Cellular Pathways in the Repair and Tolerance of Formaldehyde-Induced DNA Damage

By

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DEDICATION

I would like to dedicate this thesis to my partner Leah who put up with me when things got really tough, and I got increasingly unpleasant. To my whole family, all the aunts, uncles, and cousins for their unconditional support. To my parents for giving up their dreams and immigrating to this country. I know it's been a rough and sometimes impossible ride, but I assure you, I would not be here without you. To my brother who for the past 27 years has always lent an ear or a shoulder. I dedicate this, finally, to my grandmothers who showed me the strength of the Latina women; who with scarce resources and no formal education raised children and grandchildren who have excelled beyond their wildest dreams. Si Se Pudo!

LIST OF ABBREVIATIONS

OSHA	Occupational Safety and Health Administration
DNA	Deoxyribonucleic Acid
IARC	International Agency for Research on Cancer
EPA	Environmental Protection Agency
DPC	DNA-protein Crosslinks
NPC	Nasopharyngeal Cancer
FEMA	Federal Emergency Management Agency
NER	Nucleotide Excision Repair
HR	Homologous Recombination
CHO	Chinese Hamster Ovary
DSB	Double Strand Break
SSB	Single Strand Break
UV	Ultraviolet
CDP	Cyclobutane Pyrimidine Dimers
NHEJ	Non-homologous End Joining

DDR	DNA Damage Response
OD	Optical Density
RT-PCR	Real-time Polymerase Chain Reaction
WT	Wild-Type
BLM	Bloom-Syndrome RecQ Like Helicase
AUC	Area Under the Curve
GO	Gene Ontology
IR	Ionizing Radiation

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ABSTRACT

Formaldehyde is a highly reactive compound produced extensively worldwide. People are exposed to formaldehyde in both industrial and occupational settings, and environmentally via off-gassing of vehicle exhaust, cigarette smoke, and home production materials. Formaldehyde has been implicated extensively in human carcinogenicity studies, and thus is classified as a class I human DNA carcinogen. Formaldehyde has been shown to have severe detrimental effects on cellular processes, likely due to induction of its predominant DNA lesion, DNA-protein crosslinks. This covalent linkage of proteins to DNA has been demonstrated to occur with a plethora of proteins, implicated in a wide variety of cellular process. Consequently, studies have identified several DNA repair pathways that play a role in mitigating cytotoxicity and genotoxicity after formaldehyde exposure. In this study, we choose to further define and reconcile discrepancies in the literature by using a high-throughput systems approach to discern the conserved pathways necessary for survival following chronic formaldehyde exposure, across cell type and species. We found that though there was heterogeneity across cell types in the genes elicited for survival following chronic formaldehyde exposure, the Homologous Recombination, Double-strand Break Repair, and Response to Ionizing Radiation, and DNA Replication pathways were conserved. Importantly, these pathways were also shown to be important for cell survival following chronic low-dose formaldehyde exposure in S. cerevisiae. Given the importance of chromatin remodeling in all of the identified pathways and our previous findings demonstrating that deletion of chromatin remodeling genes

in yeast results in elevated cytotoxicity after low-dose chronic exposure, we chose to also investigate the role of chromatin remodeling in the repair of formaldehyde-induced DNA damage. We found that the SWI/SNF chromatin remodeling complex is working in conjunction with the Homologous Recombination pathway to mitigate cytotoxicity and the repair of formaldehyde-induced double strand breaks. We also demonstrated a severe cell cycle delay in strains deficient in components of the SWI/SNF complex following acute high-dose formaldehyde exposure. In conclusion, our findings highlight that the Homologous Recombination pathway is crucially important for cell survival across cell types and species following chronic formaldehyde exposure. Moreover, the Homologous Recombination pathway is working with the SWI/SNF complex, in yeast, to mitigate cell death, effective double-stand break repair, an appropriate cell cycle checkpoint, and DNA damage response.

INTRODUCTION

Formaldehyde Overview

Formaldehyde is a high production volume chemical with over 20 million tons produced per year worldwide [1,2]. Commercially, formaldehyde is manufactured as an aqueous solution called formalin, which is commonly used as a tissue preservative and as a bactericide in embalming fluid and medical laboratories. It is primarily utilized in the production of casein, phenol-, and urea-formaldehyde resins, synthetic plastics, and chemical intermediates [3]. The Occupational Safety and Health Administration (OSHA) estimates that approximately 2.1 million workers in the United States, and many more in developing countries, are exposed to formaldehyde. The highest levels of formaldehyde occur in occupational settings [1]. Exposed workers are commonly found in industrial settings that manufacture formaldehyde and formaldehyde-based resins, textiles, wood composites/furniture, fiberglass, automobiles, and home appliances [3–6]. In addition, people working in laboratories, pathology departments, as embalmers, and in agriculture are also exposed to formaldehyde. Although environmental exposure to formaldehyde typically occurs at much lower levels than occupational exposure, a greater number of people are exposed to formaldehyde in their daily lives. Environmental sources of formaldehyde include, but are not limited to, automobile engines, cigarettes and e-cigarettes, cosmetic products, burning of forest and manufactured wood products, urea formaldehyde home insulation (used mainly in 1970-1980s homes), off-gassing from furniture, carpeting and flooring [7,8]. Surprisingly, there are also numerous sources of endogenous formaldehyde

including the one carbon pool, amino acid and alcohol metabolism, lipid peroxidation, and p450 dependent demethylation [2]. Interestingly, elevated levels of formaldehyde have also been found in human tumors [9]. Given the vast array of occupational, environmental, and endogenous sources of formaldehyde exposure it is of critical importance that further studies delineate the biological and epidemiological consequences of formaldehyde.

Carcinogenicity Studies

Both chronic and acute exposure to formaldehyde has been associated with a plethora of human health conditions including eye and upper respiratory irritation, contact dermatitis, occupational asthma and altered lung function [5,6,10]. Many epidemiological studies have also evaluated the relationship between formaldehyde and human malignancies. These studies are segregated into 1) historical cohort studies that evaluate workers in a variety of industries that manufacture or use formaldehyde 2) historical cohort studies of health professionals including physicians, embalmers, and pathologists, 3) population or cancer registry based studies, and 4) population studies on the mortality or cancer incidence in an occupation. Based on these epidemiological studies formaldehyde has been classified as a class I human DNA carcinogen by both the International Agency for Research on Cancer (IARC) and the United States Environmental Protection Agency (EPA) [4].

Early mode of action inhalation studies between 1978 and 1983 in F344 rats showed a dose dependent increase in the incidence of nasal squamous cell

carcinomas. This caused simultaneous concern across the Food and Drug Administration, Consumer Product Safety Commission and EPA since nasal squamous cell carcinomas are very rare neoplasia in rats [2]. The Battelle study in 1983 showed rhinitis, squamous metaplasia, and epithelial dysplasia in all formaldehyde-exposed groups of rats which regressed with increasing post-exposure recovery times. Of the 240 total (120 male, 120 female) F344 rats exposed to 14.3 ppm formaldehyde, 103 developed squamous cell carcinoma of the nose [11]. A more detailed study in 1996 showed increased cellular proliferation and tumor incidence in a dose dependent manner with a 60% tumor formation at the highest exposure dose of 14.3 ppm formaldehyde [12]. Though the doses in these studies far exceed the highest continuous exposure (2-5 ppm) doses measured in humans, short-term exposures at levels as high as 12.3 ppm (9.3 ppm measured) have been predicted in embalmers [13,14].

Subsequent studies established the non-linear relationship between DNA-protein crosslinks (DPC) and airborne formaldehyde concentrations [15,16]. A later study provided clear evidence that inhaled formaldehyde was in fact reaching the nasal epithelium, but not distinct tissues, by tracing a stable isotope-labelled formaldehyde in rats [17]. Their data conflicted with previous studies which correlated high formaldehyde exposure with leukemia, particularly myeloid leukemia [3,18–20]. Other cohort studies have also associated formaldehyde exposure with nasopharyngeal cancer (NPC) in formaldehyde exposed workers [21]. Surprisingly, a later, larger, cohort study did not find increased NPC incidence in plant workers. Nonetheless, further studies did confirm the original Hauptmann findings of

increased NPC incidence, but were unable to show any relationship between dose-dependent exposure and NPC. Importantly, it was later noted that the affected plant workers were previously employed in other industries such as metal work and brass plating which had previously been shown to correlate with increased NPC incidence. Ultimately, though it is plausible that formaldehyde causes NPC, since it is the initial site of exposure, a clear and definite relationship between formaldehyde exposure and cancer in epidemiological studies has not been established. Interpretation of these studies may be confounded by several environmental and genetic factors, and relatively small study cohorts for statistical analysis.

More recently, environmental exposure to formaldehyde made headlines after a 42.6-million-dollar class action suit was filed, and later settled, against the Federal Emergency Management Agency (FEMA) by the victims of Hurricanes Katrina and Rita after mobile homes and trailers provided by FEMA were shown to off-gas very high levels of formaldehyde. Residents reported headaches, nosebleeds, and shortness of breath after moving into the trailers and mobile homes. These same nose bleeds and severe headaches have also been reported by cosmetologists that apply hair-smoothing products. Investigations by the FDA and OSHA found that hair-smoothing products, particularly the Brazilian Blow Out, contained methylene glycol, which, when heated, releases formaldehyde into the air. Most recently, 60 Minutes also reported high-levels of formaldehyde in Lumber Liquidators' Chinese-made laminate flooring [22]. They independently tested 150 different boxes of laminate flooring and found that none of them met formaldehyde

emission standards set by California state law. It should be noted that these standards are not set, or adhered to, throughout the United States, raising concerns about home indoor air quality standards nationwide. Currently, there have been 138 lawsuits filed against Lumber Liquidators for selling products containing excessive levels of formaldehyde.

OSHA currently sets its formaldehyde standards for permissible exposure limits (PLE) in the workplace at 0.75 ppm measured as an 8-hour time-weighted average (TWA) [23]. The standard for short-term exposure limit is 2 ppm over a 15-minute period. Occupational exposure limits in Canada vary by jurisdiction, but range from 0.3-2 ppm [24].

Therefore, though there is conflicting evidence in the literature, formaldehyde's contributions to human malignancies cannot and should not be ruled out given the overwhelming amount of peer-reviewed research, summarized below, which shows its mutagenicity and cytotoxicity.

Formaldehyde Mutagenicity and Cytotoxicity

The genotoxic effects of formaldehyde were first reviewed in 1988 by Te-Hsiu Ma and Mary Harris. They highlighted that not only was there epidemiological evidence for the detrimental effects of formaldehyde, but that mutagenicity studies in bacteria, yeast, nematodes, drosophila, mouse, and human cell lines supported formaldehydes' mutagenic potential [25]. Further studies showed in a mouse lymphoma assay that formaldehyde exposure induced mutations likely through its induction of chromosomal aberrations [26]. Kawanishi et all showed that

formaldehyde exposure could induce intra-strand crosslinks leading to tandem base substitutions, a unique mutation feature of formaldehyde [27]. More recent studies have shown, in S. cerevisiae, that formaldehyde exposure induced single-base-pair insertions in Nucleotide Excision Repair (NER) and translesion synthesis deficient strains [28]. These findings support the mutagenic potential of formaldehyde and potential contributions to carcinogenicity in humans.

DNA-Protein Crosslinks Overview

The covalent crosslinking of proteins to DNA presents a major challenge to multiple cellular processes. DPCs have been shown to be the predominant lesion induced by formaldehyde [25,29]. DPCs are induced by a wide variety of environmental, occupational, and endogenous sources. Paradoxically, they have both been implicated in cancer incidence and progression, as well as been used in chemotherapeutics regimens to treat human malignancies with drugs such as cisplastin, melphalan, and mytomycin C [30]. DPCs can be formed through several different chemistries, which can not only affect the stability of the lesion but likely also the DNA damage pathways that are elicited for repair. Proteins can be crosslinked to DNA via oxidative free radical mechanisms, directly through chemical or drug linkers, such as formaldehyde, or through metal atoms [31,32].

Formaldehyde forms DPCs by reacting with amino and imino groups of proteins to form a Schiff base, which subsequently reacts with another amino group (Figure 1) [33,34]. Several groups have tried to identify the proteins that are crosslinked to DNA; understanding the plethora of proteins that are crosslinked to

DNA by the various DPC inducing agents may help further discern the biological consequences and repair mechanisms necessary to mitigate DPC cytotoxicity.

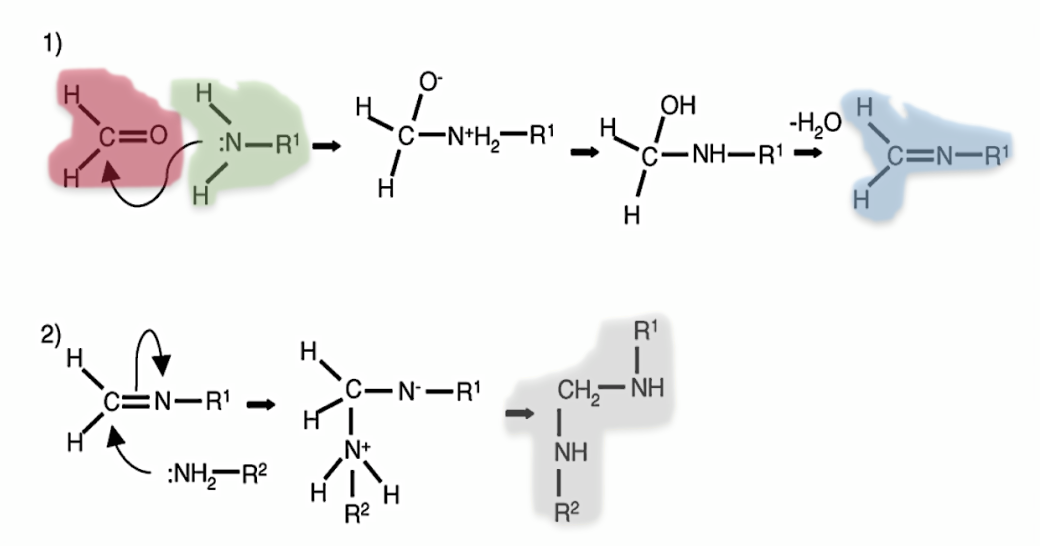

Figure 1. Formaldehyde Crosslinking Mechanism. The steps in the reaction of formaldehyde (red) with an amino group of a putative protein side chain (green) to form a Schiff base (blue). This can then react with another amino group to form a DPC (grey). [Reprinted with permission from S. Barker et al.]

Biologically relevant proteins that have been shown to be crosslinked to DNA in vivo by various agents include actin, histones, lectin, aminoglycoside nucleotidyl transferase, GRP78 (a heat shock protein), cytokeratins, vimentin, protein disulfide isomerase, and transcription factors and co-factors (Table 1) [30]. Given the variety of proteins crosslinked to DNA, their biological relevance, and the diversity of the DNA repair pathways that have been implicated in DPC repair, further investigations are necessary.

DNA-Protein Crosslink Repair

Studies have shown that DPCs can be longed lived and can persist through several replication cycles [35,36]. Unrepaired DPCs and repair intermediates can have permanent and serious consequences on genome integrity. Several studies have identified specific cellular pathways important for cell survival via repair of DPC lesions [30,37], including NER [37,38], proteosomal degradation [29], the Fanconi anemia pathway [39–41], and Homologous Recombination (HR) [37,42].

Proteins identified in DNA–protein crosslinks

Protein	Crosslinking agent
Actin	Chromium
	Cisplatin
	Mitomycin C
	Pyrrolizidine Alkaloids
Lectin	Chromium
Aminoglycoside nucleotidyl transferase	Chromium
Histones H1, H2A, H2B, H4	Formaldehyde
Histone H3	Formaldehyde
	Gilvocarcin V
Glucose regulated protein 78	Gilvocarcin V
Cytokeratins	Arsenic
Vimentin	Formaldehyde
	Metabolic byproducts
Protein disulfide isomerase	Cisplatin
Estrogen receptor	Cisplatin
HET/SAF-B	
hnRNP K	
Histone deacetylase 1	

Table 1. Proteins Identified in DNA-Protein Crosslinks. Proteins that have been shown to be crosslinked to DNA by various crosslinking agents. [Reprinted with permission from S. Baker et al.]

Nucleotide Excision Repair

Our lab showed, via systematic screens of the S. cerevisiae non-essential gene deletion library, that components of the NER pathway are necessary for cell survival after high-dose acute formaldehyde exposure. Nakano et al showed in bacteria that NER could repair DPCs, but only those smaller than 12-14 kDa [43]. Our lab also demonstrated increased cell death in 5 different NER-deficient Chinese Hamster Ovary (CHO) cell lines following exposure to formaldehyde-induced DPCs, of which XPF and ERCC1 deficient cells were the most sensitive to formaldehyde [38]. These deficiencies lead to replication-dependent upregulation of double-strand breaks (DSBs), radial formation, altered cell ploidy, and apoptosis [38]. Others have also determined that chromium induced DPCs could be repaired by NER in humans [35]. Groehler et al also showed that XPA deficient human cells were more sensitive to phosphoramide mustard, a DPC inducer, than wild type cells [44]. Given all this evidence, the current model for DPC removal may include proteosomal degradation of proteins [29], followed likely by damage detection by the three subunit complex Rad4/23 and Cdc31, in yeast. After recognition and binding at the lesion site, DNA is unwound by the TFIIH-complex (NEF3 group). The damaged DNA is then excised at the 5' end by Rad1/10/14 complex and at the 3' end via Rad2. Rpa then binds and stabilizes the exposed single stranded DNA. This gap is then quickly filled via DNA synthesis by polymerase delta/epsilon, and ligated by Cdc9 (Figure 2) [45–47].

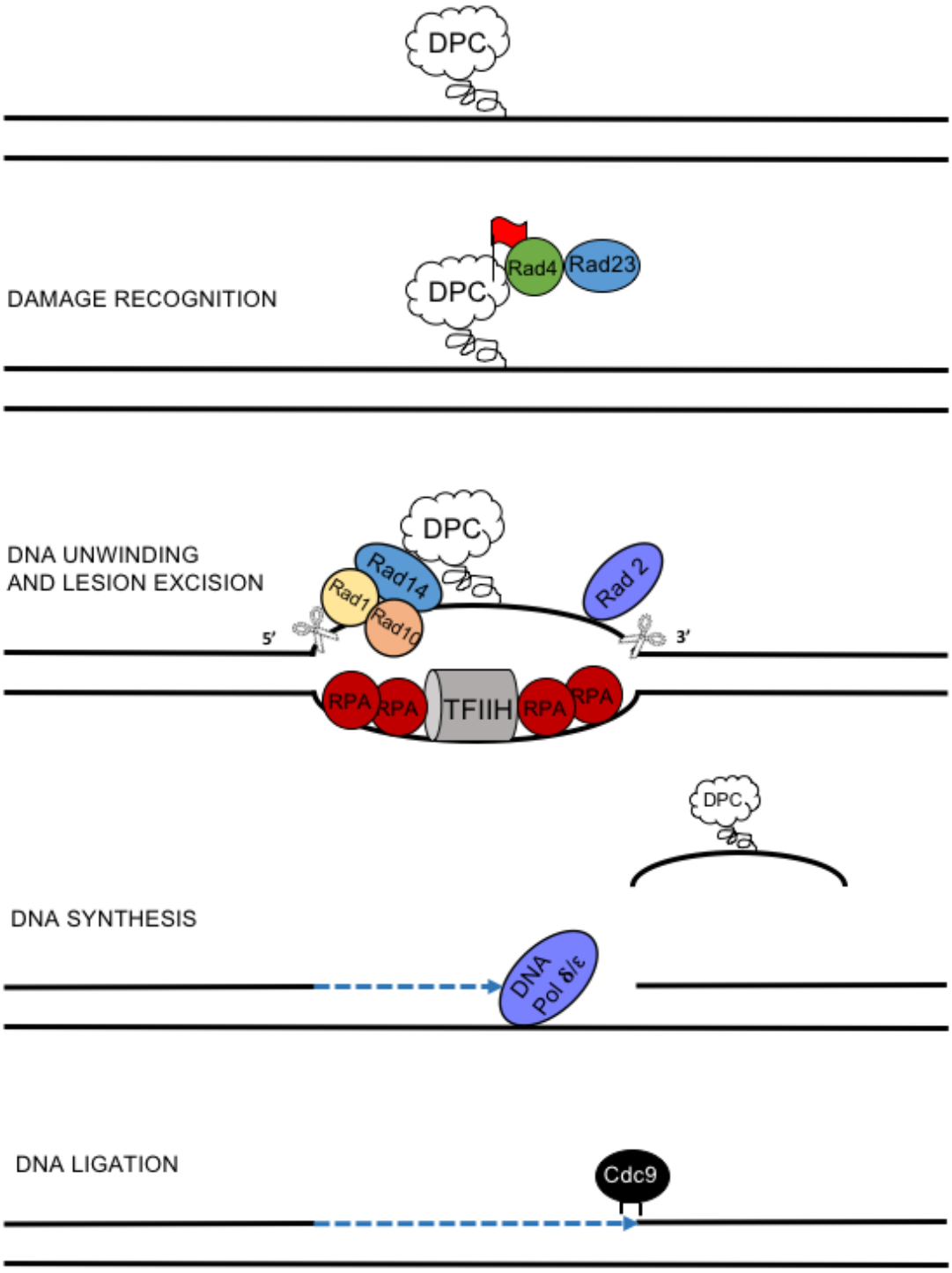

Figure 2. Nucleotide Excision Repair Pathway in S. Cerevisiae

Homologous Recombination

Even though NER has been implicated in DPC repair, there is evidence that suggests that the HR pathway may also be involved. These distinct pathway involvements may be, in part, due to chemically distinct crosslinks induced by different DPC-inducing agents. In addition, unlike NER, which has a protein size limitation for repair, HR is thought to be involved in the repair of oversized DPCs. Nakano et al showed that large DPCs are processed exclusively by RecBCD-dependent HR [43]. Moreover, we and others, have shown that DPC induction via exposure to several genotoxic agents results in dose-dependent formation of DSBs [4,4849]. We hypothesize that DSB formation occurs when DPCs are excised from DNA creating single-strand breaks (SSB). Subsequent DNA replication and/or replication-fork collapse leads to DSB formation. Repair of DSBs by HR is not only the preferred repair method in yeast, but also the most efficient and effective at restoring chromosomal integrity. In yeast, DSB detection and DNA end-resection is carried out by the Mrx complex (Mre11-Rad50-Xrs2) in conjunction with Sae2, Exo1, Sgs1 and Dna2. Immediately, the exposed single stranded DNA is protected and stabilized by Rpa (Rfa1/2) [50–52]. This is followed by filament formation and homology search by Rad51, Rad55/57, Sem1, and Rad 52 [53]. Next, is the crucial strand invasion step in which Rad59 and Rad54/51 create a displacement-loop with the complementary homologous DNA strand [54]. Finally, DNA is synthesized by DNA Pol δ and Srs2p, and ligated (Figure 3) [54].

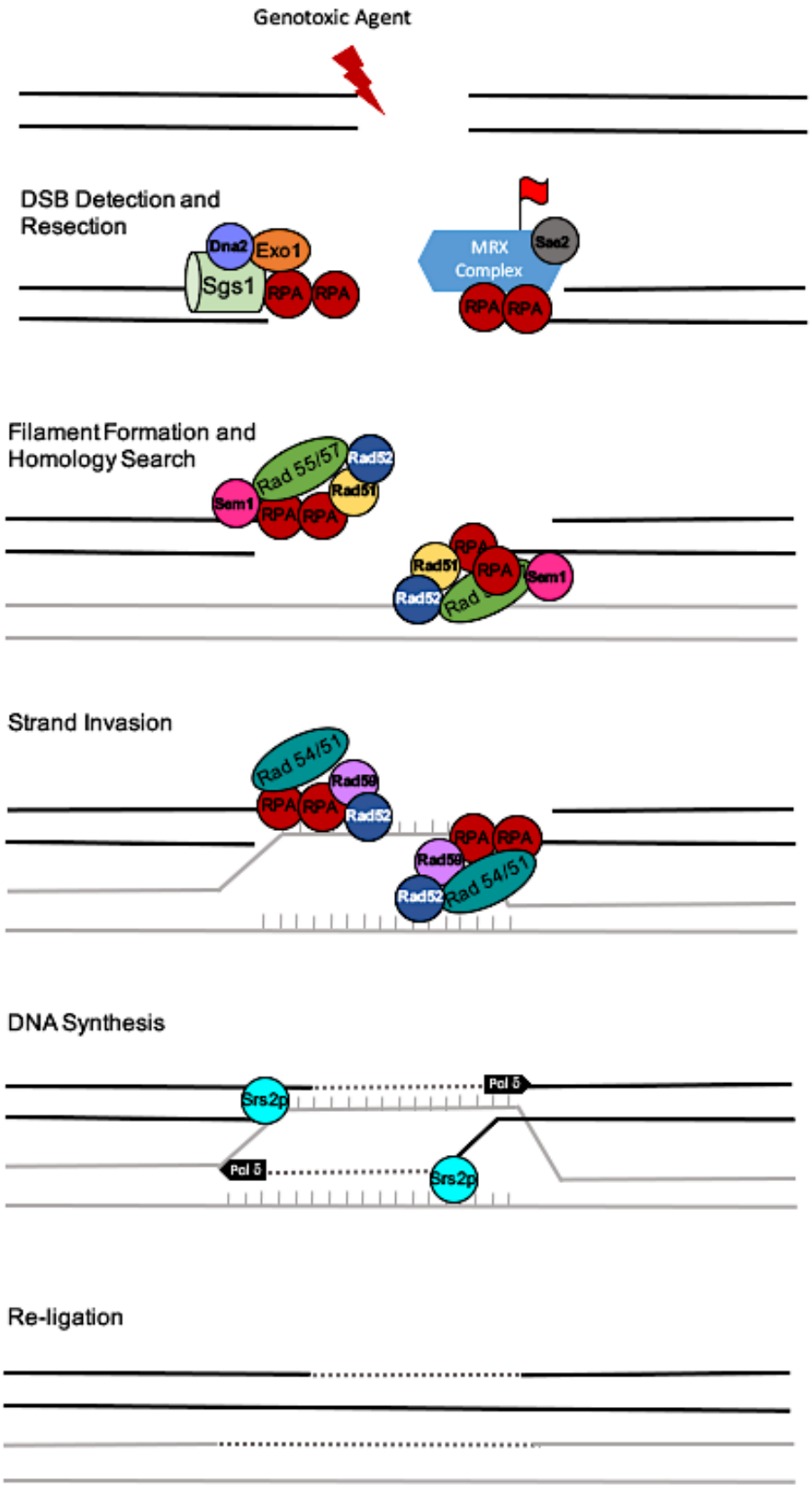

Figure 3. Homologous Recombination Pathway in S. Cerevisiae

Chromatin Remodeling in DNA Repair

It has previously been shown that chromatin remodeling is important for DNA repair, since chromatin intrinsically restricts accessibility of DNA repair proteins to damaged DNA [55–62]. Much of our mechanistic understanding of DNA repair has been derived from biochemical studies that investigate DNA repair reactions in non-physiological conditions which are not representative of the compact state of DNA in living cells. Recently, efforts to understand the role of chromatin remodelers in DNA repair process has elucidated that not only does chromatin decrease the rate of repair, but also that genomic repair rates display a distinctive pattern, suggesting that DNA repair is highly organized throughout the genome. Yu et al found that after ultraviolet (UV) irradiation global genome-NER is organized and initiated from specific genomic locations, and that deficiencies in chromatin modifiers, proteins that facilitate this carefully orchestrated repair, can have direct effects on the spatial distribution of mutations [63]. Extensive studies have been conducted on the role of chromatin remodeling in NER [56,58,59]. ATP-dependent chromatin remodelers, SWI/SNF and INO80, have been found to be important for the NER-mediated repair of cyclobutane-pyrimidine dimers (CPD) in the heterochromatic regions of the yeast genome [64]. Investigations into the role of chromatin remodelers in the maintenance of genome stability and DSB repair have identified that DNA repair pathway choice is mediated by cell cycle stage. Bennett et al showed that DSB recruitment of a host of chromatin remodelers was inhibited by non-homologous end joining (NEHJ) machinery, and that conversely, recruitment was facilitated by the HR pathway in G2/M [55]. The current

understanding of the role of chromatin remodeling in DSB repair has been extensively reviewed and studied [65]. Mammalian cells lacking H2AX have been shown to be hypersensitive to ionizing radiation, a DSB inducer, and to have defects in both NHEJ and HR directed repair [57]. Others have also shown that the chromatin remodeling complex INO80 is recruited to DSBs via H2AX phosphorylation, and that INO80 deficiency results in impaired recruitment of 53BP1 foci. This implicates INO80 in DNA strand resection, an early step of DSB repair [66,67]. Collectively, it can be concluded that chromatin remodeling promotes the formation of open, euchromatic DNA at DNA lesions which allows for the proper recruitment of DNA repair machinery and the timely repair of DNA damage in otherwise spatially confined regions of the genome.

The SWI/SNF Chromatin Remodeling Complex Overview

In our *S.cerevisiae* gene deletion screen, we observed that deletion of the chromatin remodeling genes SWI3, SNF6, SNF2, and ARP5 resulted in increased cytotoxicity following formaldehyde exposure under chronic conditions [37]. To the best of our knowledge this is the first report of chromatin remodeling being important for cell survival following formaldehyde exposure. The SWI/SNF chromatin remodeling complex initially identified in yeast 22 years ago, belongs to a family of multi subunit complexes that use ATP for active remodeling of nucleosomes [60]. In humans, the complex is comprised of two mutually exclusive ATPase core subunits, BRG1 and BRM. It can form into a multitude of unique combinations of core and accessory proteins; several have been implicated to play a role in DNA repair (Table 1). There are two-well defined complex subsets, BAF

and PBAF, which contain BRG1/BRM or BRG1 only, respectively. Both complexes have been shown to be important for an appropriate DNA Damage Response (DDR) and maintenance of sister chromatid cohesion [68].

SWI/SNF subunits.

Protein	Gene name	Complex(es)	Features	Notes
BRG1	*SMARCA4*	BAF and PBAF	Bromodomain, ATPase	Roles in NHEJ, HR, repression of transcription after DNA damage
BRM	*SMARCA2*	BAF	Bromodomain, ATPase	Role in NHEJ
BAF250a/b	*ARID1A/B*	BAF	ARID domain	Role in NHEJ, promotes BRM association with damaged chromatin, interaction with TopoIIa
BAF200	*ARID2*	PBAF	RFX-type winged helix, Zinc finger, ARID domain	
BAF180	*PBRM1*	PBAF	6 Bromodomains, 2 BAH domains, 1HMGB domain	Promotes sister chromatid cohesion, repression of transcription after DNA damage
BRD7	*BRD7*	PBAF	Bromodomain	
BAF170	*SMARCC2*	BAF and PBAF	Chromo, SWIRM and SANT domains	Roles in HR and NHEJ, not needed for BRM accumulation at damaged chromatin, interaction with BRIT1
BAF155	*SMARCC1*	BAF and PBAF	Chromo, SWIRM and SANT domains	Roles in HR and NHEJ, not needed for BRM accumulation at damaged chromatin, interaction with BRIT1
BAF60a/b/c	*SMARCD1/2/3*	BAF and PBAF	SWIB	Role in NHEJ, promotes BRM association with damaged chromatin
BAF57	*SMARCE1*	BAF and PBAF	HMGB	No NHEJ defect when depleted
BAF53a/b	*ACTL6A/B*	BAF and PBAF	Actin related protein	No NHEJ defect when depleted
Actin	*ACTB*	BAF and PBAF	Actin	
BAF47 (SNF5)	*SMARCB1*	BAF and PBAF		Role in NHEJ, promotes BRM association with damaged chromatin
BAF45a/b/c/d	*PHF10/DPF1/DPF3/DPF2*	BAF and PBAF	2 PHD fingers	

Table 2. SWI/SNF Subunits and Their Role in DNA Repair

The SWI/SNF complex has been shown to have a large variety of cellular roles and has been widely implicated in tumorigenesis [60,68–70]. Reports have demonstrated that components of the SWI/SNF complex can act as both a bona fide tumor suppressor and an oncogene [69,71–75]. Astonishingly, it was recently demonstrated, that 92% of undifferentiated/rhabdoid carcinomas of the gastrointestinal tract had mutations in at least one component of the SWI/SNF complex [72]. Given the strong implications of the SWI/SNF complex in human malignancy it should be of no surprise that the complex has also been demonstrated to play a role in several DDR and repair pathways. Smith-Roe et al showed that depletion of BRG1/BRM followed by treatment with 6 separate

genotoxic agents resulted in differential cell death. They concluded that SWI/SNF deficiency led to genome instability due to an inability to properly repair DSBs, but only those associated with stalled/collapsed replication forks [76]. SWI/SNF is thought to be recruited to DSBs by the NuA4 and Gcn5 histone acetyltransferase, to promote phosphorylation of H2AX by ATM/ATR and subsequent DSB repair [77–79]. These findings have also been corroborated in yeast studies showing that loss of SNF2 (Brg1) results in decreased levels and activity of Mec1 (ATR) [80]. In addition, the SWI/SNF complex has been shown to play a role in NER in both yeast and human cancer cells following UV exposure [64,81]. All-together, these findings support the profound impact of the SWI/SNF complex in mitigating genome instability and DNA repair which likely contributes to its role in human malignancies.

Preface: Chapter 1

Decades of epidemiological studies have highlighted the potential detrimental effects of formaldehyde exposure on human health. In addition, mutagenicity studies have shown the damaging effects of formaldehyde exposure on DNA in a myriad of model systems. Therefore, in this study we chose to investigate the effects of low-dose chronic formaldehyde exposure using several human cell lines derived from different tissue types. Moreover, given current evidence which suggests different DNA repair pathways may be participating in the formaldehyde-induced DNA damage response, perhaps due to different crosslinking chemistries/DPC size, exposure conditions, or tissue types, we chose to investigate the roles of DNA repair genes in preventing cellular toxicity. Finally, our goal is to discern the cellular pathways that are mitigating formaldehyde-induced DNA damage.

Chapter 1: A Cell Line Specific siRNA Screen of Candidate DNA Repair Genes Mediating Cytotoxicity Following Formaldehyde Exposure

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Abstract

Human exposure to formaldehyde occurs through commercial uses in the chemical industry, as a byproduct of combustion, from off-gassing of various building products, and as a fixative for pathologists and embalmers. It has been classified as a class I human carcinogen by the International Agency for Research on Cancer and a known human carcinogen by the National Toxicology Program. Thus, it is important to not only delineate the biological pathways necessary for repair of formaldehyde-induced DNA damage, but also to understand how cells cope with exposure through repair and tolerance. The covalent crosslinking of proteins to DNA is the predominant lesion caused by formaldehyde exposure, and this presents a major challenge to multiple cellular processes. In addition to formaldehyde, DNA-protein crosslinks are also induced by a variety of environmental and endogenous agents including ultraviolet light, metals, aldehydes, chemotherapeutics, cigarette smoke, and physiological metabolites. Thus, understanding pathways for cellular responses to these DNA adducts will have broad impact. In this study, we sought to determine the genes necessary for survival following chronic formaldehyde exposure using a DNA-damage response siRNA library in three human cell lines: GM00639, U2OS, and SW480. Here we show that different genes mitigate cytotoxicity following formaldehyde exposure in a cell line specific manner, likely due to genetic variance across cell lines and tissue types. We demonstrate that though there is variance in the genes necessary for survival, four cellular pathways were universally necessary to mediate formaldehyde toxicity following chronic

exposure: the homologous recombination pathway, double-strand break repair, ionizing radiation response pathway, and DNA replication. These findings highlight the importance of understanding genetic context and heterogeneity when performing high-throughput genotoxic assays. Importantly, we also show that the pathways necessary to mitigate cytotoxicity following formaldehyde exposure are not only conserved across cell lines, but also, species.

Introduction

Environmental and endogenous exposure to chemicals that produce DNA-protein and DNA-peptide crosslinks are correlated with an increased risk of several cancers, asthma, and other diseases [2,13,19,21]. Currently, many individuals in the US population are exposed to one of the most common DNA-protein crosslink (DPC) inducing agents, formaldehyde [5,14,82]. Exposures to formaldehyde take place in both occupational and in-home settings, and affected individuals often experience multi-year chronic exposures that are far in excess of typical indoor air quality standards [2]. The highest levels of formaldehyde have been shown to occur in occupational settings [1]. Exposed workers are commonly found in industries that manufacture formaldehyde and formaldehyde-based resins, textiles, wood composites/furniture, fiberglass, automobiles, and home appliances [3–6]. In addition, people working in laboratories, pathology departments, as embalmers, and in agriculture are also exposed to formaldehyde. Although environmental exposure to formaldehyde typically occurs at much lower levels than occupational exposures, a greater number of people are exposed to formaldehyde in their daily lives. Environmental sources of formaldehyde include, but are not limited to, automobile exhaust, cigarettes and e-cigarettes, cosmetic products, burning of forest and manufactured wood products, urea formaldehyde home insulation (used mainly in homes built in the 1970-1980s), off-gassing from furniture, carpeting and flooring [7,8]. There are also numerous sources of endogenous formaldehyde including the one carbon pool, amino acid and alcohol metabolism, lipid peroxidation, and P450 dependent demethylation [2]. Interestingly, elevated levels

of formaldehyde have also been found in some human tumors [9]. Given the vast array of occupational, environmental, and endogenous sources of formaldehyde exposure it is of critical importance to investigate the biological and epidemiological consequences of formaldehyde.

Several studies have identified specific cellular pathways important for cell survival via repair of DPC lesions [30,37], including Nucleotide Excision Repair (NER) [37,38], proteosomal degradation [29], the Fanconi Anemia pathway [39–41], and Homologous Recombination (HR) [37,42,48]. Our lab, and others, have shown that formaldehyde exposure leads to cell cycle perturbations, cytotoxicity, and aberrant gene expression [37,38,48,83–86]. We demonstrated via systematic screens of the Saccharomyces cerevisiae non-essential gene deletion library, that components of the NER pathway were necessary for cell survival after high-dose acute formaldehyde exposure. Conversely, components of the HR pathway were necessary for survival following low-dose chronic exposure [37]. These results indicated that *S.cerevisiae* responds in fundamentally different ways to formaldehyde, dependent on exposure conditions, and suggested that DPC repair was highly regulated and dose dependent. We also demonstrated that NER-deficient Chinese Hamster Ovary cell lines UV41 (XPF-deficient) and UV20 (ERCC1-deficient) were highly sensitive to acute formaldehyde exposure [38]. We later showed that human HR deficient Bloom-Syndrome RecQ Like Helicase (BLM) cells had increased sensitivity to formaldehyde, and an immediate G2/M cell cycle arrest followed by an accumulation of chromosomal breaks and radials, in a replication-dependent manner [48].

Our previous findings highlight the importance of understanding dosing and cell cycle timing when performing genotoxic studies. Several publications have also underscored the significance of discerning the role of genetic and gene expression heterogeneity when addressing discordant data in the literature, variable chemotherapeutic response, as well as acquired drug resistance [87–92]. In addition, others have also demonstrated, that the DNA damage response can vary greatly across tissues types and species [93–95]. Collectively, these data suggest that "background" mutations in cell lines, that are inherently genomically unstable, and tissue specific gene expression may affect response to genotoxic agents. Ultimately, this accentuates the importance of cross species and cross cell line/tissue analyses when performing genotoxic studies that may inform patient care and occupational hazard guidelines.

Given the vast array of pathways implicated in DPC repair and the environmental and epidemiological implication of formaldehyde exposure, we sought to identify the roles of individual DNA damage response (DDR) gene products in modulating cytotoxicity following chronic formaldehyde exposure. Using a custom DDR siRNA library, we surveyed 318 genes representing proteins of interest in pathways such as HR, double-strand break (DSB) repair, non-homologous end joining (NHEJ), and cell death. In addition, understanding that human cell lines exhibit both genetic and phenotypic heterogeneity, we assayed all 318 genes across three different human cell lines: GM00639, SW480, and U2OS. These cells lines are commonly used in genotoxic studies and are derived from different tissues; human fibroblast, epithelial

adenocarcinoma, and epithelial osteosarcoma, respectively [48,96–98].

In the present study, we report heterogeneity in the genes that were necessary for survival following chronic formaldehyde exposure across our three cell lines. Importantly, we also found four pathways, HR, DSB repair, ionizing radiation (IR) response, and DNA replication, that were profoundly important for cell survival across all three cell lines and tissue types. Our findings reveal the importance of genetic context and cell origin when performing high-throughput genotoxic studies. Further, these insights may also help explain conflicting reports in the literature regarding which DNA repair pathways are critical, and the severity of the detrimental effects of formaldehyde exposure on humans.

Methods

Cell Lines and Culture Conditions

The following human cell lines were used: GM00639, U2OS, and SW480. GM00639 and U2OS were a kind gift from Dr. Robb E Moses (OHSU). SW480 was a kind gift from Dr. Owen McCarthy. Cells were grown in DMEM supplemented with 10% fetal bovine serum and anti/anti (penicillin, streptomycin, and Amphotericin B) (Gibco) at 37 °C in a 5% CO_2 incubator.

IC_{50} Determinations

For all IC_{50} experiments, sub-confluent cultures were plated in 96 well-plates in triplicate and allowed to adhere overnight. Cells were then treated with various concentrations of formaldehyde (Fisher Scientific) as noted throughout. After a 5-day continuous formaldehyde treatment, cells were assayed for viability by

CellTiter-Glo® assay (CTG) following the manufacturer's instructions. CTG is a method used to determine the number of viable cells in culture based on quantification of the ATP present, which signals the presence of metabolically active cells. Briefly, 100 µl of CTG reagent was added to each well and placed on a shaker for 10-15 min to allow for homogenous mixture. Luminescence output for each well was measured using a Tecan plate reader (Infinite M200). IC_{25-75} calculations for each cell line were made using Graph Pad Prism 7 software (La Jolla, CA, USA) with a sigmoidal, 4PL, log curve.

High-throughput RNA Interference Screen

A custom-designed DNA Damage Response-Repair library for our siRNA screen was used [99]. The screen was performed with cell viability as the phenotypic endpoint, as measured by CTG assay, on three cell lines: SW480, U2OS, and GM00639. siRNA screens were performed in a 384-well format using existing automation within the Quellos High Throughput Screening Core (University of Washington). Transfection feasibility for each cell line was established using a duplex targeting kif11 (kinesis-like protein) that arrests cells in mitosis, as a positive control, with CTG readout for viability. Minimum metrics for viability based screening are at least 50% loss of viability upon kif11 target engagement, while lethality associated with non-targeting duplexes and mock treatment are less than 25% absolute deviation when using a population of untreated wells (cells plus media and Optimem only) as a reference. Mock and non-targeting universal siRNA controls were used as negative controls. Putative IC_0, IC_{10}, IC_{25}, IC_{50}, IC_{75} and IC_{90} dosimetry curves for each cell line with formaldehyde were

established via automated titration in the range of 0 to 100 μM, using our previously established dose ranges as a guide. Compound addition methods yielded very similar signal intensities using either peri pumps or capillary pins. Therefore, peri pumps were used at a volume of addition of 5 μL, and because they limit the ambient exposure time of cells making them ideal for bulk dispensing. All reagent conditions were statistically evaluated using a simple Z-factor score (all scores $\geq$ 0.5) to determine differences and variability among replicates, and to identify optimal transfection and treatment conditions for each cell line.

Briefly, the siRNA library targeting 318 DDR genes was synthesized on 0.25 μmol scale (Qiagen) with 4 siRNAs to each gene target pooled in a single well. siRNA pools for each gene were tested in triplicate with each replicate on a separate plate to establish experimental variability and statistical validity. Cells were plated in opaque 384-well dishes and concurrently transfected with siRNA pool or mock solution. After 24 hours, cells were treated with varying concentrations of formaldehyde or PBS (untreated). A CTG assay was performed to access viability using an Envision Multilable detector/plate reader (Perkin Elmer). CTG reagent alone (blank) was subtracted from all wells to establish final luminescence values. The scramble siRNA negative control was used to monitor off target effects and results were standardized as percent survival of siRNA transfected compared to mock transfected on the same plate.

siRNA Screen Quality Analysis

We expected some siRNA transfections alone to be cytotoxic without formaldehyde treatment. To identify these cases, exclusion plots for each cell line were generated to determine the effects of siRNA alone knockdown (without formaldehyde) on cell viability with ≥ 20% cell viability (≤ 80% cell death) as a cut off in an effort to remain within the assay detection limits and biological relevance (data not shown). Next, we determined acceptable reproducibility across replicates for each formaldehyde treatment and calculated the standard deviation to determine the variation across the sample replicates. Z Scores for the standard deviation were calculated using the equation

$$\mathrm{Z-score\ (SD)} = \frac{\mathrm{XSD} - \mu}{\sigma} \qquad \text{Equation 1}$$

where μ is the mean for the standard deviations, and σ standard deviation for the standard deviations. This determined acceptable reproducibility across replicates for each formaldehyde treatment for our assay. Genes with z-scores ≥ +2.0 or those for which an untreated data point did not meet the quality minimums outlined above were excluded.

siRNA Screen Data Analysis

The cell survival mean (μ_t) from three replicates, for each gene and dose, was determined across all cell lines. The mean for each dose treatment (μ_t) was then normalized to the untreated mean (μ_u) to calculate normalized survival or mean (μ_n).

$$(\mu n) = \frac{\mu t}{\mu u}$$ Equation 2

Normalized data were then used to calculate area under the curve (AUC) using GraphPad Prism 7 software (La Jolla, CA, USA). Z scores for AUC were calculated using the equation below, where μ_a is the mean for the AUCs, and σ_a standard deviation for the AUCs.

$$Z - \text{score (AUC)} = \frac{\text{AUC} - \mu a}{\sigma a}$$ Equation 3

In each cell line, all genes with Z scores ≤ 0 were considered sensitive. This cut-off score was chosen because we used a biased siRNA library highly enriched for genes sensitive to genotoxic agents, and for which sensitivities were expected to be less dynamic across genes. Highly sensitive genes were classified as genes with Z score ≤ -1.0 for each cell line. Genes with a Z score ≥ +2.0 were classified as protective genes.

Results

Identification of DNA Damage Response Pathways that are Important for Survival Following Formaldehyde Exposure

The following cell lines were used in these studies: GM00639, U2OS, and SW480. GM00639 is a 'normal' SV40 transformed human fibroblast line; SW480 a human

epithelial colorectal adenocarcinoma cell line; U2OS is a human epithelial osteosarcoma cell line, and all have been extensively used in genotoxicity studies [48,96–98]. Prior to the siRNA screen, dose-response curves for each cell line following chronic formaldehyde treatment were determined. Cell lines were grown in 96-well plates and treated with increasing doses of formaldehyde for five continuous days (Figure 4A). Cell viability was assessed using CTG assays. The extent of cytotoxicity following treatment with various concentrations of formaldehyde was cell line-specific (Figure 4B-D). Though there were obvious differences in cytotoxicity between cell lines, all cell lines had a similar dose range response supporting their use in our high-throughput assays and validating the appropriateness of our conditions for comparisons across cell lines (Figure 4E). Therefore, a DDR RNAi viability screen was performed on our set of human cell lines described above. A total of 318 genes were interrogated using an arrayed siRNA platform that quantified cell viability after knockdown with pooled siRNAs. All assays were performed in triplicate. Cell viability was assessed after 5 days of continuous formaldehyde treatment using the CTG assay. Quality assessment was performed on the cell viability data as described in the Materials and Methods section.

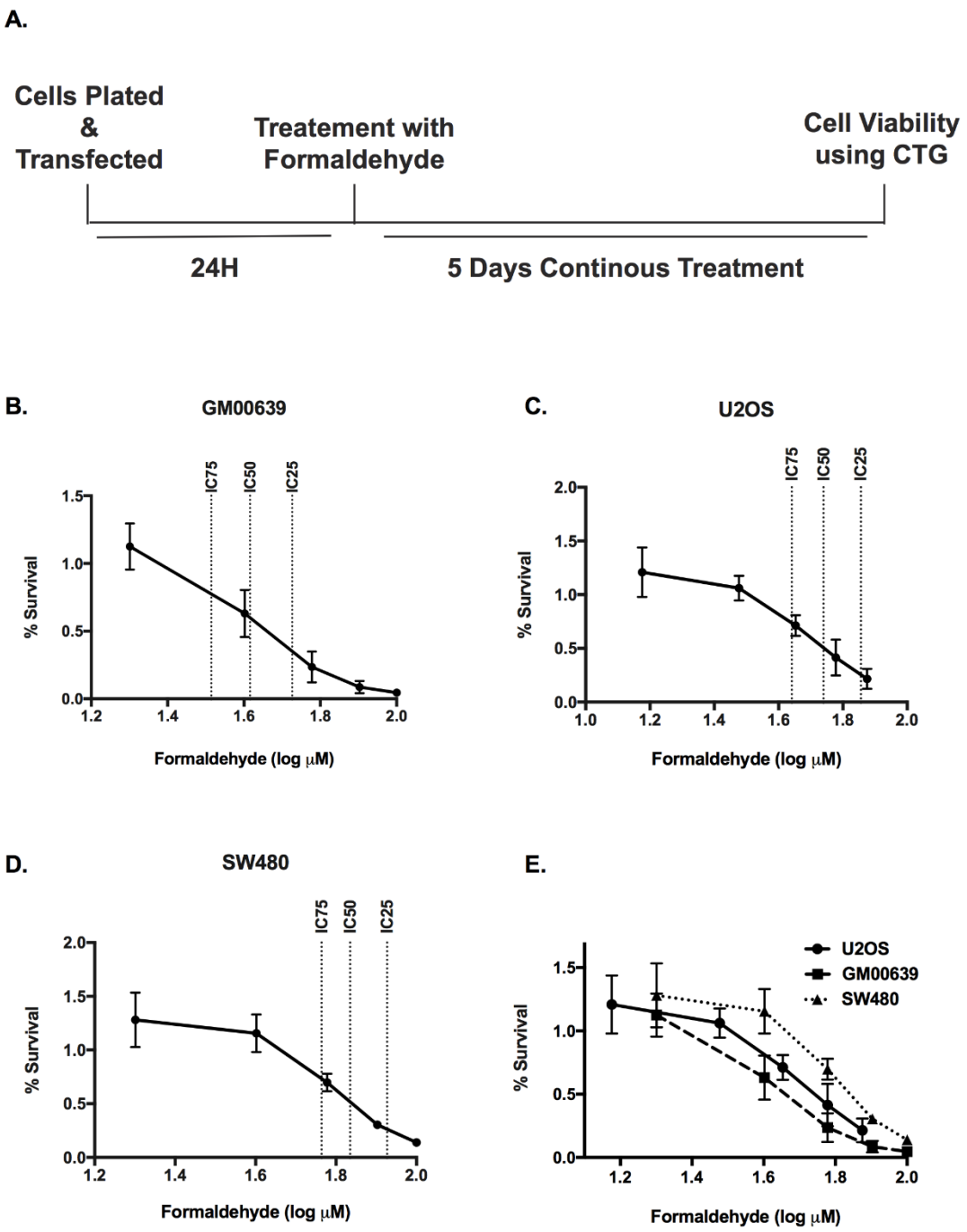


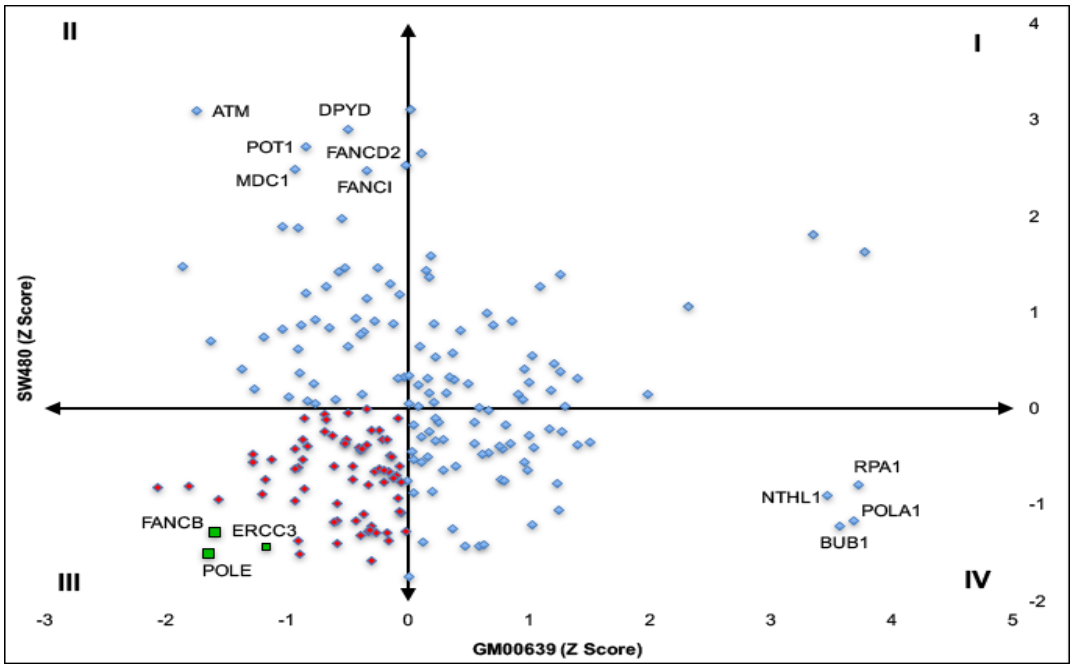
Figure 4. GM0639, U2OS, and SW480 have similar dose response curves following chronic formaldehyde exposure. A) Schematic of our siRNA approach. Dose response curves following 5 days continuous formaldehyde exposure (uM). B) GM639 C) U2OS D) SW480 E) All cell lines.

Heatmaps for all data that met quality metrics for each cell line were created using Cluster 3.0 software and Java Tree View; hierarchical clustering across genes using a complete linkage method was performed (Figure 5). These heatmaps accurately depict the different dose responses for genes across cells lines. They also show the overall sensitivity to formaldehyde for each cell line, and the heterogeneity in genes sensitive and protective following formaldehyde exposure across each cell line. Cartesian plots with cross cell line comparisons of cell viability Z-scores for all genes, for all possible iterations, were created (Figure 6). These plots highlight the genes that were sensitive, protective, and disparate in sensitivity among different cell line comparisons. Collectively, we found that though a large portion of genes were sensitive across at least two cell lines, only 23 genes were sensitive across all cell lines (Figure 7A). These 23 genes likely represent pathways that are essential for mitigating DNA damage induced by formaldehyde exposure. Further, no genes were highly sensitive (Z-score ≤ -1) or protective (Z-score ≥ 2) across all cell lines (Figure 7B-C). This emphasizes how tissue or cell type can drastically impact which pathways and genes are important to modulate a DDR

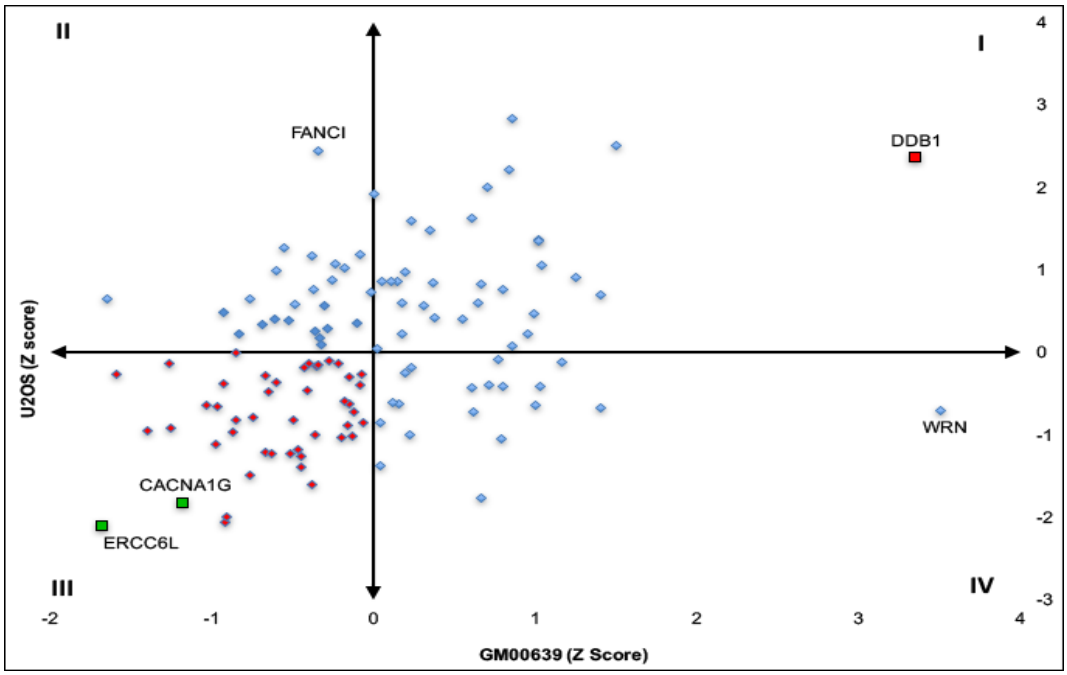
Figure 5. Heat maps depict the dose-dependent cell death following gene knockdown and formaldehyde exposure for all genes within each cell line.

Heatmaps for all genes for each cell line depicting the severity of cell death compared to siRNA alone.

A.


II
I
III
IV
ATM
DPYD
POT1
FANCD2
MDC1
FANCI
FANCB
ERCC3
POLE
RPA1
NTHL1
POLA1
BUB1
SW480 (Z Score)
GM00639 (Z Score)
-3
-2
-1
0
1
2
3
4
5
-2
-1
0
1
2
3
4

B.


II
I
III
IV
FANCI
DDB1
WRN
CACNA1G
ERCC6L
U2OS (Z score)
GM00639 (Z Score)
-2
-1
0
1
2
3
4
-3
-2
-1
0
1
2
3
4

C.

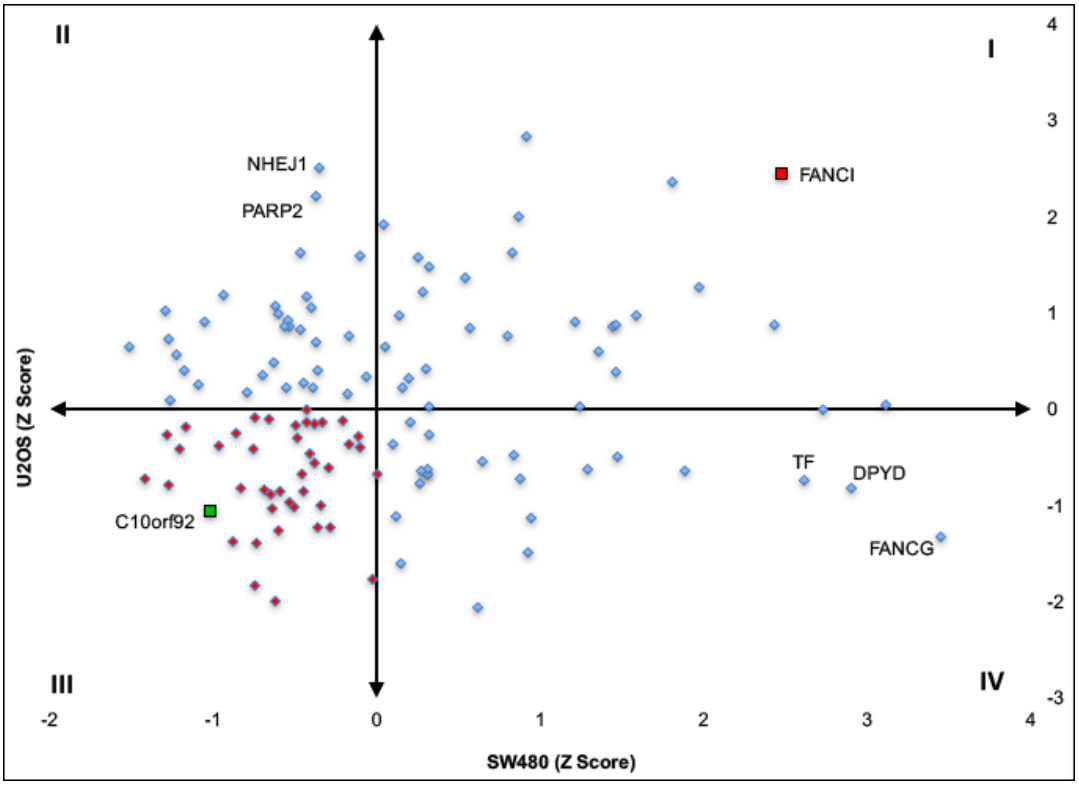


Figure 6. Comparisons across cell lines show heterogeneity in the genes that are necessary for protection and cytotoxicity following formaldehyde exposure. Cartesian plots of Z-scores depicting gene targets that had the highest impact on cell viability. A) GM00639 v SW480, B) GM00639 v U2OS, C) SW480 v U2OS (Red diamond: sensitive in both cell lines; Green square: Z-score ≤ -1; Labeled in quadrants II and IV: disparate genes; Red square: protective in both cell lines).

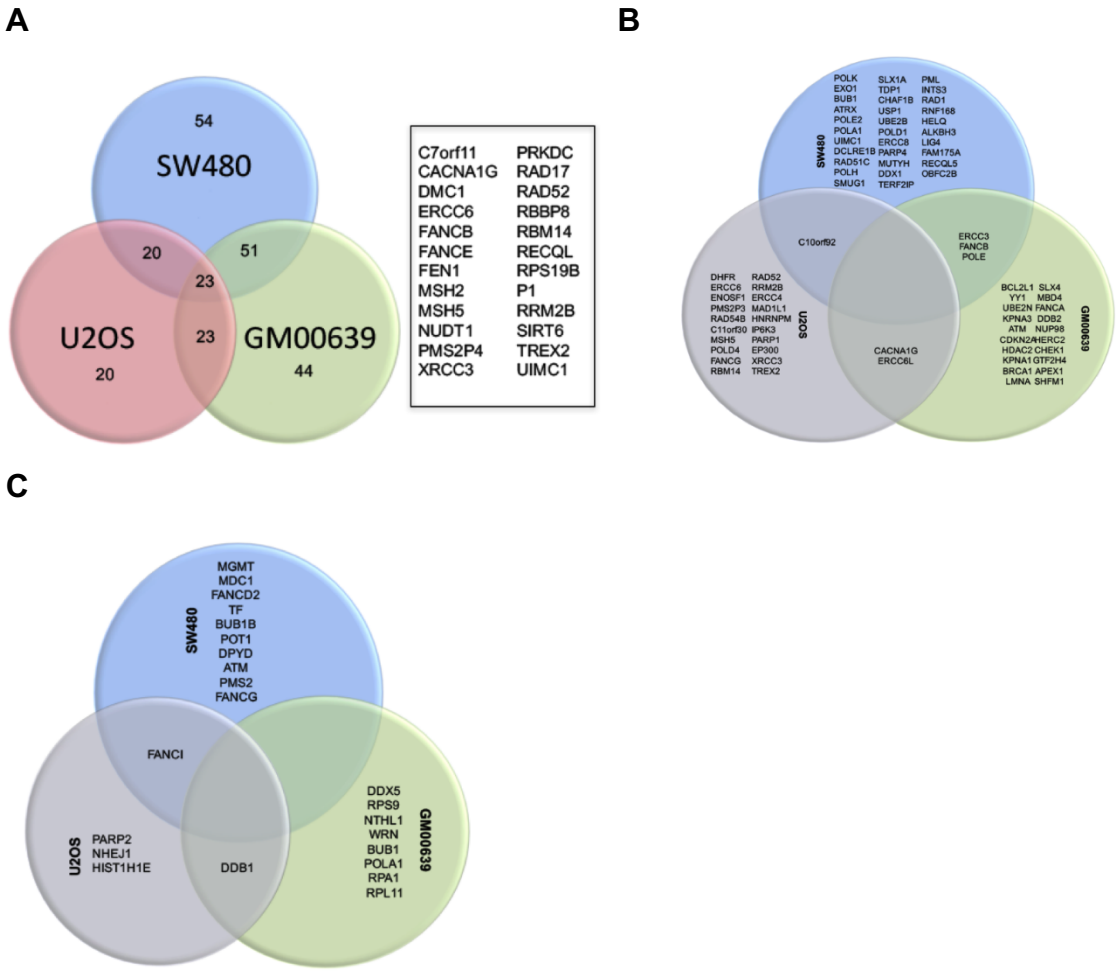
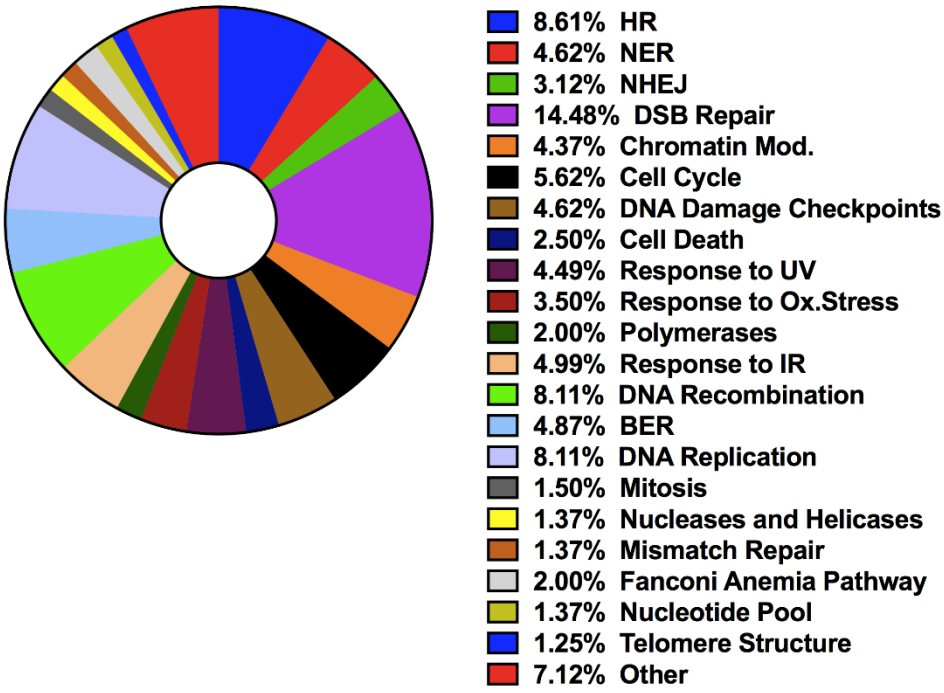


Figure 7. Twenty-three genes were sensitive across all three cell lines. Venn diagrams with A) Sensitive genes (Z-score ≤ 0). Depicted are the 23 genes that were sensitive in all three cell lines. B) Genes highly sensitive (Z-score ≤ -1) to formaldehyde exposure for all three cell lines. C) Genes that were protective following formaldehyde exposure, resulting in less cell death than formaldehyde alone (Z-score ≥ +2) for all three cell lines.

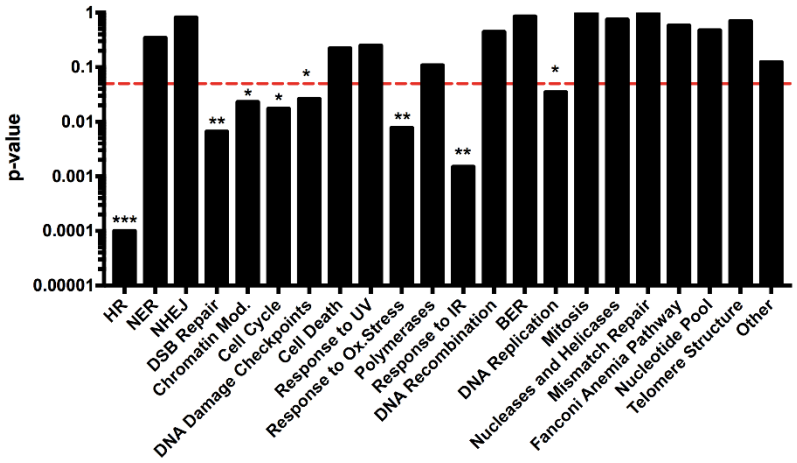
Homologous Recombination, Replication, and Double-Strand Break Repair Pathways Mitigate Cytotoxicity Following Formaldehyde Exposure

To investigate if networks or pathways may be conserved across cell lines, genes in our DDR siRNA screen were analyzed using the Gene Ontology (GO) consortium terms and manual literature searches to classify them into DDR pathways. It should be noted that a single gene can be, and was, classified in more than one pathway when appropriate. In totality, 318 genes were interrogated and assigned to 22 different DDR pathways (Fig 8A). Using these pathway classifications, we sought to identify if our highly sensitive genes belonged predominantly to any pathways that were represented greater than expected by chance, given the distribution of the pathway in our library. We performed a well-established siRNA screen statistical methodology, two-tailed Fisher's exact test, and found that 8 DDR pathways were highly sensitive to formaldehyde exposure across all three cell lines. These pathways represent HR, DSB Repair, Chromatin Modification, Cell Cycle, DNA Damage Checkpoints, Response to Oxidative Stress, Response to IR, and DNA replication (Fig 8B). Given our small and biased sample size, we chose to also perform bootstrapping with replacement, a more rigorous and robust statistical test. This stringent analysis identified 4 pathways that were significantly over-represented within our highly sensitive gene set: HR, DSB Repair, Response to IR, and DNA replication (Fig 8C). These results indicate that these pathways are crucial for mitigating formaldehyde-induced toxicity following chronic exposure. These findings also highlight that although there is significant heterogeneity in the genes necessary

A


8.61% HR
4.62% NER
3.12% NHEJ
14.48% DSB Repair
4.37% Chromatin Mod.
5.62% Cell Cycle
4.62% DNA Damage Checkpoints
2.50% Cell Death
4.49% Response to UV
3.50% Response to Ox.Stress
2.00% Polymerases
4.99% Response to IR
8.11% DNA Recombination
4.87% BER
8.11% DNA Replication
1.50% Mitosis
1.37% Nucleases and Helicases
1.37% Mismatch Repair
2.00% Fanconi Anemia Pathway
1.37% Nucleotide Pool
1.25% Telomere Structure
7.12% Other

B


p-value
1
0.1
0.01
0.001
0.0001
0.00001
HR
NER
NHEJ
DSB Repair
Chromatin Mod.
Cell Cycle
DNA Damage Checkpoints
Cell Death
Response to UV
Response to Ox.Stress
Polymerases
Response to IR
DNA Recombination
BER
DNA Replication
Mitosis
Nucleases and Helicases
Mismatch Repair
Fanconi Anemia Pathway
Nucleotide Pool
Telomere Structure
Other

C

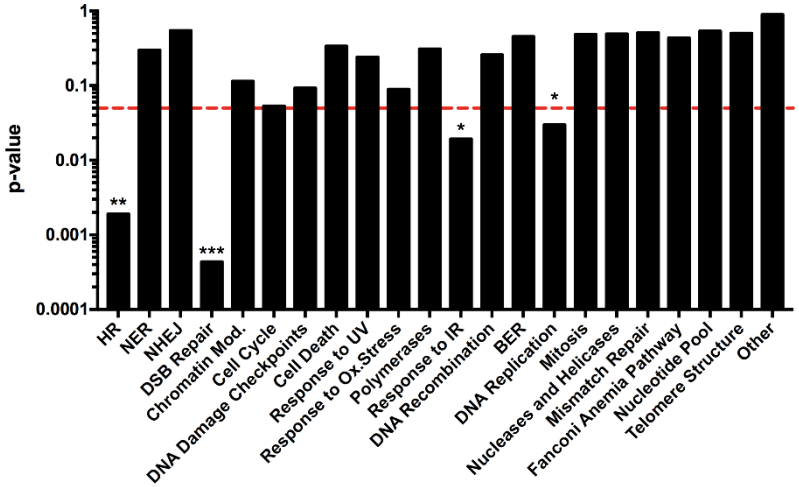

p-value
1
0.1
0.01
0.001
0.0001
HR
NER
NHEJ
DSB Repair
Chromatin Mod.
Cell Cycle
DNA Damage Checkpoints
Cell Death
Response to UV
Response to Ox.Stress
Polymerases
Response to IR
DNA Recombination
BER
DNA Replication
Mitosis
Nucleases and Helicases
Mismatch Repair
Fanconi Anemia Pathway
Nucleotide Pool
Telomere Structure
Other

Fig 8. Pathway comparison identified several pathways significantly necessary to mitigate cytotoxicity following formaldehyde exposure.

Genes were curated into pathways using GO and manual literature searches. (A) Depiction of the 22 pathways and their percentage of representation within our siRNA pathway. Genes that exist in more than one pathway were counted multiple times for these percentages. (B) Plotting of p-values derived using a Fisher's exact test. (C) Plotting of p-value derived using bootstrapping with replacement with 1000 iterations. Dotted red line represents the p-value ≤ 0.05

to mitigate cell survival following chronic formaldehyde exposure, likely due to cell intrinsic genetic variability, the HR, DSB repair, IR Response, and replication pathways are conserved in this response and necessary irrespective of cell type or origin.

Genes Sensitive Across All Cell Lines Show Differential Cytotoxicity at Low and High Chronic Doses of Formaldehyde

Our previously published findings showed that, in yeast, different DDR response genes and pathways are necessary for survival following chronic low-dose versus acute high-dose formaldehyde exposure. Moreover, epidemiological studies have also shown that low-dose exposure can sometime be more detrimental to human health than high doses. In addition, given current efforts to use formaldehyde as a possible combinatorial chemotherapeutic, understanding the genes necessary for cytotoxicity following low-dose versus high-dose formaldehyde exposure will be crucial when trying to target tumors deficient genes and pathways for treatment. Therefore, we chose to investigate the impact of dose on the genes that were

necessary for survival. This analysis was limited to the 23 genes that were sensitive in all three cell lines. We found that, for example, knockdown of RBPP8 (CtIP) resulted in increased cytotoxicity at a low dose in SW480, at a high dose in U2OS, and showed transient sensitivity in GM00639, further highlighting the importance of understanding genetic context when performing large genotoxic screens. Knockdown of genes like FANCE and XRCC3 consistently resulted in cell death at high and low doses respectively (Figure 9). It should also be noted that though SW480 was the most refractory to formaldehyde requiring a higher dosage for its IC^{50} compared to the two other cell lines, gene specific dosages for cytotoxicity were not high across the board. This emphasizes that other mutations and genetic alterations within the cell likely contribute not only to gene expression changes, but also protein and pathway recruitment following formaldehyde exposure.

Genes Necessary to Prevent Cell Death Following Formaldehyde Exposure are Conserved Across Species

Previously, our lab showed via a systemic screen of the S. cerevisiae non-essential gene deletion library that components of the NER pathway are necessary for survival after high-dose acute formaldehyde exposure. Conversely, we found that components of the HR pathway were necessary for survival following low-dose chronic formaldehyde exposure [37] . These results showed that S. cerevisiae responds in fundamentally different ways to DPC induction dependent on exposure conditions. In this study, we sought to elucidate the genes and pathways necessary for cell survival following chronic formaldehyde exposure in human

cells. We found that similar to the S. cerevisiae screen, the human HR pathway was necessary for survival following chronic formaldehyde exposure (Figure 6). We then investigated if there was overlap between the 23 genes sensitive across all cell lines and our yeast screen. We found that 17 genes in our DDR library had yeast homologue; one gene was part of the yeast essential gene library and hence was not included in our yeast screen. Of the 16 remaining genes from the human DDR screen, 9 genes were also designated as sensitive in our yeast screen (Table 3). Interestingly, genes that were sensitive in both screens, belonged to pathways that we identified in our human screen to be significantly sensitive to formaldehyde: HR, DSB, replication, DNA damage checkpoints, and cell cycle pathways. Conversely, genes that were not sensitive in both screens belonged to DNA repair pathways that were not found to be sensitive to chronic formaldehyde exposure in our human screen, such as mismatch repair and base-excision repair. This further strengthens our conclusion that though there may be some heterogeneity in the genes that are necessary for survival following chronic formaldehyde exposure across different cell lines, it is clear, that the HR, DSB repair, and DNA replication pathways are essential for cell survival across cell lines and species.

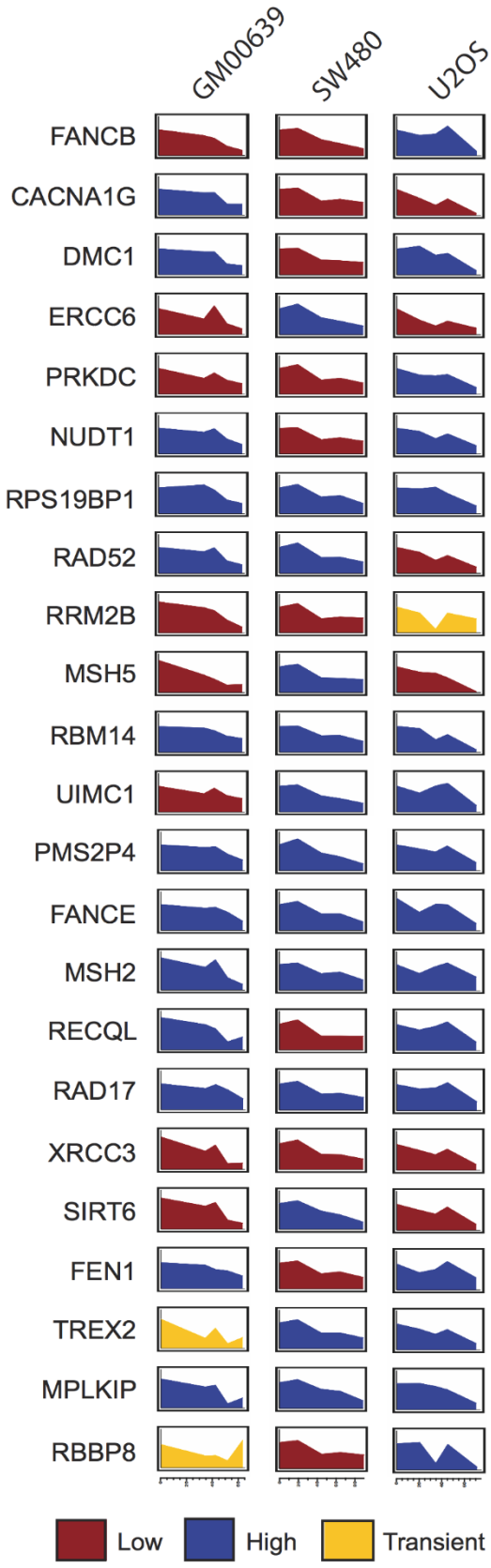


Figure 9. Genes respond differentially to low and high doses of formaldehyde across different cell lines. We identified 23 genes sensitive across all cell lines. A) Area-under the curve graphs were constructed for all 23 sensitive genes. Curves were labeled low (red), high (blue), or transient (yellow) corresponding to the doses necessary to induce cell death following chronic formaldehyde exposure.

	Yeast Comparison			
Human	**Homologue**	**Systemic Name**	**Sensitivity**	**Description**
MPLKIP	CDC5P	YMR001C	Essential	Cell cycle/ Mitosis
DMC1	ECM30	YLR436C	Sensitive	HR/DSB repair/Mitosis
ERCC6	RAD26	YJR035W	Not Sensitive	NER/BER
FANCB	-	-	-	Fanconi Anemia
FANCE	-	-	-	Fanconi Anemia
FEN1	RAD27	YKL113C	Moderately	BER/DNA replication
MSH2	MSH2	YOL090W	Moderately	Mismatch Repair
MSH5	MSH5	YDL154W	Not Sensitive	Mismatch Repair
NUDT1	NPY1	YGL067W	Not Sensitive	BER
PMS2P4	PMS1	YNL082W	Not Sensitive	Mismatch Repair
PRKDC	-	-	-	NHEJ/DSB Repair
RAD17	RAD24	YER173W	Sensitive	Cell Cycle/DNA Damage Checkpoint
RAD52	RAD52	YML032C	Sensitive	HR/DSB repair/DNA Replication
RBBP8	SAE2	YGL175C	Not Sensitive	Cell Cycle/DNA Damage Checkpoint
RBM14	PSP2	YML017W	Sensitive	Mitochondrial mRNA splicing
RECQL	SGS1	YMR190C	Sensitive	HR/DSB Repair/Replication
RPS19BP1	-	-	-	Ribosomal Subunit
RRM2B	RNR4	YGR180C	Sensitive	DNA Damage Checkpoint/ Replication
SIRT6	SIR2	YDL042C	Moderately	Chromatin Modification/DNA Replication
TREX2	-	-	-	HR/DSB Repair
UIMC1	-	-	-	HR/DSB Repair/Chromatin Modification
XRCC3	RAD57	YDR004W	Not Sensitive	HR/DSB Repair

Table 3. Cross species analysis identified conserved genes and pathways necessary for mitigating cell death following formaldehyde exposure. Human and S. cerevisiae gene names, along with the yeast systemic name are provided. A brief description of the gene pathway involvement as well as the level of sensitivity in our previously published yeast screen are noted [37].

Discussion

It is important to perform comprehensive DDR genotoxic studies to elucidate the specific contributions of cellular pathways following genotoxic exposures. In this study, we demonstrated that the genes necessary for cellular responses to chronic formaldehyde exposure are heterogeneous. Irrespective, at the pathway level, the HR, DSB repair, IR Response and DNA replication pathways are important for cell survival and conserved across cell lines. These findings are in accordance with previously published work that has shown that different components of the HR pathway are critical for repair of formaldehyde induced DNA damage [37,42,48,100,101]. Previously, we showed that loss of BLM protein, an integral component of the HR pathway, leads to persistent and un-repaired formaldehyde-induced DSB formation. In addition, BLM deficiency resulted in an immediate and pronounced G2/M cell cycle arrest and this arrest was exacerbated when cells were treated in S phase, indicating that formaldehyde induced cell cycle perturbations occur in a replication-dependent manner [48]. Further validating our findings that the HR, DSB, and DNA replication repair pathways are important for a proper DDR following formaldehyde exposure. This emphasizes that the cellular responses and biochemical mechanisms associated with mitigating formaldehyde-induced DNA damage are complex, multi-gene and multi-pathway processes.

To identify the contribution of genetic context, “background” mutations and gene expression difference in different cell lines and tissue types, we performed our siRNA DDR response screen with three human cell lines derived from different

tissues. We found that 23 genes were sensitive across all three cell lines; no genes were protective or highly sensitive in all the cell lines. These findings provided strong evidence that understanding the full spectrum of mutations harbored within a cell line and having isogenic paired controls whenever feasible is of paramount importance when performing genotoxic studies in both normal and cancer cell lines. These results are also important to occupational and environmental formaldehyde exposure studies, since the tissue of origin of each cell line, in part, contributes to this heterogeneity. These findings could both confound results and lead to an underestimation of exposure risk in epidemiological studies, if studies are not performed in tissues relevant to means of exposure. Our work further highlights the complexity behind chemotherapeutic resistance given both intra and inter tumor heterogeneity, and strengthens the importance of developing new methods of personalized medicine that account for genetic variability in humans. By understanding the complex and changing genetic landscape of cell lines as a model system, we can identify the crucial pathways for cellular response and increase the number of actionable cellular targets.

In addition, in this study we show cell line specific differences in the dose necessary to elicit cytotoxicity following gene knockdown and formaldehyde treatment. Knockdown of genes such as RBBP8 (CtIP), recently shown to associate with BRCA1 and MRN to repair topoisomerase II-DNA adducts (DPCs), resulted in cell death following intermediate, low, and high doses of formaldehyde in GM00639, SW480, and U2OS, respectively[102]. In fact, others have shown that low doses of compounds may exhibit more cell death than high doses, and that

this process often depends on the metabolic activity of enzymes necessary to activate or clear compounds within that tissue [87]. These findings point to the importance of carefully selecting doses, particularly in transient responding genes, in toxicological experiments because this could lead to an underestimation of the exposure risk. This demonstrates that not only is timing of the insult and genetic context preeminent, but that dose is also an integral component dictating cellular response, and should be emphasized when performing high-throughput genotoxic studies.

Finally, new avenues are currently being explored to exploit the genotoxicity of formaldehyde and use it in combination with chemotherapeutic drugs for treatment of human cancers [103]. Therefore, it is important to delineate the cellular processes necessary to mitigate formaldehyde DDR. Identifying the genes and pathways necessary can help inform treatment regimens by, targeting cancers that are deficient in these genes and pathways.

In conclusion, this study demonstrates the importance of performing genotoxic studies informed of the diverse and dynamic genetic landscape of cell lines and tumor models. Additionally, it provides strong evidence that the HR, DSB repair, IR Response and replication pathways are conserved and critically important for mitigating a DDR following chronic formaldehyde exposure. Further studies are required to validate these findings and to address the specific role of these genes in repair of formaldehyde-induced DPCs and DNA damage.

Preface: Chapter 2

With the advent and advancement of next-generation sequencing, studies that delve into the role that genetic background may play on disease incidence, progression, and response to pharmaceuticals are becoming more accessible. Performing these types of studies are also of paramount importance to genotoxic investigations since mode and site of exposure varies widely across agents. In our previous study, we chose to interrogate the cellular pathways important to mitigate formaldehyde-induced DNA damage across 3 different human cells lines which are widely used in genotoxic studies. We found that 23 genes were important to mitigate formaldehyde induced cytotoxicity across all of our cell lines, and that the HR, DSB repair, IR Response, and DNA replication pathways were crucial for survival. The following study seeks to discern the molecular mechanisms underlying the importance of these pathways for DPC repair and cell survival. We hope to elucidate the interplay between these 4 DDR pathways, and how they may be working in conjunction with other cellular pathways, particularly chromatin remodeling, to mitigate DNA damage. Given the variable and often bulky size of DPCs, we hypothesized that chromatin remodeling may play a vital role in accessibility of DNA repair proteins to the lesion for initiation of repair. Our approach will use a yeast model system, since genetic manipulation, as well as other molecular biology endpoints, are more accessible in yeast and DNA repair and chromatin remodeling pathways are highly conserved from yeast to humans.

Chapter 2: Homologous Recombination and the SWI/SNF Chromatin Remodeling Pathways are Necessary for the Repair of Formaldehyde-Induced DNA Damage

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Abstract

The SWI/SNF complexes are a family of ATP dependent multi-subunit complexes that actively remodel nucleosomes. The involvement of the SWI/SNF complex in carcinogenesis was first demonstrated by the identification of biallelic, truncating mutations of the complex's subunit, SMARCB1, in the highly aggressive childhood cancer, malignant rhabdoid tumors. Subsequently, genome-wide sequencing studies have identified mutations in several other subunits of the SWI/SNF complex across several cancer types. The SWI/SNF complex is thought to play a critical role in DNA repair since chromatin intrinsically restricts accessibility of DNA repair proteins to damaged DNA. Recently, others have shown that its roles in transcriptional regulation, protein activation, and DNA strand invasion, may also contribute to its involvement in DNA repair processes. The mechanisms by which mutations in this complex specifically drive tumorigenesis are unclear. Here we investigated the importance of SWI/SNF-mediated chromatin remodeling for the effective repair of formaldehyde induced DNA damage. We found that the SWI/SNF complex and Homologous Recombination pathway were sensitive to chronic, but not acute formaldehyde exposure. We also showed that these two pathways are working together to mitigate repair of formaldehyde-induced double-strand breaks, and that repair deficiency contributes to subsequent cell death. Interestingly, though very limited cell death was noted following acute formaldehyde exposure in the snf2Δ strain, cells exhibited a severe growth delay and delayed DNA damage response. All in all, these findings support that the SWI/SNF complex is working with the homologous recombination pathway

to mitigate cytotoxicity and DNA damage induced by formaldehyde exposure. The roles of the SWI/SNF complex in DNA damage repair may ultimately contribute to its involvement in carcinogenesis and putative function as a bono-fide tumor suppressor.

Introduction

Abnormalities in chromatin remodeling, specifically due to dysregulation of the SWI/SNF complex, have recently been intricately linked to human tumorigenesis. An increasing number of studies show subunits of the SWI/SNF complex to be commonly mutated across several cancer types and subtypes, accounting for mutations in 20% of all cancers [104]. In fact, alterations of the SWI/SNF complex are currently used as diagnostic and prognostic biomarkers in several tumor types [60,61,104–109]. Though not completely understood, it is apparent from the frequency and diversity of mutated subunits that the SWI/SNF complex encourages tumorigenesis through its pleiotropic roles in the regulation of the cell cycle, oncogenic pathways, cellular metabolism, and DNA repair.

The SWI/SNF chromatin regulatory family was first identified in yeast exhibiting mating-type switching and sucrose non-fermenting defective phenotypes [110]. The SWI/SNF complexes are a family of ATP dependent multi-subunit complexes comprised of 12 genes in yeast and 28 in mammalian cells, which generate an extensive array of functional protein subunits [81]. Both the yeast and mammalian complexes are composed of an ATPase and multiple core subunits. In addition to these core subunits, the SWI/SNF complex forms with 7-15 accessory proteins [60]. The variability in the protein composition of the complex and diversity of its interchangeable subunits is reflected in its diverse cellular roles. It has previously been established that chromatin remodeling is important for DNA repair [55–58,68,111,112]. Recent publications have also shown that the human ATPase core subunit, BRG1, of the SWI/SNF complex, may play a role in Homologous

Recombination (HR) mediated Double Strand Break (DSB) repair. They showed that BRG1 is recruited to repair foci and that depletion of BRG1 leads to defective RAD51 filament assembly and increased RPA retention, suggesting that BRG1 promotes the exchange of RPA with RAD51 on single-stranded DNA; a crucial step in HR-mediated DSB repair [68,113]. In contrast, others showed that SMARCL1, a member of the SWI/SNF family of proteins, was important in mitigating topoisomerase 2-inhibitor induced DNA damage in G1, via non-homologous end-joining-mediated repair of DSBs [112]. Finally, others found that BRG1 may also be crucial for Nucleotide Excision (NER) mediated repair of UV-induced DNA lesions [64]. In Saccharomyces cerevisiae, F. Gong showed that Snf6 and Snf5, two subunits of the SWI/SNF chromatin-remodeling complex, co-purify with the NER damage-recognition heterodimer Rad4–Rad23. This interaction between SWI/SNF and Rad4–Rad23 was stimulated by UV irradiation, indicating that the SWI/SNF chromatin-remodeling complex is recruited to DNA lesions by damage-recognition proteins to increase DNA accessibility for NER in chromatin following UV radiation [81]. Given the suggested DNA lesion-dependent roles of the SWI/SNF complex in DNA repair in both yeast and humans, we investigated the role of the SWI/SNF complex in the repair of formaldehyde-induced DNA damage.

The covalent crosslinking of proteins to DNA presents a major challenge to multiple cellular processes. DPCs are induced by a variety of environmental and endogenous agents including ultraviolet light, metals, aldehydes, chemotherapeutics, cigarette smoke, and physiological metabolites [5]. Therefore, it is important to delineate the biological consequences of DPC lesions and to

understand how cells cope with exposure to DPC-inducing agents through repair and tolerance. In these studies, we used formaldehyde as a model compound for DPC-induction as it has been shown to predominantly cause DPCs [114]. Several studies have identified specific cellular pathways important for cell survival via repair of DPC lesions [30,37], including NER [37,38], proteosomal degradation [29], the Fanconi Anemia pathway [39,40,60,61,115], and HR [37,64]. In fact, despite evidence that chromatin-remodeling genes are important for DNA repair processes, the role of chromatin remodeling pathways in DPC repair has not been investigated [37,56,58,64,111].

Our lab previously showed, via systematic screens of the S. cerevisiae non-essential gene deletion library, that components of the NER pathway are necessary for cell survival after high-dose acute formaldehyde exposure. Conversely, we found that components of the HR pathway were more necessary for survival following low-dose chronic exposure. These results showed that S. Cerevisiae responds in fundamentally different ways to DPC induction dependent on exposure conditions. We also observed that deletion of SWI3, SNF6, SNF2, and ARP5 chromatin remodeling genes resulted in increased cytotoxicity following formaldehyde exposure under chronic conditions [37].

In this study, we show that the SWI/SNF chromatin remodeling complex is important for survival following chronic, but not acute, formaldehyde exposure. Moreover, we demonstrate that the HR pathway and the SWI/SNF complex are working together to mitigate DSBs induced by chronic formaldehyde exposure. Our findings highlight the interplay between chromatin remodeling and DNA repair

processes, and illustrate that the pathways elicited for a DNA Damage Response (DDR) are lesion and dose dependent.

Methods

Yeast Strains and Chemicals

Formaldehyde was purchase from Sigma (F8775). All the S. cerevisiae MAT-a BY4741 (his3Δ1 leu2Δ0 met15Δ0 ura3Δ0) S288C-derivative laboratory deletion strains were obtained from the European S. cerevisiae archives (EUROSCARF). The SNF2 (PSY2) MATa (his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 snf2Δ snf2-2FLAG-9 amino acids-LEU2) strain was a kind gift from Dr. Blaine Bartholomew (MD Anderson Cancer Center). Briefly, double deletion strains were generated by standard gap repair methodology, where two PCR products flanking the domain of interest were made with pRS416 plasmid for URA3 amplification. PCR product was transfected via lithium acetate methodology. 50mL of pre-warmed YPD were inoculated with overnight cultures and allowed to grow for 3-4 hours. Cells where then spun down and re-suspended in water to a dilution of $10x10^8$ cells per 1mL. Finally, 50uL of the yeast dilution were mixed with PCR product, single stranded salmon sperm carrier DNA, 50% polyethylene glycol, and 1M lithium acetate. Cells were then incubated at 30˚C for 1 hour. Finally, cell suspensions were plated on YPD-URA+Kanamycin plates for clonal selection.

Cell Survival Assays

For rapid semi-quantitative survival analysis, cells were cultured in YPD or selection media overnight and diluted to optical density (OD) 0.33 (1x10^7 cells).

For chronic formaldehyde exposure, cells were plated on agar plates containing the indicated concentrations of formaldehyde ranging from 0 to 1.75mM. For acute formaldehyde exposure, cells where exposed to formaldehyde at indicated concentrations (0 to 60mM) in liquid YPD for 15 minutes. After the exposure, cells were collected by centrifugation, washed twice in YPD, and re-suspended in YPD at a concentration of 1x10^7 cells/ml. Each strain was serially diluted (1:10) in YPD before plating 2µl of each suspension onto their respective plates as indicated. Cells were grown for 2-5 days at 30˚C and images captured on an AlphaInnotech imaging system.

To assess survival following both chronic and acute formaldehyde exposure quantitatively, colony-forming assays were performed. For both chronic and acute exposure, yeast strains were grown overnight at 30˚C, with vigorous shaking, resulting in log phase cultures. Cells were diluted to 1x10^7 cells (early exponential phase) and allowed to grow for 30 minutes at 30˚C. For acute formaldehyde exposure, cells were treated with indicated concentrations of formaldehyde (0 to 60 mM) for 15 minutes. Cells were then washed twice with YPD and plated on agar plates. For chronic exposures, formaldehyde was added to the plates at various concentrations (0–1.75 mM). For all experiments, cells were plated such that the total number of surviving colonies ranged from 50 to 250 cells per plate, and colonies were counted after 2–5 days of growth at 30˚C. All experiments were repeated at least three independent times.

To access cell viability at any given time following formaldehyde exposure we used the alamarBlue® Cell Viability Assay. AlamarBlue® cell viability reagent

functions as a cell health indicator by using the reducing power of living cells to quantitatively measure the proliferation of various human and animal cell lines, bacteria, plant, and fungi. Briefly, alamarBlue® reagent was added 4 hours prior to reading at a 1:10 dilution in a 96-well plate. Fluorescence was read using a TECAN i-control Infinite 200 plate reader using an excitation wavelength of 545nm and emission of 590nm; Absorbance was not read because yeast cells interfere with readings at the manufacturers indicated wavelength. Controls for YPD alone (negative control) and fully reduced reagent in YPD (positive control) were also included in each plate. Relative fluorescence units were normalized to respective controls, as per the manufacturer's instructions. Finally, values were normalized to their respective untreated controls were indicated.

Cell Growth Assays

To investigate growth delay induced by formaldehyde, growth curves were quantified using OD 600nM absorbance. Cell cultures were grown overnight to a saturated log phase in YPD. Cells were diluted to an OD = 0.33 (early-exponential phase) and incubated at 30˚C for 30 minutes. Cells were then treated with appropriate concentrations of formaldehyde for 15 minutes; cells were washed 2x with YPD. Finally, 1.2 million cells were plated per well in a 96-well flat bottom transparent plate with a total volume of 150µl per well. The OD for each well was taken every 15 minutes on a TECAN i-control Infinite 200 plate reader at 30˚C with 1 minute of shaking prior to measurement. Three blank wells (YPD only) were also read on each plate and subtracted from the final absorbance for each well. Plates

were read continuously for 20 hours.

Gene Expression Analysis

For analysis of gene expression levels of MEC1 and TEL1, DDR kinases, cells were treated for 15 minutes with 40mM formaldehyde. Cells were then washed 2x with YPD, and incubated in YPD at 30˚C for indicated recovery times. Total RNA was isolated from each yeast culture using YeaStar™ RNA Kit (ZymoResearch). RNA was then DNAase treated, to remove any DNA contaminates, using DNAse1 (Invitrogen). 100-400 ng of RNA from each sample were reverse transcribed using iScript cDNA Synthesis Kit (BioRad), as per manufacturer's instructions. Real-time polymerase chain reaction (RT-PCR) amplification was done for 40 cycles with gene specific primers using iQ Sybr Green Supermix (BioRad) in a iCycler iQ (BioRad).

Comet Assay

To quantify DSBs induced by formaldehyde we performed a time-course dose-response neutral comet assay. Briefly, overnight cultures were diluted to OD = 0.33 and incubated for 30 minutes at 30˚C. Cells were then treated with 1.5mM formaldehyde continuously for the time indicated in YPD. Cells were then harvested and re-suspended in ice cold S-buffer (1M sorbitol, 25mM KH_2PO_4, pH 6.5). Cells were mixed with low-melting agarose and Zymolase 1000U (ZymoResearch) at 2mg/mL concentration, and loaded on Comet slides. Slides were then incubated at 30˚C for 30 min; Cells were then lysed overnight at 4˚C.

Next, slides were drained and placed in Neutral Electrophoresis Buffer at 4°C for 30 min before undergoing electrophoresis for 15 min at 25V in Neutral Electrophoresis Buffer at 4°C. Slides were placed in DNA precipitation solution for 30 min at room temperature, then 70% ethanol for 30 minutes at room temperature. Slides were dried for 15 min prior to being stained with Gel Red Nucleic Acid Stain 10,000x (PhENIX). Cells were imaged using a Axioskop 2 microscope (Zeiss) and images were processed using Axiovision Software (Zeiss) fluorescence microscope and analyzed using Image J software. For each experiment, 100 individual comets were scored and each experiment was done in triplicate. A positive control was included by treating cells with 75 μM H_2O_2 for 20 min after Zymolase incubation at 4°C.

Western Blot

For Western immunoblot analyses, overnight cultures were diluted to OD = 0.33 and incubated for 30 min at 30˚C. Cells were then treated with 1.5mM formaldehyde continuously in YPD. For western blot analyses cells were harvested at indicated time points and disrupted in a lysis buffer (50 mM Tris–HCl, pH 8.0, 0.5% NP-40, 5 mM EDTA, 150 mM NaCl, 1 mM pepstatin A, and 1 mM PMSF) with glass beads on a vortex for 5 min. Aliquots of total cell lysate (25 μg) were run on a 4–20% Mini Protean SDS-PAGE gel (Bio-Rad) at 15 mA constant current for stacking and at 25 mA for protein separation. Gel was then electro transferred onto PVDF membranes at 250 mA for 2 hr. The membranes were immunoblotted independently with the following primary antibodies: overnight incubation at 4° C

with anti-Histone H2A phosphor S129 (ab15083:Abcam) and 2-hour incubation with actin (GTX109639:GeneTex). The membranes were then incubated for 1 hour with secondary antibodies conjugated to HRP in 5% milk, and proteins were detected by the enhanced chemiluminescence detection system (Clarity Western ECL Substrate from Bio-Rad) using a FluorChem M System (Protein Simple).

Results

Chromatin Remodelers are Differentially Sensitive to Formaldehyde Exposure

Our lab and others have identified cellular pathways critical for cell survival following DPC induction [30,37,116]. Specifically, we demonstrated via a screen of the S. cerevisiae haploid essential gene deletion library, that the NER pathway was necessary for cell survival after high-dose acute exposure (rad4Δ), and the HR pathway was necessary for survival following low-dose chronic formaldehyde exposure (mre11Δ). In addition, we showed that deletion of chromatin remodeling genes resulted in increased cytotoxicity following chronic low-dose formaldehyde exposure [37]. To the best of our knowledge, this was the first report of chromatin remodeling proteins being important for the repair of DPCs. Therefore; we choose to further investigate the sensitivity of different chromatin remodelers to formaldehyde, and to assess if they had differential sensitivity to acute versus chronic formaldehyde exposure. We exposed different chromatin remodeler deletion strains to both chronic and acute formaldehyde doses. Following chronic formaldehyde exposure, strains with deletions in the ATPase core subunit (SNF2) and core component (SNF6) of the SWI/SNF complex had increased cytotoxicity.

Conversely, the SWI/SNF deletion strains were not sensitive to acute formaldehyde exposure, compared to Wild-Type (WT) and rad4Δ, to acute formaldehyde exposure. Interestingly, the isw1Δ strain was not sensitive under chronic, but was highly sensitive under acute formaldehyde exposure (Figure 10).

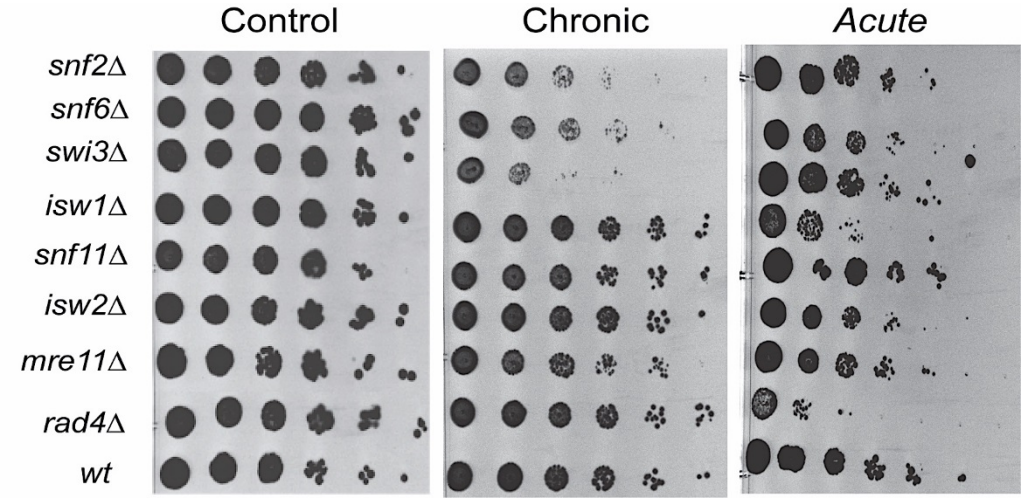
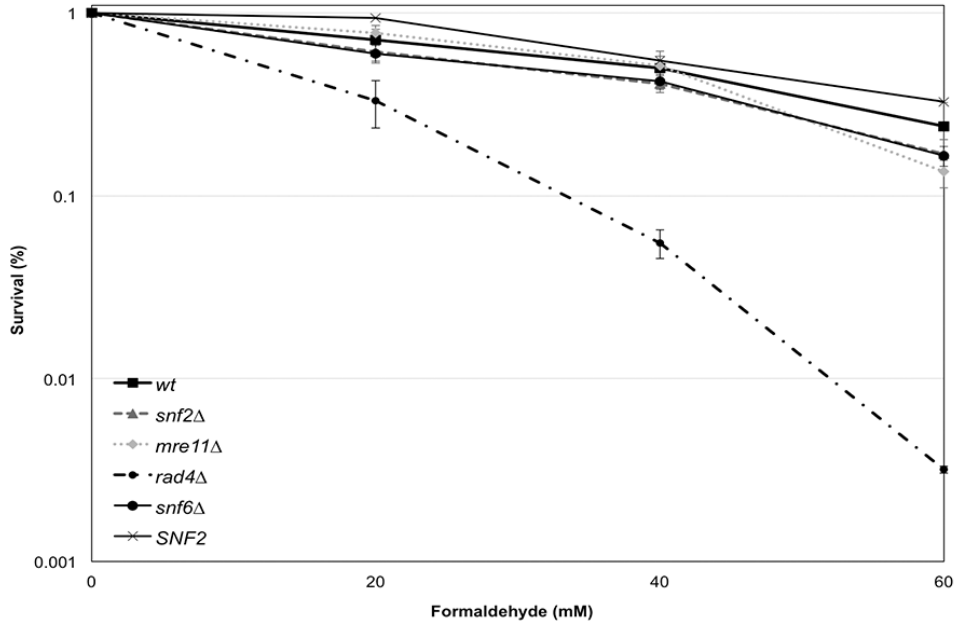


Figure 10. Chromatin remodelers are differentially sensitive to formaldehyde exposure. Cell survival spot assays were performed. Untreated (Left panel). Chronic 1.5mM continuous formaldehyde exposure (Middle panel). Acute 40mM 15-minute formaldehyde exposure (Right panel).

To more accurately quantify the sensitivity of components of the SWI/SNF complex to formaldehyde colony forming assays with increasing doses of formaldehyde were performed. Following acute formaldehyde exposure rad4Δ, as previously shown, was highly sensitive to formaldehyde in a dose dependent manner. The WT, mre11Δ, snf2Δ, and snf6Δ strains showed comparable and limited cytotoxicity to formaldehyde following acute exposure (Figure 11A). In

contrast, these deletions strains exhibited increased and comparable sensitivity following chronic formaldehyde exposure (Figure 11B). In addition, complementation of the snf2Δ deletion strain with SNF2 gene leads to a rescue of the cytotoxicity phenotype following chronic formaldehyde exposure. The rad4Δ deletion strain showed intermediate sensitivity to chronic exposure (Figure 11B). Collectively these data demonstrate that loss of the SWI/SNF complex results in sensitivity under chronic, and not acute formaldehyde exposure. Moreover, it suggests that the SWI/SNF complex may be working in a similar pathway as the HR pathway in the repair of DNA damage induced by chronic low-dose formaldehyde exposure.

A.



B.

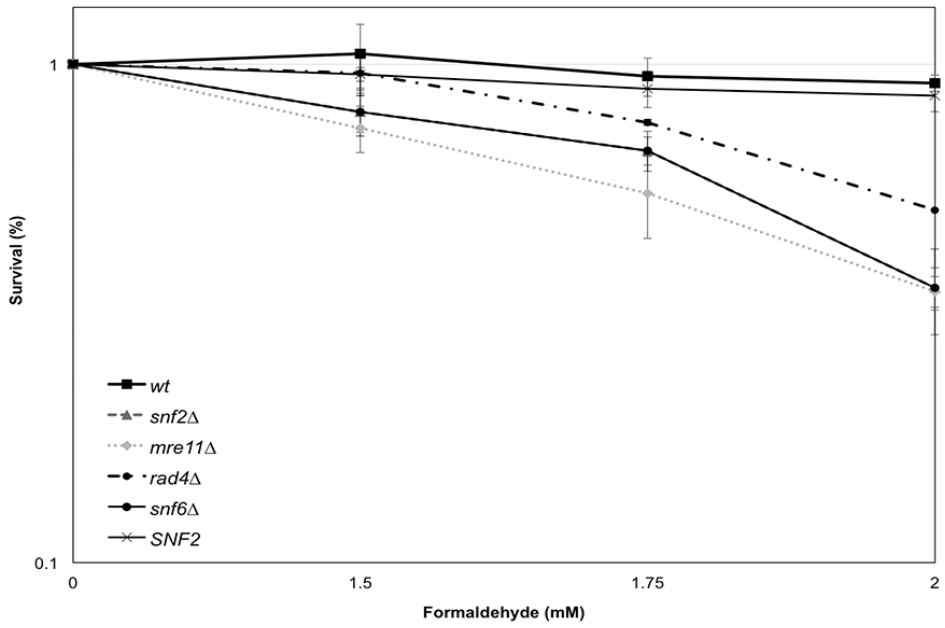


***Figure 11. Yeast strains deficient in the SWI/SNF complex and the Homologous Recombination pathway have increased cytotoxicity under chronic, but not acute formaldehyde* exposure.** Colony forming assays to quantify cell survival. A) Acute-15 minute. B) Chronic continuous formaldehyde exposure.

Acute Formaldehyde Exposure Results in Delayed Cell Growth

While performing colony forming assays following acute formaldehyde exposure, it was noted that treated cells showed a delay in their ability to form colonies, and that this delay increased in a dose-dependent manner. To further quantify this, we first measured the growth rate of each strain under untreated conditions every 45 minutes for 17 hours. We found that inherently the snf2Δ strain exhibited a slow growth phenotype; mre11Δ also had a minor growth delay (Figure

12A). We then treated cells with increasing acute doses of formaldehyde and measured their growth rate (Table 4).

	Formaldehyde (mM)		
	0	20	40
WT	293 ± 14.1	807 ± 42.4	1432 ± 15.6
Δrad4	331 ± 3.5	1530 ± 14.1	2230 ± 530.3
Δsnf2	646 ± 37.8	1315 ± 82.6	1880 ± 82.6
Δmre11	390 ± 62.6	780 ± 30	1498 ± 32.1
Δrad4Δsnf2	870 ± 20	2427 ± 154.2	2685 ± 105
Δsnf2Δmre11	903 ± 24.7	1292 ± 326.8	1986 ± 96.3

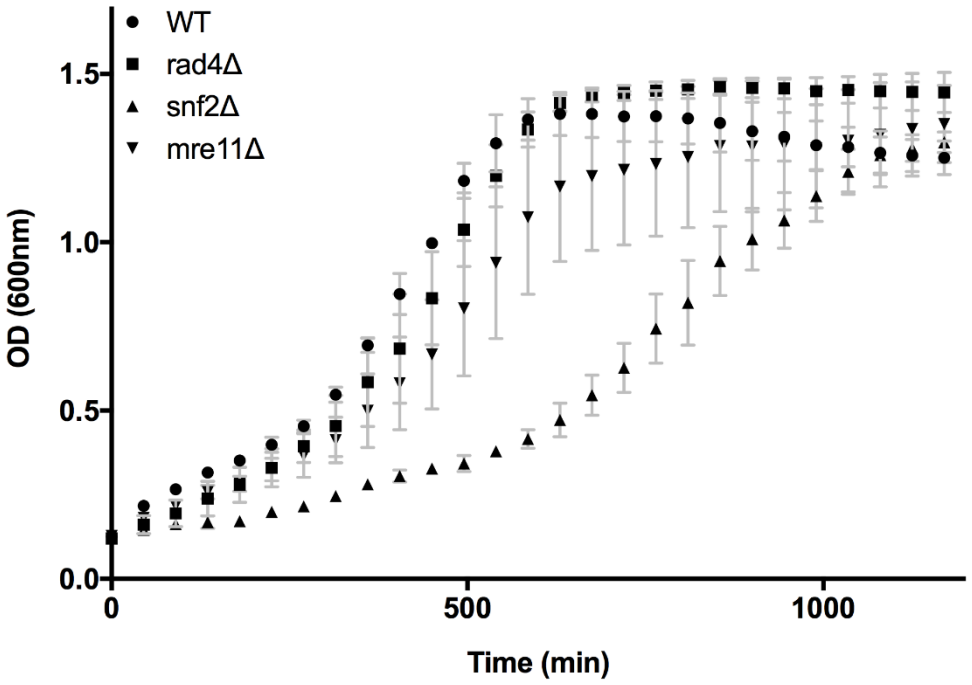
Table 4. All strains show a dose-dependent growth delay following acute formaldehyde exposure. Time (in minutes) each strain takes to complete half of its exponential growth (OD=0.5). Data are mean ± SD.

Compared to the untreated control, WT had a 2.7 and 4.9-fold growth delay in response to 20 and 40 mM formaldehyde exposure, respectively. While snf2Δ and mre11Δ strains showed a more modest fold change. The snf2Δ strain took the longest to form colonies because of its intrinsic slow-growth phenotype (Figure 12B). It should be noted that we did not measure this fold change for the rad4Δ strain because this assay cannot differentiate between dead/live cells, and we previously showed that acute formaldehyde exposure was highly cytotoxic to this strain (Figure 11). Therefore, we used an alamar blue assay to quantify cell viability at different time points following acute formaldehyde exposure. We wanted to investigate the initial time point and the duration of this growth delay, and determine the time point at which cell death occurred. We noted that both WT and

snf2Δ had a prolonged growth delay directly after treatment, but no cell death, that lasted for 6 and 12 hours respectively. Interestingly, the rad4Δ deletion strain did not show an initial pause or delay in growth (Figure 12C). Cell death occurred 6 hours after treatment in the rad4Δ strain.

Given the severity of the delayed growth phenotype in the snf2Δ strain, we chose to investigate if these cells also had a delayed DDR. We found that the snf2Δ strain had both a delayed and aberrant DDR response following acute formaldehyde exposure. MEC1, yeast orthologue of human ATR, showed a delayed upregulation of the gene transcript compared to WT, and a blunted rate of transcript degradation (Figure 13A). The TEL1, yeast orthologue of human ATM, gene transcript showed a blunted and delayed DDR response (Figure 13B). This data validates our previous findings that the rad4Δ deletion strain is highly sensitive

A.



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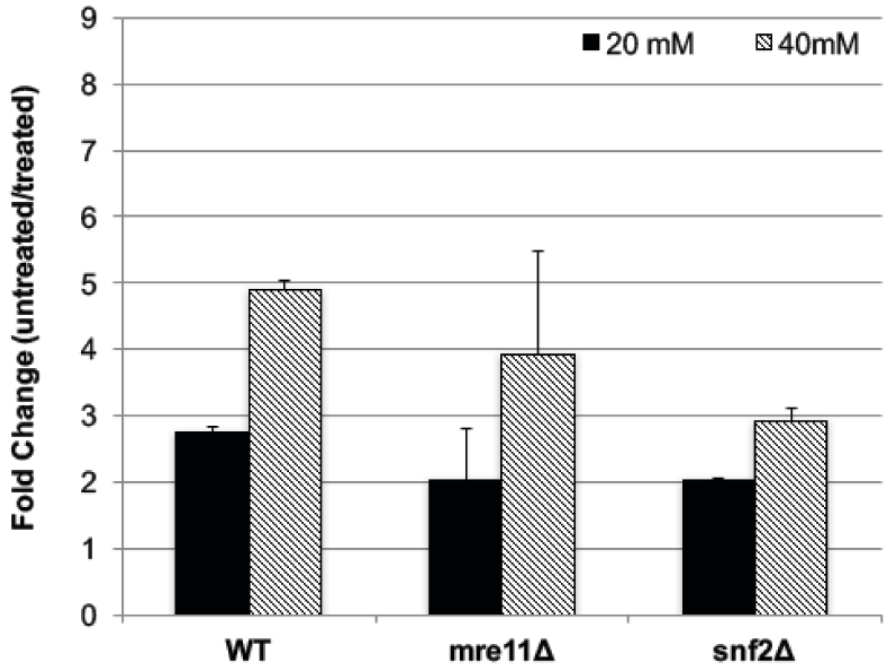

20 mM
40mM
Fold Change (untreated/treated)
0
1
2
3
4
5
6
7
8
9
WT
mre11Δ
snf2Δ

C.

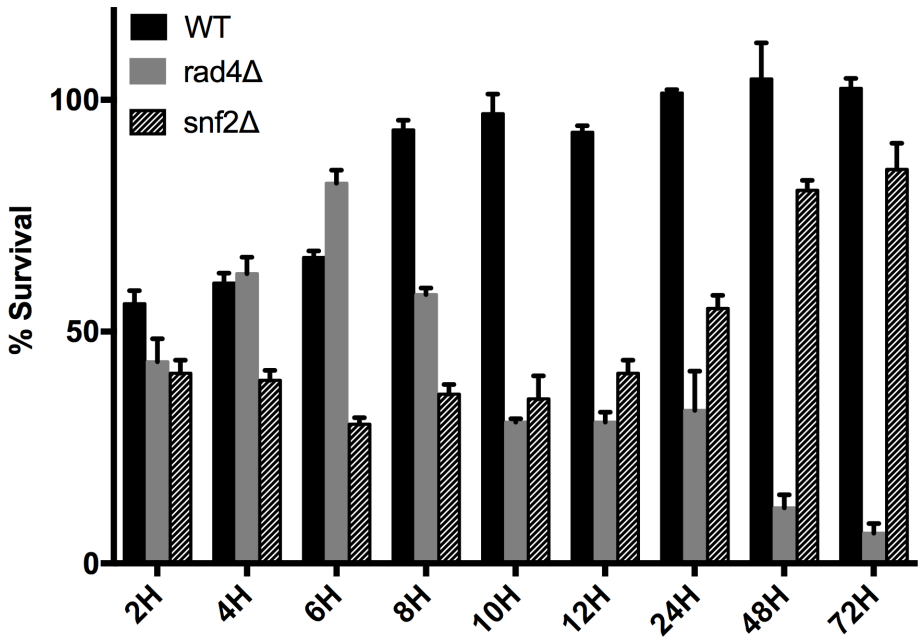
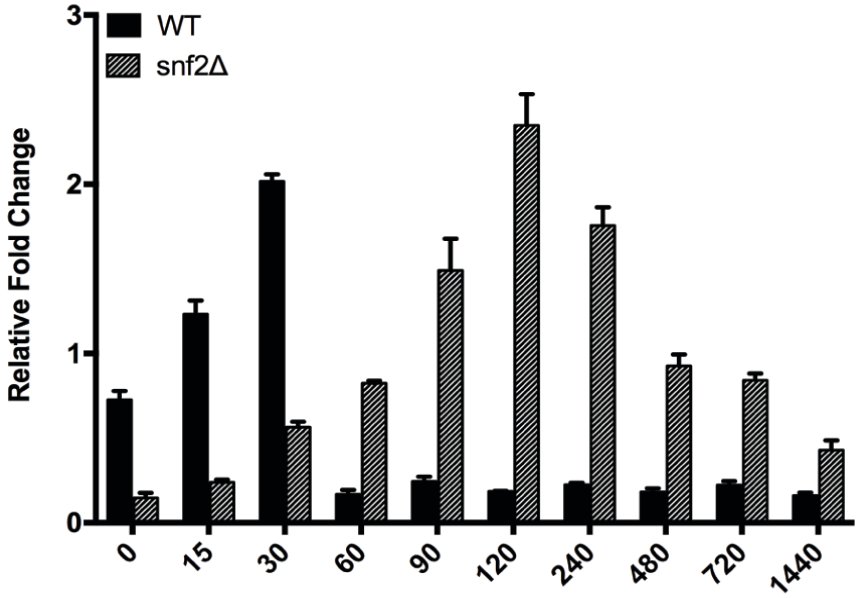

WT
rad4Δ
snf2Δ
% Survival
0
50
100
2H
4H
6H
8H
10H
12H
24H
48H
72H

Figure 12. Formaldehyde exposure results in delayed cell growth and aberrant cell cycle progression. A) Time course (minutes) cell growth assay following 40mM acute 15-minute formaldehyde exposure. B) Quantification of cell growth fold change compared to untreated after 15 minute acute 20 and 40mM formaldehyde treatment. C) Percent survival following an acute 15-minute 40mM formaldehyde exposure.

to formaldehyde, and here we show that deletion of the RAD4 gene does not result in delayed cell growth immediately following acute formaldehyde exposure. Conversely, we showed that WT and snf2Δ strains do not show cytotoxicity following acute formaldehyde exposure, but do have a severe and prolonged cell growth delay phenotype. Finally, we demonstrated that the snf2Δ deletion strain has a delayed and aberrant DDR compared to WT.

A.



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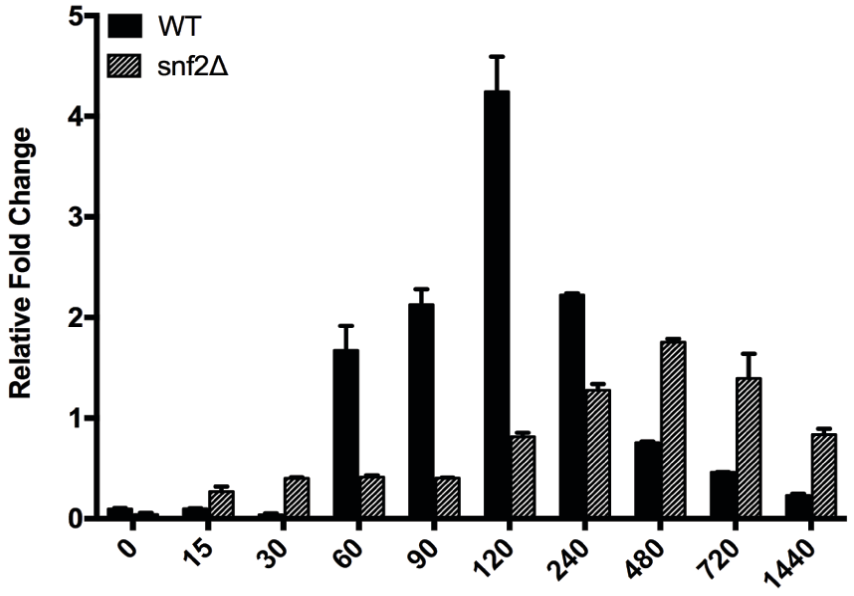

Figure 13. SWI/SNF deficient strains have a delayed and aberrant DNA damage response. Real-time PCR analysis following acute 15-minute 40 mM formaldehyde exposure at indicated recovery time points. A) *MEC1* (ATR) B) *TEL1* (ATM).

Homologous Recombination and the SWI/SNF Complex are Epistatic Following Formaldehyde Exposure

To interrogate if the SWI/SNF complex is working with the HR pathway to mitigate cytotoxicity following chronic low-dose formaldehyde exposure, we created double knockouts of the ATPase core subunit of the SWI/SNF complex, SNF2, in combination with a component of either the NER pathway (RAD4) or the HR pathway (MRE11). The snf2Δ/rad4Δ double mutant strain showed additive cytotoxicity following both chronic and acute formaldehyde exposure, suggesting

that these genes are working in distinct pathways for DPC repair irrespective of exposure conditions (Figure 14). This double mutant also displayed an additive growth delay phenotype, further validating that these two pathways are working separately to mitigate formaldehyde-induced DNA damage (data not shown). In contrast, the snf2Δ/mre11Δ double mutant exhibited an epistatic relationship with very similar sensitivity for the double and each of the single mutants following chronic and acute formaldehyde exposure (Figure 14). These findings further support that the SWI/SNF complex is working with HR pathway in mitigating cytotoxicity following chronic exposure.

SWI/SNF Complex Deficiency Results in an Accumulation of Double-strand Breaks

We next wanted to investigate if SWI/SNF deficiency resulted in persistent and unrepaired DSBs, a hallmark of HR deficiency. Using a neutral comet assay which measures DSBs, we observed that in the WT strain DSB formation peaked at 24 hours, and that these breaks had resolved to almost basal levels by 48 hours. In contrast, the snf2Δ and mre11Δ strains showed an accumulation of DBS 24 hours after initial chronic exposure that persisted even after 72 hours. Interestingly, the rad4Δ strain showed a minimal accumulation of DSBs at these time points (Figure 15A). To interrogate this further, we performed western blot analysis for yH2Ax, a DSB marker. We found that DBSs in both the WT and rad4Δ strains peaked at 8 hours after initial chronic exposure. We also found that the snf2Δ and mre11Δ strains had persistent unrepaired DSBs, further validating our comet assay findings (Figure 15B). We finally wanted to interrogate if these persistent DSBs were

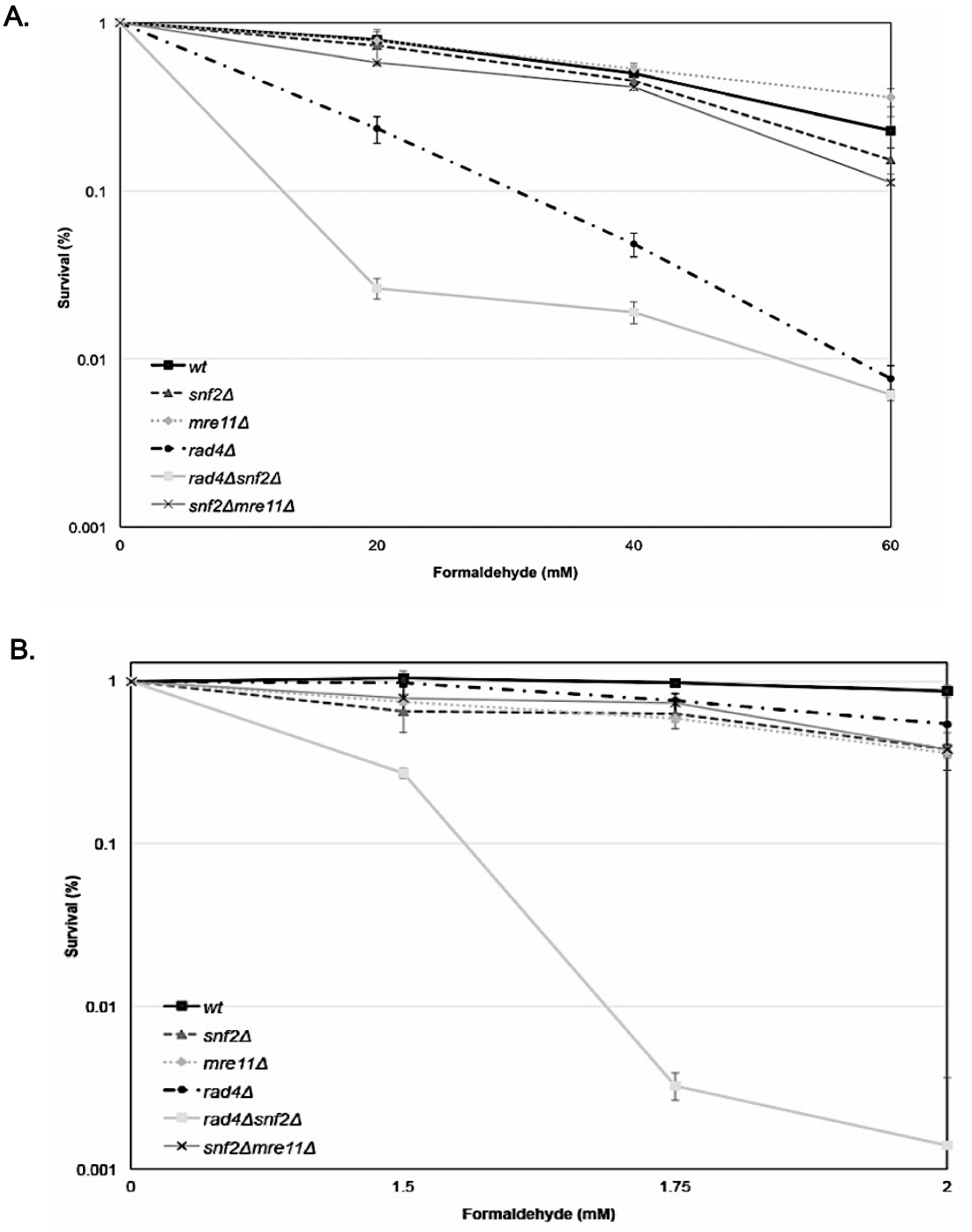

Figure 14. The SWI/SNF and Homologous Recombination pathway are epistatic following chronic formaldehyde exposure. Colony forming assays to quantify cell survival. A) Acute 15-minute B) Chronic continuous formaldehyde exposure.

contributing to the increased cell death following chronic formaldehyde exposure in the SWI/SNF deficient strain, snf2Δ. Using our alamar blue assay, we found that induction of cell death occurred 26-27 hours following initial chronic formaldehyde exposure, corresponding to the accumulation of DSBs 24 hours after exposure (Figure 16). These findings support that the SWI/SNF complex, in conjunction with the HR pathway, is mitigating both the repair of formaldehyde-induced DSBs and resulting cytotoxicity following chronic formaldehyde exposures.

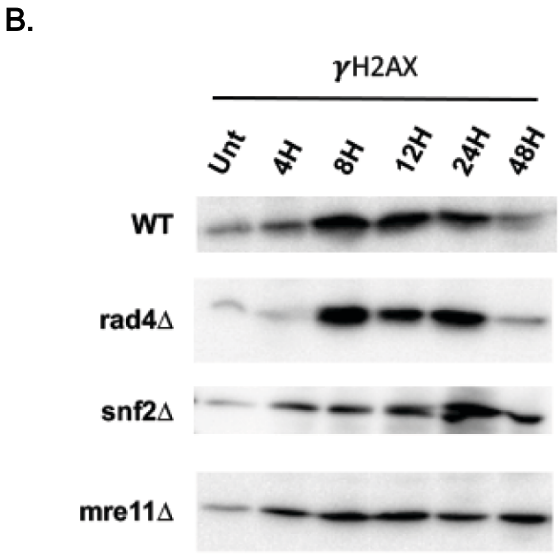

Figure 15. Homologous Recombination and SWI/SNF deficiency result in increased and persistent double-strand break formation.

A) Neutral comet assay to quantify double strand break formation B) Western blot analysis for yH2AX during continuous chronic 1.5mM

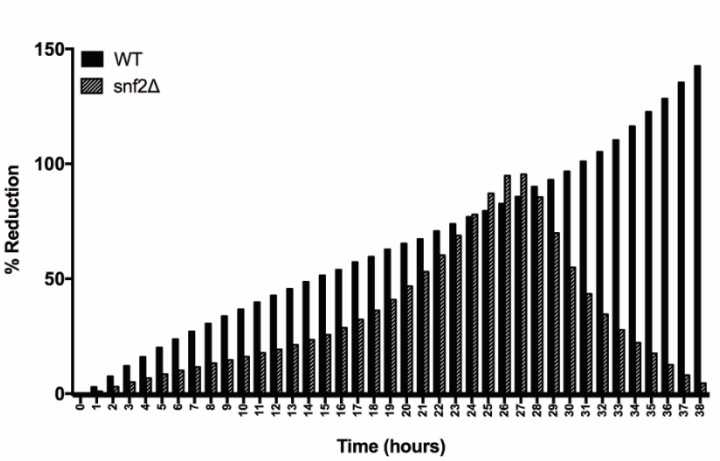

Figure 16. SWI/SNF deficiency results in cell death following double strand breaks accumulation during chronic formaldehyde exposure. Percent reduction of alamar Blue reagent during continues 1.5 mM formaldehyde exposure.

Discussion

The cellular pathways and responses associated with a DDR following genotoxic exposure are complex, multi factorial, and often DNA lesion dependent. In this study, we sought to identify the role of chromatin remodeling in the repair of formaldehyde induced DNA damage. Others have previously shown, in S.cerevisiae, that the SWI/SNF chromatin remodeling complex plays a role in NER-mediated repair of UV lesions [64,81]. Here we utilized *S.cerevisiae* deletion strains and found that the SWI/SNF chromatin remodeling complex is working in conjunction with the HR DNA repair pathway, and not NER, to repair DSBs in a timely manner and to prevent formaldehyde induced cytotoxicity following low-dose chronic formaldehyde exposure. Our findings are also in accordance with our

previously published work that showed that Bloom-Syndrome RecQ Like Helicase (BLM) protein, an integral component of the HR pathway, is important for cell survival and repair of DSBs following formaldehyde exposure in human cells [48]. These results further validate the importance of delineating the involvement of different DDR pathways in a lesion and genotoxic-dependent context.

It is clear from our study that the SWI/SNF complex and HR pathway are working together to mediate cytotoxicity and DNA damage induced by formaldehyde exposure. SWI/SNF had been shown to be important for DNA damage recognition and DNA repair protein accessibility to the sites of damage [77,81,117]. It has also been identified as important for γH2AX DSB signal propagation in both HR and NHEJ [75,78–80]. Others have found a more systemic role of the SWI/SNF complex in DDR response by modulating changes in gene expression of DDR proteins [60,69,70,118]. Finally, some have linked the SWI/SNF complex to DNA repair via transcription-independent pathways. Qi et al showed that in human cells the ATPase core subunit of the SWI/SNF complex promotes replacement of RPA with RAD51 in the repair of DNA double strand breaks [113]. While Chai et al showed in yeast that the SWI/SNF complex is required for proper strand invasion following DSB induction [119].

SWI/SNF deficiency also resulted in delayed cell growth following acute formaldehyde exposure. We showed that both WT and snf2Δ strains had a severe growth delay immediately after formaldehyde exposure, likely to allow for proper DNA repair since we did not observe an abundance of cell death in these strains. Interestingly, the rad4, yeast orthologue of human XPC, deletion strain did not

result in an overt cell growth delay following acute formaldehyde exposure (Figure 12). We previously showed that formaldehyde induced DSB formation and cell cycle perturbations occur in a replication dependent manner [48]. In addition, others have also characterized that XPC-defective human cell lines cannot initiate a proper DNA damage treatment-mediated signal transduction process. This defect results in decreased activation of the p53 pathways and the cell cycle arrest necessary to allow for DNA repair to take place [120]. Therefore, the lack of cell growth arrest in the rad4Δ strain likely results in accumulation of replication-dependent DNA damage, due to a defective cell cycle checkpoint that would otherwise allow for proper repair. This subsequently leads to the severe and intermediate cytotoxicity observed in the rad4Δ strain following acute and chronic formaldehyde exposure, respectively.

The snf2Δ strain had a profound cell growth arrest phenotype, which resulted in a delayed and aberrant DDR. Deletion of the SNF2 gene resulted in aberrant MEC1 and TEL1 transcript upregulation following acute formaldehyde exposure. This is in congruence with previously published work that found that loss of SWI/SNF resulted in reduced Mec1 activity, and that this process was mediated by direct binding of the SWI/SNF ATPase core subunit, Snf2, to Mec1. Moreover, they also showed that both snf2Δ and mre11Δ strains had decreased levels of yH2AX. This explains our findings that show decreased, but persistent yH2AX protein levels (Figure 15B).

Understanding the specific role of the SWI/SNF complex in formaldehyde-induced DSB repair and the mechanism that leads to increased cytotoxicity is of

paramount importance for future studies seeking to elucidate possible chemotherapeutics avenues for SWI/SNF deficient tumors. Our work, which shows the loss of the SWI/SNF complex phenotypically mimics defects in the HR pathway, suggest that SWI/SNF deficient tumors could possibly be treated with similar genotoxic agents that have been previously shown to be effective in treating HR deficient malignancies. In fact, formaldehyde releasing pro-drugs are currently being investigated as possible chemotherapeutic treatments [103]. Our data suggest that SWI/SNF deficient tumors may also respond to this pro-drug chemotherapeutic.

In conclusion, this study demonstrates that the SWI/SNF complex and HR DNA repair pathway are essential for mitigating cytotoxicity following chronic low-dose formaldehyde exposure. In addition, we provide strong evidence to support that SWI/SNF complex is also important for the repair of DSBs induced by formaldehyde exposure, and that persistent unrepaired breaks result in increased cell death. Further studies are required to delineate the specific role of the SWI/SNF complex in HR mediated repair of formaldehyde induced DNA damage.

CONCLUSION

The findings in this study have contributed to our understanding of the cellular pathways necessary for repair and tolerance of formaldehyde-induced DNA damage. Our work showed that genes in human cell lines are differentially needed for cell survival following chronic formaldehyde exposure. This is of importance and strengthens the notion that modeling for epidemiological studies should account for biologically relevant tissue and doses. Importantly, this also highlights the diverse response to genotoxic agents by different cell types in a multicellular organism, like humans, and how differences in both structure and function of cells can dramatically affect the cellular pathways that are recruited to mediate a proper DDR. Our data also emphasize how gene expression differences in different cell types and tissues could affect cell survival following genotoxic exposure, and demonstrates that DDR is cell type specific. These findings should be used to inform the importance of carefully selecting the doses and cell types used in larger genotoxic and epidemiological studies. Nevertheless, our work also shows that though genes important for survival following formaldehyde exposure may differ, the cellular pathways that are critical are conserved. The HR, DNA replication, IR Response, and DSB repair pathways are of the utmost importance for cell survival following chronic low-dose continuous formaldehyde exposure.

Our yeast study establishes that the SWI/SNF complex is important for cell survival following chronic formaldehyde exposure. It also supports our previous work that showed the HR pathway is critically important for cell survival following formaldehyde exposure [37,48]. We show, for the first time, that the SWI/SNF

complex is important to mitigate cytotoxicity following formaldehyde exposure. It would be of interest to discern if the importance of the SWI/SNF complex for HR-mediated repair is through 1) DNA remodeling to allow DNA proteins access to DNA lesions, 2) gene expression changes in apoptotic or DDR proteins, or 3) playing an active role in DSB repair within the HR pathway.

Given that the SWI/SNF complex has been demonstrated to be highly mutated in human malignancies, our findings could prove crucial in determining efficacious chemotherapeutics in tumors deficient in component of the SWI/SNF complex; our findings also suggest that drugs which induce DPCs or DSBs or have previously been demonstrated to be effective in HR-deficient tumors, could be promising. Further, our data implies that chemotherapeutics that induce DPCs could be effective treatments for cancers deficient in HR, DNA replication, IR radiation response genes, and DSB repair.

Finally, our work supports our proposed model (Figure 17) in which DPCs are induced (a) and then recognized by an unknown pathway. Chromatin surrounding the DPC is then remodeled to allow access to repair proteins likely by the SWI/SNF complex (b). The DPC is then excised (c) creating a SSB (d). If DNA replication is not arrested, to allow for repair, this results in an accumulation of replication-induced DSBs or replication fork collapse (e). These DSBs in S-phase would then be preferentially repaired by Homologous Recombination. Recruitment of the HR pathway (f) and subsequent steps may also be facilitated by the SWI/SNF complex, as previously reported (g) [119]. Proper HR-mediated repair allows the cells to be released from the G2/M checkpoint, and to continue

with faithful cell division. This is a well characterized phenotype in cells with an intrinsic HR-defect, which have been shown to have elevated numbers of cells arrested at that the G2/M checkpoint. Importantly, our data gives rise to new and important questions regarding the specific mechanisms that make the pathways we identified critically important for mitigating cytotoxicity following formaldehyde exposure.

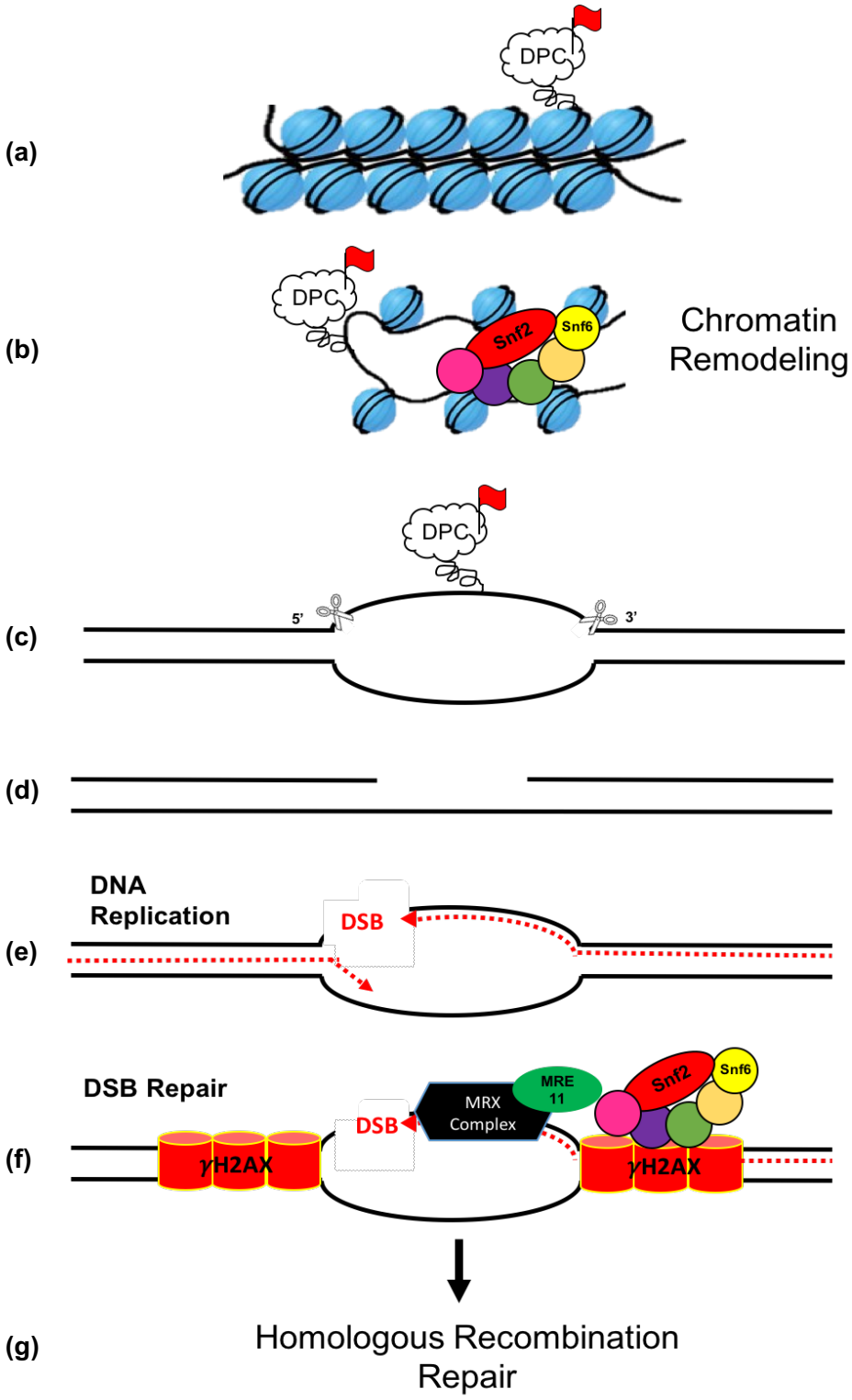

Figure 17. Proposed DPC Repair Model

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