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Molecular mechanisms of hexavalent chromium-induced centrosome amplification.

Jennifer Haruka Toyoda
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MOLECULAR MECHANISMS OF

HEXAVALENT CHROMIUM-INDUCED

CENTROSOME AMPLIFICATION

By

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B.A., University of Massachusetts Amherst, 2005
B.S., University of Kentucky, 2011
M.S., University of Louisville, 2018

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A Dissertation Approved on
June 21, 2022

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DEDICATION

This dissertation is dedicated to everyone who has a dream they are taking courage to chase. Be proud of the unique shape of your life's path. It is never too late to start something new. Whatever you do, believe in yourself, and listen to the voice inside.

There is a voice inside of you
That whispers all day long,
"I feel this is right for me,
I know that this is wrong."
No teacher, preacher, parent, friend
Or wise man can decide
What's right for you–just listen to
The voice that speaks inside.

- Shel Silverstein

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ABSTRACT

MOLECULAR MECHANISMS OF
HEXAVALENT CHROMIUM-INDUCED
CENTROSOME AMPLIFICATION

Jennifer H. Toyoda

June 21, 2022

Lung cancer is the deadliest form of cancer and resulted in 1.8 million deaths worldwide in 2020. While cigarette smoking is the most familiar cause of lung cancer, up to 25% of cases occur in non-smokers, thus other environmental agents are also causative. Hexavalent chromium [Cr(VI)] is a known lung carcinogen and poses occupational and environmental exposure risks relevant to humans, wildlife, and ecosystems. This dissertation considers the carcinogenic mechanisms of a highly potent, particulate, hexavalent chromium compound, zinc chromate.

The molecular mechanism of carcinogenesis induced by Cr(VI) is not fully understood, but it is known that chromosome instability is a key effect. Chromosome instability refers to structural instability characterized by breaks and translocations, and numerical instability characterized by changing numbers of

chromosomes. This dissertation focuses on how hexavalent chromium causes numerical chromosome instability in human lung cells and uses the One Environmental Health approach to gain insights into the associated mechanism using whale cells as a comparative model. The hypothesis of this project is: Prolonged hexavalent chromium exposure targets securin in human lung cells, leading to centrosome amplification and numerical chromosome instability, while the ability of whale cells to retain normal securin levels confers resistance to these effects.

A main driver of numerical chromosome instability is centrosome amplification, defined in this study as a single cell with more than two centrosomes. We previously found Cr(VI) induces centrosome amplification, which increased with duration and concentration of exposure and correlated with Cr(VI)-induced aneuploidy. In Aim 1 we focused on a novel potential target of Cr(VI), securin. Securin is an important centrosome regulator because it is the canonical inhibitor of separase. Separase is the enzyme that causes centriole disengagement and permits centrosome duplication. Prolonged Cr(VI) exposure decreased securin protein levels in a dose-dependent manner. Securin protein loss was not due to changes in protein degradation, but rather a loss of securin mRNA. Three measures of securin function were analyzed to determine if decreased securin levels were sufficient to control separase activity. Separase cleaves itself, kendrin, and cohesin. Prolonged Cr(VI) exposure caused increased separase autocleavage, increased kendrin cleavage, and increased separation at centromeres caused by cohesin cleavage. Securin knockdown increased levels of

aneuploidy after acute Cr(VI) exposure, in contrast to untransfected cells which retained normal background levels. Together these data showed Cr(VI) disrupts securin, a key protein in the maintenance of numerical chromosome stability.

Aim 2 sought to uncover the mechanism of Cr(VI)-induced securin loss described in Aim 1. Transcription factors bind to gene promoter regions to enhance or inhibit gene transcription and have been shown to be disrupted by Cr(VI). Thus, we measured three securin-promoting transcription factors, Sp1, NF-YA, and E2F1, and two repressing transcription factors, KLF6 and p53. Cr(VI) elevated levels of securin-promoting factors in nuclear fractions, indicating Cr(VI) is not repressing their levels to decrease securin expression. E2F1 was previously shown to decrease with prolonged Cr(VI) exposure, but experimental knockdown revealed E2F1 is not a driving factor for Cr(VI)-induced securin loss. Securin expression loss was also not explained by p53 activation, as protein levels and phosphorylation did not correlate with securin loss. KLF6 nuclear protein levels were increased at prolonged timepoints, which may begin to explain reduction in securin levels. MicroRNA (miRNA) regulation is altered by Cr(VI) and is a possible mechanism for securin loss. Several miRNAs were found to be significantly up- and down-regulated at all tested concentrations and timepoints. Securin was not specifically targeted by any of the altered miRNAs. However, several centrosome-associated proteins are putative targets of Cr(VI)-induced miRNA alteration.

The One Environmental Health approach acknowledges humans, wildlife, and ecosystems are connected by a shared environment and common toxicants such as Cr(VI). Aim 3 characterizes the effects of prolonged Cr(VI) exposure on

whale cells. We measured securin levels and function in bowhead whale cells and found securin levels remained normal under all exposure conditions and neither premature centromere separation nor centrosome amplification were increased. We found Cr(VI) did not cause aneuploidy in sperm whale or bowhead whale cells. Interestingly, chromosome damage still occurred, indicating Cr(VI) is active inside whale cells. These data indicate maintenance of securin under Cr(VI) exposure may protect whale cells from developing centrosome amplification and numerical chromosome instability.

Overall, this dissertation shows securin is targeted by prolonged Cr(VI) exposure in human lung cells and centrosome regulation pathways are central in the mechanism of Cr(VI) carcinogenesis. We showed whales are resistant to securin loss and the proposed downstream phenotypes, centrosome amplification and numerical chromosome instability. Together these data support the hypothesis Cr(VI)-induced securin loss leads to numerical chromosome instability.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1: INTRODUCTION

Chromium Use and Exposure Routes

Chromium is one of the top 10 most abundant minerals in Earth's crust and is most often encountered at one of its two most stable forms, trivalent [Cr(III)] or hexavalent [Cr(VI)] (Jacobs & Testa, 2005). Naturally-occurring chromium is largely in the form of Cr(III) and enters the environment through natural processes such as volcanic eruption and erosion (Tchounwou et al., 2012). Cr(VI) is mostly man-made and used for industrial purposes. Due to the unique chemical and physical properties of Cr(VI) compounds, discontinuation of their use or replacement is challenging, if not impossible. The volume of production and use of Cr(VI) continues to increase and consequently, waste, by-products, and leaching from commercial products cause Cr(VI) to become ubiquitous in the environment. The human health risks of Cr(VI) was first studied in chromate workers (Langård and Vigander, 1983; Lindberg and Hedenstierna, 1983; Moller et al., 1986; Ishikawa et al., 1994). Evaluation of health impacts related to environmental levels of Cr(VI) contamination are still in need of investigation.

Chromate compounds provide resistance to corrosion and fouling and produce salts of bright colors. They have broad industrial usage including in pigment production, paints, anti-corrosives, leather tanning, wood preservatives,

cement mixtures, electroplating, stainless steel welding, and metal processing. Contamination arises from manufacturing wastes, mining waste, toxic dust, and degradation of paints and coatings (Jacobs & Testa, 2005; NIOSH, 2013). In addition to primary chromate use, Cr(VI) is released as a by-product from waste incineration, diesel fuel and coal combustion, and cooling towers (Kingston et al., 2005; Wang et al., 2003; Astrup et al., 2005; Parr et al., 1976).

Exposure can occur through inhalation, dermal contact, or ingestion of contaminated water or soil. Carcinogenicity by oral exposure is controversial. Some have suggested high rates of reduction to Cr(III) in saliva and stomach acid should reduce absorption of Cr(VI) (Proctor et al., 2002; Paustenbach et al., 2003). Indeed, some studies have shown lower rates of Cr absorption and DNA damage biomarkers after oral exposure (De Flora et al., 2008; Kuykendall et al., 1996; Costa, 1997). However, these results may be due to the timeframe of the studies, as prolonged exposure to animals caused hyperplasia and metaplasia of stomach and small intestine (Bucher, 2007) and human populations exposed to contaminated water had high rates of cancer deaths (Linos et al., 2011; Beaumont et al., 2008). Dermal exposure is known to cause allergic contact dermatitis, but serious injury by this route is rare (Lin et al., 2009; Paustenbach et al., 2003). Cr(VI) primarily targets the respiratory system. Over 1.3 million workers are reportedly exposed to airborne Cr(VI) in Europe and the United States alone (NIOSH, 2013; IARC, 2012). Many countries do not have adequate reporting to estimate worker risks and do not regulate occupational exposure as in the United States and Europe. Additional to occupation risks, in the United States, industrial sources

release up to 2,900 tons of chromium into the atmosphere (ATSDR, 2012), and total global atmospheric emission is estimated at 58,000 to 112,000 tons per year (Johnson et al., 2006). Approximately one third of these emissions are in the hexavalent state (Johnson et al., 2006). Thus, potential Cr(VI) exposure is a concern for anyone living near an industrial area, and effects of low, chronic levels of airborne-Cr(VI) are relatively unknown.

Physical and Chemical Properties of Chromium

Chromium exists in valence states from chromium (0) to chromium (VI). Cr(0), Cr(III), and Cr(VI) are environmentally stable. Cr(0) is metallic chrome, used for making steel. Cr(III) and Cr(VI) form brightly colored salts of variable solubilities. The reason human health risks center around Cr(VI) exposure and not Cr(III) is due to chemical and toxicokinetic differences of the valence states. Cr(III) readily binds to extracellular molecules and evidence suggests this hinders its entry into cells (Wetterhahn and Hamilton, 1989; Cohen et al., 1993). However, due to structural mimicry of phosphate and sulfate, Cr(VI) enters cells via facilitated diffusion through anion channels (Jennette, 1981). Gao et al. (1993) measured Cr distribution in various rat tissues after intratracheal instillation and found elevated Cr levels in whole blood, plasma, and urine after Cr(VI) but not Cr(III) treatment, lending evidence to lower absorption of Cr(III) compared to Cr(VI). The Cr(VI) anion specifically generated outside of the cell has been shown to be responsible for clastogenesis. Cr(VI) particles can be phagocytosed by cells, but particle internalization without extracellular dissolution does not contribute to clastogenesis

(Xie et al., 2004). Also, after extracellular dissolution, the cation of the chromate salt can enter the cell, but it has no apparent contribution to the carcinogenic effect of the Cr(VI) compound (Wise et al., 2004).

Chromium is proposed to damage cells by two approaches. First, inside the cell, Cr(VI) is rapidly reduced to Cr(III) by agents such as ascorbate and glutathione, producing chromium intermediate species and reactive oxygen species that can damage intracellular molecules (Standeven and Wetterhahn, 1989; Suzuki, 1990; Hu et al., 2016). Secondly, the ability of Cr(III) to bind to proteins and guanine bases raises the possibility for direct interactions to damage molecular targets (Brown et al., 2020; Hneihen et al., 1993; Peterson et al., 2008). Bound Cr(III) is unable to leave the cell or penetrate the plasma membrane (OSHA, 2006). Intracellular depletion of Cr(VI) by reduction favors Cr(VI) diffusion into the cell. Chromium-biomolecule complexes have been implicated in protein interference as well as severe DNA damage.

Cr(VI) compounds vary in solubility which influences their carcinogenic potential. Fully soluble chromates such as sodium chromate and potassium chromate are relatively less carcinogenic than water-insoluble chromates such as lead chromate and barium chromate (Bragt and van Dura, 1983; Levy et al., 1986). Differences in toxicity seem to correlate to the residence time and elimination time of the various compounds. Insoluble Cr(VI) particles lodge at bronchial bifurcation sites where they persist and release ions over a long period of time (ATSDR, 2012; OSHA, 2006). The efficiency of particle elimination by the mucociliary clearance mechanism depends on the size of the particle, location of deposition, and the

health of the individual (OSHA, 2006). Bragt and van Dura (1983) tested a range of soluble and insoluble chromates by intratracheal instillation in rats. They found soluble compounds are absorbed more quickly and thus soluble chromate levels in the lung decrease more rapidly than particulate chromates. They also concluded slightly soluble compounds such as zinc chromate were more potent than soluble sodium chromate or insoluble lead chromate. Compounds classified as “slightly soluble” possess the particulate nature that incurs long residence times in the lung, but increased solubility that causes greater local ion concentrations than highly insoluble particulates. Cell culture studies also showed insoluble Cr(VI) is more potent than soluble chromates (Patierno et al., 1989; Wise et al., 2006).

Chromium Carcinogenesis

Cr(VI) is a category 1 carcinogen recognized by the IARC, meaning sufficient evidence concludes that it is carcinogenic to humans. It is listed among the top 20 on the Agency for Toxic Substances and Disease Registry (ATSDR) Substance Priority List, which prioritizes substances based not only on their toxicity, but on their abundance and risk of human exposure (ATSDR, 2020). In 2006, the Occupational Safety and Health Administration (OSHA) recognized the dangers of Cr(VI) and reduced occupational exposure limits to 5 $\mu g/m^3$ of air (OSHA, 2006).

Cr(VI) is a potent carcinogen, as demonstrated by numerous epidemiological, animal, and cell culture studies. High rates of lung cancer have been observed in chromate pigment workers since the 1930s (Machle and

Gregorius, 1948; Baetjer, 1950). Follow-up studies of workers employed at a Norwegian zinc chromate pigment plant between 1948 and 1972 show that 6 of 24 workers employed over 3 years, and 6 of 18 workers exposed for over 5 years developed bronchial carcinomas in excess of the expected local rates (Langård & Vigander, 1983). Intrabronchial pellet implantation of hexavalent chromates in rat lungs produced bronchial carcinomas and demonstrated the carcinogenic potential of several species of Cr(VI) (Levy et al., 1986). Cell culture studies show that Cr(VI) causes DNA breaks, suppresses DNA repair, and contributes to aneuploidy (Xie et al., 2005; Holmes et al., 2006; Wise and Wise, 2012; Browning et al., 2016). Particulate Cr(VI) caused loss of contact inhibition and anchorage-independent growth in human lung fibroblasts (Xie et al., 2008) and human lung epithelial cells (Xie et al., 2007).

Cr(VI)-Induced Chromosome Instability

A major theory of Cr(VI) carcinogenesis is chromosome instability (CIN) This theory is based on evidence that low rates of common oncogene mutations are found in Cr(VI)-induced lung tumors, yet CIN is a common and early effect of Cr(VI) exposure (Hirose et al., 2002; Kondo et al., 1997; Holmes et al., 2008; Proctor et al., 2014; Wise et al., 2018; Wise et al., 2010). CIN has two main categories: 1) structural CIN, featuring chromosome breaks and translocations, and 2) numerical instability characterized by the loss or gain of entire chromosomes (Funk et al., 2016; Palumbo and Russo, 2016; Negrini et al., 2010). CIN entails a dynamic process manifesting as a changing karyotype (Duesberg et

al., 1998). The outcome of CIN can be measured as alterations in chromosome structure or copy number, representing snapshots in time that evidence the process of CIN. Structural or numerical aberrations alone are not synonymous with CIN (Valind et al., 2013). For example, the immortalized human bronchial epithelial cell line, BEP2D, features stable monosomy of chromosomes 12 and 13 and a stable marker chromosome of the translocation of 12q and 13q (Willey et al., 1991, Weaver et al., 1997). Such a stable translocation does not constitute structural CIN. Similarly, in the condition of Down Syndrome stable trisomy 21 causes an aneuploid karyotype of 47 chromosomes but does not cause dynamic mis-segregation of chromosomes as in numerical CIN (Valind et al., 2013, Schukken and Fiojer, 2018). In this dissertation, we will use the term aneuploidy to refer to a singular measurement of non-diploid chromosome number, and the term numerical CIN refers to fluctuating chromosome numbers.

It is unknown precisely how Cr(VI) causes CIN, but it is an early event after Cr(VI) exposure, and a hallmark of cancer (Holmes et al., 2006; Wise et al., 2010; Hanahan and Weinberg, 2011). Cr(VI) causes DNA strand breaks, chromosome breaks, gaps, and translocations, measured by single-cell gel electrophoresis, chromosome aberration assay, and gamma-H2Ax immunofluorescence (Wise et al., 2002; Xie et al., 2005; Wise et al., 2006; Xie et al., 2009; Ha et al., 2004). Mechanisms of DNA damage are not entirely understood. Cr(VI)-induced structural chromosome damage has been linked to formation of DNA lesions, abasic sites, potential replication fork stalling, and loss of DNA repair mechanisms

(Slade et al., 2005; Messer et al., 2006; Ha et al., 2004, Browning et al., 2016; Browning et al., 2017).

How numerical instability arises is not fully understood but it is known that mitotic disruption can cause asymmetrical segregation of chromosomes, resulting in aneuploid daughter cells. Numerical CIN is the most common form of CIN in cancer (Vasudevan et al., 2021; Brinkley, 2001). While aneuploidy can confer loss of fitness, the dynamic nature of CIN allows genetic variation within cell populations from which cancer hallmarks can emerge (Stingele et al., 2012; Duijf and Benezra, 2013). Gain or loss of chromosomes causes gene imbalances that perturb pathways critical to genomic stability, such as DNA repair, cell cycle regulation, and DNA segregation (Vasudevan et al., 2021; Pavelka et al., 2010; Stingele et al., 2012; Duijf and Benezra, 2013). Rate of tumor progression is influenced by dramatic shifts in ratios of tumor suppressor genes or oncogenes incurred by chromosome gains and losses (Fukasawa, 2005).

Centrosome Amplification Drives Numerical Chromosome Instability

Aneuploidy is usually caused by chromosome segregation errors, which result from failure of cytokinesis, or from mitotic disruptions such as loss of checkpoint control, kinetochore attachment errors, and centrosome amplification (Fukasawa, 2005). Centrosome amplification in this study is defined as a single cell having more than two centrosomes. Centrosome amplification has not been investigated in tumors caused by Cr(VI), but it is observed in most solid and hematological cancers (Chan, 2011). Cell culture studies show Cr(VI)-induced

centrosome amplification is the likely driver of numerical CIN (Holmes et al., 2006; Holmes et al., 2010; Bian et al., 2022). Aberrant mitosis and centrosome amplification correlate with Cr(VI)-induced numerical CIN in human lung fibroblasts (Holmes et al., 2006; Martino et al., 2015). Cr(VI)-transformed BEP2D cells, identified by loss of contact inhibition and gain of anchorage-independent growth, exhibited numerical CIN as well as centrosome amplification (Xie et al., 2007). Other known metal carcinogens such as arsenic, and cadmium also induce centrosome amplification and numerical CIN (Wise & Wise, 2010; Zhang et al., 2019; Ochi, 2002; Yih et al., 2006). Thus, numerical CIN is a key event in Cr(VI) carcinogenesis, and metal carcinogenesis in general, and centrosome amplification should be investigated further as a mechanism for its induction.

Centrosomes in Carcinogenesis

Cr(VI) causes numerical chromosome instability, likely through its ability to induce centrosome amplification. How Cr(VI) causes centrosome disruption is unknown and is the focus of this dissertation. Previous work shows Cr(VI) causes premature centriole disengagement which is known to cause centrosome overduplication (Martino et al., 2015; Tsou and Stearns, 2006a,b). Centrosomes are a relatively recent focus of Cr(VI) research. Only four studies address centrosome-specific effects of Cr(VI) (Holmes et al., 2006; Holmes et al., 2010; Martino et al., 2015; Bian et al., 2022). To fill knowledge gaps in the molecular mechanisms of Cr(VI)-induced centrosome disruption this study focuses on regulators of centriole engagement, and most intensely the protein securin, to

uncover the source of centrosome amplification. Background on centrosome structure and function is presented here, along with relevant findings in Cr(VI) research.

Centrosomes have been implicated in cancer for decades. In 1902, Theodor Boveri published a theory of cancer, including "the suggestion that malignant tumors might be the result of a certain abnormal condition of the chromosomes, which may arise from multipolar mitosis," and became the father of the chromosome theory of cancer and the first to implicate centrosomes in carcinogenesis (McKusick, 1985). Since then, other authors have supported the theory that centrosome amplification is central to chromosome instability in cancer (Lingle et al., 2002; Sato et al., 2001; Skyldberg et al., 2001). Centrosome amplification is observed in the majority of solid and hematological cancers and is associated with late-stage tumors, early neoplasia, and is an early effect in cell culture studies (Chan, 2011; Nigg et al., 2002), indicating its role in both initiation and progression of cancer. In breast cancers, 60-80% of tumors have centrosome amplification and Lingle et al. (2001) found *in situ* ductal carcinomas showed centrosome numbers correlating with aneuploidy and chromosome instability. Levine et al. (2017) reported *in vivo* induction of supernumerary centrosomes was sufficient to cause mammary tumors in mice.

The centrosome is the microtubule organizing center of the cell. It functions to nucleate microtubules and plays roles in cell processes such as ciliogenesis, cell motility, cell signaling, Golgi organization and mitotic spindle formation (Bettencourt-Dias and Glover, 2007; Rios, 2014; Bornens, 2002). The mature

centrosome is a membrane-free organelle composed of two centrioles surrounded by a protein cloud known as the pericentriolar material (Moritz et al., 1995). The centrioles are cylindrical tubes formed of nine triplet stacks of microtubules. They are approximately 0.5 um in length and 0.2 um wide (Agircan et al., 2014). The pericentriolar material contains multiple associated proteins, including gamma-tubulin that acts as the nucleation seed for microtubule generation. We used gamma-tubulin as a marker to visualize and count centrosomes. While the pericentriolar material imparts functionality to the centrosomes, the centrioles determine the replication status of the centrosome (Agircan et al., 2014). The structure of the centrosome is illustrated in Figure 1.

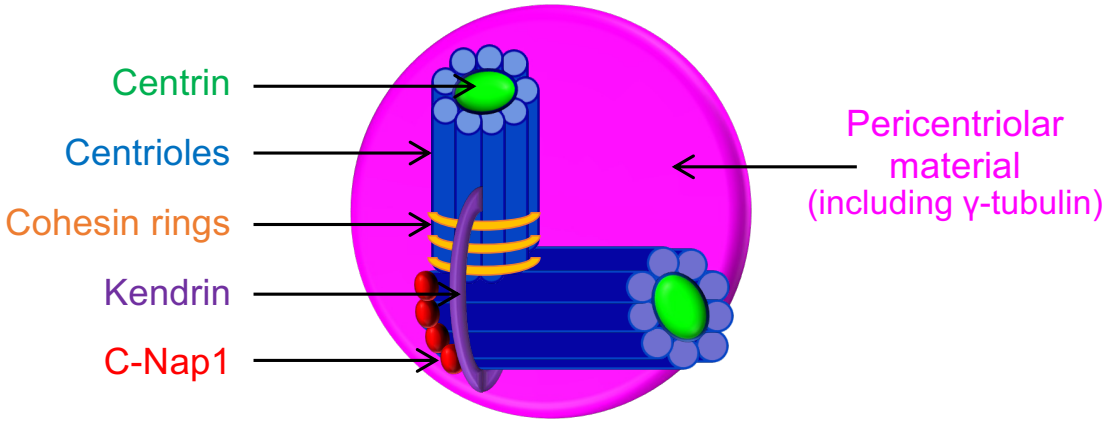


Figure 1. Centrosome structure. The mature centrosome is composed of two centrioles engaged in perpendicular arrangement. The centrioles are embedded in the pericentriolar material, a protein matrix including gamma-tubulin. The centrioles are supported in engagement by cohesin rings and kendrin protein. The distal lumen of the centrioles contain centrin and C-Nap1 is localized to the proximal ends.

The focus on centrosomes in cancer lies in their powerful influence over chromosome segregation. In culture, multipolar cells featuring more than two centrosomes are observed to segregate chromosomes asymmetrically or into more than two daughter cells (Ochi, 2016; Weaver et al., 2007). For the most part, severe asymmetry and multicellular cytokinesis are fatal to the daughter cells (Weaver et al., 2007). However, centrosome amplification can be tolerated by clustering or inactivating supernumerary centrosomes (Brinkley, 2001; Vitre et al., 2020). While clustering aids cell survival by promoting bipolar spindle formation, it does not guarantee against aneuploid daughter cells. Clustered centrosomes can block one another to interfere with proper microtubule-kinetochore attachment so that bipolar division with supernumerary centrosomes causes asymmetrical chromosome division (Ganen et al., 2009; Silkworth et al., 2009).

Centrosome amplification can occur by various avenues. Failure of cytokinesis results in cells that have a 4N complement of DNA and inherit two centrosomes instead of one per cell (Normand and King, 2010). These centrosomes can then duplicate during S phase, giving the cell four mature centrosomes (Fukasawa, 2005). *De novo* centrosome amplification occurs when centriolar synthesis-associated proteins are overexpressed and form centrosomes independently of mother centrioles (Godinho & Pellman, 2014; Tsou & Stearns, 2006a). Another scenario involves the fragmentation of the pericentriolar material which then is able to function as an acentriolar centrosome (Fukasawa, 2005). Cr(VI)-exposed cells do not show a large number of acentriolar centrosomes and amplification occurs in cells with diploid DNA content, indicating that cytokinesis

failure is not a prominent source of centrosome amplification (Holmes et al., 2010). The most likely mechanism for Cr(VI)-induced centrosome amplification is premature reduplication during interphase. Evidence points to premature disengagement and loss of reduplication blocks as key steps in Cr(VI)-induced centrosome amplification (Martino et al., 2015).

Normally, in late mitosis or early G1, the centriole pair disengages. Disengagement is the licensing step for duplication (Tsou & Stearns, 2006a,b). Engagement blocks the recruitment of proteins involved in centriolar synthesis, including Plk4, SAS6, Cep135, and STIL, required to form the daughter centriole cartwheel on the side of the mother centriole (Conduit et al., 2015; Nigg & Stearns, 2011; Wang et al., 2014). Disengagement is caused by cleavage of the centriole linkers, kendrin and cohesin, by the enzyme, separase (Nakamura et al., 2009; Karki et al., 2017; Matsuo et al., 2012). Securin is the regulator of separase and thus key in controlling the timing of centriole disengagement (Lee and Rhee, 2012). Centriole disengagement by separase is illustrated in Figure 2. Centrosome overduplication along with centriole disengagement has been shown to occur during extended G2 arrest in the presence of DNA damaging agents (Dodson et al., 2004; Douthwright & Sluder, 2014; Inanc et al., 2010; Karki et al., 2017). Martino et al. reported significant premature centriole disengagement after exposure of human lung cells to zinc chromate (Martino et al., 2015). Increase in centriole disengagement followed a similar pattern over time and treatment concentrations as centrosome amplification in those cells, supporting the theory of centriole disengagement as a key process in Cr(VI)-induced centrosome

amplification (Martino et al., 2015). Yet, it is unknown how centriole engagement becomes dysregulated by Cr(VI). Current knowledge points to the integrity of kendrin and cohesin and the timing of separase activity as critical points of regulation. In this dissertation, we focus on securin, separase, and their influence on centriole linkers as potential targets of Cr(VI) exposure.

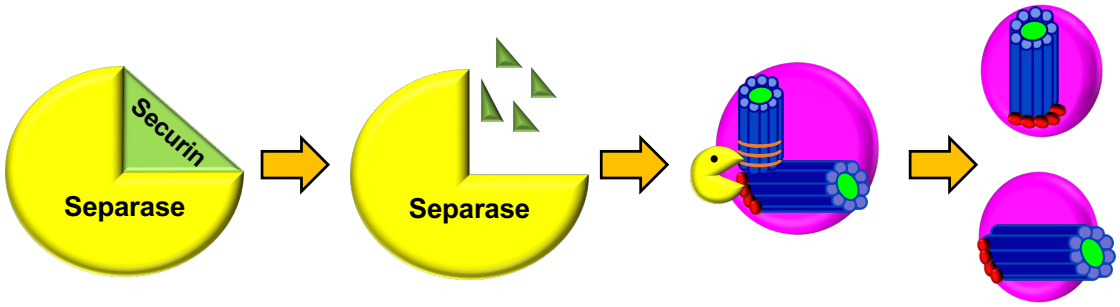


Figure 2. Centriole disengagement is caused by separase activity. Securin inhibits separase. When securin is degraded, separase is released and its enzyme activity cleaves centriole linkers, securin and kendrin, to cause centriole disengagement. Centriole disengagement licenses centrosome duplication.

Securin and Separase

Separase is a large cysteine protease with roles in chromosome segregation, DNA repair, centriole disengagement and centrosome duplication (Baum et al., 1988; Uhlmann et al., 1999; Nagao et al., 2004; Hellmuth et al., 2018; Tsou et al., 2009; Lee et al., 2012). Human separase is approximately 233 kDa (UniProt.org, 2010). It has a catalytic domain at the C-terminal end that is highly conserved among eukaryotes, an unstructured middle hinge region, and a helical N-terminal region which varies with species (Luo and Tong, 2021; Viadiu et al., 2005). Separase is largely localized in the cytoplasm in normal, undamaged cells (Sun et al., 2006). It is excluded from the nucleus both by virtue of its large size and by a nuclear export signal on its C-terminal end that ensures the catalytic activity is not present in interphase cells (Sun et al., 2006). The catalytic domain is responsible for endopeptidase activity causing cleavage of its substrate proteins at conserved recognition sites (Sullivan, et al., 2004; Waizenegger et al., 2000; Hauf et al., 2001; Waizenegger et al., 2002). The substrates of separase relevant to this project are separase, kendrin, and the SCC1 subunit of cohesin (Matsuo et al., 2012; Rosen et al., 2019; Waizenegger et al., 2002).

Separase cleaves proteins after an arginine (R) residue in the motif E-X-X-R, in which X is any amino acid (Waizenegger et al., 2000; Hauf et al., 2001; Waizenegger et al., 2002). Separase contains three cleavage sequences that serve as substrates of its own catalytic site and upon activation separase can cleave itself (Waizenegger et al., 2002). Though the cleavage status does not alter

enzyme function (Waizenegger et al., 2002), it is used as a marker of separase activity.

Kendrin is a huge, 378 kDa coiled-coil protein that localizes in the pericentriolar region of centrosomes to support and protect centriole engagement (Doxsey et al., 1994; Matsuo et al., 2012). Kendrin, also named pericentrin, contains a separase cleavage sequence at R_{2231} which if mutated causes kendrin to escape separase cleavage and prevents centriole disengagement (Matsuo et al., 2012). Separase activity is distinctly controlled at the centrosome to cause kendrin cleavage (Agircan and Schiebel, 2014) and allow centriole disengagement.

Separase is responsible for cleaving the cohesin ring, which holds the centromeres of sister chromatids together. The cohesin ring is a complex composed of subunits SMC1, SMC3, SCC1/RAD21, and SA1 or SA2, depending on the isoform (Losada et al., 2000; Sumara et al., 2000; Sumara et al., 2002). Most cohesin rings are located along the arms of chromosomes and are released in prophase by a separase-independent mechanism (Losada et al, 2000; Losada et al., 2001; Kueng et al., 2006). However, cohesin at the centromeres is protected by the protein shugoshin (Watanabe and Kitajima, 2005) and only released upon separase activity (Hauf et al., 2001). SCC1, also named RAD21, is the cohesin subunit cleaved by separase (Uhlmann et al., 1999; Waizenegger et al., 2000). Cleavage of SCC1 opens the cohesin ring (Uhlmann et al., 1999). Cleavage of centromeric cohesin allows separation of chromatids into daughter cells during mitosis, and this function of separase is critical for progression through anaphase

and cytokinesis (Hauf et al., 2001; Funabiki et al., 1996; Ciosk et al., 1998). Cohesin is present also at the centrosomes where it helps hold centriole pairs in engagement. Separase activity reporters show cohesin cleavage at centrosomes occurs before cohesin cleavage at the centromeres, indicating location-specific timing of separase activity (Agircan and Schiebel, 2014).

Separase regulation is critical to control the timing of its cleavage activity. Multiple control mechanisms exist, including inhibitory binding by securin and the cyclin B1-cdk1 complex and inhibitory phosphorylation (Huang et al., 2008; Yu et al., 2021; Stemmann et al., 2001; Ciosk et al., 1998, Gorr et al., 2005). Securin is the main inhibitor and binds to separase throughout the cell cycle (Ciosk et al., 1998; Hornig et al., 2002). After the discovery of separase function, the yeast securin homolog, Pds1, was found to be the regulator of separase activity, which was released by degradation mediated by the anaphase-promoting complex (APC) (Cohen-Fix et al., 1996). Vertebrate securin was discovered in 1999 and found to be the same product of PTTG (pituitary tumor-transforming gene) and thus linked to carcinogenesis via its effects on chromosome disjunction (Zhou et al., 1999). Human securin is approximately 22 kDa and features a KEN-box region and a D-box (destruction-box) region, both of which are required for anaphase promoting complex/cyclosome (APC/C) recognition and degradation (Zur and Brandeis, 2001). The pseudo-substrate region is where it interacts with separase. The securin sequence varies among eukaryotes, but its function remains conserved. Securin associates with nascent separase and acts as a chaperone that enables its proper folding and solubility (Hornig et al., 2002). Securin binding

to separase primes it for activity and securin absence depresses separase activity (Hornig et al., 2002; Jallepalli et al., 2001; Mei et al., 2001). Securin occupies the active site via its pseudo-substrate sequence and prevents cleavage activity until it is ubiquitinated at anaphase by APC/C and subsequently degraded by the proteasome (Cohen-Fix et al., 1996; Zhou et al., 1999; Zur and Brandeis, 2001; Hagting et al., 2002).

Since securin is the main inhibitor of separase, and separase function is so critical to chromosome stability, it is surprising that knockout mouse and human cell studies show securin is not essential to the normal function of cells (Mei et al., 2001; Jallepalli et al., 2001; Pfleghaar et al., 2005). Inhibitory phosphorylation contributes to separase regulation (Stemmann et al., 2001; Gorr et al., 2005; Helmuth et al., 2015) but is not sufficient alone for inhibition. Cyclin B1 can bind to separase and inhibit its activity. Cdk1 partnered to cyclin B1 phosphorylates separase, which causes it to aggregate and thus deactivate (Stemmann et al., 2001; Gorr et al., 2005). Association of the cdk1-cyclin B1 complex with separase keeps it soluble and ready to activate, similar to how securin maintains separase in an inactive but ready state (Helmuth et al., 2015; Gorr et al., 2005). Cyclin B1 is degraded by the APC/C pathway also at the anaphase transition, allowing it to compensate for securin loss (Hellmuth et al., 2015).

It was first discovered in *Saccharomyces cerevisiae* that separase deregulation leads to centrosome amplification, but this defect was prevented if cells were held in G1 or G0 phase (Baum et al., 1988). Separase activity is implicated in breast cancer, as it is a marker for tumor progression (Gurvits at al.,

2017), and modification of separase can induce or suppress tumorigenic outcomes in experimental animals and cells (Zhang and Pati, 2018; Zhang et al., 2018; Mukherjee et al., 2014). In lung cancer, securin dysregulation is implicated in promoting invasion and migration of non-small cell cancer cells (Li, et al, 2013). In patients with acute myeloid leukemia (AML), the transition from myelodysplastic syndrome to AML was correlated to separase activity and centrosome aberrations (Ruppenthal et al., 2018).

In this summary, we highlight the carcinogenic implications of centrosome amplification and specifically the regulatory proteins, securin and separase, and centriole linkers, kendrin and cohesin. In our pursuit of molecular targets of Cr(VI), we focus on these centriole-associated regulatory proteins. These key regulation points are widely studied in cell biology research but are understudied in the field of Cr(VI) carcinogenesis.

One Environmental Health Approach for Environmental Toxicology

The One Health philosophy proposes that the health of all living inhabitants on earth is interconnected and insights from any human, animal, or ecosystem health field can contribute to the enhancement of the others. The One Environmental Health approach specifically focuses on the shared toxicants relevant to human, animal, and ecosystem health (Pérez and Wise, 2018). This approach extends the advancements made by the systems biology paradigm to holistic consideration of multidisciplinary research scaling from molecules, cells, and organisms to ecosystems (Zinsstag et al., 2011). In the context of this

dissertation, we use a species comparison to tease apart the differences in molecular responses to Cr(VI) and gain insights into key events in the carcinogenic mechanism.

Cr(VI) is a global environmental contaminant and thus a relevant toxicant to One Environmental Health studies. Cr(VI) can form naturally from oxidation reactions with Cr(III) (Jacobs & Testa, 2005), but Cr(VI) in the environment largely arises from industrial activities.

Geisler and Schmidt (1992) provided an overview of marine chromium. They reported the thermodynamically stable valence state and the dominant species of chromium in sea water is Cr(VI). Erosion and industrial runoff contribute to chromium input into the ocean, but another large source is deposition from the air, which indicates that both marine waters and atmosphere are contaminated with Cr(VI) (Geisler and Schmidt, 1992; Jacobs & Testa, 2005; Tchounwou et al., 2012). The range of chromium levels in sea water have been reported as 5 to 800 µg/L (Jacobs and Testa, 2005) and 2 to 5 nmol/kg (Geisler and Schmidt, 1992). Environmental reduction of Cr(VI) to Cr(III) can occur under acid conditions, for example in anoxic marine zones with hydrogen sulfide present (Geisler and Schmidt, 1992).

Whales are of particular interest in environmental toxicology because they are the closest marine relative to humans. Whales have long life spans, breathe air, and are potentially exposed to Cr(VI) by skin, ingestion, and inhalation. Chromium accumulates in whale skin and levels vary among geographically diverse populations. Wise et al. (2009) analyzed sperm whale skin biopsies from

361 individuals across 16 ocean regions (Wise et al., 2009). Chromium levels ranged from 0.9 to 122.6 μg/g of tissue, with a global mean of 8.8 ± 0.9 μg/g, which is 28-fold higher than the mean Cr levels in human skin without occupational exposure. Regional means ranged from 3.3 ± 0.4 μg/g to 44.3 ± 4.4 μg/g. Fin whale skin biopsies from the Gulf of Maine had mean Cr levels of 10.07 μg/g tissue (Wise et al., 2015).

Interestingly, cell culture studies have shown that particulate Cr(VI) is less genotoxic to whale cells than human cells (Browning et al., 2017; Li Chen et al., 2012; Li Chen et al., 2009). Fewer instances of structural chromosome damage occurred to North Atlantic right whale lung cells versus human lung fibroblasts exposed to lead chromate (Li Chen et al., 2009). Lead chromate produced 3- to 5-fold fewer damaged metaphases in sperm whale skin cells compared to human skin cells (Li Chen et al., 2012). Thus, it appears that whales have protective mechanisms against Cr(VI)-induced structural chromosome damage. This dissertation investigates Cr(VI)-induced numerical chromosome instability in whale cells. By comparing whale-specific cellular and molecular responses to those of humans, we can further elucidate key mechanistic events of Cr(VI) carcinogenesis and uncover how whales may resist cancer.

Summary and Dissertation Aims

Lung cancer is the leading cause of cancer deaths worldwide. While the most familiar risk factor is cigarette smoking, up to 25% of cases occur in individuals who never smoked (Samet et al., 2009, Sung et al., 2021; Couraud et

al., 2012). Thus, understanding occupational and environmental carcinogens is critical in the battle against lung cancer. Metals are a major cause of lung cancer. Hexavalent chromium [Cr(VI)], which is both an occupational and environmental contaminant, is a known carcinogen and among the top 20 on ATSDR's Substance Priority List (ATSDR, 2020). Lung tumors feature high incidences of aneuploidy and centrosome amplification, and this phenotype has been reproduced by Cr(VI) exposure *in vitro* (Holmes et al., 2006; Holmes et al., 2010; Martino et al., 2015). Centrosome amplification is gaining recognition as not only a hallmark of many cancers, but a potential key in carcinogenesis because it can lead to aneuploidy. The goal of this dissertation is to identify the mechanism of Cr(VI)-induced centrosome amplification. The central hypothesis of this project is: Cr(VI) causes loss of the key centrosome regulator, securin, causing aberrant separase activity, which leads to premature centriole disengagement, centrosome amplification, and numerical chromosome instability. Figure 3 shows a graphical representation of the hypothesis. I will examine the molecular changes that underlie this phenotype in three aims.

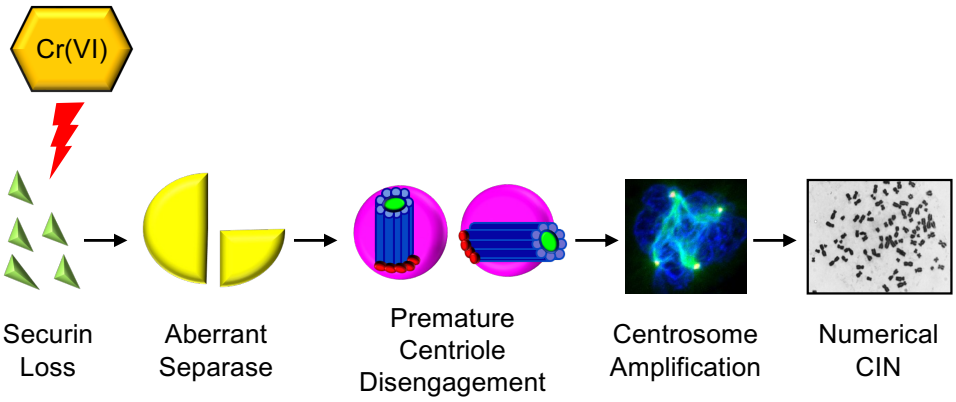


Figure 3. The central hypothesis of this project. Cr(VI) causes loss of the key centrosome regulator, securin, causing aberrant separase activity, which leads to premature centriole disengagement, centrosome amplification, and numerical chromosome instability.

Aim 1. Particulate Hexavalent Chromium-Induced Securin Disruption Drives Numerical Chromosome Instability in Human Lung Cells. Aim 1 investigates the proteins involved in Cr(VI)-induced premature centriole disengagement. Centriole disengagement is the licensing step to centrosome duplication and untimely release of this blocking mechanism allows multiple rounds of centriole synthesis (Tsou et al., 2006a; Tsou et al., 2006b). Centriole disengagement has been observed by our lab after Cr(VI) exposure to normal human lung cells (Holmes et al., 2006; Holmes et al., 2010; Martino et al., 2015) and thus is a likely cause of centrosome amplification in Cr(VI)-exposed cells. Separase cleaves cohesin and kendrin at the centrosomes, implicating it as a key factor in centriole disengagement. Securin is the canonical separase inhibitor and cyclin B1 is a secondary inhibitor. Thus, this project focuses on securin loss in normal human lung fibroblasts, which could result in increased separase activity, and addresses the role of cyclin B1 as a secondary inhibitor. We measure securin protein levels, and address two possible avenues of securin loss, including increased protein degradation and decreased securin messenger RNA (mRNA) levels. We also measure cyclin B1 protein after Cr(VI) exposure. Next, we seek to confirm the roles of securin and separase using siRNA-induced gene knockdown to induce or rescue Cr(VI)-associated phenotypes.

Aim 2. Mechanisms of Hexavalent Chromium-Induced Securin Disruption. We have seen that Cr(VI) causes a wide variety of protein alterations in the cell and decreased protein levels will have cascading effects across several pathways. Aim 2 dives deeper into how Cr(VI) alters securin protein expression. It

is important to assess if securin transcription factor protein levels are also altered. This Aim focuses on pre-transcriptional disruption by two avenues: 1) Loss of transcription factors that promote securin transcription and 2) increased levels of transcription factors that inhibit securin transcription.

This aim measures Cr(VI) effects on levels of three transcription factors that bind to the securin promoter region and enhance transcription of the securin gene: Sp1, NF-YA, and in certain cases E2F1. Binding of both Sp1 and NF-YA at the securin promoter is required for securin transcription (Clem et al., 2003). Therefore, if Cr(VI) alters either of these proteins, securin expression would be hindered. We have shown E2F1 is targeted by Cr(VI) (Speer at al., 2021) and will investigate E2F1 as a possible mechanism for securin expression loss. Cr(VI) exposure not only changes protein levels, but can also alter protein localization (Speer at al., 2021) and so we also measure transcription factor levels in nuclear and cytoplasmic fractions to determine if nuclear function may be maintained after Cr(VI) exposure. In addition to transcription factors that promote securin transcription, we also investigate transcription factors that are known to have an inhibitory effect, namely KLF6 and p53 (Lee at al., 2010; Chen et al., 2013; Zhou et al., 2003). We measure alteration in protein levels and localization after Cr(VI) exposure.

In the pursuit of the mechanism by which Cr(VI) induces loss of securin mRNA, we also investigate potential effects of microRNA (miRNA) regulation. MiRNAs are increasingly recognized for their potential roles in environmental carcinogenesis (Wu et al., 2019; Pratap et al., 2018; Gonzalez et al., 2015; Li et

al., 2014). MicroRNA sequencing shows Cr(VI) causes global miRNA dysregulation. Speer et al. (2022) found miRNAs were significantly affected in all tested timepoints and concentrations, including up- and down-regulation of multiple miRNAs involved in several cancer pathways. We analyzed the miRNAs that are significantly altered by Cr(VI), to identify potential targets in the pathways of securin and other centrosome regulators.

Aim 3. Whale Cells Resist Hexavalent Chromium-Induced Securin Disruption. In Aim 3 we undertake the first study of centrosome amplification in whale cells. Whales are long-lived mammals that are exposed to significant levels of environmental chromium. We show whale cells are resistant to Cr(VI)-induced securin loss, centrosome amplification, and numerical chromosome instability. Comparative data from whales yield supportive evidence for key mechanisms of centrosome amplification and provide insights into molecular strategies against metal-induced carcinogenesis.

Figure 4 shows an overview of the project, including the mechanism of particulate Cr(VI) dissolution and uptake and the hypothesis of this project.

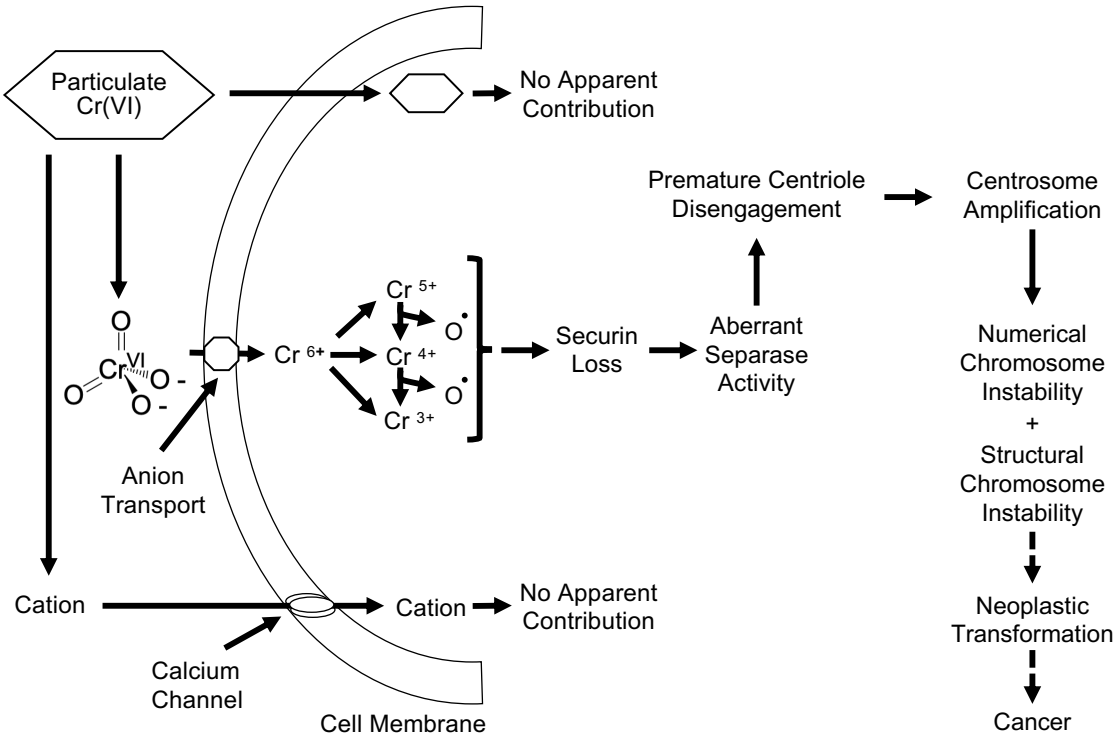


Figure 4. Overview of Cr(VI) uptake and proposed mechanism of carcinogenesis. Cr(VI) particles can be phagocytosed, but have no apparent contribution to Cr(VI) carcinogenesis. Particulate Cr(VI) dissociates outside the cell into the chromate anion and the cation. The cation can enter the cell via calcium channels, but has no apparent contribution to Cr(VI) carcinogenesis. The Cr(VI) anion enters the cell membrane via anion transport channels. Inside the cell Cr(VI) is reduced to Cr(V), Cr(IV), and Cr(III) and the reduction process generates reactive oxygen species. We propose one or more of these products induces securin protein loss, which causes aberrant separase activity, leading to premature centriole disengagement. Centriole disengagement causes centrosome amplification, which drives numerical CIN which causes neoplastic transformation and ultimately leads to cancer.

CHAPTER 2: METHODS AND MATERIALS

Chemicals and Reagents

DMEM and Ham's F-12 (DMEM/F-12) 50:50 media, glutagro 200 mM L-alanyl-L-glutamine supplement, sodium pyruvate, and Dulbecco's phosphate-buffered saline (DPBS), tissue culture flasks, dishes and plasticware were purchased from Corning, Inc. (Manassas, VA). Cosmic calf serum and penicillin/streptomycin was purchased from HyClone (Logan, UT). Sodium pyruvate (100 mM) and MycoAlert kit were purchased from Lonza (Allendale, NJ). Trypsin-EDTA (0.25%) and KaryoMAX® Colcemid Solution (10 μg/ml) was purchased from Gibco. Zinc chromate (CAS# 13530-65-9, 99.7% purity) was purchased from Pfaltz and Bauer (lot Z00277, Waterbury, CT). HALT protease and phosphatase inhibitor cocktail, RIPA buffer, NE-PER nuclear and cytoplasmic extraction reagents, mirVana miRNA isolation kit, High Capacity cDNA Reverse Transcription, TaqMan Assays, glass chamber slides, Super Up Rite slides were purchased from Thermo Fisher Scientific Inc. (Waltham, MA). Dharmacon ON-TARGET siRNAs (siESPL1, siPTTG1, siCCNB1, siE2F1, siNF-YA, siSp1, and non-targeting siRNA), DharmaFECT transfection reagent, and 5X siRNA buffer were purchased from Horizon Discovery (St. Louis, MO). Mini-Protean TGX gels, 4X protein sample loading buffer, Odyssey blocking buffer (TBS), IRDye® 800CW

and IRDye® 680RD near-infrared fluorescent secondary antibodies were purchased from Li-Cor (Lincoln, NE). FNC Coating Mix® (fibronectin, collagen, albumin mix) was purchased from Athena Environmental Sciences, Inc. (Baltimore, MD). Tween-20, cycloheximide (CHX), methanol, sodium dodecyl sulfate (SDS), potassium chloride, nitric acid and micro cover glass were purchased from VWR International (Radnor, PA). Acetic acid was purchased from Avantor (Center Valley, PA). Gurr's buffer and 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) were purchased from Life Technologies Corporation (Grand Island, NY). Giemsa stain was purchased from Ricca Chemical Company (Arlington, TX). Ethylene glycol tetraacetic acid (EGTA), fish skin gelatin, neocarzinostatin (NCS), and glycerol were purchased from Sigma-Aldrich, Inc. (St. Louis, MO). Normal goat serum was purchased from Abcam (Eugene, OR). Piperazine-N,N′-bis(2-ethanesulfonic acid) (PIPES) was purchased from Alfa Aesar (Ward Hill, MA). Magnesium sulfate was purchased from J.T. Baker (Phillipsburg, NJ). Bovine serum albumin was purchased from EMD Millipore Corporation (Billerica, MA). Sodium azide was purchased from Amresco, Inc. (Solon, OH). Prolong Diamond Antifade Reagent with DAPI and Alexa Fluor secondary antibodies were purchased from Invitrogen (Eugene, OR). Anti-centrin rabbit monoclonal antibody and anti-alpha-tubulin mouse antibody with FITC conjugation were purchased from EMD Millipore Corporation (Temecula, CA). Anti-CNAP1 (CEP250) rabbit polyclonal antibody was purchased from Proteintech (Rosemont, IL). DyLight secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA). Anti-separase and anti-gamma-tubulin

monoclonal mouse antibodies were purchased from Abcam (Eugene, OR). Anti-securin rabbit monoclonal, anti-alpha-tubulin mouse monoclonal, anti-cyclin B1 rabbit polyclonal, anti-p53 (phospho-Ser 15) rabbit, and anti-Sp1 rabbit polyclonal antibodies were purchased from Cell Signaling Technology (Danvers, MA). Anti-p53 mouse monoclonal antibody was purchased from BD Biosciences (Franklin Lakes, NJ). Anti-alpha-tubulin and anti-GAPDH mouse monoclonal antibodies were purchased from GeneTex (Irvine, CA). Kendrin/pericentrin rabbit antibody was purchased from Bethyl Laboratories (Montgomery, TX). Anti-NF-YA Rabbit polyclonal antibody, anti-lamin B1mouse monoclonal antibody were purchased from Invitrogen/Thermo Fisher (Rockford, IL). Anti-KLF6 mouse monoclonal antibody was purchased from Santa Cruz Biotechnology (Dallas, TX). Anti-Scc1 monoclonal guinea pig antibody was a gift from Dr. Olaf Stemmann (University of Bayreuth, Germany).

Human Cell Culture

The human lung cell line used was WTHBF-6 cells, a bronchial fibroblast cell line developed from normal primary human bronchial fibroblasts, as previously published (Wise et al., 2004). This clonal cell line has an hTERT-extended lifespan with a normal, stable karyotype and displays the same growth rate and cytotoxic and clastogenic response to metals as the primary parent cells (Wise et al., 2004). Using this immortalized cell line enables consistent cell passaging and prolonged exposure periods used in our toxicological assays. Fibroblast cell lines are relevant cell models for Cr(VI)-induced lung cancer due to observations that chromium

deposits in the bronchial stroma of chromate workers, but not in the epithelium (Kondo et al., 2003). Human fibroblast cells are typically employed in toxicological assays concerning aneuploidy given that currently available epithelial cell lines demonstrate high background aneuploidy. Our study focuses on chromosomal stability and thus a stable control karyotype is an important prerequisite.

WTHBF-6 cells were maintained according to our published methods (Wise et al., 2004) as an adherent, sub-confluent layer in DMEM/F-12 media, supplemented with 15% cosmic calf serum, 0.2 mM L-alanyl-L-glutamine, 100 IU/ml penicillin, 100 mg/ml streptomycin and 0.1 mM sodium pyruvate. Cells were maintained in a humidified incubator at 37º C, 5% CO_2. Cells were authenticated through karyotyping when thawed for use and after every 3 months of continuous culture. Short tandem repeat analysis was conducted approximately yearly to confirm authenticity. Monthly mycoplasma screening was performed and cells were monitored for any growth or morphological changes during maintenance.

Whale Cell Culture

Primary whale cell lines developed from two whale species were employed in our studies. SPW457sk is a primary skin fibroblast cell line derived from a female sperm whale. Skin biopsy was obtained from a free ranging, healthy adult in the Gulf of Mexico. BHW200Lu and BHW24Lu are a primary bowhead whale lung fibroblast cell lines derived from two male whales obtained during subsistence hunts in Barrow, Alaska. Whale fibroblasts were isolated from tissue and maintained according to our published methods (Wise et al., 2011). Tissue was

immersed for at least 30 minutes in tissue buffer containing 2% penicillin/streptomycin and 0.4% gentamicin. Tissue was sliced into small pieces using sterile scalpel and placed in tissue culture flasks with 500 ml of L-15 media containing 2% penicillin/streptomycin and 0.4% gentamicin. Flasks were incubated at 33º C. After 48 hours, flasks were flooded with media and monitored for fibroblast migration from explants. After colonization of the tissue culture flask, tissue was removed and cell were released with 0.25% trypsin. Cell lines were maintained at 33º C in a humidified chamber with 5% CO_2. Adherent, sub-confluent cells were maintained in DMEM/F-12 media, supplemented with 15% cosmic calf serum, 0.2 mM L-alanyl-L-glutamine, 100 IU/ml penicillin, 100 mg/ml streptomycin and 0.1 mM sodium pyruvate. Cells were frozen in growth media plus 10% DMSO for long-term storage at -140º C.

Preparation of Zinc Chromate and Cell Treatments

All experiments are performed on logarithmically growing cells. WTHBF-6 cells have a doubling time of approximately 24 h (Wise et al., 2004) and were allowed 48 hours after seeding to enter logarithmic growth phase before beginning treatments. Whale cells have an approximate doubling time of 36 h (Wise at al., 2011) and were allowed 72 hours to enter logarithmic growth phase before experiment start time. According to published methods (Xie et al, 2009), zinc chromate was prepared by washing twice with deionized H_2O to remove water soluble contaminants, rinsed twice with acetone to remove organic contaminants, and thoroughly dried. Washed zinc chromate was suspended in sterile water and

stirred overnight at 4ºC. Before treatment, fresh media was added to cell dishes and zinc chromate suspension was applied at a concentration of 0, 0.1, 0.2, 0.3, or 0.4 μg/cm^2, as specified for experiment and cell type. Treatment durations were 24 and 120 hours. Cells were maintained during treatment period at 5% CO_2 in a humidified incubator. Human cells were maintained at 37º C and whale cells were maintained at 33º C.

Clonogenic Survival Assay

Clonogenic survival assay was performed as previously published (Wise et al., 1992) to measure cytotoxicity. This assay determines relative plating efficiency of treated cells compared to untreated cells. Cell viability assays based on enzyme or metabolic activities are unsuitable because Cr(VI) treatments can alter these markers independent of cell survival. The clonogenic survival assay is well-established in the literature for Cr(VI) toxicity and is useful for evaluating optimal chemical exposure levels and cell seeding density that enable prolonged toxicological exposures. Cells were seeded on 6-well plates and treated as described above. At the end of the treatment period, media were removed and cells were rinsed with 1X DPBS and released from the plate with 0.25% trypsin-EDTA. From each treatment condition, 2000 cells were seeded onto each of four 100 mm tissue culture dishes. Cells were maintained in culture, without any further treatment, and media were changed every 5 days until colonies formed. Colonies were stained with crystal violet. Colonies were counted in each dish and averaged

across all dishes for each treatment. Average colony growth per treatment group are reported relative to the control group. Three experiments were performed.

Chromosome Instability Analysis

Chromosomes were prepared and analyzed according to previously published methods with some modifications (Wise et al., 1992). Cells were seeded into 100 mm tissue culture dishes and treated with zinc chromate as described above. Before harvesting, 0.1 µg/mL demecolcine was added to each dish to arrest cells in metaphase. Demecolcine was added for 1 hour for human cells and for five hours for whale cells. Demecolcine treatment period is optimized for various cell lines and doubling times to ensure the cell population is enriched with metaphase cells without over-condensation of chromatids that will impede chromosome visualization. After 24 hours or 120 hours of exposure, media and treatment were rinsed from the dishes and cells were released from the plate with 0.25% trypsin-EDTA. Cells were washed in PBS, treated with hypotonic 0.075 M potassium chloride for 17 minutes, and fixed in Carnoy's fixative for 20 minutes. Fixative was changed twice before preparing slides. Fixed cells were then dropped onto wet glass slides and dried at 30º C, 30% humidity. Slides were stained with Giemsa and glass cover slips were applied. Chromosomes were counted in at least 100 metaphases per concentration. Normal human lung cells contain 46 chromosomes. Normal diploid sperm whale and bowhead whale cells contain 42 chromosomes. Any metaphases with greater or fewer than the normal chromosome complement were counted as aneuploid.

Evidence of spindle assembly bypass including centromere spreading, premature centromere division, and premature anaphase was recorded. A minimum of 100 diploid metaphases per concentration were analyzed and abnormal centromere separation events were recorded in all metaphases encountered during analysis. Three experiments were analyzed.

Protein Analysis

Cells were seeded in tissue culture dishes and treated with zinc chromate as described above. Cell-equivalent whole cell protein analysis was performed as previously published (Speer et al., 2021). At the end of Cr(VI) exposure period, cells were released from dishes by incubation in 0.25% trypsin-EDTA. Cells were collected, washed in PBS, and counted with Beckman Coulter Multisizer 4e. Cells were lysed in RIPA buffer supplemented with 100X HALT protease and phosphatase inhibitor cocktail. Cell lysis was centrifuged 10 minutes at 14,000 rpm and supernatant was collected and prepared with 4X protein sample loading buffer. Protein samples in loading buffer were heated for 10 minutes at 70º C and stored at -20º C. Prepared sample volumes equivalent to 50,000-75,000 lysed cells (antibody-dependent) was loaded into Mini-Protean TGX gels. After electrophoresis, protein was transferred onto 0.45 µm nitrocellulose membrane in ice cold transfer buffer with 10-20% methanol (antibody-dependent). Membrane was probed with primary antibodies and secondary near-infrared antibodies. Membranes were scanned using Odyssey CLx scanner and analyzed with Li-Cor

Image Studio software. All primary antibodies were verified using positive and negative controls.

Cytoplasmic and nuclear protein extractions were performed with NE-PER nuclear and cytoplasmic extraction reagents according to manufacturer's instructions. Cells were harvested and counted as described above for whole cell extraction. Cell pellets were re-suspended ice-cold CERI (cytoplasmic extraction reagent I) supplemented with 100X Halt protease and phosphatase inhibitor, vortexed vigorously for 15 seconds and incubated on ice 15 minutes. Ice-cold CERII (cytoplasmic extraction reagent II) was added and tube was vortexed for 5 seconds, incubated on ice for 1 minute, vortexed for 5 seconds, and centrifuged at 14,000 RPM for 5 minutes. Cytoplasmic supernatant was isolated and stored on ice until preparation with 4X protein sample loading buffer as for whole cell protein samples. Nuclear fraction was resuspended in NER (nuclear extraction reagent) supplemented with 100X Halt protease and phosphatase inhibitor. Tubes were vortexed on highest setting for 15 seconds every 10 minutes for a total incubation time of 40 minutes. Samples were centrifuged at 14,000 RPM and nuclear fraction was prepared with 4X protein sample loading buffer as for whole cell extractions and stored at -20º C.

Protein Half-Life Analysis

Protein half-life analysis was performed as previously published (Speer et al., 2021) with some modifications. Cells were seeded in 100 mm tissue culture dishes and treated with zinc chromate as described above at the final

concentrations of 0 or 0.2 μg/cm^2. Treatment durations were 24, 72, and 120 hours. At the end of exposure time, cells were treated with cycloheximide at final concentration of 10 μM. Cells were harvested at 0, 2, 4, 6, and 8 hours after cycloheximide addition and whole cell protein was analyzed according to the Protein Analysis method above. Protein half-life was calculated from the exponential best fit curve of protein levels after 0, 2, 4, 6, and 8 h cycloheximide using the equation, Rate = (LN(0.5))/b, where the best fit line equation is $y=ae^{bx}$.

RNA Extraction, cDNA synthesis, and RT-qPCR

RNA analysis was performed as previously published (Speer et al., 2021). After cell seeding and treatment as described for protein analysis, cells were released from dishes by incubation in 0.25% trypsin-EDTA. Cells were collected and washed in PBS. Total RNA was extracted using mirVana miRNA isolation kit according to manufacturer's instructions. Cells were lysed with lysis binding buffer included in the kit, lysate was kept on ice, and homogenized. RNA was extracted with acid-phenol:chloroform, isolated by glass-fiber filter, washed with mirVana kit reagents, and RNA was eluted from the filter. Total RNA quality and quantity was measured by spectrophotometry on NanoDrop instrument. Samples were stored at -80º C.

Total RNA was reverse transcribed to single-stranded cDNA using a High-Capacity cDNA Reverse Transcription Kit according to manufacturer's instructions. 2X master mix was prepared containing random primers and reverse transcriptase. An equivalent amount of RNA across all experiment conditions, up to the maximum

of 2 µg per reaction, was added to master mix for each sample. Control samples with no reverse transcriptase and no input RNA were included with each experiment. Reverse transcription was performed on a Biometra thermocycler. cDNA was stored at -20ºC for no longer than one week before RT-qPCR analysis.

RT-qPCR was performed using TaqMan™ 20X RNA Assays and TaqMan™ Universal Master Mix II, with UNG or TaqMan Fast Advanced Master Mix, with UNG. Endogenous mRNA (GAPD) and target mRNA were analyzed in duplex. RT-qPCR reactions were performed in triplicate and controls for no reverse transcriptase, no RNA input, and no cDNA template were included for each of three independent experiments. Quantitation was performed on StepOne Plus Real-Time PCR System. Results were normalized by ΔΔCt method and expressed as relative quantification compared to untreated control (ΔCt = Ct gene target – Ct endogenous control; ΔΔCt = ΔCt sample 1 - ΔCt calibrator (untreated control); Fold change = 2- ΔΔCt). The calibrator has a relative quantification of 1. Relative quantification value of 10 means the gene is 10 times more expressed, while a value of 0.1 means 10 times less expressed.

MicroRNA Analysis

MicroRNA was analyzed by RNA sequencing as previously published (Speer et al., 2022) Total RNA samples were extracted as described above and analyzed by University of Louisville CGeMM DNA Facility Core. Library preparation was performed using the TruSeq Small RNA Library Prep Kit v2 with gel purification followed by library validation and quantification to create miRNA

libraries from 0.5–2 μg total RNA. 1 × 75 bp sequencing was performed using the NextSeq 500 High Output v2 (75 cycles) kit on the Illumina NextSeq500 instrument. At least ten million reads per sample were generated.

Bioinformatics was performed as described in Speer et al., 2022. Fastx-toolkit was used to filter reads (http://hannonlab.cshl.edu/fastx_toolkit/) (Hannon, 2009). Base calls that show a Phred score < 20 (i.e., base call error rate > 1%) were subsequently trimmed from both ends of a sequencing read to preserve the longest section of a high-quality sequence read. Reads with base call quality <20 for over 25% of the remaining base calls were removed. For all data filtering steps, sequencing reads with lengths <15 nucleotides were discarded. Sequencing reads were mapped to human non-coding RNA reference transcriptome (Ensembl GRCh38) using the Bowtie2 (Kim et al., 2013) 'end-to-end' mode, followed by discarding transcriptome mapping results that yielded mapping scores <20 (i.e., > 1% error rate) using SamTools (Li and Durbin, 2009; Li et al., 2009). Expression profiles for each sample were made using custom Perl scripts. Transcripts identified as "miRNA" in the Ensembl "transcript_biotype" database (Ensembl Version 98) were used in analyses.

Four technical replicates were analyzed for each experimental timepoint and Cr(VI) concentration. All miRNAs with fewer than 10 total reads were removed from analysis. Raw reads were normalized by multiplying the ratio of 1,000,000 over the sum of all its read counts. Linear mixed effect models were used to identify differentially expressed miRNAs. Cr(VI) concentration and exposure time were set as fixed effects and experiment as random effects. Each treatment concentration

was compared to the 0 μg/cm^2 zinc chromate control at its respective time point. For each miRNA, diagnostic plots were used to ensure the assumption of heterogeneity of the variance and normality were maintained. If not, log-transformation of the miRNA expression was used for statistical analysis. Adjusted *p*-values were determined using the Benjamini-Hochberg false discovery rate (FDR). All statistical analyses were carried out in R software (Version 4.0.0) using the R "nlme" package. Significantly different regulation was determined by an adjusted *p* value <0.01. In some cases, the mean counts of miRNAs were reported as '0' in the control or treatment groups and so 0.1 was added to all miRNA means to avoid errors in Log_2(Fold change) calculation. Also, some adjusted *p*-values were reported as 0, therefore, "0.0001" was added to all values to allow for Log(adjusted *p*-value) calculation.

Centrosome-related genes were identified using the Kyoto Encyclopedia of Genes and Genetics (KEGG) online database (https://www.genome.jp/kegg/) and relevant publications. For each gene of interest, potential targeting miRNAs were searched using miRSystem database (Lu et al., 2012). All potential gene-targeting miRNAs were then compared to the data of significantly up- and down-regulated miRNAs.

siRNA Transfection

Cells were seeded into 60 mm tissue culture dishes and allowed to plate for 24 h. Transfection was performed using the Dharmacon DharmaFECT 1 reagent as previously published (Speer et al., 2021). The selected siRNA stock was diluted to 5 μM with 1X siRNA buffer. In separate tubes, siRNA (Tube 1) and

DharmaFECT transfection reagent (Tube 2) were diluted in serum-free, antibiotic-free media and incubated for 5 minutes. The contents of Tube 1 and Tube 2 were mixed and incubated for 20 minutes. Antibiotic-free medium was added at the appropriate volume and transfection solution containing siRNA and transfection reagent was applied to each well. The final concentration of siRNA was 25 nM and the transfection reagent final concentration in the dish was 1 µl/ml. Cells were incubated for 24 h to allow transfection, then dishes were aspirated and fresh media were added and cells were treated with zinc chromate for 24 h (48 h total transfection time) or 120 h (144 h total transfection time). After treatment period, cells were harvested for protein analysis or chromosome analysis. Protein knockdown was verified using protein analysis method above.

Centrosome Analysis

Centrosomes were analyzed by immunofluorescence as previously published (Holmes et al., 2006). Bowhead whale cells were seeded on glass, FNC-coated chamber slides. Cells were allowed 72 hours to enter logarithmic growth before treatment with zinc chromate at final concentration of 0 or 0.2 µg/cm^2 zinc chromate. After 24 h or 120 h exposure, media were aspirated and cells were washed twice with microtubule stabilizing buffer (3 mM EGTA, 50 mM PIPES, 1 mM $MgSO_4$, 25 mM KCl), fixed with -20 ºC methanol for 10 minutes and allowed to air dry completely. Cells were rehydrated for 3 minutes in 0.05% Triton X-100, followed by 30 minutes of blocking with centrosome buffer (5% normal goat serum, 1% glycerol, 0.1% bovine serum albumin, 0.1% fish skin gelatin, 0.04% sodium

azide in PBS). Cells were incubated with anti-γ-tubulin antibody and α-tubulin-FITC antibody for 1 hour each, washing in PBS three times between each incubation. Cells were incubated with isotype-specific Alexa Fluor 555 for γ-tubulin visualization. Cells were washed and aged overnight before mounting coverslips with Prolong Diamond Antifade Mountant with DAPI. Slides were analyzed by fluorescent microscopy on Olympus BX51 and representative images were obtained by Applied Spectral Imaging GenASIS software version 7.1.0.1277. Centrosome number was counted in 1000 interphase and 100 mitotic cells per treatment. Two experiments were analyzed.

Chromium Uptake Assay

Chromium uptake was measured as previously reported (Speer et al., 2019). Whale cells were seeded into 60 mm dishes and treated as described above. Harvests were performed at the time of treatment (0 h) and after 24 h and 120 h of exposure. Extracellular chromium was analyzed from culture media passed through a 0.2 µm filter. To obtain intracellular samples, plates were rinsed with 1X PBS and cells were released using 0.25% trypsin-EDTA. Cell counts and cell diameter were recorded. Cells were washed twice in 1X PBS and suspended in 1 ml 0.075 M hypotonic potassium chloride for 5 minutes. One milliliter 2% sodium dodecyl sulfate was added for 15 minutes to rupture the cell membrane and the suspension was sheared through an 18G needle 7 times. Lysate was filtered through a 0.2 µm filter. All samples were diluted in 2% nitric acid. Extracellular and intracellular chromium was analyzed by atomic absorption

spectroscopy with a Perkin Elmer 900Z graphite furnace atomic absorption spectrometer (GFAAS) using Syngistix Software. Calibration was performed using chromium standards at 0, 5, 10, 20, 40 and 80 ppb of chromium (PerkinElmer Pure,1,000 µg/ml, 2% HNO_3). The wavelength used for chromium was 357.87 nm. All points were within the range of detection and trace free 2%. The limit of detection for chromium was 0.004 µg/l. Three experiments were analyzed per cell line.

Cell Cycle Analysis

Cell cycle analysis was performed using flow cytometry using published methods (Xie et al., 2009) with modifications. Cells were seeded in 100 mm tissue culture dishes and treated with zinc chromate as described above for 24 h or 120 h. One hour before end of treatment, NCS (400 ng/ml final concentration) was applied to the positive control dish and incubated for 30 minutes in the dark, followed by 30 minutes recovery time in fresh media. At the end of treatment time, media was collected in 50 ml conical tubes to ensure analysis of all cells. Adherent cells were released from dishes with 0.25% trypsin-EDTA and collected and combined with harvested media. Cells were centrifuged to a pellet at 1,000 RPM for 5 minutes, then resuspended in 5 ml PBS and counted. One million cells for each treatment condition was saved in a new tube and resuspended in 0.5 ml of PBS. Cells were fixed in 4% PFA (paraformaldehyde) on ice, 15 minutes. Cells were pelleted by centrifugation, washed with PBS, and centrifuged again. Cells were resuspended in 0.5 ml PBS and combined with 70% ethanol, added drop-

wise during gentle vortexing. Fixed cells were centrifuged, resuspended in 1 ml 70% ethanol and stored at -20º C until analysis. Cell cycle analysis was performed using propidium iodide to quantify DNA content. Flow cytometry was analyzed using FlowJo software to determine percentage of cells in G1, S, or G2/M phase.

Karyotype Analysis

Karyotype analysis using Giemsa banding (g-banding) was used to confirm cell line identity. G-banding creates signature staining patterns on chromosomes to enable identification of each chromosome and to characterize a cell line. Cells were seeded into a tissue culture flask and at 70-80% confluency cells were arrested in metaphase by demecolcine. Media was collected and cells were released using 0.25% trypsin-EDTA. Cells were centrifuged at 1,000 RPM, media was aspirated, and cells were resuspended in hypotonic 0.075 M potassium chloride for 17 minutes, then fixed in Carnoy's fixative for 20 minutes. Fixative was changed twice before preparing slides. Fixed cells were then dropped onto wet glass slides and dried at 30º C, 30% humidity. Cells were baked in 95º C oven for 45 minutes. Chromosomes were stained by digesting the cells with trypsin (1:250) in Gurr's buffer approximately 30 seconds – 1 minute and then neutralized in Gurr's buffer with 2% fetal bovine serum. Slides were dipped 3-4 times in Gurr's buffer (pH 7.0) then 70% ethanol, 95% ethanol and Gurr's buffer (pH 6.8) before staining approximately 3 minutes in Geimsa stain. Slides were cover-slipped and imaged using an Applied Spectral Imaging microscope and software. Ten metaphases were assessed per analysis.

Statistical Analysis

All values are expressed as the mean ± SEM (standard error of the mean). Clonogenic survival, aneuploidy, spindle assembly checkpoint bypass, cell cycle analysis, and chromium uptake were analyzed by single factor ANOVA (α=0.05) to determine the significance of zinc chromate treatments within each time point. For cell cycle analysis, Dunnett's multiple comparisons test (α=0.05) was used to assess differences between treatments and controls in each cell phase. Two-tailed Student's t-tests (unequal variance) were performed for all other assays to determine differences between each chromate concentration and the untreated control for each time point.

CHAPTER 3: RESULTS

AIM 1: PARTICULATE HEXAVALENT CHROMIUM-INDUCED SECURIN DYSREGULATION DRIVES NUMERICAL CHROMOSOME INSTABILITY IN HUMAN LUNG CELLS

BACKGROUND

Particulate hexavalent chromium [Cr(VI)] is a potent lung carcinogen. Widespread industrial usage causes Cr(VI) to be both an occupational exposure risk as well as an environmental contaminant whose dangers are recognized by prominent placement on the ATSDR Substance Priority List (ATSDR, 2019). However, despite well-known cancer risks posed by Cr(VI), its carcinogenic mechanisms are not well understood. A driving mechanism in Cr(VI) carcinogenesis is chromosome instability (Kondo et al., 1997; Hirose et al., 2002; Holmes et al., 2008; Salnikow and Zhitkovich, 2008; Wise et al., 2010; Proctor et al., 2014). We previously reported particulate Cr(VI) induces numerical chromosome instability in human lung cells after prolonged (> 48 h) exposure but not after acute (24 h) exposure (Holmes et al., 2006; Holmes et al., 2010). We also found Cr(VI)-induced numerical chromosome instability was heritable at a cellular level, and consistent with our previous reports, required at least 48 h of exposure

to occur (Wise et al., 2018). How Cr(VI) causes numerical chromosome instability is currently unknown.

A central regulator for numerical chromosome instability is securin (Zhou et al., 1999; Jallepalli et al., 2001; Chao et al., 2006). Securin regulates separase activity through inhibitory binding and the securin-separase complex is present in yeast, plants, and animals (Cohen-Fix et al., 1996; Funabiki et al., 1996; Zhou et al., 1999). Securin is best known as an anaphase inhibitor that regulates separase activity at centromeres and thus protects faithful chromosome segregation (Yamamoto et al., 1996; Funabiki et al., 1996a; Cohen-Fix et al., 1996; Jallepalli et al., 2001; Waizenegger et al., 2002). Indeed, securin has been well-described for inhibiting sister chromatid disjunction (Uhlmann et al., 1999; Ciosk et al., 1998). Interaction with securin inhibits the active site of separase until timely degradation of securin releases this block (Funabiki et al., 1996; Cohen-Fix et al., 1996; Zhou et al., 1999; Hornig et al., 2003; Waizenegger et al., 2000). At the metaphase-anaphase transition, separase-bound securin is degraded, inducing separase protease activity to cleave the SCC1/RAD21 subunit of cohesin. The opening of the cohesin ring releases sister chromatid conjunction and allows chromatid segregation into daughter cells. Uncontrolled separase activity at the centromeres causes abnormal mitosis and aneuploidy (Zhang et al., 2008; Shindo et al., 2021).

Securin has another key role in maintaining numerical chromosome fidelity through centrosome duplication control (Inanç et al., 2010; Tsou and Stearns, 2006). The securin-separase complex localizes to centrosomes and separase activity there causes centriole disengagement (Agircan and Schiebel, 2014; Tsou

and Stearns, 2006). Centrioles are held together by cohesin and kendrin, both of which are separase substrates. Daughter centriole disengagement is the licensing step for centrosome duplication (Tsou and Stearns, 2006) and maintenance of centrosome number requires controlled timing of this occurrence. At the centrosomes, loss of securin inhibitory function on separase activity leads to centrosome amplification. Centrosome amplification drives numerical chromosome instability by generating aberrant spindle poles, kinetochore-microtubule disruption, and asymmetrical chromatid segregation (Baum et al., 1988; Sluder and Nordberg, 2004; Fukasawa, 2009; Jusino et al., 2018).

We previously demonstrated prolonged Cr(VI) exposure induces premature chromatid separation, premature centriole disengagement, and centrosome amplification (Holmes et al., 2006; Wise et al., 2006; Holmes et al., 2007; Martino et al., 2015). These defects can lead to numerical chromosome instability, yet the mechanistic cause is unknown. Securin has been shown to be vulnerable to DNA-damaging agents (Zhou et al., 2003; Chou et al., 2005) and experimental securin modulation can induce centrosome amplification and CIN (Yu et al., 2003; Chou et al., 2005; Kim et al., 2007; Tsou and Stearns, 2006; Mora-Santos et al., 2013). We investigate securin as a target of Cr(VI) and as a potential key in Cr(VI)-induced premature chromatid separation, premature centriole disengagement, centrosome amplification, and numerical chromosome instability.

RESULTS

Prolonged Cr(VI) exposure causes securin protein loss.

We previously reported prolonged (120 h) exposure to zinc chromate induces centrosome amplification and abnormal centrioles (Holmes et al., 2009; Martino et al., 2015). Acute (24 h) exposure did not induce changes in centrosomes or chromosome instability. Securin is a central regulator for centrosome maintenance and prevents abnormal centriole splitting, thus we investigated if securin is affected by acute or prolonged zinc chromate exposure. After 24 h and 120 h zinc chromate exposure, securin protein levels were measured by western blot. We found 24 h exposure caused securin increase. Cells exposed to 120 h concentrations of 0.1, 0.2, and 0.3 ug/cm^2 zinc chromate showed reductions of securin levels to 48.5%, 31%, and 15.3% relative to untreated controls (Figure 5).

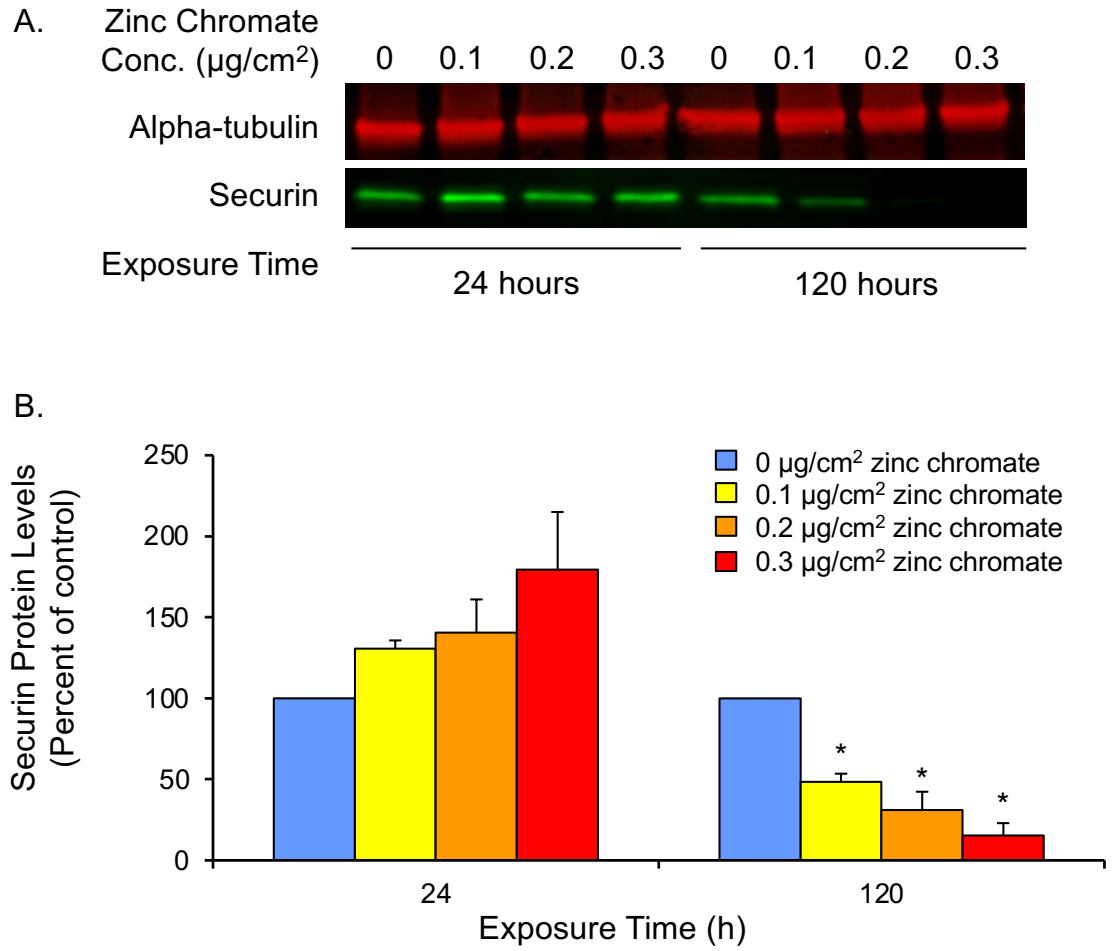
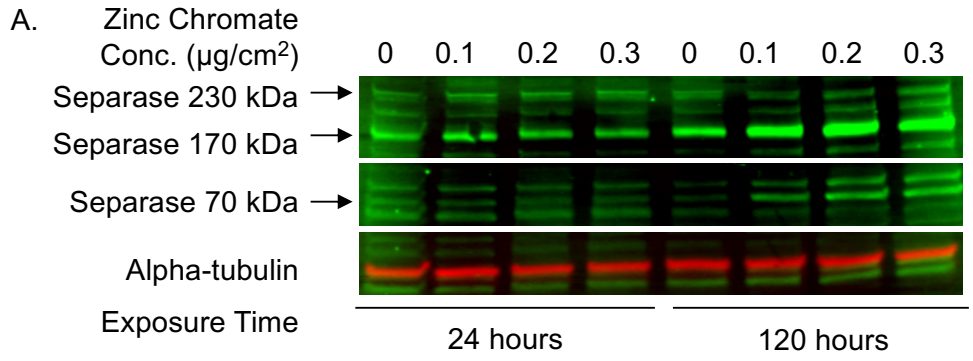
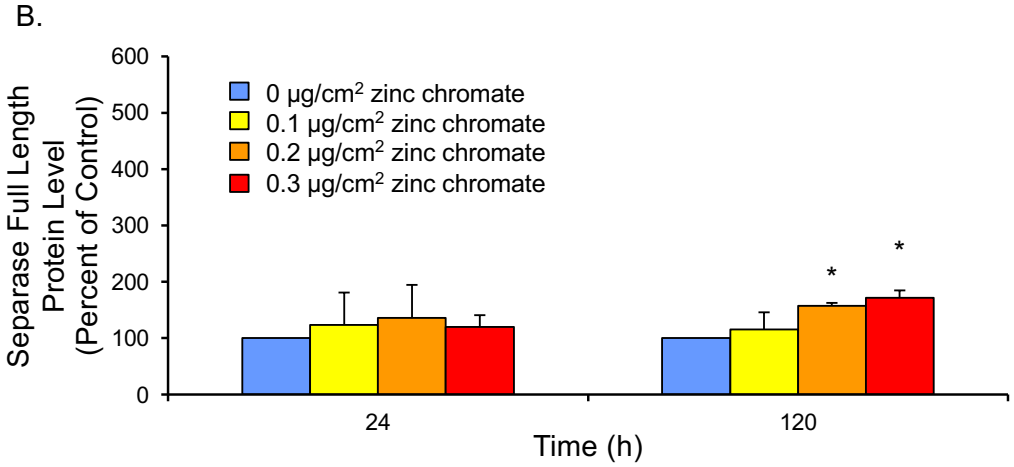


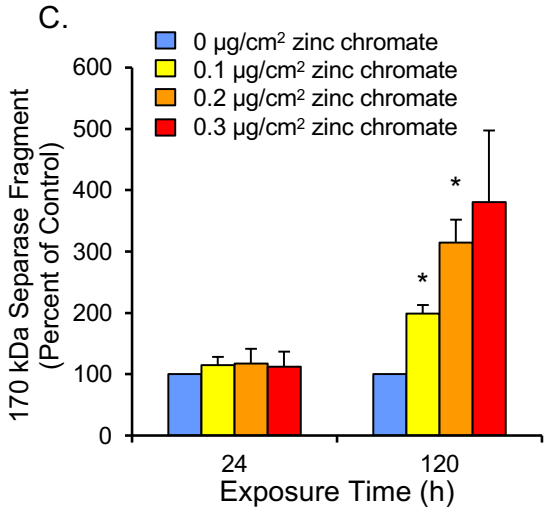
Figure 5. Securin protein levels in human lung cells after zinc chromate exposure. This figure shows prolonged Cr(VI) exposure decreased securin levels. (A) Representative western blot for securin. Alpha-tubulin was used as a loading control. (B) Securin whole cell protein levels decreased after 120 h Cr(VI) exposure. Data are expressed as percent of untreated control cells and reflect the mean of three independent experiments. Error bars = SEM. *Significantly different from control group ($p < 0.05$).

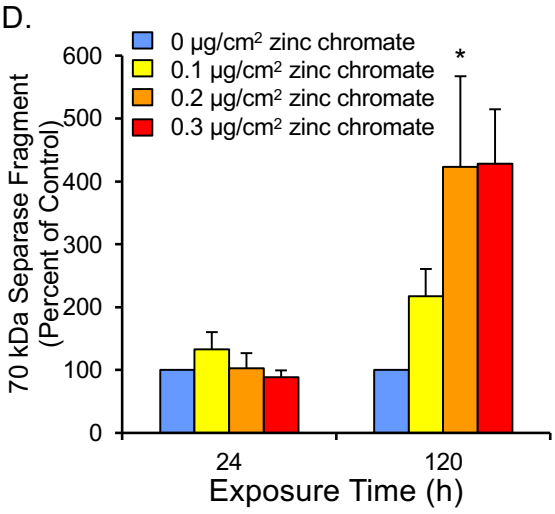
Prolonged Cr(VI) exposure causes loss of securin function.

We have shown 120 h zinc chromate exposure to human lung cells causes a loss of securin protein which becomes more severe at higher concentrations. Securin and separase function are critical to proper cell function and thus are tightly controlled and protected by redundant pathways (Stemmann et al., 2001; Nagao et al., 2002; Hellmuth et al., 2015). We sought to determine if cells cope with zinc chromate exposure by retaining securin function under lower protein levels. Securin function restrains separase enzyme activity, and so we investigated three measures of separase activity: 1) separase autocleavage, 2) kendrin cleavage, 3) Scc1 cleavage. When securin is released from separase, separase becomes its own substrate and cleaves itself (Waizenegger et al., 2000). Separase autocleavage is observable on western blots as a full length, 220 kDa, band and quickly migrating bands of smaller protein fragments (Figure 6A). After 24 h, separase 220 kDa, 170 kDa, and 70 kDa bands are similar to control levels at all zinc chromate concentrations we tested (Figures 6B, 6C, 6D). However, 120 h exposure caused the 170 kDa protein fragment to increase to 200%, 310%, and 380% of control levels with 0.1, 0.2, and 0.3 ug/cm^2 zinc chromate concentrations, respectively (Figure 6C). Separase fragments of 70 kDa increased to 220%, 420% and 430% of control levels, respectively (Figure 6D). These data indicate autocleavage increases with prolonged Cr(VI) exposure, thus securin inhibition of separase cleavage activity is not maintained at these zinc chromate concentrations. Figure 6E shows the levels of separase full-length and 170-kDa fragment relative to control levels.


A.
Zinc Chromate
Conc. (μg/cm²)
0 0.1 0.2 0.3 0 0.1 0.2 0.3
Separase 230 kDa
Separase 170 kDa
Separase 70 kDa
Alpha-tubulin
Exposure Time
24 hours
120 hours


B.
Separase Full Length Protein Level (Percent of Control)
0 μg/cm² zinc chromate
0.1 μg/cm² zinc chromate
0.2 μg/cm² zinc chromate
0.3 μg/cm² zinc chromate
600
500
400
300
200
100
0
24
120
Time (h)
*
*


C.
0 μg/cm² zinc chromate
0.1 μg/cm² zinc chromate
0.2 μg/cm² zinc chromate
0.3 μg/cm² zinc chromate
170 kDa Separase Fragment (Percent of Control)
600
500
400
300
200
100
0
24
120
Exposure Time (h)
*
*


D.
0 μg/cm² zinc chromate
0.1 μg/cm² zinc chromate
0.2 μg/cm² zinc chromate
0.3 μg/cm² zinc chromate
70 kDa Separase Fragment (Percent of Control)
600
500
400
300
200
100
0
24
120
Exposure Time (h)
*

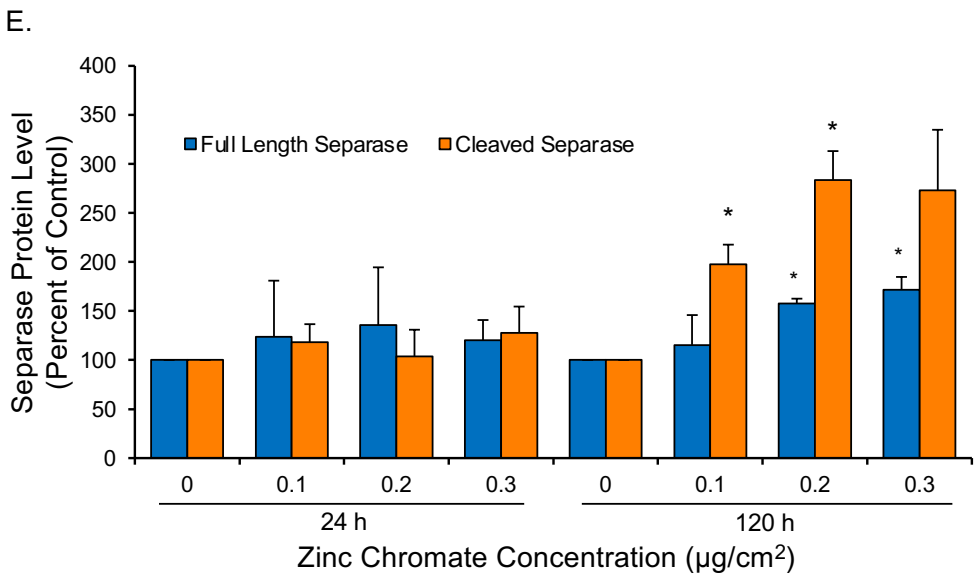


Figure 6. Separase protein levels in human lung cells after zinc chromate exposure. This figure shows prolonged Cr(VI) exposure induced separase cleavage. (A) Representative western blot for separase. Alpha-tubulin was used as a loading control. (B) Separase full length protein levels increased slightly after 120 h Cr(VI). (C-D) Cleaved separase protein levels increased after 120 h Cr(VI). (E) Comparing full length and cleaved levels showed differences in cleavage activity between 24 h and 120 h exposures. Data are expressed as percent of untreated control cells and reflect the mean of three independent experiments. Error bars = SEM. *Significantly different from control group ($p < 0.05$).

The specificity of western blot antibody visualization does not allow us to measure total separase levels. To determine if increased cleavage products are a consequence of increased separase expression, we measured separase mRNA levels after 24 and 120 h of zinc chromate exposure. RT-qPCR results show separase mRNA levels were reduced at all time points and concentrations of zinc chromate exposure (Figure 7). 24 h exposure to 0.1, 0.2, and 0.3 ug/cm^2 zinc chromate resulted in 0.761, 0.683, and 0.564 expression relative to compared to controls, versus 0.412, 0.145, and 0.085 of control levels at 120 h, respectively, indicating a downregulation. Thus, observed increases in separase cleavage products are not likely caused by increased separase protein production and can properly serve as an indicator of increased separase autocleavage.

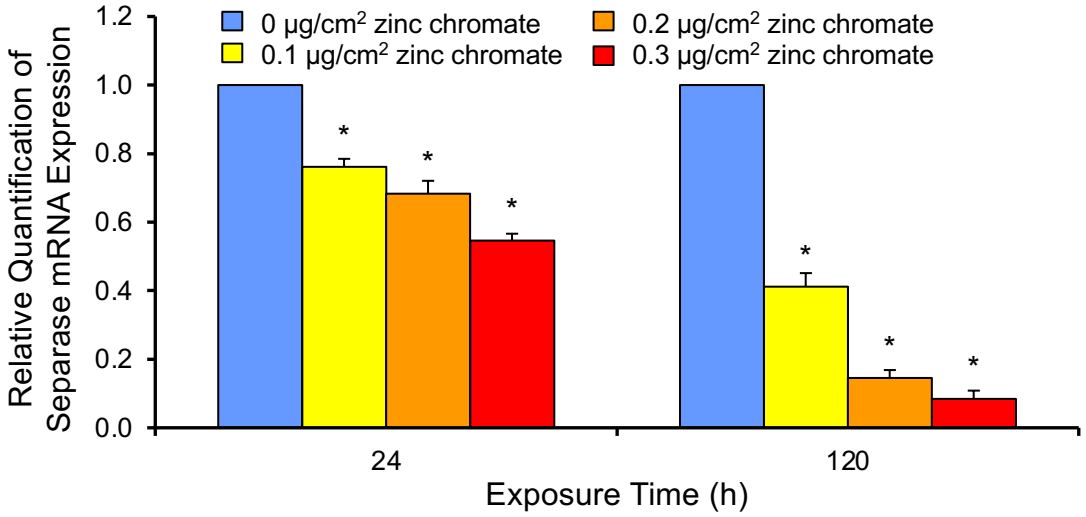


Figure 7. Separase mRNA quantified by RT-qPCR after 24 and 120 h zinc chromate exposure. This figure shows Cr(VI) exposure decreased separase mRNA after 24 h and 120 h. *Significantly different from control group ($p > 0.05$). Data are expressed as relative expression compared to untreated control cells and reflect the mean of three independent experiments with three technical replicates each. Error bars = SEM. *Significantly different from control group ($p < 0.05$).

Securin functions to control cleavage of kendrin by separase. Kendrin is a large coiled-coil protein with the essential role in centrosome maintenance of supporting centriole engagement (Matsuo et al., 2010; Matsuo et al., 2012). Centrioles must remain engaged throughout the cell cycle until after anaphase when they split for the purpose of centrosome duplication. Aberrant centriole separation during G2 phase can cause overduplication of centrosomes and lead to centrosome amplification (Tsou et al., 2009; Inanç et al., 2010; Loncarek et al., 2010). Kendrin cleavage can be observed by western blot as a full-length band of 360 kDa and a 125 kDa N-terminal fragment (Figure 8A). Figure 8B shows full length kendrin protein levels. 24 h exposure induced slight increases in protein, but 120 h exposure to 0.1, 0.2, and 0.3 ug/cm^2 zinc chromate resulted in full length protein levels of 75.7%, 56.4% and 55.8% of control levels. Cleaved protein at 120 h increased to 118.3%, 159.9% and 152.2%. of control levels (Figure 8C). Thus, 120 h zinc chromate exposure resulted in higher cleaved versus full-length kendrin levels, indicating increased separase activity and loss of securin function.

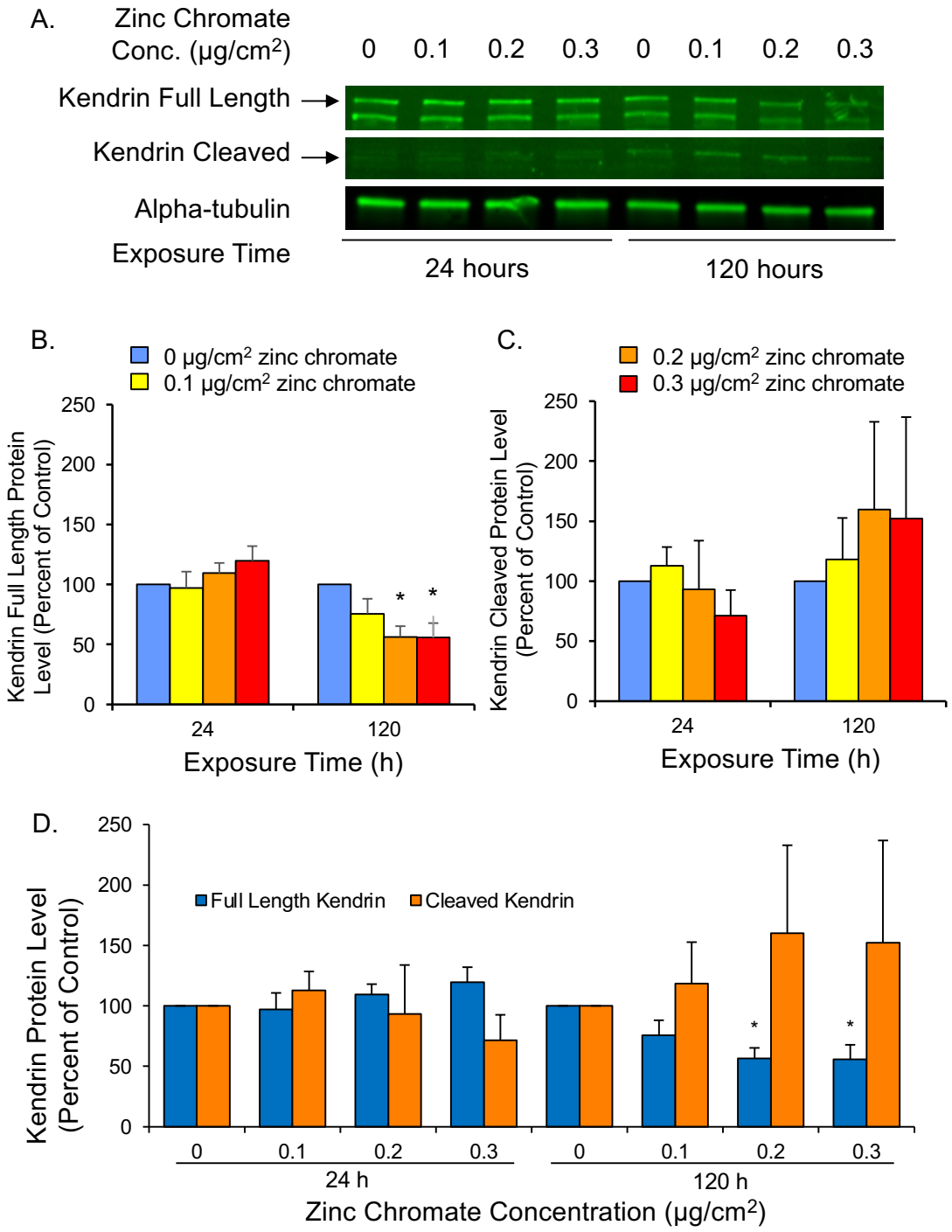


Figure 8. Full Length and cleaved kendrin protein after 24 and 120 h zinc chromate exposure. This figure shows prolonged Cr(VI) induced kendrin cleavage. (A) Representative western blot showing full-length and cleaved kendrin bands.

Alpha-tubulin was used as a loading control. (B) Full length kendrin levels decreased after 120 h Cr(VI). (C) Cleaved kendrin protein levels increased after 120 h. (D) Comparing full-length and cleaved protein levels shows cleavage activity changed after 120 h compared to 24 h. *Significantly different from control group ($p > 0.05$). Data are expressed as percent of untreated control cells and reflect the mean of three independent experiments. Error bars = SEM. *Significantly different from control group ($p < 0.05$).

Securin functions to inhibit separase from cleaving the SCC1 subunit of cohesin. Cohesin rings are present at the centrioles to link them together during interphase and prevent premature disengagement (Schockel et al., 2011). The full length SCC1 protein appears as a 130 kDa band, while its cleavage product migrates at 90 kDa. We measured SCC1 cleavage as a ratio of cleaved to full protein. In whole cell extracts, zinc chromate did not significantly alter SCC1 cleavage at any timepoint or concentration (Figure 9B). This outcome may be because the vast majority of cohesin is sequestered in the nucleus and not cleaved by separase during interphase. We propose centriole-associated cohesin is cleaved by prematurely active separase in interphase cytoplasm, thus we used cytoplasmic protein fractions to analyze SCC1 cleavage, but protein levels were too low to be detected by western blot (data not shown). We measured SCC1 in nuclear fragments and found prolonged Cr(VI) exposure increased the ratio of cleaved/full-length protein (Figure 9C). Though the result was not statistically significant, 120 h exposure to 0.1, 0.2, and 0.3 μg/cm^2 zinc chromate increased the ratio of cleaved/full-length protein to 102.9%, 148.1%, and 152.0% compared to control, respectively.

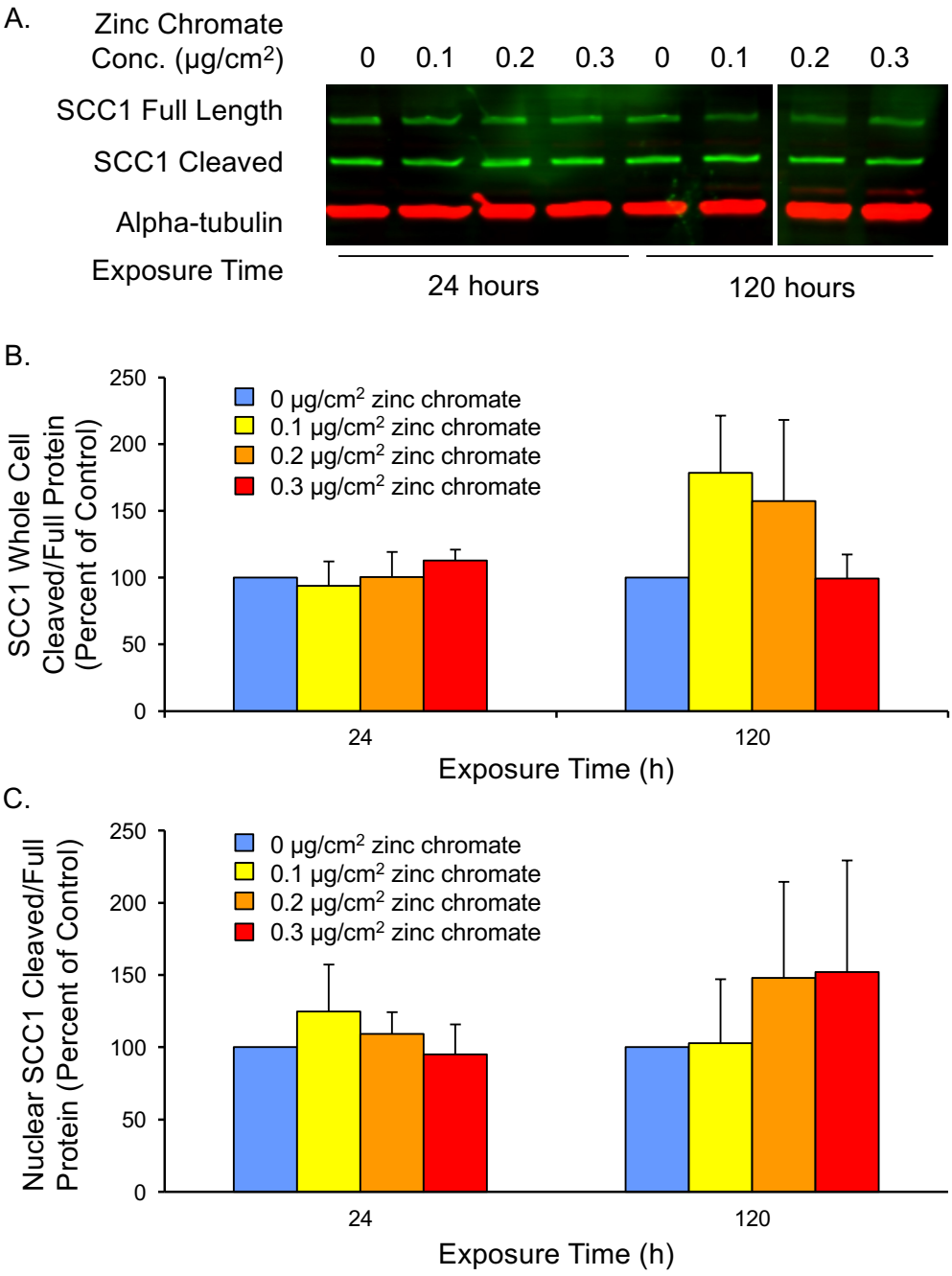


Figure 9. Full-length and cleaved SCC1 protein after 24 and 120 h zinc chromate exposure. SCC1 cleavage was measured in Cr(VI)-exposed cells versus control cells. (A) Representative western blot showing full-length and cleaved SCC1 bands from whole cell extract. Alpha-tubulin was used as a loading control. (B) The

ratio of cleaved/full-length SCC1 was not significantly altered by Cr(VI) in whole cell lysates. (C) The ratio of cleaved/full-length nuclear SCC1 was not significantly altered but show a strong increasing trend, indicating increased cohesin cleavage. Data are expressed as percentage of untreated control cells and reflect the mean of two independent experiments. Error bars = SEM. No condition was significantly different from control group.

Kendrin and cohesin are responsible for maintaining centriole engagement throughout the cell cycle, until timely release in late mitosis or early G1 phase (Agircan et al., 2014). We previously demonstrated zinc chromate induces centriole disruption, including abnormal centriole disengagement in G2 phase (Martino et al., 2015). Thus, our results showing kendrin cleavage increased and nuclear cohesin cleavage increased while securin levels decreased are consistent and indeed help to explain previous results indicating zinc chromate targets centriole linker maintenance.

Prolonged Cr(VI) exposure causes loss of cyclin B1 protein.

Securin is the canonical separase inhibitor. However, timely separase activation is so critical to cellular integrity that multiple redundant mechanisms exist to control separase. Redundant inhibition is evidenced by studies showing securin knockout in human and mouse cells can persist and grow relatively unaffected (Mei et al, 2001; Pfleghaar et al., 2005). Cyclin B1 acts as a secondary separase inhibitor (Gorr et al., 2005; Hellmuth et al., 2015). Cyclin-dependent kinase 1 (Cdk1) is activated by its partner, cyclin B1, to phosphorylate separase, making separase susceptible to aggregation and precipitation and thus inactive (Hellmuth et al., 2015). Also, the Cdk1-cyclin B1 complex binds to separase which also renders it inactive but primed for activation (Hellmuth et al., 2015). Thus, we measured cyclin B1 protein levels after 24 h and 120 h of 0.1, 0.2, and 0.3 ug/cm^2 zinc chromate exposure. Again, no significant change was observed after acute exposure, but after prolonged 120 h exposure, cyclin B1 levels were reduced to

43.7%, 24.5%, and 12.7% of control values (Figure 10). These data indicate Cr(VI) reduces securin and also inhibits secondary compensation by cyclin B1.

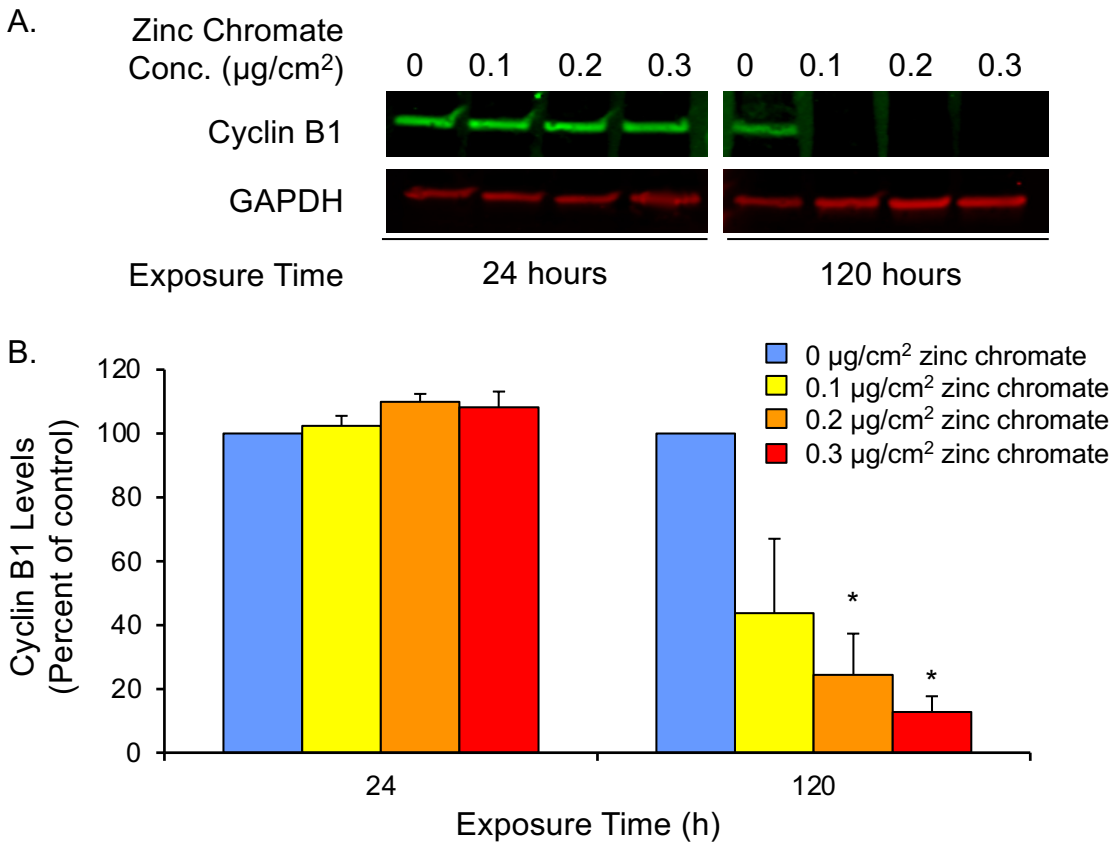


Figure 10. Cyclin B1 protein levels in human lung cells after zinc chromate exposure. This figure shows prolonged Cr(VI) exposure decreased cyclin B1 levels. (A) Representative western blot for cyclin B1. GAPDH was used as a loading control. (B) Cyclin B1 whole cell protein levels decreased after 120 h Cr(VI) exposure. Data are expressed as percent of untreated control cells and reflect the mean of three independent experiments. Error bars = SEM. *Significantly different from control group ($p < 0.05$).

Cyclin B1 levels fluctuate with the phases of the cell cycle such that levels are low in G1, begin to rise in S, peak in early mitosis, and drop with degradation at the metaphase-to-anaphase transition. Securin levels rise during S phase and are depleted at the initiation of anaphase. One interpretation of observed securin and cyclin B1 losses is these data may be evidence of a cell cycle effect in which Cr(VI) causes a large proportion of cells to accumulate in G1 phase. To pursue this question, we performed cell cycle analysis by flow cytometry and measured cell cycle phase by the DNA content. Cr(VI) exposure for 24 h and 120 h did not enrich the population of G1 cells. In fact, consistent with a DNA damage exposure, Cr(VI) caused increase in G2/M populations (Figure 11). G2 and M are the phases at which securin and cyclin B1 levels should be the highest, providing confidence that measured protein loss is not due to cell cycle disruption.

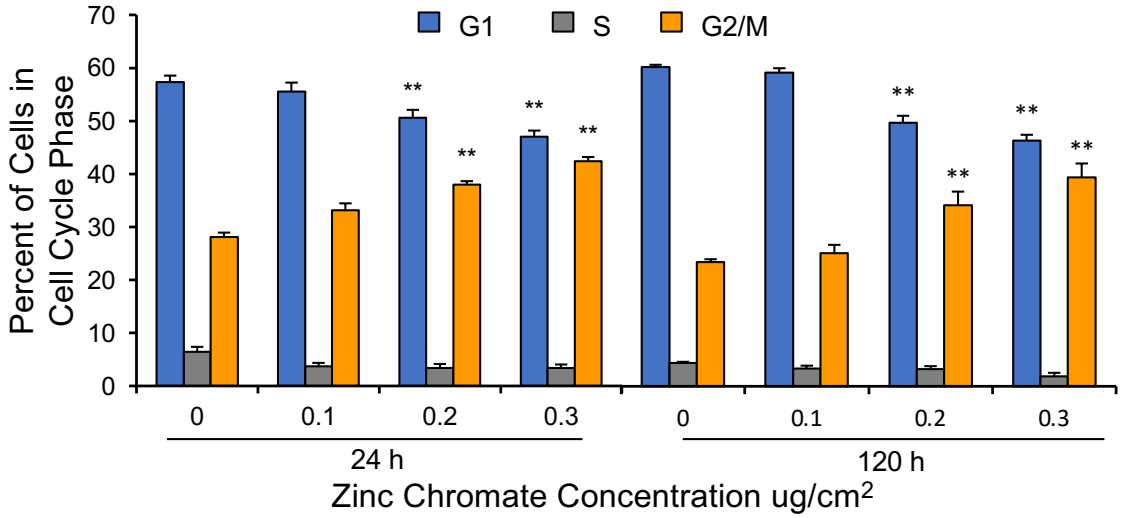


Figure 11. Percent of human lung cells in each cell phase after zinc chromate exposure. This figure shows Cr(VI) decreased the percentage of cells in G1 and increased the percentage of cells in G2/M. Data are expressed as percent of untreated control cells and reflect the mean of three or four independent experiments. Error bars = SEM. **Significantly different from control group (p < 0.001).

Securin knockdown with acute Cr(VI) exposure recapitulates aneuploidy observed after prolonged Cr(VI) exposure.

We previously reported 120 h exposure to 0.2 ug/cm^2 zinc chromate caused aneuploid metaphases to increase to 44% compared with control levels of 8-13% (Holmes et al., 2010). Our hypothesis is Cr(VI)-induced aneuploidy is caused by loss of securin protein. Zinc chromate exposure for 24 h did not reduce securin protein levels or induce aneuploid metaphases (Holmes et al., 2010). By artificially reducing securin alongside 24 h Cr(VI) exposure, numerical CIN may be induced similarly to prolonged Cr(VI) exposures which incur securin loss. We used securin-targeting siRNAs to knockdown securin protein levels and then exposed human lung cells to 0.2 ug/cm^2 zinc chromate for 24 h. Figure 12 shows securin levels after knockdown by two different siRNAs. Aneuploidy was not significantly increased by 24 h zinc chromate exposure alone, consistent with our earlier findings. 24 h exposure did increase the percent of aneuploid metaphases to 15.9% and 20.2% after knockdown with siRNAs #1 and #2, respectively. Securin knockdown alone increased aneuploidy slightly. Of note, non-targeting siRNA transfection caused cell death and increased aneuploidy rates compared with control, which could be interpreted as an effect of transfection.

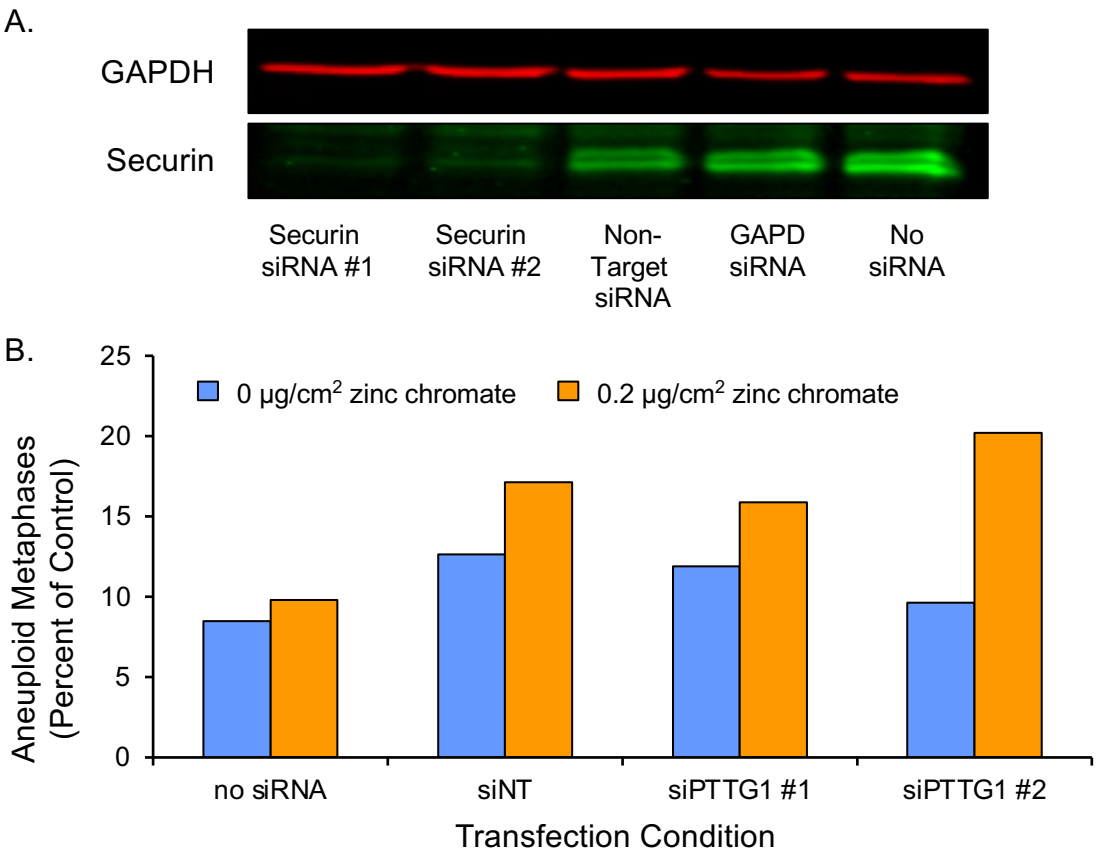


Figure 12. Aneuploidy after 48 h securin knockdown and 24 h zinc chromate exposure. This figure shows securin knockdown increases aneuploidy after acute Cr(VI) exposure. (A) Securin protein loss by siRNA targeting was confirmed. GAPDH was used as a loading control. (B) Percent of aneuploid metaphases increased after securin knockdown and acute Cr(VI) exposure. Data reflect one experiment.

Securin knockdown with acute Cr(VI) exposure recapitulates spindle assembly checkpoint bypass observed after prolonged Cr(VI) exposure.

Spindle assembly checkpoint prevents cell cycle progression into anaphase until all kinetochores are properly attached to microtubules. Proper separase regulation will inhibit enzyme activity towards centromeric cohesin until anaphase. Thus, metaphase abnormalities including centromere spreading, premature centromere division, and premature anaphase are evidence of spindle assembly checkpoint bypass and aberrant separase activity, both of which cause aneuploidy. As described by Holmes et al. (2010), centromere spreading is defined as dissociation of chromatids at the centromere but not at the rest of the chromosome; premature centromere division is characterized by at least one chromosome in a metaphase spread that is separated from its sister chromatid while at least one chromosome is connected to its sister chromatid; premature anaphase is defined as all chromosomes separated from their sister chromatids. We previously reported 120 h exposure to 0.2 ug/cm^2 zinc chromate caused 5% centromere spreading, 21% premature centromere division, and 13% premature anaphase while controls displayed 1% premature centromere division and 1% premature anaphase (Holmes et al., 2010). Our hypothesis is loss of securin protein leads to premature separase activity, which can result in cleavage of centromeric cohesin before metaphase to anaphase transition. We used siRNA to knockdown securin levels and then treated human lung cells for 24 h with 0.2 ug/cm^2 zinc chromate. Previous findings (Holmes et al., 2010) revealed no increase in SAC bypass was measured after 24 h exposure. Consistent with Holmes et al., we measured 1%

premature centromere division and 1% premature anaphase in both untreated and treated cells without transfection. Securin knockdown increased the percentage of metaphases exhibiting SAC bypass, and this was an effect consistent with each siRNA (Figure 13). Securin siRNA #1 caused 2.9% premature centromere division and 4.8% premature anaphase. Securin siRNA #2 caused 6% centromere spreading and 2% premature anaphase. Non-targeting siRNA also caused increased SAC bypass with 2% centromere spreading, 1% premature centromere division, and 1% premature anaphase. Interestingly, zinc chromate exposure with securin knockdown resulted in SAC bypass as low as control levels.

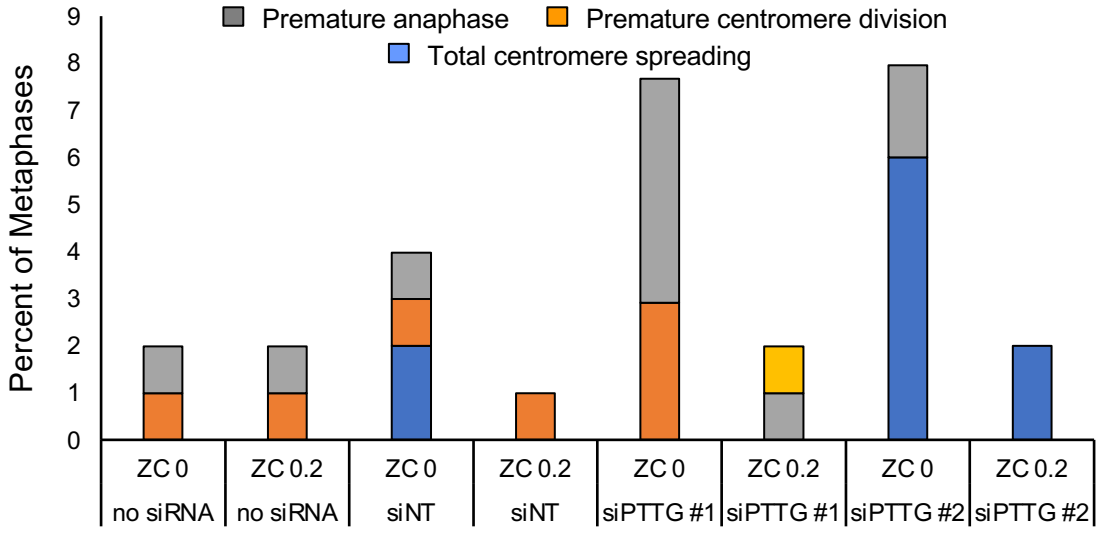


Figure 13. Spindle assembly checkpoint bypass after 48 h securin knockdown and 24 h zinc chromate exposure. This figure shows securin knockdown increases SAC bypass. This effect was not retained with addition of acute Cr(VI) exposure. Percent of metaphases showing SAC bypass increased after securin knockdown with both siRNAs. Data reflect one experiment.

Prolonged Cr(VI) targets securin via a pre-translational mechanism.

In considering how zinc chromate reduces securin protein levels, one possibility is that securin degradation is affected. Securin is degraded via the anaphase-promoting complex/cyclosome (APC/C) during anaphase (Cohen-Fix et al., 1996; Yamamoto et al., 1996; Tsou et al., 2006b), allowing activation of separase. To measure securin protein degradation rates, we performed a cycloheximide chase assay after 24, 72, and 120 h zinc chromate exposure. We focused on 0.2 ug/cm^2 zinc chromate exposure because it shows a strong response in securin reduction after 120 h. We added the intermediate time point of 72 h to detect potential key events in degradation leading up to the observed 120 h reduction of securin protein level. Securin protein half-life did not differ between control and treated cells after 24, 72, or 120 h zinc chromate exposure (Figure 14).

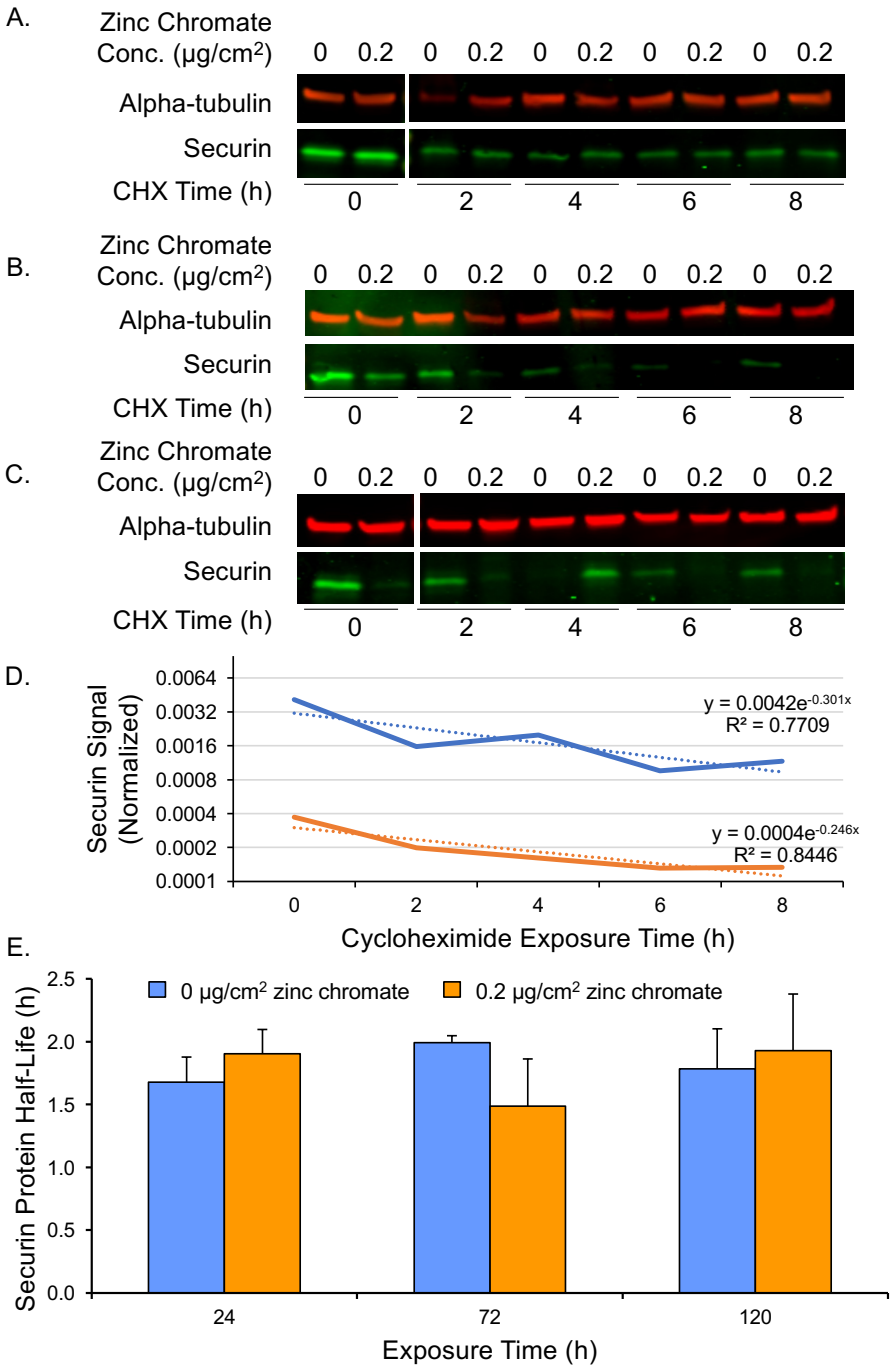


Figure 14. Securin protein half-life in human lung cells after zinc chromate exposure. This figure shows Cr(VI) did not change rates of securin degradation. (A-C) Representative western blots for securin after cycloheximide (CHX) treatment. GAPDH was used as a loading control. (A) Securin protein after 24 h

Cr(VI). (B) Securin protein after 72 h Cr(VI). (C) Securin protein after 120 h Cr(VI). (D) Representative plot of securin degradation over time on log base 2 scale (120 h zinc chromate exposure example). Solid line represents the securin signal and dotted line represents the best fit line used for calculating the protein half-life. (E) Securin half-life did not significantly change after zinc chromate exposure. Data reflect the mean of three independent experiments. Error bars = SEM. No condition was significantly different from the untreated control group.

Our data show securin loss is not due to Cr(VI)-induced protein degradation, thus we measured effects on securin at the pre-translational level by quantifying securin mRNA using RT-qPCR after 24 and 120 h zinc chromate exposures. Slight decreases in mRNA levels after 24 h were not statistically significant. However, 0.1, 0.2, and 0.3 ug/cm^2 zinc chromate exposures at 120 h caused securin mRNA expression levels of -0.52, 0.143, and 0.012, respectively (Figure 15). Together these data show zinc chromate targets securin via pre-translational mechanisms and does not change protein stability.

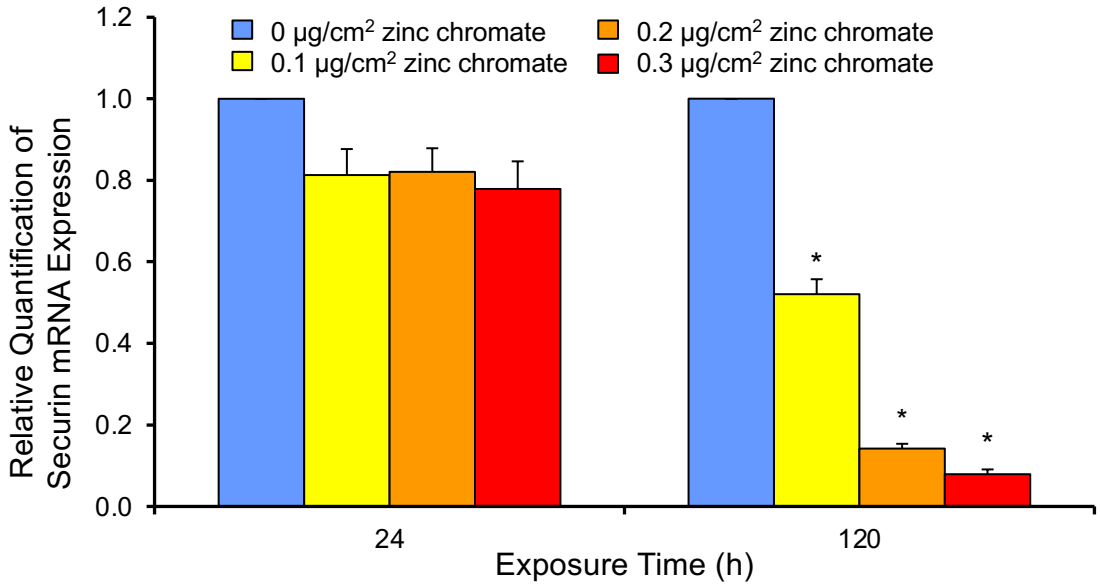


Figure 15. Securin mRNA quantified by RT-qPCR after 24 and 120 h zinc chromate exposure. This figure shows Cr(VI) exposure decreased securin mRNA after 24 h and 120 h. *Significantly different from control group ($p > 0.05$). Data are expressed as relative expression compared to untreated control cells and reflect the mean of three independent experiments with three technical replicates each. Error bars = SEM. *Significantly different from control group ($p < 0.05$).

Summary

Cr(VI) is a well-known human carcinogen. Despite evidence of genotoxic effects in humans, rodent studies, and cell culture, the molecular mechanism of carcinogenesis remains unknown. Cr(VI) exposure causes SAC bypass, centriole disengagement, centrosome amplification, and numerical CIN indicated by aneuploid metaphases (Martino et al., 2015; Holmes et al., 2010; Wise et al., 2006; Holmes et al, 2006). Studies show centriole disengagement leads to centrosome amplification. Centrosome amplification is a driver of numerical chromosome instability. Separase is the enzyme responsible for both centriole disengagement and cohesin cleavage at centromeres which permits SAC bypass. This study investigates how numerical chromosome instability may be induced through disruption of the main separase regulatory protein, securin. Securin inhibition of separase controls timing of cohesin and kendrin cleavage at centrioles. Thus securin regulates centriole disengagement and cohesin cleavage at centromeres. Disruption of securin can cause premature centromere spreading, centromere division, and premature anaphase.

We show here 120 h zinc chromate exposure in human lung cells causes dramatic decrease in securin protein levels. Protein half-life measurements show securin degradation is not affected by Cr(VI) and cell cycle analysis shows changes in securin protein are not explained by changes in the cell cycle. RT-qPCR analysis of securin mRNA correlate with protein loss after 120 h, indicating Cr(VI) causes reduction in securin gene expression.

Depressed levels of securin protein may still be sufficient to control separase, thus we used three markers of separase activity to analyze loss of securin function. Upon securin release, separase becomes active and causes 1) separase autocleavage, 2) kendrin cleavage, and 3) Scc1 cleavage. We show separase autocleavage increases after 120 h zinc chromate exposure, indicating separase activation. Separase mRNA quantification shows increased separase activity is not due to increased separase expression, and separase mRNA actually decreased after 120 h Cr(VI) exposure.

We found 120 h Cr(VI) caused increased kendrin cleavage. Kendrin is a separase substrate present at the centrioles to support engagement and prevent untimely centriole disengagement. Thus, abnormal kendrin cleavage provides evidence for premature separase activity at the centrosomes. Variation in the levels of cleaved kendrin fragments reduced statistical significance and may be due to generation of fragments that run at variable speeds. Kendrin has A and B isoforms that differ slightly in total molecular weights and whose cleavage products have different sizes. Furthermore, Lee and Rhee (2015) identified 22 sites at which Plk1 phosphorylates kendrin. Variation in protein phosphorylation will alter band migration upon electrophoresis. A useful measurement of kendrin cleavage can be performed by fluorescent reporter. Agircan et al. (2014) developed a peptide consisting of a centrosome-localizing sequence and kendrin cleavage site flanked by mCherry and eGFP peptides that caused an observable color change after substrate cleavage by separase. This reporter would be a useful addition, but we were not able to replicate this reporter for our study.

We measured cleavage of the cohesin subunit, SCC1, after Cr(VI). We did not find significant alteration of SCC1 cleavage. This result is consistent with current knowledge regarding separase activity on cohesin. The portion of total cohesin cleaved by separase is relatively small. Firstly, we propose premature separase activity causes centriole disengagement in interphase cells, specifically during Cr(VI)-induced G2 arrest. The majority of cohesin in the cell is associated with chromosomes. Separase is largely excluded from the nucleus during interphase due to the large size of the protein and a nuclear exclusion sequence at its C-terminal (Sun et al., 2006) which prevents its activity in the nucleus. DNA damage induces translocation of separase into the nucleus to aid in DNA damage repair, and thus a small portion of cohesin specifically at DNA break sites may be cleaved by separase (Kueng et al., 2006). Securin enables separase import into the nucleus (Hornig et al., 2002) and so under lower securin levels, intranuclear separase may be lower than usual after Cr(VI) exposure. Secondly, we observed the effects of separase activity in mitotic cells through premature cohesin cleavage at centromeres. The portion of cohesin cleaved by separase at mitotic chromosomes is again relatively small. The majority of cohesin release along chromosome arms is caused by separase-independent dissociation (Sumara et al., 2002; Kueng et al., 2006) leaving only centromeric cohesin to be cleaved by separase. Therefore, separase targets only a small percentage of total SCC1 in both interphase and mitotic cells and changes in cleavage of this small portion are difficult to measure by western blots in either whole cell or nuclear extractions. We did observe a trend in increased cohesin nuclear cohesin cleavage. Perhaps by

repeating nuclear cohesin measurements several more times, the trend might become more prominent. Peptide reporters can be designed to measure SCC1 cleavage by spectrometry (Basu et al., 2009; Haass et al., 2015; Zhang et al., 2014) but the production of these peptides was not feasible for this study. In our cell system, evidence for premature separase activity at centromeres is best demonstrated using SAC bypass analysis.

The role of securin in separase activity inhibition was shown with chromosome analyses after securin knockdown by siRNA. Predictably, SAC bypass was markedly increased after securin knockdown, but we were surprised this effect was not enhanced with Cr(VI) exposure. Cr(VI)-treated knockdown cells had SAC bypass events at the same level as untransfected control cells. Centromeric cohesin is protected by shugoshin protein, so one possible explanation is this protection from separase cleavage remained intact after 24 h exposure. Consistent with the hypothesis securin loss causes numerical chromosome instability, aneuploidy increased in securin-knockdown cells after only 24 h Cr(VI) exposure. Securin siRNA #2 caused a 2-fold increase in aneuploidy after zinc chromate exposure.

Securin knockout studies in human and mouse cells show securin is not required for cell survival. Securin knockout mouse embryonic stems cells have been shown to grow normally compared to wild type and efficiently arrest upon colcemid exposure, indicating functional separase control in securin knockout cells (Mei et al., 2001). Jallepalli et al. (2001) showed securin knockout in human colorectal carcinoma cells (HCT116) causes chromosome instability. Pfleghaar et

al. (2005) demonstrated that although securin-knockout HCT116 initially lose chromosome stability, after several passages they regain chromosome stability despite reduced separase activity and protein levels. However, results in this p53-deficient cancer cell line are not necessarily representative of normal cell response. Securin is dispensable because cells have redundant mechanisms for separase control. Cyclin B1 is a secondary inhibitor that enables cells to cope with securin loss. The Cdk1-cyclin B1 complex phosphorylates separase to inactivate it, and also stably binds to separase to control its activity. We showed 120 h Cr(VI) exposure reduced cyclin B1 levels, which helps explain how securin knockout cells can cope relatively better than Cr(VI)-exposed cells. Overall, gene knockdown is a helpful tool to provide support for the role of securin in human lung cells, but does not precisely align with Cr(VI)-induced effects. Cr(VI)-induced cellular changes are complex and likely synergistic, yet securin knockdown at 24 h mimics the critical effects observed after 120 h Cr(VI) exposure. We attempted to rescue the effects of 120 h Cr(VI) using siRNA knockdown of separase. However, separase knockdown induced severe aneuploidy in untreated cells, including polyploidy and endoreduplication (data not shown). Separase knockdown is hence unsuitable for evaluating the role of securin disruption at prolonged timepoints.

In Aim 1 we show Cr(VI) targets securin by disrupting mRNA levels. Reduced securin levels are not sufficient to control separase activity, evidenced by increased substrate cleavage and consistent with previous reports of centromere division and centriole disengagement. Additionally, securin loss is not compensated by cyclin B1, as it is also lost upon Cr(VI) exposure. As a critical

centrosome regulatory protein, securin emerges as a key target of Cr(VI). Numerical chromosome instability and centrosome amplification are among the most common cancer hallmarks. Uncovering protein players that contribute to the mechanism of Cr(VI) carcinogenesis enhances understanding of environmental causes of lung cancer.

AIM 2: INVESTIGATING HEXAVALENT CHROMIUM-INDUCED SECURIN LOSS

BACKGROUND

In Aim 1 we showed Cr(VI) reduces securin protein levels in human lung cells after 120 h exposure. Securin loss was not explained by degradation because protein half-life did not change between treated and untreated cells. However, along with protein decrease, securin mRNA levels dropped significantly after 120 h of zinc chromate exposure, indicating securin is targeted at a pre-translational level. To more fully understand the molecular mechanisms of Cr(VI)-induced cellular changes, we seek to discover how Cr(VI) targets gene regulation. Transcription factors are key regulatory tools for gene transcription. These proteins contain DNA-binding domains that interact with gene promoter regions. Transcription factors can promote or repress gene transcription by enabling or hindering binding of RNA polymerase to the promoter region. Chromium alters global gene expression and causes transcription inhibition (Raja et al., 2008; Zablon et al., 2019; Wetterhahn et al., 1989); however, specific Cr(VI) effects on securin transcription are not well known. Here we test the hypothesis, Cr(VI) causes securin loss through alterations of transcription factor protein levels.

Securin protein is the product of the PTTG1 gene. PTTG1 is located at chromosome 5q35.1 (Kakar, 1998). Its 5' region contains no TATA box near the transcription start site, but it does have a CAAT sequence at -474 bp and three Sp1 box sites (Kakar, 1999). Securin gene promotion has been attributed to the

transcription factors nuclear transcription factor Y (NF-Y), specificity protein 1 (Sp1), and E2 factor 1 (E2F1) (Clem et al., 2003; Zhou et al., 2009). NF-Y is composed of three subunits, including NF-YA, NF-YB, and NF-YC. The NF-Y complex binds to CCAAT sequences at gene promoter regions. The CCAAT region was discovered to be over-represented in cancer-associated genes (Dolfini and Mantovani, 2013) and thus NF-Y is an important regulator of cancer development. All three subunits are required for NF-Y function, and NF-YA is the regulatory subunit. NF-YA contains the DNA-binding region and is overexpressed in breast carcinoma (Dolfini et al., 2019), gastric adenocarcinoma (Gallo et al, 2021), cervical cancer (Yang et al., 2020), and lung squamous cell carcinoma (Bezzecchi et al., 2019). Furthermore, Priest et al. (2021) found YB and YC to be in excess while NF-YA is the limiting subunit. Thus, we focus on NF-YA as the sensitive marker for NF-Y complex. Sp1 binds to CG-rich binding sites and mediates cell growth, differentiation, angiogenesis, and tumorigenesis, and has been implicated in multiple cancers, including lung cancer (Vellingiri et al., 2020; Vizcaino et al., 2015; Wang et al., 2003; Hsu et al., 2012). Both NF-Y and Sp1 are required for securin expression (Zhou et al., 2003). Mutation of either Sp1 or NF-YA binding sites caused 70% and 25% loss in promoter activity, respectively, while mutation of both sites caused 90% reduction in promoter activity (Clem et al., 2003). E2F proteins are overexpressed in tumor cells and contribute to cancer progression (Yan et al., 2014). E2F1 was the first-discovered E2F transcription factor. It activates gene transcription and is modified by Cr(VI) exposure (Speer et al., 2021). E2F1 binds to the PTTG1 promoter region, causing securin induction (Zhou

et al., 2009). We consider the potential of Cr(VI) to alter NF-YA, Sp1, and E2F1 levels to reduce securin expression.

In addition to DNA-binding elements that promote securin transcription, there are repressive transcription factors as well. Securin transcription is repressed by p53 and Kruppel-like factor 6 (KLF6). The p53 protein is a well-known tumor suppressor that alters gene regulation, and its function is central to carcinogenesis. p53 inhibits NF-Y binding to the securin promoter, thereby reducing securin protein expression (Bernal et al., 2002; Hamid et al., 2004; Zhou et al., 2003). KLF6 is a repressive transcription factor with a binding site in the securin promoter region that directly inhibits securin transcription (Chen et al., 2013; Lee et al., 2010). Lee at al. (2010) identified securin as the most upregulated gene in the KLF6(+/-) mouse liver compared with control samples and showed KLF6 overexpression reduces securin promoter activity. Chen et al. (2013) showed KLF6 interacts with the securin promoter and KLF6 knockdown released securin repression. We evaluated p53 activation and KLF6 protein levels as potential events that lead to securin downregulation after Cr(VI) exposure.

An alternative hypothesis is securin mRNA loss is a post-transcriptional event. MicroRNAs (miRNAs) are small (21-25 nucleotides), non-coding RNA molecules that regulate mRNA translation (Pasquinelli and Ruvkun, 2002). MiRNAs bind to partial sequence matches of mRNAs and either impede their translation or induce their degradation (Lee et al., 1993; Wightman et al., 1993; Bartel, 2004). They are increasingly recognized for their roles in cancer, including gene regulation, potential cancer biomarkers, and potential therapeutic benefit

(Macfarlane et al., 2010; Hata et al., 2015; Borralho et al., 2011; Hayes et al., 2014). Environmental chemicals, including carcinogenic metals, have been shown to alter miRNAs (Wu et al., 2019; Hu et al., 2011; Humphries et al., 2016). He et al. (2013) found Cr(VI)-transformed epithelial lung cells highly expressed miR-143, whose suppression inhibited Cr(VI)-induced transformation and angiogenesis. Securin-targeting miRNAs have been confirmed and shown to affect proliferation and migration in pituitary tumor cells (Liang et al., 2015). However, it is unknown if Cr(VI) exposure specifically alters securin-targeting miRNAs. Therefore, we investigated Cr(VI)-induced effects on miRNA regulation and potential targeting of centrosome-associated genes.

RESULTS

Cr(VI) increases nuclear levels of NF-YA and Sp1.

Human lung cells were treated with 0, 0.1, 0.2, and 0.3 ug/cm^2 zinc chromate for 24 and 120 h. Whole cell lysates were probed for the transcription factor subunit NF-YA. No change in protein expression was observed at any tested concentration after 24 h or 120 h (Figure 16). In addition to altering protein levels, Cr(VI) can cause changes in subcellular protein localization. Speer et al. (2021) showed Cr(VI) exposure causes cytoplasmic accumulation of the homologous repair protein RAD51, which prevents it from proper intranuclear function. Transcription factors must be abundant specifically in the nucleus to have their gene-regulatory effect. Thus, we measured protein levels of NF-YA in nuclear extracts from human lung cells after 24 h and 120 h of zinc chromate exposure.

After 24 h exposure to 0.1, 0.2, and 0.3 ug/cm^2 zinc chromate, NF-YA levels increased to 120.3%, 135.6%, and 142.5% compared with untreated cells, respectively. 120 h exposure induced increases to 162.6%, 261.4%, and 419.3% increase, respectively (Figure 17).

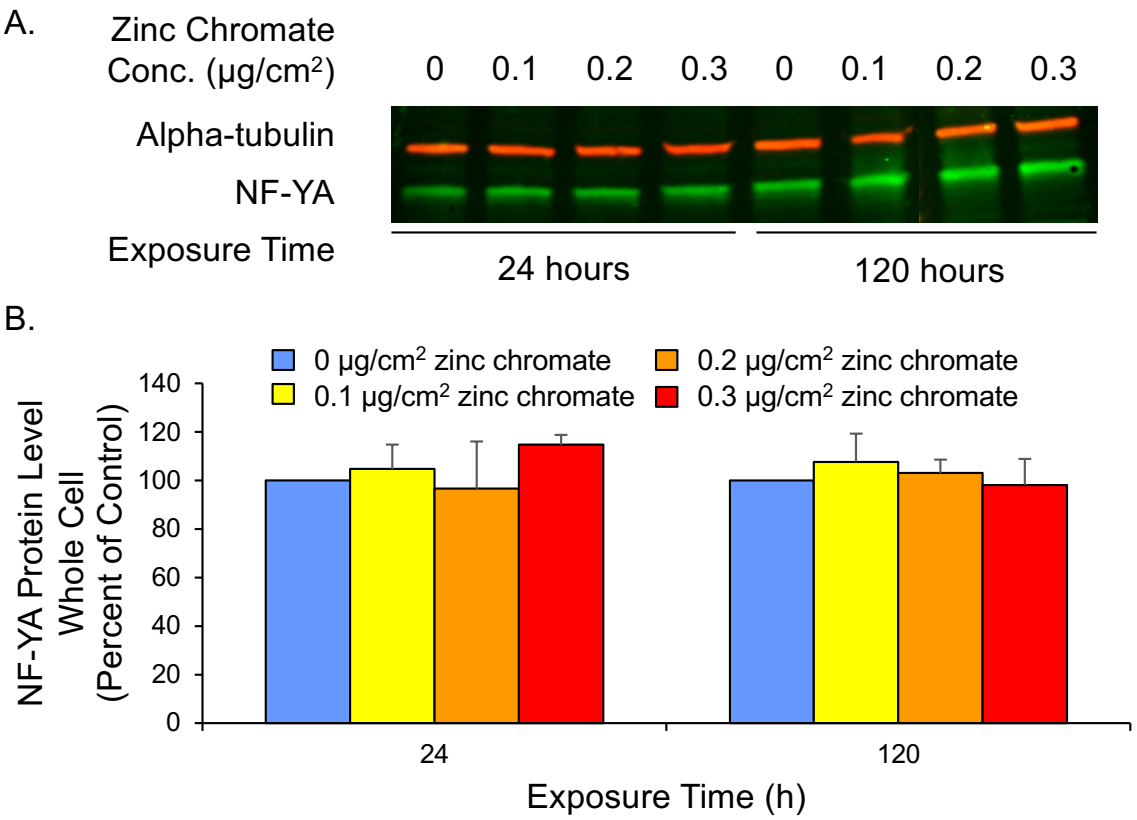


Figure 16. Whole cell NF-YA protein levels in human lung cells after zinc chromate exposure. This figure shows Cr(VI) exposure did not alter NF-YA whole cell protein levels. (A) Representative western blot for NF-YA. Alpha-tubulin was used as a loading control. (B) NF-YA whole cell protein levels were unchanged at any concentration or timepoint. Data are expressed as percent of untreated control cells and reflect the mean of three independent experiments. Error bars = SEM.

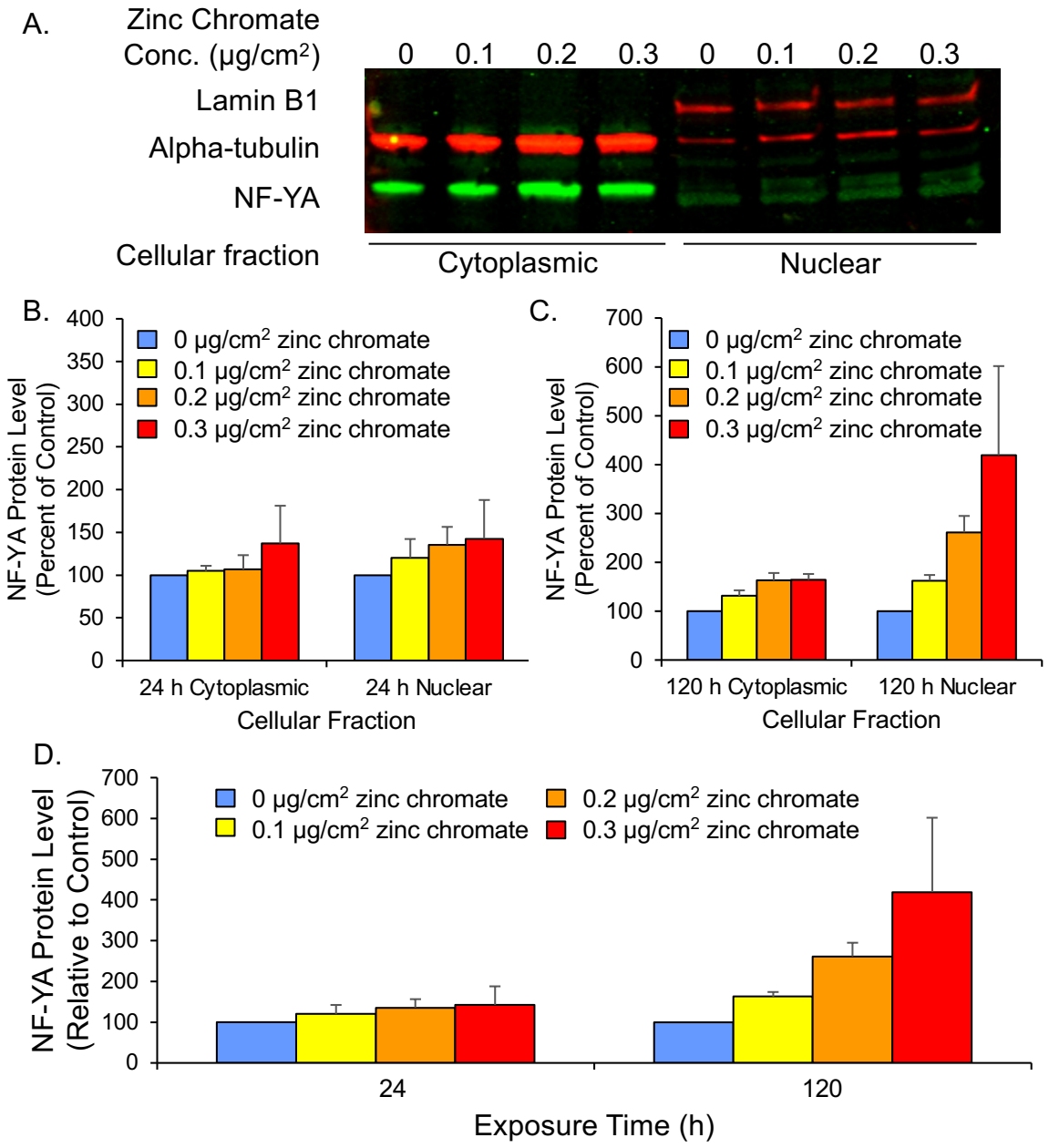


Figure 17. Nuclear and cytoplasmic NF-YA protein levels in human lung cells after zinc chromate exposure. This figure shows Cr(VI) exposure increased NF-YA nuclear protein levels after prolonged exposure. (A) Representative western blot for NF-YA. Alpha-tubulin was used as a loading control in cytosolic fractions. Lamin B1 was used as a loading control in nuclear fractions. (B) NF-YA nuclear protein levels increased slightly after 24 h Cr(VI). (C) NF-YA nuclear protein levels

increased after 120 h Cr(VI). (D) Changes in NF-YA nuclear levels were more apparent after 120 h exposure. Data are expressed as percent of untreated control cells and reflect the mean of three independent experiments. Error bars = SEM. No results were significantly different from untreated control groups.

Sp1 protein levels measured in whole cell protein did not significantly change after zinc chromate exposure (Figure 18), however 120 h exposure caused a trend of Sp1 decrease which was dose dependent. To measure Sp1 nuclear localization, we extracted nuclear protein fractions after Cr(VI) exposure and analyzed them using western blot. Sp1 nuclear levels after 24 h exposures were 62.5%, 84.4%, and 90.7%, and increased after 120 h to 233.2%, 247.3%, and 403% above control levels, respectively (Figure 19). Together these data show Cr(VI) causes nuclear levels of NF-YA and Sp1 to increase after acute and prolonged exposures.

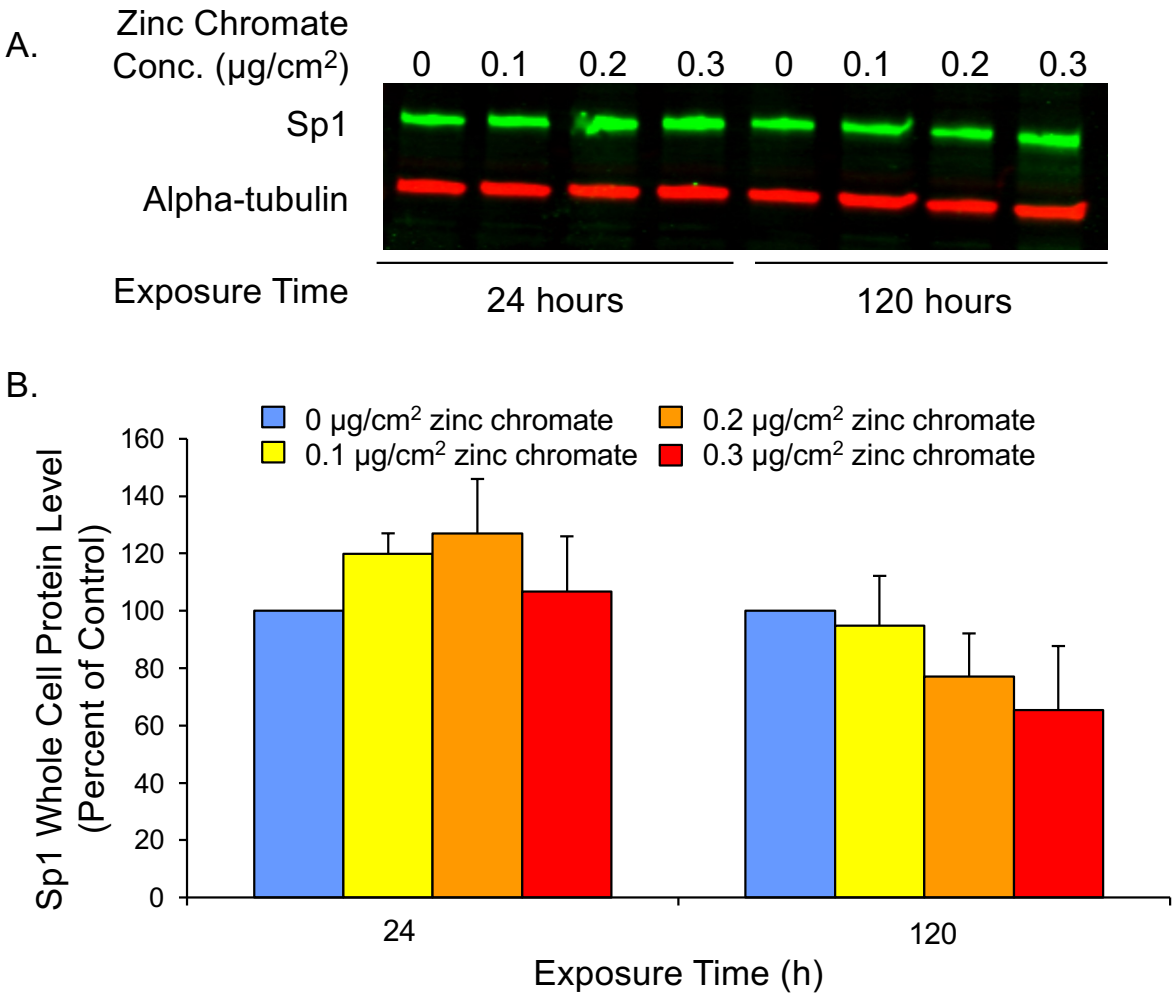


Figure 18. Whole cell Sp1 protein levels in human lung cells after zinc chromate exposure. This figure shows Cr(VI) exposure did not significantly alter Sp1 whole cell protein levels, though 120 h exposure induced a trend towards decreasing Sp1. (A) Representative western blot for Sp1. Alpha-tubulin was used as a loading control. (B) Sp1 whole cell protein levels nonsignificantly reduced after 120 h zinc chromate. Data are expressed as percent of untreated control cells and reflect the mean of three independent experiments. Error bars = SEM.

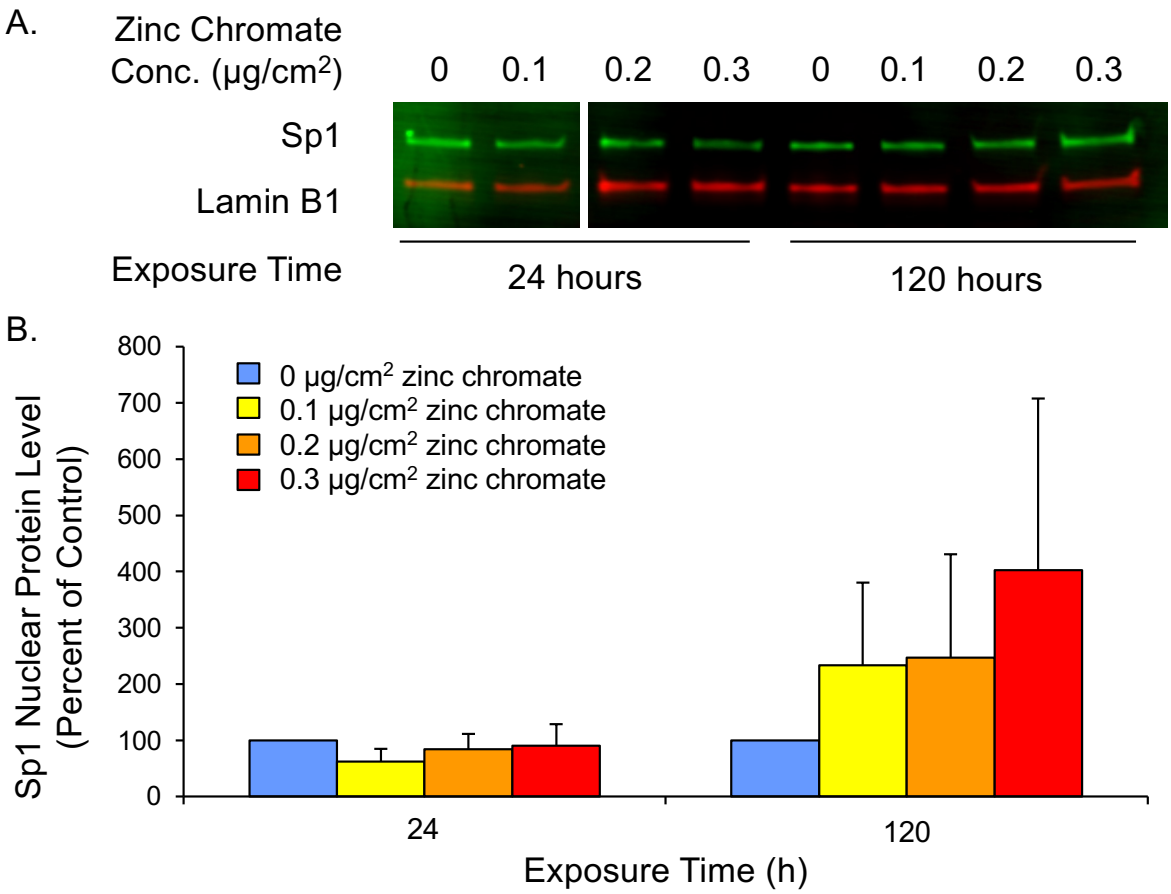


Figure 19. Nuclear Sp1 protein levels in human lung cells after zinc chromate exposure. This figure shows Cr(VI) exposure increased Sp1 nuclear protein levels after prolonged exposure. (A) Representative western blot for Sp1. Lamin B1 was used as a loading control in nuclear fractions. (B) Sp1 nuclear protein levels increased after 120 h Cr(VI). Data are expressed as percent of untreated control cells and reflect the mean of three independent experiments. Error bars = SEM. No results were significantly different from untreated control groups.

Acute Cr(VI) exposure with NF-YA or Sp1 knockdown does not cause securin loss.

To help elucidate the potential roles of NF-YA and Sp1 in Cr(VI)-treated cells, we used siRNAs to separately knockdown each transcription factor. If NF-YA or Sp1 plays a role in Cr(VI)-induced securin loss, we expect 48 h knockdown accompanied by 24 h zinc chromate exposure to cause securin loss similar to 120 h zinc chromate exposure seen in Aim 1. Securin levels increased slightly after 24 h of zinc chromate exposure in each transfection condition, in agreement with 24 h 0.2 μg/cm^2 exposure in Aim 1 (Figure 20). Non-targeting siRNA cells had securin levels of 93% compared with untransfected cells. NF-YA knockdown did not decrease securin levels significantly, with 86% of control cells. Sp1 knockdown alone decreased securin to 67% of the control cells, but 24 h zinc chromate exposure again slightly increased securin levels. Together these data indicate Cr(VI) does not target securin via the NF-YA and Sp1 proteins.

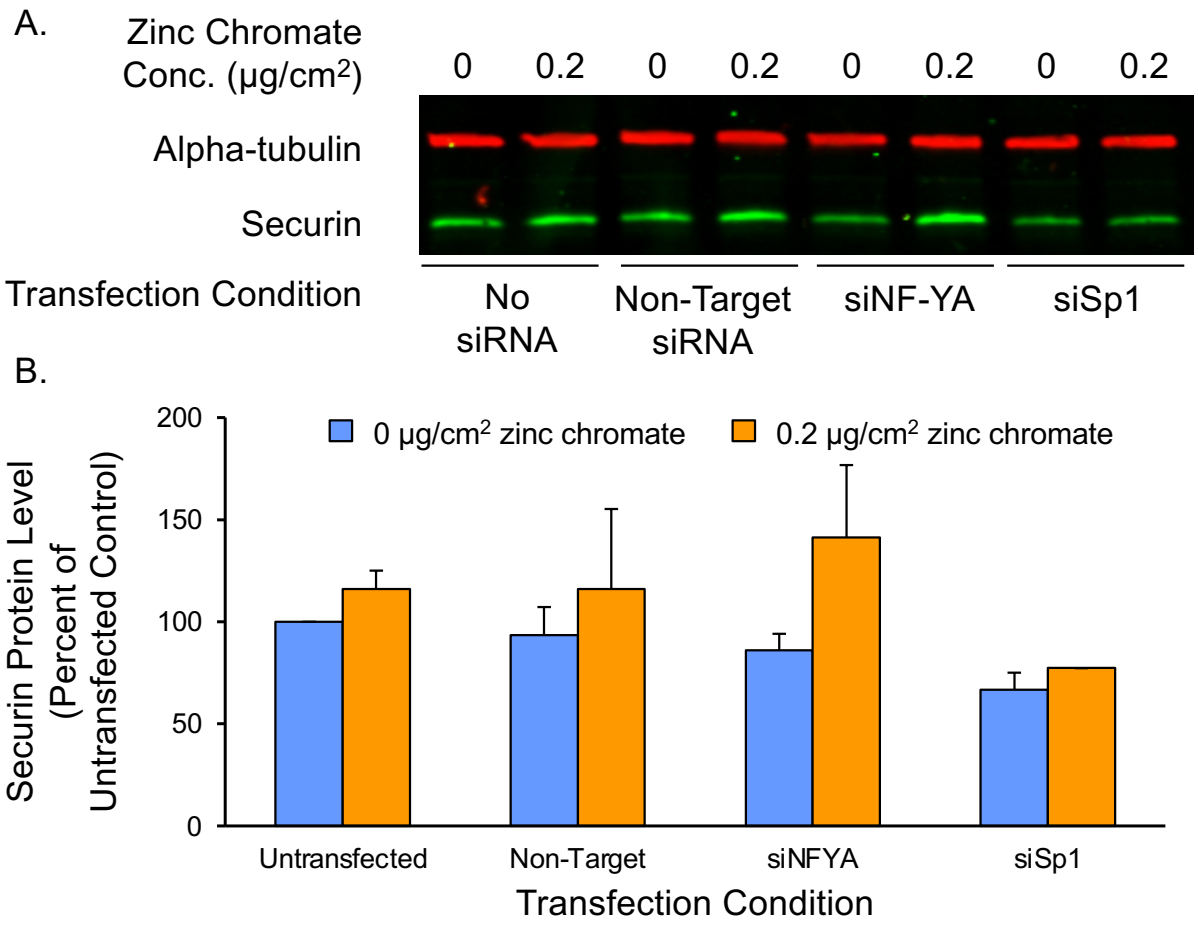


Figure 20. Securin protein levels after transcription factor knockdown and acute zinc chromate exposure. This figure shows 24 h Cr(VI) did not enhance securin protein loss with transcription factor knockdown. (A) Representative western blot for securin. Alpha-tubulin was used as a loading control. (B) Securin levels were not markedly decreased by NF-YA or Sp1 knockdown and 24 h Cr(VI). Data are expressed as percent of untransfected control cells and reflect the mean of two independent experiments. Error bars = SEM. No results were significantly different from untreated control groups.

E2F1 knockdown does not induce securin loss after acute Cr(VI) exposure.

E2F1 is a securin promoting transcription factor (Zhou et al., 2009) and we previously published Cr(VI) decreases E2F1 protein and mRNA levels in human lung cells at prolonged exposure times (Speer et al., 2021). E2F1 loss was not observed after 24 h Cr(VI) exposure, in agreement with retention of securin after acute exposure. To determine if securin loss is caused by E2F1 reduction, we transfected human lung cells with E2F1-targeting siRNAs, along with zinc chromate exposure. If E2F1 plays a role in Cr(VI)-induced securin loss, we expect 48 h siE2F1 knockdown accompanied by 24 h zinc chromate exposure to cause securin loss similar to 120 h zinc chromate exposure. Securin levels increased slightly after 24 h zinc chromate exposure in each transfection condition, as observed with 24 h 0.2 μg/cm^2 exposure in Aim 1 (Figure 21). Non-targeting siRNA decreased securin levels to 71% compared with untransfected cells, indicating a potential effect of the transfection process or a potential off-target effect of our non-targeting siRNA. Comparing E2F1 knockdown by two different siRNAs to the non-targeting siRNA, there was no significant change in securin levels. Thus, Cr(VI)-induced E2F1 depression does not explain securin loss.

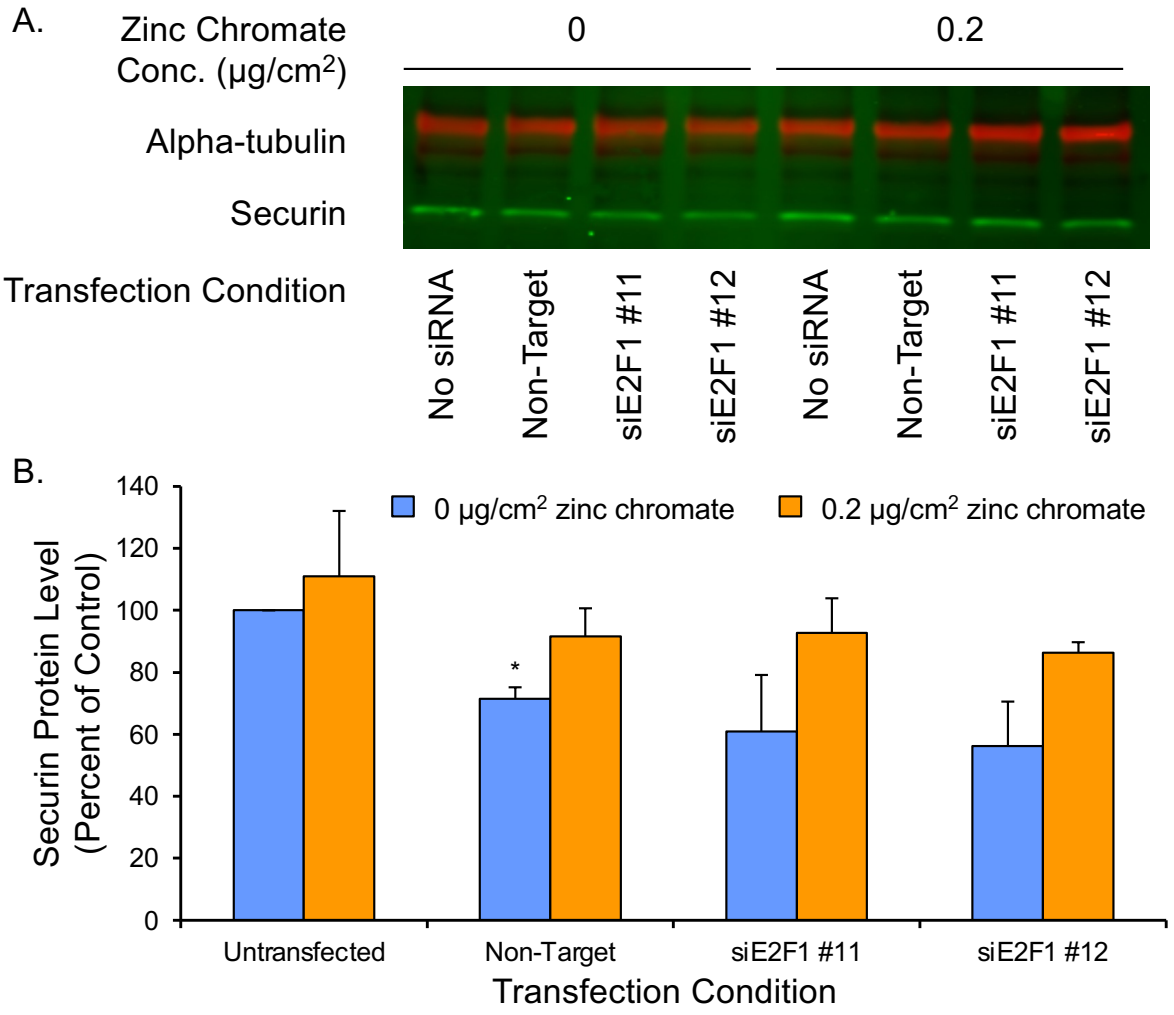


Figure 21. Securin protein levels after E2F1 knockdown and acute zinc chromate exposure. This figure shows E2F1 factor knockdown did not recapitulate 120 h securin loss with 24 h Cr(VI) exposure. (A) Representative western blot for securin. Alpha-tubulin was used as a loading control. (B) Securin levels were not markedly decreased by E2F1 knockdown with 24 h Cr(VI). Data are expressed as percent of untransfected control cells and reflect the mean of two independent experiments. Error bars = SEM. Significantly different from untransfected control group ($p < 0.05$).

Cr(VI) does not induce securin transcription repressors, p53 and KLF6.

Securin is a target of the tumor suppressor protein p53. p53 interacts directly with securin and inhibits its transcription (Bernal et al., 2002; Zhou et al., 2003; Yu et al., 2000). DNA-damaging agents cause p53 phosphorylation at serine 15 (Ser15), which stabilizes the protein and is indicative of p53 activation (Saito et al., 2003; Zhao et al., 2008). We measured levels of p53 and phospho-p53(Ser15). p53 activation is assessed by the ratio of phospho-p53/pan-p53 levels. No significant alterations of p53 whole cell levels or levels of phosphorylated p53 were observed (Figure 22B-C). Exposure for 120 h did not significantly increase phosphorylated/pan-p53 ratio (Figure 22D). Overall, Cr(VI)-induced securin loss is not explained by effects on p53.

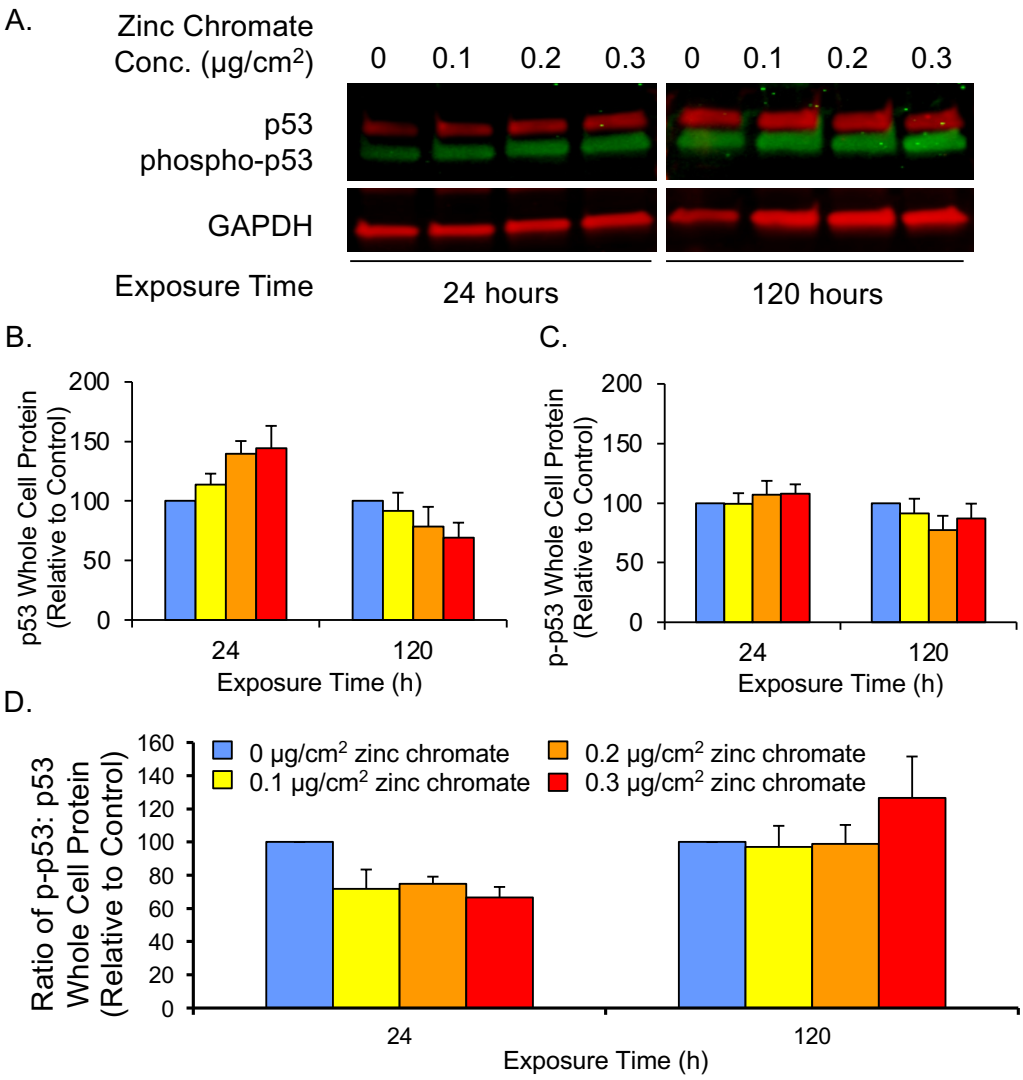


Figure 22. p53 protein levels after zinc chromate exposure. This figure shows Cr(VI) did not significantly alter p53 levels or phosphorylation status. (A) Representative western blot for p53. GAPDH was used as a loading control. (B) Pan-p53 levels (C) phosphor-p53 (Ser15) levels were not significantly changed by Cr(VI). (D) The ratio of pan-p53/phospho-p53 did not indicate activation by Cr(VI) exposure. Data are expressed as percent of untransfected control cells and reflect the mean of two independent experiments. Error bars = SEM. No result was significantly different from control group.

KLF6 is a transcription factor, often dysregulated in cancers (Narla et al., 2001; Reeves et al., 2004), which acts at the securin promoter site to repress its transcription (Lee at al., 2010; Chen et al., 2013). Increased KLF6 levels in Cr(VI)-exposed cells could help explain loss of securin expression. Thus, we measured nuclear KLF6 protein levels in human lung cells. 24 h exposure did not change KLF6 nuclear levels, but 120 h exposure to 0.2 and 0.3 ug/cm^2 zinc chromate increased KLF6 to 160% and 240% of control levels, respectively (Figure 23), which supports the hypothesis Cr(VI) causes securin loss via transcription factor regulation.

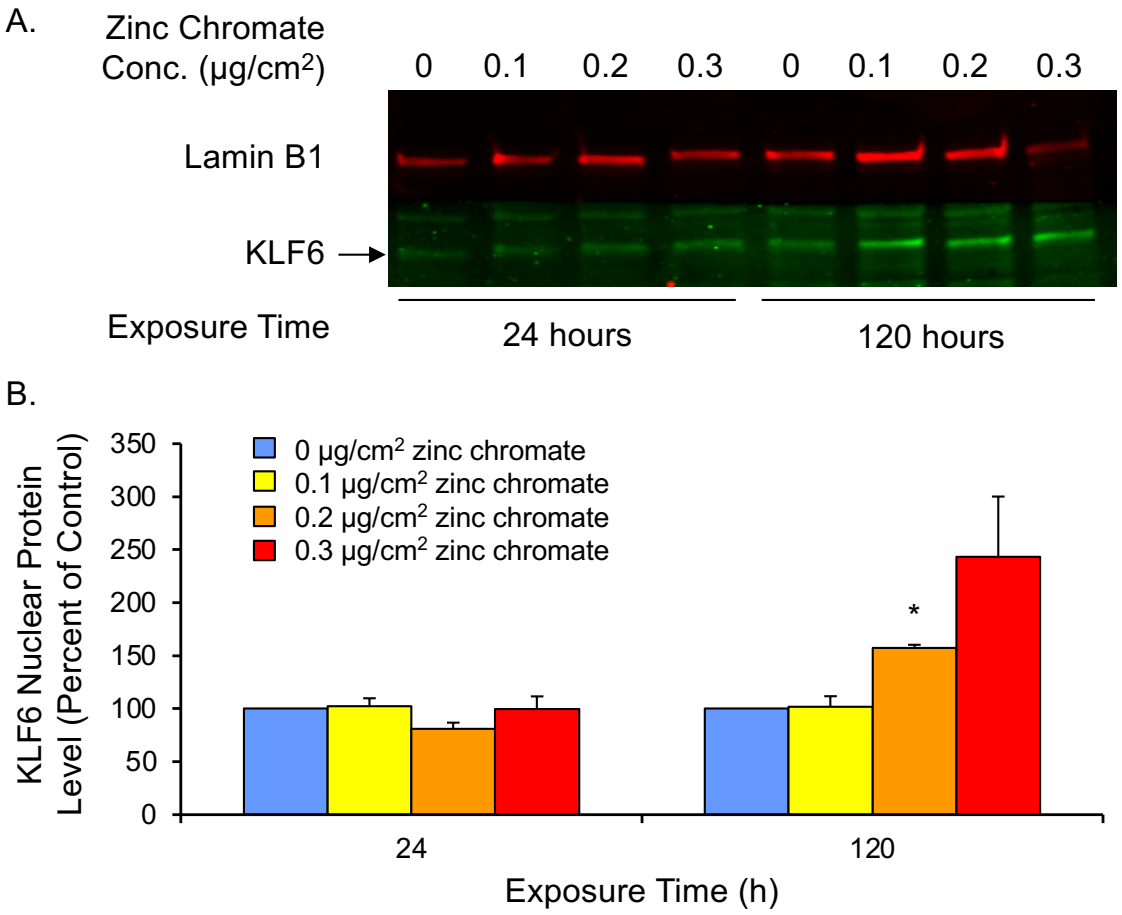


Figure 23. KLF6 nuclear protein levels after zinc chromate exposure. This figure shows KLF6 nuclear levels increased after prolonged Cr(VI) exposure. (A) Representative western blot for KLF6. Lamin B1 was used as a loading control. (B) KLF6 protein levels increased in the nucleus after 120 h Cr(VI). Data are expressed as percent of untreated control levels and reflect the mean of three independent experiments. Error bars = SEM. Significantly different from control group ($p < 0.05$).

Cr(VI) alters miRNA expression

Environmental chemicals, including carcinogenic metals, have been shown to alter miRNA regulation and play a role in carcinogenesis. To determine how Cr(VI) influences miRNA expression, we performed miRNA sequencing (miRNA-seq) after 24 h, 72 h, and 120 h exposures to 0, 0.1, 0.2, and 0.3 ug/cm^2 zinc chromate. In total, 958 miRNAs were identified and analyzed. MiRNA reads were compared relative to untreated control cells at each exposure time. At all tested time points and Cr(VI) concentrations, significantly up- and down-regulated miRNAs were identified ($p < 0.01$) (Table 1). At each time point the number of differentially expressed miRNAs increased with Cr(VI) concentration, with the exception of 24 h samples, which showed high numbers of both up- and down-regulated miRNAs in the 0.2 ug/cm^2 zinc chromate condition. With each zinc chromate concentration, miRNA alteration increased with prolonged exposure time, except for a peak at the 0.2 ug/cm^2 zinc chromate 24 h exposure. This study was the first to measure Cr(VI)-induced global miRNA expression changes in human lung cells (Speer at el., 2022).

	24h			72h			120h		
Zinc Chromate Concentration (ug/cm²)	**0.1**	**0.2**	**0.3**	**0.1**	**0.2**	**0.3**	**0.1**	**0.2**	**0.3**
Total upregulated	23	154	65	19	19	55	35	52	54
Total downregulated	15	55	47	70	100	107	110	123	138
Total altered	38	209	112	89	119	162	145	175	192

Table 1. Significantly up-regulated and down-regulated miRNAs after 24 h, 72 h, and 120 h zinc chromate exposures (adjusted p-value < 0.01). This table show the number of significantly altered miRNAs at each timepoint and concentration of Cr(VI). Data represent the mean of three independent experiments and four technical replicates.

To determine if Cr(VI)-induced securin mRNA decrease can be explained by miRNA interaction, we investigated several securin-targeting miRNAs. A “target gene to miRNA” search was performed in the miRSystem database (Lu et al., 2012) and returned six predicted securin-targeting miRNAs (miR-186, miR-655, miR-105, miR-19b, miR-300, and miR-495). In addition, Liang et al. (2015) confirmed securin targeting by miR-329 and miR-381. We searched our miRNA-seq data for these eight miRNAs. They appeared as significantly altered in only three of our test conditions as apparently isolated incidents. No miRNA was consistently altered by zinc chromate. After 24 h, miR-186 and miR-655 were upregulated with 0.3 μg/cm^2 exposure and miR-381 was upregulated with 0.2 μg/cm^2 exposure. None of the eight miRNAs were significantly altered after 72 h. After 120 h, miR-186 was downregulated by 0.3 μg/cm^2 exposure.

Although securin-targeting miRNAs were not altered by Cr(VI), miRNAs could potentially impact other genes in pathways of centrosome regulation and contribute to centrosome amplification. Centrosome pathways can be found within the family of chromosome-associated genes in the KEGG BRITE database hierarchies, which classifies genes based on biological function. Using KEGG BRITE hierarchies combined with relevant publications, we identified 37 genes across 7 functional categories, including centriole biogenesis, centriole disengagement, securin degradation, microtubule nucleation, centriole maturation, extra centrosome monitoring, and securin transcription factors. For each gene of interest, we used miRSystem to create lists of potential gene-targeting miRNAs. Our miRNA-seq data were then searched for all identified miRNAs. To evaluate

potential gene targeting, any miRNAs that were consistently up- or down-regulated at all three zinc chromate concentrations within the same time point were counted. Table 2 shows the numbers of differentially expressed miRNAs predicted to target each gene. Putative miRNA interactions increased with higher concentrations and longer exposure times. These data indicate miRNAs may play roles in Cr(VI) effects on centrosomes. Many of the altered miRNAs were altered at both 72 h and 120 h, and several were observed to target multiple genes in our list.

Centriole Biogenesis						
Target gene	up 24 h	down 24	up 72 h	down 72	up 120 h	down 120
Plk4	0	0	0	1	0	3
SAS6	0	1	0	20	2	28
STIL	1	2	3	12	3	12
CPAP/CENPJ	0	0	0	1	0	2
CEP135	0	3	1	25	7	31
CEP152	0	1	0	9	1	11
CEP192	0	0	0	3	1	3
Centriole Disengagement						
Target gene	up 24 h	down 24	up 72 h	down 72	up 120 h	down 120
PLK1	0	0	0	1	0	2
PCNT	1	2	5	26	7	41
ESPL1	0	0	1	0	3	0
PTTG1	0	0	0	0	0	0
RAD21	1	0	2	5	1	14
CDK1	1	0	0	3	1	7
CCNB1	0	2	0	5	0	8
Securin Degradation						
Target gene	up 24 h	down 24	up 72 h	down 72	up 120 h	down 120
Cdh1	0	1	1	11	6	18
UBB	0	0	0	0	1	0
UBA52	0	0	0	0	1	0
UBC	0	1	0	12	0	12
RPS27A	0	0	0	0	0	0
APC3/CDC27	0	1	0	5	1	13
ANAPC7	0	0	0	5	0	7
PP2A/PPP2CA	1	1	0	19	1	29
SKP1	1	2	1	13	3	23
Cul1	0	1	1	15	2	18
FBXO5	0	1	0	4	0	5
FBXO43	0	0	0	3	0	5
Emi1	0	1	0	4	0	5
Microtubule Nucleation						
Target gene	up 24 h	down 24	up 72 h	down 72	up 120 h	down 120
NLP	0	1	1	3	1	4
Centriole Maturation						
Target gene	up 24 h	down 24	up 72 h	down 72	up 120 h	down 120
AURKA	1	0	1	9	1	11

Extra Centrosome Monitoring						
Target gene	up 24 h	down 24	up 72 h	down 72	up 120 h	down 120
LATS2	0	1	1	23	2	30
p53	1	2	1	17	5	26
Securin Transcription Factors						
Target gene	up 24 h	down 24	up 72 h	down 72	up 120 h	down 120
Sp1	1	5	3	44	15	62
NF-YA	0	3	2	30	3	34
NF-YB	1	1	2	28	4	41
NF-YC	0	0	1	10	1	15
E2F1	0	2	1	19	4	23
KLF6	0	2	2	13	4	20

Table 2. This table shows the number of putative targeting miRNAs that are significantly (adj p-value ≤ 0.01) up- and down-regulated by zinc chromate at each timepoint and exposure concentration. miRNAs were identified by miRSystem and searched within our miRNA-seq data. Data represent the number of unique miRNAs that were significantly altered and potential target each gene of interest.

Summary

Gene regulation is frequently altered in cancer and represents a known effect of Cr(VI) exposure, contributing to its carcinogenic mechanism (Raja et al., 2008; Zablon et al., 2019). While Cr(VI) can broadly alter gene transcription, its effect specifically on securin transcription is unknown. We investigated three promoters of securin transcription, NF-YA, Sp1, and E2F1 for their roles in Cr(VI)-induced securin loss. NF-YA and Sp1 are the two main transcription factors of securin and loss of either one decreases securin promoter activity (Clem et al., 2003). Our study tested the hypothesis that Cr(VI) decreases securin transcription promoters. Cr(VI) exposure did not significantly change whole cell protein levels of either NY-YA or Sp1. Measuring nuclear levels, we found both promoters increased in nuclear fractions at prolonged time points in a concentration-dependent manner. We observed a strong response in nuclear localization after 120 h exposure and thus these promoters are not only present, but apparently activated upon Cr(VI) and does not explain the securin loss we observed.

We also used siRNA knockdown of NF-YA or Sp1 in combination with 24 h zinc chromate exposure in an attempt to mimic the 120 h phenotype. If transcription promoter loss was central to Cr(VI)-induced protein loss, we would expect premature knockdown to shift the response earlier. However, as reported in Aim 1, 24 h zinc chromate exposure increased securin levels compared with unexposed cells. NF-YA knockdown validation was not confirmed by western blot, so it may require more than 48 h transfection time for levels to decrease, or the mRNA may not have been appropriately targeted by the siRNA. Sp1 siRNA

induced the greatest decrease in securin protein. However, 24 h Cr(VI) exposure with Sp1 knockdown produced a slight increase in securin, indicating a possible compensatory response to 24 h Cr(VI) exposure that is retained despite gene knockdown.

We previously reported Cr(VI) inhibits E2F1, which is also a securin-promoting transcription factor. 48 h E2F1 knockdown with 24 h zinc chromate exposure did not significantly reduce securin levels. Zhou et al. (2009) reported E2F1 knockdown decreased securin only in p53-deficient cells, and not in p53-competent cells. 24 h and 120 h Cr(VI) exposures did not induce p53 loss, so our results are consistent with previous evidence that E2F1 modification of securin is p53-dependent.

Our conclusion is Cr(VI) does not target securin by reducing protein levels or nuclear localization of promoting transcription factors. This does not necessarily mean securin is not targeted at the transcriptional level. It is possible transcription factors are prevented from binding to the promoter. Cr(VI) and Cr(III) complexes have been reported to both activate and repress nuclear binding of transcription factors, including Sp1 (Raja et al., 2008; Kaltreider et al., 1999). Changes in DNA methylation could also prevent access to promoter regions (Lou et al., 2003).

Another path to transcription inhibition involves repressive transcription factors. We measured p53 and KLF6, as they are reported to reduce securin transcription. We did not measure significantly increased p53 levels after Cr(VI) exposure. Although Cr(VI) has been reported to activate p53 in cell culture and elevated p53 protein has been measured in Cr(VI)-exposed workers (Ye et al.,

1999; Hanaoka et al., 1997), limited or borderline p53 activation has been observed in lung cells (Luczak et al., 2019) and rat lung (D'Agostini et al., 2002). Out studies confirm p53 is not a major target of Cr(VI). We measured p53 activation by the relative phosphorylation of Ser15. Ser15 is phosphorylated upon DNA damage detection (Saito et al., 2003; Zhao et al., 2008), but activation of p53 is complex. Multiple post-translational modifications interact to modulate p53 activation, interaction, and stabilization (Lavin et al., 2006). A complete assessment of the role of Cr(VI)-induced p53 interaction with securin would require measurements of more subtle combinations of p53 modifications. KLF6 nuclear levels were increased after 120 h Cr(VI) exposure, which may explain securin loss. KLF6 knockdown, as was performed with NF-YA, Sp1, and E2F1, would help clarify the role of Cr(VI) altered KLF6.

Finally, we explored the alternate hypothesis securin loss occurs by a post-translational mechanism. MiRNAs are altered by environmental chemicals, including Cr(VI) and other metals (Wu et al., 2019; Hu et al., 2011; Humphries et al., 2016; He et al., 2013; Liang et al., 2015). However, previous studies only investigated selected Cr(VI)-altered miRNAs. In this Aim we show zinc chromate caused global alterations in miRNA levels at all tested concentrations and time points, as measured by miRNA-seq. We queried miRSystem to identify miRNAs that provide predicted targeting for several genes of interest. Securin-targeting miRNAs were not significantly changed according to our data, but several other genes related to centrosome regulation are potentially affected. Of note, all the identified transcription factors, Sp1, NY-Y subunits, E2F1 and KLF6, had relatively

high numbers of putative miRNAs that were down-regulated. MiRNAs typically repress gene transcription and thus miRNA down-regulation could potentially contribute to increased NF-YA and Sp1 levels we observed. However, decreased E2F1 levels after prolonged Cr(VI) (Speer et al., 2021) can not be explained in light of several positive hits on potential miRNA associations noted here. Proteins involved in securin degradation, PP2A, SKP1, and Cul1, are also potential miRNA targets. Upregulation of these proteins by miRNA down-regulation would be expected to cause increased securin and cyclin B1 degradation. Our data showed securin degradation was not significantly changed after prolonged Cr(VI). Investigating premature upregulation by the ubiquitination pathway as a means to decrease cyclin B1 would be informative.

This Aim shows Cr(VI) disruption of securin and other proteins relevant to centrosome function and chromosome stability may occur by multi-pronged and complex mechanisms. Cr(VI) effects may not be explained or recapitulated by isolated events. A combination of effects on transcription factor function, protein localization, and miRNAs should be explored in tandem to reveal the mechanisms of Cr(VI) carcinogenesis in finer detail.

AIM 3: WHALE CELLS RESIST HEXAVALENT CHROMIUM-INDUCED SECURIN DISRUPTION AND NUMERICAL CHROMOSOME INSTABILITY

BACKGROUND

According to the multistage theory of carcinogenesis, accumulation of detrimental and inheritable molecular events in a single cell can develop into cancer (Nunney, 2016). The likelihood of a cell accumulating the requisite amount of aberrant changes leading to cancer increases with cell number and time (Nordling, 1952). Thus, large species would be expected to have higher rates of cancer and long-lived animals, with more time to accumulate mutations and longer exposure periods to environmental carcinogens, would be expected to have greater rates of cancer than species with small bodies and shorter lifespans. However, these principles do not hold up to observation, an incongruity known as "Peto's Paradox" (Nunney, 1999; Caulin and Mauley, 2011; Peto et al., 1975; Peto, 1977). Whales, having 1000-fold the number of cells humans have, would be expected to have higher rates of cancer than humans. Yet, sperm whales live 60-70 years and bowhead whales have been estimated to live over 200 years (Keane, 2015) and reports of cetacean cancers are rare (Newman and Smith, 2006).

In this Aim we use sperm whale and bowhead whale cells as comparative toxicological models. Sperm whales have the distinction of being deep divers. Unlike baleen whales that feed mostly at surface, sperm whales hunt at depths of 1000 meters and have been recorded at depths over 2000 meters (Watkins et al., 2002; Zimmer et al., 2003). They typically remain submerged for 45 minutes and

can hold their breath for longer than 90 minutes (Watkins et al., 2002; Watwood et al., 2006). Reports of chromium speciation in oceans has found that Cr(VI) concentrations increase in deep waters (Geisler, 1992) and thermal vents emit heavy metals, making the deep ocean an interesting place to study metal toxicity. The bowhead whale is one of the largest whale species and with estimated lifespans over 200 years it is suspected to be the longest living mammal (Keane et al., 2015). Bowhead whale genome sequencing and comparative analysis shows gene duplication and loss in genes associated with DNA repair, cell cycle regulation, cancer, and aging (Keane et al., 2015). Greater understanding of how whales maintain genomic stability can advance prevention and treatment of human cancers.

Whales are exposed to environmental carcinogens, such as hexavalent chromium. Cr(VI) is the dominant speciation of chromium in sea water (Geisler and Schmidt, 1992) and whale skin sampling show high levels of chromium accumulation. Sperm whales sampled across the globe had 0.9 to 122.6 μg Cr/g tissue with a global mean of 8.8 μg/g (Wise et al., 2009), North Atlantic right whales had mean Cr levels of 7.1 μg/g tissue (Wise et al., 2008), and fin whales sampled from the Gulf of Maine showed Cr levels of 1.71 to 19.6 μg/g. These levels are high compared to data on non-occupational human chromium accumulation of 0.31 μg/g (Schroeder et al., 1970) and occupation-associated Cr-induced lung cancer accumulation levels of 0.4-132 μg/g with a mean of 20.4 μg/g (Tsuneta et al., 1980). Elevated levels of environmental carcinogens could pose health risks to exposed wildlife, and indeed studies find the St. Lawrence Estuary population of

beluga whales have high cancer rates, likely due to contamination by organochlorines, polycyclic aromatic hydrocarbons, and heavy metals (Newman and Smith, 2006; Martineau et al., 2002; De Guise et al., 1994). Reports of high Cr levels in whale biopsies inspired toxicological investigations of Cr(VI) in whales. Soluble and particulate chromates induced cytotoxicity and genotoxicity in North Atlantic right whale lung and testes fibroblasts (Wise et al., 2008; Li Chen et al., 2009). Cell culture studies confirmed Cr(VI)-induced cytotoxicity and genotoxicity in sperm whale (Wise et al., 2001), and fin whale cells as well (Wise et al., 2015).

Measurements of cytotoxicity and genotoxicity in whale cells however were lower than those of Cr(VI)-exposed human cells (Li Chen et al., 2009; Li Chen et al., 2012.). Li Chen et al. (2009) compared cytotoxicity of particulate lead chromate in human and North Atlantic right whale lung cells. Whale lung cells had significantly higher survival rates than human lung cells at the same administered concentrations. Structural chromosome damage was significantly higher in human cells than in whale cells. Intracellular chromium levels were higher in humans, but even after correcting for uptake, human cells still had significantly higher rates of damage than whale cells. Similarly, when comparing human and sperm whale skin cells, whale cells had higher survival rates and lower chromosome damage than human cells (Li Chen et al., 2012). These data show whale cell resistance to Cr(VI) toxicity is not organ-specific and is observed in both baleen whale and toothed whale species, which occupy distinct trophic categories.

In the effort to discover how whale cells resist Cr(VI)-induced genotoxicity, molecular investigations revealed important species-specific differences in DNA

repair response. Browning et al. (2017) showed homologous recombination repair, the mechanism that corrects DNA double strand breaks incurred by Cr(VI), was retained in North Atlantic right whale cells after Cr(VI) exposure. This outcome is in stark contrast to the earlier finding Cr(VI) induces loss of homologous recombination repair in human cells (Browning et al., 2016; Qin et al., 2014). Transcriptome analysis from whale cells exposed to Cr(VI) show upregulation of DNA repair pathways at moderate exposure levels and induction of apoptosis pathways at highest exposure levels (Pabuwal et al., 2013).

Comparisons of whale and human molecular responses to Cr(VI) shed light on key events in the carcinogenic pathway. Previous studies reveal how whale cells avoid structural chromosome instability (Browning et al., 2017), however, no studies have explored numerical CIN in whale cells. Numerical chromosome instability is a hallmark of cancer and observed in the majority of solid tumors (Tweats et al., 2019; Farkas et al., 2016; Duijf and Benezra, 2013). Numerical chromosome instability and centrosome amplification are key effects of Cr(VI) exposure (Martino et al., 2015; Holmes et al., 2010; Holmes et al., 2006) and in Aim 1 we show centrosome regulation disruption, including securin protein loss, underpins these effects. Here we translate our Aim 1 findings of Cr(VI)-induced centrosome dysregulation in human cells to whale cells, and for the first time characterize centrosome effects and numerical chromosome instability in whale cells.

RESULTS

Particulate hexavalent chromium is cytotoxic to whale cells.

After 24 h of zinc chromate treatment at 0.1, 0.15, 0.2, 0.3, and 0.4 μg/cm^2, sperm whale skin fibroblasts produced colony numbers 89.3%, 80.8%, 70.3%, 66.3%, and 51.1% relative to untreated cells (Figure 24A). After 120 h of treatment at the same concentrations, relative survival was 85.2%, 80.9%, 64.7%, 47.6%, and 19.6% respectively. Survival was statistically less than controls at 0.2, 0.3 and 0.4 μg/cm^2 for both time points. After 24 h of zinc chromate treatment at 0.1, 0.15, 0.2, 0.3, and 0.4 μg/cm^2, bowhead whale lung fibroblasts produced colonies at 96.7%, 92.5%, 90.3%, 86.8% and 79.3% relative to untreated cells (Figure 24B), with the highest concentration producing significantly different results compared to control. Relative survival after 120 h of exposure was similar to 24 h exposures at 94.1%, 90.2%, 80.6%, 79.3%, and 79.5%. The difference compared to control was significant for 0.2, 0.3 and 0.4 μg/cm^2 zinc chromate treatments. Sperm whale and bowhead whale cells show different responses to prolonged Cr(VI) exposure. While survival after 120 h decreased in sperm whale cells, survival rates for bowhead whale cells were not significantly less than after 24 h exposures.

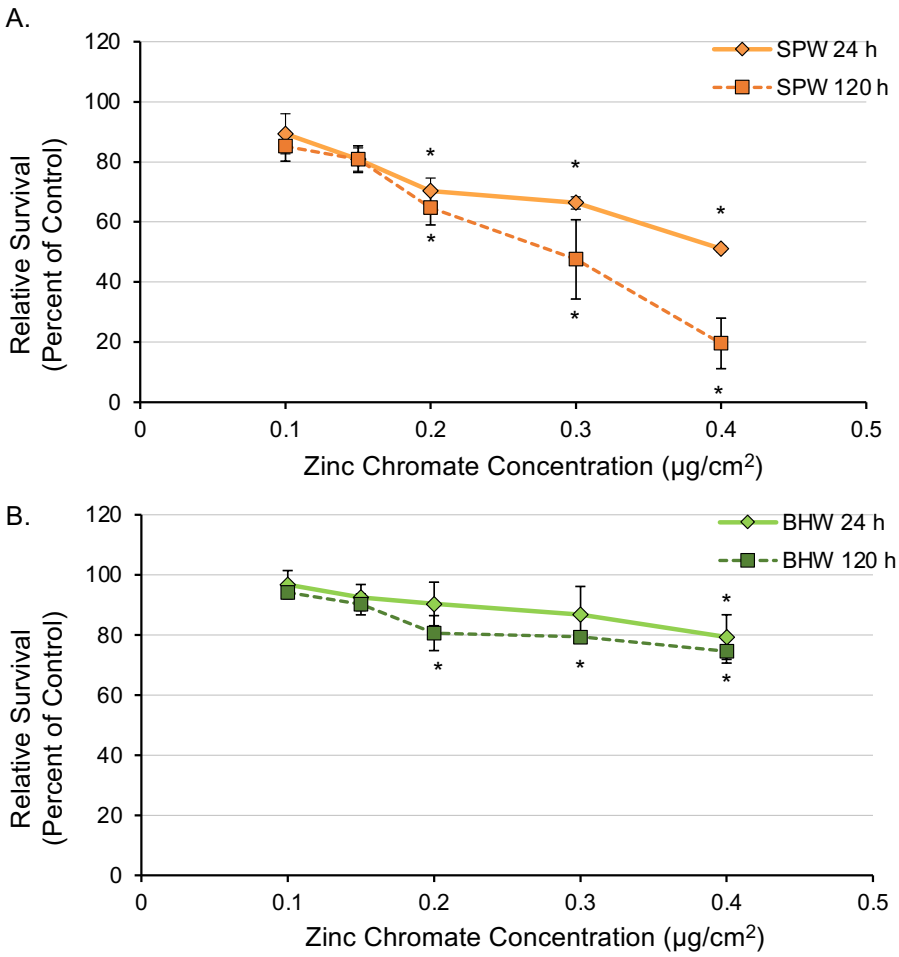


Figure 24. Cr(VI)-induced cytotoxicity in whale cells. This figure shows Cr(VI) is cytotoxic to sperm whale and bowhead whale cells. (A) Colony survival of sperm whale cells after 24 h and 120 h zinc chromate treatment, relative to control. (B) Colony survival of bowhead whale cells after 24 h and 120 h zinc chromate treatment, relative to control. Data reflects the mean of three independent experiments. Error bars = SEM. *Significantly different from untreated cells at the same timepoint ($p < 0.05$).

Cr(VI) induces chromosome breaks and cell cycle arrest in bowhead whale cells.

Cr(VI) causes chromosome damage and cell cycle arrest in human cells (Xie et al., 2005; Xie et al., 2009), thus we characterized these toxic effects in bowhead whale cells. Metaphase chromosomes were analyzed for chromosome aberrations such as breaks and gaps and spreading centromeres (Figure 25). After 24 h exposure to 0.1, 0.15, 0.2, 0.3, and 0.4 μg/cm^2 whale lung cells had 10%, 8.7%, 7.7%, 14%, and 17.3% metaphases with at least one damaged chromosome, while untreated cells had 2.7%. 120 h exposure caused 6.3%, 6.7%, 9.3%, 7%, and 9.7%, of metaphases to exhibit damage, with control cells measuring 4%. Lower levels of damage after 120 h may be due to cell cycle arrest of damaged cells.

Changes in cell cycle phase were analyzed by flow cytometry. Analysis of DNA content shows the proportion of cells that are in each cell phase. 24 h exposure did not change cell cycle distribution, while 120 h exposure decreased G1 cell populations slightly and enriched G2/M phase cells, although this change is not statistically significant (Figure 27). One drawback to using flow cytometry for cell cycle analysis is G2 and M phases can not be distinguished by DNA content alone. Mitotic index analysis was performed using light microscopy to determine if changes in G2 and M phase were occurring (Table 3). Mitotic cell counts show 24 h exposure of 0, 0.1, 0.2, 0.3, and 0.4 μg/cm^2 zinc chromate yielded 83, 64.7, 50, 42.3 and 38 mitotic cells per 5000 cells scored. 120 h exposure produced 50.7, 45, 32.3 27.3, and 20.7 mitotic cells per 5000 cells scored. This indicates depression of mitosis and combined with the flow cytometry data suggest Cr(VI) causes G2

cell cycle arrest in bowhead whale cells. These data show zinc chromate causes cell cycle arrest in bowhead whale cells after 120 exposure.

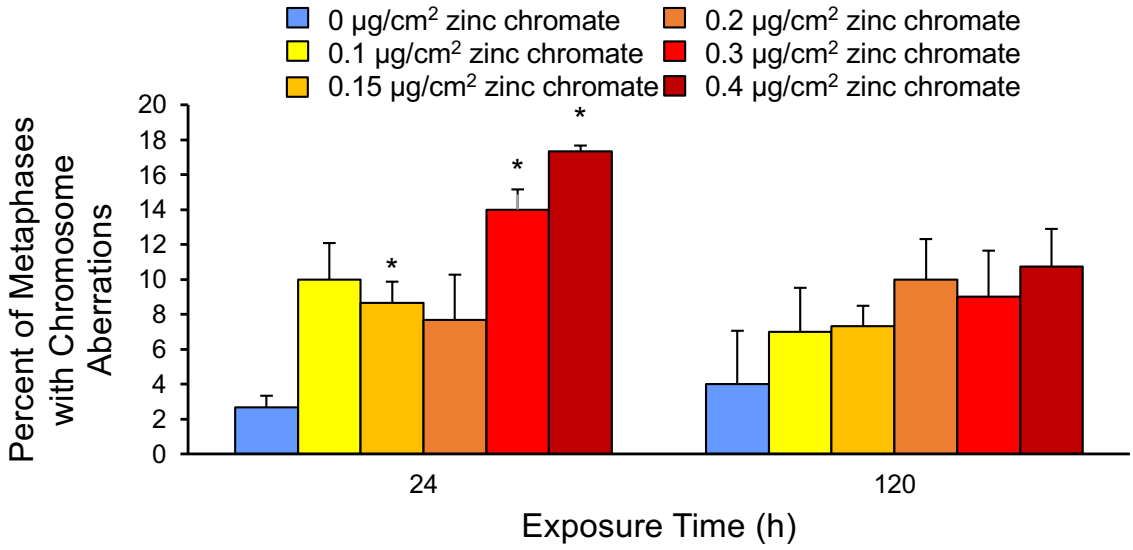


Figure 25. Cr(VI)-induced chromosome aberrations in bowhead whale cells. This figure shows Cr(VI) is caused chromosome damage in bowhead whale cells. Data reflects the mean of three independent experiments. Error bars = SEM. *Significantly different from control group ($p < 0.05$).

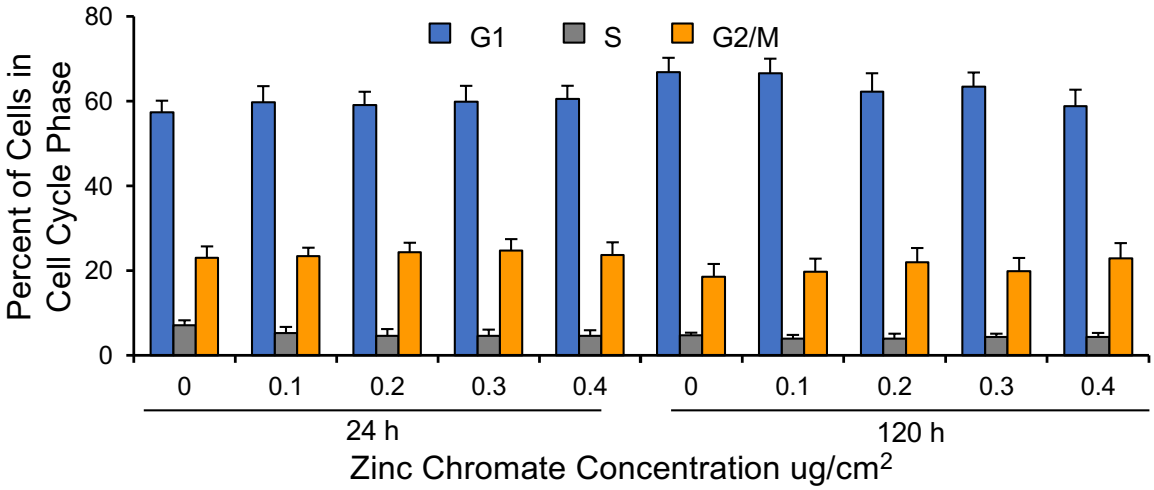


Figure 26. Cell cycle analysis in bowhead whale cells after 24 h and 120 h zinc chromate exposure. This figure shows Cr(VI) caused a slight decrease in the percentage of G1 phase bowhead whale cells and a corresponding increase in G2/M after prolonged exposure. Data reflects the mean of three independent experiments. Error bars = SEM. No groups were significantly different from control group.

Mitotic Index Analysis				
	24 h		120 h	
Zinc Chromate (ug/cm^2)	Average	SEM	Average	SEM
0	83.0	4.0	50.7	12.7
0.1	64.7	4.4	45.0	13.0
0.2	50.0	13.9	32.3	11.6
0.3	42.3	5.4	27.3	11.9
0.4	38.0	3.1	20.7	11.6

Table 3. Mitotic index in bowhead whale cells after 24 h and 120 h zinc chromate exposure. This table show the number of mitotic cells per 5000 total cells after Cr(VI) exposure. The mitotic index decreases after acute and prolonged Cr(VI). Data reflects the mean of three independent experiments.

Cr(VI) does not induce securin loss in whale cells.

We measured securin levels in bowhead whale cells after 24 and 120 h exposure to 0.1, 0.2, and 0.3 ug/cm^2 zinc chromate (Figure 27). The greatest reduction in securin levels was observed after 120 h exposure to 0.3 ug/cm^2 resulting in 85.6% of untreated control levels, which was not statistically significant. In Aim 1 we show 120 h exposure to 0.1, 0.2, and 0.3 ug/cm^2 zinc chromate causes securin levels to drop to 48.5%, 31%, and 15.3%, respectively, in human cells.

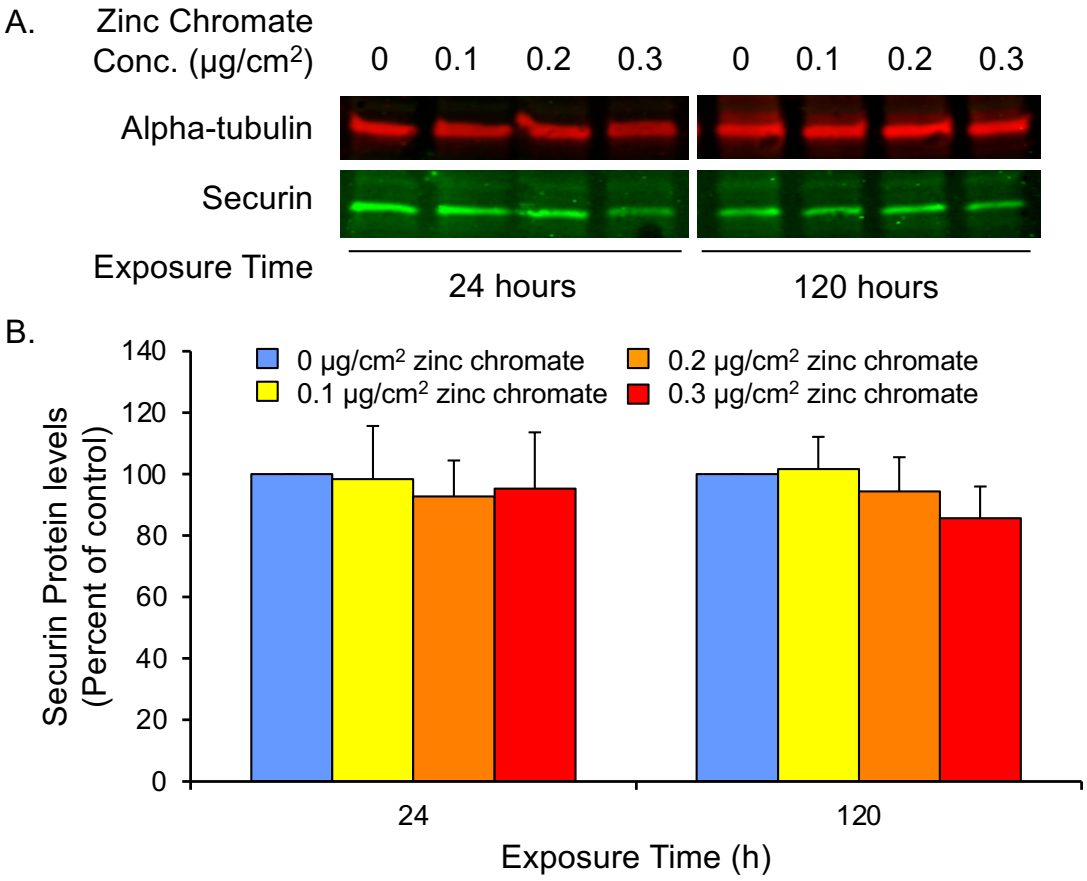


Figure 27. Securin protein levels in bowhead whale lung cells after 24 h and 120 h zinc chromate exposure. This figure shows prolonged Cr(VI) exposure did not alter securin levels in whale cells. (A) Representative western blot for securin. Alpha-tubulin was used as a loading control. (B) Securin whole cell protein levels were unchanged at all concentrations and timepoints. Data are expressed as percent of untreated control cells and reflect the mean of three independent experiments. Error bars = SEM. No results were significantly different from control group.

Cr(VI) does not induce spindle assembly checkpoint bypass in whale cells.

The retention of securin levels by whale cells after 120 h Cr(VI) is a notable difference compared to the human cell response. Spindle assembly checkpoint (SAC) is a mechanism that prevents progression from metaphase to anaphase until all kinetochores are properly attached to spindle fibers. The SAC is a protective mechanism against aneuploidy. SAC bypass allows cell division to occur in conditions of improper kinetochore-microtubule attachments, resulting in lagging chromosomes at anaphase and asymmetrical chromosome segregation. Centrosome amplification can cause abnormal mitosis via improper kinetochore attachments and thus SAC is a protective measure against this disruption. SAC bypass serves as an indicator of securin dysfunction, as aberrant separase activity is responsible for cleaving the centromeres before anaphase. Particulate chromate has been shown to cause SAC bypass in human lung fibroblasts, observed as centromere spreading, premature centromere division, and premature anaphase (Holmes et al., 2010; Wise et al., 2006). Definitions of these phenomena are described in Wise et al. (2006) and followed here. Centromere spreading entails separation of the chromatids at the centromere only and not the entire length of the chromosome. Premature centromere division is defined as at least one chromosome fully dissociated from its sister chromatid, while at least one other chromosome in the same metaphase is still attached. Premature anaphase is defined as all chromosomes being completely separated.

Strikingly, sperm whale skin fibroblasts show resistance to particulate chromate-induced SAC bypass (Figure 28A). All treatments showed zero increase

in centromere spreading after 24 and 120 h. After 24 and 120 h, premature centromere division and premature anaphase occurred in 0.3 to 1.0% of metaphases in a non-dose dependent fashion.

Bowhead whale lung fibroblasts also show resistance to SAC bypass (Figure 28B). No centromere spreading was observed in any treatment concentrations after either 24 or 120 h. Premature centromere division occurred in 0.5% of cells after both 24 and 120h at 0.4 μg/cm^2 zinc chromate concentration.

Separase is the protein that cleaves cohesin at the centromeres, and thus SAC bypass and abnormal centromere division is also evidence of aberrant separase activity. We demonstrate in Aim 1 Cr(VI)-induced securin loss leads to abnormal separase activity in human lung cells. Lack of SAC bypass in whale cells indicates normal separase regulation in whale cells even after prolonged Cr(VI) exposure. In contrast to human cells (Wise et al., 2006) whale cells do not exhibit Cr(VI)-induced SAC bypass or loss of securin function.

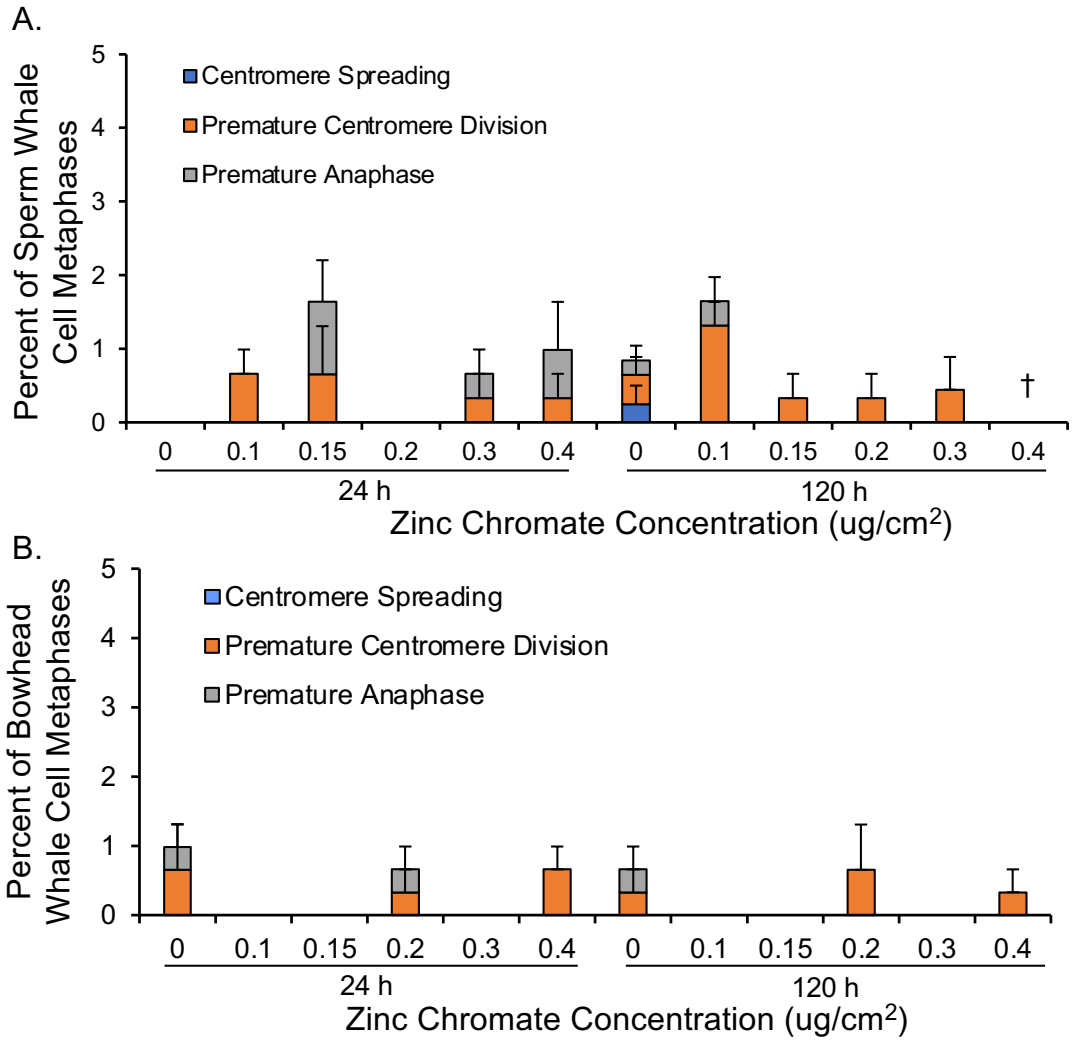


Figure 28. Spindle assembly checkpoint bypass in whale cells after 24 h and 120 h zinc chromate exposure. This figure shows Cr(VI) does not induce SAC bypass in whale cells. (A) Sperm whale metaphases do not display centromere spreading, premature centromere division, or premature anaphase, shown in stacked columns. (B) Bowhead whale metaphases do not display centromere spreading, premature centromere division, or premature anaphase, shown in stacked columns. Data reflect the mean of three independent experiments. † = Fewer than 100 metaphases were produced for analysis. Error bars = SEM. No results were significantly different from control group.

Cr(VI) does not induce centrosome amplification in interphase whale cells.

Previous studies show centrosome amplification increases with Cr(VI) exposure time and concentration and correlates with increased numbers of aneuploid metaphases in human lung cells (Martino et al., 2015). Centrosome amplification has been shown to occur in prolonged G2 phase induced by DNA damage, indicating interphase is when centrosome amplification arises (Dodson et al., 2004; Holmes et al., 2010; Inanç et al., 2010). To determine if observed DNA damage and cell cycle arrest in Cr(VI)-exposed whale cells induces centrosome amplification, we analyzed centrosomes in interphase cells. Centrosomes were counted in 100 interphase cells per treatment concentration. Bowhead whale interphase cells (Figure 29A) exposed to 0, 0.2, 0.3, and 0.4 μg/cm^2 zinc chromate for 24 h had 1.5%, 3.5%, 2.0%, and 2.5% centrosome amplification. After 120 h, percent of centrosome amplification was 2.0%, 0.5%, 6.0% and 4.0%. Sperm whale interphase cells (Figure 29B) exposed to 0, 0.2, 0.3, and 0.4 μg/cm^2 zinc chromate for 24 h had greater than 2 centrosomes in 2.5%, 2.0%, 3.0% and 3.0% of cells respectively. After 120 h exposure, sperm whale cells showed 4.5%, 4.5%, 3.5%, and 1.5% centrosome amplification. No treatments showed significant increase in centrosome amplification in either cell line or exposure time point.

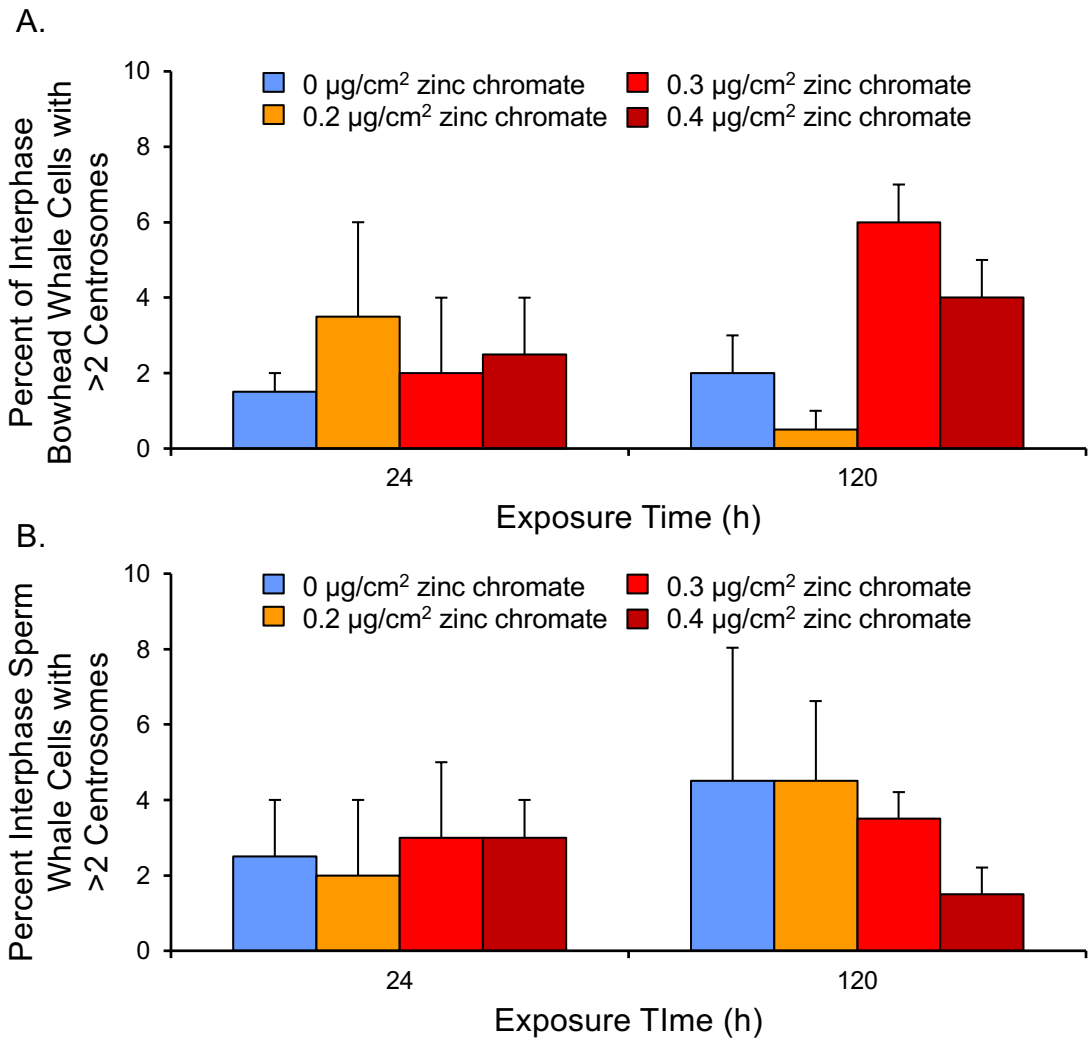


Figure 29. Centrosome amplification in interphase whale cells after 24 h and 120 h zinc chromate exposure. This figure shows Cr(VI) does not induce centrosome amplification in interphase whale cells. (A) Bowhead whale cells and (B) Sperm whale cells do not experience Cr(VI)-induced centrosome amplification. 100 cells scored per experimental condition. Data reflect the mean of two independent experiments. Error bars = SEM. No results were significantly different from the control group.

Cr(VI) does not induce centrosome amplification in mitotic whale cells.

Rare cells with supernumerary centrosomes may progress to mitosis and produce aneuploid daughter cells (Brinkley, 2001; Nigg et al., 2014). It is during mitosis that centrosome amplification can cause chromosomal instability, so we also analyzed mitotic cells. Centrosomes were counted in 50 mitotic cells per treatment concentration. After 24 h, an average of 2.19% untreated mitotic bowhead whale cells (Figure 30A) had centrosome amplification. Treatment with 0.2, 0.3, and 0.4 μg/cm^2 zinc chromate produced 3.13%, 3.17%, and 1.52% centrosome amplification. After 120 h, 2% of untreated mitotic cells had greater than 2 centrosomes, while treated cells showed centrosome amplification in 0%, 2%, and 2.13% of mitotic cells.

After 24 h, untreated sperm whale cells (Figure 30B) contained greater than 2 centrosomes in 7% of mitotic cells, while 0.2, 0.3, and 0.4 μg/cm^2 zinc chromate treatment produced 5.2%, 1.2% and 0% centrosome amplification. Fewer than 50 mitotic cells were found after 24 h exposures of both 0.3 and 0.4 μg/cm^2 zinc chromate. After 120 h, 2% of untreated mitotic cells had centrosome amplification, and treatment resulted in 1%, 2%, and 2.9% centrosome amplification. After 120 h fewer than 50 mitotic cells were found in the highest concentration of 0.4 μg/cm^2 zinc chromate. No treatments showed significant increase in centrosome amplification in either cell line or exposure time point.

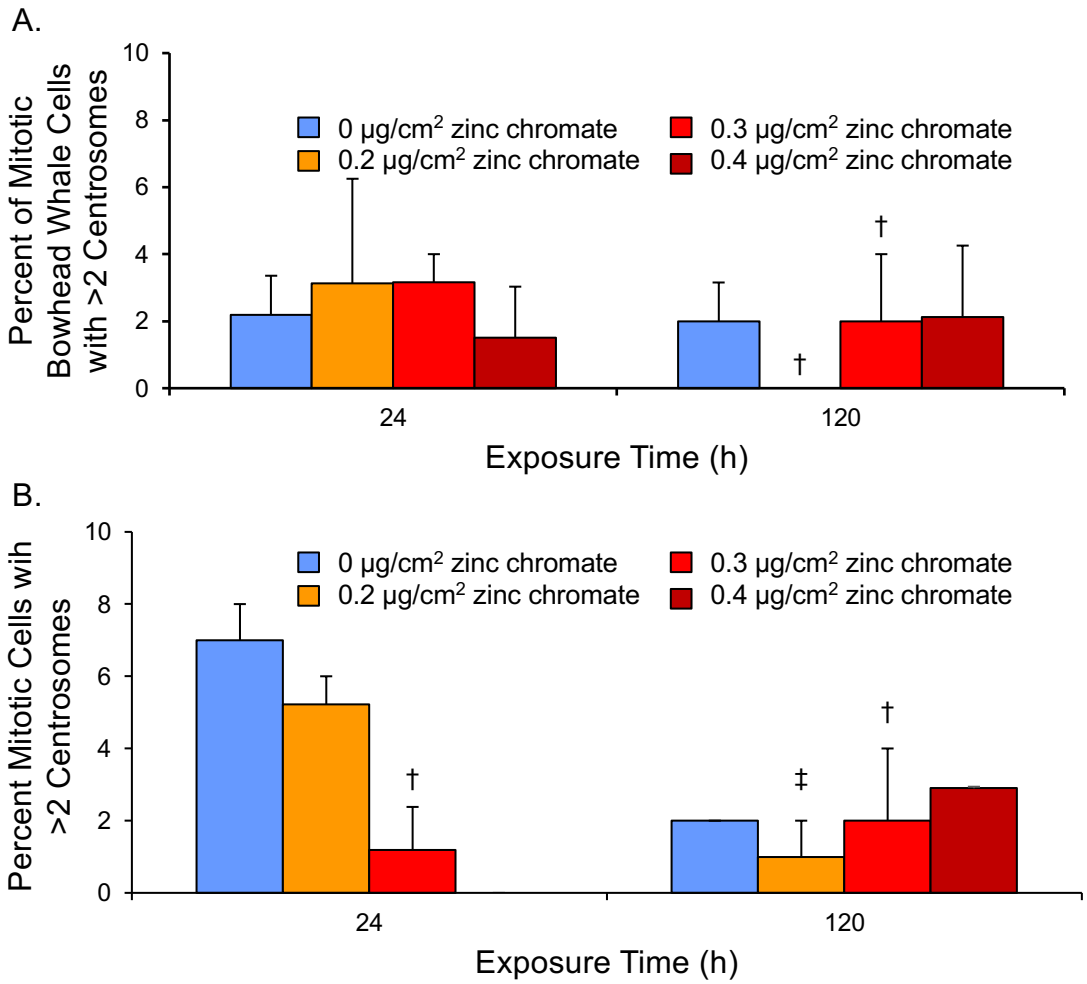


Figure 30. Centrosome amplification in mitotic whale cells after 24 h and 120 h zinc chromate exposure. This figure shows Cr(VI) does not induce centrosome amplification in mitotic whale cells. (A) Bowhead whale cells and (B) sperm whale cells do not display Cr(VI)-induced centrosome amplification in mitotic cells. 50 cells scored per experimental condition. Data reflect the mean of two independent experiments. † = Fewer than 45 mitotic cells present per experiment. ‡ = Fewer than 25 mitotic cells present per experiment. Error bars = SEM. No results were significantly different from the control group.

Whale cells are resistant to Cr(VI)-induced numerical CIN.

Aneuploidy is defined as the loss or gain of entire chromosomes. Numerical chromosome instability is a dynamic process characterized by changing numbers of chromosomes. Changes in polidy from the stable chromosome number are measured as evidence of numerical chromosome instability. Numerical chromosome instability is a key effect of Cr(VI) in human cells. Exposure of 0.1, 0.15, and 0.2 μg/cm^2 zinc chromate for 120 h induced 28%, 40%, and 44% aneuploid metaphases, respectively, in human lung cells (Holmes et al., 2010). We analyzed aneuploidy in whale cells by metaphase analysis, counting chromosomes per metaphase and classifying those with greater or fewer than 42 chromosomes as aneuploid. We scored 100 metaphases with 40–44 chromosomes and any hyper- or hypodiploid metaphases were also added to the aneuploidy analysis. Metaphases were analyzed for aneuploidy after 24 and 120 h of zinc chromate treatment.

For the 24 h time point, bowhead whale control group showed 20.3% aneuploid metaphases while 0.1, 0.15, 0.2, 0.3, and 0.4 μg/cm^2 treatment resulted in 21.6%, 26.0%, 21.0%, 21.3%, and 22.5% aneuploid metaphases (Figure 31A). After 120 h, control cells showed 23.2% aneuploid metaphases and treatment resulted in 23.3%, 22.0%, 30.8%, 25.1%, and 29.0% aneuploid metaphases. None of the treatment results were significantly different from the control bowhead whale cells.

Sperm whale control cells from the 24 h treatment group had 13.8% aneuploid metaphases and 0.1, 0.15, 0.2, 0.3, and 0.4 μg/cm^2 treatment resulted

in 16.5%, 11.5%, 11.9%, 12.3%, and 15.5% aneuploid metaphases, respectively (Figure 31B). The 120 h control had 12.9% aneuploid metaphases while the treatments had 10.2%, 17.3%, 12.0%, 18.6%, and 22.6% respectively. The elevated percentages at the two highest concentrations are in treatments that failed to produce 100 metaphases in each experiment. The two highest concentrations, 0.3 and 0.4 μg/cm^2 zinc chromate, were significantly different from the control bowhead whale cells (p = 0.024 and p = 0.012 respectively).

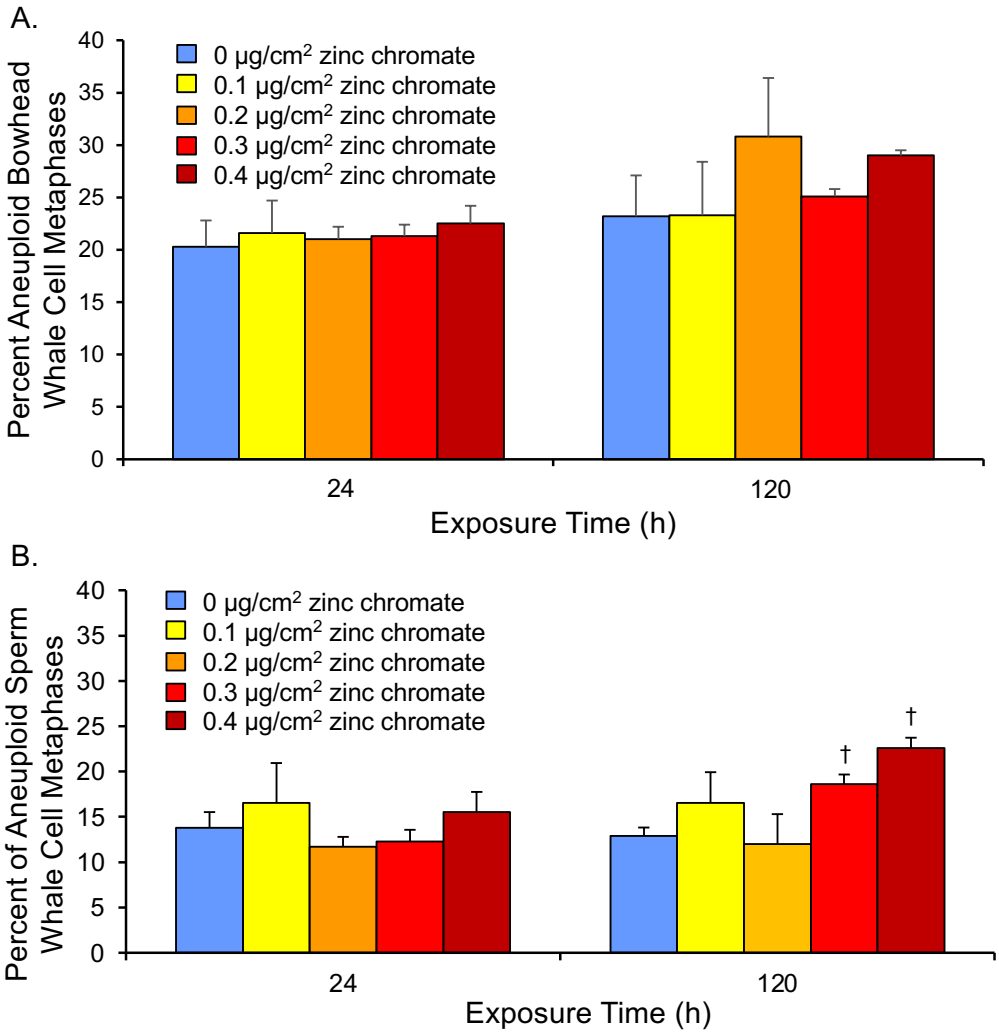


Figure 31. Percent of aneuploid metaphase in whale cells after 24 h and 120 h zinc chromate exposure. This figure shows Cr(VI) does not induce aneuploidy in whale cells. (A) Percent of aneuploid bowhead whale metaphases do not increase with Cr(VI). (B) Percent of aneuploid sperm whale metaphase do not increase with Cr(VI). 100 metaphases scored per experimental condition. Data reflect the mean of three independent experiments. † = Fewer than 100 metaphase present per experiment. Error bars = SEM. No results were significantly different from the control group.

Chromium uptake differs between sperm whale and bowhead whale cells.

The difference in cytotoxicity between sperm whale and bowhead whale cells may be caused by differences in Cr(VI) uptake by the cells. We measured intracellular chromium after treatment at all experimental concentrations for 24 and 120 h. These data show that intracellular chromium concentration is higher in sperm whale cells than bowhead whale cells (Figure 32) after equal administrations and at both time points. In bowhead whale cells (Figure 32A), 24 h of exposure to 0, 0.1, 0.15, 0.2, 0.3, and 0.4 μg/cm^2 zinc chromate administration caused average intracellular chromium concentrations of 0, 68, 94, 89, 129, and 247 μM. Exposure of 120 h at the same administered concentrations resulted in 0, 78, 107, 159, 278, and 338 μM chromium. In sperm whale cells (Figure 32B), 24 h of exposure to 0, 0.1, 0.15, 0.2, 0.3, and 0.4 μg/cm^2 zinc chromate administration resulted in average intracellular chromium concentrations of 0, 109, 198, 302, 427, and 532 μM. Exposure of 120 h at the same administered concentrations resulted in 0, 101, 195, 323, 404, 862 μM chromium. Comparing intracellular concentrations between 24 and 120 h exposures did not reveal significant increases for either cell line.

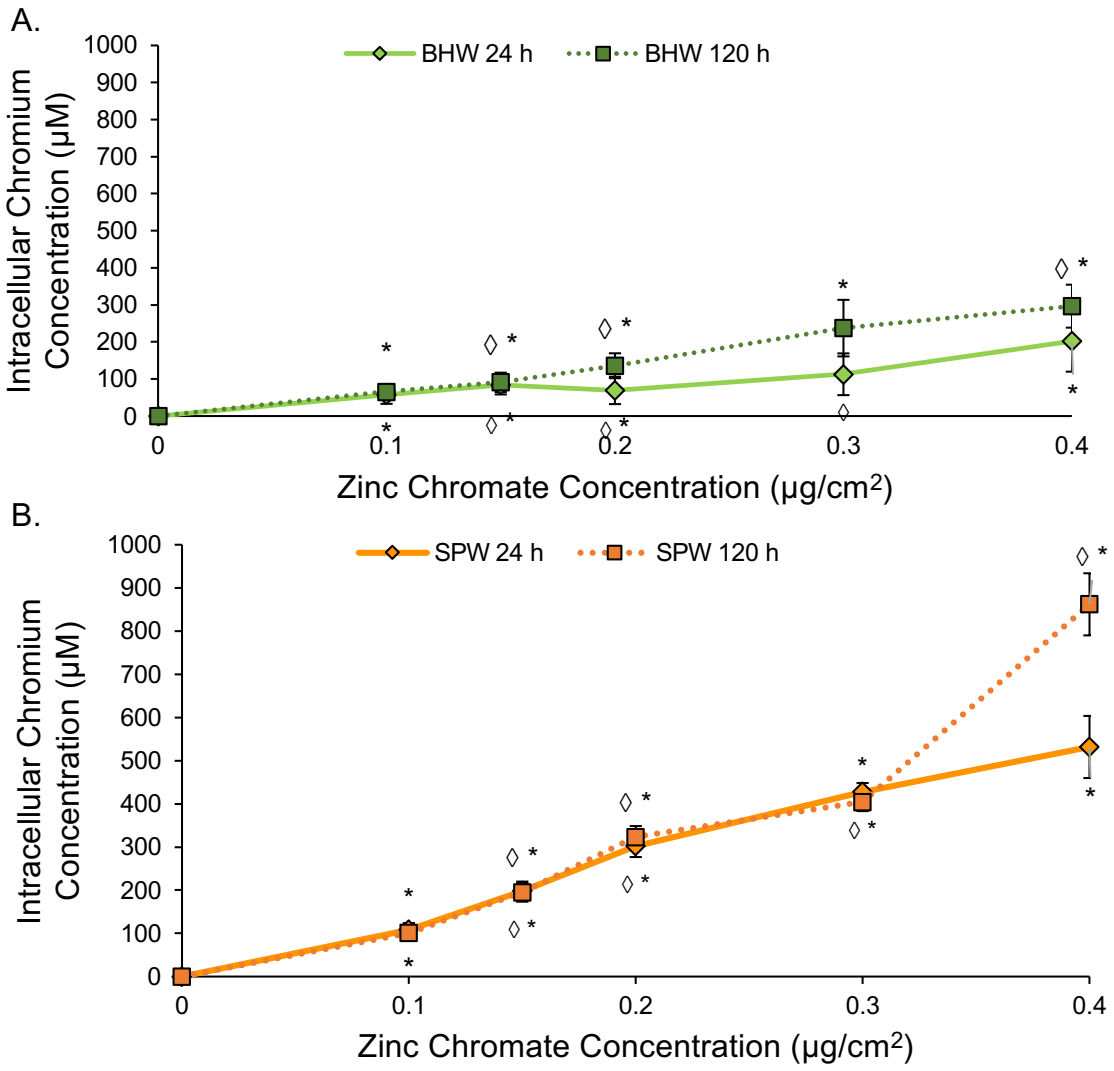


Figure 32. Intracellular chromium concentration (µM) in whale cells after 24 and 120 h of zinc chromate exposure. This figure shows intracellular chromium levels increase after 24 h and 120 h of Cr(VI) exposure. (A) Bowhead whale cells take up less chromium than (B) sperm whale cells. Data reflect the mean of three independent experiments. Error bars = SEM. ◊ = Data significantly different between cell lines ($p < 0.05$). *Significantly different significantly different from the control group within the same cell line and timepoint ($p < 0.05$).

SUMMARY

Previous studies demonstrate that prolonged particulate Cr(VI) exposure causes significant aneuploidy in human lung fibroblasts which correlates with centrosome amplification (Holmes et al., 2010; Martino et al., 2015). Whales are long-lived air breathers and are exposed to Cr(VI) (Wise et al., 2015; Wise et al., 2009), however the rates of cancer in these animals appear to be much lower than in humans (Caulin & Maley, 2011). Comparative investigations between human and whale cells revealed that while Cr(VI) is cytotoxic and genotoxic to both species, whale cells were more resistant to cytotoxicity and to structural chromosome damage compared to human cells (Li Chen et al., 2012; Li Chen et al., 2009). However, no previous studies have investigated numerical chromosome instability or centrosome amplification in whales. Interspecies differences in these key promoters of carcinogenesis can illuminate the mechanism of Cr(VI)-induced genomic instability. Here we found, as opposed to human cell responses shown in Aim 1, Cr(VI) did not induce securin loss and separase dysfunction in whale cells. In addition, Cr(VI) did not induce aneuploidy or centrosome amplification in whale cells, which supports our hypothesis securin loss is a central event in Cr(VI) carcinogenesis.

Cytotoxicity assays show administered concentrations of 0.2, 0.3, and 0.4 $\mu g/cm^2$ zinc chromate cause statistically significant decreases in colony formation compared to control in both sperm whale and bowhead whale cell lines after 120 h of exposure. Zinc chromate at these levels was cytotoxic to sperm whale skin

fibroblasts after 24 h of exposure, whereas bowhead whales showed significant cytotoxicity after 24 h only at the highest concentration of 0.4 μg/cm^2. The difference in survival between 24 and 120 h was significant in sperm whales at 0.1, 0.2, 0.3, and 0.4 μg/cm^2 zinc chromate concentrations, however zinc chromate was not significantly more toxic to bowhead whale cells after 120 h compared to 24 h at the same concentration. Percent survival relative to control was not significantly different between cell lines, except at the highest concentration (Student's t-test, $p > 0.05$). At both time points sperm whale cells were more sensitive to cytotoxicity upon 0.4 μg/cm^2 zinc chromate administration.

Relative survival after 24 h shown here are in line with published cytotoxicity in sperm whale skin fibroblasts after 24 h treatments of similar levels of lead chromate, another particulate form of Cr(VI) (Wise et al., 2011). At similar administered concentrations of lead chromate, zinc chromate appears to induce similar colony reduction in sperm whale cells. Concentrations of 0.1 and 0.5 μg/cm^2 lead chromate induce 86 and 63% relative survival (Wise et al., 2011) while 0.1 and 0.4 μg/cm^2 zinc chromate induce 89% and 51% relative survival after 24 h.

While sperm whale cell survival decreased after 120 h of exposure, intracellular chromium concentrations were not significantly increased after 120 h compared to 24 h for either cell line. Since intracellular chromium levels increase with administered concentration, it is apparent that the cell is not saturated with chromium at lower experimental concentrations, and it is expected that with prolonged exposure time the cell continues to take up chromium, especially as it is brought out of solution by binding to intracellular molecules. It is possible that

prolonged exposure to low intracellular chromium causes increased cytotoxicity at 120 h.

Securin is the protein that controls separase activity. In Aim 1 we show Cr(VI) causes loss of securin in human cells and abnormal separase activity. We measured securin levels in bowhead whale lung cells and found protein is retained at normal levels after even 120 h of Cr(VI) exposure. Spindle assembly checkpoint protects against aneuploidy by preventing progression to anaphase until all kinetochores are properly attached to spindle fibers. Centromere spreading, premature centromere division, and premature anaphase are consequences of particulate Cr(VI) exposure in human lung fibroblasts (Holmes et al., 2010). These phenotypes produced in metaphase-arrested cells are evidence of spindle assembly checkpoint bypass. Neither sperm whale nor bowhead whale cells showed evidence of spindle assembly checkpoint bypass at any treatment conditions, demonstrating that these whale species somehow maintain regulation of spindle assembly checkpoint proteins under conditions in which human lung cells do not. Separase cleaves cohesin at centromeres and thus these data indicate separase is not prematurely active in metaphase whale cells after Cr(VI) exposure.

Zinc chromate treatment did not induce centrosome amplification in bowhead or sperm whale interphase cells at any treatment concentration or time point. The point at which centrosome amplification becomes critical is during mitosis, as aberrant segregation of chromosomes results in aneuploidy (Ganem et al., 2009). Cells can overcome multipolar arrangements by clustering centrosomes

to form pseudo-bipolar spindle poles (Ganem et al., 2009). However, this coping mechanism does not ensure faithful chromosome segregation because excess centrosomes block one another and may also form erroneous kinetochore attachments before moving into bipolar positions. Normal mitotic cells have 2 centrosomes. During our study, no zinc chromate treatment conditions caused aberrant centrosomes to elevate above control percentages in either cell line. Consistent with the metaphase assays, 0.4 μg/cm^2 zinc chromate caused depressed mitotic numbers in sperm whale cells, suggesting cell cycle arrest. Background levels of centrosome amplification were low in both mitotic and interphase whale cells.

It is possible that whale cells with centrosome amplification are culled by apoptosis. However, cytotoxicity data for bowhead whale lung fibroblasts reveal only mild decreases in relative survival with increasing zinc chromate concentrations. Mitotic arrest seen at the high end of our experimental concentrations could be a strategy to prevent aberrant cells from dividing and producing aneuploid cells, however research has identified G2 arrest to be causative in centrosome amplification and these data prove that interphase centrosome amplification does not increase in whale cells exposed to Cr(VI). Thus, these data suggest that whales have evolved strategies to combat chromosome instability induced by Cr(VI) which are lacking in human cells.

The mechanisms of centrosome amplification are unknown. Hypotheses center on protein dysregulations that allow the centrosome duplication cycle to become desynchronized with cell cycle progression (Agircan et al., 2014; Bolgioni

& Ganem, 2016; Hatano & Sluder, 2012). The fact that zinc chromate does not cause centrosome overduplication in whale cells make them a useful comparative model for studying the molecular components that regulate centrosome duplication. This result supports the hypothesis whale cells avoid Cr(VI)-induced centrosome amplification and SAC bypass by retaining sufficient securin protein

Aneuploidy was evaluated after zinc chromate treatment in sperm whale and bowhead whale cells. Notably, after 120 h of exposure to 0.4 μg/cm^2 zinc chromate, sperm whale cells experienced cell cycle arrest, failing to yield enough metaphases to analyze. No treatment condition produced aneuploidy in excess of control cells. Bowhead whale cells had slightly higher background levels of aneuploidy (20.3%) compared to sperm whale cells (13.8%). Published results show that 0.1, 0.15, and 0.2 μg/cm^2 zinc chromate caused 28%, 40%, and 44% aneuploidy in human lung cells, which is significantly higher than the background rates of 8-13% (Holmes et al., 2010). Our current study suggests that whale cells are resistant to Cr(VI)-induced numerical chromosome instability.

A tempting explanation for how whale cells avoid the consequences of Cr(VI) exposure that human cells exhibit is that Cr(VI) uptake is less in whale cells. Indeed, comparative studies show that chromium uptake differs between whale and human cells. Lead chromate experiments show human cells achieve higher intracellular concentrations than whale cells after equal administrations. However, Li Chen et al. (2012) corrected for differential uptake and showed that uptake differences did not fully explain clastogenic differences such as structural chromosome damage. While whale cells show extremely low occurrences of

numerical chromosome instability and centrosome amplification, we do observe cytotoxicity, cell cycle arrest, and DNA double strand breaks which indicate Cr(VI) presence in the cell is having many of the same toxic indications as in human cells. Thus, uptake does not sufficiently explain how whales evade Cr(VI)-induced centrosome amplification and numerical CIN.

Whale cell resistance to Cr(VI)-induced centrosome amplification and numerical chromosome instability highlights the important link between these two phenotypes which are proposed to underlie carcinogenesis. Species comparisons between human and whales can help to elucidate the molecular mechanisms of Cr(VI) carcinogenesis and indeed the stark difference in securin protein response in human cells, illustrated in Aim1, and whale cells, illustrated in Aim 3, support the hypothesis securin is a key centrosome regulator which is targeted by Cr(VI).

CHAPTER 4: DISCUSSION

Overview

Lung cancer is by far the deadliest cancer worldwide and resulted in 1.8 million deaths in 2020 (Sung et al., 2021). The most well-known cause of lung cancer is cigarette smoking, but the World Health Organization estimated up to 25% of deaths occur to patients who never smoked, indicating other causes such as occupational exposures and air pollution are important contributors (Sung et al., 2021; Couraud et al., 2012). Hexavalent chromium [Cr(VI)] is a lung carcinogen with widespread environmental and occupational exposure risks. Despite decades of study on Cr(VI) in lung cancer through occupational exposures, *in vivo* animal studies, and cell culture experiments, its molecular mechanism of carcinogenesis is not fully understood (Tsuneta et al., 1980; Langård and Vigander, 1983; Levy et al., 1986; Takahashi et al., 2005; Wise et al., 2002; Xie et al., 2007).The prevailing theory implicates Cr(VI)-induced chromosomal instability. However, little is known about how Cr(VI) induces numerical chromosome instability.

Aneuploidy is characterized by loss or gain of whole chromosomes per cell and it is the most common form of chromosomal instability observed in cancers (Chan, 2011; Compton, 2011). One proposed mechanism by which numerical instability arises is through centrosome amplification (Brinkley, 2001; Compton, 2011; D'Assoro et al., 2002; Ganem et al., 2009). Centrosome amplification has

been observed in a wide range of solid and hematological cancers and has been identified as an early event in carcinogenesis (Chan, 2011; Wise & Wise, 2010). Centrosome amplification increases with tumor aggressiveness and correlates with poor prognoses (Chan, 2011). It is an observed outcome of exposure to carcinogenic metals such as Cr(VI), cadmium, and arsenic (Holmes et al., 2010; Zhang et al., 2019; Yi et al., 2006). Thus, centrosome amplification is an important cancer phenotype as well as a potential key mechanism of metal carcinogenesis. This dissertation investigates potential mechanisms of centrosome amplification and numerical chromosome instability after Cr(VI) exposure to build on previous work that shows these are potential keys to its carcinogenic mechanism.

Securin is an important regulatory protein involved in numerical chromosome stability and centrosome maintenance (Zhou et al., 1999; Jallepalli et al., 2001; Chao et al., 2006). DNA-damaging agents have been found to alter securin levels (Zhou et al., 2003; Zhou et al., 2005) and its role in centrosome amplification and chromosome instability has been confirmed by gene modulation experiments (Yu et al., 2003; Zhou et al., 2005; Kim et al., 2007; Tsou and Stearns, 2006; Mora-Santos et al., 2013). Securin dysregulation has been observed in several cancers (Kakar, 2006). However, no investigations focus on the impact of Cr(VI) exposure on securin regulation. This dissertation tests the hypothesis Cr(VI) causes securin disruption, which alters separase activity, and leads to centrosome amplification and numerical chromosome instability. As a ubiquitous environmental contaminant, Cr(VI) poses potential threats to wildlife and humans. Thus, we explore our hypothesis using the One Environmental Health approach. We

conducted our investigation in three aims showing: 1) Cr(VI) targets securin causing loss of expression in human lung cells, 2) Cr(VI) alters nuclear levels of transcription factors and miRNA expression in human lung cells, and 3) whale cells are resistant to Cr(VI) induced effects observed in human cells.

Prolonged Cr(VI) exposure targets securin, causing loss of protein levels and function.

In Aim 1 we tested the hypothesis particulate Cr(VI) causes securin disruption. Securin is a key protein in numerical chromosome stability because 1) it regulates separase cleavage at centromeres to restrict chromatid division until proper spindle assembly and anaphase progression and 2) it regulates separase activity at centrosomes to restrict centrosome duplication to once per cell cycle. Securin disruption could explain previous reports of Cr(VI)-induced SAC bypass, premature centriole division, centrosome amplification, and numerical chromosome instability (Wise et al., 2006; Holmes et al., 2010; Martino et al., 2015).

The first step to investigating the effects of Cr(VI) on securin was to measure protein levels. After 24 h exposure, securin protein levels showed a trend toward increased levels with Cr(VI) concentration which was not statistically significant, but levels significantly decreased after 120 h. Despite known securin involvement in chromosome instability and carcinogenesis, the role of securin in carcinogenic metal exposure is not well known. Chao et al. (2006) showed 24 h arsenite treatment suppressed securin expression in both mouse endothelial cells

and human colorectal epithelial cancer cells. It is possible securin is abundant in human lung cells and the remaining protein is sufficient to control separase activity. It is also possible the cell could respond to securin loss with compensatory regulation to maintain securin functions. Our previous reports of SAC bypass and centriole disengagement (Wise et al., 2006; Holmes et al, 2006; Martino et al., 2015) suggest securin failure at 120 h, though to determine if securin inhibition of separase activity was maintained, we directly investigated cleavage of separase substrates.

Separase is an endopeptidase which cleaves proteins at conserved recognition sites. When uninhibited by securin and active it cleaves itself, kendrin, and the cohesin subunit, SCC1. When probing these proteins by western blot, a full-length protein band can be observed, along with one or more quickly-migrating, smaller fragments. After 24 h zinc chromate exposure, no change in the percentage of full length or cleaved separase was observed. After 120 h exposure, we measured increased protein levels in all separase bands. The increases in cleaved protein were greater than increases in full-length protein, showing up to 430% and 380% increase for the two cleaved protein bands while full length protein increased to 170% of control cells. These data indicate separase cleavage is increased after prolonged Cr(VI) exposure and securin function is not sufficient at lower levels to control separase activity.

While measurements of increased separase activity were in agreement with our securin data, another interpretation was that separase activity was increased by upregulated separase expression. Western blot assay only allows

measurement of protein fragments that contain the antibody-recognized epitope. Therefore, any cleaved fragments missing the epitope will not be visualized and thus it is impossible to measure total protein levels using this method. We used RT-qPCR to measure separase mRNA expression after Cr(VI) exposure and better understand if separase levels could be increasing in treated cells. We found separase expression was reduced by Cr(VI). This outcome could be a potential feedback response to the loss of securin and supports findings by Pfleghaar et al. (2005) and Jallepalli et al. (2001) that securin(-/-) cells had depressed separase protein levels.

Next, we analyzed kendrin cleavage. Kendrin is a separase substrate which supports centriole engagement. Kendrin is localized to the centrosome and thus provides a measure of centrosome-specific separase activity. After 24 h Cr(VI) exposure, no significant changes in kendrin levels were observed. After prolonged 120 h Cr(VI) exposure, full length kendrin protein decreased and cleaved kendrin levels increased compared to untreated cells, adding evidence for increased Cr(VI)-induced separase activity. This activity, which is localized to the centrosomes and directed toward the centriole linkers further implicates centriole disengagement as a route to centrosome amplification after prolonged Cr(VI) exposure. There are several potential routes to centrosome amplification, including failure of cytokinesis, *de novo* centrosome synthesis, centrosome fragmentation, and over-duplication (Fukasawa, 2005; Godinho & Pellman, 2014; Tsou & Stearns, 2006a). Our data supports Martino et al. (2015) in proposing centrosome over-

duplication, by way of premature centriole disengagement, is the most likely cause of Cr(VI)-induced centrosome amplification.

Cohesin is the best-known separase substrate and has been investigated widely for its role in sister chromatid cohesion. Cohesin also plays a role in supporting centriole engagement. Cohesin is a ring complex made of three subunits and separase specifically targets the SCC1 subunit to open the cohesin ring. Western blot revealed two bands for SCC1, representing the full-length and cleaved proteins. Our results indicate prolonged zinc chromate exposure caused a trend towards increasing SCC1 cleavage in nuclear extracts. Repeated experiments could further strengthen this trend. SCC1 cleavage is difficult to detect by western blot because the proportion of cohesin that is cleaved by separase is relatively small. In interphase cells, cohesin cleavage may occur at sites of DNA damage (Sun et al., 2006). Centrosome-associated cohesin is a small amount of protein in the cytoplasm. Even in mitotic cells, when separase is known to play a critical role in chromatid disjunction, the majority of cohesin along chromatid arms is released by separase-independent mechanisms, including WAPL protein, and only the cohesin that remains at the centromeres is cleaved by separase. (Sumara et al., 2002; Kueng et al., 2006). Thus, separase is responsible for approximately 10% of cohesin cleavage (Sun et al., 2006) and western blot imaging of total SCC1 may be insufficient to demonstrate separase-specific cleavage.

While securin is the main regulator of separase, its function is protected by compensatory mechanisms in the cell. Studies show separase control can be retained even after securin knockout (Mei et al., 2001; Pfleghaar et al., 2005),

leading to questions of how Cr(VI) might circumvent compensation for securin loss. We investigated the secondary separase inhibitor, cyclin B1. Cyclin B1, in complex with its partner, cdk1, interacts with separase to inhibit its activity. After prolonged Cr(VI) exposure we found cyclin B1 protein was also dramatically decreased in human lung cells, showing Cr(VI) disruption causes a multipronged disruption of centrosome regulation. Indeed, Cr(VI) carcinogenesis is complex and has been shown to alter various proteins but the targeting on securin and cyclin B1 indicates a concentration of effects in pathways of centrosome and chromosome stability that reach a tipping point in the relatively short period of 120 h.

To confirm the role of securin in Cr(VI)-induced chromosome instability, we employed siRNA knockdown in human lung cells. Exposure to 24 h Cr(VI) does not produce effects on securin levels and does not induce numerical chromosome instability (Wise et al., 2006; Holmes et al., 2010; Martino et al., 2015). We used securin-targeting siRNAs to experimentally reduce securin levels at 24 h Cr(VI) exposure times. Our hypothesis was securin reduction will shift the characteristic 120 h phenotype earlier. This hypothesis was supported by the data siRNA transfection alone did not elevate numerical chromosome instability but addition of 24 h zinc chromate exposure raised levels up to 20.2%, which represented more than doubling of the control level of 8.5%.

After showing securin is targeted by Cr(VI), protein levels are lost, and securin function is inhibited, we pursued the mechanism by which securin is targeted. Cr(VI) can disrupt cellular pathways thorough translational or post-translational processes, such as altered ubiquitin signaling and protein

degradation (Bruno et al, 2016). Securin destruction is mediated by ubiquitination and proteosome degradation. However, our data show loss of securin levels was not explained by altered degradation rates. Cr(VI) causes cell cycle arrest or delay (Montiero et al., 2019; Lou et al., 2013; Xiao et al., 2012) so another possibility was altered protein levels were artifacts of cells becoming stalled in G1 when low levels of securin are expressed. However, cell cycle analysis confirmed proteins levels could not be explained by cell cycle effects. RT-qPCR analysis showed Cr(VI) decreased securin mRNA levels, and thus the mechanism of disruption was narrowed in to the pre-translational level.

Securin is a novel target of Cr(VI) and while no studies have measured separase activity after metal exposure, nor specifically in lung cancers, increased separase activity has been observed in several cancers and it is a topic of emerging interest in carcinogenesis (Ruppenthal et al., 2018; Gurvits et al., 2017; Haass et al., 2012). Our data support investigation into securin loss and separase disruption as a potential step in the Cr(VI) carcinogenic mechanism and as a potential event in lung cancers.

Cr(VI)-induced alteration of transcription factors does not explain its action on securin

Mechanisms of Cr(VI)-induced protein disruption are diverse, and thus we sought to define how Cr(VI) targets securin expression. Cr(VI) has been shown to inhibit transcription (Raja et al., 2008; Zablon et al., 2019; Wetterhahn et al., 1989) which aligns with our observations of decreased securin mRNA levels. We tested

the hypothesis Cr(VI) alters securin-targeting transcription factor levels. Three transcription factors are reported to promote securin expression, NF-Y, Sp1, and E2F1 (Zhou et al., 2009, Pappas et al., 2017; Clem et al., 2003). Each of these is connected with cancer pathways and commonly disrupted in cancer (Gallo et al., 2021; Yang et al., 2020; Dolfini et al., 2019; Bezzecchi et al., 2019; Vellingiri et al., 2020; Vizcaino et al., 2015; Hsu et al., 2012; Zhou et al., 2009). No published studies were identified that address effects of Cr(VI) exposure on NF-Y, though one study showed cadmium increased NF-Y levels (Ghosh et al., 2020). Raja et al. (2008) showed Cr(III)-ligand complexes inhibit Sp1 binding at DNA binding sites and inhibit transcription. These effects were dependent on ligand and concentration and do not confirm the ability to Cr(III) to disrupt binding *in vivo*. In contrast, Kaltreider et al. (1999) observed increased Sp1 binding activity in Cr(VI)-exposed melanoma cells, though this effect was transient and only occurred after 1 h exposure. E2F1 dysregulation has been reported after Cr(VI) exposure (Speer et al., 2021), as well as other metals (Ghosh et al., 2020; Li et al., 2008).

After 24 h and 120 h exposures, Cr(VI) did not induce any changes in whole cell protein levels of NF-YA or Sp1. Previous findings show Cr(VI) can change subcellular protein localization, for example sequestering it in the cytoplasm and thus hindering its function in the nucleus (Speer et al., 2021). Since both NF-YA and Sp1 are required for securin expression, if either was decreased in the nucleus, protein levels would decline. Nuclear protein fractions were isolated and analyzed. Both NF-YA and Sp1 levels increased in the nucleus with prolonged exposure, indicating Cr(VI) activated nuclear localization of transcription factors.

We conclude NF-YA and Sp1 levels are not inhibited by Cr(VI) and are actually increased in the nucleus.

Increased transcription factor levels in the nucleus did not correlate with our observations of securin mRNA loss. This conclusion does not eliminate the possibility of Cr(VI) disruption of NF-YA and Sp1 function. It is possible Cr(VI) affects transcription factor binding or access of transcription machinery to the DNA. Results from Raja et al. (2008) showed Cr(III) complexes can prevent transcription factor-DNA binding and depress transcription, though it is unknown if this occurs at the exposure levels relevant to our study and in human lung cells. Nuclear binding may be prevented by direct interaction of Cr ions with chromatin, causing DNA-protein cross-links and DNA breaks (Xu et al., 2004; Zablon et al., 2019). Indirect Cr actions can include altered DNA and/or histone methylation and acetylation, preventing access to promoter sites. Cr(VI) globally alters DNA and histone methylation, impeding gene expression (Lou et al., 2013; Sun et al., 2009; Schnekenburger et al., 2007). Interestingly, one study examined CpG methylation in three cancer cell lines and thyroid carcinomas and found no epigenetic alterations to the securin gene (Hidalgo et al., 2008). This study differs from our model in that it investigated securin overexpression and the cause of cell transformation and thyroid may not be due to environmental chemicals. Cr(VI) has also been shown to alter chromatin architecture, which could disrupt transcription through displacement of enhancer regions relative to inducible promoters (VonHandorf et al., 2018; Ovesen et al., 2014).

We have previously shown whole cell protein levels of the securin-promoter, E2F1, are decreased in Cr(VI)-exposed human lung cells (Speer et al., 2021). Speer et al. (2021) showed E2F1 protein loss after prolonged exposure, which could explain securin loss. In Aim 2, E2F1 knockdown in addition to acute Cr(VI) exposure was expected to induce securin loss. However, securin levels increased non-significantly with 24 h Cr(VI) treatment in E2F1 knockdown cells, as in untransfected cells. This result is in concordance with results from Zhou et al. (2009) showing E2F1 modulation changes securin levels in p53-deficient cells but not in p53-competent cells. Knockdown experiments are useful to pinpoint critical targets, yet the mechanism of Cr(VI) disruption is proving to be multifaceted. Our results do not exclude the possibility that E2F1 loss plays a role in some way, yet it is not alone sufficient to explain how Cr(VI) causes dramatic securin loss.

In addition to securin-promoting transcription factors, we investigated two identified transcription repressors, p53 and KLF6. Whole cell levels and activation of p53 were not significantly altered. Cr(VI) is reported to increase, stabilize and phosphorylate p53 (Hill et al., 2008; Hanaoka et al., 1997; Ye et al., 1999). However, an explanation for this discrepancy is published reports use higher Cr(VI) levels and often employ cancer cell lines. For example, Ye et al. (1999) used 75-500 μM Cr(VI). If completely dissolved, 0.1, 0.2, and 0.3 μg/cm^2 is approximately equivalent to 0.66, 1.34, and 2.00 μM. Chromosome instability and centrosome amplification are often attributed at least partially to p53 inactivation (Manning et al., 2014; Fukasawa et al., 1996; Kawamura et al., 2004). In contrast, Luczak et al. (2019) showed limited activation of p53 in human lung cells and D'Agostini et al.

(2002) showed marginal p53 induction in rat lungs. Our data show p53 inactivation or protein loss is not a central effect of Cr(VI).

Prolonged Cr(VI) increased KLF6 nuclear levels, which may partially explain securin loss, however this effect was only seen at the two highest concentrations and does not fully explain securin loss. Securin targeting by KLF6 is reported in two known studies (Chen et al., 2013; Lee at al., 2010) and has been shown to be targeted by DNA-damaging agents (Gehrau et al., 2011). KLF6 has not been shown to be altered by metal exposure, though other kruppel-like family members are (Sharma et al., 2013; Zhu et al., 2018). While KLF6 nuclear reduction is not the sole source of securin decrease, it could be a contributing factor and KLF6 loss at prolonged and higher concentrations may have implications for Cr(VI) carcinogenesis.

Cr(VI)-induced securin loss is not caused by differential miRNA expression

Cr(VI)-induced decrease in securin mRNA was not fully explained by transcription factor disturbances. Another mechanism for mRNA loss is miRNA regulation. Typically, miRNAs alter gene expression by inhibiting translation of their target mRNAs. Few reports have investigated Cr(VI) effects on miRNA. Li et al. (2014) showed plasma miR-3940-5p in chromate workers correlated with micronuclei frequency and levels of DNA repair proteins XRCC2 and BRCC3. Dioni et al. (2016) analyzed 377 miRNAs from human peripheral blood samples and identified nine miRNAs with negative correlations to urinary Cr. These studies

demonstrate human Cr exposure can induce changes in miRNAs. Other carcinogenic metals, such as arsenic and cadmium, have also been shown to cause miRNA alterations (He at al., 2013; Humphries et al., 2017; Wang et al., 2020; Liu et al., 2015; Gonzalez et a., 2015). We used miRNA-seq to assess global miRNA expression after 24, 72, and 120h in human lung cells and found Cr(VI) up- and down-regulated miRNAs at each concentration and time point. Predicted securin-targeting miRNAs were not altered in our study and thus observed miRNA changes do not explain securin loss. However, several proteins involved in centrosome and chromosome maintenance are predicted targets of Cr(VI)-induced miRNA alteration. Notably, Sp1, NF-Y subunits, E2F1 and KLF6 are putative targets for miRNAs down-regulated by Cr(VI). MiRNA downregulation could explain the increase of Sp1, NF-YA, and KLF6 we measured, though it is incongruent with E2F1 loss previously observed (Speer et al., 2021). PP2A, SKP1 and CUL1 are involved in degradation of securin and cyclin B1. Our data showed potential miRNA interactions could be downregulated by Cr(VI), theoretically increasing degradation. Our data showed securin degradation rates were unchanged, but cyclin B1 is degraded by the same pathway and thus miRNA regulation could play a role in cyclin B1 loss. Overall, we found after 72 h and 120 h Cr(VI) exposure, miRNAs were more frequently down-regulated than up-regulated.

Whales resist Cr(VI)-induced numerical chromosome instability through retention of normal securin and centrosome regulation

Cr(VI) is a widespread environmental carcinogen. The One Environmental Health philosophy acknowledges humans and wildlife are exposed to the same environmental contaminants and research into toxic effects on one species is informative to other species and to ecosystem health (Pérez and Wise, 2018). In Aim 3 we showed whale cells exposed to zinc chromate experience cytotoxicity after acute and prolonged exposure. Sperm whale skin cells had lower survival rates compared to bowhead whale lung cells, which may be explained by differences in Cr uptake. It is unknown whether this is due to the difference in species, organ, or individual variation. Xie et al. (2015) show human skin cells are more sensitive to Cr(VI) toxicity. Peto (1977) considered a line of reasoning to explain cancer differences among various organ types, suggesting perhaps certain cells might be better equipped to cope with proliferative changes, given their inherent differences in life cycle.

Several published findings demonstrate whale cells are susceptible to Cr(VI)-induced cytotoxicity and genotoxicity (Wise et al., 2008; Li Chen et al., 2009; Wise et al., 2011; Wise et al., 2015). While bowhead whales experienced relatively lower levels of cytotoxicity than sperm whale cells, they nevertheless displayed DNA double strand breaks and chromosome damage, indicating toxic effects of Cr(VI) are induced at the intracellular levels achieved in bowhead whale cells. Remarkably, as opposed to human lung cells, pressure of Cr(VI) exposure and DNA damage did not decrease securin protein levels in whale lung cells, even at

prolonged timepoints. We measured SAC bypass as a proxy for securin function and found no increase in metaphase centromere defects in whale cells. Browning et al. (2017) showed North Atlantic right whale cells resisted loss of RAD51 protein, in contrast to the human cell response (Browning et al., 2016; Qin et al., 2014). Thus, evidence suggests whale cells maintain normal protein expression under Cr(VI). It would be insightful to learn if this ability is due to differences in epigenetic control.

We propose securin loss leads to centrosome amplification in human lung cells. We measured centrosome amplification in whale cells and found no increase in centrosome amplification in either mitotic or interphase cells. We propose Cr(VI)-induced centrosome amplification drive numerical chromosome instability in human lung cells. Whale cells had no increase in aneuploidy after 24 or 120 h. This is the first study to consider centrosomes or numerical chromosome instability in whale cells and aligns with Browning et al. (2017) in showing carcinogenic pathways are avoided in whale cells despite Cr(VI) exposure.

Our results support the hypothesis whale cells are resistant to Cr(VI)-induced structural and numerical chromosome instability. These data are the first to consider numerical chromosome instability and centrosome amplification in whale cells and contribute to mechanistic insights in Cr(VI) genotoxicity. Previous research showed whale cells resist Cr(VI)-induced disruption to homologous repair, making them resistant to structural chromosome instability (Browning et al., 2017) and this finding helped further solidify the essential role of homologous repair in Cr(VI) genotoxicity in human cells. Likewise, by showing prevention of

securin loss and centrosome amplification in whale cells correlates to resistance against numerical chromosome instability, we promote the importance of securin function and centrosome maintenance to Cr(VI) toxicity to humans.

Conclusions

This dissertation investigates a hypothesis of molecular mechanisms central to Cr(VI)-induced centrosome amplification. Together, these data show Cr(VI) targets securin expression, leading to loss of mRNA levels, protein levels, and compromises securin's inhibitory function of separase. Loss of separase inhibition leads to elevated cleavage of its substrates, including centriole linkers. These outcomes support the conclusion premature cleavage of centriole linkers causes centriole disengagement that promotes centrosome overduplication. Supernumerary centrosomes result in numerical chromosome instability, which confers genotypic plasticity advantageous to Cr(VI)-induced transformation and carcinogenesis.

CHAPTER 5: FUTURE DIRECTIONS

This dissertation describes new findings in the carcinogenic mechanism of Cr(VI) and specifically identifies securin as a novel target that leads to centrosome amplification and numerical chromosome instability. Our results introduce new directions for research that enhance the field of knowledge about cancer and metal toxicology. The following suggestions highlight future work which could build upon our understanding of the mechanisms proposed.

In Aim 1 we report prolonged Cr(VI) exposure caused increased kendrin cleavage, indicating elevated separase activity. We also measured cleavage of the cohesin subunit, SCC1. However, western blots showed variable results in kendrin cleavage product bands, which might be explained by variable phosphorylation. In the case of SCC1 western blots, the amount of cohesin product specifically cleaved by separase is actually a low percentage of total cohesin. It is possible to synthesize reporter peptides that would be useful in defining separase activity on kendrin and SCC1 with greater resolution. Peptides constructed to mimic separase cleavage sites can be integrated with fluorescent dyes to generate a color-changing signal upon cleavage activity (Agircan et al., 2014; Basu et al., 2009; Haass et al., 2015; Zhang et al., 2014). These reporters can be designed for use in lysates and measured by spectrometry. Addition of dyes for cell uptake control

allows reporters to be measured in flow cytometry and, theoretically, in adherent cells. Reporter peptides offer a more sensitive measure of separase activity. Plus, with localization sequences, they can indicate separase activity specific to the centromeres or centrosomes. These reporters have never been used in Cr(VI) research and would be useful not only to confirm alterations in separase activity but could indicate timing and cellular location of cleavage.

In Aim1 we show securin knockdown increased 24 h Cr(VI)-induced numerical chromosome instability above untransfected Cr(VI)-exposed cells. The increase was in the same direction, but less severe than cells exposed to 120 h Cr(VI). Given that the secondary separase inhibitor, cyclin B1, is also reduced after 120 h exposure, it is likely that the magnitude of Cr(VI)-induced effects is a combination of factors. Future studies that knockdown both securin and cyclin B1, and cyclin B1 only, can show the relative importance of securin loss in the context of Cr(VI) exposure.

In Aim 2 we measured effects on two transcription factors that promote securin transcription. We found whole cell levels were not significantly altered, and nuclear levels were induced. Thus, transcription factor levels are not implicated in securin loss. Yet, other effects on transcription factor function could be occurring. To further address this question, chromatin immunoprecipitation can determine if NF-YA and Sp1 are binding to the securin promoter region. In the case binding is lost, epigenetic assays can then direct research to answer how Cr(VI) may alter DNA methylation, histone modifications, or chromatin architecture. Broadly, Cr(VI)

is known to alter epigenetics, but if these effects play a role in centrosome amplification is unknown.

Loss of KLF6 was observed in nuclear protein fractions at the highest concentrations and exposure times. Whether KLF6 is a contributing factor to securin loss is not completely understood based on our results. Securin measurement after KLF6 knockdown would help clarify the role of KLF6 in Cr(VI)-induced securin loss.

No securin-targeting miRNAs were found to be altered in our miRNA-seq data. However, several other proteins associated with centrosome maintenance and chromosome stability are potential targets of Cr(VI)-altered miRNAs. Future work exploring post-transcriptional deregulation of these genes could break ground in discovering key miRNAs involved in Cr(VI)-induced numerical chromosome instability. Additionally, measuring mRNA stability would help focus research on the precise level of expression that is intercepted.

In Aim 3, whale cells are shown to be resistant to Cr(VI)-induced securin loss, centrosome amplification, and chromosome instability. These data support earlier findings that show proteins key to homologous recombination repair are retained in Cr(VI)-exposed whale cells. Our work shows species comparisons help elucidate the mechanisms of toxicity in humans as well. Examining DNA methylation, transcription binding, and mRNA stability in whale cells would help identify how whale cells avoid a toxic fate and aid understanding Cr(VI) carcinogenesis. Wildlife work can be hampered by lack of appropriate reagents

such as antibodies, but the sperm whale genome is highly annotated and protein and gene sequences can be identified in many cases.

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APPENDIX: ABBREVIATIONS

APC: Anaphase promoting complex

APC/C: Anaphase promoting complex/cyclosome

ATSDR: Agency for Toxic Substances and Disease Registry

CIN: Chromosome instability

Cr(VI): Hexavalent chromium

IARC: International Agency for Research on Cancer

kDa: Kilodalton

mRNA: Messenger RNA

miRNA: MicroRNA

NIOSH: National Institute for Occupational Safety and Health

OSHA: Occupational Safety and Health Administration

RNA: Ribonucleic acid

RT-qPCR: Real-time quantitative polymerase chain reaction

SAC: Spindle assembly checkpoint

siRNA: Small interfering RNA

CURRICULUM VITAE

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Education

Doctor of Philosophy

2018 – 2022 University of Louisville, Louisville, KY
Pharmacology & Toxicology Graduate Program

Master of Science

2016 – 2018 University of Louisville, Louisville, KY
Pharmacology & Toxicology Graduate Program

Bachelor of Science

2008 – 2011 University of Kentucky, Lexington, KY
Biology Major

Summer 2010 Gulf Coast Research Laboratories, Ocean Springs, MS
Summer Field Courses

Bachelor of Arts

2002 – 2005 University of Massachusetts, Amherst, Massachusetts
Studio Art Major

Research Experience

2016 – 2022
Wise Laboratory of Environmental and Genetic Toxicology, University of Louisville
PhD research in the molecular mechanisms of hexavalent chromium-induced carcinogenesis. Cell culture, *in vivo* rodent, human tissue, and wildlife studies provide a One Environmental Health perspective of the mechanisms of hexavalent chromium-induced centrosome disruption and numerical chromosome instability.

2009 – 2011
Philip Crowley Lab, University of Kentucky
Performed a series of treatments and behavioral experiments to test effects of the estrogenizing herbicide atrazine on assortative mating in guppies (*Poecilia reticulata*). Exposures conducted *in utero* or after sexual maturity. Care, maintainance, and euthanasia of experimental fish.

Training Grants, Fellowships, Scholarships

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Integrated Programs in Biomedical Sciences (IPIBS) Fellowship
August 2016 – June 2018
University of Louisville School of Medicine

Doctoral Dissertation Completion Scholarship Award
January 2022 – May 2022
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Publications

1. Meaza, I., Speer, R.M., **Toyoda, J.H.**, Lu, H., Wise, S.S., Croom-Perez, T.J., Aboueissa, A. and Wise, Sr., J.P. Prolonged Exposure to Particulate Cr(VI) is Cytotoxic and Genotoxic to Fin Whale Cells. Journal of Trace Elements in Medicine and Biology, 62:126562, 2020. PMID: 32570008; PMCID: PMC7655514.
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Extramural abstracts

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2. Croom-Pérez, T.J., **Toyoda, J.H.**, Wise, S.S. and Wise, Sr., J.P. Chronic Exposure to Particulate Hexavalent Chromium Induces Centrosome Abnormalities and Disrupts Mitosis in both Sea Turtle and Alligator Primary Lung Cells. Presented at the annual meeting of the Ohio Valley Regional Chapter of the Society of Toxicology (OVSOT), December 2017.

3. **Toyoda, J.H.,** Martino, J. and Wise, Sr., J.P. Mechanisms of Hexavalent Chromium-Induced Centriole Disengagement and Centrosome Amplification. Presented at the Graduate Student Regional Research Conference, Louisville, KY, February 2018.

4. Croom-Pérez, T.J., **Toyoda, J.H.**, Wise, S.S. and Wise, Sr., J.P. Chronic Exposure to Particulate Hexavalent Chromium Induces Centrosome Abnormalities and Disrupts Mitosis in both Sea Turtle and Alligator Primary Lung Cells. Toxicological Sciences, 150(1): 480, 2018.

5. Wise, Sr., J.P., Wise, S.S., Browning, C.L., **Toyoda, J.H.,** Martino, J., and Xie, H. Chromosomal Instability Is a Key Step in the Carcinogenic Mechanism of Hexavalent Chromium. Presented at the EMBO Conference: DNA replication, chromosome segregation and fate decisions, Kyllini, Greece, September 2018. P88, page 154

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28. Meaza I., Speer M.R., **Toyoda, J.H.**, Lu H., Wise S.S. Croom-Perez J.T., El-Makarim, A. and Wise, Sr., J. P. Prolonged exposure to particulate Cr(VI) is cytotoxic and genotoxic to fin whale cells. Presented at the summer meeting of the Ohio Valley Regional Chapter of the Society of Toxicology (OVSOT), July 2020.

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

30. **Toyoda, J.H.**, Martino, J., Speer, R.M., and Wise, Sr., J.P. Hexavalent Chromium Decreases Securin Expression and Increases Separase Substrate Cleavage in Human Lung Cells. Presented at the annual meeting of the Ohio Valley Regional Chapter of the Society of Toxicology (OVSOT), November 2020.

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of miRNAs Involved in Carcinogenesis Pathways. Presented at the annual meeting of the Ohio Valley Regional Chapter of the Society of Toxicology (OVSOT), November 2020.

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34. **Toyoda, J.H.**, Martino, J., Speer, R.M., Wise, S.S, Wise, Sr., J.P. Divided We Fall: Particulate Hexavalent Chromium Targets Securin Driving Premature Centriole Separation. Presented at the Graduate Student Regional Research Conference, March 2021.

35. **Toyoda, J.H.**, Martino, J., Wise, S.S., Wise, Sr., J.P. "When Your Chainsaw Loses Its Safety Lock: A Hypothesis for Metal-Induced Lung Cancer." Presented at the annual meeting of the Society of Toxicology (SOT), March 2021.

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

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38. Meaza, I., Speer, R.M., **Toyoda, J.H.**, Lu, Y., Xu, Q., Walter, R., Kong, M. and Wise, Sr., J.P. Particulate Hexavalent Chromium Altered the Expression of miRNAs Involved in Carcinogenesis Pathways. Toxicological Sciences, 180:2073, 2021.

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40. Lu, H., Wise, S.S., **Toyoda, J.H.**, Speer, R.M., Bolt, A., and Wise, Sr., J.P. Whale Cells Resist Cr(VI)-Induced Loss of Homologous Recombination Repair. Toxicological Sciences, 180:2087, 2021.

41. **Toyoda, J.H.**, Martino, J., Speer, R.M., Wise, S.S., Wise, Sr., J.P. Particulate Hexavalent Chromium Targets Securin, Disrupts Centriole Engagement, and Induces Chromosome Instability. Presented at the annual meeting of the Genetic Toxicology Association, May 2021.

42. Lu, H., Wise, S.S., **Toyoda, J.H.**, Speer, R.M, Bolt, A., and Wise, Sr., J.P. Whale cells are resistant to Cr(VI)-induced chromosome instability. Presented at the annual meeting of the Genetic Toxicology Association, May 2021.

43. Williams, A.R., Speer, R.M., Browning, C.L., Meaza, I., **Toyoda, J.H.**, 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.

44. Meaza, I., Speer, R.M., **Toyoda, J.H.**, Lu, Y., Xu, Q., Walter, R., Kong, M. and Wise, Sr., J.P. Particulate Hexavalent Chromium Induces Global miRNA Downregulation and Altered the Expression of miRNAs Involved in Carcinogenesis Pathways. Presented at the annual meeting of the Genetic Toxicology Association, May 2021.

45. Meaza, I., Speer, R.M., **Toyoda, J.H.**, Lu, Y., Xu, Q., Walter, R., Kong, M. and Wise, Sr., J.P. Particulate Hexavalent Chromium [Cr(VI)] Exposure Alters miRNA Profiles and Targets miRNAs Involved in Pathways of Cr(VI) Carcinogenesis. Presented at the summer meeting of the Ohio Valley Chapter of the Society of Toxicology (OVSOT), July 2021

46. **Toyoda, J.H.**, Cahill, C.R., Wise, S.S., and Wise, Sr., J.P. Chronic Hexavalent Chromium Exposure Causes Persistent Securin Disruption, and Induces Chromosome Instability. Environmental and Molecular Mutagenesis Volume 62, Issue S1, p.44, 2021.

47. Williams, A.R., Speer, R.M., Browning, C.L., Meaza, I., **Toyoda, J.H.**, 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.

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the Environmental Mutagenesis and Genomic Society (EMGS), September 2021.

51. **Toyoda, J.H.,** Cahill, C.R., Wise, S.S., Speer, R.M., Lu, H., Kouokam, J.C., and Wise, Sr., J.P. Securin Disruption and Chromosome Instability Persist After Chronic Hexavalent Chromium Exposure. Presented at the annual meeting of the Ohio Valley Chapter of the Society of Toxicology (OVSOT), November 2021.

52. Wise, Jr., J.P., Young, J.L., Lu, H., Meaza, I., **Toyoda, J.H.**, Wise, S.S., Speer, R.M., Croom-Perez, T.J., Cai, L., and Wise, Sr., J.P. A Toxic Aging Coin: Cr(VI) Neurotoxicity and Gerontogenicity. Presented at the annual meeting of the Ohio Valley Chapter of the Society of Toxicology (OVSOT), November 2021.

53. Williams, A.R., Meaza, I., **Toyoda, J.H.**, Wise, S.S., Kouokam, J.C., Speer, R.M., Browning, C., and Wise, Sr., J.P. Particulate Hexavalent Chromium Induces Loss of 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.

54. Lu, H., Wise, S.S., Hoyle, G., **Toyoda, J.H.**, Speer, R.M., Croom-Perez, T.J., Meaza, I., Wise, Jr., J.P., Kouokam, J.C., Young, J.L., Cai, L., Kondo, K., and Wise, Sr., J.P. Particulate Hexavalent Chromium Inhibits Homologous Recombination Repair in Rat Lung. Presented at the annual meeting of the Ohio Valley Chapter of the Society of Toxicology (OVSOT), November 2021.

55. Meaza, I., Wise, S.S., **Toyoda, J.H.**, Lu, H., Williams, A.R., Wise, Sr., J.P. Chromate-Induced Loss of RAD51 and 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.

56. 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. Annual Meeting of the Society of Toxicology (SOT), March 2022.

57. Lu, H., Wise, S.S., Hoyle, G., **Toyoda, J.H.**, Speer, R.M, Croom-Perez, T.J., Meaza, I., Wise, Jr., J.P., Kouokam, J.C., Young J.L., Cai, L., Kondo, K., and Wise, Sr., J.P. Particulate hexavalent chromium inhibits homologous recombination repair in rat lung. Annual Meeting of the Society of Toxicology (SOT), March 2022.

58. Meaza I., **Toyoda, J.H.**, Lu, H., Williams, A.R., and Wise Sr., J.P. Chromate-Induced Loss of RAD51 and Increased Chromosome Instability in Human Bronchial Epithelial Cells. Annual Meeting of the Society of Toxicology (SOT), March 2022.

59. Kouokam, J.C., Speer, R.M., Meaza, I., **Toyoda, J.H.**, Lu, H., Kong, M. and Wise, Sr., J.P. Analysis of the effects of particulate hexavalent chromium on global gene expression in human fibroblasts reveal the involvement of inflammation. Annual Meeting of the Society of Toxicology (SOT), March 2022.

60. Lu, H., Wise, S.S., **Toyoda, J.H.**, Wise Jr. J.P., Speer, R.M, Bolt, A.M., Meaza, I., Wise, C.F., Wise, J.T.F., Young, J.L., and Wise Sr. J.P. Of Whales and Men, How Great Whales Evade Metal Induced Cancer. Society of Toxicology (SOT) Annual Meeting, March 2022.

61. Williams, A.R., Speer, R.M., Browning, C.L., Meaza, I., **Toyoda, J.H.**, and Wise, Sr., J.P. Particulate Hexavalent Chromium Exposure Suppresses BCDX2 Complex Response in Human Lung Cells. Society of Toxicology (SOT) Annual Meeting, March 2022.

62. Wise, Jr., J.P., Young, J.L., Lu, H., Meaza, I.I., **Toyoda, J.H.**, Wise, S.S., Speer, R., Croom-Perez, T., Cai, L., and Wise, Sr., J.P. A Toxic Aging Coin: Cr(VI) Neurotoxicity and Gerontogenicity. Society of Toxicology (SOT) Annual Meeting, March 2022.

63. Wise, S.S., **Toyoda, J.H.**, Lu, H., Meaza, I., Wise, Sr., J.P. Chromosome Instability and Cellular Transformation of Human Lung Cells Chronically Treated with Particulate Hexavalent Chromium. Society of Toxicology (SOT) Annual Meeting, March 2022.

64. 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. Society of Toxicology (SOT) Annual Meeting, March 2022.

65. Wise, Sr., J.P., Lu, H., Wise, S.S., **Toyoda, J.H.**, Wise, Jr., J.P., Bolt, A. Of Whales and Men: How Great Whales Evade Metal-Induced Cancer. Annual Meeting of the Society of Toxicology (SOT), March 2022.

<u>Intramural abstracts</u>

1. **Toyoda, J.H.** and Shenoy, K. *In Utero* Exposure to Endocrine Disruptors and Mate Choice in Female Guppies. Showcase of Undergraduate Scholars, University of Kentucky, 2011.

2. **Toyoda, J.H.**, Martino, J. and Wise, Sr., J.P. Mechanisms of Hexavalent Chromium-Induced Centriole Disengagement and Centrosome Amplification. Presented at Research!Louisville, University of Louisville, September 2017.

3. **Toyoda, J. H.**, Croom-Pérez, T. J., Wise, S. S., and Wise, Sr., J. P. Particulate Hexavalent Chromium Does Not Induce Centrosome

Amplification in Sperm Whale and Bowhead Whale Cells. Presented at Research!Louisville, University of Louisville, 2018.

4. Lu, H., Browning, C.L, Wise, S. S., **Toyoda, J. H.**, and Wise, Sr., J. P. Homologous Recombination Repair Protects Against Genomic Instability in Bowhead Whale Lung Cells After Prolonged Particulate Chromate Exposure. Presented at Research!Louisville, University of Louisville, 2018.

5. Geisen, M.E., **Toyoda, J.H.**, Croom-Pérez, T.J., and Wise, Sr., J.P. Comparative Effects of Particulate Hexavalent Chromium on Mitotic Stages in Human and Whale Lung Fibroblasts. Presented at Research!Louisville, University of Louisville, 2018.

6. **Toyoda, J.H.**, Martino, J., Kondo, K., and Wise, Sr., J.P. Prolonged Particulate Hexavalent Chromium Exposure Disrupts Centrosome Regulation Proteins and Causes Centrosome Amplification. Presented at Research!Louisville, University of Louisville, September 2019.

7. Wise, S.S., Miller, E., Daniel, S., Meaza, I., **Toyoda, J.H.**, Lu, H., Speer, R. M., Young, J. L., Isakov, R., Jaggers, H., Wise, Jr., J. P., Croom-Perez, T. J., Cai, L., Hoyle, G., Wise, Sr., J. P. Effects of Chronic Exposure to Particulate Chromate in Rat Lungs. Presented at Research!Louisville, University of Louisville, September 2019.

8. Meaza, I., Speer, R.M., **Toyoda, J.H.**, Wise, Sr., J.P. Particulate Hexavalent Chromium Induces Cytotoxicity and Genotoxicity in Female and Male Fin Whale Primary Fibroblasts. Presented at Research!Louisville, University of Louisville, September 2019.

9. **Toyoda, J.H.**, Cahill, C.R., Wise, S.S., Wise, Sr., J.P. Securin Deregulation and Chromosome Instability Persist After Chronic Hexavalent Chromium Exposure. Presented at Research!Louisville, Louisville, KY, October 2021.

10. Kouokam, J.C., Speer, R.M., Meaza, I., **Toyoda, J.H.**, Lu, H., Kong, M. and Wise, Sr., J.P. The involvement of the inflammatory response in particulate hexavalent chromium-induced toxicity. Presented at Research!Louisville, October 2021.

11. Lu, H., **Toyoda, J.H.**, Williams, A.R., Meaza, I., Wise, S.S., Wise, Sr., J.P. Particulate Hexavalent Chromium Induces DNA Double Strand Breaks in Rat Lung. Presented at Research!Louisville, Louisville, KY, October 2021.

12. Williams, A.R., **Toyoda, J.H.**, Meaza, I., Lu, H.,Wise, S.S., Wise, Sr., J.P. Particulate Hexavalent Chromium Targets the BCDX2 Complex in Homologous Recombination Repair. Presented at Research!Louisville, Louisville, KY, October 2021.

13. Meaza, I., **Toyoda, J.H.**, Lu, H.,Wise, Williams, A.R., S.S., Wise, Sr., J.P. Particulate Hexavalent Chromium Targets RAD51 in Human Lung Epithelial Cells, Leading to Increased Chromosome Instability, a Driver of Carcinogenesis. Presented at Research!Louisville, Louisville, KY, October 2021.

14. Cahill, C.R., **Toyoda, J.H.**, Wise, S.S., Wise, Sr., J.P. Securin Deregulation Persists After Chronic Hexavalent Chromium Exposure. Presented at Research!Louisville, Louisville, KY, October 2021.

Oral Presentations

1. Three-Minute Thesis: “Securin Disruption and Chromosome Instability Persist After Chronic Hexavalent Chromium Exposure.” Ohio Valley Society of Toxicology Annual Meeting, November 2021.

2. Platform: “Chronic Hexavalent Chromium Exposure Causes Persistent Securin Disruption and Induces Chromosome Instability.” Environmental Mutagenesis and Genomic Society (EMGS) 2021 Annual Meeting, September 2021.

3. Guest Lecture: “A Whale Tale: The Story Whale Biopsies Tell About Global Marine Pollution.” University of Southen Mississippi Gulf Coast Research Laboratory, May 2021.

4. Poster Talk: “Particulate Hexavalent Chromium Targets Securin, Disrupts Centriole Engagement, and Induces Chromosome Instability.” Genetic Toxicology Association Annual Meeting, May 2021.

5. Oral Presentation: “Divided We Fall: Particulate Hexavalent Chromium Targets Securin Driving Premature Centriole Separation.” Society of Toxicology Metals Specialty Section Reception, March 2021

6. Three-Minute Thesis: “When Your Chainsaw Loses Its Safety Lock: A Hypothesis for Metal-Induced Lung Cancer.” Society of Toxicology, March 2021

7. Three-Minute Thesis: “When Your Chainsaw Loses Its Safety Lock: a Hypothesis for Metal-Induced Lung Cancer.” University of Louisville, March 2021

8. Seminar: “Divided We Fall: Particulate Hexavalent Chromium Targets Securin and Drives Premature Centriole Separation.” Department of Pharmacology and Toxicology, January 2021.

9. PhD Platform: “Prolonged Particulate Hexavalent Chromium Exposure Disrupts Centrosome Regulation Proteins and Causes Centrosome Amplification.” Ohio Valley Society of Toxicology (OVSOT) Annual Meeting, November 2020.

10. PhD Platform: “Hexavalent Chromium Decreases Securin Expression and Increases Separase Substrate Cleavage in Human Lung Cells.” Ohio Valley Society of Toxicology (OVSOT) Student/Postdoc Meeting, August 2020.

11. Seminar: “Investigating the Role of Securin in Hexavalent Chromium Carcinogenesis.” Department of Pharmacology and Toxicology, January 2020.

12. Oral Presentation: “Particulate Hexavalent Chromium Does Not Induce Centrosome Amplification in Sperm Whale and Bowhead Whale Cells: Metals Carcinogenesis from a One Environmental Health Perspective.” Graduate Student Regional Research Conference, February 2019.

13. PhD Platform: “Particulate Hexavalent Chromium Does Not Induce Centrosome Amplification in Sperm Whale and Bowhead Whale Cells: Metals Carcinogenesis from a One Environmental Health Perspective.” Ohio Valley Regional Chapter of the Society of Toxicology, Annual Meeting, November 2018.

14. Master Thesis Defense and Ph.D. Proposal: “Molecular Mechanisms of Particulate Hexavalent Chromium-Induced Centrosome Amplification.” Department of Pharmacology and Toxicology Seminar, June 2018.

15. Seminar: “Mechanisms of Hexavalent Chromium-Induced Centriole Disengagement and Centrosome Amplification.” Department of Pharmacology and Toxicology, March 2017.

Meetings Attended (current year and previous 5 years)

2017 Annual Meeting, Ohio Valley Chapter of the Society of Toxicology (OVSOT)
Research!Louisville
Graduate Student Regional Research Conference

2018 Annual Meeting, Ohio Valley Chapter of the Society of Toxicology (OVSOT)
Research!Louisville
Graduate Student Regional Research Conference

2019 Annual Meeting of the Society of Toxicology (SOT)
Annual Meeting, Ohio Valley Chapter of the Society of Toxicology

Research!Louisville
Graduate Student Regional Research Conference

2020 Annual Meeting of the Society of Toxicology (SOT)
Annual Meeting, Ohio Valley Chapter of the Society of Toxicology (OVSOT)
Summer Meeting, Ohio Valley Chapter of the Society of Toxicology (OVSOT)
American Society for Cell Biology
University of Rhode Island STEEP (Sources, Transport, Exposure & Effects of PFAS)
Graduate Student Regional Research Conference

2021 Annual Meeting of the Society of Toxicology (SOT)
Annual Meeting, Ohio Valley Chapter of the Society of Toxicology (OVSOT)
Summer Meeting, Ohio Valley Chapter of the Society of Toxicology (OVSOT)
Annual Meeting of Genetic Toxicology Association (GTA)
Annual Meeting of the Environmental Mutagenesis and Genomics Society (EMGS)
NIEHS Superfund Research Program Annual Meeting

2022 Annual Meeting of the Society of Toxicology (SOT)

Honors and Awards

2022 Environmental Carcinogenesis Merit Award, Carcinogenesis Specialty Section, Society of Toxicology

2021 Research Grant, University of Louisville, $500 for custom siRNAs

2021 First Place, “Tox on the Clock” Competition, Ohio Valley Society of Toxicology Annual Meeting

2021 Best Graphical Abstract, “Tox on the Clock” Competition, Ohio Valley Society of Toxicology Annual Meeting

2021 Environmental Mutagenesis and Genomics Society (EMGS) Emerging Scientist Award

2021 Exemplary Abstract Award, Genetic Toxicology Association Annual Meeting

2021 First Place, Graduate Student Abstract/Poster Award, Metals Specialty Section, Society of Toxicology Annual Meeting

2021 First Place, Three Minute Thesis (3MT), University of Louisville

2020 Graduate Student Poster Award, NIEHS Superfund Research Program Annual Meeting

2020 First Place, PhD Platform, Ohio Valley Society of Toxicology Annual Meeting

2020 First Place, PhD Platform, Ohio Valley Society of Toxicology Student/Postdoc Meeting

2020 Second Place, Dharm V. Singh Graduate Student Award, Carcinogenesis Specialty Section, Society of Toxicology

2020 First Place, PhD Poster Session, Graduate Student Council Regional Research Conference

2020 Travel Award, University of Louisville, $350 for travel to 2020 Annual Meeting of the Society of Toxicology

2019 First Place, "Tox on the Clock" Competition, Ohio Valley Society of Toxicology Annual Meeting

2019 Travel Award, University of Louisville, $350 for travel to 2019 Annual Meeting of the Society of Toxicology

2018 Second place, PhD Platform, Ohio Valley Society of Toxicology Annual Meeting

2018 Research Grant, University of Louisville, $500 for whale sample collection

2017 Research Grant, University of Louisville, $500 for whale sample collection

Leadership and Service Positions

2021-2022 Chair – Communications Subcommittee, Society of Toxicology, Graduate Student Leadership Committee

Graduate Student Representative – Metals Specialty Section, Society of Toxicology

Graduate Student Representative – Ohio Valley Regional Chapter, Society of Toxicology

2020-2021 Secretary – Communications Subcommittee, Society of Toxicology Graduate Student Leadership Committee

Graduate Student Representative – Metals Specialty Section, Society of Toxicology

Director of Finance – Graduate Student Council, University of Louisville (UofL)

Chair – Graduate Student Council Research Grant Committee, UofL

Student Senator – Student Government Association & Senate Appropriations Committee, UofL

Department Representative – Graduate Student Council, UofL

President – Department of Pharmacology and Toxicology Student Organization

2019-2020 Department Representative – Graduate Student Council, UofL

Committee Member – Graduate Student Council Research Grant Committee, UofL

President – Department of Pharmacology and Toxicology Student Organization, UofL

Secretary & Social Media Coordinator – Science Policy Outreach Group, UofL

2016 – 2019 Class Representative – Department of Pharmacology and Toxicology Student Organization

Professional and Academic Memberships

2016 – Present Graduate Student Member – Science Policy Outreach Group, UofL

2016 – Present Graduate Student Member – Society of Toxicology
Ohio Valley Regional Chapter, Metals Specialty Section,
Carcinogenesis Specialty Section,
Women in Toxicology Special Interest Group

Research Mentorship Experience

2021 Undergraduate: Caitlin Cahill

2019 Visiting Scholar: Emma Martinez Lopez, PhD

2019 Visiting Master's Student: Chester Gan

2018 Undergraduate: Mariah Geisen

Volunteer and Community Work

2022 Science Fair Mentor, Louisville Regional Science and Engineering Fair
Charlotte Connally, First Place Cellular and Molecular Biology, Middle School

2021 Judge, Louisville Regional Science and Engineering Fair
Judge, Wyoming State Science Fair

2020 Science Fair Mentor, Central High School
Science Fair Mentor, Kentucky Science and Engineering Fair
Judge, Louisville Regional Science and Engineering Fair
Science Fair Mentor, Central High School

2019 Science Fair Mentor, Central High School
Facilitator Assistant, Girls Rule STEM+Health Summit
Continuing Education Course Volunteer, Annual Meeting of the Society of Toxicology
Science Fair Ambassador, Louisville Regional Science and Engineering Fair
Science Fair Mentor, Central High School

Field Work

2017-2019 Alligator sampling expeditions at Kennedy Space Center and other locations in Florida.

2018-2021 Whale biopsy collection in the Gulf of Maine

2011-2012 Fish sampling in the Bering Sea, Gulf of Alaska, North Pacific Ocean

Techniques and Skills

Cell culture

Western blot analysis

Immunofluorescence

Confocal microscopy

Chromosome analysis

Mitotic stage analysis

Atomic absorption spectroscopy

Single cell gel electrophoresis ("Comet" assay)

Flow cytometry

RT-qPCR

RNA sequencing

Rat chemical inhalation exposures

Rat and mouse lung inflation, anesthesia, euthanasia, handling

Tissue staining (hematoxylin and eosin, picosirius red)

Fluorescence *In Situ* Hybridization (FISH)

Wildlife primary cell line establishment

Whale ID and biopsy processing

Alligator blood, skin, and urine sampling