Characterizing The Cellular Response To Nitrogen-doped Carbon Nanocups For Potential Application In Understanding The Abscission Delay Pathway In Mammalian Cells

by

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Cytokinesis is the separation of two daughter cells after successful chromosome segregation. Occasionally cells face challenges during the end of cell division. I specifically investigate how cells deal with the challenge of chromatin trapped in the cleavage plane. It is understood that cells have developed a mechanism where they delay cytokinesis until the chromatin is cleared. This mechanism is conserved in yeast, mammalian cells and drosophila[1-4]. In mammalian cells, Aurora B is thought to be a key protein in delaying cytokinesis[4, 5]. What is yet to be understood is how trapped chromatin leads to the reactivation of Aurora B at the end of cytokinesis. I have explored the nature of this pathway, using fluorescence microscopy and live cell imaging. My data suggest that post-translational modifications on Aurora B changes when lagging chromatin is trapped in the cleavage plane. To understand the mechanism in more detail, I propose using carbon nanoparticles, specifically carbon nanotubes, to mimic lagging chromatin and help further characterize the pathway allowing cells to modulate cytokinesis in response to chromatin in the cleavage furrow.

In collaboration with Dr. Star in the chemistry department by using their synthesized material, I investigate how cells react to the exposure of nitrogen-doped carbon nanocups (NCNCs). Originally, NCNCs were developed to aid in delivering drug to tumors, and have been studies in mouse systems and in neutrophils[6, 7]. In my study, I have demonstrated that NCNCs are capable of entering cells, they have minimal cytotoxic effects, and protein-conjugated NCNCs

can successfully enter cells. This primes NCNCs as a potential tool to be used as a signaling platform to recapitulate the abscission delay pathway in cells undergoing normal cytokinesis.

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Preface

I would like to thank Dr. William Saunders for welcoming me into his lab. Through his mentorship I have grown to be the scientist that I am today. I would also like to thank former members of the lab, Dr. Lingxing Xu and Thomas Zhang, BS. They are familiar faces I know I can go to on days when experimentation would get the best of me. I would also like to thank the faculty members at the Department of Biological Sciences, their feedback and guidance has really helped me along in my tenure at the University of Pittsburgh. I owe all of my success to Philippa Carter and the faculty members who developed the Hot Metal Bridge post-baccalaureate program in the Department of Biological Sciences. This program really showed me that it was my calling to become a biologist. I'd also like to thank all the graduate students, those who's already graduated and those who are still in the journey, without you all as my peers my time at the University of Pittsburgh would not be as memorable.

I'd like to thank my committee members Dr. Karen Arndt, Dr. Jeffrey Hildebrand, Dr. Kirill Kiselyov for their unwavering support during my dissertation work. I especially would like to thank Dr. Alexander Star and Dr. Seth Burkert for their time and commitment in our collaboration during my dissertation work. Lastly, I would like to thank my family for supporting me throughout this process. They saw the greatness within me during the times I had doubted myself. I am forever grateful to have their support during this journey.

1.0 Cytokinesis

At this very moment, about 100 million cells in the human body are undergoing mitosis[1]. At the end of this stage, these cells will undergo a process called cytokinesis. This is the process where the daughter cells are physically separated from each other after the segregation of their genetic material. This process is highly regulated and can generally be divided into two parts, furrow ingression and abscission[8].

Furrow ingression occurs after anaphase and it is characterized by the bending of the plasma membrane at the area termed the cleavage plane, which is located in between the segregated chromosomes[8, 9]. The cleavage plane contains an array of proteins and structures, with a key structure being the central spindle[10]. The central spindle contains microtubules, kinesins, and kinases, and these proteins help coordinate the morphological changes of the plasma membrane for furrow ingression[10, 11]. This process requires Citron kinase, Aurora B Kinase, RhoA and Rac1 to activate the constriction of the acto-myosin ring[10, 12-14]. The central spindle also keeps the chromosomes apart and away from the cleavage plane[9]. As the central spindle matures, the distance between the plasma membrane reduces and the width of the spindle shortens to about 1 to 1.5 μm[10, 15]. At this point, the structure transitions to the structure termed the midbody[12].

The midbody is a structure that is important for abscission, the second part of cytokinesis. It helps recruit proteins required for the separation of the plasma membrane between the daughter cells[1, 9]. Key regulators for abscission include proteins that originated from the central spindle and transport proteins such as ESCRT family proteins[9, 12, 15, 16]. The polymerization of ESCRT-III allows the plasma membrane to further constrict on either side of the midbody to 0.2

μm (Figure 1a)[9, 17]. Once this occurs, an AAA-ATPase VSP4 is recruited to this constriction site and aids in the scission of the plasma membrane to complete abscission (Figure 1a)[18].

It is important that cytokinesis occurs with a central spindle that contains no obstructions. One out of every 100 mitotic cells have a chromosome missegregation event during cell division[4, 14]. This can lead to nuclear material left in the cleavage plane. It has been noted in yeast, plants and mammalian cells that chromatin can cause detrimental effects to cells if the chromatin is caught during cytokinesis. In yeast and plants, it has been shown that the cytokinetic machinery is capable of cutting through the genetic material that is trapped[14, 19, 20]. In mammalian cells, it appears that cells have several pathways to deal with trapped chromatin during cytokinesis. Cells can potentially cut through the chromatin, like yeasts and plants, and other times the cleavage furrow regresses giving rise to tetraploid cells[1, 8, 14, 16, 19, 20]. Before going into further detail on the consequences of trapped chromatin in cytokinesis, I would like to discuss the origins of chromatin in the cleavage plane.

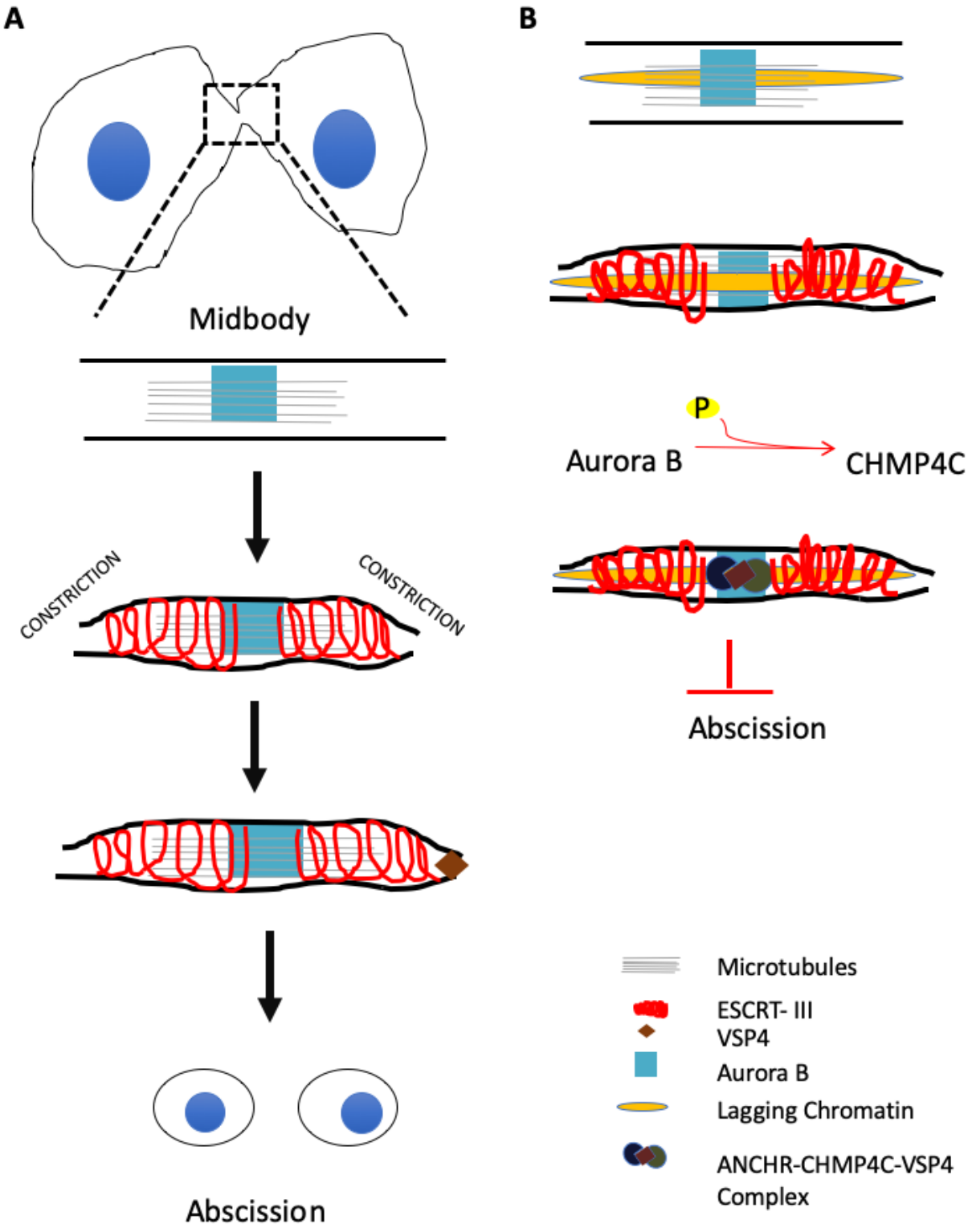

A
Midbody
CONSTRICTION
CONSTRICTION
Abscission
B
P
Aurora B
CHMP4C
Abscission
Microtubules
ESCRT- III
VSP4
Aurora B
Lagging Chromatin
ANCHR-CHMP4C-VSP4
Complex

Figure 1 Schematic of abscission

A) Midbody during normal cytokinesis. Aurora B is inactivated, ESCRT-III is polymerized to constrict the intercellular bridge, and VSP4 is localized to the consticted edge of the midbody to separate the daughter cells. B) Midbody with trapped chromatin. Aurora B is activated, it phosphorylated CHMP-4C to form a complex with ANCHR and VSP4 to sequester VSP4 at the midbody to delay abscission.

1.1 Formation Of Lagging Chromatin

There are three prominent ways DNA can get left behind in anaphase. They can come in the form of whole chromosomes, chromosome fragments and chromatin bridges[14]. Lagging whole chromosomes are the result of merotelic attachments of kinetochores to both spindle poles. This is insidious because this type of attachment does not activate the surveillance mechanism, Spindle Assembly Checkpoint (SAC), to fix this attachment[14, 20-22]. Because the kinetochore of the chromosome is attached to both spindle poles, at the time of anaphase, the equal tension pulling on the centromere by the microtubules prevents the sister chromatid from segregating to its rightful daughter cell. This keeps the chromosome in the center of the cleavage plane. Studies have suggested that these chromosomes eventually segregate into their rightful daughter cells, although they do not rejoin the main nucleus and reside in micronuclei[14].

Chromatin bridges are defined in two categories; pathological chromatin bridges and ultra-fine chromatin bridges (UFB)[14, 23]. UFBs are found in cells that incompletely decatenate entangled DNA, which usually occurs at the centromeres of chromosomes[14, 22]. UFBs can also form when cells undergo replication stress in S phase, forming conjoining DNA, or hemi-

catenannes[22, 23]. It has been suggested that UFBs are usually resolved by late anaphase[24]. Pathological chromatin bridges (PCB) are dicentric chromosomes[14, 18]. These chromatin structures contain more than one centromere and are the result of improperly repaired double strand breaks or fusing of critically shortened telomeres[14, 23]. When these chromosomes are in mitosis, each of the centromeres can get attached to opposite spindle poles. During anaphase, each centromere segregates to its designated pole, which causes the chromatin in between the centromeres to span across the cleavage plane. Unlike lagging whole chromosomes, PCB and UCB cannot avoid the cytokinetic machinery once cytokinesis is initiated. Lastly, chromosome fragments are formed from unrepaired double strand breaks. These fragments can potentially lack centromeres, which would prevent them from properly segregating in anaphase[25].

1.2 NoCut Pathway

As mentioned earlier, chromatin that is trapped during cytokinesis can lead to chromosome cleavage and tetraploidization, depending on the model system. In subsequent cell cycles, this can be catastrophic for cells. The cleavage of chromosomes ultimately results in DNA damage. Aneuploidy due to chromosome cleavage can potentiate the misregulation of up to hundreds or thousands of genes, depending on the location of the damage. In terms of mammalian cells, this can lead to the loss of tumor suppressors, or the gain in expression of oncogenes[14]. Inefficient repair of the damage can lead to amplification and translocation of genes on chromosomes[14, 21].

On the other hand, if cells undergo furrow regression when chromatin is trapped, this leads to tetraploidization and its own set of problems. Once a tetraploid cell enters the second round of cell division, it has twice the number of centrosomes. These extra centrosomes can increase the

frequency of merotelic attachments in metaphase. This begins a vicious cycle of lagging chromosomes and aneuploidy for future divisions, a pre-requisite to genome instability.

The concept of preventing cytokinesis in the presence of chromatin is not unique to just eukaryotes. Bacteria developed a system that coordinates nuclear segregation with fission. Bacteria utilize FtsZ, a protein similar to tubulin to form a Z-ring, an important factor in their cytokinetic machinery[26-28]. Like plants and yeast, bacterial cytokinetic machinery has the potential to cut through genetic material if caught in the cleavage plane[26, 28]. As a result, bacteria have developed a system to regulate the initiation of cytokinesis with the segregation of their genetic material. The pathway, termed nucleoid occlusion, utilizes the proteins Noc and SlmA to prevent the polymerization of FtsZ when genetic material is in the cleavage plane[26-28]. In rod-shaped, cells the Min pathway utilizes MinC and MinD proteins to prevent the formation of the Z-ring at the poles of cells, which also assists in localizing the cytokinetic machinery to an area that is free of DNA[26, 27].

An inaugural study done in budding yeast has shown that yeast cells developed a pathway (NoCut) to delay division when chromatin is trapped[3]. The Aurora B kinase homolog Ipl1 was determined to be a key regulator in this pathway, as it is recruited to the spindle midzone. A later study from the same lab found that the histone acetyl-transferase Ahc1 is important for the upstream activation of Ipl1[29]. They suggested that acetylation events may trigger the activation of Ipl1, which then goes to phosphorylate Boi1 and Boi2 to sequester them at the bud neck to stall abscission. In the same year, Steigemann and colleagues discovered a similar pathway in mammalian cells, with Aurora B as a key kinase activator of abscission delay in the presence of lagging chromatin[4]. Similar mechanisms have also been found in *Drosophila*[1, 2].

Over recent years, the abscission pathway in mammalian cells has been further characterized. Like yeast, Aurora B is localized to the midbody during abscission[4, 24, 30-35]. It was determined that Aurora B kinase activity is essential for the activation of the abscission delay pathway. It then phosphorylates CHMP4C, a chromatin modifying protein, CHMP4C forms a complex with ANCHR and VSP4 to sequester VSP4 at the center of the midbody (Figure 1b). This prevents it from concentrating at the constriction site to complete abscission[5, 13, 18, 34]. It is yet to be understood how the presence of lagging chromatin activates Aurora B to delay abscission.[1, 16]

1.3 Aurora B Regulation

During mitosis, transcription is silenced, and RNA translation is inhibited[36-38]. In order to regulate various proteins in this stage, cells utilize post-translational modification for regulation during mitosis[37, 38]. Aurora B is an integral kinase during mitosis. Its kinase activity is required in chromosome congression in prophase, proper kinetochore bi-orientation in metaphase, and the initiation and completion of cytokinesis[11, 12, 24, 31, 39-43]. Because Aurora B has many roles during mitosis, its activity must be tightly regulated. Aurora B is subjected to several post-translational modifications throughout mitosis. It is capable of autophosphorylation at T232, this modification activates the protein's kinase activity[35, 44]. Once Aurora B is no longer needed at the kinetochore of properly aligned chromosomes, it is ubiquitinated by Cul3 E3 ligase to assist its translocation from the centromeres to the central spindle[35, 45]. At the end of mitosis, Aurora B is targeted for degradation via ubiquitylation by APC/C-Cdh1[35]. Aurora B is also SUMOylated at the end of metaphase, where SUMOylation ensures proper function Aurora B in

the spindle assembly checkpoint (SAC). It is suggested that Aurora B SUMOylation assists in its translocation from the centromeres to the central spindle at the metaphase-anaphase transition[35].

Fadri-Moskwik and colleagues have shown that Aurora B is acetylated in prophase, then deacetylated during SAC and then acetylated again at the metaphase-anaphase transition[37]. They suggest that the acetylation of Aurora B decreases its kinase activity, and they demonstrated that Aurora B interacts with histone deacetylase HDAC3 in these stages. It is noted that the group mainly studied this modification in earlier stages of mitosis.

Based on this data, I propose that the link to activate Aurora B to the activation of abscission delay is a post-translational modification on Aurora B. To investigate this, I would like to monitor how Aurora B is modified during abscission in the presence of lagging chromatin. One challenge that I will encounter is that Aurora B is essential for stages in mitosis that precedes abscission. Inhibition via siRNA knockdown, overexpression of kinase dead Aurora B, or even chemical inhibition leads to similar phenotypes i.e. mitotic arrest, chromosome segregation errors and cytokinesis failure[31, 46]. This may obscure roles in downstream events that affect abscission delay. I propose the use of emerging technology to further characterize the upstream factors linking Aurora B activation to the presence of lagging chromatin trapped in the cleavage plane.

1.4 Carbon Nanotubes

Over several years, carbon nanomaterial have caught the attention of biologists. Carbon nanomaterials, specifically carbon nanotubes (CNTs), are sheets of carbon that roll onto itself to form hollow tubes. They come as two varieties, either as single-walled carbon nanotubes

(SWNCTs), or multi-walled carbon nanotubes (MWCNTs). In MWCNTs, there are multiple layers of carbon rolled onto itself to form a tube. SWCNTs tend to have a diameter of a few nm, whereas MWCNTS have a diameter of 10-100nm. CNTs are attractive for biological use because they have unique properties. They can be chemically modified, and their small length leads to a large aspect ratio, meaning that CNTs have a large surface area for its size[47, 48]. This aids in conjugating more compounds and molecules on the surfaces of CNTs.

Scientist have hesitations for using CNTs in biological applications because CNTs have a potential toxicity to living tissue. Unmodified CNTs tend to aggregate in aqueous solution due to their hydrophobic nature, thus forming large agglomerates[47, 49]. In addition, the method in which CNTs are synthesized influences cellular tolerance for the material[49-51]. With this information, it is detrimental to assume that all synthesized CNTs behave in the same manner. Scientists have been able to circumvent some of these issues by chemically modifying the surfaces of CNTs. The addition of carboxy and/or amino groups to the outer surfaces of CNTs seems to ameliorate their toxic effect on cells[7, 47, 52, 53]. This modification has led investigators to explore biological applications for CNTs.

Uses for CNTs for biological applications include using CNTs for targeting drugs to cancer cells for more effective treatments using chemotherapeutic drugs[6, 47]. There have also been emerging studies where CNTs are targeted to specific subcellular compartments[47, 54]. I propose that targeting CNTs to subcellular domains can be a useful tool in answering basic science questions in biology. Ultimately, I would like to use CNTs to recapitulate the abscission delay pathway with candidate proteins. Using this system, I would like to target CNTs modified with candidate proteins to the cleavage plane of normally dividing cells. Here, I can conduct assays to observe abscission timing to discover which factors are important for activating Aurora B in the

presence of lagging chromatin during abscission. This would be a more simplified system to study abscission delay because I will be leaving Aurora B kinase activity intact, as opposed to using siRNA or chemical inhibition. This will give me greater control of how cells are manipulated to study this pathway.

In a collaboration with the Star lab in the Department of Chemistry at the University of Pittsburgh, I obtained nitrogen-doped carbon nanocups for use to explore its possible application in further understanding the abscission delay pathway. Nitrogen-doped carbon nanocups (NCNCs) take a cup-like shape where one end of the nanotube is closed off[7]. This material ranges in length between 50-400 nm. This material can exist as individual cups or stacks of cups to form rods. NCNCs contain carboxy groups on the outer surfaces and amine groups on the opening of the cup. Previous studies on this material have been done in mouse models to demonstrate NCNCs as an effective anticancer drug delivery mechanism[6]. Another study that investigated NCNC exposure to neutrophils, a type of immune cell, showed that these immune cells are capable of degrading the material[7]. I would first like to determine if NCNCs pose a cytotoxic effect on cultured human cells. If they are deemed non-cytotoxic, I then would ultimately like to use NCNCS as a tool to aid in understanding abscission delay. I can conjugate candidate abscission delay proteins, such as PTM modified Aurora B, to the outer surfaces of NCNCs and target the material to the cleavage plane of normally dividing cells. From there, I can conduct assays to determine the fate of cytokinesis (Figure 2).

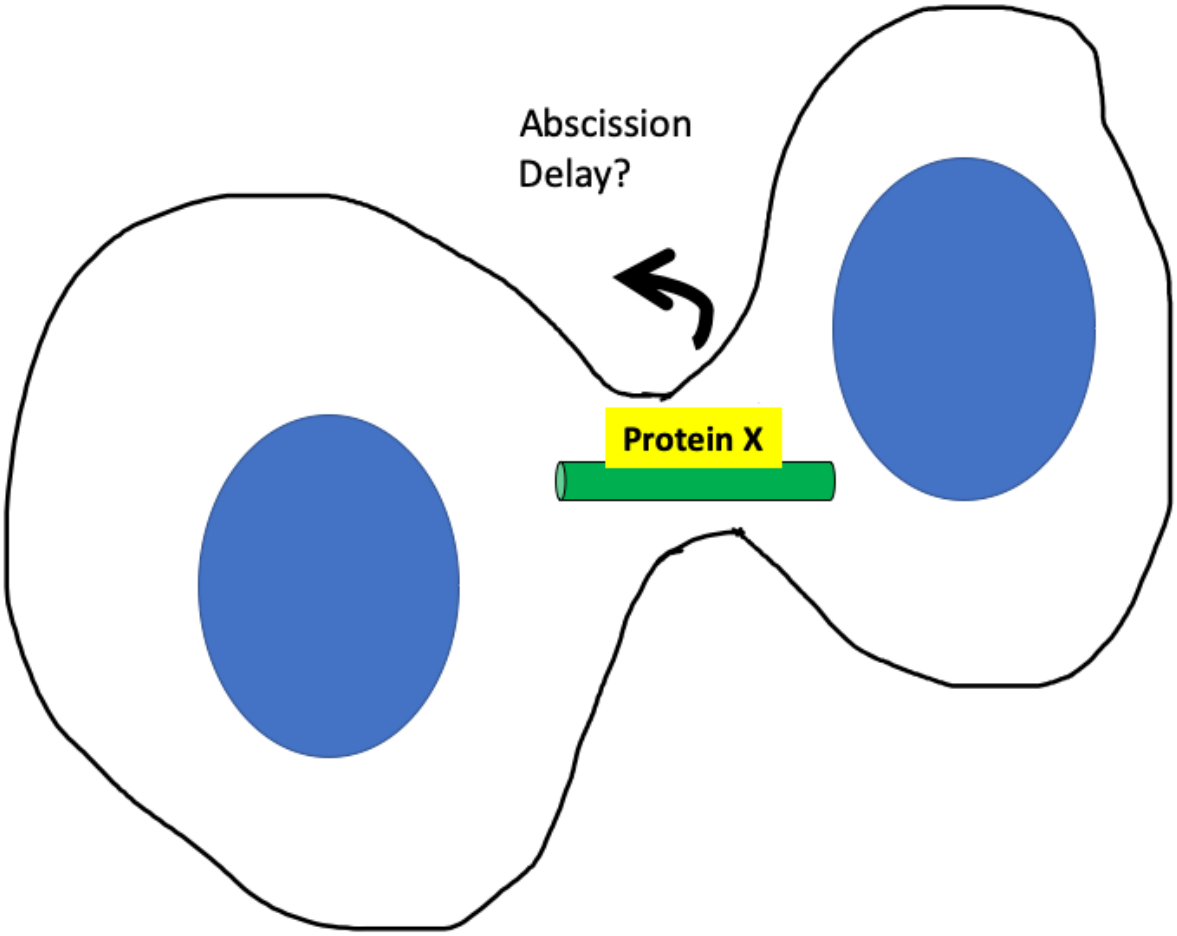


Figure 2 Schematic of CNT signaling platform in cytokinetic cell

The green bar represents a carbon nanotube with the yellow box of candidate protein attached to the outer surface.

2.0 Exploring Abscission Delay In Mammalian Cells

Since there is variability in how mammalian cells handle lagging chromatin during cytokinesis, I wanted to determine if the type of chromatin trapped in the cleavage plane influences how cultured cells complete cytokinesis. I utilized live cell imaging and fluorescence microscopy to determine the preferred pathway. I then used fluorescence microscopy to investigate the potential markers for linking the activation of Aurora B to the cellular response to lagging chromatin.

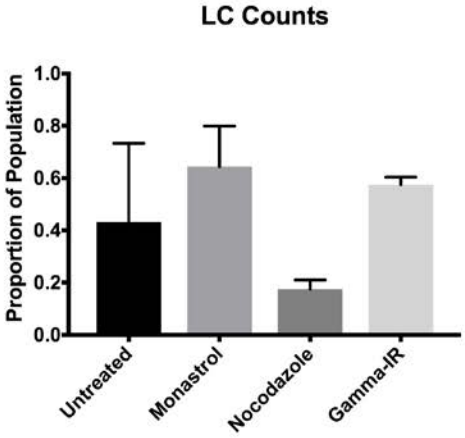
2.1 Cellular Response To Lagging Chromatin

I hypothesized that cells undergo furrow regression when challenged with lagging chromatin when undergoing cytokinesis, leading to the formation of multinucleated cells. I reasoned this because it would be in the best interest of cells to prevent DNA damage during mitosis. I implemented three different methods to induce lagging chromatin in dividing cells and I measured the frequency of multinucleation. I used nocodazole, a microtubule disruptor that stalls cells at metaphase, and monastrol, an Eg5 kinesin inhibitor that disrupts spindle pole bi-polarity[37]. These two drugs prevent cells from transitioning through mitosis by the activation of the spindle assembly checkpoint. When cells are released from these drugs, they are more inclined to produce merotelic attachment of chromosomes to the spindle poles[55, 56]. This will cause whole chromosomes to be left in the cleavage plane during anaphase. Irradiation by

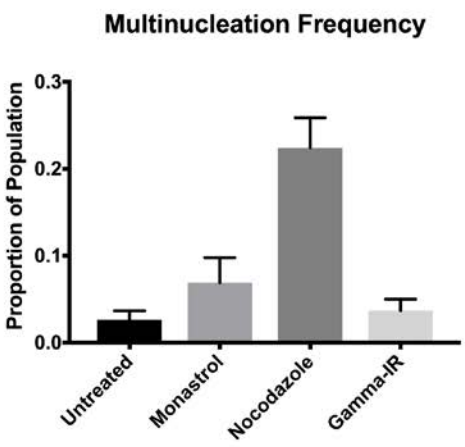
exposure cesium (Cs^{137}) was used to introduce chromatin bridges and chromosome fragments[57, 58].

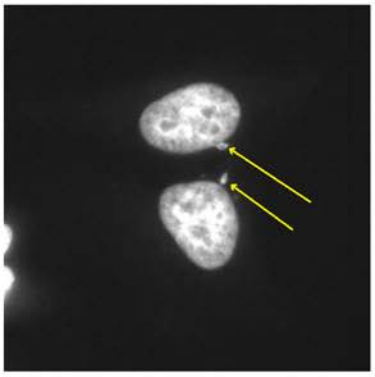
HeLa cells were exposed to 3 Gy radiation or treated with 50ng/mL nocodazole for 12 hours and then released in normal serum for 24 hours. Alternatively, HeLa cells were treated with 100 μM monastrol for 16 hours and then released for 2 hours. After treatment, cells were fixed and stained with N-cadherin to delineate cell boundaries, and DAPI to visualize chromatin. To ensure the efficacy of the induction of lagging chromatin, I first looked at cells during anaphase to determine how frequently cells left chromatin in the cleavage plane during anaphase. In untreated cells, 43.25% of the anaphases contained lagging chromatin ($n \geq 25$ anaphase cells, 8 replicates). After nocodazole treatment, the number of anaphases containing lagging chromatin was 17.5% ($n = 20$ anaphase cells, 2 replicates) and for monastrol the percentage was 64.43% ($n \geq 25$ anaphase cells, 5 replicates). Lastly, the percentage of anaphases containing lagging chromatin after irradiation was 57.5% ($n = 40$ anaphase cells, 4 replicates). One-way ANOVA demonstrated that there was no significant difference between the treatments in regard to lagging chromatin during anaphase (Figure 3a). This suggested that cells did not have a significant increase in lagging chromatin after these treatments. Since monastrol, nocodazole and gamma irradiation has been shown to promote lagging chromatin in literature, I concluded that my method of using lagging chromatin during anaphase to assay induction of chromatin by these methods was not fully reliable.

A


LC Counts
Proportion of Population
1.0
0.8
0.6
0.4
0.2
0.0
Untreated
Monastrol
Nocodazole
Gamma-IR

B


Multinucleation Frequency
Proportion of Population
0.3
0.2
0.1
0.0
Untreated
Monastrol
Nocodazole
Gamma-IR



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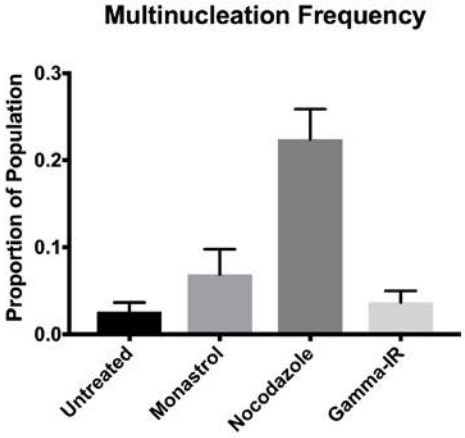
Multinucleation Frequency
Proportion of Population
0.3
0.2
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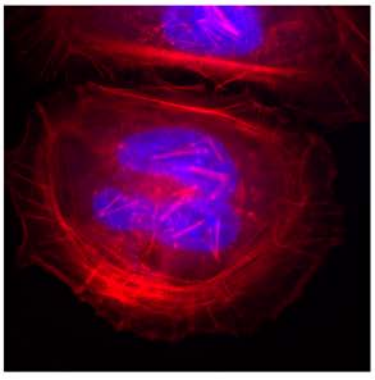


Figure 3 Induction of LC and quantification of lagging chromatin and cytokinesis failure

HeLa cells were exposed to 50 ng/mL nocodazole, 100 μM monastrol or 3 Gy irradiation and allowed to recover. Cells were then fixed and stained with N-cadherin and DAPI for imaging. A) Anaphases in each treatment were tallied for the frequency of lagging chromatin. N ≥ 20 anaphases, and ≥ 2 experiments for each treatment B) The frequency of micronuclei were counted for each treatment. N ≥ 200 cells and ≥ 2 experiments for each treatment. Arrows indicate micronuclei. C) Multinucleation frequency was counted for each treatment. Error bars indicate standard deviation for all panels. N ≥ 100 cells and ≥ 3 experiments for each treatment.

I then investigated the formation of micronuclei, as an alternative means of monitoring lagging chromatin during cell division. Micronuclei form when lagging chromatin cannot join the main mass of chromosomes during nuclear envelope reformation. As a result, the lagging chromatin is encapsulated in its own nuclear envelope[59]. Cells were treated with lagging chromatin inducers, allowed to recover and were then fixed and stained for chromatin using DAPI to visualize chromatin. Interphase cells were viewed to determine the frequency of micronuclei (Figure 3b insert). Untreated cells had a micronuclei frequency of 18.08% (n= 200 cells, 4 replicates). The micronuclei frequency for monastrol and nocodazole was 18.54% (n = 200 cells, 2 replicates) and 11.11% (n ≥ 206 cells, 3 replicates respectively. Irradiated cells had a micronuclei frequency of 34.98% (n ≥ 210 cells, 4 replicates). One-way ANOVA determined that the micronuclei frequency of irradiated cells was significantly higher than untreated cells (P-value = 0.0006) (Figure 3b). This suggests that gamma irradiation significantly increases the frequency of lagging chromatin in HeLa cells.

Next, I counted the multinucleation frequency in all of the treatments. Here, I hypothesized that cells will regress their cleavage furrow when faced with lagging chromatin. Based on the

micronuclei counts, I would expect to see a significant population of multinucleated cells in the irradiation treatment. Cells were treated with inducers of lagging chromatin, fixed and then stained with N-cadherin to delineate cell boundaries. An example of a multinucleated cell is demonstrated in Figure 3c. Untreated HeLa cells had a multinucleation frequency of 2.61% (n = 200 cells, 4 replicates). The multinucleation frequency for monastrol and irradiated cells were 6.88% (n = 200 cells, 2 replicates) and 3.68% (n ≥ 210 cells 4 replicates) respectively. Interestingly, nocodazole treated HeLa cells had the highest induction of multinucleation at 22.41% (n = 200 cells, 4 replicates). One-way ANOVA determined that the monastrol and nocodazole treatment have multinucleation frequencies significantly higher than untreated cells (P-value = 0.02 for monastrol and P-value = 0.001 for nocodazole) (Figure 3c). A caveat to using nocodazole to induce lagging chromosomes is that it disrupts the microtubules. Microtubules are essential for cytokinesis and studies have suggested that prolonged exposure to nocodazole during cell division may lead to furrow regression due to the absence of a stabilized midzone[60, 61]. This may also be the case for monastrol, since it influences spindle formation.

From this data, I determined that exposing cells to 3 Gy irradiation is the most efficient way to induce lagging chromatin because this treatment produced the highest frequency in micronuclei. When investigating the cellular response to lagging chromatin, it appears that cells are still able to successfully complete cytokinesis because the multinucleation frequency did not change significantly when the rates of micronuclei increased by almost 2-fold. This could be due to the cytokinetic machinery cutting through the material left in the cleavage plane, or that cells are delaying the completing of cytokinesis until the structure is cleared.

2.2 Live Cell Imaging Of Cells With Lagging Chromatin

After analyzing the results from my analysis on fixed cells, I concluded that I can obtain a more complete understanding of how cytokinesis progressed when challenged with lagging chromatin by looking at cells dividing in real time. It may be possible that cells may not tolerate trapped chromatin during cytokinesis. Cell death can then skew the data towards a population with a low mitotic index or multinucleation frequency. I hypothesized that this could be demonstrated as a mitotic arrest, cell death or a delay in cytokinesis. I tracked the progression of cytokinesis by live imaging OSCC cells that were stably expressing GFP-conjugated H2B protein to visualize chromatin. To delineate the cell boundary. I infected cells with an adenoviral plasmid containing a GPI-anchored fluorescently tagged protein (GPI-FAP) that localized to the plasma membrane[62]. During live cell imaging, the cells are incubated with a cell impermeant fluorophore that fluoresces when it binds to the FAP. This allows for stable staining of the plasma membrane during live cell imagining[63].

Based on my fixed cell analysis, I used exposure to 3 Gy irradiation to induce lagging chromatin in OSCC cells. Images of cells undergoing late mitosis were collected on untreated and irradiated cells. Images were taken every 3 minutes starting at metaphase until the end of cytokinesis, indicated by the closing of the membrane between the two daughter cells. The fist image that contained chromosomes in an anaphase formation was denoted as the start point for the timing of the completion of cytokinesis. In the untreated cells, I followed 14 cells that completed cytokinesis in the absence of lagging chromatin. The average time for cells to transition from anaphase to cytokinesis was 8.6 minutes. In the irradiated cells, all but one (12/13) cells underwent cytokinesis in the presence of lagging chromatin. This shows lagging chromatin does not generally cause furrow regression in these cells. The average transition time from anaphase to cytokinesis

completion was 9.9 minutes. Student's T-test determined that the time increase in cells containing lagging chromatin was not significant (Figure 4). Although the delay in cytokinesis did not show significance, it does hint that cells may potentially recognize that lagging chromatin is trapped in the cleavage plane.

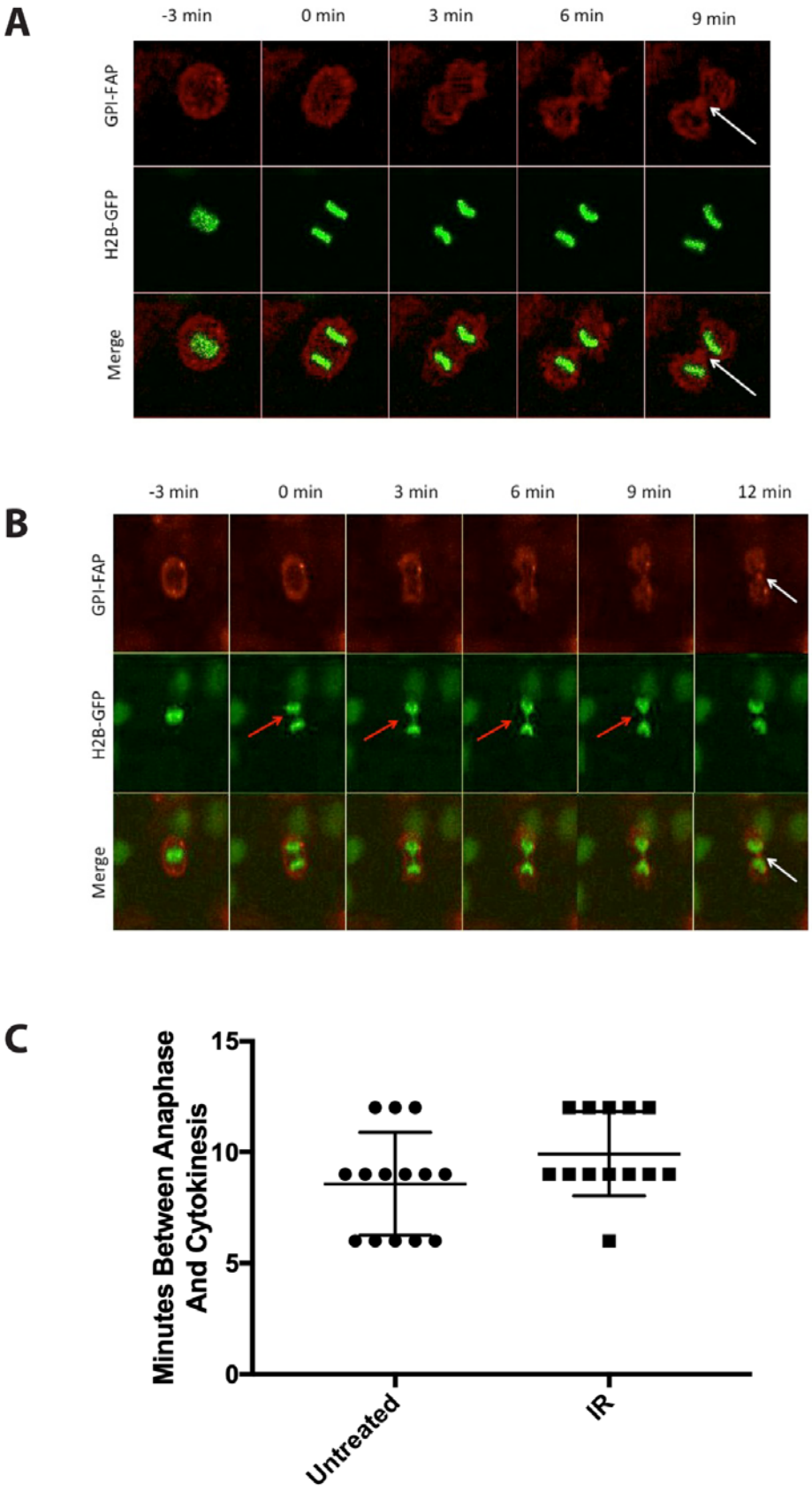

A
-3 min
0 min
3 min
6 min
9 min
GPI-FAP
H2B-GFP
Merge
B
-3 min
0 min
3 min
6 min
9 min
12 min
GPI-FAP
H2B-GFP
Merge
C
Minutes Between Anaphase And Cytokinesis
15
10
5
0
Untreated
IR

Figure 4 Live cell imaging of cytokinesis with lagging chromatin

OSCC40 cells stably expressing GFP-H2B were infected with GPI-FAP and irradiated for 24 hours. Images were taken every 3 minutes. A) Representative example of cytokinesis with no lagging chromatin. B) representative example of cytokinesis with lagging chromatin. C) Comparison of time elapsed between membrane closeure and anaphase for untreated and irradiated cells. (IR). White arrow indicates full closure of the plasma membrane.

2.3 Post-Translational Modification Analysis On Aurora B

Since I determined that cells were potentially able to sense lagging chromatin through live cell microscopy and that they do not fail at cytokinesis during this challenge via multinucleation counts, my data is congruent with recent papers that characterizes that the abscission delay pathway is the preferred mechanism in which cells deal with trapped chromatin[3, 4, 18, 29]. In this pathway, it was determined that Aurora B, a mitotic kinase, is a key factor in activating abscission delay in the presence of lagging chromatin What is yet to be understood is the method in which Aurora B is triggered to start the pathway. I aimed to study the signaling mechanism that links Aurora B activation to the trapped chromatin. Because Aurora B is present throughout mitosis, I hypothesized that Aurora B is post-translationally modified upon activation of the abscission pathway.

I investigated four different types of post-translational modifications of Aurora B in late state mitosis using fluorescence microscopy. I first investigated the phosphorylation of Aurora B during cytokinesis in the presence or absence of lagging chromatin. This post-translational modification at T232 is important for maintaining kinase activity of Aurora B. I induced lagging chromatin via 3 Gy irradiation and allowed cells to recover for 24 hours. I then immunolabeled

cells with antibodies against Aurora B and phospho-Aurora B and compared their localization. I found that the phospho-marker for Aurora B co-localized with Aurora B 100% regardless of the presence of lagging chromatin (n = 21 midbodies for cells without LC and 27 midbodies for cells containing LC) (Table 1). Examples of phospho- modifications and other PTMs co-localizing with Aurora B is demonstrated in Figure 5. The changes in the presence of SUMOylation and acetylation at the midbody when trapped chromatin is present suggest that Aurora B post-translational modifications may play a role in abscission delay activation.

Table 1 Aurora B Co-Localization With PTMs At The Midbody

	Phospho-Aurora B	SUMO-2/3	Acetylated Lysine	HDAC3
No LC	100% n = 21 midbodies	41.6% n = 24 midbodies	33.3% n = 24 midbodies	13% n = 23 midbodies
LC	100% n = 27 midbodies	26.1% n = 23 midbodies	20.8% n = 24 midbodies	26.1% n = 23 midbodies

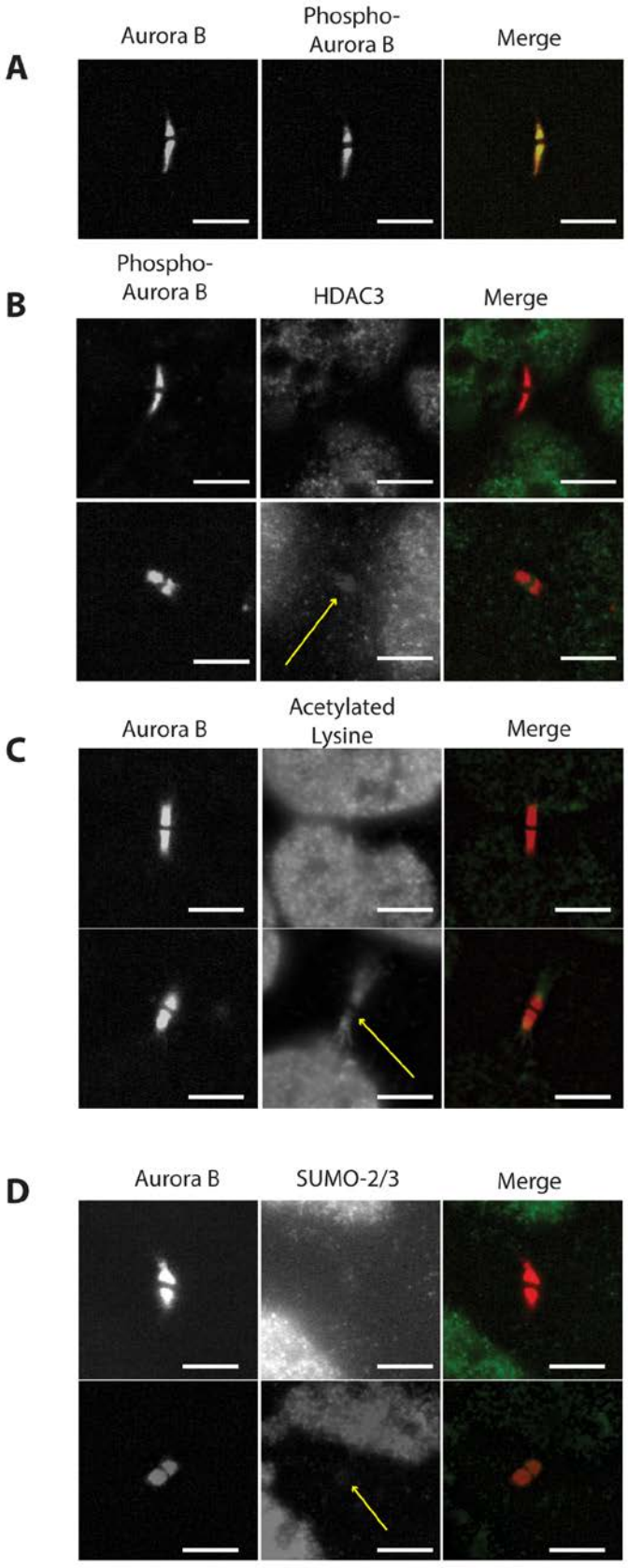

A
Aurora B
Phospho-
Aurora B
Merge
B
Phospho-
Aurora B
HDAC3
Merge
C
Aurora B
Acetylated
Lysine
Merge
D
Aurora B
SUMO-2/3
Merge

Figure 5 Examples of positive and negative PTM colocalization with Aurora B at the midbody

HeLa cells were fixed and stained with antibodies against A) Aurora B and phoshpho-Aurora B, B) Phospho-Aurora B and HDAC3, C) Aurora B and antibodies against acetylated lysine, or D) Aurora B and antibodies against SUMO-2/3. Scale bar indicates 5 microns, yellow arro indicates location of positive signal.

Recently it has been discovered that Aurora B can be SUMOylated and acetylated. The acetylation of Aurora B leads to the reduction of its kinase activity, and that HDAC3, a deacetylase enzyme, physically interacts with Aurora B to remove the acetylation modification[37, 64]. Another study suggested that the covalent addition of SUMO to Aurora B facilitates its localization in the transition from metaphase to anaphase by assisting in its disassociation from chromosomes[35]. Both of these studies investigated these modifications in earlier stages in mitosis so the status of these modifications in cytokinesis is unknown. I proposed that under normal conditions, Aurora B would be acetylated in cytokinesis to inhibit its kinase activity. When chromatin is trapped during cytokinesis, Aurora B would interact with HDAC3 to then become de-acetylated, and Aurora B would be SUMOylated to potentially become more associated with the trapped chromatin.

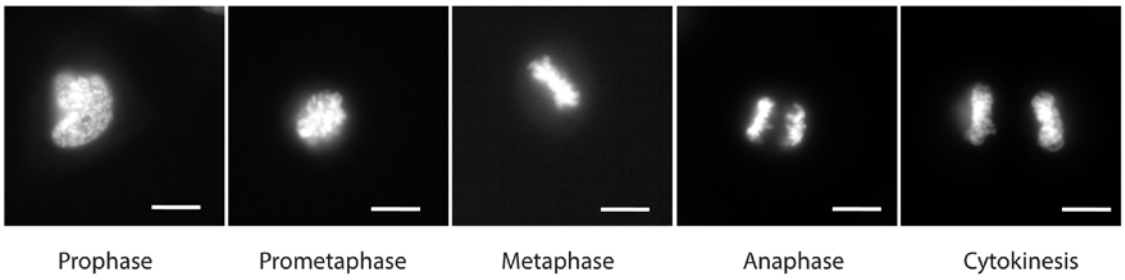
I irradiated HeLa cells with 3 Gy radiation and allowed them to recover for 24 hours. I then either stained cells with antibodies against Aurora B and a maker for acetylated lysine, antibodies against phospho- Aurora B and HDAC3, or Aurora B and a marker for SUMOylation. I used phospho-Aurora B with HDAC3 because the species for the antibody against HDAC3 conflicted with the antibody against Aurora B. Since the co-localization of phospho- Aurora B and Aurora B is 100%, it served as a good marker for Aurora B with an antibody of a compatible species to the HDAC3 antibody. I then compared the localization of the markers with Aurora B in the presence or absence of lagging chromatin. In normal cytokinesis, acetylation markers co-

localized with Aurora B 33.3% of the time (n = 24 midbodies). When lagging chromatin is present, the lysine marker co-localization was reduced to 20.8% (n = 24 midbodies) (Table 1). Looking at the co-localization of HDAC3 with Aurora B at the midbody, I found that during normal cytokinesis the co-localization was at 13% (n = 23 midbodies), and in midbodies that contained lagging chromatin, the co-colocalization of HDAC3 with phospho-Aurora B increased to 26.1% (n = 23 midbodies) (Table 1). Lastly, I investigated the co-localization of Aurora B with SUMO modifications. Cells undergoing normal cytokinesis had SUMO co-localizing with Aurora B 41.7% of the time (n = 24 midbodies). This number reduced to 26.1% when cells were challenged with lagging chromatin during cytokinesis (n = 23 midbodies) (Table 1).

Taken together, this data supports my hypothesis that the post-translational modifications of Aurora B may change when cells are challenged with lagging chromatin during cytokinesis. The reduction of acetylated lysine and the increase in co-localization of HDAC3 with Aurora B may suggest a mechanism for re-activating Aurora B's kinase activity for the propagation of abscission delay. Surprisingly, there was a reduction in the co-localization of SUMO modification with Aurora B. In the paper that investigated SUMOylation of Aurora B, they did mention that SUMOylation of Aurora B slightly reduced its kinase activity[35]. The reduction of SUMOylation at the midbody when chromatin is trapped may further enhance Aurora B's kinase ability to activate abscission delay. A caveat to these findings is that there are no specific antibodies against acetylated and SUMOylated Aurora B, so for these experiments, I used an antibody against the actual modification. So, although they appear in the same area as Aurora B, it is hard to determine if this modification is actually on the protein.

2.4 Purification Of Aurora B During Cytokinesis

To confirm the changes in PTMs that I have seen on Aurora B in cells undergoing cytokinesis with lagging chromatin, I implemented the use of immunoprecipitation and western blotting. I reasoned that if I can purify Aurora B from cells and probe with the antibodies against the modification, it would provide a clearer relationship between Aurora B and the PTMs. I first synchronized cells using thymidine and nocodazole to enrich for cells undergoing cytokinesis. I treated HeLa cells with thymidine for 16 hours, then released them in nocodazole for 2 hours to pause them in mitosis. The cells were then washed and incubated in growth medium to allow them to continue and complete mitosis. To check the efficacy of this protocol, I fixed cells on coverslips over several time points up to 2 hours after the release of nocodazole and analyzed the shape of chromatin with DAPI to determine their progress through mitosis. I found that immediately after nocodazole release, the mitotic cells were mainly in a prophase/prometaphase state. By 90 minutes after release the majority of mitotic cells were in cytokinesis (Figure 6).



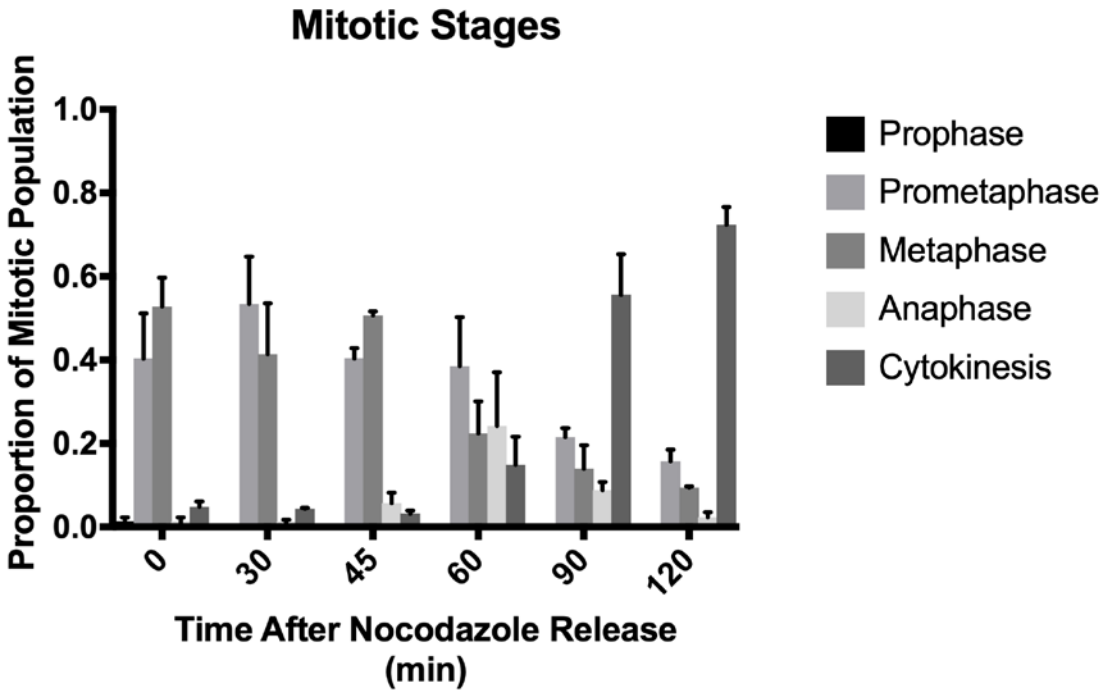


Figure 6 Time course of mitotic stages after thymidine-nocodazole arrest

HeLa cells were synchronized with a single thymidine block and released into nocodazole for 4 hours. Afterwards cells were washed and incubated in fresh medium. Each time point represents the given time allowed for cells to release from nocodazole before fixation for analysis. N ≥ 50 mitotics, 2 experiments. Scale bar indicates 10 microns.

For my western blots, I expected to see peak Aurora B acetylation at 45 min when the cells transition from metaphase because it was demonstrated that Aurora B is acetylated to assist with deactivating the spindle assembly checkpoint. I would then expect to see another acetylation peak at the 90 min and 120 min time points in cells undergoing normal cytokinesis. I performed an immunoprecipitation assay of Aurora B across the same time points I used in the immunofluorescence assay and probed for acetylation on my pulldowns. My pulldown efficiency for Aurora B was about 28% from several western blots. Based on my blots, it did not appear that Aurora B was acetylated throughout the stages of mitosis (Figure 7a). This was not consistent with the other study that suggested that Aurora B was acetylated during metaphase[37]. This could be due to my pulldown efficiency being low. I would also like to stress that although I was able to synchronize my cells to have the majority of mitotics in cytokinesis by 90 minutes, it is still unclear as to which part of cytokinesis these cells were in. I have also tried to probe for Aurora B SUMOylation after immunoprecipitation, but this as well produced inconclusive results (Figure 7b). As mentioned in the introduction, cytokinesis can be divided into two parts, furrow ingression, and abscission. The PTMs I am particularly interested in occurs during abscission, and it is uncertain as to which stage of cytokinesis these particular cells are at the 90, and 120-minute intervals when I conducted my immunoprecipitations.

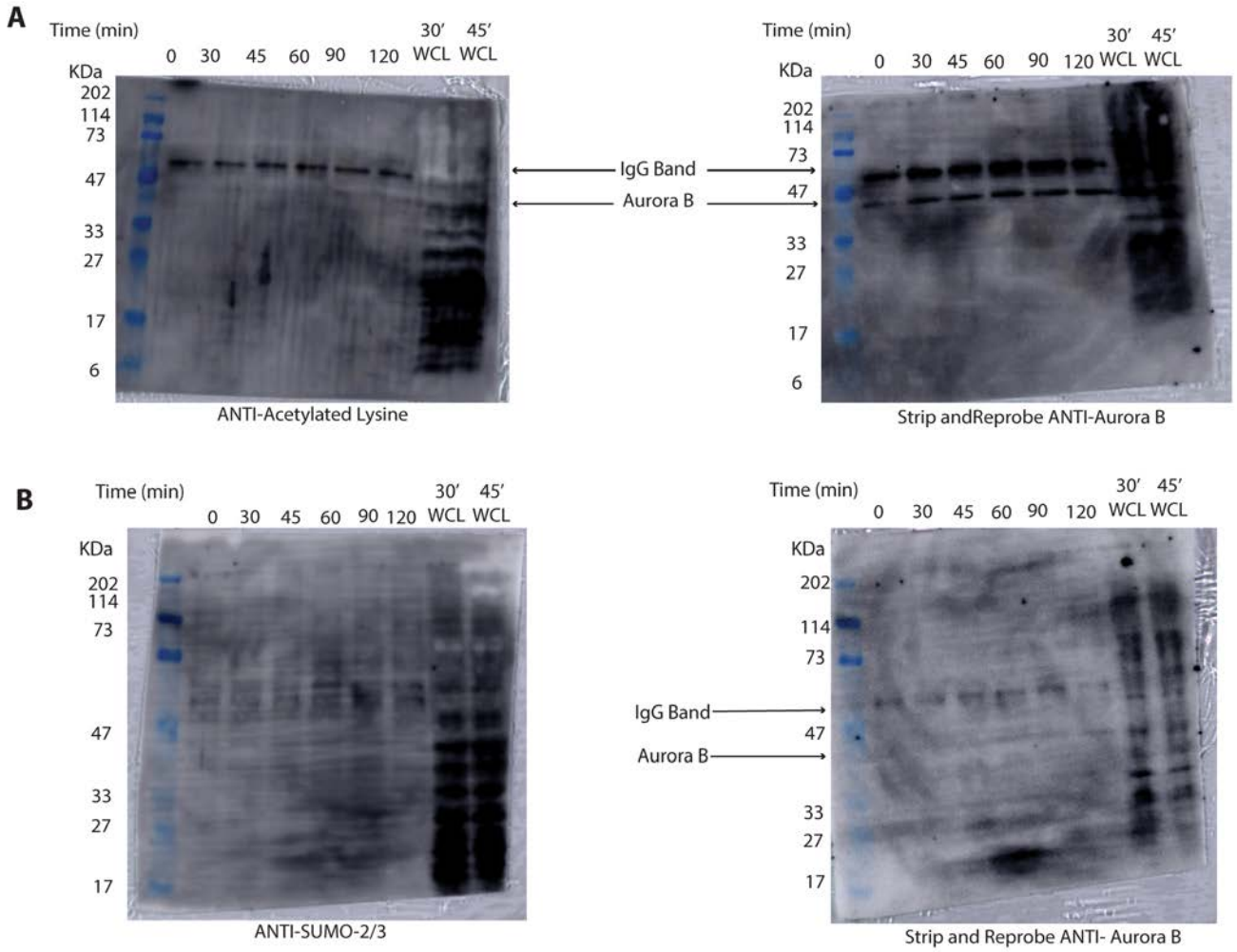


Figure 7 Western blot probing for acetylated and SUMOylated Aurora B extracted from cells

Hela cells were synchronized with a single thymidine and nocodaloze release. Cells were collected at various times up to two hours after the removal of nocodazole. Lysates were collected and incubated with Protein A beads conjugated to Aurora B for immunoprecipitation. A) Immunoprecitated Aurora B on PVDF membrane was intitally probed for acetylated lysine using a pan-acetylated lysine antibody(left). It was then stripped and repobed using antibodies against Aurora B (right). B) Immunoprecipitated Aurora B on PVDF membrane was initially probed for SUMOylation using antibodies agains SUMO-2/3 (left). The membrane was then stripped and reprobed using antibodies against Aurora B (right). WCL stands for whole cell lysate.

Because this assay produced inconclusive results, I thought it would be better to approach the activation of Aurora B in the abscission delay via alternative means. Instead of attempting to purify Aurora B with its modifications during cytokinesis with lagging chromatin, I wanted to overexpress Aurora B with candidate post-translational modifications at the midbody during normal cytokinesis. Using this method, I can parse apart the link of lagging chromatin and the activation of abscission delay in a more simplified form. To do this, I needed a substrate where I can conjugate modified Aurora B and target it to the midbody. Carbon nanotubes, with their modifiable surfaces, seem to be a prime substrate for cellular manipulation during cytokinesis.

3.0 Exploring The Cellular Response to NCNCs

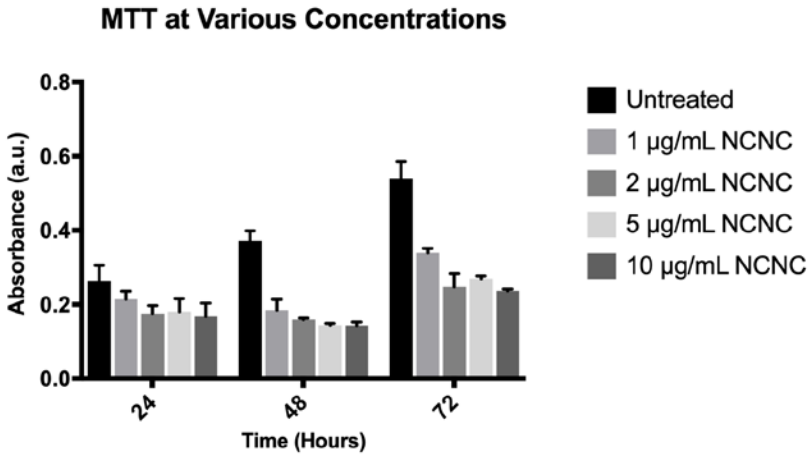
Ultimately, I would like to recapitulate the abscission delay pathway using carbon nanotubes that are conjugated with candidate proteins. Here, I anticipate targeting the conjugated material containing candidate factors to the cleavage plane of normally dividing cells and use it as a signaling platform to elicit a lagging chromatin response. This will allow me to explore the upstream factors of abscission delay in a more simplified system. In a collaboration with the Star lab in the chemistry department at the University of Pittsburgh, I was able to obtain nitrogen-doped carbon nanocups (NCNCs) that were functionalized with carboxy groups along the outer walls. This material was previously studied in mouse models and neutrophils, I wanted to investigate whether this material would be suitable for my anticipated application as a signaling platform[6, 7]. I investigated the cytotoxicity and cellular response to NCNCs on cultured human cell lines and implemented the use of fluorescence and transmission electron microscopy to determine the localization of NCNCs within cells.

3.1 Investigating NCNC Toxicity

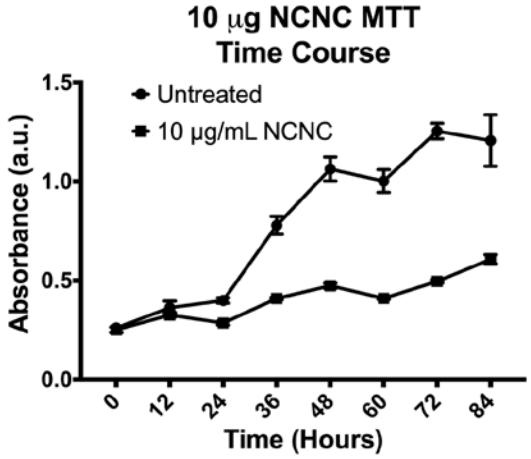
I first wanted to determine if NCNCs had detrimental effects on cells. I first tested the metabolic rate of HeLa cells with an MTT assay. This assay measures the viability of cells using

the conversion of tetrazolium salt by mitochondria. The more conversion of tetrazolium, the more cells are present on the plate[66]. I exposed cells HeLa to varying concentrations of NCNCs up to 10 μg/mL for a duration of 72 hours and an MTT was performed every 24 hours. The metabolic activity in cells treated with NCNCs appeared to lag, starting at 24 hours compared to untreated cells (two-way ANOVA P-value < 0.0001) (Figure 8a and 8b). I also conducted an MTT assay on cells treated with 2 μg/mL NCNC over a span of 72 hours, this time with intervals every 12 hours (Figure 8c). Like the previous experiment, the metabolic activity of cells treated with NCNCS began to lag behind untreated cells, this time starting at 24 hours (two-way ANOVA P-value < 0.0001). I also conducted these experiments in RPE-1 cells, an immortalized diploid epithelial cell line. Treatment of RPE-1 cells with NCNCs at varying concentrations yielded a similar response as HeLa cells (two-way ANOVA P-value < 0.0001) (Figure 9a and 9b). This suggested that treatment with NCNCs were interfering with the metabolic activity of cells.

A


MTT at Various Concentrations
Absorbance (a.u.)
0.0
0.2
0.4
0.6
0.8
24
48
72
Time (Hours)
Untreated
1 µg/mL NCNC
2 µg/mL NCNC
5 µg/mL NCNC
10 µg/mL NCNC

B


10 µg NCNC MTT
Time Course
Absorbance (a.u.)
0.0
0.5
1.0
1.5
0
12
24
36
48
60
72
84
Time (Hours)
Untreated
10 µg/mL NCNC

C

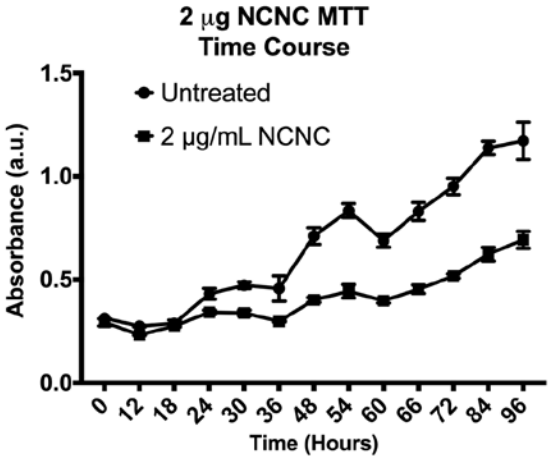

2 µg NCNC MTT
Time Course
Absorbance (a.u.)
0.0
0.5
1.0
1.5
0
12
18
24
30
36
48
54
60
66
72
84
96
Time (Hours)
Untreated
2 µg/mL NCNC

Figure 8 MTT assay on NCNC treated HeLa cells

A) HeLa cells were treated with various concentration of NCNCs and assayed using MTT over a span of 72 hours. N = three readings for each time point. B) HeLa cells were treated with 10 μg/mL NCNC and assayed using MTT every 12 hours over a span of 84 hours. N = three readings for each time point. C) HeLa cells were treated with 2 μg/mL NCNC and assayed using MTT every 12 hours over a span of 84 hours. N = three readings for each time point. Error bars indicate standard deviation for all panels.

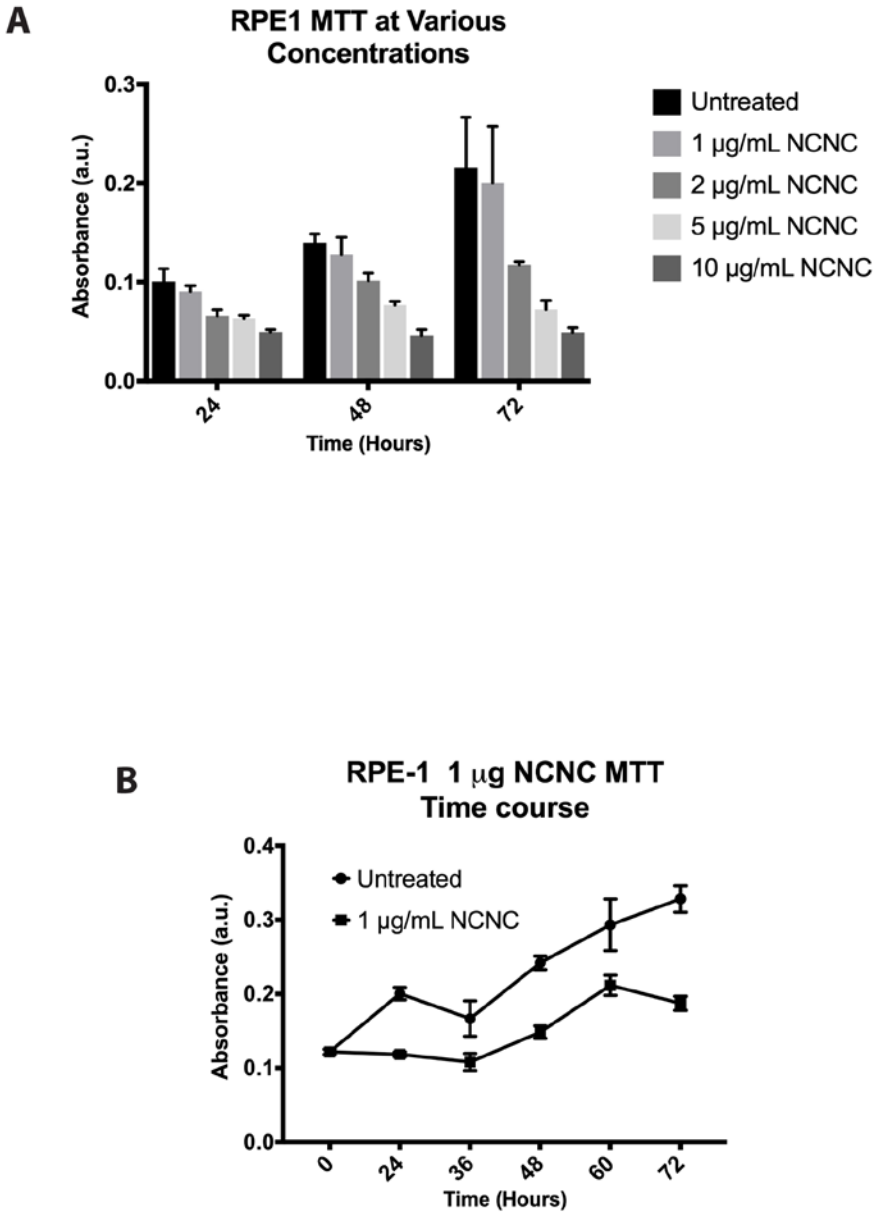
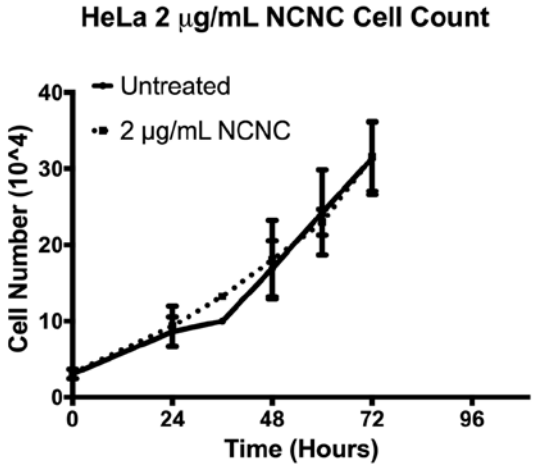


Figure 9 MTT assay on NCNC treated RPE-1 cells

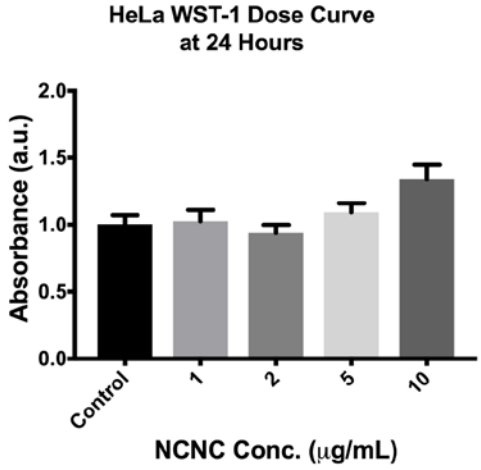
A) **RPE-1 cells were treated with varying concnetrations of NCNCs and assayed with MTT over a span of 72 hours. N = three readings for each time point. B) RPE-1 cells were treated with 1 µg/mL NCNC and assayed with MTT every 12 hours over a span of 24 hours. N = three readings for each time point. Eror bars indicate standard deviation for all panels.**

Interestingly, as I was conducting this experiment, I noticed that the population of cells in dishes containing NCNCs were similar in population to untreated cells when viewed on the microscope, which is paradoxical to the results obtained from the MTT assay. I then proceeded to quantify this observation by counting cells with a hemocytometer for both untreated and cells incubated with 2 μg/mL NCNCs over a span of 72 hours with intervals every 12 hours. Confirming my suspicion, the population of cells treated with NCNCs was similar in comparison to untreated cells (Figure 10a). In a literature search, I found an article that discussed the issues of using MTT assays on cells that are treated with single-walled carbon nanotubes (SWCNTs)[66, 67]. In this paper, they noted that SWNCTs interfere with the solubility of the formazan crystals from the assay. The SWNCTS would prevent the formazan crystals from dissolving in SDS, which would provide a false reading of reduced metabolic activity. This paper also suggested utilizing an alternative compound to measure metabolic activity, WST-1. WST-1 works in a manner similar to MTT, but instead of forming a water-insoluble crystal, the formazan in WST-1 is water soluble. I then exposed HeLa cells to increasing concentrations of NCNCs up to 10 μg/mL for 24 hours. The data from the WST-1 assay indicates that the metabolic activity of cells treated with NCNCs is not affected (Figure 10b). I also measured HeLa cells treated with 2 μg/mL NCNCs over a span of 96 hours (Figure 10c). This trend was also similar in RPE-1 cells (Figure 11), which also confirmed that NCNCs does not have a detectable effect on the metabolic activity of cells. Of note, RPE-1 cells appeared to be more sensitive to higher concentrations of NCNCs (two-way ANOVA P-value < 0.001).

A


HeLa 2 μg/mL NCNC Cell Count
Untreated
2 μg/mL NCNC
Cell Number (10^4)
40
30
20
10
0
0
24
48
72
96
Time (Hours)

B


HeLa WST-1 Dose Curve
at 24 Hours
Absorbance (a.u.)
2.0
1.5
1.0
0.5
0.0
Control
1
2
5
10
NCNC Conc. (μg/mL)

C

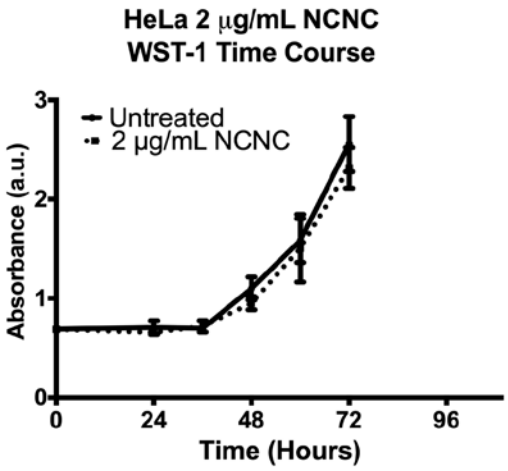

HeLa 2 μg/mL NCNC
WST-1 Time Course
Untreated
2 μg/mL NCNC
Absorbance (a.u.)
3
2
1
0
0
24
48
72
96
Time (Hours)

Figure 10 Cell counts and WST-1 assay on HeLa cells

A) HeLa cells incubated with 2 μg/mL NCNCs over a span of 72 hours. Cells were counted using a hemocytometer every 12 hours. N = 2 counts for each time point. B) HeLa cells were treated with varying concentrations of NCNCs for 24 hours and assayed with WST-1. N = three readings for each concentration. C) HeLa cells were treated with 2 μg/mL NCNCs and assayed every 12 hours with WST-1 over a span of 24 hours. N = three readings for each time point. Error bars indicate standard deviation for all panels.

I next wanted to determine if NCNCs interfere with cell division. Due to the structure of NCNCs, I had concerns that the NCNCs would act as a physical barrier to the cytokinetic machinery, akin to asbestos fibers[68]. If this is the case, then NCNCs may not be a good candidate for use as an artificial signaling platform, especially since I will be using it to study cytokinesis. To test this, I counted the multinucleation frequency and mitotic index in cells treated with NCNCs. I hypothesized that if NCNCs were to interfere with cytokinesis, I would expect to see an increase in the multinucleation frequency. In addition, if cells were stalled in mitosis, I would expect to see an increase in mitotic index. Another outcome would be that NCNCS are preventing cells from entering mitosis. If this occurs, I would expect to see a reduction of cells undergoing mitosis; i.e. a lower mitotic index. HeLa cells were treated with 2 μg/mL NCNCs over a span of 72 hours. They were then fixed and stained with phalloidin to delineate cell boundary via the actin cytoskeleton. Multinucleated cells were classified as cells containing more than one nuclei within a continuous single-cell array of actin cytoskeleton. As a positive control for cytokinesis failure, I used psychosine, a sphingolipid that is known to promote multinucleation. I determined that the multinucleation frequency of NCNCs were not significantly different from untreated cells (Figure 12a). Next was to determine if NCNCs interrupt progression through mitosis. For an inducer of mitotic arrest, I used 25 ng/mL nocodazole treatment for 24 hours (Figure 12b). Based on the data,

it appears that the mitotic index of cells was not affected by NCNC exposure. Together this suggests that NCNCs do not influence cells undergoing division.

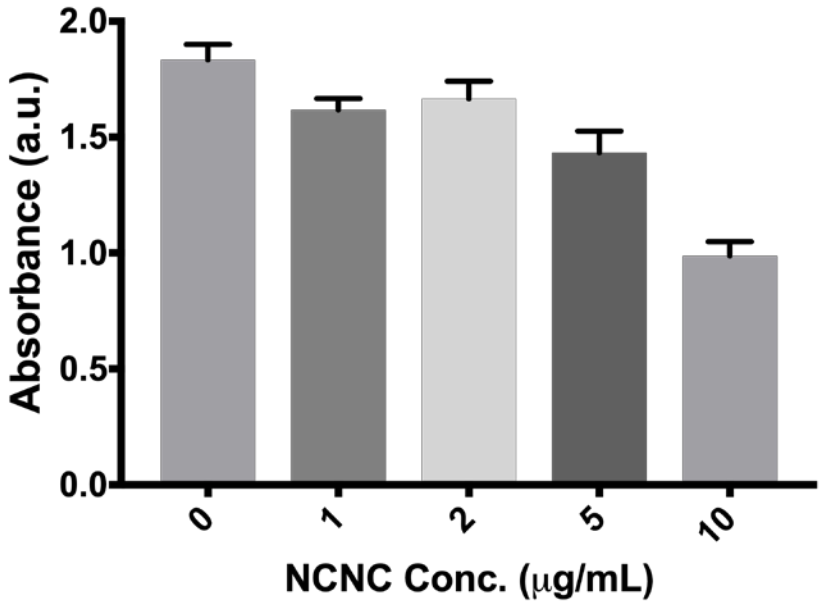


Figure 11 WST-1 assay on RPE-1 cells

RPE-1 cells were incubated with varying concentrations of NCNCs for 24 hours and then assayed using WST-1. Error bars indicate standard deviation. N = three readings for each concentration.

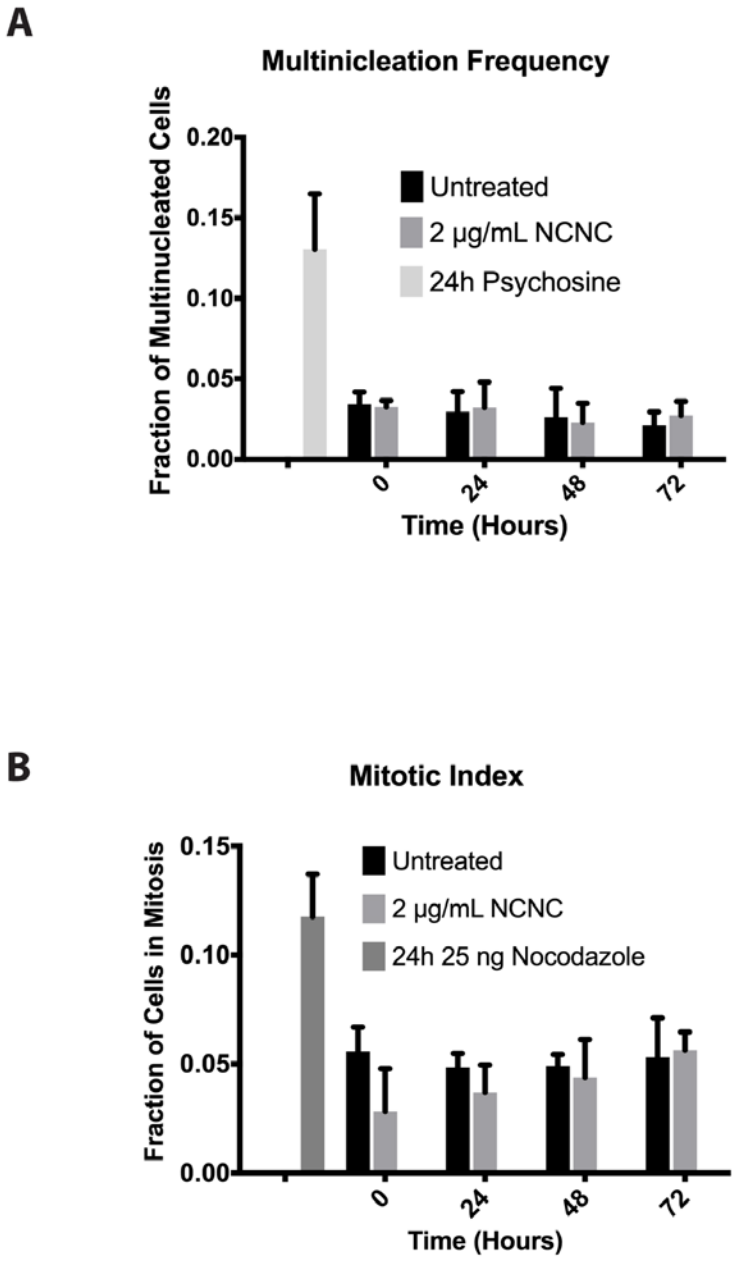


Figure 12 Multinucleation counts and mitotic index on NCNC treated HeLa cells

A) HeLa cells were treated with 2 µg/mL NCNCs over a span of 72 hours. Binucleation frequency was counted at 24-hour intervals. N = three experiments. B) HeLa cells were treated with 2 µg/mL NCNC over a span of 72 hours. Mitotic indices were counted at 24-hour intervals. HeLa cells were treated with 25 ng nocodazole for 24 hours as a positive inducer for mitotic arrest. N = three experiments. Error bars indicate standard deviation for all panels.

Other studies on carbon nanotubes (CNTs) have suggested that cells exposed to CNTs elicit an inflammatory response via secretions of inflammatory cytokines such as interleukins -6 and -8 (IL-6 and IL-8)[69, 70]. I wanted to determine if cultured cells respond in the same way to NCNCs. Hela cells were incubated with varying concentrations of NCNCs for 24 hours, and as a control, TNFα was used to induce IL-6. I then conducted an ELISA on the cell media to probe for secreted IL-6. None of the cells that were exposed to NCNCs had a significant increase of IL-6 when compared to control cells (figure 13). Taken altogether, the data suggest that NCNCs do not have cytotoxic effects on cells.

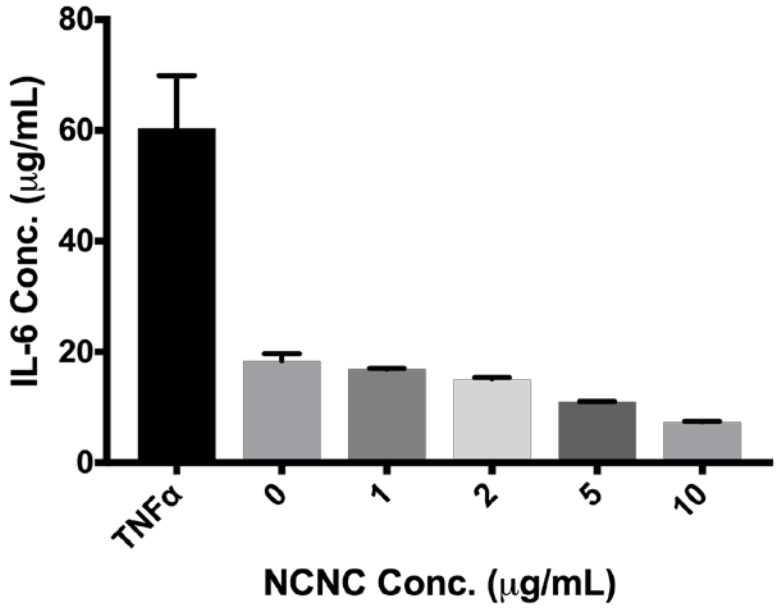


Figure 13 IL-6 ELISA on NCNC treated HeLa cells

HeLa cells were treated with increasing concentrations of NCNCs for 24 hours. The supernatants were collected and assayed using an ELISA to probe for IL-6 secretion. 1 ng/mL of TNFα was used as a positive control for secretion of IL-6. Error bars indicate standard deviation. N = two readings for each concentration.

3.2 NCNC Entry Into Cells

I next wanted to ascertain if NCNCs can enter cells. I found that NCNCs were visible, and potentially in cells, through the use of bright field microscopy. After HeLa cells were incubated with 2 μg/mL NCNCs for 24 hours, I found that the NCNCs often gathered on one side of the nucleus (Figure 14). The pattern in which they were clustering suggested that the NCNCs may be associating with the centrosome. I then stained cells with antibodies against the centrosome marker γ-tubulin (Figure 15a). The clusters of NCNCs did, in fact, localize in the same vicinity of the centrosome marker. I hypothesized that since they are gathering around the centrosome, they were potentially targeted to the centrosome via microtubules and disrupting microtubules would lead to the dispersal of NCNC clusters. Fluorescent images of cells treated with NCNCs and nocodazole showed that the NCNCs appeared to be more dispersed (Figure 15b). Next, I proceeded to quantify the distances of NCNC clusters from the centrosome under normal conditions and in the presence of microtubule disrupter nocodazole (Figure 15c). If I encountered more than one centrosome in a cell, I measured the distance of the puncta from each centrosome and took the average of the measurements. In cells that were only incubated with NCNCs, the NCNCS clusters were on average 5.6 microns away from centrosomes. In nocodazole treated cells, the distance for the NCNC clusters was significantly further away from the centrosome at an average of 8.7 microns in distance (t-test P-value < 0.0001 $n \geq 100$ NCNC puncta per treatment group) (Figure 15c). This would suggest that NCNCs are trafficked in the direction of the centrosome in a microtubule-dependent manner.

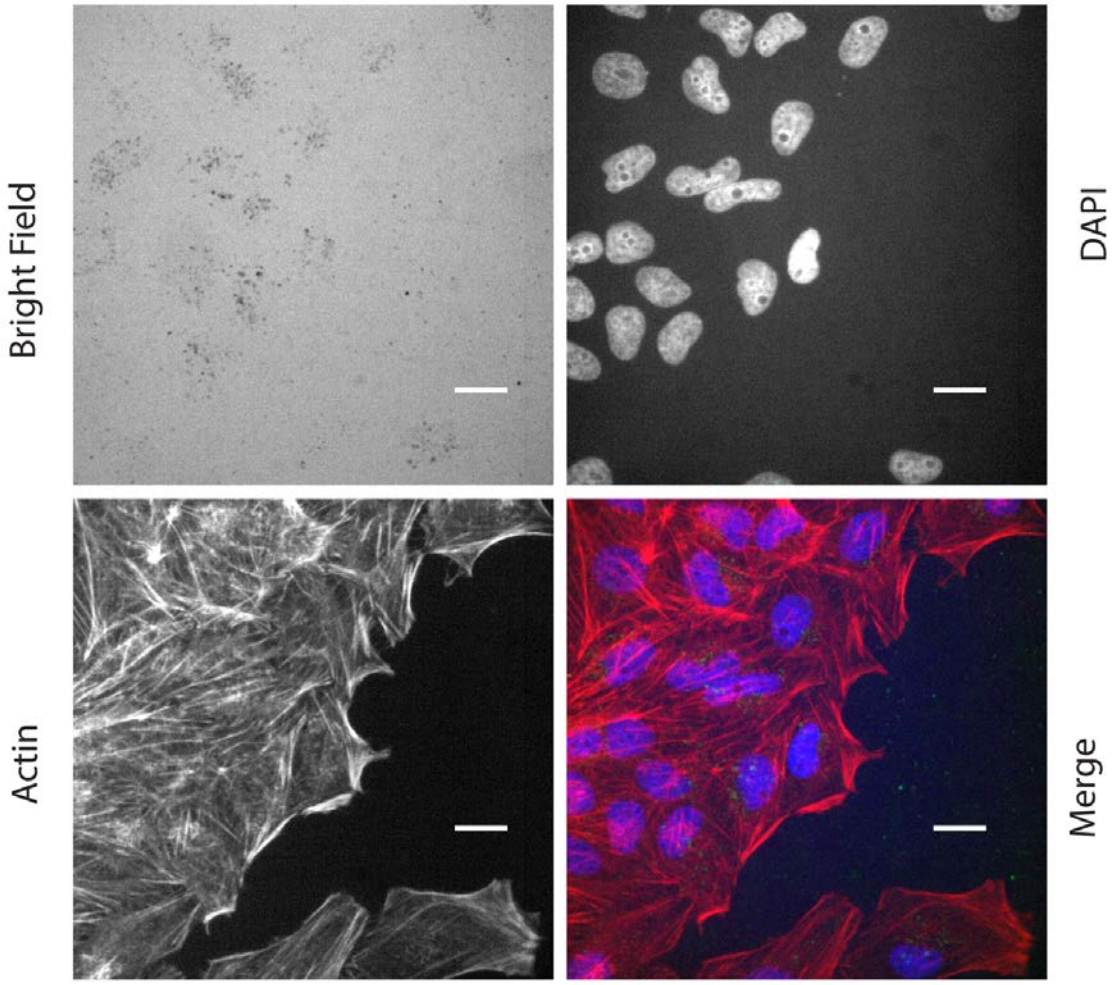


Figure 14 Bright field microscopy to visualize NCNCs

HeLa cells were treated with 2μg/mL NCNCs for 24 h, they were then washed and stained for actin using rhodamine conjugated phalloidin. The nucleus was stained with DAPI. Green in the merge channel represents the NCNCs from bright field. Scale bar indicates 10 microns.

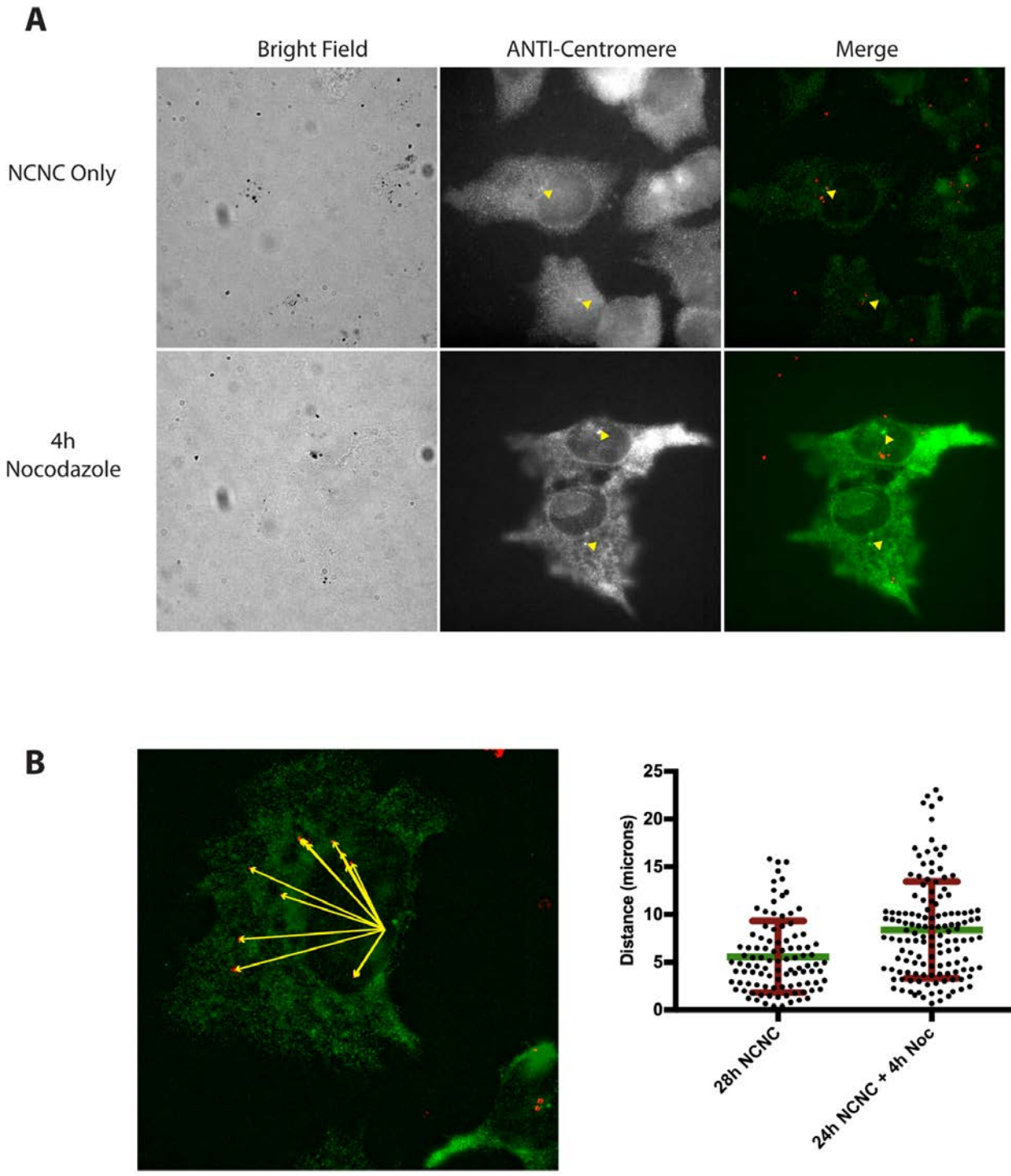

A
Bright Field
ANTI-Centromere
Merge
NCNC Only
4h
Nocodazole
B
Distance (microns)
25
20
15
10
5
0
28h NCNC
24h NCNC + 4h Noc

Figure 15 Fluorescence microscopy and cluster distance of NCNCs from centrosomes

A) HeLa cells were incubated with 2 μg/mL NCNC for 24 hours, they were then washed and incubated for 4 hours with fresh medium and were fixed and stained using antibodies against γ-tubulin. Yellow arrowheads indicate location of centrosomes. Red puncta in merge image represents the NCNCs imaged in bright field. B) HeLa cells were incubated with 2 μg/mL NCNCs for 24 hours, washed and incubated with the microtubule disruptor nocodazole for 4 hours. The cells were then fixed and stained using antibodies against γ-tubulin. Yellow arrowheads indicate the location of centrosomes. Red puncta in merge image represents the NCNCs imaged in bright field. C) NCNC puncta distance was calculated using ImageJ software for cells in treatment groups from panels A and B. Yellow arrows show how distance was determined. Green line indicates mean distance and red bars indicate standard deviation.

In addition to fluorescence microscopy, I conducted imaging using a transmission electron microscope. I found that about 50-60% of NCNCs were encapsulated in vesicles, while the remainder resided in the cytoplasm (Figure 16a and 16b). This number was constant over a duration of 48 hours. Work that has been done on other CNTs have suggested their entry into cells requires endocytosis, other studies have suggested that CNTs have the potential to enter cells passively across the plasma membrane. To verify the identity of the vesicles that contain NCNCs, I incubated cells with NCNC in the presence of colloidal gold. It has been confirmed that colloidal gold is uptaken in cells via endocytosis, so I hypothesized that if the NCNCs are entering via this pathway, I would expect to see the vesicles contain colloidal gold and NCNCs with the co-incubation[71, 72].

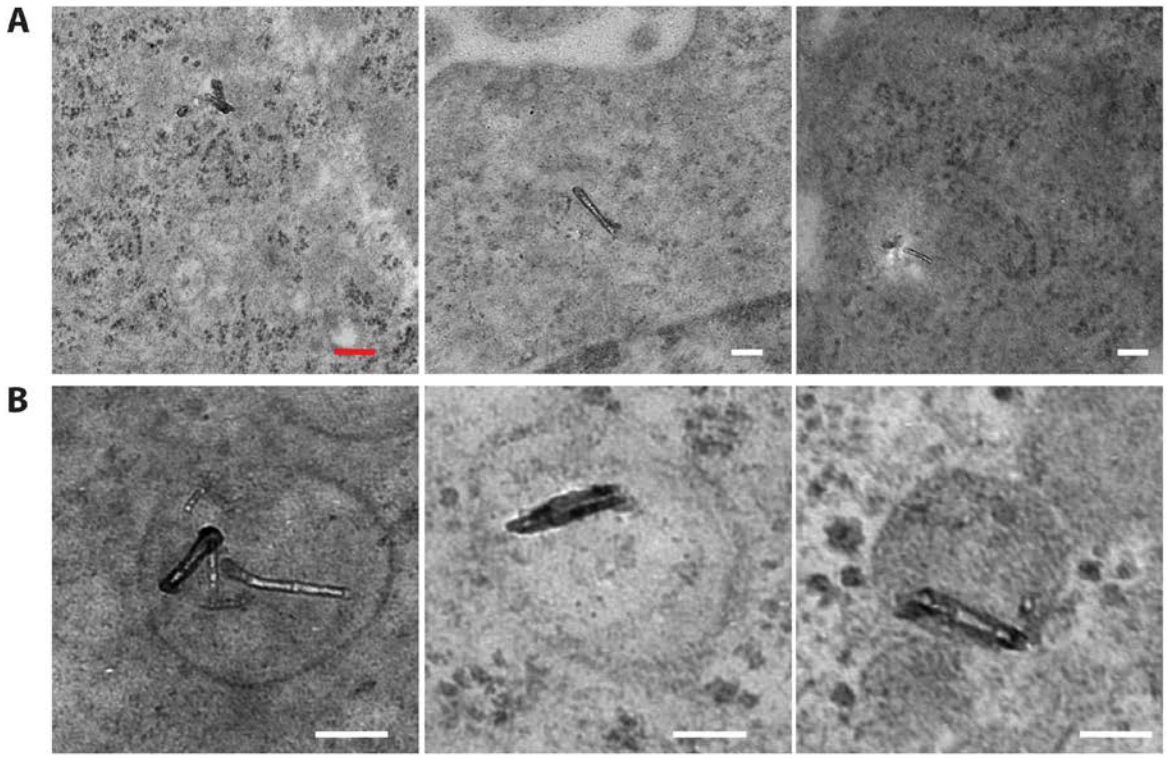


Figure 16 TEM visualization of NCNCs inside of cells

A) **Examples of NCNCs residing in the cytoplasm of HeLa Cells. Cells were treated with 5 μg/mL NCNCs for 6 hours. B) Examples of NCNCs residing in vesicles within HeLa Cells. Cells were treated with 5 μg/mL NCNCs for 6 hours. White scale bar indicates 100 nm, red scale bar indicates 200 nm.**

HeLa cells were treated with 5 μg/mL NCNCs and 5 nM of 20 nm colloidal gold for 6 hours. The cells were then fixed, embedded in plastic and prepped for TEM viewing. My hypothesis was confirmed as I found vesicles that contained both colloidal gold and NCNCs (Figure 17a). To further verify entry via endocytosis, I incubated HeLa cells with NCNCs for 6 hours in the presence of methyl-beta cyclodextrin (MβCD) a compound that inhibits both clathrin-dependent and caveolae endocytosis through sequestering cholesterol from the plasma membrane[73]. As a control, I established that MβCD was inhibiting endocytosis by monitoring the uptake of dextran in the presence of the drug (Figure 18a and 18b). In untreated cells, on

average there were 8.7 NCNCs per cell section. In the presence of MβCD, on average the number of NCNCs per cell section was 1.4, significantly lower than control cells (t-test P-value < 0.0058 n = 2 experiments) (Figure 17b). In my TEM images, I found examples of NCNCs that appeared to penetrate the plasma membrane, which may account for the residual uptake of NCNCs in the presence of MβCD (Figure 19a). I also noticed that some of the vesicles containing NCNCs appeared to be distorted, and in some instances NCNCs appear to be in the process of escaping vesicles (Figure 19b). This can account for the almost 50-50% distribution of NCNCs in vesicles although the majority of its entry appears to be through endocytosis.

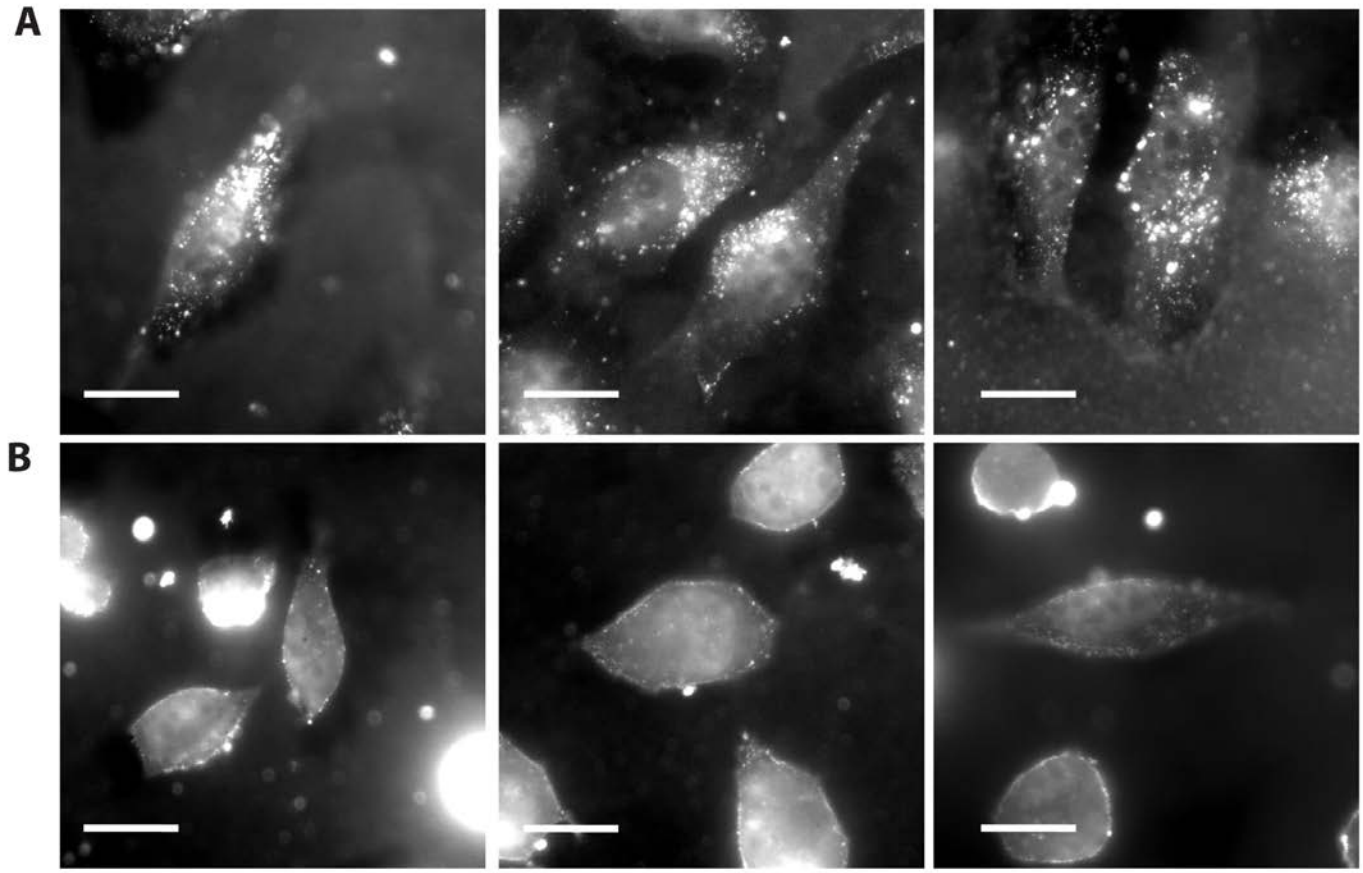


Figure 17 Dextran uptake in the pesence of MβCD

A) HeLa Cells incubated with Texas red dextran for 6 hours. B) HeLa cells were pre-incubated with 5 mM of endocytosis inhibitor MβCD for 30 min and then incubated with Texas red dextran for 6 hours in the presences of the inhibitor.

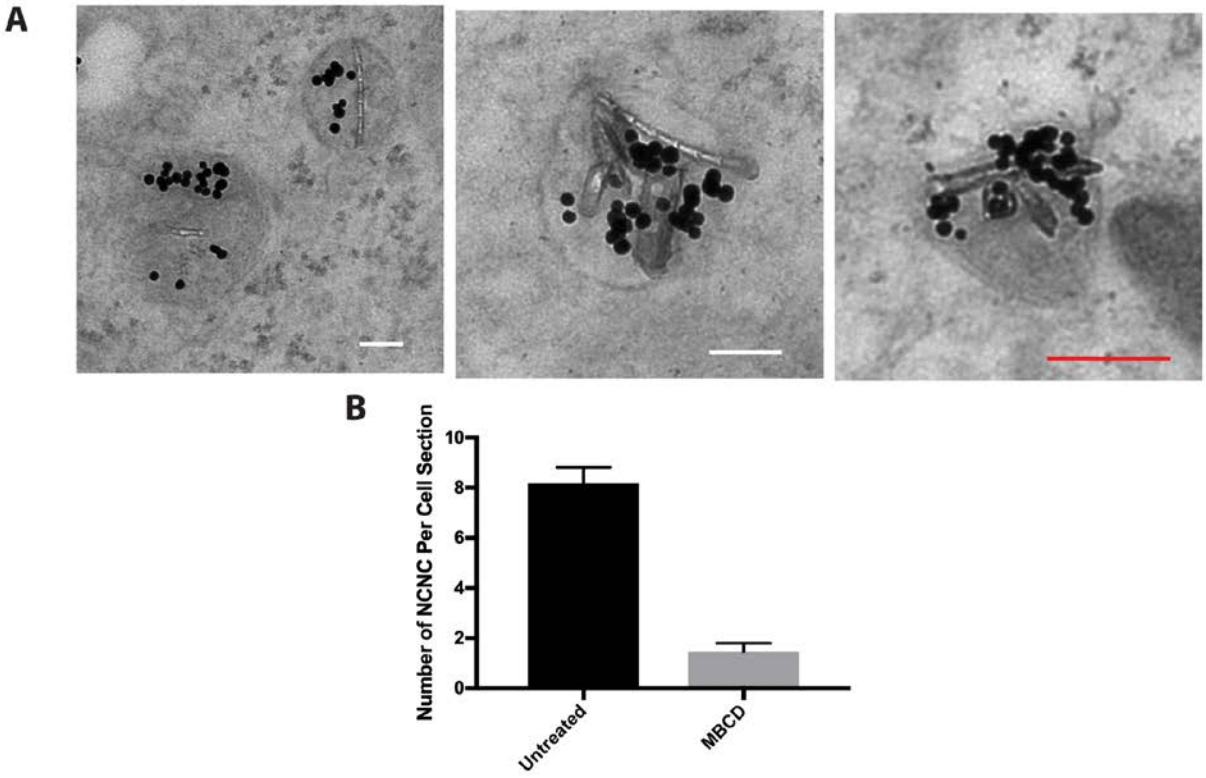


Figure 18 Colloidal gold co-incubation with NCNCs and endocytosis inhibition

A) **Examples of colloidal gold and NCNCs residing in the same vesicle. HeLa cells were treated with 5 μg/mL NCNCs and 5 nM 20 nm colloidal gold for 6 hours. B) HeLa cells were treated with 5 μg/mL NCNCs in the absence (untreated) or presence of endocytosis inhibitor MβCD for 6 hours. Number of NCNCs per cell section was counted for each treatment. N = two experiments. Error bar indicates standard deviation. White scale bar indicates 100 nm, red scale bar indicates 200 nm.**

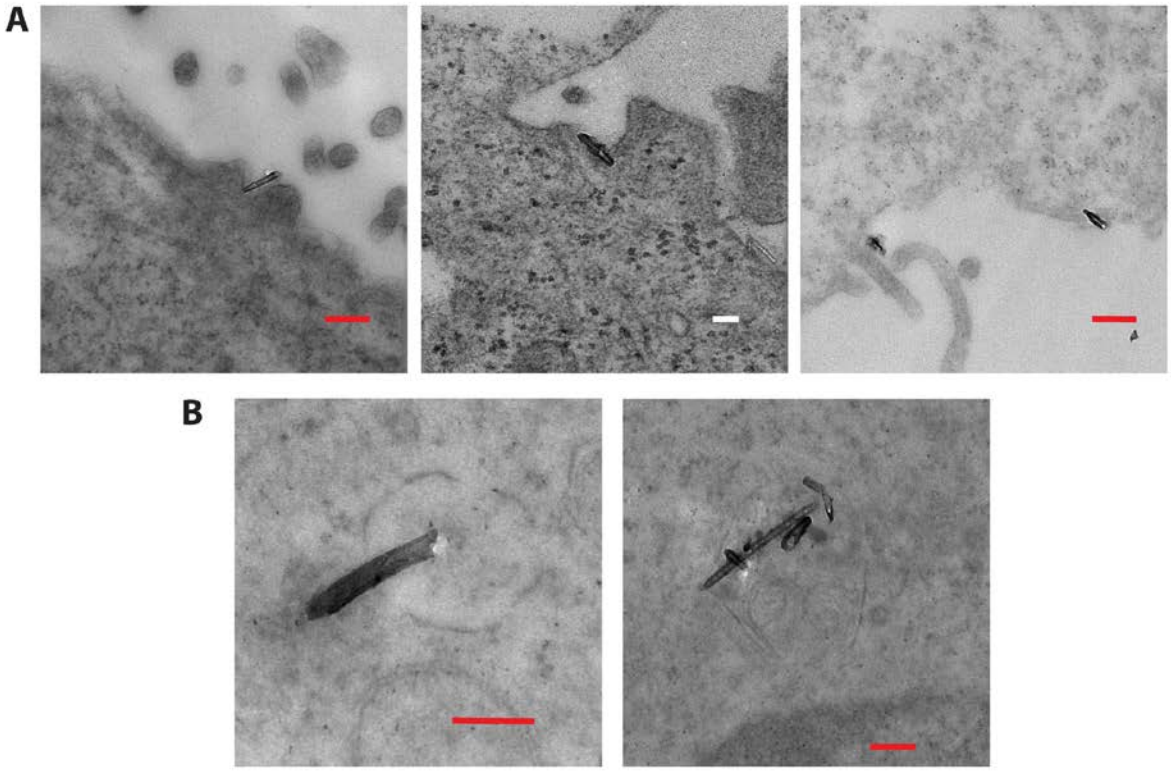


Figure 19 TEM visualization of NCNCs penetrating plasma membrane

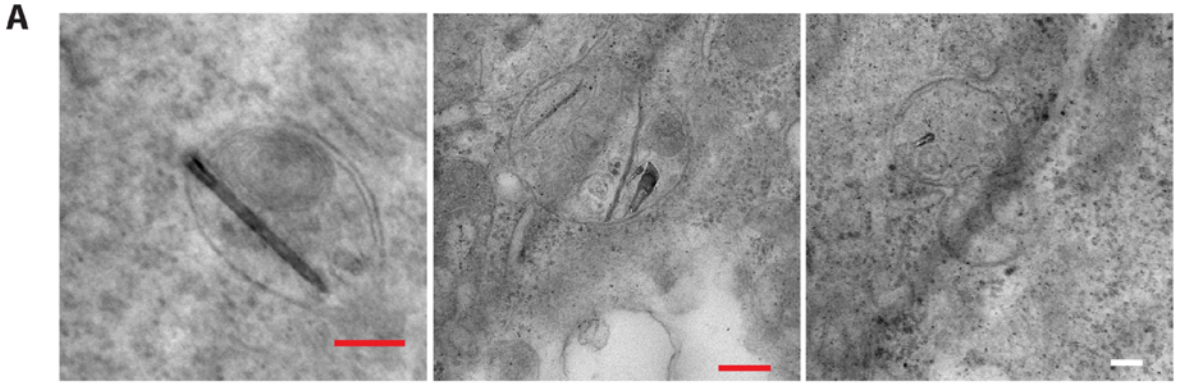
HeLa cells were treated with 5 μg/mL NCNCs and imaged using TEM. A) Examples of NCNCs penetrating the plasma membrane. B) Examples of NCNCs escaping and distorting vesicle membranes. White scale bar indicates 100 nm, red scale bar indicates 200 nm.

3.3 Autophagy Induction

Upon closer inspection of the vesicles in TEM images, I noticed that a portion of the vesicles had the morphology of autophagic vesicles (Figure 20a). The content of the vesicles had a morphology similar to the cytoplasm, and occasionally a double-membrane can be seen. I was curious to determine if NCNCs induced an autophagic response when incubated with cells. Autophagy is a dynamic process where long-lived proteins and organelles are incorporated into double membraned vesicles. These vesicles are then destined to fuse with the lysosome for degradation and the broken-down material is then recycled back into the cytoplasm for cellular use. This process requires the lipidation of a protein called LC-3 for the formation of autophagic vesicles and occurs at a low basal level in cells[74].

I hypothesized that if cells were upregulating the formation of autophagosomes, I would expect to see an increase in the level of lipidated LC-3. Alternatively, if cells are increasing the turnover of autophagosomes, also known as autophagic flux, then I would expect to have an enhanced enrichment of lipidated LC-3 in the presence of lysosomal inhibitor when compared to control cells. I incubated RPE-1 cells with 2 μg/mL NCNCs for 6 and 24 hours. The cells were then lysed and blotted for lipidated LC-3, which runs as a band lower than unmodified LC-1. I investigated the levels of lipidated in the presence of lysosomal hydrolase inhibitor bafilomycin A. This grants me the opportunity to observe the autophagic flux within cells. As a positive control, I incubated cells in starvation medium for 4 hours. Cells that were treated with NCNCs for 6 and 24 hours were compared to untreated and starved cells. Analysis of the western blot

showed that the levels of lipidated LC-3 was similar for untreated and NCNC treated cells. Looking at lysosome inhibited cells also shown that there was no enhanced enrichment of lipidated LC-3 for NCNC treated cells (Figure 20b). This data suggests that NCNCs do not induce an autophagic response and that the NCNCs that are found in autophagic vesicles were potentially uptaken via basal levels of autophagy that occurs in cells.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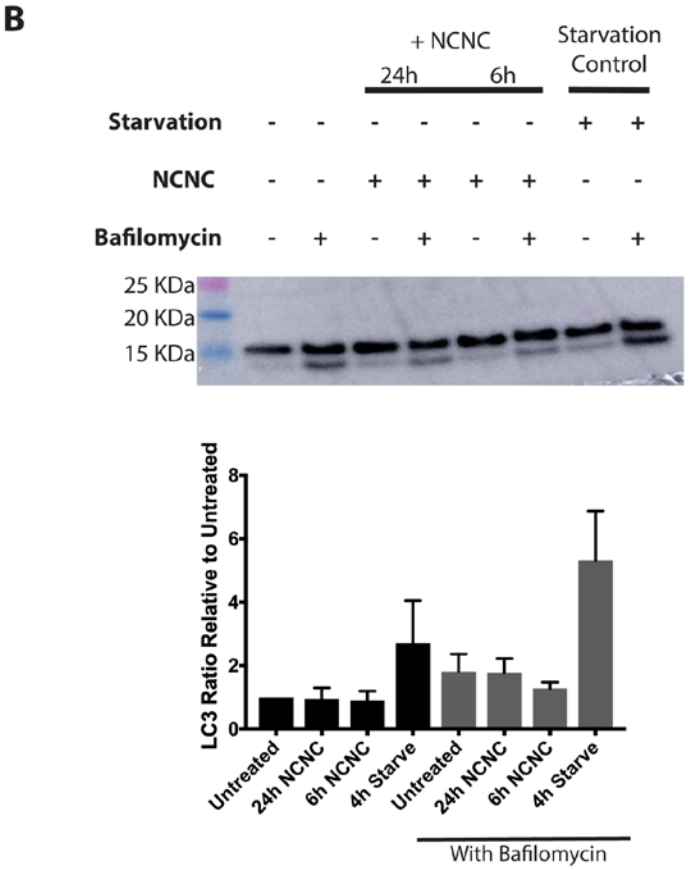
B
+ NCNC
24h
6h
Starvation Control
Starvation
NCNC
Bafilomycin
25 KDa
20 KDa
15 KDa
LC3 Ratio Relative to Untreated
Untreated
24h NCNC
6h NCNC
4h Starve
With Bafilomycin

Figure 20 TEM visualization of autophagic vesicles containing NCNCs and western blotting for autophagy induction

A) Examples of double membrane autophagic vesicles containing NCNCs in HeLa cells treated with 5 μg/mL NCNCs for 6 hours (left) and 48 hours (right). White scale bar indicates 100 nm, red scale bar indicates 200 nm. B) Representative western blot proving for autophagy marker LC3. For NCNC treatments, HeLa cells were incubated with 2 μg/mL of NCNCs for either 6 hours or 24 hours. For starvation treatment, cells were nutrient starved for a duration of 4 hours. 100 nM bafilomycin was added the last 2 hours of treatment in specified conditions. Graph is a quantification of bottom band/top band ratios of 4 western blot experiments. White scale bar indicates 100 nm, red scale bar indicates 200 nm.

3.4 NCNC Conjugation And Entry Into Cells

After determining that NCNCs do not have any detrimental effects on cells, I worked with an undergraduate researcher, Thomas Zhang, to determine if NCNCs could be used as a scaffold to manipulate cellular behavior. We obtained NCNCs that were covalently conjugated with 488 AlexaFluor IgG antibodies from the Star lab. This conjugation was added to the amine groups on the outer edges of NCNCs using EDC NHS cross-linking reactions. The conjugation of NCNCs to the fluorescent antibody was confirmed using fluorescence microscopy. HeLa cells were then incubated with antibody-conjugated NCNCs for 24 hours and fixed for imaging on the confocal microscope. Cells were stained with wheat germ agglutin to delineate cell boundaries. The images confirmed that the conjugated-NCNCs were within the boundaries of cells (Figure 21). Like the unmodified NCNCs, conjugated-NCNCs were found residing around the nucleus. Confocal microscopy also confirmed the antibodies remained attached to NCNCs while inside of cells based

on the colocalization of green fluorescence and bright field images of NCNCs. This shows that NCNCs are a good candidate for use as a scaffold for cellular manipulation.

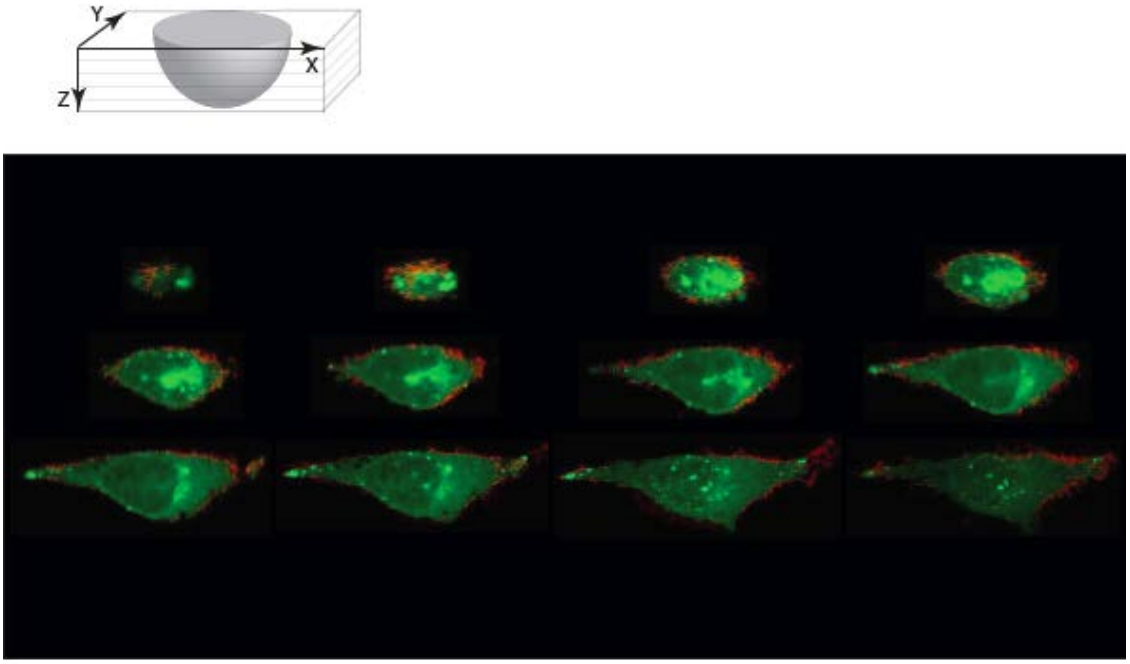

Figure 21 Confocal microscopy of conjugated-NCNCs in cells

HeLa cells were incubated with fluorescently labeled antibodies (Alexa Fluor 488) conjugated to NCNC for 24 h and stained with wheat germ agglutinin. Z stack images were collected of a representative cell as shown. NCNC-IgG conjugates (green) are localized within the boundaries of the cell as shown by WGA (Red).

4.0 Discussion

In this study, I have demonstrated that cells do not fail at cytokinesis when challenged with lagging chromatin. I have also shown using live cell imaging that cells with lagging chromatin slightly delay the completion of cytokinesis. Based on my experiments, it appears that gamma irradiation is the best method to induce lagging chromatin in HeLa cells. The question still remains as to whether the type of lagging chromatin is important for the activation of abscission delay. Is it chromatin in general that needs to be trapped, or do certain parts of the chromosome elicit a response from cells? In the example of nucleoid occlusion in bacterial cells, the cytokinetic machinery does not polymerize until DNA that is bound with Noc is away from the cleavage plane, which is generally the terminal end of the bacteria's genome. Also, studies in *Drosophila* have shown that cells are able to sense that chromatid arms are still in the cleavage plane and as a response, cells will elongate to ensure that its cytokinetic machinery does not cut into the ends of segregated chromosomes[2]. To further characterize the abscission delay pathway in mammalian cells, one can target regions of chromatin i.e. centromeres or telomeres, to the cleavage plane and determine if abscission delay is activated.

Because mitosis requires extensive movement of chromatin, it is in the best interest of cells to silence transcription during this process. In addition, protein synthesis is also inhibited during mitosis. How do cells modulate the activity of proteins if gene and protein expression are inhibited? Cells can utilize post-translational modifications (PTMs), to modulate protein-protein interactions and kinase activity. Examples of these modifications include phosphorylation, ubiquitylation, and SUMOylation[37]. More recently, modifications like acetylation and

methylation have been expanded from an exclusive histone modification to a form of regulation for non-histone proteins[75].

PTMs prove to be especially relevant for a protein that has multiple unique responsibilities. As mentioned earlier, Aurora B is a kinase that is integral in the progress of mitosis. The post-translational modifications of this kinase have been explored in various stages of mitosis. It is well understood that Aurora B phosphorylation is required for its activation[35, 44]. Recently it's been shown that PTMs like ubiquitylation and SUMOylation is required for translocating the kinase to the central spindle to initiate cleavage furrow ingression during anaphase. It is also known that Aurora B activity can be terminated through dephosphorylation by protein phosphatases 1 and 2[34]. Fadri-Moskwik and colleagues have shown that Aurora B is acetylated in early mitosis and that this modification inhibits its kinase activity[37]. They also have shown that HDAC3 was the enzyme that removes this modification from Aurora B.

For many of the known PTMs Aurora B undergoes, they have been exclusively studied before the onset of anaphase. In regard to abscission delay, I hypothesized that the reactivation of Aurora B to delay abscission would be dependent on PTMs. I proposed that since Aurora B will need to be deactivated for cytokinesis completion, I would expect to see unphosphorylated and possibly acetylated Aurora B in the midbodies without trapped chromatin. Under normal cytokinesis, Aurora B is localized to the outer portions of the midbody. If activation of abscission delay required Aurora B to relocalize to a different area within the midbody, I proposed that the cell may use SUMOylation to elicit this potential translocation.

I investigated potential acetylation, phosphorylation and SUMOylation of Aurora B in midbodies in the presence or absence of lagging chromatin. I found that phosphorylation of Aurora B in the midbody was insensitive to trapped chromatin. Because this modification is important for

Aurora B activity, this suggests that other PTMs may play a role in modulating Aurora B activity at the midbody. I also determined that both SUMOylation and acetylation of Aurora B decreases when chromatin is trapped in the midbody and that HDAC3 increases its presence at these midbodies. The fact that the decrease in acetylated lysine and increase in HDAC3 at these midbodies may suggest that acetylation may play a role in reactivating Aurora B for abscission delay. In an attempted to quantify these findings, I attempted immunoprecipitation assays and western blot probing to look for acetylated Aurora B in cytokinesis. Unfortunately, these experiments yielded inconclusive results.

Interestingly, in yeast cells, it has been shown that histone acetyl-transferase Ahc1 is required for NoCut when chromatin is trapped in the cleavage plane[29]. It is not yet known which acetyl-transferase modifies Aurora B in mitosis, elucidating this enzyme may get us one step closer to linking trapped chromatin to Aurora B activation. Since acetyl-transferases and deacetylases are known to associate with chromatin in term of histone modifications, it may be possible that the reactivation of transcription and translation after the nuclear envelope reforms may provide a local elevation of these proteins on chromatin that spans across the cleavage plane. As a result, Aurora B may be reactivated to engage the abscission delay pathway. Along the lines of Aurora B activation as a result of reinvigorated transcription, I propose that another potential activator for Aurora B with trapped chromatin can come in the form of RNA. Jambhekar and colleagues have demonstrated in Xenopus that Aurora B kinase activity was enhanced by mRNAs[76]. Interestingly, the mRNA was derived from cytoskeleton and transcriptional factors. This group suggests that these particular mRNAs are concentrated at the spindle and that their localization helps bolster Aurora B's activity in ensuring proper chromosome bi-orientation during metaphase.

It would be interesting to determine if the formation of RNAs from transcription of trapped chromatin is the link to the activation of abscission delay.

My investigation of PTMs activating abscission delay exclusively focused on the protein Aurora B. it should be kept in mind that Aurora B is part of a protein complex in cells. It includes the scaffolding protein INCENP, Borealin, and Survivin, and together it forms the chromosome passenger complex (CPC) [12, 31, 40, 41]. In addition, Aurora B can exist in a complex containing INCENP only, or as a holo-complex with Borealin and Survivin included[31]. INCENP has been found to be necessary for the activation of Aurora B via phosphorylation either by Aurora B or Citron kinase[33, 40, 77]. Studies have also shown that Borealin and Survivin can modulate Aurora B activity and localization. The phosphorylation of Borealin by MPS1 and association of Survivin with Aurora B has been linked to increased Aurora B kinase activity[31, 40]. Survivin association also assists in translocating Aurora B to centromeres during metaphase[40]. In addition, it has been suggested that Borealin can be SUMOylated during mitosis[30]. Taken together, this opens the possibility that Aurora B reactivation for abscission delay may not be restricted to PTMs only on Aurora B. Moving forward, one should consider investigating the modulations of PTMs on other CPC proteins when chromatin is trapped during cytokinesis.

A major challenge to investigating the factors upstream of Aurora B in the abscission delay pathway is that Aurora B is needed in practically all stages of mitosis. Traditional methods, which includes siRNA knockdown, or chemical inhibition of Aurora B tends to be a bit ambiguous when interpreting the phenotypes of cells under these conditions because these methods inhibit Aurora B kinase activity before cytokinesis. The inhibition of Aurora B must be precisely timed, because if its activity is essential for the completion of cytokinesis. Inhibiting Aurora B before it is finished its job at cytokinesis will yield abscission failure and multinucleated cells[31, 46]. To circumvent

this conundrum, I wanted to find a way to target candidate proteins upstream of Aurora B to the cleavage plane of cells undergoing normal cytokinesis.

I proposed potentially using carbon nanotubes as a signaling platform to further study abscission delay. This material has recently picked up steam for potential application in the biological field. Currently, CNTs are being used as biosensors and delivery tools for chemotherapeutic drugs[47, 50]. With its application in biological systems, many scientists were concerned about their effects on biological tissue. Pristine CNTs were found to be cytotoxic to cells and tended to aggregate due to their hydrophobic nature[47, 49]. Developments in synthesizing CNTs introduced functionalization of the outer surfaces of CNTs. Although these modifications mitigated the negative effects of CNTs on biological systems, the variety in cellular responses to various CNTs emphasizes that how they are synthesized renders each material as unique[49-51].

Collaborating with the Star Lab at the University of Pittsburgh, I wanted to investigate how cells interfaced with NCNCs, a carbon nanomaterial that was synthesized by this group. In this study, I have shown that NCNCs do not interfere with the metabolic activity or proliferation. Studies on SWCNTs have shown that they interfered with mitosis[78]. Multinucleation frequency and mitotic counts on HeLa cells treated with NCNCs demonstrate that unlike SWNCTs, NCNCs does not interfere with cell division. Another setback encountered with SWNTS and MWCNTs is that they can elicit an inflammatory response to cells that are exposed to them[69, 70]. An ELISA assay conducted to measure IL-6 secretion has shown that cells do not secrete inflammatory cytokine IL-6 while incubated with NCNCs. Taken together, this substantiates the notion that NCNCs are not cytotoxic to cells, which is in accordance with studies that have been done on other nitrogen-doped CNTs[7, 53, 79].

I have also shown that NCNCs are capable of entering cells and that they localize near the centrosome. This spatial arrangement is disrupted when microtubules are depolymerized, suggesting that NCNCs are trafficked in the direction to the centrosome via microtubules. Using transmission electron microscopy I have also shown that, NCNCs primarily enter cells via endocytosis I have also found that about half of the NCNCs in cells resided in vesicles, The percentage of NCNCs that were vesiculated was about 55-60%. This number seem incongruent with the main pathway for NCNC entry into cells, but this could be due to potential vesicle escape into the cytoplasm (Figure 19b). Further investigation is needed to fully parse apart how NCNCs enter cells. I have also shown that NCNCs conjugated with fluorescent antibodies are capable of entering cells. This finding is in agreement with the entry of other types of CNTs[80-82].

Interestingly, I found NCNCs to reside in vesicles that had a morphology similar to autophagosomes. For other nanomaterials, specifically, titanium oxide nanoparticles and carboxylated MWCNTs, cells treated with these materials have upregulated autophagy as stress response[69, 81, 83]. Looking at lipidated LC-3 levels via western blotting either in the absence or presence of lysosomal inhibitor demonstrates that cells do not upregulate autophagy when exposed to NCNCs. The presence of NCNCs potentially in autophagic vesicles could be due to the material being uptaken in basal autophagy that occurs within cells. Studies with NCNCs in neutrophils have shown that this cell type is capable of degrading NCNCs[7]. Further investigation needs to be done to determine if other cell types can clear NCNCs via degradation.

Taken together, my data suggests that NCNCs are not bioactive in cells. This makes NCNCs a prime material to be used as a signaling platform. The next step would be to determine if proteins with catalytic activity can successfully cross the plasma membrane while maintaining the integrity of the protein. A suggested experiment would be to attach luciferase to NCNCs, allow

them to incubate with cells and then conduct a fluorescence assay to determine the activity of the protein. If this proves to be successful, the use for NCNCs as a signaling platform can be expanded for use in studying other pathways. Next, I would want to target NCNCs to specific regions within cells. Other groups have shown that it is possible to target CNTs to subcellular compartments. This required the conjugation of CNTs with targeting molecules. For example, Battigelli et al. attached a mitochondrial targeting sequence to MWCNTs to deliver them into mitochondria[84]. Another group targeted CNTs to the nucleus by conjugating estradiol, a hormonal steroid, to their nanoparticles[85].

Having NCNCs encapsulated in vesicles can be a hindrance to targeting the material to desired locations within cells. Depending on how NCNCs are utilized, the 40-45% of NCNCs residing in the cytoplasm may be enough to manipulate cellular behavior to our liking. On the other hand, the potential trafficking of NCNCs through the autophagic pathway can be useful to scientist studying lysosomal storage disorders. Currently, a rising treatment for diseases such as Gaucher's disease and Fabry's disease is enzyme replacement therapy (ERT)[86, 87]. With ERT, the patient is administered recombinant lysosomal enzyme intravenously to replace the misfunctioning protein in the lysosomes of cells in the patient. The protein is then picked up by cells via receptors and then transported to the lysosome[86]. A limitation for this type of treatment is that the recombinant protein has a hard time passing through the blood-brain barrier (BBB), which prevents the treatment from ameliorating the neurological symptoms of these diseases[86].

Loading NCNCs with recombinant protein and administering it to patients systemically can be an improvement for ERT. With this method, NCNCs can potentially administer a more concentrated dose of recombinant protein to the lysosome as it transports through autophagy. It's been suggested that CNTs have the capability of crossing the BBB, it would be interesting to

investigate whether NCNCs have this capability as well[88]. If they do possess the ability to cross the BBB, they can be a valuable tool in treatment for lysosomal storage disorders.

In order to use NCNCs as a tool to study abscission delay, I must find a way to target them at the cleavage plane during cytokinesis. At the central spindle, where cytokinesis occurs, there are bundles of microtubules that are organized with their plus ends stacked on each other. To target NCNCs to the cleavage plane I could try attaching a SxIP motif, a segment found in the EB1 protein that targets it to the plus ends of microtubules[89]. This would place the NCNCs at the center of the midbody. As I mentioned earlier, Jambhekar et al. have shown that Aurora B kinase activity can be activated with RNA[76]. I think it would be interesting to investigate if the presence of RNA at the cleavage plane can activate Aurora B for abscission delay. To do this, I would need to conjugate RNA constructs to NCNCs. Other groups have shown that CNTs can be conjugated with nucleic acids for application in gene delivery[52]. I think it would be a good step forward to adapt this method of conjugation to investigate the impact of RNA at the cleavage plane in regards to abscission delay. Also, I think it would be good to explore functionalizing NCNCs with multiple factors of the abscission delay pathway. For example, adding both RNA and Aurora B to individual NCNCs for targeting to the midbody of normally dividing cells. Moving forward, investigating whether NCNCs can be multifunctionalized using the method by Ménard-Moyon and colleagues can really prime NCNCs as a powerful tool in understanding the link between trapped chromatin and Aurora B activation in the abscission delay pathway[90].

5.0 Materials And Methods

5.1 Cell Culture And Materials

OSCC40 cells were grown in Minimum Essential Medium, supplemented with 10% fetal bovine serum and 1% non-essential amino acids. OSCC40 cells were selected for cells expressing H2B-GFP through the use of Blasticidin S HCl (Invitrogen) in 2 ng/mL concentrations. HeLa cells were grown in Dulbecco's Minimum Essential Medium and supplemented with 10% fetal bovine serum. RPE-1 cells were grown in DMEM/F-12 1:1 Medium with 10 % fetal bovine serum. All cells were incubated in a 37°C incubator with 5% CO_2. Nocodazole, monastrol, and methyl-beta cyclodextrin were purchased from Sigma Aldrich. NCNCs and antibody-conjugated NCNCs were a kind gift from the Star lab at the University of Pittsburgh. Cells that were incubated with NCNCs were treated with 1% penicillin and streptomycin. TEM fixation materials were obtained from Electron Microscopy Sciences. Recombinant TNFα was purchased from Thermo Fisher. Adenovirus plasmid containing GPI-anchored Fluorescently Tagged Protein (GPI-FAP) and accompanying dye was a kind gift from the Center for Biological Imaging (CBI) at the University of Pittsburgh. A table of all antibodies used is provided below.

Table 2 Table Of Antibodies Used For Experiementation

Antibody	Brand	Species	Dilution
N-Cadherin	Invitrogen	mouse	1:250
Aurora B	Cell Signaling	mouse	1:250 (1:1000 for WB)
Phospho-Aurora B	Cell Signaling	rabbit	1:250
Acetylated Lysine	Cell Signaling	rabbit	1:100 (1:1000 for WB)
SUMO-2/3	Cell Signaling	rabbit	1:250 (1:1000 for WB)
HDAC3	Cell Signaling	mouse	1:250
Gamma Tubulin	Sigma Aldrich	mouse	1:2000
LC-3	Cell Signaling	rabbit	1:1000

5.2 Methods

5.2.1 Lagging Chromatin Induction

To induce chromatin bridges and fragments, cells were exposed to 3 Gy of cesium and allowed to recover in fresh medium for 24 hours. To induce lagging whole chromosomes, cells were either incubated with 50ng/mL nocodazole for 12 hours and then released into fresh media for 24 hours or incubated with 100 μM monastrol for 16 hours and then released in fresh media for 2 hours. The cells were then fixed in the method described in fluorescence microscopy and immunostained with antibodies against N-cadherin to delineate cell boundaries.

5.2.2 Immunoprecipitation Assay

Hela cells were synchronized by incubation with 2 mM thymidine for 16 hours, then released in culture media for 10 hours. Cells were then blocked in 100 ng/mL nocodazole for 4 hours. The cells were then rinsed with fresh media and allowed to continue mitosis. Cells were collected a various time points over a span of two hours. The cells were lysed in RIPA buffer and put on ice. Protein A beads were coated with antibodies against Aurora B and incubated with lysates overnight at 4°C with constant shaking. The beads were then pelleted via centrifugation and ran on a polyacrylamide gel. The proteins were transferred to PVDF membrane and blotted using antibodies against Aurora B and acetylated lysine.

5.2.3 Live Cell Imaging

OSCC40 cells stably expressing GFP-H2B were grown on matek glass bottom coverslips. They were transiently infected with an adenovirus plasmid containing GPI-FAP. After infection, the cells were subjected to 3 Gy irradiation and allowed to recover for 24 hours. Cells were then incubated with a cell impermeant dye that reacts with the GPI-FAP and were imaged every three minutes for a duration of one hour on a confocal microscope with a temperature controlled staged at 37°C[62, 63].

5.2.4 Cell Viability Assay

For WST-1 Assay, cells were seeded at (3*10^3) per well in triplicates on a 96 well plate and allowed to settle overnight. At each designated time point the media was removed and WST-1 at a dilution of 1:10 was added and incubated for 2hours. Plates were then analyzed on an EL800 microplate reader at 450 nm. For MTT assay, cells were seeded (5*10^3) per well in triplicates on 96 well plates and allowed to settle overnight. At each designated time point, the media was removed and MTT was added at a dilution of 0.5 mg/mL and incubated for 4 hours. The solution was removed and 10% SDS with HCl was added to each well and incubated for 16 hours. The plates were then read on an EL800 microplate reader at 562 nm.

5.2.5 TEM Preparation

HeLa cells were incubated with 5 μg/mL NCNCs at designated time points. Cells were then trypsinized and fixed in 2% glutaraldehyde and 2% paraformaldehyde in phosphate buffer for 2 hours. Cells were then washed 3 times for 10 min each in phosphate buffer, then incubated in 1% osmium tetroxide in phosphate buffer for 1h. Cells were then washed 5 times 10 min each in phosphate buffer and then incubated in 50% ethanol in deionized water for 10 min. Cells were then incubated in 70% ethanol for 10 min and 100% ethanol twice for 15 minutes. Cells were then incubated in 100% propylene oxide for 15 min each and then allowed to incubate in a 50% mixture of embedding epoxy and propylene oxide with rotation overnight. The next day the solution was replaced with 100% embedding epoxy and allowed to incubate for several hours. Samples were then allowed to cure overnight in BEEM capsules in a 60°C oven.

Samples were then cut into 70nm sections using an ultramicrotome with a diamond knife and placed on copper EM grids. Samples were imaged on a Morgagni TEM microscope with a camera.

5.2.6 Fluorescence Microscopy

HeLa cells were seeded on glass coverslips and allowed to recover overnight. Cells were then treated with monastrol, nocodazole or 3Gy irradiation and allowed to recover at various time points. The cells were then fixed with 2% paraformaldehyde with 0.2% triton for 15 minutes. Cells were then washed three times 5 minutes each with 1X PBS. They were then incubated with 5% BSA in 1X PBS and 0.5% TWEEN for 30 minutes. Cells were washed again three times with

1X PBS and incubated with primary antibodies for a minimum of 30 minutes. They were then washed three times, 5 minutes each and incubated with fluorophore conjugated secondary antibodies for a minimum of 30 minutes. Cells were washed two times, 5 minutes each with 1X PBS, incubated in autoclaved water for 5 minutes and then mounted onto slides using mounting solution that contains DAPI. Images were taken on an Olympus microscope using a 100X oil emersion objective.

Cells were grown on glass coverslips and treated with NCNC's at various time points. They were then fixed with 4% paraformaldehyde in 1X PBS for 15 minutes. Cells were then washed three times in 1X PBS and then incubated with rhodamine phalloidin (Cytoskeleton) at a 1:1000 dilution for 15 minutes and washed twice with 1X PBS. Cells were then washed with deionized water for 5 min and then mounted on to glass slides with a mounting solution with DAPI included (Life Technologies). Slides were imaged on an Olympus microscope using a 100X oil emersion objective.

To determine the efficacy of endocytosis inhibition, HeLa cells were seeded on glass coverslips and treated with 5 mM methyl-beta cyclodextrin for 6.5 hours at a concentration of 5 mM. After 30 min pre-incubation, 4 μL of 25 mg/mL Texas red dextran per mL of media was added to the coverslips and incubated for 6 hours. Cells were then fixed with 4% paraformaldehyde and washed with 1X PBS three times and mounted on glass slides using mounting solution with DAPI.

5.2.7 Centrosome Staining

Cells were grown on glass coverslips and incubated with NCNCs for 24 hours. The media was then removed and replaced with 1 μg/mL nocodazole for 4 hours. Cells were then fixed with 100% methanol at -20°C for 15 minutes. They were then washed 3 times with 1X PBS and incubated in blocking solution containing 1.5% BSA and 0.1% Tween in 1X PBS for 30 minutes. Coverslips were then incubated with primary mouse antibodies against γ-tubulin (Sigma Aldrich) at a dilution of 1:2000 in blocking buffer for 1 hour. After washing 3 times in 1X PBS, coverslips were incubated with 488-Alexa fluorophore-conjugated secondary antibodies (Life Technologies) for 30 min. The cells were then washed twice, 10 min each in 1X PBS, once with deionized water for 5 min and mounted on glass slides using mounting solution with DAPI.

5.2.8 Western Blotting

Cells were treated with NCNCs at various time points and then lysed with RIPA lysis buffer. The lysate was then spun down at high speed for 10 min and the supernatant was collected and electrophoresed on a 4-12% polyacrylamide gel. Protein was transferred to PVDF membrane and blocked in 5% milk in TBST and then incubated overnight with rabbit primary antibodies against LC3 (Cell Signaling). The membrane was then washed with 1X TBST and incubated with HRP conjugated secondary antibodies (GE Life Sciences) for 1 hour. The membrane was then washed with 1X TBST and incubated with luminol for 5 min and imaged on an Amersham western blot imager.

5.2.9 Autophagy Induction and Inhibition

RPE-1 cells were induced to undergo autophagy using a starvation medium containing 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 g/L glucose, and 10 mM HEPES at a pH of 7.4 and sterile filtered. Cells were incubated in starvation media for 6 hours and then incubated either in the absence or presence of bafilomycin for 2 hours. Cells were then lysed for western blotting. Autophagy was inhibited using 500 nM wortmannin for 6 hours.

5.2.10 Inflammatory Cytokine Detection

IL-6 secretion was assayed using an IL-6 ELISA kit from BD Scientific. Hela cells were seeded 3*10^3 cells per well in a 96 well plate and allowed to attach overnight. Cells were then treated with increasing concentrations of NCNC, or 1 ng/mL TNFα for a duration of 24 hours. The supernatants were collected and spun down at 8 krpms for 5 minutes to remove particulate material. 100 μL of supernatant was used for analysis in the ELISA.

5.2.11 Confocal Microscopy Of NCNC-IgG Conjugates

Cells were grown on a glass coverslip and incubated with NCNC-IgG conjugates for 24 hours. The media was removed and washed in 1X PBS five times. Cells were fixed with 4% paraformaldehyde in 1x PBS for 15 min. Cells were then washed s times in 1X PBS and then incubated with wheat germ agglutinin at 1:1000 dilution for 30 min and washed with 1x PBS two times. Cells were then mounted onto glass slides a mounting solution with DAPI included (Life Technologies). Slides were imaged on a Leica TCS SP5 confocal and multi-Photon microscope.

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