



HHS Public Access

Author manuscript

J Carcinog Mutagen. Author manuscript; available in PMC 2020 December 11.

Published in final edited form as:

J Carcinog Mutagen. 2013 ; 4(2): . doi:10.4172/2157-2518.1000140.

Chronic Exposure to Particulate Hexavalent Chromium Alters Cdc20 Protein Localization, Interactions and Expression

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Abstract

Hexavalent chromium [Cr(VI)] compounds are well established human lung carcinogens, but it is unknown how they cause lung cancer in humans. Recent data indicate that Cr(VI) induces chromosome instability in human lung cells, and genomic instability is considered a leading mechanism to explain chromate carcinogenesis. The Spindle Assembly Checkpoint (SAC) is a critical regulator of the metaphase-to-anaphase transition and ensures genome stability by preventing chromosomal missegregation events. Bypass of the SAC can lead to genomic instability, manifested as aneuploidy, which eventually leads to tumor formation and cancer. Recent studies in our laboratory demonstrated that chronic exposure to zinc chromate induces SAC bypass in a concentration- and time-dependent manner in human lung fibroblasts. To further study these events, we focused on the cell division cycle 20 (Cdc20) protein, a downstream effector protein in the SAC. Cdc20 has not been studied after Cr(VI) exposure, but other studies show that experimentally induced alterations of Cdc20 localization to kinetochores or of Cdc20 protein expression leads to aneuploidy. Here, we investigated the effects of zinc chromate, a particulate Cr(VI) compound, on Cdc20 localization, protein expression and interactions. Our data show Cdc20 is a target for particulate Cr(VI). Chronic zinc chromate exposure altered Cdc20 kinetochore localization and reduced the interaction of phosphorylated Cdc20 with Mad2, which may underlie zinc chromate-induced SAC bypass.

Keywords

Hexavalent chromium; Zinc chromate; Cdc20; Mad2; Spindle assembly checkpoint; Spindle assembly checkpoint bypass

Introduction

Hexavalent chromium [Cr(VI)] is a known human lung carcinogen and the solubility of Cr(VI) plays an important role in its carcinogenicity [1–3]. Epidemiologic studies show a

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higher lung cancer risk for particulate-Cr(VI)-exposed workers [2], and experimental studies show that only particulate Cr(VI) compounds induce tumors in animal models and neoplastic transformation of cultured mouse embryo cells [2,4]. However, despite its potent carcinogenicity, the carcinogenic mechanism for Cr(VI) is poorly understood.

Chromosome Instability (CIN) consisting of alterations of both chromosome number and structure is a hallmark of human lung cancer [5]. Cell culture studies show that both particulate and soluble Cr(VI) compounds can induce CIN in human lung cells and that human bronchial epithelial cells malignantly transformed by Cr(VI) exhibit an aneuploid phenotype [6,7]. Further data show that mechanisms underlying Cr(VI)-induced numerical CIN involve centrosome amplification and bypass of the Spindle Assembly Checkpoint (SAC) [7,8].

The SAC acts as a quality control mechanism for accurate chromosome segregation during mitosis [9]. It delays anaphase until all chromosomes are correctly attached to the mitotic spindle. Bypassing the SAC can lead to chromosome missegregation and aneuploidy. We previously showed that chronic exposure to particulate Cr(VI) compounds leads to SAC bypass manifested as centromere spreading, premature centromere division, and premature anaphase [7,8]. These metaphase disruptions occurred despite the presence of an intact, functioning SAC indicating that particulate Cr(VI) is able to bypass the induction of the SAC and allow metaphase cells premature entry into anaphase. However, the mechanism that underlies how Cr(VI) exposure disrupts the SAC is unknown.

The primary effector in the SAC is Cdc20, which is an essential activator of the anaphase promoting complex/cyclosome (APC/C). As a mitotic cell becomes organized in metaphase, Cdc20 is recruited and localized to the kinetochore and combined with the Mad2, BubR1 and Bub3, proteins into a mitotic checkpoint complex (MCC). This complex then prevents Cdc20 from activating APC/C, preventing the start of anaphase [10]. When a metaphase cell is ready to progress into anaphase, the MCC releases Cdc20 so it can bind and activate APC/C. Once activated, APC/C ubiquitinates securin causing securin to release separase, which then cleaves cohesin separating the chromatid arms and anaphase ensues.

Data show that disrupting Cdc20 can lead to aneuploidy and cancer. For example, alteration of Cdc20 expression is associated with impairment of the SAC and aneuploidy in cancer [11]. Mutations or/and dysregulated expression of Cdc20, has been found in breast cancer cells [12]. Loss of Mad2-Cdc20 interactions due to mutant Cdc20 promotes tumor formation in mice [13]. Myeloma cells are highly aneuploid and have very low levels of Cdc20 and Mad2 protein [14]. Female mice that express low level of Cdc20 produce aneuploid oocytes and embryos [15]. Cadmium, a human carcinogen, reduces Cdc20 protein levels leading to disruption of early mitosis progression [16]. However, the impact of Cr(VI) on Cdc20 has not been studied. Thus, this study determined the impact of Cr(VI) on Cdc20 considering its effects Cdc20 kinetochore localization, its interactions with Mad2 and its protein expression levels.

Material and Methods

Chemicals and reagents

Zinc chromate was purchased from Alfa Aesar (Ward Hill, MA). Demecolchicine and nocodazole were purchased from Sigma/Aldrich (St. Louis, MO). Dulbecco's modified eagle's medium/Ham's F-12 (DMEM/F-12) 50:50 mixture and Phosphate-Buffered Saline (PBS) were purchased from Mediatech Inc. (Herndon, VA). Cosmic Calf Serum (CCS) was purchased from Hyclone (Logan, UT). GlutaMAX, sodium pyruvate, trypsin/EDTA and penicillin/streptomycin were purchased from Invitrogen Corporation (Carlsbad, CA). Tissue culture flasks, dishes and plasticware were purchased from BD (Franklin Lakes, NJ).

Cells and cell culture

Human lung fibroblasts were used as the cell model system for this study, specifically, WTHBF-6 cells, a clonal cell line derived from normal human bronchial fibroblasts. These cells exhibit a stable, normal diploid chromosome complement and similar clastogenic and cytotoxic responses to metals compared to their parent cells [17]. These cells ectopically express human telomerase and thus, have an extended lifespan. Cells were cultured as subconfluent monolayers in DMEM/F-12 media supplemented with 15% CCS, 100 U/ml penicillin/100 ug/ml streptomycin, 2 mM glutaMAX and 0.1 mM sodium pyruvate. They were incubated at 37°C and in 5% carbon dioxide. They were fed every two days and subcultured at least once a week using 0.25% trypsin/1mM EDTA solution. Experiments were performed on logarithmically growing cells.

Metal preparation

Zinc chromate was used as a representative particulate Cr(VI) compound and administered as a suspension in cold water, as previously described [18]. Cells were treated with 0, 0.1, 0.15 and 0.2 ug/cm² zinc chromate for 24, 48, 72, 96 and 120 h.

Antibodies

Rabbit anti-human Cdc20, mouse anti-human Mad2, rabbit anti-phosphoserine and mouse anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody were purchased from Abcam (Cambridge, MA). Mouse anti-human Cdc20 antibody was purchased from Millipore (Billerica, MA). Mouse Anti-β-actin antibody was purchased from Rockland (Gilbertsville, PA). Human anti-centromere serum was provided as a gift from Dr. William Earnshaw from the University of Edinburg (Edinburg, Scotland, UK). AlexaFluor 555 goat anti-rabbit IgG and AlexaFluor 488 goat anti-human antibodies were purchased from Molecular Probes (Eugene, OR). Goat anti-rabbit IgG (H+L)-HRP and goat anti-mouse IgG (H+L)-HRP secondary antibody conjugate were purchased from Bio-Rad (Hercules, CA).

Western blot analysis

Western blot analysis was performed according to our published methods [18]. Briefly, after zinc chromate treatment, whole cell population or mitotic cell population by mitotic shake off were lysed with RIPA lysis buffer for 15 minutes on ice. Protein concentrations were analyzed by the Bradford protein assay using a Nanodrop spectrophotometer. Equal protein

was resolved using 10% SDS-polyacrylamide gel electrophoresis (PAGE) and then transferred on to a 0.45 um pure nitrocellulose membrane. The membrane was then blocked with 5% non-fat milk and probed with anti-Cdc20 or anti-Mad2 antibody overnight at 4°C, followed by 1 h incubation with horseradish peroxidase conjugated anti-rabbit or anti-mouse IgG secondary antibody. The blots were visualized using ECL plus detection reagent and images were taken using the Storm 840. GAPDH was used as a loading control. At least three independent experiments were performed.

Immunofluorescence analysis

Immunofluorescence analysis was performed according to our published methods [18]. Briefly, cells were seeded onto one-well chamber slides and treated with 0–0.2 ug/cm² of zinc chromate for 24–120 h. After treatment, cells were fixed with 4% paraformaldehyde, permeabilized with 0.25% Triton X-100 and blocked with blocking buffer. Cells were then incubated with anti-Cdc20 and anti-centromere serum for 1 h. Cells were washed and then incubated with anti-mouse Alexa 555 and anti-human Alexa 488 for 1 h. Cells were washed and coverslips were then mounted on slides with Prolong anti-fade with DAPI. The first 50 mitotic cells were analyzed per concentration using fluorescence microscopy. At least three independent experiments were performed.

Immunoprecipitation analysis

Cell lysates were prepared in RIPA buffer as described above. 500 ug of protein were incubated with anti-Cdc20 antibody-bound beads for two hours using the Crosslink Immunoprecipitation Kit (Pierce-Thermo Fisher Scientific) according to the manufacturer's instructions. Precipitated proteins were released by elution buffer, resolved by SDS-PAGE, and specifically detected by immunoblot as described above.

Statistics

The Student's t-test was used to compute p-values to further determine the statistical significance of the difference in means, with a p value < 0.05 or <0.01 considered as statistically significant. No adjustment was made for multiple comparisons.

Results

Chronic exposure to zinc chromate induces decreased Cdc20 localization to the kinetochores

During SAC activation in mitosis, Cdc20 localizes to unattached kinetochores binding with other checkpoint proteins and enzymes to inhibit the metaphase to anaphase transition [19–21]. Studies show that disrupting Cdc20 localization to the kinetochores can attenuate the checkpoint [13,22]. Thus, we evaluated the impact of zinc chromate on Cdc20 localization. We found that chronic exposure to zinc chromate induced a time-dependent decrease in Cdc20 localization to the kinetochores (Figure 1) after 48–120 h of exposure, with a pronounced effect starting at 72 h. For example, at 24 h, cells treated with 0.2 ug/cm² zinc chromate none of the cells had altered Cdc20 localization. But altered localization was 0.7% at 48 h and increased to 22% at 72 h, 38% at 96 h and 47% at 120 h (Figure 1B). We also found that chronic exposure to Cr(VI) induced a concentration-dependent decrease in Cdc20

localization to the kinetochores (Figure 1). For example, at 72 h the amount of altered localization was 0, 4, 18.7 and 22 percent of cells after exposure to 0, 0.1, 0.15 and 2 $\mu\text{g}/\text{cm}^2$ zinc chromate, respectively (Figure 1B).

Chronic exposure to zinc chromate induces decreased Cdc20 and Mad2 protein levels

A growing body of evidence shows that overexpression or underexpression [22–24] of Cdc20 protein leads to premature anaphase promotion, missegregation of chromosomes and aneuploidy, which indicates that maintaining appropriate amounts of Cdc20 is essential in mitosis. Cdc20 protein is constantly being synthesized and degraded with a half life of approximately 30 minutes in human cells [25]. We found that zinc chromate induced a concentration-dependent decrease in protein levels starting after 48 h of exposure (Figures 2A and 2B). This effect was still evident when we limited our analysis to only those cells in mitosis (Figures 2C and 2D). Using an anti-phosphate antibody, we also found a consistent decrease in phosphorylated Cdc20 levels at both 24 h and 120 h (Figure 3A). 0.2 $\mu\text{g}/\text{cm}^2$ zinc chromate decreased levels of phosphorylated Cdc20 by 20% relative to control at both 24 and 120 h.

We also considered the effect of zinc chromate on Mad2 protein levels. Mad2 is one of the proteins that interacts with Cdc20 at the kinetochore and sequesters it in the MCC complex preventing Cdc20 from activating APC/C (26–28). We found that zinc chromate also induced a concentration-dependent decrease in Mad2 protein levels beginning at 48 h (Figures 4A and 4B). For example, a 96 h exposure to 0.1, 0.15 and 0.2 $\mu\text{g}/\text{cm}^2$ zinc chromate decreased Mad2 levels to 30, 50 and 70% relative to control, respectively. This effect was also still evident when we limited our analysis to only those cells in mitosis (Figures 4C and 4D).

Chronic exposure to zinc chromate decreases interactions between phosphorylated Cdc20 and Mad2

Given that both Cdc20 and Mad2 protein levels were decreased, we considered the interaction of the two proteins as Mad2 binds to phosphorylated-Cdc20 and sequesters it in the MCC complex preventing Cdc20 from activating APC/C [26–29]. Since zinc chromate decreased Cdc20 localization, we determined whether it also decreased phosphorylated-Cdc20-Mad2 interactions. We found that chronic zinc chromate exposure reduced the interaction of phosphorylated-Cdc20 with Mad2 (Figures 5A and 5B). For example, exposure to 0.2 $\mu\text{g}/\text{cm}^2$ zinc chromate decreased the ratio of Mad2/pCdc20 from 90% at 24 h to 40% at 120 h indicating less protein was binding together.

SAC Bypass

Our previous reports indicated that Cr(VI) was bypassing the SAC and not eliminating it. Specifically, we found the SAC to remain functional during Cr(VI) exposure as triggering the SAC by adding colchicine, reduced the amount of SAC bypass caused by zinc chromate [7,8]. In this study, we further investigated whether the SAC was functional by again triggering it with either demecolchicine or nocodazole. We found that triggering the checkpoint did reverse the effects of zinc chromate on Cdc20-kinetochore localization, but not on its effects on Cdc20 or Mad2 protein levels (Figures 6 A–E).

Discussion

Particulate Cr(IV) compounds are established human lung carcinogens, however, their underlying carcinogenic mechanisms are not well understood. The best explanation appears to be based on their ability to induced CIN including structural and numerical chromosomal changes [7,8]. Our previous studies show that chronic exposure to particulate Cr(VI) induces concentration- and time-dependent increase in CIN manifested as aneuploidy, centromere spreading, premature centromere division and premature anaphase [7,8]. Further, we found that bypassing the SAC was part of the mechanism for particulate Cr(VI)-induced CIN [7,8]. Here we extend those findings and begin defining how Cr(VI) bypasses the SAC on a cellular and molecular basis.

In mitosis, Cdc20 localizes to the kinetochore during checkpoint activation where it is bound and inhibited until the kinetochore is properly attached to microtubules. Disrupting Cdc20 localization to the kinetochores could attenuate the checkpoint. One study has shown that Cdc20 hypomorphic mice exhibit a decreased Cdc20 localization to the kinetochores which led to progressive aneuploidy and premature sister chromatid separation. Our data are the first report of a chemical carcinogen disrupting Cdc20 localization and the observations that particulate Cr(VI) disrupts this localization in a time and concentration-dependent manner are consistent with our previous reports of SAC bypass at these concentrations [7,8].

In general, it is unknown how the lack of Cdc20 kinetochore localization leads to SAC bypass. When the SAC is activated, Cdc20 is sequestered by binding to Mad2 at kinetochores to inhibit the activity of the APC by forming an inactive complex with Cdc20 and APC. Thus, one possibility is that decreasing Cdc20 localization to the kinetochores could lead to decreased interactions of Cdc20 with Mad2 and/or other checkpoint proteins at the kinetochores, resulting in more Mad2-free Cdc20 to prematurely activate APC. Consistent with this possibility, we did find that zinc chromate decreased the interaction of Mad2 and phosphorylated Cdc20. These data are also consistent with observations that arsenic, another human carcinogen, decreased interactions of Mad2 with the APC/Cdc20 complex, although that study did not measure the direct interaction between Mad2 and Cdc20 [30].

The role of phosphorylated Cdc20 is also not well understood. It has been reported that phosphorylated Cdc20 binds to Mad2 to regulate the spindle checkpoint function [26,31]. Consistent with that finding, we find that zinc chromate decreases the interactions of phosphorylated Cdc20 with Mad2, potentially freeing phosphorylated Cdc20 to activate APC. How Cr(VI) interferes with the Cdc20/Mad2 interactions is unknown. There is a metal binding motif on Cdc20 that is near the Mad2-binding domain [32]. Thus, one possibility, albeit untested, is that Cr binds to that metal binding motif and interferes with Mad2 binding.

Zinc chromate decreased Mad2 levels. These data are consistent with our previous study showing that lead chromate decreased Mad2 protein levels and suggest this outcome may be a common mechanism for particulate chromates. It is also consistent with other studies showing that arsenic induces SAC bypass and decreases Mad2 expression levels [33] and

that 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), suppresses the expression of Mad2 resulting in increasing CIN [34]. Altogether, these data suggest that Mad2 disruption may be a common mechanism for chemical carcinogens to induce CIN.

We continue to find that particulate Cr(VI) is bypassing the SAC rather than damaging or eliminating it. Previously, we reported that triggering the checkpoint with spindle poisons in particulate Cr(VI)-treated cells decreases the observed effects indicating the SAC was functional and being bypassed [7,8]. This study supports that conclusion as our data show that triggering the checkpoint with spindle poisons in zinc chromate-treated cells reverses the disruption of Cdc20 kinetochore localization. The decrease in Cdc20 protein levels we found may also indicate that the checkpoint is functional and being bypassed. A previous study shows that spindle poisons prevent the SAC from being satisfied and arrest the cells in metaphase and while doing so decrease Cdc20 protein levels [34]. This outcome suggests that decreased Cdc20 levels may be a specific response to mitotic spindle checkpoint activation [34]. This possibility is also consistent with another previous report that cadmium decreases Cdc20 protein levels leading to prometaphase arrest [35].

Thus, we hypothesize that the mechanism for particulate chromate-induced carcinogenesis starts with Cr(VI) particles impacting at bronchial bifurcation sites in the lung and dissolving outside the cell. Once inside the cell, Cr(VI) is reduced to Cr(III), which accumulates in the cell with time. Some cells function normally in mitosis, but in a subset of cells Cr(III) binds to Cdc20 and disrupts Mad2-mediated Cdc20 localization to the kinetochore and the repressive function of the mitotic checkpoint complex. This outcome allows these cells to bypass the checkpoint and enter anaphase without proper microtubule-kinetochore attachment resulting in centromere spreading, premature centromere division, premature anaphase and ultimately aneuploidy. Further work is aimed at determining the mechanistic links between Cr(III) binding and the effects on Cdc20 and the impact on other SAC proteins.

Acknowledgments

We thank Geron Corporation for the use of the hTERT materials, Dr. William Earnshaw at the Wellcome Trust Centre for Cell Biology, Edinburgh for the use of anti-centromere serum antibody. We also like to thank Christy Gianios, Jr. and Shou-ping Huang for technology and administrative support. This work is supported by NIEHS grant ES016893 (J.P.W.) and the Maine Center for Toxicology and Environmental Health. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Environmental Health Sciences or the National Institutes of Health.

Abbreviations:

Cr(VI)	Hexavalent Chromium
SAC	Spindle Assembly Checkpoint
CIN	Chromosome Instability

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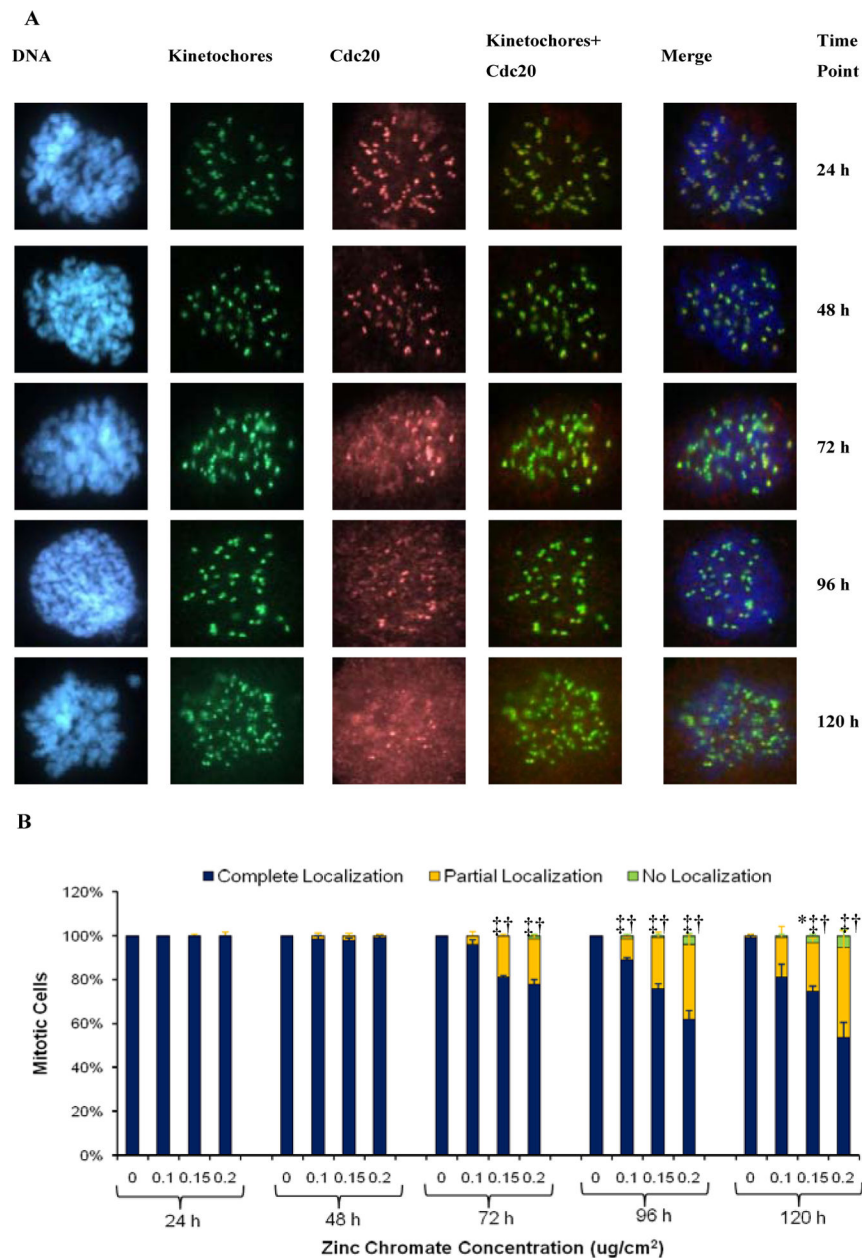


Figure 1: Particulate Cr(VI) Disrupts Cdc20 Localization to Kinetochores.

This figure shows that chronic exposure to zinc chromate induces a concentration- and time-dependent decrease in Cdc20 localization to the kinetochores. **A)** The representative images of Cdc20 localization to the kinetochores after exposure to 0.2 ug/cm² zinc chromate for 24–120 h. Blue (DAPI) represents the DNA, green (Alexa 488) represents the kinetochores (anti-centromere serum) and red (Alexa 555) represents Cdc20. **B)** This figure shows that 24 h exposure to 0–0.2 ug/cm² zinc chromate induced no changes in Cdc20 kinetochore localization while more chronic exposures (48–120 h) decreased Cdc20 kinetochore localization. Data represent an average of three independent experiments ± the standard error of the mean. *Cells with no Cdc20 localization were statistically different from control (p<0.05), †Cells with partial Cdc20 localization were statistically different from control

($p < 0.05$), ‡Cells with complete Cdc20 localization were statistically different from control ($p < 0.05$).

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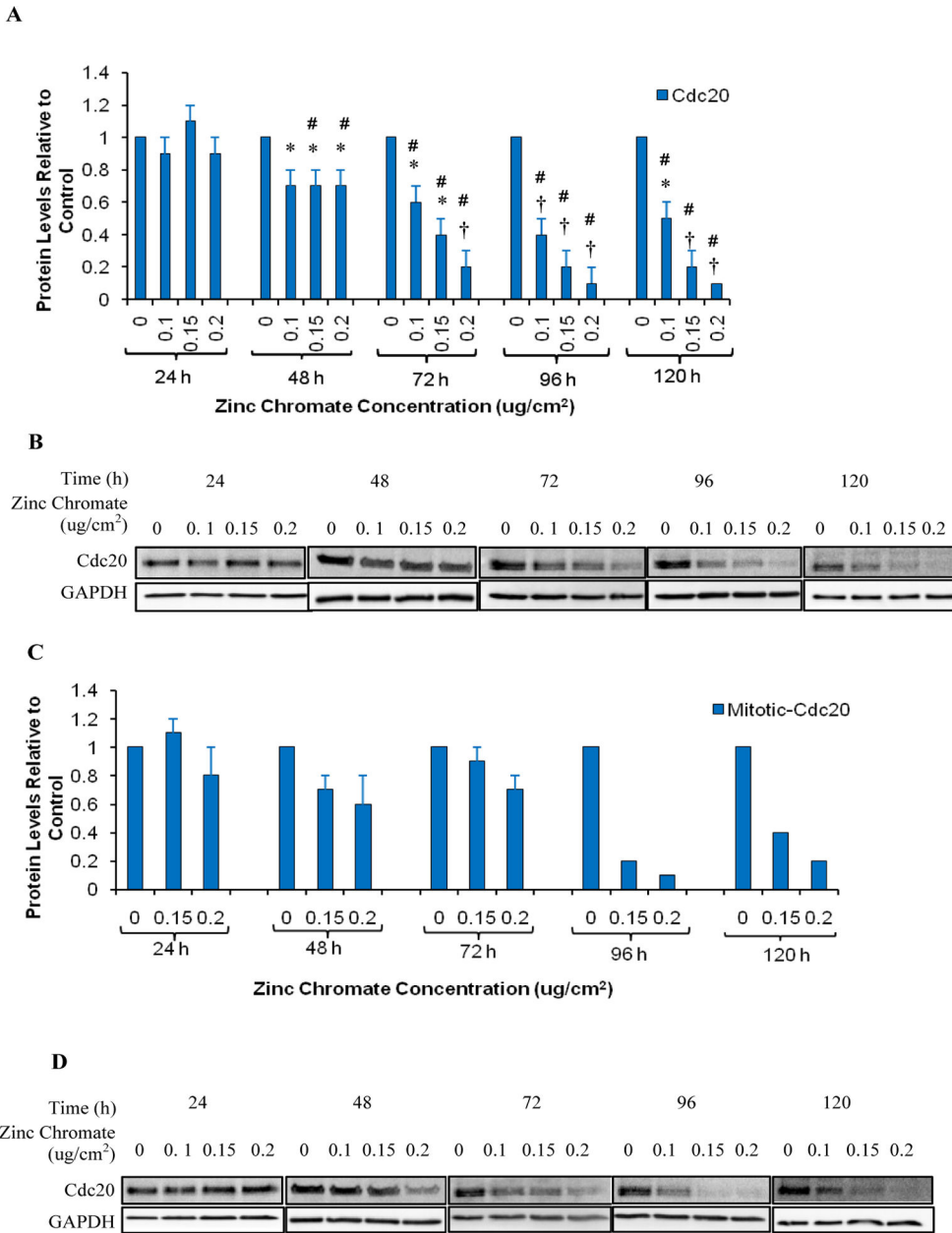
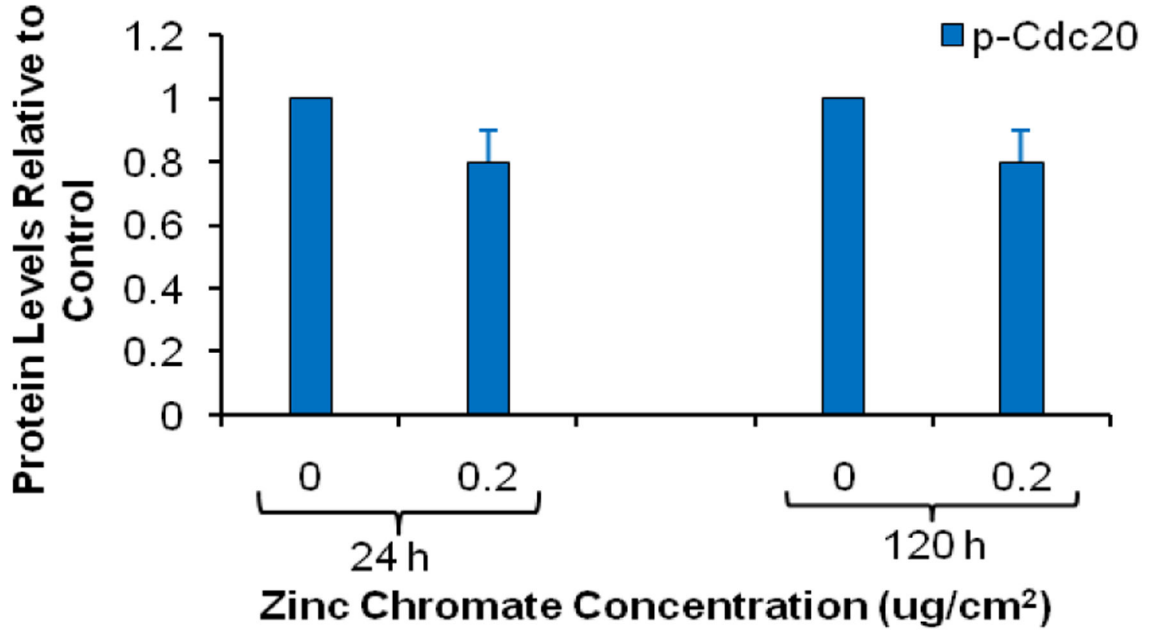


Figure 2: Particulate Cr(VI) Decreases Cdc20 Protein Levels.

This figure shows that zinc chromate decreases Cdc20 protein levels after 24–120 h exposure. Protein expression was measured with western blotting. GAPDH was used as a loading control. **A)** Protein expression level in total population of cells. **B)** Representative western blot image of Cdc20 expression in total population of cells. **C)** Protein expression level in mitotic population of cells collected with shake off. **D)** Representative western blot image of Cdc20 expression level in mitotic cells. Data represent an average of three independent experiments ± the standard error of the mean. *Statistically different from control (p<0.05). †Statistically different from control (p<0.01). #Statistically different from 24 h treatment at this concentration (p<0.05).

A



B

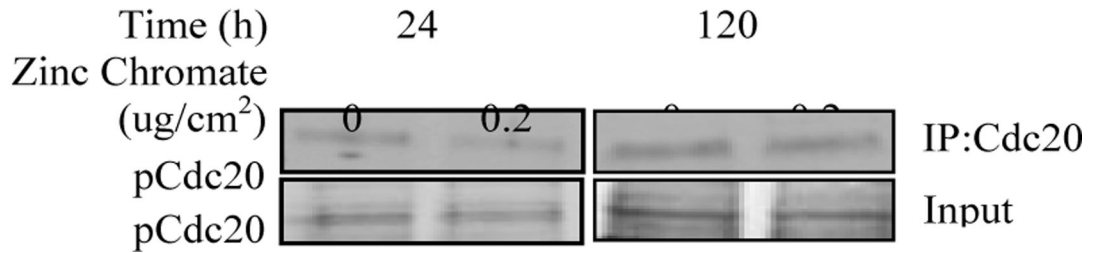


Figure 3: Particulate Cr(VI) Decreases Phosphorylated Cdc20 Protein Levels.

This figure shows that zinc chromate decreases phosphorylated Cdc20 protein levels after 24–120 h exposure. Immunoprecipitation was carried out from whole cell lysates with anti-Cdc20 beads and western blotting was performed with anti-phosphoserine antibody. **A)** Phosphorylated Cdc20 levels after a 24 or 120 h exposure to 0.2 ug/cm² zinc chromate. Data represent an average of two experiments ± standard error of the mean. **B)** Representative western blot images of phosphorylated Cdc20 protein levels after total-Cdc20 immunoprecipitation.

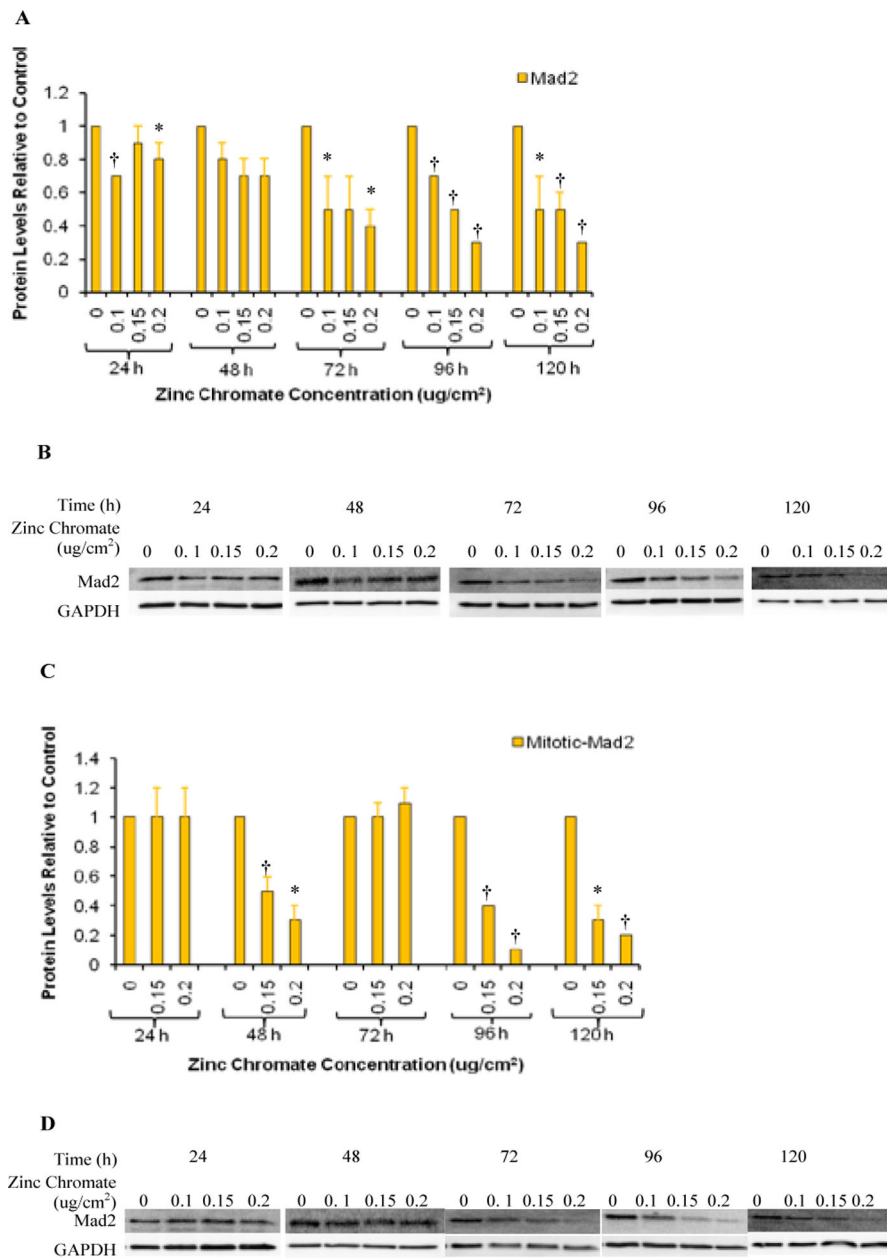
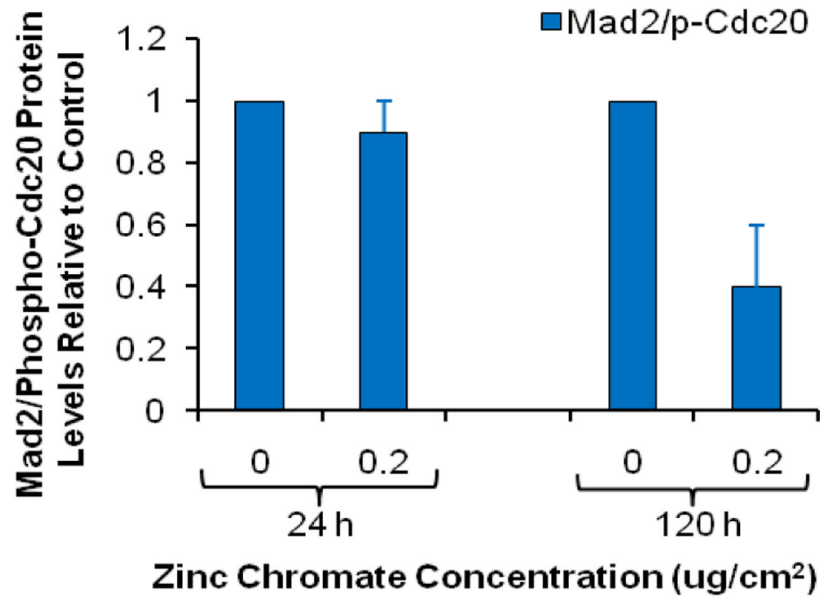


Figure 4: Particulate Cr(VI) Decreases Mad2 Protein Levels.

This figure shows that zinc chromate decreased Mad2 protein levels after 24–120 h exposure. Protein expression was measured with western blotting. GAPDH was used as a loading control. **A)** Protein expression level in total population of cells. **B)** Representative western blot image of Mad2 expression in total population of cells. **C)** Protein expression level in mitotic population of cells. **D)** Representative western blot image of Mad2 expression in mitotic cells. Data represent an average of three independent experiments ± the standard error of the mean. *Statistically different from control (p<0.05). †Statistically different from control (p<0.01).

A



B

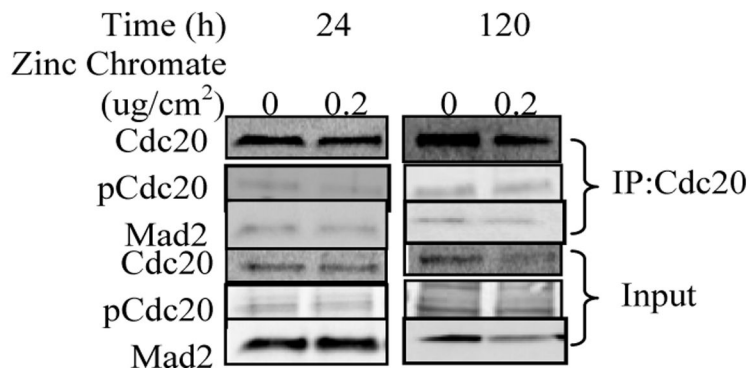


Figure 5: Particulate Cr(VI) Alters Mad2 and Cdc20 Protein Interactions.

This figure shows that zinc chromate alters Mad2-Cdc20 interactions after 24 or 120 h exposure. Co-immunoprecipitation was carried out from whole cell lysates with anti-Cdc20 beads and western blotting was performed with indicated antibodies. **A)** Mad2-phosphorylated Cdc20 interactions are decreased after both 24 and 120 h exposure with a dramatic impact at 120 h. **B)** Representative image of total-Cdc20, phosphorylated Cdc20 and total-Mad2 protein levels following Cdc20 immunoprecipitation after 24 and 120 h exposure. Data represent an average of two experiments \pm standard error of the mean.

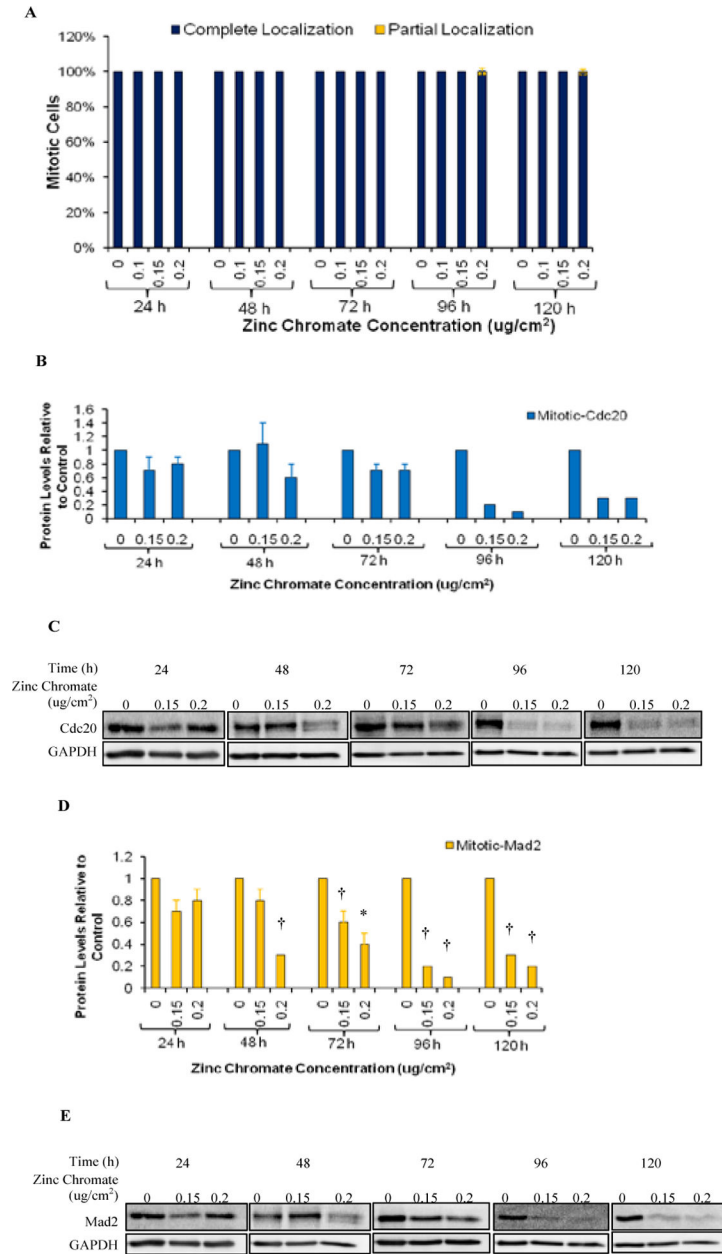


Figure 6: Particulate Cr(VI) Causes SAC Bypass not Elimination.

This figure shows the effects of triggering the SAC on zinc chromate effects on Cdc20 kinetochore localization and expression levels, and Mad2 protein expression levels. **A)** One hour before the end of zinc chromate treatment, cells were treated with 0.1 ug/ml demecolchicine. Demecolchicine reversed zinc chromate effects on localization. Cells were fixed after the treatment, followed by immunostaining using anti-Cdc20 and centromere antibodies. **B)** Sixteen hours before the end of zinc chromate treatment, cells were treated with 0.4 ug/ml nocodazole. Nocodazole had no effect on zinc chromate-induced decreases in Cdc20 protein levels in mitotic cells. **C)** Representative western blot image of Cdc20 expression in nocodazole co-treated cells. **D)** Nocodazole had no effect on zinc chromate-induced decreases in Mad2 protein levels in mitotic cells. **E)** Representative western blot

image of Mad2 expression in nocodazole co-treated cells. Protein expression was measured with western blot using anti-Cdc20 and Mad2 antibodies. GAPDH was used as a loading control. Data represent an average of three independent experiments \pm the standard error of the mean. *Statistically different from control ($p < 0.05$). †Statistically different from control ($p < 0.01$).

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