid repair assay. A detailed Materials and Methods section is included within each chapter of this work.

As a follow up and to study the mechanism of action, a selection of the confirmed candidate genes is explored in greater detail using genetic assays.

Chapter 2: A Plasmid-based screen for NHEJ-related genes reveals multiple novel candidates

Abstract

Non-homologous end-joining (NHEJ) is an important DNA repair mechanism which, we hypothesized, can be affected by a variety of cellular factors. Here, we sought to elucidate gene deletion mutants with impaired NHEJ activity using a plasmid-based NHEJ screen on a subset of the yeast gene deletion array. This assay revealed multiple factors known to be key to efficient NHEJ, such as NEJ1, LIF1 and LIG4, as well as recently identified genes such as the chromatin remodeller RSC2. Several novel candidates were also discovered, including PPH3, PSY2, HUR1, BUB1, BUB2, RTT109, SWI4, and others. Because HUR1 contains a short overlap sequence with PMR1 (not detected in our screen), we sought to determine if HUR1 or PMR1 was responsible for the observed phenotype. HUR1 partial deletion mutants also exhibited a reduced NHEJ activity, and HUR1 overexpression partially recovered the impaired chromosomal NHEJ phenotype in a JKM139 background. We therefore conclude that HUR1 is a novel NHEJ-related gene, among several other candidates.

Introduction

One of the more severe forms of DNA damage is the DSB. In a DSB, both complementary strands of DNA are cut at or near the same location, shearing the DNA into two separate parts. This form of DNA damage can have significant deleterious effects on the cell; Chromosomal rearrangement, mutation, loss of genetic information, cell death or cancer can all result from an unrepaired or misrepaired DSB (Burma *et al.* 2006, Zha *et al.* 2007). Due to the risks associated with improper or unsuccessful repair and the necessity of repairing a DSB, two well-conserved and efficient systems for DSB repair have evolved. If a homologous sequence can be found, HR can be used to reliably restore a damaged region using this homologous area as a template (reviewed in Dudáš and Chovanec 2003). In the absence of a homologous template, NHEJ can directly re-ligate two broken ends of the DSB (reviewed in Dudášová *et al.* 2004). Both pathways are significant contributors to DSB repair in the yeast *S. cerevisiae*, though HR is the dominant pathway where it is available (Siede *et al.* 1996).

The dominant model of NHEJ requires the activity of three core complexes for efficient ligation of the two broken DNA ends. The Yku70p/Yku80p heterodimer binds to each broken DNA end within seconds, protecting the ends from processing, identifying the break and serving as a recruitment factor for other NHEJ factors. The MRX complex, composed of Mre11p, Rad50p and Xrs2p (in a 2:2:1 stoichiometry), represents a scaffold for the ligase to be recruited to, as well as a bridging complex between the two broken DNA ends. The final ligase complex, composed of Lig4p, Lif1p and Nej1p, rejoins the two broken ends. Additional factors, such as Pol4p and Rad27p, process the ends in such a

way as to allow DNA joining to occur, even in the case of chemically damaged or non-complementary overhangs, where necessary.

While the core NHEJ pathway has long been elucidated, recent work has increasingly broadened the perception of this pathway's function to include much of the cellular and *in vivo* context of DSB repair. Notably, interactions with chromatin structure, histone modification, cell cycle regulation and DNA damage sensors, as well as protein behaviour in other contexts such as at telomeres or in mammalian V(D)J recombination have all been productive areas of research (de Villartay 2009). Associations between NHEJ and chromatin structure regulators (Shim *et al.* 2005), Sir proteins (Boulton and Jackson 1998), and H3K56 acetylation (Fillingham *et al.* 2006) have all recently come to light.

We hypothesized that, given recently identified relationships between NHEJ and novel genes such as Rsc8p, Rsc30p (Shim *et al.* 2005), Sir2p, Sir3p, and Sir4p (Boulton and Jackson 1998), as well as the significant portion of genes in the *S. cerevisiae* genome with uncharacterized function, there are likely as yet undiscovered NHEJ-related genes in the yeast genome. Taking a subset of the yeast deletion array (described in Winzeler *et al.* 1999), we set out to identify and characterize some of these genes. Our results show a variety of cellular processes are needed to allow NHEJ to occur at high efficiency, including histone modification, cell cycle proteins, transcription factors, and others. We have also found a protein of unknown function, Hur1p, which appears necessary for NHEJ efficiency, particularly in a chromatin context.

Materials and Methods

Media, Strains and Plasmids

Standard biological media supplies, such as glucose or yeast extract, as well as hydroxyurea, were purchased from Bioshop Canada. Other chemicals, such as calcium chloride and methyl methanesulfonate (MMS) were purchased from Sigma. Enzymes such as restriction endonucleases and Taq polymerase, as well as DNA ladders, were purchased from New England Biolabs and Invitrogen, excepting some Taq polymerase which was isolated by associates at the Willmore lab and was graciously provided to us. Distilled water was used for all solutions, except where otherwise noted. All chemical solutions and media requiring sterility were sterilized using high temperature and pressure in an autoclave, for a minimum 20 minute cycle, with the exception of heat-labile antibiotics and other chemicals that required filter sterilization, which was done using a sterile 0.2 micrometer filter. Sterile disposable plastic supplies were purchased from Diamed, Ultident, and Fischer Scientific. Plasmid isolation kits and PCR product cleaning kits were purchased from QIAgen and Fermentas, excepting the plasmid extraction kit for yeast cells, which was purchased from Omega Biotek.

All yeast cells, unless otherwise stated, were grown in standard YPD media (1% weight/volume yeast extract, 2% bacteriological-grade peptone, 2% glucose as a carbon source, with 2% laboratory grade agar for solid media). For uracil selection, a minimal media lacking uracil was prepared (6.74g/L yeast nitrogen base with ammonium sulphate without amino acids, 2g/L amino acid dropout powder [containing all essential amino acids except Uracil], 2% glucose, and 2% laboratory grade agar). For JKM139

assays, YP-GAL media in solid form was identical to YPD, except using 2% galactose as an alternate carbon source.

Yeast strains for most experiments are gene deletion variants of S288C, from a yeast gene deletion set described in (Winzeler *et al.* 1999). JKM139 strains are described in Moore and Haber (1996). Yeast mating type alpha used are strain Y7092. For plasmid maintenance and production in preparation for yeast transformation experiments, *E. coli* strain DH5 α was used.

Plasmids used in this experiment include a derivative of plasmid p416 (Krogan *et al.* 2003), with a LacZ insert following the GAL promoter region, used for the NHEJ plasmid repair assay. Overexpression plasmids (which possess a URA selection marker as well as the targeted gene sequence paired to a GAL-promoter) are described in Sopko *et al.* (2006).

Novel gene knockouts are produced by homologous recombination using PCR-produced inserts based on the NAT gene, performed as in Winzeler *et al.* (1999). Briefly, a set of oligonucleotides were ordered from Sigma-Genosys which contained at the 3' end PCR primer regions surrounding the NAT gene located on plasmid pAG25. These 20-25 nucleotide regions provided the annealing and priming activity necessary for PCR amplification of the resistance marker. The 5' end of these primer pairs constituted 55-70 nucleotide regions which shared identity upstream and downstream of the gene deletion target. Using standard lithium-acetate transformation methods (see below), the PCR product containing NAT resistance and flanked on each end by regions of sequence homology surrounding the gene deletion target was allowed to recombine

with the chromosomal DNA, inserting a resistance marker and removing the targeted gene. To confirm gene deletion, a PCR reaction was performed on isolated chromosomal DNA (isolated using glass beads to lyse cells, followed by phenol-chloroform extraction), or by colony PCR (using unprocessed cells as a PCR template, with an extended initial melting phase to disrupt cells during the PCR program). By using a forward primer upstream of the gene believed to be removed and a reverse primer within the NAT gene believed to be inserted, a PCR product of the correct size would indicate successful insertion of the NAT resistance marker at the desired location within the genome. Primer sequences are available in Appendix A.

Plasmid Repair Assay

The efficiency of NHEJ in a gene deletion mutant was assayed initially using a plasmid-based repair assay similar to Shim *et al.* (2005). In brief, plasmid p416 was isolated from DH α and prepared for transformation in two sample sets. Some of this plasmid was digested by restriction enzyme XbaI for 4 hours at 37°C in appropriate buffer (supplied by enzyme-provider) and BSA, as per provider's instructions. Samples were cleaned either by QIAgen kit (as per manufacturer's instructions) or by phenol-chloroform extraction. To confirm digestion, samples were used in gel electrophoresis in a 0.8% agarose gel for 45-60 minutes at 80V, and were visualized by ethidium bromide staining under UV light. A single band was visible in successful digestion. An equal volume of plasmid was kept undigested.

For the transformation, standard lithium acetate procedures were used.

Deletion mutants were grown to saturation (2 days at 30°C), then diluted to an OD₆₀₀ of 0.2-0.4 and were allowed to grow to an OD₆₀₀ of approximately 1. These cells were washed, treated with 0.1M lithium acetate, and were divided into 2 equal 50µl volumes. Each volume was treated with an additional 1M lithium acetate solution, 50% polyethylene glycol, a small volume of carrier ssDNA isolated from salmon testes (Sigma) and either intact plasmid DNA or an approximately equal amount of digested plasmid. After one hour at 30°C, 40µl dimethyl sulfoxide was added to each sample, and transformation occurred at 42°C for 15 minutes, after which cell solutions were plated on minimal media plates lacking Uracil. As plasmid p416 contains the URA3 gene, cells receiving an intact plasmid or cells capable of repairing a received digested plasmid would form a colony. Efficiency of NHEJ was therefore estimated as the proportion of colonies formed from the transformation receiving digested, linearized plasmid compared to colonies formed from the transformation receiving circular plasmid.

Deletion Mutant Array (DMA) Drug Sensitivity Assay

To quickly assay the entire collection of DNA damage candidate genes for sensitivity to DNA damage-inducing agents hydroxyurea (HU), methyl methanesulfonate (MMS), and UV light, a DNA damage deletion mutant array was provided from previous work in our lab, arrayed on a single 384-spot array plate. Drug sensitivity by deletion mutant array replica pinning was conducted manually as in Tong *et al.* (2004).

Approximately 380 gene deletion mutants were transferred by a 384 floating pin system

to a fresh YPD plate and allowed to incubate for 2 days at 30°C, control and test YPD plates (containing 50mM HU, 0.03% MMS, or subsequently exposed to UV light ($\sim 12\text{J}/\text{m}^2$)). Colonies were given overnight at 30°C to grow. Synthetic sick or synthetic lethal phenotypes were scored visually as a reduced growth compared to the immediately-surrounding colonies on the test plate (to control for localized pinning or plate effects) and compared to the corresponding colony on the control plate. Pinning was done in triplicate. Only reductions that occurred in at least 2 cases were considered.

Drug Sensitivity Spot Screening

To improve the accuracy of our drug sensitivity screens, a spot test assay was used on all 380 candidate genes. In brief, cells were grown to saturation at 30°C (1-2 days), then serially diluted to concentrations of 10^{-3} to 10^{-6} . 15 μl spots of each concentration were placed on plates containing 0.03% MMS, 50mM HU, or on standard YPD that was subsequently exposed to 12j/m² of UV light. Standard YPD controls were used. Growth reduction was assessed visually as fewer colonies or less colony growth than controls.

Chromosomal DSB Repair Assay

To confirm the results of the plasmid repair assay in a chromosomal context for our selected candidate genes, a JKM139-based chromosomal DSB repair assay was used. This assay was performed as in Moore and Haber(1996). Gene deletion mutations were

created as previously mentioned in a JKM139 background, and where indicated, gene overexpression plasmids were transformed in the corresponding deletion mutant to test phenotypic recovery.

Cells were grown to saturation (2 days) at 30°C in YPD. Cultures were then serially diluted to concentrations between 10^{-1} and 10^{-6} and 100µl samples were spread on petri plates containing either standard YPD or YP-GAL media, as described above. Plates were given 2-3 days incubation at 30°C to form colonies. Colony counts were acquired manually, using one YPD plate and one YP-GAL plate per set, and were normalized to the same concentration.

Results

Plasmid Repair Assay Screen Can Detect Known Key NHEJ Proteins

In this work, we used a plasmid repair assay based on transformation with linearized and circular plasmid p416 DNA to identify gene deletions which negatively affect NHEJ efficiency. The plasmid contains a URA selection marker, allowing cells to produce Uracil and therefore subsist on media lacking this amino acid. If two equal volumes of competent cells are transformed with an equal amount of plasmid, one circular, the other linearized with the enzyme XbaI, colony formation on the plate receiving the linearized plasmid, corrected to colony formation on the plate receiving circular plasmid, should represent an approximation of successful NHEJ repair events. This procedure has a significant successful history in the literature in identifying NHEJ-related proteins. It also directly measures the cell's ability to repair a specific type of

DNA lesion, in this case a DSB with short and complementary overhangs. DNA repair is also limited to NHEJ because the region containing the DSB has no complementary region within the *S. cerevisiae* genome. For a positive control, the key NHEJ gene YKU70 was used, which is known from previous research to reduce NHEJ efficiency to 2.5% of wild-type or less (Dudášová *et al.* 2004)

For the initial screen, approximately 380 genes were selected for screening (enough to fill most of one 384 colony array plate, for ease of handling and some subsequent analysis). We adopted an arbitrary cutoff of approximately 40% reduction in NHEJ efficiency to consider a gene deletion mutant as a possible candidate. Genes were given letter-number coordinates denoting their place on the array, thereby blinding the identity of the genes studied until candidates were identified (with exceptions for genes chosen subsequently for study, based on additional experimental information, such as Vps75 or Mad3 (see chapters 3-4)). A full list of genes tested and initial colony counts are given in Appendix B.

Within the 380 deletion gene set were several key NHEJ genes that were successfully identified by our screen. The significant reduction of NHEJ efficiency in the positive control YKU70 gene deletion strains conforms well to literature values previously reported. Genes identified include NEJ1, LIF1, and the ligase LIG4 (figure 1). Other possible candidate genes identified included genes recently shown to be involved in NHEJ in an indirect fashion. These genes include one member of the RSC complex, RSC2 (RSC being linked to NHEJ in Shim *et al.* 2005), as well as the SIR2 gene (SIR genes being linked to NHEJ in Boulton and Jackson (1998)) (figure 1).

Plasmid Repair Assay Screen Detects Novel Candidate Genes

Novel NHEJ possible candidate genes were also identified. These genes include PSY2, PPH3, POL4, POP2, MEK1, SWI4, MSH6, UBC13, VPS72, RXT1, RNR3, RAD2, SRO9, ARP6, HIR3, HUR1, BUB1, BUB2, and RTT109 (figure 2). These genes represent an interesting assortment of genes from multiple pathways and with varying functions. Pph3p is a protein phosphatase which forms a complex with Psy2p (Hastie *et al.* 2006). Swi4p is a transcription regulator connected with cell wall synthesis (Iyer *et al.* 2001). Bub1p and Bub2p are members of two distinct branches of the spindle assembly checkpoint (Hardwick 1998).

Not all possible candidate genes were confirmed upon further inspection. Attempts to replicate results multiple times identified several false-positives, which were discarded from further research.

Partial Overlap between Drug Sensitivity Screen and Novel Candidate List

To further examine our set of potential candidates, a drug sensitivity screen based on the gene deletion array was used. A 384-floating pin colony transfer system was used to transfer our entire set of deletions to control plates (YPD) as well as to test plates containing 50mM HU, 0.03% MMS, or to YPD plates that were subsequently exposed to $\sim 12\text{J/m}^2$ UV light. Sensitivity was assessed visually as a reduction in growth compared to the control plate in each set and compared to surrounding colonies.

Figure 2: Plasmid repair screen candidate results. Several genes identified in the screen as impaired for NHEJ efficiency are novel NHEJ factors.

UBC13 deletion mutants were impaired in NHEJ plasmid repair and also showed increased sensitivity to MMS. RAD2 and MEK1 deletion mutants were sensitive to UV light. And RTT109 deletion mutants were sensitive to both MMS and HU. The plate coordinates (row and column) for each gene deletion mutant are listed as part of Appendix B. All images of control and test plates for the pinning assays here used are provided in Appendix C.

To examine the relationship between our candidates identified in the plasmid repair assay and drug sensitivity more closely, a spot test screen was used. For each of the genes in our screen, spot tests were conducted on 0.03% MMS, 50mM HU and on YPD that was then exposed to UV irradiation ($12\text{J}/\text{m}^2$). All candidate genes identified except RNR3, HIR3 and PSY2 were found to be sensitive to at least one drug condition. Several genes, including novel candidates POP2 and RTT109, were found sensitive in all conditions, with HUR1 found to be sensitive to both UV and HU (Figure 3). Sensitivity for each gene is listed as part of Appendix B.

Limited overlap in drug or UV sensitivity conforms to previous experimental results reported elsewhere (reviewed in Dudášova *et al.* 2004). As NHEJ is not the primary DNA repair system in yeast, impairment of NHEJ may only cause limited phenotypic effects as HR or other DNA repair systems can mask any reductions in pathway efficiency. Further, none of the experimental conditions used solely create DSB's, and factors related to other DNA repair mechanisms are well-represented in our screen.

Figure 3: Venn diagram showing the candidate genes identified by plasmid repair assay and their sensitivity to selected DNA damage inducing agents HU, MMS, and UV.

Uncharacterized Gene HUR1 is identified as a NHEJ-related gene, whose activity is not due to overlap with PMR1

One of the more interesting gene deletion mutants identified in our plasmid repair assay screen was $\Delta hur1$, named as *HydroxyUrea Resistance 1*. This gene had originally been only partially characterized as a gene involved in resistance to the DNA damaging agent Hydroxyurea, only to have subsequent research identify the relevant resistance activity as being connected to a small region of overlap with PMR1 (Jordan *et al.* 2007). When only the first section of HUR1 was deleted, leaving the overlapping section of PMR1 unaffected, hydroxyurea resistance was increased to wild-type levels (Jordan *et al.* 2007). PMR1 is a calcium import gene associated with the golgi, and due to its localization and its function having no clear relevance to NHEJ, we sought to determine if HUR1 was truly a NHEJ-related gene or if the impairment of PMR1 was responsible for our plasmid repair deficiency.

In our plasmid repair assay, PMR1 did not show reduction in plasmid repair efficiency significantly different from wild-type (0.88, $p=0.48$) (figure 4a). However, a partial deletion of the first 70 amino acids of HUR1 demonstrated a reduction that was significantly different from wild-type (0.46, $p=0.011$), though not as great a reduction as the full HUR1 deletion.

In the JKM139 chromosome repair assay, a HUR1 deletion reduced the NHEJ efficiency to almost zero (0.000008% survival) (figure 4b). To determine if this effect was indeed caused by the loss of HUR1, we reintroduced the HUR1 gene on an overexpression plasmid (with HUR1 linked to a GAL promoter), and tested to see if the

Figure 4: (a) Plasmid repair assay for HUR1 and PMR1 deletion strains. HUR1 deletion reduced the efficiency of plasmid repair as indicated by reduced colony formation after transformation. This effect was not seen in PMR1 deletions, and was partially maintained in a deletion of the first 70 amino acids of HUR1.

(b) JKM139 chromosomal repair assays for HUR1, showing reduction in survivorship for the deletion mutant and partial recovery for the deletion mutant when supplied with a HUR1 overexpression plasmid.

phenotype could be recovered. While in a $\Delta hur1$ /HUR1 overexpression genetic background, JKM139 strain did exhibit a small recovery of NHEJ efficiency. We therefore concluded the effects of HUR1 on NHEJ were in fact caused by HUR1, and not the overlap with PMR1.

Discussion

Here we present results of a screening designed to find new genetic factors relevant to the efficient repair of DSB's in a NHEJ context within *S. cerevisiae*. In addition to successfully identifying known key NHEJ genes such as LIG4, LIF1 and MRE11, as well as recently-identified NHEJ-related factors such as RSC2, a series of new, previously unknown NHEJ factors have been identified. These factors include RTT109, BUB1, BUB2, and HUR1, among others. Several factors, such as HUR1, RTT109, BUB1, BUB2, PPH3, UBC13, MEK1, and others, were also found to confer resistance to DNA damage inducing agents MS, HU, and UV light, which is consistent with impairment to NHEJ.

The candidate genes that display an NHEJ-deficient phenotype are related to many different functions within the yeast cell. For example, PPH3 and PSY2 are two parts of a protein phosphatase associated with cisplatin resistance (Hastie *et al.* 2006). UBC13, RAD2, and MSH6 are involved in other known DNA repair pathways. RNR3 and POL32 are involved in DNA synthesis. MEK1 is involved in cellular checkpoints. And VPS72, ARP6, and HIR3 are related to histones.

While some factors have established roles in NHEJ, the mechanism for other genes to influence NHEJ efficiency are not immediately clear. For example, HUR1 is regarded in the *Saccharomyces* Genome Database as a protein of unknown function. Previous work indicating a role in HU resistance (Zewail *et al.* 2003) was overturned with the discovery that a short overlapping sequence with PMR1, a golgi-related calcium uptake gene (Antebi and Fink, 1992), was responsible (Jordan *et al.* 2007). This overlap effect calls into question our own drug sensitivity screen, in which our HUR1 deletion strain would also be compromised for part of PMR1. It was therefore necessary to examine this gene more closely to confirm a function in NHEJ. It was found that not only did a partial deletion of HUR1 maintain much of the NHEJ reduction phenotype despite not compromising PMR1, but an overexpression of HUR1 partially restored the NHEJ activity in JKM139 strain HUR1 deletion mutants. While this recovery was very minor, it should be noted that the expression level of HUR1 under a GAL promoter is not likely to match the wild-type expression levels, and this disruption may also cause a defect in NHEJ. However, despite confirmation of HUR1's significance, its mechanism for influencing NHEJ remains unknown.

It should be noted here that identification of a gene as a possible candidate does not constitute identification of a gene directly physically involved in NHEJ; gene deletion mutants may display a NHEJ deficient phenotype for many possible reasons, from disruption of the repair event itself to downregulation of a DNA damage identification signal or the disruption of a relevant transcription factor. Also, the use of plasmid DNA represents a somewhat artificial context for NHEJ, as the naked DNA does not contain

the same histone interactions one would expect to find in a chromosomal context.

However, both in previous work and in our own, even deletion of histone modification proteins show a significant effect on the efficiency of NHEJ (Shim *et al* 2005). The identified candidates that are successfully confirmed represent an interesting assortment of gene deletion mutants which are likely impaired for NHEJ in multiple ways.

Ultimately, the identification of factors whose genetic deletion from the cell impairs NHEJ is only a first step. Further work must examine the mechanistic role each protein has in connection to this process. Our subsequent research examined several genes of interest identified by this method of screening, RTT109, BUB1 and BUB2, in the hopes of finding a more detailed explanation for how these factors relate to NHEJ.

Chapter 3: Interacting proteins Rtt109 and Vps75 affect the efficiency of non-homologous end-joining in *Saccharomyces cerevisiae*

Abstract

One of the key pathways for DNA double-stranded break (DSB) repair is the non-homologous end-joining (NHEJ) pathway, which directly re-ligates two broken ends of DNA. Using a plasmid repair assay screen, we identified that the deletion strain for RTT109 had a reduced efficiency for NHEJ in yeast. This deletion strain also had a reduced efficiency to repair induced chromosomal DSBs *in vivo*. Tandem-affinity purification of Rtt109 recovered Vps75 as a physical interacting protein. Deletion of VPS75 was also shown to have an effect on the efficiency of NHEJ in both the plasmid repair and the chromosomal repair assays. In addition, deletion mutants for both RTT109 and VPS75 showed hypersensitivity to different DNA damaging agents. Our genetic interaction analysis supports a role for RTT109 in DNA damage repair. We propose that one function of the Rtt109-Vps75 interacting protein pair is to affect the efficiency of NHEJ in yeast. Vps75 but not Rtt109 also seem to have an effect on the efficiency of DSB repair using homologous recombination.

Introduction

Perhaps the most severe form of DNA damage is the double-strand break (DSB), in which both complementary strands of DNA are cut at or near the same region. If unrepaired, such damage can lead to the loss of genetic information, genomic instability, mutagenesis or cancer in the case of humans (Burma *et al.* 2006, Zha *et al.* 2007). Therefore, robust systems to repair this damage correctly are of great importance to the survival of the cell. Two systems in particular have evolved to deal with DSB's; homologous recombination (HR), and non-homologous end-joining (NHEJ) (reviewed in Daley *et al.* 2005, Sonoda *et al.* 2006). Of the two systems, NHEJ is the most versatile, as it does not require a homologous template to function (Daley *et al.* 2005, Dudášová *et al.* 2004). Instead, NHEJ proceeds by direct ligation of two available ends of DNA. However, without a homologous template to copy from, NHEJ has a higher degree of error and mutagenesis (Pierce *et al.* 2001). Despite this risk, repair through NHEJ remains preferable to leaving the damage unrepaired, and NHEJ has become the dominant DSB repair system in many mammals (Pierce *et al.* 2006).

Three key protein complexes are involved in the process of NHEJ in *Saccharomyces cerevisiae* (Dudášová *et al.* 2004). One is the Yku70/Yku80 heterodimer, which binds to the DNA, serving as a recruitment factor as well as protecting the broken ends of DNA. The second complex, termed the MRX complex, is composed of the proteins Mre11, Rad50, and Xrs2, and controls end-bridging of the two broken ends of DNA. The final protein complex is composed of the proteins Lig4 (a ligase), Lif1, and Nej1, the latter of which has been suggested to act as a localization factor. Further,

Polymerase Pol4 and nuclease Rad27 have also been identified as part of the end-processing apparatus of NHEJ, allowing annealing to take place between complementary overhangs. Other proteins and complexes have recently been connected to this pathway as well, such as the RSC complex (Shim *et al.* 2005) and the proteins Sir2, Sir3, and Sir4 (Boulton and Jackson 1998). Association of NHEJ with other pathways has also been the subject of numerous recent investigations (reviewed in Wysocki *et al.* 2005).

The deletion of genes involved in NHEJ has been shown to dramatically reduce the ability of the cell to repair plasmids that are cut in both strands via restriction digestion (Krogan *et al.* 2004, Sugawara *et al.* 2003, Ooi *et al.* 2001). Deletion of the YKU70 gene, for example, greatly reduced the ability of a cell to repair linearized plasmid DNA (Shim *et al.* 2005, Dudášová *et al.* 2004). Recently, other gene deletion strains have been examined for their potential reductions in NHEJ efficiency based on their ability to repair digested plasmids. Proteins such as Rsc8 and Rsc30, which belong to the RSC complex, have been identified as factors in NHEJ using this methodology (Shim *et al.* 2005).

To identify novel genes that affect the efficiency of NHEJ, we recently initiated a systematic investigation to detect yeast non-essential gene deletion mutants with reduced ability to repair linearized plasmids. In the present study, we report that in the absence of Rtt109 the efficiency of NHEJ in *S. cerevisiae* is reduced. Evidence that Rtt109-Vps75 heterodimer affects the efficiency of NHEJ is presented.

Materials and methods

Strains and plasmid

The yeast strains *OshG18 MATa ura3-1 leu2-3,112 his3-11, 15 trp1 ade2-1 YLL002W-TAP::TRP1* and *OshG19 MATa ura3-1 leu2-3,112 his3-11,15 trp1 ade2-1 YNL246W-TAP:TRP1* were used for protein purification analysis. Yeast gene deletion strains are described in Winzeler *et al.* (1999). Generation of JKM139 is described in Moore and Haber (1996). Gene deletions in JKM139 were made using a PCR transformation as explained Winzeler *et al.* (1999). For the plasmid repair assay, a derivative of plasmid p416 was used (Krogan *et al.* 2003).

Plasmid repair assay

A unique XbaI restriction site was used for plasmid linearization. Using standard protocols, approximately equal amounts of cells were transformed in parallel with equal amounts of circular and linearized plasmid DNA, and were plated onto minimal media plates lacking uracil (2% glucose, 2% agar, 6.74 yeast nitrogen base/L, 2 g amino acid powder minus uracil/L). Plates were incubated for 2–3 days at 30°C, and colonies were counted manually. Each experiment was repeated at least four times.

Plasmid recombination assay

A plasmid recombination assay was performed as described in Erdemir *et al.* (2002). Briefly, plasmid pGV256-dead was linearized at a BglII site, within a LacZ coding region containing a stop codon. This linearized plasmid was co-transformed with linear

amplified LacZ fragment obtained by PCR using plasmid pGV256-live as a template. Transformations were conducted as described above. A minimum of fifty colonies were sub-cultured and analyzed by colony-lift b-galactosidase assay as described Erdemir *et al.* (2002), to improve clarity of counts. The ratio of blue/white colonies formed was used to estimate efficiency of HR. All analyses were done in triplicate from unique transformations.

Tandem affinity purification (TAP) tagging

The affinity purification of proteins was performed as previously described Wood *et al.* (2003). In brief, the protein complexes were affinity-purified on immunoglobulin G (IgG) and calmodulin columns from whole cell extracts made from 4L of yeast cells. The purified mixture was fractionated on an SDS-PAGE and visualized (using silver staining). The protein bands were subjected to in-gel trypsin digestion followed by identification using matrix associated laser desorption ionization-time-of-flight (MALDI-TOF) mass spectrometry (MS). Each purification was repeated at least three times. The interactions that varied from one experiment to other were assumed as non-specific and were omitted.

Spot tests

Single- or double-deletion mutants were grown to saturation in YPD at 30°C, and serially diluted to concentrations of 10^{-3} to 10^{-6} . Spots of 15µl of each dilution were plated on YPD plates (1% yeast extract, 2% peptone, 2% glucose, 2% agar), or YPD plates

containing 0.03%–0.05% methyl methanesulfonate (MMS), 20 and 50 mM hydroxyurea (HU), or YPD plates that were subsequently exposed to 5 min of UV light ($420\mu\text{J}/\text{s}/\text{cm}^2$). The plates were incubated for 2–3 days at 30°C . Sensitivity was assessed manually by analyzing the number and size of the colonies formed on each plate in relation to the control.

JKM139-based analysis

Single-deletion mutants constructed in the yeast strain JKM139 were grown at 30°C to saturation in YPD. Dilutions were prepared at concentrations between 10^{-3} and 10^{-6} , and $100\mu\text{l}$ of each dilution were plated on YPD plates (containing glucose as a sugar source) as well as on YP-GAL plates (containing galactose which induces a single DSB). Plates were incubated for 2–3 days at 30°C , and colonies were counted manually. Cell survival was analyzed by dividing the number of colonies formed on the YP-GAL plates by the number formed on YPD plates, using one plate per set. Each experiment was repeated at least three times.

Genetic interaction analysis

Genetic interactions between RTT109 and a set of yeast gene deletion strains for 384 genes known or thought to be involved in DNA metabolism were assessed by synthetic genetic miniarray profiling as before (Schuldiner *et al.* 2005) and the colony growth differences were assessed as in Memarian *et al.* (2007). Possible synthetic sick or

synthetic lethal interactions were confirmed by a spotting assay explained in Tong *et al.* (2004).

Results

A plasmid repair assay identifies RTT109 as a candidate gene involved in NHEJ

To identify novel genes that influence the efficiency of NHEJ, a plasmid repair assay (Shim *et al.* 2005, Boulton and Jackson 1998) was used to screen the set of yeast non-essential gene deletion strains (~4500) for their efficiency to repair linearized DNA. In this assay, target gene deletion strains are transformed using equal amounts of intact and linearized plasmids. The plasmid used contains a URA3 gene and consequently in its intact form allows for the growth of yeast cells in a selective media (lacking uracil). When the cells are transformed using linearized plasmids, the relative number of colonies formed on the selective media is assumed to be representative of plasmid repair efficiency in that strain. The relative efficiency of plasmid repair is estimated by calculating the ratio of the number of colonies formed on the selective media using linearized and intact plasmids and then relating it to the same ratio obtained in a parallel control experiment using a wild-type strain. The plasmid is linearized in a region that has no homology to yeast chromosomal DNA, therefore the efficiency of plasmid repair is assumed to represent the efficiency of NHEJ in that strain. Using this assay we estimated the efficiency of NHEJ for deletion mutants of YKU70 and RAD27 to be 1.3% and 28.6%, respectively (figure 5). Similar results for $\Delta yku70$ have been reported previously. This result suggests the assay is capable of identifying genes known to affect the efficiency of

Figure 5: The plasmid repair assay reveals RTT109 and VPS75 deletion mutants are impaired for NHEJ.

NHEJ. A systematic screening of the gene deletion strains also identified other genes that are known to be involved in NHEJ such as part of the RSC complex, RSC2, reaffirming that the assay is capable of detecting NHEJ related genes (figure 5).

It was observed that a gene deletion strain for RTT109 ($\Delta rtt109$) had a reduced efficiency for NHEJ (figure 5). When RTT109 was deleted, the efficiency of the cells to perform NHEJ was estimated to be approximately 47%. An involvement for Rtt109 in NHEJ has not been previously reported. Therefore, we hypothesized that Rtt109 might be a novel factor that affects the efficiency of NHEJ.

Deletion of RTT109 reduces the survival of the JKM139 strain in the presence of galactose

We used the JKM139 strain (Moore and Haber 1996) to further investigate the effect of Rtt109 on the efficiency of NHEJ. This strain contains the HO site-specific endonuclease linked to a GAL-promoter. In the presence of galactose, the endonuclease is expressed and then cuts the chromosomal DNA at the MAT locus. Thus a DSB is induced within the chromosomal DNA of the yeast cell. As JKM139 does not encode a homologous region to the DSB, due to the deletion of HML and HMR regions in this strain, DSB repair is presumed to rely almost exclusively on NHEJ (Shim *et al.* 2005). Therefore, if deletion of a particular non-essential gene compromises NHEJ, the ability of JKM139 cells to survive when chromosomal DSBs are induced using galactose will be compromised (Shim *et al.* 2005).

To investigate whether RTT109 deletion would impair cell survival in a JKM139 background, equal numbers of $\Delta rtt109$ yeast cells were plated on media with or without

galactose. Colonies that formed in the presence of galactose were compared to those formed in its absence (i.e. in glucose), and numerically compared to those obtained for a wild-type strain. A deletion strain for YKU70 was used as a positive control. It was observed that the deletion of RTT109 causes an approximately four-fold reduction in the cell survival (figure 6). This reduction is comparable to that observed for $\Delta yku70$ (figure 6) and further suggests that Rtt109 functions in NHEJ in yeast cells.

It should be noted that mutagenesis caused by inaccurate NHEJ events will generate cells resistant to the HO-endonuclease cleavage. Deletion of Yku70 severely impairs not only the efficiency of NHEJ, but also the accuracy of those events (Shim *et al.* 2005). This would lead to the destruction of the restriction site, protecting the cell from further damage. Wild-type cells are more prone to recreating the restriction site, and therefore, being subject to repeated DSB stress. It is therefore not surprising that Yku70 deletion mutants would form 12% as many colonies as the wild-type in this assay, but only possess 1% of the NHEJ repair efficiency in the plasmid repair assay. The apparent increased severity in NHEJ impairment for Rtt109 (or Vps75) deletion strain in this assay can be explained similarly. If this deletion does not impair the accuracy of NHEJ, or impair accuracy to a lesser extent than deletion of Yku70, then the deletion mutant cells would face repeated DSB stress. A single colony would potentially require multiple successful NHEJ events to form, magnifying a more moderate effect on NHEJ efficiency.

Figure 6: JKM139 chromosomal break tests confirm RTT109 and VPS75 deletion mutants are impaired for NHEJ.

$\Delta rtt109$ has an increased sensitivity to DNA damaging agents

To further support the involvement of Rtt109 in DNA repair we investigated the sensitivity of its gene deletion strain ($\Delta rtt109$) with respect to different DNA-damaging agents such as MMS, HU and UV light. MMS has been shown to methylate DNA, specifically affecting adenine and guanine, stalling replication forks and causing DSBs (Chang *et al.* 2002). HU interferes with deoxynucleotide production, thereby causing the stalling and the collapse of the replication forks, which could lead to generation of DSBs (Rittberg and Wright 1989). UV light causes ionization of the DNA and could lead to the formation of thymidine dimers (Hinz *et al.* 2005, Limoli *et al.* 2002). Spot test analysis indicated that under our experimental conditions, $\Delta rtt109$ had hypersensitivity to all three DNA-damaging agents (figure 7). This is in agreement with similar recently reported findings (Han *et al.* 2007a, Schneider *et al.* 2006) in which $\Delta rtt109$ was found to be hypersensitive to MMS and HU.

To further characterize these hypersensitivities, we constructed double mutant combinations for RTT109 and RAD52 ($\Delta rtt109\text{-}\Delta rad52$) and RTT109 and YKU70 ($\Delta rtt109\text{-}\Delta yku70$). RAD52 has a key function in HR (Sugawara *et al.* 2003). Deletion of RAD52 alone ($\Delta rad52$) was observed to cause hypersensitivity to MMS, HU and UV. When combined with gene deletion for RTT109, it was observed that the double-mutant strain ($\Delta rtt109\text{-}\Delta rad52$) showed some increased hypersensitivity for all treatments tested (figure 7). This synergy may imply that the two genes repair the DNA damage caused by these agents using independent mechanisms. As deletion of RAD52 alone caused a

Figure 7: Drug sensitivity tests for RTT109 and VPS75 deletion mutants, as well as double-mutants. RTT109 deletion mutants demonstrate sensitivity to UV, HU, and MMS, while VPS75 deletion mutants show sensitivity to HU. Double mutant analysis suggests the sensitivity effect is independent of RAD52, a key HR gene.

severe hypersensitivity to 50 mM HU, concentrations of HU were reduced for the experiments with deletion strains for RAD52. Deletion of YKU70, one of the important genes in NHEJ, conferred some hypersensitivity to UV treatment only. A double-mutant strain for RTT109 and YKU70 ($\Delta rtt109\text{-}\Delta yku70$) possessed similar hypersensitivity to that which was caused by single mutation for RTT109 alone when exposed to UV and MMS (figure 7). However, $\Delta rtt109\text{-}\Delta yku70$ showed a synergy for HU treatment suggesting that the two genes may also possess other functions that do not fully overlap for repairing DNA caused by HU.

Genetic interaction analysis for RTT109

Genes that are functionally related may also genetically interact with each other (Tong *et al.* 2004). Consequently, if RTT109 is involved in DNA damage repair, it is expected that it may genetically interact with some known DNA damage repair genes. To further investigate the involvement of RTT109 in DNA damage repair, we investigated its genetic interactions using a miniaturized (Schuldiner *et al.* 2005) synthetic genetic array analysis (Tong *et al.* 2005). The list of our observed interactions that were confirmed is reported in figure 8. As indicated, RTT109 genetically interacts with the previously characterized DNA damage repair gene RAD52. In addition, RTT109 interacted with several genes that are known to be involved in NHEJ (such as YKU80, XRS2, and MRE11), but not with others (such as YKU70 and LIF1). These observations are in agreement with the observation that RTT109 affects the efficiency of NHEJ, as well as perhaps other DNA repair pathways.

Figure 8: Genetic interactions tested by synthetic genetic array for RTT109. Interacting partners include NHEJ factors, elements involved in DNA metabolism, and parts of other complexes of interest.

Other potentially interesting genetic interactions for RTT109 include components of the COMPASS complex, as well as CIN1 and CSE2 genes that have previously been shown to interact with chromatin remodeling genes SWR1 and SWI2 (Mizuguchi *et al.* 2004, Smith and Peterson 2005), and NTG1 which is associated with base excision repair (Alseth *et al.* 1999). These interactions may suggest other roles for RTT109 in DNA repair pathway.

Rtt109 physically interacts with Vps75

One way of studying the function of a target protein is by examining the interactions that it makes with other proteins (Wood *et al.* 2003). To further investigate Rtt109 function we examined its physical interactions in vivo. This was performed using Tandem Affinity Purification (TAP) tag analysis (Rigaut *et al.* 1999). In this method a target protein is double-tagged on its chromosomal locus using a one-step PCR transformation. The tagged protein and its interacting protein partners are then purified in two steps, using affinity purification. There is no overproduction of the target protein; hence the observed interactions are thought to represent genuine interactions within a cell. TAP tag analysis of Rtt109 identified Vps75 as a single interacting protein partner for the tagged-Rtt109 protein (figure 9a). Vps75 is a protein of unknown function, though deletion of this gene has been shown to cause a reduction in telomere length (Askree *et al.* 2004). To confirm the observed interaction a reciprocal TAP tag analysis for Vps75 was performed (figure 9b). In this analysis, Rtt109 was identified as a single interacting protein partner for the tagged Vps75 protein, thus reaffirming an interaction

Figure 9: Tandem Affinity Purification for Rtt109 as bait reveals an interaction with Vps75 (a). Using Vps75 as the bait, Rtt109 is recovered (b). This indicates the two proteins physically interact. A control is presented in (c).

between the two proteins. It should be noted that the data presented here is not exclusive and there might be other components involved in this complex which have not been picked up due to the technical limitations associated with TAP-tagging.

Vps75 also affects the efficiency of NHEJ

It is thought that the proteins that interact with each other are often functionally related. Vps75 interacts with Rtt109; therefore, it was hypothesized that Vps75 may also affect the efficiency of NHEJ. To examine this hypothesis, a deletion mutant strain for VPS75 was subjected to the plasmid repair assay. It was observed that like RTT109, the deletion of VPS75 caused a reduction (34%) in the efficiency of cells to repair linearized plasmid DNA (figure 5). Similarly, the deletion of VPS75 in a JKM139 strain reduced the survival of the cells by more than 60% when DSBs were induced (figure 6). When $\Delta vps75$ was examined for increased sensitivity to MMS, HU, and UV light (figure 7) it was observed that this deletion strain had an increased sensitivity to UV and HU. The analysis of double-gene-deletion mutants for VPS75 with RAD52 and YKU70 showed no synergy. Altogether, these results suggest that VPS75 may have an effect on NHEJ, similar to RTT109, but with a lesser efficacy. This further reaffirms that the two proteins (Rtt109 and Vps75) have a functional interaction.

Effects of Rtt109 and Vps75 on HR

To determine if Rtt109 or Vps75 also affect the efficiency of HR, a modified plasmid repair assay (Erdemir *et al.* 2002) was used. In this assay, plasmid pGV255-dead

was linearized with restriction enzyme BglII, within a non-functional LacZ gene. This linearized plasmid was then co-transformed with a functional LacZ PCR product. Colonies expressing b-galactosidase are formed through HR events between the plasmid and PCR product, while colonies not expressing b-galactosidase are formed through other DSB-repair events. Therefore, the ratio of colonies expressing functional b-galactosidase to the total colony count on selective media represents the efficiency of HR within the strain. It was observed that $\Delta rtt109$ demonstrated levels of HR similar to the wild-type cells (figure 10). However, in the absence of VPS75 ($\Delta vps75$), the efficiency of HR is reduced to 76% of that in the wild-type cells (figure 10). This suggests that VPS75 and not RTT109, might have an effect on the efficiency of HR.

Discussion

Here we provide evidence that Rtt109 and Vps75 physically interact with each other and the deletion of either of the two proteins affects the efficiency of NHEJ in yeast. A role in NHEJ for this complex is consistent with numerous previous findings. For example, Rtt109 has been identified as a regulator of Ty1 transposition (Scholes *et al.* 2001), as were NHEJ factors Mre11 and Rad50. Association between transposition and DSB repair is reviewed in (Haber 2005), and suggests transposition-regulating genes as reasonable candidates for affecting NHEJ. Moreover, Vps75 has previously been identified as being involved in telomere maintenance (Askree *et al.* 2004), but its molecular function has also remained unknown. This finding, in conjunction with the fact that several genes associated with DNA repair have also been shown to associate

Figure 10: A plasmid-based test for homologous recombination reveals VPS75, but not RTT109, appears to be involved in homologous recombination.

with telomeres (Goudsouzian *et al.* 2006, Zhang *et al.* 2006), is therefore in keeping with the suggestion that Vps75 might be involved in the efficiency of NHEJ. Further, both Rtt109 and Vps75 have been localized to the nucleus (Huh *et al.* 2003). Since NHEJ occurs in the nucleus, this is also in agreement with our data that the two proteins Rtt109 and Vps75 interact and affect the efficiency of NHEJ.

The deletion of Rtt109 had a stronger effect than deletion of Vps75 on the efficiency of NHEJ in both plasmid repair as well as in chromosomal break repair. Similarly, $\Delta rtt109$ showed higher sensitivity to DNA damaging agents. This may suggest that Rtt109 is perhaps the core component of the Rtt109-Vps75 protein pair within NHEJ. Alternatively, it is possible that Rtt109 can maintain a reduced functional efficiency independent of its interaction with Vps75. However, it is interesting to note that Vps75 also functions within HR. While Rtt109 may be the functional component within NHEJ activity, Vps75 may possess other, more general functions in the context of DNA damage.

Generally, it is expected that the deletions of the genes associated with DNA repair cause hypersensitivity to DNA damaging agents. For example, deletions of different NHEJ factors cause varying degrees of hypersensitivity to different DNA damaging agents (reviewed in Dudášová *et al.* 2004). $\Delta yku70$ and $\Delta yku80$ both have been indicated to show some MMS sensitivity previously, but not to the same degree as inactivation of HR through deletion of RAD52 (Milne *et al.* 1996). Deletion of LIG4 also has been demonstrated to slightly increase sensitivity to MMS and UV (Schär *et al.* 1997). In this study we show that the deletion of Rtt109 confers hypersensitivity to UV,

HU, and MMS, and that the deletion of VPS75 makes the cells hypersensitive to UV. Such variations in hypersensitivity to different DNA damaging agents for deletion strains of various NHEJ-associated genes may stem from the differences in the processing steps required to prepare the DNA ends for re-ligation. Radiation-induced DSB's often require the excision of chemically-modified nucleotides (Wyman and Kanaar 2006), whereas MMS is a known DNA methylating agent that methylates adenine and guanine (Chang *et al.* 2002) which may lead to other required forms of processing. HU, however, induces DSB's through collapsed replication forks (Rittberg and Wright 1989), rather than through chemical alteration of the DNA structure, and may therefore require less processing. It is also possible Rtt109 is involved in other DNA repair systems, as indicated by genetic interactions with a broad range of DNA repair proteins. This involvement may or may not completely overlap with Vps75, leading to numerous differences in how each deletion strain could deal with varying types of DNA damage.

Our observations indicate that the effect of neither Rtt109 nor Vps75 on the efficiency of NHEJ seem to be as strong as the core components of NHEJ such as Yku80, that are directly responsible for the repair of the damage at the site of the DNA break. Chromatin immunoprecipitation analysis did not indicate enrichments for Rtt109 at the site of the DNA damage, nor did deletion of Rtt109 reduce the level of Yku80-TAP recruitment to the site of DNA damage. Similarly our Western-blot analysis using Yku80-TAP strain did not indicate any reduction for Yku80-TAP in the absence of Rtt109. These observations may suggest a non-direct role for Rtt109 in NHEJ.

While this work was in progress it was reported that Rtt109 has a novel histone acetyltransferase activity that specifically acetylates histone H3 at lysine 56 (H3K56). The strong DNA damage sensitivity of RTT109 was implied to be related to the lack of this important histone modification (Han *et al.* 2007, Tsubota *et al.* 2007, Collins *et al.* 2007, Driscoll *et al.* 2007). It is likely that Rtt109 protects DNA in S-phase via H3K56 acetylation, and also has an independent effect in NHEJ in association with Vps75. However, previous examination of yeast strains deficient in H3K56 acetylation using a plasmid repair assay revealed greater than 80% efficiency for NHEJ (Matsumoto *et al.* 2005), much higher than what we observed for gene deletions of RTT109 and VPS75 in this study. While altered chromatin configuration caused by the absence of Rtt109 or Vps75 could undoubtedly affect NHEJ in an indirect fashion, this sole H3K56 acetylation activity is insufficient to explain our observed reduction in NHEJ activity. Since VPS75 does not appear to have a significant role in H3K56 acetylation, other acetylation targets of the Rtt109 HAT with relevance to NHEJ are likely to exist that might be specifically regulated by Vps75. Whether there exist other histone modification targets or other modifiable DNA repair proteins is currently under investigation.

Our observations indicate that Vps75 might also play a role in HR. This along with our other observations regarding the sensitivity of RTT109 and VPS75 deletion strains to different DNA damaging agents, together with our genetic interaction data for RTT109 suggest that these proteins may be involved in a more general DSB response. Further investigations are needed to identify the exact role of Rtt109 and Vps75 in DNA repair.

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Chapter 4: BUB1, BUB2, MAD3 and the Spindle Assembly Checkpoint Response are Necessary for Efficient Non-Homologous End-Joining in *Saccharomyces cerevisiae*

Abstract

The Non-Homologous End-Joining (NHEJ) DNA repair pathway requires multiple cellular functions to operate smoothly. Here we provide evidence that genes associated with the spindle assembly checkpoint (BUB1, BUB2, and MAD3) are required for efficient NHEJ repair using a plasmid-based DNA repair screen. This effect is relevant in a chromatin context and appears to be mediated through the actions of the Anaphase Promoter Complex (APC), the cyclin CLB2 and the SBF transcription factor, composed of SWI4 and SWI6. However, no other members of either branch of the spindle assembly checkpoint tested appeared to share this effect. Synthetic genetic interactions between BUB2 and other identified NHEJ-related genes strengthen the conclusion that these genes influence efficient NHEJ. Overexpression of BUB1 and BUB2 were further found to recover the DNA damage sensitivity phenotype of $\Delta rtt109$ and $\Delta xrs2$ mutants, both compromised for DNA repair activity. We hypothesize that compromising the spindle assembly checkpoint compromises DSB repair by an effect similar to impairment to the DNA damage checkpoint.

Introduction

Throughout the *Saccharomyces cerevisiae* cell cycle, spontaneous or induced DNA damage can cause a variety of negative consequences. One of the most serious types of DNA damage is the Double-Strand Break (DSB), which occurs when both strands of DNA are cut at or near the same location. This shears the DNA molecule into two separate pieces which must be rejoined. If unrepaired or misrepaired, this type of DNA damage can lead to mutation, loss of genetic information, chromosomal rearrangements, and cell death. As a result, efficient repair of DSB's is essential for the long-term survival of the cell and faithful maintenance of genetic information.

There are two pathways in *S. cerevisiae* which have evolved specifically to deal with DSB's; Homologous Recombination (HR) (reviewed in Dudáš and Chovanec 2004) and Non-Homologous End Joining (NHEJ) (reviewed in Dudášová *et al.* 2004, Daley *et al.* 2005). While HR is the preferred pathway in yeast (Siede *et al.* 1996), it is limited by a requirement for a homologous template to use as a basis for repair. Without this template, HR cannot proceed. As a result, HR is limited in its availability, and NHEJ must sometimes be used.

The primary proteins and complexes involved in NHEJ are largely known. Repair begins with the identification of the lesion's two ends by the Yku70p/Yku80p heterodimer, which is a ring-shaped complex that encircles the broken DNA ends to protect them from digestion and recruit repair factors. The MRX complex, composed of Mre11p/Rad50p/Xrs2p, serves as a scaffold and bridge. Finally, a complex of

Lig4p/Lif1p/Nej1p is recruited to the break, where Lig4p ligates the two broken ends back together (Dudášová *et al.* 2004).

During cell division, any disruption to the DNA or to chromosome structure can be particularly destructive to the cell. This necessitates cell cycle checkpoint proteins to prevent cell division until all DNA lesions, misaligned chromosomes, spindle damage, or other problems can be resolved. There are many checkpoint mechanisms involved in this checkpoint, but the Anaphase-Promoting Complex (APC) serves as a significant regulator of cell division in response to many factors such as errors in chromosome alignment (reviewed in Zich and Hardwick 2010).

In our screen for gene deletions that impaired NHEJ efficiency, we identified two genes, BUB1 and BUB2, which are known to play related but separate roles in the spindle checkpoint (Hardwick 1998). We therefore sought to confirm this effect and determine how these two genes could influence NHEJ repair efficiency, hypothesizing that the effect may be mediated through a regulatory effect in the cell cycle.

Materials and Methods

Strains and Plasmids

Yeast gene deletion strains used in plasmid repair assays and synthetic genetic array analysis are derivatives of yeast S288C, described in Winzeler *et al.* (1999). For the creation of double-mutants, a knockout procedure using PCR products containing the *NAT* gene, flanked by regions homologous to BUB1 or BUB2 flanking regions was used, as described in Winzeler *et al.* (1999). Double-mutant products were confirmed for

deletion of either BUB1 or BUB2 by PCR with primers within the *NAT* gene and upstream of the targeted gene. Generation of yeast strain JKM139 is described in Moore and Haber (1996). Gene deletion strains in JKM139 were created and confirmed as previously described. For the plasmid repair assay, a derivative of plasmid p416 was used (Krogan *et al.* 2003). Gene overexpression plasmids are described in Sopko *et al.* (2006).

Plasmid Repair Assay

The yeast NHEJ-specific plasmid repair assay was performed as described previously. Briefly, a unique XbaI site located in a region of the p416 plasmid without homologous sequence was used to linearize the plasmid. Equal volumes of cells were transformed in parallel using standard lithium acetate yeast transformation procedures. Transformants were grown on minimal media lacking Uracil (2% glucose, 2% agar, 6.74 yeast nitrogen base/L, 2 g amino acid powder minus uracil/L), an essential nutrient that can be produced by gene *URA3* on the transformed plasmid. Plates were incubated 2-3 days at 30°C and colonies were counted manually. Relative colony formation on the linearized plasmid transformation plate compared to the intact plasmid transformation plate was normalized to a wild-type. Each experiment was replicated in triplicate.

JKM139 Repair Assay

The JKM139 chromosomal NHEJ assay was performed as described previously. Briefly, deletion strains in JKM139 background were grown in YPD (1% yeast extract, 2%

peptone, 2% glucose) to saturation (2 days in 30°C). Serial dilutions from 10^{-1} to 10^{-6} were created and 100µl samples were plated on standard YPD and YP-GAL (1% yeast extract, 2% peptone, 2% galactose, 2% agar). Plates were incubated for 2-3 days at 30°C and colonies were counted manually. Repair efficiency was estimated as the number of colonies per µl on plates containing galactose divided by the number of colonies formed per µl corrected to the same dilution factor on glucose plates, using only a single plate per set. Each test was repeated in triplicate.

Drug Sensitivity Spot tests

Spot test analysis was performed as previously described in this work. Briefly, cells were grown to saturation in YPD media (2 days), serially diluted to concentrations of 10^{-3} - 10^{-6} and 15µl 'spots' were placed sequentially on YPD plates with or without 40-50mM HU, 0.02-0.03% MMS, or on YPD plates which subsequently received 12j/m² of UV light. To maintain the presence of overexpression plasmids, and to induce overexpression (where used), minimal media lacking uracil and containing galactose as a carbon source was substituted for YPD. Growth defects were scored visually based on colony count and colony size, compared to wild-type. The analysis was conducted at least 3 times.

Synthetic Genetic Array Analysis

SGA analysis for BUB2 was assayed for as in Schuldiner *et al.* (2005) using a subset of ~380 gene deletion mutants selected for this research project based on known

or suspected effects within DNA damage repair. Synthetic sick or synthetic lethal phenotypes were scored visually. The assay was repeated twice.

Phenotypic Recovery Genetic Array Analysis

To assess if overexpression of BUB1 or BUB2, an overexpression plasmid containing the genes of interest were introduced to yeast strain Y7092 mating type α , using standard lithium acetate yeast transformation procedures. Mating was conducted as in Schuldiner *et al.* (2005) with the ~380 gene deletion set previously described. Crossed plates were maintained on minimal media lacking uracil to prevent loss of plasmid.

Once a crossed set of deletion strains containing the overexpression plasmids were created, both deletion/overexpression sets (Δxxx /BUB1 overexpression and Δxxx /BUB2 overexpression) were replica pinned on minimal media with galactose containing 50mM HU, 0.03% MMS. The initial deletion set without overexpression plasmid was used as a control. Plates were allowed to incubate at 30°C for 1-2 days. Growth defects were scored visually as a reduction in colony size, compared to surrounding colonies as well as to control plates, to ensure localized effects did not bias the results. Recovery was also scored visually, as growth comparable to surrounding colonies and control plate when in the presence of BUB1 or BUB2 overexpression. The assay was repeated at least twice.

Results

Both BUB1 and BUB2, but not all associated genes, affect NHEJ efficiency in a plasmid repair assay

A plasmid repair assay was used to systematically identify novel genes whose deletion would impair NHEJ efficiency. A linearized plasmid was transformed into selected deletion strains and colony formation was compared to an equal amount of intact plasmid control. The plasmid contained the URA3 gene as a selectable marker, and therefore colony formation of the cells receiving linearized plasmid is assumed to occur only in the event of successful repair. As the plasmid was linearized in a region without homologous region within the *S. cerevisiae* genome, it is further assumed all successful repair events will be the result of NHEJ. Efficiency of NHEJ repair is calculated as the number of colonies formed from transformation with linearized plasmid divided by the number of colonies formed from intact plasmid, normalized against a wild-type transformation. This assay has been successfully used to identify NHEJ genes in the past (Shim *et al.* 2005), and was able to identify key NHEJ-impairing gene deletions in our assay such as $\Delta lig4$ (figure 11a).

Interestingly, both $\Delta bub1$ and $\Delta bub2$ show a reduced NHEJ efficiency compared to a wild-type (0.40 +/- 0.20 and 0.49 +/- 0.23, respectively) (figure 11a). Both BUB1 and BUB2 are associated with the spindle damage checkpoint and delaying the cell cycle in response to improper chromosome alignment, however, both act independently to prevent cell cycle progression. As there is no current association between these genes and NHEJ, we hypothesized that a novel role in NHEJ efficiency exists for this pathway.

Figure 11: Both plasmid repair assays (a) and JKM139 chromosomal assays (b) reveal a possible role in NHEJ efficiency for spindle checkpoint genes BUB1, BUB2, and MAD3, but not all spindle checkpoint genes.

Because both BUB1 and BUB2 operate in genetically-distinct pathway branches (Hardwick 1998), we initially sought to examine other gene deletion strains in the same two pathway branches for similar effects. Bub1p interacts physically or functionally with Bub3p, Mad1p, Mad2p, Mad3p, and several other proteins, whereas Bub2p interacts with Bfa1p. However, of the mutants tested, only MAD3 deletion mutants showed a reduced, but still statistically significant, reduction in NHEJ efficiency (0.66 ± 0.38) (figure 11a), suggesting the effect on NHEJ is a specific function of BUB1, BUB2, and MAD3, and not general to both checkpoint mechanisms.

The NHEJ efficiency effect of $\Delta bub1$ and $\Delta bub2$ is relevant in a chromosomal context

To confirm the significance of the plasmid repair assay in chromosomal DNA, a JKM139-based assay for NHEJ in the context of chromosomal DSB's was used. JKM139 is a strain of yeast (described in Moore and Haber 1996) which contains an HO endonuclease paired with a GAL-promoter, as well as the appropriate restriction site located in the *MAT* locus. This allows galactose-inducible cleavage of the DNA in a region without homologous template, which must be repaired by NHEJ for the cell to survive and form a colony on media containing galactose as a carbon source. Unlike the plasmid-based assay, this repair assay requires NHEJ to occur within the cell's chromatin, thereby mimicking a naturally-induced chromosomal DSB with all relevant histone and protein DNA packaging. In this chromosomal NHEJ assay, both $\Delta bub1$ and $\Delta bub2$ reduced colony formation to 4.6% and 7.8% of the wild-type, respectively (figure 11b).

The efficiency of NHEJ in $\Delta bub1$ and $\Delta bub2$ mutants appears much lower in a chromosomal context than in a plasmid context, however, this may be an artifact of our procedure. Survivorship of the JKM139 strain is substantially reduced (2.4% in wild-type), which reflects the fact that a successful and non-mutagenic NHEJ repair event will restore the HO endonuclease recognition site and potentially lead to re-cleavage. This causes a JKM139 wild-type or deletion mutant with no loss of repair fidelity to undergo repeated cycles of cleavage and repair, with reduced survival at each repair event. By contrast, deletion of YKU70 is known to reduce repair fidelity (Shim *et al.* 2005), and thereby reduce the likelihood of restoring the cleavage site. This leads to an artificial increased depression of the wild-type's apparent NHEJ efficiency, and potentially an increased depression of $\Delta bub1$ and $\Delta bub2$ mutant strains. Alternatively, the chromosomal DSB, combined with damage to the spindle checkpoint, may be more likely to lead to cell death in actively dividing cells, as chromosome fragments may be mis-segregated during cell division.

To further support a role for BUB1 and BUB2 in DNA repair, spot test analysis was conducted with DNA damage inducing agents MMS, HU, and UV irradiation. Serial dilutions of both $\Delta bub1$ and $\Delta bub2$ mutant strains were placed on plates containing 0.03% MMS, 40mM HU, and on YPD plates that were then exposed to 12J/m² of UV light. $\Delta bub1$ showed a slight sensitivity to both MMS and UV treatments, as measured in colony numbers, while $\Delta bub2$ showed reduced growth in the UV treatment only (figure 12). This further supports a possible role in DNA repair that is not related to the artificial presence of a plasmid.

Figure 12: Sensitivity spot tests for BUB1 and BUB2 deletion mutants in the presence of MMS and after exposure to UV light. BUB1 deletion mutants show slight sensitivity to MMS, and both show sensitivity to UV.

YPD

MMS (0.03%)

UV (12j/m²)

The Anaphase Promoting Complex is relevant to the effects of BUB1 and BUB2 in promoting NHEJ efficiency

Both BUB1 and BUB2 prevent the cell from completing cell division through the activity of the APC, which blocks progression through anaphase until lesions such as misaligned chromosomes or spindle damage can be repaired. To determine if our results were related to this activity, we repeated the plasmid repair assay on several components of the APC. $\Delta apc9$, $\Delta swm1$, and $\Delta cdc26$ all showed a reduction in NHEJ efficiency compared to wild-type (0.51 +/- 0.30, 0.62 +/- 0.10, and 0.51 +/- 0.03 respectively), however, $\Delta mnd2$ did not (0.86 +/- 0.47) (figure 13). Double-mutants with $\Delta apc9/\Delta bub2$ recovered the effect of either single mutation (1.06 +/- 0.48), suggesting a positive genetic interaction between the two genes. A double-mutation of $\Delta cdc26/\Delta bub2$ was similar to the single-mutants (0.56 +/- 0.27), suggesting the effect of the genes on NHEJ is likely within the same pathway. It should be noted that deletion of other APC proteins often results in a sick or inviable phenotype, and so an exhaustive set of plasmid repair assay results would not be possible.

The cyclin CLB2, but not APC target ASE1, is relevant to the activity of BUB1, BUB2 in promoting NHEJ efficiency

The APC regulates the concentration of numerous factors related to cell cycle control. As APC disruption was found to affect NHEJ efficiency, we chose to examine two substrates targeted by the APC; the cyclin Clb2 (primary cyclin involved in mitosis

Figure 13: Plasmid repair assay results for other components of the APC reveal the NHEJ impairment of a BUB1/BUB2 deletion may act through the APC.

(Grandin and Reed 1993)) and Ase1 (spindle elongation and stabilization factor (Schuyler *et al.* 2003)). In our plasmid repair assay, $\Delta clb2$ was found to have significantly reduced NHEJ efficiency (0.44 ± 0.19) compared to wild-type. $\Delta ase1$, however, was not detectably reduced (1.21 ± 0.06). A double-mutant of $\Delta clb2/\Delta bub2$ showed a reduction comparable to both single-mutants (0.56 ± 0.27), indicating the NHEJ disruption of each single-mutant was likely within the same pathway. (figure 14a)

As cyclin Clb2p is degraded by the APC, it is possible that disruption to the APC may cause Clb2p to accumulate. We therefore used a CLB2 overexpression plasmid (Sopko *et al.* 2006) to examine the effects of increased Clb2p concentration on NHEJ efficiency. In JKM139 WT strains overexpressing Clb2p under a gal promoter, NHEJ efficiency was reduced to 33% of wild-type (figure 14b). This indicates deletion or overexpression of CLB2 results in NHEJ loss of efficiency, and further provides evidence that this effect is present within a chromosomal context as well as within a plasmid context.

The SBF complex, a transcription factor associated with budding, is relevant to the activity of BUB1, BUB2 in promoting NHEJ efficiency

The essential protein Cdc28p is regulated by Clb2p, and in turn regulates the SBF and MBF complexes, composed of Swi4p-Swi6p and Mbp1p-Swi6p respectively. Both these factors represent significant transcription factors, with SBF believed to associate more with budding and cell wall synthesis and MBF associating more with DNA

Figure 14: Plasmid repair assay and JKM139 chromosomal break assay reveals the APC mediated effects of BUB1/BUB2 on NHEJ may be occurring through the cyclin CLB2

replication and repair (Iyer *et al.* 2001). When tested with the plasmid repair assay, both $\Delta swi4$ and $\Delta swi6$ showed significant reduction in NHEJ activity (0.38 ± 0.10 and 0.43 ± 0.24), while $\Delta mbp1$ did not (1.27 ± 0.13) (figure 15). A $\Delta swi4/\Delta bub2$ double-mutant showed similar NHEJ reduction to either single mutant (0.37 ± 0.08), but a $\Delta swi6/\Delta bub2$ double-mutant was not significantly different from wild-type, indicating a possible recovery of the phenotype (0.81 ± 0.35) (figure 15). Therefore, the SBF complex, not the MBF complex, appears related to the NHEJ efficiency effect observed in $\Delta bub2$ mutants.

Synthetic Genetic Interactions of the BUB2 deletion reveal DNA repair proteins, other NHEJ candidates, and other interactions

To further characterize the effects of $\Delta bub2$ deletion on yeast cells, a limited SGA analysis was performed. Synthetic sick or synthetic lethal phenotypes were scored visually. As expected, genes involved in budding, the spindle checkpoint, and chromosomal or plasmid maintenance were detected, consistent with BUB2's role in the spindle checkpoint and cell division. Our results also indicate several genes known to be involved in NHEJ (RAD50, XRS2), as well as a subset of the genes previously identified in our search for novel NHEJ factors (ASF1, RTT101, SWR1, RNR3, SWI6, PSY2). In addition, genes related to telomere maintenance and genes associated with other forms of DNA repair were found to genetically interact with BUB2. This further supports our claim that BUB2 has a function in NHEJ. (figure 16)

Figure 15: Plasmid repair assay reveals transcription factors SWI4 and SWI6, but not MBP1, are involved in the NHEJ efficiency mediation effects of BUB1/BUB2.

Figure 16: SGA genetic interactions for BUB1, revealing interacting partners involved in chromosome/plasmid maintenance, DNA repair, NHEJ, Chromatin remodelling, and many of our novel NHEJ mediating candidate genes.

Overexpression of Bub1p can recover DNA damage sensitivity phenotypes in $\Delta rtt109$ and $\Delta xrs2$ mutants

In an attempt to find a more specific role in NHEJ for BUB1, overexpression plasmids containing each gene connected to a GAL promoter (described in Sopko *et al.* 2006) were introduced to the Y7092 α -mating type, and were crossed with our subset of the yeast gene deletion array (~380 genes). A reduced growth phenotype was found to occur in both $\Delta xrs2$ and $\Delta rtt109$ colonies in treatments for HU and MMS, among other genes. However, this growth reduction phenotype was rescued by the induction of BUB1 through the overexpression plasmid on GAL media.

To confirm this effect, spot tests were performed on media containing 40mM HU or 0.02% MMS on minimal media plates containing galactose sugar, to induce BUB1 overexpression. While $\Delta xrs2$ and $\Delta rtt109$ both exhibited reduced growth under HU, growth under BUB1 induction returned to near wild-type levels (figure 17). When exposed to MMS, BUB1 induction also recovered the sensitivity phenotype of $\Delta xrs2$ mutants (figure 17). This demonstrates BUB1 induction can support an otherwise compromised NHEJ repair pathway.

Discussion

In this paper, we provide evidence linking the genes BUB1 and BUB2 to efficient NHEJ via an APC- and SBF-mediated pathway. Both $\Delta bub1$ and $\Delta bub2$ show a reduction in NHEJ efficiency, as does $\Delta mad3$. However, not all the BUB1- or BUB2- associated

Figure 17: Sensitivity spot tests revealing that overexpression of BUB1 can compensate for partial impairment of DNA damage tolerance induced by deletion of key NHEJ genes.

genes display this reduction, indicating the effect is specific to only some proteins in this pathway. There is no clear explanation for this selectivity in terms of cell cycle delay; *Δbub1*, *Δbub2*, and *Δmad3* lose the ability to delay cell cycle in response to spindle depolymerisation, but so do *Δbub3* and *Δbfa1* (Li and Murray 1991). Bub1p binds to the kinetochore, while Bub2p binds to the spindle, and Bub3p also binds to the kinetochore. Bub3p also complexes to, and is phosphorylated by, Bub1p (Hardwick 1998), but only Bub1p appears relevant to NHEJ. BUB1 shares some sequence identity with MAD3, but not in the kinase domain which defines BUB1's signalling activity (Hardwick *et al.* 2000). There appears to be no clear connection between the three candidates in terms of binding partners or pathway branch. While previous research has indicated a possible overlap between the DNA damage checkpoint and the spindle assembly checkpoint (Kim and Burke, 2008), this effect was independent of the kinetochore which Bub1p is binding to, and involved many proteins in the Bub1p branch of the spindle checkpoint, not the Bub2p branch. However, it may be that the relevant distinction is not specific type of activity, but overall effect on the progression of the cell cycle (see below).

As both BUB1 and BUB2 delay cell cycle progression through anaphase by affecting the APC, we examined several APC components to determine if disruption of this complex would create a similar effect. *Δapc9*, *Δswm1*, and *Δcdc26*, but not *Δmnd2*, produced a similar reduction in NHEJ activity, and a double-mutant of *Δcdc26/Δbub2* showed a reduction similar to both single-mutants. This may indicate a disruption of NHEJ in *Δbub1* or *Δbub2* strains could be a result of cell cycle regulation effects through the activity of the APC.

The APC regulates the cell cycle through the targeted degradation of a variety of proteins such as the B-type cyclin Clb2p. Our results show a disruption to CLB2, both in the case of a $\Delta clb2$ mutant and in the case of a CLB2 overexpression plasmid, creates a reduction in NHEJ efficiency, with no additional reduction for a $\Delta clb2/\Delta bub2$ double-mutant. The same is not true for all targets of APC degradation, indicating the effect is somewhat specific. It is interesting that both deletion and overexpression of CLB2 caused a reduction in NHEJ efficiency. It is unusual, though not unheard of, for a deletion and overexpression phenotype to show similar effects (Sopko *et al.* 2006). As cyclin concentration in the cell regulates cell division by repeated cycles of expression and degradation, it may be that deregulation of this expression cycle is responsible to the NHEJ effects regardless of how that disruption occurs.

Cyclin Clb2p is known to activate Cdc28p, which in turn activates the transcription factor complexes SBF and MBF. Interestingly, the MBF complex is associated more strongly with DNA repair and synthesis, but in this experiment the SBF complex was found to have a greater effect on NHEJ efficiency.

There are several possible explanations for the specificity of phenotypic effects we observe. The most obvious manner for BUB1 and BUB2 to influence NHEJ through a SBF-mediated activity is gene regulation. Differential DSB repair mechanisms are favoured in different stages of the cell cycle; with a known NHEJ effect for $\Delta swi4$ and $\Delta swi6$ strains, as well as the comparable phenotype of $\Delta swi4/\Delta bub2$ double deletion mutants to $\Delta swi4$ single deletion mutants, a DSB-repair regulatory effect for disruption of the spindle-assembly checkpoint seems reasonable. However, the SBF complex is

believed to regulate less in the way of DNA synthesis and repair pathways than the MBF complex (Iyer *et al.* 2001), and $\Delta mbp1$ deletion mutants did not show a reduced NHEJ efficiency. MBF is known to regulate Rad27p (Iyer *et al.* 2001), a gene known to be involved in NHEJ as an end-processing endonuclease (Dudášová *et al.* 2004). This activity is unnecessary in our plasmid repair screen and our JKM139-based chromosomal screen, but one would expect it to be potentially relevant in MMS sensitivity or UV sensitivity. It is therefore unexpected that a general DNA damage regulatory effect would not occur through MBF. It is also difficult to explain how upregulation of BUB1 could possibly overcome gene deletions of the MRX complex, as MRX is a key functional component of both NHEJ and HR. While MRX deletions do not completely incapacitate the NHEJ pathway, they remain a core component of efficient NHEJ. Enhancing the expression of a promoter of NHEJ activity should not restore the loss of NHEJ functionality.

An alternative explanation for the observed data is that less efficient NHEJ, such as in a $\Delta rtt109$ or $\Delta xrs2$ strain, can be partially compensated for by altering the amount of time spent in varying cell cycle stages, as caused by overexpression or deletion of the examined spindle assembly checkpoint proteins. A $\Delta bub1$ or $\Delta bub2$ mutant is known to be unable to delay cell cycle progression in response to spindle damage, and deletions of XRS2, CLB2 and BUB1 are all known to possess a higher budding index than wild-type (Zettel *et al.* 2003), indicating more cells in these deletion mutants will be in the process of active cell division. Either by increasing the proportional amount of time spent in active cell division (which favours HR, as sister chromatids are available as templates), or

by preventing a temporary 'pause' in each cycle of cell division prior to commitment to anaphase, the effects of imperfect efficiency for NHEJ may be exacerbated. By not allowing time for multiple attempts at repairing the lesion, by downregulating NHEJ to favour HR, or by not allowing a slower, less optimal repair sequence to occur, apparent NHEJ efficiency is reduced. This effect may resemble impairing cell cycle delay through the DNA damage checkpoint. As Clb2p must be degraded by the APC, targeted by Cdh1p, in order to allow cell division to complete, and given the high levels of Clb2p accumulation in APC-impaired $\Delta cdh1$ mutants (Visintin *et al.* 1997), it is reasonable that both our APC-impaired strains and our CLB2 overexpression strains can trigger a similar effect. SBF is also a known regulator of CLB2, among other cyclins (Iyer *et al.* 2001). Given that cells stressed by MMS or HU are not required to use NHEJ exclusively, or even primarily, and that the MRX complex is active in HR, this effect is not expected to be NHEJ-specific. Likely the BUB1 overexpression recovery of a MRX-compromised cell's drug sensitivity phenotype represents a more general compensation for damage to DNA repair pathways, many of which could impact HU or MMS sensitivity.

Previous research on BUB1-5 mutants (which expresses BUB1 at an unusually high level) shows a tendency to trigger cell cycle arrest despite a lack of spindle damage (Farr and Hoyt 1998). This may indicate that BUB1 overexpression is halting cell cycle progression with an effect that overlaps the DNA damage response. Essentially, the spindle assembly checkpoint (which delays cell cycle progression) has similar physiological effects in response to genotoxic stress to the DNA damage checkpoint (which delays cell cycle progression). Connections between the two pathways have

previously been proposed (Kim and Burke, 2008) and both delay cell cycle in a similar manner, by acting on the APC. This allows the hypothesis that the DNA damage checkpoint and the spindle assembly checkpoint are simply not distinct in their downstream effects beyond a certain point, at least not in respect to any APC-mediated event. By altering the dynamics of the spindle assembly checkpoint, we may be indirectly stimulating the DNA damage response checkpoint's cell cycle delay function or regulatory mechanisms, which act on the DNA damage present regardless of the cause of the pathway stimulation. In the relatively simple genetic environment of *S. cerevisiae*, this is not surprising; it will be interesting to determine if there are distinctions downstream of the APC in 'higher' eukaryotes.

Chapter 5: Concluding Remarks:

A plasmid repair assay-based screen successfully identified novel NHEJ-related candidate genes

Our objective in this study was to identify and characterize novel genes related to the NHEJ pathway in *S. cerevisiae*. To identify these genes, we used a primary screen based on a commonly used plasmid-based repair assay on a subset of the yeast gene deletion array. This screen not only identified known NHEJ factors such as LIF1, LIG4, and NEJ1, but also RSC2, which encodes for part of the RSC complex, that has recently been associated with efficient NHEJ (Shim *et al.* 2005). This allows us to confirm the screen's ability to successfully identify NHEJ-related gene candidates.

Numerous other factors were found to influence NHEJ efficiency. PSY2 and PPH3, two parts of a protein phosphatase complex associated with cisplatin resistance (Hastie *et al.* 2006), were found to decrease NHEJ efficiency in deletion mutants. Other factors include genes involved in DNA synthesis (RNR3, POL32), genes involved in other forms of DNA damage repair (MSH6, RAD2, UBC13), transcription-related genes (SWI4, POP2, RXT1, SRO9), histone related genes (ARP6, HIR3, VPS72), checkpoint related proteins (BUB1, BUB2, MEK1), and one uncharacterized gene (HUR1).

It should be noted, however, that as only a subset of the yeast gene deletion array was tested, and due to gene deletion strains being available for only ~4600 of the over 6000 genes in the *S. cerevisiae* genome, it is unlikely that this represents an exhaustive list of NHEJ-related genes. Further, the possibility exists that the effects of some NHEJ-relevant gene deletions may be concealed by compensatory effects from

other genes. In this case although they affect the efficiency of NHEJ, they would be undetectable in our screen.

Another limitation associated with our approach is the technical limitation associated with the plasmid repair assay. It is likely that the effect of gene deletion on NHEJ may fall below our detectable reduction threshold. Altogether, while this list contains some interesting protein candidates, and may direct future study to significant NHEJ-related complexes or pathways, it is by no means a complete list of all relevant genes.

Protein Hur1 is required for efficient NHEJ activity in vivo

Originally identified in our plasmid repair screen, HUR1 possesses a short region of overlap with the gene PMR1. In addition to our initial results, we have confirmed that HUR1 is responsible for the NHEJ effect by using a deletion of the first 70 amino acids of HUR1, thereby leaving the PMR1 overlap region unaffected. Further, HUR1 deletion strains in a JKM139 background reduced survivorship on galactose-containing media to almost zero. This effect was partially recovered by the introduction of a plasmid containing the HUR1 gene fused to a GAL promoter. The imperfect recovery of NHEJ activity may be due to disruption in expression levels of this protein.

Protein complex Rtt109-Vps75 is required for efficient NHEJ activity in vivo

RTT109 was found to be an important factor in NHEJ efficiency, as well as being needed for various types of DNA damage inducing agents. Interestingly, it was found to

form a complex with VPS75, which was not part of our original screen. Investigation of VPS75 revealed it also possessed a NHEJ efficiency-related effect, though not as severe as RTT109. This observation further highlights the notion that our original list of 384 gene deletion candidates are by no mean exhaustive, and there are other genes that can affect the efficiency of NHEJ but do not appear in our list. The effect of both RTT109 and VPS75 was found to be relevant in a chromosomal context through the use of a JKM139 strain. Genetic interactions and double-mutant analysis further support a role in NHEJ. Oddly, VPS75 also appeared to possess a HR-related effect, while RTT109 did not. It is believed the RTT109-VPS75 complex influences the efficiency of NHEJ through acetylation of histone 3 on lysine 56, part of a signal which allows the nucleosome to unravel, allowing DNA repair factors access to the lesion.

Rtt109p is a histone acetyltransferase responsible for H3K56 acetylation, a histone modification known to be related to NHEJ and DSB repair. While several theories have been presented as to how exactly this histone modification contributes to DNA repair, it is present at the site of DNA damage induction and seemingly must be removed as DNA damage repair progresses. If acetylation factor Rtt109p is lost, or its chaperone Vps75, there is a significant reduction in NHEJ activity. If deacetylation activity is lost, NHEJ repair proteins do not disassociate from the lesion, indicating a failure to complete repair. Clearly the carefully regulated acetylation/deacetylation throughout the cell's response to a DSB is an essential component for allowing NHEJ to act efficiently to maintain genome integrity.

Proteins Bub1 and Bub2 are required for efficient NHEJ activity in vivo

Both deletion mutants of BUB1 and BUB2 display a significant reduction of NHEJ efficiency in our plasmid repair assay, as well as significant chromosomal NHEJ impairment. As these proteins are part of different branches of the spindle assembly checkpoint, we tested other factors involved in this pathway. However, it appeared that only MAD3 demonstrated a similar effect.

As the spindle assembly checkpoint delays cell cycle progression through the activity of the APC, we tested components of the APC for similar effect. Several APC factors (APC9, SWM1, and CDC26) were found to be necessary for efficient NHEJ. The cyclin and APC target CLB2 was found to impair NHEJ both in the event of a gene deletion and in the case of gene overexpression. Another APC target, ASE1, did not show a similar effect. Finally, the SBF complex, a transcription factor composed of Swi4p and Swi6p, was discovered to be related to this NHEJ effect. This indicates a long cascade of signals, from BUB1 and BUB2 to the SBF complex, in some way regulates a cellular response to DSB's, allowing NHEJ to act efficiently to repair any detected lesions.

It appears the effects of BUB1 and BUB2 deletion, and all subsequent disruptions in this pathway, may disrupt NHEJ efficiency in a manner that resembles the DNA damage response, which involves the blocking of cell cycle progression in response to a DNA lesion in a similar manner to the spindle assembly checkpoint. BUB1 deletions exhibit a slow growth and increased budding index phenotype, which may indicate a disruption to the normal rhythm of cellular division. If cells cannot progress through the cell cycle properly, DNA repair systems that are cell cycle regulated may be affected.

Alternatively, the DNA damage response (which limits cellular division in the event of a DNA lesion) may be receiving an indirect stimulation from a normal or hyperactive spindle assembly checkpoint, as in the case of a wild-type or BUB1 overexpression strain. If this stimulation is lost, such as in the case of a $\Delta bub1$ or $\Delta bub2$ mutant, the DNA damage response, which allows a time of cell cycle delay for repair events to occur, may fail. Further characterization of this effect will be necessary.

Direction for future research

Beyond the obvious possibility of further NHEJ-related candidate genes being identified through a broader screening, there are several future projects that may be considered. These projects can enhance our overall understanding of the NHEJ pathway and its relationship to other cellular processes.

Hur1 represents an interesting candidate for future research. It has been identified as a protein of unknown function in the Saccharomyces Genome Database, and has no known biological activity. Even the name, which suggests HydroxyUrea Resistance (HUR), is rather meaningless, as the hydroxyurea sensitivity exhibited by a deletion mutant is related to the short overlapping sequence with PMR1. There are no known genetic interactions or protein-protein interactions with this short polypeptide listed in SGD. However, overlap with PMR1 cannot explain our results for reduced NHEJ activity, based on our further research using partial HUR1 deletion and on overexpression of HUR1 in a JKM139 chromosomal DSB context. A more detailed look at this protein may be fruitful.

Histone modification has become a significant area of research, and its relevance to DNA damage repair is well-accepted. Understanding and modeling DNA repair in the simplified context of naked DNA ignores a significant amount of repair machinery and required biological activity. The mechanistic role of H3K56 acetylation, what other factors may serve similar functions, and why Vps75 seems to be relevant to HR while Rtt109 is limited to NHEJ remain open questions, and will be necessary to fully characterize the *in vivo* DSB repair system.

Is the spindle assembly checkpoint effect on NHEJ dependent on a specific regulatory signal of the Bub1 or Bub2 proteins that passes through the APC, or is the NHEJ defect caused by a general disruption of this cell cycle checkpoint such as a cell cycle delay? A more detailed look at the proteins in question could reveal novel insights into NHEJ regulation. Similarly, the significance of spindle or kinetochore defects in the cell to mutation avoidance and efficient DNA repair could be a significant area for medically-relevant research. However, these results would first need to be confirmed in an animal cell model, before any clinical relevance can be hypothesized.

Can Pol32 supplement the known activity of Pol4 in NHEJ? Could new synthesis of DNA during NHEJ require or be assisted by Ubc13 and the postreplication repair pathway? Does Msh6 commonly assist in repairing mismatched base pairs at otherwise mutagenic NHEJ sites? Can a more detailed model of how histones behave near a DSB be created, which explains the activity of Arp6, Hir3, and Vps72 in a NHEJ context? Many of our identified candidate genes could lead to interesting new questions in subsequent investigations.

Final note

It is without question that a cell contains a great many interacting pathways and networks. It is also clearly the case that a disruption of one pathway or protein can have far-reaching effects on the cell beyond the immediate binding partners or direct activity of the disrupted element. For a medically-relevant pathway such as a DNA damage repair pathway, any factor that disrupts the efficient completion of the task at hand can be of tremendous significance.

Here we have identified specific proteins whose deletion impairs the cell's ability to efficiently perform NHEJ, using *S. cerevisiae* as our model organism. We have found a variety of interesting factors, including proteins involved in chromatin modification, cell cycle checkpoints, and proteins of previously unknown function. While further research is warranted in many cases to determine the exact cause of this NHEJ impairment, this work represents another step in ultimately creating a complete model of the *in vivo* process of DSB repair.

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Appendix A

Primer	Gene	Role	Sequence
MJ-YKU70-F	YKU70	Forward primer	GAATGGTTATCAGTTAATTGACTCTCGGTAGCCAAGTT GGTTTAAGGCGCAAGACTGTAAATGACCACTCTTGACG ACACGGCTTAC
MJ-YKU70-R	YKU70	Reverse primer	TGCAGGTCTTGATAATGATAGAGGTGAGCCTGAAATTT CAAATTGGGCAAACACTTGCGCTTTTTATTGTCAGTAC TGACTAC
MJ-YKU70-C	YKU70	Confirmation primer	
MJ-RTT109-F	RTT109	Forward primer	TTTGTCATAGAGTTGTCCAGTAGAGTTAAAAGGTCAA TTCAACCGGTCTTCAATAAGACATGCACATACGATTTTA GGTGACAC
MJ-RTT109-R	RTT109	Reverse primer	AAACATGCATTTTCTAAGATCGAGCTACATACGTGTACT AAATAATAAATATCAATATGAATACGACTCACTATAGG GAG
MJ-RTT109-C	RTT109	Confirmation primer	
MJ-VPS75-F	VPS75	Forward primer	TAATGATTAGGCGCTTGATGTTGACGACAAACATTTG AAGCATAACACTGCCAATGATGACCACTCTTGACGACA CGGCTTACCGG
MJ-VPS75-R	VPS75	Reverse primer	AAAGAGCAATGTCGCAACGAGGGAAACCGTTGTCCAA GCCCCGCTCCGGACCTAGACTACTACTCAGTATAGCACC AGCATTCAC
MJ-VPS75-C	VPS75	Confirmation primer	
MJ-BUB1-F	BUB1	Forward primer	TTTCATCATGGAAAGATTGACGGTTCCTATTGTTTGAAT GTTAACGCTGACCAGGAACACATACGATTTTAGGTGAC AC
MJ-BUB1-R	BUB1	Reverse primer	ACCTGCTAGTTTTATATTACCTTTTATATATTAATGTTAC AATTGGCTCTACCTGAAGTTAATACGACTCACTATAGG GAG
MJ-BUB1-C	BUB1	Confirmation primer	CTCTACTAATTAATAGCTGG
MJ-BUB2-F	BUB2	Forward primer	ATAATAGTAACGACGTAAAGGTAAAAGAAACAACAGA CTTTTAACTTGTTAACTTTTGCCACATACGATTTTAGGT GACAC
MJ-BUB2-R	BUB2	Reverse primer	GAAGGTGAAGGATATGCGTACGAGCCATTTTCATTTG TGCAATAATAGAATAATAACCTAATACGACTCACTATAG GGAG
MJ-BUB2-C	BUB2	Confirmation primer	ACCTGTCTTATGCTATAATGACACTGGAGG

MJ-HUR1-F	HUR1	Forward primer	GAAAAAGAAGGAAAGAATAAAATTTTATATTGCTTTGT ATATACGAAAAGTCGCCCATACCACATACGATTTTAGG TGACAC
MJ-HUR1-R	HUR1	Reverse primer	GTCAATCTTCGAAATCGGCTTTTTTACGAACAAAATGTT CACTACACGCCGTTGGACTGAATACGACTCACTATAGG GAG
MJ-HUR1-C	HUR1	Confirmation primer	ATATTTGTCGAAGGTATAAATGAGGAAGG
CONF R	NAT	Reverse confirmation primer	
pGV LIVE F	LACZ	Forward primer	AGACGGATCCTCCTTTGCGAATACGCCCAC
pGV LIVE R	LACZ	Reverse primer	AGACGAATTCTGTGAAAGAAAGCCTGACTG

Appendix B

Gene	ORF	ROW	COLUMN	Plasmid	Linear	Efficiency of NHEJ	HU sens	MMS sens	UV sens
NGG1	YDR176W	A	1	1200	733	0.61			
ARD1	YHR013C	A	2	280	170	0.61	low	no	high
YBR223C	YBR223C	A	3	815	252	0.31	no	no	high
PRE9	YGR135W	A	4	300	173	0.58	no	no	mod
SLX1	YBR228W	A	5	281	232	0.83	no	no	no
RTF1	YGL244W	A	6	1516	1168	0.77	no	no	mod
POL4	YCR014C	A	7	368	205	0.56	no	no	mod
RAI1	YGL246C	A	8	433	682	1.58	no	no	high
SAC3	YDR159W	A	9	388	0	0.00	no	no	high
ZIP2	YGL249W	A	10	449	0	0.00	high	no	high
HIR1	YBL008W	A	11	1000	764	0.76	high	no	high
HFM1	YGL251C	A	12	1480	950	0.64	high	no	mod
HPC2	YBR215W	A	13	450	209	0.46	high	no	mod
SPO11	YHL022C	A	14	104	332	3.19	high	no	high
FUS3	YBL016W	A	15	319	90	0.28	high	no	high
RTT107	YHR154W	A	16	332	1061	3.20	high	no	high
APN2	YBL019W	A	17	2000	2025	1.01	high	no	high
CLB1	YGR108W	A	18	107	8	0.07	mod	no	high
MRC1	YCL061C	A	19	1104	8	0.01	high	no	mod
CLB6	YGR109C	A	20	210	50	0.24	mod	no	low
SWI5	YDR146C	A	21	650	1200	1.85	low	high	mod
NDT80	YHR124W	A	22	139	42	0.30	mod	no	high
MSI1	YBR195C	A	23	50	45	0.90	mod	high	high
ARP1	YHR129C	A	24	600	864	1.44	low	no	low
HTZ1	YOL012C	B	1	4000	3500	0.88	high	no	high
HHF2	YNL030W	B	2	3000	2000	0.67	mod	low	mod
HAT1	YPL001W	B	3	5000	3500	0.70	low	no	mod
HHT2	YNL031C	B	4	4000	21	0.01	low	no	mod
VIK1	YPL253C	B	5	1000	650	0.65	low	no	high
DPB3	YBR278W	B	6	3000	2000	0.67	no	no	high
PHR1	YOR386W	B	7	4000	3000	0.75	low	no	high
PAF1	YBR279W	B	8	198	12	0.06	no	no	high
CIN2	YPL241C	B	9	542	222	0.41	low	no	high
MSH5	YDL154W	B	10	615	403	0.66	mod	no	mod
SIN3	YOL004W	B	11	647	1350	2.09	mod	no	high
CLB3	YDL155W	B	12	2226	5520	2.48	mod	high	mod
TOP1	YOL006C	B	13	128	108	0.84	mod	low	high
PPH21	YDL134C	B	14	458	940	2.05	mod	no	mod
UME1	YPL139C	B	15	4000	3800	0.95	mod	no	high
MAD3	YJL013C	B	16	896	224	0.25	mod	no	high
CTF19	YPL018W	B	17	508	276	0.54	mod	no	mod

CIN4	YMR138W	B	18	543	226	0.42	no	no	low
SPP1	YPL138C	B	19	216	199	0.92	no	no	high
CHD1	YER164W	B	20	89	237	2.66	no	no	high
CHL1	YPL008W	B	21	298	355	1.19	no	no	mod
RAD24	YER173W	B	22	402	642	1.60	no	low	mod
HHO1	YPL127C	B	23	382	686	1.80	no	no	low
BMH1	YER177W	B	24	252	794	3.15	no	no	high
HTA2	YBL003C	C	1	176	560	3.18	mod	no	high
IOC2	YLR095C	C	2	294	290	0.99	no	no	high
DPB4	YDR121W	C	3	1275	700	0.55	no	no	no
HCS1	YKL017C	C	4	1100	2000	1.82	mod	no	mod
SRO9	YCL037C	C	5	1200	10	0.01	low	no	no
ELF1	YKL160W	C	6	1350	3000	2.22	low	no	high
ARO1	YDR127W	C	7	5000	650	0.13	no	no	low
REM50	YLL002W	C	8	1210	300	0.25	low	high	high
NUP60	YAR002W	C	9	1100	269	0.24	low	no	high
ARP6	YLR085C	C	10	2860	107	0.04	no	no	mod
SWD3	YBR175W	C	11	798	722	0.90	no	no	mod
CCE1	YKL011C	C	12	170	112	0.66	no	no	low
SWD1	YAR003W	C	13	479	164	0.34	no	no	high
AAT2	YLR027C	C	14	157	279	1.78	no	high	high
DCC1	YCL016C	C	15	298	195	0.65	no	no	high
SPT8	YLR055C	C	16	444	322	0.73	no	no	high
FOB1	YDR110W	C	17	661	393	0.59	no	no	low
RAD27	YKL113C	C	18	1850	496	0.27			
NTG1	YAL015C	C	19	1450	412	0.28	no	no	no
APN1	YKL114C	C	20	1900	1020	0.54	no	no	no
DEP1	YAL013W	C	21	25	54	2.16	no	no	no
	YLR015W	C	22	1340	908	0.68	low	high	low
TEL1	YBL088C	C	23	1128	586	0.52	no	no	mod
PFD1	YJL179W	C	24	163	50	0.31	low	no	low
MEI5	YPL121C	D	1	900	830	0.92	no	no	no
VPS71	YML041C	D	2	271	27	0.10	no	no	no
RAD17	YOR368W	D	3	49	69	1.41	no	mod	mod
UBP3	YER151C	D	4	412	495	1.20	no	no	low
CLN2	YPL256C	D	5	503	1	0.00	no	no	mod
CSM3	YMR048W	D	6	495	31	0.06	no	no	no
NCE4	YPL024W	D	7	3000	3000	1.00	high	high	high
PSO2	YMR137C	D	8	2000	2000	1.00	no	no	high
RAD1	YPL022W	D	9	225	149	0.66	no	no	no
RAD4	YER162C	D	10	62	159	2.56	no	no	no
CIN1	YOR349W	D	11	59	211	3.58	no	mod	low
SPT4	YGR063C	D	12	2050	93	0.05	no	no	no
MEK1	YOR351C	D	13	3800	98	0.03	no	low	no
HIR3	YJR140C	D	14	1500	146	0.10	no	no	no
KAR9	YPL269W	D	15	404	60	0.15	no	high	no
CST9	YLR394W	D	16	160	0	0.00	no	mod	low
YPL047W	YPL047W	D	17	2200	1600	0.73	no	no	no

MAG1	YER142C	D	18	68	1500	22.06	no	no	low
REV3	YPL167C	D	19	1300	2400	1.85	no	no	low
SLX8	YER116C	D	20	640	80	0.13	low	no	high
MLH3		D	21	1500	2000	1.33	no	no	no
SOD1	YJR104C	D	22	2500	3000	1.20	high	no	mod
YOR338 W	YOR338W	D	23	2000	2000	1.00	no	no	low
	YER111C	D	24	1500	1000	0.67	high	no	mod
SWC1	YAL011W	E	1	820	2000	2.44	low	no	mod
SET2	YJL168C	E	2	694	827	1.19	no	no	no
UMP1	YBR173C	E	3	191	363	1.90	mod	no	low
DOA1	YKL213C	E	4	265	1	0.00	mod	no	mod
MSH6	YDR097C	E	5	417	6	0.01	no	no	low
VID31	YKL054C	E	6	462	433	0.94	high	no	high
UBP13	YBL067C	E	7	253	759	3.00	no	no	no
RNH1	YMR234W	E	8	1195	877	0.73	no	no	no
YAL027W	YAL027W	E	9	239	458	1.92	no	no	low
RSC2	YLR357W	E	10	542	169	0.31	no	no	no
CCR4	YAL021C	E	11	1000	1200	1.20	high	no	high
STE11	YLR362W	E	12	1600	2800	1.75	no	no	no
FUN30	YAL019W	E	13	2500	2500	1.00	no	no	low
EST1	YLR233C	E	14	1700	1400	0.82	no	no	low
RAD55	YDR076W	E	15	2200	719	0.33	mod	no	high
TOP3	YLR234W	E	16	1500	1500	1.00	mod	low	mod
BLANK	BLANK	E	17						
ESC1	YMR219W	E	18	3000	2800	0.93	no	no	low
BLANK	BLANK	E	19						
UBP8	YMR223W	E	20	225	178	0.79	no	no	low
UBC13	YDR092W	E	21	2400	0	0.00	no	high	high
MRE11	YMR224C	E	22	1300	1260	0.97	high	high	high
CLN3	YAL040C	E	23	700	1000	1.43	no	no	low
CLB4	YLR210W	E	24	1800	800	0.44	no	no	low
THP1	YOL072W	F	1	785	279	0.36	no	no	low
POL32	YJR043C	F	2	1000	196	0.20	mod	mod	high
REV1	YOR346W	F	3	5840	4800	0.82	no	mod	mod
RAD7	YJR052W	F	4	336	135	0.40	no	no	high
RXT1	YPL181W	F	5	683	78	0.11	no	no	low
BFA1	YJR053W	F	6	180	90	0.50	no	no	low
LGE1	YPL055C	F	7	16400	11200	0.68	no	mod	mod
THR1	YHR025W	F	8	2440	1000	0.41	no	no	low
GAL11	YOL051W	F	9	3	500	166.6	low	no	mod
SRB2	YHR041C	F	10	450	87	0.19	low	no	low
DDC1	YPL194W	F	11	2568	2088	0.81	no	mod	high
REC107	YJR021C	F	12	2000	603	0.30	no	no	low
NTG2	YOL043C	F	13	1734	1048	0.60	no	no	low
RAD26	YJR035W	F	14	2464	710	0.29	no	no	low
ISW2	YOR304W	F	15	7400	2280	0.31	no	high	high
BUB2	YMR055C	F	16	1264	292	0.23	no	no	low
SNU66	YOR308C	F	17	1682	970	0.58	no	no	low

BUB1	YGR188C	F	18	655	113	0.17	no	low	mod
SNF2	YOR290C	F	19			#DIV/ 0!	high	high	high
DMC1	YER179W	F	20	1540	393	0.26	no	no	low
SIR2	YDL042C	F	21	1020	100	0.10	no	no	no
MND2	YIR025W	F	22	54	675	12.50	no	no	low
UBC4	YBR082C	F	23	1800	1243	0.69	low	no	mod
RIM1	YCR028C-A	F	24	186	300	1.61	low	no	mod
AOR1	YBR231C	G	1	466	13	0.03	no	no	mod
YLR218C	YLR218C	G	2	570	522	0.92	no	no	no
ISW1	YBR245C	G	3	175	648	3.70	no	no	low
YML011C	YML011C	G	4	800	850	1.06	no	no	high
PPH3	YDR075W	G	5	897	22	0.02	no	no	low
CIK1	YMR198W	G	6	126	24	0.19	high	low	high
YBL046W	YBL046W	G	7	361	22	0.06	no	no	mod
CLN1	YMR199W	G	8	792	1636	2.07	no	no	mod
SAS3	YBL052C	G	9	924	2100	2.27	no	no	mod
RAD14	YMR201C	G	10	704	1870	2.66	no	no	high
LIF1	YGL090W	G	11	1156	98	0.08	no	no	mod
MMS22	YLR320W	G	12	123	508	4.13	low	low	high
GIM4	YEL003W	G	13	1500	1900	1.27	low	no	mod
YKE2	YLR200W	G	14	860	2000	2.33	no	no	low
MEI4	YER044C-A	G	15	3004	6000	2.00	no	no	mod
REC102	YLR329W	G	16	1400	2500	1.79	no	no	mod
MAD1	YGL086W	G	17	447	1850	4.14	no	no	high
BLANK	BLANK	G	18						
MMS2	YGL087C	G	19	92	296	3.22	no	no	mod
BLANK	BLANK	G	20						
YER049W	YER049W	G	21	43	750	17.44	no	no	mod
TSA1	YML028W	G	22	15	884	58.93	no	no	mod
RAD30	YDR419W	G	23	1328	1500	1.13	no	no	high
SGS1	YMR190C	G	24	2500	2500	1.00	high	high	high
KIM3	YPR164W	H	1	127	359	2.83	no	high	mod
HSM3	YBR272C	H	2	342	459	1.34	no	no	mod
RDH54	YBR073W	H	3	189	273	1.44	no	no	mod
PAC1	YOR269W	H	4	358	764	2.13	no	low	mod
NHP10	YDL002C	H	5	227	496	2.19	no	no	mod
RIF1	YBR275C	H	6	578	1038	1.80	no	no	low
SLX5	YDL013W	H	7	48	444	9.25	low	no	high
EAF3	YPR023C	H	8	2050	2400	1.17	no	no	low
RPN4	YDL020C	H	9	1010	380	0.38	no	no	high
NT01	YPR031W	H	10	1600	283	0.18	no	no	mod
CTF4	YPR135W	H	11	5000	5000	1.00	high	high	high
YAF9	YNL107W	H	12	844	942	1.12	no	no	low
KAR3	YPR141C	H	13	300	942	3.14	no	no	high
POP2	YNR052C	H	14	5000	0	0.00	high	low	high

MUM2	YBR057C	H	15	580	5600	9.66	no	no	low
SPO69	YPR007C	H	16	490	790	1.61	no	no	mod
CLB2	YPR119W	H	17	2362	1528	0.65	no	no	no
PHO23	YNL097C	H	18	1139	868	0.76	no	no	low
CLB5	YPR120C	H	19	1442	265	0.18	no	no	low
SIR3	YLR442C	H	20	3480	1680	0.48	no	no	no
CDC26	YFR036W	H	21				no	no	low
YLR455w	YLR455w	H	22	1568	123	0.08	no	no	high
SEM1	YDR363W-A	H	23	1063	576	0.54	no	no	mod
FAB1	YFR019W	H	24	211	173	0.82	no	no	mod
RTT103	YDR289C	I	1	712	952	1.34	no	no	high
SWI6	YLR182W	I	2	752	2000	2.66	high	no	high
BIM1	YER016W	I	3	289	1048	3.63	no	no	high
MLH1	YMR167W	I	4	179	192	1.07	no	no	high
ISC1	YER019W	I	5	2000	2250	1.13	no	no	mod
CDC73	YLR418C	I	6	626	141	0.23	high	no	high
RAD6	YGL058W	I	7	82	5	0.06	no	no	high
RPN13	YLR421C	I	8	276	332	1.20	no	no	low
YER030W	YER030W	I	9	358	308	0.86	no	no	mod
SRC1	YML034W	I	10	592	408	0.69	no	no	no
SGF73	YGL066W	I	11	68	76	1.12	mod	mod	low
RAD52	YML032C	I	12	106	572	5.40	high	high	high
RPB9	YGL070C	I	13	386	358	0.93	high	no	high
TPP1	YMR156C	I	14	396	301	0.76	low	no	mod
DIN7	YDR263C	I	15	448	243	0.54	low	no	high
SFP1	YLR403W	I	16	560	488	0.87	low	no	high
SIZ1	YDR409W	I	17	468	480	1.03	no	no	mod
SLX4	YLR135W	I	18	792	756	0.95	low	no	low
HOP2	YGL033W	I	19	1122	1074	0.96	no	no	no
YLR385C	YLR385C	I	20	906	858	0.95	no	no	high
DST1	YGL043W	I	21	968	724	0.75	no	no	high
IOC4	YMR044W	I	22	937	908	0.97	no	no	mod
PAC2	YER007W	I	23	926	1127	1.22	no	no	mod
RED1	YLR263W	I	24	1432	1336	0.93	no	no	mod
IML3	YBR107C	J	1	4500	4000	0.89	no	no	mod
RNH35	YNL072W	J	2	1124	2000	1.78	no	no	mod
HHF1	YBR009C	J	3	3500	5000	1.43	no	no	mod
PMS1	YNL082W	J	4	928	1032	1.11	no	no	high
HHT1	YBR010W	J	5	4000	5500	1.38	no	no	mod
IOC3	YFR013W	J	6	982	1400	1.43	no	no	mod
RAD16	YBR114W	J	7	962	1200	1.25	no	no	high
RAD18	YCR066W	J	8	744	191	0.26	no	no	mod
MSH3	YCR092C	J	9	1416	1284	0.91	no	no	low
SPT7	YBR081C	J	10	455	365	0.80	no	no	low
CDC50	YCR094W	J	11	562	440	0.78	no	no	mod
TOF2	YKR010C	J	12	889	1200	1.35	no	no	mod
MED1	YPR070W	J	13	150	600	4.00	no	no	mod

RAD5	YLR032W	J	14	263	700	2.66	mod	mod	high
YBR094 W	YBR094W	J	15	76	600	7.89	high	no	high
NDJ1	YOL104C	J	16	1300	1500	1.15	no	no	high
RXT2	YBR095C	J	17	408	700	1.72	no	no	high
RAD50	YNL250W	J	18	710	815	1.15	mod	low	high
MMS4	YBR098W	J	19	262	235	0.90	no	no	mod
ABF2	YMR072W	J	20	794	988	1.24	no	no	low
SIF2	YBR103W	J	21	2400	2600	1.08	no	no	low
TRF4	YOL115W	J	22	3200	3200	1.00	no	no	low
MCM16	YPR046W	J	23	728	2000	2.75	no	no	low
GIM5	YML094W	J	24	1593	2200	1.38	no	no	high
CHL4	YDR254W	K	1	46	103	2.24	no	no	low
NEJ1	YLR265C	K	2	621	53	0.09	no	no	high
MUS81	YDR386W	K	3	561	2200	3.92	no	high	high
VID22	YLR373C	K	4	272	889	3.27	low	low	mod
SPT3	YDR392W	K	5	9	357	39.67	no	no	high
YLR376C	YLR376C	K	6	273	1000	3.66	no	no	high
HAT2	YEL056W	K	7	63	207	3.29	no	no	mod
LYS7	YMR038C	K	8	33	1400	42.42	low	no	high
CKB1	YGL019W	K	9	1510	1600	1.06	no	no	high
CTF3	YLR381W	K	10	649	1025	1.58	no	no	high
CIN8	YEL061C	K	11	1340	1600	1.19	mod	mod	high
APC9	YLR102C	K	12	767	1200	1.56	no	no	high
PGD1	YGL025C	K	13	1247	1500	1.20	high	high	high
LEO1	YOR123C	K	14	850	1300	1.53	mod	high	high
ESC2	YDR363W	K	15	1048	657	0.63	low	high	high
GIM3	YNL153C	K	16	259	1152	4.45	mod	high	high
XRS2	YDR369C	K	17	360	460	1.28	high	high	high
RRP6	YOR001W	K	18	890	458	0.51	no	no	mod
LSM6	YDR378C	K	19	84	702	8.36	high	no	high
DNL4	YOR005C	K	20	433	0	0.00	no	no	mod
IES6	YEL044W	K	21	563	281	0.50	high	no	high
MCK1	YNL307C	K	22	1100	282	0.26	low	no	high
RPN14	YGL004C	K	23	911	1400	1.54	no	no	mod
TRF5	YNL299W	K	24	245	194	0.79	no	no	mod
DUN1	YDL101C	L	1	1300	1060	0.82	no	no	high
RAD10	YML095C	L	2	549	900	1.64	no	no	high
EAF6	YJR082C	L	3	907	990	1.09	no	no	mod
CAC2	YML102W	L	4	550	838	1.52	no	no	low
NUP84	YDL116W	L	5	233	246	1.06	no	no	high
DOT6	YER088C	L	6	1650	1447	0.88	no	no	mod
SOH1	YGL127C	L	7	230	611	2.66	no	no	high
IES5	YER092W	L	8	438	678	1.55	no	no	high
ITC1	YGL133W	L	9	711	645	0.91	no	no	low
RAD51	YER095W	L	10	349	316	0.91	high	no	high
PSP1	YDR505C	L	11	309	317	1.03	no	no	mod
RCO1	YMR075W	L	12	319	74	0.23	no	no	low
MFT1	YML062C	L	13	1046	964	0.92	no	no	high

CTF18	YMR078C	L	14	447	38	0.09	no	no	high
MPH1	YIR002C	L	15	1038	1342	1.29	no	no	high
YKU80	YMR106C	L	16	588	5	0.01	no	no	high
OGG1	YML060W	L	17	293	647	2.21	no	no	high
CTF8	YHR191C	L	18	394	1000	2.54	no	no	low
VPS72	YDR485C	L	19	956	139	0.15	low	no	low
NPL6	YMR091C	L	20				low	no	high
PAC11	YDR488C	L	21	702	909	1.29	no	no	low
MLH2	YLR035C	L	22	964	1004	1.04	no	no	mod
SDC1	YDR469W	L	23	312	270	0.87	no	no	mod
SAS2	YMR127C	L	24	1150	2500	2.17	no	no	mod
RAD9	YDR217C	M	1	534	1064	1.99	no	no	high
CLA4	YNL298W	M	2	132	1144	8.67	no	no	mod
HTA1	YDR225W	M	3	2000	4000	2.00	no	no	high
SGO1	YOR073W	M	4	569	2800	4.92	high	no	mod
SIR4	YDR227W	M	5	1392	89	0.06	no	no	mod
SAS5	YOR213C	M	6	2324	4500	1.94	no	no	mod
VID21	YDR359C	M	7	928	2976	3.21	no	low	high
YNL201C	YNL201C	M	8	5572	2280	0.41	no	high	high
RAD23	YEL037C	M	9	436	872	2.00	no	no	mod
IES4	YOR189W	M	10	728	335	0.46	no	no	mod
SWR1	YDR334W	M	11	1924	21	0.01	no	mod	high
ASE1	YOR058C	M	12	1750	2000	1.14	no	no	high
UME6	YDR207C	M	13	1500	1850	1.23	mod	low	mod
CKA2	YOR061W	M	14	1068	1012	0.95	no	no	high
SAS4	YDR181C	M	15	2500	4000	1.60	no	no	mod
SLK19	YOR195W	M	16	1060	2600	2.45	no	no	mod
YDR314C	YDR314C	M	17	2125	3500	1.65	no	no	mod
MGS1	YNL218W	M	18	2250	3500	1.56	no	no	mod
HST4	YDR191W	M	19	1780	4000	2.25	no	no	mod
IES2	YNL215W	M	20	800	2000	2.50	no	no	low
MCM21	YDR318W	M	21	1288	2000	1.55	no	no	mod
UBP15	YMR304W	M	22	372	506	1.36	no	no	mod
REF2	YDR195W	M	23	825	1025	1.24	low	low	high
HIR2	YOR038C	M	24	1072	1950	1.82	no	no	mod
UBP6	YFR010W	N	1	1800	1500	0.83	no	mod	high
IES3	YLR052W	N	2	1400	986	0.70	no	no	high
MET18	YIL128W	N	3	863	2500	2.90	low	mod	high
REC114	YMR133W	N	4	1250	2100	1.68	no	no	low
RNR3	YIL066C	N	5	485	0	0.00	no	no	no
MSH2	YOL090W	N	6	628	105	0.17	no	no	low
REV7	YIL139C	N	7	985	1502	1.52	no	low	mod
RTT101	YJL047C	N	8	6000	6000	1.00	low	low	mod
DOT1	YDR440W	N	9	679	1800	2.65	no	no	mod
MAD2	YJL030W	N	10	1085	3000	2.76	no	no	high
SAE2	YGL175C	N	11	432	1700	3.94	low	no	high
RIF2	YLR453C	N	12	321	1428	4.45	no	no	mod
RXT3	YDL076C	N	13	680	408	0.60	no	no	low

UNG1	YML021C	N	14	996	828	0.83	no	no	mod
MCM22	YJR135C	N	15	504	528	1.05	no	no	mod
BRE5	YNR051C	N	16	249	204	0.82	no	no	low
RAD54	YGL163C	N	17	548	542	0.99	mod	high	high
LSM1	YJL124C	N	18	427	1475	3.45	mod	low	high
HUR1	YGL168W	N	19	988	34	0.03	high	no	mod
ASF1	YJL115W	N	20	2500	1600	0.64	mod	high	high
KEM1	YGL173C	N	21	274	355	1.30	mod	mod	mod
HO	YDL227C	N	22	436	375	0.86	no	no	low
MBP1	YDL056W	N	23	468	652	1.39	no	no	low
HPR5	YJL092W	N	24	865	958	1.11	low	no	high
HOP1	YIL072W	O	1	196	308	1.57	no	no	mod
CKB2	YOR039W	O	2	587	510	0.87	no	no	high
SDS3	YIL084C	O	3	690	184	0.27	no	no	mod
WHI2	YOR043W	O	4	668	135	0.20	no	no	mod
YGR071C	YGR071C	O	5	288	110	0.38	no	no	mod
	YMR284W	O	6	344	2	0.01	no	no	mod
PAC10	YGR078C	O	7	1064	1300	1.22	mod	no	high
ARP8	YOR141C	O	8	305	800	2.62	low	no	high
CKA1	YIL035C	O	9	942	1065	1.13	no	no	mod
ELG1	YOR144C	O	10	1036	1305	1.26	no	no	high
LRP1	YHR081W	O	11	928	1100	1.19	no	no	mod
HST3	YOR025W	O	12	133	316	2.38	no	no	no
TIM13	YGR181W	O	13	169	201	1.19	no	low	high
BUB3	YOR026W	O	14	408	400	0.98	no	no	high
UBR1	YGR184C	O	15	208	368	1.77	no	no	no
SAP30	YMR263W	O	16	812	651	0.80	no	no	mod
RPN10	YHR200W	O	17	127	324	2.55	no	no	mod
YNL253W	YNL253W	O	18	142	438	3.08	no	no	mod
RMR3	YHR031C	O	19	236	392	1.66	no	no	low
RTS1	YOR014W	O	20	87	678	7.79	no	no	mod
REC104	YHR157W	O	21	208	596	2.87	no	no	mod
TOF1	YNL273W	O	22	210	179	0.85	no	no	mod
THP2	YHR167W	O	23	52	265	5.10	mod	no	low
RLF2	YPR018W	O	24	403	518	1.29	no	no	mod
RAD59	YDL059C	P	1	44	921	20.93	no	low	mod
RAD57	YDR004W	P	2	624	554	0.89	mod	low	high
SAE3	YHR079C-B	P	3	486	268	0.55	no	no	low
SIT4	YDL047W	P	4	0	4240		low	no	mod
RTT102	YGR275W	P	5	730	145	0.20	no	no	high
SIR1	YKR101W	P	6	621	341	0.55	no	no	mod
BLANK	BLANK	P	7						
RAD61	YDR014W	P	8	1444	2500	1.73	no	no	no
BLANK	BLANK	P	9						
BRE1	YDL074C	P	10	13	552	42.46	low	no	high
PAT1	YCR077C	P	11	503	816	1.62	low	no	high
MSH4	YFL003C	P	12	1444	2500	1.73	no	no	low
CSM1	YCR086W	P	13	976	1021	1.05	no	no	no

IES1	YFL013C	P	14	2200	2500	1.14	no	no	high
RLR1	YNL139C	P	15	479	536	1.12	no	no	mod
SPT20	YOL148C	P	16	121	750	6.20	no	no	high
YNL136W	YNL136W	P	17	126	224	1.78	low	no	mod
RAD2	YGR258C	P	18	870	163	0.19	no	no	high
CSE2	YNR010W	P	19	254	328	1.29	no	no	mod
RAD53-11	YPL153C	P	20				high	no	high
PPH22	YDL188C	P	21	455	810	1.78	no	no	no
BLANK	BLANK								
CHK1	YBR274W	P	23	526	475	0.90	no	no	no
BLANK	BLANK								

Appendix C

All arrays are presented in an orientation where the upper left corner represents coordinate A-1. Columns are designated 1-24 counting across. Rows are designated A-P, progressing down. Coordinates of any spot matches the gene name and coordinate in Appendix B.

Control and drug plates are presented together for ease of comparison. Images of control plates are internally labelled 'CON', test plates are labelled 'TEST'. Both plates are labelled according to the trial and condition being tested.

UV Can 2

UV Test 2

uv Test 3

uv Can 3