THESIS

AURORA A KINASE PHOSPHORYLATES SERINE 62 ON HEC1 TO AFFECT MITOTIC KINETOCHORE MICROTUBULE INTERACTIONS

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ABSTRACT

AURORA A KINASE PHOSPHORYLATES SERINE 62 ON HEC1 TO AFFECT MITOTIC KINETOCHORE MICROTUBULE INTERACTIONS

The Hec1 protein plays an important role in ensuring successful chromosome segregation during cell division. Its 80 amino acid, unstructured, "tail" region is critical for kinetochore-microtubule attachment regulation, which is mediated through Aurora kinase phosphorylation. At least nine phosphorylation target sites within this domain have been identified, including the recently confirmed target site, serine 62 (S62). However, the functional significance of phosphorylation of this residue remains elusive. Here, we selectively target Aurora A and Aurora B kinase protein activities using the inhibitors MLN8054 and ZM447439, respectively, and study their effects on the dynamics of serine 62 phosphorylation in the Hec1 tail. Utilizing immunofluorescence, we demonstrated that inhibition of Aurora A kinase activity leads to a significant reduction in phosphorylation levels at serine 62. Additionally, using phospho-null mutants, we studied the effect of serine 62 phosphorylation on the creation of stable, tension-generating kinetochore-microtubule attachments by measuring the distance between sister kinetochores. Our findings reveal that alterations in serine 62 phosphorylation status result in subtle changes in interkinetochore distances showcasing the functional relevance of this phosphorylation event in regulating kinetochore-microtubule attachments. Furthermore, under conditions of nocodazole-induced mitotic arrest, we observe a marked decrease in phosphorylation at serine 62 suggesting a microtubule dependent regulation of this phosphorylation. These findings provide evidence supporting the role of Aurora A kinase in

phosphorylating serine 62 of the Hec1 tail and shed light on the regulation of this critical post-translational modification during mitosis.

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INTRODUCTION

Mitosis is a fundamental process that plays a pivotal role in cell proliferation and growth. Through mitosis, a single parent cell duplicates its genetic material and divides into two genetically identical daughter cells. This precise and controlled division ensures the proper distribution of genetic information. While mitosis is a vital process, mis-segregation of the genetic material can occur, resulting in aneuploidy. This event is when a cell receives an abnormal number of chromosomes as a result of errors in mitotic division. Aneuploidy is a hallmark of cancer cells, and it is associated with genomic instability. Cells have checkpoints and error correction mechanisms that work together to ensure proper chromosome segregation. The kinetochore is a critical structure that plays a role in these processes that ensure an accurate distribution of chromosomes.

The Kinetochore and Hec1

The kinetochore is a complex protein structure that assembles on the centromere region of each chromosome just before nuclear envelope breakdown. This structure functions as an attachment point for spindle microtubules emanating from each of the two spindle poles. The attachment of microtubules to the kinetochore happens in a highly regulated way. NDC80 is a protein complex located at the kinetochore that is important for this microtubule binding activity. This protein complex is composed of Hec1, Nuf2, Spc24, and Spc25. NDC80 plays a crucial role in mediating the attachment of chromosomes to the microtubules of the mitotic spindle. Spc24 and Spc25 play a role in directing the NDC80 complex to the kinetochore and ensuring its stability (Ciferri et. al 2005; Wang et. al 2008). The N-termini of these proteins form

a coiled coil domain, facilitating interactions with the coiled coil domain of Hec1 and Nuf2 and enabling the formation of a tetrameric structure (Ciferri et. al, 2005, 2008; Wei et al., 2005; Wang et al. 2008; Valverde et al. 2016). The N-termini of Hec1 and Nuf2 each contain a globular calponin-homology (CH) domain. In the case of Hec1, this CH domain includes a region known as the “toe,” which directly binds to microtubules (Wilson-Kubalek et al., 2008; Alushin et al., 2010). In the far N terminus of Hec1, there is an unstructured tail domain which is highly basic and positively-charged. The Hec1 tail has been suggested to play a role in generating stable kinetochore-microtubule attachments, although the mechanism for this function remains unresolved. Additionally, the Hec1 tail is dynamically phosphorylated at multiple residues throughout mitosis (Figure 1). These post-translational modifications assist in maintaining the attachment of NDC80 to microtubules or facilitating its release in case of an erroneous attachment (Cheeseman et. al, 2006; DeLuca et al. 2006; Zaytsev et al. 2014, 2015). During the start of mitosis, the attachments between Hec1 and the microtubules are unstable, allowing for incorrect attachments to correct themselves. As mitosis continues, correct kinetochore-microtubule attachments are generated, the turnover of these attachments is reduced, and attachments are stabilized to allow for force to be generated for chromosome movements (Wimbish and DeLuca, 2020).

Aurora Kinases

Aurora A and B kinases are serine/threonine protein kinases that play a role in the regulation of cell division. Aurora A kinase has a well-established role in centrosome maturation and spindle assembly (Hannak et al., 2001; Asteriti et al., 2015). It is primarily found at the centrosomes and seen along the spindle microtubules; however, Aurora A kinase has also been

detected at kinetochores in both dividing human and mouse cells (Kufer et al., 2002; Chmátal et al., 2015; Katayama et al., 2008). Aurora A binds the microtubule associated protein TPX2 (Targeting protein for Xenopus kinesin-like protein 2), thereby promoting increased catalytic activity of Aurora A kinase (Kufer et al. 2002; Bayliss et al. 2003). Aurora B is a component of the Chromosomal Passenger Complex (CPC) that has roles in chromosome condensation, alignment, and segregation (Carmena et al., 2009, 2012; Hindriksen et al., 2017). Aurora B kinase is activated by its co-factor INCENP, which is also a member of the CPC (Yang et al. 2009). Both Aurora kinases are involved in the regulation of Hec1 and phosphorylate numerous target residues along the Hec1 tail. Aurora A and B have high sequence similarity as well as the ability to recognize highly similar consensus sequences (Meraldi et al., 2004; Ohashi et al., 2006; Carmena et al., 2009; Kim et al., 2010). The phosphorylation of Hec1 by these kinases affects the affinity of kinetochores to microtubules as well as regulates kinetochore microtubule dynamics in metaphase (Cheeseman et al., 2006; DeLuca et al., 2006, 2011; Zaytsev et al., 2014, 2015). Polymerized microtubules have a net negative charge, and it has been suggested that microtubules directly interact with the positively charged tail domains of Hec1 via electrostatic interactions (Alushin et al., 2012). Phosphorylation of at least 9 sites along the Hec1 tail introduces a negative charge to the amino acids being modified, promoting dissociation of Hec1 from microtubules, and thus facilitating kinetochore-microtubule error correction (Figure 1 C) (Cheeseman et al., 2006; Umbreit et al., 2012, Zaytsev et al., 2015; DeLuca et al., 2006; Sundin et al., 2011; Kucharski et al., 2022). Previous studies have utilized phospho-specific antibodies to characterize phosphorylation dynamics of specific residues along the Hec1 tail (DeLuca et al., 2011, 2018). These antibodies bind to the Hec1 tail if

phosphorylation is present at the specific residue. Using these antibodies, it was determined that Aurora B kinase is responsible for phosphorylating serines 8 (S8), 15 (S15), 44 (S44), and 55 (S55), while Aurora A kinase is responsible for phosphorylation of S55 (together with Aurora B) and S69. These results give rise to a model in which the Aurora kinases may each phosphorylate specific regions of the tail domain with Aurora B kinase targeting residues at the far N-terminus of the Hec1 tail, and Aurora A kinase targeting residues closer to the CH domain. These phospho-antibodies were also used to determine phosphorylation trends throughout mitosis, and it was determined that the Aurora B sites are phosphorylated early in prometaphase and levels decrease as cells proceed to metaphase (DeLuca et al., 2011). This idea aligns with the model that suggests phosphorylation along the Hec1 tail plays a role in modulating attachment stability. During prometaphase, higher phosphorylation levels are needed to allow for the correction of erroneous attachments while in metaphase correct attachments are established resulting in a decrease in phosphorylation levels. It was also determined that the Aurora A site S69 remains highly phosphorylated throughout mitosis and that removing Aurora A phosphorylation resulted in a lower level of metaphase kinetochore oscillations (DeLuca et al., 2018). These findings support a model suggesting that in addition to high levels of phosphorylation on the Hec1 tail being required for error correction in early mitosis, low levels of phosphorylation are required in late mitosis to support kinetochore oscillations and error-free chromosome segregation.

Tension Generation at the Kinetochore-Microtubule Interface

During mitosis, as cells align at the metaphase plate and correct attachments are made, tension is generated at the kinetochore-microtubule attachment interface. Tension arises from

the opposing forces exerted by dynamic microtubules at the kinetochores and is essential for chromosome alignment and segregation (Waters et al., 1996). Once correct attachments are made, tension is generated, cells align their chromosomes, and then progress into anaphase; however, if an attachment error is made and correct tension isn't achieved, the cell will employ error correction mechanisms to correct the error and restore proper tension (Stern and Murray, 2001; Pinsky and Biggins, 2005; Maresca and Salmon, 2009). Several models have been proposed that explain how tension impacts both error correction and Aurora kinase activity at the kinetochore. A prominent model states that as tension is generated, sister kinetochores are stretched away from a centromere-localized pool of Aurora B kinase, such that Aurora B becomes spatially separated from its kinetochore substrates, resulting in Aurora B no longer being able to phosphorylate Hec1 (Liu et al., 2009; Welburn et al., 2010). This model explains how correct, tension-generating attachments produce a situation in which Hec1 is stably attached to spindle microtubules and can't be phosphorylated to promote microtubule release. Another model, however, states that correct, tension-generating attachments result in structural changes within the kinetochore, which facilitate the eviction of Aurora B kinase, thus preventing its phosphorylation of the Hec1 tail (Campbell and Desai 2013; de Regt et al. 2022). Specifically, the model suggests that when Hec1 is not stably attached to microtubules, the kinetochore contains a binding site for Aurora B kinase; however, when Hec1 properly attaches to microtubules and generates tension, this generates changes in the kinetochore that mask the Aurora B binding site, thus resulting in low Hec1 phosphorylation (de Regt et al. 2022). The precise mechanism of how Aurora kinases interact with kinetochores while under varying degrees of tension is still up for debate; however, a large body of literature strongly suggests

that as stable kinetochore-microtubule attachments are formed, tension is generated across kinetochore pairs, and Aurora B kinase activity decreases at kinetochores.

Phosphorylation of Hec1 at Serine 62

As mentioned above, it remains unclear how and why Aurora A and B phosphorylate different residues on the Hec1 tail, but we have suggested a spatial model in which the sites in the N-terminal half of the tail are accessible primarily to Aurora B kinase, and the sites in the C-terminal half of the tail (closer to the CH domain) are accessible primarily to Aurora A kinase**.** In this study, our objective is to characterize the phosphorylation dynamics of Hec1 at S62, identify the kinase responsible for phosphorylation, and begin assessing the biological function of phosphorylation on this residue. Our hypothesis posits that Aurora A kinase, rather than Aurora B kinase, will phosphorylate S62, as this residue resides in the C-terminal half of the Hec1 tail. We hypothesize that the phosphorylation dynamics of S69 are similar to those of S62, given their close proximity to each other, and specifically, that phosphorylation will remain high throughout the duration of mitosis to facilitate normal kinetochore oscillations in metaphase. Our approach involves fixed cell analysis of cells fluorescently stained with an antibody to detect phosphorylation at site 62, allowing us to identify phosphorylation trends. Additionally, we use Aurora kinase inhibitors to discern the kinase responsible for phosphorylating this residue. To begin exploring the functional aspects, we generated Hec1 tail constructs with phospho-null mutations at various sites along the Hec1 tail. These constructs allow us to investigate how the phosphorylation/dephosphorylation of S62 contributes to formation of stable kinetochore-microtubule attachments and the generation of kinetochore tension, assessed through interkinetochore distance measurements.

RESULTS

Previous findings indicate that low, persistent levels of phosphorylation on the Hec1 tail are essential for kinetochore oscillations in metaphase and error-free chromosome segregation (DeLuca et al. 2018). It has been established that phosphorylation of S69 persists throughout mitosis to assist in these oscillations by providing this sustained level of phosphorylation that appears to be integral for normal kinetochore movements (DeLuca et al. 2018). However, the role of pS62 in this process remains unclear. To address this, we generated a phospho-specific antibody targeting this site (pS62), aiming to discern its phosphorylation pattern and gain insights into its impact on Hec1 activity and function. Hela cells were grown, fixed, and immuno-stained using the antibody, imaged using a fluorescence microscope, and then kinetochore fluorescence intensities were quantified (Deluca et. al 2018). Quantification revealed that S62 was phosphorylated at kinetochores in early prometaphase, and kinetochores sustained this level of phosphorylation throughout all stages of mitosis (Figure 2). These results are consistent with the pattern of phosphorylation of pS69 observed previous in our lab and differ from results obtained with antibodies to other well-characterized phosphorylation sites on the Hec1 tail, in which phosphorylation levels significantly decrease at kinetochores in metaphase and anaphase, as cells progress through mitosis (DeLuca et al. 2011, 2018). To confirm the specificity of the antibody, we immuno-stained cells in which Hec1 was knocked down using siRNA. This experiment ensures that when Hec1 is absent from the cell, the antibody will not bind at kinetochores. The fluorescence intensity of pS62 at kinetochores was significantly decreased upon Hec1 knockdown to undetectable levels, indicating that the antibody was indeed specific for Hec1 (Figure 3).

We subsequently investigated the dependency of S62 phosphorylation on either Aurora A or Aurora B kinase. Most sites along the Hec1 tail show specificity for Aurora B kinase despite the kinases sharing nearly identical consensus sequences (DeLuca et al. 2011). Aurora A, however, has been shown to be the primary kinase responsible for phosphorylation of S69, and has also been shown to contribute to the phosphorylation of S55 (i.e. both Aurora A and Aurora B kinase phosphorylate this site) (DeLuca et al. 2011, 2018). To determine the kinase responsible for S62 phosphorylation, we treated cells with 0.5 µM MLN8054 or 2 µM ZM447439. These are small molecule inhibitors that target Aurora A kinase or Aurora B kinase, respectively (Bavetsias and Linardopoulos 2015). Both inhibitors at the given concentration prevent the activation of their respective kinase. Aurora A kinase is phosphorylated at Thr288 and Aurora B kinase at Thr232, and these sites are required for the kinase's activation (Yasui et al., 2004; Ohashi et al., 2006; Tavernier et al., 2021). Once treated with inhibitors, the phosphorylation at these sites decreases and the kinases will be inactive and unable to phosphorylate Hec1. We first ensured the inhibitors had minimal effect on the opposite kinase by staining for phosphorylated Aurora A and Aurora B kinase after kinase inhibition treatment. Although there was some low level of cross-inhibition, Aurora A kinase remained highly phosphorylated after ZM447439 treatment (Figure 4) and Aurora B remained highly phosphorylated after MLN8054 treatment (Figure 5). Next, we immuno-stained cells treated with the kinase inhibitors using our pS62 antibody and found that pS62 fluorescence intensity at kinetochores significantly decreased after MLN8054 treatment, but not ZM447439 treatment, demonstrating that S62 is preferentially phosphorylated by Aurora A kinase, similar to what was found previously for S69 (Figure 6) (DeLuca et al. 2018).

Previous studies have demonstrated that microtubules play a role in promoting the activity of Aurora A kinase, and the levels of phosphorylation at residue S69 decreased significantly in the absence of microtubules (Silva and Cassimeris, 2013; DeLuca et al., 2018). Thus, we aimed to investigate the impact of microtubule depolymerization on the phosphorylation levels of residue S62. To achieve this, we treated cells with 1 µM nocodazole to depolymerize spindle microtubules and measured the kinetochore fluorescence intensity of phosphorylated S62 using our pS62 antibody. Following nocodazole treatment, we observed a significant decrease in the intensity of kinetochore-associated pS62 (Figure 7), similar to the previously observed decrease in S69 phosphorylation levels upon similar treatment (DeLuca et al., 2018). To confirm the absence of microtubules, cells were stained with a tubulin antibody after treatment, and as expected, reduced polymerized microtubules were detected (Figure 7). In addition, we immuno-stained nocodazole-treated cells with an antibody to Mad2, which is a component of the mitotic checkpoint complex (MCC) and accumulates at unattached kinetochores (Waters et al. 1998). After kinetochore-microtubule attachments are properly formed, Mad2 is released from kinetochores, satisfying the checkpoint so cells can proceed to anaphase. In nocodazole-treated cells, Mad2 levels remained high at kinetochores, demonstrating that kinetochore-microtubule attachments were not formed due to the absence of microtubules (Figure 7). Finally, we assessed levels of phosphorylated Aurora A kinase, and as expected, Aurora A kinase fluorescence intensity at Thr288 decreased significantly (Figure 7).

We next investigated the functionality of S62 phosphorylation on the Hec1 tail by assessing the stability of kinetochore-microtubule attachments in cells experimentally manipulated to prevent phosphorylation at this site. This was done by measuring the distance

between sister kinetochore of aligned chromosome (i.e. interkinetochore distances). Once stable attachments are formed between the microtubule and the kinetochore, tension is generated causing the kinetochores to stretch apart. This tension can be assessed by measuring inter-kinetochore distances, in which the distance between two sister kinetochores is determined, which is a read-out for how stretched apart the kinetochores are. It has been shown the more stable kinetochore-microtubule attachments are, the higher the tension generation is between the two sister kinetochores, which leads to increased stretch and greater inter-kinetochore distances (Stern and Murray, 2001; Pinsky and Biggins, 2005; Maresca and Salmon 2009). To assess the functionality of phosphorylation on residue S62, mutants of the Hec1 tail were made. Specifically, residue 62 was mutated from serine to alanine (S62A) to create a phospho-null mutant, which inhibits phosphorylation on this site. This construct, which also contains a GFP moiety (for identifying transfected cells), was generated and tested along with three other mutants: (1) S69A; (2) a double mutant, S62A/S69A; and (3) a "9A" mutant, in which all 9 Aurora kinase phosphorylation sites are mutated to alanine (S4, S5, S8, S15, S44, T49, S55, S62, and S69), and whose expression has been previously shown to produce hyper-stable kinetochore-microtubule attachments with increased inter-kinetochore distances (Tauchman et al. 2015). These mutants were transfected into HeLa cells depleted of endogenous Hec1 using siRNA, and cells were fixed and immuno-stained with antibodies to tubulin and a kinetochore marker. Cells expressing the mutants were identified via their GFP fluorescence and imaged using fluorescence microscopy. These images were then used to measure inter-kinetochore distances in cells with aligned chromosomes. Interestingly, we found that as expected, cells expressing the 9A mutant demonstrated the highest average inter-

kinetochore distance, indicating hyper-stable kinetochore-microtubule attachments and high levels of tension generation at the kinetochore-microtubule interface (Figure 8). Cells expressing the single alanine mutants both showed a slight, but statistically significant increase in average inter-kinetochore distance compared to wild-type Hec1 tail (Figure 8). Cells expressing the double mutant also showed a slight increase in average inter-kinetochore distance compared to cells expressing wild-type Hec1, but these distances were not statistically significantly different compared to cells expressing either of the single mutants (Figure 8). Interestingly, cells expressing another double mutant, in which two known Aurora B sites were mutated to alanine (S8A/S15A) exhibited similar inter-kinetochore distances as cells expressing S62A, S69A, and S62A/S69A (Figure 8).

DISCUSSION

Aurora A kinase has a well-documented role in centrosome maturation and spindle assembly, but its role at the kinetochore has yet to be fully discovered. Interestingly, although Aurora B has long-been considered the primary regulator of kinetochore-microtubule attachment through phosphorylation of the Hec1 tail, recent work from multiple labs has demonstrated that Aurora A kinase also phosphorylates the Hec1 tail in mitosis (Kettenback et al. 2011; Ye et al. 2015). In addition, Aurora A kinase phosphorylates additional kinetochore substrates including CENP-E and CENP-A (Kunitoku et al. 2003; Kapoor et al. 2006). A prominent model suggests that Aurora A phosphorylation of kinetochore substrates only occurs early in mitosis, when kinetochores are in close proximity to the spindle poles, typically just after nuclear envelope breakdown (Barisic et al., 2014). In contrast to the predictions of this model; however, it has recently been demonstrated that Hec1 is, in fact, phosphorylated by Aurora A at site S69 continuously throughout mitosis, suggesting that Aurora A has an unexplored and unexpected role at the kinetochores (DeLuca et al. 2018). Aurora A has also been found to interact directly with INCENP, a well-known component of the Chromosome Passenger Complex that plays a well-established role in regulating Aurora B activity (DeLuca et al. 2018). These findings lead to numerous important questions. For example, why are both Aurora A and Aurora B utilized for phosphorylating Hec1; and does Aurora A have additional roles other than phosphorylating S69 at kinetochores?

Similar to residue S69, the neighboring residue S62 remains highly phosphorylated during the entirety of mitosis. However, it remains unclear why these sites are a target for reversible modification when they showcase a need to remain consistently phosphorylated

where other sites earlier in the tail, like S44 and S55, are not. A previous study has implicated phosphorylation of S69 in metaphase oscillations, as cells expressing a S69A Hec1 construct exhibit significantly dampened kinetochore oscillations (DeLuca et al. 2018). Conversely, in cells expressing an 8A/S69 Hec1 construct, in which S69 is left unperturbed and the remaining 8 Aurora target sites are mutated to alanine, phosphorylation of S69 alone is able to rescue metaphase kinetochore oscillations to near wild-type values (DeLuca et al. 2018). Importantly, phosphorylation of S44 or S55 alone does not rescue kinetochore oscillations (i.e. in cells expressing 8A/S44 or 8A/S55) (DeLuca et al. 2018). This finding demonstrates a need for specific sites to remain highly phosphorylated throughout mitosis to facilitate kinetochore oscillations instead of solely contributing to kinetochore-microtubule error correction. This function is critical for cell and organismal health, since kinetochore oscillations have been implicated in promoting successful, error-free chromosome segregation (Skibbens et al., 1993; Stumpff et al., 2008).

In this study, we found that upon inhibition of Aurora A kinase, phosphorylation at residue S62 decreases significantly, whereas upon Aurora B inhibition, phosphorylation remains high. This result is consistent with our proposed tail-domain specificity model of Hec1 tail phosphorylation. This model posits that residues located in the far N-terminal region of the tail are preferentially phosphorylated by Aurora B and sites in the C-terminal region of tail, closer to the CH domain, are phosphorylated by Aurora A. This model is based on a previous study demonstrating that S44 is primarily phosphorylated by Aurora B kinase, S55 is phosphorylated by both kinases, and S69 is phosphorylated primarily by Aurora A kinase (DeLuca et al., 2011, 2018). Again, the finding that S62 is phosphorylated primarily by Aurora A follows this trend and

shows a similar phosphorylation pattern to S69 further supports the tail-domain specificity model. However, why this spatial specificity exists is still yet to be determined, but we speculate that the N-terminal sites are integral for maintaining proper kinetochore-microtubule attachment stability and error correction in early mitosis, whereas the C-terminal sites are important for proper chromosome movements during late mitosis.

In a microtubule-free system using nocodazole, cells typically have two different classes of morphologies. The first class consists of cells that were in the process of cell division at the time of nocodazole treatment. These cells display more compact chromosomes, as they had already initiated alignment at the metaphase plate and formed attachments, so upon microtubule depolymerization the chromosomes become condensed. The second class involves cells that initiate entry into mitosis after nocodazole treatment. In these cells, chromosomes appear more dispersed due to the absence of attachments, hindering their alignment and organization. Phosphorylation at residue S62 is significantly decreased in both classes of morphology after nocodazole treatment, indicating that the presence of polymerized microtubules is required for Aurora A-mediated phosphorylation of S62. This is not unexpected, since it has been previously established that activation of Aurora A by co-factors requires microtubules (Tsai et al. ,2003 ; Bayliss et al., 2003). This relationship between Aurora A activity and the presence of microtubules was further supported by staining for phosphorylated Aurora A. This revealed that after nocodazole treatment, phosphorylation of Aurora A kinase on T288 was significantly reduced, indicative of reduced activity. As such, cells that displayed a dispersed morphology did not have active Aurora A to first phosphorylate Hec1 at the onset of mitosis and compact cells were unable to maintain S62 phosphorylation.

By measuring inter-kinetochore distances in metaphase cells, we observed that introducing phospho-null alanine mutations in the Hec1 tail at sites S62 and/or S69 leads to subtle alterations in kinetochore-microtubule attachment stability. Interestingly, when one or two alanine mutations are introduced, the inter-kinetochore distances remain relatively consistent but are increased compared to the wild type. Notably, this trend holds for both Aurora B and Aurora A sites investigated. However, we only tested sites in close proximity. It is unknown what happens to inter-kinetochore distances if two sites on the opposite sides of the tail are mutated to alanine and if this change would alter the results. It is also important to note that the sites are not additive in the manner that phosphomimetic mutations are (Zaytsev et. al, 2014).

FUTURE DIRECTIONS

In vitro kinase assays show Aurora A kinase's ability to phosphorylate serine 44 on Hec1, despite Aurora As inability to do so in cellular contexts (DeLuca et al., 2018). These results could suggest that Aurora A is capable of phosphorylating Aurora B targets but is unable to due to low levels of Aurora A at the kinetochore. Mutations such as Aurora A G198N and Δ120 demonstrate enhanced kinetochore localization, deviating from the usual centrosome localization (Fu et al., 2009; Hans et al., 2009). Aurora A G198N, generated by substituting G198 of the Aurora A catalytic domain with the equivalent residue in Aurora B, N158. This mutant was hypothesized to impair the binding affinity of Aurora A to TPX2 and upon expression, this mutant exhibits co-localization with Aurora B at the centromere while losing its spindle localization entirely (Fu et. al 2009). Importantly, in Aurora B knockdown experiments, G198N demonstrates functionality and partially restores Aurora B loss (Fu et al., 2009). Upon siRNA knockdown of Aurora B, cells were shown to have an increase in early mitotic cells and a decrease of cells in anaphase leading to an early exit of mitosis but upon expression of G198N this decrease was partially restored (Fu et. al 2009). The N terminus of Aurora A has also been implicated in Aurora As ability to bind centrosomes (Li et al., 2015). Truncation experiments removing the last 120 amino acids of the N-terminus result in Aurora A localizing to the kinetochores instead of the centrosomes with no INCENP binding being observed (Hans et. al 2009). Both Δ120 and G198N have been shown to phosphorylate histone H3, a typical substrate of Aurora B, indicating functional similarity between Aurora A and B (Hans et al., 2009).

To further investigate Aurora A's potential to rescue Aurora B activity, overexpression of Aurora A G198N, Δ120, and wild type Aurora A in Hela cells followed by ZM447439 and

MLN8054 mediated inhibition can be performed. Subsequent analysis using pS44, pS55, pS62, and pS69 antibodies to detect Hec1 tail phosphorylation can determine whether Aurora A localized at centromeres or kinetochores can rescue Aurora B function. This experimental approach may clarify whether the inability of Aurora A to phosphorylate Hec1 Aurora B targets in cells is due to localization or other properties of these sites yet to be determined. Moreover, it may discern a distinction between centromeric and kinetochore populations of Aurora kinases, highlighting the role of INCENP-bound Aurora kinase populations.

MATERIALS AND METHODS

Cell Culture

HeLa Kyoto cells were maintained in 5% CO_2 at 37°C and cultured in Dulbecco's Modified Eagle Medium supplemented with 10% FBS and 1% Antibiotic/Antimycotic solution. For fixed cell analysis cells were cultured on sterile acid washed cover-slips in six well plates and grown to 50% confluency before treatment.

Cell treatments and Transfections

HeLa Kyoto cells were transfected with 2uL of a 20uM stock Hec1 siRNA solution (5'-CCCUGGGUCGUGUCAGGAA-3') and 4ug of plasmid DNA. Cells were transfected using Lipofectamine 3000 and reduced serum Opti-MEM according to manufacturer's instruction. Cells were incubated for 24h and media replaced with DMEM supplemented with 10% FBS and a 1% Antibiotic/Antimycotic solution. Cells were fixed 48h post transfection. For drug treatments media was aspirated from the six well dishes and replaced with media containing 2uM ZM447439 or 0.5uM MLN8054 1h prior to fixing. For microtubule perturbation experiments 1uM nocodazole was added to cells and incubated overnight.

Immunofluorescence

Cells were washed in 37°C PHEM buffer (60 mM PIPES, 25mM HEPES, 10mM EGTA, 4Mm $MgCl_2$, pH 7.0) and permeabilized in lysis buffer (PHEM with 0.5% Triton-X 100) for 10s. Followed by fixation for 20 minutes at room temperature in 37°C 4% paraformaldehyde in PHEM buffer. After fixation cells were washed three times for five minutes in PHEM-T Buffer (PHEM buffer with

0.1% Triton-X 100), washed with PHEM, and then blocked in 10% boiled donkey serum (BDS) with 0.05% Sodium Azide in PHEM for 1h at room temperature. Primary antibodies diluted in 5% BDS in PEHM were added to cells and incubated for 15h at 4°C. The following primary antibody dilutions were made:

After primary antibody incubation cells were washed three times for five minutes in PHEM-T Buffer and washed in PHEM. Secondary antibodies were diluted 1:1000 in 5% BDS in PHEM and were added to cells and incubated for 45min at room temperature. Secondary antibodies were conjugated to either Alexa Fluor 488, Alexa Fluor 568, or Alexa Fluor 647 (Jackson ImmunoResearch Laboratories, Inc.). Coverslips were washed three times for five minutes in PHEM-T Buffer and incubated in a 2ng/ml DAPI solution in PHEM for 30s. Cells were washed twice for 5 minutes in PHEM-T followed by a wash in PHEM and then mounted onto glass slides in VECTASHADE antifade mounting media. Coverslips were then sealed in nail polish and stored at 4°C. For Interkinetochore distance experiments cells were washed in 37°C PHEM buffer and fixed for 20 minutes at room temperature in 37°C 4% paraformaldehyde in PHEM buffer followed by permeabilized in lysis buffer for 10s. After permeabilization cells were treated as described above.

Imaging and Analysis

Images were acquired using a DeltaVision Personal DV Imaging system (GE Healthcare) on an IX71 inverted microscope (Olympus) using SoftWoRx software (GE Healthcare). Experiments were imaged using a 60X 1.42 NA differential interference contrast Plan Apochromat oil immersion lens (Olympus) with final magnification of 107.6nm/pixel at the camera sensor

(edge4.2;PCO). Fluorescence intensity measurements were analyzed on a custom made MatLab program (Mathworks) courtesy of X. Wan (Wan et al., 2009) using nondeconvolved uncompressed images. Interkinetochore distances were done on ImageJ software using nondeconvolved uncompressed images on kinetochore pairs that resided in a single focal plane. Statistical analyses were done in SigmaPlot Software.

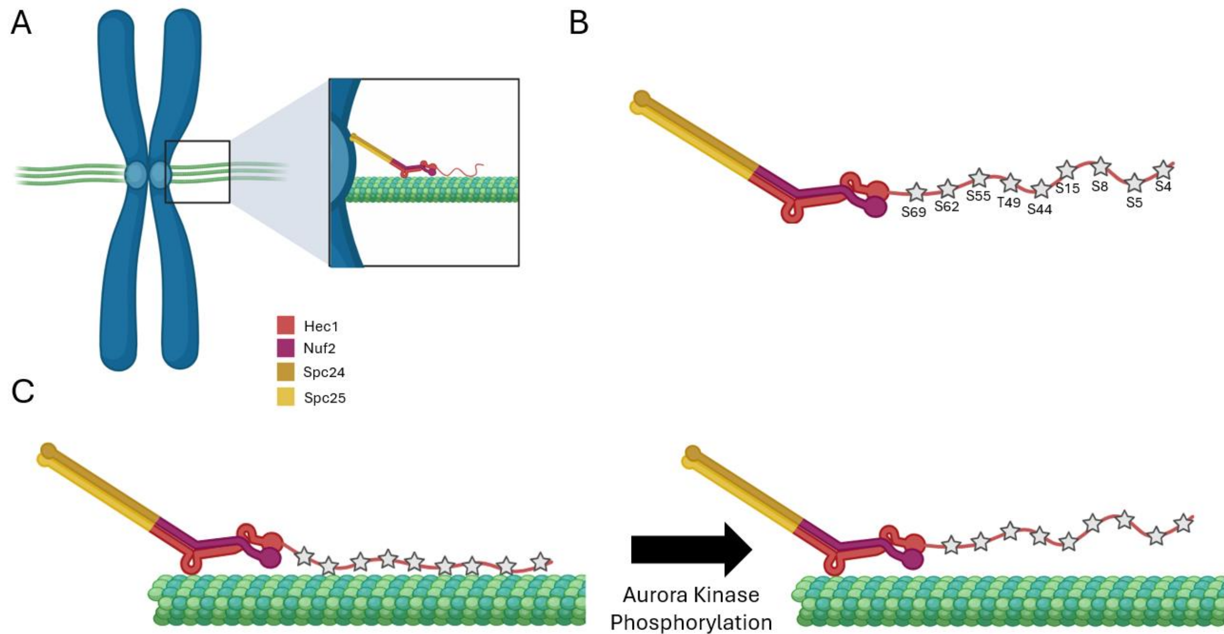


Figure 1. NDC80 and the function of Hec1 A) Diagram of the NDC80 complex at the kinetochore binding to microtubules. Other kinetochore proteins not diagramed. **B)** Diagram showing the 9 Aurora kinase phosphorylation sites on the Hec1 tail in humans. Sites shown are S4, S5, S8, S15, S44, T49, S55, S62, S69. **C)** A model for Hec1 tail function showing the Hec1 tail directly binding to the microtubule followed by phosphorylation by Aurura Kinases releasing the tail domain from the microtubule lattice.

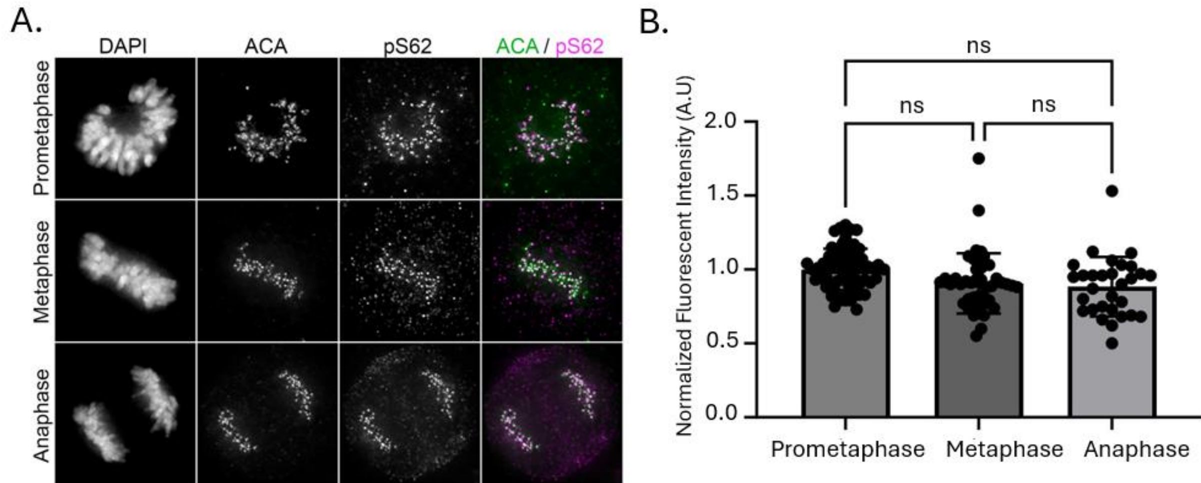


Figure 2. Hec1 S62 is phosphorylated throughout mitosis A) Immunofluorescence images of HeLa cells stained with phosphospecific antibodies to Hec1 pS62 and an anticentromere antibody (ACA) depict the kinetochore localization of pS62 during mitosis. **B)** Quantitative data is presented, with ≥30 cells measured for each depicted phase.

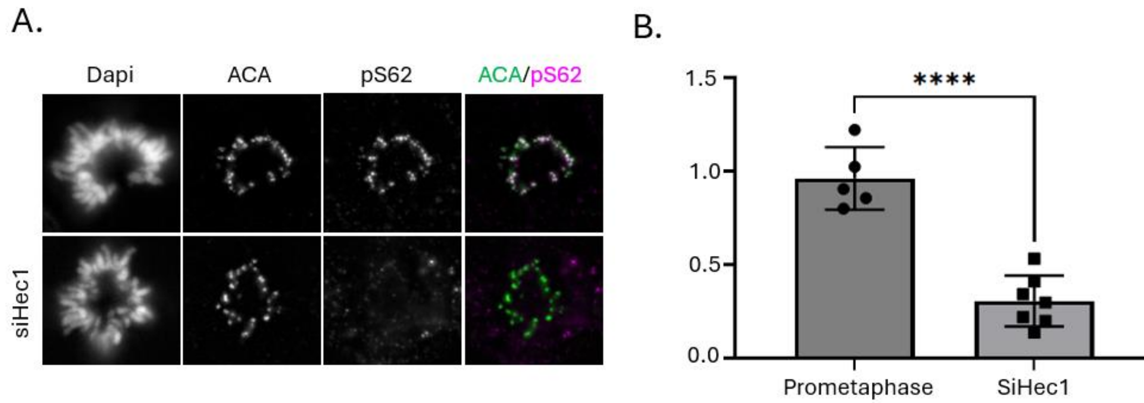
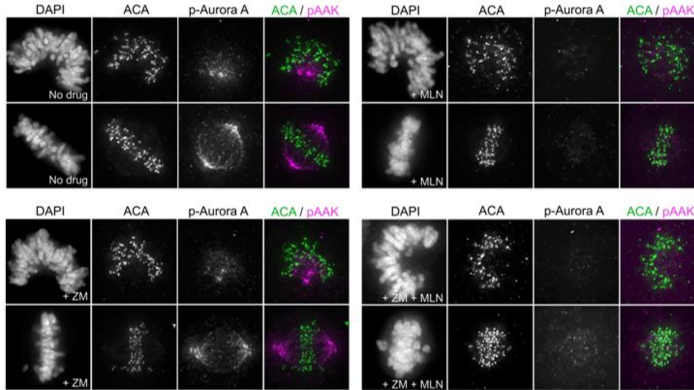


Figure 3. pS62 fluorescence only in presence of Hec1 A) immunofluorescence images of HeLa cells stained with phosphospecific antibody to Hec1 pS62 after depletion of Hec1. Knockdown of Hec1 shows depletion of pS62 staining at kinetochores. **B)** Quantitative data is presented, with ≥5 cells measured for each depicted phase.

A.



B.

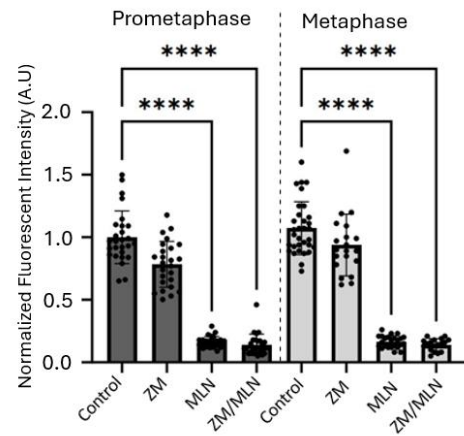


Figure 4. Aurora A activity is reduced in cells treated with MLN8054 A) HeLa cells after one hour treatment in either 2 µM ZM447439, 0.5 µM MLN8054, or both inhibitors and stained with phopspho-Aurora A kinase antibody and an anticentromere antibody (ACA).**B)** Quantitative data is presented , with ≥18 cells measured for each depicted phase.

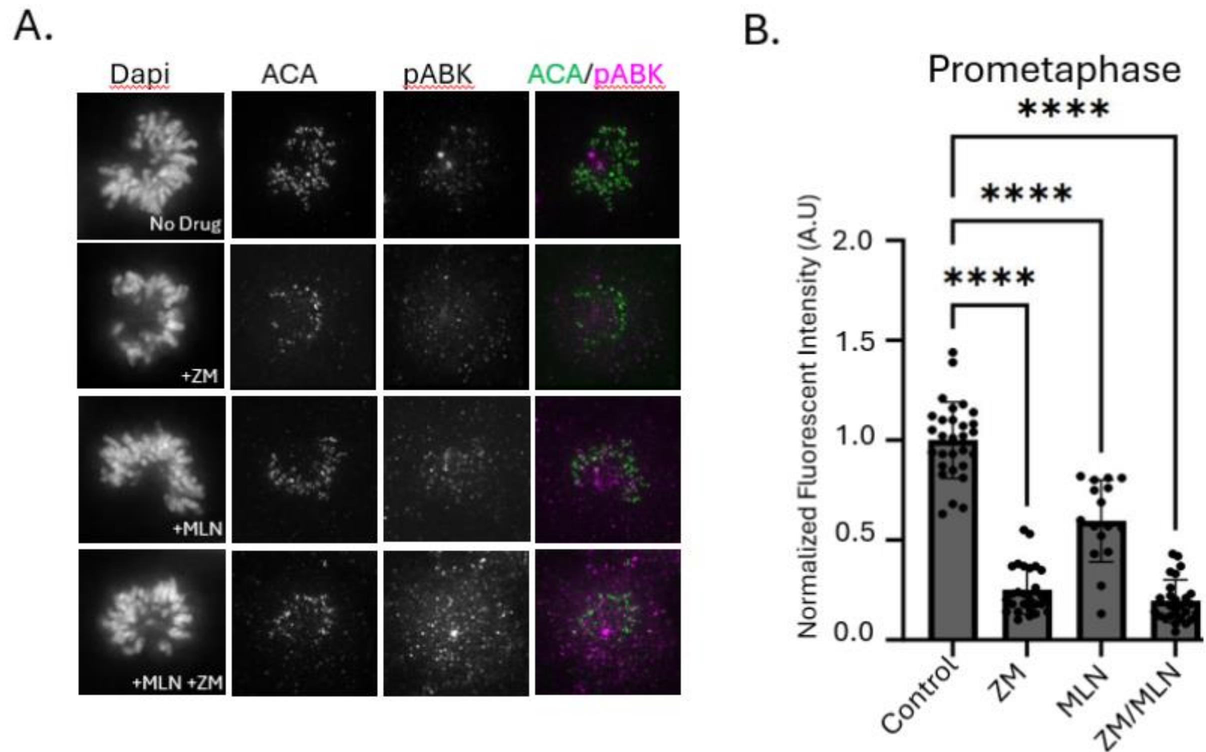


Figure 5. Aurora B activity is reduced in cells treated with ZM447439 A) HeLa cells after one hour treatment in either 2 µM ZM447439, 0.5 µM MLN8054, or both inhibitors and stained with phopspho-Aurora B kinase antibody and an anticentromere antibody (ACA).**B)** Quantitative data is presented , with ≥16 cells measured for each depicted phase.

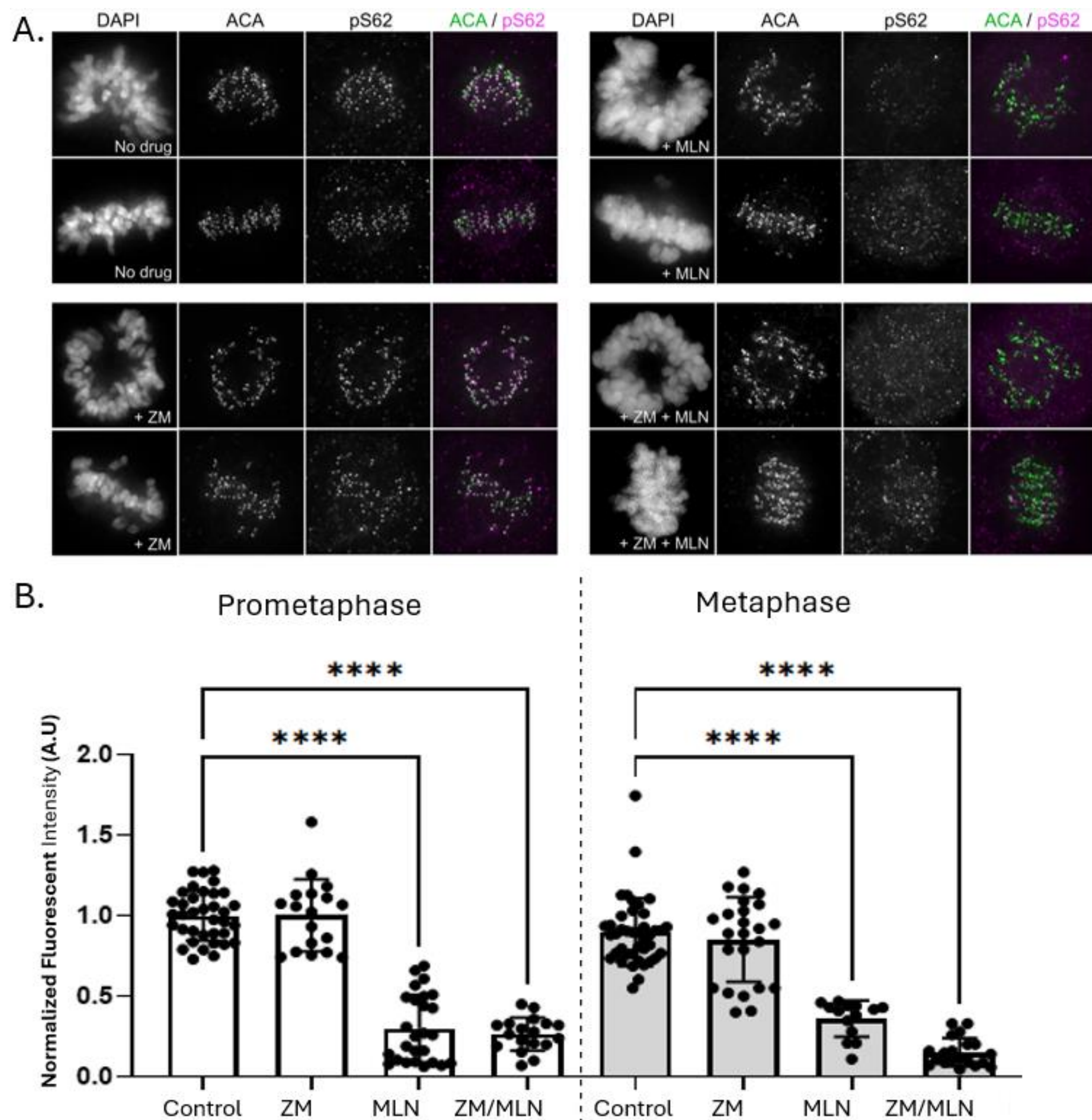


Figure 6. pS62 is phosphorylated by Aurora A in cells A) HeLa cells after one hour treatment in either 2 µM ZM447439, 0.5 µM MLN8054, or both inhibitors and stained with phosphospecific antibodies to Hec1 pS62 and an anticentromere antibody (ACA). **B)** Quantitative data is presented, with ≥18 cells measured for each depicted phase.

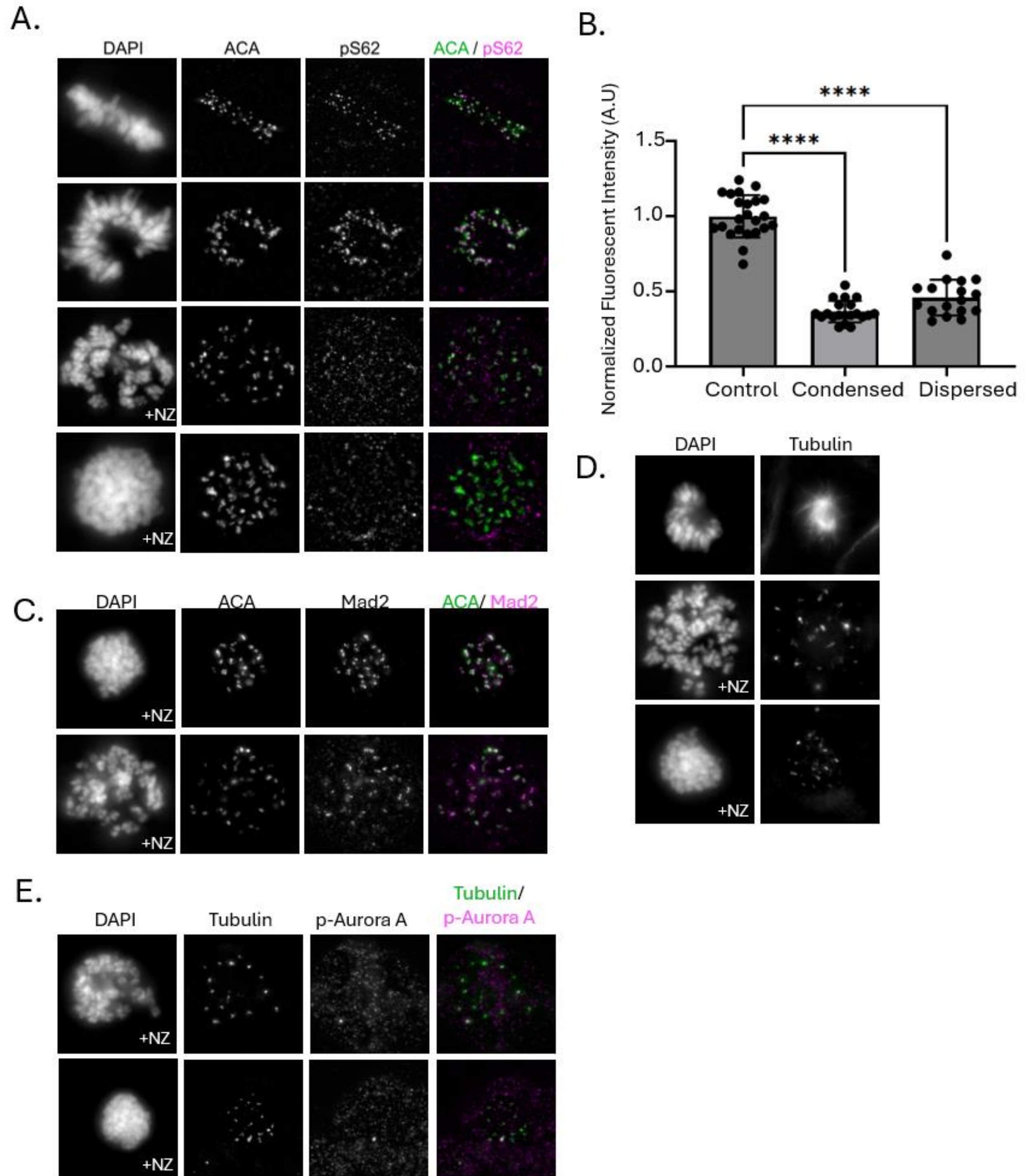


Figure 7. pS62 phosphorylation decreases in absence of microtubules A) HeLa cells after overnight treatment in 1 μM nocodazole and stained with phosphospecific antibodies to Hec1 pS62 and an anticentromere antibody (ACA). **B)** Quantitative data is presented, with ≥17 cells measured for each depicted phase. **C)** HeLa cells after nocodozole treatment stained with ACA and Mad2. **D)** HeLa cells treated with nocodozole and stained with a tubulin antibody. **E)** HeLa cells treated with nocodozole stained for phosphorylated Aurora A kinase and tubulin.

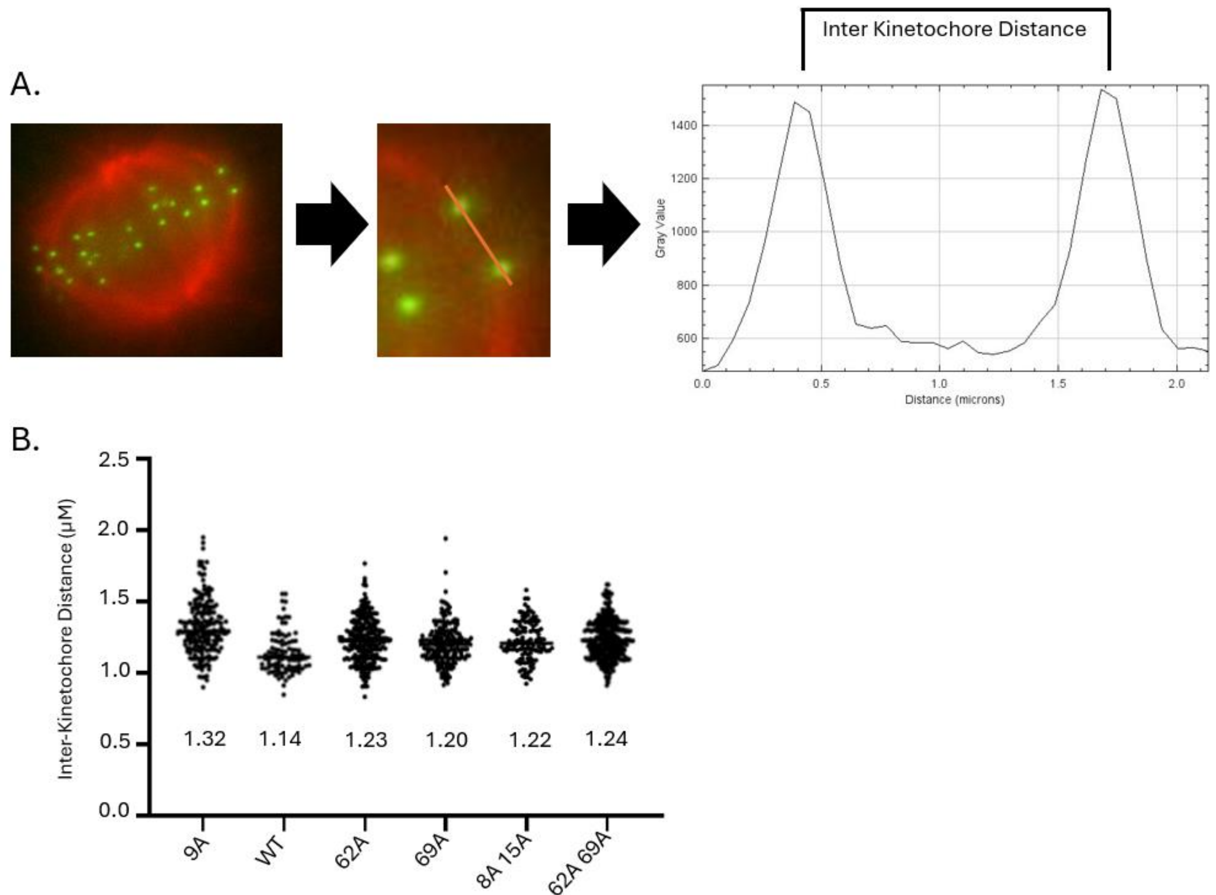


Figure 8. Inter-kinetochore distance increase in response to phospho-mutation of S62. A) Depiction of inter-kinetochore distance. **B)** Inter-kinetochore distance measurements from HeLa cells transfected with Hec1 tail mutants. Each point represents a measured kinetochore distance for a pair of sister kinetochore, with ≥100 kinetochore pairs measured for each depicted construct.

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