PARTICULATE HEXAVALENT CHROMIUM INHIBITS HOMOLOGOUS RECOMBINATION REPAIR BY TARGETING RAD51 PARALOGS IN HUMAN LUNG FIBROBLASTS

By

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B.A., Chemistry, Pre-Pharmacy, Eastern Kentucky University, 2018

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DEDICATION

I dedicate this thesis to my mother and father for always supporting me and making it possible for me to pursue and complete my degree

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First and foremost, I would like to thank the almighty God who has granted countless blessings, knowledge, and opportunities to be able to accomplish this thesis. I would like to show my greatest appreciation to my mentor, Dr. John Wise for his patience and endless support with my project. Special thanks to my committee members, Dr. Sandra Wise, Dr. David Hein, Dr. Calvin Kouokam, Dr. Lu Cai and Dr. Jim Liu for their insights and suggestions. I would also like to thank my lab members, Haiyan Lu, Idoia Meaza, and Jennifer Toyoda for their help and guidance with my work. Furthermore, my enormous appreciation to my beloved parents Deniece and Abraham Williams, for giving me unfailing support and encouragement. Finally, the love, guidance and support I've received from my family, pastor, boyfriend, and friends, throughout my academic career. I could not have successfully made it through without you guys. Lots of love and thanks all of you.

ABSTRACT

PARTICULATE HEXAVALENT CHROMIUM INHIBITS HOMOLOGOUS RECOMBINATION REPAIR BY TARGETING RAD51 PARALOGS IN HUMAN LUNG FIBROBLASTS

Aggie R. Williams

March 4, 2022

Hexavalent Chromium [Cr(VI)] is a known human lung carcinogen and general health hazard. The mechanism of carcinogenesis remains poorly understood, but chromosome instability (CIN) is the major theory in its carcinogenic mechanism. Homologous recombination (HR) repair is a DNA repair pathway that prevents CIN by repairing DNA double-strand breaks. RAD51, a key mediator protein of HR repair, along with the RAD51 paralogs (RAD51B, C, D, XRCC2, and 3) are required for HR repair. During HR, RAD51 loads and forms a helical nucleoprotein filament structure to promote DNA strand exchange and stimulate pairing activity of DNA. Cr(VI) exposures have been shown to target RAD51 and prevent its loading in lung fibroblasts. The mechanism by which Cr(VI) impacts RAD51 paralogs to cause RAD51 dysfunction remains unknown. In this study, we investigate the effects of Cr(VI) on these paralogs and their complexes in

human lung cells. This study found both acute and prolonged Cr(VI) exposure inhibits RAD51D repair response evidenced by decreased RAD51D foci formation, protein levels and gene expression. In contrast, Cr(VI) had minimal effect on XRCC3 repair function, suggesting RAD51D as a part of the BCDX2 complex may be a key initial target in Cr(VI)-induced loss of RAD51 function and HR repair.

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INTRODUCTION

Carcinogenicity of Chromium

Chromium Exposure

Chromium (Cr) is a metal commonly found in Earth's crust. Cr's desirable properties include its hardness, bright pigmentation, non-corrosive, and non-oxidative properties. Cr is used to build and plate objects to protect them from oxygen and corrosion. On a larger scale, Cr is used in the metallurgical and chemical industry to produce stainless steels, alloys, and plating steel, as well as coloring agents for pigments, paints and dyes, wood preservation, and leather tanning. Cr's potential for widespread industrial and commercial use continues to expand for companies around the globe.

Cr occurs in two environmentally stable valence states: trivalent chromium Cr(III) and hexavalent chromium Cr(VI). The trivalent form of chromium readily binds to extracellular molecules and is prevented from entering the cells. Cr(III) was initially believed to be a nontoxic and essential element for humans due to its effects on insulin action. Further research indicated that although Cr(III) may have pharmacological benefits, it is not essential. In 2014, the European Food Safety Authority Panel on Dietetic Products, Nutrition, and Allergies concluded there is no evidence suggesting chromium is an essential nutrient and, therefore, setting chromium intake recommendations would be inappropriate (EFSA, 2014).

Cr(VI) is also not essential, but unlike Cr(III), Cr(VI) poses a major level of concern to human health, particularly because of its ability to induce lung cancer. Cr(VI) is more toxic than Cr(III) because of uptake. Cr(VI) gets transported easily into the cell as chromate, which structurally resembles sulfate and phosphate. Thus, chromate can use the same channels as sulfate and phosphate and enters by the process of facilitated diffusion. By contrast, Cr(III) binds many available ligands extracellularly, which makes it too structurally bulky to use these transporters and it can only enter very slowly by simple diffusion (Figure 1). Once inside the cell, Cr(VI) gets rapidly reduced to Cr(III) generating Cr(IV), Cr(V) as well as reactive oxygen species in the process. Although Cr(VI) is toxic, it is not the ultimate toxicant. The actual species that is the ultimate carcinogen remains unknown with evidence supporting Cr(III), Cr(V), Cr(IV) and reactive oxygen species or possibly some combination of them.

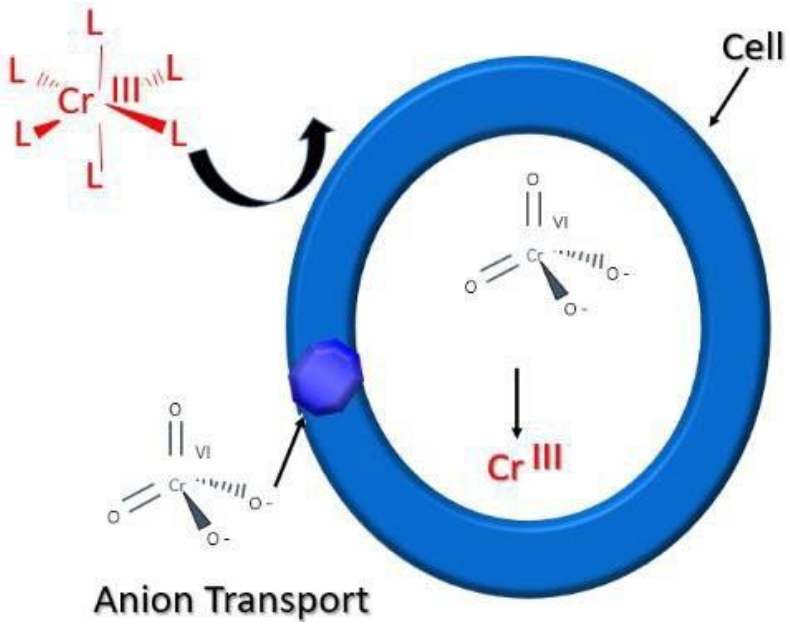


Figure 1: Properties of Chromium
This figure shows the two environmentally stable valence states of chromium that determine its toxicological effects. Cr(III) does not readily enter the cell because it binds to extracellular molecules. Cr(VI), however, uses anion transport to readily enter cells where it is reduced to Cr(III).

Evidence that Cr(VI) Causes Cancer

According to the Occupational Safety and Health Administration (OSHA 2006), all Cr(VI) compounds are considered carcinogenic to workers and the risk of developing lung cancer increases with the amount of Cr(VI) inhaled and the length of individual exposure. The Environmental Protection Agency (EPA 1999) classifies Cr(VI) as an agent within Group A: a known human carcinogen. Cr(VI) is listed as 17th most hazardous substance posing significant threat to human health by the Agency for Toxic Substances and Disease Registry (ATSDR 2012). This agency prioritizes substances based on toxicity and their potential for human exposure. All these regulations were conclusions based on epidemiology, animal and cell culture studies which indicate Cr(VI) compounds, when inhaled, cause lung cancer in humans. Cr(VI) poses a significant environmental health risk but despite well- known toxic effects, its carcinogenic mechanism remains poorly understood.

Epidemiological studies consistently show increased lung cancer rates in workers exposed to varying levels of Cr(VI) in the air. Pathology studies show the principal tumor type induced by Cr(VI) are bronchial carcinomas (Langard & Vigander 1983). Kishi et al., (1987) measured exposure to specific amounts of chromium over a decade in people who worked at a chromate chemical manufacturing plant. The amount of chromium in the lungs of workers for over 10 years averaged 51.5

ug/g (range 24.8-210 ug/g), while Cr levels in the lungs of non-exposed controls were 0.07-1.01 ug/g. Interestingly, chromate cancers found in the tumors exhibited molecular features different than those cancers induced by smoking, such as microsatellite instability and a specific pattern of methylation of tumor suppressor genes (Urbano et al., 2012). Human pathology studies also reveal both Cr-induced tumors and Cr accumulation occur at bronchial bifurcation sites (Ishikawa 1994). In addition, these studies show Cr(VI) persists in the lungs years after exposures have ceased. For example, a follow up study done by Mancuso & Hueper (1951) conducted on 332 chromate workers employed from 1931 to 1951 in Painesville, OH, showed 50% of mortality by 1974. Of the 50% of the men that died, 63.6%, 62.5% and 58.3% of cancer deaths for the ones employed in 1931-1932, 1933-1934 and 1935-1937 were due to lung cancer. The lungs measured from employees showed lung cancer death rates increased with increased exposure, and the deposition of chromium found in the lungs of workers were long after exposure ceased.

Cr(VI) compounds cause tumors in experimental animals. For example, Levy et al., 1986 used intrabronchial pellet implantation of several species of hexavalent chromates in rat lungs that produced bronchial carcinomas. Another study done by Farris (2014) showed Cr(VI) compounds administered long-term by inhalation were found to produce lung tumors. These whole animal studies confirm epidemiological studies and establish that Cr(VI) can cause tumors. Thus, Cr(VI)induced animal and human tumors have been further investigated in cell culture studies to confirm Cr(VI) compounds are carcinogenic with respect to lung

cancer. The results of these studies support the classification of Cr(VI) compounds as carcinogens.

Cell culture studies were also used to confirm the carcinogenicity of Cr(VI) compounds showing they induced neoplastic transformation (Xie et al., 2007). For example, Wise et al, investigated neoplastic transformation caused by Cr(VI) exposure in clonal cell lines using an anchorage independent assay to detect transformation of cultured cells (Wise et al., 2018). The ability for cells to grow in soft agar is a property acquired by neoplastic cells. Data showed 14.4% of first-generation clones grew in agar, 12.5% of second-generation clones grew in agar, and 20.5% of third generation clones grew in soft agar. None of the control clones grew in agar.

Role of Solubility in Cr(VI) Carcinogenesis

Another key factor of Cr(VI) carcinogenicity is water solubility. While all Cr(VI) compounds are classified as carcinogens, not all Cr(VI) compounds have equivalent carcinogenic potencies. Cr(VI) compounds can either be water soluble (i.e., sodium and potassium chromates) or water insoluble (i.e., lead and zinc chromates). Numerous available data from epidemiological, animal, and cell culture studies show water insoluble (particulate) salts are more potent than water soluble ones (Holmes et al., 2008). Zinc chromate for example, a water insoluble 'particulate' Cr(VI) compound was shown to be carcinogenic in epidemiology studies and to cause tumors in experimental animals. The underlying explanation

for why the particulates are more potent comes from pathology studies, which show increased levels of Cr levels and tumor formation at lung bifurcation sites where particles are expected to impact and persist, and from culture data that show the particle-cell contact leads to extracellular dissolution in the immediate microenvironment of the cell indicating the particles can act like a localized Cr(VI) sink, while soluble compounds are more readily cleared.

Mechanism of Cr(VI) Carcinogenesis

The mechanism of carcinogenesis by Cr(VI) remains poorly understood. However, the dominant theory is that Cr(VI) drives the formation of lung tumors by inducing chromosome instability, including structural and numerical alterations in the chromosomes (Hirose et al., 2002). Structural chromosome instability results from Cr(VI) inducing DNA double strand breaks that, when unrepaired, progress to structural chromosomal changes. Notably, Cr(VI) also inhibits DNA double strand break repair (Qin et al., 2014). How, this inhibition occurs is poorly understood and is the focus of this thesis.

Cr(VI)-induced DNA Double Strand Breaks

Cell culture studies show Cr(VI) causes DNA double strand breaks after acute and prolonged exposures (Holmes et al., 2008; Wise and Wise 2012; Wise et al., 2008; Xie et al., 2008). DNA double strand breaks were measured by gamma H2AX foci formation or with single gel electrophoresis assay (neutral comet assay). The breaks formed in the late S and G2 phases of the cell cycle (Ha et al., 2004; Xie et al., 2005). The cause of the double strand breaks likely involve several factors.

Some breaks forms as a result of crosslinks/ternary adducts from either their repair by excision repair or from the collapse of stalled replication forks. Breaks can also form from an unrepaired single strand break entering S or G2 and being converted to a DNA double strand break or from futile mismatch repair (Holmes et al., 2004, Wise et al., 2008).

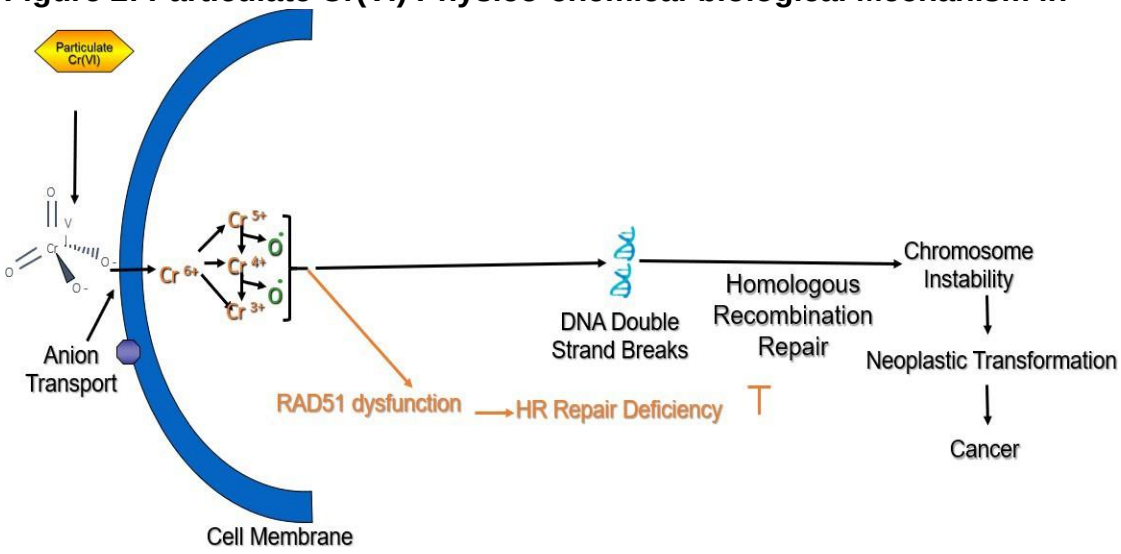
Cr(VI) Targets RAD51 to Inhibit DNA Double Strand Break Repair

When a DNA double strand break occurs, the break is recognized and primarily repaired by one or the other of two different pathways: homologous recombination (HR) and non-homologous end joining repair (NHEJ). Of these two, HR is the pathway that protects against Cr(VI) carcinogenesis and chromosome instability. (Stackpole et al., 2007)

Cr(VI) inhibits HR repair, allowing unrepaired breaks to cause chromosome instability (Figure 2). Browning et al. 2016 observed cells after prolonged exposure to Cr(VI) (>72 h) and found reduced HR repair (Browning et al., 2016). HR signaling and repair involves several steps, including sensing the damage (sensor), transducing, and amplifying the repair signal (transducer), resection, and carrying out the repair (effector). Several proteins, including H2A.X, MRE11, RAD50, NBS1, ATM and RAD51, among others play significant roles in HR. After a break occurs, the initial processing involves the MRN (MRE11, RAD50, NBS1) complex as a part of the sensing step. ATM is recruited during the transducing step to signal proteins downstream for repair. RAD51 is the signature downstream effector protein in HR. Qin et al (2014) found proteins early in the HR signaling

pathway responded normally after Cr(VI) exposure. In contrast, they found the later effector steps were altered by Cr(VI), specifically altering RAD51. In particular, the data show inhibition of RAD51 foci formation, protein levels, and nucleoprotein filament formation after prolonged exposure (Qin et al., 2014; Browning et al., 2016) . However, how Cr(VI) alters RAD51 function is unknown.

Figure 2: Particulate Cr(VI) Physico-chemical-biological Mechanism in human cells



This figure shows Cr(VI) induces DNA double strand breaks while simultaneously inhibiting the repair of those breaks, which results in CIN, neoplastic transformation and ultimately cancer.

RAD51 Paralogs as Possible Targets to Explain the Loss of RAD51 Function

RAD51 initiates single strand invasion and homology pairing in sister chromatids during the effector stage of HR (Sung and Robberson., 1995). The key function of RAD51 is to form a helical nucleoprotein filament which promotes repair activities. However, prolonged exposure to particulate Cr(VI) inhibits the formation of this RAD51 filament (Browning et al., 2016). Loss of this filament is thought to be a key event in Cr(VI) carcinogenesis resulting in loss of HR repair and allowing the

unrepaired breaks to destabilize the chromosomes. However, how Cr(VI) induces the loss of this filament is unknown.

There are several proteins involved in forming the RAD51 nucleofilament. These proteins are referred to as RAD51 paralogs because they share 20-30% amino acid sequence identity with RAD51 (Thacker 1999). They are thought to originate from a gene duplication of the ancestral RADA protein and have maintained their structural resemblance to RAD51. The five classical RAD51 paralogs are RAD51B, RAD51C, RAD51D, XRCC2 and XRCC3. Each is an important component of the HR pathway. In the early stages of HR, the paralogs act by regulating either the assembly or stability of the RAD51 nucleoprotein filaments. Deficiency in any of these paralogs results in chromosomal abnormalities, decreased DNA damage-induced sister chromatid exchanges, reduced RAD51 focus formation, and deficiency in replication fork protection (Garcin et al., 2019)

The paralogs exist in two distinct complexes: the BCDX2 complex (RAD51B-RAD51C-RAD51D-XRCC2) and the CX3 complex (RAD51C-XRCC3) (Masson et al., 2001) (Figure 3). These two complexes act during different stages of the HR pathway. The BCDX2 complex acts during RAD51 recruitment (Yonetani et al., 2005; Chun et al., 2013) and plays a role in RAD51 filament formation and stabilization. By contrast, the CX3 complex acts downstream of RAD51 recruitment. promoting RAD51 nucleofilament remodeling, stability, and strand invasion.

How Cr(VI) affects the paralogs, and these complexes is poorly understood. The only available data show Cr(VI) can inhibit RAD51C foci formation after prolonged exposures, but had no effect on RAD51C whole cell protein levels (Browning et al., 2016, Browning et al., 2018). No studies so far have investigated the effect of Cr(VI) on the remaining paralogs. Therefore, this study focuses on the effects of Cr(VI) on two additional RAD51 paralogs RAD51D and XRCC3, representing the BCDX2 and CX3 complexes, respectively .

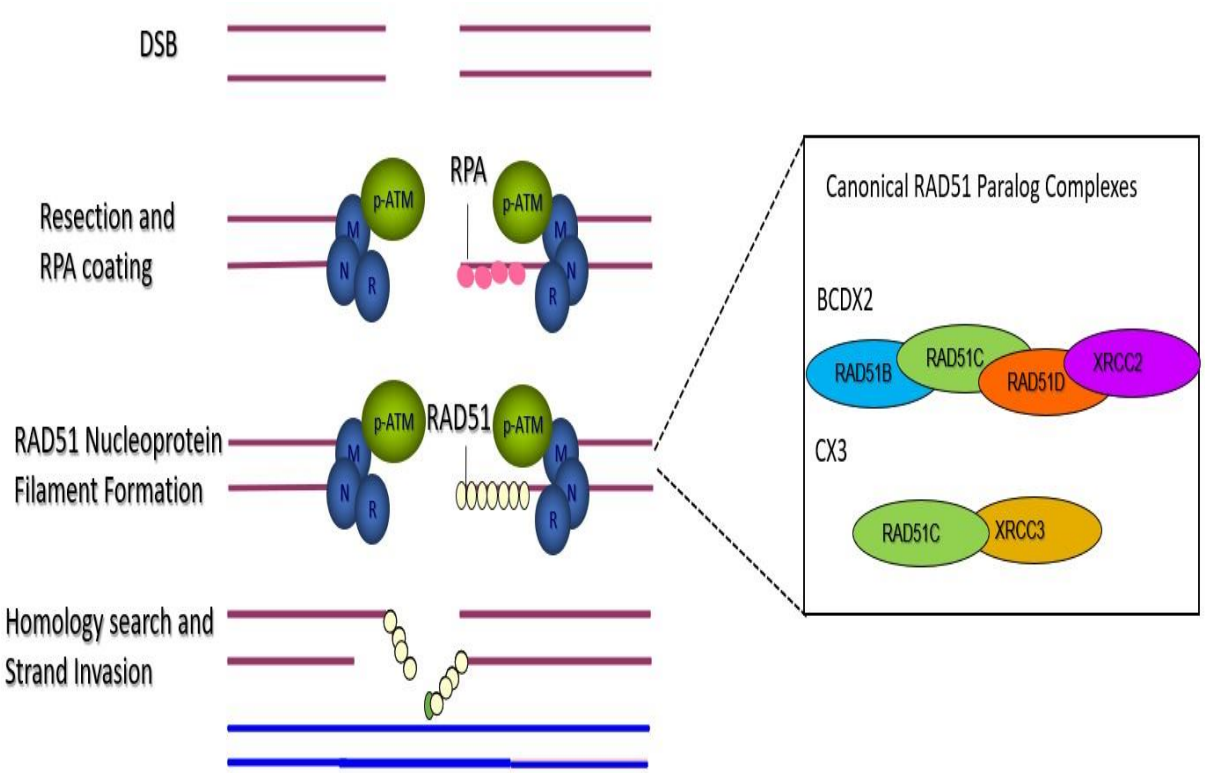


Figure 3: Schematic of Canonical RAD51 paralog Complexes This figure shows the steps of DSB repair through HR and complexes BCDX2 (consisting of RAD51B, RAD51C, RAD51D, and XRCC2) and CX3 (consisting of RAD51C and XRCC3).

MATERIALS & METHODS

Chemicals and reagents

Cell Culture

WTHBF-6 cells, an hTERT immortalized clonal cell line derived from human bronchial fibroblasts, were used as a representative human lung cell line. The bronchial cell strain, isolated from normal lung of a 67-year-old Caucasian male and this cell line exhibits normal growth parameters and a normal stable karyotype. WTHBF-6 cells were maintained as a monolayer in Dulbecco's Modified Eagle Medium/Nutrient Mixture F- 12 (DMEM/F- 12) supplemented with 15% Cosmic calf serum (CCS), 1% Corning glutaGRO supplement, 0.1 mM sodium pyruvate, and 1% penicillin/streptomycin and fed every other day. Cells were sub-cultured every three to four days using 0.25% trypsin-EDTA. All experiments were maintained in a 37°C, humidified incubator with 5% CO_2.

Chromium Preparation

Suspensions of zinc chromate particles were prepared by rinsing twice in double-distilled water to remove any water-soluble contaminants. Then the particles were washed twice in acetone to remove any organic contaminants. The washed particles were air-dried, weighed, and placed in double-distilled water in a borosilicate scintillation vial and stirred overnight with a magnetic stir bar at 4°C.

In previous studies by the Wise Laboratory these methods were shown to result in particles in the size range of 0.2-2.3 um with a mean size of 2.7 um. During the preparation of the appropriate dilutions and during the treatment procedure the particles were kept in suspension using a vortex mixer. Final chromate concentrations in cell culture ranged from 0-0.3 ug/cm^2 for the zinc chromate treatments. The dilutions were dispensed directly into cultures from these suspensions which are environmentally relevant ranges to which humans may be exposed.

Cell Treatments

For all experiments, cells were seeded and allowed 48 h to enter logarithmic growth before treatment. Before treatment, the medium was changed and the zinc

chromate suspension was added at a concentration of 0, 0.1, 0.2, or 0.3 ug/cm^2 to fresh media unless otherwise specified. Treatment durations were for 24 or 120 h.

Immunofluorescence Assay

For the immunofluorescence assay, cells were seeded on four well glass slides, fibronectin (FNC) coating mix-coated chamber slides. For the 24 h treatment conditions (0, 0.1, 0.2 and 0.3 ug/cm^2), 12,000 cells were seeded in each well of the four well glass. For 120 h 0-0.3ug/cm^2 were seeded with 3000 cells whereas 0.2 ug/cm^2 wells were treated with 4000 cells and 6000 for 0.3 ug/cm^2. Cells were allowed 48 h to enter logarithmic growth before treatment with zinc chromate. At harvest, after

24 or 120 h exposure, media was aspirated, and cells were rinsed with Dulbecco's phosphate-buffered saline (DPBS).

For RAD51D foci, cells were fixed with 4% paraformaldehyde for 5 min, permeabilized with 0.2% Triton X-100 for 5 min and blocked with 10% goat serum and 5% Bovine serum albumin (BSA) in Phosphate buffer saline (PBS) for 30 min. Cells were then incubated with anti-RAD51D (NB100-178; 1:500) antibody for 1 h, washed with PBS and incubated with Alexa Fluor 488 goat anti-mouse igG (2066710; 1:1500) for 1 h in the dark. For XRCC3 foci, cells were fixed with 100% methanol at -20°C for 5 min and blocked with PBS containing 4% BSA at 4°C overnight, Cells were then incubated with XRCC3 (NB100-10F1/6; 1: 500)

antibody for 1 h at 37°C in a humid environment using a slide warmer. washed with PBS and incubated with Alexa Fluor 488 goat anti-mouse igG (2066710; 1:1000) for 1 h at 37°C in a humid environment using slide warmer in the dark. Cells were washed with PBS and coverslips were mounted with a simple fluorescent stain, 4',6-diamidino-2-phenylindole (DAPI).

Nuclear foci were scored in 100 cells per concentration/timepoint using fluorescence microscopy. Results were expressed as the percentage of cells with >20, >5 or >11 foci based on background levels such that negative controls had 5% or less of cells with this level. Images of cells per concentration/timepoint were obtained with an Olympus confocal microscope.

Western Blot Assay

Cells were seeded on 100 mm dishes and allowed 48 h to enter logarithmic growth phase. Cells were treated as described above. At the end of the treatment period, after 24 h or 120 h, media was removed, and cells were rinsed with PBS and released from the plate with 0.25% trypsin-EDTA. Cell pellet were collected in 1.5 ml microfuge tubes. Whole cell extracts were obtained by washing, collecting and incubating cells in 500 µl of extraction buffer (Pierce RIPA Buffer (Thermo 89900)) with protease and phosphatase inhibitors 100x (Thermo 78440) for 20 min on ice. Cells were centrifuged at 14 000 rpm for 10 min. Supernatant was collected and stored at −80 °C. Immunoblots were probed with anti-Rad51 (Santa Cruz sc-38819; 1:1000) or anti-RAD51D (Santa Cruz SC-38819; 1:1000). Equal loading

was confirmed by Alpha-tubulin (Cell Signaling 11H10; 1:1000). Nuclear protein extract was resolved on either 12% Bis-Tris SDS-PAGE gels or precast gel for use with tris/glycine buffers and transferred to either PVDF membranes or nitrocellulose membranes. Immunoblots were incubated with Alexa680 or Alexa800 (1:3000, 1:15000) secondary antibodies and fluorescence detected using an Odyssey Imager (LiCor, Lincoln, Nebraska).

Quantitative reverse transcription PCR (RT-qPCR)

Cells were seeded on 100 mm dishes and allowed 48 h to enter logarithmic growth phase. Cells were treated as described above. At the end of the treatment period, after 24 or 120 h, media was removed, and cells were rinsed with PBS and released from the plate with 0.25% trypsin-EDTA. Cell pellet were collected in 1.5 ml microfuge tubes. Total RNA extracts were obtained, and cDNA was prepared using a mirVana miRNA isolation kit, with phenol, Thermo Fisher; (AM1560) and TaqMan assay probes using manufacturer's instructions. Briefly, cells were lysed directly in the culture plates and homogenized. RNA was extracted using acid-phenol chloroform and the aqueous phase was transferred to filter cartridges. Total RNA was washed several times using ethanol and eluted into a fresh tube. RNA quality and concentration were measured using a Nanodrop ND-1000 spectrophotometer. cDNA synthesis was carried out using a High-Capacity cDNA Reverse Transcription Kit per the manufacturer's instructions with slight modifications. Briefly, 2 RT master mix was prepared using random primers,

combined with 2 lg total RNA (per 20ul reaction). TaqMan RNA primers (Hs00864094_m1; RAD51D- Hs00979562_m1; GAPDH-Hs027899_) were combined with TaqMan Universal PCR Master mix II (Thermo Fisher Inc) and cDNA in triplicate. The no RNA and no reverse transcriptase controls from cDNA synthesis and a no cDNA control were included in all qPCR runs. Protocols were utilized designed to use with STepOne Plus quantitative polymerase chain reaction (qPCR) machine. The CT threshold was set by instrument's calculations and results are displayed as ΔΔCT values relative to the untreated (0 ug/cm^2 zinc chromate) control for each time point, respectively.

Statistical Analysis

Student's t-test were conducted to determine statistical significance between data points. ANOVA was also used for comparisons between timepoints. Results were expressed as the mean ± SEM (standard error of the mean) of 3 independent experiments. Statistical significance was determined to be a p value less than 0.05.

RESULTS

Particulate Cr(VI) Inhibits RAD51D Foci Formation

We focused on RAD51D as a representative of the BCDX2 complex. We evaluated the ability of Cr(VI) to inhibit the function of RAD51D through foci formation using immunofluorescence (Figure 4A). Particulate Cr(VI) inhibited RAD51D foci formation in a concentration dependent manner after 24 and 120 h exposure (Figure 4B). Specifically, 0.2, and 0.3 ug/cm^2 zinc chromate reduced the percent of cells with more than 11 RAD51D foci to 3.9, and 2.4 percent, respectively compared to 5.8 percent in untreated controls. A somewhat greater inhibition was seen after a 120 h exposure as 0.1, 0.2, and 0.3 ug/cm^2 zinc chromate reduced the levels to 6.3, 3.3, and 2.3 percent, respectively, compared to 7.4 percent in untreated controls. At both time points, both 0.2, and 0.3 ug/cm^2 were significantly lower than their respective controls ($P<0.05$).

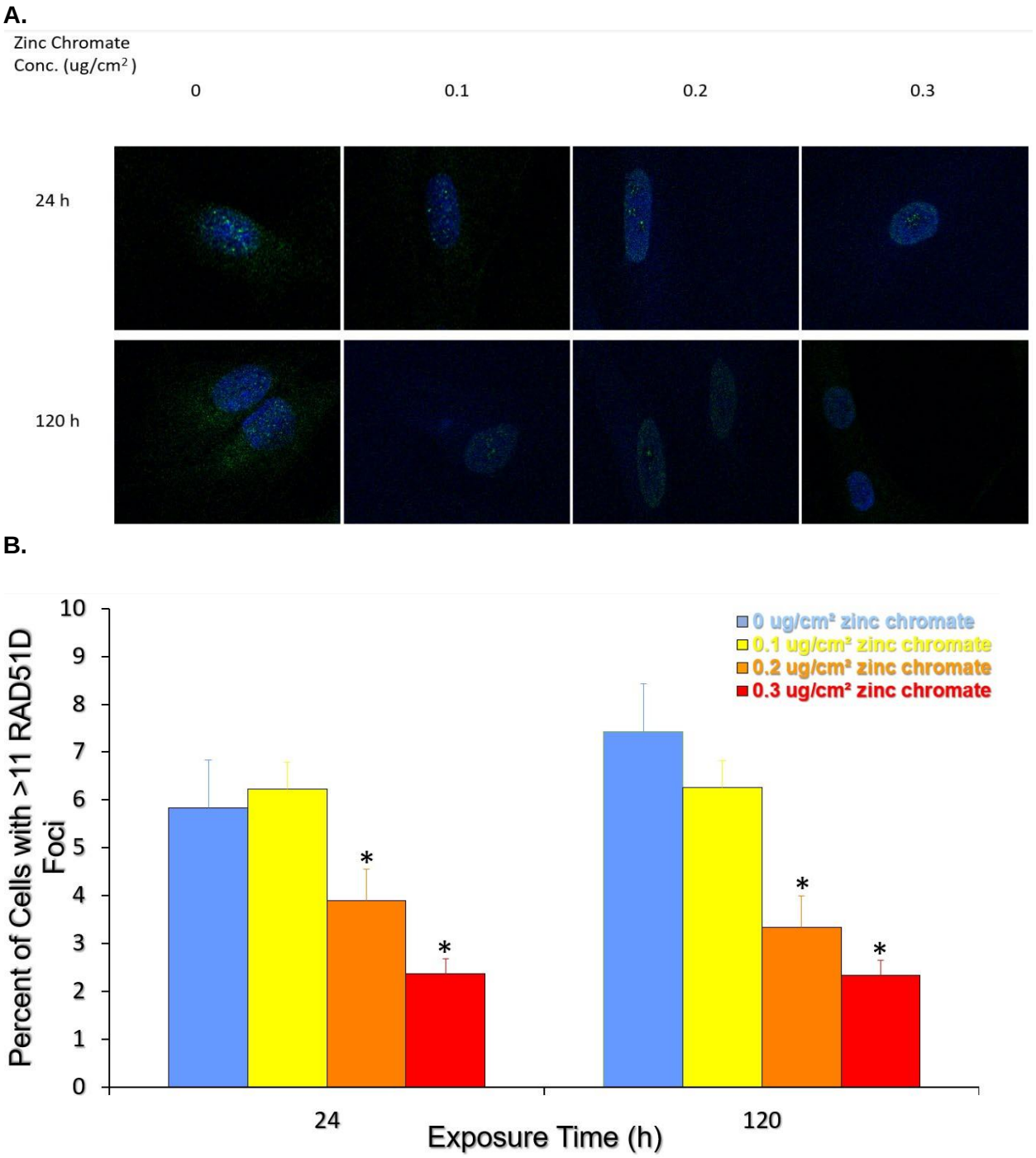
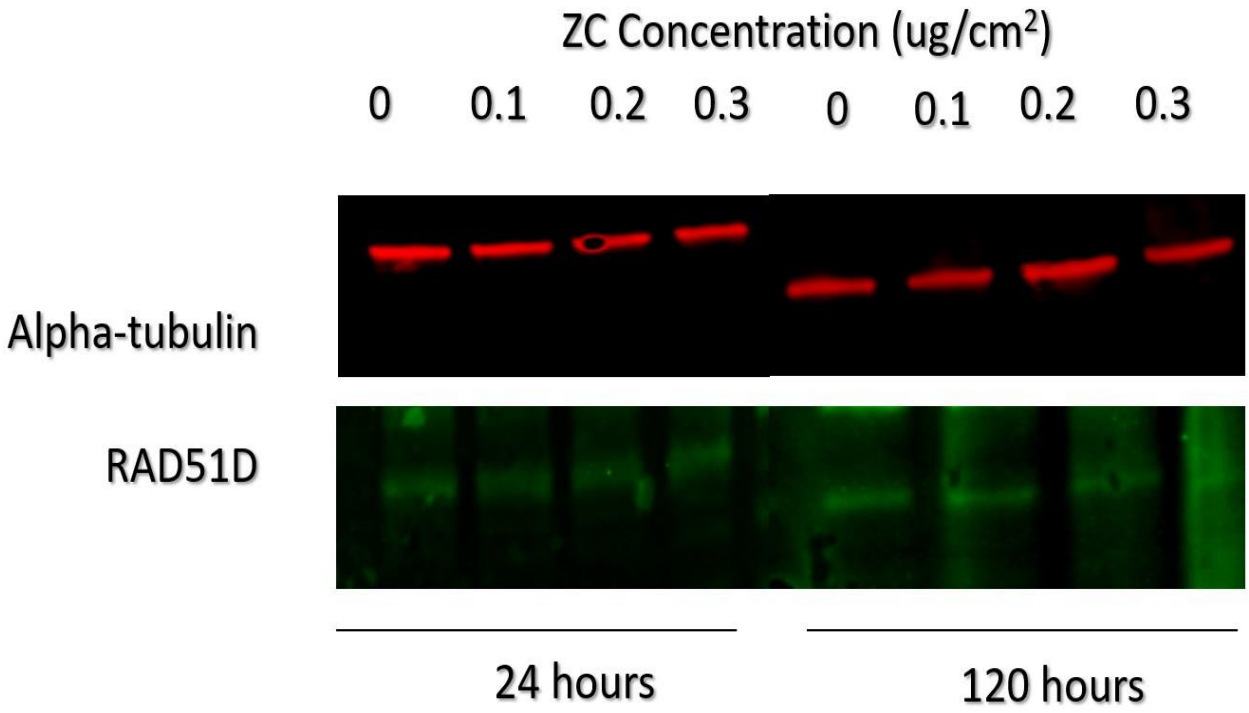


Figure 4. Particulate Cr(VI) Inhibits RAD51D Foci Formation.
This figure shows acute and prolonged zinc chromate exposure reduced RAD51D foci formation in a time and concentration-dependent manner. (**A**) representative immunofluorescence images of RAD51D foci. (**B**) Quantification of the percent of cells with RAD51D foci. Data represent the mean of three experiments. Error bars = standard error of the mean. *Statistically significant compared to control $p < 0.05$.

Particulate Cr(VI) Inhibits RAD51D Whole Cell Protein

To determine if the lack of RAD51D foci was due to a decrease in protein expression, whole cell RAD51D protein was measured by western blot (Figure 5A). Particulate Cr(VI) reduced whole cell RAD51D protein levels in a concentration-dependent manner after 24 and 120 h exposure (Figure 5B). After 24 h exposure, RAD51D protein levels decreased at 0.2, and 0.3 ug/cm^2 zinc chromate to 83 and 76% of control. An even greater reduction was seen after 120 h exposure as 0.1, 0.2, and 0.3 ug/cm^2 zinc chromate reduced protein levels to 60, 56 and 43%, respectively. At both time points, 0.2, and 0.3 ug/cm^2 were significantly lower than their respective controls ($P<0.05$).

A.



B.

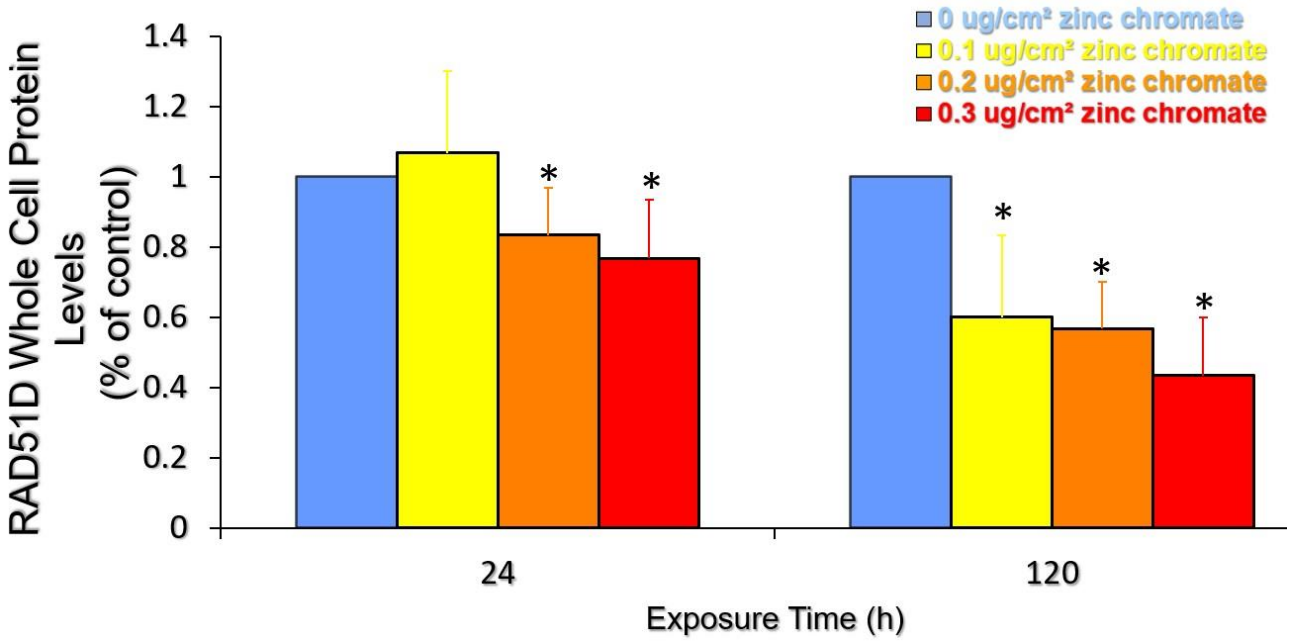


Figure 5. Particulate Cr(VI) Inhibits RAD51D Whole Cell Protein
This figure shows acute and prolonged zinc chromate exposure reduces RAD51D protein levels in a time and concentration-dependent manner. (**A**) representative western blot images of whole cell RAD51D protein expression (cropped from original image). (**B**) Quantification of RAD51D whole cell protein (relative to control). Data represent the mean of three experiments Error bars = standard error of the mean, *statistically significant compared to control $p< 0.05$.

Particulate Cr(VI) Inhibits RAD51D mRNA Levels.
We tested if Cr(VI) inhibits RAD51D mRNA levels using quantitative polymerase chain reaction (qPCR) following particulate Cr(VI) exposure. Particulate Cr(VI) inhibited RAD51D mRNA levels in a concentration-dependent manner after 24 and 120 h exposure (Figure 6). After 24 h, RAD51D mRNA was reduced to 0.66, 0.51, and 0.43 relative to control following 0.1, 0.2, and 0.3 ug/cm^2 zinc chromate. RAD51D mRNA remained low after 120 h decreasing to 0.54, 0.27, and 0.24 relative to control. After prolonged exposure of 120 h RAD51D mRNA was significantly reduced at all concentrations compared to control ($P<0.05$).

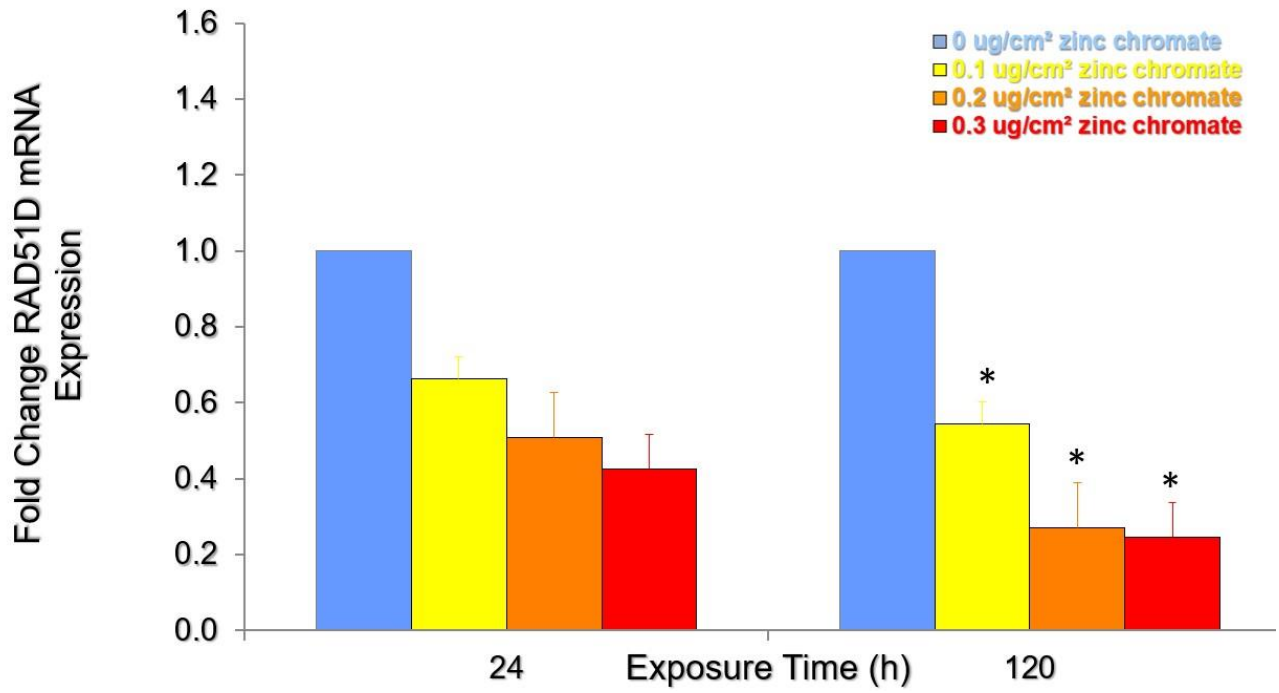


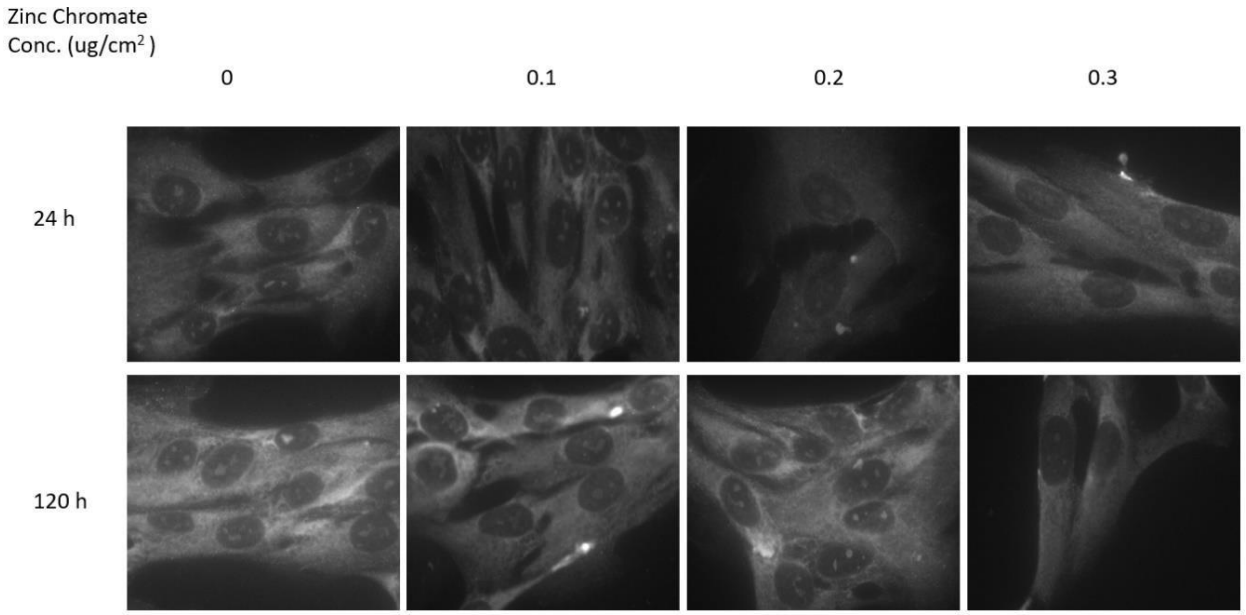
Figure 6. Particulate Cr(VI) Inhibits RAD51D mRNA Levels.
This figure shows acute and prolonged zinc chromate exposure reduces RAD51D mRNA levels in a time and concentration-dependent manner. Data represent the mean of three experiments Error bars = standard error of the mean, *statistically significant compared to control $p < 0.05$. Quantification of RAD51D whole cell protein (relative to control).

Particulate Cr(VI) Slightly Inhibits XRCC3 Foci Formation

We focused on XRCC3 as a representative of the CX3 complex. We evaluated the ability of Cr(VI) to inhibit the function of XRCC3 through foci formation using immunofluorescence (Figure 7A). Particulate Cr(VI) did not affect XRCC3 foci formation after 24 h exposure (2.9, 2.8, 2.6 percent) but slightly inhibited XRCC3 foci formation in a concentration-dependent manner after 120 h exposure (Figure 7B). Specifically, 0.2, and 0.3 ug/cm^2 zinc chromate reduced the percent of cells with more than 5 XRCC3 foci to 2.2, and 1.6 percent, respectively, compared to

2.8 percent in untreated controls. Although these small reductions were not statistically significant compared to control.

A.



B.

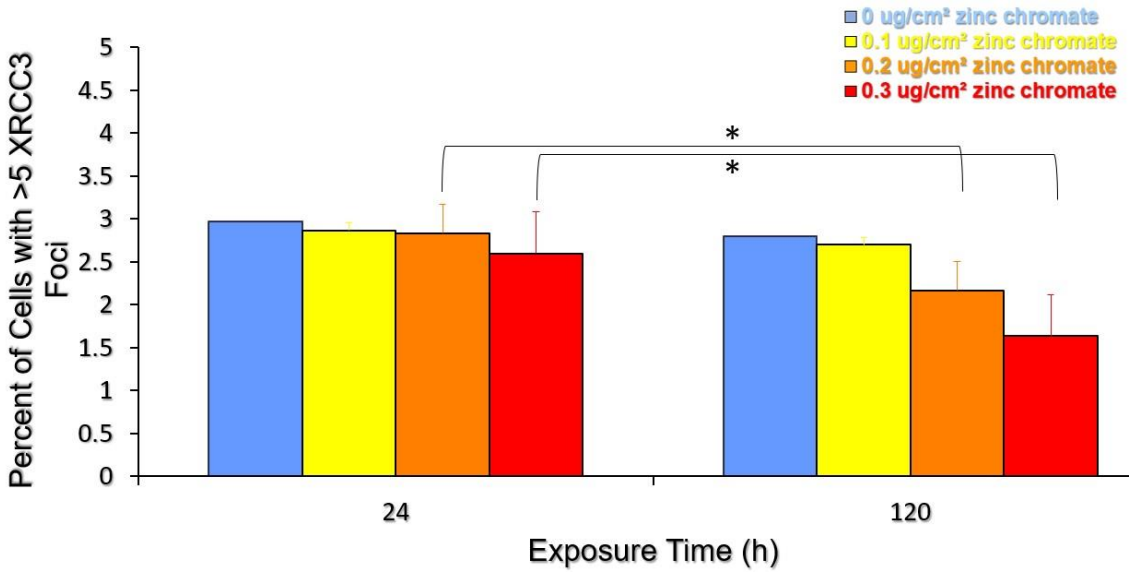


Figure 7. Particulate Cr(VI) Slightly Inhibits XRCC3 Foci Formation

This figure shows prolonged zinc chromate exposure slightly reduces XRCC3 foci formation in a concentration-dependent manner. **A**) representative immunofluorescence images of XRCC3 foci. (**B**) Quantification of the percent of cells with XRCC3 foci. Data represent the mean of three experiments Error bars = standard error of the mean.

DISCUSSION

Particulate hexavalent chromium Cr(VI), a human lung carcinogen, is an environmental contaminant that poses harm to human health; however, the mechanism of Cr(VI) carcinogenesis remains poorly understood. Cr(VI) induces chromosome instability, a hallmark event in lung cancer. The formation of DNA double strand breaks combined with DNA double strand break repair failure are major underlying events that lead to chromosome instability.

Previous studies show homologous recombination repair is the preferred mechanism for repairing Cr(VI) induced double strand breaks in DNA double strand breaks (Bryant et al., 2006; Stackpole el al., 2007; Xie et al., 2009; Tamblyn et al., 2009). Notably, particulate Cr(VI) causes DNA double strand breaks and prolonged exposure impairs homologous recombination by targeting the key protein, RAD51, in the effector step of this pathway (Qin et al., 2014). During homologous recombination, RAD51 is loaded onto single-stranded DNA, creating a helical nucleoprotein filament responsible for carrying out repair. Data show prolonged exposure to particulate Cr(VI) prevents RAD51 nucleofilament formation (Browning et al., 2016), however, how it causes the loss of this filament is poorly understood.

Because of the observations of loss of RAD51 nucleofilament formation, the first goal of this study investigated how Cr(VI) targets key complexes in homologous recombination repair critically important for the loading and stability of RAD51 onto the filament in human lung cells. We focused on RAD51D, because its depletion would represent a loss of function in the BCDX2 complex, which is the central mediator of RAD51 loading (Chun et al. 2013). We showed RAD51D foci formation decreased following both acute and prolonged particulate Cr(VI) exposure indicating a loss of function as early as 24 h after exposure. This finding is notable because RAD51 functions appropriately to 24 h Cr(VI) exposure, evidenced by an increase in the number and complexity of RAD51 nucleoprotein filaments (Browning et al., 2016). This outcome suggests there is sufficient RAD51 loaded onto the filament after 24 h, which agrees with the previous report of increased RAD51 foci formation following acute Cr(VI) exposure (Browning et al., 2016, Qin et al., 2014). Both RAD51D and RAD51 are suppressed after 120 h of exposure indicating the outcome worsens with longer exposure. RAD51D is responsible for the formation and stabilization of RAD51 nucleofilament formation. This protein is also involved in protecting the integrity of sequences during double strand breaks, and specifically protects against large deletion events. RAD51D stabilizes the RAD51 nucleoprotein filament upstream of its formation (Chun et al., 2013). Therefore, the observed inhibition of RAD51D's response suggests the defects in RAD51 nucleofilament formation results from Cr(VI) targeting RAD51D.

Only one other study showed Cr(VI)-induced inhibition of HR repair looking at

RAD51 paralogs in the BCDX2 complex. That study focused on RAD51C and found Cr(VI) exposure did not inhibit RAD51C after 24 h exposure as RAD51C foci increased in a concentration dependent manner after 24 h just as RAD51 did (Browning et al. 2016). However, with longer exposures RAD51C was also inhibited and suppressed. These data, when combined with the data in this study, suggest that RAD51D is targeted first followed by RAD51C with longer exposures. The loss of both proteins may exacerbate the RAD51 loss on the filament as data showed double depletion of components in the BCDX2 complex lead to additional loss of RAD51 foci formation (Chun et al., 2013). The BCDX2 complex has been reported to form subcomplexes (BC and DX2) (Sigurdsson et a1., 2001). Data indicate the DX2 subcomplex may have a greater role than the BC subcomplex in RAD51 filament stabilization (Chun et al., 2013), which would be consistent with our results showing RAD51D as the primary target.

How Cr(VI) impairs RAD51D is uncertain. We showed RAD51D protein expression decreases following both acute and prolonged exposures. The decrease in RAD51D protein levels could be a result from either decreased protein production or protein degradation. However, we also found RAD51D mRNA expression decreased following both exposures, which suggests loss of protein synthesis is the likely explanation.

The second goal of this study was to investigate XRCC3 as a representative of the CX3 complex in human lung cells, which functions downstream of RAD51

recruitment. We focused on XRCC3, because its depletion or deletion would represent a loss of function in the CX3 complex (Chun et al. 2013). Our understanding of the interaction between RAD51C and XRCC3 is limited due to very few studies reported. One genetic study looking at CX3 complex in treated DT40 cells confirmed RAD51C/XRCC3 functions at later stages of replication dependent repair induced by camptothecin and cisplatin (Yonetani et al., 2005). Another study showed the CX3 catalyzes strand exchange *in vitro* suggesting this complex may be important for the catalysis of homologous pairing between homologous chromosomes during recombination repair (Kurumizaka et al., 2001). We found no significant effects on XRCC3 following Cr(VI) exposure suggesting this protein is not a major target for Cr(VI). However, RAD51C is part of the CX3 and data show RAD51C depletion has a more pronounced effect on the CX3 complex than on the BCDX2 complex in U2OS cells (Chun et al., 2013). Thus, considering the results previously reported (Chun et al., 2013), it is likely the CX3 complex is targeted by Cr(VI) due to Rad51C loss, which would impact the repair of any cells that escape the loss of the BCDX2 complex.

SUMMARY AND CONCLUSIONS

Strengths of this work

This work is pioneering in that currently there are no data investigating the effects of Cr(VI) on RAD51 paralog complexes. This is the first study to evaluate RAD51 paralogs function after Cr(VI) exposure in human cells. The primary target of particulate Cr(VI) exposure is the lung. We evaluated how particulate Cr(VI) altered RAD51 paralogs in human lung cells after acute and prolonged Cr(VI) exposures.

Future Directions

This work requires more mechanistic data to gain better understanding of how Cr(VI) affects RAD51 and its paralogs in humans. More mechanistic data investigating how Cr(VI) exposure affects other members of the BCDX2 complex and consequences of impaired RAD51D will be further investigated. Future work will consider the impact of Cr(VI) on other members of the RAD51 paralog complexes and the mechanism of Cr(VI)- induced BCDX2 inhibition of homologous recombination repair in metal carcinogenesis.

Conclusions

Overall, our data modify the mechanism for Cr(VI) carcinogenesis to show first Cr(VI) targets RAD51D resulting in loss of RAD51 leading to reduced homologous recombination repair response resulting in chromosome instability and ultimately carcinogenesis.

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CURRICULUM VITAE
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Education

2019-current Ph.D. in Pharmacology, University of Louisville, Louisville, KY.

2014-2018 B.A. in Chemistry with a focus in Pre-pharmacy, Eastern Kentucky University, Richmond, KY

Current Appointments

Member, Society of Toxicology (SOT)

Member, Ohio Valley Chapter, Society of Toxicology (OVSOT)

Member, Metals Journal Club

Member, Environmental Mutagenesis & Genomics Society (EMGS)

Professional Experience

2019-Present Graduate Fellow, Wise Lab, Pharmacology and Toxicology, University of Louisville

2018-2019 Manufacturing Peptide Chemist, Creosalus Advanced Chem-Tech, Louisville

2017-2018 Independent Study Lab Assistant, Eastern Kentucky University

Student Mentoring

2021 Mentor, Cates Doyle, Georgetown University

Honors, Awards and Professional Activities:

Awards

2022 Center for Integrative Environmental Health Sciences Travel Award

2022 2nd Place SOT Metals Specialty Section Graduate Student Research Award

2022 Bristol Myers Squibb Graduate Student Research Training Award to Promote Diversity in Toxicology, Society of Toxicology (SOT)

2021 Toxicology Excellence for Risk Assessment (TERA), University of Cincinnati

2021- current Predoctoral T32 Environmental Health Sciences Training Grant Fellowship, National Institute of Environmental Health Sciences

2021 Graduate Student Council Fall Research Travel Grant, University of Louisville

2021 Graduate Student Travel Award, University of Louisville

2021 Graduate Student Council Spring Research Travel Grant, University of Louisville

2021 GTA "Exemplary Abstract" Award, Genetic Toxicology Association (GTA) Education Award

2021 First place, People Choice Award "3 Minute Thesis" Competition, Society of Toxicology (SOT)

2020 Graduate Student Council Fall Research Travel Grant, University of Louisville

2020 First Place, Masters Student Poster Presentation Award, Ohio valley Chapter, Society of Toxicology (OVSOT)

2019-2021 IPIBS Fellowship (full tuition and stipend), University of Louisville

2018 Licensed Pharmacy Technician

2016-2018 **McNair-EKU Presidential Scholarship Eastern Kentucky University, Richmond KY.**

Awards Received by Student During My Mentorship

2021 Third Place (Cates Doyle), 90 Second Elevator Pitch, University of Louisville

2021 Finalist (Cates Doyle), Poster Presentation, University of Louisville

Abstracts

Extramural Abstracts

1. **Williams, A. R,** Collins, K., Calderon, L. Dose Dependent Decrease in Tumor Size bt Pt-Mal-LHRH Treatment. Presented poster at the 23rd Annual Ronald E. McNair Undergraduate research conference at the University of Buffalo. Niagara Falls, NY August 2017

2. **Williams, A. R.** Collins, K., Calderon, L. Correlation with Reduced Tumor Levels of Pt-Mal-LHRH. Presented at Minority Access 18th annual Conference for STEM research at the Marriott Marquis conference Center, Washington, DC. September 2017

3. **Williams, A. R.** Collins, K., Calderon, L. Attenuating Breast Cancer Tumor Growth Mediated by Dose Dependent Treatment of Pt-Mal-LHRH. Presented poster at the 17th annual Posters at the Capitol, Capitol Building, Frankfort, Kentucky. February 2018

4. **Williams, A.R.,** Speer, R.M., Browning, C. Meaza, I., Toyoda J., and Wise, Sr., J. P. Particulate Hexavalent Chromium Inhibits DNA Repair by Targeting RAD51 Paralogs. Presented at the summer meeting of the Ohio Valley Society of Toxicology (OVSOT), July 2020.

5. **Williams, A.R.,** Browning, C.L. Meaza, I., Toyoda, J., and Wise, Sr., J.P. RAD51 Paralogs Are Key Targets for Loss of Homologous Recombination Repair in Metal
   Carcinogenesis. Presented at the annual meeting of the Ohio Valley Society of Toxicology (OVSOT), November 2020.

6. **Williams, A.R.,** Speer, R.M., Browning, C.L., Meaza, I., Toyoda, J., and Wise, Sr., J.P. Particulate Hexavalent Chromium Inhibits DNA Repair by Targeting RAD51 Paralogs. Toxicological Sciences, 180:2081, 2021.

7. **Williams, A.R.,** Speer, R.M., Browning, C.L., Meaza, I., Toyoda, J., and Wise, Sr., J.P. Lung Cancer Secret Revealed! RAD51D: Culprit Protein Found! Presented at the annual meeting of the Society of Toxicology (SOT), April 2021

8. **Williams, A.R.,** Speer, R.M., Browning, C.L., Meaza, I., Toyoda, J., and Wise, Sr.,
   J.P. Particulate Hexavalent Chromium Inhibits RAD51 Paralog Proteins-Key to Lung Cancer Progression. Presented at the annual meeting of the Genetic Toxicology Association, May 2021.

9. Meaza, I., Toyoda, J.H., Lu, H., **Williams, A.R.,** Wise, S.S., and Wise Sr. J.P. Particulate Hexavalent Chromium Induces Loss of RAD51 Leading to Increased Genomic Instability, A Driver of Carcinogenesis. Environmental and Molecular Mutagenesis Volume 62, Issue S1, p57, 2021.

10. **Williams, A.R.,** Speer, R.M., Browning, C.L., Meaza, I., Toyoda, J., and Wise, Sr.,
    J.P. Particulate Hexavalent Chromium Targets RAD51 Paralogs Leading to Loss
    of Homologous Recombination Repair in Metal Carcinogenesis. Environmental and Molecular Mutagenesis Volume 62, Issue S1, p.63, 2021.

11. **Williams, A.R.,** Speer, R.M., Browning, C.L., Meaza, I., Toyoda, J., and Wise, Sr.,
    J.P. Particulate Hexavalent Chromium Induces Loss of the BCDX2 Complex Leading to Loss of Homologous Recombination Repair. Presented at the annual meeting of the Ohio Valley Chapter of the Society of Toxicology (OVSOT), November 2021.

12. Meaza I., Toyoda, J.H., Lu, H., **Williams, A.R.,** and Wise Sr. J.P. Particulate Hexavalent Chromium Causes DNA Repair Inhibition Leading to Increased Chromosome Instability in Human Bronchial Epithelial Cells. Presented at the annual meeting of the Ohio Valley Chapter of the Society of Toxicology (OVSOT), November 2021.

13. **Williams, A.R.,** Speer, R.M., Browning, C.L., Meaza, I., Toyoda, J., and Wise, Sr.,

J.P. Particulate Hexavalent Chromium Exposure Suppresses BCDX2 Complex Response in Human Lung Cells. To be presented at the annual meeting of the Society of Toxicology (SOT), March 2022.

14. Meaza I., Toyoda, J.H., Lu, H., **Williams, A.R.,** and Wise Sr. J.P. ChromateInduced Loss of RAD51 and Increased Chromosome Instability in Human Bronchial Epithelial Cells. To be presented at the annual meeting of the Society of Toxicology (SOT), March 2022.

15. Toyoda, J.H., Speer, R.M., Meaza, I., Lu, H., Kouokam, J.C., **Williams, A.R.,** and
Wise, Sr., J.P. Hexavalent Chromium Induces Numerical Chromosome Instability Via Securin Disruption in Human Cells but Not in Whale Cells. To be presented at the annual meeting of the Society of Toxicology (SOT), March 2022.

16. Meaza, I., Toyoda, J.H., Lu. H., **Williams, A.R.,** Kouokam, J.C., and Wise, Sr., J.P. Missing Protein! Have You Seen it? Reward: to Cure Cancer. To be presented at the Annual Meeting of the Society of Toxicology (SOT), March 2022.

17. **Williams, A.R.,** Toyoda, J.H., Speer, R.M., Wise, S.S. and Wise, Sr., J.P. Particulate Hexavalent Chromium Targets RAD51D to Inhibit Homologous Recombination Repair. In Preparation.

18. Meaza, I., **Williams, A.R.,** Toyoda, J.H., Wise, S.S. and Wise, Sr., J.P. Particulate Hexavalent Chromium Induces Chromosome Instability, and Inhibits Homologous Recombination Repair in Human Lung Epithelial Cells. In Preparation.

Intramural Abstracts

1. Doyle, C., **Williams, A.R.,** Browning, C.L., Wise, S. and Wise, Sr., J.P. Particulate Hexavalent Chromium Exposure Induces Persistent and Heritable Loss of RAD51D. Presented at Research!Louisville, July 2021.

2. Meaza, I., Toyoda, J.H., Lu, H., **Williams, A.R.,** Wise, S.S. and Wise Sr. J.P. Particulate Hexavalent Chromium Targets RAD51, the Key Protein in Homologous
Recombination Repair, Leading to Increased Genomic Instability, A Driver of Carcinogenesis at Research Louisville! Presented at Research!Louisville, October 2021.

3. **Williams, A.R.,** Meaza, I., Toyoda, J.H., Speer, R.M. Browning, C.L. and Wise, Sr., J.P. Particulate Hexavalent Chromium Targets the BCDX2

Complex in Homologous Recombination Repair. Presented at Research!Louisville, October 2021.

Seminars/Presentations

1. Speaker: “Correlation with Reduced Tumor Levels of Pt-Mal-LHRH” Presented at Minority Access 18th annual Conference for STEM research at the Marriott Marquis conference Center, Washington, DC. September 2017

2. Speaker: 19th Annual University of Maryland National Conference for McNair scholars and Undergraduate Research College Park, MD, March 2018

3. Speaker: “Chromium Exposure Disrupts RAD51 Nucleoprotein Filament Formation: A Defining Step in Homologous Recombination Repair Pathway”. Presented at the
University of Louisville Pharmacology and Toxicology Departmental Seminar, April 2020

4. Speaker: “Particulate Hexavalent Chromium Exposure Inhibits Homologous Recombination Repair by Targeting RAD51 paralog proteins in Human Lung Fibroblast”. Presented at the University of Louisville Pharmacology and Toxicology Departmental Seminar, April 2021

Meetings Attended

2020 Ohio Valley Chapter, Society of Toxicology (SOT) Annual Meeting

2021 Society of Toxicology (SOT) Annual Meeting
Ohio Valley Chapter, Society of Toxicology (SOT) Annual Meeting
Environmental Mutagenesis & Genomics Society (EMGS)
Genetic Toxicology Association Education Award, (GTA)
Annual Biomedical Research Conference for Minority Students (ABRCMS).