THESIS

MAPPING THE RECRUITMENT PATHWAYS OF CORE SPINDLE ASSEMBLY CHECKPOINT PROTEINS

Submitted by

Daniel Mallal

Department of Biochemistry and Molecular Biology

In partial fulfillment of the requirements

For the Degree of Master of Science

Colorado State University

Fort Collins, Colorado

Summer 2017

Master's Committee:

Advisor: Jennifer DeLuca
Co-Advisor: Steven Markus

John Spencer

Copyright by Daniel Mallal 2017

All Rights Reserved

ABSTRACT

MAPPING THE RECRUITMENT PATHWAYS OF CORE SPINDLE ASSEMBLY CHECKPOINT PROTEINS

The Spindle Assembly Checkpoint (SAC) is a vital regulatory pathway in eukaryotic cells to ensure proper division of duplicated chromosomes such that each daughter cell receives a complete and equal copy of genetic material. The SAC specifically ensures that kinetochores form proper attachments to spindle microtubules by preventing anaphase until every chromosome is bi-oriented and attached at each pair of kinetochores to microtubules emanating from opposite spindle poles. The SAC is a highly regulated and intricate network of proteins which allows for a robust inhibitory signal to be produced in the presence of erroneous attachments, halting cells in anaphase allowing for error correction. An important set of interactions occurs surrounding the proteins Bub1, BubR1, BuGZ binding to Bub3 mediated through a GLEBS domain binding Bub3. The precise nature of the interplay between these proteins binding to Bub3 is rather unclear and requires further characterization. Here we set out to characterize the direct recruitment sufficiency of each of these proteins. In order to distinguish the direct recruitment sufficiency of each individual protein, we targeted Bub1, BubR1, Bub3, and BuGZ individually to an ectopic site on chromosomes away from the kinetochore. We find that Bub1, BubR1, and Bub3 are sufficient to recruit each other as well as BuGZ, however BuGZ is only able to recruit Bub3 indicating that the Bub3-BuGZ GLEBS interaction is the strongest of the three. Interestingly, we also find that BuGZ is able to recruit Bub3 less efficiently in mitotic cells, suggesting a regulatory mechanism that decreases the affinity of BuGZ for Bub3 as cells transition into mitosis. Together, these data support a model in which BuGZ is exchanged for Bub1 to bind Bub3 at kinetochores in mitosis to promote efficient SAC signaling.

TABLE OF CONTENTS

Introduction and Background

Cell division is a highly regulated, intricate cellular process, comprised of many pathways that are that is well conserved across many forms of life, especially amongst higher eukaryotes. When a cell divides, it must faithfully segregate equal numbers of sister chromatids to each daughter cell, ensuring that each cell receives a complete set of genomic DNA. When it is time for a cell to enter mitosis, mitotic spindles are constructed from dynamic microtubules nucleated from the two centrosomes, forming the spindle poles from which microtubules emanate towards the chromosomes (Winey, 1991). These spindle microtubules then form attachments to chromosomes at a specialized structure called the kinetochore. The kinetochore is a large macromolecular structure that is built upon centromeric chromatin of each sister chromatid, functioning as a docking site and sensor for microtubule attachment (Musacchio and Desai, 2017). The polymerization and depolymerization forces of microtubules are responsible for aligning chromosomes along the metaphase plate (Inoue and Salmon, 1995). Kinetochore-microtubule attachments are highly labile early in mitosis, and become more stable as mitosis progresses and correct attachments are formed (Gonzalez 2012). It is critical that all chromosomes become bioriented at the spindle equator and form proper attachments to microtubules at the kinetochore, otherwise daughter cells may receive an uneven distribution of genetic material (Santaguida and Amon, 2015).

Erroneous Attachments and Activation of SAC

Several types of erroneous kinetochore-microtubule attachments, including syntelic, monotelic, and merotelic attachments are known to occur during mitosis. In the case of syntelic attachments, both kinetochores of a sister pair form attachments to microtubules that are generated from the same spindle pole. Monotelic attachments are a case in which only one kinetochore has attached to a spindle

microtubule, and the other is lacking attachment altogether. Merotelic attachments occur when a single kinetochore forms attachments to microtubules emanating from both spindle poles (Gregan et al., 2011). Each of these types of incorrect kinetochore-microtubule attachments may lead to an unequal distribution of genetic material to daughter cells (Thompson and Compton, 2011). Correct attachments, known as amphitelic attachments, occur when kinetochores are bioriented, and each sister kinetochore forms proper attachments solely to their respective spindle pole, generating tension forces and stabilization of the attachment (Pinsky and Biggins, 2005). An important contingency pathway exists in cells to ensure that mitotic cells do not progress into anaphase until all chromosomes have established correct kinetochore-microtubule attachments: the spindle assembly checkpoint (SAC). The SAC is a robust signaling mechanism which, in the presence of unattached or erroneously attached kinetochores, is activated to send out a "wait anaphase" signal called the Mitotic Checkpoint Complex (MCC). By sequentially recruiting checkpoint proteins in a cascading fashion, an unattached kinetochore rapidly and robustly amplifies this "wait anaphase" signal within the cell to prevent anaphase onset and arrest cells in mitosis (De Antoni et al., 2005). Importantly, the SAC is activated both in the case of completely unattached kinetochores, as well as in the presence of attachments such as syntelic and monotelic, but the SAC is typically not activated by merotelic attachments (Rieder, 1995; Thompson and Compton, 2011). The SAC stalls cells in metaphase until attachment errors are corrected and the chromosomes become properly bioriented (Nezi and Musacchio, 2009).

Aurora B Kinase Mediated Error Correction

A secondary mechanism exists in cells in order to correct erroneous attachments, ensuring cells do not progress through mitosis with such attachments. This pathway is primarily dependent on a serine/threonine kinase called Aurora B Kinase (ABK) (DeLuca et al., 2011). ABK forms a complex with the proteins Borealin, Survivin, and INCENP to form what is known as the chromosomal passenger complex (CPC) (Gassmann et al., 2004). The CPC accumulates at kinetochores during mitosis when there

are incorrect kinetochore-microtubule attachments present (Xu et al., 2009). ABK, the primary catalytic subunit of the CPC, then phosphorylates many targets. Importantly, ABK phosphorylates the NDC80 complex subunit Hec1 early in mitosis, destabilizing kinetochore-microtubule attachments (DeLuca et al., 2011). This is an important error correction mechanism, as erroneous attachments often occur early on in mitosis, and by destabilizing kinetochore microtubules, ABK dependent phosphorylation of Hec1 ensures that cells do not progress through mitosis with such attachments. Importantly, by destabilizing these attachments, the resulting unattached kinetochores are also then able to trigger the SAC, preventing progression of mitosis (Biggins and Murray, 2001). Correct bipolar attachments, however, reduce the levels of ABK phosphorylation on mitotic substrates, leading to stabilization of proper kinetochore-microtubule attachments. There is also evidence that ABK has functions in regulation of the SAC, independent of its role in error correction, specifically playing a role in recruiting Mps1 kinase to kinetochores (Zhu et al., 2013).

Molecular Mechanism of SAC activation

If a single kinetochore has not formed proper attachments with microtubules, a series of signaling events occurs, leading to a sequential accumulation of SAC proteins at kinetochores, sending out a 'wait-anaphase' signal to the cell (Rieder et al., 1995). In the absence of microtubule attachments, a kinetochore protein, KNL1, is first phosphorylated at several residues on threonine residues within multiple conserved "MELT" motifs (Methionine-Glutamate-Leucine-Threonine) by Mps1 kinase (London et al., 2012). This phosphorylation event triggers the sequential recruitment of SAC proteins to the kinetochore (Figure 1). Bub3 binds to phosphorylated MELT motifs on KNL1, and brings with it the proteins Bub1 and BubR1 (Overlack et al., 2015; Primorac et al., 2013), as well as possibly the newly identified kinetochore-associated, Bub3 binding protein, BuGZ (Toledo et al., 2014). Mad1 and Mad2 are then recruited to the kinetochore as a hetero-tetrameric complex by kinetochore bound Bub1 (Ji et al., 2017; Alfieri et al., 2016). Mad1 and Mad2 are also recruited to the by a recursive pathway in a

mechanistically unclear manner by the RZZ complex. The RZZ complex is an important element for Mad1 and Mad2 kinetochore recruitment through a KNL1 dependent pathway (Caldas et al., 2015). As SAC signaling is crucial to proper cell division, it is possible that, evolutionarily, multiple pathways of SAC protein recruitment evolved as a contingency in case of the ablation of one mechanism of kinetochore recruitment. In addition to phosphorylating the MELT motifs in KNL1, Mps1 kinase has recently been shown to have additional roles in building a functional checkpoint. Specifically, the kinase phosphorylates Mad1 and Bub1 in order to create a catalytic platform for the assembly of a functional MCC, as well as a phosphorylation dependent interaction between Mad1 and Cdc20 for MCC assembly (Ji et al., 2017; Faesen et al, 2017). It is evident that many of the SAC proteins have a multitude of roles and interactions that are important in the culmination of a highly regulated signal.

Mitotic Checkpoint Complex

The inhibitory "wait anaphase" signal is a multi-protein complex known as the MCC which directly functions to inhibit the Anaphase Promoting Complex/Cyclosome (APC/C) (Herzog et al., 2009). The MCC is a diffusible signaling complex comprised of the proteins Mad2, CDC20, BubR1, and Bub3 which function to bind to, and inhibit the ability of CDC20 to activate the APC/C (Tipton et al., 2011; Izawa and Pines, 2014). The MCC is also formed through a series of signaling events, starting with kinetochore accumulation of its components. Mad2 has two conformations, open (or low affinity for CDC20), or closed (high affinity for CDC20), and the majority of cytosolic free Mad2 exists in the open conformation (De Antoni et al., 2005). Upon binding to Mad1, Mad2 undergoes a conformational change from open to its closed formation (Izawa and Pines, 2014). This kinetochore bound Mad1:Mad2 complex serves as a template to promote the conversion of free open-Mad2 to closed-Mad2 (De Antoni et al., 2005). Both BubR1 (in complex with Bub3), and closed-Mad2 bind to and sequester CDC20, forming the MCC (Izawa and Pines, 2014; Han et al., 2013). The APC/C is an E3 ubiquitin ligase which relies upon CDC20 as a necessary cofactor for proper function, and as its name suggests, the activity of the APC/C is

primarily responsible for the onset of anaphase in eukaryotes (Peters, 2002; Fang et al., 1998). The APC/C has two key mitotic substrates: Securin and Cyclin B, both of which are important for keeping the cell in mitosis, preventing the onset of anaphase (Fang et al., 1998; Kraft et al., 2005). The checkpoint may presumably be activated by a single unattached kinetochore *in vivo* due to the diffusible nature of the MCC (Heasley et al., 2017). Once correct, stable attachments are formed at every sister kinetochore pair, the checkpoint is silenced, and the cell progresses through anaphase and cytokinesis (Mussachio and Salmon 2007).

Consequences of a Perturbed Spindle Assembly Checkpoint

Abrogation of the spindle checkpoint leads to critical errors in chromosome segregation, which ultimately gives rise to aneuploidy (Li et al., 2009). Aneuploidy is a condition in which cells contain an abnormal number of chromosomes. Having an abnormal number of chromosomes leads to a slew of downstream issues within the cell. Aneuploidy is seen in virtually all human cancers, as well as many other birth defects and diseases (Rajagopalan and Lengauer, 2004). Many cancer tissues have shown an increased requirement for SAC proteins in order to maintain a steady state of aneuploidy chromosomes. A certain level of aneuploidy is inevitably cytotoxic, and not even the heartiest of cancers survive. To avert this, many cancerous tissues overexpress certain SAC proteins to fortify the pathway (Ding et al 2013; Herman et al., 2015). Consequently, many proteins involved with the SAC have been shown as putative targets for selective oncogenic therapies (Ding et al., 2013; Toledo et al., 2014; Marques et al., 2015).

Molecular Interactions of Core SAC Proteins/Aims of Study

Bub1, BubR1, and BuGZ are important SAC proteins containing a highly conserved GLEBS domain. The GLEBS domain facilitates Bub1, BubR1, and BuGZ binding to Bub3 at its WD40 motif. These three proteins all require their GLEBS domains for efficient kinetochore recruitment (Toledo et al., 2014; Overlack et al., 2015). It has previously been shown that BuGZ is necessary for stabilization of Bub3, and

co-localizes with Bub3 to kinetochores in early mitosis prior to nuclear envelope breakdown (Toledo et al., 2014). After nuclear envelope breakdown, Bub1, and BubR1 are then recruited to kinetochores by way of a poorly understood Bub3 dependent mechanism (Overlack et al., 2015). The exact nature of the interplay between these three GLEBS domain-containing proteins and their interaction with Bub3 is unclear and requires further investigation. More specifically, it is of interest to explore what function BuGZ has in kinetochore recruitment of Bub1 and BubR1, as Bub1 and BubR1 have been shown to be interdependent in their recruitment to kinetochores (Overlack et al., 2015). Here, we set out to characterize the interdependencies of recruitment patterns of several core SAC proteins: Bub1, BubR1, Bub3, BuGZ, and Mad1. The actual sub-complexes that are formed in cells between these proteins are unclear, hence, we wanted to gain further insight into, and clarify the interactions and interdependencies that are seen amongst these proteins in cells. We show that Bub1, BubR1, and Bub3 are all sufficient to recruit each other as well as BuGZ. BuGZ, however IS able to recruit Bub3 only. Furthermore, BuGZ recruitment of Bub3 is decreased in mitotic cells. This leads us to hypothesize that a regulatory mechanism such as post-translational modification decreases the affinity of BuGZ for Bub3 as cells transition into mitosis, supporting a model in which BuGZ is exchanged for Bub1 to bind Bub3 at kinetochores in mitosis to promote efficient SAC signaling.

Results and Discussion

Approaches and Methods

The kinetochore is a very large macromolecular assembly comprising of several copies of approximately 30 subunits assembled upon centromeric chromatin (Klare et al., 2015). There presents a multitude of complexities whilst studying kinetochore and kinetochore associated proteins such as SAC components when considering there are >100 proteins present in vertebrate kinetochores (Santaguida and Musacchio, 2009). Hence, in order to study the extent of recruitment dependencies and interactions seen between individual SAC proteins in cells, we have targeted several of these proteins, to an ectopic location on chromosome arms, away from kinetochores. This will provide insight into how these proteins directly compete, and interact amongst one another in cells away from the kinetochore by removing spurious factors that arise from the complexities of kinetochores, allowing for the direct study of the contribution that a single SAC protein has upon the recruitment of others. The aim of this study is to create a map of the recruitment dependencies seen between the SAC proteins Bub1, BubR1, Bub3, BuGZ, and Mad1 at an ectopic location in cells. To do so, we employed the use of a bone marrow cancer cell line, U2OS, that have been genetically altered to contain approximately 200 tandem repeats of the Lac Operon (henceforth referred to as U2OS-LacO cells) inserted at a site away from kinetochores in a euchromatic region at 1p36 on chromosome 1 (Janicki et al., 2004). We utilized a plasmid construct containing the Lac repressor (LacI) fused to GFP. This LacI-GFP construct was then individually fused to each one of the following proteins: Bub1, BubR1, Bub3, and BuGZ; creating a total of four different expression plasmids with LacI-GFP fused to a SAC protein (Figure 2A). The LacI repressor protein strongly co-localizes to the LacO array in cells, thus transient expression of these constructs transfected into the U2OS-LacO cells effectively targets a large pool of the LacI-GFP fusion proteins to the ectopic LacO array

such that recruitment patterns may be analyzed away from kinetochores by immunofluorescence (Figure 2B and C). Immunofluorescence was then used to analyze whether or not each LacI-GFP fusion protein sufficient to recruit Bub1, Bub3, BuGZ, and Mad1 by co-staining with antibodies specific to each. Co-localization analysis showed whether or not a LacI-GFP fusion protein co-localized with the antibody specific to each SAC protein. The LacI-GFP construct that did not have a SAC protein fused to it was used to control for nonspecific antibody binding (referred to as LacI-GFP-Control). Control cells were transfected alongside test conditions and stained for using the same antibodies. Fluorescence intensity of the antibody at the LacO array was quantified using ImageJ quantification software and compared between cells transfected with the LacI-GFP fused to SAC proteins and those that were transfected with the LacI-GFP control construct. If the average immunofluorescence signal at the LacO array was greater than that of the corresponding control condition, we concluded that the LacI-GFP fusion SAC protein was sufficient to recruit the protein that was stained for.

LacI-GFP-Bub1

When Bub1 was ectopically targeted to the LacO array, robust co-recruitment of Bub3 was seen in nearly 100% of cells analyzed (Figure 3A and C). As Bub3 is necessary for Bub1 to localize to kinetochores as well as the fact that Bub1-Bub3 complexes form throughout the cell cycle (Overlack et al., 2014), it was to be expected that Bub1 is reciprocally able to recruit Bub3 to an ectopic site. This indicates evidence of a strong interaction between Bub1 and Bub3 in cells in the absence of any post-translational modifications, conformational changes, or other protein-protein interactions that might be seen solely at the kinetochore. LacI-GFP-Bub1 was also able to recruit BuGZ, albeit less efficiently than Bub3, to the array as well. It has previously been shown that LacI-Bub1 is able to recruit BubR1 ectopically to a LacO array in a similar experiment to those done here (Overlack et al., 2014). Importantly, a GLEBS domain mutant of LacI-Bub1 (LacI-Bub1$^{\Delta GLEBS}$) was not able to recruit either Bub3 or BubR1 to the array indicating that Bub1-Bub3 is likely binding to BubR1-Bub3 to form a ternary

complex (Overlack et al., 2014). Since BuGZ and BubR1 are recruited in a similar fashion to the LacO array by LacI-Bub1, it is likely that, here, BuGZ is forming a complex where the LacI-Bub1 recruits a Bub3 molecule, which in turn recruits a Bub3-BuGZ heterodimer, similar to the ternary complex seen by Overlack et al., 2014 between Bub1 and BubR1 (Figure 3B). Further validation is required in this experimentation to rule out the possibility that BuGZ is directly interacting with Bub1. This could be tested by using a LacI-Bub1$^{\Delta GLEBS}$ mutant to show the insufficiency for both Bub3 and BuGZ recruitment, thus showing no direct interaction between BuGZ and Bub1 here.

Mad1 recruitment was also seen at the array by LacI-Bub1 (Figure 3A and C), which was to be expected as Bub1 is known to recruit Mad1 to the kinetochore (Ji et al., 2017; Alfieri et al., 2016). As Mad1 and Mad2 form a hetero-tetrameric complex, it is likely that Mad2 is also recruited to the array in these experiments, however, several Mad2 antibodies commercially available, as well as a homemade antibody for Mad2 nonspecifically localized to the LacO array in the presence of LacI-GFP in a large population of cells, and thus was not able to be analyzed. We also could not validate the presence of BubR1 seen at the LacO array by Overlack et al., 2014 due to similar nonspecific antibody binding issues. Only about 40% of cells, both mitotic and interphase, had Mad1 co-recruited to the LacO array when transfected with LacI-Bub1. This low frequency of recruitment to the LacO array could possibly be explained by the endogenous RZZ complex recruiting Mad1 and Mad2 to kinetochores here (Caldas et al., 2015). The RZZ complex thus could be sequestering free Mad1 and Mad2 away from the LacO array, allowing it to only localize to the array occasionally. The interaction between Bub1 and Mad1 may not be as strong as the interaction between RZZ and Bub1, so LacI-Bub1 was not always able to recruit Mad1 to the ectopic site. It has previously been shown that Mps1 phosphorylation of Bub1 facilitates the binding of Mad1-Mad2 hetero-tetramers to Bub1. It is possible that this post-translational modification where Mps1 phosphorylates Bub1 does not occur ectopically at the LacO array. This could explain why Mad1 is only sometimes able to be recruited to the LacI-Bub1 array. The phosphorylation status of LacI-

Bub1 requires further investigation. We note that there was significant discrepancy in the recruitment of Mad1 to LacI-Bub1 between the two experiments conducted, (shown by large error bars in figure 3A). This discrepancy expresses the need for further investigation into the nature of this recruitment of Mad1 to LacI-Bub1. This error could've arisen from many sources. It's possible that for some reason cells in the experiment with more Mad1 recruited to the array either also recruited RZZ, or some post-translational modification of Bub1 or Mad1 increased their affinity for each other in these cells.

LacI-GFP-Bub3

As expected, when Bub3 was targeted to the LacO array, it was sufficient for the recruitment of both Bub1 and BuGZ. LacI-Bub3 was also sufficient to recruit Mad1 to the array (Figure 5A and C). Mad1 is likely recruited to the array via either Bub1 or BubR1 co-recruitment. Interestingly, Mad1 was only efficiently recruited to the array in mitotic cells transfected with LacI-Bub3, and was rarely seen in interphase cells. This indicates that there is likely some post-translational modification that mediates mitotic recruitment of Mad1 to Bub3, likely mediated through Bub1 (Figure 5A). We would predict that BubR1 might be recruited to the array under this condition as well because it appears that LacI-Bub3 is not the limiting factor at the array. That is to say, there is enough Bub3 present at the array to recruit some level of Bub1, BubR1, and BuGZ. If Bub3 were the limiting factor, we would expect to see solely BuGZ recruitment to the spot similar to the reciprocal condition where LacI-BuGZ could only recruit Bub3 and not Bub1. The interaction between BuGZ and Bub3 is likely stronger than Bub1 binding Bub3 in cells, but since Bub3 appears not to be limiting when it is targeted to the LacO array, it is able to co-recruit both Bub1 and BuGZ. Several potential complexes could be formed at the array in this condition (Figure 5B). LacI-Bub3 could be recruiting Bub1 and BuGZ (and BubR1) independently of each other, or it is also possible that some sort of macromolecular complex is formed here similar to that what was seen with the ternary complex of LacIBub1-Bub3 binding BuGZ-Bub3 before (Figure 5B). Mad1 might be integrated into this complex either through a BubR1-CDC20 mediated interaction, or directly binding to

Bub1. It is also possible that populations of BuGZ and Bub1 are recruited to the LacI-Bub3 via soluble Bub3 recruited to LacI-Bub3, bringing with it a molecule of Bub1 or BuGZ, forming a complex between LacI-Bub3 with a Bub3-Bub1 or Bub3-BuGZ dimer (Figure 5B).

Mutating the Bub3 binding domain (GLEBS) on Bub1 and BuGZ labeled with an mCherry tag and co-transfecting them in tandem with LacI-Bub3 would be an interesting experiment to further explore the dependencies of recruitment on the GLEBS domain to Bub3. If either Bub1 or BuGZ is able to be recruited to Bub3 in the absence of a consensus GLEBS domain, this would indicate that some other interaction is occurring. For example if mCherry labelled BuGZ$^{\Delta GLEBS}$ is recruited to LacI-Bub3, this would indicate some other sub-complex with either Bub1 or BubR1 formed to recruit BuGZ$^{\Delta GLEBS}$ to the array. It would also be interesting to stain for CDC20 with this LacI-Bub3 construct transfected into the U2OS cells to clarify whether Mad1 localization to the array was mediated by BubR1 binding CDC20 and Mad1 or simply Bub1 directly co-recruiting Mad1 to the LacI-Bub3 pool. An important experiment to be done with this construct is to do three color imaging of LacI-Bub3 (GFP) and then pairwise staining or fluorescent imaging of combinations of Bub1, BuGZ, and BubR1. Preferably this would be done in live cells to reveal dynamic behavior, but this experiment would elucidate whether or not one protein was ever recruited to the LacI-Bub3 array without the other, or if one was recruited, the other was always also recruited. Live cell imaging would prove challenging due to the difficulty of transfecting multiple constructs into a single cell and controlling for the amount of protein produced between the three constructs transfected. This could lead to inaccurate results in that if one was expressed at greater levels than the other as it could localize more strongly solely due to amount of protein in the cell.

LacI-GFP-BuGZ

We next wanted to look at the ability of BuGZ to recruit other SAC proteins to an ectopic site. When LacI-GFP-BuGZ was transfected into U2OS LacO cells, it was able to robustly recruit Bub3 to the array in almost all interphase cells (97% of interphase cells), however the number of cells dropped off

significantly to ~62% of mitotic cells with Bub3 recruited to the array (Figure 4A). This indicates that BuGZ binding affinity for Bub3 decreases as cells enter mitosis, supporting a model where BuGZ helps to stabilize Bub3 recruitment to the kinetochore, then is exchanged so that Bub1 is able to bind Bub3 (Toledo et al., 2014). Interestingly, LacI-BuGZ was insufficient to recruit either Bub1 or Mad1 either in interphase or mitotic cells (Figure 4A). It is possible that BubR1 was able to be recruited, but unlikely due to the lack of both Bub1 and Mad1 recruitment seen. At the array, it is likely that LacI-BuGZ only recruits Bub3, and possibly soluble endogenous BuGZ in a ternary complex where LacI-BuGZ recruits a molecule of Bub3, in turn recruiting a heterodimer of BuGZ-Bub3 (Figure 4B). Taken together with the difference in recruitment seen between LacI-Bub1 for BuGZ and LacI-BuGZ for Bub1, these data might suggest that BuGZ has a stronger interaction with Bub3 through its GLEBS domain than Bub1 does, especially in interphase. We expected to see that as BuGZ's affinity for Bub3 goes down in mitosis, Bub1 would be recruited to the array at some frequency, forming a ternary complex where a heterodimer of LacI-BuGZ-Bub3 binds a heterodimer of Bub1-Bub3, however this interaction was not seen in these experiments (Figure 4C). This result was surprising, and requires further investigation to clarify the nature of these interactions. The fact that Bub1 cannot form a similar ternary complex could be explained either by BuGZ having a greater affinity for Bub3, or Bub1 requiring a modification that might only occur at kinetochores via a kinase such as Mps1 in order to robustly bind to Bub3. We saw that Bub1 localizes strongly to kinetochores in these cells (Figure 4C). Hence, another possibility of why Bub1 is unable to be recruited to LacI-BuGZ is that the majority of endogenous Bub1 localizes to kinetochores, thus there is little Bub1 available to be recruited to the LacI-BuGZ array. This differs from the condition where LacI-Bub3 was targeted to the array. At the LacO array, we hypothesize that LacI-BuGZ as well as endogenous BuGZ is binding to Bub3 strongly, not leaving enough free Bub3 at the array able to bind to Bub1.

It is likely that some sort of modification occurs on BuGZ as the cell transitions into mitosis that decreases its affinity for Bub3, and thus the mitotic LacI-BuGZ pool wasn't always able to recruit Bub3

ectopically. More exact timing and characterization of this decrease in affinity is necessary to further explain this, and how the kinetochore exchange of BuGZ-Bub3 for Bub1-Bub3 complexes occurs. Live cell experiments in this system would reveal more about the precise nature of these interactions. Another interesting experiment would be to analyze a chimeric mutant of LacI-BuGZ where the GLEBS domain was swapped with the GLEBS domain of Bub1, or truncation analysis of BuGZ to map the domains necessary for these interactions. Targeting Mps1 kinase to the array in addition to BuGZ might lead to some interesting differences in the results seen here, as Mps1 is known to phosphorylate many targets, including Bub1 (Ji et al., 2017), which could be an important event for modulating the affinity of Bub1 for Bub3.

LacI-GFP-BubR1

Finally, we looked at the recruitment sufficiency of ectopically targeted BubR1. LacI-BubR1 was able to recruit Mad1 to the LacO array at a significant extent. LacI-BubR1 was also able to recruit BuGZ, Bub3, and Bub1 robustly in both interphase and mitotic cells (Figure 6A). Mad1 recruitment was somewhat surprising, as the main pathways of Mad1 recruitment in cells are mediated through Bub1 or RZZ (Caldas et al., 2015; Ji et al., 2017; Alfieri et al., 2016). We speculate that this interaction of LacI-BubR1 to the recruitment of Mad1 is mediated two-fold: through the Bub1 pathway of Mad1 recruitment, and also through BubR1 binding CDC20 and then subsequent recruitment of Mad1 (Figure 6B). BuGZ recruitment to the LacI-BubR1 site was also unexpected, however could be explained by a ternary complex similar to that seen between Bub1-Bub3 and BubR1-Bub3 (Figure 6B). It is also possible that there is a dynamic interaction occurring between Bub1 and BuGZ binding to BubR1 where some population of BuGZ is exchanged with Bub1 forming a dynamic ternary complex (Figure 6C). This model is supported by BuGZ only recruited to the array in ~50% of cells both mitotic and interphase (Figure 6A). It is possible that BuGZ mediates a ternary complex similar to that seen with Bub1 (Overlack et al., 2014), stabilizing the interaction of BubR1 with Bub3 (Figure 6C). The model of dynamic exchange of

Bub1 and BuGZ rather than a direct ternary complex forming also supports the idea that BuGZ has a stronger binding affinity for Bub3 than that of the affinity of Bub1 for Bub3 (Figure 6B and C). These results are consistent with previous work (Toledo et al., 2014), where BuGZ depletion was shown to have a greater effect on depletion of kinetochore localized Bub1 than that of BubR1, indicating that BubR1 also has a greater affinity for Bub3 than Bub1.

Bub1 and Bub3 recruitment to LacI-BubR1 arrays was expected, as it has been shown that Bub1 and BubR1 directly interact in cells when both are bound to Bub3 (Overlack et al., 2014). It is likely that LacI-BubR1 recruits Bub3, forming a BubR1-Bub3 complex which can, in turn, recruit a complex of Bub1-Bub3, forming a ternary complex. It is interesting that BubR1 is sufficient to recruit Bub1 ectopically, as Overlack et al. showed that a Bub3 binding domain mutant LacI-Bub1$^{\Delta GLEBS}$ construct could not recruit Bub3 nor BubR1 to a LacO array (Overlack et al., 2014). This indicates that, here, it is likely that LacI-BubR1 must be bound to Bub3 in order to recruit Bub1 ectopically. This also raises the question of whether LacI-BubR1 must also bind Bub3 in order to recruit BuGZ to the array. Testing the ability of a Bub3 binding domain mutant LacI-BubR1$^{\Delta GLEBS}$ to recruit Bub1 and Bub3 is an important experiment to demonstrate the necessity of Bub3 binding for the recruitment of both Bub1 and BuGZ to the ectopic array. If BuGZ is actually able to be recruited in the absence of Bub3, this would indicate a direct interaction between BubR1 and BuGZ. Live cell imaging with multiple proteins tagged would also be an informative experiment. LacI-GFP-BubR1 transfected pairwise with a Bub3-mCherry and either Bub1 or BuGZ fused with an IR fluorescent protein would elucidate dynamics between these four proteins at the LacO array, as well as the dependency on each for co-recruitment.

Interestingly, LacI-BubR1 showed a decrease in Bub3 recruitment to the LacO array in mitotic cells from ~82% of interphase cells down to ~57% of mitotic cells (Figure 6A). This result was unexpected, because normally in cells, BubR1 doesn't localize to kinetochores until nuclear envelope breakdown (Howell et al., 2004), and also because BubR1 requires Bub3 binding for efficient

kinetochore localization (Overlack et al., 2014). This indicates that there is some sort of kinetochore based modification to either BubR1 or Bub3 that helps to mediate this interaction in mitosis that does not occur on the ectopically targeted LacI-BubR1. In this scenario, the affinity of BubR1-Bub3 is not modulated properly in mitosis, resulting in a loss of Bub3 binding.

Conclusion

To summarize the results from this study, LacI-Bub3 was sufficient to recruit Mad1, and robustly recruited Bub1 and BuGZ ectopically. LacI-Bub1 was sufficient to recruit Mad1 and BuGZ in some cells, and able to robustly recruit Bub3 ectopically. LacI-BubR1 strongly recruited Mad1 and Bub3, and recruited both BuGZ and Bub1 in many cells (~50%). LacI-BuGZ was able to only recruit Bub3 to a significant extent, and this recruitment decreased in mitotic cells. It is significant that LacI-BuGZ was only able to recruit Bub3 to the ectopic LacO array, as this might suggest that BuGZ has a stronger binding affinity for Bub3 through its GLEBS domain than the GLEBS domain of Bub1 binding Bub3 WD40 repeats. The results from this study support a model in which BuGZ strongly binds Bub3 in interphase cells, but in mitosis this affinity decreases, facilitating the exchange for Bub1 to bind Bub3 at kinetochores. BubR1 and BuGZ likely have a stronger affinity for Bub3 than Bub1, and thus BuGZ likely mediates the exchange of Bub1, and BubR1 is able to localize to kinetochores independently of BuGZ. The experiments done here have laid the groundwork for additional future experiments that may be done to further dissect the precise nature of these interactions. Future experiments will be important for elucidating the manner by which, and the dependency of BuGZ on the facilitation for an exchange of Bub1 and/or BubR1 binding to Bub3 at kinetochores.

Analyzing the recruitment sufficiency of SAC proteins ectopically has its advantages, but it also has several limitations. This method particularly highlights the direct requirement for a single protein upon the recruitment of others, isolating it from its normal environment, but still examining interactions specifically seen in the context of cells, avoiding potential confounds that may stem from *in vitro* analysis. On the other hand, however, ectopic analysis of these proteins presents potential problems as well. Particularly, it is extremely difficult to control for the amount of LacI-fusion protein that is

expressed in the cells, how much of that actually localizes to the LacO array, and furthermore how much of that protein is able to interact with the same efficacy of native state protein. For example, when targeted to the LacO array, the protein of interest may fold in a way such that it may not be able to interact with normal binding partners as some residues may be positioned abnormally. Different LacI-fusion proteins may localize and fold in varying manners, making it difficult to compare between two constructs. The large concentration of protein that is targeted to the LacO array is much greater than that seen endogenously at kinetochores, thus interactions seen may be a result of this abnormally high local concentration. It is also important to note that U2OS cells inherently exhibit chromosome instability (CIN), and thus each cell may exhibit peculiar phenotypic differences, as well as maintaining a various amount or location of the LacO inserts.

Highlighting the importance of further characterization of the exact mechanism and dynamics of Bub1, BubR1, and BuGZ binding to Bub3, targeting GLEBS domain interactions with Bub3 has been shown to be a very promising putative therapeutic target for the selective treatment of Glioblastoma Multiforme tumors (Ding et al., 2013; Toledo et al., 2014; Herman et al., 2015). Dissection of the nature and requirement of Bub3 interactions with these important SAC proteins will aid in the drug development process, allowing for extremely precise targeting of interactions that are crucial to the viability of these cancer cells. It is also important for understanding why these specific cancers have an increased requirement for this interaction as opposed to other cancers which are not sensitive to GLEBS inhibition.

Spindle assembly checkpoint timing and regulation is an extremely precise and dynamic process in cells that requires multiple levels of control for proper function. Many proteins of the SAC also moonlight as mediators of chromosome alignment, a related, but distinct functionality from the SAC functions (Akera, & Watanabe, 2016). There exists an important dynamic tug-of-war between phosphorylation and de-phosphorylation of many different kinetochore and SAC proteins by the

multiple phosphatases and kinases that are involved (Manic et al., 2017; Funabiki and Wynne, 2013). This leads to extremely precise regulation of SAC activity depending on the level of various substrate phosphorylation, allowing the SAC to be turned on robustly in the presence of unattached kinetochores, and quickly turned off once all proper kinetochore-microtubule attachments are formed. This precise regulation ensures that dividing eukaryotic cells equally distribute genetic material through many generations with a very low incidence of errors. Characterization and understanding of the multiple levels of regulation that are crucial to SAC function and chromosome alignment is important for understanding how and why this process sometimes goes awry. The consequences of defective SAC signaling and improper chromosome alignment leads to aneuploidy, a characteristic common to most all human cancers (Rajagopalan and Lengauer, 2004). Interestingly, many cancers also show a dependency on increased activity of SAC proteins (Ding et al., 2013; Yuan, 2006), highlighting the importance, and even necessity for the regulation of proper chromosome segregation even in cells which have previously foregone this to ensure that there is not further abrogation of the division of genetic material to a fatal degree. The regulation of these processes has been studied for decades, and there is still a vast deal that is unclear, requiring further characterization.

Methods & Materials

Cell culture and transfections

U2OS-LacO cell line (gift from S. Janicki) were grown in McCoy's 5a® Growth Media (Gibco®) supplemented with 10% Fetal Bovine Serum (FBS), 1% Penicillin/Streptomycin, and 0.1mg/mL Hygromycin. The LacI_GFP_Bub1, LacI_GFP_BubR1, LacI_GFP_Bub3, and LacI_GFP_BuGZ plasmids were generated through PCR amplification of wild type Bub1, BubR1, Bub3, and BuGZ fragments, and cloned into the LacI-GFP parent vector through isothermal assembly cloning. Cells were incubated at 37°C in 5% CO_2. Cells were detached from plates using 0.5% Trypsin (HyClone™), and seeded into a 6-well dish onto glass coverslips at a count of 150,000 cells per well such that they would be ~50% confluence the next day for transfection. Transfections were performed using TransIT x2 Lipid Transfection Reagent (MirusBio), according to manufacturer's instructions with 250 ng of the respective LacI fusion protein plasmid, 250 µL of OptiMEM, and 5 µL (per well) of TransIT x2 Lipid Transfection Reagent (MirusBio). Samples were then incubated for 20 minutes at room temperature, then subsequently added dropwise into the 6-well dish containing 1.75 mL of OptiMEM (Gibco®) supplemented with 10% FBS. In order to synchronize cells, and increase the percentage of cells in early mitosis, an RO-3306 washout method was utilized. 24 hours post-transfection, transfection media was replaced with 9 µM RO-3306 (Sigma-Aldrich) in Mckoy's 5A® media (Gibco®), in order arrest the population in G2, then incubated for 20 hours at 37°C. After 20 hours, the RO-3306 drug containing media was replaced with 1x PBS (140 mM NaCl, 2.5 mM KCl, 1.6 mM KH_2PO_4, 15 mM Na_2HPO_4, pH 7.0), for 5 minutes at 37°C to wash away drug from the cells, and subsequently replaced with normal U2OS growth media and incubated for ~20-30 minutes at 37°C until cells started to round up and enter mitosis (as visually determined by phase contrast microscopy).

Immunofluorescence

Post-transfection, coverslips were rinsed with PHEM (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 8 mM $MgSO_4$, pH 7.0). Cells were then lysed with PHEM+0.5% Triton X-100 for 5 min. Following lysis, cells were fixed in 4% paraformaldehyde in PHEM for 20 min. The cells were then rinsed 3 x 5 min in PHEM + 0.1% TritonX-100 and blocked for 1 hour with 10% boiled donkey serum (BDS) in PHEM. Primary antibodies were diluted in 5% BDS in PHEM as follows: mouse anti-Bub1 (Abcam ab54893) 1:500, mouse anti-Bub3 (Fisher BDB611731) 1:300, rabbit anti-BuGZ (Genetex GTX 116214) 1:500, rabbit anti-Mad1 (Genetex GTX109519) 1:500. Cells were incubated with primary antibodies overnight at 4°C then rinsed 3 x 5 min in PHEM + 0.1% TritonX-100. Cells were then incubated with secondary antibodies conjugated to either Donkey anti-Mouse Alexa 647 ® (Jackson ImmunoResearch Laboratories, 715-605-150) or Donkey anti-Rabbit Alexa 647 ® (Jackson ImmunoReseach Laboratories 711-606-152) diluted 1:750 in 5% BDS in PHEM for 45 minutes at room temperature. Cells were rinsed 3 x 5 min in PHEM + 0.1% Triton X-100 then stained with 2 ng/mL 4', 6-diamidino-2-phenylindole (DAPI) diluted in PHEM (1 µg/mL) for one minute. Cells were then mounted onto slides with ~10 mL of an anti-fade mounting media (20 mM Tris, pH 8.0, 0.5% *N*-propyl gallate, and 90% glycerol).

Image acquisition and analysis

Cell images were acquired using a DeltaVision Personal DV (Applied Precision) imaging system equipped with a CoolSNAP HQ2 (Photometrics/Roper Scientific) camera, a 60X/1.42 NA PlanApochromat oil immersion objective lens (Olympus) and SoftWorx acquisition software (Applied Precision). All images for immunofluorescence experiments were collected as z-stacks at 200nm intervals. The phase of the cell was determined visually by DAPI staining, with onset of mitosis being defined by clear nuclear envelope breakdown. The locus of the LacO array was determined by LacI-GFP position, which created a very clear high fluorescence intensity 'spot' in cells. Fluorescence intensities were determined using ImageJ quantification software. Images were converted to maximal intensity projections, then the area

of brightest intensity within the LacI-GFP spot was chosen, and local fluorescence intensities were quantified. Background values were corrected for by subtracting an average background intensity value for each individual image. SAC proteins were stained with Alexa647 conjugated secondary antibodies. The same pixel areas as measured in the GFP channel were measured in the 647 channel to determine co-localization of other SAC proteins to the LacO spot. These intensity values were then compared to that of a set of control experiments that were carried out to ensure that there is not bleed-through of fluorescence or non-specific binding of antibodies. In these control experiments, cells were transfected with the parent vector: LacI-GFP. When expressed in cells, this construct strongly localizes to the LacO spot. These control experiments were carried out on the same day, using the same antibody conditions, and immunofluorescence staining protocols as their respective test conditions. The fluorescent intensities at the LacO spot were quantified for each respective control condition and then compared to the test conditions. If the intensity at the LacO spot in the 647 channel was greater in the test condition than the minimum threshold determined from a histogram of the control conditions' intensities, it was deemed positive scoring for co-localization. Quantification was carried out separately for mitotic cells and interphase cells. Two separate experiments were conducted for all conditions (except for LacI-GFP-BuGZ stained for Bub1 had only one experiment). Each experiment had an n of ~40 cells, approximately 20 mitotic and 20 interphase cells for each condition.

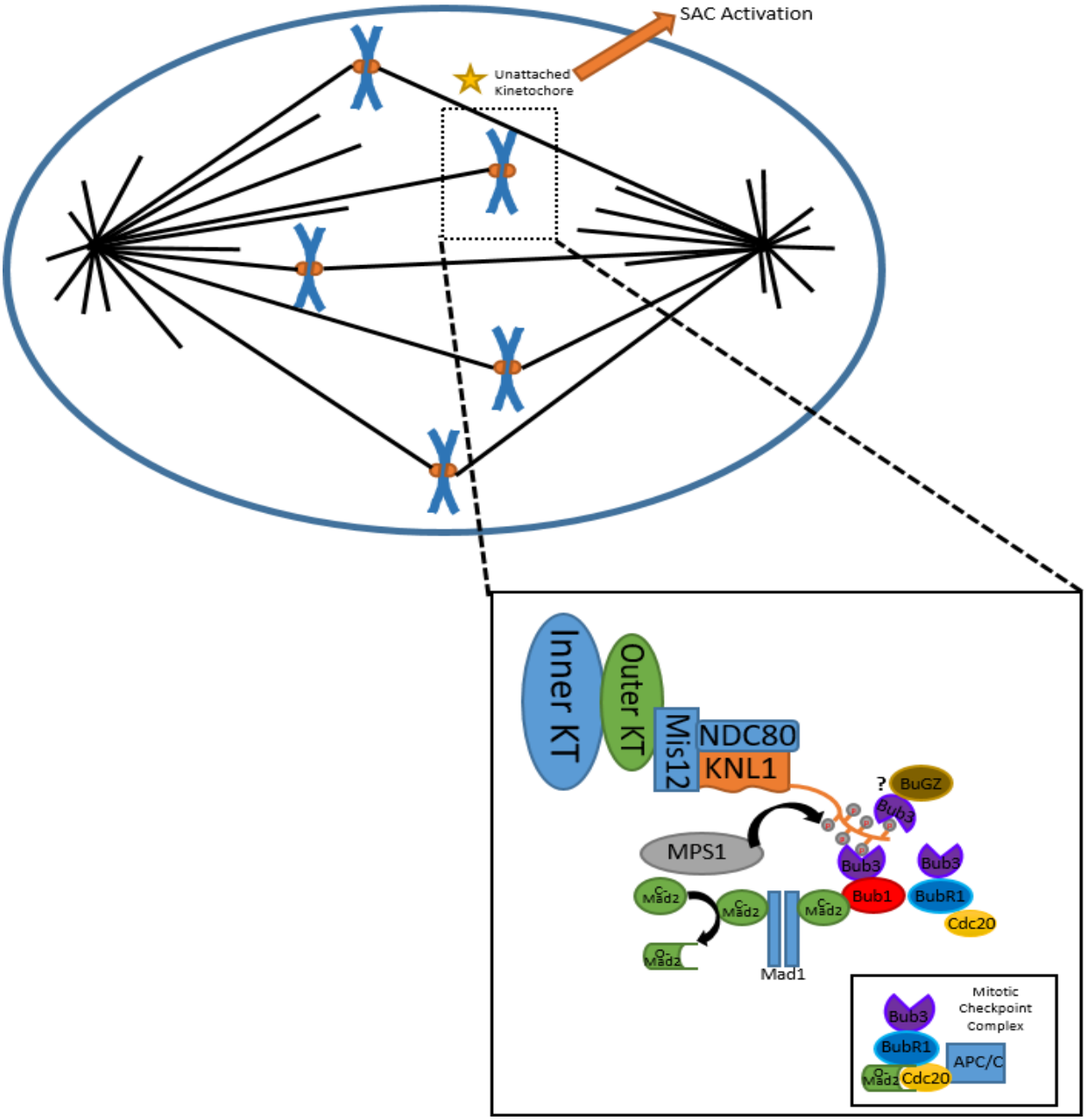


Figure 1. Schematic representation of SAC signal originating from an unattached kinetochore. Mps1 Kinase is recruited to the KT and phosphorylates MELT motifs on KNL1. Phosphorylated MELT motifs then recruit Bub3 in complex with Bub1 and possibly BuGZ. BubR1 bound to Bub3 is recruited by Bub1-Bub3. Bub1-Bub3 also recruits Closed-Mad2 as a heterotetrameric complex with Mad1. Mad1 bound to C-Mad2 serves as a template for the conversion of C-Mad2 to O-Mad2 which is then associated into the MCC along with BubR1-Bub3, and Cdc20 to form the inhibitory signal that halts anaphase until correct attachments are formed.

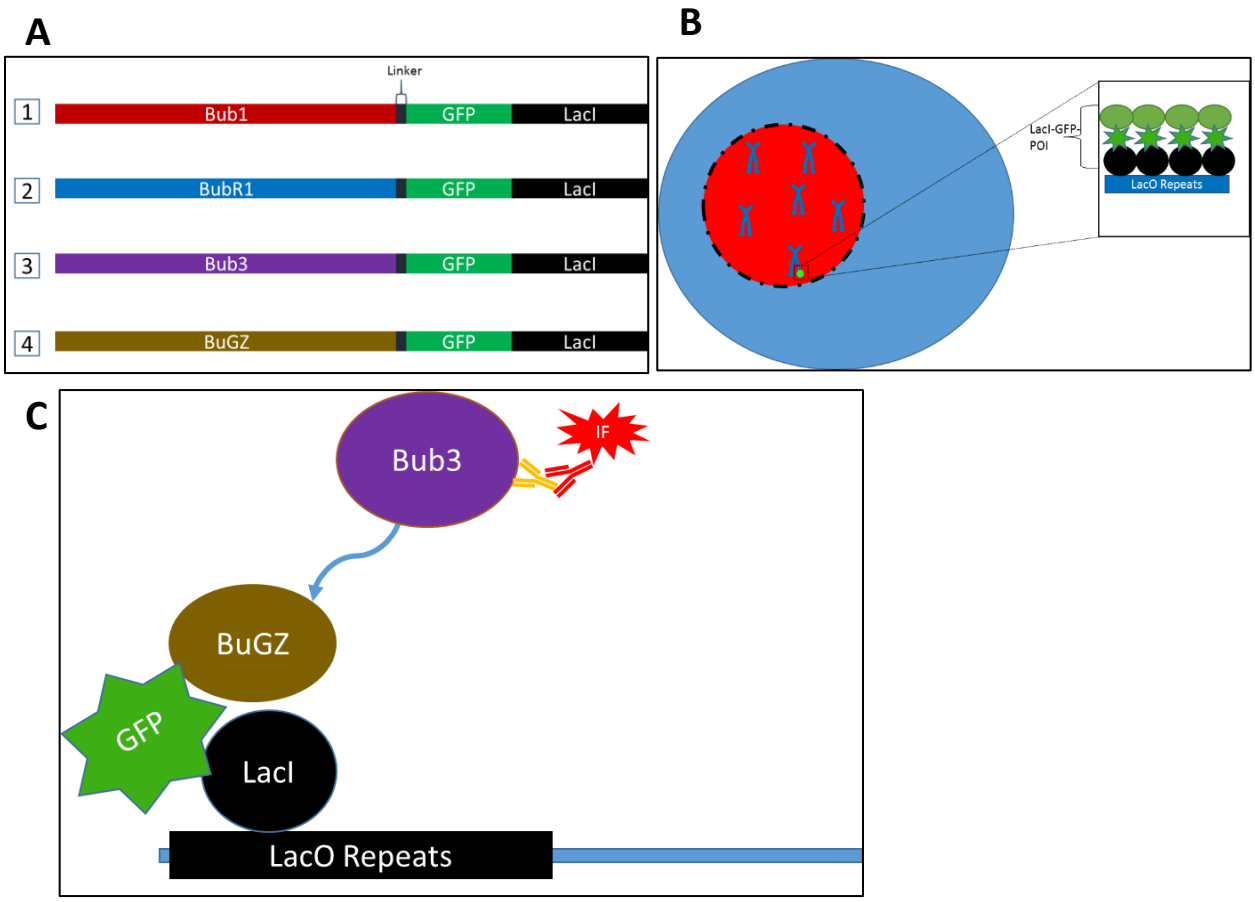


Figure 2. Cartoon representations of the methodology used in these experiments

(A.) Diagram of the four LacI-GFP-SAC plasmids that were transfected into U2OS cells. Gene of interest (Bub1, BubR1, Bub3 or BuGZ) followed by a short linker, GFP, and LacI Repressor gene. Plasmid containing LacI and GFP but no SAC protein were used as controls to ensure antibodies did not bind non-specifically to the LacI-LacO array.

(B.) Representation of how this system works in cells. LacI-GFP-ProteinofInterest is transfected into U2OS cells, and strongly localizes to the LacO repeats in the cells (represented by a green circle ectopically on a chromosome arm).

(C.) Representative example of how this system is used here to detect co-recruitment. First a protein of interest (here BuGZ), is targeted to the LacO repeats with the LacI repressor. Then, said protein of interest may also recruit to the LacI-LacO array other proteins that interact in cells (here Bub3), and may be detected using immunofluorescence.

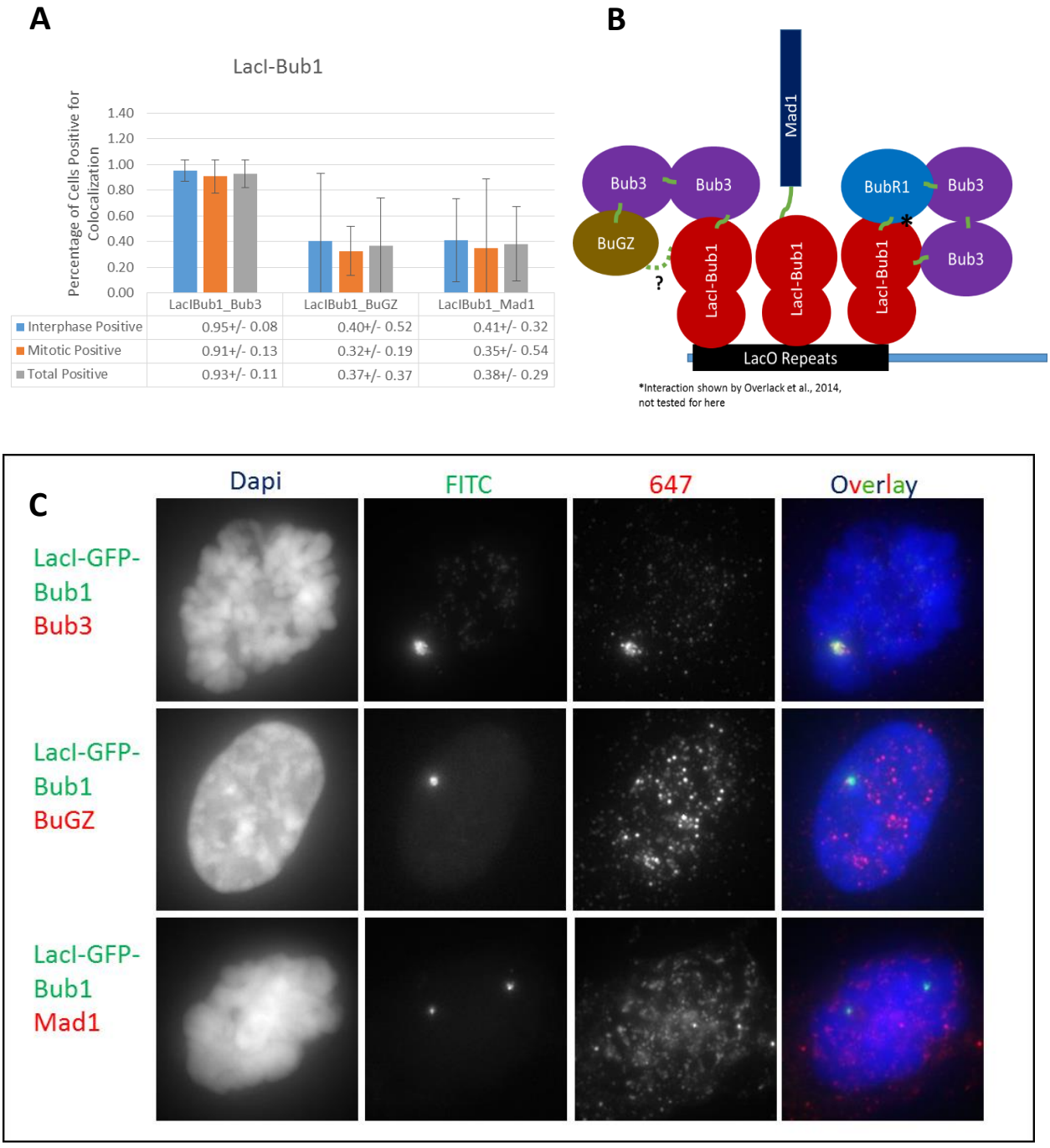


Figure 3. LacI-GFP-Bub1 is sufficient to recruit Bub3, BuGZ, and Mad1

(Figure legend continued on next page)

Figure 3. (Continued)

(A.) Quantification of LacI-GFP-Bub1 co-stained with Bub3, BuGZ, or Mad1. Graphs show percentage of cells positive for co-localization at the LacO array. Cells were treated with RO-3306 to synchronize then washed out to enrich mitotic population. Two separate experiments for each antibody with n = ~40 per experiment (~20 Mitotic, ~20 Interphase cells per experiment) were conducted, error bars represent standard deviation between the two experiments. Datum compared to control condition for each antibody (Figure 7 A). LacI-Bub1 was able to recruit to the LacO array: Bub3, BuGZ, and Mad1 to an extent significant compared to the control.

(B.) Cartoon representation of the interactions seen/expected at the LacO array with LacI-Bub1. It is likely that Bub1 directly interacts with Mad1. Bub3 is likely also recruited in a direct manner, and forms a ternary complex to recruit BuGZ, unknown if BuGZ directly interacts with Bub1. As shown by Overlack et al., 2014, BubR1 forms a ternary complex with LacI-Bub1/Bub3 binding BubR1/Bub3 in complex.

(C.) Representative immunofluorescence images for LacI-GFP-Bub1 (FITC Channel) co-stained with Bub3, BuGZ, or Mad1 antibodies (647 Channel), and chromosomes (DAPI). Some co-localization seen with each Bub3, BuGZ, and Mad1.

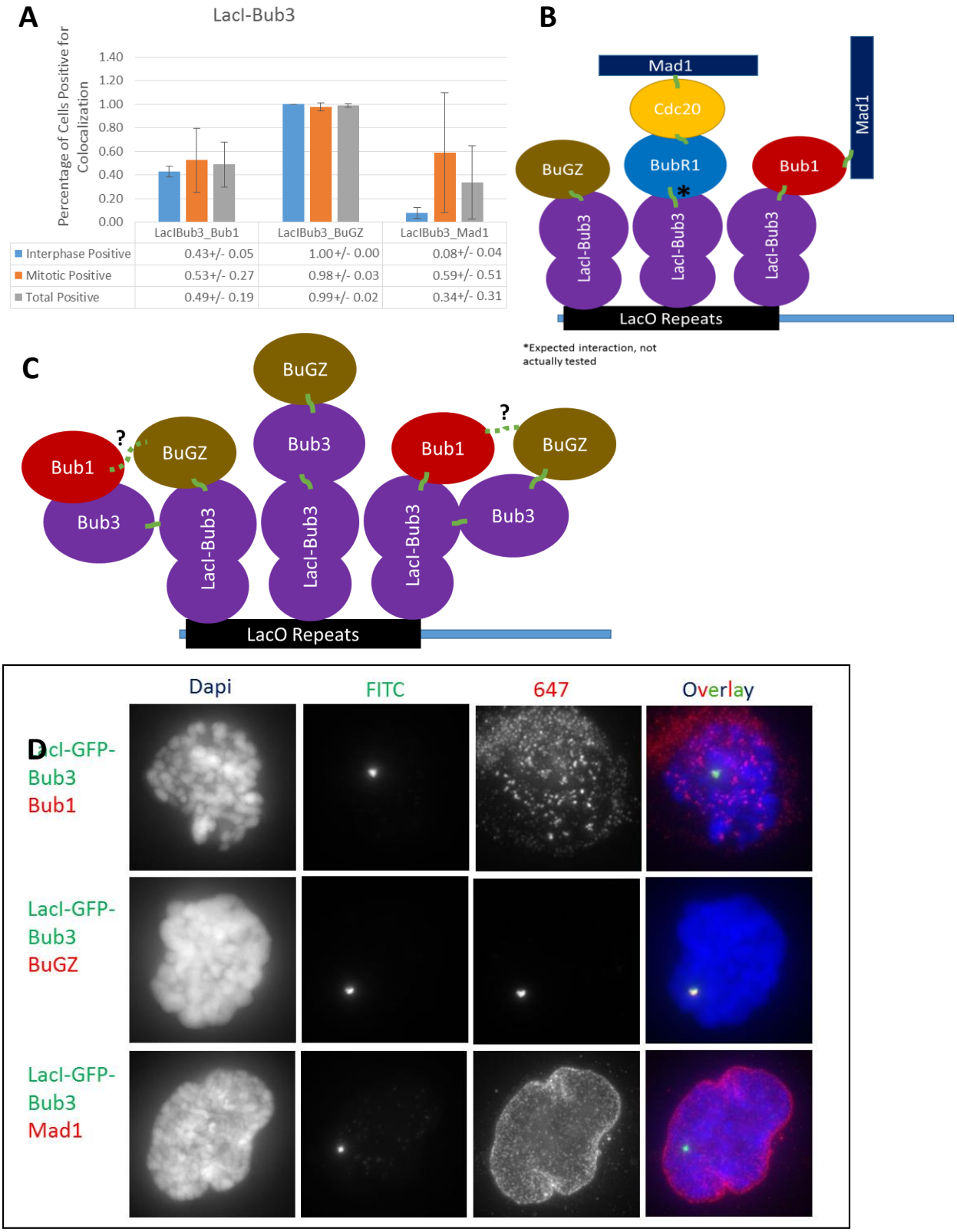


Figure 4. LacI-GFP-Bub3 is sufficient to recruit Bub1, BubR1, BuGZ, and Mad1

(Figure legend continued on next page)

Figure 4. (Continued)

(A.) Quantification of LacI-GFP-Bub3 co-stained with antibodies specific to Bub1, BuGZ, or Mad1. Graphs show percentage of cells positive for co-localization at the LacO array. Cells were treated with RO-3306 to synchronize then washed out to enrich mitotic population. Two separate experiments for each antibody with n = ~40 per experiment (~20 Mitotic, ~20 Interphase cells per experiment) were conducted, error bars represent standard deviation between the two experiments. Datum compared to control condition for each antibody (Figure 7 A). LacI-Bub3 was able to recruit to the LacO array: Each Bub1, BuGZ, and Mad1 to a significant extent.

(B.) Cartoon representation of the interactions seen/expected at the LacO array with LacI-Bub3. It is likely that BuGZ and Bub1 are bound directly through a GLEBS domain interaction. Mad1 is likely recruited to LacI-Bub3 in an indirect manner mediated either through Bub1 binding Mad1 directly or BubR1 binding CDC20 and then Mad1. LacI-Bub3 is expected to recruit BubR1, but couldn't be tested for here.

(C.) Model of some of the likely sub-complexes formed at the LacO array when LacI-Bub3 is present. It is likely that LacI-Bub3 directly binds BuGZ, which in turn may recruit a dimer of Bub3/Bub1 (left). This complex could also be reciprocally formed with Bub1 directly binding LacI-Bub3, recruiting a dimer of Bub3/BuGZ to form a ternary complex (right). It is also likely that LacI-Bub3 may directly bind a dimer of Bub3/BuGZ. BubR1 is also likely to be forming similar complexes (not tested for here), and the total population of these four proteins is likely in some dynamic equilibrium of all of these complexes.

(D.) Representative immunofluorescence images for LacI-GFP-Bub3 (FITC channel) co-stained with Bub1, BuGZ, or Mad1 antibodies (647 Channel), and chromosomes (DAPI). Co-Localization seen with Bub1, BuGZ, and Mad1

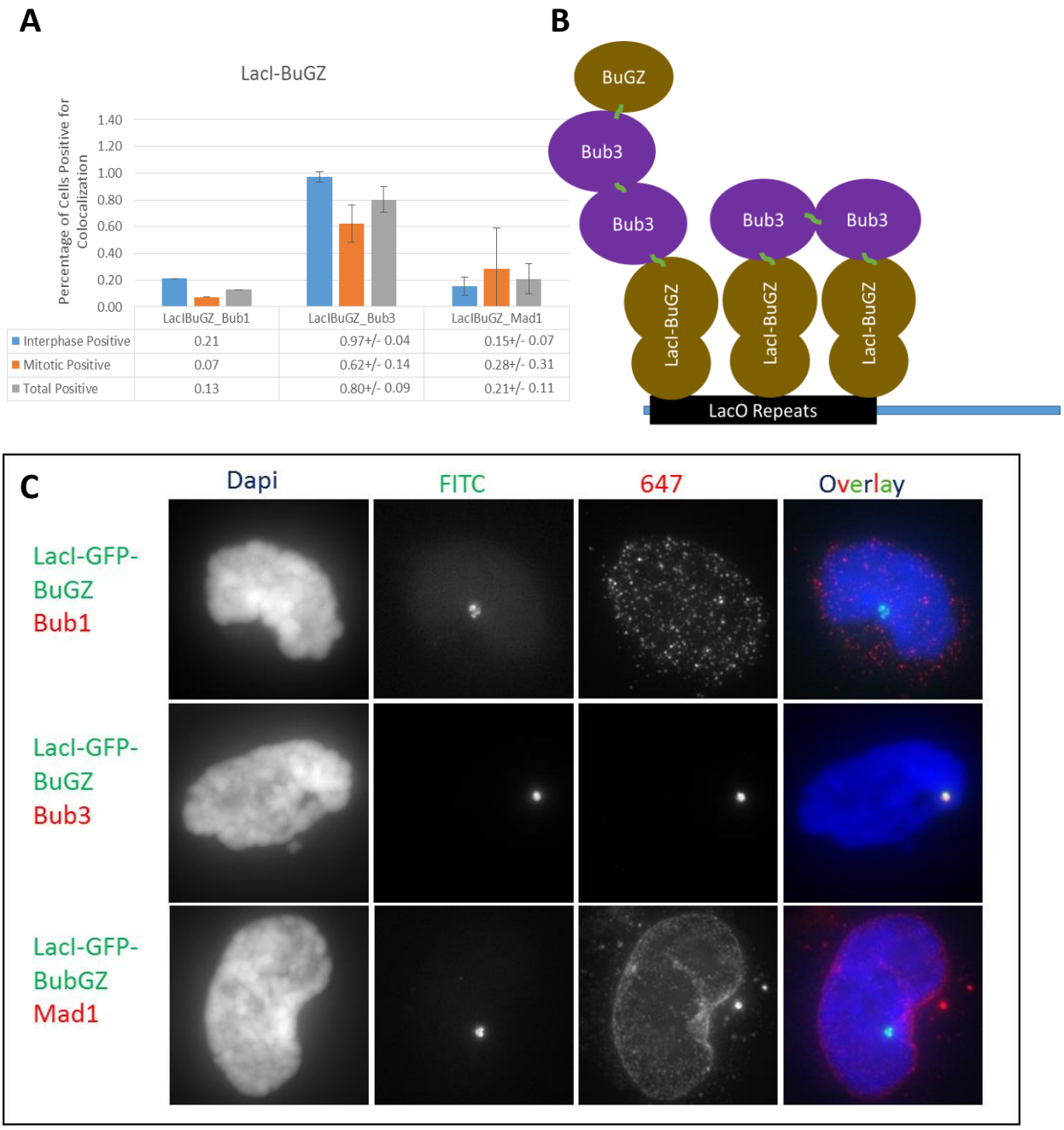


Figure 5. LacI-GFP-BuGZ is sufficient to recruit Bub3 only

(Figure legend continued on next page)

Figure 5. (Continued)

(A.) Quantification of LacI-GFP-BuGZ co-stained with Bub1, Bub3, or Mad1. Graphs show percentage of cells positive for co-localization at the LacO array. Cells were treated with RO-3306 to synchronize then washed out to enrich mitotic population. Two separate experiments for each antibody with n = ~40 per experiment (~20 Mitotic, ~20 Interphase cells per experiment) were conducted (Only one experiment conducted for LacI-GFP-BuGZ_Bub1), error bars represent standard deviation between the two experiments. Datum compared to control condition for each antibody (Figure 7 A). LacI-BuGZ was able to recruit to the LacO array: Bub3 only at any appreciable amount.

(B.) Cartoon representation of the interactions seen/expected at the LacO array with LacI-BuGZ. LacI-BuGZ can recruit Bub3 robustly, which may in turn recruit a complex of Bub3 bound to a molecule of soluble endogenous BuGZ to the array, or Bub3 could form a ternary complex with LacI-BuGZ and a second molecule of Bub3 binding another molecule of LacI-BuGZ on the array.

(C.) Representative immunofluorescence images for LacI-GFP-BuGZ (FITC Channel) co-stained with Bub1, Bub3, or Mad1 antibodies (647 Channel), and chromosomes (DAPI). Co-Localization only seen with Bub3.

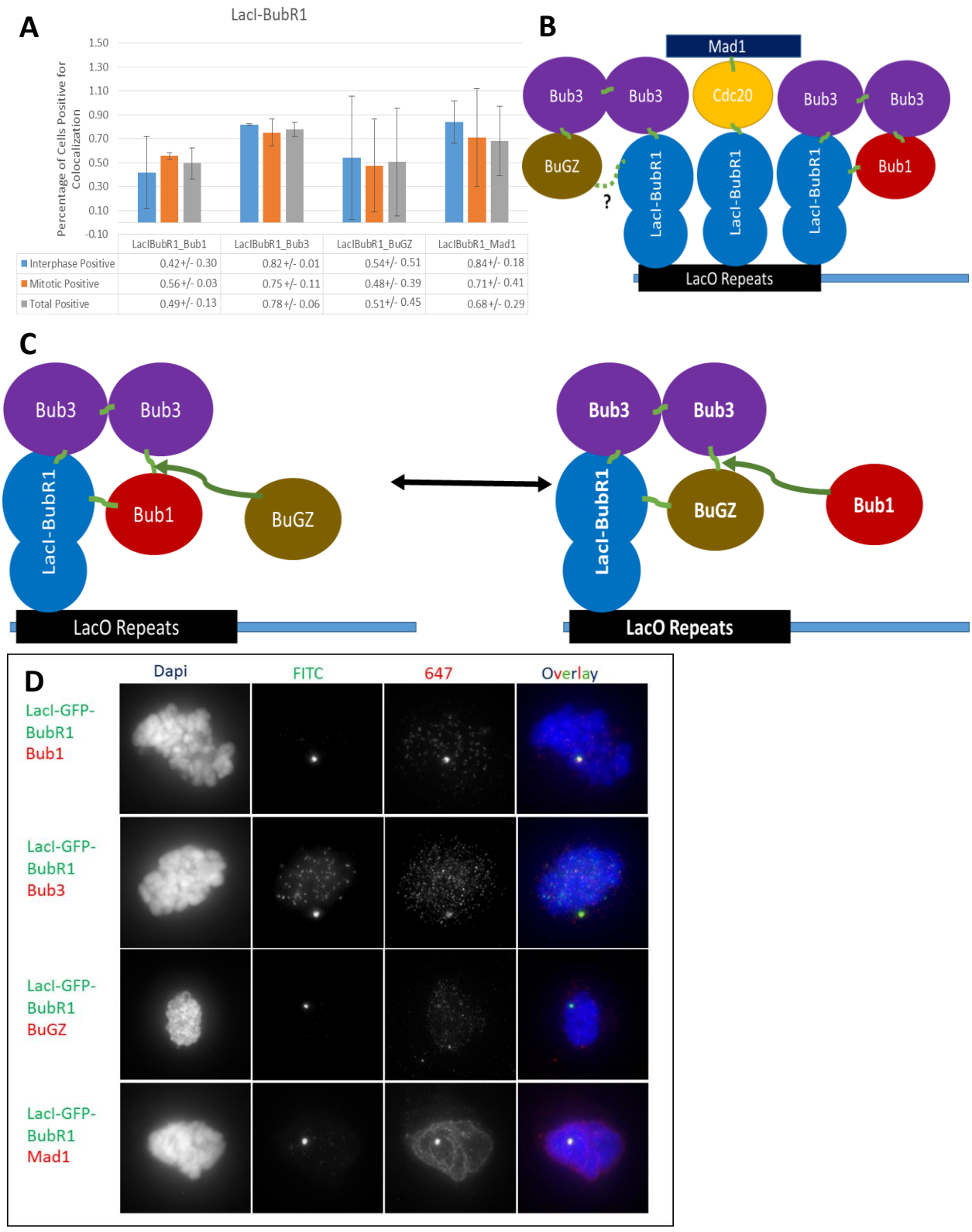


Figure 6. LacI-GFP-BubR1 is sufficient to recruit Bub1, Bub3, BuGZ, and Mad1

(Figure legend continued on next page)

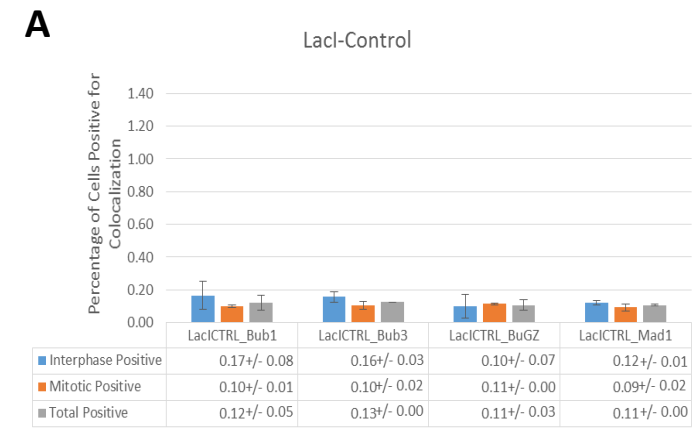
Figure 6. (Continued)

(A.) Quantification of LacI-GFP-BubR1 co-stained with antibodies specific to Bub1, Bub3, BuGZ, or Mad1. Graphs show percentage of cells positive for co-localization at the LacO array. Cells were treated with RO-3306 to synchronize then washed out to enrich mitotic population. Two separate experiments for each antibody with n = ~40 per experiment (~20 Mitotic, ~20 Interphase cells per experiment) were conducted, error bars represent standard deviation between the two experiments. Datum compared to control condition for each antibody (Figure 7 A). LacI-BubR1 was able to recruit to the LacO array: Bub1, Bub3, BuGZ, and Mad1 to a significant degree.

(B.) Cartoon representation of the interactions seen/expected at the LacO array with LacI-BubR1. Likely forming a ternary complex similar to that seen with the other conditions where LacI-BubR1 binds Bub3 which in turn recruits a Bub3/Bub1 (right) or a Bub3/BuGZ (left) dimer. Mad1 recruitment is likely mediated through Cdc20 binding (Cdc20 not stained for here)

(C.) Model of dynamic exchange of ternary complex formed between LacIBubR1-Bub3 and Bub3/Bub1 and Bub3/BuGZ. It is likely that some population of Bub1 and BuGZ are in competition for binding Bub3 and undergo a dynamic exchange where Bub1 is replaced by BuGZ, or BuGZ is replaced by Bub1.

(D.) Representative immunofluorescence images for LacI-GFP-BubR1 (FITC channel) co-stained with Bub1, Bub3, BuGZ, or Mad1 antibodies (647 Channel), and chromosomes (DAPI). Co-Localization seen with Bub1, Bub3, BuGZ, and Mad1



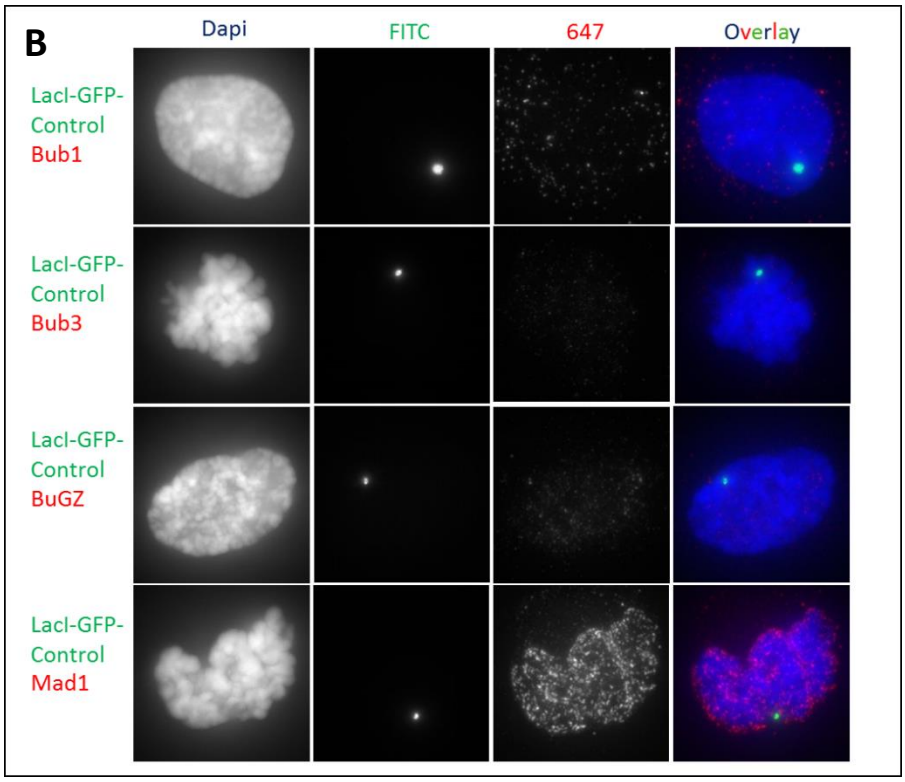


Figure 7. LacI-GFP is unable to recruit Bub1, Bub3, BuGZ, or Mad1 (Control)

(A.) Quantification of LacI-GFP-Control (No SAC fusion protein) co-stained with antibodies specific to Bub1, Bub3, BuGZ, or Mad1. Graphs show percentage of cells positive for co-localization at the LacO array. Cells were treated with RO-3306 to synchronize then washed out to enrich mitotic population. Two separate experiments for each antibody with n = ~40 per experiment (~20 Mitotic, ~20 Interphase cells per experiment) were conducted, error bars represent standard deviation between the two experiments. Controls used to compare test conditions ensuring that antibodies do not nonspecifically localize to the array as well as to provide a threshold for positive co-localization.

(B.) Representative immunofluorescence images for LacI-GFP-Control (FITC channel) co-stained with Bub1, Bub3, BuGZ, or Mad1 antibodies (647 Channel), and Chromosomes (Dapi).

References

Akera, T., & Watanabe, Y. (2016). The spindle assembly checkpoint promotes chromosome bi-orientation: A novel Mad1 role in chromosome alignment. *Cell Cycle*, *15*(4), 493-497. http://dx.doi.org/10.1080/15384101.2015.1128596

Alfieri, C., Chang, L., Zhang, Z., Yang, J., Maslen, S., Skehel, M., & Barford, D. (2016). Molecular basis of APC/C regulation by the spindle assembly checkpoint. *Nature*, *536*(7617), 431-436. http://dx.doi.org/10.1038/nature19083

Biggins, S., & Murray, A. (2001). The budding yeast protein kinase Ipl1/Aurora allows the absence of tension to activate the spindle checkpoint. *Genes & Development*, *15*(23), 3118-3129. http://dx.doi.org/10.1101/gad.934801

Caldas, G., Lynch, T., Anderson, R., Afreen, S., Varma, D., & DeLuca, J. (2015). The RZZ complex requires the N-terminus of KNL1 to mediate optimal Mad1 kinetochore localization in human cells. *Open Biology*, *5*(11), 150160. http://dx.doi.org/10.1098/rsob.150160

Cheeseman, I., & Desai, A. (2008). Molecular architecture of the kinetochore–microtubule interface. *Nature Reviews Molecular Cell Biology*, *9*(1), 33-46. http://dx.doi.org/10.1038/nrm2310

De Antoni, A., Pearson, C., Cimini, D., Canman, J., Sala, V., & Nezi, L. et al. (2005). The Mad1/Mad2 Complex as a Template for Mad2 Activation in the Spindle Assembly Checkpoint. *Current Biology*, *15*(3), 214-225. http://dx.doi.org/10.1016/j.cub.2005.01.038

DeLuca, K., Lens, S., & DeLuca, J. (2011). Temporal changes in Hec1 phosphorylation control kinetochore-microtubule attachment stability during mitosis. *Journal Of Cell Science*, *124*(4), 622-634. http://dx.doi.org/10.1242/jcs.072629

Ding, Y., Hubert, C., Herman, J., Corrin, P., Toledo, C., & Skutt-Kakaria, K. et al. (2012). Cancer-Specific Requirement for BUB1B/BUBR1 in Human Brain Tumor Isolates and Genetically Transformed Cells. *Cancer Discovery*, *3*(2), 198-211. http://dx.doi.org/10.1158/2159-8290.cd-12-0353

Etemad, B., Kuijt, T., & Kops, G. (2015). Kinetochore–microtubule attachment is sufficient to satisfy the human spindle assembly checkpoint. *Nature Communications*, *6*, 8987. http://dx.doi.org/10.1038/ncomms9987

Faesen, A., Thanasoula, M., Maffini, S., Breit, C., Müller, F., & van Gerwen, S. et al. (2017). Basis of catalytic assembly of the mitotic checkpoint complex. *Nature*, *542*(7642), 498-502. http://dx.doi.org/10.1038/nature21384

Fang, G., Yu, H., & Kirschner, M. (1998). *Direct Binding of CDC20 Protein Family Members Activates the Anaphase-Promoting Complex in Mitosis and G1*.

Funabiki, H., & Wynne, D. (2013). Making an effective switch at the kinetochore by phosphorylation and dephosphorylation. *Chromosoma*, *122*(3), 135-158. http://dx.doi.org/10.1007/s00412-013-0401-5

Gassmann, R., Carvalho, A., Henzing, A., Ruchaud, S., Hudson, D., & Honda, R. et al. (2004). Borealin a novel chromosomal passenger required for stability of the bipolar mitotic spindle. *The Journal Of Cell Biology*, *166*(2), 179-191. http://dx.doi.org/10.1083/jcb.200404001

Gregan, J., Polakova, S., Zhang, L., Tolić-Nørrelykke, I., & Cimini, D. (2011). Merotelic kinetochore attachment: causes and effects. *Trends In Cell Biology*, *21*(6), 374-381. http://dx.doi.org/10.1016/j.tcb.2011.01.003

Gurden, M., Anderhub, S., Faisal, A., & Linardopoulos, S. (2014). Aurora B prevents premature removal of spindle assembly checkpoint proteins from the kinetochore: A key role for Aurora B in mitosis. *Oncotarget*. http://dx.doi.org/10.18632/oncotarget.10657

Han, J., Holland, A., Fachinetti, D., Kulukian, A., Cetin, B., & Cleveland, D. (2013). Catalytic Assembly of the Mitotic Checkpoint Inhibitor BubR1-Cdc20 by a Mad2-Induced Functional Switch in Cdc20. *Molecular Cell*, *51*(1), 92-104. http://dx.doi.org/10.1016/j.molcel.2013.05.019

Hauf, S., Cole, R., LaTerra, S., Zimmer, C., Schnapp, G., & Walter, R. et al. (2003). The small molecule Hesperadin reveals a role for Aurora B in correcting kinetochore–microtubule attachment and in maintaining the spindle assembly checkpoint. *The Journal Of Cell Biology*, *161*(2), 281-294. http://dx.doi.org/10.1083/jcb.200208092

Heasley, L., Markus, S., & DeLuca, J. (2017). “Wait anaphase” signals are not confined to the mitotic spindle. *Molecular Biology Of The Cell*, *28*(9), 1186-1194. http://dx.doi.org/10.1091/mbc.e17-01-0036

Herman, J., Toledo, C., Olson, J., DeLuca, J., & Paddison, P. (2014). Molecular Pathways: Regulation and Targeting of Kinetochore-Microtubule Attachment in Cancer. *Clinical Cancer Research*, *21*(2), 233-239. http://dx.doi.org/10.1158/1078-0432.ccr-13-0645

Herzog, F., Primorac, I., Dube, P., Lenart, P., Sander, B., & Mechtler, K. et al. (2009). Structure of the Anaphase-Promoting Complex/Cyclosome Interacting with a Mitotic Checkpoint Complex. *Science*, *323*(5920), 1477-1481. http://dx.doi.org/10.1126/science.1163300

Inoue, S., & Salmon, E. (1995). Force Generation by Microtubule Assembly/Disassembly in Mitosis and Related Movements. *Molecular Biology Of The Cell*, *6*(12), 1619-1640. http://dx.doi.org/10.1091/mbc.6.12.1619

Izawa, D., & Pines, J. (2014). The mitotic checkpoint complex binds a second CDC20 to inhibit active APC/C. *Nature*, *517*(7536), 631-634. http://dx.doi.org/10.1038/nature13911

Janicki, S., Tsukamoto, T., Salghetti, S., Tansey, W., Sachidanandam, R., & Prasanth, K. et al. (2004). From Silencing to Gene Expression. *Cell*, *116*(5), 683-698. http://dx.doi.org/10.1016/s0092-8674(04)00171-0

Ji, Z., Gao, H., Jia, L., Li, B., & Yu, H. (2017). A sequential multi-target Mps1 phosphorylation cascade promotes spindle checkpoint signaling. *Elife*, *6*. http://dx.doi.org/10.7554/elife.22513

Kraft, C., Vodermaier, H., Maurer-Stroh, S., Eisenhaber, F., & Peters, J. (2005). The WD40 Propeller Domain of Cdh1 Functions as a Destruction Box Receptor for APC/C Substrates. *Molecular Cell*, *18*(5), 543-553. http://dx.doi.org/10.1016/j.molcel.2005.04.023

Lara-Gonzalez, P., Westhorpe, F., & Taylor, S. (2012). The Spindle Assembly Checkpoint. *Current Biology*, *22*(22), R966-R980. http://dx.doi.org/10.1016/j.cub.2012.10.006

Larsen, N., Al-Bassam, J., Wei, R., & Harrison, S. (2007). Structural analysis of Bub3 interactions in the mitotic spindle checkpoint. *Proceedings Of The National Academy Of Sciences*, *104*(4), 1201-1206. http://dx.doi.org/10.1073/pnas.0610358104

Li, M., Fang, X., Wei, Z., York, J., & Zhang, P. (2009). Loss of spindle assembly checkpoint–mediated inhibition of Cdc20 promotes tumorigenesis in mice. *The Journal Of Cell Biology*, *185*(6), 983-994. http://dx.doi.org/10.1083/jcb.200904020

Manic, G., Corradi, F., Sistigu, A., Siteni, S., & Vitale, I. (2017). Molecular Regulation of the Spindle Assembly Checkpoint by Kinases and Phosphatases. *International Review Of Cell And Molecular Biology*, 105-161. http://dx.doi.org/10.1016/bs.ircmb.2016.08.004

Marques, S., Fonseca, J., Silva, P., & Bousbaa, H. (2015). Targeting the Spindle Assembly Checkpoint for Breast Cancer Treatment. *Current Cancer Drug Targets*, *15*(4), 272-281. http://dx.doi.org/10.2174/1568009615666150302130010

Mora-Santos, M., Hervas-Aguilar, A., Sewart, K., Lancaster, T., Meadows, J., & Millar, J. (2016). Bub3-Bub1 Binding to Spc7/KNL1 Toggles the Spindle Checkpoint Switch by Licensing the Interaction of Bub1 with Mad1-Mad2. *Current Biology*, *26*(19), 2642-2650. http://dx.doi.org/10.1016/j.cub.2016.07.040

Musacchio, A., & Desai, A. (2017). A Molecular View of Kinetochore Assembly and Function. *Biology*, *6*(1), 5. http://dx.doi.org/10.3390/biology6010005

Musacchio, A., & Salmon, E. (2007). The spindle-assembly checkpoint in space and time. *Nature Reviews Molecular Cell Biology*, *8*(5), 379-393. http://dx.doi.org/10.1038/nrm2163

Nezi, L., & Musacchio, A. (2009). Sister chromatid tension and the spindle assembly checkpoint. *Current Opinion In Cell Biology*, *21*(6), 785-795. http://dx.doi.org/10.1016/j.ceb.2009.09.007

Overlack, K., Primorac, I., Vleugel, M., Krenn, V., Maffini, S., & Hoffmann, I. et al. (2015). A molecular basis for the differential roles of Bub1 and BubR1 in the spindle assembly checkpoint. *Elife*, *4*. http://dx.doi.org/10.7554/elife.05269

Peters, J. (2002). The Anaphase-Promoting Complex. *Molecular Cell*, *9*(5), 931-943. http://dx.doi.org/10.1016/s1097-2765(02)00540-3

Pinsky, B., & Biggins, S. (2005). The spindle checkpoint: tension versus attachment. *Trends In Cell Biology*, *15*(9), 486-493. http://dx.doi.org/10.1016/j.tcb.2005.07.005

Primorac, I., Weir, J., Chiroli, E., Gross, F., Hoffmann, I., & van Gerwen, S. et al. (2017). *Bub3 reads phosphorylated MELT repeats to promote spindle assembly checkpoint signaling*.

Prinz, F., Puetter, V., Holton, S., Andres, D., Stegmann, C., & Kwiatkowski, D. et al. (2016). Functional and Structural Characterization of Bub3·BubR1 Interactions Required for Spindle Assembly Checkpoint Signaling in Human Cells. *Journal Of Biological Chemistry*, *291*(21), 11252-11267. http://dx.doi.org/10.1074/jbc.m115.702142

Rajagopalan, H., & Lengauer, C. (2004). Aneuploidy and cancer. *Nature*, *432*(7015), 338-341. http://dx.doi.org/10.1038/nature03099

Rieder, C. (1995). The checkpoint delaying anaphase in response to chromosome monoorientation is mediated by an inhibitory signal produced by unattached kinetochores. *The Journal Of Cell Biology*, *130*(4), 941-948. http://dx.doi.org/10.1083/jcb.130.4.941

Rieder, C., & Salmon, E. (1998). The vertebrate cell kinetochore and its roles during mitosis. *Trends In Cell Biology*, *8*(8), 310-318. http://dx.doi.org/10.1016/s0962-8924(98)01299-9

Santaguida, S., & Amon, A. (2015). Short- and long-term effects of chromosome mis-segregation and aneuploidy. *Nature Reviews Molecular Cell Biology*, *16*(8), 473-485. http://dx.doi.org/10.1038/nrm4025

Santaguida, S., & Musacchio, A. (2009). The life and miracles of kinetochores. *The EMBO Journal*, *28*(17), 2511-2531. http://dx.doi.org/10.1038/emboj.2009.173

Thompson, S., & Compton, D. (2011). Chromosome missegregation in human cells arises through specific types of kinetochore-microtubule attachment errors. *Proceedings Of The National Academy Of Sciences*, *108*(44), 17974-17978. http://dx.doi.org/10.1073/pnas.1109720108

Tipton, A., Wang, K., Link, L., Bellizzi, J., Huang, H., Yen, T., & Liu, S. (2011). BUBR1 and Closed MAD2 (C-MAD2) Interact Directly to Assemble a Functional Mitotic Checkpoint Complex. *Journal Of Biological Chemistry*, *286*(24), 21173-21179. http://dx.doi.org/10.1074/jbc.m111.238543

Toledo, C., Herman, J., Olsen, J., Ding, Y., Corrin, P., & Girard, E. et al. (2014). BuGZ Is Required for Bub3 Stability, Bub1 Kinetochore Function, and Chromosome Alignment. *Developmental Cell*, *28*(3), 282-294. http://dx.doi.org/10.1016/j.devcel.2013.12.014

Vleugel, M., Omerzu, M., Groenewold, V., Hadders, M., Lens, S., & Kops, G. (2017). *Sequential Multisite Phospho-Regulation of KNL1-BUB3 Interfaces at Mitotic Kinetochores*.

Winey, M. (1991). MPS1 and MPS2: novel yeast genes defining distinct steps of spindle pole body duplication. *The Journal Of Cell Biology*, *114*(4), 745-754. http://dx.doi.org/10.1083/jcb.114.4.745

Xu, Z., Ogawa, H., Vagnarelli, P., Bergmann, J., Hudson, D., & Ruchaud, S. et al. (2009). INCENP–aurora B interactions modulate kinase activity and chromosome passenger complex localization. *The Journal Of Cell Biology*, *187*(5), 637-653. http://dx.doi.org/10.1083/jcb.200906053

Yuan, B. (2006). Increased Expression of Mitotic Checkpoint Genes in Breast Cancer Cells with Chromosomal Instability. *Clinical Cancer Research*, *12*(2), 405-410. http://dx.doi.org/10.1158/1078-0432.ccr-05-0903

Zhang, G., Lischetti, T., Hayward, D., & Nilsson, J. (2015). Distinct domains in Bub1 localize RZZ and BubR1 to kinetochores to regulate the checkpoint. *Nature Communications*, *6*, 7162. http://dx.doi.org/10.1038/ncomms8162

Zhang, G., Mendez, B., Sedgwick, G., & Nilsson, J. (2016). Two functionally distinct kinetochore pools of BubR1 ensure accurate chromosome segregation. *Nature Communications*, *7*, 12256. http://dx.doi.org/10.1038/ncomms12256

Zhu, T., Dou, Z., Qin, B., Jin, C., Wang, X., & Xu, L. et al. (2013). Phosphorylation of Microtubule-binding Protein Hec1 by Mitotic Kinase Aurora B Specifies Spindle Checkpoint Kinase Mps1 Signaling at the Kinetochore. *Journal Of Biological Chemistry*, *288*(50), 36149-36159. http://dx.doi.org/10.1074/jbc.m113.507970