Suv420H2 Localization during Mitosis is Sensitive to Phosphorylation

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Abstract

Epigenetic regulation of histones through phosphorylation, methylation, and acetylation is crucial for regulating chromosome organization and gene expression. The histone methyltransferase Suv420H2 is responsible for placing di- and tri- methyl marks on the lysine 20 residue of histone 4. H4K20me2/3 is a repressive mark that is enriched at centromeres and telomeres, as well as at silenced genes and its loss or misplacement can compromise gene expression, repair of DNA damage, and chromosome segregation during mitosis. However, the mechanism that regulates the localization and activity of Suv420 is not well understood. Motif analysis performed with the database ELM identified putative binding motifs for several kinases and phosphatases, including the mitotic kinase Nek2. In this project, I have tested the hypothesis that, during mitosis, the association of Suv420H2 with chromatin is regulated by the Nek2 kinase. I have used cell fractionation assays and immunofluorescence imaging of cells to monitor the subcellular localization of Suv420H2 in control mitotic cells and those following inhibition of Nek2 or protein phosphatase I (PP1). My data indicate that the localization of Suv420H2 in mitosis is sensitive to its phosphorylation state.

Background

Mitotic Cell Division is Highly Regulated

Mitosis is the process by which one cell divides into two new daughter cells. In preparation for mitosis, the cell first increases its size (G1 or Gap phase 1), then duplicates its genome (S or Synthesis phase), checks for and corrects errors (G2 or Gap phase 2), before finally committing to division (M phase or mitosis) (Mitchison & Salmon, 2001). In early mitosis the DNA is compacted into chromosomes then lined up along the center of the cell. This movement of chromosomes is coordinated by the attachment of the bipolar spindle to kinetochores, a protein complex attached to the centromere of a chromosome. The bipolar spindle provides the force needed for chromosome movement due to associated motor proteins. Once the chromosomes are properly attached to the spindle, one copy of each chromosome is pulled to each side of the dividing cell. As the cell completes division to separate the cytoplasm into two new daughter cells, each daughter forms a new nuclear envelope around the chromatin it has inherited. Mitotic cell division in normal cells is highly regulated to ensure accuracy of chromosome segregation and the generation of genetically identical daughter cells. However, in rare occasions when random errors occur, or in disease contexts where the mitotic spindle or kinetochores do not form correctly improper chromosome segregation can occur. Defects in chromosome segregation result in the daughter cells containing abnormal chromosome numbers, known as aneuploidy, and can also result in mutation-causing DNA damage (Crasta et al., 2012) (Potapova & Gorbsky, 2017).

Kinetochores

Kinetochores are protein complexes that are built upon the centromere of each chromosome and act as an anchor point for the microtubules to attach during mitosis and as mechanosensors to align the chromosomes at the equator of the cell. Kinetochores also regulate the spindle assembly checkpoint (SAC), which functions to prevent a premature exit from metaphase. They produce a signal that delays mitotic exit until kinetochores have fully attached to microtubules a, resulting in chromosome biorientation and alignment (Cleveland et al., 2003). This system is sensitive enough that it will prevent mitotic exit if even one kinetochore or microtubule is unattached (Cleveland et al., 2003).

Centromeres are Epigenetically Defined

The centromere is a highly repetitive sequence that is epigenetically defined on each chromosome. Centromeres in different species can vary in size and sequence. However, all centromeres perform the same critical function of directing the formation of kinetochores, which serve as a functional link between the chromosome and the mitotic spindle that enables chromosome movement and accurate segregation during cell division (Smith & Maddox, 2017) (Westhorpe & Straight, 2015). One of the main epigenetic markers for the centromere is the centromeric protein CENP-A, a histone 3 variant (Black et al., 2007). When CENP-A is moved artificially it creates a neocentromere where there wasn't a centromere before. Properly defining the centromere region of the chromosome is important for proper chromosome segregation during mitosis (Fioriniello et al., 2020).

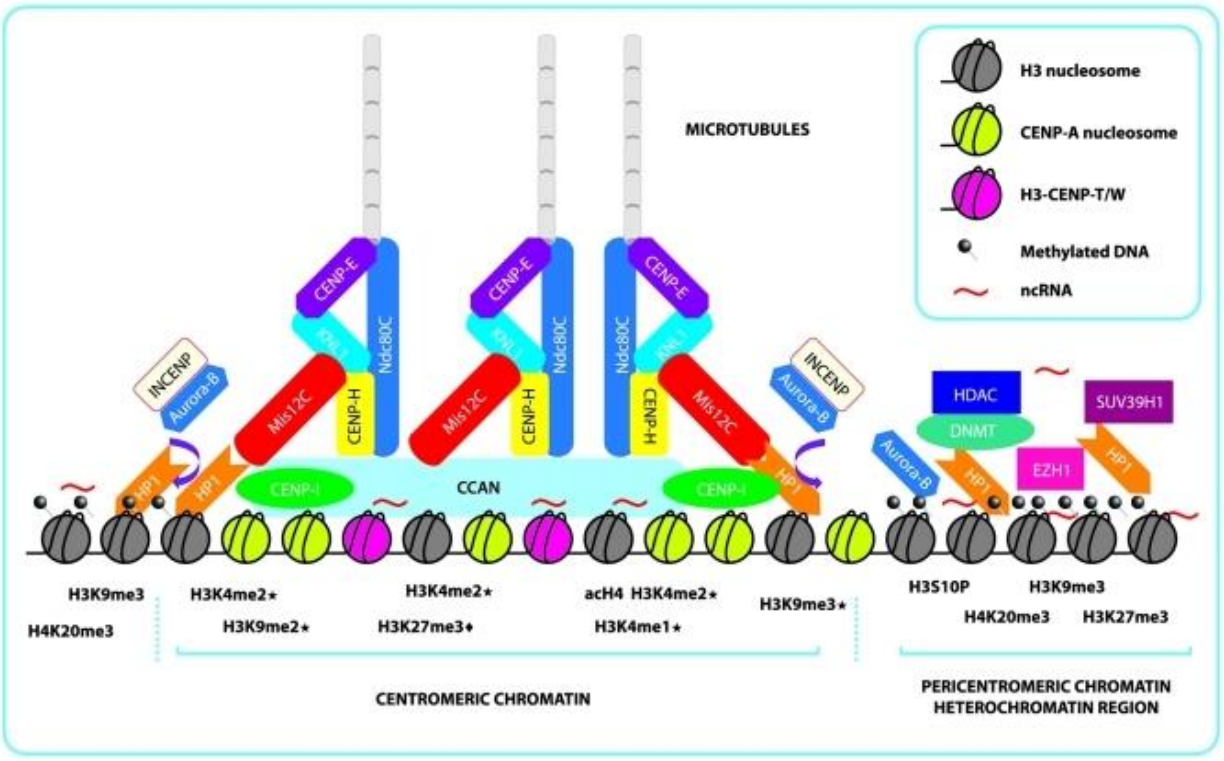

Fig 1 Diagram showing location and interactions of centromeric and percentromeric proteins and the epigenetic marks present. (González-Barrios et al., 2012)

Another region of the chromosome controlled by epigenetic marks is the pericentromeric region which sits on either side of the centromere. This area is defined by the satellite DNAs (satDNAs) β-satellite and γ-satellite, and satellites I, II, and III (Fioriniello et al., 2020). This region is crucial in preserving the genome, repressing gene movement, and proper chromosome segregation. Some other common features of the pericentromeric region found in both mice and humans are DNA methylation, low acetylation of histones, and enriched repressive histone marks. Some crucial epigenetic marks of this region are H3K9me2/3, H4K20me3 (Schotta et al., 2004), H3K27me1, H3K64me3 (Rice et al., 2003), and non-coding RNAs. H3K9me2 domains border CENP-A chromatin, while H3K9me3 domains are further away (Fioriniello et al., 2020).

H3K9me2/3 is the di- and tri- methylated form of histone 3 at the lysine residue 9 and is methylated by Suv39 methytransferase. This modified histone is important in the organization of pericentromeric heterochromatin and provides a binding site for HP1. HP1 then recruits Suv420 methytransferase and enriches the area with H4K20me3, a binding site for HP1. Reduction of Suv39 leads to significant impairment of DNA methylation in the pericentromeric heterochromatin.

The modified histone H4K20me3, methylated by Suv420, acts as a docking site for other specific factors. H3K64me3 stabilizes DNA-histone interactions. It also recruits more histones and DNA methyltransferases and might be involved in ensuring the proper epigenetic state of the pericentromeric region. H3K64me3 is dependent on H3K9me3 being present (Fioriniello et al., 2020).

The DNA repeats in the pericentromeric are mostly methylated at cytosine 5 of 5meC dinucleotides. In somatic cells the DNA is hypermethylated and hypomethylation can lead to cell senescence and cancers. Hypermethylation on the other hand is a platform for the binding of methyl-binding domain protein family members. An example of one of these proteins is MeCP2 that modulates chromatin structure and is enriched in the pericentromeric region. MeCP2 is mutated in a severe neurological disorder called Rett syndrome (Fioriniello et al., 2020).

Centromere Transcription Promotes Accurate Chromosome Segregation

Despite being heterochromatin, satDNA is actively transcribed using RNA polymerase I (Biamonti & Vourc'h, 2010). These satellite transcripts are involved in high order pericentromeric organization by anchoring related proteins to chromocenters (Almouzni &

Probst, 2011) (Jagannathan et al., 2019). There can be abnormal ncRNAs transcribed from centromere and pericentromere sequences as a response to cell stress and senescence as well as in diseases, including cancers (Fioriniello et al., 2020). One hypothesis for how satRNA molecules function is that they act like sponges to bind and modulate available epigenetic factors (Biamonti & Vourc'h, 2010). In this way, excess transcription under disease conditions could sequester critical components of molecular machinery needed for accurate chromosome segregation.

Heterochromatin found in the pericentromeric region maintains the boundary between euchromatin and the centromere (Scott et al., 2006) (Sullivan & Karpen, 2004). It also helps centromere function by serving as an enrichment point for the cohesin complex. The cohesin complex plays important roles in sister chromatid cohesion and chromosome segregation (Park et al., 2018). The formation of pericentromeric heterochromatin can also suppress recombination during meiosis, thereby promoting centromere integrity (Chen et al., 2022).

Suv4-20H2 Methyltransferase is a Critical Regulator of Pericentromere Identity

In mammalian cells, the Suv4-20 methyltransferase has two homologs that di- and tri-methylate histone 4 at the Lysine-20 residue (Southall et al., 2014). It is specific for the substrate H4K20me and catalyzes the addition of one or two methyl groups to form the further modified histone product (Southall et al., 2014). The number of methylations on H4K20 is important to protein binding sites. Suv420H2 is normally found at or around the pericentromeric region of the chromosome during mitosis. Changing the localization of Suv420 to the centromeres lead to more micronuclei and missegregation (Herlihy et al., 2021).

Increasing Suv39 and Suv420 at the centromere suppresses centromere transcription and compromises localization of Aurora B, a mitotic kinase that is the master regulator of error correction (Willems et al., 2018). In the research article by Herlihy et al. they used The Cancer Genome Atlas and found that Suv39 and Suv420 have increased expression in a cancer context and that Suv420H2 has increased average expression compared to normal tissue samples in 11 out of 14 cancer subtypes. This high expression of Suv39 and Suv420 significantly positively correlates to aneuploidy which leads to more aggressive cancer and poor patient outcomes. They depleted H3K9me3 demethylase (KDM4A) with siRNA and found it compromised mitotic

fidelity using live cell imaging. The cells were able to align in metaphase, however 25% of anaphase showed lagging chromosomes. Lagging chromosomes can end up outside of the reforming nuclear envelope and for micronuclei (Herlihy et al., 2021). They then targeted Suv39H1 onto centromeres and saw an increase in metaphase alignment defects and lagging chromosomes. This experiment also indirectly implicated the deposition of H4K20me3 onto centromeres. This was repeated with Suv420H2 and there was an increase of metaphase alignment defects and anaphase segregation error and the authors concluded that increasing H4k20me3 at centromeres corrupts kinetochore regulation. Looking into mitotic error correction mechanism efficiency they found that with the KDM4A depletion, and targeted Suv39 and Suv420 cell lines there were delays and metaphase alignment indicating a decrease in correction efficiency (Herlihy et al., 2021). The authors also looked at Aurora B kinase and INCENP at centromeres and saw a decrease with the depletion of KDM4A, Suv420 and Suv39 targeted to the centromeres (Herlihy et al., 2021). In experiments without H4K20me3 enrichment at the centromeres there was no change in Aurora B localization and is sensitive to Suv420/H4k20me3 localization to the centromeres (Herlihy et al., 2021). The inhibition of Suv420 with A196 (Bromberg et al., 2017) partially resources Aurora B localization and restores efficient chromosomal alignment. An increase of H4K20me3 contributes to chromosomal instability.

Phosphorylation Regulates Proteins

Many proteins are regulated by phosphorylation. Phosphorylation can be envisioned to work like a switch where addition or removal of a positively charged phosphate group can turn a protein:protein interactions ‘on’ or ‘off’(Ubersax & Ferrell Jr, 2007). Since protein: protein interactions drive localization and function, phosphorylation can be a powerful way to regulate protein activity. The enzymes that catalyze the addition of phosphate groups are called kinases. Kinases use their catalytic cleft to phosphorylate protein targets, known as substrates (Ubersax & Ferrell Jr, 2007). Substrate binding and phosphorylation by kinases is sequence dependent and prior studies of kinases and their known substrates have led to the identification of preferred phosphorylation sequences, known as motifs (Ubersax & Ferrell Jr, 2007). Sequence analysis can be used to predict kinase-specific phosphorylation of new proteins. Using the linear amino acid sequence of Suv420H2 and predictive software, in this case The Eukaryotic Linear Motif

(ELM) resource, we can predict possible phosphorylation sites on Suv420 that could regulate Suv420.

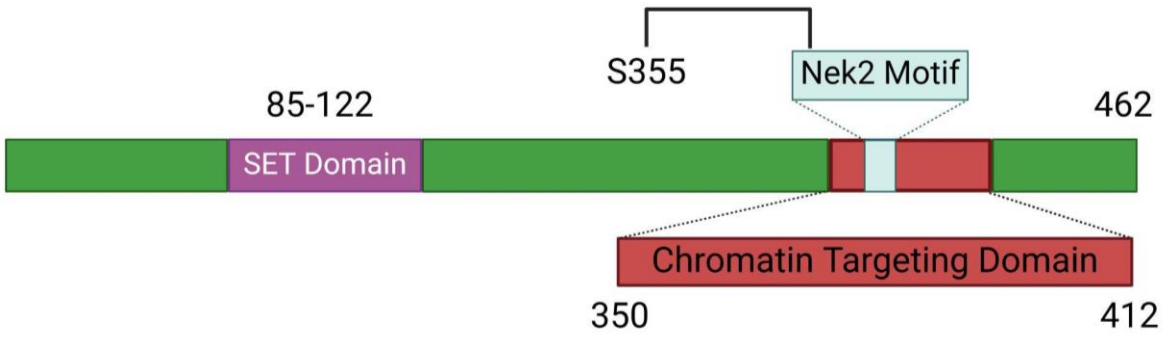

Fig 2 Suv420H2 linear protein diagram with important domains and motifs. Image created with Biorender.com

The EL M database identified 4 different sites where Nek2 kinase could phosphorylate Suv420H2 including one in the chromatin targeting domain of Suv420H2. Nek2 kinase plays a role in chromosome condition and segregation, centrosome segregation, microtubule stability, kinetochore attachment and SAC. When Nek2 is overexpressed, it can lead to tumorigenesis and drug resistance by activating several oncogenic pathways (McCrury et al., 2024).

I hypothesize that Suv420H2 is phosphorylated by Nek2 kinase, and that this phosphorylation event regulates Suv420's localization at centromeres and pericentromeres. Given previous data showing that increased localization of Suv420H2 to the centromere causes mitotic defects, I predict that phosphorylation by Nek2 may be a key regulatory step to keep Suv420H2 localization at centromeres low, thereby ensuring proper chromosome segregation.

Methodology

Small Scale Fractionation

To begin my project, I used retinal pigment epithelial cells transformed with TetR Suv420-Halo. This is an adherent cell line that has an inducible gene added for Suv420 tagged with the Halo tag on the C-terminus of the protein. I also worked with 2 mutant Suv420 lines that were also tagged with Halo. One of these mutant lines switches the amino acid serine 355 for alanine, to make prevent phosphorylation at this site, and the other mutation is swapping the serine for aspartic acid, an amino acid that mimics the size and charge of a phosphorylated serine residue.

I first seeded the cell lines into T75 flasks to ensure enough cells to pick up a protein signal when immunoblotting. The flasks are then treated with doxycycline (2ug/ml) 24 hours prior to fractionation to induce the expression of Suv420-Halo. I then treated with nocodazole (at least 16 hours before the shake off step to arrest cells in mitosis. Nocodazole, which disrupts microtubule formation. The lack of microtubules activates an important checkpoint that prevents the cell from progressing and arrest cells in mitosis.

16-18 hours after the addition of nocodazole I checked that the cells are arrested in mitosis and begin shaking off the mitotic cells which are round versus the adhered flat non mitotic cells. The force of hitting the flasks on a table is enough to suspend a mitotic cell in the media while leaving any non-mitotic still adhered to the flask. After shake off, I took samples of each cell line and counted the cell concentration while centrifuging the rest of the cells down to pellets. I then resuspended the pellets with media and equalized the cell concentration of each line so that 2 ml of each line should have about the same number of cells in them. In separated samples of 2ml I treated the cells with a protein phosphatase 1 inhibitor Calyculin A at 1:1000 dilution (10uM) (Ishihara et al., 1989) and treated with a Nek2 kinase inhibitor, Rac-CCT, at 1:1000 dilution (100uM) (Innocenti et al., 2012). After a short treatment time of 15 minutes which has proven effective to see a phenotype (summer experiments in the Manning Lab). I prepped the hypotonic buffer with 2.5ul protease inhibitor and a quarter tablet of phosphatase inhibitor and kept on ice throughout the fractionation. I then spun down the samples and removed the media then resuspended with 66ul of the prepped hypotonic buffer and placed samples onto ice to incubate for 15 minutes. I then removed 16ul for my whole cell lysate and added in 5.3ul Laemmli 4x sample buffer and placed the whole cell lysate samples back on ice.

To the remaining sample I added 3.13ul of 10% NP40 and quickly vortexed the samples for 10 seconds and placed them onto the rotator in the 4 degree Celsius fridge for 15 minutes. During this wait time I moved the whole cell lysate samples to the hot plate to boil for 8 minutes and then moved them into the -20 degree Celsius fridge to store. After removing the samples from the rotator, they were placed in the centrifuge at top speed for 5 minutes to create a chromatin pellet and soluble fraction. I then removed the supernatant which is my soluble fraction leaving behind the chromatin pellet. It is important to remove as much of the soluble fraction to prevent mixing the chromatin bound fraction with a small amount of the soluble fraction. To the soluble fraction I added 17.7 ul of Laemmli 4x sample buffer and placed the sample on ice. I then resuspended the chromatin pellet with 70.8 ul of Laemmli 1x sample buffer. I then boiled the rest of the samples and moved them into the -20 degree Celsius fridge to store. All these steps after introducing the hypotonic buffer are done on ice to preserve the integrity of the protein and phosphorylation states within the sample.

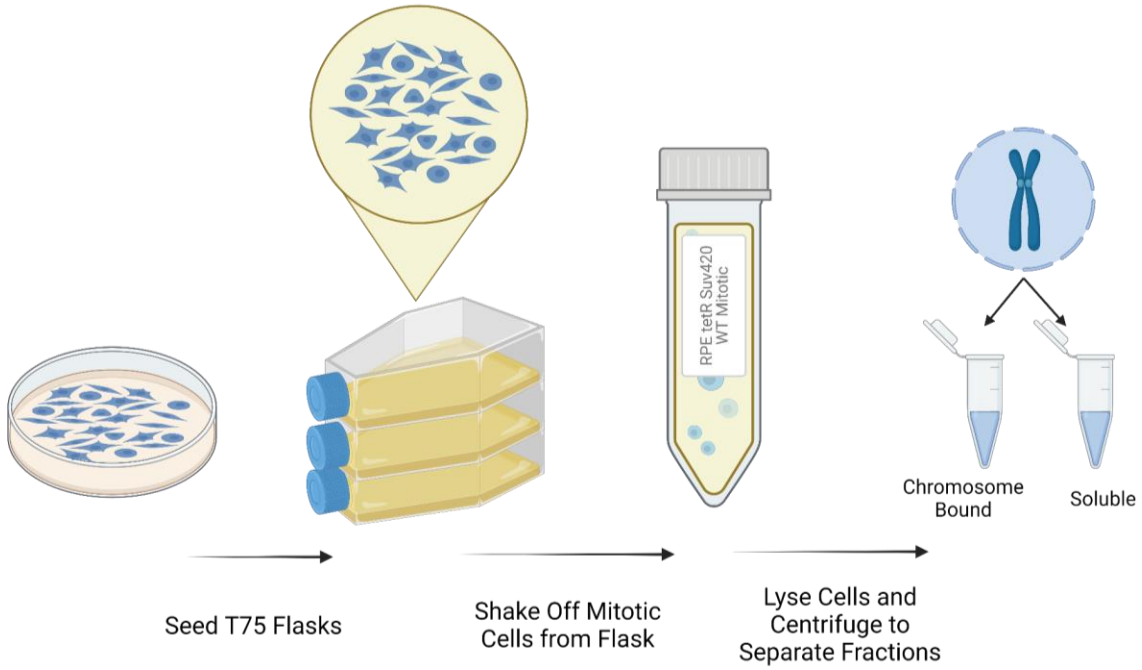

Fig 3 Fractionation experimental process diagram. Image created with Biorender.com

Gel Electrophoresis, Western Blotting and Autoradiography

To separate the proteins within my fractionation samples, I used SDS-page gel electrophoresis using a 10% gel. I poured the gels before running the blot and loaded the sample alongside the Precision Plus Protein All Blue Standards Ladder. I ran the gels at 95V until the samples hit the separating gel which took about 90 minutes and then upped the voltage to 120 V to efficiently separate the proteins.

After running the gel for about 1.5 hours, I transferred the proteins onto nitrocellulose membranes and confirmed efficient transfer using Ponceau stain. Ponceau is a reagent used to stain proteins to make the bands visible before probing with antibodies and was washed out with water.

At this point I continued and probed for Halo (G921A) and GAPDH (60004-1g). Both are mouse antibodies and were used on cut membranes to separate the protein bands. To do this I first blocked the membrane in 5% milk in TBST for 45 minutes while rocking at room temperature. I then cut the membranes and placed them in heat sealed baggies and added 2 ml 5% milk in TBST to the top section and 2 ul of the Halo antibody and sealed the bag.
To the bottom section I added 3 ml 5% milk in TBST and 0.6 ul GAPDH antibody and then sealed the bag. I placed these bags on the rocker in the 4 degree Celsius fridge overnight. In the morning, I washed the membranes in TBST for 5 minutes 3 times. I placed the membranes in fresh heat-sealed bags and added 2ml 5% milk in TBST and 10 ul HRP mouse secondary antibody and sealed the bags. They were left to rock at room temperature for 1 hour and 15 minutes.

15 minutes before use the autoradiography machine was turned on in order to warm up. The membranes were then washed with TBST for 5, 10 and 15 minutes in that order on the rocker. The membranes were then placed in a development cassette and 750 ml of prosignal pico:peroxide in a 1:1 ratio was added to each membrane and allowed to sit for about 30 seconds. Extra reagent was then removed with kimwipes. I then moved the cassette into the developing room. Using the autoradiograph (Kodak M35A X-OMAT Processor) and Blue Devil autoradiography film sheets I developed the blots for 30 seconds, 1 minute, 5 minutes, and 15 minutes. I labeled and scanned the 15 minute developed film.

Fixing and Staining

The second half of the project was dedicated to a complementary assay to the fractionation assay described above. I switched cell lines to RPE TetR Suv420H2-FLAG that has Suv420H2 tagged with FLAG on the N terminus as the anti-FLAG antibody is great for immunofluorescence imaging. I was still treating the cells with the Nek2i and PP1i drugs. Instead of using Nocodazole to arrest cells in mitosis I used the drug MG132 to arrest cells in metaphase as it inhibits the proteasome necessary to cleave cohesion and leaves chromosomes attached and unable to separate and move into anaphase (Zeng et al., 2010).

To begin this experiment, I first added a round 18mmm #1 ½ thickness round coverslip that had been rinsed in 70% ethanol and then dabbed dry on a kimwipe to a 12 well dish. While trypsinizing the cells I let the coverslips dry and then rinsed with sterile 1x PBS two times. I then seeded my cells with 100,000 to 150,000 hTertRPE-1 Flag Suv420H2 cells and added media to bring the volume to 1 ml media with 2 ug/ml doxycycline concentration to induce the cells. After adding cells, I used a pipet tip to make sure that the coverslip stuck to the bottom of the well to ensure that the cells adhere to the coverslip and not underneath the coverslip which would make imaging more difficult. The well dish was then placed in the incubator set to 37 degrees Celsius and 5% CO2 levels. On the second day I added MG132 for 20uM final concentration. This was then incubated for 4 hours. I treated the cells with PP1i at 200nM for 5 minutes and Nek2i at 20uM for 10 minutes. I quickly checked that the cells were still adhered and proceeded to quickly appropriate the media off and wash with 1x PBS. 1 ml of prewarmed 4% paraformaldehyde was added to each well and incubated at room temp for 10 minutes. The paraformaldehyde was removed, and coverslips were washed with 1x PBS. I then extracted with 1 ml PBS/0.2% triton x100 for 10 minutes at room temperature. This was again washed with 1x PBS. Coverslips were then blocked with 1 ml TBS/BSA for 1 hour at room temperature. The coverslips were then blotted on kimwipes and placed onto the cover of a 12 well dish covered with parafilm in a tupperware humid container with wet blot paper underneath. They were then incubated with 100ul of diluted primary antibody (mouse anti-FLAG (M2 Millipore 1804 1:200) diluted in TBS/BSA for 3 hours at room temperature. I again blotted the liquid off of the coverslips using a kimwipe and then placed back into a 12 well dish and washed with TBS/BSA for 5-10 minutes. I removed excess liquid by blotting with kimwipes and placed the coverslips back into humid tupperware and incubated with secondary antibody mouse green (1:1000 diluted in TBS/BSA plus DAPI) for 1 hour in the dark at room temperature. The coverslips were placed

back in wells and washed in TBS/BSA 5-10 minutes. I blotted off excess liquid with kimwipes and mounted the coverslips with 1 drop of Prolong Gold Antifade and let it cure flat overnight at room temperature in the dark before putting them in the 4 degree Celsius fridge to store.

Immunofluorescence Microscopy

Once cells were fixed and stained, I used the Nikon Ti-E microscope on the 4th floor to image my coverslips using the 60x Plan Apo oil immersion objective. I captured mitotic cells and was aiming for mostly metaphase, but also captured early mitotic and anaphase cells as well. I set the Zyla sCMOS mounted camera ROI to 500 x 500 to capture one to two mitotic cells per frame. Each experiment was imaged on the same day to prevent any differences in intensity due to fluorophore decay. I autoset the exposure on the Nek2i treated cells as they should be the brightest coverslips. This was to prevent overexposing my images of the other conditions. Once I had captured about 35-55 cell images, I then transferred the images to the server to later analyze.

Image Analysis

In order to quantify the intensity of my cell images I first open up my images on NIS elements. Using the ROI autoselect tool I gate on the DAPI channel (chromatin) and select the XY coordinate. I am then able to measure the FITC channel intensity (FLAG) of a single cell. I do this for all mitotic cells that are in focus and in frame. I purposefully left out far apart anaphase cells as quantifying those mitotics can be tricky and I focused on early mitotic cells instead. After exporting the intensity data to an Excel sheet. I then average Z frame intensity measurements for each cell to find the average intensity of each cell. Once done getting the individual cell intensity averages, I then take the average of the untreated control and use this to normalize the treatment cells to see how different from the control the treatments intensities are. I repeated this analysis for all my IF replicates. Once I had all my data, I then was able to plot each individual cell average into a superplot to better understand the distribution of cell intensities across the replicates.

Results

To understand how Suv420H2 is normally localized in mitotic cells, I performed cell fractionation experiments. These experiments use cell lysis and centrifugation to separate chromatin and associated proteins from soluble proteins located in the cytoplasmic fraction of mitotic cells. I first treated cells and then fractionated them into chromatin bound and soluble fractions. I next ran the samples on SDS-page gels to separate the proteins and afterwards probed for the Halo tag and GAPDH. This data shows that Suv420H2 is localized primarily in the chromatin bound fraction of mitotic cells. To understand how Nek2 dependent phosphorylation may influence Suv420H2 localization, I performed, in parallel, fractionation experiments where cells were treated with either a Nek2-specific small molecule inhibitor (Rac-CCT) to prevent phosphorylation, or with Calyculin A, a phosphatase inhibitor, to lock Suv420 in a phosphorylated state. Western blot analysis shows that, compared to Calyculin A and untreated conditions, more Suv420H2 remains associated with chromatin following Nek2 inhibition. In contrast, Calyculin A treatment results in reduced chromatin bound Suv420H2, when compared to either untreated cells or those treated with a Nek2 inhibitor. My western blot is consistent with the previous MQP data (Shell, 2023).

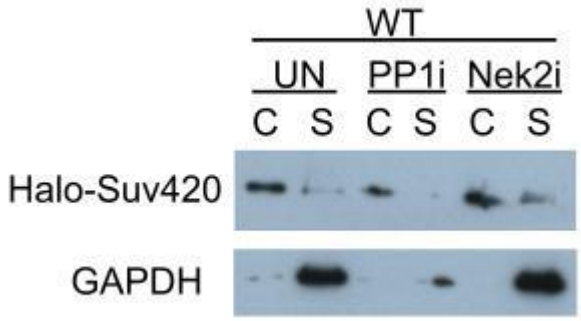

Fig 4 Suv420-Halo fractionation western blot

To understand how individual putative Nek2 phosphorylation sites might influence Suv420H2 localization, I next looked at site specific phosphorylation because Suv420 localization during mitosis has been shown to be sensitive to changes in phosphorylation. The Suv420-Halo cell line was mutated so that Suv420 has the mutation S355A, mimicking the unphosphorylated state, and S355D, mimicking the phosphorylated state. What I see from my western blots is that the S355D untreated shifts over to the soluble fraction. As for the S355A

mutation, when untreated it is hard to see a super noticeable shift. However, when treated with PP1i there is some Suv420-Halo in the soluble fraction compared to the almost no protein in the wild type Suv420-Halo. The Nek2i treated S355A cells show a slight shift of protein onto chromatin but still have quite a bit of soluble Suv420-Halo when compared to the wild type of fractionation results.

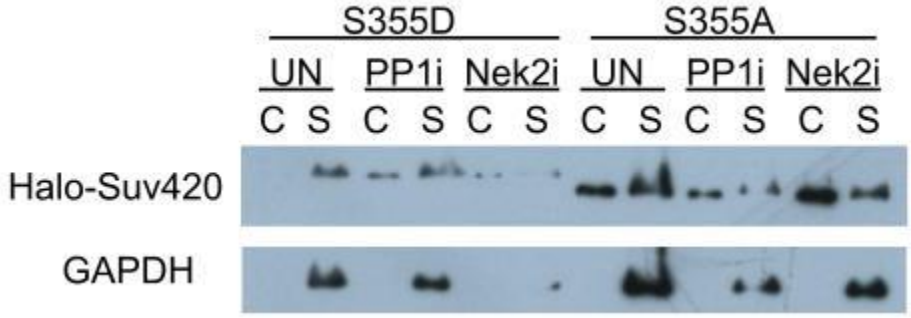

Fig 5 Site specific mutation western blot experiment

To further investigate the localization of Suv420H2 I next used immunofluorescence imaging to capture mitotic cell images and visualize Suv420 localization within a cell. By staining for DNA and the FLAG tag I can visualize and quantify the staining intensity of the protein. There is a clear increase of Suv420-FLAG intensity on chromatin with the Nek2i treatments. The treatment of CalcA shifted Suv420-FLAG off of chromatin and decreased the intensity levels on chromatin. From the plotted data just looking at mitotic cells has a large spread between replicates and within replicates (Fig 6b). In parallel to the above experiment, I also included cells treated with MG132 to arrest cells in metaphase to synchronize the mitotic cells I captured. This decreased the amount of spread within replicates, however there is still some spread between replicates. The data shows a clearer shift of Suv420-FLAG onto chromatin when treated with Nek2i and a shift off of chromatin when treated with CalcA (Fig 6c).

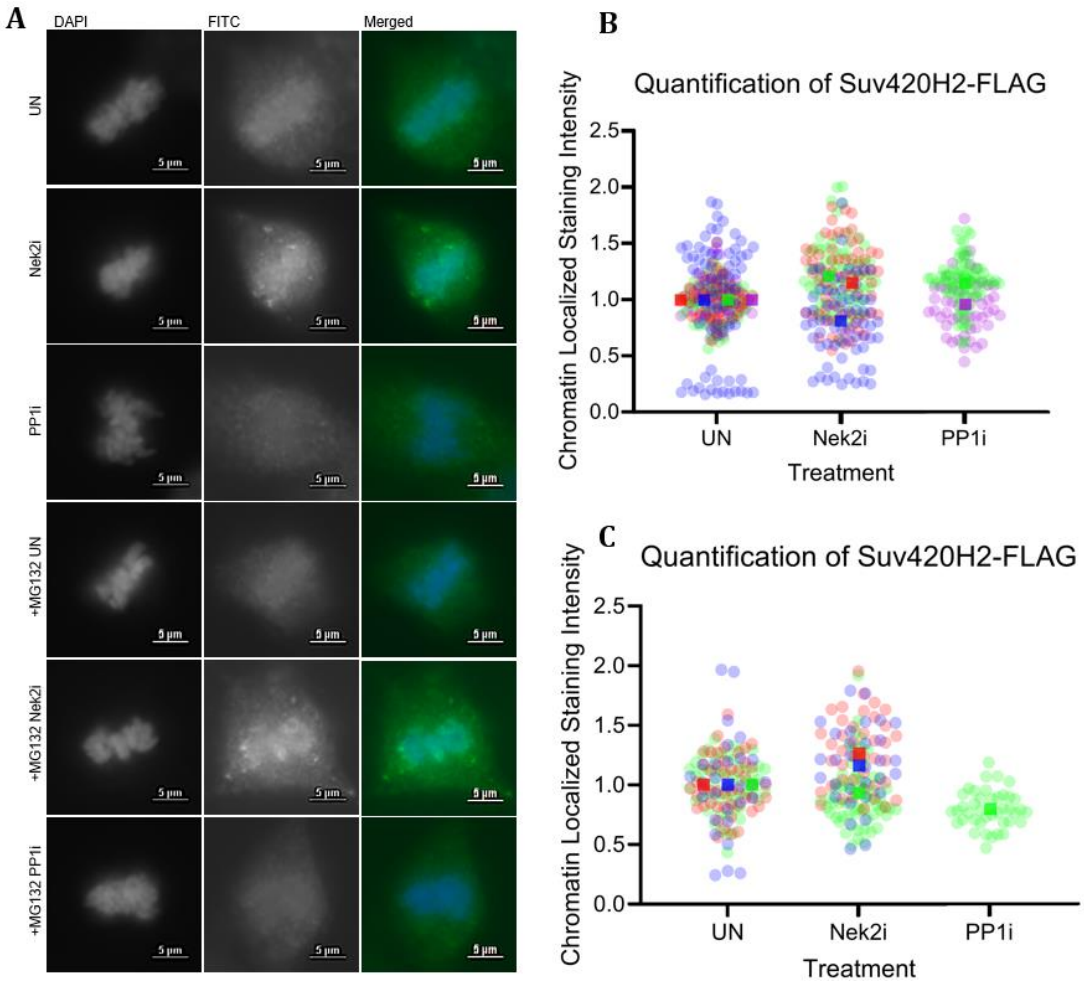

Fig 6 Suv420H2-FLAG RPE cell imaging and quantification. a) Representative images of IF experiments staining for FLAG in FITC. b-c) Individual cells (circles) normalized to the untreated average for each replicate with replicate averages (squares). Rep 1 is blue, Rep 2 is red, Rep 3 is green, and Rep 4 is pink.

Both the fractionation experiment and immunofluorescence imaging show Nek2 inhibition caused more Suv420H2-Halo and Suv420-FLAG to localize on chromatin and that it is still functional even tagged on different terminus. The inhibition of protein phosphatase 1 (PP1) with Calyculin A (CalcA) treatment leaves Suv420H2 in a more highly phosphorylated state and this CalcA treatment caused a shift in Suv420-Halo and Suv420H–FLAG off of chromatin. Suv420H2 association with chromatin is sensitive to its phosphorylation state.

Discussion and Future Directions

The fractionation and western blot data would be more convincing if there was an obvious shift between the chromatin bound and soluble fractions as well as having a loading control for the chromatin bound fraction (Fig 4). It is also important to note the load control GAPDH shows the amount of protein is not equal between the samples loaded. This data has been seen in the previous MQP student's western blots giving more confidence to our data (Fig 4) (Shell, 2023). Having different people performing the same experiment and having similar observations gives more confidence that this phenotype is there.

Looking at site specific mutations for the S355D untreated we expect to see similar results as the wild type PP1i treated samples if this is an important phosphorylation site. The S355D untreated shifted over to the soluble fraction (Fig 5). As for the S355A mutation, when treated with PP1i there is some Suv420-Halo in the soluble fraction compared to the almost no protein in the wild type Suv420-Halo. The Nek2i treated S355A cells show a slight shift of protein onto chromatin and still have quite a bit of soluble Suv420-Halo when compared to the wild type fractionation results. These fractionation results suggest that this site might be responsible for localization of Suv420 and more strongly suggests that Suv420 is sensitive to changes in Nek2 kinase phosphorylation. From these we can reasonably conclude that Suv420H2 localization is sensitive to changes in phosphorylation state (Fig 4 and 5).

The complementary assay, immunofluorescence imaging, backs up the data from the western blots images that Suv420H2 localization is sensitive to changes in phosphorylation state and floors the predicted changes (Fig 6a).

During drug treatment for immunofluorescence, I was having issues keeping the cells treated with Calyculin A (CalcA) adhered to the coverslips even after spinning down the cells in a centrifuge. I found that the PP1i treatment was extremely time sensitive and if off by half a minute the cells would not adhere to the coverslips properly due to CalcA having multiple targets and a wide range of effects on cells and not just the phosphorylation we are looking into. It would be great to get more replicates of CalcA treated cells to be able to perform statistical analysis on the data.

Throughout this process I moved quickly and found over time I got more efficient with my movements throughout the protocol. Another issue was not using another antibody as a staining control as I wanted to be able to use the DM1α antibody, however we ran out of the

version that was made in rabbit. This is important because the anti-FLAG antibody was made in mice, and I want to prevent cross staining between light channels so having a different species for the antibody would prevent this. Having microtubules stained with the DM1α antibody would make the data more robust and make it easier to find mitotics that are aligned and can be used to normalize the intensity of the FLAG staining. It would also be good to look at the site-specific mutants with immunofluorescence to have more data with a consistent assay.

It might also be a good idea to think about trying out ELISAs to quantify the amount of Suv420 within a fractionation sample. Future directions this research can take is looking into new mutations in predicted phosphorylation sites in Suv420 and start to combine these mutations together to see what the combined effect of these mutations will do to change Suv420's localization patterns.

Knowing what kinases phosphorylate and regulate Suv420 can help us find possible drug targets for aneuploidy diseases and cancer as increased Suv420 was found to be positivity correlated to aggressive cancer (Herlihy et al., 2021). Nek2 is a known mitotic kinase (Fry, 2002) and when overexpressed can lead to tumorigenesis and drug resistance by activating several oncogenic pathways (McCrury et al., 2024). Both the location and known effect on chromosome segregation is a great kinase to look at and my data supports that it regulates Suv420H2 localization during mitosis. The inhibition of Nek2 led to changes in Suv420H2 localization in both my western blots and cell imaging experiments.

The regulation of Suv420 expression and activity are still not well described in literature even with many examining changes in Suv420 expression driving cancer development and progression. Studies find that Suv420 is either overexpressed/overactive and silences tumor suppressor genes, or that Suv420 is inactivated in cancer, and results in an overall loss of H4K20me3 at the telomeres and on many oncogenic genes (Gabellini & Pedrotti, 2022).

The elevation of Nek2 is correlated with malignant transformation of tumors in cancer and progression of tumors. This could mean that in cancer Suv420 is more highly phosphorylated and shifted off of chromatin leading to the loss of H4K20me3 markers. Decreased H4K20me3 has been shown to lead to poor patient prognosis in breast cancer patients (Yokoyama et al., 2014). The changes in regulation of Suv420H2 localization both more on chromatin and off of chromatin lead to poor patient outcomes (Herlihy et al., 2021) (Yokoyama

et al., 2014) so understanding this mechanism and being able to target it could lead to better cancer patient outcomes in the future.

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