Investigating the Role of SUV4-20 Inhibition in Mitosis

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Abstract

SUV4-20 is a lysine methyltransferase responsible for the trimethylation of Histone 4 Lysine 20 (H4K20). Overexpression of SUV4-20 has been linked to cancer and mitotic defects. Preliminary data from the lab demonstrates that the regulation of Aurora B, a master regulator of mitotic chromosome segregation, is compromised when Suv4-20 is enriched at mitotic centromeres. In order to better understand the mechanism of SUV4-20 in mitotic regulation, a small molecule inhibitor called A196 was used to prevent the methyltransferase activity of the enzyme. My data suggest that the addition of the inhibitor decreased the trimethylation of H4K20. However, decreased H4K20 methylation did not correspond with a reduction in the number of mitotic defects that occurred. Consistent with this, inhibition of SUV4-20 methyltransferase activity has no effect on the level of Aurora B at centromeres. This suggests that SUV4-20 regulation of Aurora B is independent of its methyltransferase activity.

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I would like to thank Professor Amity Manning who was my advisor on this project. Without her guidance and support, this project would not have been possible. I have learned so much about the experimental process and design from you while doing this project and I am so glad to have obtained these useful skills. Furthermore, Professor Manning also provided immense support outside of this project by taking an interest in my aspiration and future plans and helping me find applicable opportunities. I am so thankful to have gotten to work on this project with her as an advisor.

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1. Introduction

1.1. Genomic Instability

Mitosis is the final stage of cell division in which a cell physically divides itself to produce two identical daughter cells[1]. The cell division cycle is a vital part of growth and repair in many different organisms. The cycle consists of interphase, in which the cell grows and replicates its DNA, and then mitosis, in which the cell actually divides[1]. Errors in mitosis can lead to unequal distribution of genetic material in the daughter cells[1]. Genomic instability refers to when the process of mitosis is prone to genomic alterations[2]. There are four main mechanisms that the cell uses to maintain genomic integrity which include; high fidelity replication of DNA, equal distribution of chromosomes to daughter cells, DNA damage repair throughout the cell cycle, and regulation of the cell cycle through checkpoints[2]. Errors in any of these processes can result in problems such as DNA damage, structural changes to chromosomes, or aneuploidy[2].

While aneuploidy is often detrimental to the cell's fitness it is also a characteristic of cancer cells[3]. The loss or gain of genetic material that occurs in aneuploidy can lead to missing tumor suppressors or additional copies of proto-oncogenes[3]. Cells that have underlying defects in mitotic chromosome segregation are considered to have chromosome instability (CIN). CIN is defined as cells that have mutations that interfere with its ability to accurately segregate its chromosomes, resulting in high levels of aneuploidy as well as genomic heterogeneity within the cell population[4]. Many cancer cells exhibit CIN and the resulting genomic heterogeneity promotes an ability for these cells to adapt quickly to selective pressures, which can aid cancer cells in growing in conditions they would not normally be fit to grow in.

Often unequal distribution of genetic material between daughter cells results from defects in how microtubules attach to chromosomes. There are two main classes of chromosome mis-attachments that have the potential to lead to chromosome segregation errors, merotelic and syntelic attachments. Merotelic attachments are characterized by the attachment of microtubule bundles from different spindle poles to the same kinetochore[5]. On the other hand, syntelic attachments are when both of the sister kinetochores are attached to microtubule bundles from the same spindle pole[5]. Syntelic attachments activate the spindle assembly checkpoint, preventing cells from progressing to anaphase. However, merotelic attachments satisfy the spindle assembly checkpoint and cells that proceed into anaphase with uncorrected merotelic attachments may exhibit a lagging chromosome in which the chromosome is located in the middle of the two anaphase plates. Ultimately, this lagging chromosome is at risk of being mis-segregated into the same daughter cell as its replicated sister, resulting in two aneuploid daughter cells.

1.2. Epigenetic regulation

Epigenetic regulation is a type of heritable, non-genetic regulation, characterized by the addition of chemical marks to the chromatin. By serving as a docking site for the binding of various chromatin-associated proteins, these marks serve many functions, including helping to regulate gene expression[6] , cellular differentiation[7], and other cellular functions. Furthermore, many types of disease have been found to have errors in epigenetic regulation, making it an important area of study[8].

Histone modifications can range from a wide variety of marks added to the tails of histones including; methylation, acetylation, ubiquitination, and phosphorylation[6] (Figure 1A & 1B). When epigenetic marks are added to histone tails they can cause the chromatin to wrap tighter or looser around the histones, resulting in silencing or expression of the genes in that area[7]. Enzymes that place epigenetic marks are called writers, those that remove epigenetic marks are called erasers, and those that bind to epigenetic marks are called readers.

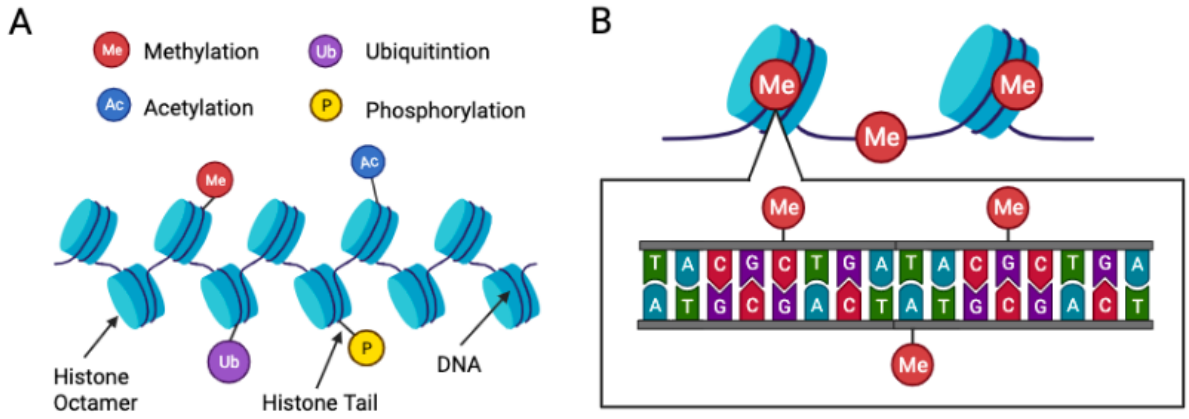

Figure 1: Epigenetic marks. (A)A diagram of some types of histone modification marks that can be added to the histone tails. (B) A diagram of methylation of DNA. Created with BioRender.com.

1.3. Aurora B Kinase

Aurora B is a kinase that is part of the chromosomal passenger complex (CPC), which is responsible for regulating mitosis and correcting microtubule misattachments[9]. Aurora B works by phosphorylating its protein substrates, which include the kinetochore proteins Hec1 and Ndc80[9]. These substrates are subunits of the kinetochore-microtubule-network (KMN) that functions to link microtubules to the kinetochore. Phosphorylation of these substrates by Aurora B kinase causes the release of kinetochore microtubules[9]. Aurora B is able to selectively phosphorylate its substrates in a tension-dependent manner. When sister chromatids are

connected to opposite spindle poles (or bioriented) there is tension between the microtubules and kinetochores. This tension moves centromere-localized Aurora B further from the kinetochore-localized substrates[10]. When Aurora B is further away from its substrate it is unable to phosphorylate it, resulting in selectivity to only kinetochore-microtubule attachments that have less tension. Chromatids with microtubule attachments that are under less tension are often ones that are not bioriented, for example, merotelic and syntelic attachments. When there is less tension Aurora B is able to phosphorylate kinetochore substrates, destabilizing the microtubule attachments and providing an error correction mechanism[10].

Because Aurora B is an immobile kinase, it must rely on other proteins in the CPC in order to localize at the centromeres. The CPC is made up of four proteins, Aurora B, INCENP, Borealin, and Survivin[11]. Before the cell enters mitosis the CPC is located on the arms of chromosomes, but then once mitosis begins epigenetic marks help the complex locate to the centromere[12]. Survivin helps the CPC localize to the centromere during mitosis by interacting with phosphorylation mark of histone three, threonine three (H3-pT3) that is added by an enzyme called Haspin[12] (Figure 2). The phosphorylation of H2A serine 121 (H2A-S121), which is mediated by the enzyme Bub1, is also important for the localization of CPC to the centromere[12] (Figure 2). An enzyme called Sgo2 is responsible for mediating the CPC to the centromere and Sgo2 requires H2A-S121 in order to properly localize the CPC[12] (Figure 2). Studies have shown that both H3-pT3 and H2A-S121 are able to independently localize CPC to the centromere, however when mutations occur preventing these marks from being added, the CPC is not able to localize properly[12].

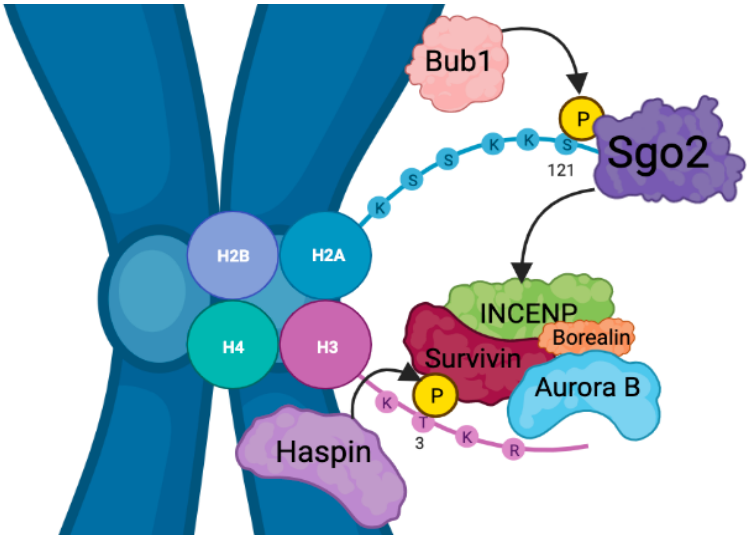

Figure 2: Epigenetic regulation of the chromosomal passenger complex. Haspin adds a phosphate group to histone 3 threonine 3. Survivn interacts with this mark which helps the CPC locate to the centromere. Bub1 adds a phosphate group to histone 2A serine 121 which is necessary for Sgo localization. Sgo helps the CPC localize to the centromere. Adapted from "Histone Modification", by Biorender.com (2021).Retrieved from https://app.biorender.com/biorender-templates

1.4. SUV4-20

SUV4-20 is an enzyme in the protein lysine methyltransferase (PKMTs) family that has been shown to be involved in regulating genomic integrity[13]. In a normal cell, SUV4-20 is involved in the catalysis of the di- and tri-methylation of histone 4 lysine 20 (H4K20). These added methyl marks serve as a docking site to recruit various proteins to chromatin. For example, the addition of a dimethylation mark by SUV4020 acts as a binding site for p53-binding protein 1 (53BP1) which is necessary for nonhomologous end-joining (NHEJ)[14]. When SUV4 20 is dysregulated or lost it can result in a decrease in proliferation as well as defects in the cell cycle and NHEJ [13].

The methylation of H4K20 changes dynamically throughout the cell cycle with trimethylation occurring mostly during late M phase, early G1 phase, and G2 phase[15]. Suv4-20-dependent trimethylation of H4K20 is responsive to the interaction of SUV4-20 with heterochromatin protein 1 (HP1) and retinoblastoma protein (pRB)[16]. When HP1 or RB1 are knocked out, trimethylation levels of H4K20 are decreased, indicating that these two proteins may play a role in regulating SUV4-20[16]. Loss of pRB or HP1 results in chromosome segregation errors during mitosis[17], suggesting that mitotic fidelity may be sensitive to the regulation of Suv4-20.

Furthermore, the overexpression of SUV4-20 has been shown to be prevalent in many cancer types[18,19]. In cancer, the overexpression of this enzyme is also correlated with aneuploidy and worse patient prognosis[18,20]. A reason for these harmful phenotypes may be due to the fact that when SUV4-20 is over-expressed, higher levels of mitotic defects such as merotilic and syntelic attachments are seen. Previous studies have shown that when SUV4-20 is over-expressed and enriched at the centromere it results in a decreased ability of Aurora B containing CPC to localize at the centromere. In this context, mis-attachments go uncorrected and chromosome segregation errors are prevalent[18].

2. Methods

2.1. Cell Culture

Both the parental RPE cell line and the RPE cell line carrying the cenSUV4-20-GFP construct were cultured for this project. Cells were split 1:5 every 72 hours. To pass the cells the old media was removed and then 2mLs of 1xPBS were used to rinse the cells and remove any trypsin inhibitors. 2 mL of Trypsin was then added and the cells were incubated at 37℃ for three to five minutes. After the incubation period, the trypsin was quenched with complete DMEM in a 1:5 ratio. Then the trypsin and cells were collected. A fifth of the volume was removed and added to a new plate. More DMEM was added to bring the plate to 10 mL. Cells were monitored regularly to ensure they were not more than 90% confluent.

In order to better define the role of SUV4-20 at centromeres, a construct was designed to allow inducible overexpression of this enzyme that could be tethered to kinetochores. The expression construct consists of three parts: the DNA binding domain of the centromere protein CENP-B, the coding sequence of SUV4-20, and a C-terminal GFP tag[18](called cen-Suv4-20-GFP; Figure 3B). The fusion protein was put under the regulation of a tetracycline response element (TRE) promoter that would be active when a tetracycline analog such as doxycycline is present,[21] such that when the TRE promoter is activated it will induce the expression of the Suv4-20 fusion protein (Figure 3A). Microscopy based observation of the GFP fluorescence tag allows for the localization of the fusion protein to be monitored (Figure 3C). The hTERT-immortalized human retinal pigment epithelial (RPE) cell line was used to express this construct because these cells are non-transformed human cells that are convenient to culture and image due to their adhesive nature The cell line also has a stable genome, exhibits normal mitoses, and are amenable to genetic engineering approaches.

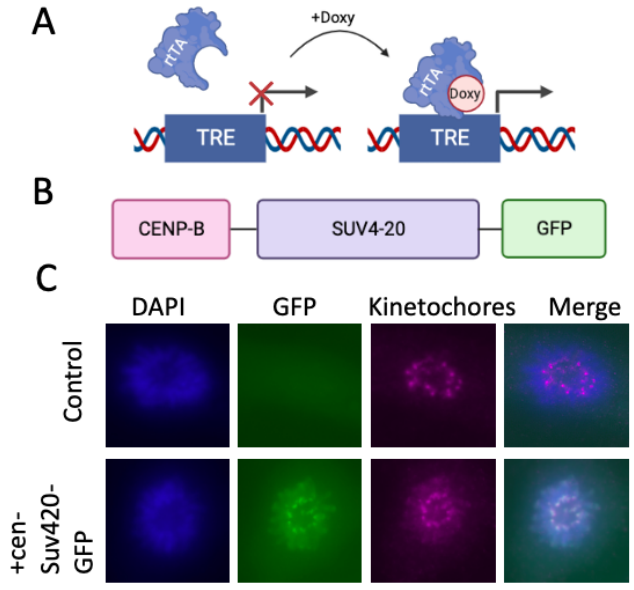

Figure 3: The cen-SUV4-20-GFP construct. (A) A schematic image showing the tetracycline-inducible promoter used to induce expression of the fusion protein. (B) A diagram of the three parts of the fusion protein; the CENP-B binding domain, SUV4-20, and GFP. (C) Representative images of the cenSUV4-20-GFP construct when doxycycline is absent (top row) or present (bottom row). When doxycycline is present the cen-Suv4-20-GFP fusion protein is expressed. The cen-fusion tethers Suv4-20-GFP to the centromere, as confirmed by immunofluorescence imaging. Antigen-specific antibodies were used to detect GFP (green) and anti-centromere antigen (ACA: kinetochores, magenta). DNA was stained with DAPI (blue). Panels A and B were created with BioRender.com.

2.2. Experimental Setup

2.2.1. Anaphase Defects Experiment

The RPE cenSUV4-20-GFP cell line was used to analyze mitotic defects. 25,000 cells were seeded into each cell of a 6 well dish. 1:1000 of 2 mg/mL doxycycline and 1:1000 of 10mM A196 was added to the wells so that there are four conditions consisting of; no drug addition, doxycycline only, A196 only, and Doxycycline and A196 (Figure 4). 24 hours after the addition of the drugs the cells were fixed and stained using methanol fixation.

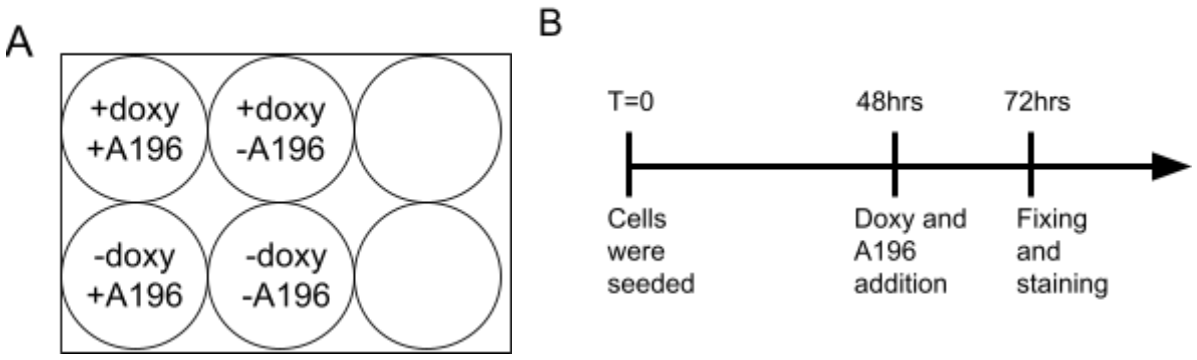

Figure 4: Experimental set up for anaphase defects experiments. (A) A six well plate was used to set up the experiment with four wells being used, four conditions were added as shown doxycycline and A196, Doxycycline only, A196 only, and no drug addition. (B) A timeline for the experiment

2.2.2. Mitotic Spreads

RPE-1 cells were seeded into T-25 dishes at 50,000 cells per mL (250,000 cells per flask) and 1:1000 of 2 mg/mL doxycycline and 1:1000 of 10 mM A196 were added to the cells the next

day for a total of 24 hours (Figure 5). Three hours prior to harvest, 1:1000 of 0.1 mg/mL nocodazole was added to the cells (Figure 5). The mitotic cells were collected by using a mitotic shake-off method in which the dishes were hit against the table 3 times and tapped on the sides 3 times. The resulting cell suspension was then placed in a 50mL conical tube and the dish was rinsed with 5-10 mL of PBS. The PBS was then added to the same conical tube as the media. Mitotic spreads were prepared as described in 21. Briefly, the conical tube was centrifuged at 800 RPM for 5 minutes and then the cells were resuspended in 75mM KCl for 10 minutes at 37℃. Next 100µL of the cells were added to each chamber on the poly lysine treated chamber slide. The chambers were then spun down at 340xg for 5 minutes. The supernatant was then removed. The unfixed immunofluorescence was processed by incubating the cells in KCM buffer (10mM Tris pH8.0; 120mM KCl; 20mM NaCl; 0.5 mM EDTA; 0.1% Triton X-100 buffer) for 10 minutes. Next, the antibodies were prepared by adding 1:500 human ACA (Antibodies Inc. 15-234), 1:1000 goat GFP (ab6662, Abcam), and 1:2000 H4K20me3 rabbit (rabbit ab9053) into 1%BSA in KCM buffer. 100µL was added to each chamber and then left overnight in the dark at 4°C. The next morning the cells were washed once with KCM buffer. The secondary antibodies were prepared by adding 1:100 human far red (Thermo Fisher Scientific A21445) and 1:1000 rabbit red (Thermo Fisher Scientific A10040) to 1%BSA in the KCM. 100µL was added to each chamber and then left for 2 hours in the dark at room temperature. The chamber slide was washed once with KCM buffer and fixed with 4% PFA for 10 minutes. The slide was then counterstained with DAPI for 20 minutes, washed once with TBS-BSA, and then mounted with anti-fade solution.

Figure 5: Time course for mitotic spreads

2.2.3. A196 Time Course Experiment

RPE cells were used for the A196 time-course experiment. 10,000 cells were plated in each well. The cells were allowed to grow for 6 days and 1:1000 of 10 mM A196 was added to the respective well every 24 hours so the conditions were as follows; 1 day, 2 days, 3 days, 4 days, 5 days, and 6 days of A196 addition (Figure 6). A control group was also prepared by

adding 1:1000 DMSO to the well. 24 hours after the addition of A196 to the day 1 well all of the cells were fixed and stained using the PFA/methanol fixation procedure (Figure 6).

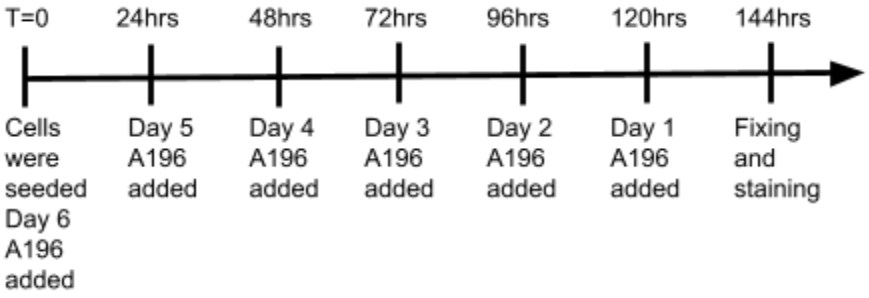

Figure 6: Timeline for A196 time course experiment

2.3. Microscopy

All images in this paper were taken using a Zyla sCMOS camera that was attached to a Nikon Ti-E microscope. A 60x Plan Apo oil immersion objective was used to view the slides. Immunofluorescence imaging of fixed cells was used to visualize and analyze structures in mitotic cells. Images were captured of 30 mitotic cells from each condition. Eleven 0.3 uM Z stacks were captured of each cell. Exposure time was adjusted such that the exposure was the same for all conditions in which fluorescence level would be quantitatively compared between conditions.

2.3.1. Fixing and Staining

Immunofluorescence imaging of fixed cells was used to visualize and analyze structures in mitotic cells. Two types of fixing and staining techniques were used; methanol fixation and Paraformaldehyde fixation.

2.3.1.1. Methanol Fixation

To begin the fixation process the media was first aspirated from the wells and cells were washed briefly with PBS. After PBS was removed each coverslip was placed in 1 mL of methanol at -20℃ for 10 minutes before blocking with TBS-BSA for 20-30 minutes. Primary antibodies were prepared in TBS-BSA as follows; 1:500 human ACA (Antibodies Inc. 15-234), 1:1000 goat GFP (ab6662, Abcam), 1:1000 mouse tubulin(dm1α, Sigma). 100µL of the primary antibody was placed on each coverslip and allowed to sit in the dark for 1-2 hours. After 1-2 hours the coverslips were washed with TBS-BSA for 5-10 minutes before the secondary antibody was added. The secondary antibody was prepared by adding 1:1000 mouse red (Thermo Fisher Scientific A11003) and 1:1000 human far red (Thermo Fisher Scientific A21445) to

TBS-BSA+DAPI (to stain DNA). 100 µL of the secondary antibody was added to each coverslip and incubated for 1-2 hours. The coverslips were then washed with TBS-BSA again before mounting them on the slide with prolong gold antifade mounting reagent. DAPI was visualized with a UV filter cube. Fluorophore-conjugated secondary antibodies specific to the primary antibodies used (described above) were visualized using FITC, TxRed, and Cy5 filter cubes.

2.3.1.2. PFA/methanol fixation

Aurora B staining was prepared by aspirating the media and washing the coverslips with PBS. Then room temperature paraformaldehyde was added to the coverslips for 15 minutes. Once the paraformaldehyde was removed ammonium chloride was added to the coverslips for 10 minutes before removing. Then methanol was added to each of the coverslips and allowed to fix for 5 minutes at -20^{0}C. After the methanol was removed the coverslips were washed with TBS-BSA for 20 minutes. Primary antibodies were prepared by adding 1:500 human ACA (Antibodies Inc. 15-234) and 1:1000 mouse Aurora B(AIM-1, BD Biosciences) to TBS-BSA + 0.5% Triton X-100. 100µL of the primary antibody was placed on each coverslip and allowed to sit in the dark at 4℃ overnight. The next morning a 10 minute TBS-BSA wash was done before adding the secondary. The secondary was prepared by adding 1:1000 mouse red(Thermo Fisher Scientific A11003) and 1:1000 human far red(Thermo Fisher Scientific A21445) to TBS-BSA + DAPI. 100 µL of the secondary antibody was added to each coverslip and was allowed to sit for 1.5 hours. Then a 5 minute TBS-BSA wash was performed before mounting the coverslips onto the slide.

2.3.2. Analysis

Images were viewed and analyzed using both the NIS elements and NIS viewer software.

2.3.2.1. Measuring Methylation at the Centromere

The methylation level at the centromeres was performed by measuring a line across kinetochore pairs and then measuring the area under the curve (between the kinetochores) of the intensity profile (Figure 7). When kinetochores would be measured the intensity of ACA would form two Peaks. The intensity of the area under the curve was measured for both trimethylation and ACA between the two peaks of the ACA intensity. Three kinetochores from each cell were measured and 30 cells from each condition were scored.

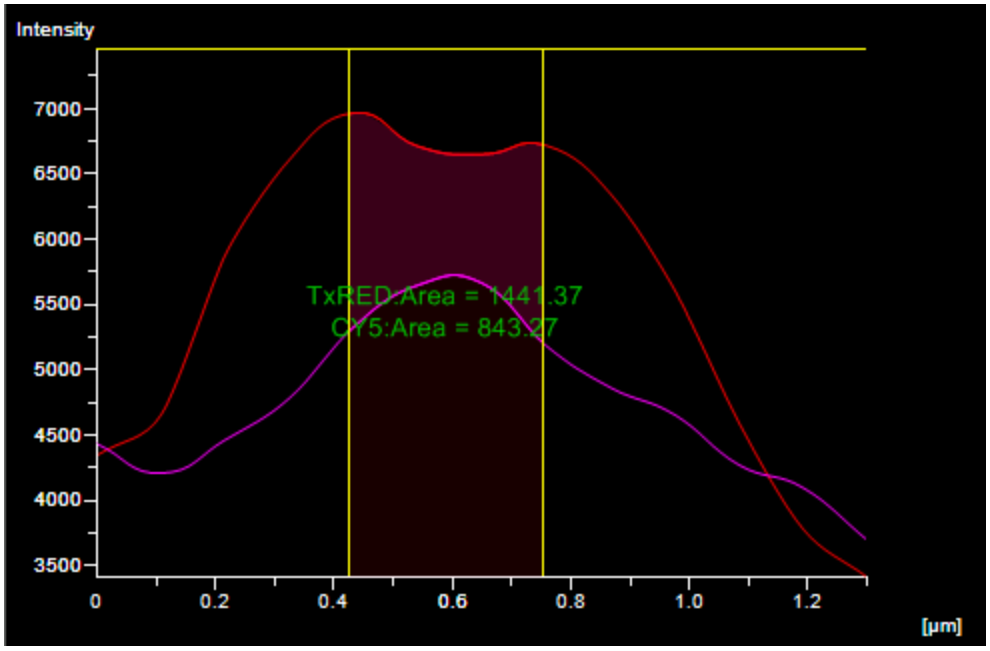

Figure 7: Measurement of trimethylation at kinetochores. Example of how the measurement of trimethylation of kinetochores was measured. Red lines indicate the intensity of ACA staining while the pink line indicates the intensity of the trimethylation staining.

2.3.2.2. Anaphase Defects Scoring

Cells were scored based on the presence of normal anaphase cells or the presence of one or more lagging chromosomes. Tubulin was observed to ensure that the cells were anaphase cells. Any cells that began to have nuclear envelope formation were considered to be telophase cells and were not scored (Figure 8). Lagging chromosomes were determined by looking at the DAPI staining to see if there was DNA between the two metaphase plates. To distinguish between normal chromosomes that are large in size and lagging chromosomes, the kinetochores were also observed. If the kinetochore was at least the length of the metaphase plate away from the metaphase plate, then the chromosome was considered to be lagging (Figure 8). Cells with one or more lagging chromosomes were scored as 1 while normal anaphase cells were scored as 0. The percent of cells with lagging chromosomes was calculated.

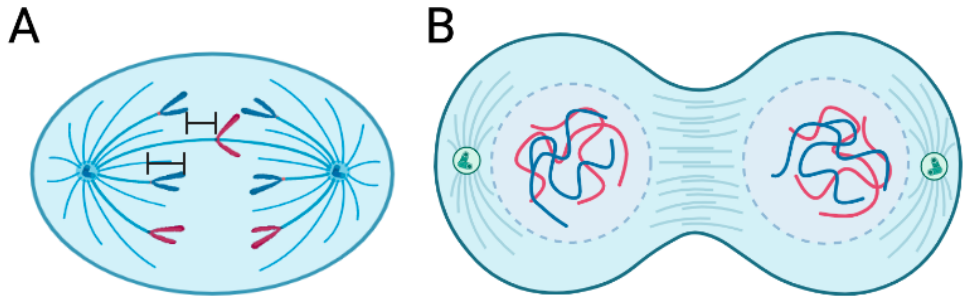

Figure 8: Parameters for Scoring Anaphase lagging chromosomes. Lagging chromosomes were only counted if the kinetochore was at least the length of the metaphase plate away from the metaphase plate (A). Cells that had started developing a nuclear envelope were not scored because lagging chromosomes may not be able to be seen in this stage of the cell cycle (B). Created with BioRender.com.

2.3.2.3. Micronuclei Defects Scoring

Cells were scored looking for the presence or absence of micronuclei. Micronuclei were counted when a cell had a small bubble of DNA that was not incorporated into the DNA. Only DNA fragments in close proximity to a cell were scored to ensure that it was micronuclei being scored and not cellular debris (Figure 9). Cells with micronuclei were scored as a 1 and normal cells were scored as zero. The percent of cells with micronuclei was calculated. A thousand cells from each condition were scored.

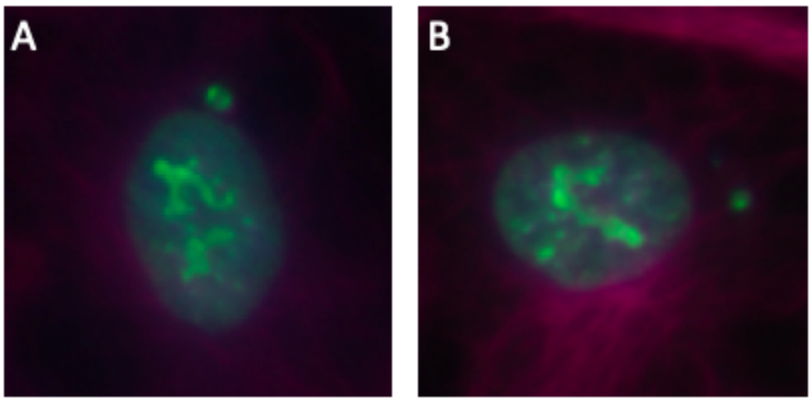

Figure 9: Parameters for scoring micronuclei. Micronuclei were only counted if there was a small region of DNA in close proximity to the cell (A) A cell which contains micronuclei that is close to the cell (B) A cell with a fluorescent object outside of the cell boundary. This is likely cellular debris and not a micronuclei due to the distance of the fragment from the nucleus.

2.3.2.1. Range of Interest Measurements

Measurements of ACA and Aurora B intensity were determined by first setting a region of interest (ROI) measurements in the NIS Elements software. Auto-detection of the region of interest was determined by gating on the DAPI channel and measuring pixel intensity in the other channels. The sum intensity of the fluorescence for kinetochores and aurora B in that range of interest was measured. Three Z-stacks selected from the central plane of each cell were measured. 30 cells from each condition of each replicate were analyzed and compared in this manner.

3. Results and Discussion

3.1. SUV4-20 inhibition decreases levels of H4K20 trimethylation at the kinetochores

Increased SUV4-20 localization to the centromere has been shown to decrease Aurora B centromere localization. It is unknown whether the decreased level of methylation is due to the addition of the epigenetic mark (H4K20me3) or due to other binding partners of SUV4-20 that may regulate Aurora B, such as RB1[16]. In order to test the hypothesis that the methyltransferase activity of SUV 4-20 was responsible for the decreased Aurora B activity, it's methyltransferase activity was inhibited by a small molecule called A196. A196 has been shown to selectively inhibit the activity of SUV4-20 methyltransferase by competitively binding to the enzyme's active site [13](Figure 10). Combined with the construct developed to overexpress SUV4-20 these conditions allow for the experimental manipulation of Suv4-20 activity through overexpression and/or enzymatic inhibition as means to better understand the enzyme's role in the cell.

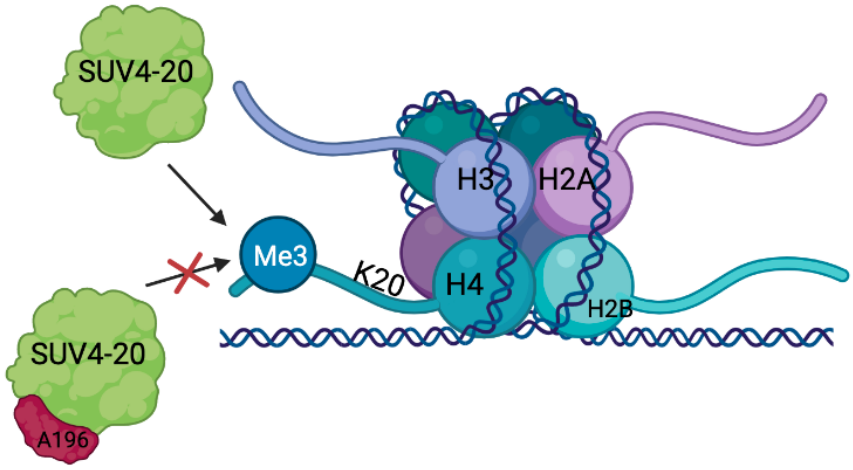

Figure 10: The addition of a trimethyl mark on histone 4 lysine 20 by SUV4-20. SUV4-20 adds the trimethylation mark of histone 4 lysine 20. When A196 is present it inhibits SUV4-20's methyltransferase activity. Created with BioRender.com.

In order to validate A196's ability to inhibit Suv4-20 methyltransferase activity, mitotic spreads were prepared to analyze H4K20me3 levels when SUV4-20 overexpression was inhibited by A196. Doxycycline was added to the RPEcenSUV4-20-GFP cell line to induce cen-SUV4-20-GFP expression and tethering to the centromeres. Concurrently, A196 was added to inhibit methyltransferase activity. Immunofluorescence staining was used to detect DNA, GFP, the Kinetochore marker ACA, and trimethylation of H4K20 (Figure 11). These fluorescence microscopy images were then used to measure the level of H4K20me3 at the kinetochores. The quantification of the fluorescence levels showed that when only the A196 inhibitor was added there were lower levels of H4K20me3 compared to the condition with no drug addition (Figure

12). Likewise, when A196 and doxycycline were present there was also a decrease in H4K20 compared to the condition with only doxycycline. This data demonstrates that A196 is able to decrease the trimethylation of H4K20. However, the addition of doxycycline did not result in higher levels of H4K20me3 compared to the control, as would be expected if cen-Suv4-20 expression were induced (Figure 12). Nevertheless, GFP localization at the centromeres suggests that cen-Suv4-20-GFP has been expressed and is localized properly. (Figure 11). A possible explanation for the lack of increased methylation could be the time course of this experiment. Doxycycline was added to the cells 24 hours before fixing and staining which appears to have been enough time to get expression and localization of the construct, however, it may not have been enough time for the enzyme to have a detectable increase in the amount of trimethyl mark to H4K20. Unfortunately, the inability to detect increased H4K20me3 upon induction of the cenSUV4-20-GFP construct limits our ability to determine if the concentration of A196 would be sufficient to limit methyltransferase activity in the presence of high SUV4-20 activity[22]. Regardless, the experiment did show that A196 is able to reduce centromere levels of H4K20 trimethylation, even if it is just acting on the endogenous Suv4-20.

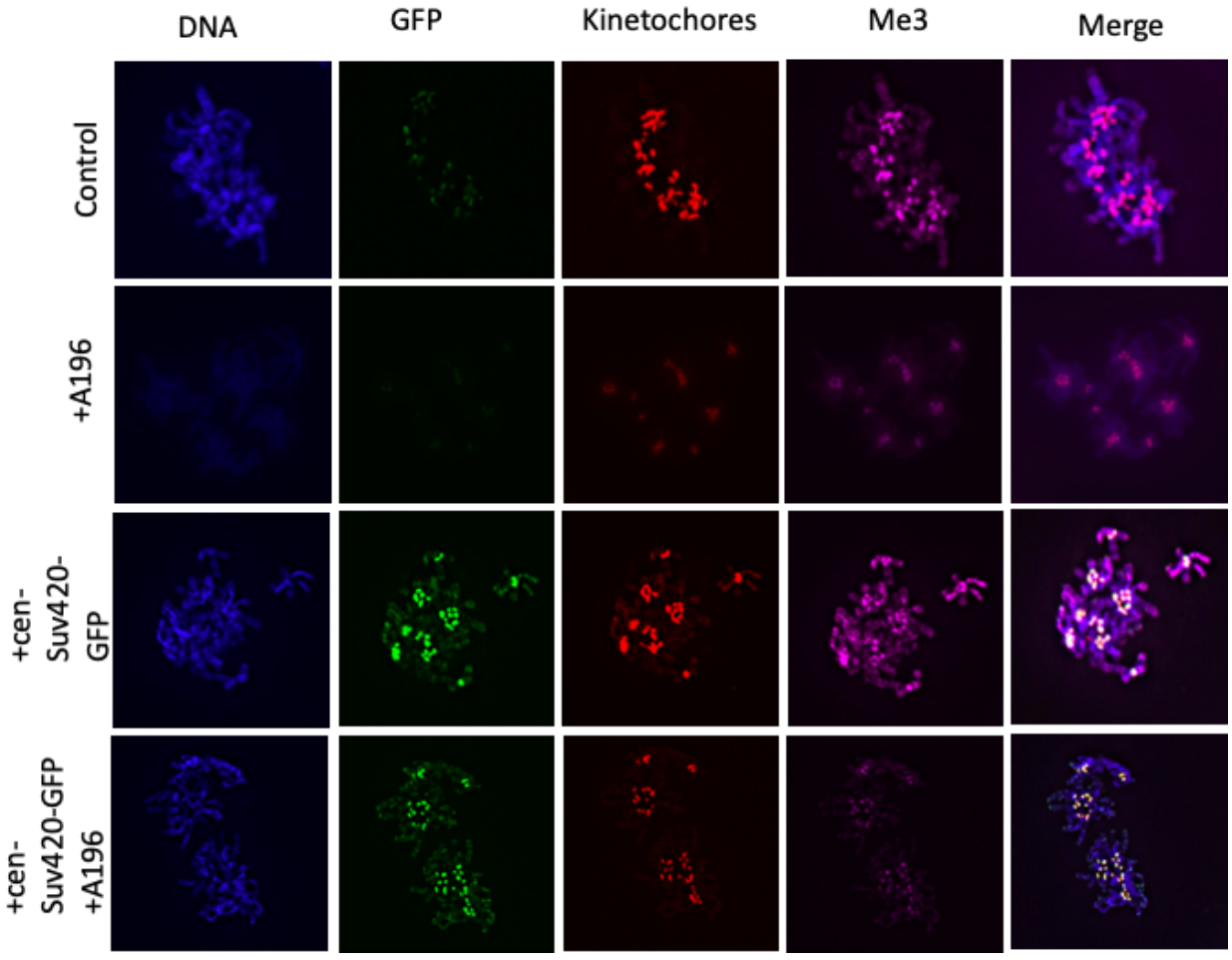

Figure 11: Fluorescence microscopy of mitotic spreads showing H4K20 trimethylation. Fluorescence microscopy images of cells with cenSUV4-20-GFP expression and A196 inhibition. Doxycycline and A196 were added to the cell 24 hours before fixing and staining. DNA, GFP, Kinetochores, and H4K20me3 were all observed using immunofluorescence.

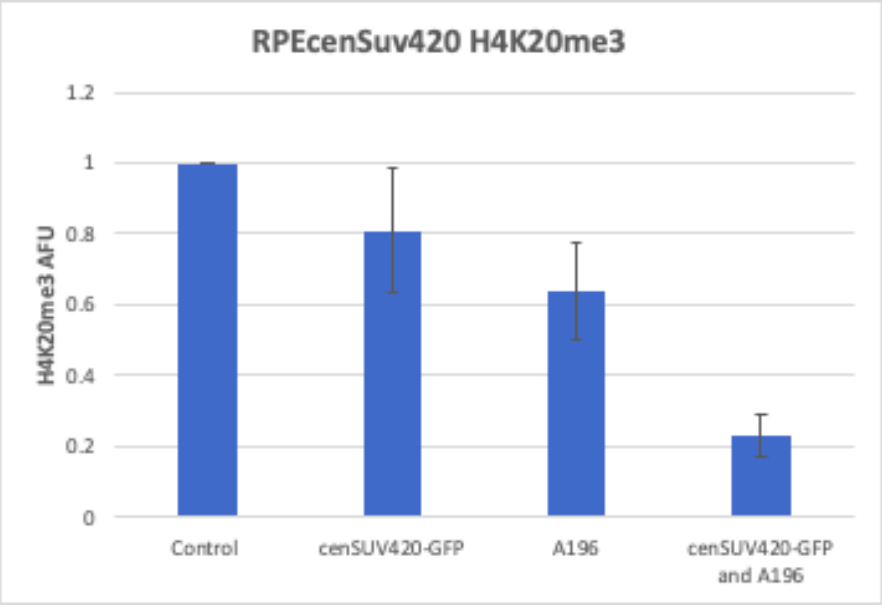

Figure 12: Quantification of H4K20 trimethylation. Fluorescence measurements of H4K20 trimethylation at the kinetochores. Doxycycline was used to induce cenSUV4-20GFP expression while A196 was used to inhibit SUV4-20's lysine methyltransferase activity. Thirty spreads from each of two biological replicates were measured with three kinetochore pairs being measured from each spread.

3.2. Inhibition of SUV4-20's methyltransferase activity does not reduce mitotic defects in cen-SUV4-20-GFP expressing cells

The overexpression of SUV4-20 has been linked to increased levels of mitotic defects[18]. A potential cause for these increased defects could be high levels of H4K20 trimethylation at the centromere. Because A196 was shown to inhibit SUV4-20 methyltransferase activity and decrease the trimethylation levels of H4K20, it was proposed that the addition of A196 may prevent mitotic defects when SUV4-20 is over-expressed. To test this hypothesis, doxycycline was used to induce the overexpression of the cenSUV4-20-GFP fusion protein and mitotic defects were scored with and without the addition of A196.

3.2.1. Anaphase defects

Anaphase cells were scored in each condition to determine the number of cells that had lagging chromosomes (Figure 13). Both the uninduced control with no drug added and the uninduced condition with only A196 had low levels of lagging chromosomes (Figure 14). As previously described, the condition with doxycycline added to induce cenSUV4-20-GFP expression had an increased number of lagging chromosomes[18]. The condition with both doxycycline and A196 had similarly high levels of anaphase defects to the condition with doxycycline alone (Figure 14). Only two replicates were scorable so statistical data could not be obtained. However, due to the moderate increase in anaphase defects observed in the doxycycline only condition, together with the lack of increase in H4K20me3 in this same condition (Figure 12), the interpretation of this data is limited. The trends shown in Figure 14 indicate that A196 was not able to lower the number of cells with lagging chromosomes and thus was likely unable to restore the localization of Aurora B. It is also interesting to note that the trend showed the highest levels of lagging chromosomes in the condition that had both doxycycline and A196, possibly indicating that A196 may cooperate with Suv4-20 overexpression to create more mitotic defects. A potential caveat of using A196 to inhibit Suv4-20 methyltransferase activity lies in the role of Suv4-20 throughout the genome, not just at mitotic centromeres[13]. Because A196 would act on SUV4-20 globally and not just at the centromeres, it may result in other unexpected problems in the cell[13]. While the primary focus of this research is to determine how SUV4-20's activity regulates Aurora B, SUV4-20 is also involved in other epigenetic regulation throughout the chromatin. When A196 inhibits SUV4-20's epigenetic regulation globally it may cause problems due to the loss of SUV4-20's epigenetic regulation abilities at regions distinct from the centromere.

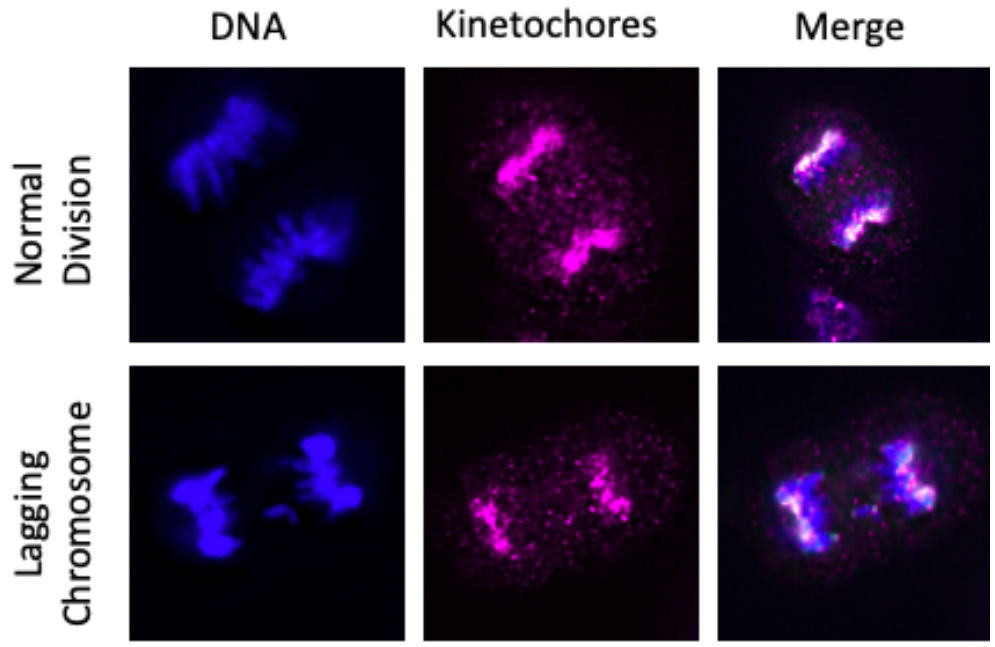

Figure 13: Scoring Parameters for Lagging chromosomes. Immunofluorescence imaging showing a cell undergoing normal division and a cell with a lagging chromosome in anaphase. The presence of DNA and an associated kinetochore separate from the mitotic plate was used to characterize a lagging chromosome.

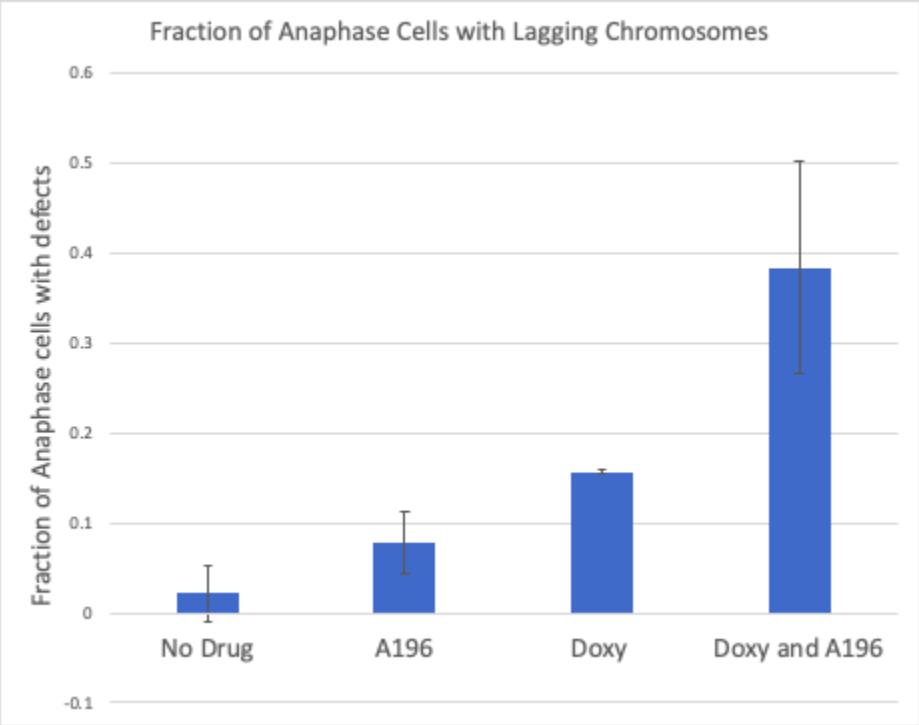

Figure 14: Quantification of Lagging chromosomes. The ratio of cells with lagging chromosomes to normal cells was determined when doxycycline was used to induce cenSUV4-20-GFP expression and A196 was present. Two replicates were performed with thirty anaphase cells from each condition being scored.

3.2.2. Micronuclei data confirming results

To independently test the propensity of each condition to result in mitotic defects, I next scored for the appearance of micronuclei. Micronuclei form when lagging chromosomes fail to be incorporated into the main nucleus when the nuclear envelope reform[23]. Cells from each condition were scored and the fraction of nuclei with adjacent micronuclei was calculated (Figure 15). A similar pattern to that seen in the anaphase defects was observed with the control and A196 only conditions having the lowest levels of micronuclei, followed by the doxycycline condition, and then the doxycycline and A196 condition (Figure 16). There was no statistical difference between any of the conditions but the frequency of micronuclei formation appears consistent with the pattern of anaphase defects. The lack of statistical evidence between the conditions could be a result of two factors: the sensitivity of the assay, or the endogenous

variability in the cellular populations. More replicates of the experiment could be beneficial in determining the cause of the variability. Together these data support a model whereby loss of Suv4-20's methyltransferase activity does not alter the frequency of mitotic defects.

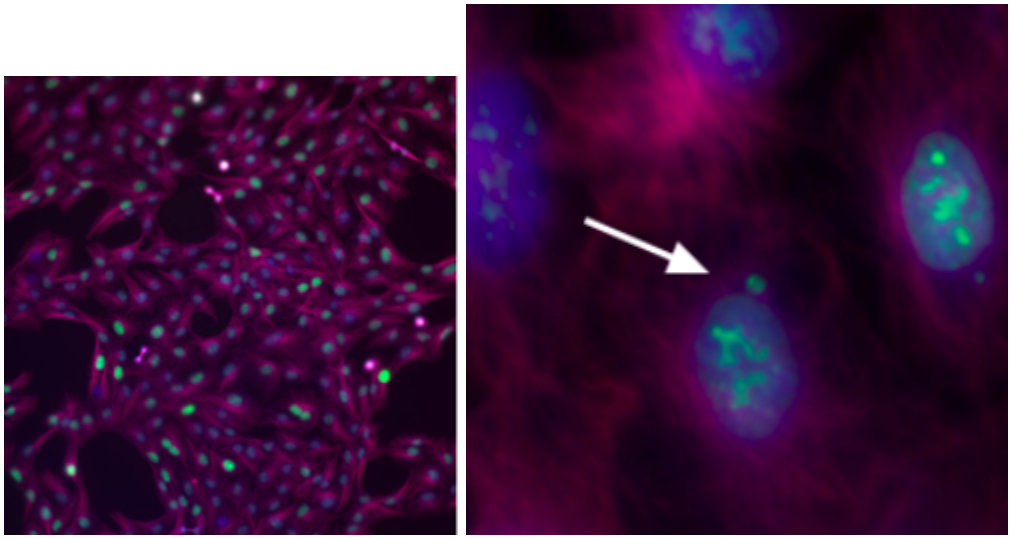

Figure 15: Scoring Parameters for Micronuclei. Immunofluorescence imaging showing a cell with micronuclei (pointed to by arrow). A thousand cells from each condition were scored as either normal cells or cells with micronuclei.

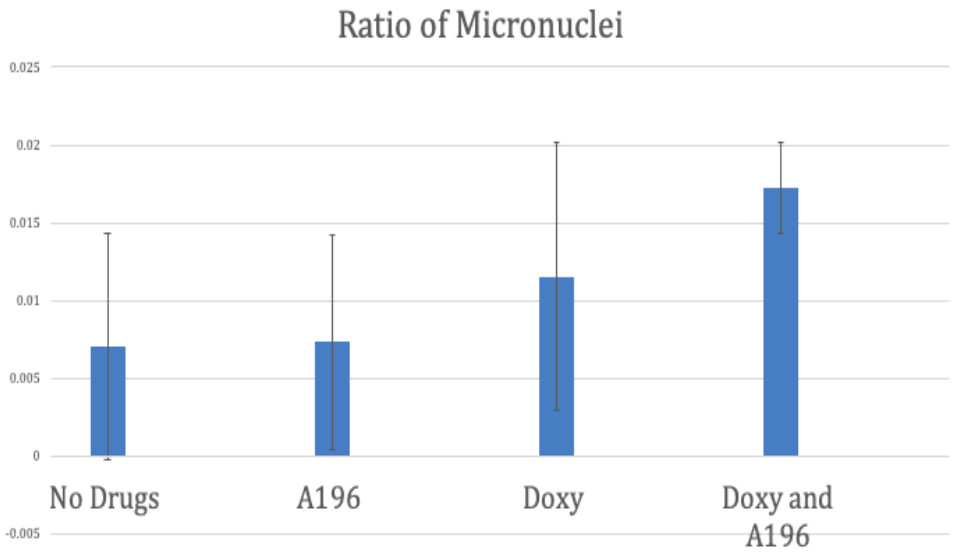

Figure 16: Quantification of Micronuclei. The ratio of cells with micronuclei to normal cells was determined when doxycycline was used to induce cenSUV4-20-GFP expression and A196 was present. A thousand cells from each condition were scored.

3.3. Inhibition of SUV4-20's methyltransferase activity does not affect Aurora B levels in RPE cells

To further explore a potential role for H4K20me3 in the regulation of Aurora B, I next monitored the localization of Aurora B at mitotic kinetochores following a time course of A196 treatment. A196 was added to RPE cells over 6 days and then the level of Aurora B was measured in one-day intervals using fluorescence microscopy (Figure 17). It was expected that if A196 inhibition did not prevent mitotic defects, then the inhibition of H4K20 trimethylation may not be involved in regulating Aurora B. Quantification of the centromere-localized levels of Aurora B after A196 was added, showed that there was no significant change in Aurora B levels (Figure 18). The plot shows that there was great variability among measurements in each condition which indicates that there may be variability of Aurora B localization to centromeres as cells proceed through mitosis (Figure 18) and that a more controlled synchronization of cells will need to be used to minimize variability. If true that the addition of A196 does not change Aurora B levels, it would indicate that Aurora B is not regulated by the trimethylation of H4K20 and is instead sensitive to Suv4-20 localization itself. An important caveat of this interpretation is that, although I have shown that A196 is able to lower the trimethylation of H4K20 at the centromeres (Figure 12), the staining techniques were not compatible to allow concurrent measures of H4K20me3 and Aurora B and thus we can not confirm that the cells in which Aurora B is measured have the expected reduction in levels of H4K20me3.

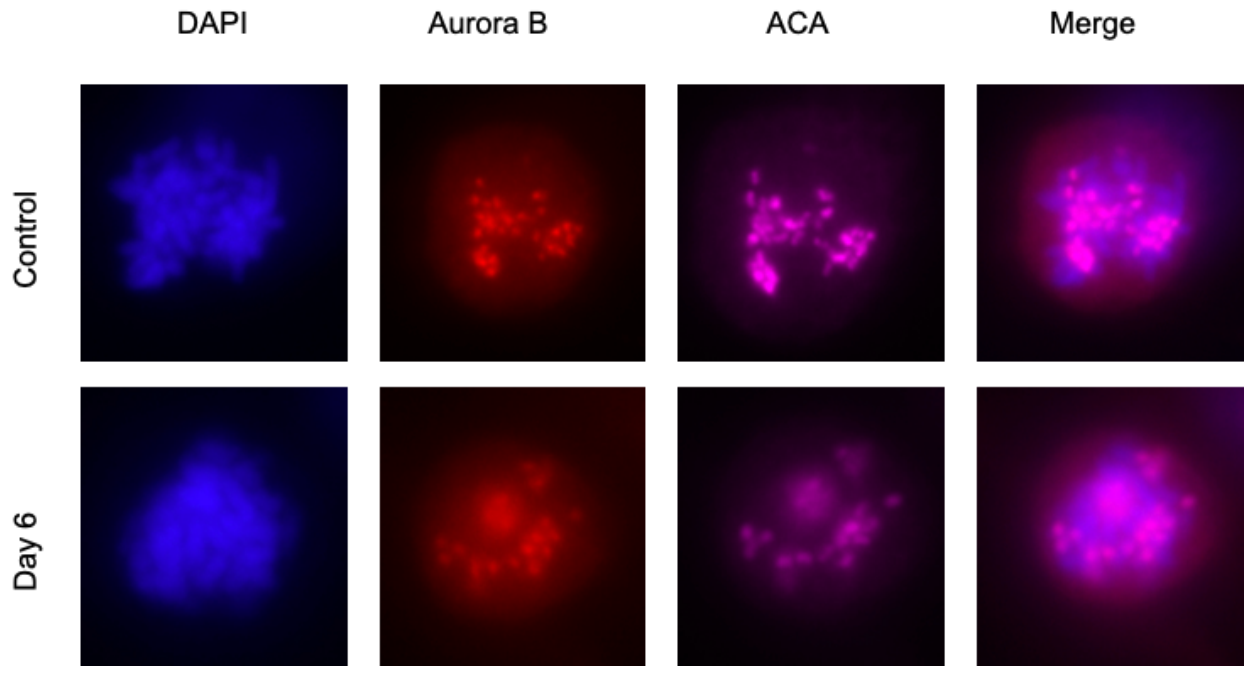

Figure 17: Fluorescence Microscopy of Aurora B after A196 Addition. Fluorescence microscopy images of a time-course experiment with A196 addition. DNA, Aurora B, and kinetochores observed using immunofluorescence.

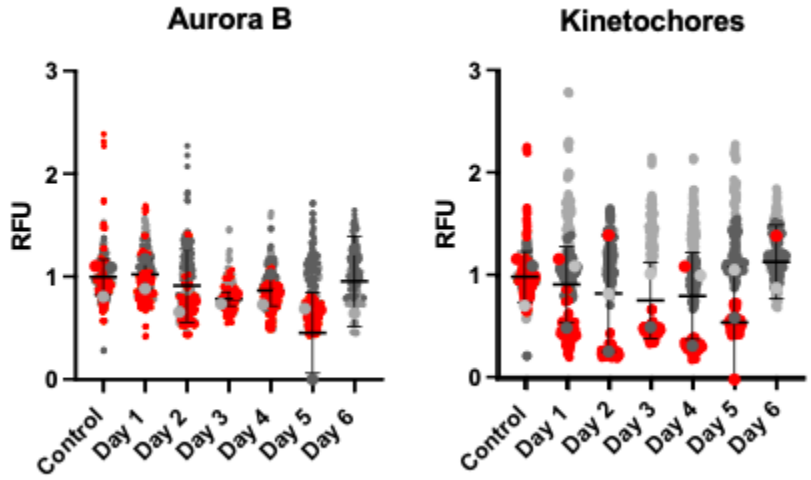

Figure 18: Quantification of H4K20 trimethylation. Range of interest fluorescence measurements of Aurora B and kinetochores over time. A16 was added each day to determine if the level of aura B was affected by A196 over different times. Thirty metaphase cells were measured from each condition.

4. Conclusions and Future Work

Aurora B is a master regulator of mitosis involved in error correction. Its misregulation in a cell can have detrimental effects on chromosome segregation, resulting in CIN and aneuploidy. SUV4-20 is an enzyme that plays a role in regulating Aurora B localization[18]. Overexpression of SUV4-20 is prevalent in cancer and is associated with aneuploidy and poor patient prognosis [18] [19] [24]. Furthermore, when SUV4-20 is overexpressed using the RPEcenSUV4-20-GFP construct there are found to be decreased levels of Aurora B at the centromeres[18]. This evidence supports the hypothesis that SUV4-20 is a regulator of Aurora B. In this project, the effect of inhibiting SUV4-20's methyltransferase activity was studied and showed that inhibiting this function was not able to restore Aurora B levels or prevent mitotic defects. These results were surprising since SUV4-20's function as a methyltransferase is thought to be key to its regulation of centromeres[14]. The data showed evidence that the level of trimethylation of H4K20 did not correlate with levels of Aurora B at centromeres. These findings suggest that Aurora B is not regulated by SUV4-20's methyltransferase activity. Instead, SUV4-20 may function as a scaffold to recruit other centromere components that are responsible for regulating Aurora B.

While the data from this paper indicates that A196 inhibition of SUV4-20's methyltransferase activity does not perturb Aurora B's ability to localize at the centromere, some of the experiments lacked sufficient controls to show that the experimental system worked as designed. Of significant concern is that the expression of the RPE cenSUV4-20-GFP construct did not result in an increase of H4K20 trimethylation at kinetochores, or mitotic defects. The GFP tag on this construct was localized to the kinetochores which indicates that the construct was being expressed and localized correctly, however, the induction time course of this construct may have been insufficient for increased SUV4-20 levels to catalyze a detectable increase in the addition of methyl groups to H4K20. In order to determine if the experimental timeline was insufficient to allow for increased methylation, a time-course experiment could be performed to determine if an increased amount of time between RPE cenSUV4-20-GFP induction, using doxycycline, and cell fixation results in increased mitotic defects or H4K20me3. Once the amount of time is determined for doxycycline to produce the expected phenotype, this time course could be used to repeat the experiments in this paper looking at A196's ability to inhibit trimethylation and prevent mitotic defects. If the evidence still showed that A196 was not involved in preventing mitotic defects it would support the hypothesis that SUV4-20's methyltransferase ability is not the cause of Aurora B misregulation.

It has been shown that the overexpression of SUV4-20 is associated with the decreased localization of Aurora B at the kinetochores[18]. If the regulation of Aurora B is not related to SUV4-20's methyltransferase activity then another potential cause may be the other proteins that SUV4-20 binds to. Therefore the misregulation of Aurora B's localization when SUV4-20 is over-expressed is likely due to another protein interaction that SUV4-20 is involved with. Using a co-immunoprecipitation assay to pull down Suv4-20 interacting protein partners of SUV4-20 could be identified. By identifying potential binding partners of SUV4-20, candidates for the protein involved in limiting Aurora B localization, when SUV4-20 is overexpressed, could be determined. Once specific candidates are identified, quantitative fluorescence microscopy, as performed in figure 7, could be used to analyze the relative quantity and location of those proteins when SUV4-20 is over-expressed. If the levels or location of these binding partners changed when SUV4-20 was overexpressed it may indicate that SUV4-20 is regulating that protein. Furthermore, if SUV4-20 is regulating that protein it may be involved in the regulation of Aurora B. By potentially unlocking new steps in the regulation pathway of Aurora B, new methods for restoring Aurora B's function could be investigated.

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