THESIS

INVESTIGATING THE ROLE OF KINETOCHORE DYNEIN-DYNACTIN IN SPINDLE ASSEMBLY CHECKPOINT FUNCTION

Submitted by

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ABSTRACT

INVESTIGATING THE ROLE OF KINETOCHORE DYNEIN-DYNACTIN IN SPINDLE ASSEMBLY CHECKPOINT FUNCTION

When a cell divides it is essential that its chromosomes are equally divided into two new daughter cells, thus ensuring that each cell receives an identical copy of genetic information. The importance of this process is emphasized by the fact that a hallmark of cancer cells is erroneous chromosome segregation, leading to uncontrolled proliferation. A key cellular structure involved in maintaining genomic integrity is the kinetochore, a large proteinaceous structure that assembles upon centromeric chromatin during cell division. This complex structure is involved in linking mitotic chromosomes to spindle microtubules, as well as detecting and correcting erroneous kinetochore-microtubule attachments to ensure faithful chromosome segregation. Monitoring of kinetochore-microtubule attachments is carried out by the spindle assembly checkpoint (SAC), a surveillance system that generates a “wait anaphase” signal at unattached kinetochores, with the goal of delaying cell division until every kinetochore has attached to a spindle microtubule. The checkpoint signal is propagated by SAC effector proteins that accumulate at the outer surface of unattached kinetochores during mitosis. In metazoan cells, the minus end-directed motor protein cytoplasmic dynein-1 (dynein) is known to facilitate eviction of SAC effectors from the kinetochore upon stable microtubule attachment, effectively silencing the checkpoint and allowing for anaphase progression. It has been suggested that dynein-mediated

eviction of checkpoint proteins is dependent on dynein's microtubule-based motor activity, with the prevailing model depicting SAC effectors transported as cargo toward the poles by the dynein motor along spindle microtubules. However, data supporting this model is lacking and the process is poorly understood. Here we have identified a subset of SAC effectors that require dynein for their removal from the kinetochore upon stable microtubule attachment. Additionally, we have generated a CRISPR cell line in which dynein is endogenously tagged, allowing us to characterize activity of kinetochore dynein in a manner not previously possible. Using this cell line in conjunction with small molecule-based inhibition of mitotic processes, we sought to investigate the role of spindle microtubules in dynein-mediated SAC silencing. Interestingly, our data show that dynein-mediated removal of key checkpoint proteins from kinetochores can occur in the complete absence of microtubules, suggesting a motility-independent role for the dynein motor in SAC silencing.

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INTRODUCTION

The ability of a cell to properly divide its contents into two new daughter cells is a well-characterized and fundamental process of life. This process is critical for cell proliferation as well as organismal growth and survival. When a cell undergoes mitotic division, it duplicates all of its contents including its DNA and distributes them equally between two new cells. It is of paramount importance that the parent cell's genome is maintained in the two nascent daughter cells. The importance of this process is underscored by the fact that aneuploidy, a result of chromosome mis-segregation, is a major hallmark of cancer cells. Because of this, cells have myriad mechanisms in place to ensure that genomic integrity is maintained throughout cell division. A critical player in this process is a structure called the kinetochore.

The kinetochore and the spindle assembly checkpoint

As a human cell transitions from interphase into mitosis its nuclear envelope breaks down, spilling the condensed chromosomes into the cytoplasm to commence an open mitosis. At this point the kinetochore, a large proteinaceous complex, begins to assemble upon the centromeric chromatin of mitotic chromosomes. The vertebrate kinetochore is a complex macromolecular machine composed of over a hundred proteins. It is responsible for linking mitotic chromosomes to spindle microtubules, as well as identifying and correcting erroneous kinetochore-microtubule attachments to ensure accurate chromosome segregation.

Early in mitosis kinetochore-microtubule attachments are labile and have high turnover, and as mitosis progresses these attachments become corrected and stabilized for progression into anaphase (Hara and Fukagawa, 2020). Monitoring of these attachments is carried out by the spindle assembly checkpoint (SAC), a critical function of the kinetochore. The SAC acts as a molecular surveillance system, preventing the cell from progressing through mitosis until all chromosomes have become properly bioriented at the metaphase plate (Figure 1). The checkpoint signal is propagated by SAC effectors that accumulate at the outer surface of unattached kinetochores, generating a diffusible “wait anaphase” signal that inhibits the Anaphase Promoting Complex/Cyclosome (APC/C), an E3 ubiquitin ligase (Sudakin et al., 1995). Once all kinetochores have become attached to microtubules, inhibition of the APC/C is relieved, allowing for anaphase progression (Li et al 1997). The checkpoint is sensitive enough that SAC signal emanating from a single unattached kinetochore is enough to inhibit the APC/C and prevent anaphase progression (Rieder et al., 1995).

Dynein at the kinetochore

When a stable end-on kinetochore-microtubule attachment is made, SAC effectors are evicted from the kinetochore, silencing the checkpoint and allowing for progression through mitosis. The exact mechanism of this eviction remains unknown, but it has been shown that a key player in this process is the motor protein cytoplasmic dynein-1, hereafter referred to as dynein (Sacristan et al., 2018). Dynein is a minus end-directed motor protein that is responsible for transporting cellular cargoes toward microtubule minus ends (Schlager et al., 2014). The pool of dynein that is recruited to

the kinetochore is thought to be involved in SAC silencing, promoting accurate chromosome segregation and thus genomic stability (Sacristan et al., 2018).

Human dynein is not highly motile on its own – it must be in complex with a 1.2 MDa multi-subunit complex called dynactin, so named for its function as a dynein activator (Schlager et al., 2014). Also required for optimal motility is a molecule called a cargo adaptor, which facilitates the dynein-dynactin interaction and promotes cargo binding (Schlager et al., 2014). The dynein adaptor at the kinetochore is Spindly, a coiled-coil protein that shares similarities with other well-characterized dynein adaptors (Figure 3A) (Gassmann et al., 2010).

Dynein is recruited to the kinetochore indirectly via the heterotrimeric Rod-Zwilch-ZW10 (RZZ) complex (Mosalaganti et al., 2017). An important molecule, RZZ has roles in both amplifying and silencing the SAC. At the outer kinetochore, RZZ interacts directly with the dynein adaptor Spindly (Mosalaganti et al., 2017). Spindly in turn recruits dynein to the kinetochore via its conserved dynein-interaction motifs (Figure 3A) (Gassmann et al., 2010).

Role of the RZZS-DD module in regulating dynamic kinetochore size

The metazoan kinetochore is organized into three distinct domains: the constitutive centromere-associated network (CCAN), the Knl1-Mis12-Ndc80 complex network (KMN), and the fibrous corona. The CCAN is a stable 16-subunit complex that is directly associated with centromeric chromatin in all phases of the cell cycle (Hara and Fukagawa, 2020). The KMN network is a module that assembles upon the CCAN during M phase. Ndc80 of the KMN network acts as the attachment interface between

kinetochores and microtubules (DeLuca and Musacchio, 2012). Knl1, another component of the KMN network, is an important molecule that functions as a scaffold upon which the rest of the kinetochore can assemble; phosphorylation of sites within Knl1 in early mitosis by Mps1 kinase promotes recruitment of key SAC effectors (Hara and Fukagawa, 2020). Importantly, it is thought that Knl1, along with the SAC protein Bub1, is involved in the recruitment of RZZ (Caldas et al., 2015).

The fibrous corona is a dynamic module at the outer surface of the kinetochore comprised of SAC components, dynein, RZZ, Spindly, CENP-E, and microtubule-associated proteins (Sacristan et al., 2018). In early mitosis when kinetochore-microtubule attachments have not yet been established, this structure expands into a crescent-like shape surrounding the kinetochore. This effect can be seen dramatically in cells that have been treated with a microtubule-depolymerizing agent such as nocodazole, where the expanded corona structure can form a ring fully engulfing the core kinetochore (Rodriguez-Rodriguez et al., 2018). As mitosis progresses and microtubule attachments are made, the corona structure decreases in volume and is compacted to a puncta-like morphology, a process shown to be facilitated by dynein (Sacristan et al., 2018).

It has been suggested that interactions between RZZ and Spindly at the outer kinetochore drive expansion of the corona via oligomerization, forming a RZZS meshwork upon which other corona components can assemble (Pereira et al., 2018). Interestingly, Spindly must be farnesylated at its C-terminus in order to interact with the Rod subunit and form oligomers (Sacristan et al., 2018). The kinase Mps1 is also required for kinetochore expansion in two ways: 1) Mps1 phosphorylation of Spindly's

N-terminus is proposed to release Spindly autoinhibition, facilitating interaction and oligomerization with Rod (Sacristan et al., 2018), and 2) Mps1 phosphorylation of Rod's N-terminus is required for corona expansion, as demonstrated by phospho-null mutants of Rod that display compact kinetochores even in nocodazole-treated cells (Rodriguez-Rodriguez et al., 2018).

The dynamic expansion of unattached kinetochores serves both to facilitate microtubule capture in early mitosis, and to amplify the SAC signal (Rodriguez-Rodriguez et al., 2018). In addition to forming stable end-on attachments with microtubules, kinetochores can also attach laterally to the microtubule lattice (Itoh et al., 2018). Lateral attachments are highly prevalent in early mitosis and are thought to facilitate microtubule capture and conversion to stable end-on attachments (Itoh et al., 2018). An expansive outer kinetochore domain containing microtubule-binding proteins could act as a molecular "sticky net" of sorts in early mitosis, grabbing hold of nearby microtubules and promoting formation of kinetochore attachments (Rodriguez-Rodriguez et al., 2018). The expanded corona domain could also be serving to amplify the "wait anaphase" signal from unattached kinetochores, simply due to the increased volume of checkpoint signaling molecules accumulated in the meshwork of the expanded kinetochore module. Preliminary results from our group suggest that cells display a weaker checkpoint signal when they are mutagenically prevented from forming expanded coronas at unattached kinetochores, as demonstrated by a shortened mitotic arrest in mutant cells when the checkpoint was challenged (data not shown). Thus, kinetochore expansion seems to be important in both attachment and SAC signaling.

Dynein-mediated SAC silencing

Upon establishment of a stable end-on kinetochore-microtubule attachment, dynein is thought to mediate the eviction of SAC proteins from the kinetochore (Sacristan et al., 2018). The exact molecular mechanism by which dynein facilitates eviction of SAC components remains unknown. It has been proposed that dynein utilizes its microtubule-based motor activity to transport SAC components as cargo along spindle microtubules away from the kinetochore and toward the poles, silencing the checkpoint and allowing for anaphase progression (Figure 2A). However, this model lacks substantial evidence – such motility-driven eviction of checkpoint proteins by dynein along spindle microtubules has never been directly observed in mammalian cells. Additionally, the molecular mechanism that triggers dynein-mediated eviction of checkpoint proteins upon establishment of a stable kinetochore-microtubule attachment remains unknown.

Here we set out to investigate the role of kinetochore dynein in spindle assembly checkpoint function. We hypothesize that dynein facilitates removal of SAC effectors from attached kinetochores in a motility-independent manner, rather than actively transporting SAC cargoes along spindle microtubules. We utilized fixed-cell analysis of cells expressing a mutant form of Spindly to identify a subset of SAC components that require dynein for eviction from bioriented kinetochores. We also generated an endogenously-tagged dynein cell line that allows us to directly visualize the dynamics of kinetochore dynein in living cells. Using this cell line, we established a live-cell SAC eviction assay that allows us to examine what components are required for dynein-mediated eviction of checkpoint proteins *in vivo*. Using this assay, we generated

exciting preliminary data that challenges the currently established paradigm for dynein-mediated SAC eviction (Figure 2B).

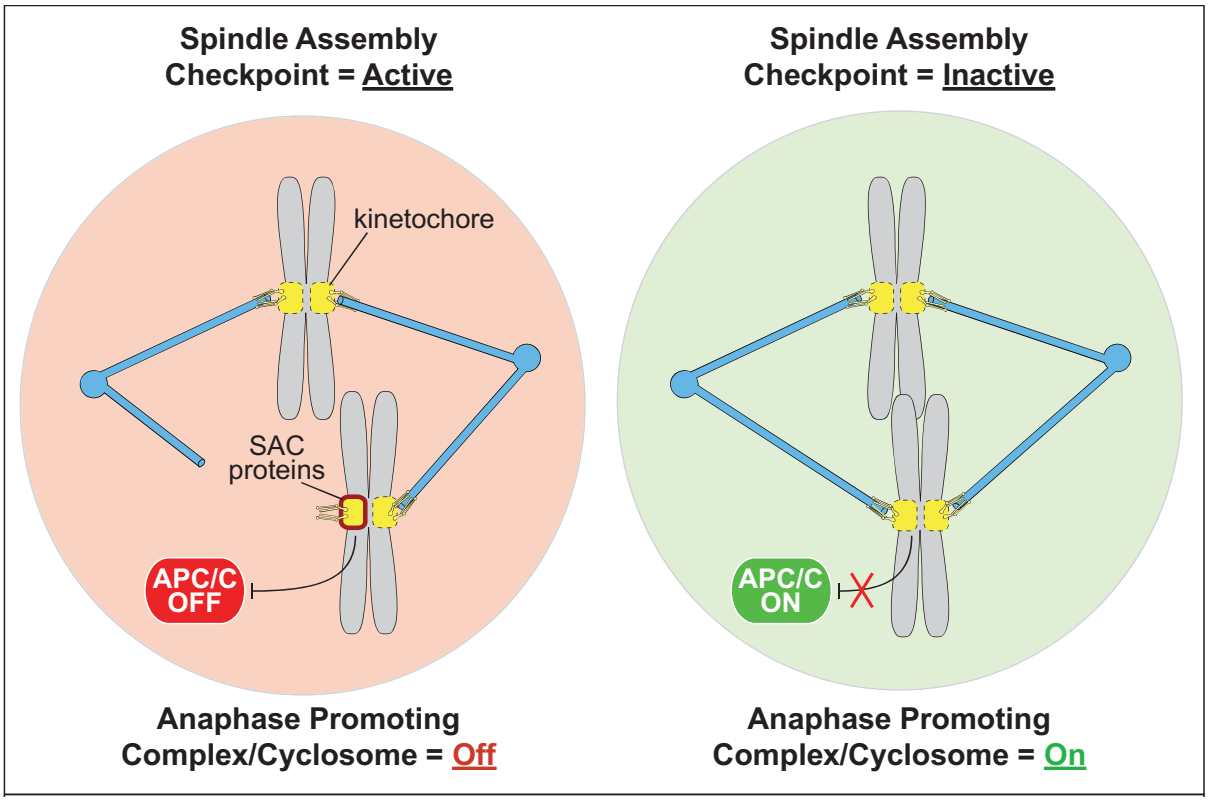


Figure 1. Spindle Assembly Checkpoint function

Schematic depicting Spindle Assembly Checkpoint function. The red cell on the left depicts an active SAC. Checkpoint effectors accumulate at unattached kinetochores, designated here as a red outline surrounding the kinetochore. This creates a diffusible “wait anaphase” signal that inhibits the APC/C, an E3 ubiquitin ligase. The green cell on the right depicts an inactive SAC. Once all kinetochores have established microtubule attachments, the SAC is silenced. This relieves inhibition of the APC/C, allowing degradation of Securin and cyclin B which promotes anaphase onset and chromosome segregation.

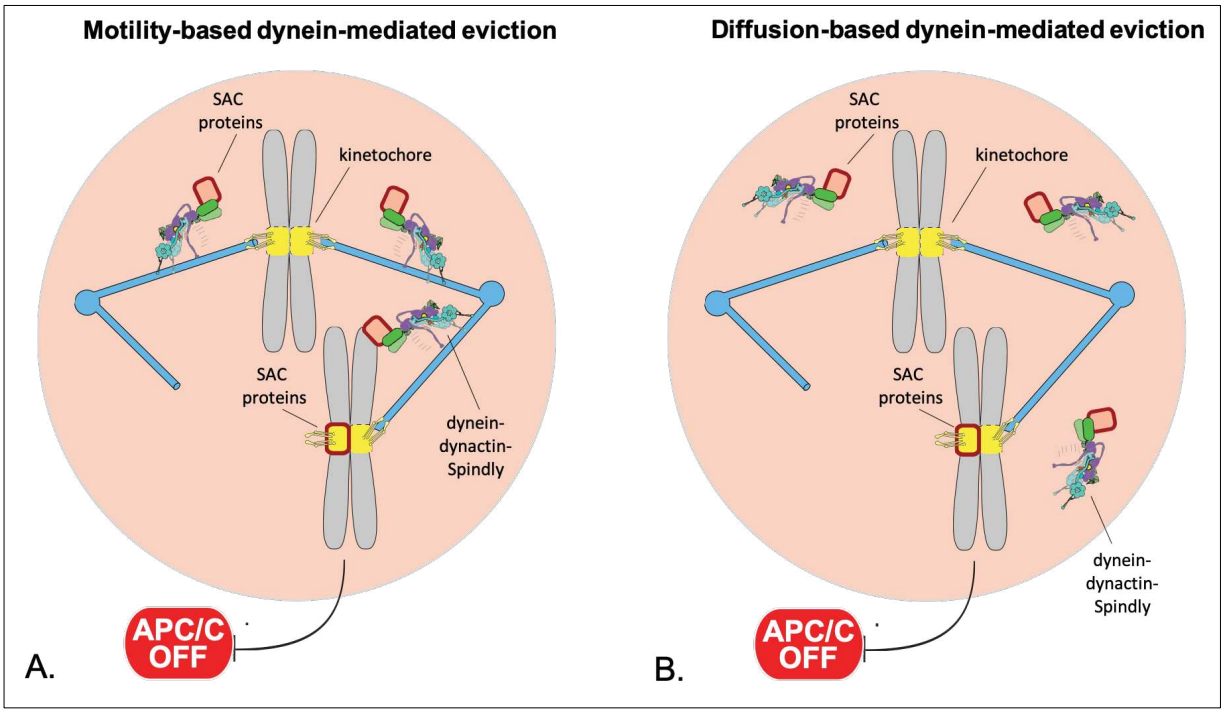


Figure 2. Possible mechanisms of dynein-mediated SAC silencing

A. Schematic depicting dynein, in complex with dynactin and Spindly (DDS), utilizing its minus end-directed microtubule-based motor activity to silence the checkpoint via active poleward transport of SAC effectors along spindle microtubules.
B. Schematic depicting DDS facilitating SAC silencing via diffusion. Upon stable end-on attachment, an unknown molecular mechanism triggers release of DDS from its kinetochore binding site, causing eviction of SAC effectors from the kinetochore and subsequent checkpoint silencing.

RESULTS

Identification of SAC effectors that undergo dynein-mediated eviction

The dynein-dynactin complex is targeted to the kinetochore via its cargo adaptor Spindly. Together with the RZZ complex, they make up the RZZS-DD module, which is a component of the fibrous corona at the outer kinetochore. Spindly, a 605 amino acid coiled-coil protein, possesses three conserved motifs that facilitate interaction with dynein: the CC1 box, the CC2 box, and the Spindly box (Figure 3A) (Sacristan et al., 2018). As mentioned previously, it is known that dynein is required at the kinetochore to silence the spindle assembly checkpoint by facilitating eviction of certain SAC effectors from attached kinetochores (Gassmann et al., 2010). Here we sought to better understand the interaction between dynein and Spindly, and to further elucidate their roles in checkpoint silencing.

We speculated that inhibiting dynein at the kinetochore could shed insight into its SAC silencing functions. However, seeing as dynein has many important cellular functions beyond its role in mitosis, global inhibition of the motor yields a wide range of effects, many of which could be confounding. With this in mind, we obtained stable cell lines as a generous gift from Reto Gassmann that stably express a mutant form of Spindly that cannot recruit dynein to the kinetochore. This mutant Spindly construct can robustly localize to unattached kinetochores while its ability to target dynein to the kinetochore is severely diminished, thus providing us an opportunity to specifically inhibit kinetochore dynein.

These cell lines were generated using Flp-mediated genomic recombination to mutate the conserved residue F258 of the Spindly box motif to an alanine, which perturbs interaction with dynein-dynactin (Gassmann et al., 2010) (Figure 3A). The construct is fused with a tetracycline-inducible RNAi-resistant GFP construct to allow for depletion of endogenous Spindly, over-expression of the Spindly transgene, and visualization of the construct in cells (Gassmann et al., 2010). Using these cell lines, we wanted to investigate which SAC effectors rely on dynein for eviction from kinetochores. HeLa cells stably expressing wildtype Spindly or the F258A motif mutant were treated with Spindly siRNA to deplete endogenous Spindly, then induced to express transgenic Spindly. 48 hours later, cells were fixed and stained with antibodies for various checkpoint proteins. Fluorescence intensities of transgenic Spindly and checkpoint proteins were quantified from aligned metaphase kinetochores (Figure 3B).

We found that cells expressing the Spindly motif mutant that disrupts kinetochore dynein recruitment displayed significantly increased retention of Spindly and certain checkpoint proteins at fully aligned metaphase kinetochores. This is compared to cells expressing wildtype Spindly, in which we saw expected decreased levels of Spindly and checkpoint proteins at metaphase kinetochores due to proper dynein-mediated eviction (Figure 3B). These results suggest that Spindly-mediated recruitment of kinetochore dynein is required for evicting a subset of SAC effectors from attached kinetochores (Figure 4B). These data are consistent with findings published by Reto Gassmann (Gassmann et al., 2010).

Additionally, we performed a correlation analysis in which asynchronous RPE-1 cells were fixed and stained with antibodies to p150 (a subunit of dynactin, used here as

a proxy for dynein) and the checkpoint proteins tested in the motif mutant experiment. Fluorescence intensities of p150 and checkpoint proteins were quantified from prometaphase and metaphase kinetochores, and their levels correlated. Our results show a high degree of temporal correlation between p150 and the SAC effectors identified as dynein cargoes in the motif mutant experiment, with levels being high at prometaphase kinetochores and declining in metaphase as kinetochores establish stable microtubule attachments (Figure 4A). Furthermore, we saw a low degree of correlation between p150 and the SAC components identified as not relying on dynein for removal (Figure 4A).

Taken together, these results suggest that targeting of dynein to the kinetochore by its cargo adaptor Spindly is required for eviction of a subset of SAC effectors from aligned metaphase kinetochores.

Investigating the mechanism of dynein-mediated SAC silencing

It is well-established that dynein is required for the timely eviction of SAC components from attached kinetochores, allowing for anaphase onset and faithful chromosome segregation. What is less well understood are the molecular mechanisms regulating dynein-mediated SAC eviction. Upon kinetochore-microtubule attachment, it is proposed that dynein utilizes its microtubule-based motor activity to actively transport SAC effectors along spindle microtubules toward the poles, silencing the checkpoint and allowing for mitotic progression. However, such motility-driven transport of checkpoint proteins by dynein has never been directly observed in mammalian cells. Additionally, the molecular mechanism that triggers dynein eviction upon kinetochore-

microtubule attachment remains elusive. With this study we have set out to characterize native dynein activity at the kinetochore, and to explore the role of spindle microtubules in dynein-mediated SAC silencing.

DHC-GFP CRISPR cell line

Due to the immense size and complexity of the human dynein-dynactin complex, experimental approaches involving manipulation or visualization of the complex *in vivo* have proved challenging. In light of this technical limitation, with the help of O'Neil Wiggan we generated a CRISPR/Cas9-engineered HeLa cell line in which endogenous dynein is tagged with a GFP fluorophore, allowing us to directly visualize native dynein at the kinetochore. CRISPR/Cas9 gene editing technology was leveraged to insert a GFP tag at the C-terminus of the dynein heavy chain (DHC) coding sequence in the HeLa cell genome via homology-directed recombination. Clonal colonies expressing the transgene were selected for and expanded.

Using this DHC-GFP cell line we confirmed that dynein localizes to the nuclear envelope in prophase and loads to kinetochores in high amounts upon nuclear envelope breakdown (NEBD), when all kinetochores are in an unattached state (Figure 5A). As mitosis progresses and microtubule attachments are made, dynein levels at the kinetochore diminish gradually until they are undetectable by metaphase, as expected (Figure 5A). In an attempt to visualize motility-based "stripping" of SAC effectors along spindle microtubules by dynein, we utilized confocal microscopy to image DHC-GFP cells in late prometaphase, when such stripping should occur. Despite imaging at the highest possible frequency (every 0.05 seconds) with 100x magnification, we were not

able to observe any evidence of dynein particles streaming along spindle microtubules (Figure 5B). This finding led us to speculate that dynein-mediated eviction of SAC effectors may be occurring in a manner independent of dynein's microtubule-based motor activity.

Kinetochore eviction assay

As previously mentioned, dynein is targeted to the kinetochore via the RZZS complex. The method of RZZ recruitment to the kinetochore is poorly understood, but is potentially thought to involve the scaffolding protein Knl1, a component of the KMN network (Caldas et al., 2015). Knl1 undergoes phosphorylation of its "MELT" motifs in early mitosis by the kinase Mps1, initiating its role as a platform upon which the rest of the kinetochore can assemble. This Mps1-mediated phosphorylation event promotes recruitment of many key proteins to the kinetochore, including SAC effectors. As mitosis progresses and attachments are made, a shift in the kinase-phosphatase balance occurs at the kinetochore, leading to dephosphorylation of Knl1 MELT motifs which promotes eviction of SAC proteins and subsequent anaphase onset (Hara and Fukagawa, 2020). Dephosphorylation of Knl1 MELT motifs can be artificially induced by a small molecule drug called reversine, which acts by inhibiting Mps1 kinase (Santaguida et al., 2010). Treating cells with reversine causes eviction of SAC effectors from kinetochores regardless of microtubule attachment status, and subsequent anaphase onset.

In the current study, we leverage the SAC eviction-inducing properties of reversine in combination with our DHC-GFP cell line in an effort to better understand

how dynein eviction is coupled to SAC protein removal, and the role that kinetochore-microtubule attachments play in this process. We have established a “kinetochore eviction” assay, in which we express fluorescently tagged checkpoint proteins of interest in HeLa cells. The cells are treated with nocodazole, a spindle poison that completely depolymerizes all microtubules in the cell, causing SAC proteins to accumulate in high amounts at unattached kinetochores. Using confocal microscopy, the live cells are imaged immediately before addition of reversine, and then after at a frequency of every 5 minutes for 2 hours. Fluorescence intensities of the tagged proteins are quantified from each frame throughout the time-lapse movie in order to analyze the effect of Mps1 inhibition on candidate checkpoint proteins.

Using this assay, we have been able to analyze the eviction kinetics of candidate checkpoint proteins and how they relate to those of dynein. Corroborating our previous findings, we found that SAC effectors that are known dynein cargoes, including RZZS and Mad2, display similar eviction kinetics to those of dynein itself – a gradual decline of 40-80% of kinetochore signal upon Mps1 addition (Figure 6A). In contrast, effectors that are not dynein cargoes display differing kinetics, such as BubR1 whose kinetochore signal completely diminishes immediately upon Mps1 inhibition, suggesting a dynein-independent mode of eviction (Figure 6A,B).

Interestingly, we noted a difference in eviction kinetics when cells were treated with low dose nocodazole (0.5uM) versus high dose (5uM), most notably for Mad2. Upon reversine-mediated Mps1 inhibition, Mad2 signal diminishes by almost 100% in 0.5uM nocodazole as compared to diminishing by 80% in 5uM nocodazole (Figure 6A,B). Cells were fixed and stained with an antibody for tubulin to confirm that the

nocodazole concentrations used were completely depolymerizing spindle microtubules. Treating cells overnight with 0.5uM nocodazole actually resulted in residual microtubule "tufts", which have been shown under certain conditions to form end-on attachments that can silence the SAC (Tauchman et al., 2015). We further showed that microtubules are in fact completely depolymerized when cells are treated with 5uM nocodazole overnight. The ability of kinetochore-microtubule attachments to form in cells treated with 0.5uM nocodazole explains the discrepancy in Mad2 eviction observed, and suggests that microtubule attachments are required for complete eviction of certain SAC components from the kinetochore. Interestingly, BubR1, a SAC effector previously shown not to be a dynein cargo, displayed complete and rapid eviction upon reversine treatment in both high and low-dose nocodazole conditions (Figure 6A.B), suggesting that BubR1 does not rely on microtubule attachment for eviction but instead relies on dephosphorylation of Knl1 MELT motifs. Taken together, these data suggest that establishment of a stable kinetochore-microtubule attachment confers some kind of molecular change at the kinetochore that can facilitate dynein-mediated SAC eviction.

In an effort to further elucidate the role of kinetochore dynein binding partners in SAC silencing, we implemented this assay in our DHC-GFP cells depleted of endogenous Spindly and expressing a mutant form of Spindly, in which all three of its dynein recruitment motifs (CC1, CC2, Spindly Box) have been mutated, called "Spindly-ΔCCS" (Figure 3A). We find that under these conditions, dynein recruitment to kinetochores is severely diminished (Figure 7A).

Using this system in conjunction with our kinetochore eviction assay, we interrogated the mechanism of removal of key checkpoint effectors by kinetochore

dynein, and what effect microtubules have on this process. In other words, can dynein-mediated SAC eviction occur even in the absence of microtubules? Cells expressing GFP-tagged Mad2 (a key checkpoint effector) and mCherry-tagged Spindly-ΔCCS were subjected to our kinetochore eviction assay with the goal of observing Mad2 kinetics in the absence (or presence) of dynein, when no microtubules are present. Strikingly, our results show that in cells that cannot recruit dynein to the kinetochore, Mad2 levels decrease by ~40% upon Mps1 inhibition, as compared to decreasing by ~80% in cells that can recruit dynein to the kinetochore (Figure 7B). These data suggest that dynein is required in a motility-independent manner to facilitate Mad2 eviction from kinetochores – the first demonstration of a function of dynein that is independent of its motor activity.

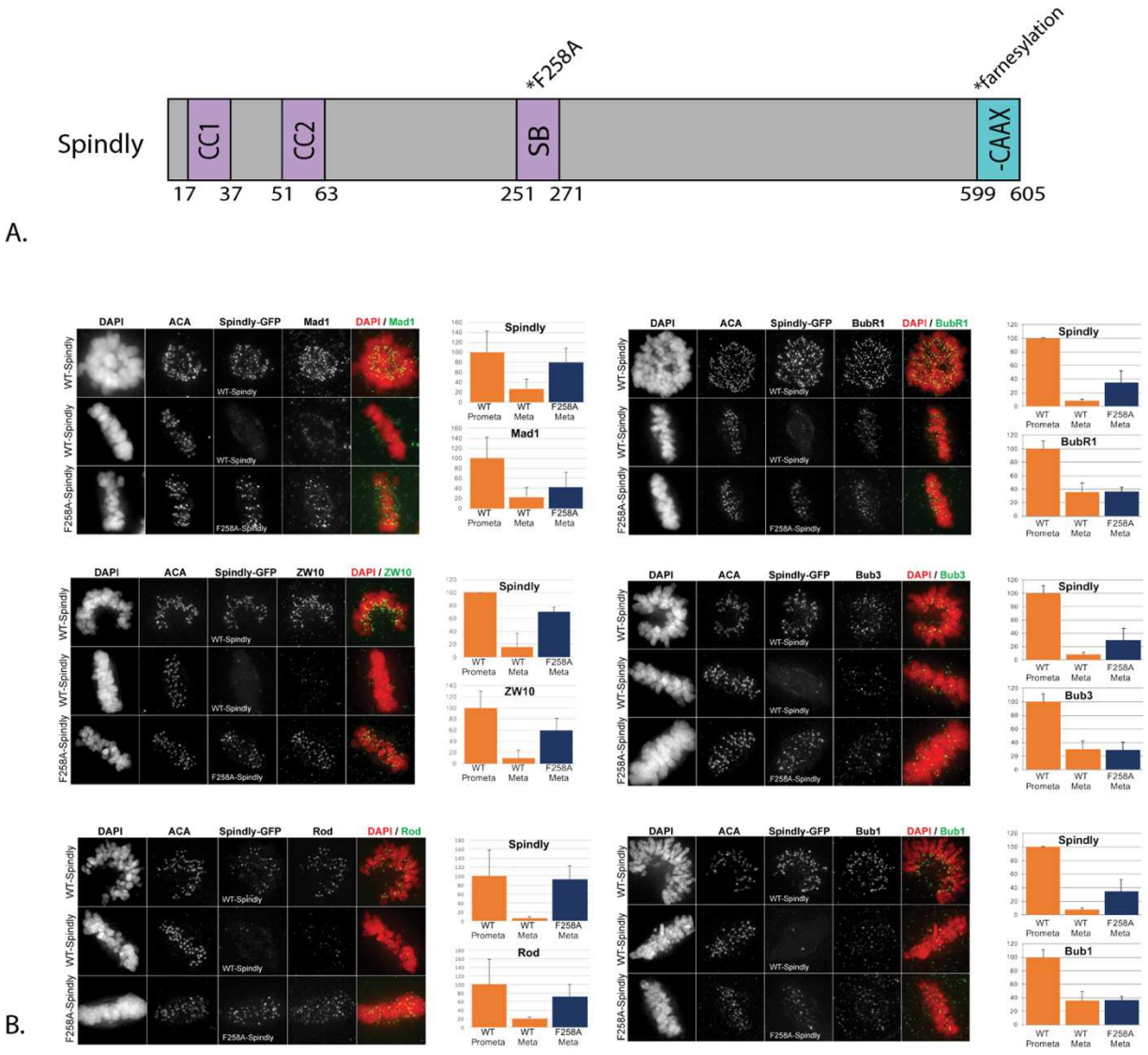
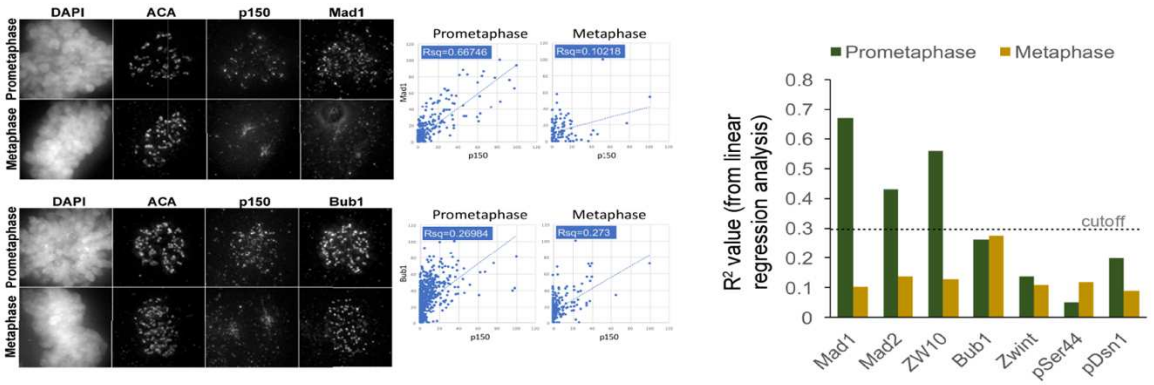


Figure 3. Cells expressing Spindly mutant that disrupts kinetochore dynein recruitment retain dynein cargoes

A. Schematic of human Spindly, depicting three dynein recruitment motifs in lavender (CC1 box, CC2 box, Spindly box) and the CAAX box in teal, which undergoes farnesylation to promote interaction with RZZ. Asterisk indicates F258A mutation used in these experiments.
B. Immunofluorescence images of HeLa cells stably expressing inducible WT or F258A Spindly. Cells were depleted of endogenous Spindly and induced to express transgenic Spindly. 48hrs later, cells were fixed and stained with indicated antibody for checkpoint proteins. Fluorescence intensities were measured from >150 kinetochores from >5 cells.

A.



B.

Dynein cargo?

Yes	No
Mad1*	BubR1*
Mad2*	Bub1
Rod	Bub3
ZW10	

*Consistent with Gassmann *et al.*, *Genes Dev* 2010
(Note: Gassmann data suggested Zwilch-1 is not cargo)

Figure 4. A subset of SAC effectors requires dynein for eviction from attached kinetochores

A. Correlation analysis corroborates dynein cargo at kinetochores. Asynchronous RPE-1 cells were fixed and stained with antibodies to p150 and the indicated antibody. Fluorescence intensity values for p150 and indicated test protein at individual kinetochores were measured and are plotted as shown on left. Linear regression analysis was performed on each data set to determine degree of correlation between dynactin and test protein. A value of 0.3 was chosen as our cutoff for positive correlation based on control analysis. Zwint is a negative control whose values do not change during mitotic progression.
B. List of SAC effectors confirmed or denied as bona fide dynein cargoes

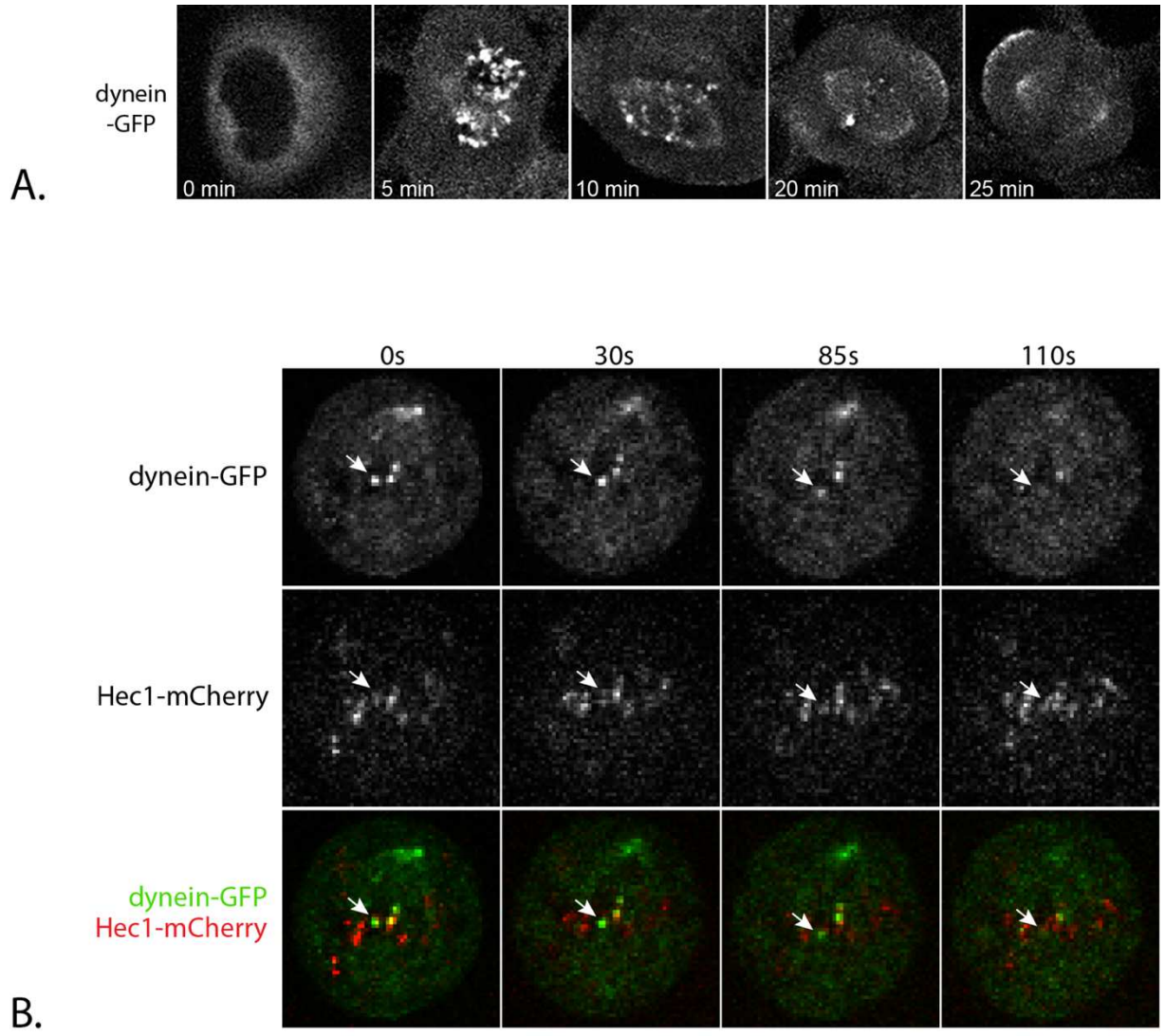
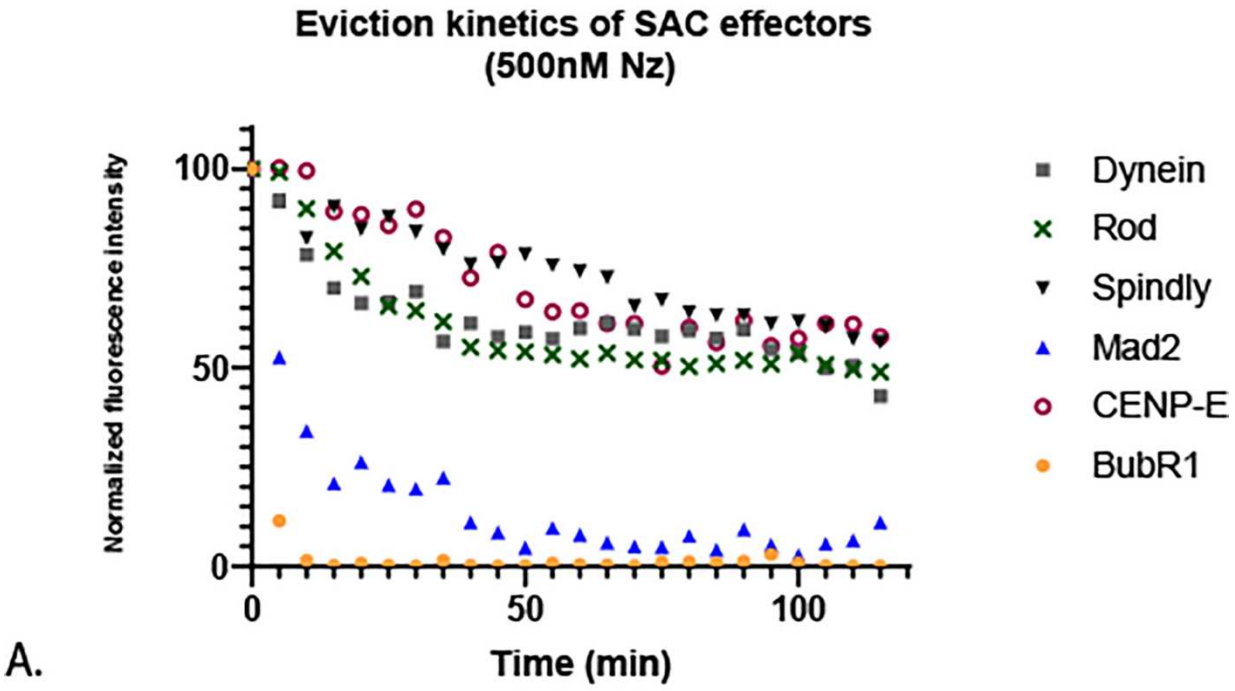


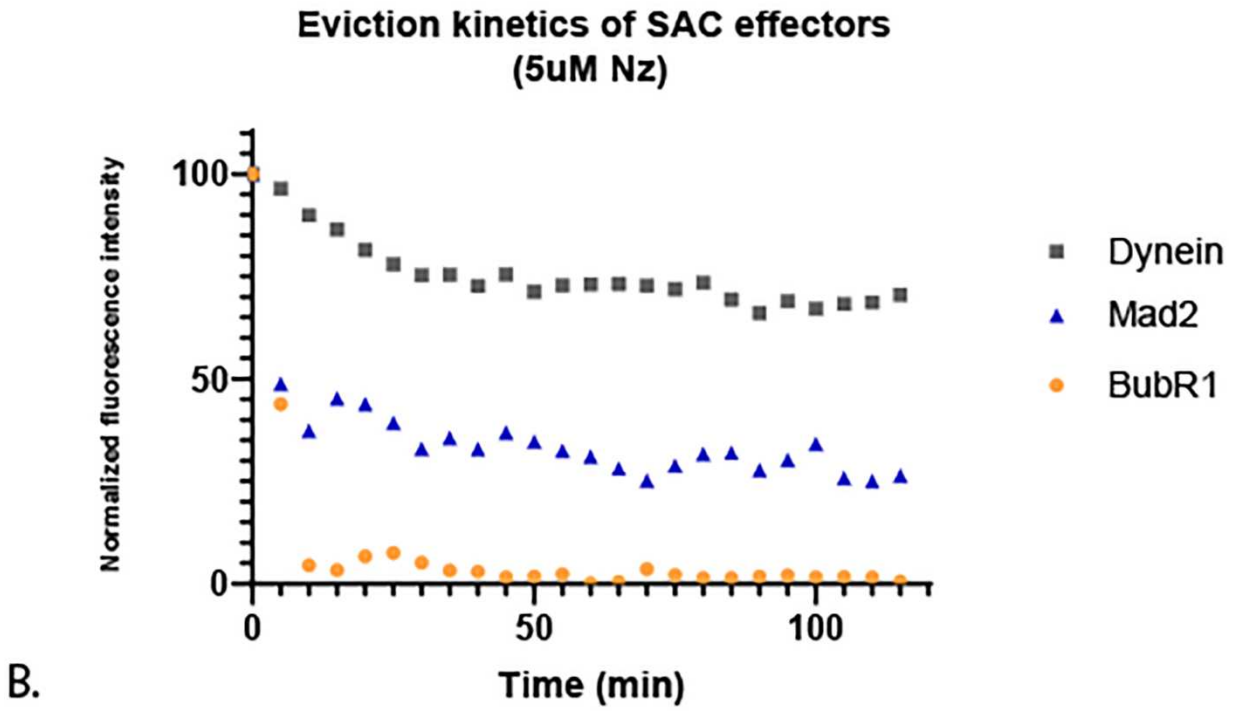
Figure 5. Characterizing native dynein at the kinetochore

A. Images from a time-lapse imaging sequence of CRISPR-engineered cells expressing endogenously-tagged DHC-GFP. Dynein localizes to the nuclear envelope pre-NEBD, and then loads to kinetochores in high concentration upon NEBD. As mitosis progresses and KT-MT attachments are made, kinetochore dynein signal gradually diminishes until it is gone by metaphase (25min image). Dynein can also be seen localizing to the spindle and poles here.
B. Removal of dynein from kinetochores may occur in a motility-independent manner. Images are from a time-lapse imaging sequence of a DHC-GFP CRISPR cell expressing Hec1-mCherry as a kinetochore marker. Arrow marks a kinetochore whose DHC-GFP signal diminishes upon KT-MT attachment, with no apparent evidence of

poleward transport along spindle fibers; dynein eviction appears to be occurring in a diffusion-mediated manner.


Eviction kinetics of SAC effectors
(500nM Nz)
Normalized fluorescence intensity
100
50
0
0
50
100
Time (min)
Dynein
Rod
Spindly
Mad2
CENP-E
BubR1

A.


Eviction kinetics of SAC effectors
(5uM Nz)
Normalized fluorescence intensity
100
50
0
0
50
100
Time (min)
Dynein
Mad2
BubR1

B.

Figure 6. Eviction kinetochores of SAC effectors in the presence or absence of KT-MT attachments

A. Graph displaying eviction kinetics of SAC effectors in response to Mps1 inhibition in cells treated with 0.5uM nocodazole (Nz). Cells expressing fluorescent proteins of interest were treated with 0.5uM Nz overnight to generate microtubule tufts. Before imaging, MG132 was added to media to keep cells from exiting mitosis. Live cells were imaged for one time frame prior to adding reversine, and then imaged every 5min for 2 hours after adding reversine. Fluorescence intensity for whole-cell checkpoint signal was quantified from each time frame and corrected for photobleaching to generate a curve representing checkpoint eviction kinetics.
B. Graph displaying eviction kinetics of SAC effectors in response to Mps1 inhibition in cells treated with 5uM Nz. Cells expressing fluorescent proteins of interest were treated with 5uM Nz overnight to depolymerize all microtubules. Before imaging, MG132 was added to media to keep cells from exiting mitosis. Live cells were imaged for one time frame prior to adding reversine, and then imaged every 5min for 2 hours after adding reversine. Fluorescence intensity for whole-cell checkpoint signal was quantified from each time frame and corrected for photobleaching to generate a curve representing checkpoint eviction kinetics.

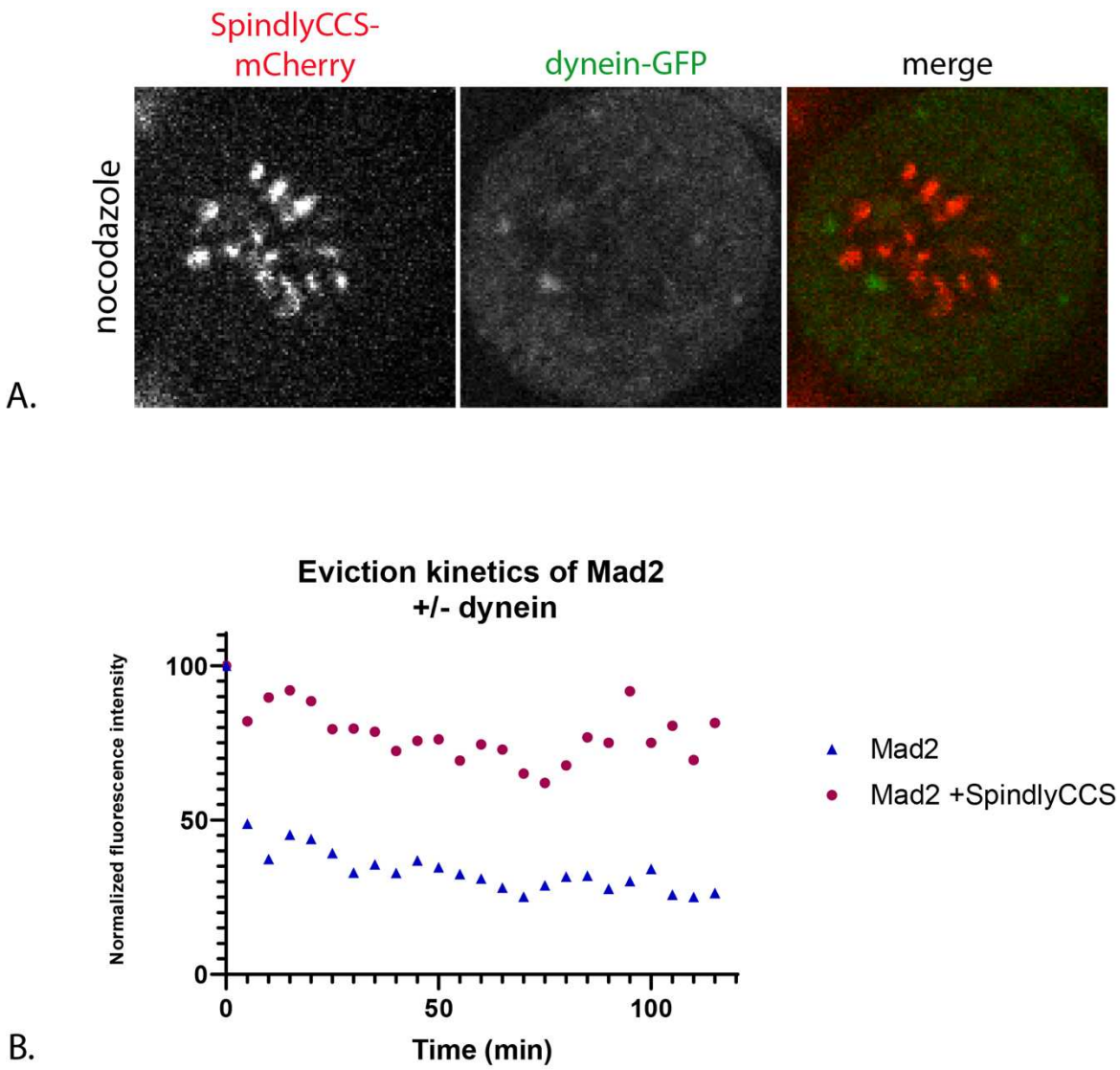


Figure 7. Preventing dynein recruitment to the kinetochore causes increased retention of Mad2 in the absence of microtubules

A. Image of a DHC-GFP CRISPR cell depleted of endogenous Spindly and expressing SpindlyΔCCS-mCherry and treated with 5uM nocodazole, demonstrating that the Spindly motif mutant prevents localization of dynein to the kinetochore.
B. Graph displaying eviction kinetics of Mad2 in the presence or absence of dynein. HeLa kyotos were transfected with Mad2-GFP alone, or Mad2-GFP and SpindlyΔCCS-mCherry. Cells were treated with 5uM Nz overnight to depolymerize all microtubules. Before imaging, MG132 was added to media to keep cells from exiting mitosis. Live cells were imaged for one time frame prior to adding reversine, and then imaged every 5min for 2 hours after adding reversine. Fluorescence intensity for whole-cell Mad2

signal was quantified from each time frame and corrected for photobleaching to generate a curve representing Mad2 eviction kinetics.

DISCUSSION

While our understanding of basic kinetochore structure and function is well-established, there is still much to be learned about the dynamic relationship between kinetochore-microtubule attachments and silencing of the spindle assembly checkpoint. Here we attempted to elucidate the function of the minus end-directed motor protein dynein at the kinetochore in human cells. Specifically, we sought to understand the manner in which dynein facilitates SAC silencing to allow for faithful chromosome segregation. We hypothesized that dynein facilitates eviction of SAC effectors from attached kinetochores in a motility-independent manner, rather than actively transporting checkpoint cargoes along spindle microtubules to silence the checkpoint. From these studies, we confirmed a subset of SAC effectors as bona fide dynein cargoes. Additionally, we developed tools to study the kinetics of SAC removal from kinetochores, which we used to demonstrate that dynein-mediated eviction of SAC effectors can occur in a motility-independent manner, supporting our hypothesis and challenging the established paradigm of kinetochore dynein function.

A subset of SAC effectors requires dynein-mediated eviction from attached kinetochores

Through our experiments involving the Spindly motif mutant, we confirmed Mad1, Mad2, Spindly, Rod, and ZW10 as SAC effectors requiring dynein for their removal from attached kinetochores. This is corroborated by work from other groups (Gassmann et al., 2010), as well as *in vivo* data from our kinetochore eviction assays. Other SAC

effectors such as Bub1, Bub3, and BubR1 did not display increased retention at metaphase kinetochores when dynein recruitment was prevented, suggesting a dynein-independent mechanism of eviction for those effectors. These proteins are recruited to the kinetochore through a pathway dependent on Mps1-mediated phosphorylation of Knl1 MELT motifs in early mitosis. As mitosis progresses and attachments are established, a shift in the kinase-phosphatase balance at the kinetochore occurs, leading to dephosphorylation of Knl1 and subsequent eviction of Bub1, Bub3, and BubR1. This is supported by data from our kinetochore eviction assay, which demonstrates rapid removal of BubR1 from kinetochores immediately upon treating cells with the drug reversine which inhibits Mps1 kinase. These eviction kinetics differ significantly from those of bona fide dynein cargoes, and dynein itself, which display more of a gradual removal from the kinetochore upon Mps1 inhibition.

It is important to note that depleting cells of endogenous Spindly also prevents dynein localization to the kinetochore. However, it has been shown that cells depleted of Spindly undergo a delay in chromosome alignment, followed by successful silencing of the checkpoint and eventual anaphase progression (Gassmann et al., 2010). Thus, it is proposed that there is a dynein-independent mechanism for satisfying the checkpoint that becomes active in the absence of the Spindly-dynein-dynactin module (Gassmann et al., 2010). Cells expressing the Spindly motif mutant demonstrate an increased frequency of failure to satisfy the checkpoint, suggesting that in the presence of Spindly and absence of dynein, the dynein-independent mechanism of SAC silencing is not activated (Gassmann et al., 2010). Thus, the Spindly-dynein interaction is critical for dynein-mediated SAC silencing.

As mentioned previously, Spindly possesses three conserved motifs that promote interaction with dynein (Figure 3A) (Sacristan et al., 2018). Our Spindly F258A motif mutant has only one of these motifs disrupted – the Spindly box. Moving forward, we plan to repeat this experiment using a Spindly construct that has all three dynein recruitment motifs mutated, called Spindly-ΔCCS, to obtain a more penetrant phenotype due to a more complete prevention of dynein recruitment to the kinetochore.

Dynein-mediated eviction of SAC effectors does not require microtubules

Generation of an endogenously-tagged dynein CRISPR cell line facilitated interrogation of kinetochore dynein behavior in a manner not previously possible. Using this exciting new tool, we confirmed dynein's dynamic localization patterns throughout mitosis (Figure 5A). Dynein loads onto unattached kinetochores in high concentrations upon nuclear envelope breakdown, which itself is a dynein-mediated process. Dynein signal gradually diminishes at kinetochores as they make attachments with microtubules, and by metaphase dynein is completely absent from all kinetochores. At this point there is visible dynein signal localizing on spindle microtubules, which could lead one to reason that dynein is utilizing its microtubule-based motor activity to strip SAC components from aligned kinetochores. However, despite extensive imaging of our DHC-GFP cells with many different magnifications, exposures, and conditions, we did not observe any active poleward streaming of dynein particles along spindle fibers (Figure 5B). Thus, we speculate that any dynein signal observed upon mitotic spindle microtubules can be explained by the microtubule-binding capabilities of diffuse cytoplasmic dynein.

It is possible that our imaging conditions were not able to resolve single molecules of endogenously-tagged dynein streaming along the spindle. However, the only evidence supporting a model of motility-based dynein-mediated SAC silencing are time lapse movies of Drosophila cells in metaphase, where checkpoint proteins can be seen streaming along spindle microtubules toward the poles (Défachelles et al., 2015) (Griffis et al., 2007). However, plus end-directed movement of checkpoint proteins is also observed in these movies, leading us to question the validity of the dynein transport model. Given the significant difference in mitotic processes between Drosophila and human cells, and the fact that active dynein-mediated transport of SAC effectors has never been directly observed in mammalian cells, a motility-independent mechanism of dynein-mediated SAC silencing is possible.

Supporting this hypothesis are the data from our kinetochore eviction assays. Using our Spindly-ΔCCS mutant that prevents recruitment of kinetochore dynein, we observed Mad2 eviction kinetics upon dephosphorylation of the kinetochore, while in the complete absence of microtubules. We saw a significant increase in Mad2 retention at the kinetochore in cells that could not recruit dynein as compared to cells that could (Figure 7B). Strikingly, these data indicate that dynein is capable of removing Mad2 from kinetochores in a microtubule-independent manner, further supporting our hypothesis that dynein facilitates dissociation of checkpoint proteins without the use of its motor activity. If true, this would be the first instance of a motility-independent function for dynein.

In light of these findings, future directions will explore the role of Spindly as a cargo adaptor, with a particular focus on investigating its impact on dynein-dynactin

motility. To approach this question, *in vitro* reconstituted motility assays will be implemented in which purified dynein, dynactin, Spindly, and other candidate molecules will be incubated together and flowed into a chamber containing adhered microtubules. Total Internal Reflection Fluorescence (TIRF) microscopy will be used to visualize the fluorescently-tagged purified proteins in order to assess the motility of the DDS complex by measuring velocity of the complex, as well as the length it can travel along a microtubule track before dissociating. Full-length Spindly constructs, as well as various truncations of Spindly will be tested to investigate the role that the Spindly-dynein interaction has on motility of the complex. Purified RZZ will also be used to explore RZZ-mediated regulation of Spindly activity. Additionally, the assay will be performed with “dead” adaptors, or adaptors known not to impart motility, as well as active adaptors to see how motility compares to Spindly-mediated motility. The assay can also be performed using cell extracts instead of purified protein in an attempt to identify unknown components involved in DDS function.

The molecular mechanism that triggers dynein removal upon kinetochore-microtubule attachment remains elusive. It has been proposed that the structure of the kinetochore is altered upon end-on attachment to a microtubule, which may induce a conformational change that promotes dissociation of dynein from the kinetochore, along with its SAC cargo (Hara and Fukagawa, 2020). To better understand the relationship between kinetochore dynein and end-on microtubule attachments, we will perform cell-based experiments to observe dynein retention in response to different microtubule attachment statuses. In order to do this, mutants of the protein Hec1, a subunit of the Ndc80 complex that acts as the core linkage between microtubules and kinetochores

(DeLuca and Musacchio, 2012), will be utilized in conjunction with our CRISPR DHC-GFP cells. A mutant of Hec1 that forms hyper-stable attachments with microtubules early in mitosis (Hec1-9A), as well as a mutant that cannot form stable end-on attachments at all (Hec1-K166D) will be expressed in the CRISPR DHC-GFP cells. We expect to see lower amounts of dynein at the kinetochores of Hec1-9A-expressing cells, and increased dynein in K166D-expressing cells, supporting the hypothesis that dynein requires end-on attachments to be removed from kinetochores.

METHODS/MATERIALS

Cell culture

All cell lines used in this manuscript were maintained at 37°C in a 5% CO2 atmosphere in Dulbecco's Modified Eagle Medium supplemented with 10% FBS and 1% antibiotic/antimycotic solution and low glutamine. HeLa FlpIn Trex SpindlyWT-GFP and HeLa FlpIn Trex SpindlyF258A-GFP cell lines were a gift from Reto Gassmann (Gassmann et al., 2010).

Motif mutant experiments

Cell treatments and transfections

For all fixed-cell experiments, HeLa FlpIn Trex SpindlyWT-GFP and HeLa FlpIn Trex SpindlyF258A-GFP cell lines were grown on sterile, acid-washed coverslips in six well plates. Cells were transfected using Oligofectamine and reduced-serum Opti-MEM according to the manufacturer's instructions. A predesigned siRNA for Spindly (GA AAGGGUCUCAAACUGAA) was used at a final concentration of 100 nM. After incubation for 5–6 h, 1 vol of medium and fetal bovine serum (10% final) was added. After 24 h, the transfection mixture was replaced with fresh medium. Transgene expression was induced with doxycycline 24 h post-transfection and cells were fixed 48 h post-transfection. Cells were treated with 10uM MG132 for 1hr prior to fixing to accumulate metaphase cells for analysis.

Immunofluorescence

Cells were rinsed in PHEM buffer (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 4 mM MgCl2, pH 7.0) and permeabilized in lysis buffer (PHEM + 0.5% Triton-X 100) for 3 minutes at 37°C. Post-lysis, cells were quickly washed in PHEM and subsequently fixed in freshly made fixative solution (4% paraformaldehyde in PHEM) for 20 minutes at 37°C. After fixation, cells were subjected to three five-minute washes in PHEM-T (PHEM + 0.1% Triton-X 100), quickly rinsed in PHEM, and blocked in 10% boiled donkey serum (BDS) in PHEM for 1 hour at room temperature. Following blocking, primary antibodies diluted in 5% BDS in PHEM were added to cells and incubated for 1 hour at room temperature followed by 16 hours at 4°C. Primary antibodies were used as follows: human anti-centromere antibody (ACA) at 1:300 (Antibodies, Inc.), mouse anti-Bub1 at 1:200 (Abcam), mouse anti-Bub3 at 1:300, rabbit anti-Mad1 at 1:700 (GeneTex), mouse anti-BubR1 at 1:200 (Millipore), rabbit anti-Mad2 at 1:1500 (homemade), mouse anti-p150 at 1:500 (BD), mouse anti-Rod at 1:500, and rabbit anti-ZW10 at 1:1000. After primary antibody incubation, unbound antibody was washed off using three five-minute PHEM-T rinses, followed by a quick wash in PHEM. Secondary antibodies (conjugated to Alexa 488, Cy3 dye, or Alexa 647, Jackson ImmunoResearch) were diluted 1:750 in 5% BDS. Cells were incubated in secondary antibody for 45 minutes at room temperature, and unbound antibody was washed off with 3 x 5 min PHEM-T washes followed by a quick rinse in PHEM. Cells were then incubated in a 2 ng/ml DAPI solution (diluted in PHEM) for 30 seconds, subjected to two five-minute PHEM-T washes, quickly rinsed in PHEM, and mounted onto glass slides

using an antifade solution (90% glycerol + 0.5% N-propyl gallate). Following mounting, coverslip edges were sealed with nail polish and slides were stored at 4°C

Imaging and analysis

All fixed cell images were acquired using a DeltaVision Personal DV Imaging system (GE Healthcare) on an IX71 inverted microscope (Olympus) using SoftWoRx software (GE Healthcare). All fixed cell experiments were imaged using a 60X 1.42 NA differential interference contrast Plan Achromat oil immersion lens (Olympus). Images were acquired using a CoolSNAP HQ2 camera (Photometrics/Roper Technologies) for a final magnification of 107 nm/pixel. Measurement of kinetochore fluorescence intensity in fixed cells was measured from non-deconvolved, non-compressed images using a custom program in MatLab (Mathworks) courtesy of X. Wan (Wan et al., 2009). Signal intensities of Spindly and checkpoint proteins were normalized to ACA signal.

CRISPR DHC-GFP cell line

Cloning

Cloning of Cas9/gRNA vector

Candidate guide RNAs targeting the C-terminus of the human genomic dynein heavy chain (DHC) coding sequence were identified using the CCTop website (Webmaster, COS). Optimal guide RNA was chosen, meaning the guide RNA that targets the Cas9 enzyme as close to the desired cut site as possible while having the lowest amount of off-target genomic effects – this is limited by natural placement of PAM sequences (NGG). Guide RNA targets Cas9 to cleave 3 nucleotides upstream of the ‘N’ of the PAM

sequence. Top and bottom strands of the guide RNA were ordered, with overhangs for ligation into the Cas9 plasmid (CCTop provides these overhangs). Guide RNA strands were annealed together and ligated into the Cas9 vector.

Cloning of donor vector

A gBlock was ordered from IDT encoding “homology arms”, which are 500bp flanking the genomic DHC C-terminal cut site (250bp before and after the cut site) with a BamHI restriction site in the middle. These arms act as sites of homology to facilitate homology directed recombination (HDR) of our modification into the genomic locus. Homology arm gBlock is ligated into pBluescript vector and digested with BamHI. The GFP “selection cassette”, which contains the sequence for a GFP fluorophore as well as a hygromycin resistance gene for selection, was ligated into the digested homology arm vector. Final result was a plasmid containing the selection cassette flanked on either side with sequences homologous to the C-terminal genomic locus of DHC.

Transfection and selection

$1.5e^6$ HeLa Kyoto cells were co-transfected with 2ug of both the gRNA/Cas9 vector and the donor vector using Lipofectamine 3000. 48 hrs post-transfection cells were changed into selection media of DMEM containing 300ug/ml hygromycin. Colonies were allowed to form in selection media, and then were individually transferred to a 24-well dish. Clones were screened for successful HDR-facilitated incorporation of the GFP tag into the genome at the C-terminal locus of the DHC gene. Positive clones were expanded.

Imaging DHC-GFP cells for streaming

DHC-GFP CRISPR cells over-expressing Hec1-mCherry were seeded in a 35mm live-cell dish. Cells were imaged on a Nikon Ti-E microscope equipped with a Piezo Z-control (Physik Instrumente), stage top incubation system (Okolab), spinning disc confocal scanner unit (CSUX1; Yokogawa) using a 100X objective and an iXon DU888 EM-CCD camera (Andor). Single-plane images were acquired using 488 nm and 594 nm lasers on a triggered setting to excite GFP and mCherry, respectively. Perfect focus was not used to allow for rapid imaging.

Kinetochore eviction assay

Transfection, drugs treatments

HeLa Kyoto cells or DHC-GFP cells were transfected using Lipofectamine 3000 as follows: 100nM Spindly siRNA with 2.5ug SpindlyΔCCS-mCherry for 48hrs, 1ug Mad2-GFP for 48hrs, 1ug BubR1-GFP for 24 hrs, 1ug CENPE-GFP for 48hrs, 100nM Rod siRNA for 60 hrs with 1ugRod-GFP for 48hrs. 16-24 hrs prior to imaging, cells in a 35mm live cell dish were treated with 500nM or 5uM nocodazole to accumulate mitotic cells with unattached kinetochores. Immediately prior to imaging, media was replaced with filming media containing 500nM or 5uM nocodazole and 10uM MG132. Cells were imaged for one time frame, then 10uM reversine was added to the media.

Imaging conditions

Cells were imaged on a Nikon Ti-E microscope equipped with a Piezo Z-control (Physik Instrumente), stage top incubation system (Okolab), spinning disc confocal scanner unit (CSUX1; Yokogawa) using a 60X objective and an iXon DU888 EM-CCD camera (Andor). Z-stacks were acquired taking 3 planes at 1.25 μm steps using 488 nm and 594 nm lasers to excite GFP and mCherry, respectively.

Analysis

To generate curves representing eviction kinetics of checkpoint proteins, whole-cell fluorescence intensities of fluorescent checkpoint proteins were measured from each time frame of a time-lapse movie for 10-15 cells per protein using Nikon Elements software. Data from cells treated with nocodazole, MG132, and reversine were normalized to data from cells treated with nocodazole and MG132 to correct for photobleaching.

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