Investigation into the Role of DNA Damage and Repair During Influenza Infection and Inflammation

by

Marcus Curtis Parrish
B.S., Yale University (2010)

Submitted to the Department of Biological Engineering in partial fulfillment of the requirements for the degree of Doctor of Philosophy at the MASSACHUSETTS INSTITUTE OF TECHNOLOGY

February 2017

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Signature redacted

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September 20, 2016

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Thesis Supervisor

Accepted by ........................................

Forest M. White
Chair, Biological Engineering Graduate Committee

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Professor Steven R. Tannenbaum
Dedicated to my loving parents

Mr. Harry Parrish and Dr. Lorraine Fleming

Thank you for your unconditional love and support
Preface

The work presented in Chapter 2 was published in *Cellular and Molecular Life Sciences* in March of 2015. The *in vitro* experiments were performed and completed by the author. The development of the mouse model was done in collaboration with Dr. Na Li, Dr. Prashant Rai, Liang Li, Dr. Yoshiyuki Yamada, and Dr. Vincent Chow at the Singapore-MIT Alliance for Research and Technology (SMART) Infectious Disease-Interdisciplinary Research Group (ID-IRG) and the National University of Singapore. The mass spectrometry analysis was performed with Dr. Nona Albohassani from the Laboratory of Dr. Leona Samson with guidance from Dr. Koli Tagahizadeh from the Center for Environmental Health Sciences. All other data were collected by the author.

At the time of writing, Chapter 3 has been drafted as a manuscript for submission. Gene Set Enrichment Analysis was performed with the patient support of Charles Whittaker of the MIT BioMicro Center. Kai Sen Tan of the SMART ID-IRG and the National University of Singapore performed infections and histological processing of wild type mice infected with influenza. I would also like to acknowledge Dr. Bogdan Fedele and Dr. Laura Trudel for their training in the exposure of cells to SIN-1 and HOCl, respectively. In addition, I would like to acknowledge Dr. Jennifer Calvo of the Laboratory of Dr. Leona Samson for her assistance in the generation and breeding of the $Aag^{-/-}/Alkbh2^{-/-}/Alkbh3^{-/-}$ mice and support in data analysis. Technical assistance with immunofluorescent staining and Metafer quantification of fluorescent slides was graciously provided by Dr. Takafumi Kimoto from Teijin Pharmaceuticals Ltd., Dr. Na Li of the SMART ID-IRG and Dr. Prashant Rai of the SMART ID-IRG. Dr. Yin Lu from the SMART ID-IRG analyzed the Hematoxylin & Eosin stains for cellular infiltration. All other data were generated by the author.
At the time of writing, Chapter 4 has been drafted as a manuscript for submission. Wild type, \( Aag^{+/+} \), \( AagTg \) mouse embryonic fibroblasts were kindly provided by Dr. Leona Samson. I would like to acknowledge the technical support of Dr. Jing Ge in performing CometChip Analysis. Host Cell Reactivation Assay experiments were performed in collaboration with Drs. Isaac Chaim and Zachary Nagel of the Laboratory of Dr. Leona Samson. I would also like to acknowledge Dr. Carrie Margulies and Joshua Corrigan of the Laboratory of Dr. Leona Samson for their training in the colony forming assay and abasic site quantification respectively. All other data were collected by the author.

The work done was supported, in part, by the Lemelson-MIT Presidential Fellowship, the Siebel Scholars Foundation, the National Science Foundation Graduate Research Fellowship Program, National Institute of Environmental Health Sciences (NIEHS) Training Grant in Environmental Toxicology (#T32-ES007020), Singapore-MIT Alliance for Research and Technology, the MIT Nitric Oxide Program Project Grant (National Cancer Institute Grant #P01-CA026731), and the MIT Center for Environmental Health Sciences (NIEHS Grant P30-ES002109).
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I would be remiss not to mention my Singaporean colleagues with whom I had the pleasure of working at the National University of Singapore during the summer of 2011 and then later in Cambridge. Drs. Na Li, Prashant Rai, Kai Sen Tan, Tze Khee Chan, Yamada Yoshiyuki, and Vincent Chow – thank you for all of your technical and scientific support. Your assistance has been vital to a large portion of this thesis.
Moreover I would like to thank some amazingly patient and generous collaborators I’ve had over the years from the BioMicro Center, the Dedon Lab, the Langer Lab, the Runstadler Lab, the Samson Lab, the So Lab, the Tannenbaum Lab, and the Division of Comparative Medicine. Your assistance and friendship has been enormously helpful. In addition, I’d like to thank the BE and Broad Communication Labs for helping me write this thesis. Interacting with you helped me challenge my assumptions and produce a more polished product. I would also like to thank the entire Department of Biological Engineering and the Center for Environmental Health Sciences for making my experience in graduate school such a pleasant one.

I would like to thank my friends over the years: my sisters Arlesa and Colleen, my Brothers from Landon, my Bulldogs from Yale, and my BE-ers from MIT. Thank you for the laughs and the joy throughout my tenure in grad school and life. Every day was like a battle, but every night with us was like a dream.

Finally, I would like to thank my parents. I am lucky to be the son of two people who understood what it meant to go to grad school and get a PhD. Thank you for being there during the good times and the bad times. The I-want-to-quit-grad-school days and the I-am-definitely-winning-a-Nobel-prize days. You two are such amazing engineers, educators, and parents. There is no one in my corner shouting louder for me, and for that I am forever grateful. I love you.
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Role of DNA Damage and Repair during
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Abstract
The DNA in every cell accrues nearly 100,000 lesions daily from both endogenous and exogenous sources. The accumulated damage, e.g. strand breaks and base lesions, can lead to mutations, cell death, and cancer if not repaired efficiently. To protect genome integrity, organisms have evolved multiple DNA repair processes. A deeper comprehension of DNA damage and repair during disease pathogenesis can aid the development of novel therapeutics to reduce the damage and ameliorate the disease. Here, we studied DNA damage and repair in two inflammatory contexts. First, we investigated the role of DNA damage and repair during influenza infection, a common viral respiratory disease with an active inflammatory response. Second, we examined the effects of S-nitrosation, a post-translational modification that is common in inflammatory regions, on repair of alkylation damage.

Influenza induces an excessive inflammatory response in the host and a reduction in inflammation reduces morbidity. While inflammation can cause DNA damage and induce DNA repair in other inflammatory contexts, there has been minimal analysis on the existence and function of DNA damage and repair during influenza infection. Utilizing immunofluorescent analysis of double strand break markers, we observed an increase in strand breaks both in vitro and in vivo. Influenza infected mice also displayed a significant increase in homologous recombination (HR) gene and protein expression during the recovery phase of infection in multiple virus and mouse backgrounds. Moreover, influenza infected mice deficient in DNA repair proteins AAG, ALKBH2, and ALKBH3, displayed increased morbidity and HR protein expression
when compared to wild type. Together, these results raise the possibility of a role for DNA repair and more specifically HR during influenza infection.

To study the effects of inflammation on DNA repair protein function, we analyzed the capacity of cells treated with S-nitrosoglutathione (GSNO), a nitrosating agent, to repair alkylation damage. GSNO-exposed cells displayed dysregulation in the activities of base excision repair (BER) proteins. Following challenge with an alkylating agent, GSNO-exposed cells had an increase in repair intermediates and reduced viability, suggesting that GSNO exposure inhibits BER completion. The knowledge gained from these studies lays the groundwork for new prevention strategies and novel therapeutics.

Thesis Supervisor: Bevin P. Engelward
Title: Professor of Biological Engineering
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<tr>
<td>5'-dRP</td>
<td>5'-deoxyribophosphate</td>
</tr>
<tr>
<td>53BP1</td>
<td>p53-binding protein 1</td>
</tr>
<tr>
<td>8-oxoG</td>
<td>8-oxoguanine</td>
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<tr>
<td>AAG</td>
<td>Alkyladenine glycosylase</td>
</tr>
<tr>
<td>ALKBH2</td>
<td>AlkB homolog 2</td>
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<tr>
<td>ALKBH3</td>
<td>AlkB homolog 3</td>
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<td>AP</td>
<td>Apurinic/Apyrimidinic Site</td>
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<td>APE1</td>
<td>AP endonuclease 1</td>
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<td>ATM</td>
<td>Ataxia telangiectasia mutated</td>
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<td>ATR</td>
<td>Ataxia telangiectasia Rad3-related protein</td>
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<td>ATRIP</td>
<td>ATR Interacting Protein</td>
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<td>BER</td>
<td>Base Excision Repair</td>
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<td><em>C. trachomatis</em></td>
<td><em>Chlamydia trachomatis</em></td>
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<td>CHK1</td>
<td>Checkpoint kinase 1</td>
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<td>Checkpoint kinase 2</td>
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<td>DDR</td>
<td>DNA damage response</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>DNA-PK</td>
<td>DNA protein kinase</td>
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<td>DNA-PKcs</td>
<td>DNA-PK catalytic subunit</td>
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<td>DPI</td>
<td>Days post infection</td>
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<td>DR</td>
<td>Direct reversal</td>
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<td>DSB</td>
<td>Double strand break</td>
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<td>EBV</td>
<td>Epstein-Barr Virus</td>
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<td>EGFP</td>
<td>Enhanced green fluorescent protein</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
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<td>EXO1</td>
<td>Exonuclease 1</td>
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<td>FDR</td>
<td>False discovery rate</td>
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<td>FEN1</td>
<td>Flap endonuclease 1</td>
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<td>FM-HCR</td>
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<td>S-nitrosoglutathione</td>
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<td>S-nitrosoglutathione reductase</td>
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<td>H. pylori</td>
<td>Helicobacter pylori</td>
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<td>Hematoxylin &amp; Eosin</td>
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<td>Hemagglutinin</td>
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<td>Hypochlorous acid</td>
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<td>Homologous recombination</td>
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<td>Interferon β</td>
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<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
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<td>KEGG</td>
<td>Kyoto Encylopeda of Genes and Genomes</td>
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<td>mAAG</td>
<td>Modified AAG</td>
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<td>Monocyte chemoattractant protein-1</td>
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<td>Mouse embryonic fibroblasts</td>
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<td>MGMT</td>
<td>6-methylguanine methyltransferase</td>
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<td>MMR</td>
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<td>MMS</td>
<td>Methylmethane sulfonate</td>
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<td>MRN</td>
<td>Mre11/Rad50/Nbs1</td>
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<tr>
<td>NA</td>
<td>Neuraminidase</td>
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<td>Nucleotide excision repair</td>
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<td>Non-homologous end joining</td>
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<td>Polydimethylsiloxane</td>
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<td>Polynucleotide kinase phosphatase</td>
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<tr>
<td>POLβ</td>
<td>Polymerase Beta</td>
</tr>
<tr>
<td>PR8</td>
<td>Influenza A/Puerto Rico/8/1934</td>
</tr>
<tr>
<td>RONS</td>
<td>Reactive oxygen and nitrogen species</td>
</tr>
<tr>
<td>RPA</td>
<td>Replication protein A</td>
</tr>
<tr>
<td><em>S. pneumoniae</em></td>
<td><em>Streptococcus pneumoniae</em></td>
</tr>
<tr>
<td>SIN1</td>
<td>3-morpholinosydnonimine</td>
</tr>
<tr>
<td>SNOTRAP</td>
<td>SNO trapping by triaryl phosphine</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>SSB</td>
<td>Single strand break</td>
</tr>
<tr>
<td>ssGSEA</td>
<td>Single sample GSEA</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
</tr>
<tr>
<td>TKO</td>
<td>Triple knockout ($Aag^{/-}, Alkbh2^{/-}, Alkbh3^{/-}$)</td>
</tr>
<tr>
<td>TNF-alpha</td>
<td>Tumor necrosis factor - alpha</td>
</tr>
<tr>
<td>TOPBP1</td>
<td>Topoisomerase II binding protein 1</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
<tr>
<td>$\gamma$H2AX</td>
<td>Phosphorylated Serine-139 on Histone H2AX</td>
</tr>
<tr>
<td>$\varepsilon$A</td>
<td>$1, N^{\varepsilon}$-ethenoadenine</td>
</tr>
<tr>
<td>$\varepsilon$G</td>
<td>$1, N^{\varepsilon}$-ethenoguanine</td>
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Chapter 1

Introduction

DNA, the blueprint of life, is under constant assault from both exogenous agents (e.g. UV irradiation, diet, and pollution) and endogenous agents (e.g. reactive oxygen and nitrogen species (RONS) from inflammation and cellular metabolism). It is estimated that more than $10^4$ strand breaks and base losses occur every day in each mammalian cell [1, 2]. In addition to strand breaks, damaging agents can cause other types of damage including base lesions and crosslinks. If unrepaired, DNA damage can lead to cancer and aging [2]. Thus, the study of DNA damage and DNA damaging agents is vital to further understanding disease pathogenesis. Similarly, it is important to understand the mechanisms by which cells combat the incessant onslaught of DNA damage. Cells contain six major pathways that together repair a wide variety of damages: Base Excision Repair (BER), Direct Reversal (DR), Homologous Recombination (HR), Mismatch Repair (MMR), Non-homologous End Joining (NHEJ), and Nucleotide Excision Repair (NER). Each of these pathways repairs a specific class of damages; deficiencies in any of these pathways can lead to mutations or cell death [2]. A deeper understanding of DNA repair mechanisms during the disease process can potentially reveal new therapeutic targets and identify susceptible populations.

In this thesis, one will find analyses of DNA damage and repair in two inflammatory contexts: during influenza infection, a prevalent viral disease with an
active inflammatory response, and S-nitrosation, a post-translational modification that can be dysregulated during inflammation. Before delving into the work, there will first be a brief primer on the DNA repair pathways most relevant to the studies: BER, DR, HR, and MMR, and the DNA damage response. Subsequently, the role of DNA damage and repair in other infectious diseases will be reviewed and background on influenza viral pathogenesis will be provided. Finally, transitioning to inflammation, the current knowledge of S-nitrosation’s effects on DNA repair will be summarized. It is hoped that the knowledge gained from these studies will help others understand influenza and inflammation more thoroughly and could potentially aid in the development of new therapies.

1.1 DNA Damage and Repair

The broad spectrum of DNA damages that occur in cells can be repaired by multiple DNA repair pathways. For example, both HR and NHEJ can repair double strand breaks. Alkylation damage can be repaired by BER and DR, among other pathways. However, though DNA repair pathways can overlap in targets, deficiencies in one pathway can be very problematic for organisms. For instance, people with the genetic disorder xeroderma pigmentosum are deficient in NER genes, which lead to an increased sensitivity to ultraviolet light and a higher risk of skin cancer [3]. In addition, deficiencies in the HR protein BRCA2 has been linked to multiple types of cancer including breast and ovarian cancer [2]. Here, one will find a brief overview of the DNA repair pathways most relevant to the work: BER, HR, DR, MMR, and the DNA damage response. The overview includes information on the specific damages repaired by each pathway, the mechanism of repair, and the role of the pathway in human disease.
1.1.1 Base Excision Repair

BER is a pathway applicable to the repair of multiple types of damages including single strand breaks (SSBs), abasic sites, alkylation base lesions, and oxidative base lesions. If unrepaired, these damages can be mutagenic, toxic, or both [4]. For instance, 8-oxoguanine, the most commonly damaged purine generated under oxidative stress [5], can mispair with adenine during replication leading to a GC to TA mutation (Fig. 1.1) [6]. Furthermore, alkylation lesions like 3-methyladenine, produced endogenously by S-adenylmethione and exogenously by chemotherapeutics like methyl methanesulfonate [7], can block replication machinery and induce double strand breaks and cell death [4]. Thus, BER plays an important role in surviving damages.

The BER pathway is initiated by one of 11 glycosylases excising damaged or improper bases [8]. The glycosylases, which are each specific for a group of base lesions, are classified into two broad categories. The first category, the monofunctional glycosylases, includes alkyladenine glycosylase (AAG) and excises the base lesion leaving an apurinic/apyrimidinic (AP) site (Fig. 1.2). The AP site is subsequently cleaved by AP endonuclease 1 (APE1) in mammalian cells, leaving a 5’-deoxyribos phosphophate (5’-dRP) and a 3’-hydroxyl group [8]. Bifunctional glycosylases, the second category of glycosylases including 8-oxoguanine glycosylase (OGG1), have AP lyase activities, which enable them to remove the damaged base and cleave the backbone, leaving a gap with a 5’-phosphate and either a 3’-aldehyde or a 3’-phosphate group. The 3’ groups are cleaved by APE1 and polynucleotide kinase phosphatase (PNKP) respectively, leaving a 3’-hydroxyl group [9]. Subsequently, poly-(ADP-ribose) polymerase-1 (PARP1) adds ADP-ribose chains to itself and other proteins near the SSBs generated from either glycosylase, recruiting downstream BER enzymes. The SSBs can subsequently be repaired through the short patch or long patch branch of the BER pathway. During
short patch BER, Polymerase β (POLβ) using its dRP-lyase activity, cleaves the 5’-dRP and inserts a new base. Finally, a Ligase IIIα/XRCC1 complex seals the backbone [4, 10]. During long patch BER, polymerases β, δ, or ε synthesizes multiple new bases, displacing the 5’-phosphate and 2 to 10 nucleotides. Flap endonuclease 1 (FEN1) in complex with proliferating cell nuclear antigen (PCNA) excises the 5’-dRP and the displaced DNA, and the backbone is sealed by Ligase I [9].

Effective initiation and completion of the BER pathway is necessary for cells to survive alkylation and oxidative damage. Deficiency in Aag, one of the main glycosylases for excising 7-methylguanine [11], 1,N6-etheneoadenine [12], 1-methylguanine [13], hypoxanthine [14], 3-methyladenine [15], and many other lesions, leads to increased sensitivity to alkylating agents due to reduced ability to begin BER [16, 17]. Similarly, cells deficient in Polβ have lower viability following alkylation treatment compared to wild type due to their inability to complete BER resulting in an accumulation of toxic repair intermediates [18, 19]. In vivo, mice with low levels of AAG have increased sensitivity to colon inflammation and increased levels of mutations [20], while mice with high levels of AAG activity have high sensitivity to alkylating agents inducing increased retinal degeneration [21]. Therefore, the impact of alteration in AAG activity can be severe. Similarly, downstream proteins like APE1, XRCC1, and POLβ are also vital to cells; deficiencies in any of these three proteins is embryonically lethal [22]. Studies in humans have detected increased expression of both AAG and APE-1 in breast cancer tissues and cell lines [23, 24]. Furthermore, thirty percent of human tumors contain a low fidelity variant of Polβ [25]. Taken together, these data demonstrates that alterations or imbalances in the activity of BER proteins can be toxic and are characteristic of some tumors.
1.1.2 Direct Reversal of Alkylation Damage

In contrast to BER, which requires multiple proteins to repair base lesions, DR needs only a single protein to process damages. While there exists multiple DR proteins which can repair a variety of DNA damages including cyclobutane pyrimidine dimers [26], here we focus on three direct reversal proteins most pertinent to the following studies: O\textsuperscript{6}-methylguanine methyltransferase (MGMT), AlkB homolog 2 (ALKBH2), and AlkB homolog 3 (ALKBH3). The most common lesion MGMT repairs is O\textsuperscript{6}-methylguanine. If the lesion is unrepaired by MGMT or other pathways, it can lead to a G to A mutation (Fig. 1.3A). MGMT transfers the alkyl damage directly onto its cysteine residue (Fig. 1.3B), causing the protein to be inactivated, ubiquinated, and degraded [27, 28]. Studies have shown that deficiencies in MGMT cause tissues and animals to have increased sensitivity to alkylating agents and have increased mutations [29-31]. Furthermore, MGMT polymorphisms have been associated with increased cancer risk [32] and altered activities of MGMT has been detected in multiple tumor type including colon tumors and melanomas [33, 34].

ALKBH2 and 3 are two of nine α-ketoglutarate-dependent dioxygenase enzymes that are homologs of the *E. coli* protein AlkB [26]. Alkbh2/3 repair alkylation damages such as 1-methyladenine and 3-methylcytosine, by hydroxylating the alkyl groups which leads to the release of an aldehyde from the base (Fig. 1.4A) [35]. In addition, AlkB and its homologs repair cyclic ethenoadducts (e.g. 1, N\textsuperscript{6}-ethenoadenine, and 3, N\textsuperscript{4}-ethenocytosine) by oxidizing the double bond resulting in the generation of a glyoxal group (Fig. 1.4B) [36, 37]. If unrepaired by AlkB, the lesions can be mutagenic and potentially cytotoxic through replication blockage [36, 38]. At the organism level, mice deficient in Alkbh2 display an increased sensitivity to alkylating agents and an accumulation of base lesions [39]. Furthermore, mice deficient in Alkbh2/3 have been observed to have higher levels of tissue damage and increased tumor burden in the colon
following inflammation [40]. Thus, the DR proteins ALKBH2/3 and MGMT are vital for the viability and well being of organisms.

1.1.3 Homologous Recombination

One of the primary mechanisms by which base lesions and strand breaks induce toxicity in cells and tissues is by inhibiting replication progression [41]. Replication machinery can encounter the lesion or strand break leading to replication fork breakdown and a double strand break (DSB) (Fig. 1.5) [42]. Two-ended DSBs are primarily repaired through two mechanisms: non-homologous end joining, which functions mostly during the G1 phase of the cell cycle, and homologous recombination, which is most active during the S/G2 phase [43]. HR is the primary repair pathway for one-ended DSB caused by replication fork breakdown due to the need for homology searching in the repair process [44]. Repair of broken forks begins with the MRE11-RAD50-NBS1 (MRN) complex excising nucleotides at the 5' end of the broken DNA enabling Exonuclease 1 (EXO1) to resect the DNA in a 5' to 3' direction (Fig. 1.5) [45, 46]. Subsequently, BRCA2 loads Rad51 onto the 3' overhang forming a nucleoprotein filament, which is able to invade the intact strands of DNA searching for homology [47, 48]. Thereafter, the X-shaped Holliday Junction is cleaved and the replication fork is restored.

Studies have shown a link between HR and inflammation. E. coli strains exposed to nitric oxide, a reactive nitrogen species prevalent in inflammatory environments, were observed to have increased levels of recombination [49]. Similar increases in recombination have also been observed in mammalian cells exposed to peroxynitrite, another reactive nitrogen species generated during inflammation [50]. Blocking lesions and strand breaks induced by the inflammatory chemicals are likely causing replication fork breakdown leading to an increase in HR. In addition, bacteria and mammalian cells
deficient in recombinational repair have increased sensitivity to the inflammatory chemicals nitric oxide and hydrogen peroxide when compared to WT cells [49, 51]. Thus, HR may be necessary to survive in inflammatory regions. HR has also been shown to play a role in inflammation on the organismal level. Mice exposed to overlapping bouts of pancreatic inflammation displayed high levels of recombination [52]. If mice are challenged with DNA damage during inflammation-associated cell proliferation, then the level of recombination is significantly increased [52], suggesting that damage during proliferation can lead to fork breakdown and HR. Therefore, HR plays a major role during inflammation and recovery from inflammation.

There have been many studies indicating the necessity of HR for survival. Brca2 null cells and tumors are increasingly sensitive to ionizing radiation (IR), X-rays, and alkylating agents and display increased chromosome instability [53, 54]. Similarly, Brca2 mutant cells display a 6-100 fold decrease in recombination [55]. Furthermore, deficiencies in either Brca2 or Rad51 are embryonically lethal [56, 57]. In humans, Brca2 deficiency has been linked to breast and ovarian cancers [58, 59] and observed in the D1 complementation of group of Fanconi anemia, a leukemia-prone genetic disorder [60]. New cancer therapeutics capitalize on the Brca2 deficiencies in tumors [61] as Brca2 deficient cells are unable to safely repair broken replication forks. By treating Brca2−/− tumors with PARP-1 inhibitors, there will be an increase in BER intermediates that will induce DSBs. Since the DSBs are unable to be effectively repaired through HR, the cells will die [62]. Thus, HR plays a vital role in survival and deficiency in this pathway can be toxic to both normal cells and tumors.

1.1.4 Mismatch Repair

One of the most common types of errors in DNA is the base mismatch; a polymerase places the wrong base across from its hydrogen bond partner. To combat mismatches,
the cell utilizes the mismatch repair pathway (MMR). The pathway is initiated by heterodimers of MutS homologs (MSH2-MSH6 forming MutSα or MSH2-MSH3 forming MutSβ) and heterodimers of MutL homologs (MLH1, MLH3, PMS1, or PMS2) recognizing the mismatch and nicking the backbone [63]. EXO1 digests the DNA before and after the mismatch and DNA polymerase δ replaces the DNA nucleotides [64]. Finally, Ligase 1 seals the backbone [65]. Mutations in MSH2, MSH6, and MLH1 have been shown to play a major role in colorectal cancer and reduced MSH2 and MLH1 gene expression have been found in nearly half of all lung adenocarcinomas [66-69]. Thus, an active and effective MMR pathway is necessary for proper homeostasis.

1.1.5 DNA Damage Response

The DNA damage response (DDR) is a collective term for the signaling cascades induced by DNA damage that results in multiple cell processes including cell cycle arrest, DNA repair, and apoptosis. Deficiencies in DDR, such as the autosomal disorder ataxia-telangiectasia, can lead to neurodegeneration, genome instability, and cancer predisposition [70]. The main mediators of DDR are three phosphoinositide 3-kinase related protein kinases: ataxia-telangiectasia mutated (ATM), ataxia-telangiectasia Rad3 related (ATR), and DNA-Protein Kinase (DNA-PK). Both ATM and DNA-PK are primarily induced in response to DSBs, while ATR is primarily activated by single-stranded DNA. Following DNA damage, ATM autophosphorylates and dissociates from an inactive dimer into an activated monomer. The ATM monomer is recruited to the DNA breaks by the MRN complex [70, 71]. Subsequently, ATM phosphorylates many targets including Ser-139 of histones H2A near the breaks, forming γH2AX, which can recruit MDC1. Immunofluorescent detection of γH2AX is often used as a marker for DSBs [72]. After phosphorylation by ATM, MDC1 can recruit multiple downstream signaling proteins including p53-binding Protein 1 (53BP1), another common marker for
DSBs, which aid in repairing DSBs [70]. In addition, activated ATM is able to phosphorylate p53 and cell cycle checkpoint kinase 2 (CHK2) through a signaling cascade, which can lead to cell cycle arrest [71].

ATR is recruited to Replication protein A (RPA) coated single-stranded DNA by ATR interacting protein (ATRIP) [73]. Upon activation by the colocalized complex of topoisomerase II binding protein 1 (TOPBP1) and Rad9-Rad1-Hus1, ATR can phosphorylate hundreds of substrates including cell cycle checkpoint kinase 1 (CHK1) [74]. The downstream signaling proteins inhibit the cell from entering mitosis, reduces the rate of DNA replication, and induces DNA repair [74]. ATR is vital for cell survival, as ATR deficiencies are embryonically lethal.

DNA-PK catalytic subunit (DNA-PKcs) is recruited to DSBs by the heterodimers Ku70/80, and subsequently autophosphorylates [73]. Together, they form DNA-PK and initiate the non-homologous end joining pathway, a potentially error-prone DSB repair pathway that is most active during the G1 phase of the cell cycle [43]. Upon the DNA-PK unit binding, end processing enzymes, such as Artemis, excise the ends of the break to make them suitable for ligation [75]. Subsequently, DNA ligase IV with XRCC4 and XLF ligate the DNA ends together, repairing the DSB. Together with the DNA repair pathways, DDR protects the DNA from genomic instability and potential mutation. Below, we will analyze the role of DNA damage and repair during infectious disease.

1.2 Infectious Disease and Genomic Instability

There are billions of organisms living inside all humans. If these organisms migrate to sterile compartments, they can induce an active inflammatory response. Innate immune cells like neutrophils and macrophages invade the infected tissue to clear the pathogen with RONS such as nitric oxide and superoxide and in the process, damage the DNA of
surrounding tissues. The DNA damage generated by the pathogen and its induced-immune response increases the potential for mutations and cancer [76]. Here, we discuss four infectious bacterial and viral pathogens that have been analyzed on their properties to induce DNA damage and the role of DNA repair during that infection.

1.2.1 Bacterial Infections and DNA Damage

*Helicobacter pylori* (H. pylori) is a gram negative bacteria that colonizes the stomach epithelium of approximately two-thirds of the world’s population [77]. The Center for Disease Control and Prevention classifies it as a Type 1 carcinogen and in 2005 Barry Marshall and Robin Warren received the Nobel Prize for their discovery of its role in gastritis [78]. One of the important factors in *H. pylori*’s carcinogenic potential is its ability to induce chronic inflammation in the stomach. Increased levels of oxidative lesions and GC to TA mutations were observed in mice and humans infected with *H. pylori* further suggesting that the oxidative inflammatory environment is inducing mutations [79, 80].

Studies have shown that *H. pylori* can also cause DNA damage and affect DNA repair without the assistance of an immune system [79]. Cells treated with *H. pylori* display an increase in physical DNA strand breaks and an increase in the response to the strand breaks as observed through γH2AX and 53BP1 foci [79]. Strand break increase was observed in a contact dependent manner and was reduced in *H. pylori* infected cells treated with ATM inhibitors [79]. Therefore, *H. pylori* can induce DNA damage that is causing a response from the cell. The exact mechanism by which *H. pylori* induces DSBs is still unknown given that infecting cells deficient in the nitric oxide synthase protein iNOS and NADPH Oxidase 2 (NOX2), which produces superoxide, did not cause any effect, suggesting that the strand breaks were not a result
of RONS [79]. Thus, both the bacterium and its immune response can cause DNA damage.

Furthermore, *H. pylori* and its immune response also affect DNA repair. Mice infected with *H. pylori* display a decrease in MSH2, MSH3, and MSH6 mRNA and protein levels [81]. In addition, humans who have had *H. pylori* eradicated from their stomachs observe an increase in MMR proteins as well [82]. One model for this effect is that *H. pylori* could activate or repress transcription of the proteins by affecting methylation of CpG islands in their promoter regions [83]. *H. pylori* has also been shown to affect BER. Cells treated with *H. pylori* display a decrease in APE1 protein expression, but display consistent levels of OGG1 [81]. An imbalance in BER proteins can be toxic due to an increase in strand breaks and can be problematic for cells and organisms [84]. Furthermore, *Aag*−/− mice infected with *H. pylori* have already been observed to have more lesions leading to cancer than wild type mice [20], suggesting that BER capacity may be an exacerbating factor in the ability of *H. pylori* to cause cancer. Taken together, *H. pylori* and its immune response have the potential to induce gastric cancer by causing DNA damage and reducing DNA repair capacity.

The role of DNA damage and repair during *Chlamydia trachomatis* (*C. trachomatis*) infection, a gram-negative bacterial strain that is a leading cause of sexually transmitted diseases and has been associated with cervical cancer [85], is not as deeply studied as *H. pylori*’s. However, a recent report has demonstrated that *C. trachomatis* can induce oxidative stress which contribute to DSBs in cells [86]. Through γH2AX analysis and neutral comet assay, a measure of physical DSBs, the study observed an increase in strand breaks. In addition, an increase in 8-oxoguanine was observed [86]. NOX2 inhibitors reduced the levels of γH2AX, but also reduced the amount of bacteria propagated, suggesting that the bacteria are using the oxidative stress to proliferate. Interestingly, MRE11, ATM, and 53BP1 were not colocalized to
areas where γH2AX occurred [86]. Therefore, *C. trachomatis* may be inducing DNA damage through ROS to propagate, and inhibiting the DDR simultaneously. Analysis of the effects of *C. trachomatis* infections on cells and mice deficient in DNA repair proteins, such as DNA-PKcs or OGG1, would be a very informative study. The current model suggests that further increasing the levels of DNA damage in the cell would further help the bacteria to grow and proliferate; using repair deficient systems would be an easy way to test this model. In addition, determination of the specific part of *C. trachomatis* inhibiting the DDR could be lead to novel cancer therapeutics.

The Engelward Laboratory has recently studied the DNA damaging effects of another bacteria, *Streptococcus pneumoniae* (*S. pneumoniae*), which is known to induce pneumonia in the upper respiratory tract of humans. *S. pneumoniae* has previously been observed to use the enzyme pyruvate oxidase to produce H2O2 by pyruvate [87]. In a recent paper, they observed that the expelled hydrogen peroxide from *S. pneumoniae* could cause DNA damage in both cells and mouse lungs [88]. By treating cells with the antioxidant catalase, the levels of γH2AX were significantly decreased. Similarly, treating cells with bacteria deficient in the gene for pyruvate oxidase also reduced the levels of γH2AX [88]. In addition, *S. pneumoniae* produces pneumolysin, another virulence factor that can induce DNA damage. Pneumolysin perforates the host cell membrane, and that can increase γH2AX and 53BP1 levels in the infected cells [89]. Treating cells with DNA-PKcs and ATM inhibitors reduced γH2AX production heavily, as expected given that DNA-PKcs and ATM are two of the main kinases of Ser-139 of H2AX. It would be interesting to determine whether physical DSBs are also reduced with the inhibitors. Taken together, one can imagine a novel therapeutic that inhibits pneumolysin and scavenges ROS in lung tissues. New therapeutics are drastically needed for pneumonia, given that 30% of pneumococcal infections are resistant to at least one antibiotic [90].
1.2.2 Viral Infections and DNA Damage

Numerous viral infections have been observed to cause cancer. As such the role of DNA damage and DDR for many viruses, such as Hepatitis C virus [91], Human Papillomavirus [92], and Simian Virus 40 [93] has been deeply studied. One prime example is the Epstein-Barr virus (EBV), a DNA virus that infects 90% of individuals [94]. Associated with Hodgkin’s lymphomas, EBV latently infects B lymphocytes; when the virus turns lytic, it begins to amplify production of new viral particles [94]. Both latent and lytic EBV infections have been observed to cause DNA damage. During the latent phase, nine EBV proteins are generated in infected cells. Three proteins, Epstein-Barr nuclear antigen 1 (E1), latent membrane protein 1 (LMP1), and Epstein-Barr nuclear antigen 3C (E3C) have been observed to induce genomic instability [95]. E1 induces physical strand breaks and an increase in γH2AX and phosphorylated ATM levels [96]. It has also been observed to increase ROS and 8-oxoguanine levels in cells. Inhibiting NOX2 or treating cells with antioxidants decreases the levels of γH2AX in the system [96]. Interestingly, LMP1 does not directly cause DNA damage like E1, but rather reduces ATM expression in the cells, causing a decrease in DNA repair activity [95]. Cells infected with LMP1 were slower to repair gamma irradiation and UV radiation. E3C does not cause DNA damage or slow repair. Instead, it increases chromosomal aberrations by suppressing expression of BubR1, a mitotic checkpoint protein [95]. In summary, EBV can cause genetic instability through a variety of mechanisms during its latent phase.

Upon converting to the lytic phase, EBV begins to replicate the viral genomes in small compartments. Studies have shown an increase in γH2AX, pATM, and pChk2 levels in lytic phase cells [94]. In addition, pATM, MRE11, and NBS1, and RAD51 all colocalize with viral replication areas suggesting that the cell’s DNA repair machinery is
being recruited to repair damage in the virus’s DNA [94, 97]. Studies have shown that MMR proteins are also recruited to the virus’s DNA [98]. The observation that viral infections in cells with Rad51 knocked down have a reduced virus production also supports this model [97]. Taken together, EBV cells induce genomic instability during the latent phase of infection and coopts host repair machinery during its own replication process. Thus, host cells may be less able to repair the damage in their own DNA, resulting in mutations and increasing the likelihood of cancer.

While the role of DNA damage and repair during the prevalent EBV infection and the immunoreactive H. pylori infection has been deeply studied, the number of studies analyzing role of DNA damage and repair during influenza infection is significantly fewer. Influenza infection affects nearly 1 billion people in the world annually and can induce an active inflammatory response [99]. A few of the limited influenza infection studies have shown an increase in 8-oxoguanine and the antioxidant superoxide dismutase (SOD) in patients [100] and one study demonstrated that influenza can induce strand breaks in vitro [101], yet a systems analysis of DNA damage and repair during influenza infection and its inflammatory response has yet to be performed. In Chapters 2 and 3, we investigate DNA damage and repair during influenza infection both in vitro and in vivo. The necessary information on influenza pathogenesis to understand the work will be covered in Section 1.3.

1.3 Influenza and the Inflammatory Response

1.3.1 Societal and Economic Effects of Influenza Infection

Influenza, a single-stranded negative RNA virus that infects the respiratory tract of humans, is a prevalent disease that causes not only biological issues for the individuals afflicted, but also economic and societal problems for the United States and the world
every year. The annual seasonal influenza epidemic affects approximately one billion people in the world and cause five to seven million people to have a severe illness [102]. 500,000 people succumb to the disease annually; the majority of them are elderly, immunocompromised, or very young. While every year the influenza virus mutates leading to a new seasonal epidemic, occasionally the virus can have a major mutational event leading to a global pandemic with very high morbidity. The most devastating global influenza pandemic occurred in 1918, when the “Spanish Flu” killed over 50 million people [103]. Interestingly, the 1918 pandemic had a very high mortality rate people between the ages of 18-45, with a peak mortality at age 28 [104]. While one of the major causes of mortality from the 1918 infection was a secondary bacterial infection and while the world has made major advances in public health, vaccines, and antibiotics, pandemic viruses still cause problems today [105]. The 2009 “Swine Flu” Pandemic was the most destructive pandemic of the 21st century. It led to a seven-fold increase in hospitalization of children compared to seasonal infections [106]. Furthermore, individuals aged 18-64 had a four-fold increase in hospitalizations and a twelve-fold increase in death compared to seasonal infections [106]. Thus, influenza infections have the potential to negatively impact large segments of the population.

One of the primary methods to combat influenza epidemics is through vaccination. While vaccinations have averted nearly 40,000 deaths in the past decade [107], they are not always successful, given that their effectiveness can range annually from 49% in 2012-13 [108] to 23% in 2014-15 [109]. Further exacerbating the ineffectiveness of vaccines is the fact that there exists disparities in influenza vaccination and preparation [110]. While 43.7% of all American get vaccinated annually, only 32% of individuals below the poverty line and only 30% of those between the ages of 18 and 44 receive the prophylactic treatment [110]. Furthermore, there is a wide range in vaccine administration in developing countries which leads to triple the number of
children hospitalized from influenza [111]. In addition, administration of the influenza vaccine through the nasal spray has recently been ended due to the injected vaccine being found 20 times more effective [112]. Therefore, the vaccination rate is likely to decrease further in coming years. Studies have shown that each influenza episode leads to a loss of 23 work-hours [113]. Given that hourly workers already have twice as many episodes as waged workers, a new pandemic or a severe seasonal epidemic could have grave consequences for workers [113]. Therefore, society needs novel therapeutics to influenza infection or else the $3.7 billion spent on influenza healthcare annually [110] will increase and more influenza-induced deaths can be expected.

1.3.2 Influenza, the virus

There are three genera of the Influenza virus, a member of Orthomyxoviridae family: Influenza virus A, B, and C. While Influenza B and C are found primarily in humans, Influenza A is also found in many warm-blooded animals like birds, pigs, and seals [99]. The diversity of hosts for influenza A enables gene reassortment among different virus strains in one species, which can induce pandemics in another [114]. For example, the 2009 H1N1 pandemic occurred from antigenic evolution and drift in wild aquatic birds and swine and spread to humans [115]. Specific virus strains are categorized by two glycoproteins found on the virus surface: hemagglutinin (HA) of which there are 18 subtypes and neuraminidase (NA) of which there are 11 subtypes. Each virus strain is referred to by the glycoproteins it possesses, i.e. H1N1 [116].

HA is required for viral entry into epithelial cells. In human cells, HA binds α2,6 linked-sialic acid residues inducing endocytosis of the virus [116]. The low pH of the endosome enables a conformational change in HA inducing a fusion of the virus and the endosome membranes and the release of the viral genome into the cytoplasm [117]. The eight negatively stranded RNA segments, encoding 10 proteins, are encapsulated by
nucleoprotein (NP) and are connected to three viral polymerases, polymerase acidic protein (PA), polymerase basic protein 1 (PB1), and polymerase basic protein 2 (PB2), which initiate production of new viral RNA and proteins [116]. Upon completion of virus production, NA is utilized for viral exit. Newly budding viruses from the host cell membrane are connected to sialic acid residues by HA. NA cleaves the HA-sialic acid residue bond of the newly formed viruses releasing them to infect new cells [118]. Due to its vital role in virus propagation, NA has been a major target for antiviral medications including the over the counter medicine oseltamivir (Tamiflu) [118].

Two other proteins used by the virus for pathogenesis are NS1 (non-structural protein 1) and PB1-F2, an additional protein encoded in the PB1 gene segment. NS1 is a defense mechanism by the virus to inhibit the antiviral response. It prevents interferon-β (IFNβ) from being synthesized and thus reduces the cell's inflammatory response [119]. NS1 null viral strains are only able to grow cells deficient in IFN [120]. In addition, NS1 blocks endogenous mRNA export into the cytoplasm inducing the cell to only produce viral proteins [121]. PB1-F2 assists the virus by inducing apoptosis in the host cell [116]. It is targeted to the mitochondria and induces cytochrome c release by puncturing the mitochondrial membrane [122, 123]. Studies have shown that infecting mice with viruses deficient in PB1-F2 induce less morbidity and a smaller viral in the host [124].

Many viral proteins have been implicated in pandemics. Infecting mice with influenza virus containing the HA protein from any of the major pandemics of the last century (1918, 1957, 1968, or 2009) causes significantly higher tissue damage and increased morbidity compared to mice infected with seasonal virus [125]. Furthermore, there appeared to be a marked increase in pro-inflammatory cytokines in mice infected with transgenic viruses [125]. Similarly, animals infected with virus containing the 1918 PB1-F2 had more inflammation compared to mice infected with seasonal infections.
In all of these studies, an overactive immune response appears to be characteristic of pandemic infections. In Section 1.3.3 we will further explore the role of the inflammatory response during influenza infection.

1.3.3 Influenza Pathogenesis

Individuals typically contract influenza infection either through airborne transmission or through physical contact with a person or an object containing viral particles on their surface [126]. Forty-eight hours after the virus has infected the alveolar epithelial cells, peak viral replication occurs [127, 128]. Studies have shown that symptoms of the infection generally peak at 72 hours [128], suggesting that there is a 24 hour lag between viral infection and its systemic effects. Influenza infection can cause many symptoms in individuals including high fever, cough, headaches and malaise. These irritating symptoms can last in humans 7 – 10 days [127].

In order to study influenza mechanisms and pathogenesis, many researchers use the animal model Mus musculus or mice, though they are suboptimal. Mice have a different set of symptoms than humans following influenza challenge. Mice display anorexia, hypothermia, dehydration, and hunched posture upon influenza infection, none of which are common in influenza-infected humans [129]. In addition, mice have varied capabilities to transmit virus depending on the virus and mouse strain making them poor for communicability studies [129]. Furthermore, many virus strains are not able to directly infect mice, due to the abundance of α2,3 sialic acid residues in mouse airways in contrast to the α2,6 residues in human lungs [129]. For example, the most common influenza virus for mouse studies, Influenza A/Puerto Rico/8/1934 (PR8), is used not because of its similarity to human disease, but because it has been adapted to mice over multiple generations and is effective at inducing infection [129]. Moreover, some mouse strains like C57/Bl6 and BALB/c mice are more resistant to influenza
infection when compared to DBA/2J mice which die within seven days of infection [130]. Thus, one can obtain drastically different results based on animal strain. Yet, due to their size, relatively low cost, compared to ferrets, a mammal with a flu pathogenesis more consistent with humans, and the ability to easily perform genetic alterations, mice are still the most used model for influenza infection [129].

Multiple studies have shown that one of the major problems with influenza infection is an overactive immune response. Tissue sections of people who succumbed to the 1918 influenza virus display a large amount of inflammation markers [131]. Both mice [132] and macaques [133] infected with the 1918 virus also display high levels of inflammation compared to seasonal viruses. Mice and humans infected with pathogenic H5N1 viruses have high levels of proinflammatory cytokines like tumor necrosis factor α (TNF-α) [134] and monocyte chemoattractant protein 1 (MCP-1) [135], which recruits macrophages to the site of the infection. Studies have also shown that a superabundance of neutrophils is one of the major differences between lethal and sublethal infections [136]. Innate immune cells like neutrophils and macrophages infiltrate the lung to clear the virus and in that process they produce a large amount of RONS [137, 138]. Macrophages produce superoxide and nitric oxide which can directly damage host DNA and tissues in other inflammatory environments [139]. Superoxide and nitric oxide can also react with each other to form peroxynitrite, another particularly toxic RONS [50]. In addition, neutrophils produce myeloperoxidase, an enzyme that catalyze the reaction between hydrogen peroxide and chloride ions to produce hypochlorous acid (HOCl), which also can be quite damaging [139-141]. Studies by the Maeda Laboratory at Kumamoto University School of Medicine in Japan have shown that a reduction in RONS can mollify influenza-induced morbidity [142, 143]. By treating animals with the superoxide scavenger, superoxide dismutase, or the nitric oxide inhibitor, n-methyl arginine, one can reduce the levels of inflammation and tissue damage, decrease
morbidity, and rescue mice [142-144]. Similarly, mice deficient in inducible nitric oxide synthase (iNOS) infected with influenza displayed increased survival and reduced pneumonitis compared to wild type infected mice [145]. Mice deficient is NOX2, a subunit of NADH, which produces superoxide, also display less inflammation, fewer RONS, and enhanced viral clearance compared to wild type mice infected with influenza [146]. Thus, influenza induced inflammation can be toxic to the system and a reduction in RONS can alleviate morbidity in animals.

While reducing the levels of RONS in the system has been observed to be protective to influenza infected mice, completely inhibiting the existence of immune cells in infected lungs has been shown to be problematic. Treating influenza-infected animals with antibodies specific to neutrophils that significantly reduces the neutrophils in the system, leads to lower body weight, increased virus titer, and decreased survival compared to untreated mice [147]. Furthermore, mice deficient in MCP-1 displayed lower body weight and higher viral loads compared to wild type influenza-infected mice [148]. Taken together, the data suggests that while an overactive inflammatory response exacerbates influenza infection, inflammation is necessary to clear the virus and survive the infection.

Yet, the fact that a reduction in RONS alleviates influenza infection suggests that RONS and the inflammatory response play a key role in aggravating influenza-induced morbidity. Given the expression of RONS during influenza infection and the fact that RONS can damage DNA [137-139, 149, 150], we hypothesize that DNA damage occurs during influenza infection and that the ability to repair DNA damage contributes to the morbidity of infection. In Chapters 2 and 3, we will test these hypotheses. Moreover, studies have shown that RONS produced by inflammation can induce DNA damage, and also affect DNA repair capacity. We will analyze the effects of RONS, specifically
nitric oxide, on DNA repair in Chapter 4. Previous studies demonstrating the effects of nitric oxide on DNA repair are described in the following section.

1.4 Nitric Oxide and DNA Repair

Studies have shown that DNA repair deficiency makes cells and animals more sensitive to inflammation and RONS. For example, Aag<sup>−/−</sup> mice treated with DSS to induce colon inflammation display a significant increase in mutation and tissue damage, and a decrease in survival [20, 40]. However, inflammation can also affect DNA repair activity causing the organism to have increased susceptibility to damage. Mice infected with *Helicobacter hepaticus* display significant decreases in gene expression of multiple DNA repair enzymes including, *Mgmt*, *Aag*, and *Ogg1* in the colon [151]. Similarly, mice infected with *H. pylori* have decreased levels of *Ape1, Msh2*, and *Msh3* gene expression that coincide with increased genomic instability [81]. Moreover, studies in colons from ulcerative colitis (UC) patients, a chronic inflammatory disease of the colon that often leads to colon cancer, have observed an increase in AAG and APE1 protein expression suggesting that the imbalance in BER protein expression contributes to the characteristic genome instability in UC [152]. Studies have also shown that RONS produced during inflammation can affect DNA repair. The Boland Laboratory has demonstrated through *in vitro* assays that hydrogen peroxide can directly inactivate MMR [153]. However, the RONS that is most thoroughly investigated in its capacity to directly affect DNA repair is nitric oxide.

Nitric oxide (NO) is a biological signaling molecule that can have both physiological and pathological effects depending on the concentration and the cellular context. It is generated by a enzymatic reaction between L-arginine and oxygen, catalyzed by the nitric oxide synthase (NOS), of which there are three primary proteins: neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS) [154, 155].
nNOS and eNOS primarily generate NO transiently at nanomolar levels for physiological processes such as neurotransmission and blood pressure control, though both NOSs are able to become dysregulated during disease [139]. iNOS is mainly activated in innate immune cells (e.g. macrophages) to combat foreign substances and pathogens during an inflammatory response by producing micromolar concentrations of NO [156]. Studies have shown that iNOS and generated NO can be vital to surviving infections from pathogens such as mycobacterium tuberculosis, leishmania major, and listeria monocytogenes [157]. However, persistently high levels of NO produced during a chronic inflammatory response can be detrimental to the host organism through the induction of DNA damage, which can lead to mutations and cancer [50, 137, 138, 150, 158-160].

In addition to reacting with DNA, NO can also react with the cysteine residues of DNA repair proteins through a process known as S-nitrosation. Many proteins can become S-nitrosated resulting in both physiological and pathological effects. S-nitrosothiols, S-nitrosated proteins, have been observed to aid in vasodilation and inhibition of platelet relaxation [161]. S-nitrosothiols can also contribute to the clearance of pathogens. For example, by S-nitrosating a coxsackievirus protease in its active site, NO can inhibit its activity and alter the life cycle of the pathogen [162]. Mice treated with iNOS inhibitors displayed increased viral titer and reduced survival when infected with coxsackievirus, likely due to the lack of nitric oxide production [163]. Yet, alteration of the basal level of S-nitrosation in a tissue has been linked to numerous diseases [164]. For instance, lower than normal levels of nNOS-produced nitric oxide have been observed to induce arrhythmias in mice [164]. This is due to the lack of physiological levels of NO to S-nitrosate cardiac receptors causing contraction.

One of the mechanisms of S-nitrosation is through a reaction with S-nitrosoglutathione (GSNO) [165]. The S-nitrosated form of glutathione (GSH) [166],
GSNO can transfer its nitric oxide moiety to the cysteine residue of another protein (Fig. 1.6). Moreover, the levels of S-nitrosothiols in a cell are regulated by the GSNO reductase (GSNOR), a formaldehyde dehydrogenase, that reduces GSNO into a glutathione disulfide (Fig. 1.6) [167]. GSNOR is conserved from bacteria to humans and by reducing the cellular levels of GSNO, it can lower the overall level of S-nitrosothiols in the cell [167, 168]. Studies have shown that genetic deficiencies in GSNOR in yeast and mice display increased levels of GSNO and S-nitrosothiols [168]. This alteration in GSNOR activity can contribute to disease. Various SNPs in the gene for GSNOR are related to both increased and decreased risk for childhood asthma in humans [164]. While the exact connection between the SNPs and GSNOR activity is currently unknown, adults with asthma display increased GSNOR expression and fewer S-nitrosothiols suggesting that high GSNOR activity may be connected to asthma [169]. There are currently multiple labs developing GSNOR inhibitors to ameliorate diseases through S-nitrosation [167, 170].

The effects of S-nitrosation and GSNO on multiple DNA repair proteins have been studied, most thoroughly MGMT. GSNO can transnitrosate the protein MGMT on the cysteine residue that normally reacts with the alkyl damage on the base lesion [171]. S-nitrosated MGMT is subsequently degraded by the proteasome leading to an increase in unrepaired base lesions and cell death. GSNOR−/− mice display decreased levels of MGMT and increased hepatocellular tumor burden [172]. Moreover, 50% of hepatocellular carcinoma tumors were found to have mutations significantly reducing GSNOR expression and activity potentially implicating S-nitrosation in the promotion of cancer [172].

Another repair protein known to be affected by nitric oxide is OGG1. OGG1 is inhibited by nitric oxide through a process that can be blocked with nitric oxide scavengers [173, 174]. OGG1 is likely inhibited through S-nitrosation. Proteins derived
from an OGG1 polymorphic variant containing a cysteine residue displayed reduced activity compared to a variant containing serine, following exposure to nitric oxide [175]. Given that cysteine residues are the amino acids most likely to react with NO, it is likely that OGG1 is S-nitrosated in this experiment [166]. In addition, the cysteine variant cells also displayed an increase in genetic instability suggesting that the S-nitrosation of OGG1 leads to potentially carcinogenic effects.

The effects of nitric oxide on many DNA repair proteins have not been thoroughly studied. For instance, while both Ligase I and PARP-1 proteins have been observed to be inhibited by nitric oxide biochemically, the mechanism by which they are affected by nitric oxide is yet to be determined [176, 177]. In addition, the effects of nitric oxide on their repair activities in the context of a cell have not been investigated. Other proteins such as DNA-PKcs [178] are known to get S-nitrosated in cells, yet the effect of nitric oxide on the NHEJ pathway is still undetermined. One pathway worthy of future study is the BER pathway. Biochemically, studies have shown that S-nitrosated AAG has increased excision activity [179] and cellular studies have observed APE-1 export into the cytoplasm following S-nitrosation [180]. Yet, there is a lack of knowledge on how S-nitrosation affects these proteins in the context of the entire pathway. In Chapter 4, we query the effects of GSNO on the ability of cells to repair alkylation damage through the BER pathway.

1.5 Overview of the Presented Study

In this work, we analyze DNA damage and repair in the context of influenza infection and its inflammatory response and in the context of the post-translational modification common to inflammatory regions, S-nitrosation. In the Chapter 2, we investigate the ability of the influenza virus to induce strand breaks in cells and in animal tissues. We
also characterize our influenza infection mouse model and determine whether influenza infection leads to an increase in base lesions. We observed strand breaks in both environments, yet we did not observe an increase in base lesions. Together this indicates that influenza can induce DNA damage and that the DNA repair machinery is able to repair the damage. In Chapter 3, we study the role of DNA repair during influenza infection. We observed an increase in HR gene and protein expression, suggesting that HR occurs after influenza infection. In addition, we analyzed mice deficient in AAG, ALKBH2, and ALKBH3 and detected an increase in morbidity and Rad51 levels compared to WT suggesting that DNA repair plays a role in recovery and that HR is induced to compensate for the DNA repair deficiency. Finally, in Chapter 4 we query the effects of S-nitrosation on the repair of alkylation damage. GSNO-exposed cells displayed an imbalance in BER protein activity. When challenged with MMS, GSNO exposed cells displayed an increase in BER intermediates and a reduction in cell viability demonstrating that GSNO and S-nitrosation impairs the activity of the BER pathway. The results and potential for future studies will be discussed in Chapter 5.
1.6 References


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Fig. 1-1: Mechanism of oxidation induced G to T Mutation
Fig. 1-2: Simplified Model of the Base Excision Repair Pathway
(Adapted from Jing Ge)
Fig. 1-3: Mechanism of $\textit{O}^{6}$-methylguanine-induced mutation (A) and MGMT repair (B)
Fig. 1-4: Mechanisms of AlkB repair of (A) 1-methyladenine and (B) 1, $N^6$ethenoadenine
1. Replication
2. Replication Fork Breakdown
3. Resection
4. Rad51 Loading
5. Strand Invasion
6. Progression
7. Holliday Junction Resolution
8. Replication Restart

Fig. 1-5: Simplified Model of the Homologous Recombination Pathway
Represents few of the over 20 proteins in the HR pathway
Fig. 1-6: Mechanism of GSNO induced transnitrosation and GSNO reduction by GSNOR.
Chapter 2

Influenza Induces DNA Damage in Cells and Tissues

2.1 Abstract

Nearly five hundred thousand people die each year from influenza, a single-stranded RNA virus that infects the epithelial cells of the respiratory tract. Inflammation, triggered by the virus, is thought to be a major cause of the morbidity and severity of influenza infection. In inflammatory environments, macrophages and neutrophils produce reactive oxygen and nitrogen species (RONS) to attack the foreign species and, in the process, cause damage to the surrounding tissues and DNA. Yet, there have been minimal studies on whether influenza or its inflammatory response can cause damage to the DNA on its own. Here, we tested the ability of influenza and its inflammatory response to induce DNA damage. Through *in vitro* immunofluorescent analysis and CometChip analysis, we observed a marked increase in the level of strand breaks in influenza-infected cells. Furthermore, analysis of influenza infection in mice demonstrated that peak tissue damage and apoptosis occurs after peak viral levels. Finally, mice infected with influenza displayed an increase in double strand break marker, γH2AX, and did not display a differential increase in base lesions. Through
these results, we propose that DNA damage affects the morbidity of influenza infection and hypothesize that DNA repair may also play a role during influenza infection.

2.2 Introduction

Influenza, a single-stranded RNA respiratory virus, is a very common pathogen that infects nearly 1 billion people in the world each year and causes an annual $16 billion economic burden on the United States [1, 2]. While the virus can induce cytotoxic effects on its own, an overactive inflammatory response has been shown to be a driving force of morbidity and mortality. Studies analyzing highly pathogenic influenza infection in mice [3], macaques [4], and humans [5, 6] have all found increased amounts of inflammatory cells and proinflammatory cytokines, which can lead to increased tissue damage.

Inflammation is the primary method by which organisms respond to foreign substances and repair damaged tissues. One mechanism by which inflammation clears foreign pathogens is through the release of reactive oxygen and nitrogen species (RONS) by macrophages and neutrophils [7]. While RONS can combat pathogens, they can also cause great harm by inducing collateral damage to surrounding tissues. Researchers observed increased survival and reduced morbidity in influenza-infected animals treated with antioxidants, compounds that reduce the levels of RONS [8, 9]. Studies in other inflammatory environments have shown that RONS, such as hydrogen peroxide and nitric oxide, can lead to strand breaks and base lesions in the DNA [10]. Strand breaks, particularly double strand breaks (DSBs), are very toxic to the cell and can induce apoptosis [11]. Unrepaired base lesions can be mutagenic if they mispair and they can be cytotoxic if they block replication machinery leading to a DSB [12]. Base lesions can be induced by oxidation (e.g. 8-oxoguanine), by deamination (e.g. inosine), and indirectly
by lipid peroxidation (e.g. 1, N\textsuperscript{6}-ethenoadenine and 1, N\textsuperscript{2}-ethenoguanine) [10]. Interestingly, while influenza is known to induce a strong inflammatory response, and inflammation has been observed to elicit DNA damage, the existence and role of DNA damage during influenza infection specifically is worthy of more study.

There are currently three main techniques to analyze strand breaks and base lesions in cells and tissues: γH2AX detection, the comet assay, and mass spectrometry. γH2AX is a phosphorylation modification of histone H2AX. When a DSB occurs, PI3K-like kinases phosphorylate H2AX at Ser-139 (γH2AX) which helps recruit repair proteins [13]. Antibody detection of γH2AX by western blot or immunohistochemistry is a common marker for DSBs, though it’s specificity to DSBs has come under debate recently [14, 15]. While γH2AX is a marker of the response to DSBs, the comet assay detects physical strand breaks. The comet assay is based on the principle that damaged DNA travels more readily through a gel matrix than tightly coiled, unbroken DNA [16]. Under neutral conditions, one can detect DSBs; while under alkaline conditions, one can detect multiple types of breakages including single stand breaks (SSBs), alkali labile sites, and abasic sites due to the alkaline pH enabling increased unwinding from histones [17, 18]. Previously, the Engelward Lab has developed a high-throughput version of the comet assay called the CometChip, which can be performed under alkaline and neutral conditions [19, 20]. While γH2AX and comet assays allow the assessment of strand breaks, mass spectrometry allows the detection of base lesions. By extracting the DNA from the cell, digesting it into individual nucleotides and running it through a mass spectrometer, one can determine the exact amount of base lesions such as hypoxanthine and 8-oxoguanine among others in the DNA [21].

Here, we demonstrate that influenza infection can lead to strand breaks \textit{in vitro} through the use of γH2AX staining and alkaline and neutral CometChip analysis. In agreement with literature, we also observed that influenza-infected mice display high
levels of tissue damage, apoptosis, and body weight loss, after viral peak. Finally, while there is not an increase in base lesions in influenza-infected mice, there was an increased in γH2AX markers in infected mouse lungs. Taken together, these studies suggest that DNA damage may play a role during influenza infection. If so, then we hypothesize that DNA repair may impact the morbidity from influenza infection.

2.3 Materials and Methods

2.3.1 Cells and Cell Culture
Madin-Darby canine kidney (MDCK) cells were maintained in Dulbecco’s Modified Eagle Medium (ThermoFisher Scientific) containing 10% fetal bovine serum (Atlanta Biologicals).

2.3.2 In vitro Influenza Infection and Immunofluorescent Staining
MDCK cells were cultured on gelatinized coverslips overnight, and subsequently infected with PR8 influenza at multiplicity of infection (MOI) of one, diluted in 2 mg/mL bovine serum albumin (BSA) (Sigma) and 2 μg/mL tosyl phenylalanyl chloromethyl ketone treated (TPCK-) trypsin (Sigma) in minimum essential medium (MEM) (Invitrogen) for 3, 6, 9, or 12 h. Non-treated cells were incubated with 2 mg/mL BSA and 2 μg/mL TPCK-trypsin in MEM for 12 h. Cells were then fixed and incubated with 2 μg/ml mouse anti γH2AX (Millipore) overnight at 4° C. Stained cells were then incubated with FITC-conjugated anti-mouse antibody (Santa-Cruz), mounted with ProLong Antifade containing DAPI (Invitrogen) and imaged with a Nikon 80i upright microscope under 609 magnification. At least ten images were taken per time-point in a blinded fashion. To quantify γH2AX-positive cells, images were “blinded” and counted manually for DAPI-positive nuclei. At least 100 cells were counted for each sample, with the
exception of three samples for which 82-99 cells were quantified. Nuclei harboring 5 or more \( \gamma \text{H2AX} \) foci were considered positive for \( \gamma \text{H2AX} \). Three independent biological replicates were performed for each condition and time-point.

### 2.3.3 Alkaline and Neutral CometChip

CometChip was fabricated using a polydimethylsiloxane (PDMS, Dow Corning) mold as described previously [22]. Briefly, molten 1 % normal melting point agarose (Invitrogen) was applied to a sheet of GelBond film (Lonza), and allowed to gel with the PDMS mold on top. Removal of the PDMS mold revealed a \( \sim 300 \) \( \mu \)m thick gel with arrayed microwells. The micowell gel was then clamped between a glass plate and either a bottomless 24-well or 96-well titer plate (Greiner BioOne) to create the CometChip. Cells were added to each well of the CometChip, and allowed to settle by gravity in complete growth media at 37°C, 5 % CO₂. Excess cells were aspirated after 15 min and the bottomless plate was removed to capture the arrayed cells in a layer of 1 % low melting point agarose (Invitrogen). After encapsulation in agarose, the bottomless plate was re-aligned to the original position on the CometChip. Wells were infected with 50 \( \mu \)L of PR8 influenza virus at MOI of \( \sim 1 \) in virus medium (0.2 % bovine serum albumin, 2 \( \mu \)g/mL TPCK-trypsin in minimum essential medium) at 37°C. Negative controls were treated with 50 \( \mu \)L of virus medium under the same conditions. After 1 h, the bottomless plate was removed, and all wells were incubated with 0.2 % bovine serum albumin and 2 \( \mu \)g/mL TPCK-trypsin in Opti-MEM at 37 °C. At 3, 6 and 9 h after influenza exposure, at least three influenza virus infected wells were processed according to either the alkaline or neutral comet assay described in literature [22].
2.3.4 Fluorescent Imaging and Comet Analysis

After electrophoresis, alkaline comet and neutral comet gels were neutralized in 0.4 M Tris, pH 7.5 (2 x 15 min) and stained with SYBR Gold (Invitrogen). Images were captured using an automated epifluorescent microscope, and analyzed using custom software written in MATLAB (Mathworks) [22].

2.3.5 Animals

All animal experiments were conducted according to the Guide for the Care and Use of Laboratory Animals, and were approved by the MIT Committee on Animal Care.

2.3.6 In vivo Influenza Infection

Six- to eight-week-old female C57/Bl6 J mice (Jackson Labs) were anesthetized with ketamine and xylazine (100mg/kg and 10mg/kg respectively). Upon anesthetization, the mice were intratracheally infected with 20 PFU of H1N1 Influenza A/Puerto Rico/8/34 (PR8) in 50 μL of sterile PBS. Uninfected controls were treated with the same volume of sterile PBS. Mice were weighed throughout the experiment to monitor the course of the infection in the animals. Mice that were below 70% of original body weight were euthanized through CO₂ asphyxiation. At 0, 3, 5, 7, 9, 11, 13, 15, and 17 days post infection, infected mice were euthanized through CO₂ asphyxiation. Uninfected mice were sacrificed at the end of the experiment. Left lobes of the lungs were fixed in 10% formalin.

2.3.7 In vivo Immunofluorescent Staining

Four micron sections of formalin-fixed, paraffin-embedded lung tissues were deparaffinized, rehydrated and boiled in antigen retrieval solution (S1700, Dako) for 30
min. After blocking with 3% BSA in Tris-buffered Saline, tissues were incubated overnight at 4°C with either 1:100 anti-γH2AX (Millipore), 1:100 anti-NS1, or 1:100 anti-cleaved caspase 3 (Cell Signaling Technology), in 0.3%BSA in TBS. After washing, sections were incubated for 1 hour with 1:200 Goat anti-Rabbit Alexafluor-488 (A11008, ThermoFisher Scientific) at room temperature. Sections were mounted and counterstained with ProLong Gold Antifade Mountant with DAPI (P36931, ThermoFisher Scientific).

2.3.8 Cleaved Caspase 3 Analysis
Caspase 3 stains were analyzed using the Metafer/RCDetect automated image analysis system as previously described [23]. Briefly, using predefined search parameters a high-resolution camera scanned the entire area of the lung section. The system detected all green fluorescence events that colocalized with DAPI. The proportion of FITC positive nuclei and the estimated total number of nuclei in the scanned area was given.

2.3.9 Mass Spectrometry
Six- to 8 week- old female C57/Bl6 (Jackson Labs) mice were instilled intra-tracheally with PBS or 20 PFU of PR8 influenza virus in PBS. The mice were euthanized at 0, 3, 5, 7, 9, 11, 13, 15, or 17 days following treatment. The lungs were harvested and flash frozen in liquid nitrogen. The tissues were transferred to a 13 ml round tube, mechanically homogenized in lysis buffer and the DNA extracted through centrifugation for genomic DNA isolation (Roche). Antioxidants and deaminase inhibitors were added to the lysis buffer, protein precipitation solution, and water: THU (50 μg/mL), DFO (0.1 mM), BHT (100 μM), and pentostatin (0.2 μg/mL). DNA etheno (ε) adducts 1, N6-Etheno-2’-deoxyadenosine (εdA) and 1, N2-Etheno-2’-deoxyguanosine (εdG), 8-OH-dG,
and hypoxanthine were quantified as described previously [21]. Measurements were on DNA prepared from 3 animals per group, with the following exceptions: 3 dpi was from two samples for εdA, 8-OH-dG, and εdG, 13 dpi was from four samples for εdA, and 0, 5 and 9 dpi for hypoxanthine were from 2 samples.

2.3.10 Statistical Analysis

GraphPad Prism was used for all unpaired and paired Student’s t tests. A p value of less than 0.05 was considered significant.

2.4 Results

2.4.1 Influenza infection induces γH2AX foci in vitro

When DSBs are formed, the H2AX histones within two megabases of the break can become phosphorylated at Serine 139 (γH2AX), which can be detected with antibodies [13]. To test whether influenza infection causes DNA damage in vitro, we incubated Madin Darby Canine Kidney (MDCK) cells, cells known to be able to propagate influenza [24], with H1N1 Puerto Rico/8/A/1934 (PR8) virus at a multiplicity of infection of 1 at 37°C. After one hour, the cells were washed and incubated in media to allow the virus to propagate. At the indicated times, the cells were fixed and analyzed for γH2AX.

Figure 2-1A shows representative images of γH2AX in the cells. Studies have shown that γH2AX can be visualized as punctate foci and that each foci indicates a single DSB. Cells not infected with PR8 virus (NT) show few cells with punctate foci. Cells that were infected for varying times of incubation have higher levels of foci compared to NT cells. γH2AX was quantified in a blinded fashion by determining the number of nuclei with greater than 5 foci per cell (Fig. 2-1B). Our analysis shows that
infected cells display nearly twice as many γH2AX positive cells as NT cells. In addition, three hours after infection, nearly half of all cells are positive of γH2AX. Together, the γH2AX results suggest that influenza can cause DSBs in vitro.

2.4.2 Influenza infection causes physical single and double strand breaks in vitro

While γH2AX is predominantly analyzed as a marker of the response to DSBs, it has previously been shown to occasionally occur in the absence of DNA damage [14, 15, 25]. Furthermore, the kinetics of γH2AX phosphorylation and dephosphorylation has been observed to be markedly slower than the kinetics of physical DSB induction and repair [20]. To analyze whether influenza infection can cause physical SSBs and DSBs and to analyze their kinetics, we used the CometChip under neutral and alkaline conditions. Under neutral conditions, only DSBs are observed in the tail of the comet and are quantified by the length of the comet tail. Alkaline conditions allows increased relaxation of broken DNA around histones and enable the detection of single strand breaks, abasic sites, and alkali labile sites [18] which are quantified by percentage total DNA in the tail.

Figure 2-2A shows our CometChip analysis of MDCK cells infected with PR8 under neutral comet conditions. Infected cells had a significant increase in Tail Length at 3 hours post infection when compared to NT cells. The difference between the infected cells 6 and 9 hours post infection and the NT cells were statistically insignificant. Infected cells under alkaline conditions also displayed a significant increase in alkali labile sites 3 hours post infection (Fig. 2-2B). The increase was reduced to baseline at 6 and 9 hours post-infection. Taken together, these results suggest that influenza infection does indeed induce a physical increase in strand breaks, however the
strand breaks are less persistent than the γH2AX signal. The decrease in neutral and alkaline signal in influenza-infected cells could be due to the comparably low sensitivity of the CometChip or due to repair of the damaged DNA.

2.4.3 Influenza infection induces tissue damage and apoptosis after virus clearance

Given that influenza infection can cause DNA damage in vitro, we decided to analyze whether influenza infection can cause DNA damage in mouse models. However, prior to performing DNA damage analysis, we analyzed the course of infection in mice. Anesthetized C57/Bl6 mice were exposed intratracheally to equal volumes of either PBS or 20 PFU of PR8 virus. The morbidity and the recovery of the animals were quantified through daily body weight measurements (Fig. 2-3A). The body weights of mice exposed to PBS remained within 5% of original body weight throughout the course of the experiment. Influenza infected mice displayed a severe drop in body weights and reached a nadir at 9 DPI. By 15 DPI, the influenza infected mice recovered most if not all of their body weight. If a mouse weighed less than 70% of its original body weight, it was immediately euthanized to minimize suffering.

To analyze virus levels in influenza-infected mice, we performed immunohistochemical analysis for the viral protein Nonstructural protein 1 (NS1). Infected mice displayed an increase in NS1 at 3 DPI that was reduced by 8 DPI suggesting that overall morbidity of animals continues to worsen (Fig. 2-3A) after the virus has been completely cleared (Fig. 2-3B). The immunohistochemical stains in Figure 2-3C support this model. Each image in Figure 2-3C represents the region in that lung with heaviest consolidation. The lungs of mice mock infected with PBS are full of white air pockets for gas exchange. As the infection worsens from 3 DPI to 9 DPI, cells
invade the lung leading to increased consolidation and a reduction in gas exchange. The highest levels of consolidation and tissue damage appears to occur at \(^{\sim}13\) DPI and is cleared by 17 DPI. Therefore, high levels of tissue damage occur in animal lungs far after the virus has cleared from the system. Apoptosis levels in influenza-infected mice were analyzed through antibody detection of the apoptosis signal Cleaved caspase 3 (Fig. 2-3D). We observed significantly high levels of apoptosis at 9 DPI in the infected mice. Taken together, it appears that tissue damage and apoptosis occur after influenza infection has cleared the system.

2.4.4 Base lesions do not accumulate in influenza-infected mice displaying \(\gamma\)H2AX signal

To analyze the levels of DNA damage in influenza infected mice, lung sections of mice were stained for \(\gamma\)H2AX. Figure 2-4A shows the \(\gamma\)H2AX stain of a mouse mock infected with PBS (left) and infected with 20 PFU of PR8 at 9 DPI (right). The infected mice display substantially higher levels of \(\gamma\)H2AX foci compared to the mock treated mice. The high level of DNA damage occurs in the animal after the virus is cleared from the lungs suggesting that the inflammatory response may be involved in the production of DSBs in following influenza infection. Thus, influenza infection can induce DNA DSBs \textit{in vivo}.

In addition to strand breaks, we also analyzed the levels of base lesions during influenza infection. Here we utilized mass spectrometry to measure the levels of four base lesions representing three types of base modifications: 8-oxoguanine, which is formed by oxidation, inosine, which can be produced by deamination, and 1, \(N^\alpha\)-ethenoguanine and 1, \(N^\beta\)-ethenoadenine, which can be generated by DNA reacting with
lipid peroxides. All four of these lesions are mutagenic and have been found to block replication [10].

Figure 2-4B-E show the results of the experiment. There was no significant increase in any of the base lesions in influenza-infected mice. The lack of accumulation of base lesions in the mice suggests that DNA repair is keeping up with the base lesions as they are being formed. As such, one would predict that base lesions would accumulate in DNA repair deficient mice infected with influenza.

2.5 Discussion

Three to five million people in the world each year are afflicted by severe influenza virus infection [1]. One of the main causes of the severity of influenza infection is an overactive inflammatory response [8, 9]. Infiltrating immune cells produce reactive oxygen and nitrogen species (RONS), which can induce DNA damage in surrounding tissues [26]. While influenza has been observed to induce inflammation and inflammation induced by other foreign particles is known to cause DNA damage, the existence of DNA damage during influenza infection has not been analyzed. Furthermore, the possibility of influenza virus to induce DNA damage in the absence of an inflammatory response warrants further investigation. Here we show that influenza virus can induce DNA damage in vitro. In addition, while we did not observe an increase in base lesions in influenza-infected mice, we did see the existence of strand breaks in infected lungs. The strand breaks occurred following viral peak, suggesting that the immune response potentially contributes to DNA damage induction. Taken together, this study suggests that influenza infection can induce DNA damage in cells and tissues and that DNA damage may play a role in the morbidity of influenza infection.
Influenza virus A2/HK/68 has been observed to induce strand breaks in human lymphocytes in vitro [27]. We investigated whether PR8 virus can also directly induce strand breaks in MDCK cells through immunohistochemical staining of the DSB marker γH2AX and through the CometChip assay under alkaline and neutral conditions. The use of the CometChip and γH2AX assay together allows detection of physical strand breaks (through CometChip) and the cell’s response to strand breaks (γH2AX). γH2AX can only be detected if Ser-139 is phosphorylated by phosphatidylinositol 3-kinase-related kinases such as ATM or DNA-PK respond to the DNA damage [28]. In addition, using the CometChip under neutral and alkaline conditions enables discrimination of the damage as DSBs (neutral) or all alkali labile sites such as SSBs, DSBs, and abasic sites (alkaline). In all three methods, we observed an increase in signal three hours post-infection compared to non-treated cells suggesting that influenza infection induces strand breaks and that the breaks induce a DNA damage response. Studies have shown that 1Gy of irradiation can induce strand breaks with the ratio of SSBs to DSBs of ~25:1 [29]; thus, since there is an increase in CometChip signal under both neutral and alkaline conditions, it would suggest that influenza infection leads to both DSBs and SSBs. Furthermore, the increased γH2AX signal indicates that the breaks are eliciting a response from the cell. Some studies have observed the induction of γH2AX without DNA damage [15]. Given that we observe strand breaks and γH2AX in infected cells suggests that γH2AX is being induced by DNA damage in the cell.

The source of the DNA damage in influenza-infected cells warrants further investigation, however numerous studies have observed that infection with RNA viruses, including influenza virus, can induce an increase in reactive oxygen species (ROS) [30-32]. In addition, a recent study has shown that influenza can increase ROS in cells while simultaneously reducing the expression of antioxidants like glutathione and superoxide
dismutase [33]. Therefore, even a small increase in ROS could have damaging effect for the DNA due to the reduced ability to clear the ROS.

The method by which the virus induces an increase in ROS is still unknown, however unpublished studies by Yamada Yoshiyuki show that γH2AX can be induced by transfecting cells with plasmids containing individual viral RNA segments. The segments for PB1 and PB1-F2, RNA polymerase proteins for replicating the viral genome [34], generated the strongest increase in γH2AX signal. PB1-F2 is known to permeabilize mitochondrial membranes of host cells [35, 36], and therefore may be the cause of the increase of ROS in the cell. Another potential cause of the ROS increase is a stress response induced by the presence of viral RNA in the cell. Taken together, our current model suggests that either the presence of viral RNA or PB1-F2 expression induces an upregulation in ROS production, which elicits DNA damage in infected cells.

Analysis of strand breaks at six and nine hours post-infection through CometChip indicates a reduction of strand breaks to baseline while analysis through γH2AX shows a consistently higher level of strand breaks when compared to NT. While higher throughput and more quantitative, the comet assay has been found to be less sensitive to DSBs, when compared to γH2AX analysis [37]. Studies have shown that a cell needs ~50 DSBs to produce a signal through the neutral comet assay [16], while each γH2AX focus has been found to correspond to a single DSB [38]. In addition, analysis of MDCK cells irradiated with 50 Gy displayed only double the tail length of influenza-infected cells three hours post infection. One would predict a high irradiation dose such as 50 Gy to cause a markedly higher increase in neutral comet assay signal. Thus, the discrepancy in results may be due to variations in assay sensitivity.

Through both assays, a decrease in signal from three to nine hours post infection is observed. We hypothesize that the reduction in signal is due to influenza-induced apoptosis, which has been observed to occur in culture. Studies have shown that γH2AX
foci exist during the early stages of apoptosis and are subsequently reduced over time [39, 40]. Thus the lower levels of γH2AX foci at 9 and 12 hours are likely due to the cells proceeding in apoptosis. In addition, neutral comet assay would show apoptosis-induced decreased signal due to its natural limitations. Apoptosis causes DNA fragmentation and studies have shown that when analyzed through the neutral comet assay, influenza-infected cells undergoing apoptosis produce oddly shaped comets [41]. Instead of the broken DNA flowing from the head of the comet in a straight line (as most comets do), influenza-infected cells going through apoptosis display a small head with a blooming cloud of DNA fragments behind it. The non-standard shape of the comet makes it more difficult to accurately analyze the tail length through the computational program. Thus, the decrease in signal at six and nine hours post infection, may be due to some of the cells initiating the process of apoptosis eliciting an inaccurate reduced signal.

Approximately nine days post infection appears to be the pathophysiologically worst time period for mice following sublethal influenza infection. At 9 DPI, mice are at their lowest body weight, have very high levels of tissue damage, and display peak apoptosis levels. In addition, we and others have found that severely infected animals also display ruffled fur and experience dyspnea when very sick [42]. Interestingly, at 9 DPI the majority of the virus has cleared the system supporting previous studies, which have also found that peak inflammation occurs after viral peak [43]. The worsening of the infection after viral clearance is likely due to immune cell infiltration. Studies have shown that influenza induces an overactive inflammatory response in many models. Na Li has also seen the highest levels of immune cell infiltration and oxidative stress after viral clearance suggesting that the immune cells infiltrate the lung to clear the virus and subsequently linger while producing RONS [43]. Studies have shown that after treating influenza-infected animals with antioxidants to reduce RONS in the system, mice
display increased survival and decreased morbidity [8, 9]. Thus, it is likely that the high levels of morbidity following viral peak is caused by the immune response to the infection.

We also propose that the overactive inflammatory response to influenza infection induces DNA damage during influenza infection. We observed increased levels of γH2AX in influenza-infected mice, which complement work performed by Na Li. She has observed a significant increase γH2AX signal from 7 to 17 DPI in influenza infected lungs and has detected signal in the lung parenchyma, infiltrating T cells and macrophages, and in the bronchoepithelium. The increase in signal coincides with oxidative stress in the mouse lungs suggesting that the RONS produced by the inflammatory cells may cause the DNA damage. Additional studies of the DNA damage levels in antioxidant-treated, influenza-infected mice would help in determining whether the inflammatory response is one of the major inducers of the DNA damage.

Interestingly, while we did observe an increase in strand breaks, we did not see an increase in base lesions during influenza infection. If RONS play a major role in inducing strand breaks, one would predict them to also induce formation of oxidation, deamination and lipid peroxidation-induced lesions. The lack of base lesion accumulation is likely due to the cells efficient DNA repair processes. Studies by the Samson Laboratory have shown that chronic inflammation in colon can produce a significantly higher increase in base lesions in a DNA repair deficient background than in a wild type background [26], suggesting that the inability of cells to repair lesions leads to an observable accumulation of lesions. Thus, while there was no significant increase in DNA repair lesions in wild type infected mice, infection of DNA repair deficient mice would potentially lead to a detectable increase in base lesions.

Here we have shown that influenza infection can cause DNA damage in both cells and mice and that influenza infection leads to markedly increased morbidity after viral
clearance suggesting an overactive inflammatory response. The knowledge gained from this study suggests a model wherein influenza infection leads to an increase in DNA damage, which is exacerbated by infiltrating inflammatory cells. Given that DNA damage occurs in the context of influenza infection, it would be interesting to determine how DNA damage affects morbidity and mortality. Perhaps an alteration in DNA repair capacity would affect the severity of influenza infection. Additional studies must be performed to determine the role of DNA repair during influenza infection. If DNA damage and repair does affect morbidity of infection, it would suggest that these factors may be a cause of inter-individual variation in response to influenza infection.
2.6 References


Fig. 2-1: Influenza infection leads to an increase in γH2AX foci in vitro
(A) Representative images of untreated (left, NT) and influenza infected (right) MDCK cells at 3 hours post infection (hpi) stained for γH2AX (green) and DAPI (blue). Cells were infected with PR8 virus at an MOI of 1 (B) Quantification of percent γH2AX positive MDCK cells infected with PR8 or NT. Positive cells contained > 5 γH2AX foci/nucleus (For statistical analysis, n = 3 independent experiments; data points represent mean ± SEM; *p < 0.05 for paired Student’s t test)
Fig. 2-2: Influenza infection leads to an increase in strand breaks
Neutral (A) and Alkaline (B) CometChip analysis of MDCK cells not treated with PR8 virus (NT) or infected with an MOI 1 of PR8 at 3, 6, and 9 hours post infection. (For statistical analysis, n = 3 independent experiments; data points represent mean ± SEM; *p < 0.05 for paired Student’s t test)
Fig. 2-3: Characterization of influenza infection in C57/Bl6 Mice

(A) Body weight analysis mice exposed to PBS (blue) or 20 PFU of PR8 (red). $n \geq 5$ individual mice. (B) Representative images of PBS exposed (mock) or PR8 infected wild type lungs stained for NS1 (green) and DAPI (blue). (For statistical analysis, $n = 3$ mice; data points represent mean ± SEM; *$p < 0.05$ for paired Student’s $t$ test) (C) Representative H&E images of the most infiltrated regions of the lungs of PBS exposed (mock) or PR8 infected wild type lungs at 3, 9, 13, and 17 DPI. (D) Analysis of cleaved caspase 3 staining in PBS exposed (mock) or PR8 infected wild type lung. (For statistical analysis, $n = 3$ mice; data points represent mean ± SEM; *$p < 0.05$ for unpaired Student’s $t$ test)
Fig. 2-4: Characterization of DNA damage during influenza infection in C57/Bl6 Mice

(A) Representative images of PBS exposed (mock) or PR8 infected wild type lungs (9 DPI) stained for γH2AX (green) and DAPI (blue). (B-E) Analysis of base lesions, 8-oxoguanine (8-OH-dG, B), inosine (dI, C), 1, N²-ethenoguanine (εdG, D) and 1, N⁶-ethenoadenine (εdA, E) by mass spectrometry. For statistical analysis, n = 3 mice; data points represent mean ± SEM; *p < 0.05 for unpaired Student’s t test)
Chapter 3

Systems Analysis of DNA Repair
During Influenza Infection

3.1 Abstract

Influenza, a prevalent RNA virus that infects the respiratory tract, induces a strong inflammatory response accompanied by excessive production of reactive oxygen and nitrogen species (RONS). Previously, we have shown that both influenza infection and associated inflammatory responses coincide with DNA damage in the lungs of mice. However, studies of the roles of DNA repair pathways following influenza infection are lacking. Here, we applied gene set enrichment analysis (GSEA) and immunohistochemical techniques to study the expression of DNA repair genes and proteins following influenza infection. Through traditional GSEA and single sample GSEA methods, we observed a significant increase in the expression of genes in multiple DNA repair pathways during the recovery stage of infection, including Homologous Recombination (HR), which had the most significant q value of all the DNA repair gene sets investigated. The observed enrichment of certain DNA repair gene sets was conserved among mice of various backgrounds infected with several different influenza virus strains. Additionally, immunohistochemistry shows that the HR protein RAD51
was significantly increased in infected mice. We also found that cells deficient in the HR gene Brca2 compared to WT cells, have increased sensitivity to the inflammatory RONS, peroxynitrite and hydrogen peroxide. Finally, to further investigate the role of DNA repair during influenza infection in vivo, we studied the impact of influenza on mice deficient in the DNA repair proteins AAG, ALKBH2, and ALKBH3, which have been previously shown to have reduced survival under inflammatory conditions. The DNA repair deficient mice took longer to recover from influenza infection compared to WT mice and had persistently increased levels of DNA double strand breaks as detected through γH2AX and increased levels of RAD51. Taken together, these studies point to a role for DNA repair, and in particular HR, in recovery from influenza infection.

3.2 Introduction

Influenza A is in the orthomyxoviridae family of single stranded RNA viruses. Influenza affects approximately 1 billion people in the world each year and causes high morbidity and mortality in children, the elderly and the immunocompromised [1]. One of the driving forces of the morbidity is an overactive immune response to the virus. Mice [2], macaques [3], and humans [4] have all been found to have elevated levels of inflammatory cells and proinflammatory cytokines following infection with highly pathogenic virus strains. While infiltrating neutrophils and macrophages produce reactive oxygen and nitrogen species (RONS) to combat pathogens, RONS can also cause great harm by inducing collateral damage to surrounding tissues [5, 6]. RONS derived from nitric oxide and/or superoxide, including hydrogen peroxide and peroxynitrite, can directly induce DNA base modifications, such as 8-oxoguanine, and can indirectly produce lesions such as 1-\(N^6\)-ethenoadenine through lipid peroxidation [7].
RONS can also lead to DNA double strand breaks (DSBs), one of the most toxic forms of DNA damage [8].

DSBs are repaired predominantly by two pathways, non-homologous end joining (NHEJ) and homologous recombination (HR). HR is primarily active during the S/G2 phase when cell division occurs [9]. During cell division, replication forks can breakdown upon encountering a blocking lesion or a strand break, leading to a DSB [10]. HR is the main pathway for fixing these broken replication forks, due to the need for homology searching in order to align sequences to repair the broken fork [11].

In order to participate in DNA repair, HR proteins must localize to the sites of DSBs. The process of HR repair protein localization is initiated by a phosphorylation event at the Ser-139 of the H2AX histone near the site of the break (γH2AX) [12]. Mediator of DNA damage checkpoint protein 1 (MDC1) binds to γH2AX, enabling recruitment of BRCA1 and the Mre11/Rad50/Nbs1 (MRN) complex, which promotes the resection of DNA to generate a 3’ single-strand overhang [13, 14]. BRCA2 brings RAD51 to the damage and loads it onto the single-stranded DNA to form the nucleoprotein filament [15], which searches for homology and performs strand invasion [16]. Following strand invasion, the resulting Holliday Junction is cleaved and the replication fork is restored, thus preventing cytotoxicity [17].

Previously, we have shown that influenza infection induces an increase in the levels of DNA double strand breaks (DSBs) (analyzed by visualization of γH2AX foci) [18]. The level of γH2AX foci increases and persists, in some cases for days after the virus has been cleared from the system. During the recovery phase of infection, γH2AX foci were found to occur concurrently with cell division and oxidative stress in the lungs [18], suggesting that inflammation may be a source of the DNA damage in replicating cells. In addition, during similar periods of oxidative stress in the lungs, we observed an increase in RAD51 protein levels via Western Blot analysis [18]. While these results
point to a possible role for DNA repair, an unbiased analysis of gene expression for major DNA repair pathways in the context of influenza infection, and a deeper analysis of HR during influenza infection, have yet to be performed.

To analyze the effects of a disease on multiple pathways and processes in an unbiased fashion we used gene set enrichment analysis (GSEA) [19]. GSEA is a robust method that extends gene expression data beyond differential expression of individual genes to the analysis of gene sets or pathways that are enriched in a particular phenotype [20]. Experiments performed in cells \textit{in vitro} [21] or with assorted virus strains [22] in mice of various backgrounds [23] can lead to erratic results in terms of the pathogenesis of influenza infection. In addition, transcription data from distinct laboratories utilizing various microarray platforms can lead inconsistent conclusions. A GSEA meta-analysis of transcriptome data of influenza-infected mice employing multiple viruses and mouse strains enabled robust identification of pathways that are enriched in infected animals [24, 25].

Here we combined computational, animal, and cellular studies to analyze the role of DNA repair following influenza infection. Through GSEA, we saw that influenza-infected mice display altered gene expression of multiple DNA repair pathways, including HR. We found that HR protein levels are increased in infected mice, and that cells deficient in the HR protein BRCA2 have increased sensitivity to inflammatory chemicals. Finally, we discovered that mice deficient in the base excision repair gene, \textit{Aag}, and the direct reversal genes, \textit{Alkbh2} and \textit{Alkbh3} have delayed recovery from influenza infection, display persistent DSBs, and have increased expression of HR proteins. Taken together, these findings shed light on the role of DNA repair, and in particular HR, during influenza infection and suggest that impaired DNA repair capacity could be a modulator of influenza-induced morbidity.
3.3 Results

3.3.1 Homologous Recombination and Mismatch Repair KEGG gene sets are significantly enriched in influenza infected mouse lungs

To query the effects of influenza infection on gene expression, we analyzed transcriptome data of influenza-infected mice from two previously published studies (Table 3-1). The first study, published by our laboratory [26], contains data from BALB/c mice infected with 0.4 LD_{50} of highly pathogenic H1N1 virus A/Puerto Rico/8/34 (PR8) (Cohort #1). The other study, published by the Germain Lab [22], contains data of C57Bl/6 mice infected with 0.2 LD_{50} PR8 (Cohort #2), 0.6 LD_{50} PR8 (Cohort #3), or 10^{6} PFU of seasonal H1N1 virus A/Texas/36/91 (Tx91) (Cohort #4). We used all four previously published cohorts for our gene expression analyses. Phenotypic analysis of the cohorts is published elsewhere [22, 26].

We utilized Gene Set Enrichment Analysis (GSEA) to analyze the 186 gene sets of the Kyoto Encyclopedia of Gene and Genomes (KEGG) database [27] to reveal the gene sets that were enriched in the influenza infected animals compared to mock infected animals at Day 10 and Day 15 post infection. Table 3-2 shows the 27 gene sets that were enriched at a False Discovery Rate (FDR) q value of less than 0.05 in all four cohorts, and these are ranked by their average FDR q value. While many of the top gene sets are related to host defense (e.g., Natural Killer Cell Mediated Cytotoxicity, Cytokine-Cytokine Receptor Interaction, T Cell Receptor Signaling Pathway), two DNA repair gene sets, Homologous Recombination and Mismatch Repair, were significantly enriched as well. In addition, the Cell Cycle and DNA Replication gene sets were also enriched in the lungs of infected animals. It is important to note that for some gene sets, there is an enrichment of genes that are present in multiple gene sets. For example,
Polδ1 and Pcnα are present in and increase the GSEA score of multiple gene sets, including the Cell Cycle and DNA Replication gene sets. Therefore, high GSEA signals can be caused by an enrichment of both pathway specific and nonspecific genes.

### 3.3.2 Multiple DNA repair gene sets are enriched during the recovery phase of infection

To further analyze the role of DNA repair during influenza infection, we performed single sample gene set enrichment analysis (ssGSEA) on the five major DNA repair gene sets in the KEGG database: Base Excision Repair gene set (BER), Homologous Recombination gene set (HR), Mismatch Repair gene set (MMR), Nucleotide Excision Repair gene set (NER), and Non-homologous End Joining gene set (NHEJ) (Fig. 3-1 and 2). The ssGSEA approach allows the comparison of multiple cohorts by generating a GSEA score for each subject (animal) [28]. Using ssGSEA, we observed that PR8 infected BALB/c mice at 15 days post infection (dpi) had significantly higher BER, HR, MMR, NHEJ, and NER ssGSEA scores (Fig. 3-1) compared to mock treated animals at Day 15. In addition, the 15 dpi infected animals had higher BER, HR, MMR, and NER ssGSEA scores than mock treated and infected animals at 7 dpi (Fig. 3-1A-C and E).

We also analyzed the DNA repair ssGSEA scores of C57/Bl6 mice infected with seasonal T991 virus and two doses of pathogenic PR8 virus. Similar to the BALB/c mice, the C57/Bl6 mice infected with PR8 displayed higher ssGSEA scores at 10 dpi compared to uninfected animals at that time point in all five DNA repair pathways (Fig. 3-2A and B). In addition, these infected cohorts at 10 dpi display a significantly higher BER, HR, MMR, and NHEJ ssGSEA score when compared to uninfected and infected cohorts at 3 dpi. Interestingly, C57/Bl6 mice exposed to seasonal T991 virus not only displayed a significant increase in BER, HR, and MMR ssGSEA values at 10
dpi, but also at 3 dpi when compared to the mock treated controls at their specific time points (Fig. 3-2C). In addition, the NHEJ ssGSEA scores of the seasonally infected mice were only found to be significantly higher than the mock infected controls at 3 dpi. These results suggest that for BER, HR, MMR, and NHEJ transcription may be induced at an earlier time point during seasonal infection when compared to pathogenic infections. Intriguingly, while there was little to no differential effect in the NER ssGSEA score between the mock infected and the seasonal infected mice at any time point, infected mice at 3 dpi displayed a significantly higher signal than infected mice at 10 dpi, suggesting that NER transcription may also be induced at an earlier time point compared to pathogenic infections. Taken together, the ssGSEA analysis of influenza infected mice suggests that there is a difference in gene expression for the BER, HR, and MMR gene sets between mock infected and infected mice during the recovery phase from influenza infection, in multiple virus and mouse strains; differential gene expression in the NHEJ and NER gene sets only occurs during the recovery phase following pathogenic infections.

3.3.3 HR GSEA signal in influenza-infected mice is induced by pathway-specific genes

The genes of a particular gene set that have the highest signal and account for a significant impact on enrichment in an experimental cohort are called the leading-edge subset genes (Fig. 3-3) [19]. To further probe DNA repair genes that contribute to the high HR and MMR GSEA values in influenza infected mice (Table 3-2), we analyzed the leading-edge subset genes. Table 3-3 shows the leading-edge subset genes in the HR and MMR gene sets that occur in all four cohorts in infected animals at either 10 or 15 dpi. The top 4 leading-edge subset genes of the 28-member HR gene set (Rad51, Rad54,
Blm, Brca2) are specific to the HR pathway. Rad54l and Blm solely appear in the HR gene set and are not present in the other 185 gene sets in the KEGG database. Rad51 and Brca2 are found in the HR gene set as well as two disease-specific gene sets (Pathways in Cancer and Pancreatic Cancer), which is consistent with the known role of HR in cancer, and in particular, for pancreatic cancer [29, 30]. Conversely, many of the leading-edge subset MMR genes are common to multiple pathways (Lig1, Rfc3, Rfc4, Rfc5). For example, Lig1, the second highest ranked gene in the 24 member MMR pathway, is common to the BER, NER, and DNA Replication gene sets in the KEGG database. In addition, many MMR-specific genes (Mlh1, Mlh2, Msh3) have a much lower ranking in the gene rank list (Table 3-4). The minimal effect of influenza infection on MMR-specific genes suggests that the high GSEA score for the MMR pathway is mainly due to a few significantly enriched common genes and not due to a specific induction of MMR. Conversely, the pathway specificity of HR leading-edge subset genes suggests that HR pathway is specifically upregulated in infected animals.

In addition, we analyzed the leading-edge subset genes of the three remaining DNA repair gene sets in the KEGG database, BER, NHEJ, and NER. Many of the leading-edge subset genes (Table 3-5) in the BER gene set are not pathway specific. However, the existence of BER-specific genes Neil3, a bifunctional glycosylase known to excise oxidative lesions [31, 32] and Parp1, a single strand break sensor thought to initiate the recruitment of BER enzymes [33, 34], is very interesting and warrants further investigation. While the genes in NHEJ gene set are few in number and have a much lower rank compared to the genes in the HR and MMR pathways, the leading-edge subset does contain Xrcc6 and Dclre1c, two genes that code for the vital NHEJ proteins Ku80 and ARTEMIS, respectively, suggesting possible involvement of NHEJ. Finally, none of the leading-edge subset genes in the NER gene set are pathway specific. Many of them occur in multiple pathways including DNA Replication and Cell Cycle.
Taken together, the aforementioned results suggest that due to its significant FDR q-value (Table 3-2) and the pathway specificity of its leading-edge subset genes, there is strong evidence for a role for HR during influenza infection in animals. Further, although the impact is less apparent, it will be interesting to learn more about the role of BER and NHEJ genes in the context of influenza infection. In contrast, there is little evidence for the involvement of NER and MMR.

### 3.3.4 Influenza infection leads to increased RAD51 protein levels in mouse lungs

Gene expression has been found to have only ~12-40% correlation with protein expression [35, 36]. To further determine whether the observed increase in HR gene expression correlates with protein levels, we analyzed lung tissues from the BALB/c mice infected with 0.4 LD$_{50}$ PR8 by performing immunohistochemistry using antibodies against RAD51, an integral protein of HR and the highest ranked gene in the HR gene set in infected animals (Table 3-4) [37]. The lung of a mouse exposed to bleomycin, an agent known to induce DSBs that are repaired by HR [38, 39], showed an increase in RAD51 24 hours after exposure (Fig. 3-4A). Influenza infected animals also showed higher levels of RAD51 when compared to mock treated animals (Fig. 3-4B). Indeed, the level of RAD51 in infected mice was similar to that observed in the lung of the bleomycin-exposed mouse (Fig. 3-4C). Quantification of RAD51 showed significantly higher levels in infected mice when compared to uninfected mice (Fig. 3-4C), consistent with HR protein expression being increased during influenza infection and playing a role following influenza infection.
3.3.5 Cells deficient in BRCA2 have increased susceptibility to the inflammatory chemicals hydrogen peroxide and peroxynitrite

Previous studies have shown that high levels of inflammatory RONS occur during the late stages of influenza infection and that RONS drive the morbidity of the infection at these time points [40, 41]. Given the observed increase in HR gene and protein expression levels in the lungs of infected mice at 15 dpi, we set out to determine the effect of an inflammatory environment on cells deficient in HR. Due to the embryonic lethality of many HR deficiencies [42-44], we performed the experiment *in vitro* with BRCA2 deficient and wild type Chinese Hamster Lung Fibroblasts, VC8 and V79. BRCA2 is necessary for loading RAD51 onto the single-stranded DNA for HR. Previously, VC8 cells challenged with cross-linking agents have been found to have a lower ability to perform conservative RAD51-dependent HR, and to have a lower cell viability following challenge with cross-linking agents [42, 45].

To analyze whether HR plays a role in modulating cytotoxicity in an inflammatory environment, cells were exposed to three different inflammatory chemicals: peroxynitrite (ONOO-, which is created by SIN-1), hydrogen peroxide (H$_2$O$_2$), and hypochlorous acid (HOCl). SIN-1 produces superoxide and nitric oxide, which react to form the oxidizing and nitrating chemical peroxynitrite. Peroxynitrite exposure can lead to lesions such as 8-nitroguanine, and is generated quickly in inflammatory environments by activated macrophages producing superoxide and nitric oxide [7]. H$_2$O$_2$ exposure is a commonly used model of oxidative stress *in vitro* and leads to oxidative DNA base lesions (e.g. 8-oxoguanine) and strand breaks in regions of high inflammation [46]. HOCl is a powerful oxidizing and chlorinating agent that produces chlorinated lesions (e.g. 5-chlorocytosine) in DNA [47]. It is generated during inflammation by the reaction between hydrogen
peroxide with chloride ions, catalyzed by the neutrophil-secreted enzyme, myeloperoxidase [48]. After culturing in media for 72 hours at 37°C and 5% CO₂, samples were analyzed using the XTT assay, a tetrazolium-based growth assay [49]. BRCA2 deficient cells had increased sensitivity to SIN-1 at 0.5 mM (Fig. 3-5A). Upon exposure to higher doses of SIN-1 known to be toxic in WT cells [50], both cell lines demonstrated significantly reduced cell growth when compared to non-treated cells. Hydrogen peroxide induced significantly more growth inhibition in BRCA2 deficient versus wild type cells at 25 and 50 μM (Fig. 3-5B). In contrast, hypochlorous acid did not induce a differential effect in cell growth between the two cell lines (Fig. 3-5C). The sensitivity of BRCA2 deficient cells to H₂O₂ is consistent with previous literature [51], while the effects of SIN-1 and HOCl exposure on HR deficient cells are novel. Together our data demonstrate that inflammatory chemical exposure can lead to enhanced cytotoxicity in HR deficient cells.

3.3.6 Aag/Alkbh2/Alkbh3 null mice display delayed recovery from influenza infection

When the replication machinery encounters a blocking lesion and the replication fork collapses, homologous recombination can repair the broken fork [11, 52]. Inflammatory environments have been shown to have increased levels of blocking lesions and a high frequency of broken replication forks [6, 50, 53]. One such blocking lesion previously shown to exist in inflammatory regions is 1,N⁶-ethenoadenine (εA) [6, 54]. During inflammation, RONS can react with fatty acids to generate aldehydes, which can damage DNA producing etheno lesions such as εA [55]. In systems with high levels of εA, one would predict an increase in replication fork breakdown and higher demand on HR. To study the impact of unrepaired εA during influenza, we infected a mouse strain
known to be deficient in εA repair, namely, \textit{Aag/Alkbh2/Alkbh3} null mice (triple knock out or TKO) [56]. TKO mice are deficient for \textit{Aag}, a monofunctional glycosylase in the base excision repair pathway that can excise many oxidative and alkylation damages, including εA [6], and \textit{Alkbh2} and \textit{Alkbh3}, primarily repairs alkylation damage, and in particular, etheno adducts via direct reversal (Fig. 3-6A) [54, 57]. Through a deficiency in all three enzymes, the TKO mice have a significant reduction in the ability to repair εA (Fig. 3-6A) [54, 58]. Previously, TKO mice have been shown to be very sensitive to acute colonic inflammation [56]. Here, we infected the WT and TKO mice with 0.4 LD_{50} of PR8 strain of influenza and tracked their body weights over the course of the infection (Fig. 3-6B). Both strains of mice displayed similar weight loss until 10 dpi. Thereafter, the TKO mice displayed a slower recovery in body weight compared to WT mice. TKO mice had significantly lower body weight than WT on 16-19 dpi and did not reach 100% body weight until 30 dpi, four days slower than that for WT mice.

To analyze whether influenza infection had effects on the lung tissue of TKO mice, we performed immunohistochemistry on mouse lungs to measure tissue damage, as determined by density of infiltrating cells (analyzed via H&E), and apoptosis (as determined by TUNEL analysis). We also performed immunohistochemistry for γH2AX and RAD51. There was no significant difference in tissue damage or apoptosis levels between the two strains (Fig. 3-7) and, consistent with our previous work, both strains showed an increase in γH2AX signal at 9 dpi when compared to mock treated animals (Fig. 3-6C). However, the WT mice had a significant reduction in γH2AX from 9 to 15 dpi, while TKO mice displayed minimal reduction in γH2AX levels over the same time period. One model for the steady levels of γH2AX in TKO mice is that the TKO mice have more replication blocking lesions than WT mice, leading to an increase in DSBs from replication fork breakdown. Analysis of RAD51 levels in infected animals displayed a higher level in TKO mice compared to WT mice at 15 dpi (Fig. 3-6D). Thus, infecting
TKO mice with influenza leads to slower recovery, persistent DSBs, and higher levels of HR-related proteins.

3.4 Discussion

Influenza infection is a prevalent disease causing ~500,000 deaths each year [59]. While influenza has been shown to induce oxidative stress and DNA damage inside cells and tissues [18, 60], the role of DNA repair during influenza infection is less well understood. Through GSEA, we show here that mice infected with influenza virus have an increase in the gene expression for several DNA repair pathways, most significantly HR during the regenerative phase of the infection. Immunofluorescent analysis of the HR protein RAD51 demonstrated that HR protein levels are also increased in infected lungs compared to uninfected lungs. Consistent with a model that HR is necessary for cell survival in inflammatory conditions, lung fibroblast cells deficient in BRCA2 had reduced viability compared to WT cells when challenged with the inflammatory chemicals, peroxynitrite from SIN-1 and hydrogen peroxide in vitro. Finally, when mice deficient in Aag, Alkbh2, and Alkbh3 were infected with influenza, we found them to have significantly slower body weight recovery than WT, persistently high levels of DNA double strand breaks (as determined through γH2AX quantification) and increased RAD51 levels. These studies are consistent with our previous work [18] demonstrating that DNA damage is induced during infection.

Inflammation-induced cell proliferation has previously been shown to lead to DNA damage and homologous recombination activity (e.g., [30, 61, 62]). During S phase, the replication machinery can encounter blocking lesions and single strand breaks, leading to broken replication forks that are repaired by HR [9]. Here, we observed a significant increase in the expression of the gene sets for DNA Replication
and Cell Cycle, which is consistent with our prior studies showing increased PCNA expression during the recovery phase of the infection [26]. In addition, previous studies have shown an increase in DNA damage during the recovery phase of the infection, concurrent with high levels of oxidative stress and inflammation in lung tissues [18]. Thus, our observations of increased HR gene expression and increased HR protein levels in influenza infected mouse lungs compared to uninfected lungs support a model wherein inflammation-induced cell proliferation leads to increased HR.

Interestingly, although we observed a significant enrichment of the KEGG Mismatch Repair gene set (MMR) in infected animals, genes central to the MMR pathway are not enriched in influenza-infected mice. The six of the seven leading-edge subset genes that contributed to the high GSEA score are common to multiple gene sets including DNA Replication, Base Excision Repair, and Nucleotide Excision Repair. Furthermore, the leading-edge subset genes did not include MMR specific genes, such as Mlh1, Mlh2, Msh2, and Msh3. In comparison, four of the six leading-edge subset HR genes are specific to the HR pathway (Rad51, Brca2, Rad54l, and Blm) and the two remaining leading-edge subset genes (Mre11a and Polδ1) overlapped with another gene set for additional cellular processes. Taken together, our GSEA data suggest that the observed KEGG HR GSEA signal is specifically due to HR gene expression, while the observed KEGG MMR GSEA signal is due to commonly enriched genes.

The ssGSEA signals for KEGG BER and NHEJ gene sets suggest that these repair pathways may also play a role during influenza infection. Given the observed increase in DSBs and oxidative stress during the recovery phase of influenza infection [18], one would hypothesize that genes for pathways repairing oxidative damage (BER) and DSBs (NHEJ) to be upregulated. However, their insignificant GSEA scores suggest that the role of BER and NHEJ may be reduced compared to HR. In addition, the result that all of the leading-edge subset genes of the KEGG NER gene set are common
to multiple other pathways suggests that influenza infection and its inflammatory response is not specifically inducing NER genes. NER is the primary pathway for repairing bulky lesions, which are not common during inflammation. Thus, one would not expect NER to play a role following influenza infection.

Through immunohistochemical techniques, one can detect specific cell types in fixed tissue sections. As such, we stained our tissue sections with antibodies specific to Clara cells and alveolar epithelial type II cells in the lung parenchyma and did not detect colocalization with RAD51 (data not shown). One possibility is that the RAD51 positive cells are other cells in the lung including endothelial cells and alveolar epithelial type I cells, but it is also possible that the RAD51 positive cells are immune cells. Influenza infected mouse lungs have a nearly four-fold increase in cell number following infection, with the majority of those cells being leukocytes [63]. Furthermore, we previously observed DSBs in the macrophages, neutrophils, and T cells of infected mouse lungs [18]. The observed RAD51 protein signal is likely not due to VDJ recombination in the immune cells, since V(D)J recombination only occurs in lymphoid tissues such as in the bone marrow, lymph nodes, and thymus [64, 65]. Thus, the data suggests that following influenza infection immune cells are being damaged by the inflammatory milieu they generated and are potentially using HR to tolerate the damage. It would be very interesting for the immune cells to repair the DNA damage rather than succumb to it, given that a recent study has found that immune cells typically only survive in inflammatory environments for two hours [66]. The effect of DNA damage and repair on immune cells is worthy of further study.

Previous in vitro studies have shown that RAD51 does not increase in expression, but rather relocates in cells into punctate foci, e.g. [67-71]. However, there have been multiple in vivo studies demonstrating through protein level analysis that there is an increase in RAD51 expression following irradiation [72], asthma-induced inflammation
and influenza infection [18]. In addition, it is quite interesting that multiple studies, including studies analyzing RAD51 expression in human tumors, show pan-nuclear staining in tissues [74-77]. These results indicate that it is either quite difficult to achieve high enough resolution to observe discrete foci in the context of intact tissues or that there is such a high level of RAD51 in cells, that the fluorescent signal overtales the entire nucleus. The RAD51 immunofluorescent stains shown here support our previous data [18] indicating an increase in RAD51 expression following influenza infection and suggest that RAD51 does not produce foci in tissues.

Deficiency in BRCA2 has previously been found to be sensitizing to multiple damaging agents including irradiation [78] and DNA crosslinking agents [42]. In our in vitro model system of inflammation, we revealed the importance of BRCA2 for cell survival under inflammatory conditions in vitro. Specifically, Chinese hamster lung fibroblasts deficient in BRCA2 showed significantly less cell viability than WT cells following exposure to ONOO⁻ (from SIN-1) and H₂O₂, supporting previous studies and suggesting that HR plays a role in protecting cells from the cytotoxic effects of inflammatory chemicals [51]. Interestingly, hypochlorous acid (HOCl) did not lead to a differential effect between the viability of WT and BRCA2 deficient cells. The repair mechanism for HOCl and its primary lesion, 5-Cl-Cytosine, has not been thoroughly studied. It has previously been found to weakly block replication in E. coli [79], and the comparable sensitivity of WT and Brca2 deficient cells to HOCl suggests that in mammalian cells 5-CIC is a weak inducer of recombination. Thus, HR is likely not a protective pathway for HOCl exposure. Taken together, our in vitro experiments suggest that BRCA2 and HR are beneficial for survival from oxidizing and nitrating agents released during inflammation, specifically ONOO⁻ and H₂O₂.

Compared to 5-CIC, the repair of 1, N⁶-ethenoadenine (εA) has been well studied. εA is a replication-blocking lesion [80] produced by the reaction of RONS-induced fatty
acid aldehydes with adenine [55]. The εA lesion can be repaired by alkyladenine glycosylase (AAG) through the base excision repair pathway [81] and by ALKBH2 and ALKBH3 through direct reversal (e.g. [54]). Studies have shown that the exposure of animals deficient in Aag, Alkbh2, and Alkbh3 to DSS-induced colonic inflammation leads to a significant reduction in survival compared to WT mice [56]. Thus, we infected TKO animals with influenza to determine if influenza-induced inflammation can lead to higher RAD51 protein levels and a more severe disease process. Our observations show a significant reduction in body weight and a decreased rate of recovery in TKO mice compared to WT mice, which supports a model, wherein DNA repair affects influenza-induced morbidity.

We observed an increased level of double strand breaks in infected TKO mice compared to uninfected mice during the recovery phase (15 dpi), simultaneous with increased levels of RAD51 in TKO mice compared to WT. The result of a reduced rate of recovery and higher levels of RAD51 in in Aag, Alkbh2, and Alkbh3 suggests two models for HR during influenza infection. First, HR may protect host lung tissue from infectious diseases and their inflammatory responses. It may be that the replication machinery in TKO mouse lungs encounters unrepaired, εA, leading to broken replication forks and HR. An alternative model is that HR protects host immune cells from their self-generated RONS. Thus, the TKO mice display increased RAD51 signal compared to WT because TKO immune cells require HR to fix the unrepaired εA. While the RAD51 signal may be associated with the immune cells or with lung tissue, the observed increase in morbidity and delayed recovery of the severely DNA repair deficient TKO mice suggests that DNA repair plays a critical role during influenza infection.

Taken together, the results shown here suggest that DNA repair, and more specifically HR, has increased gene and protein expression following influenza infection.
and potentially plays a role in recovery from the infection. Whether increased HR activity is protecting immune cells or resident lung cells, results from this study suggest that variation in DNA repair capacity could predict the severity of influenza-induced morbidity. There is a large inter-individual variation in the response to influenza infection [82]; these studies suggest that an epidemiological screen of DNA repair capacities and influenza infection severities in diverse populations could aid in determining whether the spectrum of DNA repair capacities in individuals could be one of the causes of the variation in response to influenza. An increased understanding of the potential connection between influenza infection severity and DNA repair capacity in humans would call attention to the value of developing therapeutics to alleviate the severity of influenza infection by mitigating DNA damage through the reduction of influenza-induced inflammation.

3.5 Materials and Methods

3.5.1 Animals

All animal experiment were conducted according to the Guide for the Care and Use of Laboratory Animals, and were approved by the MIT Committee on Animal Care.

3.5.2 Gene Set Enrichment Analysis and Single Sample Gene Set Enrichment Analysis

GSEA was performed utilizing the Kyoto Encyclopedia of Genes and Genomes (KEGG) database of 186 gene sets with the software from the Broad Institute [19]. Previously published array data were analyzed independently [22, 26]. Leading-edge subset genes of enriched gene sets were determined from the generated GSEA results. ssGSEA was performed using the ssGSEA module from the Broad Institute as previously described.
[28]. For data analyses, ssGSEA scores were normalized to a mean of 0 and a variance of 1.

3.5.3 Immunofluorescence

Four micron sections of formalin-fixed, paraffin-embedded lung tissues were deparaffinized, rehydrated and boiled in antigen retrieval solution (S1700, Dako) for 30 min. After blocking with 3% BSA in Tris-buffered Saline, tissues were incubated overnight at 4°C with either 1:100 anti-γH2AX (05-636, Millipore), or 1:100 anti-Rad51 (14961, Proteintech), in 0.3%BSA in TBS. After washing, sections were incubated for 1 hour with 1:200 Goat anti-Rabbit Alexafluor-488 (A11008, ThermoFisher Scientific) at room temperature. Sections were mounted and counterstained with ProLong Gold Antifade Mountant with DAPI (P36931, ThermoFisher Scientific). Bleomycin treated samples were stained and imaged separately from test samples.

3.5.4 RAD51 Analysis

RAD51 Stains were imaged on a Nikon 80i epifluorescent microscope (Nikon) with a CCD camera (CoolSNAP EZ, Photometric) using a 4x objective at a fixed exposure time (3s) under the FITC channel. Multipoint images were captured using an automated stage (ProScann II, Prior Scientific) and NIS Elements software (Nikon) and were stitched automatically. Blinded RAD51 images were quantified utilizing ImageJ software (NIH). After measuring the area of the lung tissues through manually tracing the lung outline, the “Adjust Threshold” function was used to discriminate between RAD51 positive fluorescence and non-specific binding. The stains were subsequently converted to binary images and the number of particles was counted through the “Analyze Particles” function.
3.5.5 Cell Culture

Wild type and BRCA2 mutant Chinese hamster lung fibroblasts (V79 and VC8 respectively) were maintain in Dulbecco’s Modified Eagle Medium (11965, ThermoFisher Scientific) containing 10% fetal bovine serum (S11150, Atlanta Biologicals).

3.5.6 Cell Exposure to SIN-1

V79 and VC8 cells were seeded at a density of 100,000 cells/mL in a 96-well plate in growth media overnight. Triplicate wells were exposed to indicated doses of SIN-1 (BML-CN245, Enzo Life Sciences) in Dulbecco’s PBS for 90 min at 37°C. Every 10 minutes, the plate was exposed to the air and shaken for 5 minutes as described previously [50]. After exposure the cultures were incubated with fresh medium.

3.5.7 Cell Exposure to H₂O₂

V79 and VC8 were seeded at a density of 100,000 cells/mL in a 96-well plate in growth media overnight. Triplicate wells were exposed to indicated doses of H₂O₂ (216763, Sigma Aldrich) in Dulbecco’s PBS (14190, ThermoFisher Scientific) for 60 min at 37°C. After washing with PBS, cultures were incubated with fresh medium.

3.5.8 Cell Exposure to HOCl

V79 and VC8 were seeded at a density of 100,000 cells/mL in a 96-well plate in growth media overnight. Sodium hypochlorite stock solution (425044, Sigma Aldrich) was adjusted to pH 7.4 and the concentration of OCl⁻ was measured spectrophotometrically
at 290 nm with a molar extinction coefficient of 350 M$^{-1}$cm$^{-1}$. The pKa of HOCl is 7.46, thus all references of HOCl in this study represent an equal mixture of HOCl and hypochlorite ion[83]. Triplicate wells were exposed to indicated doses of HOCl in Dulbecco’s PBS for 10 min at 37°C. After washing with PBS, cultures were incubated with fresh medium.

### 3.5.9 XTT Assay

Forty-eight hours after the end of chemical exposure, growth media was replaced with fresh media plus XTT Reagent and Electron Coupling Solution according to kit instructions (9095, Cell Signaling Technology). Plates were incubated for 24 hours at 37°C and then read at 450nm on VersaMax Tunable Microplate Reader (Molecular Devices).

### 3.5.10 Influenza Infection and Body Weight Analysis

Eight-week-old female WT and $Aag^{-/-} Alkbh2^{-/-} Alkbh3^{-/-}$ mice on mixed B6/129 backgrounds were anesthetized with ketamine and xylazine (100mg/kg and 10mg/kg respectively). Upon anesthetization, the mice were intratracheally infected with 20 PFU of H1N1 Influenza A/Puerto Rico/8/34 (PR8) in 50 µL of sterile PBS. Uninfected controls were treated with the same volume of sterile PBS. Mice were weighed throughout the experiment to monitor the course of the infection in the animals. Mice that were below 70% of original body weight were euthanized through CO$_2$ asphyxiation. At 9, 15, and 30 days post infection, infected mice were euthanized through CO$_2$ asphyxiation. Uninfected mice were sacrificed at day 30. Left lobes of the lungs were fixed in 10% formalin.
3.5.11 TUNEL Detection

Four micron section of formalin-fixed, paraffin-embedded lung tissues were deparaffinized, rehydrated, and incubated with 10µg/mL Proteinase K in 100mM Tris pH 8.0 for 30 min at 37°C (25530049, ThermoFisher Scientific). After washing, sections were incubated with TUNEL reaction mixture for 60 minutes at 37°C according the manufacturers’ instructions (11684795910, Roche). Sections were mounted and counterstained with ProLong Gold Antifade Mountant with DAPI (P36931, ThermoFisher Scientific).

3.5.12 γH2AX and TUNEL Analysis

TUNEL and γH2AX stains were analyzed using the Metafer/RCDetect automated image analysis system as previously described [84]. Briefly, using predefined search parameters a high-resolution camera scanned the entire area of the lung section. The system detected all green fluorescence events that colocalized with DAPI. The proportion of FITC positive nuclei and the estimated total number of nuclei in the scanned area was given.

3.5.13 Statistical Analysis

GraphPad Prism was used for all unpaired and paired Student’s t tests. A p value of less than 0.05 was considered significant. For GSEA, a false discovery rate q value of less than 0.05 was considered significant.
3.6 References


32. Svilar D, Goellner EM, Almeida KH, Sobol RW. Base excision repair and lesion-dependent subpathways for repair of oxidative DNA damage. Antioxid Redox Signal.


38. Chen J, Ghorai MK, Kenney G, Stubbe J. Mechanistic studies on bleomycin-mediated DNA damage: multiple binding modes can result in double-stranded DNA


57. Basu AK, Wood ML, Niedernhofer LJ, Ramos LA, Essigmann JM. Mutagenic and genotoxic effects of three vinyl chloride-induced DNA lesions: 1,N6-ethenoadenine,


Table 3-1: Cohorts of influenza infected mice analyzed for GSEA

PFU – Plaque Forming Units; DPI – Days post infection.

<table>
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<th>Cohort</th>
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<th>#3</th>
<th>#4</th>
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<td>GSE42641</td>
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<td>Brandes</td>
<td>Brandes</td>
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<td>GPL13912</td>
<td>GPL6887</td>
<td>GPL6887</td>
<td>GPL6887</td>
</tr>
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<td>Source</td>
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<td><em>Mus musculus</em> C57BL/6</td>
<td><em>Mus musculus</em> C57BL/6</td>
<td><em>Mus musculus</em> C57BL/6</td>
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<td>Virus Strain and Dose</td>
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<td>0.2 LD&lt;sub&gt;50&lt;/sub&gt; PR8</td>
<td>0.6 LD&lt;sub&gt;50&lt;/sub&gt; PR8</td>
<td>10&lt;sup&gt;6&lt;/sup&gt;PFU TX91</td>
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<tr>
<td>Analysis Time Points</td>
<td>7 and 15 DPI</td>
<td>3 and 10 DPI</td>
<td>1, 3, 10 DPI</td>
<td>1, 2, 3, 10 DPI</td>
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<td>Replicates per condition</td>
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Table 3-2 Top KEGG Gene Sets Enriched in Influenza Infected Mice at 10 and 15 dpi

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<th>Gene Set</th>
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<td>Natural Killer Cell Mediated Cytotoxicity</td>
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<td>Cytokine-Cytokine Receptor Interaction</td>
<td>3.73E-05</td>
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<td>T Cell Receptor Signaling Pathway</td>
<td>5.20E-05</td>
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<tr>
<td>Chemokine Signaling Pathway</td>
<td>7.50E-05</td>
</tr>
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<td>Leishmania Infection</td>
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<td>Toll Like Receptor Signaling Pathway</td>
<td>2.08E-04</td>
</tr>
<tr>
<td>Type 1 Diabetes Mellitus</td>
<td>3.99E-04</td>
</tr>
<tr>
<td>Cell Cycle</td>
<td>5.13E-04</td>
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<tr>
<td>B Cell Receptor Signaling Pathway</td>
<td>8.57E-04</td>
</tr>
<tr>
<td>Hematopoietic Cell Lineage</td>
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</tr>
<tr>
<td>Cytosolic DNA Sensing Pathway</td>
<td>1.94E-03</td>
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<td>Cell Adhesion Molecules, CAMS</td>
<td>2.33E-03</td>
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<tr>
<td>Intestinal Immune Network for IgA Production</td>
<td>2.35E-03</td>
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<tr>
<td>Systemic Lupus Erythematosus</td>
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<td>NOD Like Receptor Signaling Pathway</td>
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<td>Primary Immunodeficiency</td>
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<td>Asthma</td>
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<td>Antigen Processing and Presentation</td>
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<td>Homologous Recombination</td>
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<td>Graft versus Host Disease</td>
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<td>ECM Receptor Interaction</td>
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<td>Prion Diseases</td>
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<td>Complement and Coagulation Cascades</td>
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<td>Mismatch Repair</td>
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<td>Progesterone Mediated Oocyte Maturation</td>
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Table 3-3: Ranked Common Leading-Edge Subset Genes of HR and MMR Gene Sets in Influenza Infected Animals

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<th>HR Genes</th>
<th>Average Rank</th>
<th>MMR Genes</th>
<th>Average Rank</th>
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<td>Rad51</td>
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<td>Rad54L</td>
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<td>Lig1</td>
<td>630</td>
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<td>Blm</td>
<td>559</td>
<td>Rfc4</td>
<td>816</td>
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<td>Brca2</td>
<td>665</td>
<td>Rfc5</td>
<td>956</td>
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<td>Mre11a</td>
<td>1353</td>
<td>Rfc3</td>
<td>1104</td>
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<tr>
<td>Polδ1</td>
<td>1448</td>
<td>Polδ1</td>
<td>1448</td>
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<tr>
<td></td>
<td></td>
<td>Pcna</td>
<td>1452</td>
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</tbody>
</table>
Table 3-4: HR and MMR gene sets ordered by average rank of enrichment in influenza infected mice

Genes that are in the leading-edge subset of each gene set are shown in red

<table>
<thead>
<tr>
<th>Homologous Recombination</th>
<th>Mismatch Repair</th>
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</thead>
<tbody>
<tr>
<td><strong>Gene</strong></td>
<td><strong>Average Rank</strong></td>
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<tr>
<td>1. Rad51</td>
<td>124</td>
</tr>
<tr>
<td>2. Rad54l</td>
<td>263</td>
</tr>
<tr>
<td>4. Brca2</td>
<td>665</td>
</tr>
<tr>
<td>5. Mre11a</td>
<td>1353</td>
</tr>
<tr>
<td>6. Polδ1</td>
<td>1448</td>
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<tr>
<td>7. Xrcc3</td>
<td>2352</td>
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<tr>
<td>8. Rad51C</td>
<td>2495</td>
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<td>9. Rpa1</td>
<td>2734</td>
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<td>11. Polδ3</td>
<td>3862</td>
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<td>12. Polδ2</td>
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<td>13. Top3A</td>
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<td>15. Xrcc2</td>
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<td>16. Top3B</td>
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<td>17. Shfm1</td>
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<td>18. Rpa2</td>
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<td>20. Rad50</td>
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<td>21. Rad52</td>
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<td>22. Mus81</td>
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<td>23. Nbn</td>
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Table 3-5: BER, NHEJ, and NER gene sets ordered by average rank of enrichment in influenza infected mice

Genes that are in the leading-edge subset of each gene set are shown in red

<table>
<thead>
<tr>
<th>Base Excision Repair</th>
<th>Non-homologous End Joining</th>
<th>Nucleotide Excision Repair</th>
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<td>2. Pole</td>
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<td>2. Mre11a</td>
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<td>3. Fen1</td>
<td>587</td>
<td>3. Xrcc6</td>
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<tr>
<td>4. Lig1</td>
<td>630</td>
<td>4. Dclre1c</td>
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<td>5. Pole2</td>
<td>1280</td>
<td>5. Nhej1</td>
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Fig. 3-1: Influenza infection leads to an increase in DNA repair gene expression

Normalized ssGSEA analysis of BER (A), HR (B), MMR (C), NHEJ (D), and NER (E) gene sets of BALB/c mice mock infected with PBS (red circles) and infected with 0.4 LD$_{50}$ PR8 virus (blue squares) at 7 and 15 Days Post Infection. (Data in each graph normalized to a mean of 0 and a variance of 1; Each symbol represents one animal; *p < 0.05 for unpaired Student’s t test)
Fig. 3-2: Influenza infection leads to a virus strain-dependent increase in BER, NER and NHEJ gene sets

Normalized ssGSEA analysis of BER, HR, MMR, NHEJ, and NER gene sets of C57/Bl6 mice. Red bars represent mock infected with PBS. Blue bars represent mice infected with 0.2 LD_{50} PR8 (A), 0.6 LD_{50} PR8 (B), or 10^6 PFU Tx91 (C). n=7 mice per time point. dpi - Days Post Infection (Data in each graph normalized to a mean of 0 and a variance of 1; *p < 0.05 for unpaired Student’s t test).
Fig. 3-3: Enrichment plot of HR in Influenza Infected Mice
Example enrichment plot of the KEGG-Homologous Recombination Gene Set analyzed through Gene Set Enrichment Analysis. The GSEA score is calculated by walking down a ranked list of genes from C57/Bl6 mice infected with 0.4 LD50 at 15 DPI (left) to C57/Bl6 mice mock infected with PBS at 15 DPI (right). When encountering a gene in the gene set (vertical black lines), the running score increases. When encountering a gene not included in the gene set, the running score decreases. Dashed line indicates peak enrichment signal. Genes in the bracket that induce the enrichment signal.
Fig. 3-4: Influenza infection leads to an increase in RAD51 protein levels

(A) Representative images of untreated (left) and bleomycin treated mouse lungs one day post exposure stained for RAD51 (green) and DAPI (blue). (B) Representative images of mock (left) and PR8 influenza treated mouse lungs stained for RAD51 (green) and DAPI (blue). (C) Quantification of RAD51 signal over entire lung sections relative to lung area. Mock (n=8), Bleomycin (n=1), 7 and 15 dpi (n=4). dpi – Days Post Infection (For statistical analysis, data points represent mean ± SEM; *p < 0.05 for unpaired Student’s t test)
Fig. 3-5: BRCA2 Deficiency leads to reduced viability after treatment with SIN-1 and H₂O₂
Viability of WT (V79) and BRCA2 Deficient (VC8) cells were analyzed through the XTT assay after treatment with increasing doses of (A) SIN-1, (B) H₂O₂, and (C) HOCl. All values relative to untreated control samples. Each data point represents mean ± SEM for three experiments; *p < 0.05 for paired Student’s t test.
Fig. 3-6: Influenza infection induces delayed recovery, prolonged DNA damage and increased repair in $Aag^{-/-}$,$Alkbh2^{-/-}$,$Alkbh3^{-/-}$ triple knockout (TKO) mice

(A) Substrates for AAG, ALKBH2, and ALKBH3 enzymes that have been shown in vitro or in vivo are the lesions: 3-methyadenine (3MeA); 7-methylguanine (7MeG); hypoxanthine (Hx); 8-oxoguanine (8oxoG); $1,N^2$-ethenoguanine ($1,N^2$-eG); $1,N^6$-ethanoadenine (EA); 1-methylguanine (1MeG); $1,N^6$-ethenoadenine ($\varepsilon$A); 3-methylthymine (3MeT); 1-methyladenine (1MeA); 3-methylcytosine (3MeC). Asterisk shows that AAG is able to bind, but not remove $\varepsilon$C. Lesions in yellow are known to be substrates for bacterial AlkB, but have not been analyzed with regards to ALKBH2 or ALKBH3. $ds =$ double strand; $ss =$ single strand. (Adapted from Calvo et al. 2012) (B) Body weight measurements of WT (red circles) and TKO (blue squares) mice infected with PR8 influenza virus. Each data point represents mean ± SEM for $n > 3$ mice. *$p < 0.05$ for unpaired Student's $t$ test. (C and D) Quantification of (C) $\gamma$H2AX positive cells and (D) RAD51 signal relative to mock in WT and TKO mice mock treated (M)
or influenza infected at 9, 15, and 30 days post infection (DPI). Each data point represents mean ± SEM for $n = 3$-4 mice. *$p < 0.05$ for unpaired student’s $t$ test.
Fig. 3-7: Influenza infection induces minimal difference in tissue damage and apoptosis levels in Aag"/"Alkbh2"/" Alkbh3"/" mice
(A and B) Quantification of (A) tissue damage and (B) TUNEL signal in WT and TKO mice mock treated or influenza infected at 9, 15, and 30 days post infection. Each data point represents mean ± SEM for n = 3-4 mice; *p < 0.05 for unpaired Student’s t test.
Chapter 4

S-Nitrosation Induces Base Excision Repair Imbalance

4.1 Abstract

Nitric oxide, a reactive nitrogen species released by macrophages during inflammation, has been shown to react with DNA, proteins, and cells. Protein S-nitrosation, the process by which nitric oxide reacts with cysteine residues on proteins, has been found to affect DNA repair. However, relatively little is known about the role of S-nitrosation in the repair of alkylation damage through the base excision repair pathway. Here, we analyzed the effects of the transnitrosating protein S-nitrosoglutathione (GSNO) on the repair of methylmethane sulfonate (MMS) induced DNA damage. Through the use of the CometChip, a high-throughput version of the comet assay that measures strand breaks, we observed an AAG-dependent increase in BER intermediates in GSNO-exposed mouse embryonic fibroblasts after MMS challenge. Through the use of the Fluorescence Multiplexed Host Cell Reactivation Assay, a high-throughput assay used to measure DNA repair capacity, GSNO exposure was detected to alter the activities of BER proteins and reduce overall BER capacity. Furthermore, cells exposed to both GSNO and MMS displayed reduced viability. Given that unrepaired BER intermediates
are toxic, these results suggest that the altered activities of BER proteins following GSNO exposure induces an increase in BER intermediates that leads to increased cell death. Taken together, this study indicates the detrimental effects of S-nitrosation on the BER pathway and suggests the potential effectiveness of alkylating agents as a cytotoxic therapeutic in inflammatory regions.

4.2 Introduction

DNA is under constant attack from exogenous agents (e.g. UV irradiation and smoking) and endogenous agents (e.g. inflammation and metabolism) that induce strand breaks, base lesions, and crosslinks. Unrepaired damages can lead to mutation, which in turn can lead to aging and cancer [1]. Alkylating agents are a source of DNA damage, which are found both endogenously, from methyl donors like S-adenosylmethionine, and exogenously from chemotherapeutics [2]. There are multiple pathways to repair alkylation-induced base lesions and strand breaks, however the primary pathway is the base excision repair (BER) pathway (Fig. 4-1A) [2, 3]. The following is an example of the BER pathway with a monofunctional glycosylase. The BER pathway can be initiated by alkyladenine glycosylase (AAG) excising the lesion from the backbone, leaving an abasic site [2]. Subsequently, AP-endonuclease-1 (APE-1) cuts the backbone 5’ to the lesion leaving a 5’-deoxyribosephosphate (5-dRP) and a 3’ hydroxyl radical. Poly-(ADP-ribose) polymerase-1 (PARP1) adds ADP-ribose chains to itself and other nuclear proteins recruiting downstream BER enzymes including Polymerase β (POLβ), the scaffold protein XRCC1, and Ligase III. The lyase activity of POLβ next excises the 5-dRP and inserts a new base. Finally, Ligase I or the XRCC1-Ligase III complex seals the backbone [4, 5]. If unrepaired, each of these intermediate repair steps (Fig. 4-1A, shown in the gray box) can be toxic to the cell [6-8]. Imbalances and deficiencies in
the BER pathway have been implicated in many processes, including increased sensitivity to alkylating agents and vulnerability to inflammation [9-13].

During inflammation, immune cells produce reactive oxygen and nitrogen species (RONS), which can damage the DNA [14, 15]. In addition to damaging DNA, RONS can also affect proteins. Nitric oxide (NO) is generated by macrophages through the reaction of L-arginine with a nitric oxide synthase [16], and can react with the cysteine residues of proteins through a process known as S-nitrosation [17]. One way in which proteins can become S-nitrosated is through the transfer of NO from S-nitrosoglutathione (GSNO), a nitrosated form of glutathione (Fig. 4-1B), to a cysteine residue on another protein [18]. S-nitrosated proteins have been found to have altered activities and modified cellular localizations when compared to their non-nitrosated forms [19].

One protein that is affected by S-nitrosation is the direct reversal protein O\(^6\)-methylguanine methyltransferase (MGMT), the primary repair mechanism for alkylated base lesions, including O\(^6\)-methylguanine (O\(^6\)-meG), which is toxic and mutagenic. Under non-nitrosating conditions, MGMT transfers the methyl group of the damaged lesion onto one of its cysteine residues (Fig. 4-1C) [20]. However, GSNO can also transfer its nitric oxide moiety onto the cysteine of MGMT rendering it unable to repair O\(^6\)-meG (Fig. 4-1D). The GSNO-induced inactivation of MGMT can lead to increased levels of O\(^6\)-meG in cells and cell death [21]. In addition, animals with an inability to reduce GSNO have lower levels of MGMT and increased tumor levels [22].

While the role of GSNO in the context of direct reversal has been deeply analyzed, the effect of GSNO on the BER pathway is less understood. Previous reports have shown biochemically that nitrosation of AAG on cysteine 167, a residue in the active site of AAG, induces increased excision of 1, N\(^\text{\theta}\)-ethenoadenine (εA) [23]. Mutation of cysteine 167 abrogates the increased AAG activity. To our knowledge, the
exact mechanism by which nitrosated C167 induces increased AAG excision activity is unknown, however it may cause the AAG binding site to be less discerning in the bases it excises and enabling it to remove undamaged bases. Another report has observed that S-nitrosation of APE-1 on cysteines 93 and 310 leads to the export of APE-1 into the cytoplasm; mutations of both cysteines eliminated the effect [24]. The report suggests that the nitrosation of the cysteines may induce a conformational change exposing a nuclear export sequence, however additional studies are necessary to prove this model. While these studies are mechanistically informative, an analysis of the effect of S-nitrosation on the intact BER pathway in the context of the cell has not been performed.

To study the effect of S-nitrosation on the BER pathway in cells, we utilized two recently developed techniques: the CometChip Assay [25] and the fluorescence-based multiplex host cell reactivation (FM-HCR) assay [26]. The CometChip assay is a high-throughput version of the comet assay, which is based on the principle that damaged DNA travels more readily through a matrix when electrophoresed than undamaged DNA [27-29]. By incubating damaged cells in media and lysing at various time points, one is able to analyze the kinetics of repair in the cells and determine if the cells have repaired the damage [25, 30]. The CometChip is an improvement over former comet technologies due to its robustness and its optimized analysis platform [31]. The assay begins by loading cells previously exposed to control solutions or damaging agents onto an array of 40 micron diameter wells. The cells are subsequently lysed and electrophoresed. Thereafter, the DNA can be imaged and analyzed through the use of an epifluorescent microscope to determine the percentage of the nuclear DNA that is able to migrate away from the nucleus [31].

While the CometChip allows the analysis of the BER pathway in aggregate, the FM-HCR assay enables the analysis of specific protein activities [26]. The analysis of
glycosylase activities is based on the principle of transcriptional mutagenesis. Cells transfected with plasmids containing enzyme-specific DNA lesions in fluorescent reporter genes, only produce fluorescent proteins if the DNA lesion is unrepaired. RNA polymerase can accidentally transcribe the incorrect base across from the lesion leading to fluorescence. If the glycosylase excises the lesion, reduced fluorescence is observed. Thus variation in the activity of the glycosylase changes fluorescence levels. In addition, the analysis of endonuclease activities is based on transcription blocking. Cells are transfected with tetrahydrofuran (THF), an abasic site analog, in a fluorescent reporter gene. THF blocks transcription of the gene, which inhibits fluorescent expression, however upon repair initiated by an endonuclease, the gene can be transcribed and produce fluorescence. In both assays, the level of fluorescence reporter expression can be analyzed through flow cytometry. In addition, FM-HCR allows the simultaneous analysis of multiple enzymes and pathways by using various pathway-specific lesions in fluorescent reporters of different colors. Together, the FM-HCR and CometChip assays allow the detection of BER kinetics and individual protein activities.

Here we investigate the effect of GSNO exposure on the repair of alkylation damage utilizing the CometChip and FM-HCR assays. We show that GSNO exposure induces an imbalance in the BER pathway leading to an increase in toxic BER intermediates. The high levels of toxic intermediates are associated with the viability of cells following exposure to an alkylating agent. This study suggests a novel mechanism for nitric oxide induced toxicity in inflammatory environments.
4.3 Material and Methods

4.3.1 Cells and Cell Culture

Wild type and Ag⁻/⁻ fibroblasts were prepared in our laboratory from mouse lines and maintained in Dulbecco’s Modified Eagle Medium (ThermoFisher Scientific) containing 10% fetal bovine serum (Atlanta Biologicals).

4.3.2 GSNO Preparation

S-nitrosogluthathione was prepared as described previously [24, 32]. Briefly, 0.1 M glutathione in 0.1M HCl was incubated with 0.1 M sodium nitrite at 4°C for 45 min. The resulting solution was neutralized to pH 7.4 with sodium hydroxide. The concentration of GSNO was measured spectrophotometrically (ε₃₃₅ = 902 cm⁻¹ M⁻¹). GSNO was prepared fresh for each experiment.

4.3.3 CometChip Assay

The CometChip experimental setup has been described previously [25, 30, 31]. Briefly, MEFs were trypsinized and cultured overnight in 24 well plates with 100,000 cells/well in complete growth media at 37°C, 5% CO₂. The next day, cells were incubated with various concentrations of freshly prepared GSNO in growth media for four hours at 37°C. Cells were subsequently incubated with the indicated concentrations of MMS for 30 minutes at 37°C in media containing the appropriate concentration of GSNO. Following MMS exposure, cells were rinsed with PBS and incubated in media containing the indicated concentration of GSNO for 0, 30 or 60 minutes. At the aforementioned times, cell were trypsinized and added to the CometChip [25] and
allowed to settle by gravity in growth media at 37°C. Cells not treated with MMS were trypsinized and loaded onto the CometChip at the 0 minute time point. The CometChip was then lysed overnight at 4°C in standard alkaline lysis solution. The CometChip was subsequently processed and analyzed under alkaline conditions as explained previously [33]. All data represents at least independent biological replicates.

4.3.4 Fluorescence Multiplexed Host Cell Reactivation Assay
The host cell reactivation assay has been described in great detail previously [26]. Briefly, cells seeded at a concentration of 100,000 cells/well in 12 well plates were incubated overnight and subsequently exposed to the indicated concentrations of GSNO for 3 hours at 37°C in 5% CO₂. Subsequently, Lipofectamine LTX (ThermoFisher Scientific) was used to transfect the cells with 2.5 μg of total plasmid DNA. DNA cocktails included undamaged reporter plasmids containing blue fluorescent protein and site-specific DNA damage reporter plasmids, which were generated as previously described. Transfected cells were incubated in the indicated concentrations of GSNO for 5 additional hours at 37°C 5% CO₂. Subsequently, the GSNO solutions were replaced with fresh growth media for 13 hours. Cells were then trypsinized and resuspended in growth media containing TO-PRO-3 and transferred to 75 mm Falcon tubes with cell strainer caps (ThermoFisher Scientific). Flow cytometry analysis and the calculation of percent fluorescent reporter expression was performed as previously published [26]. Four independent biological experiments were performed for each condition.

4.3.5 Immunofluorescent Staining
Cells seeded the previous night in 24 well plates at a concentration of 100,000 cells/well were incubated with the indicated concentrations of GSNO for 4 hours at 37°C in 5%
CO₂. The cells were fixed and incubated with 1:100 rabbit anit-APE-1 (Novus Biologicals) overnight at 4°C. Stained cells were incubated with Goat anti-Rabbit AlexaFluor 488 antibody (ThermoFisher Scientific) and mounted with ProLong Gold Antifade containing DAPI (ThermoFisher Scientific). At least five images were taken per concentration in a blinded fashion using ImagePro Plus software (Media Cybernetics). To quantify cells with APE-1 in the cytoplasm, images were blinded and counted manually for DAPI-positive nuclei. At least 100 cells were counted for each condition. Cells showing green fluorescence outside the nucleus were considered positive for cytoplasmic APE-1. Four independent biological replicates were performed for each condition.

4.3.6 Abasic Site Quantification

Cells seeded the previous night in 6 well plates at a concentration of 1 million/well were incubated with the indicated concentrations of GSNO for 4 hours at 37°C in 5% CO₂. Cells were subsequently incubated with the indicated concentrations of MMS for 30 minutes at 37°C in media containing the appropriate concentration of GSNO. Following MMS exposure, cells were rinsed with PBS and incubated in media containing the indicated concentration of GSNO for 0 or 60 minutes. Cells were then trypsinized and the DNA was extracted using the Purelink Genomic DNA Mini Kit (ThermoFisher Scientific). After abasic sites were quantified through the DNA Damage Quantification Kit – AP Site Counting (Dojindo Molecular Technologies).

4.3.7 Colony Forming Assay

Colony forming assay was performed through standard protocols. Fifteen hours after cells were seeded in 60 mm plates, cells were incubated various concentrations of freshly
prepared GSNO in growth media for four hours at 37°C. Cells were subsequently incubated with the indicated concentrations of MMS for one hour at 37°C in media containing the appropriate concentration of GSNO. Following MMS exposure, cells were rinsed with PBS and incubated in media containing the indicated concentration of GSNO for four additional hours. GSNO-containing media was exchanged for standard growth media and cells were allowed to grow for 13 days to form colonies. After washing plates with PBS and allowing the plates to dry overnight, cells were stained with 1% crystal violet solution. Plates were rinsed with de-ionized water, allowed to dry and then counted in a blinded fashion. The data presented here represents three independent biological experiments.

4.3.8 Statistical Analysis
GraphPad Prism was used for all unpaired and paired Student’s t tests. A $p < 0.05$ was considered significant.

4.4 Results
4.4.1 GSNO leads to increased BER intermediates following MMS exposure
To analyze whether GSNO exposure alters the activity of the BER pathway, we utilized the CometChip [25]. The CometChip is a high-throughput version of the comet assay that allows the detection of abasic sites, single strand breaks and alkali sensitive sites. Using this approach, it is possible to monitor the levels of toxic BER intermediates formed and cleared during the repair of alkylation damage (Fig. 4-1A). If GSNO exposure increases or decreases BER protein activities, then one would expect a change in the formation and clearance of BER intermediates. To study these repair
intermediates, we incubated wild type mouse embryonic fibroblasts (WT MEFs) with GSNO for four hours before exposing the cells to methyl methanesulfonate (MMS), an alkylating agent known to produce lesions such as 1-methyladenine and 7-methylguanine, that can be repaired by BER [2]. Following MMS challenge, cells were allowed to repair the damage in the indicated concentration of GSNO for 0, 30, or 60 minutes.

Control WT MEFs exposed to 0 mM GSNO (Figure 4-2A, white bars) have low percent tail values indicating low levels of BER intermediates. After MMS challenge, the levels of BER intermediates in the 0 mM GSNO cells increase at time 0. After incubating cells in media following MMS challenge, the levels of BER intermediates (white bars) decrease over the course of 60 minutes, thus indicating that BER intermediates are being resolved.

The WT MEFs exposed to 0.25 mM (Figure 4-2A, gray bars), and 0.5 mM (Figure 4-2A, black bars) GSNO display altered BER intermediate levels when compared to 0 mM GSNO MEFs. There is an insignificant increase in repair intermediates in GSNO-exposed cells when compared to the 0 mM GSNO MEFs immediately after MMS challenge (0 minutes repair). However, at 30 and 60 minutes after MMS challenge, both concentrations of GSNO displayed significantly higher amounts of BER intermediates when compared to the 0 mM GSNO MEFs (Fig. 4-2A). In addition, MEFs unchallenged by MMS and only exposed to various concentrations of GSNO show similar percent tail values (Fig. 4-2B) which is consistent with GSNO exposure not inducing a differential effect in the basal level of BER intermediates under spontaneous conditions. Given that CometChip detects strand breaks and BER intermediates and that GSNO exposure does not alter the basal level of BER intermediates, the data suggests that GSNO exposure is increasing the levels of BER
intermediates in MMS-challenged cells and potentially altering the activity of the BER proteins to repair the MMS-induced DNA damage.

4.4.2 GSNO exposure increases AAG activity

Given the increase in levels of BER intermediates in GSNO-exposed cells, we set out to investigate the effect of GSNO exposure on individual proteins in the BER pathway. Previous studies performed biochemically have shown that nitrosation of the cysteine residues in AAG increases its excision activity [23]. We set out to test whether a similar effect could be observed in cells exposed to GSNO.

To analyze AAG activity, we utilized the Fluorescence Multiplexed Host Cell Reactivation (FM-HCR) assay developed in our laboratory [26, 34]. Cells were exposed to three concentrations of GSNO and subsequently transfected with a plasmid containing hypoxanthine in the sequence for enhanced green fluorescent protein (EGFP) and a control plasmid expressing the blue fluorescent protein (BFP). Hypoxanthine is a DNA lesion that is primarily excised by AAG [11, 35]. The assay is based on the principle that if hypoxanthine is not excised by AAG, then during transcription, RNA polymerase can incorrectly place cytosine across from hypoxanthine (Fig. 4-3A - top panel) [36]. In this assay, the transcript of the EGFP gene can only lead to the production of EGFP, if cytosine is inserted. However, if hypoxanthine is excised by AAG, initiating the BER pathway, then the insertion of cytosine is less likely to occur (Fig. 4-3A - bottom panel). Reduced levels of cytosine containing transcripts will subsequently lead to lower levels of green fluorescence. Thus, green fluorescent signal is inversely correlated to AAG activity. In these experiments, all fluorescence values were calculated relative to the control BFP fluorescent plasmid transfected in the cells.

The basal levels of AAG activity in MEFs isolated from mice with normal AAG activity (WT MEFs), AAG deficiency (Aag<sup>/</sup> MEFs) [11], and modified AAG activity
(mAAG MEFs) [10] were analyzed through the FM-HCR assay (Fig. 4-3B). The mAAG MEFs constitutively express AAG. As expected, MEFs deficient in AAG display increased levels of green fluorescence compared to WT, supporting previous data that Aag^-/- MEFs have reduced AAG activity and reduced ability to excise hypoxanthine lesions compared to WT [11]. Conversely, mAAG MEFs have a small, but significant decrease in fluorescence compared to WT MEFs indicating higher AAG activity. Therefore, fluorescent signal is connected to AAG activity in FM-HCR; lower levels of fluorescence suggest higher AAG activity.

The effects of GSNO exposure on AAG activity in WT MEFs were also analyzed through FM-HCR (Fig. 4-3C). Cells exposed to 0.25 and 0.5 mM GSNO displayed significantly lower green fluorescence compared to cells not exposed to GSNO. Cells exposed to 0.5 mM GSNO have ~50% of the fluorescence of the 0 mM GSNO MEFs. Given that FM-HCR fluorescence and AAG activity are inversely correlated, this result suggests that reduced fluorescence in the GSNO-exposed cells is due to increased AAG activity. Thus, GSNO exposure increases AAG activity in cells.

In addition, we analyzed the effects of GSNO on AAG activity in mAAG MEFs (Fig. 4-2D). mAAG MEFs showed no significant change in fluorescence and thus no change in AAG activity at any concentration of GSNO, suggesting that in cells with constitutive AAG expression, GSNO exposure has minimal effect on AAG activity. These results suggest that GSNO exposure increases the activity of AAG, however GSNO's effect is minimal in the context of constitutively expressed AAG.
4.4.3. CometChip analysis shows that GSNO exposure does not alter BER kinetics in Aag\(^{-/-}\) cells

To study the effects of GSNO in cells with altered BER capacity, we also performed the CometChip analysis on WT, Aag\(^{-/-}\), and mAAG MEFs exposed to GSNO and MMS (Fig. 4-4). Through CometChip analysis, we can ascertain the role of AAG in the production of BER intermediates under nitrosating conditions. WT MEFs exposed to 0.25 mM GSNO display a significant increase in BER intermediates 30 and 60 minutes after challenge with 1 mM MMS when compared to the control MEFs exposed to 0 mM GSNO (Fig. 4-4A). The increase in BER intermediates is consistent with results from Fig. 4-3C showing increased Aag activity following GSNO exposure. Interestingly, there was little difference between Aag\(^{-/-}\) cells that were exposed to GSNO and those unexposed (Fig. 4-4B), which is consistent with GSNO's effect on AAG contributing to the increase in BER intermediates. In addition, we analyzed the effect of GSNO and MMS exposure on mAAG MEFs (Fig. 4-4C) and observed a significant increase in repair intermediates 30 and 60 minutes after MMS challenge, similar to WT MEFs. Given that the hypoxanthine reporter assay in Figure 4-3D indicated that GSNO exposure does not affect the activity of AAG in mAAG MEFs, the CometChip results suggest that there is another cause of the increase in BER intermediates following GSNO exposure in the WT and mAAG MEFs in addition to AAG.

4.4.4 GSNO exposure induces APE-1 translocation and increased abasic sites

Given the increased activity of AAG following GSNO exposure, we decided to investigate whether GSNO can affect the activity of downstream BER proteins. To that end, we utilized the FM-HCR assay with a plasmid containing tetrahydrofuran (THF)
in the EGFP gene cassette (Fig. 4-5A). THF is an analog of abasic sites, which is the product of AAG's excision of base lesions, and APE-1 cleaves the abasic sites produced by AAG [37]. Subsequently, downstream BER proteins insert the correct base in the DNA and complete the repair process. In this assay, if the abasic site, which blocks transcription, is not repaired by APE-1 and the downstream BER proteins, then EGFP protein will not be generated (Fig. 4-5A, top panel). However, if APE-1 and the downstream proteins repair the THF plasmid, then the EGFP gene can be fully transcribed and the cell will exhibit green fluorescence (Fig. 4-5A, bottom panel). WT MEFs exposed to GSNO showed a small but significant decrease in fluorescence, indicating a decrease in activity of BER proteins downstream of AAG (Fig. 4-5B).

To further study additional causes of the observed increase in BER intermediates in GSNO-exposed cells, we analyzed the localization of APE-1. Previous studies have shown that S-nitrosation of APE-1 induces its export from the nucleus to the cytoplasm in human kidney cells [24]. Here, we show a similar increase in cytoplasmic APE-1 after GSNO exposure in WT MEFs (Fig. 4-5C). Specifically, GSNO exposure induced a greater than 50% increase in the number of cells with cytoplasmic APE-1 (Fig. 4-5D).

Studies have shown that cells with reduced APE-1 activity display an accumulation of abasic sites [6]. To further test whether GSNO exposure diminishes APE-1 activity and increases the levels of abasic sites in the DNA, we quantified abasic sites in GSNO-exposed WT MEFs. The abasic sites were detected by incubating the DNA of the GSNO and MMS exposed cells with an aldehyde-reactive probe. This probe can be colorimetrically detected and quantified [38]. We observed a significant increase in absorbance indicating an increase in abasic sites in GSNO-exposed cells immediately after MMS challenge (Fig. 4-5E). However, after an hour of repair in media containing GSNO, there was an insignificant difference in the levels of abasic sites between the
unexposed and GSNO-exposed cells. These results show that GSNO exposure reduces APE-1 activity, however the generated abasic sites are still ultimately being repaired.

High levels of unrepaired BER intermediates and strand breaks have been observed to be toxic to cells [7, 13]. Here, we tested whether GSNO-exposed cells display reduced cell viability compared to non-GSNO exposed cells following MMS challenge. WT MEFs were incubated with media containing various concentrations of GSNO and subsequently challenged with either 0 or 1 mM MMS. After incubating in media containing the indicated concentrations of GSNO for four hours, the cells were allowed to grow for 13 days. The surviving fraction for each GSNO concentration was calculated by dividing the surviving fraction of cells challenged with 1 mM MMS by the surviving fraction of cells challenged with 0 mM MMS. MMS challenge induced a significantly lower level of cell viability in GSNO-exposed MEFs compared to non-GSNO exposed MEFs (Fig. 4-5F). This effect was observed at both 0.25 and 0.5 mM concentrations of GSNO. Therefore, GSNO exposure can reduce the viability of cells following MMS challenge.

4.5 Discussion and Conclusions

4.5.1 Discussion

S-nitrosation, the process by which the inflammatory chemical nitric oxide reacts with cysteine residues on proteins, has been shown to affect the activity of MGMT, a DNA repair protein that primarily repairs $\eta^\beta$-alkylguanine lesions [39]. While the effect of S-nitrosation on the activity of MGMT has been studied, the impact of S-nitrosation on the repair of other alkylation lesions in mammalian cells warrants further investigation. Here, we analyzed in MEFs the capacity of the BER pathway to repair alkylation damage under nitrosating conditions. Exposure to GSNO, an agent that can induce S-
nitrosation, was observed to modulate the activities of AAG and downstream BER proteins and induce an increase in BER intermediates in cells following alkylation damage. Furthermore, GSNO-exposed cells have reduced viability compared to nonexposed cells following challenge with alkylating agents. Taken together, the data reveal that GSNO induces an imbalance in the BER pathway and suggest that this imbalance leads to increased alkylation-induced toxicity.

Previous studies have shown that S-nitrosation can alter the activity of AAG [23] and the localization of APE-1 [24]. Analysis of the kinetics of BER through detection of single strand breaks and BER intermediates demonstrates that S-nitrosation not only affects individual steps of the BER pathway, but it can alter the ability of the BER pathway as a whole to repair alkylation damage. Both GSNO exposed and unexposed cells have similarly high levels of BER intermediates immediately after MMS challenge. However, 30 and 60 minutes after MMS challenge, GSNO exposed cells display significantly higher amounts of BER intermediates compared to unexposed cells suggesting that GSNO exposure alters the activities of the BER proteins. Thus, GSNO is modifying the ability of cells to repair alkylation damage.

We hypothesized that the accumulation of BER repair intermediates in the WT MEFs exposed to GSNO could be caused by alteration in the activities of AAG and downstream BER proteins. Through the use of the FM-HCR assay, we observed that GSNO exposure increases the activity of AAG to remove its mutagenic substrate, hypoxanthine. While hypoxanthine is not generated during MMS challenge, our observation of increased AAG activity following GSNO exposure is applicable to MMS conditions due to the fact that AAG also has a high catalytic activity with many lesions including alkylated lesions like 7-methylguanine and 3-methyladenine, and lipid peroxidation lesions such as 1, N\textsuperscript{6}-ethenoadenine [40][11]. Furthermore, our data concur with previous reports by the Wyatt Laboratory showing that S-nitrosated AAG has an
increased ability to excise 1, $N^6$-ethenoadenine [23]. Thus, the results from two independent assays analyzing the AAG excision activity on two unique base lesions have both demonstrated that AAG has an increased ability to repair base lesions after GSNO exposure.

To further test the hypothesis that the increase in BER intermediates in GSNO-exposed cells is influenced by increased AAG activity, we used the CometChip to analyze BER intermediates in cells with altered AAG activity. GSNO exposure had a minimal effect on the ability of $Aag^{-/-}$ cells to repair alkylation damage, suggesting that GSNO requires AAG to affect BER capacity. Furthermore, GSNO-exposed mAAG cells showed similar MMS repair kinetics as the GSNO-exposed WT cells. Combined with the observation that GSNO exposure does not alter AAG activity in mAAG cells due to high expression of AAG, the similarity in repair kinetics between GSNO-exposed WT and mAAG cells suggest that other BER proteins may be playing a role in the increase in BER repair intermediates. Therefore, while AAG is needed to initiate production of BER intermediates, there may be additional downstream factors eliciting the effect of continued high repair intermediates.

An alternative approach to analyze the effects of GSNO exposure on proteins downstream of AAG is FM-HCR. To perform this assay, we transfected GSNO-exposed cells with plasmids containing an abasic site analog (tetrahydrofuran) in the EGFP cassette. The THF plasmid requires APE-1 to cleave the abasic site and for downstream intermediates to be completely resolved in order for the EGFP protein to be expressed. In our system, we observed a significant decrease in the reporter expression in GSNO-exposed cells, indicating a decrease in the activity of APE-1 and/or downstream enzymes remaining in the BER pathway. The observed decrease in activity is perhaps due to the effects of S-nitrosation on multiple proteins. For example, previous studies have shown that ligases can be inactivated by nitric oxide, suggesting that Ligase I or
III may have reduced activity in a GSNO environment [41]. In addition, studies on PARP-1 have shown that S-nitrosation reduces its activity [42]. While, to our knowledge, there have not been studies on the effects of S-nitrosation on other BER proteins such as XRCC1 and POLβ, there is a possibility that these proteins might be degraded, translocated, or inactivated by S-nitrosation. The results of THF reporter assay suggest that in aggregate, the BER proteins downstream of AAG have reduced activity following GSNO exposure.

Here, we extended upon previous studies [24] to analyze the localization and activity of APE-1 following GSNO exposure. We observed an increase in the percentage of cells with APE-1 protein in the cytoplasm following GSNO exposure. Previous studies analyzing the effects of GSNO on APE-1 have shown that GSNO exposure can induce site-specific S-nitrosation of APE-1 and induces the protein to export from the nucleus into the cytoplasm [24]. The concentration of GSNO used and the methodology of GSNO exposure in the published studies are similar to those methodologies presented here, suggesting that APE-1 is S-nitrosated in our system and S-nitrosation is inducing a significant percentage of APE-1 protein to translocate into the cytoplasm. While the exact mechanism by which S-nitrosation induces APE-1 export is unknown, current models suggest that the nitrosation causes a conformational change that exposes a nuclear export sequence [24].

If the GSNO-induced export of APE-1 had affected its activity to cleave abasic sites, one would predict the amount of abasic sites in the cell to accumulate. High levels of abasic sites have been previously observed when there is an imbalance in the activities of AAG and APE-1, e.g. in APE-1 null cells with constitutive AAG expression [6]. Here, we indeed detected an increase in abasic sites in GSNO-exposed cells immediately after MMS challenge suggesting an initial imbalance in AAG and APE-1.
activities. Therefore, GSNO exposure induces translocation of APE-1 into the cytoplasm and reduced APE-1 activity in cells.

Taken together, the aforementioned results suggest that GSNO exposure induces a synergistic effect on BER proteins. While increasing the excision activity of AAG, S-nitrosation also reduces the activity of downstream BER proteins resulting in a BER imbalance and an accumulation of repair intermediates. Previous studies have shown that BER imbalances are toxic to cells and tissues [6, 9, 10, 13, 43]. Here, we have revealed that GSNO exposure causes increased toxicity in cells challenged with MMS compared to unexposed cells, suggesting that the BER imbalance and the BER intermediate accumulation contributes to the toxicity in the cell.

4.5.2 Conclusions

In summary, the observations that GSNO exposure alters the activities of BER proteins leading to an increase in repair intermediates and reduced cell viability following MMS challenge, suggest that S-nitrosation reduces the cell’s ability to repair and survive alkylation-induced damage. While previous studies have shown that S-nitrosation can affect the activity of Ogg1 [44], the bifunctional glycosylase that repairs oxidative lesions, our work suggests that additional proteins in the BER pathway are affected by S-nitrosation, leading to higher levels of intermediates. Furthermore, numerous studies have shown that genetic BER deficiencies in cells and animals cause highly sensitivity to inflammation and inflammatory chemicals [9, 45]. Our results here suggest the potential for an inverse mechanism where inflammation causes altered BER activity, which in turn can cause increased susceptibility to alkylating agents. Thus, alkylating agents may be increasingly useful as cytotoxic therapeutics in inflammatory regions with high levels of nitric oxide.
4.6 References


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Fig. 4-1: BER and MGMT Repair Processes

(A) Simplified schematic of the Base Excision Repair pathway. The BER pathway is initiated by alkyladenine glycosylase (AAG), which excises the damaged base (black) leaving an abasic site. AP endonuclease-1 cleaves the phosphate-sugar backbone producing a 3'OHN and a 5'-deoxyribose phosphate (5'-dRP). Polymerase β (POLβ) uses its dRPase activity to remove the dRP and subsequently inserts the correct base. Ligase 3 (LIG3) seals the backbone with XRCC1 acting as a scaffold. All repair intermediates shown in the gray box are detected through CometChip analysis. (B) Nitric oxide (red) can react with glutathione (GSH) to produce S-nitrosogluthathione (GSNO). (C) depicts how O₆-methylguanine methyltransferase (MGMT) repairs O₆-methylguanine by transferring the methyl lesion (blue) to its cysteine. (D) GSNO can transfer the nitric oxide moiety (red) to the cysteine of MGMT to form the inactive SNO-MGMT.
Fig. 4-2: GSNO exposure induces increase in repair intermediates in MMS challenged cells

(A and B) CometChip analysis of WT MEFs exposed to GSNO and 0.5 mM MMS (A) and 0 mM MMS (B). Not treated MEF data (NT) in (A) is the same as the zero minute repair in (B). Each data point represents mean ± SEM for three independent experiments; *p < 0.05 for paired Student’s t test.
Fig. 4-3: GSNO exposure induces increased AAG activity
(A) Simplified schematic of the hypoxanthine reporter (Hx) of the FM-HCR assay. Cells with transfected with the Hx reporter will display high fluorescence if RNA polymerase incorrectly inserts a cytosine in the transcript (top). If the Hx is repaired/cleaved, cells will not fluoresce (bottom). (B) Hx Reporter assay tested in WT MEFs, Aag<sup>−/−</sup> MEFs, and constitutively active AAG, mAAG MEFs. (C and D) Hx Reporter assay tested in WT (C) and mAAG (D) MEFs exposed to GSNO. Each data point represents mean ± SEM for three independent experiments; *p < 0.05 for paired Student’s t test.
Fig. 4-4: GSNO exposed Aag<sup>−/−</sup> MEFs display minimal increase MMS-induced BER intermediates

CometChip analysis of WT (A), Aag<sup>−/−</sup> (B), and mAAG (C) MEFs exposed to 0 or 0.25 mM GSNO and challenged with 1 mM MMS. NT refers to cells no challenged with MMS, lysed at 0 minutes. Each data point represents mean ± SEM for seven independent experiments; *p < 0.05 for paired Student’s t test.
Fig. 4-5: GSNO affects activity and localization of BER proteins and reduces cell viability after MMS challenge

(A) Simplified schematic of the tetrahydrofuran (THF) reporter of the FM-HCR assay. If unrepaired, THF will block transcription and inhibit fluorescence (top). If THF is fully repaired, cells will display higher fluorescence (bottom). (B) THF reporter assay in WT MEFs exposed to GSNO. (C) Representative immunofluorescent stains for APE-1 (yellow) and Nuclei (Blue) of WT MEFs exposed to 0 mM (top) and 0.25 mM (bottom) GSNO. White box indicates inset image. Arrows indicates cells with cytoplasmic APE-1. (D) Blinded visual quantification of APE-1 in the cytoplasm of cells exposed to GSNO. (E) Abasic site analysis of GSNO exposed cells. NT = non-treated cells challenged with...
MMS and lysed at 0 min. Other bars treated with indicated concentration of GSNO and 1 mM MMS. 60 minute allowed to repair abasic sites for 60 minutes at 37°C in media with indicated GSNO concentration. Data is relative NT. (F) Analysis of the colony forming assay of MEFs exposed to GSNO. Each bar represents the ratio of the surviving fraction of the MMS challenged cells to the non challenged cells at each GSNO concentration. Each data point represents mean ± SEM for three independent experiments; *p < 0.05 for paired Student’s t test.
Chapter 5

Conclusions and Future Work

The integrity of the human genome, which is under constant assault from numerous agents, is maintained by multiple comprehensive DNA repair mechanisms. In this thesis, we analyzed the effects of DNA damage and repair in two pathological contexts. First, we learned that influenza, a prevalent respiratory disease, induces DNA damage both in vitro and in vivo. In addition, we discovered that DNA repair proteins and pathways are upregulated and play a role during influenza infection. Second, we leveraged cutting-edge DNA damage and repair assays to analyze the effects of S-nitrosation, a cellular process that is upregulated during inflammation, on the DNA repair of alkylation damage and determined that S-nitrosation can induce a BER imbalance. Here, the results of these studies, the implications of the data, and potential future directions will be reviewed.

5.1 DNA Damage and Repair During Influenza Infection

We began our investigation into DNA damage and repair during influenza infection by assaying influenza-infected epithelial cells for strand breaks through the CometChip and γH2AX immunofluorescent staining. Previous studies had previously shown an increase
in strand breaks in influenza-infected lymphoblastoid cells [1]. This finding is supported by our results that influenza infection induces a significant increase in strand breaks through both methodologies. The observation of increased γH2AX signal is particularly noteworthy as it suggests that the strand breaks are inducing a DNA damage response in the infected cells. Supporting this model are preliminary results, which demonstrated an increase in ATM phosphorylation in cells infected with influenza. The implication of these findings is that γH2AX signal observed during influenza infection in vivo could be the cumulative damage produced by both the virus and the induced inflammatory response. Therefore, a therapeutic that reduces the amount of DNA damage in the infected tissue could have an ameliorative effect early in the disease pathogenesis by reducing the levels of strand breaks and tissue damage in vivo. In addition, continuing studies analyzing the exact cause of the DNA damage in vitro could also be informative for therapeutic development. Influenza viral protein PB1-F2 could be a cause of the damage given that plasmids expressing the PB1-F2 gene segments increase γH2AX signals in vitro and that PB1-F2 generates pores in the mitochondrial membrane, which potentially releases ROS. Thus, PB1-F2 is potentially one of the causes of DNA damage. Analysis of the γH2AX levels in cells infected with influenza virus and treated with catalase or infected with influenza lacking PB1-F2 would be a beneficial experiment to understand the disease process further.

Through our analysis of DNA damage during influenza infection in mice, we detected an increase in γH2AX signal and an insignificant change in the levels of Hx, 8-oxoG, εA, and εG base lesions in the lungs of infected mice. The γH2AX increase is supported by Li Na et al.’s thorough work, which also characterized the kinetics of γH2AX and the cell types damaged [2]. Therefore, DNA damage is very likely occurring in vivo. The observation of insignificant changes in various base lesions is odd given that the inflammatory response to influenza induces high levels of RONS [3, 4] and that
other studies have observed an increase in 8-oxoguanine through ELISA [2]. There are two potential causes for the minimal change in base lesion levels in influenza infection. First, the DNA repair machinery in wild type mice may be effective in repairing the induced damage in infected animals. Second, influenza infection may be producing other base lesions observed in inflammatory tissues such as 8-nitroguanine, xanthine, and 5-chlorocytosine [5]. An investigation into the levels of multiple base lesions produced by RONS in wild type and DNA repair deficient mice infected with influenza would be an optimal method to test both models.

To investigate the role of DNA repair during influenza infection, we performed GSEA analysis on transcripts from mice infected with both pandemic and seasonal influenza viruses. We detected a significant enrichment of HR genes during the recovery phase of influenza infection in multiple mouse and virus strains. The mice also displayed an increase in RAD51 protein levels in the lungs. These results are supported by previous research indicating an increase in overall RAD51 levels in influenza-infected mice and suggest that HR occurs during recovery from influenza infection [2]. Moreover, we analyzed influenza infection in mice deficient in Aag, Alkbh2, and Alkbh3, which displayed significantly more morbidity and higher levels of RAD51 compared to wild type mice. In totality, these findings imply that DNA repair occurs during influenza infection and that a deficiency in DNA repair leads to increased severity of infection. More specifically, the results suggest that HR plays a role during influenza infection. Increased analysis of influenza infection in an HR deficient animal would test the necessity of HR to survive infection.

A recent review by Zachary Nagel et al. emphasizes the necessity of research analyzing the interindividual variation in DNA repair capacity [6]. These studies can help determine susceptibility to various agents and could help the development of personalized therapeutics. Our findings here suggest that the epidemiological research
proposed could be beneficial for the prevention of influenza-induced morbidity. Our animal studies suggest an association between reduced DNA repair capacity and morbidity during influenza infection. Epidemiological analysis in large groups of people for their DNA repair capacity and past influenza infection severity should be performed to test this hypothesis. If our findings translate to human populations, then perhaps we can detect individuals most susceptible to influenza infection and strongly encourage them to get an annual vaccination. This work has the potential to save many lives in the context of a global pandemic.

To combat a global pandemic, we must not only determine those most susceptible to influenza-induced morbidity, but also treat the afflicted. Our results implicate DNA damage and repair during pathogenesis of influenza infection. A study analyzing the connection between the RONS and the damage is a worthy future endeavor. Given that RONS has been observed to cause DNA damage in other contexts, we hypothesize that treating mice with RONS scavengers/inhibitors such as N-methyl arginine or SOD could reduce the levels of γH2AX in the lungs. Similar results have been observed in mice infected with *S. pneumoniae* and EBV [7, 8]. Moreover, studies have shown that treating influenza-infected animals with RONS scavengers/inhibitors alleviates infections [3, 4]. Performing similar experiments in DNA repair deficient mice could further demonstrate the applicability of antioxidant treatment as a straightforward mechanism to treat influenza infection in multiple populations and potentially save millions of lives during severe pandemics like the 1918 “Spanish Flu”.

5.2 GSNO and DNA Repair

To analyze the effects of S-nitrosation on the repair of alkylation damage, we utilized the FM-HCR assay and the CometChip assay. Through the FM-HCR assay, we
determined that cells exposed to GSNO, a S-nitrosating agent, displayed a synergistic increase in AAG activity and decrease in the activity of downstream BER proteins compared to unexposed cells. In addition, GSNO-exposed cells challenged with MMS displayed an increase in BER intermediates and strand breaks compared to cells unexposed to GSNO, as assayed through the CometChip. Together, these results suggest that GSNO exposure leads to an imbalance in BER activity, which in turn leads to an accumulation of BER intermediates. Our viability results indicate that GSNO exposed cells display reduced survival following MMS challenge, which supports previous findings that unrepaired BER intermediates are toxic to the cell [9].

Our findings implicate GSNO and S-nitrosation in dysregulation of the BER pathway. Previous biochemical studies have found that S-nitrosation increases AAG activity and that GSNO-exposure leads to APE-1 export from the nucleus [10, 11]. However, to our knowledge, our study is the first to demonstrate that S-nitrosation affects the entire BER repair process and not just individual steps; by increasing AAG activity and decreasing the activity of downstream BER proteins, S-nitrosation leads to a BER imbalance and an accumulation of toxic repair intermediates. Given that S-nitrosation affects BER, the activity of other DNA repair pathways following S-nitrosation are worthy of deeper scrutiny. Mass spectrometry analysis for S-nitrosated DNA repair proteins utilizing the SNO trapping by triaryl phosphine method (SNOTRAP) developed by the Tannenbaum Laboratory would be an effective technique to determine additional DNA repair proteins that can be S-nitrosated [12].

The results suggest that in inflammatory regions with high levels of nitric oxide, there is an imbalance in BER protein activity. If this effect also occurs during the repair of oxidative damage through BER, then the S-nitrosation-induced BER imbalance could be one of the causes of cell death during inflammation. By reducing nitric oxide levels or increasing levels of GSNO reductase (GSNOR), the primary enzyme for reducing levels
of GSNO in cells, in inflammatory regions, the effects of S-nitrosation on BER could be reduced; thus mitigating cell death. A study of the potential S-nitrosation of DNA repair proteins during inflammation in *in vivo* contexts would be a potentially informative future study.

Our results also have implications for potential new therapeutics. Studies with PARP-1 inhibitors have shown that inhibition of the BER pathway can lead to cell death in *Brca2*-null tumors [13]. Our findings suggest that inhibiting GSNOR activity, could lead to an increase in S-nitrosation of BER proteins and inhibition of the pathway. This treatment could lead to cell death in HR deficient tumors as well. In addition, GSNOR inhibitors could be a potential combination therapy with alkylating agents in tumors. Alkylating agents can promote DNA damage in tumors and the increased GSNO can inhibit their repair. Thus, GSNO-induced imbalance of the BER pathway could be novel mechanism to treat various tumors.

5.3 Conclusions

In summary, the studies presented here have revealed that DNA damage and repair occurs and plays a role during influenza infection. Additionally, we have demonstrated that S-nitrosation can lead to a reduction in the capacity of the BER pathway to repair alkylation damage. It is our hope that the findings presented here will aid others in the development of new prevention strategies and novel therapeutics against both infectious disease and cancer.
5.4 References


Appendix A

Development of a Methylation-Based CometChip Assay: The EpiCometChip

A.1 Background and Motivation

- Cytosine methylation is an epigenetic mark that can be passed down for generations (Fig. A1-1)

![Cytosine Methylation](image)

**Fig. A-1: Cytosine Methylation**

- Methylation of cytosines in CpG islands, cytosine-guanine rich areas of the DNA, can inhibit transcription of genes [1] (Fig. A1-2)
Methylation can be dysregulated during diseases like leukemia and affected of environmental agents such as diet and lifestyle [3]

Analysis of the global methylation levels of cells in high throughput manner could help determine whether agents are epigenetically toxic

A.2 Proposal

- Modified Cytosine Restriction BC endonuclease (McrBC) is an enzyme that digest DNA at methylated cytosines
- We propose to use the CometChip setup to analyze overall methylation levels in cells.
- In addition, we propose that by treating cells with 5-aza-2’deoxycytidiine (5aza), an analog of cytosine that is unable to be methylated (Fig. A2-1), we will observe a reduction in the CometChip signal in the cell indicating that the assay is specific for methylation.
A.3 Procedure

- If testing specificity of assay, incubate cells with 2 μM (5aza) for 4 cell cycle doublings
- Load cells into a 40 micron a solidified agarose matrix containing 40 micro wells and cover with low melting point agarose – now referred to as “The Chip”
- Lyse cells overnight in alkaline lysis buffer at 4 °C [4]
- Place bottomless 96 well plate on top of The Chip and equalize all wells for 1 hour at room temperature with McrBC Enzyme Buffer (0.5mM GTP, 200 μg/mL bovine serum albumin, New England Biolabs Buffer 2)
- Incubate wells with McrBC Enzyme Buffer and McrBC (50 units/mL) for 2 hours at 37 °C
- Place gels in electrophoresis box and run for 1Hr in Alkaline Electrophoresis buffer at 4 °C [4]
- Neutralize Gels in 0.4M Tris-HCl for 30 minutes at 4 °C
- Stain gels with 1:1000 SyBr Gold in PBS and image and analyze using the CometChip program

A.4 Preliminary Results

- TK6 cells treated with McrBC display increased comet tail length compared to NT cells
- Suggest that TK6 cells contain a basal level of methylation in the genome
Fig. A-4: EpiCometChip Analysis of TK6 cells

* $p < 0.05$ Paired Student’s $t$-test; SEM for 3 independent experiments

- TK6 Cells exposed to 5aza and digested with McrBC display fewer strand breaks compared to cells unexposed to 5aza
- TK6 cells exposed to 5aza and not treated with McrBC have significantly fewer strand breaks compared to 5aza cells treated with McrBC

Fig. A-5: Analysis of the Methylation-Specificity of EpiCometChip

* $p < 0.05$ Paired Student’s $t$-test; SEM for 3 independent experiments
A.5 References


Appendix B

High-Throughput Quantification of γH2AX Foci

B.1 Background and Motivation

- The current method of quantifying γH2AX foci by manually counting the number of cells with greater than 5 foci is very time-consuming and subjective
- Imaging foci on a 2D plane can potentially lead to an imprecise analysis of DNA damage observed
- Analyzing >100 cells/condition (unfeasible through manual analysis) could reveal novel trends and a more complete analysis

B.2 Proposal

- Optimize experimentation with the novel microscope developed by Dushan Wadduwage in the Laboratory of Peter So which can analyze subnuclear foci in three dimensions in a high-throughput manner
- Test the microscope through analysis of the kinetics of irradiation in cells
Fig. B-1: Examples of Subnuclear Quantification

A. 3D quantification of foci in an individual nucleus.

B. Three dimensional visualization of foci in a nucleus

B.3 Preliminary Results

- Optimized the fluorophores for use with the So Lab microscope

<table>
<thead>
<tr>
<th>Color</th>
<th>Laser</th>
<th>Fluorophore</th>
<th>Excitation Maximum</th>
<th>Emission Maximum</th>
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<tr>
<td>Green</td>
<td>473 nm</td>
<td>AlexaFluor488</td>
<td>490</td>
<td>525</td>
</tr>
<tr>
<td>Red</td>
<td>561 nm</td>
<td>AlexFluor594</td>
<td>590</td>
<td>617</td>
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<tr>
<td>Far Red</td>
<td>660 nm</td>
<td>ToProIII</td>
<td>640</td>
<td>658</td>
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A549 cells irradiated with 1Gy of irradiation display a relative increase in \(\gamma\text{H2AX}\) foci, peaking at 30 min post irradiation and decreasing over the remainder of the experiment.

![Manual \(\gamma\text{H2AX}\) Analysis](image)

**Figure B-2: Manual \(\gamma\text{H2AX}\) Analysis of A549 cells**

SEM of two independent experiments

Values are percentage of \(\gamma\text{H2AX}\) positive cells (>5 foci) normalized to NT cells at each time point.

### B.4 Future Studies

- Compare manual \(\gamma\text{H2AX}\) analysis to high-throughput analysis
- Analyze the kinetics of \(\gamma\text{H2AX}\) foci levels at different points in the cell cycle
- Investigate the effect of irradiation on DNA-PKcs-null or inhibited cell lines to determine whether the observed damage is \(\gamma\text{H2AX}\)
Appendix C

Investigation into the DNA Damaging Effects of Cellular Squeezing

C.1 Background and Motivation

- The Laboratories of Robert Langer and Klavs Jensen have developed a microfluidic delivery platform (CellSqueeze) based on cell constriction enabling diffusion of materials into the cytoplasm [1].

![CellSqueeze Mechanism](image)

**Figure C-1: CellSqueeze Mechanism**

(Adapted from Sharei et al. [2])

- CellSqueeze was found to more effective than electroporation and enables the delivery of proteins and siRNA to immune cells [2].
- Mechanical stress on cells has previously been observed to induce DNA damage and apoptosis [3].
- Studies have yet to determine whether CellSqueeze has any effect on the integrity of DNA.
C.2 Proposal

Investigate the levels of DNA damage on cells used in the CellSqueeze at diameter of 4 and 6 μm through γH2AX staining and CometChip assay.

C.3 Preliminary Results

- Cells were unable to be stained for γH2AX, due to the squeezed cells inability to attach to glass slides.
- Western Blot analysis for γH2AX displayed no signal from squeezed cells at 4 or 24 hours post squeeze.

<table>
<thead>
<tr>
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<th>4 HPS</th>
<th>24 HPS</th>
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<tr>
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<td>β-actin</td>
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<td>γH2AX</td>
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Figure C-2: γH2AX Analysis of Cells following CellSqueeze

HPS(Hours Post Squeeze) HeLa cells were squeezed in devices with a diameter of 4 or 6 μm. 10Gy cells are TK6 lymphoblastoid cells irradiated with 10 Gy and lysed after 30 minutes repair.

- Squeezed cells displayed increased levels of alkali labile site at 2, 3, and 5 hours post squeeze.
• Cells squeezed with 4 μm chamber displayed higher DNA damage than 6 μm chamber

• It is difficult to determine whether the squeezed cells are alive or dead

A. B.

Fig. C-3: CometChip Analysis of HeLa Cells following CellSqueeze

(A) 4 μm diameter Squeeze chamber. (B) 6 μm Squeeze chamber.

SEM of four independent experiments. * p < 0.05 Student’s t-test

C.4 Future Studies

• Determine whether cells are dying post squeeze
• Test whether antioxidants can reduce level of DNA damage
• Compare levels of DNA damage between CellSqueeze and electroporation

C.5 References


Appendix D

Protocols

D.1 In vitro Immunofluorescent Analysis

Adapted from NextGen Protocols

D.1A Materials

- Falcon 12-well Flat Bottom Culture Plate with lid (Corning 353043)
- 12 mm #1 Circular Coverslips (Electron Microscopy Sciences 72231-01)
- Gelatin from Porcine skin (Sigma G1890)
- Trypsinized cell solution
- Cell specific media
- 4% Paraformaldehyde aqueous solution (Electron Microscope Sciences #157-4)
- Sterile Dulbecco’s Phosphate Buffered Saline (Life Technologies 14190-144)
- Fine forceps
- Triton x-100 (Sigma-Aldrich X100)
- Tris Buffered Saline (BioRad 170-6435)
- Bovine Serum Albumin (Sigma-Aldrich A3059)
- Anti-phospho-Histone H2A.X(Ser139) Antibody, clone JBW301 (Millipore 05-636)
- Goat anti-mouse IgG-Fitc (Santa Cruz sc-2010)
- 26G x ½ Regular Bevel Needles (Becton Dickinson 305111)
- 1 mL Tuberculin Slip Tip Syringe (Becton Dickinson 309569)
- 75X25 mm Single End Frosted Slides (VWR 16004-368)
- Prolong Gold Antifade with DAPI (Life Technologies P36935)
- Fluorescent microscope
D.1B Gelatinizing the Coverslips

- Mix 5 g of Gelatin with 500 mL of distilled water
- Autoclave the 1% Gelatin solution until sterile
- Pipet 1 mL of 1% gelatin solution to well in 24-well plate
- Place individual coverslip into each well with gelatin
- Use pipet to press coverslip to bottom of well
- Rotate plate to ensure gelatin covers entire coverslip
- Place 12-well plate into 37°C incubator for 1 hour
  - Tip: Plate can be in incubator for 1-6 hours without effect

D.1C Plating the Cells

- Quantify the number of cells in the cell solution
- Centrifuge vial of trypsinized cells at 1000 RPM for 5 minutes at 4°C
- Aspirate trypsin solution from the cells
  - Tip: Be careful not to aspirate the pellet
- Resuspend the cells to 1x $10^6$ Cells/mL
- Remove 12-well plate from incubator and aspirate gelatin
- Plate 1 mL of cell-specific media to each gelatinized well in 24-well plate
- Pipet $0.1 \times 10^6$ cells to each well
- Rotate plate 360° to ensure complete coverage of area with cells
- Place plate in 37°C incubator overnight

D.1D Experimentation and Fixation

- Perform desired experiment on cells
  - Note: This experiment can be any procedure that does not require the removal of the cells from the plate.
- Upon completion of the experiment, aspirate liquid in wells of 12-well plate
- Pipet 0.5 mL of 4% paraformaldehyde to each well
  - Note: Paraformaldehyde is extremely toxic and should be used inside a ventilated fume hood.
Note: You cannot fix wells independently of one another, because 4% paraformaldehyde on one side of the slide fixes cells on the other side of the plate

- Incubate wells at room temperature for 10 min
- Removed paraformaldehyde and wash wells twice with PBS
  - Note: At this point, the cells can be incubated with 1mL PBS and placed in 4°C for two weeks or until further use

**D.1E Immunofluorescent Staining**

- Dilute 20 μL of Triton X-100 in 10 mL of TBS to produce permeabilization buffer (0.2% Triton X-100 in TBS)
- Dissolve 0.1g of BSA in 10 mL TBS to produce blocking buffer (1%BSA in TBS)
  - Note: This is enough blocking buffer to perform stains on 8 wells. Scale volume accordingly.
- Aspirate PBS from wells of the 24 well plate
- Incubate each well with 0.5mL of permeabilization Buffer for 10 min at room temperature
- Aspirate permeabilization Buffer
- Incubate each well with 0.5mL of Blocking Buffer for 1 hour at room temperature
- Dilute 6 μL of anti-γH2AX Antibody in 3 mL of Blocking Buffer (1:500 Dilution)
- Aspirate Blocking Buffer
- Pipet 500 μL of Primary Antibody solution to each well in plate
- Incubate each plate overnight at 4°C with gentle rocking
- Store blocking buffer at 4°C

Next Day:

- Aspirate Primary Cell Solutions
- Wash all wells with 0.5mL of TBS to each well twice aspirating after each wash
- Dilute 6 μL Goat-anti-mouse-FITC in blocking buffer (1:500 Dilution)
- Wash all wells with 0.5 mL of TBS twice aspirating after each wash
- Bend Syringe needle to a 90° angle on counter with tip facing down (Video #2)
- Pipet 5 μL of ProLong Antifade onto glass slide
- Using Bent Syringe and forceps, pickup coverslip and flip onto slide (Video #3)
- Image slide as soon as possible

**D.1F Imaging and Quantification**

201
• Place slide on microscope stage and visualize at 10x under DAPI filter
• Switch to 60x magnification and focus
• Capture images of the slide first under DAPI filter and subsequently under Fitc filter
• Using your image software program, create a Dapi/Fitc merged image
• Take 5-10 images per slide throughout the entire slide area
  o  Note: To be properly unbiased, search under DAPI filter
• Upon capturing images for every slide, have a colleague blind you to the name of every image
• Count the number of Dapi positive cells in that image
• Count the number of cells with >5 yH2AX foci in that image
• Repeat for remaining images.
• Unblind yourself to the images names.
• Sum the total number of DAPI positive cells and cells with >5 yH2AX foci for each original slide
• Finally quantify the percentage yH2AX positive using the equation below:
  \[
  \frac{\text{Total \# of cells with } > 5 \text{ yH2AX foci}}{\text{Total \# of DAPI Positive Cells}} \times 100\% = \text{Percentage yH2AX Positive Cells Per Condition}
  \]

D.2 In vivo Immunofluorescent Analysis

D.2A Materials
• Xylene
• 100, 90, and 70 % EtOH
• Dako Antigen retrieval buffer (S1700 Dako)
• Wax Pen
• 3% BSA in TBS
• 0.3% BSA in TBS
• 0.05% Tween in PBS (PBS-T)
• ProLong Antifade Mounting Medium with DAPI (ThermoFisher Scientific)
• Coverslips

D.2B Deparaffinization
• Incubate slides in Xylene 3x 15 min
• Incubate in 100% EtOH 2X 10 min
• Incubate in 90% EtOH 1X 10 min
• Incubate in 70% EtOH 1X 10min
• Wash in dH₂O 2x in 5 min

D.2B Antigen retrieval

• During 90% EtOH wash, heat Dako buffer in slide rack and bottle of dH₂O at Power 4 for 30Min
• Assuming buffer is actively boiling, place slides in Dako buffer and microwave on Power 7 for 30 min
  o Make sure antigen retrieval buffer is actively boiling when adding in slides
• Every 5 Min check to make sure tissue sections are covered by buffer; if the liquid is getting low, fill up container with boiling water found in microwave
• Remove slide rack and container from microwave and let cool for 20 min
• Wash slide 1X PBS 5 Min

D.2C Block and Primary

• Circle sections with wax pen
• Incubate sections with 3% BSA in TBS for 5 Hrs at RT in humidified chamber
• Washed 2x 5 min in PBS
• Incubated slides with 1:100 primary antibody in 0.3%BSA in TBS overnight at 4°C in humidified chamber

D.2D Washing and Secondary

• Incubate slide in PBS-T 3x 2 min with shaking
• Recircle sections and incubate with 1:200 secondary antibodies for 1 Hr at RT
• Incubate slides in PBS-T 5x3 min with shaking
• Wipe of wax pen and mount with ProLong Antifade with DAPI mounting medium and pressing out all bubbles
• Let cure in dark overnight and clean with 70% EtOH before imaging
• Store at RT for short periods of time, and at -20°C for long term

D.3 EpiCometChip Protocol

D.3A Materials

• CometChip Material
  o 96 well Bottomless Plate
  o Glass Slide
  o Binder Clips

203
- Normal Melting Point Agarose
- PDMS Mold
- Trypsinized Cell Solution
- Low Melting Point Agarose
- Electrophoresis Box
- Alkaline Lysis Buffer
- Alkaline Electrophoresis Buffer
- 0.4M Tris
- SyBr Gold

- Equalization Buffer: 1:10 NEB Buffer 2; 1:200 GTP, 1:100 BSA
- Digestion Buffer: Equalization Buffer + 1:100 McrBC
- PBS
- Parafilm

D.3B Day 1
- Load cells in agarose chip in 96 well plate as in the standard CometChip assay
- Cover with Low Melting point agarose
- Lyse chip overnight in Alkaline Lysis Buffer

D.3C Day 2
- Remove chip from Alkaline Lysis Buffer
- Wash chip in 0.4M Tris at 4°C – 2x 15min
- Replace bottomless 96 well plate on chip and clamp down with binder clips
- Incubate wells in 50 μL Equalization Buffer for 30 min at room temperature
- Incubate wells in 50 μL Digestion Buffer for 2 hrs at 37°C
  - Cover plate with parafilm to reduce evaporation
- Aspirate Digestion Buffer from chip
- Rinse chip twice with PBS
- Tape chip to Electrophoresis box
- Run chip for 1 hour, 1V/cm, 300 mA in Alkaline Electrophoresis Buffer at 4°C
- Neutralize chip 0.4M Tris 3x 5min at 4°C
- Stain chip 1:1000 SyBr Gold in TE buffer at RT 20 min
- Image and analyze as if a CometChip
D.4 Western Blot Analysis

D.4A Materials

- HALT Protease Inhibitor (ThermoFisher Scientific)
- Pierce RIPA Buffer — Pierce#89900)
- PBS
- Cell Scraper/Lifter
- Biorad Mini-Protean 3 Cell—Biorad #165-3301
- Precision Plus Kaleidoscope molecular weight Standards—Biorad #161-0375
- Laemmeli sample Buffer—Biorad #161-0747
- Tris-HCl 4-20% pre-cast linear gradient gel—Biorad #161-1123
- 10x Tris/Glycine/SDS Running Buffer—Biorad #161-0732
- β-Mercaptoethanol
- Methanol
- 10x Tris buffered saline (25mM)/glycine(192mM)—Biorad #161-0771
- Powdered Skim Milk
- PVDF Membranes—Biorad #162-0174
- Filter Paper—Biorad #1703932
- Tween-20—Sigma #P9416
- 10x Tris buffered saline—Biorad # 170-6435
- ECL Plus Developing Solution – Pierce 32132

D.4B Protein Extractions from Cells

- Make Protein Lysis Solution
  - 1:100 HALT Protease Inhibitor in RIPA buffer
  - Use 500 μL of RIPA buffer for every well in 6 well plate or 1mL for T75 flask
- Aspirate culture media in plate
- Wash cells twice with 5mL cold PBS
- Add cold Protein lysis solution to each desired plate. Keep on ice for 5 minutes, while swirling plate occasionally for uniform spreading
- Gather lysate off each dish to one side using cell scraper and collect the lysate into eppendorf tube.
- Centrifuge tubes at max speed for 15minutes to pellet cell debris
- Transfer supernatant to new tube for protein quantification—tubes from the same plate can be pooled at this stage or proceed with Western Blot
D.4C Preparation of Materials and Running a Gel

- Freeze heat sink for transfer.
- Prepare 900mL SDS Running buffer by diluting 90mL of 10× Running Buffer (Tris/Glycine/SDS)
- Prepare 1.5L transfer buffer (25mM Tris, 192mM Glycine) by diluting 150mL of 10×Tris/glycine and add 20% v/v methanol and move to 4°C
- Prepare sample buffer by mixing 900 µL of Lamelli Sample Buffer with 50 µL β-Mercaptoethanol
- Dilute sample 3:1 with sample buffer.
- Open gel packet and slowly remove comb from the gel.
- Remove bottom tape from the gel and place into the slots at the bottom of each side of the Electrode assembly. The short plate must face inwards towards the notches of the U-shaped gasket.
- If only running one gel, use an empty glass plate system or plastic assembly insert on the other side of the assembly
- Lift gel cassettes into place against the green gaskets and slide into the clamping frame
- Press down on the Electrode Assembly while closing the cams of the Clamping frame to form the inner chamber to insure proper seal of the short plate to notch in gasket—this prevents leaking
- Lower the inner chamber into the Minitank. Fill the inner chamber with running buffer until the level reaches half way between the taller and shorter glass plates of the gel cassette(s)—DO NOT over fill
- Add ~200mL of running buffer to the outer chamber
- Load 5 µL of protein marker into the first and last lanes.
- Load samples slowly (30-100µg), giving sample time to settle evenly in the bottom of the well. DO NOT puncture the bottom of the well with pipette tip.
- Fill inner and outer assembly with more running buffer to make sure connections are set.
- Place lid on the Mini tank. Incorrect orientation is prevented by a stop. Align color coded banana plugs and jacks.
- Insert the electrical leads into a power supply with proper polarity
- Apply power to the Mini-PROTEAN 3 cell and begin electrophoresis; 200volts constant is recommended for SDS-PAGE. Run for 35min at room temp—until loading dye at bottom.
- After electrophoresis is complete, turn off power supply and remove all leads.
- Remove lid and carefully lift out the inner chamber assembly. Pour off and discard the running buffer—ALWAYS pour off buffer before opening cams.
D.4D Blot Transfer

- Wet the PVDF membrane in methanol for 2 minutes
- Transfer PVDF into transfer buffer and allowing it to moisten
  - NEVER touch PVDF with hands. Only forceps
- Completely saturate two filter papers and filter pads with transfer buffer. Beware of trapping air bubbles.
- Gently separate the two plates of the gel cassette using the lever in the gel box
- Remove the gel gently with tweezers and place on filter paper with correct orientation
- Rinse apparatus with distilled, deionized water after use.
- Open the gel holder and layer the pieces on the holder in the following fashion being careful not to trap bubbles and using forceps:
  - Black -> Filter pad -> Filter paper -> Gel -> Nitrocellulose membrane -> Filter paper -> Filter pad -> Clear
- After placing the second filter paper, roll with the roller to completely remove bubbles.
- Close the cassette. Hold it firmly so that the sandwich will not move and secure the latch. At this point movement of the sandwich may alter gel membrane contact, which could cause incomplete transfers or swirling transfer patterns.
- Place the gel holder in the buffer tank so that the black panel of the holder is facing the black cathode electrode.
- Repeat for 2nd blot if necessary. Fill the tank with transfer buffer to just above the level of the top row of circles on the gel holder cassette.
- Place stir bar and heat sink in the tank.
- Set the buffer tank on top of a magnetic stirrer at 4°C.
- Turn on the stirrer and close the lid gently but firmly. Be sure that the electrode wires are connected to the correct pins. Plug the unit into a power pack.
- Turn on power to initiate transfer use 100V, 250mA for 2 hours (alternatively run @30V, 40mA overnight)

D.4E Antibody Detection

- Carefully remove blot from the transfer apparatus and block non-specific sites with Blocking Reagent (5%Milk 0.05% Tween in PBS) for 1 hour at room temperature with gentle shaking.
- Dilute primary antibody to correct concentration in ~5 mL blocking buffer
• Remove the blocking reagent and add the appropriate primary antibody dilution. Incubate **overnight at 4°C** on rocker.
• Prepare 250mL Washing Buffer: 10× TBS(25mL) with 0.3% Tween-20 (0.75mL)
• Wash membrane by suspending it in Wash Buffer and shaking for ~1 hour. Replace buffer 4-6 times and repeat wash. Increasing the Wash Buffer volume and/or the number of washes may help reduce background.
• Incubate blot with the appropriate HRP-conjugate dilution for 1 hour at RT with shaking in 5mL blocking buffer
• Wash membrane as in step 2
• Prepare Developing solution by mixing 125 μL of A into 5 mL of B
• Incubate blot with Developing Solution on way
• Remove blot from working solution and place it on Fluorescent stage
• Image Blot