Metaphase Chromosome Alignment is Sensitive to Dynein-Dependent Forces at the Cell Cortex

A Major Qualifying Project

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Abstract

Mitotic cell division is the process by which one cell is divided into two. During mitosis a microtubule-based machine is built to organize and physically separate the duplicated mitotic chromosomes. Defects in the mitotic machinery, or in the way microtubules associate with the chromosomes can result in the formation of daughter cells with too many or two few chromosomes. Such changes can compromise cell viability and organismal fitness.

Formation of the microtubule-based machine, known as the mitotic spindle, is dependent on the function of molecular motors that walk along and organize the microtubule filaments into a dynamic bipolar structure. Chromosomes interact with the mitotic spindle via protein structures that are built on chromatin called kinetochores. Chromosome movement during mitosis, the satisfaction of the spindle assembly checkpoint that governs mitotic exit, and the fidelity of chromosome segregation are all dependent on the formation and maintenance of proper kinetochore- microtubule attachments.

Experimental depletion of Nuf2, a kinetochore protein that promotes stable end-on microtubule attachments, prevents chromosomes from congressing to the metaphase plate and precludes progression to anaphase. Here, I show that simultaneous depletion of Nuf2, together with inhibition of dynein-dependent pulling forces at the cell cortex, enables a partial rescue such that chromosome alignment is improved and progression into anaphase can occur. Together, this work demonstrates that stable kinetochore-microtubule attachments are antagonized by the cortex-localized motor protein dynein.

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1.0 Background

1.1 The Process of Cell Division

Cell division (mitosis) occurs every day in the human body and is essential for host survival. It is the process by which cell number and diversity is increased through symmetric and asymmetric division. Symmetric cell division occurs when a single cell creates two identical daughter cells for clonal expansion whereas asymmetric division creates distinct daughter cells for diversity. How this division occurs is dictated by the orientation and positioning of the mitotic spindle within the cell. To achieve the creation of two identical daughter cells in symmetric mitosis, the mitotic spindle must be positioned at the center of the dividing cell.[1] For the cells to successfully duplicate and transfer genetic material, two crucial events must be completed: chromosome alignment and segregation.

1.1.1 Spindle Formation is Orchestrated by Motor Proteins and Dynamic Microtubules

The formation of the mitotic spindle is essential for chromosome alignment and segregation[2]. Major components of the spindle include microtubules that extend to the kinetochores of chromosomes (K-fibers), overlap in an antiparallel fashion within the central spindle (interpolar microtubules), and extend away from the spindle towards the cell cortex (astral microtubules)[3], along with two spindle poles (centrosomes) where chromosomes are eventually pulled towards (Figure 1).[4] Microtubules assemble the spindle, forming attachments to both kinetochore regions present at the center of sister chromatids and two organelles positioned on opposite poles of the cell (centrosomes).[5] K-fibers are bundles of parallel microtubules that form stable end-on attachments to kinetochores, linking chromosomes to the spindle poles. The formation of these fibers is critical for alignment and segregation to occur[2]. Simultaneously, there are lateral microtubule attachments that can occur in which chromosomes are captured along the walls of microtubules and then later tethered to the ends of microtubules to form the end-on attachments. This conversion from lateral to end-on attachment is critical as an end-on attachment is needed to sustain attachments during microtubule growth and shrinkage that generate the pulling forces on the chromosome.[6]

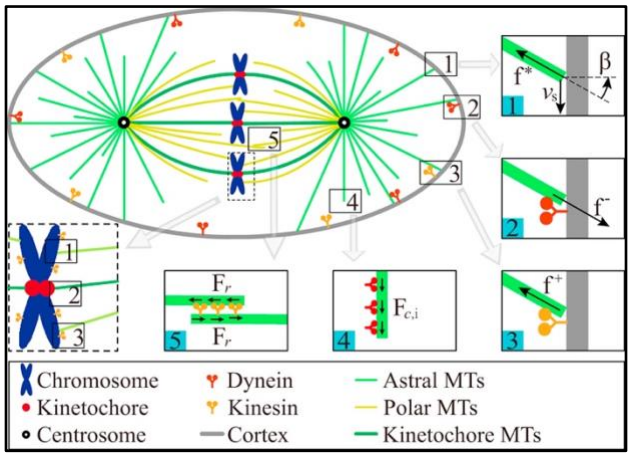

Figure 1. Schematic of the five mechanical forces acting on the mitotic spindle including three pushing forces (insets 1, 3, 5) and two pulling forces by cortical and cytoplasmic dynein (insets 2, 4).[4]

Microtubules are polar and orient with their stable minus-end at centrosomes (the spindle poles) and their dynamic plus-ends radiating outwards. The extension and dynamics of the plus-ends of microtubules contributes to bipolar structure and biorientation of chromosomes through

interaction with the cell cortex (astral), other microtubules (interpolar), and kinetochores of chromosomes (K-fibers) (Figure 1).[3] During prometaphase, the plus-ends of single K-fibers extend from a centrosome on both sides of the spindle and attach to kinetochores of duplicated chromatids. This results in biorientation of the chromosomes and congression to the center of the spindle. Once this alignment happens to all the chromosomes, the cell is in metaphase.

1.2 Dynein Motor Activity Drives Spindle Organization and Orientation

There are a variety of molecular motors acting on the microtubules while they undergo an intrinsic activity known as dynamic instability in which microtubules grow (polymerize) and shrink (depolymerize) at their plus ends in search for kinetochore attachments. This dynamic instability of microtubules is resolved upon the tension created once kinetochores are bound. At the same time, motor proteins work to create a bipolar spindle and position it in the center of the cell utilizing.[4] The five prominent force generation mechanisms exhibited in Figure 1 include the pushing force caused by the polymerization of microtubules (Inset 1), the pulling force of cortical dynein (Inset 2), the pushing force of cortical kinesin (Inset 3), the pulling force of cytoplasmic dynein (Inset 4), and the pushing force kinesin on antiparallel microtubules (Inset 5).[4]

Cortical dynein is a minus-end directed motor protein that is specifically responsible for spindle orientation (Figure 2). It is recruited and tethered to the cellular cortex via the Gαi-LGN-NuMA protein complex (Figure 2) to generate these pulling forces during metaphase.[1] Loss of LGN from this complex release's dynein, destabilizing spindle orientation and removing one of the major forces exerted on chromosomes.[4] This dynein dependent force helps drive centrosome movement and spindle positioning. Since k-fibers are anchored at spindle poles, it is reasonable to assume that forces that drive centrosome/spindle pole movement would transmit to k-fibers and their associated kinetochores.[4]

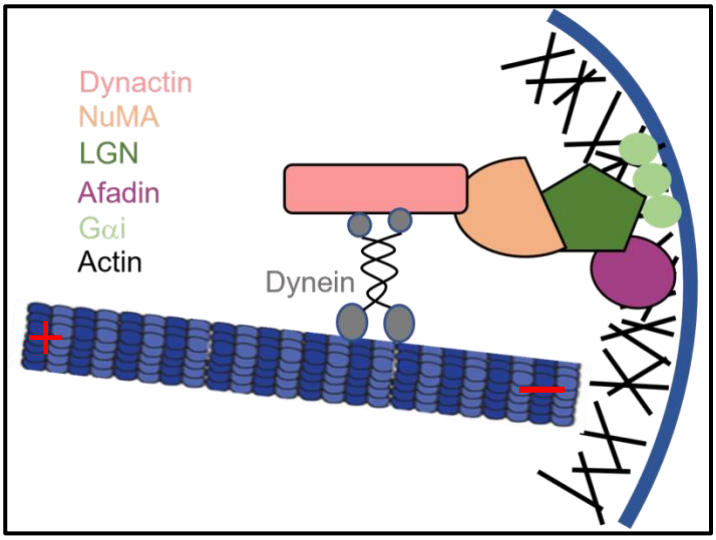

Figure 2. Cortical Dynein Interaction with Microtubules Cortical dynein (gray) is bound to a microtubule (blue) and the LGN (dark green)/NuMA(orange)/ Gαi(light green) complex tethers the dynein to the cell cortex (blue). Image created by Dayna Mercadante.

1.3 Stable End-On Microtubule Attachment with Kinetochores Drive Chromosome Movement

Chromosome alignment in metaphase and segregation in anaphase is driven primarily by the depolymerization of microtubules. Initially, molecular motors crosslink minus-ends of

microtubules which anchors them to spindle poles and induces sliding which contributes to self-organization and assembly of the spindle poles.[6] This motor-driven self-organization is needed when kinetochores actively generate forces on K-fibers.[7] Similarly, forces that drive the centrosome positioning can be transmitted via K-fibers to kinetochores/chromosomes as chromosomes transduce forces from the dynamic microtubules.[6] In addition, microtubules typically depolymerize at their minus-ends and shift between polymerizing and depolymerizing at plus-ends. ATP-independent coupling activity of kinetochore-associated motor proteins mediates chromosome attachment to plus-ends of dynamic microtubules. Thus, the combination of the depolymerization and motor coupling activity generates a pulling force strong enough to move the chromosome pulling it poleward.[8]

The poleward movement of chromosomes is countered by biorientation where the kinetochores of a cohered pair of chromosomes is attached to opposite spindle poles, allowing for a balance of forces pulling poleward and alignment of the chromosome pair at the equator of the cell. Forces generated by chromosomes interacting with astral microtubules promotes positioning that favors biorientation. Polar ejection forces occur when plus ends of astral microtubules engage with plus-end directed motors, called chromokinesins, that are localized on chromosome arms. These forces push chromosomes away from spindle poles. Interactions of kinetochore-localized plus-end directed motor proteins, like CENP-E, with the lateral side of microtubules also function to move chromosomes away from spindle poles and towards the equator of the cell. Each of these forces are transient and alone are insufficient to drive chromosome alignment but instead increase the frequency at which a monoriented (attached to microtubules from a single spindle pole) chromosome pair might become bioriented.[7]

The Nuf2 protein is part of a heterotetrametric complex that localizes to the mitotic kinetochore and is responsible for mediating end-on microtubule attachments necessary for chromosome alignment and segregation (Figure 3).[9] This heterotetramer complex, illustrated in Figure 3, contains Nuf2 and Ndc80 at the head of the complex positioned to directly bind to the microtubules.[10] Additionally, Spc24 and Spc25 are responsible for localizing the complex to the kinetochores through interactions with other kinetochore proteins (Figure 3).[10] Individual human kinetochores recruit about 240 copies of this complex (14-20 copies per microtubule, for each of 20-25 microtubules that bind at the kinetochore).[11] The Nuf2 complex helps anchor the microtubule to the kinetochore, allowing it to resist pulling forces associated with chromosome movement.[11] In the absence of Nuf2, the Ndc80 complex is unstable and mitotic kinetochores are not able to sustain end on microtubule

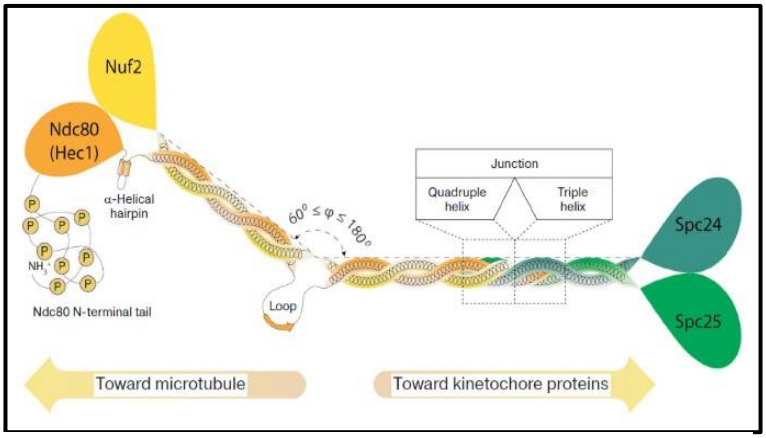

Figure 3. Ndc80 complex structure indicating the presence of four major subunits including the Nuf2 subunit on the end attaching to the microtubule.[11]

attachments.[11] The absence of stable end-on microtubule attachments precludes chromosomes biorientation and alignment.[11]

1.4 Spindle Assembly Checkpoint

The spindle assembly checkpoint (SAC) is responsible for monitoring chromosome biorientation and delaying mitotic progression until all kinetochores have formed stable end-on bioriented attachments that drive chromosome alignment during metaphase. Defects in spindle formation or K-fiber formation prevent satisfaction of the SAC and prevent activation of the anaphase-promoting complex, preventing the cell from moving forward into anaphase. Cells satisfy this checkpoint by the formation of stable end-on attachments that allow for depletion of kinetochore-localized checkpoint proteins and the recruitment of microtubule-plus end associated proteins to the kinetochore. Additionally, the formation of end-on attachments at both kinetochores of a bioriented pair of chromosomes results in tension between the centromere and the kinetochore.[12]

In early mitosis, a complex of the microtubule-depolymerizing kinesin Kif2b and CLASP1 is localized at kinetochores where it stimulates microtubule turnover and correction of microtubule attachment errors. As stable end-on microtubule attachments with kinetochores are formed, this complex is displaced from kinetochores and replaced by an astrin-CLASP1 complex. Localization of the astrin-containing complex corresponds with increased microtubule stability, chromosome congregation to the spindle equator, and satisfaction of the SAC. Together, this differential localization of Kif2b and astrin help define mitotic progression and regulate microtubule attachments.[13] Astrin requires intact Microtubules to attach to and be maintained at kinetochores, therefore astrin localization at kinetochores is an indication of stable microtubule attachment.[14]

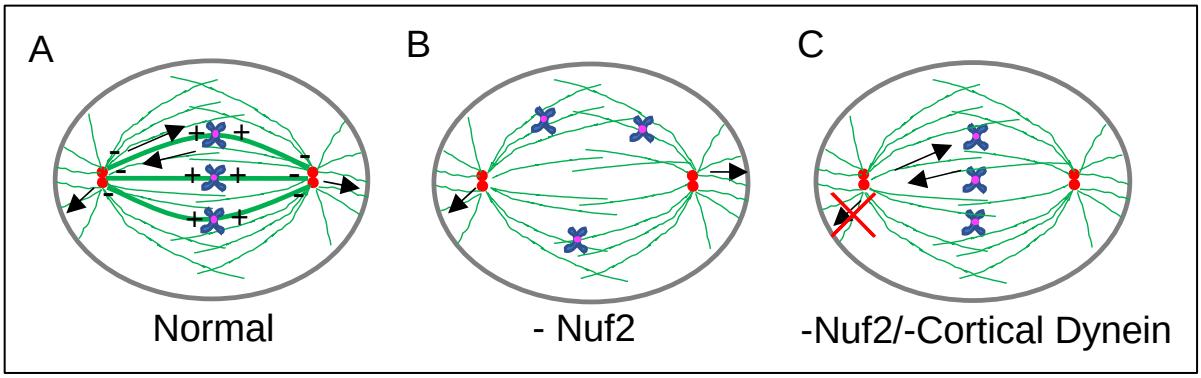

Figure 4. Cartoon Depiction of the Experimental Model to be Tested
(a) A normal cell is shown in which plus and minus-ends of microtubules are indicated along with the cell cortex (gray), K-fibers (thick green lines), astral microtubules (thin green lines at the cortex), and interpolar microtubules (thin green lines at the center), chromosomes (blue), kinetochores (pink), and centrosomes (red). The arrow at the cortex indicates cortical dynein pulling force and the arrows on the K-fiber represents the dynamic push/pull force of microtubules. (b) A Nuf2 depleted cell with no K-fiber formation. (c) A Nuf2 and cortical dynein depleted cell lacking pull forces and K-fibers.

In a normal cell, stable attachments are made at the kinetochores (the Ndc80 complex is intact and astrin is present), the attachments can withstand the pulling forces generated during spindle formation and orientation, and chromosomes can congress to the center of the cell to achieve metaphase chromosome alignment (Figure 4A).[4] Previous work has shown that, in the absence of Nuf2, K-fibers are destabilized by pole-focusing forces that pull on microtubule minus ends (Figure 4B).[7] Here, I test whether forces generated on astral microtubules by cortex-localized dynein activity might transmit, via minus ends focused at spindle poles, to kinetochore microtubules and similarly antagonize K fiber stability (Figure 4C).

2.0 Results

2.1 Disruption of Cortical Dynein Restores Chromosome Alignment in Nuf2 Deficient Cells

Stable, bioriented kinetochore attachments are necessary to drive chromosome movement and enable metaphase chromosome alignment. Cells in which kinetochore microtubule attachments have been destabilized cannot sustain attachments that drive chromosome movement and cannot achieve chromosome alignment during metaphase. To examine the role of cortical dynein dependent forces in destabilizing kinetochore fibers I first used siRNA-based approaches to deplete Nuf2 alone, or Nuf2 together with LGN from a Retinal Pigment Epithelial (RPE-1) cell line. Cells were transfected, then fixed and stained for immunofluorescence analysis following 48h of depletion. qPCR was performed to measure relative abundance of GAPDH, LGN and

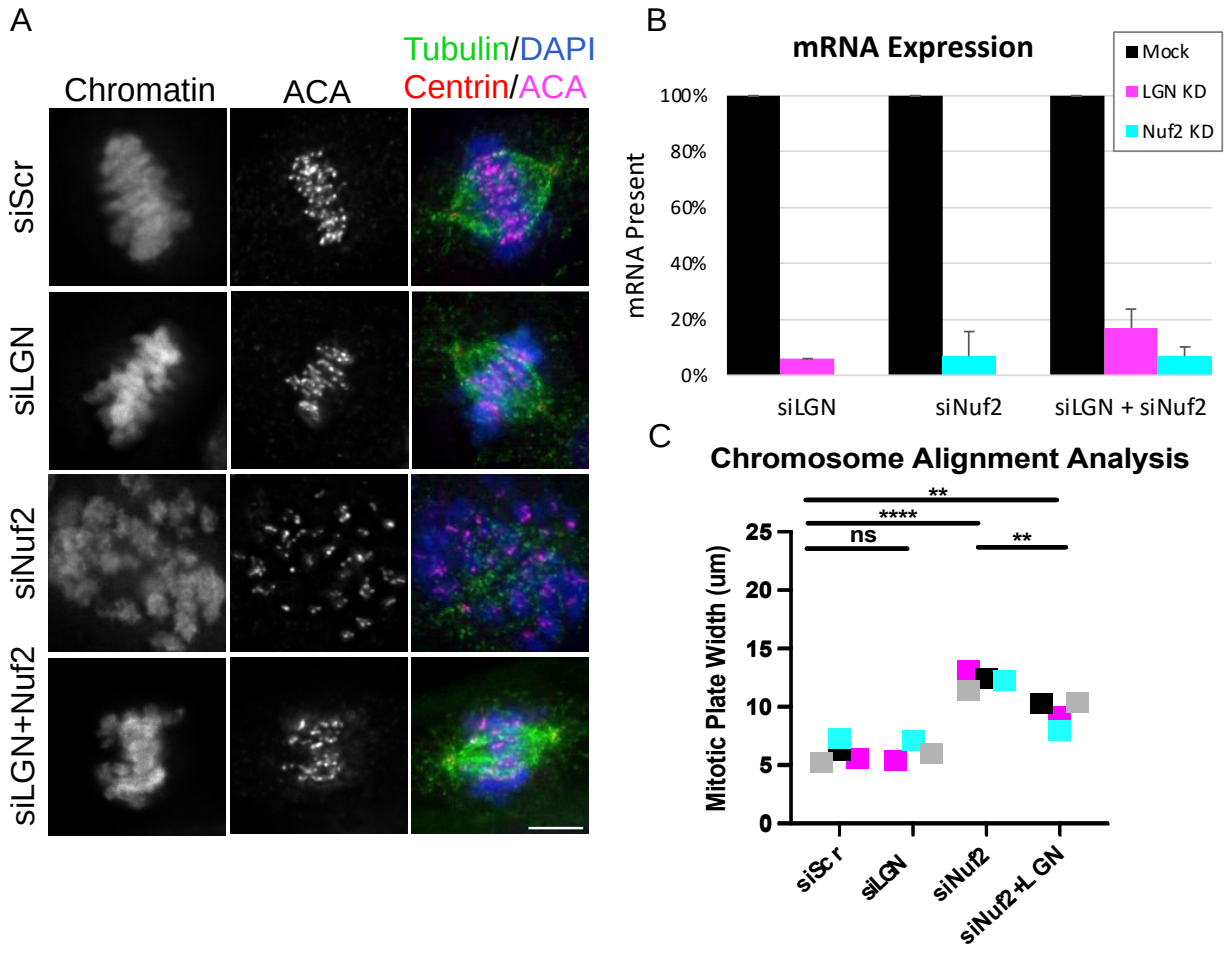

Figure 5. Chromosome Alignment Improves in the Double Knockdown Condition
(A) Immunofluorescence imaging indicates chromatin (blue) and kinetochore (pink) compaction, and varying widths is shown across single and double knockdown conditions. The scale bar represents 5uM. (B) Confirmation of single and double knockdowns was achieved through quantification of mRNA expressed through qPCR. (C) Mitotic plate widths were recorded and an unpaired t-test with n=4 was performed to indicate significant improvement in alignment.

Nuf2 mRNA in each experimental condition. The siLGN condition exhibited a 94% reduction of LGN mRNA, the siNuf2 condition exhibited a 93% reduction of the Nuf2 protein, and the siNuf2/LGN exhibited an 83% and 93% reduction in LGN and 7% Nuf2 mRNA, respectively (Figure 5B).

Next, fifty cells from each individual knockdown condition and the double knockdown condition, for each of four independent biological replicates were analyzed for chromosome alignment. (Figure 5A). The mitotic width of the metaphase plate in each cell for all four conditions was measured and recorded utilizing the farthest kinetochore from the plate on either side to mark a distance (Figure 5C). On average, the siScr control cells had a mitotic plate width of 6.02um +/- 0.91um and the siLGN condition followed a similar pattern with a width of 6.11um +/- 0.86um. As previously described[7], I found that chromosomes in cells depleted of Nuf2 failed to congress to a metaphase plate and were instead dispersed throughout the cell with an average span of 12.26um +/- 0.69um. In contrast, the double knockdown condition (siNuf2/siLGN) showed partial rescue of chromosome congregation with a metaphase plate width of 9.39um +/- 1.12um across the replicates. An unpaired t-test with n=4 was performed to determine if this difference in widths was statistically significant. The slight difference in width of the siScr and siLGN conditions was not significant, while the difference between siScr and siNuf2 was highly significant. The difference between the siNuf2 and siNuf2/siLGN conditions was significant ($p=0.0048$), however, the difference between siScr and siNuf2/siLGN conditions was also significant ($p=0.0034$), indicating that the Nuf2/LGN double depletion achieved an incomplete rescue of the deficiency in chromosome alignment.

This data denotes that in the absence of both Nuf2 and cortical dynein, there is an improvement in chromosome alignment when compared to the single Nuf2 depleted condition. However, the mechanism that allows this to occur is unknown. Whether this was due to chance positioning of chromosomes, polar ejection forces, weak lateral kinetochore microtubule attachments, or stable microtubule formation allowing for the chromosomes to align was still to be determined.

2.2 Disruption of Cortical Dynein Activity Enables Anaphase Progression in some Nuf2 Deficient Cells

To next test if improved alignment is indicative of stabilized kinetochore microtubule attachments that could promote SAC satisfaction and progression into anaphase, I examined asynchronous cell populations for evidence of mitotic progression and anaphase figures. To assess mitotic progression, fifty mitotic cells per population, for each of four biological replicates, were imaged and their mitotic stage assessed based on chromatin structure, bipolar spindle formation, centrosome placement, and kinetochore alignment (Figure 6).

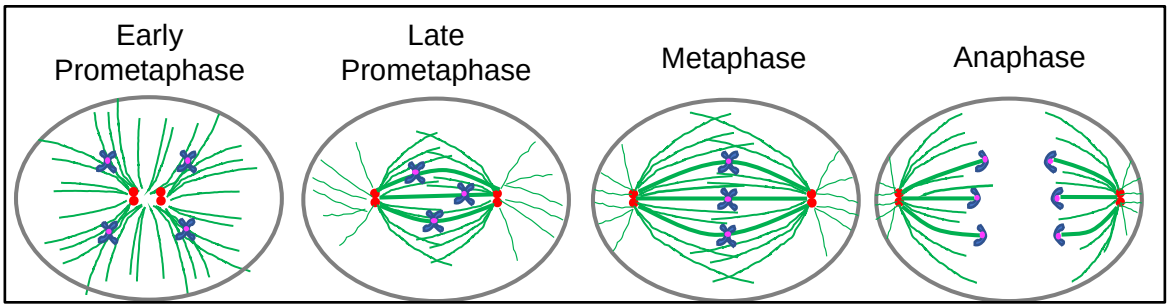

Figure 6. Schematic of Markers of the Scored Stages of Mitosis

In Figure 7A, cells in early prometaphase were identified as having chromosomes with distinct rod-like elements, often chromosomes and kinetochores oriented in a ring with chromosome arms extending outward away from the center or appear as an H-shaped mass with invaginations from two sides (i). Similarly, for late prometaphase, chromosomes begin taking the shape of the metaphase plate moving towards the center of the cell, however, kinetochores are still yet to be tightly aligned. Here a small, bipolar spindle has begun formation with thin microtubules present (ii).[15] Metaphase cells were defined as having chromosomes and kinetochores tightly aligned to the spindle equator forming the metaphase plate. Thick bundles of microtubules are apparent (K-fibers) and the centrosomes have made their way to opposing sides of the cell (iii). Lastly, anaphase cells were defined as those with their chromosomes separated, positioned on the two sides of the spindle equator. There was a distinct cluster of both chromosomes and kinetochores present at both spindle poles. The spindle is elongated and a midzone may be visible (iv).[15]

Using the criteria above, I found that approximately 30 % of mitotic cells in the siScr population exhibit a bipolar spindle with little to no chromosome alignment, indicative of prometaphase. An additional 30% of mitotic cells in this population exhibit a bipolar spindle with full chromosome alignment, indicative of metaphase, and 25% more were in anaphase, with chromosome already having begun to be segregated. The distribution of siLGN treated cells was not statistically different from that of the siScr control condition. In contrast, siNuf2 treated populations had comparatively few metaphase and anaphase cells (5% and 1%, respectively) and a corresponding increase in prometaphase cells (82%). Consistent with results showing that LGN co-depletion with Nuf2 rescued the Nuf2-depletion phenotype for chromosome alignment, assessment of mitotic distribution showed a decrease in prometaphase (70%) cells and an increase in metaphase cells (18%) in this condition. Surprisingly, I also observed a 5-fold increase in the frequency of anaphase cells in LGN/Nuf2-codepleted populations compared to Nuf2 depletion alone (Figure 7B).

A two-tailed t-test with n=4 was performed to determine if any differences were statistically significant. When comparing the Nuf2 and Nuf2/LGN co-depleted conditions, there was no significance in the difference of late prometaphase cells and metaphase cells, however there was a significant difference in the number of anaphase cells present in the two conditions ($p=0.0196$) (Figure 7B). Note that the error bars in the late prometaphase and metaphase conditions are relatively large, therefore, if more cells were to be scored, it is reasonable to expect that those differences may be significant as well.

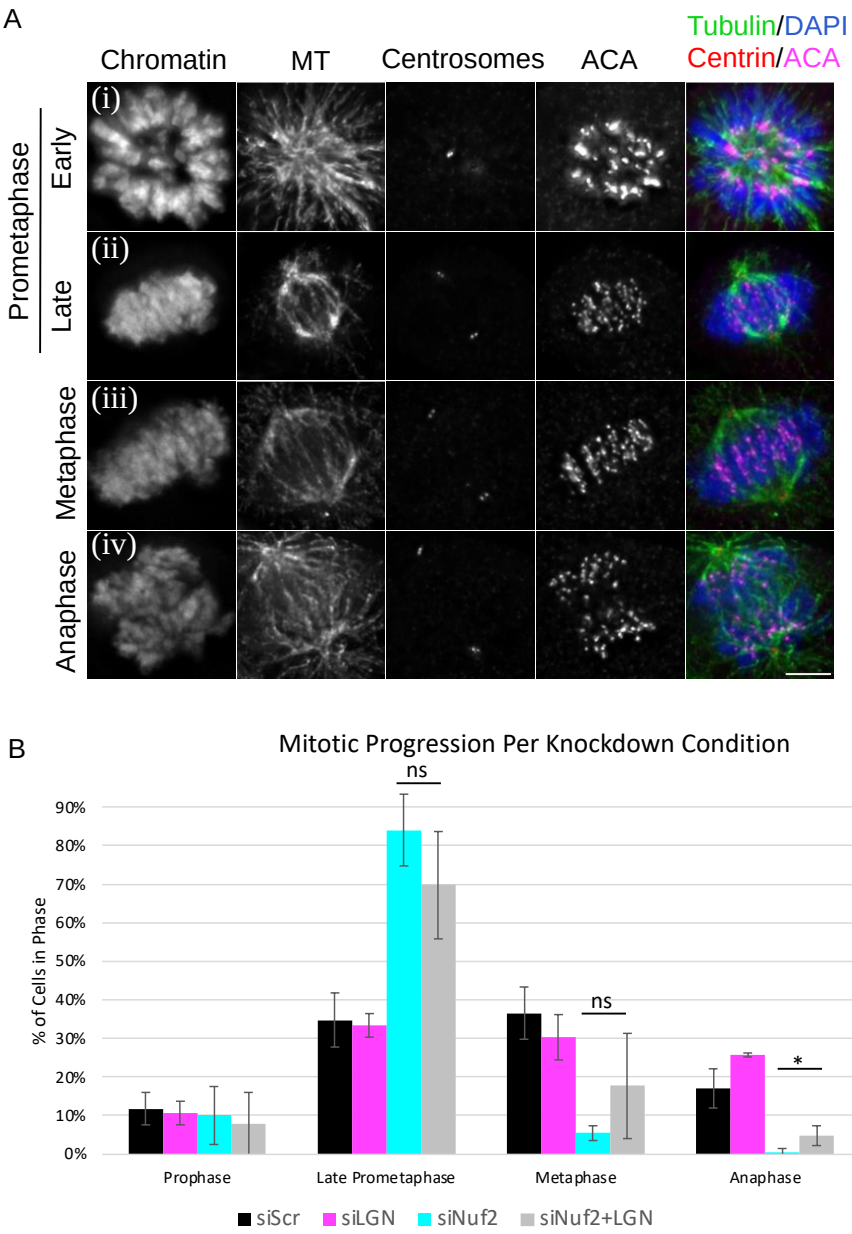

Figure 7. Increase in Anaphase Progression in Double Knockdown Condition
(A) Immunofluorescence imaging indicates scored mitotic stages visualized from early prometaphase, late prometaphase, metaphase, and anaphase cells. The first four columns represent images from individual fluorescent channels, whereas the final column shows an overlay of blue (chromatin), green (microtubules), red (centrosomes), and pink (kinetochores). The scale bar represents 5uM. (B) 50 cells per condition were scored and a two-tailed t-test was performed with n=4 to indicate significant progression into anaphase.

This data shows that in the double knockdown condition where there is an increase in alignment, more cells progressing to anaphase compared to the single Nuf2 depleted condition. This indicates that there may be stable kinetochore microtubule attachments being made that are allowing the chromosomes to move to align, satisfy the SAC, and segregate during anaphase.

Alternatively, the cells may be finding a way to bypass the spindle assembly checkpoint in the absence of stable microtubule attachments to kinetochores.

2.3 Stable Connections might not Contribute to the Increase in Segregation

The final phase of this project focused on understanding if cells in this "rescue" double knockdown condition were able to form stable microtubule attachments in the absence of the

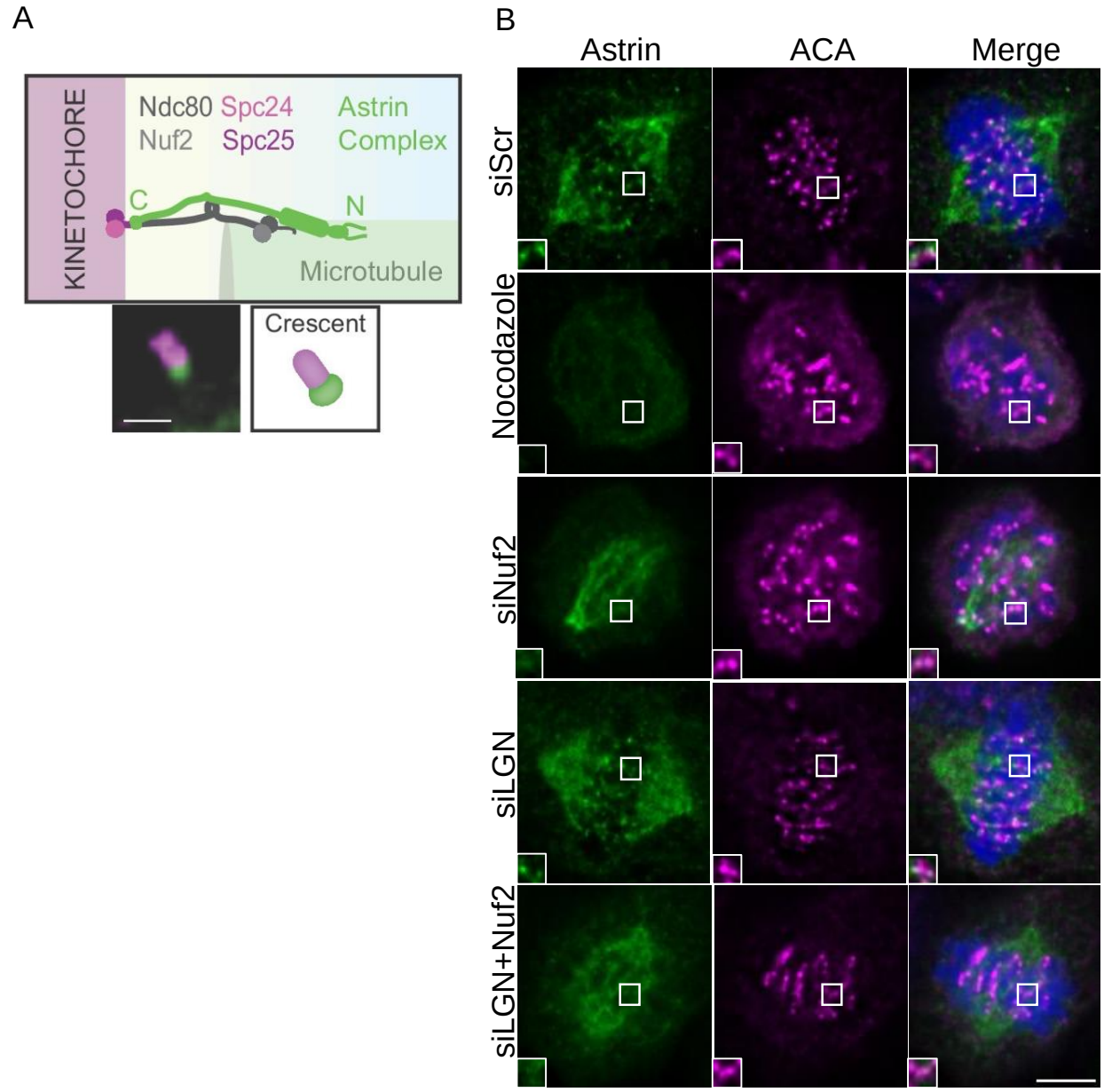

Figure 8. Astrin Foci are not Distinct in Double Knockdown Conditions
(a) Cartoon of Astrin's interaction with the Nuf2 heterotetramer and a visualization of astrin foci (green) positioning on kinetochores (pink). Figure adapted from Shrestha et al. 2017. (b) Immunofluorescence imaging indicates presence or absence of Astrin (green) at the ends of microtubules (pink) across the single and double knockdown conditions. Insets indicate single kinetochore pairings (pink) to magnify the presence or absence of Astrin foci (green). The scale bars represent 5uM.

Ndc80 complex. In other words, determine if chromosomes are aligning and segregating due to normal cellular machinery working properly or if they can do so using weak attachments such as astral or lateral microtubule attachments. I hypothesized that, in the absence of cortical dynein, a strong pulling force on the cell, kinetochore microtubule attachments may persist, even in the absence of Nuf2.

To test this hypothesis, Astrin antibody was utilized for immunofluorescence imaging to determine if it is present in the double knockdown condition. Astrin forms a bridge between the kinetochore and microtubule with the C terminus of astrin located proximal to the Nuf2/Ndc80 complex next to the kinetochore (Figure 8A).[6] Astrin is an indicator of microtubule stability and promotes proper chromosome alignment, therefore if the improvement in alignment and increased progression into anaphase is due to stable end-on kinetochore microtubule connections being made, Astrin would be expected to be present at the ends of kinetochores in the double knockdown conditions (Figure 8A).[13]

To test this hypothesis, chromosome distribution and astrin staining patterns were quantified in 30 cells per siRNA treated condition, for each of 3 biological replicates. Distinct Astrin foci were present in both the siScr control condition and the siLGN condition. These observations are consistent with Nuf2 promoting stable microtubule connections in both control cells and in cells without cortical dynein (Figure 8B). A nocodazole treated population of cells was used as a negative control for Astrin staining. Nocodazole induces depolymerization of all microtubules, including kinetochore fibers and precludes Astrin recruitment to kinetochore. As expected, the nocodazole treated cells show no kinetochore-localized Astrin foci. In the Nuf2 depleted and Nuf2/LGN co-depleted conditions, astrin staining was apparent on spindle fibers, like that seen in control cells. However, similar to the nocodazole treated negative control, no distinct Astrin foci were present at ACA-stained kinetochores in the double knockdown or single Nuf2 knockdown conditions, indicating that microtubule attachments, if stable, are not sufficiently robust to recruit Astrin localization (Figure 8B).

3.0 Conclusions and Future Directions

Nuf2 and cortical dynein are critical proteins involved in the chromosome alignment and segregation process. With Nuf2 responsible for end-on microtubule attachments and cortical dynein responsible for pulling from the cellular cortex. Absence of these proteins individually makes these processes unable to occur without error. It is hypothesized that upon pulling from cortical dynein, any weak attachments formed, such as lateral attachments, are broken and chromosomes cannot be segregated and moved towards centrosomes. However, there is potential that these weak attachments can remain intact in the absence of both Nuf2 and cortical dynein. My data suggests that in the simultaneous absence of both proteins, there is a "rescue" phenotype in which the capacity for directed chromosome movements and chromosome progression to the metaphase plate are restored. Similarly, in the double knockdown condition, this improvement in alignment does have a significant impact on the number of cells progressing into anaphase. This suggests that cells may either be forming stable microtubule attachments which are allowing them to progress, or they are bypassing the SAC checkpoint without satisfaction to progress. Thus far, it appears that cells in the double knockdown condition are not forming stable end-on attachments as Astrin is not present at bioriented kinetochores which indicates that the SAC has not been satisfied. Therefore, cells that progress to anaphase may be doing so by bypassing the checkpoint. Though this project does not demonstrate concrete mechanisms for this "rescue" phenotype, it provides preliminary data that can be used to define new hypotheses and guide future research.

First, if this project were to be continued, there should be further assessment of K-fiber formation in the double knockdown condition. I began preliminary cold stable experiments as an attempt to stabilize K-fibers, depolymerize all other Microtubules, and examine the variation in intensity of the K-fibers across conditions. I was unable to successfully visualize k fibers using either cold stable or calcium stable fixation methods. However, following optimization, these approaches may provide valuable insight into K-fiber formation. If it appears that attachments are indeed not stable, it would be important to determine what non-k fiber driven forces are responsible for chromosome alignment? One could investigate whether lateral attachments are made or if polar ejection forces acting on chromosomes may be compensating following the loss of LGN to contribute to proper alignment.

Another focus could be on the SAC and whether it remains active as the Astrin experiment suggests. The same transfection could be performed but instead assessing levels of BubR1, a centromere-localized component of the SAC, by immunofluorescence. This would determine whether kinetochores on aligned chromosomes in the double knockdown condition continue to signal the checkpoint or not. Alternatively, the Manning lab has GFP tagged MAD2 U2OS cell lines that could be utilized to answer this question. MAD2 is another SAC protein that is present when the checkpoint is not satisfied and prevents the cell from progressing into anaphase. Using this cell line, one could image the cells and assess the presence of absence of the protein at kinetochores of aligned and unaligned chromosomes.

A third future direction for this project could include a focus on mitotic progression. Since the experiments in this project indicate more cells may be progressing into anaphase in the double knockdown condition, it is important to know if such anaphase cells are competent to accurately

segregate the chromosomes. Live cell imaging of cells could be performed to enable mitotic timing, mitotic fidelity, and cell fate to be assessed. Such movies could also be used to follow individual mitotic cells to examine chromosome movement to determine if it is intentional or if some are randomly aligning. Similarly, movies could be used to assess whether attachments that enable alignment are robust enough to drive chromosome segregation during anaphase. These experiments would help determine if this “rescue” condition promotes greater cell viability and proper proliferation when compared to the single Nuf2 knockdown.

Overall, there are many questions to be asked following this project. There is much known about the process of successful cell division, however, scientists are still working to understand the mechanism behind defects to this process.

4.0 Methods

4.1 Cell Culture

4.1.1 Thawing RPE-PLK4 Inducible Cells

The vial of frozen cells was warmed in hand. Once thawed, cells were resuspended in 1mL of warmed DMEM complete (DMEM supplemented with 10% FBS and 1% Penicillin/Streptomycin). 8mL of DMEM complete was added to a conical tube along with 1mL of the resuspended cells. The cells were put into the centrifuge and set at 1000 rpm for 5 minutes. Afterwards when the cell pellet was apparent, the supernatant was aspirated, and the pellet was resuspended in 10mL of fresh DMEM complete. Cells were added to a fresh Thermo Fisher 10cm plate and incubated at 37˚C for 48 hours and not passaged until they reached 80% confluency.

4.1.2 Cell Passage

All media and reagents including PBS, Trypsin, and DMEM complete were heated 30 minutes prior to cell culture at 37˚C. Cells were examined to determine confluency (aim for 80% confluency but no more than that to prevent contact growth inhibition). Old media was aspirated out of the plate and 5mL of PBS was added to wash and then aspirated. Trypsin was then added (2mL per 10cm plate) and cells were incubated for 2 minutes at 37˚C. Four times the amount of media to Trypsin was then added to the plate (8mL DMEM complete to 2mL of Trypsin). The whole sample was collected and added to a 15mL conical tube to be centrifuged (5 minutes, 800-1000 RPM). Cells were resuspended in 10mL of media and cells were diluted 1:3 (if they were at 80% confluency and were to incubate for 48 hours). For time spans greater than 48 hours such as 72 hours, cells were split 1:7.

4.2 Nuf2 and Cortical Dynein Knockdown Experimental Design

4.2.1 Transient Transfection of siRNA

For this transfection, DMEM complete (DMEM supplemented with 10% FBS and 1% Penicillin/Streptomycin) and Pen/Strep Free DMEM (DMEM supplemented with 10% FBS) was utilized. 3uL of each siRNA (Figure 6) was added to a 1.5mL epi tube and 100uL of Optimem was added. 18uL of Lipofectamine was added to a 1.5mL epi tube and 400uL of Optimem was added (for 4 wells). Separate tubes were incubated for 5 minutes at room temperature (in hood).

siScr	siLGN	X
siNuf2	siNuf2 + siLGN	X

Figure 9. Six-Well Plate Map
The plate setup for the Nuf2 and Cortical Dynein (LGN) knockdown experiment.

Next, 100uL of Optimum+Lipofectamine was gently added dropwise to each epi tube with siRNA + Optimem in it using p1000 (this way the bubble formation caused by the siRNA becoming encapsulated by the lipid does not pop). Tubes were incubated for 20 min at room temperature. PLK4-inducible RPE cells were harvested, centrifuged at 1000rpm for 5 minutes and resuspended in 5mL of pen-strep free media for the transfection to be most effective. Cells were counted and diluted with pen-strep free media (5mL total w/ 150,000 cells/mL).

The plate was then prepared, placing the coverslips in ethanol and adding them to the four wells. The wells were then washed with PBS and aspirated. 1mL of cells was then dispensed into each individual well all while checking that the coverslips remained adhered. Lastly, 200uL of Optiumem+siRNA+Lipofectamine was added dropwise (with tip submerged in media) to the 4 wells with cells in 1mL of media. The cells were incubated at 37˚C for 48 hours. Four hours post-transfection, 1mL of DMEM complete (containing antibiotics) was added to each well containing cells.

4.2.2 RNA Extraction

The remaining media from the 6-well transfection plate was aspirated. 500uL of Trizol reagent was added to each well and collected and transferred in separate Eppendorf tubes for each well (this step should not take more than 2 minutes because RNA can begin to degrade after 5 minutes unless frozen). Cells were then frozen immediately at -80˚C or the RNA extraction process began.

The RNA extraction phase separation phase began with the addition of 0.2mL of chloroform per 1mL of Trizol reagent to each tube. Samples were vortexed for 15 seconds and incubated at room temperature for 2-3 minutes. Samples were then centrifuged at 12,000 x g for 15 minutes at 4˚C. The aqueous phase was removed and put into a new tube. The addition of chloroform step and the steps that followed were repeated with half the amount of chloroform.

The RNA precipitation portion of the extraction then began where 0.5 ml of 100% isopropyl alcohol per 1 ml of TRIZOL Reagent used for the initial homogenization. Samples were incubated at room temperature for 10 minutes and then centrifuged at 12,000 x g for 10 minutes at 4˚C.

The RNA wash then began where the remaining supernatant was removed completely leaving only the RNA pellet. The pellet was washed with 1mL of 75% ethanol per 1mL of TRIZOL used. The sample was vortexed briefly and centrifuged at 7,500 x g for 5 minutes. The wash was discarded, and the wash protocol was repeated once more. The remaining pellet was then air dried and dissolved in 10uL of DNA free-treated water. The Nanodrop was then used to determine concentration and purity (A260/280 and A260/230 values). These samples could be frozen at -20˚C if not used directly to make cDNA.

4.2.3 cDNA Synthesis

Master Mix 1was created which includes the following:

A. 1uL of 50uM random hexamers (x6 because 4 different mRNA samples present)
B. 1uL of 10mM dNTP mix (x6 because 4 different mRNA samples present)

Based on the concentrations of RNA obtained from the RNA extraction step, each RNA sample was diluted to 2ug of RNA in 11uL of nuclease free water. 2uL of Master Mix 1 was then added to each 11uL sample of diluted RNA and centrifuged briefly to move contents to the bottom of the tube. The mixture was heated at 65˚C for 5 minutes and then incubated on ice for at least 1 minute. Next, Master Mix 2 was created including the following:

A. Mix the 5x SSIV buffer and create Master Mix 2
B. 4uL of 5x SSIV buffer (x6 because 4 different mRNA)
C. 1uL of 100mM DTT (x6 because 4 different mRNA)
D. 1uL of RNaseOUT Recombinant RNase Inhibitor (x6 because 4 different mRNA)
E. 1uL of SuperScript IV Reverse Transcriptase (x6 because 4 different mRNA)

7uL of Master Mix 2 was added to each reaction tube and they were incubated at room temperature for 10 minutes. Next the reaction was incubated at 55˚C for 10 minutes, 80˚C for 10 minutes, and then used immediately for qPCR or stored at -20˚C.

4.2.4 qPCR Analysis

cDNA dilutions were made where 1uL of cDNA was added to 6uL of water per well used for cDNA. From there, 7uL of each cDNA dilution was added to their 6 corresponding wells (Figure 7).

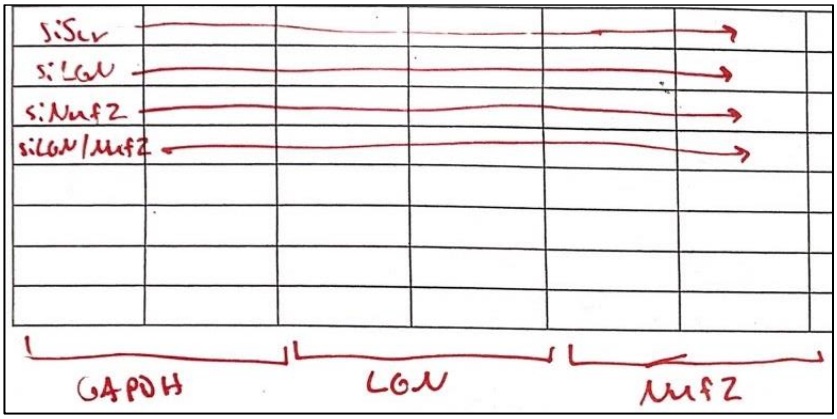

Figure 10. Plate Map used for qPCR in which 24 Wells Total Were Used.

Initial stock primer dilutions (GAPDH, LGN, and Nuf2) were then made which included 5uL of forward primer, 5uL of reverse water, and 90uL of nuclease free water. Next, primer dilutions for each well were made (enough for 10 wells since 8 were used). This included 0.5uL of primer + 7.5 uL SYBR green (multiplied by 10 = 5uL diluted primer + 75uL SYBR green).

Additionally, 8uL of the primer + SYBR green mix were added down the side of the 8 corresponding wells. Plate was brought to qPCR machine and run for 3-4 hours.

4.3 Chromosome Alignment Analysis

4.3.1 MeOH Fixing and Staining of Cells

Coverslips were removed and each placed in 1 mL MeOH at -20ºC (or on ice) for 10 minutes. Slips were then washed with PBS and blocked with TBS-BSA for 20-30 min. During this time, primary antibody was prepared in TBS-BSA and the dilutions of antibodies were as follows:

A. TBS-BSA
B. 1:500 human ACA (Kinetochores)
C. 1:1000 Mouse centrin (Centrosomes)
D. 1:1500 tubulin (Microtubules)

The coverslips were removed, placed in a dark hydration chamber, and 100uL of the primary antibody mixture was placed on each coverslip. The chamber was closed and sat at room temperature for 1-2 hours. Coverslips were then washed in TBS-BSA for 10 minutes. Secondary antibody was then prepared in TBS-BSA + DAPI and the dilutions were as follows:

A. TBS-BSA + DAPI (Chromatin)
B. 1:1000 mouse red
C. 1:1000 human far red
D. 1:1000 rabbit green

The coverslips were removed, placed in a dark hydration chamber, and 100uL of the secondary antibody mixture was placed on each coverslip. The chamber was closed and sat at room temperature for 1 hour. Coverslips were washed in TBS-BSA for 10 minutes. Coverslips were then mounted on microscope slide with antifade, put in the dark overnight, and kept at 4˚C until imaged.

4.3.2 Microscopy

A Zyla sCMOS camera that was attached to a Nikon Ti-E microscope was used to image in this experiment. A 60x objective was utilized to visualize the slides and immunofluorescence was utilized to visualize and capture cells in mitosis, specifically those with a bipolar spindle formed (pre-metaphase and metaphase). Images of 50 cells in metaphase per condition were captured with eleven 0.3uM Z stacks and exposure times adjusted such that they were the same for each condition in a replicate. DAPI was used to visualize chromatin, Tubulin for microtubules, Centrin for centrosomes, and ACA for kinetochores.

4.3.3 Analysis Criteria

All the images captured for each condition were analyzed using NIS Elements Viewer 2. Upon viewing a single cell, the two farthest kinetochores from the mitotic plate on both sides in the direction of the centrosomes were located. Kinetochores were then transposed to midline of cell based on centrosome location (Figure 8). The distance from one centered kinetochore to the next was measured using the two-point length tool, marking a point at one centered kinetochore and the second and recording the length of the line in micrometers.

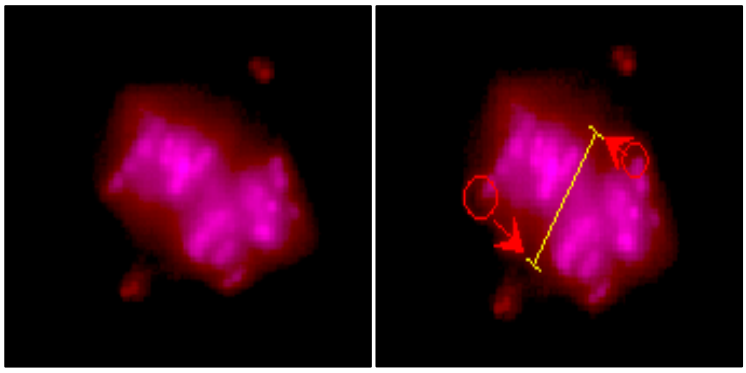

Figure 11. Chromosome Alignment Mitotic Width Analysis
The analysis is visualized on a -Nuf2/LGN condition cell. The two centrosomes are visualized in red (centrin) and the kinetochores and are present in pink (ACA). The red circles and arrows indicate the two farthest kinetochores from the mitotic plate in the direction of the centrosomes. The horizontal yellow lines represent the farthest kinetochore centered to the centrosome on each side and the vertical yellow line represents the total measured width of the mitotic plate.

There were 50 mitotic widths recorded per conditions across three replicates. The data was normalized to the siScr control and put into super plot format to compare across replicates and determine statistical significance. An unpaired T-test with n=3 was used to determine statistical significance.

4.4 Mitotic Scoring Analysis

4.4.1 Microscopy and Scoring Criteria

A Zyla sCMOS camera that was attached to a Nikon Ti-E microscope was used to image in this experiment. A 60x objective was utilized to visualize the slides and immunofluorescence was utilized to visualize and capture cells in prometaphase, late prometaphase, metaphase, and anaphase. Cells were fixed and stained using the methanol fixation protocol (Section 4.3.1).

Cells in prometaphase were identified as having chromosomes with distinct rod-like elements, often chromosomes and kinetochores oriented in a ring with chromosome arms extending outward away from the center or appear as an H-shaped mass with invaginations from two sides. There is a single spindle pole present in the center of the cell, as a bipolar spindle had not yet

formed. Similarly, for late prometaphase, chromosomes begin taking the shape of the metaphase plate moving towards the center of the cell, however, kinetochores are still yet to be tightly aligned. Here a small, bipolar spindle has begun formation with thin microtubules present (Figure 4A).

Metaphase cells were defined as having chromosomes and kinetochores tightly aligned to the spindle equator forming the metaphase plate. Thick bundles of microtubules are apparent (K-fibers) and the centrosomes have made their way to opposing sides of the cell. Lastly, anaphase cells were defined as those with their chromosomes separated, positioned on the two sides of the spindle equator. There was a distinct cluster of both chromosomes and kinetochores present at both spindle poles. The spindle is elongated and a midzone may be visible (Figure 4A).[15]

4.4.2 Analysis

The first 100 mitotic cells present on the slide were scored for each condition using the identification criteria above. Stages recorded were put into percentages for each cell and inputted into a super plot for comparison across replicates and statistical analysis. A two-tailed unpaired t-test was performed to determine statistical significance.

4.5 Microtubule Stability Analysis

4.5 Transient Transfection of siRNA

The following experiment was set up in the same fashion as described in section 4.2.1 with the following adjustments. An extra well of cells was seeded and treated with additional siScr so that it could later on be treated with Nocodazole as a negative control. Additionally, one hour prior to fixation, media was aspirated from the plate, and replenished in the siRNA treated wells. A single well of siScr treated cells was replaced with 100ng/mL Nocodazole supplemented DMEM complete media.

4.5.1 Paraformaldehyde Fixing and Staining of Cells

Media was aspirated from the plate and 3.5% paraformaldehyde warmed at 37˚C was added for five minutes at room temperature. Solution was aspirated and cells were then extracted with 0.5% Triton X-100 in TBS-BSA (10 mM Tris-Cl, pH 7.5, 150 mM NaCI, 1% bovine albumin) for 5 min at room temperature. Coverslips were then blocked with TBS-BSA for 20-30 min.

During this time, primary antibody was prepared in TBS-BSA and the dilutions of antibodies were as follows:

A. TBS-BSA
B. 1:1000 Human ACA (Kinetochores)
C. 1:1000 Rabbit Astrin

The coverslips were removed, placed in a dark hydration chamber, and 100uL of the primary antibody mixture was placed on each coverslip. The chamber was closed and sat at room temperature for at least 2 hours. Coverslips were then washed in TBS-BSA for 10 minutes. Secondary antibody was then prepared in TBS-BSA + DAPI and the dilutions were as follows:

A. TBS-BSA + DAPI (Chromatin)
B. 1:1000 Human far red
C. 1:1000 Rabbit green

The coverslips were removed, placed in a dark hydration chamber, and 100uL of the secondary antibody mixture was placed on each coverslip. The chamber was closed and sat at room temperature for 1 hour. Coverslips were then mounted on microscope slide with antifade, put in the dark overnight, and kept at 4˚C until imaged.

4.5.2 Microscopy

Coverslips were imaged as quickly as possible (up to two days post-fixation maximum) to ensure minimal loss of visible fluorescence intensity. A Zyla sCMOS camera that was attached to a Nikon Ti-E microscope was used to image in this experiment. A 60x objective was utilized to visualize the slides and immunofluorescence was utilized to visualize and capture cells in mitosis, specifically those with a bipolar spindle formed (pre-metaphase and metaphase). Images of 30 cells in metaphase per condition were captured with eleven 0.3uM Z stacks and exposure times adjusted such that they were the same for each condition in a replicate. Cells where then observed for presence or absence of Astrin foci at ends of kinetochores in each condition.

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