Cellular Response Pathways to Formaldehyde

By

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CERTIFICATE OF APPROVAL

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List of Abbreviations (In Order of Appearance)

UFFI	Urea-Formaldehyde Foam Insulation
OSHA	Occupational Safety and Health Administration
CDC	Center of Disease Control and Prevention
FEMA	Federal Emergency Management Agency
IARC	International Agency of Research on Cancer
NCI	National Cancer Institute
CA	Chromosomal Aberrations
SCE	Sister Chromatid Exchange
MN	Micronuclei
CHO	Chinese Hamster Ovary Cells
DPC	DNA-Protein Crosslinks
DNA	Deoxyribonucleic Acid
UV	Ultra Violet
NER	Nucleotide Excisions Repair
HR	Homologous Recombination
TLS	Translesion Synthesis
BER	Base Excision Repair
FAP	Familial Adenomatous Polyposis
AP	Apurinic
LIG3	DNA Ligase 3
POLB	DNA Polymerase B
POLD	DNA Polymerase
PCNA	Proliferating Cell Nuclear Antigen
FEN1	Flap Structure-specific Endonuclease
LIG1	DNA Ligase I

MMR	Mismatch Repair
IDL	Insertion/Deletion Loops
HNPCC	Hereditary Non-Polyposis Colorectal Cancer
PRR	Post Replication Repair
XPV	Xeroderma Pigmentosum Variant
TS	Translesion Synthesis
MNNG	N-Methyl-N'-Nitro-N-Nitroguanidine
CPD	Cyclobutane Pyrimidine Dimers
XP	Xeroderma Pigmentosum
CS	Cockayne Syndrome
TCR	Transcription-coupled Repair
GGR	Global Genome Repair
TTD	Trichothiodystrophy
DAR	Domain-associated Repair
RNAPII	RNA Polymerase II
(6-4) PPs	(6-4) Pyrimidine-pyrimidone Photoproduct
NEF3	Nucleotide Excision Repair Factor 3
RFC	Replication Factor C
XRCC1	X-ray repair complementing defective repair in Chinese hamster cells 1
SSB	Single Strand Breaks
DSB	Double Strand Breaks
NHEJ	Non Homologous End Joining
MMEJ	Microhomology-Mediated End Joining
MRN(X)	Mre11, Rad50, NBS1 (XRS2)
NBS	Nijmegen Breakage Syndrome
ATM	Ataxia Telangiectasia-mutated

AT	Ataxia Telangiectasia
SDSA	Synthesis-dependent Strand Annealing
D-LOOP	Displacement Loop
BLM/WRN	Bloom/Werner Helicases
BLM	Bloom Syndrome
BIR	Break Induced Repair
SSA	Single Strand Annealing
SMC	Structural Maintenance Chromosome
IR	Ionizing Radiation
ATR	Ataxia Telangiectasia and RAD3-related
DNA-Pkcs	DNA-dependent protein kinase catalytic subunit
PARP-1	Poly (ADP-ribose) Polymerase-1
PFGE	Pulsed Field Gel Electrophoresis
ICL	Intrastrand Crosslink
EUROSCARF	European *S. cerevisiae* Archives for Functional Analysis
YPD	Yeast Extract Peptone Dextrose
BSA	Bovine Serum Reaction
NCBI	National Center for Biotechnology Information
GFP	Green Fluorescent Protein
DAPI	4',6-diamidino-2-phenylindole
FACS	Fluorescence-activated Cell Sorting

List of Genes

A list of genes is provided below. The genes are sorted by the yeast names. The human ortholog, or description of the ortholog is listed.

Yeast	Human
Apn1	AP Nuclease
Apn2	HAP1
Cdc31	Centrin
Chk1	CHEK1
Chk2	CHEK2
Ddb1	DDB1/XPE
DinB1	Pol Kappa
Dnl4 (Lig4)	DNA Ligase IV
Dun1	N/A
Exo1	EXO1
Fen1	FEN1
Hus1	HUS1
Ku70	Ku70/XRCC6
Ku80	Ku80/XRCC5
Lcd1 (Ddc2)	ATRIP
Lif1	XRCC4
Lig1 (Trl1)	LIG1
Mag1	DNA Glycosylase
MEC1	ATR
Mlh1	MLH1/MUTL
Mlh2	MUTL Subunit
Mlh3	MUTL
Mms2	UBE2
Mre11	MRE11
Msh1	MSH1/MUTL Homolog 1
Msh2	MSH2/MUTS Homolog 2
Msh3	MSH3/MUTS Homolog 3
Msh4	MSH4/MUTS Homolog 4
Msh5	MSH5/MUTS Homolog 5
Msh6	MSH6/MUTS
Ntg1	AP Lyase
Ntg2	AP Lyase
N/A	DDB2/XPE
N/A	DNA Ligase 3
N/A	MUTYH
N/A	NTH1
N/A	Neil1

N/A	Pol Iota
N/A	PARP-1
N/A	MDC1
Ogg1	Ogg1, MutM
Pms1	MUTL
Pol2	POLD2
Pol30	PCNA
Pol32	Pol Delta Subunit
Pol4	POLB
Pso2 (Snm1)	DCLREIA
Rad1	XPF
Rad10	ERCC1
Rad14	XPA
Rad17	RAD1
Rad18	RAD18
Rad2	XPG
Rad23	HR23A and B
Rad24	RAD17
Rad26	CSB
Rad27 (Fen1)	FEN1
Rad28	CSA
Rad3	XPD
Rad30A/XPV	Pol Eta
Rad4	XPC
Rad5	SHPRH (DNA Helicase)
Rad50	RAD50
Rad51	RAD51
Rad52	RAD52
Rad54	RAD54/ATRX
Rad55	N/A
Rad57	N/A
Rad59	N/A
Rad6	UBE2
Rad7	N/A
Rad9	RAD9
Rdh54	N/A
Rev1	REV1
Rev3	Pol Zeta Subunit
Rev7	Pol Zeta Subunit
Rfa1, Rfa2, Rfa3	RPA
Rfc	RFC
Sae2	SAE2/UBA2
Sgs1	BLM/WRN

Spo11	SPO11
Ssl1	THIIH Complex Component
Ssl2	ERCC3
Tel1	ATM
Tfb1	THIIH Complex Component
Tfb2	THIIH Complex Component
Tfb4	THIIH Complex Component
Top3	DNA Topoisomerase III
Ubc13	UBE2
XRCC1	N/A
Xrs2	NBS1

Abstract

Although it is well established that DNA-protein crosslinks are formed as a consequence of cellular exposure to agents such as formaldehyde, transplatin, ionizing and ultraviolet radiation, the biochemical pathways that promote cellular survival *via* repair or tolerance of these lesions are poorly understood. To investigate the mechanisms that function to limit DNA-protein crosslink-induced cytotoxicity, the *Saccharomyces cerevisiae* non-essential gene deletion library was screened for increased sensitivity to formaldehyde exposure. Following low-dose, chronic exposure, strains containing deletions in genes mediating homologous recombination showed the greatest sensitivity, while under the same exposure conditions, deletions in genes associated with nucleotide excision repair conferred only low to moderate sensitivities. However, when the exposure regime was changed to a high-dose acute (short-term) formaldehyde treatment, the genes that conferred maximal survival switched to the nucleotide excision repair pathway, with little contribution of the homologous recombination genes. Data are presented which suggest that following acute formaldehyde exposure, repair and/or tolerance of DNA-protein crosslinks proceeds *via* formation of nucleotide excision repair-dependent single strand break intermediates and without a detectable accumulation of double strand breaks. In addition, the relative survivals of the formaldehyde sensitive deletion strains were assessed following exposure to other DPC-inducing agents. Not only do the exposure conditions influence the cellular response, but also the specific agent used to induce the damage. Based on the results of the genome-wide screen, the interactions of the implicated pathways were investigated. Genetic analyses were performed by creating yeast strains with deletions in genes from both the classically defined nucleotide excisions repair and homologous recombination pathways. Based on these results, it appears that in the absence of NER

and HR, the translesion synthesis pathway may function as a backup tolerance pathway to enhance cell survival after formaldehyde exposure. In addition, both Mre11 and Rad1, independent from the other homologous recombination and nucleotide excision repair proteins, play a separate role in the tolerance and/or repair of DNA-protein crosslinks. These data clearly demonstrate a differential pathway response to chronic versus acute formaldehyde exposures and may have significance and implications for risk extrapolation in human exposure studies.

1. Introduction - Formaldehyde

1.1 History of formaldehyde

Formaldehyde, CH_2O, is one of the most reactive of all aldehydes (Council 2005; NTP 2005). It was first synthesized in 1859 by Aleksandr Butlerov. Formaldehyde is produced by oxidizing methanol using a metal catalyst. In the years following its identification, *casein formaldehyde* became popular with the demand for more innovative synthetic products. It was used in everyday products like buttons, buckles and umbrellas. Shortly thereafter, the first completely synthetic plastic was made by condensing phenol with formaldehyde. Initially this plastic was used in heavy industrial products, but by the 1920-1940s, it was used in everyday consumer appliances, such as toasters and radios (Zhang, Steinmaus et al. 2009). As this plastic was formed relatively easily, other agents were used by chemists to condense with formaldehyde. This resulted in the production of *urea formaldehyde* which was commonly used in the manufacture of home appliances. In the 1970-1980s, the use of *urea formaldehyde* increased dramatically with the production of urea-formaldehyde foam insulation (UFFI) (Zhang, Steinmaus et al. 2009), used in the manufacturing of buildings. Other commonly used building materials such as plywood wall paneling, particleboard, and fiberboard all contain, and therefore emit, ambient levels of formaldehyde. Currently, the Department of Housing and Urban Development only allows the use of plywood particleboard that does not emit more than 0.4 ppm of formaldehyde in the manufacturing of buildings. In older buildings, which contain UFFI, formaldehyde levels exceeding 0.3 ppm are often found. This is relatively high compared to older buildings without UFFI, where levels

below 0.1 ppm are present (USEPA 2007). In general, these levels are relatively high as the limit set by the World Health Organization is 0.08 ppm (WHO-ROE 2006).

1.2 Formaldehyde exposure

Despite an increased awareness of the adverse health consequences of formaldehyde exposure, it is still found in household products like furniture and carpet at very low levels, and is one of the components found in cigarette smoke (Zhang, Steinmaus et al. 2009). Formaldehyde is also a contributor to outdoor air pollution, as one of many chemicals found in automobile exhaust. The levels will vary depending on the population density (i.e. up to 20 ppb was detected in Houston, TX).

Occupational exposure to formaldehyde occurs in a variety of industries, with the highest exposure (2-5 ppm) measured in workers who 1) varnish furniture and wooden floors, 2) work in the garment industry, 3) produce particle boards and 4) fabricate plywood (Cogliano, Grosse et al. 2005). In occupations such as embalmers, pathologists and paper industry workers, short term higher (>3ppm) exposures were measured (Duhayon, Hoet et al. 2008). The Occupational Safety and Health Administration (OSHA) estimated that in the U.S. approximately 2 million workers are exposed to elevated levels of formaldehyde (OSHA 1995).

More recently, non-occupational related formaldehyde exposure made the news after health issues arose with some of the residents of the >140,000 trailers and mobile homes that were provided to individuals who were displaced from their houses due to the damage caused by hurricanes Katrina and Rita. The health symptoms reported were eye and throat irritation and respiratory problems. These reports initiated an indoor air

quality assessment that resulted in the discovery of very high chronic concentrations of formaldehyde in these trailers. After these initial findings, the Center of Disease Control and Prevention (CDC) studied 519 units more extensively, and revealed average formaldehyde levels between 77 ppb and 590 ppb, the later being 50-fold above normal indoor air quality levels (Federal Emergency Management Agency (FEMA) news release 2/14/08). Based on these studies, it is fair to assume that the people that lived (>500,000) or still live (>143,000) in these units were exposed to significant doses of formaldehyde. In addition, formaldehyde is produced in most organisms, including humans, as a metabolic byproduct (NTP 2005), and is physiologically present in all bodily fluids, cells and tissues. A study on endogenous concentration in the blood of humans, monkeys and rats ranged from 2-3 mg/L (0.1 mM) (Casanova, Heck et al. 1988; Heck and Casanova 1988).

1.3 Formaldehyde as a human carcinogen

In 2004 the International Agency for Research on Cancer (IARC) reclassified formaldehyde from a probable carcinogen (Group 2) to a human carcinogen (Group 1). This classification was based on evidence provided by six major cohort studies of industrial workers and seven case-control studies of nasopharyngeal cancer (reviewed in Cogliano et al 2005). In addition, the National Cancer Institute (NCI) performed a U.S. cohort studies showing a strong exposure-response correlation between cancer mortality rate and formaldehyde exposure (Hauptmann, Lubin et al. 2004). In another study, workers manufacturing formaldehyde were shown to have an increased incidence of nasopharyngeal cancer compared with control groups (Hansen and Olsen 1995). In

addition, other nasopharyngeal cancer case control studies were done that found an elevated risk of cancer associated with formaldehyde exposure (Vaughan, Strader et al. 1986; Roush, Walrath et al. 1987; Armstrong, Imrey et al. 2000; Vaughan, Stewart et al. 2000; Hildesheim, Dosemeci et al. 2001). The IARC also concluded that formaldehyde exposure might be associated with the occurrence of leukemia (Cogliano, Grosse et al. 2005). Taken together these findings suggest that there is a correlation between formaldehyde exposure and increased cancer risk.

1.4 Cytogenetic alterations and mutagenesis

With the accumulated evidence of the adverse health effects of exposure to formaldehyde, studies have been performed to assess the effects of formaldehyde exposure at a cellular level. In studies on mice and rats that were exposed to formaldehyde, and *in vitro* research on mammalian cells in culture, increased levels of chromosomal aberrations (CA), sister chromatid exchange (SCEs) and micronuclei (MN) were observed (ATSDR 1999). Human peripheral blood lymphocytes obtained from carpet and plastic plant workers that were exposed to formaldehyde also showed an increase in CA (Lazutka, Lekevicius et al. 1999). In addition, other studies have suggested a relationship between formaldehyde exposure and the presence of chromosomal breaks and elevated dicentric and ring chromosomes (Bauchinger and Schmid 1985; He, Jin et al. 1998) and SCEs and MN in the circulating lymphocytes of exposed individuals (Suruda, Schulte et al. 1993; Shaham, Gurvich et al. 2002; Ye, Yan et al. 2005; Orsiere, Sari-Minodier et al. 2006; Iarmarcovai, Bonassi et al. 2007). Recent data from our laboratory demonstrates that breaks and radials are formed in Chinese

Hamster Ovarian (CHO) cells after formaldehyde exposure (Kumari & McCullough, unpublished).

In addition, formaldehyde appears to be mutagenic to both *Escherichia coli* and human lymphocytes, resulting in insertions, deletions and point mutations (Crosby, Richardson et al. 1988). Interestingly, in the *E. coli* study, it was reported that depending on the exposure conditions, 4 mM 1 hr versus 40 mM 1 hr exposure, different point mutations were found. At the lower dose, GC transversions were mostly found. At the higher dose, transitions at a single AT base pair were predominant (Crosby, Richardson et al. 1988).

1.5 DNA-protein crosslinks (DPCs) and formaldehyde

DNA-protein crosslinks (DPCs) are considered to be the major form of DNA damage that occurs following formaldehyde exposure. DPCs are induced by a variety of other endogenous and exogenous agents, including metals, and ultraviolet light (UV) (Barker, Weinfeld et al. 2005). Formaldehyde induced DPCs occur through a rapid initial reaction with a protein amine, and subsequent reaction with DNA to form a crosslink with the general structure, protein-NH_2-CH_2-NH_2-DNA (McGhee and von Hippel 1975; McGhee and von Hippel 1975). This reaction is illustrated in Fig. 1. The current understanding is that DPCs act as bulky helix-distorting adducts that physically block DNA replication and transcription (Barker, Weinfeld et al. 2005). In an exposure case study, elevated DPCs were detected in peripheral mono-nuclear cells of formaldehyde-exposed workers (Shaham, Bomstein et al. 1996; Costa, Zhitkovich et al. 1997). However, it should be noted that the control group showed a higher than normal

presence of DPCs in one of the studies (Costa, Zhitkovich et al. 1997). In cellular studies formaldehyde exposure induced DPCs in CHO cells (Merk and Speit 1998). It has been characterized that formaldehyde induced DPCs occur between DNA and the major histones (H1, H2a, H2b, H3 and H4) (O'Connor and Fox 1989). The exact plethora of proteins that are crosslinked to DNA following formaldehyde exposure is not known. In addition, the DNA repair mechanism(s) responsible for removal of DPCs is poorly understood.

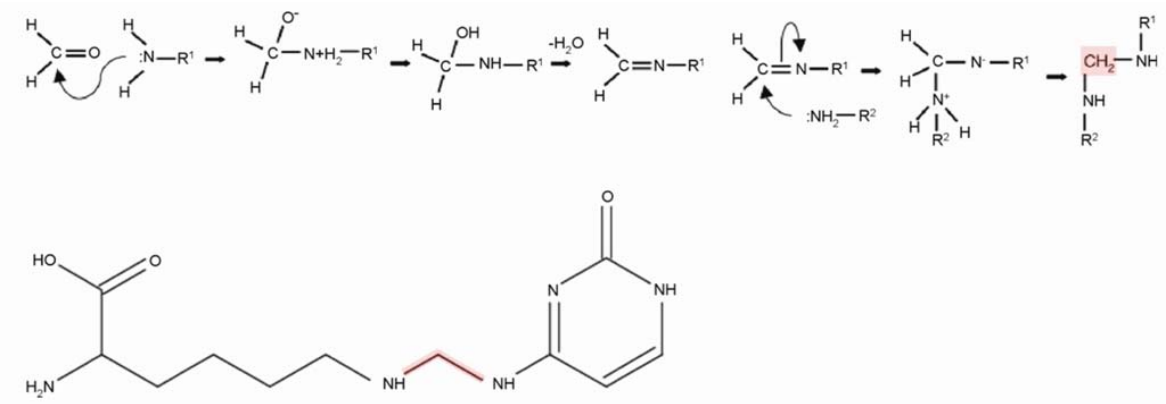

Figure 1. Schematic models of the mechanism and structure of a formaldehyde-induced DNA-protein crosslink. (Top) A schematic model in which formaldehyde reacts with an amino group (of a protein side chain) to form a Schiff base which reacts with another amino group (of a DNA base) to form the crosslink. (Bottom) A formaldehyde-induced crosslink between a cytosine and lysine. [Reprinted with permission; Zhang, Steinmaus et al. 2009].

2. Introduction - DNA repair

2.0.1 Epistasis groups

As organisms are constantly exposed to different types of DNA damaging agents, cells have evolved various complex systems of DNA repair. Many of these repair pathways have been identified initially by analyzing genetic mutants of *E. coli* and *Saccharomyces cerevisiae.* These repair pathways have been traditionally assigned to independent epistasis groups, and are thought to vary depending on the type of DNA damage i.e. RAD3/nucleotide excision repair and RAD52/homologous recombination pathways in budding yeast.

2.0.2 DPC repair

As mentioned in the previous section, the repair of DPCs is poorly understood. Several reports have implicated specific pathways in the repair of DPCs, these include nucleotide excision repair (NER) (Fornace and Seres 1982; Baker, Wuenschell et al. 2007; Nakano, Morishita et al. 2007), proteasomal degradation (Quievryn and Zhitkovich 2000; Baker, Wuenschell et al. 2007), Fanconi/BRCA pathway (Ridpath, Nakamura et al. 2007), homologous recombination (HR) (Nakano, Morishita et al. 2007; Ridpath, Nakamura et al. 2007) and translesion synthesis (TLS) (Minko, Zou et al. 2002; Ridpath, Nakamura et al. 2007). Although the importance of each pathway is not disputed, the relative contribution of each for the tolerance or repair of DPCs is unclear. To facilitate understanding of the various models proposed, a short overview of some of the major pathways is presented.

2.1 Base excision repair (BER)

2.1.1 Disease phenotype

Base excision repair (BER) is generally described as a housekeeping pahway, constitutively on, responsible for the repair of oxidized, alkylated, and deaminated bases (reviewed in Robertson, Klungland et al. 2009). The BER pathway was discovered almost 35 years ago through efforts to identify an enzymatic activity that could catalyze the removal of the mutagenic uracil DNA base (Lindahl 1974; Lindahl 1980). Later, this uracil-DNA glycosylase was shown to be a highly conserved DNA repair enzyme (Friedberg, Ganesan et al. 1975; Olsen, Aasland et al. 1989). Other mammalian DNA glycosylases have since been identified and include Ogg1, Nth1, MutyH and Neil1 (reviewed in Robertson, Klungland et al. 2009). Mutations in MutyH have been associated with the incidence of familial adenomatous polyposis (FAP)-like colon cancer designated MutyH-associated polyposis (MAP) (Wang, Baudhuin et al. 2004). In a mouse study when deleting MutyH it has been shown to result in fatty liver disease (Gao, Wei et al. 2004). Mutations in Ogg1 are associated with lung cancer (Chang, Wrensch et al. 2009) and a deletion of Neil1 in mice has been associated with a metabolic syndrome (Vartanian, Lowell et al. 2006; Chan, Ocampo-Hafalla et al. 2009). In addition, a genetic polymorphism study suggests that mutations in BER genes could be associated with increased bladder cancer risk (Figueroa, Malats et al. 2007).

2.1.2 The BER pathway

At the core of BER, four types of proteins are necessary for it to function; these include a DNA glycosylase, an apurinic (AP) endonuclease or AP DNA lyase, a DNA polymerase, and a DNA ligase.

The DNA glycosylases break the N-glycosidic bond between the base and the sugar, resulting in an AP site (Top Half Fig. 2) (O'Connor and Laval 1989; Robson and Hickson 1991). This is a substrate for the AP endonucleases which nick the damaged DNA on the 5' side of the AP site creating a free 3'-OH (Boiteux and Huisman 1989). Re-synthesis of the nucleotide of the damaged base is done by DNA polymerase beta (PolI in yeast), extending from the free 3'-OH. The resulting gap is sealed by DNA ligase 3 (LIG3) (Boiteux and Huisman 1989). For a schematic representation of this pathway see Fig. 2, left branch.

Alternatively, for some forms of base damage, the steps post-cleavage of the phosphate backbone result in a larger gap synthesis, known as long patch BER. This nick results in the recruitment of DNA polymerase beta (PolB) or DNA polymerase delta (PolD), proliferating cell nuclear antigen (PCNA), flap structure-specific endonuclease 1 (Fen1), and Lig1 DNA ligase. The long flap is displaced by PolB and PolD and further stimulated by PCNA (Klungland and Lindahl 1997). Fen1 resolves this flap. In addition, Fen1 is required for long-patch but not short-patch BER (Klungland and Lindahl 1997; Kim, Biade et al. 1998). The basic steps are illustrated in Fig. 2, right branch.

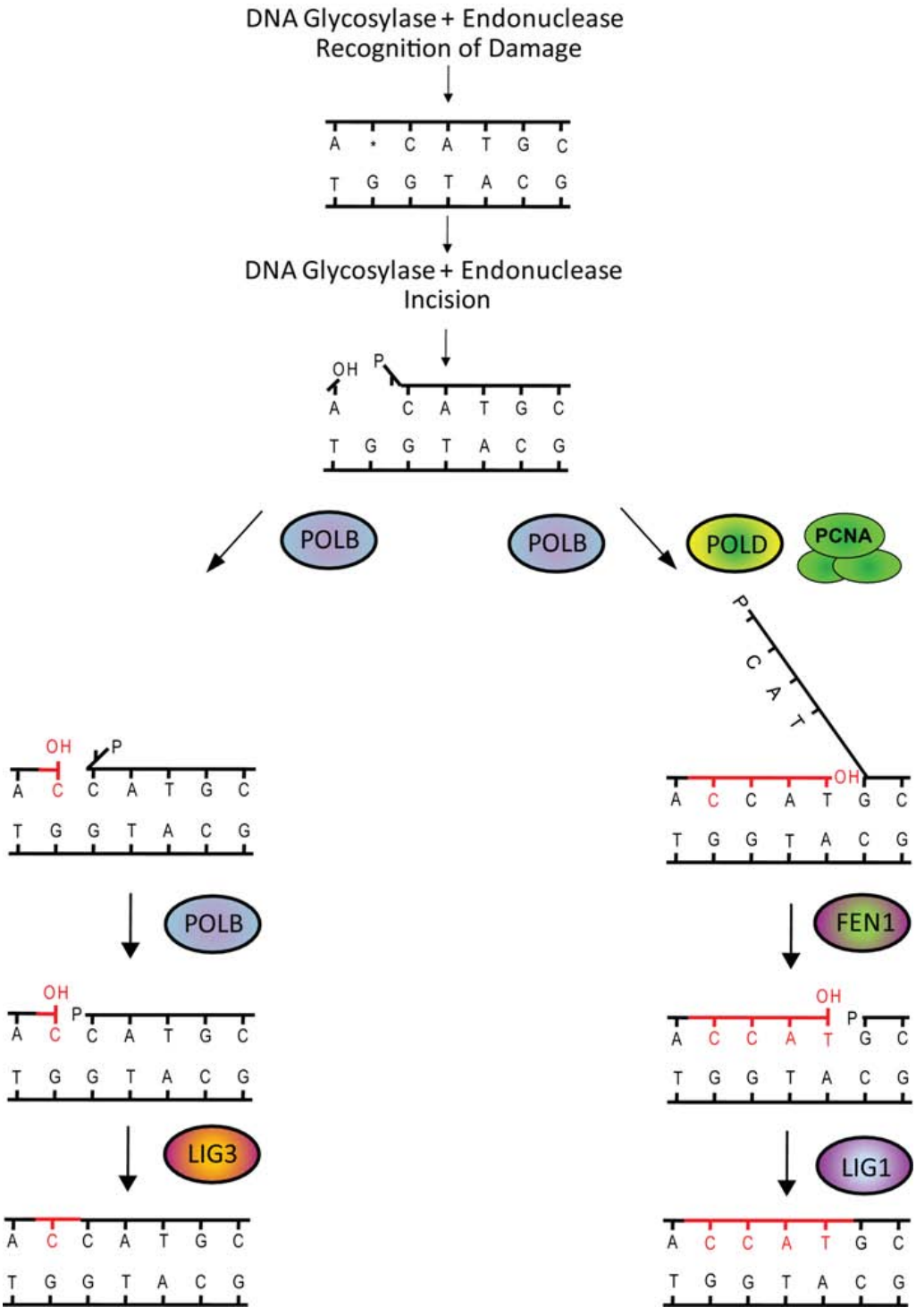

Figure 2. Model of the BER pathway. [Left Branch] Short Patch BER [Right Branch] Long Patch BER

2.2 Mismatch repair (MMR)

2.2.1 Disease phenotype

Mismatch repair (MMR) proteins are responsible for the repair of single nucleotide mismatches and small insertion/deletion loops (IDLs) of DNA (Hansen and Kelley 2000; Hoeijmakers 2001). Cells deficient in MMR show increased mutations, due to the accumulation of replication errors and inappropriate recombination events. In humans, hereditary non-polyposis colorectal cancer (HNPCC) has been associated with mutations in the mismatch repair genes, Msh2 and Mlh1 (Muller and Fishel 2002), but defects in MMR proteins have also been found in sporadic tumors (Hoeijmakers 2001). Silencing of Mlh1 due to hypermethylation of the promoter has also been observed, correlating with the resistance of anti-tumor agents (Fink, Aebi et al. 1998; Strathdee, MacKean et al. 1999).

2.2.2 The MMR pathway

Most of the mechanistic understanding on MMR comes from studies on *E. coli*. In *E. coli,* MutS recognizes mismatches and IDLs (Modrich and Lahue 1996). MutL than interacts with MutS enhancing recognition, and recruits and activates MutH. In yeast, MutL forms a heterodimer with Mlh1 or Pms1 (Prolla, Pang et al. 1994), and forms a complex with Mlh2 or Mlh3 (Harfe and Jinks-Robertson 2000).

In *S. cerevisiae,* six MutS (Msh1-6) and four MutL (Mlh1-3, Pms1) homologs have been identified, with some playing a role in meiotic recombination (Msh4, Msh5) and mitochondrial repair (Msh1) (Reenan and Kolodner 1992; Ross-Macdonald and Roeder 1994; Hollingsworth, Ponte et al. 1995). During mitotic mismatch repair, Msh2 and Msh3 or Msh6 form a heterodimer to recognize either IDLs or base substitutions and 1-2 nt loops, respectively. These complexes are known as MutS-betą (Msh2-Msh3) and MutS-alphą (Msh2-Msh6) (Genschel, Littman et al. 1998).

No eukaryotic homologs of MutH have been identified, however PCNA has been shown to interact with Msh2 and Mlh1 and is thought to participate in the discrimination of newly synthesized and template DNA strands (Harfe and Jinks-Robertson 2000). In eukaryotes, Exo1 degrades the lesion in a 5' – 3' direction, whereas DNA polymerases epsilon and delta degrade the DNA in a 3' – 5' direction (Harfe and Jinks-Robertson 2000).

Interestingly, some yeast MMR factors interact with members of the NER pathway. For example, removal of 3' non-homologous tails arising during gene conversion and single-strand annealing is done by a complex of Rad1-Rad10 and MutS-beta. In this complex MutS-beta stabilizes single-/double-strand DNA junctions enabling nicking by the Rad1-Rad10 endonuclease (Sugawara, Paques et al. 1997), thus excising larger nucleotide stretches. MutS-alpha has also been shown to have an analogous role to Msh4 and Msh5 in meiosis by binding and influencing the resolution of Holliday junctions *in vitro* (Marsischky, Lee et al. 1999). The basic understanding of MMR is illustrated in Fig. 3 from Kunz, Saito et al. (2009).

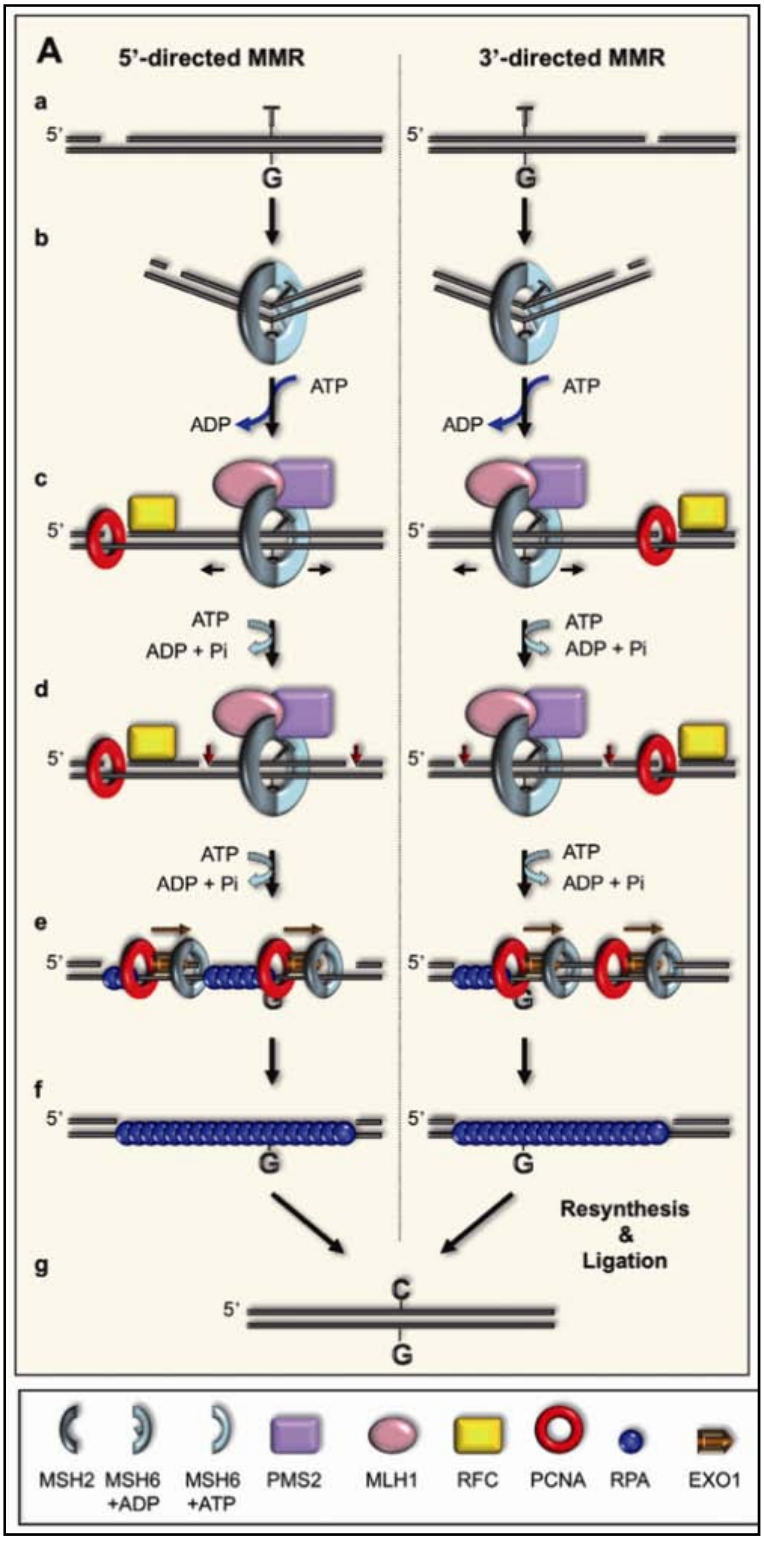
A
5'-directed MMR
3'-directed MMR
a
5'
T
G
b
ATP
ADP
c
5'
ATP
ADP + Pi
d
5'
ATP
ADP + Pi
e
5'
C
G
f
5'
G
Resynthesis
&
Ligation
g
5'
C
G
MSH2
MSH6
+ADP
MSH6
+ATP
PMS2
MLH1
RFC
PCNA
RPA
EXO1

Figure 3. Simplified overview of eukaryotic mismatch repair. (a) Bidirectional MMR requires strand discontinuities located either 5' or 3' to the mismatch. (b) MutS-alpha, a heterodimer composed of Msh2 and Msh6, binds the mismatch, (c) recruits the Mlh1-Pms2 heterodimer (MutL-alpha) and undergoes a conformational switch upon exchange of ADP with ATP, allowing sliding away from the mismatch. (d) A latent endonuclease activity in the Pms2 subunit of MutL-alpha is activated in a MutS-alpha, Rfc, PCNA and ATP dependent manner and introduces nicks in the discontinuous strand (red arrowhead). (e) This generates 5' entry points for the 5' to 3' nuclease Exo1, independently of whether the initial strand discontinuity was located 5' or 3' to the mismatch. MutS-alpha activated Exo1 subsequently degrades the nicked strand, (f) generating single stranded gaps which are protected by RPA. (g) Pol-delta then loads at the 3'-terminus and fills in the gap with help of cofactors PCNA and Rfc. Finally, DNA Ligase I completes repair by sealing the remaining nick. [Reprinted with permission; Kunz, Saito et al. 2009].

2.3 DNA damage tolerance pathway

Another way for the cell to tolerate DNA damage is to bypass the lesion, instead of repairing it. This pathway has previously also been described as post replication repair (PRR), implying repair, but it is really a mechanism of bypassing or tolerating the DNA lesion.

2.3.1 Disease phenotype

In the DNA damage tolerance pathway several polymerases play an important role, these include polymerase zeta, iota, kappa, eta and Rev1. The genes coding for these polymerases are Rev3, Dinb1, Rad30A/XPV, Rad30B and Rev1 respectively. *S. cerevisiae* does not possess both polymerase kappa and iota (reviewed in Waters, Minesinger et al. 2009). Interestingly, there is no strong correlation between deregulated TLS and cancer. One association has been made with a cancer-prone syndrome known as xeroderma pigmentosum variant (XPV). Underlying this syndrome is the deletion or mutations in Rad30 (pol eta). Characteristic are skin cancer and sensitivity to sunlight (Madril, Johnson et al. 2001; Kawamoto, Araki et al. 2005; Lehmann 2005; Leibeling, Laspe et al. 2006). In addition, studies performed on mice when deleting the gene for polymerase zeta resulted in embryonic lethal phenotype (Bemark, Khamlichi et al. 2000; Esposito, Godindagger et al. 2000; Wittschieben, Shivji et al. 2000). Contrary to the mouse studies on polymerase zeta, mouse studies on polymerase kappa did not result in any profound phenotypes, suggesting redundancy inside the cell, that might mask the effects of losing the polymerase (Schenten, Gerlach et al. 2002).

2.3.2 The DNA damage tolerance pathways

In *S. cerevisiae*, stalled replication forks are resolved by bypassing the damage either with translesion synthesis (TLS) or template switching (TS) to the nascent strand of the sister chromatid. PCNA, and whether it is mono- or poly-ubiquitinated, is the control element in deciding whether to utilize TLS or TS, respectively. PCNA is a homotrimeric complex, that functions as a clamp for DNA polymerases as well as a scaffold for many DNA repair enzymes (Moldovan, Pfander et al. 2007). PCNA is monoubiquitinated after damage such as UV, and catalyzed by Rad18 and Rad6, initiating TLS. The Rad6-Rad18 hetero-dimer plays a central role in this pathway in *S. cerevisiae* (Broomfield, Hryciw et al. 2001). Rad18 binds ssDNA gaps at stalled replication forks and recruits Rad6, an E2 ubiquitin-conjugating enzyme (Bailly, Lamb et al. 1994). Alkylating agents such as MMS that damage the DNA have been shown to trigger polyubiquitination of PCNA, which is catalyzed by Rad5, Ubc13 and Mms2 (Hoege, Pfander et al. 2002) and results in the initiation of TS. The pathways have been described as error-free (TS) and error-prone (TLS). Both pathways result in the bypass of unrepaired DNA lesions (Johnson, Washington et al. 1999) and are further described detail below.

2.3.2.1 Translesion synthesis (TLS)

TLS requires Rev1, which forms a complex with the subunits of DNA polymerase zeta, Rev3 and Rev7, to promote the incorporation of cytosine opposite an abasic site or UV photoproduct. This pathway is an error prone pathway (Nelson, Lawrence et al. 1996; Nelson, Lawrence et al. 1996).

Another mutagenic pathway has been described requiring polymerase eta after induction of damage by N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) (Haracska, Prakash et al. 2000). However, polymerase eta is also involved in the error free bypass (TS) of UV-induced cyclobutane pyrimidine dimers (CPDs), inserting two complementary adenine bases opposite the T-T dimer during DNA replication (Johnson, Washington et al. 1999; Masutani, Kusumoto et al. 1999). TLS is initiated by monoubiquitination of PCNA (K164), which is catalyzed by E3 ubiquitin ligase Rad18, and E2 ubiquitin-conjugating enzyme Rad6 (Hoege, Pfander et al. 2002). The basic steps are illustrated in Fig. 4A.

2.3.2.2 Template switching (TS)

TS is best described as being error free, and it requires ubiquitin conjugating enzymes Ubc13 and Mms2 before further branching in a pathway dependent on either Rad5, a ssDNA ATPase, or replicative DNA polymerase delta in combination with PCNA (Ulrich and Jentsch 2000; Torres-Ramos, Prakash et al. 2002). Strand-switching by DNA polymerase delta is thought to be facilitated by Ubc13-Mms2 ubiquitination of Rad5 (Torres-Ramos, Prakash et al. 2002). The activation of template switching by DNA

polymerases zeta and eta is thought to be the result of the polyubiquitination of PCNA by Rad6-Rad18 and Ubc13-Mms2 (Stelter and Ulrich 2003). The basic steps are further illustrated in Fig. 4B.

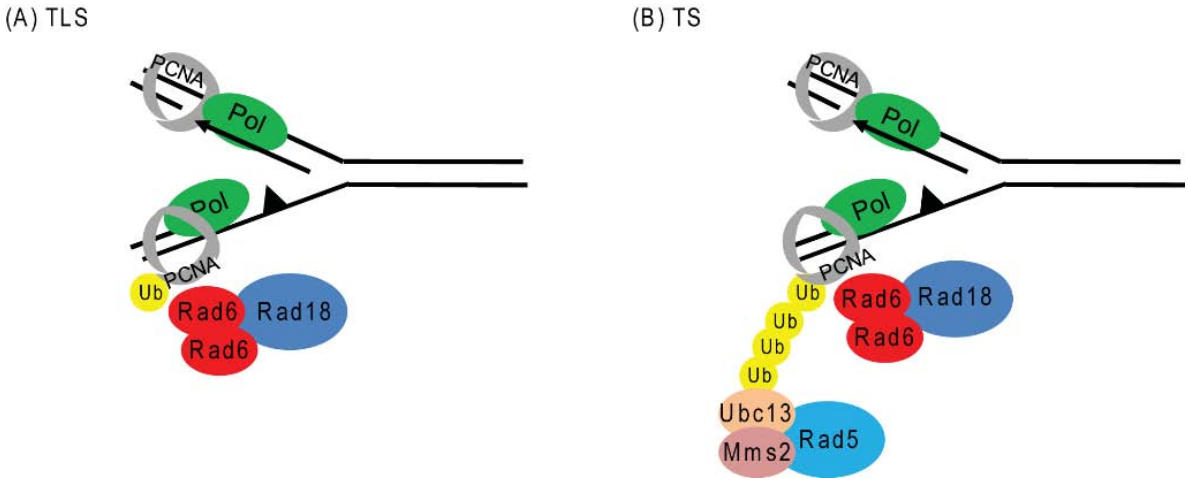

Figure 4. Schematic of two different pathways that bypass DNA damage causing stalled replication forks. (**A**) Translesion synthesis (TLS) pathway regulated by proliferating cell nuclear antigen (PCNA) monoubiquitination uses specific a TLS polymerase to bypass DNA damage. (**B**) A template switching (TS) pathway regulated by PCNA polyubiquitination bypasses DNA damage in the error-free mode. The exact molecular mechanism for the TS pathway is not clearly understood yet. The small triangle on the DNA represents DNA damage.

2.4 Nucleotide excision repair (NER)

2.4.1 Disease phenotype

Three disease phenotypes have been classically defined to be the result of mutations in NER genes. The first disease is xeroderma pigmentosum (XP), a rare hereditary disease characterized by cutaneous symptoms in the sunlight exposed areas of the skin. NER global repair is affected in XP patients cells (Cleaver 1968; Setlow, Regan et al. 1969). There are eight complementation groups in XP; these are XP-A through XP-G (Aboussekhra, Biggerstaff et al. 1995; Mu, Park et al. 1995), and a variant group XP-V referred to earlier (Masutani, Kusumoto et al. 1999). The second disease is cockayne syndrome (CS), characterized by patients that manifest a more developmental type of disease and have symptoms of neurological impairment (Nance and Berry 1992). There are five different complementation groups in CS (Rapin, Lindenbaum et al. 2000), two result from deficiencies in the CSA and CSB genes, which are needed for transcription-coupled repair (TCR) but not for global genome repair (GGR). The other three complementation groups are due to mutations in XPB, XPD and XPG, causing the more severe type II phenotype of CS (Rapin, Lindenbaum et al. 2000). The third disease is trichothiodystrophy (TTD), characterized by brittle hair (Price, Odom et al. 1980). There are four complementation groups for TTD: TTD-A (Giglia-Mari, Coin et al. 2004), XPB (Weeda, Eveno et al. 1997), XPD (Stefanini, Lagomarsini et al. 1986) and TTDN1 (Nakabayashi, Amann et al. 2005), all of which encode protein subunits of the TFIIH transcription complex. The underlying repair mechanism of these disease phenotypes is further explained in the next section.

2.4.2 Nucleotide excision repair pathway

The genes involved in yeast repair are designated *RAD* as they were initially identified as radiation sensitive. Three different sub-pathways have now been identified; global genome repair (GGR), transcription-coupled repair (TCR), and transcription domain-associated repair (DAR).

Characteristic for TCR is the fact that NER is coupled to transcription probably by stalled RNA polymerase II (RNAPII) acting as a recruitment signal. NER of nontranscribed regions is termed GGR. In addition, in several cell types that were deficient in GGR, non-transcribed strands of transcribed genes were still proficiently repaired, this lead to the identification of DAR (Nouspikel, Hyka-Nouspikel et al. 2006)

In addition to a potential role of NER in the repair of DPCs (Magana-Schwencke, Ekert et al. 1978), NER is known to be involved in the repair of other bulky DNA adducts (Wogan, Hecht et al. 2004), intra- (damage on same strand) and inter-strand (damage on both strands) crosslinks (Trimmer and Essigmann 1999). NER also repairs UV-induced lesions, including CPDs and (6-4) pyrimidine-pyrimidone photoproducts [(6-4) PPs] (Pfeifer 1997).

2.4.2.1 NER - recognition of DNA damage and binding

NER has been studied extensively using human cells, therefore the repair pathway will be reviewed in light of human NER. The yeast orthologs are listed behind the genes. In humans, three subunits form a complex that detects damage to the DNA, XPC, HR23B and centrin 2 (Araki, Masutani et al. 2001). In *S. cerevisae* these are

known as Rad4, Rad23 and Cdc31. In this complex XPC (Rad4) appears to be the protein binding the damaged DNA, and functions as a sensor for the damage (Sugasawa, Ng et al. 1998; Sugasawa, Okamoto et al. 2001; Maillard, Solyom et al. 2007). In addition, the affinity to DNA increases and degradation gets inhibited by the polyubiquitination of XPC (Rad4) (Sugasawa, Okuda et al. 2005). The exact function of HR23B (Rad23) is not known; it does interact directly with XPC (Rad4) and could play a role in the ubiquitination of XPC (Rad4) due to the presence of ubiquitin-like and ubiquitin-associated domains (Watkins, Sung et al. 1993; Sugasawa, Ng et al. 1997). Centrin 2 (Cdc31) is not essential for NER, but it stabilizes the recognition complex and stimulates NER activity (Araki, Masutani et al. 2001).

Another DNA damage-specific binding complex is a heterodimer consisting of DNA damage binding protein, DDB1 (Ddb1), and DDB2/XPE (there is no known yeast homolog), which has a high affinity for (6-4) PPs and CPDs (Payne and Chu 1994). The DDB complex is also part of a multi-subunit E3 ubiquitin ligase, namely with DDB2/XPE and CSA (Rad18) (Groisman, Polanowska et al. 2003).

2.4.2.2 NER - unwinding of the DNA

After recognition and binding of the damage the DNA is unwound around the lesion, presumably via the action of a complex of 10 subunits, the TFIIH-complex. This complex plays an important role in transcription initiation of RNAPII. Members of this complex are cyclin H, cdk7, MAT1, XPB, XPD, p62, p52, p44, p34 and TTD-A. This complex is also found in *S. cerevisiae*, and is referred to as the nucleotide excision repair factor 3 (NEF 3) group. In yeast the complex is composed of Rad3, Tfb1, Tfb2,

Ssl1, Tfb4 and associated subunit Ssl2p (also called Rad25). XPB (Ssl2) and XPD (RAD3) are helicases involved in the unwinding of the DNA (Winkler, Araujo et al. 2000; Coin, Oksenych et al. 2007). The exact function of the other proteins is not known (Schultz, Fribourg et al. 2000). It has been suggested that XPA (Rad14) identifies the lesion containing strand, although the exact function remains unknown, it is absolutely required for NER, as XPA (Rad14) patients have a severe phenotype (Sugasawa, Ng et al. 1998).

2.4.2.3 NER - incision of the DNA

After recognition and unwinding, incision of the damaged strand at either end of the denatured bubble is necessary. This is performed by three proteins, endonuclease XPG (Rad2 in yeast) cuts 3' to the damage (O'Donovan, Davies et al. 1994) and on the 5' side XPF-ERCC1 (Rad1-Rad10 in yeast) cuts (Mu, Hsu et al. 1996). In the XPF-ERCC1-complex (Rad1-Rad10) XPF (Rad1) possesses the endonuclease activity (Biggerstaff, Szymkowski et al. 1993). The incision by XPF-ERCC1 (Rad1-Rad10) requires XPG (Rad2), but XPG (Rad2) does not require XPF-ERCC1 (Rad1-Rad10) to be able to cut the DNA (Wakasugi, Reardon et al. 1997).

2.4.2.4 NER - gap filling and ligation of the DNA

Following incision and excision of the damage-containing DNA fragment there is a gap of 25 – 30 nucleotides (Svoboda, Taylor et al. 1993; Matsunaga, Mu et al. 1995; Moggs, Yarema et al. 1996). Synthesis of this DNA is performed by either DNA polymerases delta or epsilon (Popanda and Thielmann 1992) and is associated with

sliding clamp PCNA (Pol30) and replication factor C (RFC) (Nichols and Sancar 1992; Shivji, Kenny et al. 1992). It is believed that ligase III in complex with X-ray repair complementing defective repair in chinese hamster cells (XRCC1) seals the nick as the final step of NER (Moser, Kool et al. 2007). A schematic of the current model of NER is provided in Fig. 5.

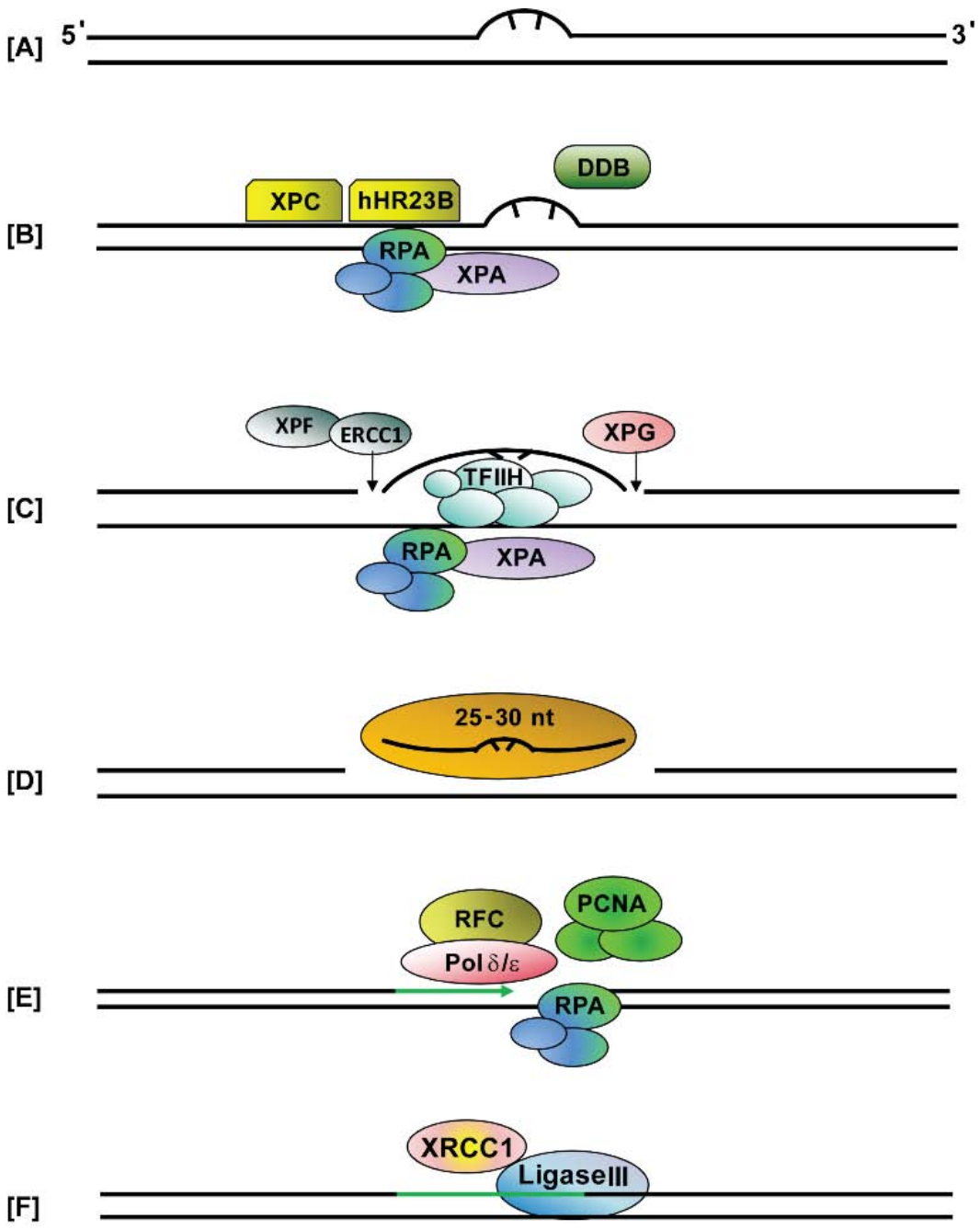

Figure 5. Model of NER. [A] Damage to the DNA [B] Recognition of the damage by XPC, hHR23B, RPA, XPA and the DDB complex. [C] Damage binding of the TFIIH-complex with RPA and XPA and incision of XPF-ERCC1 and XPG [D] excision of the damage. [E] Resynthesis of the damage by RFC, polymerase delta and polymerase epsilon, PCNA and RPA. [F] Ligation of the newly synthesized DNA with the DNA strand.

2.5 DNA double strand break (DSB) repair

2.5.1 DNA strand breaks

Single (SSBs) and double (DSBs) strand breaks in the DNA occur frequently due to endogenous and exogenous exposure of DNA damaging agents (Paques and Haber 1999). SSBs can occur during replication, it is thought that this happens due to the replication fork encountering an unrepaired adduct. SSBs are also formed after ultra violet (UV) exposure as an intermediate product due to repair, and ionizing radiation (Cox, Goodman et al. 2000). DSBs reside from exposure to ionizing radiation, from certain drugs like bleomycin, mechanical stress, endonuclease digestion, oxidative damage or as a result of unrepaired nicks during normal cellular processes and at stalled replication forks (Cox, Goodman et al. 2000). In general, strand breaks, especially DSBs, are very cytotoxic to the cell due to increased genomic instability and the inhibition of cellular processes such as replication.

During DNA replication, DSBs are thought to be the result of replication fork collapse at a DNA lesion (Paques and Haber 1999; Cox, Goodman et al. 2000). The repair of DSBs is crucial to the cell as they may lead to genome instability, gene disruption and chromosomal rearrangements which increase the risk of tumor progression. Two such cancer-prone diseases arising from defects in the repair of DSBs have been described and well studied; the Nijmegen breakage syndrome and the ataxia-telangiecstasia-like disorder (Karran 2000). Currently, there are two well-studied DSB repair pathways, non-homologous end-joining (NHEJ) and homologous recombination (HR). In addition, more recently a third DSB repair pathway named microhomology-

mediated end joining (MMEJ) has been described (Ma, Kim et al. 2003; Nussenzweig and Nussenzweig 2007).

2.5.2 Disease phenotype - Nijmegen Breakage Syndrome

In order to shed light on the different DNA DSB repair pathways, and the involvement of the MRN(X)-complex in these repair mechanisms, the MRN(X)-complex and its associated syndrome will be described first, followed by the role it has in these different DNA (double) strand break repair pathways.

A common complex found in the various DSB repair pathways is the MRN(X)-complex, and mutations in the NBS gene (Xrs2 in yeast) lead to the identification of a disease known as the “Nijmegen Breakage Syndrome” (NBS). This syndrome was first described in 1981 by a group residing in Nijmegen, The Netherlands (Weemaes, Hustinx et al. 1981). NBS is a rare autosomal recessive disease, but its actual prevalence is not known. It is characterized by microcephaly, growth retardation, a bird-like face, immmunodeciciency and predisposition to cancer. Two other important set of proteins underlying this syndrome and crucial for the sensing of DSBs and cell cycle control are Mre11, and Rad50, thus forming the MRN(X)-complex (Iijima, Komatsu et al. 2004). In addition to the identification of the syndrome, mutations in the genes coding for NBS1, Mre11 and Rad50 have also been associated with breast cancer (Heikkinen, Rapakko et al. 2006). Interestingly, studies performed on mice when disrupting Mre11, Rad50 or NBS1 resulted in embryonic lethal phenotype (Xiao and Weaver 1997; Luo, Yao et al. 1999; Zhu, Petersen et al. 2001). To date, the exact function of the MRN(X)-complex is not known, but it is known that it plays a role in sensing DSBs by recruiting ATM (ataxia

telangiestasia-mutated) to a DSB site and facilitating efficient phosphorylation of ATM substrates (Lee and Paull 2007). Mutations in ATM are associated with the autosomal recessive disorder, Ataxia-telangiectasia (AT), characterized by cerebellar ataxia, telangiectasia, immunodeficiency and a high incidence of malignancy (Shiloh 2003).

2.5.3 Homologous recombination (HR) pathway

HR is an error-free pathway for repairing DSBs. In general, proteins encoded by genes of the RAD52 epistasis group (Rad50, Rad51, Rad52, Rad54, Rad55, Rad57, Rad59, Rdh54, Mre11 and Nbs1/Xrs2) are necessary for the function of this pathway (Krogh and Symington 2004). Other proteins are required to complete specific HR reactions, these include DNA nucleases (Exo1, Sae2, Rad1-Rad10), helicases (Sgs1, Srs2), topoisomerases (Top3), polymerases (Pol32) and ligases (Krogh and Symington 2004). Classical HR can be described in three basic steps: 1) 5'-end DNA strand resection at break ends, 2) strand invasion and exchange, 3) resolution of the strand invasion and exchange of product.

2.5.3.1 Models for DSB repair through homologous recombination

Several models of homologous recombination have been suggested to repair chromosomal DSBs in mitotic yeast cells, namely gene conversion, break-induced replication, and single strand annealing. For gene conversion two separate models have been suggested. Some proposed steps are shared among the different mechanisms, these being resection of the DSB ends, strand invasion, removal of non-homologous tails, and gap repair by DNA synthesis.

2.5.3.1.1 Gene conversion

In all of the models the process is initiated by a DSB, resulting in 3' single-stranded tails. In the first proposed model for gene conversion, the Rad51 protein binds ssDNA regions at the DSB sites, preventing exonucleases from digesting the single-strand DNA ends, and facilitating invasion of the homologous duplex. The ligation of the homologous stretches of DNA results in the formation of a double Holliday junction structure. When gene conversion is successful, the Holliday junction gets resolved and genetic material is exchanged between the damaged and undamaged strand (Weng and Nickoloff 1998).

Alternatively, synthesis-dependent strand annealing (SDSA) may occur. This model has been suggested due to the low level of crossovers observed in yeast as seen in HO endonuclease-mediated mating type switching (Paques and Haber 1999). The difference being, that only one of the 3' tails invades the homologous duplex to prime DNA synthesis, which results in the formation of a displacement (D-) loop. A template for DNA synthesis from the other 3' end is provided by displacing the other strand from the donor duplex. SDSA could be responsible for the occurrence of double Holliday junctions on one side of a DSB (Haber, Ira et al. 2004). Another study suggests that SDSA is promoted *via* the helicase Srs2 by regulation of Rad51 during strand exchange (Ira, Malkova et al. 2003).

In addition, there is evidence that Sgs1 and Top3 play a role. A deletion of the Sgs1 helicase in yeast, a ortholog of the human Bloom and Werner helicases (BLM/WRN), significantly increased the frequency of crossovers. Therefore, Sgs1 in combination with topoisomerase Top3, might have an inhibitory role in the crossover

occurrence, eliminating double Holliday junction intermediates (Ira, Malkova et al. 2003). This also appears to be the case in human cells, where BLM and hTOPOIIIα act to resolve double Holliday junctions arising from HR-dependent repair or replicative strand gaps (Wu and Hickson 2003). A hallmark feature of Bloom syndrome (BLM) cells is the high frequency of sister chromatid exchange. A schematic of GC and SDSA is provided in Fig. 6A and B.

2.5.3.1.2 Break-induced replication (BIR)

Another proposed model for DSB repair is break-induced replication (BIR). It differs from gene conversion in that BIR continues to the end of the donor chromosome (Malkova, Ivanov et al. 1996). One suggested role for BIR is to maintain telomeres in the absence of telomerase enzymes (Le, Moore et al. 1999). Genetic analyses have revealed two independent branches of BIR, one for telomere maintenance and one for DSB repair in diploid cells (Malkova, Ivanov et al. 1996; Signon, Malkova et al. 2001). However, one involves the MRN(X)-complex with Rad59, while the other depends on Rad51, Rad54, Rad55 and Rad57. A basic schematic of BIR is provided in Fig. 6C.

2.5.3.1.3 Single strand annealing (SSA)

When a double strand break is present between two repeat sequences oriented in the same direction, single strand annealing (SSA) is initiated (Fishman-Lobell, Rudin et al. 1992; Symington 2002). In this process, exonucleases cut the DNA such that sufficient 3' single-stranded regions with homology at the DSB ends are present and then annealed. Noncomplementary ssDNA overhangs are removed by endonucleases

and the resulting gap is filled in by DNA synthesis and ligation (Sugawara and Haber 1992). SSA is mutagenic due to the deletion of these repeat sequences. A basic schematic of SSA is illustrated in Fig. 6D.

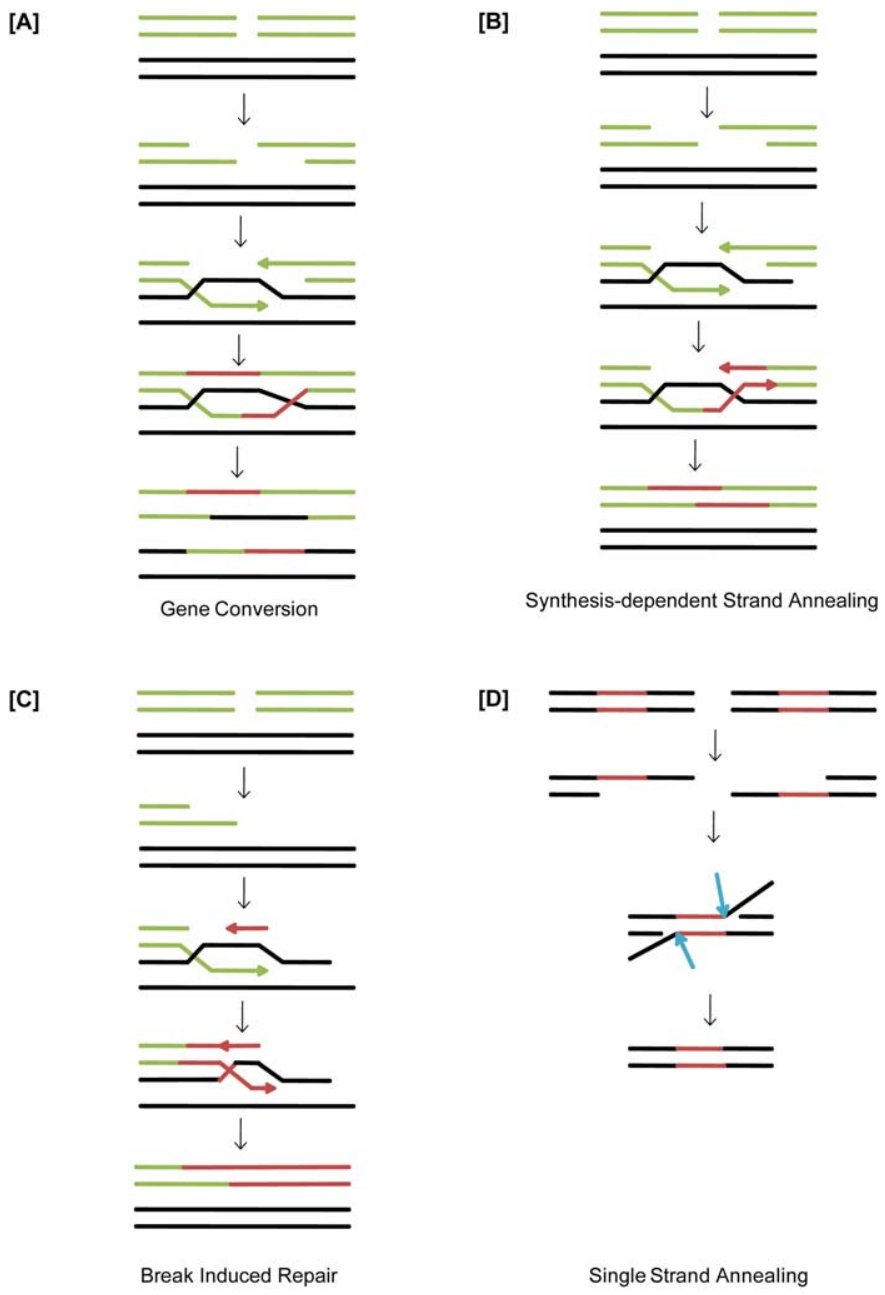

Figure 6. Models for homologous recombination. [A] Gene Conversion (GC), after DSB formation exonuclease activity exposes single strand DNA, resulting in strand invasion of both strands, followed by strand exchange, and fork resolution. [B] Synthesis-dependent Strand Annealing (SDSA) is similar to GC, but the difference is that only one of the 3' tails invades the homologous duplex. [C] Break Induced Repair (BIR) is also very similar to GC, but here repair continues to the end of the chromosome. [D] Single Strand Annealing occurs between two repeat sequences oriented in the same direction.

2.5.4 Non-homologous end-joining (NHEJ)

Another DNA DSB-repair pathway is NHEJ. As the name suggests it is the pathway responsible for religating two broken DNA ends together. This pathway is commonly described as error-prone due to the fact that this process generates small insertions and deletions (Moore and Haber 1996; Wilson, Grawunder et al. 1997). The NHEJ machinery core components are the MRN(X), Ku and DNA ligase complexes (Daley, Palmbos et al. 2005). Although the order of actions is unknown, it is believed that the MRN(X) and Ku complexes bind DSB ends shortly after DSB formation. These complexes are thought to function as a link between DSB ends, help inhibit their degradation and recruit, stabilize and stimulate the ligase complex at DSBs (Daley, Palmbos et al. 2005).

2.5.5 Microhomology-mediated end joining (MMEJ)

A more recently defined DNA DSB-repair pathway has termed microhomology MMEJ (Ma, Kim et al. 2003). Although this pathway has not been studied as extensively compared to the earlier described pathways, it is generally described as a Ku-independent end joining mediated process initiated by base pairing between microhomologous sequences of approximately 5-25 nucleotides. Chromosome abnormalities such as deletions, translocations, inversions and other complex rearrangements have also been described to result from MMEJ pathway manipulations (Chen, Umezu et al. 1998; Welcker, de Montigny et al. 2000; Yu and Gabriel 2003; Weinstock, Brunet et al. 2007). Important players in this pathway are the MRN(X)-complex, Sae2, Exo1, Rad51 and the Ku-complex.

2.6 The MRN(X)-complex

2.6.1 The MRN(X)-complex components

As described earlier the MRN(X)-complex consists of three proteins, namely Mre11, Rad50 and Nbs1 (Xrs2 in yeast). Two groups have shown that the MRX-complex is necessary to keep DSB ends associated after DSB induction (Lisby, Antunez de Mayolo et al. 2003; Lobachev, Vitriol et al. 2004)

Rad50 belongs to the family of structural maintenance of chromosome (SMC) proteins (Anderson, Trujillo et al. 2001). Of the proteins in this complex Rad50 possesses two globular ATPase domains at its N- and C-terminal ends (Usui, Ohta et al. 1998; Anderson, Trujillo et al. 2001; Wiltzius, Hohl et al. 2005). The ATPase domains mediate ATP-dependent DNA binding and unwinding, but also facilitate, and stimulate the Mre11 nuclease activity (Raymond and Kleckner 1993; Trujillo and Sung 2001; Chen, Trujillo et al. 2005). Rad50 also contains a coiled-coil region, in between the ATPase domains, that possesses a conserved zinc-binding motif known as the Rad50 hook, which mediates interaction between two Rad50 molecules (Wiltzius, Hohl et al. 2005).

The second component in this complex Xrs2, Nbs1 in mammals, binds DNA and is important for targeting the MRN(X)-complex to DNA ends (Trujillo, Roh et al. 2003). It contains a binding domain for both Mre11 and Tel1 (ATM) in the C-terminal region. Tel1 has been shown to mediate translocation of Mre11 to the nuclease and mediate phosphorylation of Xrs2 (Nbs1) (Usui, Ogawa et al. 2001). In addition, Xrs2 (Nbs1) is

able to interact with Lif1 (XRCC4), which results in the association with DNA ligase Dnl4 (Chen, Trujillo et al. 2001; Trujillo, Roh et al. 2003).

The third component of this complex is Mre11. This protein has both 3'-5' exonuclease activity and structure-specific endonuclease activity, both of which are manganese-dependent and localized at the N-terminus of the protein (Furuse, Nagase et al. 1998; Trujillo and Sung 2001). This N-terminal region is also important for maintaining the interaction with Xrs2 and Rad50. The exo- and endo-nuclease activities of Mre11 are enhanced by Xrs2 and Rad50, respectively (Trujillo and Sung 2001; Trujillo, Roh et al. 2003). The C-terminal region is important for DNA binding to both single- and double-stranded DNA in a structure and sequence specific manner (Furuse, Nagase et al. 1998; Ghosal and Muniyappa 2005). Studies using Mre11 nuclease deficient mutants have led to the conclusion that this activity is necessary for the processing of Spo11-induced DSBs during meiosis (Furuse, Nagase et al. 1998; Tsubouchi and Ogawa 1998; Usui, Ohta et al. 1998; Moreau, Ferguson et al. 1999). This is germane, as the crosslinking of Spo11 to the DNA ends during meiosis might resemble a DPC presented in the cell after formaldehyde exposure. In addition to its role in the processing of Spo11 at the DNA ends, these nuclease deficient mutants are less sensitive to ionizing radiation (IR) when compared to the complete Mre11 knockout (Krogh, Llorente et al. 2005). Interestingly, these same nuclease deficient mutants displayed no defect in the processing of HO-induced DSBs, and the telomeres were the same length as the Mre11 strains. The authors also showed that these strains do not possess any residual endonuclease activity which could have explained these results (Krogh, Llorente et al. 2005). In addition, Mre11 cleaves DNA structures that can be found at DSB ends such as hairpin structures and 3' single-strand overhangs at the

single-/double-stranded junction (Moreau, Ferguson et al. 1999; Trujillo, Roh et al. 2003). Although not essential, it has also been suggested that Mre11 could facilitate overhang pairing of DSB ends (Moreau, Ferguson et al. 1999). Taken together, these data suggest that the role of the nuclease activity is to cleave DNA structures or covalent adducts from DNA ends, and therefore hinting at a possible role in the tolerance or repair of formaldehyde induced DPCs.

2.6.2 Checkpoint signaling and the MRN(X)-complex

A short summary of the mechanisms of DNA-damage checkpoints, crucial for proper DSB repair is provided below. The classical definition of checkpoint is a delay in cell cycle transition to allow time for repair (Weinert and Hartwell 1988). It is now known that cell cycle checkpoint genes also play a role in the transcriptional induction of DNA repair genes (Aboussekhra, Vialard et al. 1996) and post-translational modifications of proteins (Weinert and Hartwell 1988).

A hallmark of the DNA-damage checkpoint pathway is the complex process of protein phosphorylation. This process is seen as one of the first signals produced after DNA-damage occurs. There are several important checkpoint genes necessary for this phosphorylation; these include ATM (Tel1), ATR (Mec1) (ataxia telangiectasia and Rad3-related) and the DNA-Pkcs (DNA-dependent protein kinase catalytic subunit). These three proteins play an essential role in triggering checkpoint response by activating effectors CHK1 and CHK2. This process also occurs in *S. cerevisiae*, where Tel1 and Mec1 (ATM and ATR orthologs) are responsible for the phosphorylation of Rad53, Chk1 and Dun1. In yeast, Rad9 protein is the mediator of checkpoint response

after DNA damage (Weinert and Hartwell 1988). In mammals, the ATM mediators are MDC1 and breast and ovarian cancer-specific tumor suppressor BRCA1 (Su 2006).

An initiating event of this checkpoint-signaling pathway is the detection of DSBs and the interaction of the MRN(X)-complex with the DSB. (Chen, Trujillo et al. 2001; Hopfner, Craig et al. 2002). In yeast, there are two ways in which checkpoint responses are evoked. The first is dependent on Mec1, where a single unrepaired DSB leads to a G2 cell cycle arrest (Zou and Elledge 2003). The second is dependent on Tel1, which requires multiple unprocessed DSBs to be present (Adams, Medhurst et al. 2006; Jazayeri, Falck et al. 2006; Dubrana, van Attikum et al. 2007). When a DSB occurs, the ssDNA generated by resection are first coated by RPA (Rfa1-3), which is recognized by Tel1/ATR with the cofactor Ddc2/ATRIP (Fig. 7E-F) (Paciotti, Clerici et al. 2000; Rouse and Jackson 2000; Zou and Elledge 2003). Further activation of ATR requires RPA-mediated recruitment of a complex composed of RAD9-RAD1-HUS1 (9-1-1-complex) in humans and Rad17-Mec3-Ddc1 in *S. cerevisiae* (Fig. 7F) (Melo, Cohen et al. 2001; Zou, Liu et al. 2003). This complex, similar to replication sliding clamp PCNA, is loaded onto the ssDNA by a replication factor C-like complex consisting of Rad24 in yeast and RAD17 in humans (Naiki, Shimomura et al. 2000).

Another important aspect of cell cycle checkpoint after formation of DSBs is the histone H2AX C-terminal tail phosphorylation, described as γH2AX (Rogakou, Pilch et al. 1998). Several proteins interact with γH2AX, including MDC1, ATM and the MRN (X)-complex (Stucki, Clapperton et al. 2005). The accumulation of γH2AX at larger chromatin domains on either side of a DSB is important for the stable accumulation of MDC1, ATM (MEC1) and the MRN(X)-complex in both mammals and yeast (Downs,

Lowndes et al. 2000; Celeste, Fernandez-Capetillo et al. 2003). This checkpoint activation is responsible for the phosphorylation of many DSB repair proteins involved in NHEJ and HR, suggesting that checkpoints may enhance the efficiency of both pathways (Herzberg, Bashkirov et al. 2006; Ahnesorg and Jackson 2007). The basic steps are illustrated in Fig. 7.

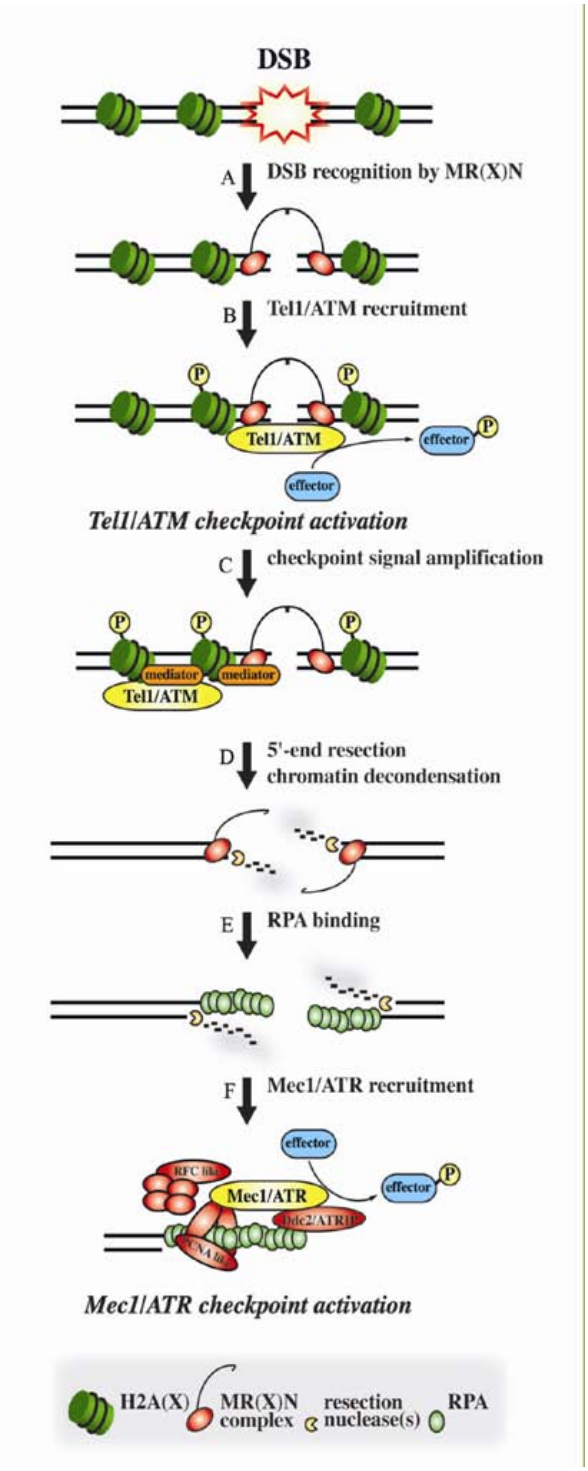
DSB
A
DSB recognition by MR(X)N
B
Tel1/ATM recruitment
P
Tel1/ATM
effector
Tel1/ATM checkpoint activation
C
checkpoint signal amplification
mediator
D
5'-end resection
chromatin decondensation
E
RPA binding
F
Mec1/ATR recruitment
Mec1/ATR
Ddc2/ATRIP
Mec1/ATR checkpoint activation
H2A(X)
MR(X)N complex
resection nuclease(s)
RPA

Figure 7. Model of DSB signaling and checkpoint activation. DNA containing a DSB is represented by a close pair of black lines. Sensors involved in checkpoint signaling are depicted in red, transducer kinases in yellow, effector kinases in light blue and mediators in orange. (A) DSB ends are recognized by the MRN(X) complex. (B) MRN(X) recruits Tel1/ATM. Tel1/ATM phosphorylates H2A/H2AX histones (gH2AX) and effector kinases that propagate the signal. (C) Mediator proteins mediate checkpoint factor propagation around the breakpoint, generating gH2AX large chromatin domains. (D) DSB 5'-ends are resected, involving MRN(X) and other nucleases and chromatin decondensates.(E) RPA binds to single-stranded overhangs generated by resection. (F) RPA-coated single-stranded DNA recruits Mec1/ ATR through its cofactor Ddc2/ATRIP. It also recruits a PCNA like complex via an RFC-like complex, which contribute to full Mec1/ATR activation. [Reprinted with permission; Pardo, Gomez-Gonzalez et al. 2009].

2.6.3 Homologous recombination and the MRN(X)-complex

The MRN(X)-complex is involved in the processing of both meiotic and mitotic DSBs (Johzuka and Ogawa 1995; Lee, Moore et al. 1998; Bressan, Baxter et al. 1999). Resection of the 5'-ended DNA strand at a DSB is a critical step of all HR reactions (Fig. 8E) (White and Haber 1990; Sun, Treco et al. 1991). Resection of the 5'-ends generates long 3' single-stranded ends which are utilized to find sequence homology in the complementary strand (Fig. 8F-G) to invade the duplex DNA (Fig. 8H) and to prime DNA synthesis. *In vitro* Mre11 has a 3' to 5' exonuclease activity, which contradicts the current models and the requirement of a 5' to 3' exonuclease in the resection of DSB ends (Fig. 8). Although this finding is confusing, the same studies showed that Mre11 is necessary for processing of meiotic or mitotic induced DSBs (Johzuka and Ogawa 1995; Lee, Moore et al. 1998; Bressan, Baxter et al. 1999).

Another interesting protein involved in the processing of DSB ends is Sae2 in yeast (CtIP in vertebra) (McKee and Kleckner 1997; Sartori, Lukas et al. 2007). Sae2 possesses endonuclease activity on single-stranded DNA and single-strand/double-strand transitions *in vitro* (Lengsfeld, Rattray et al. 2007). The MRN(X), Sae2 complex that is formed *in vivo* is necessary to process hairpin structures and meiotic DSBs (Lengsfeld, Rattray et al. 2007). This Sae2-MRN(X)-complex does not directly perform the 5'-end strand resection at DSBs, but it has been suggested that it functions as an activator or recruiter for other nucleases with 5' – 3' exonuclease activity. One candidate that could perform the 5'-end strand resection at a DSB is Exo1 (Tsubouchi and Ogawa 2000; Llorente and Symington 2004). Further support for the involvement of Exo1 comes from a study where Exo1 overexpression partially suppressed the DSB-repair defects of

MRX-depleted cells (Moreau, Ferguson et al. 1999). However, in the absence of either Exo1 or Mre11 nuclease activity, the DSB 5'-end strand resection still occurs, suggesting the involvement of yet another unidentified nuclease (Moreau, Ferguson et al. 1999).

Interestingly, DSBs induced after camptothecin treatment or during meiosis show that one strand is covalently linked to a specific protein (Spo11) (Hartsuiker, Mizuno et al. 2009; Hartsuiker, Neale et al. 2009), resembling a DNA-protein crosslink. It has been proposed that the Mre11 nuclease activity is necessary for the removal of this protein, therefore enabling 5'-end strand resection. As in NHEJ, Rad50 also appears to bridge the DNA ends keeping homologous ends close together facilitating repair, lacking the MRN(X)-complex slows down this process (Ivanov, Sugawara et al. 1994; Hopfner, Craig et al. 2002; Lisby, Antunez de Mayolo et al. 2003; Lobachev, Vitriol et al. 2004). Taken together it emphasizes the important role of the MRN(X)-complex in HR, and possible role in tolerating or repairing DPCs.

2.6.4 Non-homologous end-joining and the MRN(X)-complex

As mentioned earlier, the NHEJ machinery core components are the MRN-complex, Ku and DNA ligase complexes (Daley, Palmbos et al. 2005). It has been proposed that these complexes bridge the DSB ends together and inhibit their degradation (Fig. 8A-B). The Ku and the MRN(X)-complex further stabilize and stimulate ligase complexes to DSBs (Fig. 8C). Essential to NHEJ is the formation of heterodimeric complex (Ku) by Ku70 and Ku80 (Boulton and Jackson 1996). The MRN(X)-complex has been implicated to play a role in NHEJ due to the interaction of this complex with Ku80 (Chen, Trujillo et al. 2001; Palmbos, Daley et al. 2005). In yeast, the MRX-complex is the only protein complex found to have a role in both NHEJ and HR. In vertebrates there is no concrete evidence that the MRN-complex is involved in NHEJ. A schematic of the basic understanding of NHEJ is illustrated in Fig. 8.

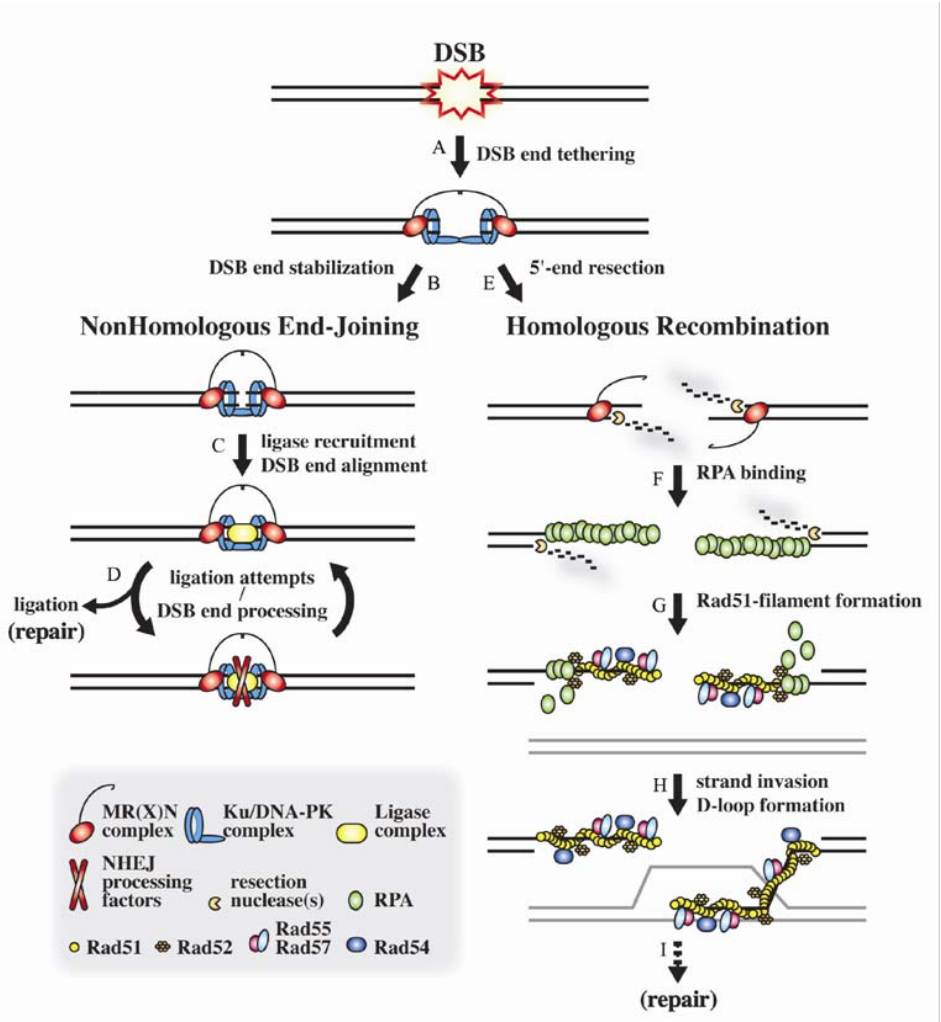

Figure 8. Model of DSB repair by NHEJ and HR pathways. DNA undergoing a DSB and the homologous template used for repair are respectively represented by a close pair of black and grey lines. (A)DSB ends are tethered by MRN(X) and Ku/DNA-PK complexes. (B) In NHEJ, DSB ends are further stabilized by MRN(X) and Ku/DNA-PK. (C) MRN(X) and Ku/DNA-PK recruit the ligase complex and DSB ends are aligned. (D)DSB ends are ligated or are processed prior to ligation (repair). (E) In HR, 5' DSB ends are resected by MRN(X) and other nucleases. (F) RPA binds to single-stranded overhangs generated by resection. (G) RPA-coated single-stranded DNA is a substrate for Rad51-filament formation, involving Rad52, Rad55-Rad57 and Rad54. (H) Rad51-filament homology search and strand invasion lead to the formation of a D-loop. (I) From the D-loop, different HR pathways can result in DSB repair. [Reprinted with permission; Pardo, Gomez-Gonzalez et al. 2009].

2.6.5 Single strand annealing and the MRN(X)-complex

This pathway is utilized when a double strand break is present between two repeat sequences oriented in the same direction. In SSA Rad52, Rad59, RPA and the MRN(X)-complex play an important role. These proteins are involved in binding ssDNA and holding broken ends close together. Making this pathway different than the others DSB repair pathways is the fact that Rad51, Rad54, Rad55, and Rad57 are not necessary. Further setting this repair pathway apart from the earlier described pathways is the necessity of mismatch repair proteins Msh2 and Msh3 and Rad1-Rad10. For the SSA pathway it is thought that the MRN(X)-complex resects the DNA, resulting in ssDNA overhangs. These overhangs can be microhomologies, or several hundred bases long. These homologies are thought to be resolved by SSA. When long(er) direct repeats are present, RPA and RAD52 facilitate pairing between the homologous strands. Next, the ERCC1/XPF (Rad1-Rad10) nuclease removes the overhanging tails, which results in deletions, and a DNA polymerase fills the gap. The basic steps of this repair pathway are best illustrated in Fig. 9.

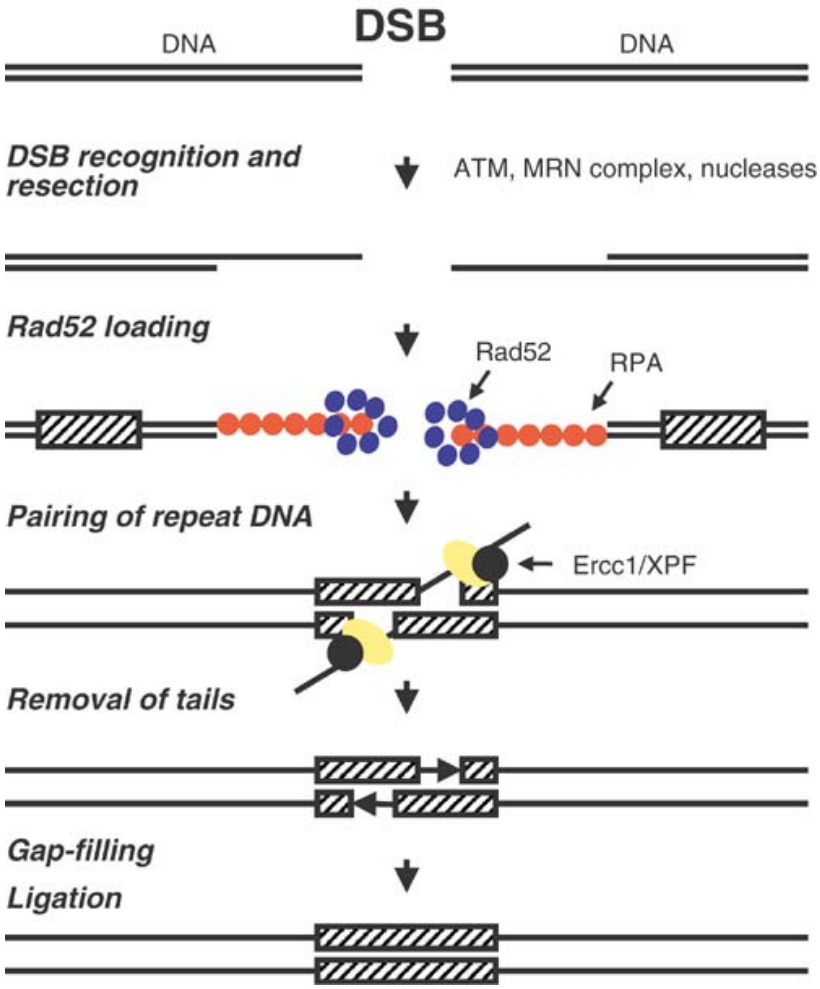

Figure 9. Single strand annealing. SSA could occur when, at a DSB, the DNA strand is resected by a nuclease, for example, MRE11 to leave ssDNA overhangs. The length of the overhangs and the extent of homology ranging from microhomologies to several hundred bases or longer most likely determine how SSA is executed. In the case of long homologies between direct repeats on the overhangs, RPA and RAD52 are necessary for facilitating DNA pairing followed by removal of the tails by ERCC1/XPF nuclease and gap filling by DNA polymerase as shown in the figure. Sensors, such as ATM, may signal and attract nucleases to the DSB. [Reprinted with permission; Valerie and Povrik 2003].

2.6.6 Microhomology-mediated end joining and the MRN(X)-complex

Another DSB repair pathway is MMEJ. When the core components of NHEJ are manipulated or depleted, MMEJ events are observed (Ma, Kim et al. 2003). More interesting is the fact that this pathway utilizes components normally associated with NHEJ and HR, these components include MRN(X)-complex, Ku, Lig4, Rad1-Rad10 and Rad51 (Ma, Kim et al. 2003; Decottignies 2007; Katsura, Sasaki et al. 2007; Lee and Lee 2007). The activation of MMEJ is not understood, but it is thought to be due to the inhibition or removal of Ku70-Ku80 and Rad51 from broken DNA ends. The pathway proceeds with the resection of DNA by the MRN(X)-complex, Sae2 and Exo1, exposing microhomology sequences at the DNA ends. If the annealing is stable, the resulting flaps are trimmed by Rad1-Rad10 (ERCC1-XPF), and the gaps filled in and ligated, resulting in a microdeletion. In yeast, two ligases are necessary, Ligase I and Ligase IV, abrogating the function of these ligase results in reduced MMEJ (Ma, Kim et al. 2003; Lee and Lee 2007). There is also evidence that DNA ligase IIIα and poly(ADP-ribose) polymerase-1 (PARP-1) might be involved in the ligation step (Audebert, Salles et al. 2004; Wang, Wu et al. 2006; Liang, Deng et al. 2008). In addition, translesion polymerases (i.e. Pol4, Pol32, Rev3 and Rad30) have been implicated to extend the annealed sequences, followed by disassociation of the initial microhomologies and realignment of other microhomologous sequences, flap removal, DNA synthesis and ligation. This results in a deletion plus insertion (Lee and Lee 2007). A schematic of this pathway is illustrated in Fig. 10.

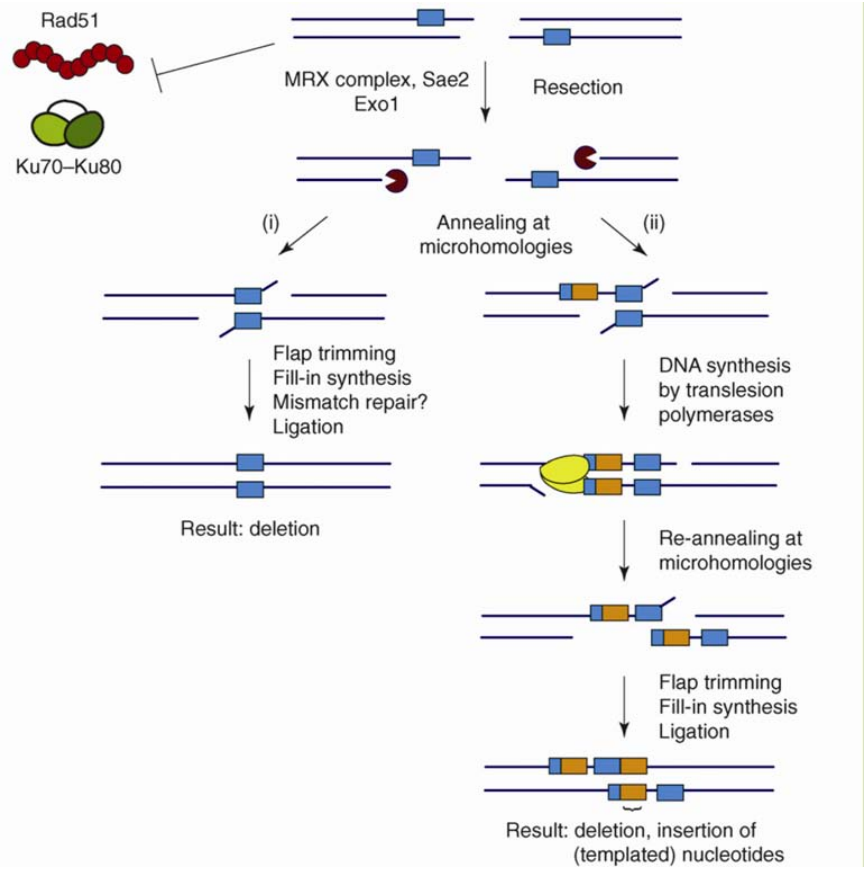

Figure 10. Model for MMEJ and alternative end-joining repair. During the initial stages of MMEJ, Ku70–Ku80 (green) and Rad51 (red), which inhibit MMEJ, are prevented from binding or are removed. This enables 50–30 resection by the MRN(X)-complex, Sae2 and Exo1 (indicated by dark red partial circle) that reveals microhomologous sequences (blue boxes). These microhomologies transiently and dynamically anneal to each other. (i) In cases in which the annealing is stable, repair is completed by flap trimming, fill-in DNA synthesis and ligation, resulting in a deletion relative to the original sequence. Mismatch repair is not required for MMEJ, although it might have a supporting role. (ii) Alternatively, one or more translesion polymerases (yellow) can extend the annealed sequences (represented here by orange–blue boxes) using templated error-prone synthesis. Dissociation of the initial microhomologies and realignment at other microhomologous sequences, followed by flap trimming, fill-in DNA synthesis and ligation completes repair, resulting in a deletion plus insertion event. Many variations and iterations of (ii) can hypothetically occur, resulting in complex insertion–deletion junctions.[Reprinted with permission; McVey and Lee 2008).

2.7 Repair of DNA-protein crosslinks

2.7.1 Cellular sensitivity to DPCs

Despite the accumulated evidence that DPCs are a threat to genomic integrity, the biochemical pathway(s) that repair these lesions are poorly understood. Research from the 1970s utilizing *S. cerevisiae,* suggested that formaldehyde induced mitotic recombination events and NER dependent single strand breaks (Chanet, Izard et al. 1975; Magana-Schwencke, Ekert et al. 1978).

Later it was suggested that NER might also be important for the repair of DPCs in human cells (Fornace 1982; Fornace and Seres 1982). The importance of this pathway was suggested by the fact that transplatin-induced DPCs were found to be more persistent in NER-deficient XPA fibroblasts when compared to normal cells (Fornace and Seres 1982). Contradicting results were found by another group showing that rapidly proliferating, SV40-transformed XPA cells were able to repair transplatin-induced DPCs, whereas the slow growing untransformed parent line was not (Gantt 1987). In addition, two other studies showed that the removal of formaldehyde-induced crosslinks is not significantly affected in XPA and XPF cells (Quievryn and Zhitkovich 2000; Speit, Schutz et al. 2000). These conflicting findings resulted one group to conclude that an undefined “active repair process” might control the removal of formaldehyde-induced DPCs (Quievryn and Zhitkovich 2000). Additional mechanisms have been found or suggested to play a role in the removal of DPCs. These include proteosomal degradation (Quievryn and Zhitkovich 2000; Baker, Wuenschell et al.

2007), the Fanconi/BRCA pathway (Ridpath, Nakamura et al. 2007), and HR (Nakano, Morishita et al. 2007; Ridpath, Nakamura et al. 2007).

2.7.2 Removal of DPCs in prokaryotes

In *E. coli,* the repair of bulky lesions is mainly initiated by the UvrABC complex, followed by UvrD helicase, DNA polymerase I and DNA ligase (Truglio, Croteau et al. 2006). In order to address whether or not this complex could remove intact covalently linked proteins, investigators created a site-specific ~ 16kDa protein adduct on DNA linked *via* a reduced abasic site (Minko, Zou et al. 2002; Minko, Kurtz et al. 2005). In these studies, it was reported that UvrABC was able to make a dual-incision on the DPC-containing strand by a mechanism identical to that shown for much smaller lesions. This suggests that the proteolytic degradation of the protein portion of the DPC was not absolutely essential for NER to occur in *E. coli*. Nevertheless, further analyses of the kinetics of UvrABC incision and the influence of the size of DNA-protein crosslinks revealed a much higher kinetics of incision when a small oligopeptide was linked to DNA (Minko, Kurtz et al. 2005). In a different study, the efficiency of UvrABC incision as a function of oligopeptide-adduct size was investigated and concluded that DPCs containing cross-linked proteins less than 12-14 kDa in size were removed by NER in bacteria (Nakano, Morishita et al. 2007).

2.7.3 Removal of DPCs in mammalian cells

Based on the evidence in *E. coli*, the role of NER in mammalian cells has been investigated using substrates similar to Minko et al (2005). In this study, the ability of the mammalian NER system to excise DPC lesions was investigated (Reardon and Sancar 2006). In concurrence with the prokaryotic findings, they demonstrated efficient removal of DNA-peptide substrates. However, the mammalian NER system could not excise the intact ~ 16 kDa linked protein (Reardon and Sancar 2006).

Several groups have also studied the role of the proteasome in the tolerance or removal of DPCs. In two independent studies, it was found that formaldehyde-induced DPC repair was negatively affected by inhibiting the proteasome with lactacystin (Quievryn and Zhitkovich 2000; Baker, Wuenschell et al. 2007). In short, they showed that there was no excision of a DNA methyltransferase covalently bound to an oligodeoxynucleotide unless the protein had been proteolyzed to an oligopeptide ~9-10 amino acids long. To further understand the role of the proteasome, the activity of the 26S proteasome was inhibited with a chemical inhibitor (MG132), which resulted in a reduction of DPC removal by more than 50% compared to proteasomal competent cells.

Another study presented evidence for the involvement of the Fanconi/BRCA pathway in the tolerance or repair of DPCs. In this study, FANCD2-deficient DT40 cell lines and two human cell lines deficient for FANCC and FANCG, all displayed hypersensitivity to formaldehyde (Ridpath, Nakamura et al. 2007). Interestingly, a moderate sensitivity to formaldehyde in cells deficient in TLS and NER was also observed.

2.7.4 DPC repair – a model

We hypothesized that formaldehyde-induced DPCs can be repaired through two independent pathways. In the first model, the DPC is degraded to a smaller peptide-DNA crosslink by a yet to be determined proteasome. Our group (Minko, Zou et al. 2002) and the findings of two other groups (Quievryn and Zhitkovich 2000; Reardon and Sancar 2006) suggest that the NER pathway might recognize this peptide crosslink as a substrate and incise around the lesion. Additional evidence suggests that certain translesion polymerases can bypass a peptide-DNA crosslink *in vitro* (Minko, Kozekov et al. 2008). A basic understanding of DPC repair at the start of this research project is illustrated in Fig. 11.

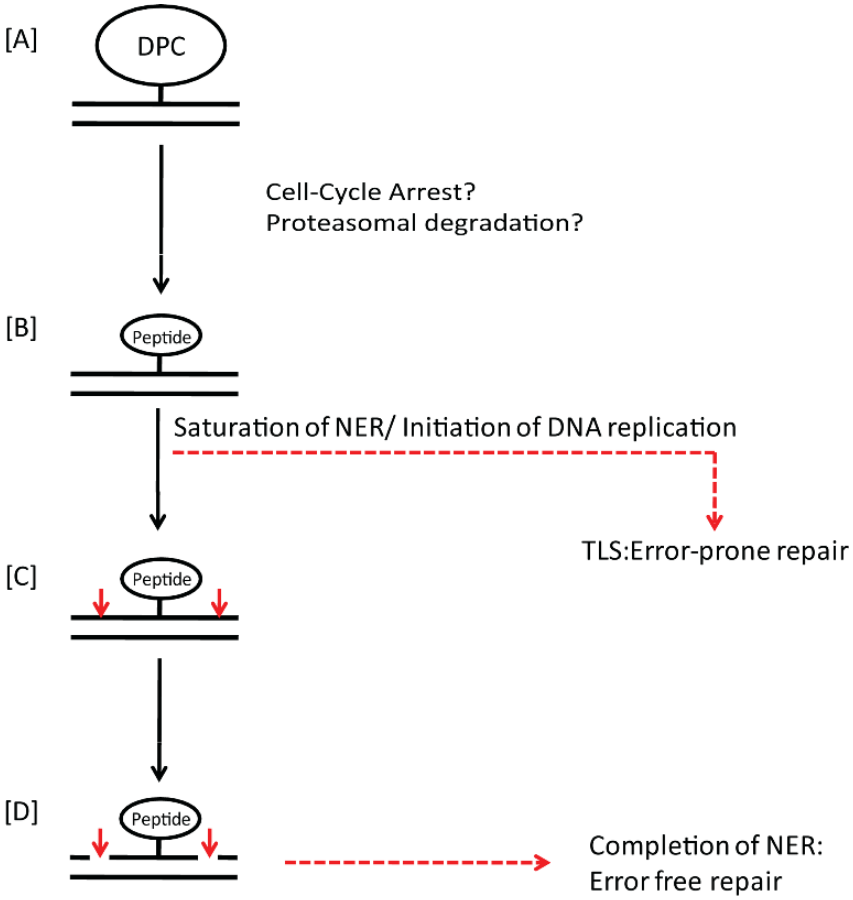

Figure 11. Model for repair DPCs. [A] DPC formation [B] DPC degradation [C] NER initiation [D] Excision of small peptide by NER

In order to more clearly define the cellular pathways or proteins involved in repair or tolerance of DPC adducts and to test the model, a genome-wide approach was utilized. Herein, results are reported on the screening of the *S. cerevisiae* non-essential gene deletion library (~5000 genes) for deletions that enhance cytotoxicity following exposure to the DPC-inducing agent, formaldehyde. Based on the screen, the involvement of various pathways were investigated and a new model for DPC repair is presented.

2.8 Objectives & Approach

2.8.1 Goal of this study

As described in the previous sections, the mechanisms for the repair or tolerance of DPCs are not well understood. The purpose of this thesis project was to elucidate the mechanisms behind DNA-protein crosslink repair.

As the predominant form of DNA damage resulting from formaldehyde exposure is thought to be DPCs, this work used formaldehyde as a model agent for the induction of DPCs in a eukaryotic system. Based on prior literature and our own preliminary data, we hypothesized that NER is responsible for a subset of DPC repair, but that there may be a second unidentified pathway involved in the processing of formaldehyde induced DNA lesions.

2.8.2 Question asked to test the hypothesis

1) What mechanisms are responsible for the tolerance and/or repair of DPCs resulting from formaldehyde exposure?

2) Does the way in which cells are exposed to formaldehyde (chronic versus acute) change the spectrum of genes necessary for tolerating formaldehyde?

3) Are SSBs or DSBs formed as intermediates in the repair of DPCs?

Point 1 – 3 have been published in B. de Graaf, et al., Cellular pathways for DNA repair and damage tolerance of formaldehyde-induced DNA-protein crosslinks, DNA Repair (2009).

4) Is the spectrum of genes/pathways required for formaldehyde tolerance/repair similar to those required for resistance to other agents known to induce DPCs, such as the topoisomerase inhibitors, camptothecin and etoposide?

5) Mre11 has been shown to have exonucleolytic and endonucleoytic activities that are postulated to be involved in the processing of DSBs and removal of Spo11 during meiosis. Therefore the question was asked: is the nuclease activity required for repair/tolerance of formaldehyde-induced DPCs?

6) Evidence suggests that classical NER or HR proteins are involved in the tolerance or repair of DPCs. In addition, TLS has been proposed to be involved in the bypass of DPCs. Using genetic approaches, can the relative importance and interactions of these pathways for the cellular response to formaldehyde be determined?

2.8.3 Approaches to test the hypothesis

1) A toxicology screen of the non-essential Mat-a gene library of *S. cerevisae* using a chronic low dose of formaldehyde. Sensitivity is based on the fact that missing an important gene results in slow or no growth after exposure.
2) The yeast mutant strains identified in the screen were tested for sensitivity after acute exposure of formaldehyde by spot assay and colony forming ability.
3) On different yeast strains such as wild-type, *mre11*Δ, *rad4*Δ*, rad52*Δ *and rad4*Δ*rad52*Δ*,* pulsed field gel electrophoresis (PFGE) analyses were performed to examine the occurrence of DNA breaks.

4) The strains identified in the screen were tested for sensitivity to etoposide and camptothecin by survival assays.
5) The Mre11 nuclease deficient strains were assessed for survival after chronic and acute formaldehyde exposure. The formation of SSBs and DSBs were also assayed by PFGE.
6) Yeast strains sensitive to ICL-inducing agents were tested for formaldehyde sensitivity. Based on the data provided by the genome-wide screen, 19 additional double mutants were created and assessed for formaldehyde sensitivity.

2.8.4 Overview of the results

The genome-wide screen of the *S. cerevisiae* gene deletion library for formaldehyde sensitivity identified 44 *S. cerevisiae* non-essential gene deletion strains that show higher sensitivity to formaldehyde compared to the isogenic wild-type strain. We observed high sensitivity of HR deletion strains, and moderate sensitivity of NER deletion strains to chronic (low dose) formaldehyde exposure. This was surprising at the time as earlier reports (Magana-Schwencke, Ekert et al. 1978) suggested a very important role of NER in the resistance to formaldehyde-induced DNA damage.

One of the interesting findings was that the method of exposure greatly influenced the outcome of the results. A comparison of the methods used in the formaldehyde literature revealed that the method of cell treatment varied considerably, i.e. either a chronic continuous exposure or an acute high dose (short) exposure. Since the deletion library screen described in this study was performed under chronic conditions, it was of interest to test the 44 mutants identified as sensitive to

formaldehyde under acute exposure conditions. Interestingly, the relative sensitivity of the yeast deletion strains switched depending on the exposure conditions. The HR deletion strains were very sensitive under chronic conditions but not after acute exposure, while NER deletion strains became more sensitive after an acute exposure, but showed only moderate sensitivity under chronic conditions.

As HR deletion strains are very sensitive under chronic exposure conditions, it was of interest to determine if DSBs are detectable following formaldehyde exposure. PFGE experiments only detected single-strand breaks, and no DSBs, both after acute and chronic formaldehyde exposure. This result is consistent with a previous study demonstrating the formation of SSBs (Magana-Schwencke, Ekert et al. 1978). Analyses using velocity sedimentation ultracentrifugation experiments confirmed the presence of SSBs. Interestingly, SSB formation appeared to be dependent on Mre11, Rad1, and Rad4 as no increase was observed after formaldehyde exposure. All of the above described results have been published in *B. de Graaf, et al., Cellular pathways for DNA repair and damage tolerance of formaldehyde-induced DNA-protein crosslinks, DNA Repair (2009).*

In addition to testing the sensitivity of the HR and NER mutants to formaldehyde, experiments were performed using other DPC inducing agents. This was done to test whether or not the sensitivity seen after formaldehyde is a general response to DPC inducing agents, or if the response is agent specific. The NER and HR strains identified in the screen were exposed to camptothecin and etoposide. In short, etoposide covalently links a TOPO2 subunit to the 5'-phosphoryl ends of the broken DNA, whereas camptothecin results in the linking of topoisomerase I to the 3' ends of broken DNA. Both

situations resemble a protein crosslinked to the DNA. Based on the survival, it was observed that the HR strains displayed similar growth rates, but that the NER deletion strains had very different survival rates after exposure. All NER deletion strains showed no increased sensitivity to camptothecin, but sensitivity to etoposide. Taken together these results suggest that the cellular response is formaldehyde-specific.

The Mre11 protein appears to be important for the formaldehyde response based on the significant sensitivity of the Mre11 deletion strain observed after chronic formaldehyde exposure. As described in the introduction, Mre11 is thought to play a role in the removal of Spo11 crosslinks at DNA ends during meiosis. Therefore, several nuclease deficient mutants (*mre11-D56A, mre11-D56N*, and *mre11-H125N*) were tested under chronic or acute conditions. Using these mutants, it was found that the nuclease activity is not essential for the tolerance or survival after formaldehyde exposure, as they displayed similar growth rates as wild-type cells. These mutants did not show a change in the formation of SSBs and DSBs compared to the *mre11*Δ strain as measured by PFGE.

In addition, the strains identified as being sensitive to ICL inducing agents were investigated. These strains were further studied due to the finding that the HR and NER pathways, or components of these pathways, are involved in the repair and/or tolerance of DPCs. Significant overlap was observed between the strains implicated in the repair of ICLs and those identified in the formaldehyde screen.

We obtained several ICL deficient strains published in Grossmann et al. 2001, and Barber et al. 2005. No evidence was found that the classically defined ICL repair pathway is involved in the DPC tolerance or repair pathway. In these experiments the

(double-) mutants displayed no significant enhanced sensitivity above the wild-type strain. In contrast, *rev3*Δ (a member of the TLS repair pathway) did display a compensatory role to *rad51*Δ when the double mutant was tested for formaldehyde sensitivity.

To further address these findings, double mutants were created to facilitate an understanding of the role of the various pathways in the repair of formaldehyde lesions. The following deletion strains were studied in more detail: *rad1*Δ*rad52*Δ*, rad4*Δ*rad52*Δ*, rad1*Δ*mre11*Δ*, and rad4*Δ*mre11*Δ. These double mutants were chosen to study, as described in the introduction, because *rad1*Δ is involved in both NER and sub-pathways of HR. Rad4 is seen as a classical NER protein, Rad52 as a classical HR protein. The Mre11 protein (the MRN-complex) plays a role in all known HR pathways. These mutants were examined under chronic and acute dose exposure. The results are summarized as follows: data presented here suggest that *rad1* and *mre11* appear epistatic, *rad1* and *rad52* additive and *rad4* and *mre11* suppressed the sensitivity phenotype after chronic and acute exposure. Interestingly, it appears that there is a difference in the *rad4*Δ*rad52*Δ mutant survival, depending on the exposure conditions.

The data generated has facilitated our understanding of the repair and tolerance pathways for DPCs and has given rise to many new and intriguing questions and models for this field of research. The experiments and results will be further described in more detail in the following chapters.

Preface

Part of this work has been recently published in collaboration with Dr. A. Clore, under supervision of Dr. McCullough in the journal DNA Repair:

B. de Graaf, et al., Cellular pathways for DNA repair and damage tolerance of formaldehyde-induced DNA-protein crosslinks, DNA Repair (2009).

The published work is described in sections 3.1-3.7, 4.1-4.6 and 5.1-5.4 and marked with an asterisk (*). Sections 3.6, 3.7, 4.4 and 4.5 (Fig. 16, 17 and 20) were performed by Dr. A Clore as part of the publication and are also included here for continuity and completeness.

3. Material & Methods

3.1 Yeast strains and chemicals*

Chemicals were purchased from Sigma unless otherwise noted. The MAT-a (BY4741) *S. cerevisiae* deletion strain library was obtained from the European *S. cerevisiae* archives for functional analysis (EUROSCARF). Additional *pso2*Δ *S. cerevisiae* strains, LBY9 (W303-1A-background) and KGY212 (A364A-background) were obtained from Peter McHugh (University of Oxford) (Barber, Ward et al. 2005) and Robb Moses (Oregon Health & Science University) (Grossmann, Ward et al. 2001), respectively. Preliminary experiments were conducted with BY4741 strains obtained from Leona Sampson (Massachusetts Institute of Technology).

3.2 Parameters determining chronic and acute formaldehyde screening conditions*

The formaldehyde concentration used in the chronic exposure was determined by growing wild-type, *rad4*Δ and *rad52*Δ strains in YPD (yeast extract, peptone, dextrose) media containing 0-10 mM formaldehyde for 48 hr. Additionally, exponentially growing cultures were serially diluted and aliquots spotted on agar-YPD plates containing formaldehyde concentrations ranging from 0-10 mM. Colonies grew for 48 hr and optimal concentrations for differential cytotoxicity were determined.

To determine the conditions for acute formaldehyde exposure, the same yeast strains as described above were harvested in exponential growth phase and resuspended in media containing 20-80 mM formaldehyde for 15 min. Cells were

collected by centrifugation, resuspended in fresh media and aliquots of serially diluted cultures spotted on agar-YPD plates. Differential survival was assessed after 48 hr at 30°C.

3.3 Formaldehyde sensitivity screen of the non-essential gene deletion library*

The master MAT-a (BY4741) *S. cerevisiae* deletion strain library was stored at -80°C in YPD + 15% glycerol. Duplicates and liquid cultures were made by growing the yeast in YPD media for 48 hr at 30°C as follows: cells were transferred (~ 1µl) with a 96 floating-pin Replicator to rich YPD agar plates containing G418 (200 µg/ml). The liquid cultures were transferred using a floating-pin replicator in triplicate onto one plate (Omnitray, NUNC plates) with no formaldehyde and another plate containing 1.5 mM formaldehyde. Strains were grown on YPD plates at 30°C and imaged at 24 and 48 hr after plating using an AlphaEase FC Imaging System (Alpha Innotech). It was predetermined that formaldehyde was stable under these conditions and plates could be used for at least 1 week after initial preparation as evidenced by comparable levels of formaldehyde-induced cytotoxicity. The entire library was independently screened twice with each deletion strain assayed in triplicate per screen. Strains were classified as sensitive when limited or no growth was observed in the two replicates of the 1.5 mM formaldehyde plating. Strains that exhibited sensitivity to formaldehyde relative to the wild-type strain were individually re-assayed from freezer stocks to verify formaldehyde sensitivity.

3.4 Cell survival assays*

For rapid semi-quantitative survival assessments, cells were cultured in YPD overnight and diluted to $1x10^7$ cells/ml. Aliquots (2 µl) of serial 10-fold dilutions of cells were spotted onto YPD agar plates containing the indicated concentrations of formaldehyde ranging from 0-2.0 mM. Cells were cultured at 30°C for 2 days (chronic exposure). For acute formaldehyde exposure, cells from an overnight culture were harvested by centrifugation, resuspended in 1 ml water and exposed to 60 mM formaldehyde for 15 min. After the exposure, cells were collected by centrifugation, washed twice in water, and resuspended in YPD at a concentration of $1x10^7$ cells/ml. Each strain was serially diluted (1:10) in YPD before plating 2 µl of each suspension onto YPD agar without formaldehyde. Cells were grown for 2 days at 30°C and images captured using an AlphaEase FC imaging system.

In order to assess the relative survival, strains representing major DNA repair pathways were chosen and colony forming assays performed. For both chronic and acute exposure, the yeast strains were grown overnight at 30°C, with vigorous shaking, resulting in a log-phase culture. Cells were diluted such that following formaldehyde exposure, the total number of surviving colonies ranged from 30-300 cells per plate under unexposed conditions. For chronic exposures, formaldehyde was added to the plates at various concentrations (0-2.0 mM), and colonies were counted after 2-3 days of growth at 30°C. For acute exposures, cells were pelleted by centrifugation, resuspended, and exposed as indicated to formaldehyde (0-80 mM) for 15 min, pelleted at 4,000 rpm for 2 min and washed twice. Cells were resuspended in media and plated

onto YPD agar without formaldehyde. All experiments were repeated at least three independent times.

3.5 Pulsed field gel electrophoresis analyses (PFGE)*

PFGE analyses were performed to determine if strand breaks accumulate following formaldehyde exposure. Assays were performed using the CHEF genomic DNA plug kit (Bio-Rad). For chronic exposure conditions the yeast strains were grown for 48 hr at 30°C in the presence of 1.5 mM formaldehyde. For acute exposure, yeast strains were incubated with 60 mM formaldehyde for 15 min, washed in water and resuspended in YPD and grown for the times indicated in the figures (0 through 24 hr) at 30°C. As a control for SSBs and DSBs, wild-type cells were also exposed to hydrogen peroxide and bleomycin, respectively. After exposure, cells were washed, pelleted and resuspended in YPD media. Additionally, PFGE was performed on cells arrested in G1 phase using α-mating factor. The arrest (>90%) was confirmed visually using a light microscope (Nikon, Eclipse E 200, 100x magnification). Following arrest, cells were exposed to 60 mM formaldehyde for 15 min, pelleted, washed twice, and resuspended in YPD containing α-mating factor (20 µg/ml) in order to maintain G1 arrest. From the inoculated media, $2x10^8$ cells were used for each plug. Plugs were made of unexposed cells or from aliquots harvested at 0, 4, 6, and 8 hr after exposure. The cells were processed for PFGE analyses according to the manufacturer's protocol. The yeast chromosomes were separated on a 2% pulsed field certified agarose gel in 0.5x TBE (90 mM Tris base, 89 mM boric acid, 10 mM EDTA pH 8.0), recirculating at 14°C, for 20 hr at 6.5 V/cm with a 60 to 120 sec switch time ramp at an included angle of 120°. Images were captured on an AlphaEase FC imaging system.

3.6 SDS/KCl precipitation to detect DPCs*

After chronic or acute treatment, $1x10^7$ cells were pelleted by centrifugation, resuspended and washed with 1 ml of TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.8). Cells were resuspended in 0.25 ml of lyticase buffer (BioRad), 8 µl of proprietary lyticase (BioRad; 170-3593), and 1 µl of proprietary yeast protease inhibitor cocktail (Sigma; p8215-1ml) for 30 min at 30°C. After confirming spheroplast formation by microscopy, 0.25 ml of 4% SDS was added, cells were frozen and then thawed at 55°C for 5 min. To the suspension, 0.5 ml of 200 mM KCl, 20 mM Tris-HCl (pH 7.5) was added and the DNA sheared by repeated pipetting. The solution was cooled on ice for 5 min. The precipitate was pelleted at 3500 x g for 5 min and the supernatant saved to measure unbound DNA. The pellet was washed in 20 mM Tris-HCl (pH 7.5), 200 mM KCl, incubated at 55°C for 5 min, cooled on ice for 5 min, followed by centrifugation at 3600 x g for 4 min. This wash procedure was repeated 3 times before a final resuspension in 0.5 ml of the same buffer. Proteins were digested by adding 0.2 mg/ml of proteinase K and incubating at 55°C for 45 min. The solution was cooled on ice and 50 µl of 10 mg/ml bovine serum albumin (BSA) (New England BioLabs) was added and placed on ice for 5 min. The precipitate was pelleted at 3500 x g for 5 min. Individually, the final supernatant (10 µl) and the supernatant from the first wash (5 µl) were separated on a 0.7% agarose gel stained with either ethidium bromide (0.001%) or a 1x solution of Sybr Gold™ (Invitrogen). Images were captured using an AlphaEase FC imaging system and the amount of DNA in each lane was quantified with ImageQuant software (GE Healthcare Lifesciences). To control for potential differential efficiencies in the extraction of DNA from formaldehyde-treated cells, the amount of cross-linked DNA was calculated

as a ratio of SDS/KCl precipitated DNA to unbound DNA. Due to the limited quantities of cross-linked DNA, the amounts of recoverable unbound DNAs were always at least in 100-fold excess over cross-linked DNAs.

3.7 Velocity sedimentation ultracentrifugation*

Overnight cultures of yeast cells were diluted 1:2 in fresh media and grown for 4 hr. Cells were arrested with α-mating factor (20 µg/ml) 2 hr prior to formaldehyde treatment. Cells were treated with 60 mM formaldehyde for 15 min, washed twice and resuspended in YPD and grown for an additional 4 hr in YPD containing α-mating factor. β-mercaptoethanol (0.2%) and 45 mg of lyticase were added and the cells incubated at 30°C for 45 min. Cells were centrifuged at 1500 x g for 10 minutes and resuspended in 2 ml TE. Sucrose gradients, 15 to 30%, were made by mixing the appropriate amount of sucrose with a buffer consisting of 15 mM EDTA, 10 mM Tris-HCl pH 7.4, 0.9 M NaCl, and 0.1 N NaOH. Linear gradients were poured with a gradient mixer into Sorvall PA ultracentrifuge tubes. Lyticase digested cells were poured on top of the gradient and 50 µl of 10% SDS was added. The gradients were placed at 4°C for 10 min to lyse cells, and centrifuged at 4°C, 11,500 rpm for 20 hr in a Sorvall AH627 rotor. After centrifugation, fractions (800 µl) were removed from the bottom of the tubes with an automated fractionator and 24 µl of 2M HCl was added to neutralize the solution. A total of 50 µl of each fraction was added to 100 µl of TE containing 1.5X Sybr Gold™ in a 96-well plate. The plate was scanned with a Spectramax Gemini XS plate reader using an excitation wavelength of 490 nm and an emission wavelength of 540 nm.

3.8 Generation of yeast double mutants

S. cerevisiae double mutants were created using a polymerase chain reaction (PCR) protocol adapted from a previously published procedure (Gietz and Woods 2002). In order to have a selectable marker, 'hybrid' primers were designed, which have homology to the URA3 gene and the gene of interest (~34bp, see Table 1 for details). Using these primers the URA gene was amplified from the pYes 2.1 plasmid (Invitrogen). The construct now also contains overhangs of the desired gene to be deleted. The following primers were ordered from Integrated DNA technologies:

Gene of Interest	Primer Sequence 5'-3'
Rad1-Del5'-URA3	AGA GCA TTT GCT AAA TGT GTA AAA ATA ATA TTG CAC TAT C**CC TGA GCG GAA GTG TAT CGT**
Rad1-Del3'-URA3	TCA CCA AAT GAA TAT TGT TAT TTT CAC TAT AGT TAA TCG C**AA TAA GGG CGA CAC GGA AAT**
Rad4-Del5'-URA3	GGA CGA CAA GCA GAG ACA TAA CGA CAC TAT TTT TCC GCT AAA ATG **CCT GAG CGG AAG TGT ATC GT**
Rad4-Del3'-URA3	AAA ACA TAC TTT CCT AAT TAT TCA AAC CGT TTC AGC CTC ATT TCA **AAT AAG GGC GAC ACG GAA AT**
Rad5-Del5'-URA3	AAA GGC CTT AGA AAC ACA CCT AAA GTC TTA CAG TAT CAC AAT ATG **CCT GAG CGG AAG TGT ATC GT**
Rad5-Del3'-URA3	ATA ATA ATA AAT AAA GTC TTT ATA TAT GAG TAT GTG GTA TGA CTA **AAT AAG GGC GAC ACG GAA AT**
Rad51-Del5'-URA3	ACG TAG TTA TTT GTT AAA GGC CTA CTA ATT TGT TAT CGT CAT ATG **CCT GAG CGG AAG TGT ATC GT**
Rad51-Del3'-URA3	AAG TAA ACC TGT GTA AAT AAA TAG AGA CAA GAG ACC AAA TAC CTA **AAT AAG GGC GAC ACG GAA AT**
Rad52-Del5'-URA3	AAA AGA CGA AAA ATA TAG CGG CGG GCG GGT TAC GCG ACC G**CC TGA GCG GAA GTG TAT CGT**
Rad52-Del3'-URA3	TGA TGC AAA TTT TTT ATT TGT TTC GGC CAG GAA GCG TTT C**AA TAA GGG CGA CAC GGA AAT**
Mre11-Del5'-URA3	CTC CAC TAT GGA CTA TCC TGA TCC AGA CAC AAT AAG GAT T**CC TGA GCG GAA GTG TAT CGT**
Mre11-Del3'-URA3	TCG ACC ATT AAG TAA ACC ATA ACT AGC GTC CTC TTC GTC A**AA TAA GGG CGA CAC GGA AAT**

Table 1. PCR construct primers for the deletion of genes. Bold lettering represents homologous sequence to the URA3 gene, normal font represents homologous sequence to the gene of interest.

Using the primers listed in Table 1, a 10 x master mix was made resulting in the following PCR reaction for each reaction:

PCR Master Mix	1x (20 µl reaction)
Phusion Polymerase (1 U/µl)	0.2
dNTPs (10 mM/Base)	0.4
5x HF Buffer	4
GeneX-Del5'-URA3 Primer (5 µM)	2
GeneX-Del3'-URA3 Primer (5 µM)	2
H_2O	10.4
Template	1µl of pYES plasmid (1:1000)

Table 2. Construct PCR reaction mix

To amplify the product the following PCR program was used:

Temperature	Time (sec)	Cycles (x)
98°C	60	1
98°C	15	30-40
55°C	15	
72°C	120	
72°C	300	1

Table 3. Construct PCR program

After the PCR was completed, 5 µl of each PCR product was analyzed on a 0.5x TBE 1-2% agarose gel, ~100V for 0.5 - 1 hr.

The background strain in which another deletion was desired was inoculated in 5 ml of 2x liquid YPD and grown overnight at 30°C and 250 rpm. Cells were counted using a hemacytometer and, 2.5 x 10^8 cells were transferred to 50 ml of 2x YPD giving a starting concentration of 5 x 10^6 cells/ml. These cells were incubated at 30°C shaken at 250 rpm. After 4 – 6 hr at a cell titer of at least 2 x 10^7 cells/ml (preferably higher, up to 8 x 10^7 cells/ml), the cells were pelleted at 3000 x g for 5 min. and washed in 25 ml of sterile water. This was followed by two washes in 1 ml sterile water. For each transformation 100 µl (ca. 10^8 cells) were transferred into a 1.5 ml micro centrifuge tube,

centrifuged at 3000 x g for 30 sec and the supernatant discarded. Carrier DNA was boiled for 5 min. and chilled on ice.

For each transformation the following reaction was mixed and put on ice:

Reagents	1x
PEG 3350 50% w/v	240 µl
LiAc 1.0 M	36 µl
Boiled SS-carrier DNA	50 µl
PCR Product	34 µl
Total	360 µl

Table 4. Transformation mix

Each transformation (total volume of 360 µl), was incubated at 42°C for 40 min. The cells were pelleted at 3000 x g for 30 sec and the supernatant discarded. The pellet was resuspended in 100-200 µl of sterile water. Depending on the expected transformation efficiency, 20-200 µl was plated on YPD plates lacking uracil (URA plates). Synthetic drop out medium was made by dissolving 4.0 g yeast nitrogen base, 12 g glucose, 0.50 g synthetic drop out mix (without Uracil) (Sigma Y1501-20G) in 600 ml water. The pH was adjusted to 5.6 with 10 M NaOH and thereafter 10 g of agar was added to the solution, and autoclaved. After plating the transformants the plates were incubated for 2-3 days at 30°C. Growth on the URA-plates selects for transformants where recombination has occurred at the site of the gene of interest. Colonies were selected and the gene deletions were confirmed by PCR. The primers necessary to confirm the deletion were designed based on the EUROSCARF primer sequence A and D from the Website (Table 5):

http://www-sequence.stanford.edu/group/yeast_deletion_project/strain_a_mating_type.txt

Primers were ordered from Integrated DNA technologies:

Gene of Interest	Primer Sequence 5'-3'
URA3 Internal 5'	CAG TAT AGA ACC GTG GAT GTG G
URA3 Internal 3'	GCA GTT GGG TTA AGA ATA CTG GGC
Rad1A	CTT TAT TTT GCG ACT TTT CTT CAT C
Rad1D	TAA TGA ATA TGA TTG TGC GCT TCT A
Rad4A	CCG ATC ATT ATC TGA CTG CTC TAT T
Rad4D	CCC TCT CAT CAA CTT TTA TTT TCA A
Rad5A	AAA TCA AAA TGA AGT AAA ACC CCT C
Rad5D	TGG CTG GAA AAC TTT CAT CTA CTA C
Rad51A	CCA ATC TAG TTT AGC TAT CCT GCA A
Rad51D	AAT TTT TCT CTT CAC TCC CCT AAA A
Rad52A	GAT TCA ACA ACT CCC TTG GCG TC
Rad52D	TAC GAC ACA TGG AGG AAA GAA AAA C
Mre11A	GTT CAC AAG CAA GCC TGT AAA TAA T
Mre11D	ATT CCT TGC TAT ACG AAC AAA AGA G

Table 5. Primers for PCR confirmation of deletions

Colonies were picked and replated on YPD and part of the colony was resuspended in 47 µl water with 3 µl Lyticase for the PCR reaction. This solution was incubated at 42°C for 30 min, followed by a 10 min. incubation at 95°C and 5 min. at -80°C, to digest and burst the cell wall of the yeast. Next a 10 x master mix was made:

PCR Master Mix	1x (20 µl reaction)
Phusion Polymerase (1U/µl)	0.2
dNTPs (10mM/Base)	0.4
5x HF Buffer	4
Gene A or D (5 µM)	2
URA3' internal 5' or 3' Primer (5 µM)	2
H2O	9.4
Template (Digested Yeast)	2 µl of the colony solution

Table 6. Yeast colony PCR reaction mix

The following PCR Program was used:

Temperature	Time (sec)	Cycles (x)
98°C	60	1
98°C	15	30-40
55°C	15	
72°C	120	
72°C	300	1

Table 7. Yeast colony PCR program

After the PCR was completed, 10 µl of the reaction were analyzed by electrophoresis through a 0.5x TBE 1-2% agarose gel ~100V. If product was observed, the remaining PCR product was used for sequencing. In order to sequence the PCR product each sequence reaction contained 2 µl DNA (PCR product), 6 µl primer (1pMol) and 12 µl water. The sequence results were analyzed by BLAST on the National Center for Biotechnology Information (NCBI) Website. The sequence confirmed colonies were stored at -80°C in 1 ml fresh YPD + 15% Glycerol.

4. Results

4.1 Genome-wide screen of *S. cerevisiae* for formaldehyde sensitivity*

In order to identify cellular pathways involved in enhancing cellular resistance to DPC-inducing agents, the *S. cerevisiae* MAT-a non-essential gene deletion library consisting of ~5000 individual strains (Kelly, Lamb et al. 2001) was screened for formaldehyde sensitivity under conditions of continuous (chronic), low-dose exposure. Prior to screening the entire library, it was necessary to establish appropriate assay conditions in which a differential in cytotoxicity was observed between the wild-type and representative strains from the major DNA repair pathways, such as NER deficient *rad4*Δ and HR deficient *rad52*Δ. Aliquots of exponentially growing cultures were spotted on agar plates that contained concentrations of formaldehyde ranging from 0-10 mM. Wild-type cells showed a marked decrease in survival at the 2.0 mM concentration, with virtually no survival observed at concentrations > 3 mM (data not shown). In contrast, repair-deficient strains showed greater cytotoxicity as compared to the wild-type strain at 1.0-1.5 mM formaldehyde (data not shown).

In order to address whether the formaldehyde was stable under the conditions used, plates containing 1.5 mM formaldehyde were prepared and stored for various times and subsequently used in screening assays. These data revealed that highly reproducible results could be obtained using plates that had been stored up to 7 days, suggesting that the effective concentration of formaldehyde does not significantly change due to interactions with the YPD media. To ensure that these doses of formaldehyde were cytotoxic, agar plugs containing the yeast spots lacking colonies

were removed from the formaldehyde-containing plates and placed in liquid YPD and allowed to grow for 48 hr. No growth was observed (data not shown).

Having established the assay conditions that gave a robust signal-to-noise ratio, the complete library was screened two independent times with each strain assayed in triplicate per screen. A representative set of plates from the screen is shown in Fig. 12. Increased cytotoxicity under the chronic exposure conditions was observed in 44 deletion strains, with the majority being genes involved in cell cycle and DNA repair. Genes involved in metabolism, transcription, protein fate and cellular transport were also identified (Table 8). All strains listed as sensitive in Table 8 were independently confirmed by re-assaying individual strains using serial dilution spot assays under chronic formaldehyde exposure. Only those deletion strains showing significant sensitivity are listed in Table 8. Interestingly, the genome-wide screen suggested that for strains involved in DNA repair and tolerance mechanisms, the strains deleted in HR genes, not NER, appeared to be the most sensitive to formaldehyde.

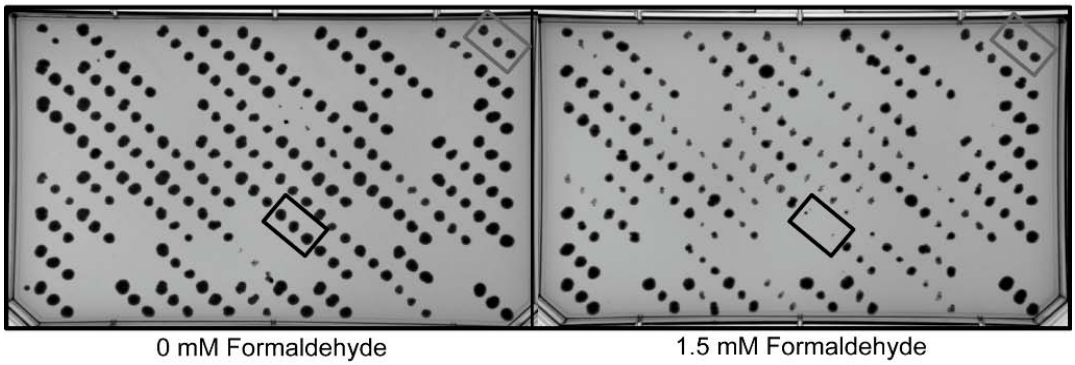

Figure 12. Identification of yeast mutant strains sensitive to formaldehyde. A representative plate (#13-4) from the complete library screen is shown. Replicates of the library master plate were grown in YPD media for 16 hr and ~ 1 µl of each well was spotted in triplicate and grown for 2 days at 30°C. The left panel shows an unexposed plate, the right panel is a duplicate plate grown in the presence of 1.5 mM formaldehyde. An example of a strain not sensitive to this concentration of formaldehyde is shown within the grey box in the upper right corner, while a strain near the middle of the plate (black box) was identified as sensitive. Areas of no growth are intentionally designed by the supplier to allow for unambiguous plate identification. From *B. de Graaf, et al., Cellular pathways for DNA repair and damage tolerance of formaldehyde-induced DNA-protein crosslinks, DNA Repair (2009).*

Functional Category	Genes/ORFs
Metabolism……....	SFA1 ERG3 ERG6 ERG5 PSD1 ADH1
Cell Cycle and DNA Processing……	SPT7 CDC50 RAD55 XRS2 RAD51 RAD4 CDC26 RAD54 MSH1 MGM101 RAD5 TOP3 MMS22 RAD52 SGS1 RAD14 MRE11 RAD50 RAD1 CTF4
Transcription……..	RPN4 SNF6 DAL81 LSM1 SWI3 SNF2 MED1
Miscellaneous……	NBP2 VID22 ARP5 NUP84 VPS9 ECM30 OPI11 NRP1 YLR235C TMA23 BEM4

Table 8. Categorization of yeast strains that display increased sensitivity following chronic formaldehyde exposure. Formaldehyde sensitive strains were categorized in functional groups according to the MIPS functional database (http://mips.gsf.de/proj/funcatDB/). These strains were identified in the two independent screens and sensitivity was confirmed by spot assay. Strains that failed these criteria were not included. From *B. de Graaf, et al., Cellular pathways for DNA repair and damage tolerance of formaldehyde-induced DNA-protein crosslinks, DNA Repair (2009).*

4.2 Homologous recombination protects against chronic formaldehyde exposure*

Following the initial genome-wide chronic screen, the relative contributions of each DNA repair and tolerance pathway in protecting the cell against formaldehyde-induced cytotoxicity were assessed in more detail. A subset of strains that had been previously characterized to be involved in DNA damage repair and tolerance mechanisms was chosen for more detailed cytotoxicity analyses based on their sensitivity to formaldehyde. In addition to those strains identified in the screen, additional strains from the NER, HR and DNA bypass pathways that were not identified as sensitive in the genome-wide screen were selected as controls (Fig. 13).

Cells were cultured in YPD and serial dilutions of these exponentially growing cells were spotted onto YPD agar plates containing 1.25 mM or 1.5 mM formaldehyde and grown at 30°C for 48 hr. Consistent with the library screen, the highest sensitivities were observed in many of the strains with gene deletions in the HR pathway, including *rad50*Δ*, rad52*Δ*, mre11*Δ *and xrs2*Δ. These strains showed high sensitivities when exposed to only 1.25 mM formaldehyde (Fig. 13A). In contrast, strains deleted for *rad51, rad54* and *rad55* displayed moderate sensitivity, while *rdh54*Δ*, rad59*Δ *and rad57*Δ were not sensitive under these conditions. Comparatively, strains deleted for the NER mutants, *rad1*Δ*, rad4*Δ*,* and *rad14*Δ demonstrated only low to moderate sensitivities to low dose formaldehyde, while other NER strains deleted in *rad2*Δ*, rad7*Δ*, rad10*Δ*,* and *rad23*Δ, were closely comparable with the wild-type strain (Fig. 13B). In addition, the cell cycle checkpoint deletion mutants *rad9*Δ*, rad17*Δ and *rad24*Δ also showed survival rates similar to the wild-type strain under these exposure conditions. Strains with

deletions in genes traditionally defined as the *RAD6* epistasis group (post-replication repair) were not sensitive to formaldehyde under these conditions, with the exception being the moderate to high sensitivity of the *rad5*Δ strain (Fig. 13C). To confirm these data, colony-forming assays were carried out on a subset of these strains (Fig. 13D). As anticipated based on the data in Fig. 13A-C, *rad18*Δ was comparable to the wild-type strain, while *rad4*Δ *and rad51*Δ were moderately sensitive and *mre11*Δ and *rad52*Δ showed marked sensitivity.

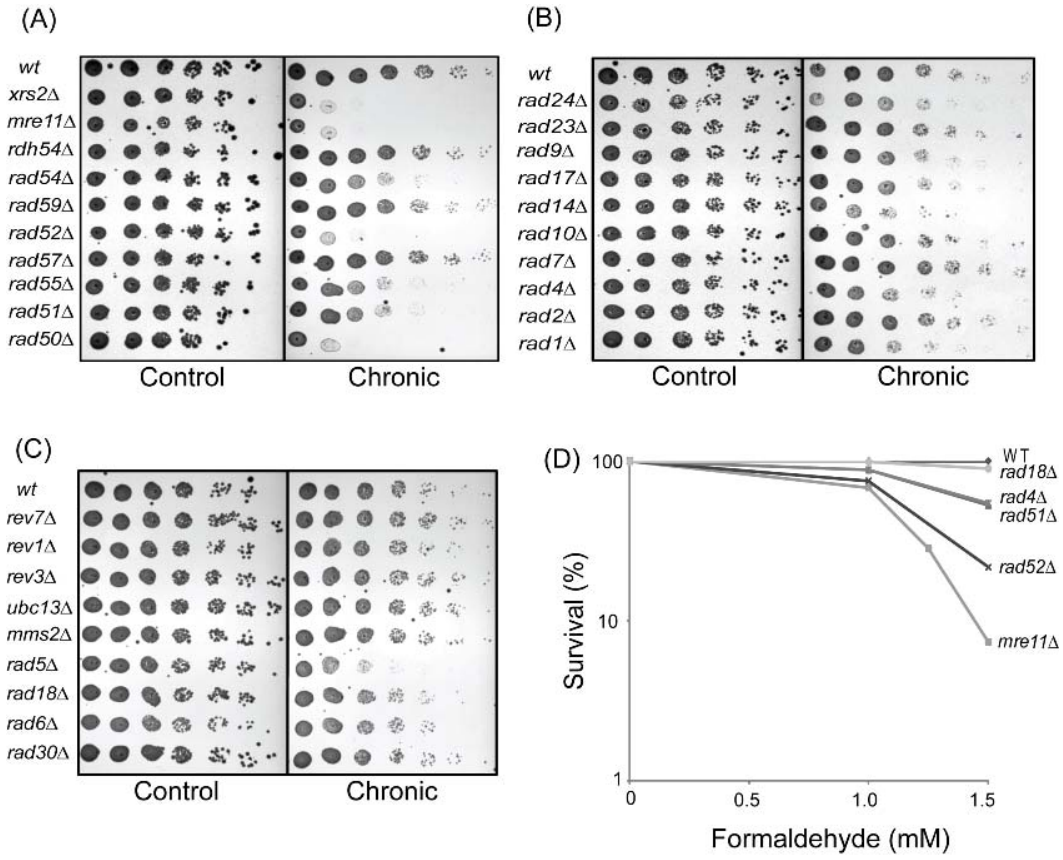

Figure 13. Survival analyses of yeast deletion strains exposed to chronic low dose formaldehyde. (A) HR deletion strains, (B) NER and cell cycle-associated deletion strains, (C) Translesion synthesis (post replication repair) strains show differential growth following formaldehyde exposure. The strains shown were grown exponentially in YPD media and 2 µl aliquots of serially diluted cells (10-fold, left to right) were spotted onto an agar plate and grown for 2 days at 30°C. In each case, the left panel is unexposed (no formaldehyde) and the right panel shows the same strains grown in the presence of (A) 1.25 mM, (B, C) 1.5 mM formaldehyde. (D) The colony forming ability of *mre11*Δ*, rad4*Δ*, rad52*Δ*, rad51*Δ*, rad18*Δ and wild-type strains after chronic exposure for 48 hr. From *B. de Graaf, et al., Cellular pathways for DNA repair and damage tolerance of formaldehyde-induced DNA-protein crosslinks, DNA Repair (2009).*

4.3 Nucleotide excision repair protects cells from acute formaldehyde exposure*

To determine if the length of time and concentration of formaldehyde exposure affected the pathway used to repair the DPCs, strains representing the major DNA repair or damage tolerance pathways were assayed for sensitivity to formaldehyde at an acute exposure (60 mM,15 min) and compared with a chronic exposure (1.5 mM, 48 hr) (Fig. 14A). It had been noted in the original screen that the *sgs1Δ* and *top3Δ* strains were highly sensitive to chronic formaldehyde exposure (Table 8). The Sgs1/Top3 complex is involved in maintenance of replisome stability and the Sgs1 RecQ-family helicase has been implicated in double-strand break processing (Bjergbaek, Cobb et al. 2005; Mimitou and Symington 2008). Thus, it was also of interest to examine the relative sensitivity of these two deletion strains following chronic versus acute exposure.

Analyses of these data revealed dramatic differences in the cellular pathway responses under the two conditions. As shown in Fig. 14A, left panel (control) all strains grew similar to the wild-type strain under non-exposed conditions, with only a modest slow growth phenotype shown in the *mre11*Δ strain. When exposed under chronic conditions (1.5 mM; middle panel), increased formaldehyde sensitivity was observed in the strains carrying deletions of genes in the HR pathway, including *rad52*Δ*, rad50*Δ*, mre11*Δ, and *xrs2*Δ*.* In addition, the *sgs1*Δ and *top3*Δ showed marked sensitivities, thus implicating these proteins in the processing of DPCs. Consistent with the data above, only modest sensitivity was seen for the NER mutants, *rad1*Δ and *rad4*Δ*,* or no sensitivity for the post-replication repair mutants, *rev3*Δ, *rad6*Δ, or *rad18*Δ.

In contrast, following acute exposures, the NER strains (*rad1*Δ and *rad4*Δ) demonstrated the highest sensitivity, whereas the HR deletion strains (*mre11*Δ*, xrs2*Δ*, rad50*Δ*, rad52*Δ) and the *sgs1*Δ and *top3*Δ strains exhibited moderate sensitivity (Fig. 14A; right panel). These data suggest that the relative contribution of DNA repair pathways to protection against formaldehyde-induced DPCs is dependent on the exposure conditions. In order to confirm these findings, colony forming assays were carried out on a subset of these strains under acute conditions. In agreement with the data shown in Fig. 14A, *rad4*Δ showed a marked sensitivity, whereas the colony forming ability of *rad52*Δ*, mre11*Δ and *top3*Δ were comparable or slightly better than that of the wild-type strain. These results were found consistently. In addition, the strains that were not identified in the screen under chronic exposure conditions as sensitive but are part of the TLS, BER and MMR pathways, were also tested under acute conditions (Fig. 15). Under these exposure conditions these strains did not display a higher sensitivity to formaldehyde compared to wild-type.

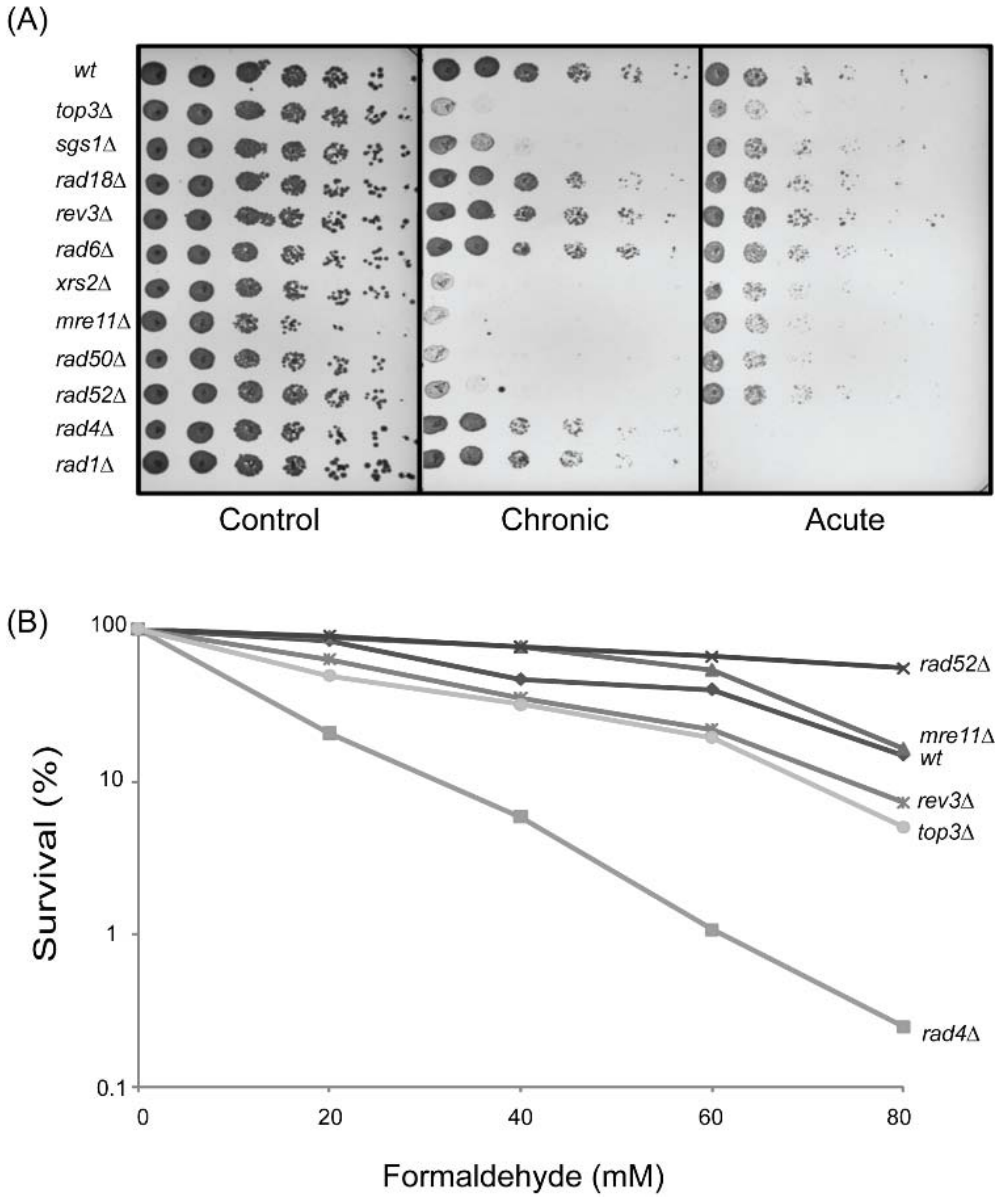

Figure 14. Survival analyses of yeast deletion strains following exposure to acute high dose formaldehyde. (A) Selected yeast deletion strains representing DNA damage response pathways were cultured for exponential growth. For the control (left) and chronic 1.5 mM formaldehyde exposure (middle), each culture was serially diluted and aliquots spotted. For the acute exposure (right), following exponential growth in liquid media, cells were exposed to 60 mM formaldehyde for 15 min and washed twice diluted and spotted onto plates. (B) The colony forming abilities of WT, *mre11*Δ, *rad4*Δ, *top3*Δ, *rev3*Δ and *rad52*Δ after acute exposure for 15 min are plotted. The cells were grown for 2 days at 30°C. From *B. de Graaf, et al., Cellular pathways for DNA repair and damage tolerance of formaldehyde-induced DNA-protein crosslinks, DNA Repair (2009).*

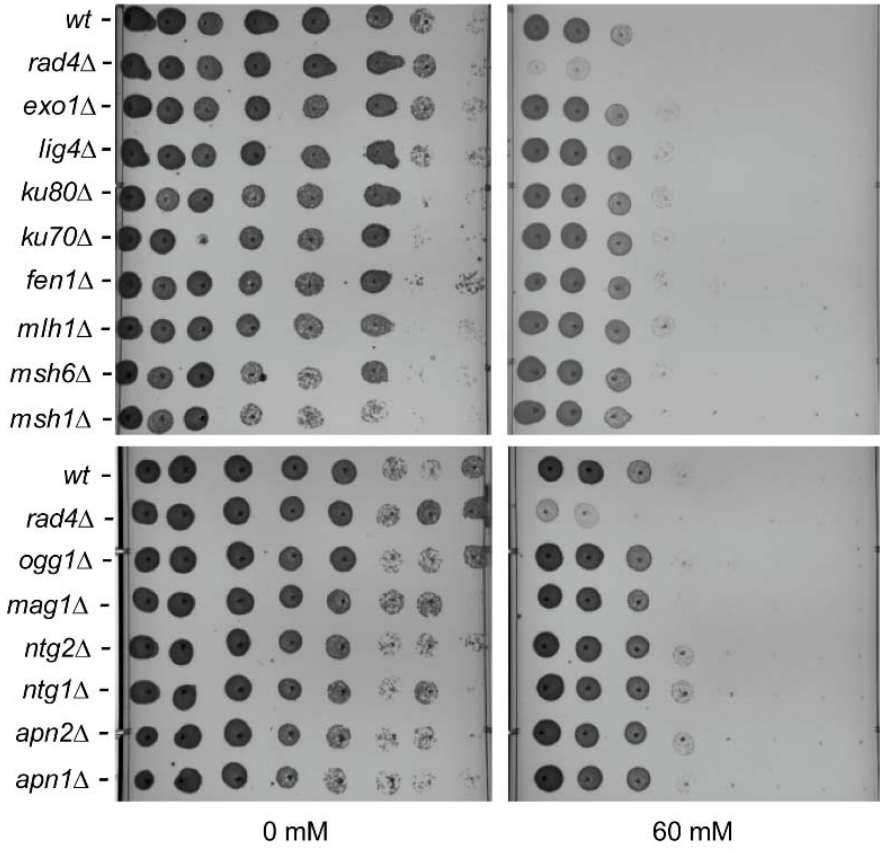

Figure 15. Survival of yeast deletion strains that are not sensitive to acute high dose formaldehyde relative to NER deficient strains. Selected yeast deletion strains representing the base excision repair, single-strand break repair, and mismatch repair pathways were cultured for exponential growth. For acute exposure, cells were incubated with 60 mM formaldehyde for 15 min, and washed twice, diluted and plated. The cells were grown for 2 days at 30°C. From *B. de Graaf, et al., Cellular pathways for DNA repair and damage tolerance of formaldehyde-induced DNA-protein crosslinks, DNA Repair (2009).*

4.4 Formaldehyde-induced DPCs in *S. cerevisiae**

Due to the differences seen in the response pathways following formaldehyde exposure, it was of interest to directly detect DPCs in yeast DNA following chronic and acute formaldehyde exposures. To assess the dose-dependent formation of DPCs, SDS/KCl precipitation of proteins from whole cell lysates was adapted for the measurement of DPC formation as previously described (Trask, DiDonato et al. 1984). This assay relies on the ability of cross-linked DNA to co-precipitate with proteins. Following a proteinase digestion, the extent of co-precipitation of DNA was measured by agarose gel electrophoresis of the DNA. As shown in Fig. 16, wild-type cells exposed to 20-40 mM formaldehyde for 15 min showed a dose-dependent accumulation of DPCs. No DPCs were detected in cells exposed to chronic doses (1.5-2 mM) of formaldehyde (data not shown). Based on the acute dose experiments, it is apparent that the lower limit of detection for this assay is between 10-20 mM exposures. Thus, DPCs may be forming at chronic doses, but are not detectable by this method.

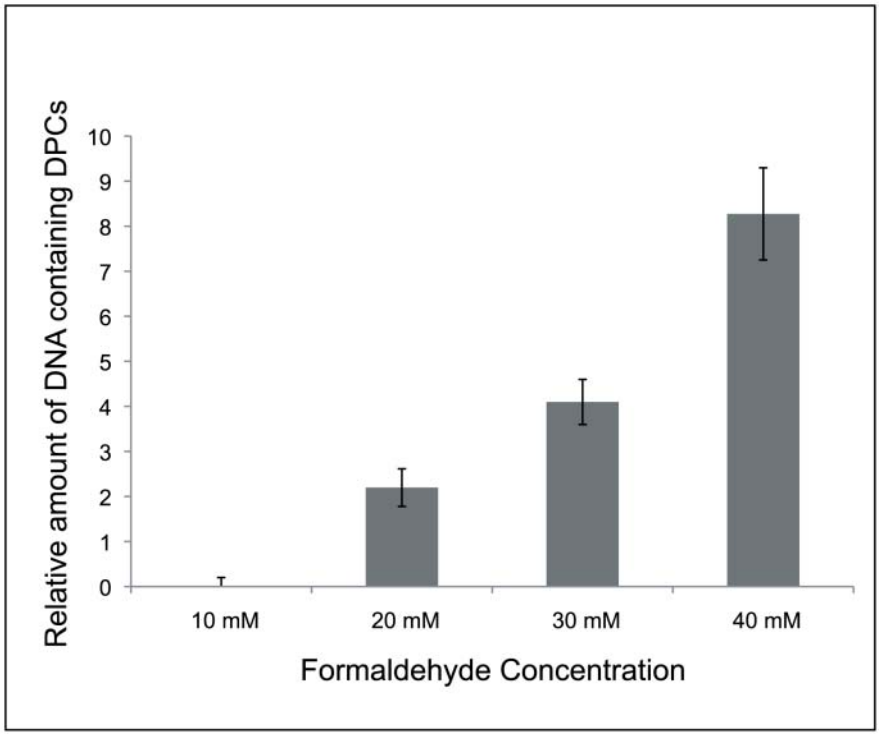

Figure 16. Accumulation of DPCs in wild-type yeast following acute formaldehyde exposure. Wild-type yeast cells were exposed to the indicated concentrations of formaldehyde for 15 min. DNA containing DPCs was isolated and analyzed as described in the methods. The data show the ratio of DNA containing DPCs compared to non-crosslinked DNA as determined by the SDS/KCl precipitation method. Each concentration was repeated in triplicate and non-treated control values were subtracted as background. [Experiment performed by Dr. A. Clore as part of the publication]. From *B. de Graaf, et al., Cellular pathways for DNA repair and damage tolerance of formaldehyde-induced DNA-protein crosslinks, DNA Repair (2009).*

4.5 The role of nucleotide excision repair in the removal of formaldehyde-induced DPCs*

After establishing that DPCs accumulate in a dose-dependent manner in cells treated with formaldehyde, the removal of these DPCs was observed at 0, 4 and 6 hr post-exposure in wild-type and NER-deficient cells. Using the SDS/KCl assay, it was observed that DPCs decrease at a linear rate in cells following acute exposure to formaldehyde, reaching the level of background approximately 6 hr post-exposure. Similar to previous results in mammalian cells (Quievryn and Zhitkovich 2000), NER-deficient yeast cells show a similar rate of removal of DPCs as wild-type cells despite the large difference in sensitivity (Fig. 17).

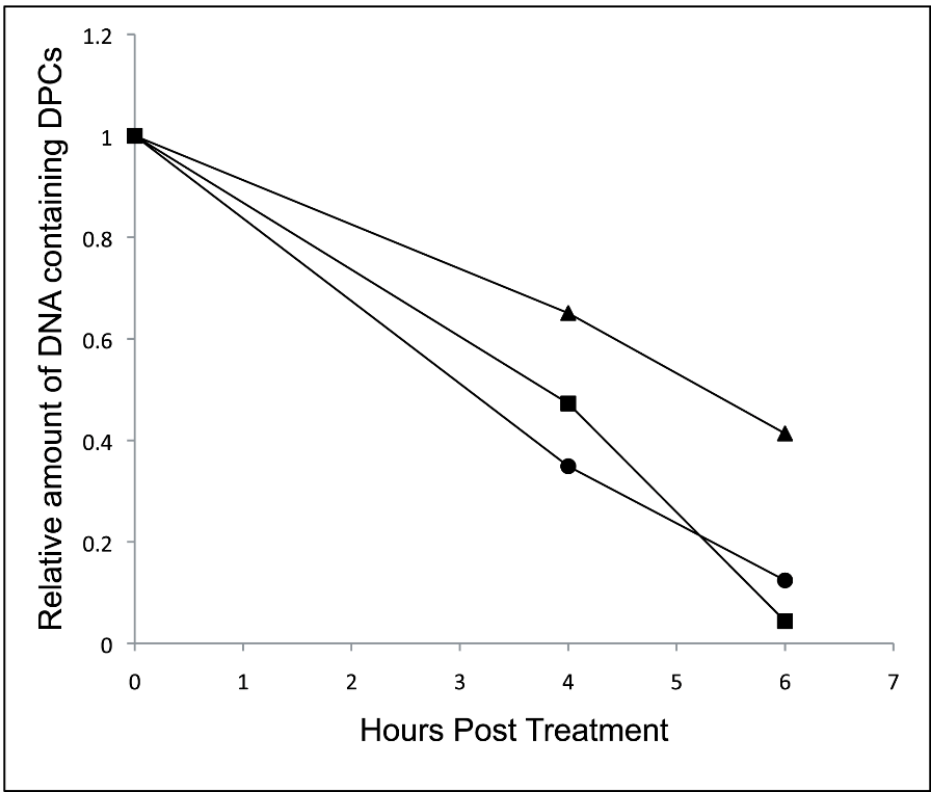

Figure 17. DPC removal following acute formaldehyde exposure. Wild-type (circles), *rad1*Δ (squares), and *rad4*Δ (triangles) yeast were exposed to 60 mM formaldehyde for 15 min. DNA containing DPCs was isolated and analyzed as described in the methods. Each concentration was independently repeated four times and non-treated control values were subtracted as background. Values were normalized so that the zero time point was 100% [Experiment performed by Dr. A. Clore as part of the publication]. From *B. de Graaf, et al., Cellular pathways for DNA repair and damage tolerance of formaldehyde-induced DNA-protein crosslinks, DNA Repair (2009).*

4.6 The occurrence of DNA breaks as an intermediate in the processing of DPCs*

Based on the sensitivity of the HR deletion strains, one plausible model invokes a DNA break intermediate in order to perform single-strand invasion to process DPCs (Baldwin, Berger et al. 2005). To test this model, PFGE analyses were performed to determine if SSBs, DSBs, or both were formed during the processing of formaldehyde-induced DPCs. PFGE analyses were performed with and without the addition of S1 nuclease, which is added to convert SSBs to DSBs that can subsequently be detected by PFGE. Analyses were performed on cells that had been exposed to chronic or acute doses of formaldehyde.

To serve as controls for the induction of DSBs or SSBs, wild-type cells were treated with bleomycin or hydrogen peroxide, respectively. Following control and experimental exposures, cells were digested with lyticase, and these lysates were subsequently incubated with proteinase K, and DNAs subjected to PFGE. The bleomycin treatment resulted in DSB formation (Fig. 18A, left panel), and the hydrogen peroxide treatment produced both DSBs and SSBs (Fig. 18A, middle panel), results that are consistent with previously published data (Moore, McKoy et al. 2000; Ribeiro, Corte-Real et al. 2006). However, cells exposed to chronic formaldehyde showed no evidence of either SSB or DSB accumulation (Fig. 18A, right panel).

A possible interpretation of the formaldehyde exposure data was that SSBs and DSBs occur only transiently, as intermediates of the repair process, and that these breaks are not detectable under the previous conditions. To increase the number of breaks occurring at any one time, cells were arrested in G1 phase with α-mating factor

prior to formaldehyde treatment. Only acute exposures could be tested on arrested cells due to the duration of a chronic exposure. Following treatment, cells were assayed for the formation of SSBs and DSBs over a 24 hr period. As shown in Fig. 18B, DSBs were not observed above background levels in PFGE after exposure at any given time point. Likewise, no DSB intermediates were observed accumulating above background levels in *rad52*Δ and *mre11*Δdeletion strains (Fig. 19).

In contrast, SSBs were observed immediately following exposure to formaldehyde accumulating up to 4 hr after exposure. However, by 8 hr, the SSBs were almost completely repaired (Fig. 18B). Since the cells were counted at each post-exposure time point and no significant increase in cell number had occurred, we conclude that there was no dilution of the damage due to cell growth. In addition, based on the library screen and survival data, NER mutants *rad1*Δ and *rad4*Δ were assayed for SSBs and DSBs.

The accumulation of SSBs following formaldehyde exposure is not evident in the *rad4*Δ strain by PFGE (Fig. 18). In addition, alkaline sucrose gradient sedimentation experiments performed by Dr. A Clore as part of the publication confirmed the lack of SSBs in both the *rad1*Δ and *rad4*Δ strains (Fig. 20).

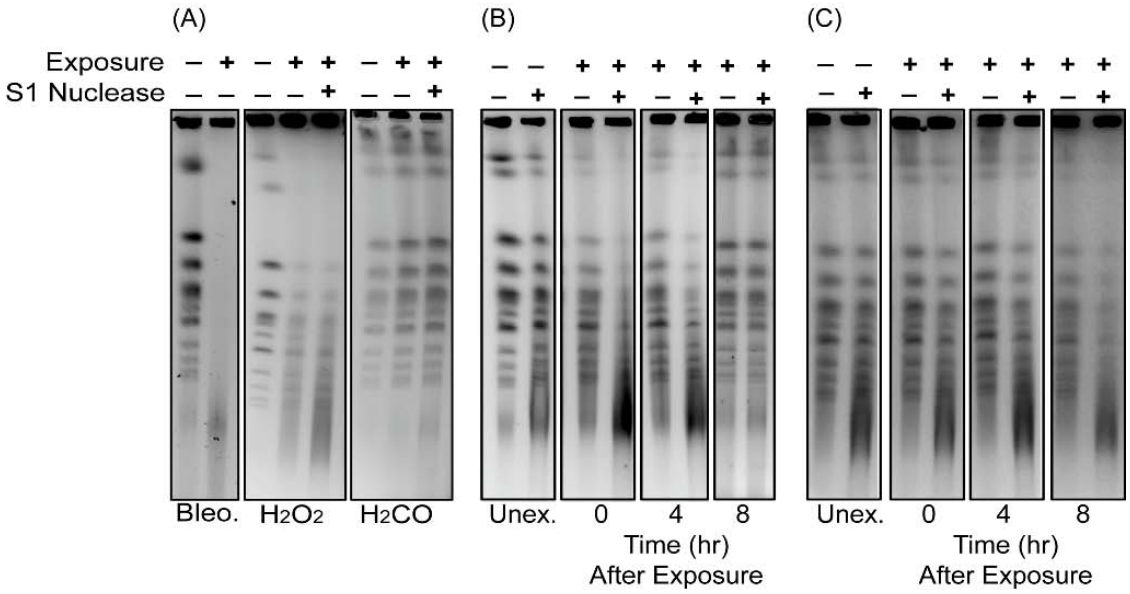

Figure 18. Pulsed field gel electrophoresis on wild-type and *rad4*Δ yeast following formaldehyde exposure. (A) 20 µg/ml bleomycin for 1 hr (left panel); 10 mM H_2O_2 for 1 hr (middle panel); 1.5 mM formaldehyde exposure for 48 hr (right panel). Wild-type cells (B) or *rad4*Δ cells (C) were arrested with α-mating factor and exposed to 60 mM formaldehyde for 15 min. Time (hr), is the time given for the cells to recover after exposure. From *B. de Graaf, et al., Cellular pathways for DNA repair and damage tolerance of formaldehyde-induced DNA-protein crosslinks, DNA Repair (2009).*

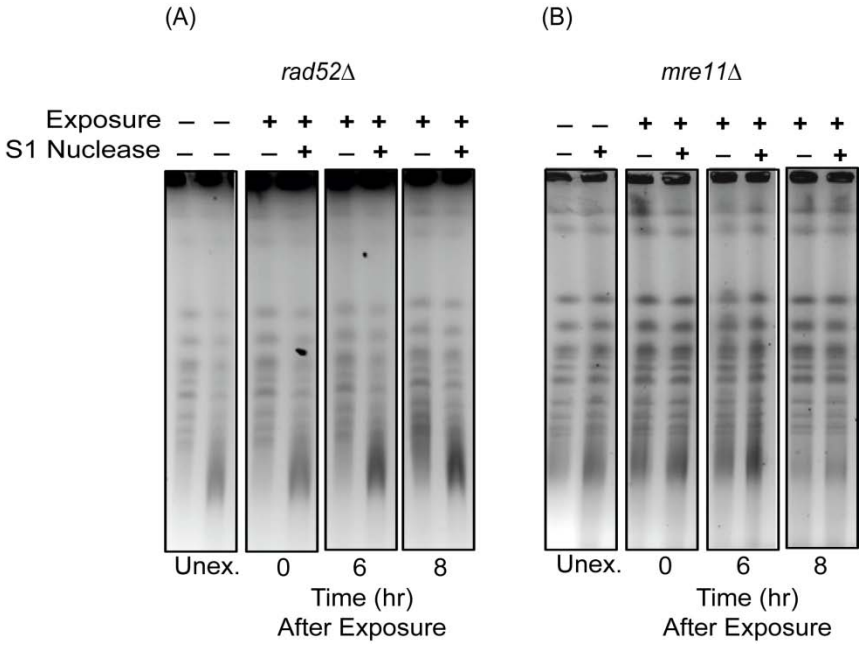

Figure 19. Pulsed field gel electrophoresis on *rad52*Δ and *mre11*Δ after exposure to formaldehyde. *rad52*Δ cells (A) or *mre11*Δ cells (B) were arrested with α-mating factor and exposed to 60 mM formaldehyde for 15 min. Time (hr), is the time given for the cells to recover after exposure. From *B. de Graaf, et al., Cellular pathways for DNA repair and damage tolerance of formaldehyde-induced DNA-protein crosslinks, DNA Repair (2009).*

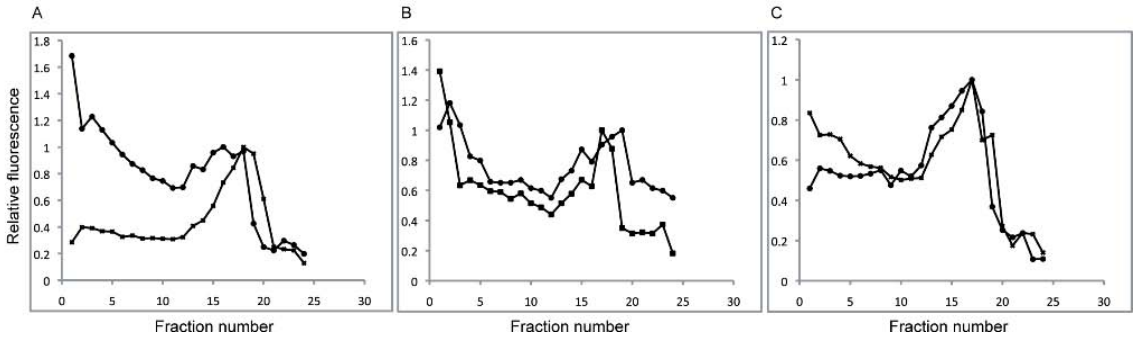

Figure 20. NER-dependent accumulation of single strand breaks following formaldehyde exposure. Yeast cells were exposed to 60 mM formaldehyde (squares) and allowed 4 hr recovery. Unexposed controls are shown (circles). The DNA from, wild-type yeast (A), *rad1*Δ (B) or *rad4*Δ (C), were analyzed by alkaline sucrose gradient sedimentation. Fractions were taken from the bottom of a 15-30% sucrose gradient that had been centrifuged for 20 hr at 11,500 rpm [Experiment performed by Dr. A. Clore as part of the publication]. From *B. de Graaf, et al., Cellular pathways for DNA repair and damage tolerance of formaldehyde-induced DNA-protein crosslinks, DNA Repair (2009).*

4.7 Cellular response to other DPC-inducing agents

Mutant strains classified to the HR or NER pathway were also tested for sensitivity to the topoisomerase inhibitors, etoposide and camptothecin. These compounds were chosen due to the fact that the mechanism of DNA-protein crosslink formation is well-understood. In addition, HR and NER have been implicated to play an important role in tolerating exposure to either of these agents. In particular, yeast mutant strains of the Rad52 epistasis group, and the Rad1-Rad10 endonuclease were of importance. Next to the importance of these pathways it has also been shown that exposure to either of these compounds results in the formation of DSBs (Vance and Wilson 2002; Huang, Traganos et al. 2003; Baldwin, Berger et al. 2005; Deng, Brown et al. 2005).

As can be seen in Fig. 21A NER mutants display no sensitivity greater than wild-type to either etoposide or camptothecin with the exception of *rad23*Δ. Checkpoint mutants, *rad9*Δ, *rad17*Δ and *rad24*Δ also show no sensitivity. On the other hand, mutants classically defined to the HR pathway show a similar sensitivity to both etoposide and camptothecin as they do to formaldehyde. In Fig. 21B all the mutants show reduced growth rates when exposed to these two compounds. Interestingly, *rdh54*Δ*, rad59*Δ and to some extend *rad54*Δ display no increased sensitivity compared to wild-type (Fig. 21).

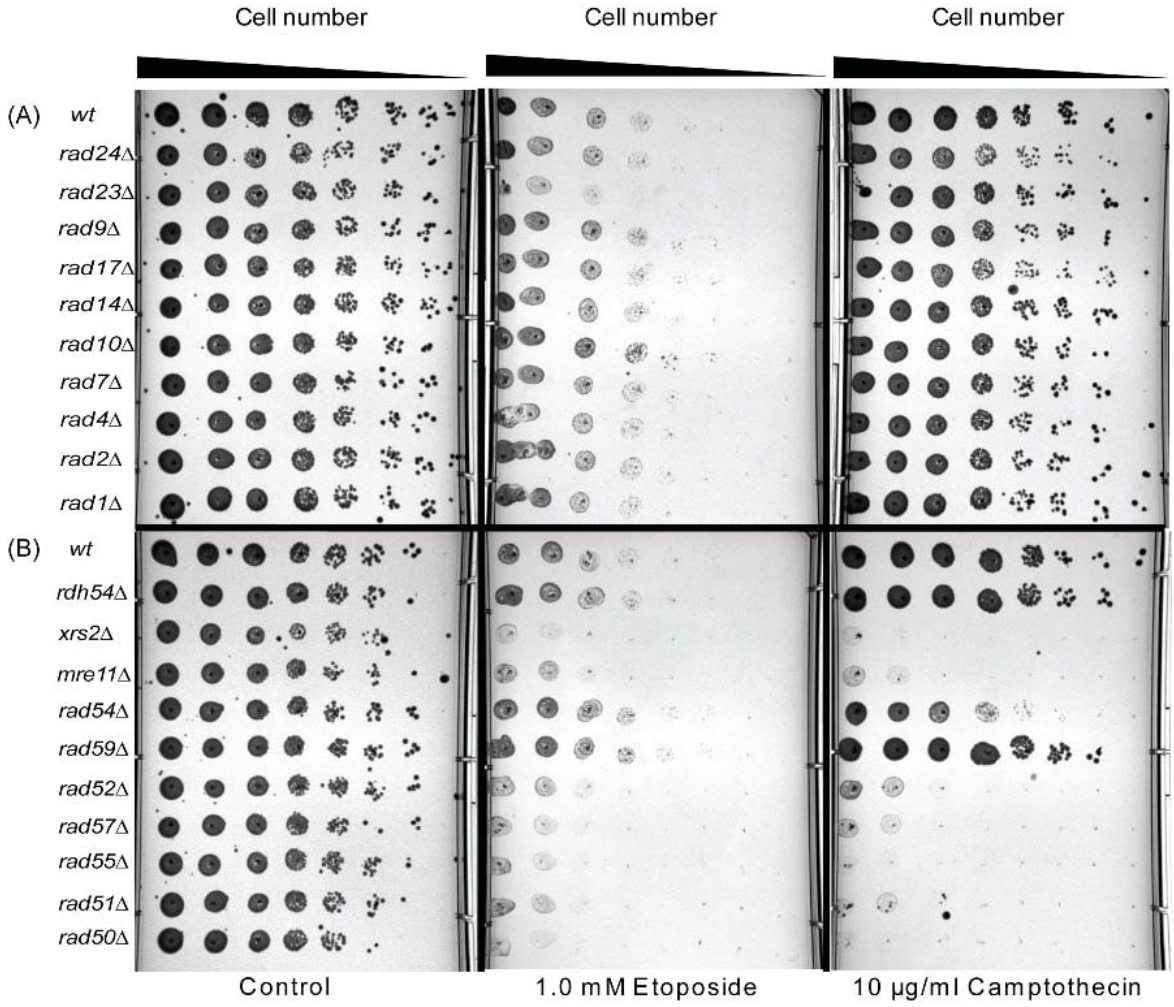

Figure 21. Sensitivity of NER and HR mutants to chronic etoposide and camptothecin exposure. The strains shown were grown exponentially in YPD media, counted, and $2x10^7$ cells were serially diluted (10-fold dilution per spot shown decreasing left to right) were spotted onto an agar plate containing the chemicals, and grown for 2 days at 30°C. In each case, the left panel is unexposed and the middle panel shows the same strains grown in the presence of 1.0 mM etoposide, the right panel the same strains after 10 µg/ml camptothecin exposure.

4.8 The role of Mre11 in the tolerance or repair of DPCs under chronic or acute conditions

After the library screen, spot assay, and a more detailed colony forming ability assay, it was observed that the *mre11*Δ strain is one of the most sensitive strains under chronic formaldehyde exposure. Therefore, the nuclease deficient strains *mre11-H125N*, *mre11-D56N*, *mre11-D56A*, *mre11*Δ, and its partner *sae2*Δ, were tested under chronic and acute conditions. These nuclease deficient mutants were tested because they have been shown to be defective in the processing of Spo11-induced DSBs in meiosis, but with no defective resection of HO-induced DSBs (Moreau, Ferguson et al. 1999; Llorente and Symington 2004; Farah, Cromie et al. 2005). It has also been reported that the Mre11 nuclease activity is necessary in order to tolerate exposure to camptothecin (Vance and Wilson 2002; Deng, Brown et al. 2005). Yeast mutant strain Sae2 was also present in the library, but was not sensitive to formaldehyde. This mutant was still included in this analysis because it is an endonuclease that processes hairpin DNA structures with the MRN(X)-complex (Rattray, McGill et al. 2001; Lengsfeld, Rattray et al. 2007).

In order to test if the nuclease plays a role in the repair of DPCs, first a spot assay was performed on the nuclease-deficient mutants. The strains were grown exponentially in YPD media, counted, and $2x10^7$ cells were serially diluted (10-fold dilution per spot shown decreasing left to right) and spotted onto an agar plate and grown for 2 days at 30°C. For a chronic exposure 1.5 mM formaldehyde was added to the plates, for the acute exposure the cells were exposure for 15 min. to 60 mM formaldehyde before spotting the yeast strains on the YPD plates. In contrast to the

*mre11*Δ strain (Fig. 13-14, 22), the Mre11 nuclease deficient mutants do not show an increased sensitivity to formaldehyde either under chronic or acute exposure conditions (Fig. 22). In addition, *sae2*Δ also displayed no sensitivity. It has to be noted that these strains are from a different background (W303), but the *mre11*Δ strain shows a similar survival rates as the *mre11*Δ from the library (Fig. 13).

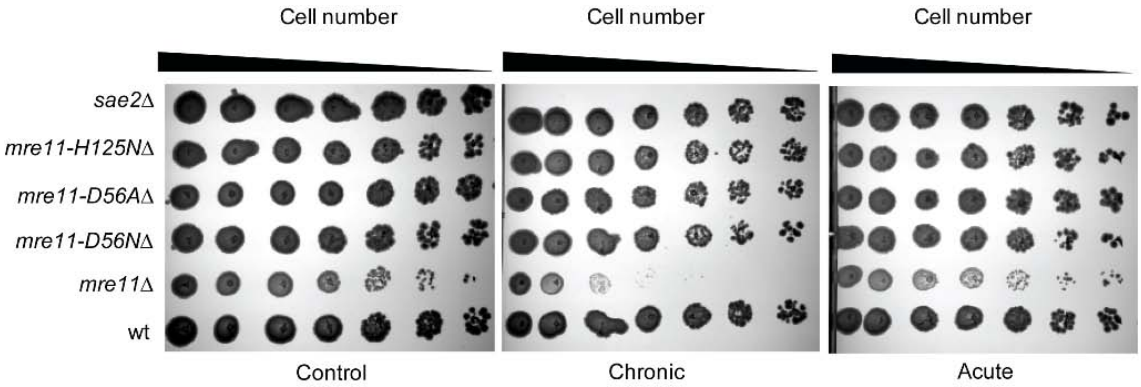

Figure 22. Sensitivity of Mre11 nuclease deficient mutants after chronic and acute formaldehyde exposure. The strains shown were grown exponentially in YPD media, counted, and $2x10^7$ cells were serially diluted (10-fold dilution per spot shown decreasing left to right), spotted onto an agar plate and grown for 2 days at 30°C. In each case, the left panel is unexposed (no formaldehyde), the middle panel shows the same strains grown in the presence of 1.5 mM formaldehyde, the right panel shows the same strains exposed to 60 mM formaldehyde for 15 min.

As described above, the nuclease activity of Mre11 plays an important role in the cell, however, the nuclease activity is not necessary for tolerance or repair of formaldehyde-induced DNA damage. In order to assess whether or not there is a difference in the formation of SSBs (as seen wild-type cells (Fig. 18) or DSBs, the Mre11 nuclease deficient strains were further studied by PFGE, as described in section 3.5. As shown in Fig. 23 no SSBs or DSBs are observed for the nuclease deficient mutants

above background levels following formaldehyde exposure. This suggests that the nuclease activity may be necessary for the formation of SSBs after formaldehyde exposure. SSBs significantly above background levels after formaldehyde exposure were also not detected in the *mre11*Δ strain (Fig. 19).

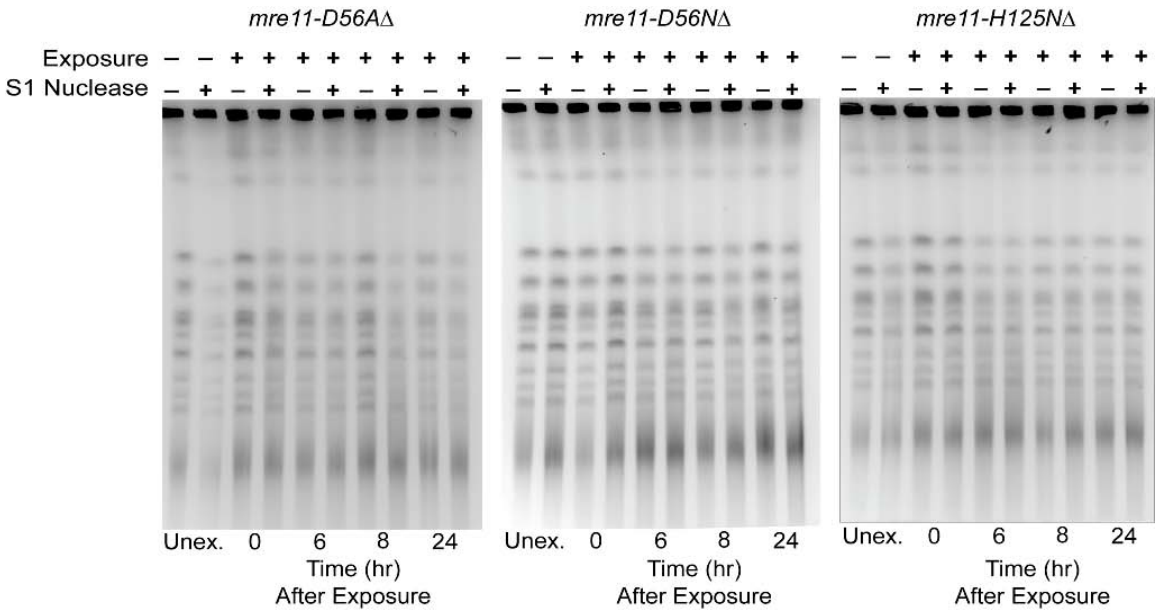

Figure 23. Pulsed field gel electrophoresis analyses of DNA from the nuclease deficient Mre11 strains. Cells were arrested with α-mating factor ($2x10^8$ cells) and exposed to 60 mM formaldehyde for 15 min. Time (hr), is the time allowed for the cells to recover after exposure.

4.9 Genes involved in ICL repair are not sensitive to formaldehyde

Our findings that the HR and NER pathways, or components of these pathways, are involved in the repair and/or tolerance of DPCs are reminiscent of the potential interplay of these two pathways in repair of ICLs. In fact, there was significant overlap between the strains implicated in the repair of ICLs and those identified in the formaldehyde screen. Several strains sensitive to mitomycin C-induced ICLs were also sensitive to formaldehyde (*rad1*Δ*, rad4*Δ*, rad51*Δ*, and rad52*Δ*)* (Grossmann, Ward et al. 2001; Barber, Ward et al. 2005). However, the mitomycin C-sensitive deletion strains *rev3*Δ and *exo1*Δ were not sensitive to formaldehyde (Fig. 13-15).

Processing of ICLs in eukaryotic systems is associated with the formation of DSBs that are hypothesized to be formed at sites of stalled replication forks (Bergstralh and Sekelsky 2008). Pso2, a 5' to 3' exonuclease, significantly contributes to cellular resistance to ICL damage. Interestingly, *pso2*Δ mutants appear to be proficient for the initial incision events for ICL repair, but fail to repair ICL-induced DSBs (Barber, Ward et al. 2005). Additionally, these mutants show decreased rates of homologous recombination, suggesting that the Pso2 nuclease acts downstream of NER in the processing of ICLs to provide a suitable substrate for HR (Grossmann, Ward et al. 2001; Barber, Ward et al. 2005). Since current models for DPC repair also predict that DSB intermediates would be formed due to replication fork collapse and since strains deleted in genes for NER and HR pathways are sensitive to formaldehyde exposures, it was of interest to determine if Pso2 may be involved in DPC repair. Although the *pso2*Δ strain in the deletion library was not sensitive to formaldehyde, additional *pso2*Δ strains with different backgrounds, previously shown to be sensitive to ICL-inducing agents, were

obtained from Dr. Peter McHugh (University of Oxford) and Dr. Robb Moses (Oregon Health & Science University) and tested under identical conditions to those described above (Table 9).

Strain	Genotype	Knockout
KGY212	*MATa ura3 trp1 leu2 his7, snm1::hisG*	*pso2*Δ
KGY216	*MATa ura3 trp1 leu2 his7, rev3::hisG*	*rev3*Δ
AWY114	*MATa ura3 trp1 leu2 his7, rad51::hisG:URA3:hisG*	*rad51*Δ
AWY118	*MATa ura3 trp1 leu2 his7, rad30::hisG*	*rad30*Δ
AWY212	*MATa ura3 trp1 leu2 his7, snm1::hisG rev3::hisG*	*pso2*Δ*rev3*Δ
AWY115	*MATa ura3 trp1 leu2 his7, snm1::hisG rad51::hisG:URA3:hisG*	*pso2*Δ*rad51*Δ
AWY116	*MATa ura3 trp1 leu2 his7, rev3::hisG rad51::hisG:URA3:hisG*	*rev3*Δ*rad51*Δ
AWY120	*MATa ura3 trp1 leu2 his7, rev3::hisG rad30::hisG*	*rev3*Δ*rad30*Δ
AWY119	*MATa ura3 trp1 leu2 his7, snm1::hisG rad30::hisG*	*rev3*Δ*pso2*Δ
AWY117	*MATa ura3 trp1 leu2 his7, snm1::hisG rev3::hisG rad51::hisG:URA3:hisG*	*rad51*Δ*pso2*Δ*rev3*Δ
LBY9	B356-7C with *pso2*::*kanMX4*	*pso2*Δ
LBY12	B356-7C with *pso2*::*kanMX4 mre11*::*HIS3*	*pso2*Δ*mre11*Δ
LBY15	B356-7C with *mre11*::*kanMX4*	*mre11*Δ
LBY17	*B356-7C* with *rad4::kanMX4*	*rad4*Δ
LBY25	B356-7C with *pso2*::*his3 rad4*::*kanMX4*	*pso2*Δ*rad4*Δ
LBY26	B356-7C with *rad1*::*kanMX4*	*rad1*Δ
LBY28	B356-7C with *pso2*::*kanMX4 rad51*::*HIS3*	*pso2*Δ*rad51*Δ
LBY50	B356-7C with *pso2*::*kanMX4 rad1*::*HIS3*	*pso2*Δ*rad1*Δ
LBY51	B356-7C with *pso2*::*kanMX4 rad52*::*HIS3*	*pso2*Δ*rad52*Δ
LSY497-9C	LSY497-9C with rad1::LEU2 rad52::TRP1	*rad1*Δ*rad52*Δ
PJM35	BY4741 with rad4::URA3 rad18::kanMX4	*rad4*Δ*rad18*Δ
PJM37	*B356-7C* with *rad4::kanMX4 rad52::HIS3*	*rad4*Δ*rad52*Δ
LSY497-9C	LSY497-9C with rad1::LEU2 rad52::TRP1	*rad1*Δ*rad52*Δ
B356-11C	B356-7C with *rad52*::*TRP1*	*rad52*Δ
B356-13D	B356-7C with *rad51*::*HIS3*	*rad51*Δ
B356-7C	W303-1A with *ade2*::*hisG his3*::*ade2-5_ _-TRP1-ade2-n*	Wild-type
WX9326	BY4741 with *rad18*::*TRP1*	*rad18*Δ
DBY747	*MATa his3-1 leu2-0 met15-0 ura3-0*	Wild-type

Table 9. Strains sensitive to ICL-inducing agents. These strains were obtained from Grossman et al 2002 (top half) and Barber et al 2005 (bottom half) and tested for formaldehyde sensitivity.

4.9.1 *rev3*Δ exhibits significant synergism with *rad51*Δ

In contrast to the sensitivity observed following treatment with nitrogen mustard, cisplatin and mitomycin C (Grossmann, Ward et al. 2001; Barber, Ward et al. 2005), no sensitivity of *rad30*Δ*, rev3*Δ*, pso2*Δ and the double mutant *rev3*Δ*rad30*Δ to formaldehyde exposure was observed (Fig. 24-25). The HR deficient strain, *rad51*Δ did show moderate sensitivity, consistent with our previous results using *rad51*Δ in the BY4741 background (Fig. 13). As can be further seen in Fig. 24 the double mutant *rad51*Δ*pso2*Δ shows moderate sensitivity to formaldehyde, but is less sensitive than *rad51*Δ alone. This suggests that *pso2*Δ, at least in part, suppresses the sensitive phenotype of *rad51*Δ. The TLS, HR double mutant, *rev3*Δ*rad51*Δ, is significantly more sensitive than *rad51*Δ alone, suggesting synergism. This synergism is interesting because the single mutant of *rev3*Δ displays wild-type resistant levels to formaldehyde. This suggests that *rev3*Δ can compensate for the loss of *rad51*Δ to some extent. The triple mutant of *pso2*Δ*rad51*Δ*rev3*Δ shows no significant increase in sensitivity compared to the double mutant *rev3*Δ*rad51*Δ, indicating that *pso2*Δ cannot suppress the phenotype of *rad51*Δ in the absence of Rev3, nor does it result in an additive survival, which would be expected for genes operating in different repair pathways.

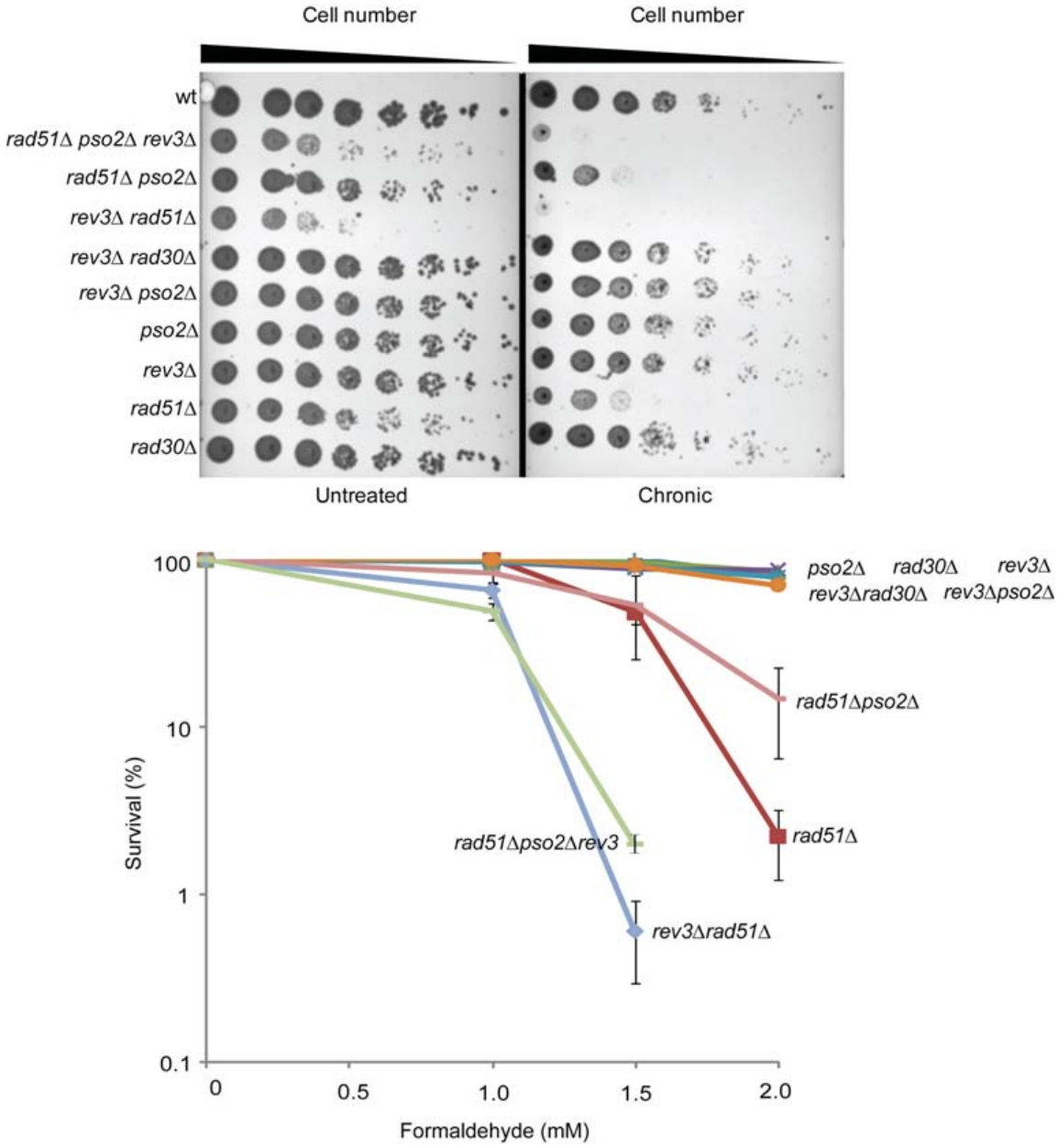

Figure 24. Sensitivity of ICL repair mutants after chronic formaldehyde exposure. The strains shown were grown exponentially in YPD media, counted, and $2x10^7$ cells were serially diluted (10-fold dilution per spot shown decreasing left to right),spotted onto an agar plate and grown for 2 days at 30°C. In each case, the left panel is unexposed (no formaldehyde) and the right panel shows the same strains grown in the presence of 1.5 mM formaldehyde. All the experiments were performed in duplicate on independent days, and repeated at least 3 times. Strains were obtained from Grossmann et al 2001 (Grossmann, Ward et al. 2001).

4.9.2 No dramatic response is observed in strains with a deletion of *pso2*, with either a NER or HR deletion after formaldehyde exposure

Although the strains from Barber et al. 2005 are from a different background (W303), following a chronic or acute formaldehyde exposure the same spectrum of sensitivity in the *mre11*Δ*, rad51*Δ*, rad1*Δ*, rad4*Δ*, rad52*Δ*, rad18*Δ and *pso2*Δ yeast strains was observed as with the strains from the library (Fig. 13 and 25). It needs to be noted that the *pso2*Δ strain is not sensitive and therefore caution needs to be taken when making conclusion, but interestingly, the double mutants of *pso2*Δ with *rad1*Δ*, rad4*Δ*, rad51*Δ*, rad52*Δ or *mre11*Δ all display a similar sensitivity to the corresponding single mutant, either showing a moderate or high sensitivity (Fig. 25). These results suggest that there is no additive response in the *pso2*Δ*rad51*Δ*, pso2*Δ*rad52*Δ*,* and *pso2*Δ*mre11*Δ strains. For the *pso2*Δ*rad1*Δ*, and pso2*Δ*rad4* strains this cannot be concluded, as both single mutant *rad1*Δ*,* and *rad4*Δ are very sensitive under acute exposure conditions. On the other hand, for all these strains it can be concluded that a suppression of phenotype is not observed. In addition, the spot assay suggests that the mutant *rad1*Δ*rad52*Δ is more sensitive than *rad4*Δ*rad52*Δ under chronic conditions (Fig. 25). To further characterize the genetic interactions of the pathways, colony forming ability was assessed for a select set of strains; *rad1*Δ*rad52*Δ, *rad4*Δ*rad52*Δ*, pso2*Δ*rad1*Δ*, pso2*Δ*rad4*Δ*, pso2*Δ*rad51*Δ*, pso2*Δ*rad52*Δ*, pso2*Δ*mre11*Δ and *rad4*Δ*rad18*Δ (Fig. 26).

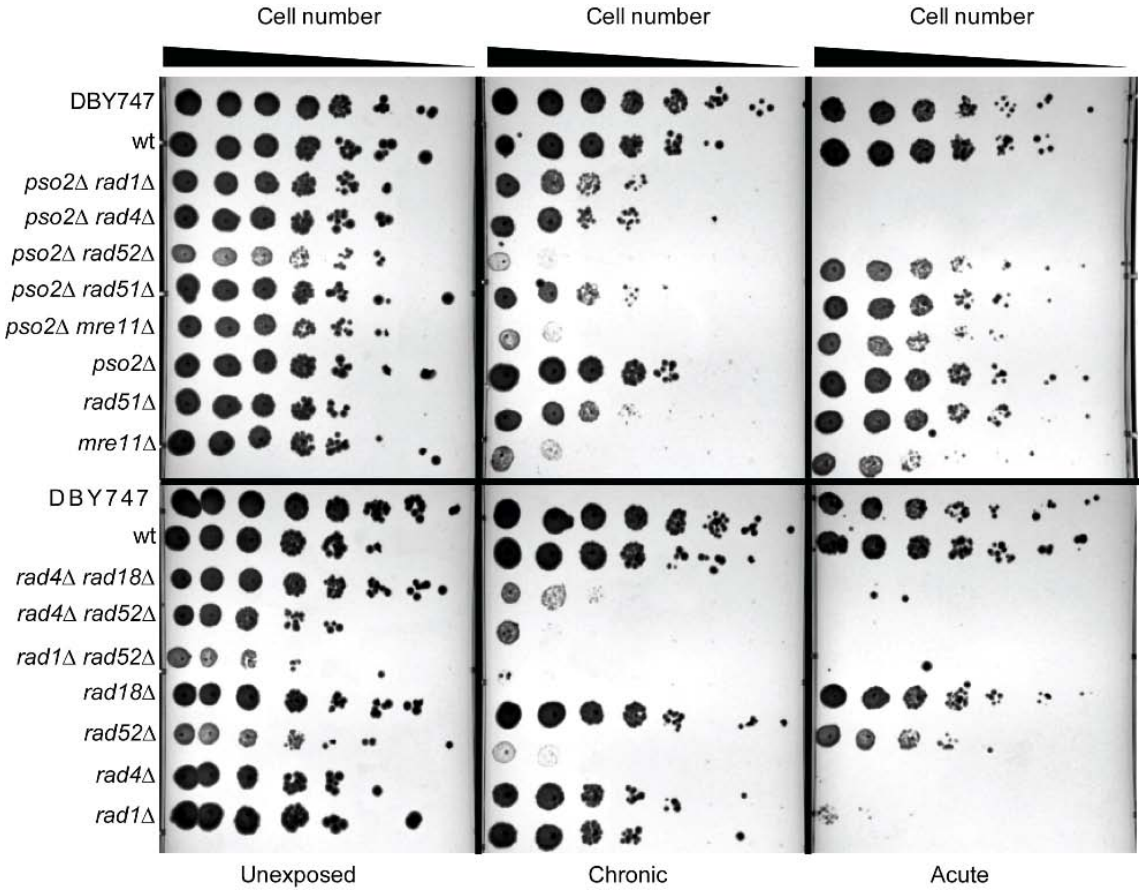

Figure 25. Sensitivity of ICL mutants after chronic and acute formaldehyde exposure. The strains shown were grown exponentially in YPD media, counted, and $2x10^7$ cells were serially diluted (10-fold dilution per spot shown decreasing left to right) were spotted onto an agar plate and grown for 2 days at 30°C. In each case, the left panel is unexposed (no formaldehyde) and the middle panel shows the same strains grown in the presence of 1.5 mM formaldehyde, the right panel the same strains after 60 mM formaldehyde for 15 minutes. Strains were from Barber et al 2005.

4.9.3 A *pso2Δrad1Δ* strain displays a different sensitivity to formaldehyde than a *pso2Δrad4Δ*

As shown in Fig. 26A-B, the *pso2*Δ has no effect on the sensitivity of *rad1*Δ, while in contrast the *pso2*Δ appears to exert (partial) suppression of the *rad4*Δ sensitive phenotype. Although *pso2*Δ was sensitive to nitrogen mustard as shown by Barber et al. 2005, they also showed that *pso2*Δ behaved epistatic with both *rad1*Δ and *rad4*Δ. These results for formaldehyde sensitivity suggest a possible separation of function of the NER proteins Rad1 and Rad4 respective to their roles in DPC repair.

4.9.4 Strains containing a deletion in *pso2* in combination with HR genes, *rad51* or *rad52,* display a different sensitivity to formaldehyde than a strain containing deletions of *pso2* and HR gene *mre11*

As described in the Introduction, Rad51, Rad52 and Mre11 are considered to be in the same epistasis group. Therefore, it would be expected that yeast strains containing deletions in these genes display similar survival rates. However, as shown in Fig. 26C-D the double mutants of *pso2*Δ*rad51*Δ and *pso2*Δ*rad52*Δ display a suppression of phenotype compared to the single mutants *rad51*Δ and *rad52*Δ*.* In other words, the *pso2* deletion appears to suppress the significant formaldehyde sensitivity seen with the *rad51*Δ and *rad52*Δ strains. In contrast, the double mutant *pso2*Δ*mre11*Δ does not display this suppression effect, but rather shows no effect (Fig. 26E). Caution needs to be taken when making conclusions, as no survival of the *pso2*Δ*mre11*Δ was seen at formaldehyde exposures greater than 1.5 mM. What is clear is that there is a difference

in the survival when *pso2*Δ*mre11*Δ is compared to *pso2*Δ*rad51*Δ and *pso2*Δ*rad52*Δ, suggesting a separation of function of Mre11 and Rad51 and Rad52 (i.e. not typical HR) after exposure to formaldehyde.

4.9.5 A *rad4*Δ*rad18*Δ strain exhibits a significant additive response

The deletion of *rad4*Δ confers moderate sensitivity to formaldehyde under chronic conditions, while *rad18*Δ exhibits wild-type levels of survival (Fig. 13, 14, 25 and 26F). On the contrary, the double mutant *rad4*Δ*rad18*Δ is very sensitive by spot assay to formaldehyde under both conditions (Fig. 25).

For a more detailed assessment of this strain deficient in both NER and TLS a colony forming ability assay was performed on *rad4*Δ*rad18*Δ (Fig. 26F). Consistent with the spot assay, *rad18*Δ shows no sensitivity and *rad4*Δ shows sensitivity at higher chronic concentrations. The double mutant *rad4*Δ*rad18*Δ is very sensitive under chronic exposure conditions. The spot assay suggests that this is also the case under acute exposure conditions (Fig. 25). These results suggest a possible compensatory or epistatic relationship. Either outcome is of interest because this suggests that these proteins work on the same substrate, or that *rad18*Δ has a compensatory role in relation to *rad4*Δ, possibly linking the two together. The sensitivity of this double mutant under acute exposure conditions could be explained due to the sensitivity of *rad4*Δ alone, either suggesting that Rad18 is not involved at all, or at least not resulting in an additive survival. Therefore, it is recommended that the experiment be repeated, and performed at lower acute concentrations to establish whether or not the interaction is compensatory or epistatic.

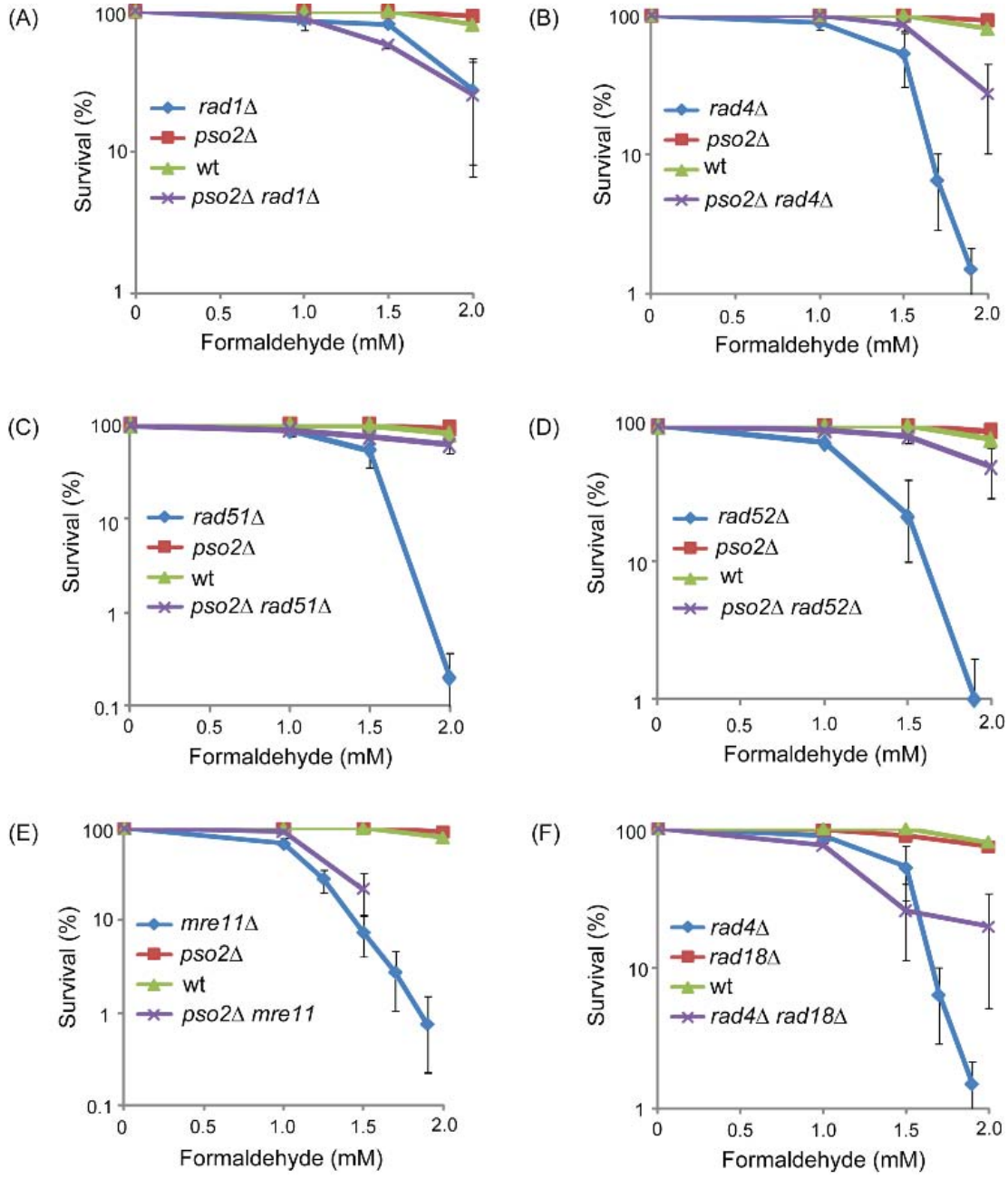

Figure 26. Survival of double mutant strains after different chronic formaldehyde exposure conditions. (A) *pso2*Δ*rad1*Δ (B) *pso2*Δ*rad4*Δ (C) *pso2*Δ*rad51*Δ (D) *pso2*Δ*rad52*Δ (E) *pso2*Δ*mre11*Δ (F) *rad4*Δ*rad18*Δ The strains shown were grown exponentially in YPD media, counted, and $2x10^7$ cells were used plated onto an agar plate and grown for 2 days at 30°C in the presence of various chronic concentrations of formaldehyde. All the experiments were performed in duplicate on independent days, and repeated at least 3 times.

4.10 Epistasis analyses of NER, HR and TLS repair mutants

The results described in the previous sections indicate an important role for HR in tolerating chronic formaldehyde exposure, NER for acute exposure, and involvement of some of the TLS repair proteins. Therefore, particular focus was made on creating double mutants missing a gene in NER, with either HR or TLS. This was done to study epistatic relationships and possible novel interactions of certain DNA repair proteins. Based on the findings of the genome-wide screen, these studies focused on *rad1*Δ, *rad4*Δ, *rad5*Δ, *rad51*Δ, *rad52*Δ, *sgs1*Δ and *top3*Δ. Using the PCR disruption method, nineteen double mutants were constructed. The yeast strains are listed in Table 10 and the deletions were confirmed by PCR and sequencing.

The double mutants were initially tested for sensitivity to formaldehyde by spot assay experiments. The strains shown were grown exponentially in YPD media, counted, and $2x10^7$ cells were serially diluted (10-fold dilution per spot shown decreasing left to right), spotted onto an agar plate and grown for 2 days at 30°C. In Fig. 27-28, the left panel is unexposed (control; no formaldehyde) and the middle panel shows the same strains grown in the presence of 1.5 mM formaldehyde, the right panel the same strains after 60 mM formaldehyde for 15 minutes.

Confirmed Doubles	Genotype	Source (Ref)
rad1Δmre11Δ	*BY4741 with rad1::kanMX4::mre11::URA3*	This Study
rad4Δrad51Δ	*BY4741 with rad4::kanMX4::rad51::URA3*	This Study
rad4Δmre11Δ	*BY4741 with rad4::kanMX4::mre11::URA3*	This Study
rad5Δrad1Δ	*BY4741 with rad5::kanMX4::rad1::URA3*	This Study
rad5Δrad4Δ	*BY4741 with rad5::kanMX4::rad4::URA3*	This Study
rad5Δrad51Δ	*BY4741 with rad5::kanMX4::rad51::URA3*	This Study
rad5Δmre11Δ	*BY4741 with rad5::kanMX4::mre11::URA3*	This Study
sgs1Δrad1Δ	*BY4741 with sgs1::kanMX4::rad1::URA3*	This Study
sgs1Δrad4Δ	*BY4741 with sgs1::kanMX4::rad4::URA3*	This Study
sgs1Δrad5Δ	*BY4741 with sgs1::kanMX4::rad5::URA3*	This Study
sgs1Δrad51Δ	*BY4741 with sgs1::kanMX4::rad51::URA3*	This Study
sgs1Δrad52Δ	*BY4741 with sgs1::kanMX4::rad52::URA3*	This Study
sgs1Δmre11Δ	*BY4741 with sgs1::kanMX4::mre11::URA3*	This Study
top3Δrad1Δ	*BY4741 with top3::kanMX4::rad1::URA3*	This Study
top3Δrad4Δ	*BY4741 with top3::kanMX4::rad4::URA3*	This Study
top3Δrad5Δ	*BY4741 with top3::kanMX4::rad5::URA3*	This Study
top3Δrad51Δ	*BY4741 with top3::kanMX4::rad51::URA3*	This Study
top3Δrad52Δ	*BY4741 with top3::kanMX4::rad52::URA3*	This Study
top3Δmre11Δ	*BY4741 with top3::kanMX4::mre11::URA3*	This Study

Table 10. The list of double mutants developed during this study. A total of nineteen double mutants were created as described in the Material & Methods. All the double mutants made in this study have been PCR and sequenced confirmed. The double mutants are described as the deletion strain used from the library listed first, and the PCR deleted gene second.

4.10.1 Spot assay analyses suggest that *top3Δ* is epistatic to *rad5Δ, rad51Δ* and *rad52Δ*, but displays additivity with *rad4Δ*

As described in previous sections Top3 is a DNA Topoisomerase, and functions in a complex with Sgs1 and plays an important role in recombination. Due to the observed sensitivity of *top3*Δ (Fig. 14) the role of this topoisomerase in tolerating formaldehyde was studied more closely by creating several double mutants (Table 10). The spot assay analysis after chronic and acute formaldehyde exposure in Fig. 27A and B suggest that *top3* is epistatic to *rad51, rad52* (* Fig. 27A) and *rad5* (* Fig 27B). This result is expected with *top3*Δ*rad51*Δ (* Fig. 27A) *and top3*Δ*rad52*Δ (* Fig. 27A) as these genes have classically been defined to the same Rad51 epistasis group and therefore both proteins would act on the same substrate. The possible epistatic relationship between *top3*Δ and *rad5*Δ (* Fig. 27B) can be explained by the fact that Rad5 has been implicated in stalled replication fork resolution. The additivity observed in the *top3*Δ*rad4*Δ (* Fig. 27B) was expected as both genes have been classified to different epistasis groups. A moderate increase in formaldehyde sensitivity compared to the corresponding single mutants is observed in the *rad5*Δ*rad51*Δ (* Fig. 27A) strain suggesting a possible role for *rad5*Δ independent of HR.

4.10.2 Spot assay analyses suggest that *sgs1Δ* suppresses the phenotype of *rad4Δ*, but that *sgs1Δ* has no effect on *rad51Δ, rad52Δ* and *rad5Δ* after formaldehyde exposure

As both Top3 and Sgs1 work together in a complex in the cell, it was also of interest to study the survival of yeast deletion strains *rad4*Δ*, rad5*Δ*, rad51*Δ and *rad52*Δ in combination with *sgs1*Δ*.* Interestingly, very different results were found compared with the *top3*Δ double deletion strains. When *sgs1* is deleted with *rad4* (# Fig. 27B)*,* it appears that *sgs1*Δ slightly suppresses the sensitive phenotype of *rad4*Δ. Another HR deletion strain, *rad51*Δ*,* also partially suppresses the *rad4*Δ (# Fig. 27A) phenotype after an acute formaldehyde exposure.

Based on the spot assay analysis no significant difference in survival after chronic or acute formaldehyde exposure is observed of the *sgs1*Δ*rad51*Δ (# Fig. 27A), *sgs1*Δ*rad52*Δ (# Fig. 27B)*,* and *sgs1*Δ*rad5*Δ (# Fig. 27B) strains compared to the corresponding single mutants. The conclusion that can be made from this is that no suppression or additivity is observed. Further colony forming ability experiments will need to be performed to confirm these results.

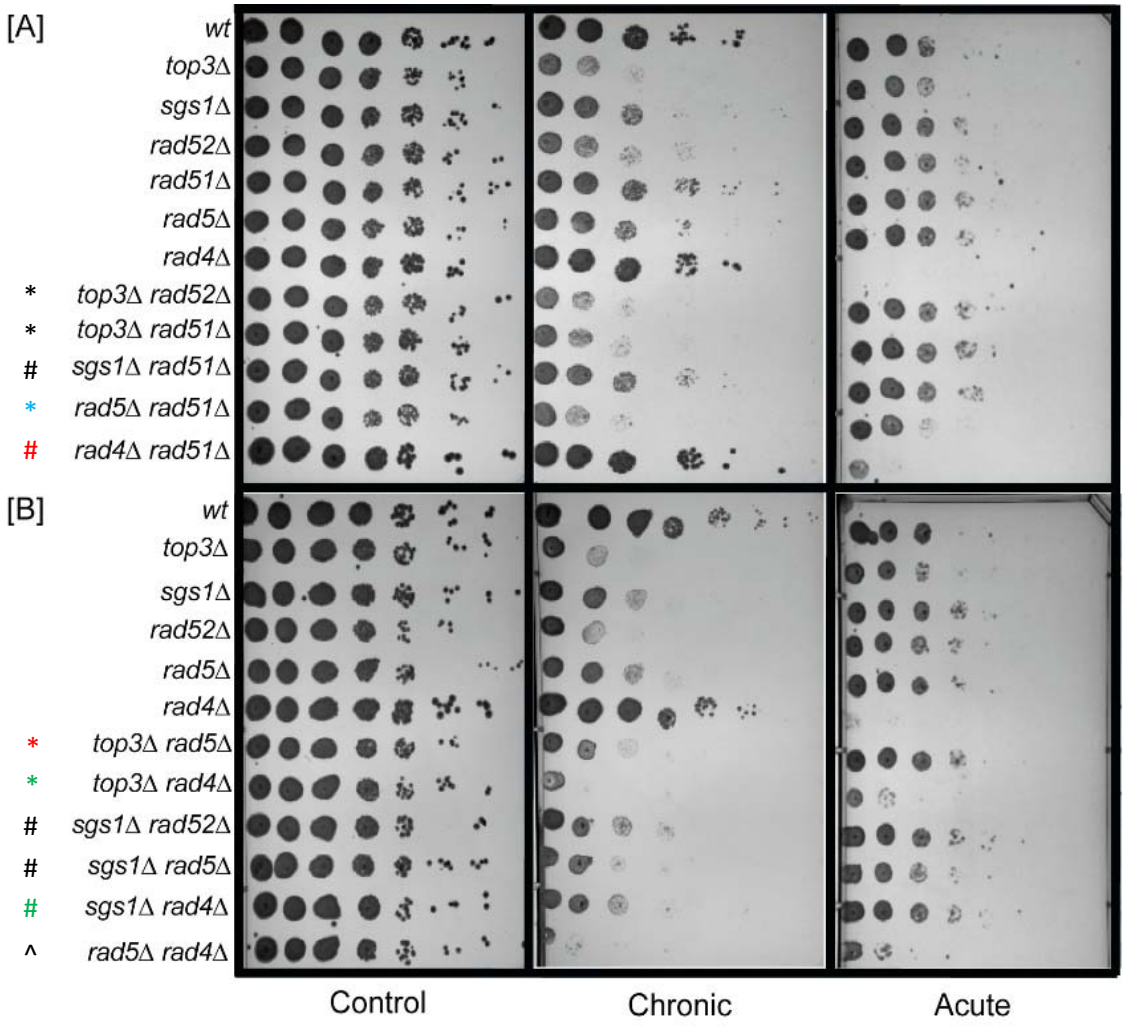

Figure 27. Spot assay on double mutants created in this study with the corresponding controls. The strains shown were grown exponentially in YPD media, counted, and 2x10^7 cells were serially diluted (10-fold dilution per spot shown decreasing left to right) were spotted onto an agar plate and grown for 2 days at 30°C. In each case, the left panel is unexposed (no formaldehyde) and the middle panel shows the same strains grown in the presence of 1.5 mM formaldehyde, the right panel the same strains after 60 mM formaldehyde for 15 minutes.

4.10.3 Spot assay analyses suggest that *rad1Δ* exhibits additivity with *top3Δ* and *sgs1Δ*, but *rad5Δ* suppresses the *rad1Δ* and *rad4Δ* phenotype

Because *rad4*Δ exhibits additivity with *top3*Δ and its sensitivity is suppressed by *sgs1*Δ under acute conditions, the survival of strains containing a deletion in *rad1* with *top3, sgs1* and *rad5* was further studied. As can be seen in Fig. 28A, the *top3*Δ*rad1*Δ and *sgs1*Δ*rad1*Δ strains become very sensitive to formaldehyde; this appears to be at least one order of magnitude greater than the single mutant *rad1*Δ. Interestingly, when the genes *rad1* and *rad5* are deleted in the same cell, the sensitivity seen in the *rad1*Δ is partially suppressed (Fig. 28A). This suggests that when Rad5 is present in the cell it can act on the formaldehyde induced DNA damage, and results in a product that normally is processed by Rad1 (i.e. NER). Now that these genes are deleted this process (that resulted in a toxic intermediate) does not occur anymore, therefore suppression of the phenotype is observed.

On the other hand, the *rad5*Δ*rad4*Δ (^ Fig. 27B) strain shows partial suppression compared of the *rad4*Δ phenotype, but this is only observed under acute conditions. Interestingly for *rad5*Δ*rad4*Δ, an additive response under chronic conditions can be observed (^ Fig. 27B). The difference in survival rates of *rad1*Δ and *rad4*Δ hints at independent roles for both proteins after formaldehyde exposure in relationship to *rad5*Δ.

4.10.4 Spot assay analyses suggest that Mre11 exhibits an epistatic relationship with Rad1, Rad5, Sgs1 and Top3, but suppression of the *rad4Δ* phenotype is observed

With *mre11* being an important gene in all of the different HR pathways (see introduction), and consistently being sensitive to chronic formaldehyde exposure, its role in tolerating formaldehyde was studied more closely by also deleting *rad1, rad4, rad5, sgs1* and *top3*. The spot assay results in Fig. 28B suggest that there might be an epistatic relationship between *mre11Δ* with *rad1Δ, rad5Δ, sgs1Δ* and *top3Δ*. The possible epistatic relationship with *sgs1Δ* and *top3Δ* is no surprise as these have already been classified to the same epistasis group (see introduction). In addition, *rad5* and *mre11* have also been linked together (Chen, Davies et al. 2005). In that study the authors state that both Mre11 and Rad5 contribute to DSB repair by means of HR, but that this repair is Rad52- and Ku-independent.

Interestingly, the phenotype of *mre11*Δ under chronic conditions is suppressed by deleting *rad4* in a *mre11*Δ background. Under acute formaldehyde exposure *mre11*Δ suppresses the *rad4*Δ phenotype. This is the first time we observe a clear separation in sensitivity of both Rad1 and Rad4. A summary of the genetic interactions resulting from the spot assay is provided in Table 11.

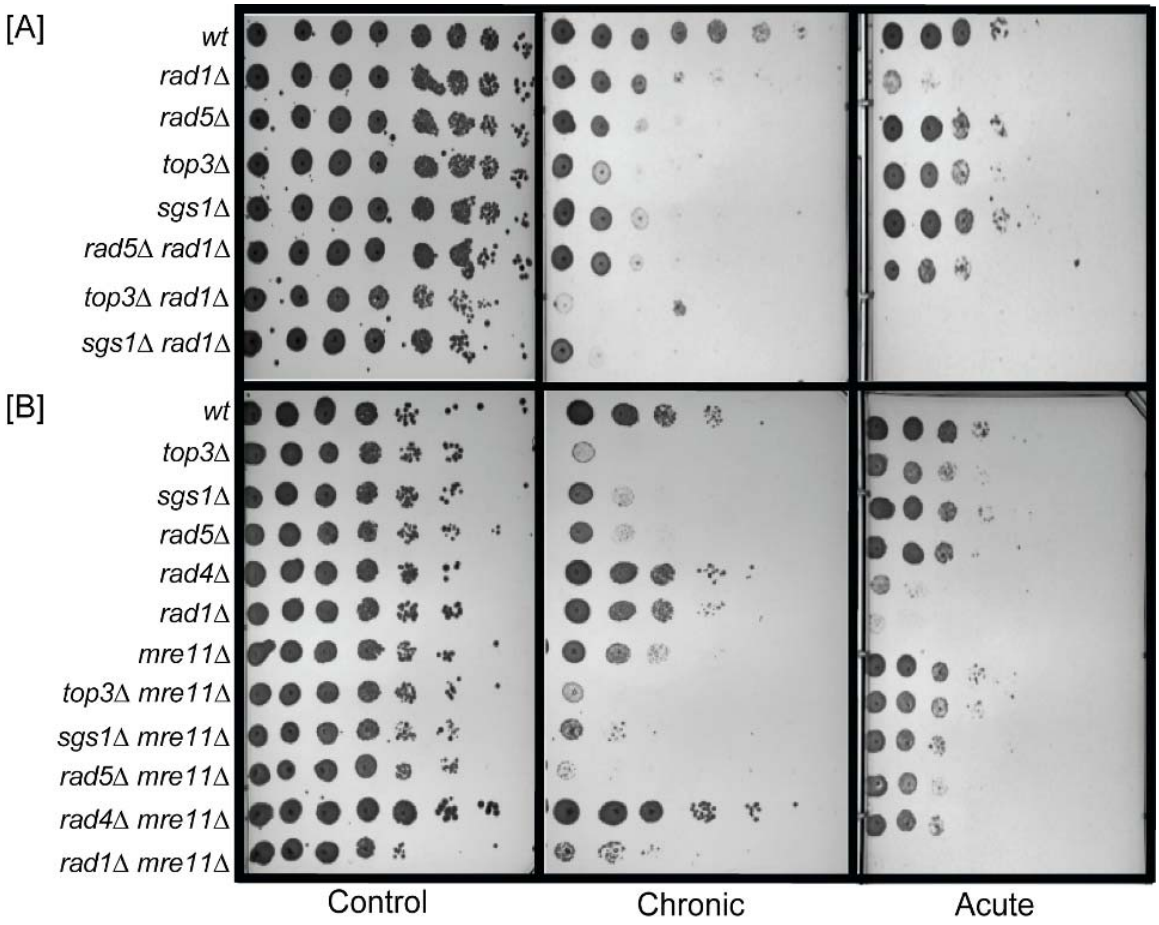

Figure 28. Spot assay on double mutants created in this study with the corresponding controls. The strains shown were grown exponentially in YPD media, counted, and $2x10^7$ cells were serially diluted (10-fold dilution per spot shown decreasing left to right) were spotted onto an agar plate and grown for 2 days at 30°C. In each case, the left panel is unexposed (no formaldehyde) and the middle panel shows the same strains grown in the presence of 1.5 mM formaldehyde, the right panel the same strains after 60 mM formaldehyde for 15 minutes.

	Spot Assay		
Confirmed Doubles	Epistatic	Additive	Suppression
*rad1*Δ *mre11*Δ	?		
*rad1*Δ *rad52*Δ*		x	
*rad4*Δ *rad51*Δ			x
*rad4*Δ *rad52*Δ*		x	
*rad4*Δ *mre11*Δ			x
*rad5*Δ *rad1*Δ			x
*rad5*Δ *rad4*Δ			?
*rad5*Δ *rad51*Δ		?	
*rad5*Δ *mre11*Δ	?		
*sgs1*Δ *rad1*Δ		x	
*sgs1*Δ *rad4*Δ			x
*sgs1*Δ *rad5*Δ	?		
*sgs1*Δ *rad51*Δ			x
*sgs1*Δ *rad52*Δ			x
*sgs1*Δ *mre11*Δ			x
*top3*Δ *rad1*Δ		x	
*top3*Δ *rad4*Δ		x	
*top3*Δ *rad5*Δ	?		
*top3*Δ *rad51*Δ	?		
*top3*Δ *rad52*Δ	?		
*top3*Δ *mre11*Δ	?		

Table 11. Summary of genetic Interactions based on the spot assay of double mutants after formaldehyde exposure. Strains marked with an asterisk (*) are the strains from Dr. McHugh. A question mark (?) suggests that this relationship might be going on. The X suggests that more definitively this relationship is going on.

4.11 Survival of NER and HR double mutants after chronic formaldehyde exposure

As a spot assay only informs you about the relative survival compared to wild-type and single mutants, a colony forming ability assay was performed on the double mutants *rad1*Δ*mre11*Δ*, rad4*Δ*mre11*Δ*, rad1*Δ*rad52*Δ*, rad4*Δ*rad52*Δ for a more detailed analyses. The study on these mutants is interesting because 1) Rad1 has been shown to have a role in both NER and HR, 2) Rad4 has been known to be involved in NER only, 3) Mre11 is thought to be involved in many pathways, but not NER and 4) Rad52 had been defined as a classical HR protein.

The yeast strains shown were grown to exponential phase in YPD media, counted, and $2x10^7$ cells were diluted resulting in 20-300 cells that were plated onto 0 – 2.0 mM formaldehyde containing plates and grown for 2-3 days at 30°C.

4.11.1 *rad1Δ* and *rad4Δ* exhibit slight differential in a *rad52Δ* background after chronic formaldehyde exposure

As predicted by the spot assay on *rad1*Δ*rad52*Δ and *rad4*Δ*rad52*Δ (Fig. 25), *rad1*Δ*rad52*Δ is more sensitive to formaldehyde under chronic conditions (Fig. 29; top half). Although it needs to be pointed out that in both cases this appears to be an additive response. The slight increase in additivity seen in the *rad1*Δ*rad52*Δ strain could be explained by the fact that Rad1 also plays a role in HR (see introduction). In other words, now two genes in HR are missing and one in NER (*rad1*Δ*rad52*Δ) versus one missing gene in HR and one missing gene in NER (*rad4*Δ*rad52*Δ). Hence you would not see true epistasis in the *rad1*Δ*rad52*Δ strain, due to the importance of both NER and HR in tolerating formaldehyde exposure.

4.11.2 Rad1 could be epistatic or synergistic to Mre11, but *rad4Δ* suppresses the sensitivity of *mre11Δ* under chronic exposure conditions

Interestingly, a difference in sensitivity in the double mutants of *rad1*Δ*mre11*Δ and *rad4*Δ*mre11*Δ is observed. The *rad1*Δ*mre11*Δ double mutant could be epistatic or a synergistic because *rad1*Δ shows only moderate sensitivity under these exposure conditions (Fig. 29; bottom half). The finding that *rad1*Δ and *mre11*Δ could be epistatic can be explained by the fact that *rad1*Δ had been shown to have a role in HR (see introduction). It is recommended to repeat this experiment at higher concentrations in order to clarify the pathway relationship of these genes.

On the other hand the rescue phenotype of *rad4*Δ*mre11*Δ back to wild-type survival levels, suggests that when both Rad4 and Mre11 are present in the cell under chronic exposure conditions it is not advantageous and in fact, attempted processing of formaldehyde-induced DNA adduct by *mre11*Δ and *rad4*Δ may produce a cytotoxic intermediate in processing DPCs.

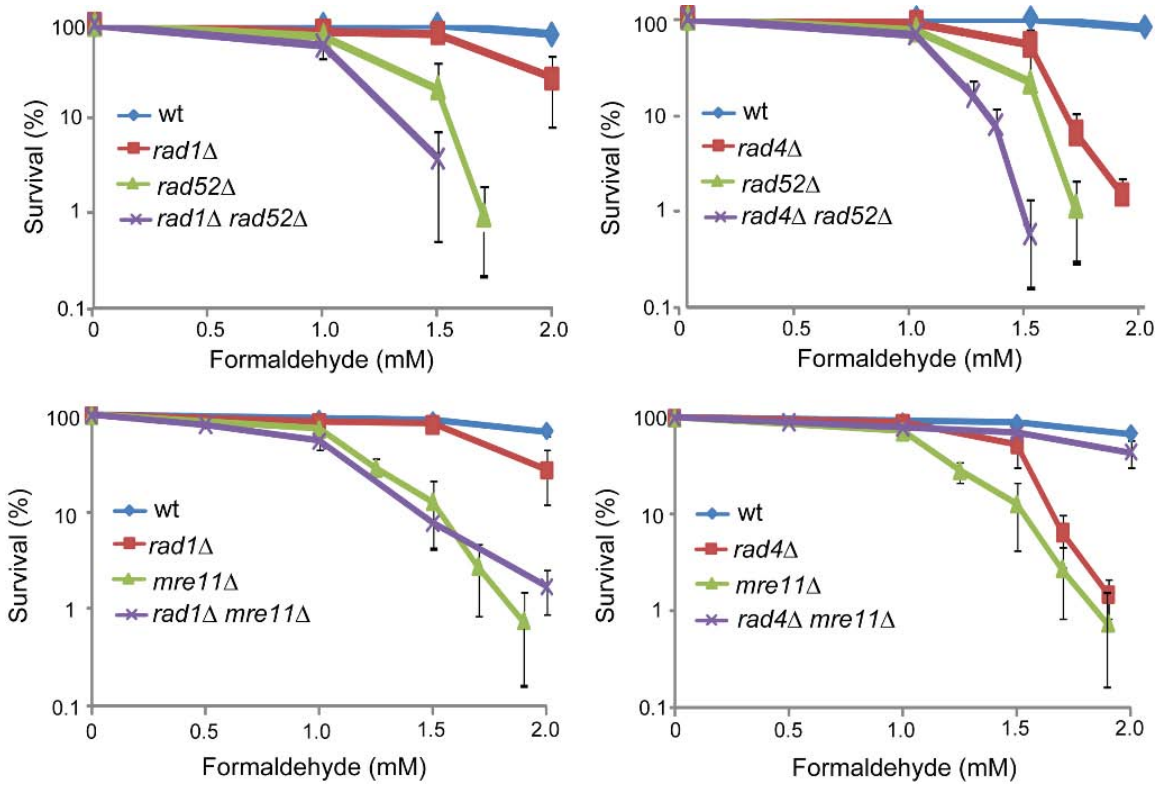

Figure 29. Colony forming ability of NER/HR double mutant strains after chronic formaldehyde exposure. The strains shown were grown exponentially in YPD media, counted, and $2x10^7$ cells were serially diluted to have in between 20-300 cells on the plates. The same amount of cells were put on YPD agar plates containing the indicated formaldehyde concentration, and grown for 2-3 days at 30°C. All the experiments were performed in duplicate on independent days, and repeated at least 3 times.

4.12 Colony forming ability of HR NER double mutants after acute formaldehyde exposure

In addition to the chronic formaldehyde exposure condition, the same mutants strains shown in Fig. 29 were tested under acute conditions. The strains were grown exponentially in YPD media, counted, exposed, washed and $2x10^7$ cells were serially diluted to have in between 20-300 cells on the plates. The same amount of cells were put on YPD agar plates containing the indicated formaldehyde concentration and grown for 2-3 days at 30°C.

4.12.1 *rad1Δ*, but not *rad4Δ*, exhibits additivity to *rad52Δ* after acute formaldehyde exposure

After an acute formaldehyde exposure no colonies were observed with the *rad1*Δ*rad52*Δ yeast strain at concentrations > 40 mM. The *rad4*Δ*rad52*Δ strain is also very sensitive to formaldehyde, the only difference being that at higher concentrations (>20 mM) around a 5% survival rate was observed (Fig 30; top half). One explanation for these results could be due to the presence of formaldehyde resistant colonies. Interestingly, *rad52*Δ consistently appears to tolerate acute formaldehyde exposure better than wild-type, but when *rad1* is also deleted additivity is observed. This would suggest that even though *rad52*Δ survives better than wild-type under these conditions, it is still very important.

4.12.2 Rad1 might exhibit epistasis to Mre11, but *mre11Δ* suppresses the *rad4Δ* phenotype

After an acute exposure the survival of *rad1*Δ*mre11*Δ suggests epistasis (Fig. 30; bottom half). Although a slight increase in sensitivity is seen in the last data point, this probably only reflects the variability in this experimental system. In addition, possible epistasis was also observed under chronic exposure conditions, and both Rad1 and Mre11 have been implicated in HR (also suggesting epistasis; see introduction). Further hinting at the fact that Rad1 and Rad4 have a different function when it comes to tolerating formaldehyde is the fact that the *rad4*Δ*mre11* yeast strain displays wild-type survival. In other words, deleting *mre11* in a *rad4*Δ background results in suppression of the phenotype after acute exposure conditions (Fig. 30; bottom half)

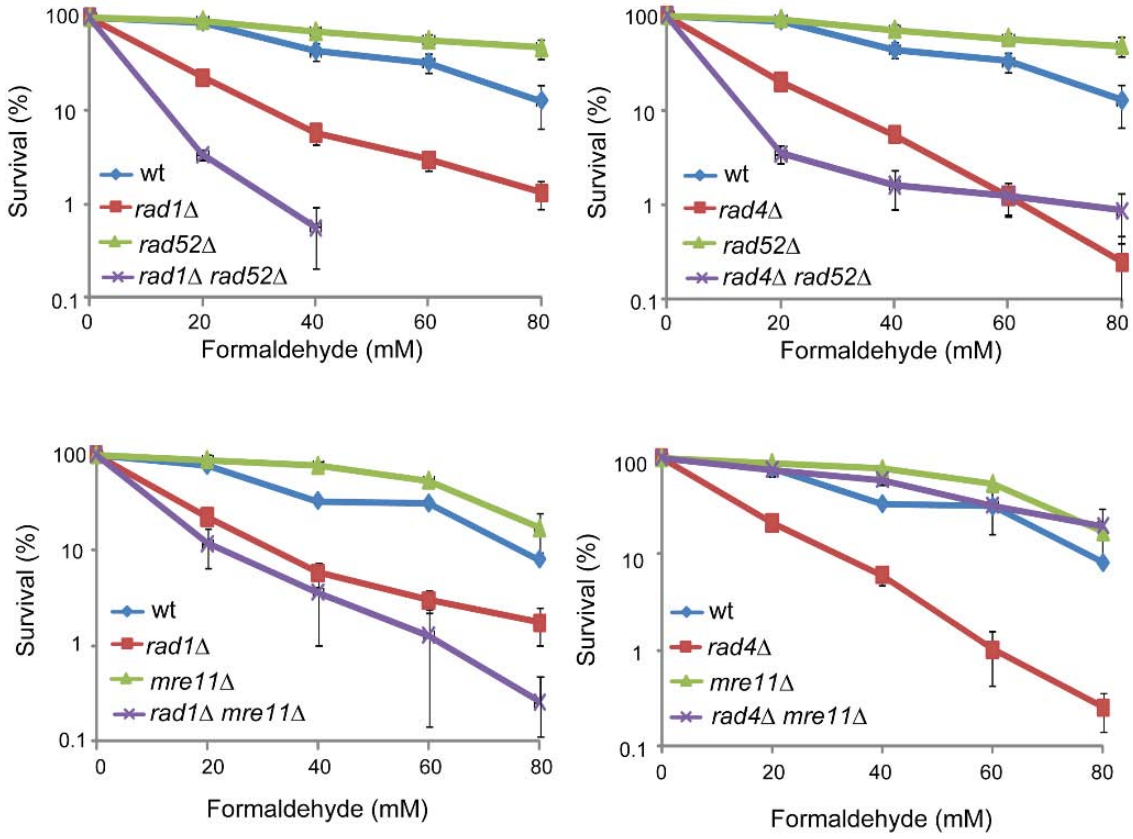

Figure 30. Colony forming ability of NER/HR double mutants strains after acute formaldehyde exposure. The strains shown were grown exponentially in YPD media, counted, and $2x10^7$ cells were serially diluted to have in between 20-300 cells on the plates. The same amount of cells were put on YPD agar plates containing the indicated formaldehyde concentration, and grown for 2-3 days at 30°C. All the experiments were performed in duplicate on independent days, and repeated at least 3 times.

4.13 DNA strand breaks in *rad4*Δ*rad52*Δ double mutant

Based on the survival data in the previous section, PFGE experiments were performed on *rad4*Δ*rad52*. This yeast strain was chosen to be studied due to the fact that 1) Rad4 only has a role in NER 2) Rad52 only has a role in HR. In addition, this double mutant is very sensitive to formaldehyde under both chronic and acute conditions, suggesting the possibility of the presence of SSBs and/or DSBs.

Interestingly, and in contrary to the strand break experiments shown in Fig. 18-20, where no increase in SSBs or DSBs were observed after formaldehyde exposure in the single mutants of *rad4*Δ*, mre11*Δ and *rad52*Δ*,* both SSBs and DSBs are observed in the *rad4*Δ*rad52*Δ double mutant. The DNA breaks appear to persist for up to 6 hours and go away at 8 hours (Fig. 31).

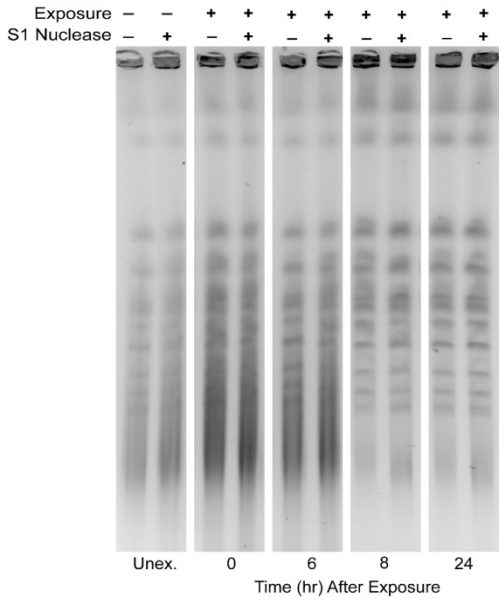

Figure 31. Pulsed field gel electrophoresis on the *rad4*Δ*rad52*Δ double mutant. α-mating factor arrested cells ($2x10^8$ cells) were exposed to 60 mM formaldehyde for 15 min. Time (hr), is the time given for the cells to recover after exposure.

5. Discussion

In addition to being the first genome-wide screen examining formaldehyde cytotoxicity, this study highlights that the exposure conditions (duration and concentration) can significantly affect the spectra of gene deletion strains that are identified as sensitive or resistant. These data clearly demonstrate a differential pathway response to chronic versus acute formaldehyde exposures and may have significance for risk extrapolation in human exposure studies. It is well recognized that the validity of such extrapolations can be challenged by the capacity of cells to activate or detoxify chemicals, as well as the steady-state DNA repair capacity to effectively remove damages as they occur. Consistent with dose-dependent pathway responses, rat formaldehyde inhalation studies have shown steep dose-dependent transitions in both cell proliferation assays and tumorigenesis (Monticello, Swenberg et al. 1996). An increased understanding of the molecular basis of transitions in the cellular response pathways may elucidate details for models of dose-dependent transitions in mechanisms of formaldehyde toxicity. The results of the work reported here will be further discussed in detail in the next session.

5.1 Homologous recombination protects against chronic formaldehyde exposure*

Interestingly, *S. cerevisiae* cells respond in fundamentally different ways to the induction of DPCs that are dependent on whether the exposure to formaldehyde was given as a chronic, low dose or acute, high dose. Following chronic exposure, cell

survival is conferred by proteins of the HR pathway including Rad50, Rad51, Rad52, Rad54, Rad55, Mre11 and Xrs2, as well as proteins associated with the resolution of stalled replication fork structures, such as Sgs1, Top3 and Rad5. These data suggest that successful tolerance and/or repair of DPCs proceed *via* intermediates that are substrates for HR. In comparison, only moderate sensitivity was observed in the NER deletion strains, suggesting a less critical contribution of NER to survival following chronic exposure. It is possible that after such formaldehyde exposures, DPCs encountered in the leading and lagging strands during DNA synthesis are dealt with in different manners. DPCs on the leading strand may block progression of the helicase complex that precedes the replisome and thus, replication complexes may stall well in advance of either polymerase reaching the site of the DNA adduct. This may allow for NER repair of the DPC and possibly explain the moderate sensitivity in the NER deletion strains. On the lagging strand, stalled replication complexes may temporarily uncouple synthesis in the two strands preventing no further lagging strand synthesis. At this point, fork regression could occur, leading to a recombinogenic structure, which when processed could effectively allow for damage tolerance and replication restart. At this time, the exact nature of these intermediates remains undetermined; however, DSBs and SSBs have not been detected following chronic exposures.

As shown in Fig. 14B, strains deficient in *mre11*Δ and *rad52*Δ show less sensitivity to formaldehyde than the wild-type strain. The enhanced resistance to formaldehyde is seen consistently in the *mre11*Δ and *rad52*Δ deletion strains, suggesting that under the acute conditions, HR is not advantageous and in fact, attempted processing of formaldehyde-induced DNA adducts by HR may produce a cytotoxic intermediate or interfere with NER processing of the DPCs. In addition, we

observed no sensitivity in either chronic or acute exposure conditions of the NHEJ genes Ku70 and Ku80, suggesting that repair does not go through NHEJ.

5.2 Nucleotide excision repair protects cells from acute formaldehyde exposure*

In the case of acute short-term exposures, cellular survival strategies appear to switch to an NER-dependent response, with Rad1, Rad4, and Rad14 all having essential roles in conferring enhanced survival. These data suggest that the signal to transition from an HR-dependent pathway to an NER-dependent pathway is related to the rapid induction of a large number of DPCs.

5.3 The role of nucleotide excision repair in the removal of formaldehyde-induced DPCs*

Based on our data showing similar rates of DPC removal in wild-type or NER-deficient arrested cells (Fig. 17), we suggest that under acute high-dose exposure, proteolytic degradation precedes NER recognition and is not initiated by stalled replication forks (replication-independent). This replication-independent, NER-dependent model for DPC repair is consistent with data reported for *E. coli* (Nakano, Morishita et al. 2007). In addition, the similar rates of removal of DPCs as measured in Fig. 17 in the WT and NER-deficient strains may be indicative of a pre-NER processing event that degrades the protein crosslink to a peptide which is not detectable by this assay.

5.4 Formation of DNA breaks as intermediates in the processing of DPCs*

Our data reveal that SSBs can be readily detected under acute exposure conditions and accumulate post-exposure, indicating that the breaks are an intermediate in the processing of DPCs. The formaldehyde-induced SSBs appear to be NER-dependent as the breaks do not accumulate post-formaldehyde exposure in *rad4Δ* or *rad1Δ* strains, suggesting that these SSBs are intermediates generated during the repair process. These results are consistent with a previous report showing a Rad3-dependent accumulation of SSBs following formaldehyde exposure (Magana-Schwencke, Ekert et al. 1978). PFGE analyses also suggest that some SSBs may be dependent on the presence of Mre11 (Fig. 19).

During the course of this study, two reports demonstrated a possible role for the HR pathway in the tolerance of DPCs (Nakano, Morishita et al. 2007; Ridpath, Nakamura et al. 2007). In both the *E. coli* study and the study using isogenic DT40 chicken cell lines, the authors proposed models for DPC processing proceeding *via* DSB intermediates. Our data demonstrating a prominent role for HR also led us to speculate that DSBs were a likely intermediate in a DPC processing pathway. However, PFGE analyses and neutral centrifugation analyses showed no detectable DSBs following exposure to formaldehyde, even in a HR-deficient background (Fig. 18-19). These data are consistent with PFGE analyses in *E. coli* and comet assays performed on V79 Hamster cells following formaldehyde exposure (Speit, Schutz et al. 2000; Nakano, Morishita et al. 2007).

Interestingly, DSBs are also not detected in cells treated with thymidine, which is known to block DNA replication. Previously, it has been shown that mammalian cells respond to hydroxyurea replication arrest differently than thymidine block (Lundin, Erixon et al. 2002), such that hydroxyurea induced DSBs and the repair of the strand breaks proceeded *via* both the NHEJ and the HR pathways. In contrast, thymidine arrest involved only HR and had no detectable DSBs. These authors concluded that the cytotoxic lesion following thymidine treatment was not persistent or transient DSBs, but a different, yet undetermined substrate for HR (Lundin, Erixon et al. 2002). Additionally, an alternative non-DSB dependent model may be that HR is initiated at a DNA nick or single-strand gap generated during replication past, or polymerase stalling at, a DPC lesion. The plausibility of this model is supported by the observation that for most mitotic HR in *S. cerevisiae*, the initiating lesion is not a DSB but potentially a SSB or single-strand gap (Lettier, Feng et al. 2006). This is consistent with our data demonstrating a lack of DSBs, but an accumulation of SSBs following formaldehyde exposure. It should be noted that our observations cannot rule out the possibility that a small amount of transient DSBs may occur which are not detectable by our methods. It is possible that the single-strand break repair pathway may have a role in preventing the cytotoxicity of DPC processing intermediates. However, the rad27 (Fen1) deletion strain was not sensitive to formaldehyde under chronic or acute conditions (Fig. 15). We cannot definitely rule out a role for this pathway since the genes for the major ligase (*cdc9*) and the polymerase (*pol delta*) for this pathway are essential and were not included in our screen.

5.5 Cellular response to other DPC-inducing agents

Etoposide and camptothecin, both topoisomerase inhibitors, were used to study the generality of the responses observed for the deletion strains identified in the formaldehyde screen. The survival of HR and NER deletion strains after formaldehyde, camptothecin and etoposide exposure were compared, and demonstrated that the spectrum of genes sensitive to formaldehyde is a specific response to this DNA damaging agent (Fig. 13 and 21). HR deletion strains were sensitive to all three agents, suggesting a role for HR in a more general response to DNA damaging agents (Bennett, Lewis et al. 2001). However, the interplay of NER and HR and the role of NER is not important for tolerating camptothecin exposure.

The lack of sensitivity of the NER deletion strains to camptothecin is consistent with previous data that have shown that Tdp1 is the regulatory element controlling resistance to chronic camptothecin exposure (Vance and Wilson 2002). This study showed that the *rad1*Δ and *tdp1*Δ strains were not sensitive to camptothecin, and that Rad1-Rad10 endonuclease serves as an alternative (backup) pathway for Tdp1. Thus, sensitivity of NER deletion strains to camptothecin would only be observed when Tdp1 was also deleted. Although modest sensitivity of these strains after chronic formaldehyde exposure, and a high sensitivity after acute formaldehyde exposure were observed, the role of NER in formaldehyde resistance could be further explored by also deleting *tdp1.* Enhanced sensitivity of the *rad1*Δ and *rad10*Δ strains after chronic formaldehyde exposure would be expected in the *tdp1*Δ background.

Consistent with our data, in a yeast genome-wide screen for camptothecin sensitivity (16 hr, 50 μM exposure) HR deletion strains were among the most sensitive

(Deng, Brown et al. 2005). In addition, Mre11 and Slx4 were shown to be important for the resistance to camptothecin. Interestingly, the *mre11-H125N* nuclease deficient strain was very sensitive to camptothecin. This is in contrast with the findings of the formaldehyde study, as no increase in sensitivity of the *mre11-H125N* strain after either chronic or acute formaldehyde exposure was observed (Fig. 22).

An etoposide-sensitivity yeast genome-wide screen has also been performed and showed that Mms22, Rtt101, Rtt107 and the HR proteins, Mre11, Xrs2, Rad50, Rad51, Rad52, Rad54, Rad55 and Rad57 play an important role in the tolerance of etoposide-induced damage (Baldwin, Berger et al. 2005). In our study, no enhanced sensitivity of Rtt101, Rtt107 and Rad57 to formaldehyde was observed.

As described in earlier sections, etoposide covalently links a Top2 subunit to the 5' phosphoryl ends of the broken DNA, whereas camptothecin results in the linking of topoisomerase I (Top1) to the 3' ends of broken DNA. Both these crosslinks are highly specific, and for formaldehyde a more random spectrum of crosslinking of proteins is expected. Indeed, Qui et al. 2009 showed by mass spectrometry on HL-60 human acute promyelocytic leukemia cells after an acute formaldehyde exposure, that various proteins were crosslinked to the DNA. This included proteins from several pathways, such as proteins involved in DNA binding, nucleotide binding, RNA binding and many others. Interestingly, among the many identified proteins were Ku70, PCNA, Top2, Xrcc6, Xrcc5, Ddb1, Lig1, Parp1 and Rad23. Of course these results, like the results presented here, could be greatly influenced by the exposure conditions, which could result in a different spectrum of proteins identified.

In addition to the formaldehyde data suggesting a heterogeneous population of crosslinked proteins to the DNA, and very specific crosslinks for camptothecin and

etoposide, it should be noted that both topoisomerase inhibitors lead to the formation of DSBs (Chen, Yang et al. 1984; Huang, Traganos et al. 2003). As can be seen in the results (Fig. 18-20), DSBs are not observed after chronic or acute formaldehyde exposure, even in HR deficient strains. The difference in DNA breaks could also help explain the difference in survival of the various strains after exposure to formaldehyde, camptothecin and etoposide. It would be of interest to test other DPC inducing agents like acetaldehyde or chromium(VI).

5.6 The role of Mre11 in the tolerance or repair of DPCs under chronic or acute conditions

The sensitivity screen on the non-essential gene deletion library indicated that HR is very important for the resistance to chronic low dose exposure to formaldehyde; *mre11*Δ in particular, was very sensitive to formaldehyde. Mre11 possesses nuclease activity, and has been implicated in the removal of Spo11 cross-linked to the DNA during meiosis and Top1 crosslinks during mitosis (Deng, Brown et al. 2005; Hartsuiker, Mizuno et al. 2009). In order to test if this nuclease activity is necessary for the processing of formaldehyde-induced DPCs, nuclease deficient mutants (*mre11-H125N*, *mre11-D56N*, *mre11-D56A)* were tested for sensitivity to formaldehyde (Fig. 22). No increase in sensitivity in any of the nuclease deficient strains after formaldehyde exposure was observed, suggesting that the nuclease activity is not necessary to process formaldehyde-induced DPCs. Although the exact role of the Mre11 protein is not known, this study suggests that Mre11 is important, but that the nuclease activity is not necessary, for the tolerance or repair of formaldehyde-induced DNA damage.

In addition to the survival data, the Mre11 nuclease-deficient strains were also studied for the formation of SSBs and DSBs. In concurrence with the *mre11*Δ strain, a slight decrease in SSBs is observed after formaldehyde exposure, and no DSBs are detected (Fig. 23). Taken together these data suggest that *mre11*Δ and/or the nuclease activity may contribute to the formation of SSBs, but that the nuclease activity is not essential for survival.

5.7 Pathway interaction analyses

The HR and NER pathways have been implicated in ICL repair (Grossmann, Ward et al. 2001; Barber, Ward et al. 2005; Bergstralh and Sekelsky 2008). Since yeast strains with a deletion in HR and NER genes were identified as being more sensitive to formaldehyde in our genome-wide screen, it was of interest to see if other ICL-specific pathway members were sensitive to formaldehyde. In addition, double mutants demonstrating ICL-specific relationships were examined.

5.7.1 The role of ICL-repair pathway proteins in formaldehyde resistance

Consistent with the results of the library screen, no sensitivity after exposure to formaldehyde in any of the other ICL repair gene deletions was observed. Interestingly, as seen in Fig. 24, the double mutant *rev3*Δ*rad51*Δ is significantly more sensitive than *rad51*Δ, while *rev3*Δ alone shows wild-type resistance to formaldehyde. This synergistic effect suggests that Rev3 can compensate for the loss of *rad51*Δ to some extent. In contrast, the survival of *rad51*Δ goes back to wild-type levels when *pso2* is knocked out.

This suggests that when Pso2 is present, it contributes to the cytotoxicity in a *rad51*Δ background. The triple mutant of *rev3*Δ*rad51*Δ*pso2*Δ shows no enhanced cell death over the double mutants; here a *pso2* deletion does not rescue *rad51*Δ in the *rev3*Δ background (Fig. 24).

An epistatic relationship between NER and Pso2, has previously been shown in response to nitrogen mustard (Barber, Ward et al. 2005). Therefore the *pso2*Δ*rad1*Δ and *pso2*Δ*rad4*Δ strains were examined for formaldehyde sensitivity. Indeed, an epistatic relationship between *pso2*Δ and *rad1*Δ was observed after chronic formaldehyde exposure. Interestingly, epistasis of *pso2*Δ with *rad4*Δ is not seen following formaldehyde exposure; in fact a *pso2*Δ partially suppresses the *rad4*Δ sensitivity (Fig. 26). A possible explanation could be that the epistatic relationship seen with the *rad1*Δ*pso2*Δ strain could be due to the role of *rad1*Δ in HR, rather than NER. The partial suppression of the phenotype seen with the *pso2*Δ*rad4*Δ mutant maybe due to the fact that a less toxic intermediate is formed during the repair of formaldehyde induced lesions, therefore enhancing survival.

The suppression of phenotype observed in for example the *pso2*Δ*rad4*Δ strain suggests that a compensatory repair mechanism might be responsible for tolerating formaldehyde exposure. One hypothesis is that in the absence of important players of both the HR and NER pathways, a less favorable backup system is initiated i.e. TLS (Rev3). Indeed when the survival of the triple mutant *pso2*Δ*rad51*Δ*rev3*Δ is assessed, suppression of *rad51*Δ is not observed anymore. In fact, the survival is similar to that of *rad51*Δ*rev3*Δ suggesting that Pso2 might not be involved or acts on the same substrate as both Rad51 and Rev3. In order to further elucidate the possible genetic interactions of

these proteins, the *pso2*Δ*rad52*Δ*rev3*Δ*, pso2*Δ*rad1*Δ*rev3*Δ*, pso2*Δ*rad4*Δ*rev3*Δ *or pso2*Δ*mre11*Δ*rev3*Δ strains should be assayed for sensitivity to formaldehyde.

The genetic relationship of Pso2 with HR genes *mre11*, *rad51* and *rad52* was also studied more closely. As Mre11 is a member of the HR epistasis group, it was expected that the double mutants with an HR and *pso2* deletion would show similar survival. Interestingly, the *pso2*Δ*mre11*Δ results in an additive phenotype, and the *pso2*Δ*rad51*Δ and *pso2*Δ*rad52*Δ strains in a suppressed phenotype. This suggests that if there is a compensatory pathway for tolerating formaldehyde exposure, it fails to be initiated when both *pso2* and *mre11* are deleted, but not when *pso2* is deleted with either *rad51* or *rad52*.

5.7.2 The role of the TLS pathway in formaldehyde resistance

From the TLS pathway, only *rad5*Δ was sensitive to chronic formaldehyde exposure (Fig. 13). The other deletion strains in this pathway did not show any significant sensitivity to formaldehyde. Rad5 is thought to play a role in stalled fork regression, therefore it is possible that Rad5 is playing a role in fork regression after formaldehyde exposure. Interestingly, Sgs1, a member of the RecQ family, has a role in stalled fork resolution and is also sensitive to formaldehyde. When the *rad5*Δ*sgs1*Δ strain was further studied it was found that Rad5 (TLS) and Sgs1 (HR) might behave epistatic or synergistic to one another after chronic or acute formaldehyde exposure (Fig. 27). Either result would be of interest, as they would both work on the same substrate or compensate for one another.

Interestingly, the double mutant of *rad5* and *mre11* results in an additive growth after formaldehyde exposure. Although Sgs1 and Mre11 have been assigned to the HR epistasis group, these results suggest separate functions in a Rad5 deficient background. Additionally the *rad5*Δ*rad4*Δ strain was very sensitive to formaldehyde (Fig. 27). This additive response is consistent with the separate pathway function of Rad4 in NER and Rad5 in TLS.

Another strain carrying a deletion in a gene assigned to the TLS pathway, *rad18*, was also assessed to sensitivity to formaldehyde. Rad18 is an E3 ubiquitin ligase, and it is epistatic with *rev3*Δ (see introduction). Although *rad18*Δ was not sensitive, the double mutant of *rad4*Δ*rad18*Δ was very sensitive under both chronic and acute exposure conditions (Fig. 25 and 26). These data suggest that TLS might be involved in tolerating formaldehyde exposure in the absence of NER.

5.7.3 The role of NER and HR in tolerating formaldehyde exposure

To further study the role of HR and Mre11 in the tolerance of formaldehyde exposure, the strains *mre11*Δ*rad1*Δ and *mre11*Δ*rad4*Δ were tested for formaldehyde sensitivity. The sensitivity of *mre11*Δ under chronic conditions appears to be suppressed by also deleting *rad4*Δ. This is not the case for the *rad1*Δ*mre11*Δ double mutant, which demonstrates an epistatic or synergistic relationship rather than suppression. This suggests under chronic formaldehyde exposure conditions that Rad1 and Mre11 might work on the same substrate or within the same pathway. Interestingly, the strains that have a deletion in HR gene *rad52,* with either *rad1*Δ or *rad4*Δ display an additive response under chronic exposure conditions (Fig. 29-30). These results also suggest that Rad52 (HR) and Rad1 or Rad4 (NER), are necessary for tolerating formaldehyde

exposure, but that both repair pathways work on different substrates due to the observed additivity in survival after formaldehyde exposure.

Under acute conditions, no survival was observed by spot assay for the *rad1*Δ*mre11*Δ strain, but *mre11*Δ appears to rescue the *rad4*Δ sensitivity under acute conditions, as the double mutant is surviving better than the single mutant *rad4*Δ (Fig. 27-28). Therefore, these mutants were tested by colony forming ability assay. With this assay it showed that *rad1*Δ and *mre11*Δ are epistatic, *rad4*Δ*mre11*Δ shows wild-type resistance to formaldehyde, and *rad1*Δ*rad52*Δ shows (more than) an additive response. Interestingly, we did not see this drastic additive response for *rad4*Δ*rad52*Δ after acute exposure as compared with the chronic exposure (Fig. 29-30). This result is intriguing as this has not been found with any other double mutant where it behaved differently depending on the exposure (i.e. the same response under chronic and acute conditions was always observed). Although consistently observed there is the possibility that formaldehyde resistant colonies are present. The fact that *rad1*Δ and *mre11*Δ are epistatic suggest that Rad1 and Mre11 might act on a same substrate or function in the same pathway.

In addition, there appears to be an epistatic relationship between *top3*Δ and *sgs1*Δ with *mre11*Δ. This is consistent with the knowledge that all these genes have a role in HR (see introduction), therefore suggesting a role of both Top3 and Sgs1 in tolerating formaldehyde exposure via an HR pathway response.

The functional/pathway assignment differences between Rad1 and Rad4 are further exemplified by the observation that the double mutants *top3*Δ*rad1*Δ and *sgs1*Δ*rad1*Δ are more sensitive to formaldehyde than *top3*Δ*rad4*Δ and *sgs1*Δ*rad4*Δ. This

suggests that the role of Rad1 in HR might be more important than its role in NER after formaldehyde exposure, as you would have expected to see similar survival rates as with *sgs1*Δ*rad4*Δ if Rad1 was exclusively functioning in NER.

To further explore relative contributions of HR and NER in the tolerance of formaldehyde exposure, the *rad4*Δ*rad52*Δ strain was assayed for strand breaks by PFGE (Fig. 31). Interestingly, the *rad4*Δ*rad52*Δ strain demonstrates both SSBs and DSBs. These breaks disappear after 6 hours to background levels, suggesting break repair in the absence of Rad4 (NER) and Rad52 (HR). This implies that both proteins could be important in the prevention of forming SSBs or DSBs, but that they are not necessary for the repair of these breaks.

In addition, we conclude that BER and MMR in their traditionally defined repair pathways are not involved in the repair of formaldehyde-induced DNA damage (Fig. 15). None of these mutants are sensitive to formaldehyde under either chronic or acute exposure conditions. Although the single mutants in the non-essential genes of these pathways do not indicate an important role in the tolerance or repair of formaldehyde induced lesions, we cannot rule out possible backup or compensatory roles of these genes.

5.7.4 Correlating the results to previously defined DNA repair pathways

Based on the results obtained in this research project, the major cellular DNA repair pathways were reviewed as described in the Introduction. One described pathway received particular attention as it is the only pathway that includes both Rad1 and Mre11. This pathway is MMEJ. The hallmark feature of this repair pathway is that it is a Ku-independent end joining mediated process initiated by base pairing between

microhomologous sequences of approximately 5-25 nucleotides. In addition to the use of Rad1 and the MRN(X)-complex, this pathway represents a potential mode for DPC repair as it results in chromosome abnormalities such as deletions, translocations, inversions and other complex rearrangements. Supporting this idea is the fact that it has been shown that formaldehyde exposure may result in DNA damage and complex chromosomal rearrangements (Kumari, McCullough Unpublished; Craft, Bermudez et al. 1987; Crosby, Richardson et al. 1988, ATSDR 1999).

However, unlike MMEJ, no DSBs were observed following formaldehyde exposure, and *exo1*Δ, *sae2*Δ, *ku70*Δ *and ku80*Δ single mutants are not sensitive to formaldehyde. It has to be noted that when defining the MMEJ pathway, sensitivity to IR of *rad1*Δ and *ku70*Δ was only observed when *rad52* was also deleted (Ma, Kim et al. 2003). In the case of *exo1*Δ, *sae2*Δ, *ku70*Δ *and ku80*Δ it might be necessary to characterize formaldehyde sensitivity in a *rad52*Δ background to see an effect. This is similar to our analysis of *rev3*Δ where *rad51* had to be deleted before any noticeable change in sensitivity was observed. Interestingly, Rev3 has also been implicated in playing a role in MMEJ, therefore it is possible that Rev3 might compensate for the loss of these genes. Taken together, these data suggest a complex mechanism for coping with formaldehyde-induced DNA damage that involved NER, HR and TLS pathway proteins.

5.8 Revisiting the model

Based on the accumulated data as described in chapter 4, the model for cellular responses to DNA damage induced by formaldehyde has been revised. As it is apparent that the exposure conditions affect the response, the model has been divided in chronic (Fig. 32) versus acute pathways (Fig. 33). Under chronic conditions, no SSBs or DSBs were detected, but HR deletion strains are very sensitive. This suggests that either the SSBs or DSBs are at undetectable levels or occur transiently, or that HR is involved in a DNA break-independent process. It does appear that in the absence of Rad51, Rev3 is compensating in some capacity for the loss of HR. Although not tested for strand breaks, Pso2 and Rad1 appear to behave epistatic or synergistic after chronic exposure, suggesting a role for Pso2 in the tolerance or repair of formaldehyde induced DPCs. Interestingly, Pso2 alone is not sensitive. Epistasis of these two genes has been shown for nitrogen mustard (Barber, Ward et al. 2005). The epistatic analysis on the *rad1*Δ*mre11*Δ strain suggests that both Rad1 and Mre11 act together on the same substrate and therefore might function in the same pathway. The only defined pathway containing both these proteins is MMEJ, and therefore these results imply that MMEJ might play an important role in tolerating chronic formaldehyde exposure. Another interesting finding is the fact that the *sgs1*Δ*rad5*Δ strain appears to behave epistatic after formaldehyde exposure. This suggests that Rad5 might play a role in fork regression and Sgs1 in collapsed fork resolution. The current model of DPC repair under chronic exposure conditions is illustrated in Fig. 32. The schematic represents two scenarios under chronic exposure conditions.

If TLS does not bypass the lesion, two scenarios could present itself during DNA replication. In one case the damage is formed in the leading strand (Fig. 32A) and the other in the lagging strand (Fig. 32B). When the damage is formed in the leading strand (Fig. 32A, gray diamond), it will result in a replication block (step 1). Synthesis of the lagging strand still proceeds as normal. Rev3 could be recruited to the site of damage and bypass the lesion (not drawn in the model) (step 2). If the damage results in a replication block, 3' to 5' helicases Rad5 and Sgs1 are necessary in order to resolve the blockage at the fork (step 3). This results in strand reversal, and hybridization of the complementary strands (step 4), creating a substrate that is extended by an unspecified DNA polymerase (step 5).

When a DPC is formed in the lagging strand (Fig. 32B, gray diamond), it will result in a replication block (step 1). In this case, the leading strand synthesis will proceed as normal, assuming the DPC on the lagging strand does not block this replication process. Rev3 might bypass the lesion, however, if Rev3 does not bypass the lesion, strand invasion and exchange might follow that requires the MRN(X)-complex and Rad52 (step 2). Next the lagging strand re-invades the DPC containing strand beyond the site of the damage and the gap is filled to a previously synthesized Okazaki fragment (step 3). The structure (step 3) has been described as a double Holliday Junction, and can be resolved by the Sgs1/Top3 complex in yeast (step 4). This is followed by gap filling between the Okazaki fragments and replication proceeds (step 5). In both the models for the leading and lagging strand the lesion remains in the DNA. The remaining lesion could potentially be a substrate for the proteasome, NER or both.

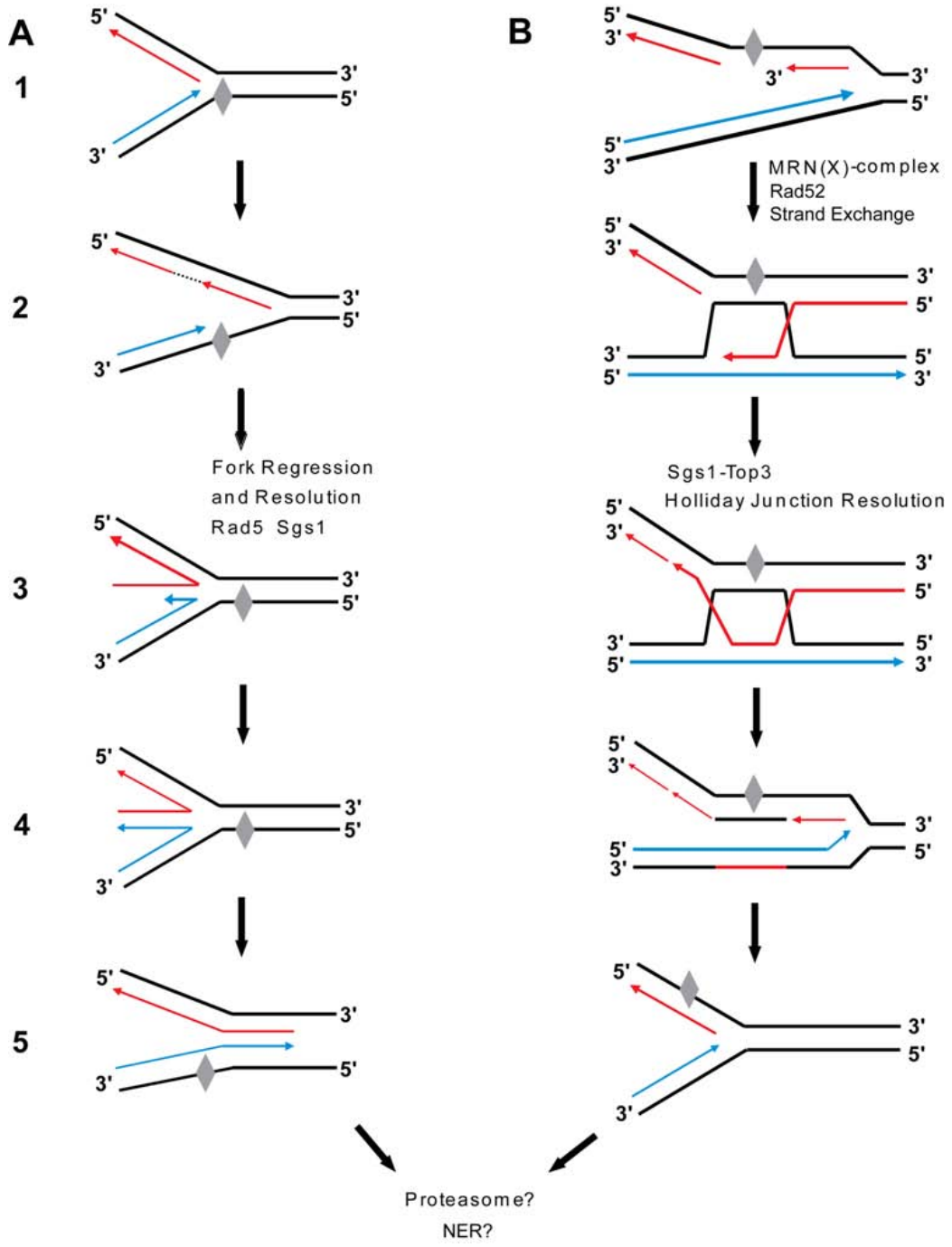

Figure 32. Revised DPC repair model after chronic formaldehyde exposure. (A) Represents a schematic in which the DPC is formed in the leading strand. **(B)** Represents a schematic in which the DPC is formed in the lagging strand.

In contrast to the chronic response, the data suggest that the acute response occurs in an NER-dependent, replication independent manner. Under acute conditions SSBs, but no DSBs were observed. The formation of SSBs appears to be dependent on NER, as SSBs were not accumulating above background levels in the *rad4*Δ strain. Described in the Introduction, proteasomal degradation might precede NER incision. In addition, it was observed that in the absence of Mre11 and Rad52 after an acute exposure, SSB formation was also reduced. Interestingly, the *rad1*Δ*mre11*Δ strain also appears to behave epistatic after an acute formaldehyde exposure, suggesting the possibility of a MMEJ or MMEJ-like pathway being involved in tolerating acute formaldehyde exposure. The idea that MMEJ or a MMEJ-like pathway is involved is only possible if replication starts before NER can repair the damage, as illustrated in the model (Fig. 33). Potentially, this initiation of replication before NER is finished, supports the observation of chromosomal rearrangements in NER deficient cell lines after formaldehyde exposure, due to i.e. replication fork collapse during replication (Kumari & McCullough, unpublished 2009).The current thinking of DPC repair after an acute formaldehyde exposure is illustrated in Fig. 33.

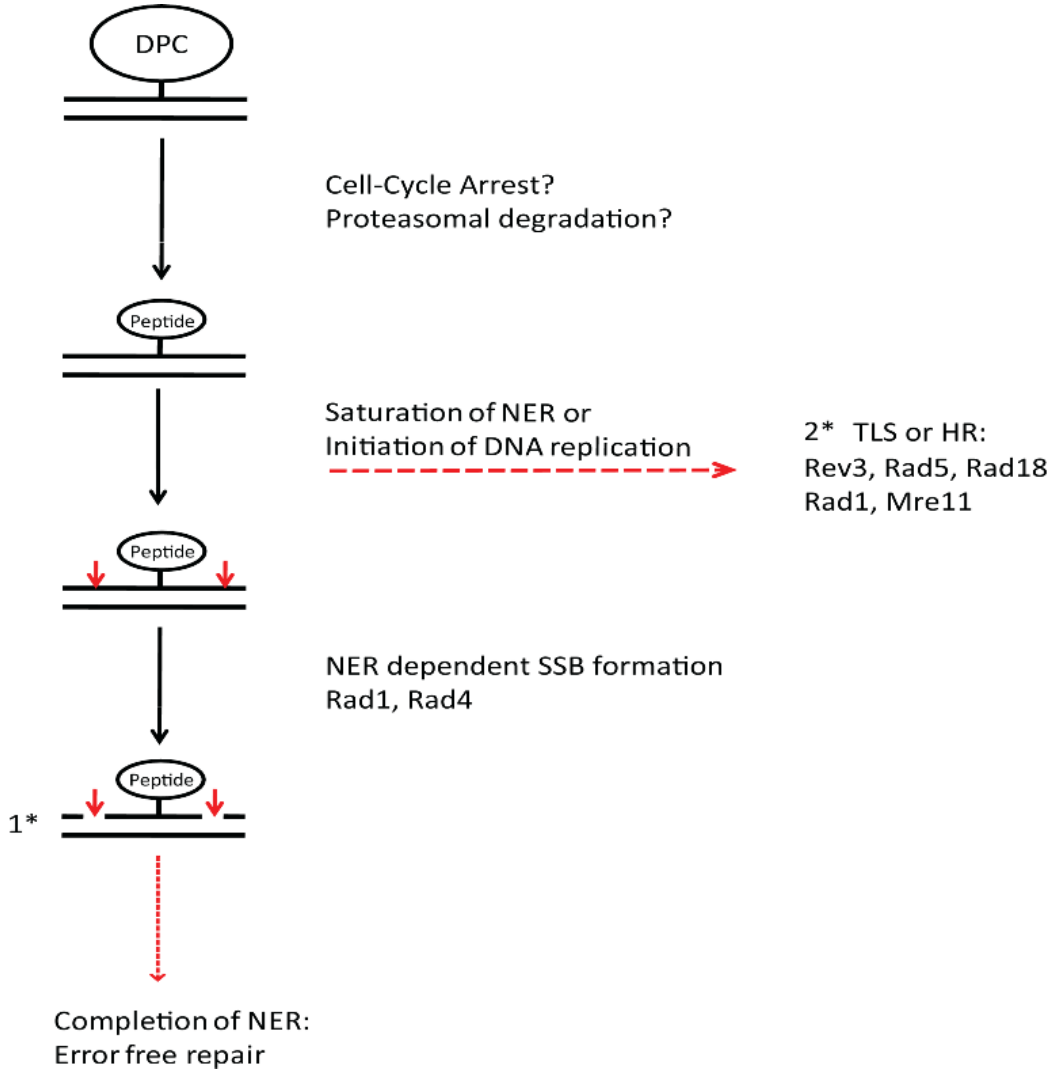

Figure 33. Revised DPC repair model after acute formaldehyde exposure. After an acute exposure the formation of a threshold dose of DPCs may lead to cell cycle arrest and the initiation of the proteasome. After the DPC is degraded to a smaller peptide, NER results in nicks around the DPC, forming SSBs. Based on previous reports (see introduction) TLS might play a role when NER is exhausted or replication starts before NER has repaired the DPC. (1*) When the incision process is interfered with it leads to cell death. (2*) If TLS is error prone, mutations in the DNA are expected to increase. If replication starts before NER is completed it could result in chromosomal aberrations.

6. Future Directions

The data in this study have helped to better understand the mechanisms contributing to the tolerance or repair of DPCs. In addition, the genome wide-screen and subsequent genetic analyses have given rise to new questions and hypotheses regarding cellular responses to formaldehyde. A few interesting directions to pursue in this area are presented below.

Based on the model, and the involvement of TLS as a backup system, it is hypothesized that the mutation frequency and spectrum may be different in certain strains. What is the mutation spectrum in the cells that display similar to wild-type or better than wild-type formaldehyde resistance?

As implied in earlier sections, Rev3 (TLS pathway) might play a compensatory role to Rad51. I hypothesize that if both NER and HR are knocked out, that the Rev3 dependent pathway might compensate for the lack of NER or HR and bypass the formaldehyde induced damage in an error-prone manner resulting in more mutations. This compensatory mechanism is supported by the synergistic response in the *rad51*Δ*rev3*Δ strain.

To study changes in mutation frequency, a forward mutation assay can be utilized. In *S. cerevisiae,* the *can1* gene encodes an arginine permease that is a selective protein channel responsible for the transport of both arginine and its toxic analog, canavanine, into the cell. Mutations in the *can1* gene can be selected for based on canavanine resistance of the formaldehyde-treated yeast strain. The measured phenotype can be the result of different types of mutations (e.g.

frameshift, base substitutions, small deletions or chromosomal rearrangements) (Chen, Umezu et al. 1998; Chen and Kolodner 1999).

Alternatively, Rad30 (Pol eta) might be involved in the error-free bypass of formaldehyde-induced damage. Similar to the *rev3*Δ strain, the *rad30*Δ strain is not sensitive to chronic formaldehyde exposure. It would be of interest to create a yeast strain with deletions in *rad51* and *rad30*. Several outcomes are possible, 1) the double mutant is not sensitive (complete suppression), 2) the double mutant displays a similar survival as the single mutant *rad51*Δ*,* 3) *rad30*Δ partially suppresses the sensitivity of *rad51*Δ*,* and 4) the double mutant is more sensitive to formaldehyde than the *rad51*Δ strain. Based on the Rev3 results, outcome 4 is expected. But if 1) is the outcome, it would mean that a yet unknown process now bypasses or repairs the lesion (potentially Rev3). If 2) and 3) are the result, it suggests that Rad30 is either not involved or that when Rad30 is present it results in a lesion that normally is more cytotoxic to the cell. If it appears that Rad30 is involved then it would be of interest to do mutational analyses on the *rad51*Δ*rad30*Δ strain utilizing the *can1* assay. If both Rad30 and Rev3 are involved in tolerating formaldehyde exposure, it is expected that in the *rad51*Δ*rad30*Δ strain the mutation in the *can1* gene would go up (because Rev3 is now involved in bypassing the lesion), but in the *rad51*Δ*rev3*Δ strain the mutation frequency would stay the same or go down (because Rad30 is now involved in bypassing the lesion). These proposals assume that Rad30 is error-free and Rev3 error-prone after formaldehyde exposure as described in the introduction.

Due to the increased sensitivity of many of the repair pathway deficient strains, it is hypothesized that a deficiency in any of these protective responses could result in gross chromosomal changes after chronic or acute formaldehyde exposure.

During the course of this study preliminary studies have been performed to examine the gross chromosomal effects of formaldehyde exposure. Microscopic experiments were done on wild-type, *rad4*Δ and *mre11*Δ yeast cells. These experiments were done staining the yeast cells with 4',6-diamidino-2-phenylindole (DAPI) and following the division of the cells microscopically, as yeast cells are easy to follow during cell division by their morphological changes. Several observations suggest further experiments are warranted; a) The cells stain less with DAPI after chronic formaldehyde exposure, but this could be due to the fact that formaldehyde might interfere with the DAPI staining, and b) after an acute exposure and giving the yeast recovery time, larger cells with increased DAPI staining were observed, suggesting that more DNA is present in the cells. Preliminary, this appeared to be more pronounced and prolonged longer in the NER and HR deficient strains.

In an effort to obtain evidence on the presence of polyploidy, fluorescence-activated cell sorting (FACS) analyses on wild-type and SWI/SNF mutants were performed. These SWI/SNF mutants were chosen to be studied due to the fact that these are impaired in remodeling the nucleosome. Unfortunately, at the time these analyses were performed, the protocol for FACS analyses on yeast was not completely optimized. However the data did suggest an increase in cell death and chromosomal decondensation after formaldehyde exposure. Interestingly, unpublished data from our group using CHO cells suggest that chromosomal breaks, radials and polyploidy occur after formaldehyde exposure. It was also found that

polyploidy was more profound in an NER-deficient background (Kumari & McCullough, unpublished).

In addition to the FACS studies and cytogenetic analyses utilizing CHO cells, similar experiments using FACS can be done with the yeast system. The FACS analyses protocol has been optimized during the course of this study, and the various strains identified in the genome-wide screen can be assayed using FACS analyses scoring for cell cycle changes. In concurrence to the FACS analyses, the cells can be followed microscopically by staining the DNA with DAPI.

A hypothesis resulting from this research is that a high, acute dose of formaldehyde induces cell cycle arrest. Are there any cell cycle changes after chronic or acute formaldehyde exposure?

Besides the microscope and FACS analyses focusing on polyploidy, the progression through the cell cycle was also studied utilizing these techniques. As mentioned above, wild-type, *rad4*Δ and *mre11*Δ yeast cells were stained with DAPI. Here no significant difference in cell division was observed after chronic formaldehyde exposure, although the cells possibly progress slower. Interestingly, these cells appear to arrest up to 6-8 hours after an acute exposure, which is consistent with when the SSBs disappear as measured by PFGE and sucrose gradient experiments. It has to be noted that *tel1*Δ (ATM homolog) was not sensitive to formaldehyde, suggesting an ATR-like cell cycle arrest. Unfortunately, ATR (Mec1) is essential in yeast, and was therefore not tested.

Preliminarily studies have been performed after formaldehyde exposure utilizing CHO cells deficient in NER (Kumari & McCullough unpublished). Consistent with the

preliminary findings in the yeast cells, formaldehyde results in an arrest in a dose-dependent manner. Interestingly, this same study suggests that the arrest of the cell correlates to the amount of chromosomal damage, and that formaldehyde induced cell death was increased by giving the cells a longer time to recover. This suggests that formaldehyde induced cytotoxicity is a result of the repair of the formaldehyde-induced DNA damage, and not the result of the initial lesion (Kumari & McCullough, unpublished). Now that the FACS protocol for yeast has been optimized, the work in the CHO-cells can potentially be supported by utilizing yeast single or double mutants, in addition double mutants could be made in a cell cycle checkpoint deficient background. As this study suggests not only a role for NER, but also HR, and potentially TLS as a backup mechanism, it is recommended to first observe cell cycle changes in these yeast cells. The advantage of performing FACS analyses on yeast is that the effects on cell cycle can be observed in double and triple mutants. In addition, the deletions will also be in an isogenic background. Based on the CHO-cell data, it is hypothesized that the cell cycle arrest will be prolonged in a dose-dependent matter, with an increase in apoptosis and polyploidy.

As suggested by the model, cell cycle arrest is hypothesized to be a prerequisite for NER. Thus, it may be of interest to create a double mutant that has a deletion in a checkpoint gene (i.e. Chk1) and NER gene. Due to the chronic exposure conditions of the genome-wide screen, no checkpoint mutants were identified. In addition, *tel1*Δ (ATM), and checkpoint deletion strains *rad9*Δ, *rad17*Δ and *rad24*Δ were not sensitive under both chronic and acute conditions.

A problem underlying making a double mutant that is checkpoint deficient and NER deficient is that the accumulation of formaldehyde-induced DNA damage could

be equal to the cytoxicity in a NER deficient cell or even more toxic to cells (because NER is not activated). This would result in a drastic accumulation of formaldehyde-induced DNA damage and therefore an increase in cytotoxicity. Based on the CHO-cell data, this is not expected, because as mentioned above, DNA damage appears to be dependent on the recovery time.

The role of MMEJ in the formaldehyde response might be an important repair pathway.

Based on the epistatic relationship between Rad1 and Mre11, I hypothesize that MMEJ could play an important role in tolerating formaldehyde exposure. The MMEJ pathway includes several important proteins, Rad1, Rad10, the MRN(X)-complex, Sae2, Exo1, Rev3 and a possible regulatory function for Ku70, Ku80 and Rad51 as described in the Introduction. Unfortunately, some of the single mutants display no sensitivity to either chronic or acute formaldehyde exposures. One potential experiment to be performed could be to increase the chronic formaldehyde concentration, which might result in an increased sensitivity of these single mutants. For example, *rad1*Δ only showed moderate sensitivity to formaldehyde at concentrations < 1.5 mM, but when the concentration was 2.0 mM or higher the sensitivity to formaldehyde increased drastically. Therefore, it would be worth performing a similar dose dependent experiment on the *sae2*Δ*, exo1*Δ*, ku70*Δ*,* and *ku80*Δ strains. Interestingly, the *rev3*Δ strain is not sensitive to formaldehyde, but Rev3 appears to compensate for *rad51*Δ as described earlier. Similar results could be expected for the *sae2*Δ*, exo1*Δ*, ku70*Δ*,* and *ku80*Δ strains. In addition, when trying to define the role of MMEJ in the cell, sensitivity to IR in these strains was only

observed when *rad52* was also deleted. All of these data suggest that for the *sae2*Δ*, exo1*Δ*, ku70*Δ*,* and *ku80*Δ strains, synergism might be found when, for example *rad1, rad51, rad52* or one of the MRN(X)-complex genes are also deleted. Alternatively, similar studies can be performed using mammalian cells and siRNA these genes and assay for difference in survival after formaldehyde exposure.

Does proteasomal degradation occur after formaldehyde response and is it a prerequisite for the activation of NER?

One hypothesis is that proteasomal degradation is a requirement to tolerate formaldehyde exposure, and that this degradation is necessary for NER activation. The role of the proteasome involvement in formaldehyde resistance remains to be demonstrated, and proteasomal activation has been suggested to be exposure dependent (see introduction). In prokaryotes it was shown that NER was more efficient in making an incision around a DPC when it was degraded to a smaller peptide, on the other hand, in mammalian cells, NER was not capable of incising around the DPC (see Introduction). In addition, it was shown that by inhibiting the proteasome with the chemicals lactacystin or MG132 that DPC repair was reduced significantly compared to proteasomal competent cells (see Introduction).

One problem with chemically inhibiting the proteasome, is that the yeast cell does not uptake these chemicals efficiently due to the cell wall. Therefore, DNA repair gene mutations would have to be made in a membrane deficient background strain, enhancing permeability. Another problem with making a strain with a more permeable membrane is that the sensitivity to formaldehyde also changes and therefore it is expected that new a chronic and acute concentration needs to be

established. Alternatively, deletions can be made in a proteasome deficient background. In order to test if this was a feasible experiment to perform, two strains with deletions in genes involved in the proteasome, *blm10* and *rpn4,* were tested for sensitivity to formaldehyde. *rpn4*Δ was identified as sensitive in the genome-wide chronic screen, but *blm10*Δ was not. This suggests that certain proteins involved in the proteasome are more important than others.

Alternatively, similar studies can be performed in mammalian cells, either in cells deficient in NER, HR, TLS, or by utilizing siRNA to knockdown a gene of interest. A potential problem with using siRNA is that complete knockdown is hard to achieve and therefore the redundant activity might be enough to result in no change in survival or DNA breaks. One advantage in using siRNA is that the experiments can be performed in an isogenic background. Preliminarily, Mre11 was successfully knocked down in our lab utilizing U2OS cells, suggesting that at least for Mre11 this technique can be used.

Using these techniques, first it needs to be established that the proteasome is important for the repair of DPCs. This would be done by chemically inhibiting the proteasome in wild-type cells and exposing these cells to formaldehyde. When the proteasome is important for tolerating formaldehyde exposure, enhanced sensitivity is expected. Hereafter, one could repeat this in DNA damage repair mutant cell lines or knockdown a gene of interest by siRNA. One problem for the chronic formaldehyde exposure studies is that the siRNAs need to be stable, due to the exposure time. Stability of the siRNA is of importance in order to maintain knock down of the gene of interest. Assuming the proteasome plays a role, changes in

survival, mutagenesis, cell cycle progression and the removal of DPCs can be measured in DNA repair deficient cell lines after formaldehyde exposure.

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