**Investigating spindle assembly checkpoint dependent phosphorylation of Mad1 on its signaling functions in *Saccharomyces cerevisiae***

Ph.D. Candidacy Examination

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**Summary of proposal**

Maintenance of genomic integrity is key for propagation of accurate genetic information to subsequent generations. Loss of genome integrity, as often characterized by a gain or loss of whole chromosomes, is a conspicuous phenotype associated with tumoregenesis and is thus detrimental to cellular and organismal viability. Consequently, intrinsic cellular surveillance mechanisms have evolved to contribute to the fidelity of processes like chromosomal segregation for preservation of genome stability. One such surveillance device, known as the spindle assembly checkpoint (SAC), monitors the state of kinetochore attachments to spindle microtubules and halts mitotic progression if such physical attachments are incorrect. Central to SAC functionality are a group of conserved of signaling molecules that function specifically to transduce a ‘wait anaphase’ signal from kinetochores lacking proper attachment to spindle microtubules. Mad1 is one such SAC protein whose signaling activity is seminal to the proper execution of the mitotic checkpoint.

Intriguingly, unlike other SAC components, the signaling functions of Mad1 extend beyond the mitotic checkpoint as exemplified by its recently elucidated role in regulating the kap121 transport pathway. While being physically positioned at nuclear pore complexes during interphase, engagement of the SAC induces a change in Mad1s activity causing it to dynamically cycle between nuclear pore complexes and unattached kinetochores. This dynamic and rapid movement between these intranuclear locations simultaneously contributes to activation and maintenance of the SAC and regulation of the Kap121 transport pathway. While much progress has been made towards defining the mechanisms that allow Mad1 to carry out these diverse signaling events, the means by which its signaling activities are regulated is poorly understood.

Concurrent with the activation of the signaling events described above is the phosphorylation of Mad1. Ironically, Mad1 phosphorylation has widely been suggested to be important for kinetochore targeting and SAC signaling, however, this hypothesis has never been directly addressed.

Here, we will begin to assess how the signaling activities of Mad1 are potentially influenced by phosphorylation using budding yeast *Saccharomyces cerevisiae* as a model*.* The following proposal outlines three specific aims that incorporate a broad range of experiments designed to provide a foundation that will address the role that phophorylation has on the signaling functions of Mad1.

**Specific Aim I:** The identity of phosphorylated amino acids in Mad1 remains elusive. Therefore, this section outlines a basic set of experiments to conclusively map the phosphorylation sites in Mad1. Following the identification of relevant sites, we will utilize oligonucleotide directedmutageneis to mutagenize specific phospho-sites to evaluate how these mutations impact the signaling functions of Mad1.

**Specific Aim II:** To further investigate the function of Mad1 phosphorylation, we will analyze how phopsho-modifications to Mad1 alter its physical interactions with known binding partners. Therefore, this section outlines a series of *in vitro* binding experiments to directly investigate how Mps1 induced phosphorylation of Mad1 potentially alters its binding interactions with components of both the spindle assembly checkpoint and nuclear transport machinery.

**Specific Aim III:** As kinase activity is crucial for activation and maintenance of spindle assembly checkpoint signaling, more evidence is emerging highlighting the importance of phosphatases in checkpoint regulation. This section therefore describes a set of experiments to investigate whether the kinetochore bound phosphatase, Glc7, is involved in regulating the signaling activities of Mad1.

**Background.**The contents of the nucleus are physically segregated from the cytoplasm by an impermeable double membrane structure called the nuclear envelope (NE). Pivotal to cellular physiology is the continual exchange of vast amounts of materials between nuclear and cytoplasmic compartments. The mediators of this exchange are selective bidirectional transporters known as nuclear pore complexes (NPCs) that form at sites where the inner and outer nuclear membranes fuse1. In addition to regulating nucleocytoplamsic transport, NPCs serve as molecular hubs for a diverse array of proteins whose functions range from regulating aspects of gene expression2 to chromosome transmission3. Therefore, not only do NPCs contribute to cellular physiology by controlling the access of effector molecules to the cells genetic material, these structures participate in a broad range of essential nuclear activities pertinent to nuclear and cellular function. Further knowledge on how NPCs regulate transport, in addition to understanding the functional and physical relationships between NPCs and peripherally associated proteins is key to understanding normal cellular function. This is underscored by the fact that mutations to components of the nuclear transport machinery can lead to a host of cellular dysfunctions that can ultimately culminate to disease including cancer4.

**Nuclear Pore Complexes and Nucleocytoplasmic Transport** NPCs are amongst the largest proteinacious assemblies in the eukaryotic cell with an estimated mass of roughly 50 million Daltons in yeast and 100 million Daltons in vertebrates5,6. Given this grand discrepancy in size, the overall structural and compositional features of NPCs are highly conserved. Roughly 450 individual polypeptides derived from 30 highly conserved nucleoporins (nups) comprise each NPC7-8. Together, each individual nup is organized into redundant subunits that coalesce together to form a cylindrical structure encircling a central conduit through which bidirectional transport occurs (Fig.1) 7. Built upon this core structure are asymmetrically organized peripheral emanations that extend off either face of the NPC known as the cytoplasmic filaments and the nucleoplasmic basket3.

Translocation of most cargos through NPCs depends upon action of soluble transport factors called karyopherins (kaps)9-10. Kaps are either specialized as importins or exportins based on their function to either import or export macromolecular cargos across the NE respectively (Fig.2)11,12. The ability of Kaps to recognize their appropriate cargos depends on the presence of specialized sequences referred to as either nuclear localization signals (NLS) or nuclear export signals (NES)13,14. Once kap/cargo complexes are formed, transport is initiated through interactions with phenylalanine-glycine FG nups that line the translocation channel9,15,16.

The small Ras-like GTPase Ran ultimately dictates directionality of kap-mediated transport11. Asymmetrical distribution of Ran-GTP provides importins and exportins with the appropriate spatial cues allowing kap-cargo complexes to form and dissociate in the appropriate compartments17,18. Ran-GTP is predominantly concentrated in the nucleus where it stably interacts with all incoming importins in complex with cargo molecules11,19. Ran-GTP binding to import kaps induces a conformational change causing the release of the NLS bearing cargo in the nucleoplasm11. Conversely, the binding of Ran-GTP to exportins in the nucleoplasm increases their affinity for cargos bearing an NES and the resulting trimeric complex is then exported. The trimeric complex dissociates once RanGAP is encountered in the cytoplasm that catalyzes the conversion of Ran-GTP to Ran-GDP11.

**Alternate functions for the nuclear transport apparatus** As highlighted above, the most well characterized function of nuclear pore complexes is regulation of nucleocytoplasmic transport. However, an ever-growing body of evidence has accumulated demonstrating a variety of ancillary roles for nuclear pore complexes. In fact, several components of nuclear pore complexes have pivotal roles required for the proper execution of mitosis. For instance, the mammalian Nup107-160 complex localizes unattached kinetochores where it interacts with the microtubule nucleator γ-TuRC to cooperatively promote spindle assembly20. At kinetochores, the Nup107-160 complex is also required for recruitment of protein complexes that are imperative for proper kinetochore functionality including Aurora B and the chromosomal passenger complex (CPC)21. Depletion of members of the Nup107/160 complex results in aberrant chromosome congression in addition to chromosomal segregation defects21-22. Parallel observations have been made in budding yeast as strains lacking the nucleoporin Nup170 show severe chromosomal segregation defects attributed to loss of kinetochore integrity23. Although it is not known whether Nup170 directly associates with centromeres, loss of kinetochore integrity could be the result of centromeric heterochromatin defects as Nup170 is known physically interact with chromatin remodeling enzymes.

In addition to making structural contributions to kinetochores, NPCs also serve as a docking site for a variety of molecules that contribute to the regulation of chromosome segregation. The observation that NPCs serve as a hub for proteins of a certain function suggests that nuclear pores may play a role in regulating some aspect of their function. Alternatively, it is possible for the converse to be true in that those proteins docked at NPCs are involved in regulating some aspect of nuclear transport. Both of the former and latter scenarios have proven to be the case for the spindle assembly checkpoint (SAC) protein Mad1.

Mad1 is one of several proteins involved in regulating the spindle assembly checkpoint, a cellular safety device that monitors both chromosome alignment and kinetochore attachment to spindle microtubules24-25. Any aberrations to kinetochore microtubule attachments, including lack of tension across sister kinetochores, causes activation of the SAC leading to a halt in mitotic progression until the correct attachments are made25-26. Checkpoint dependent metaphase arrest is achieved though inhibition of CDC20, a co-factor for the E3-ubiquitin ligase known as the anaphase promoting complex (APC)24-27. During SAC arrest, inhibition of the APC prevents precocious polyubiquitylation of two key substrates, cyclin B and securin, whose degradation is required for escape from metaphase and anaphase onset24-27 (Fig. 3). This inhibition is not relived until all chromosomes have become properly bi-oriented on the metaphase plate between separated spindle poles, a consequence of proper kinetochore-microtubule attachments24,28.

Of the identified SAC components in *Saccharomyces cerevisiae*, only Mad1 and Mad2 are known to visibly concentrate at NPCs (Fig.4)29,30. This localization pattern is highly conserved as Mad1/Mad2 localize to interphase NPCs in *Drosophila* and Human cells31-32. Characterization of the molecular basis for Mad1s interaction with yeast NPCs has revealed the existence of multiple binding sites at these structures including members of the Nup53-containing sub-complex and the nucleoplasmic basket proteins Mlp1 and Mlp229,33. The latter interaction is thought to make up the primary binding site at NPCs as loss of both protein together, or mutations that release these proteins from the NPC core structure, results in a concomitant loss of Mad1 from the nuclear periphery34. Studies in flies and humans complement this data as TPR and Mtor, the counterparts to the Mlp proteins in Humans and flies respectively, have been shown to interact with the Mad1-Mad2 complex31. Although the functional significance of these interactions is not completely understood, the idea that NPC components are modulating the activity of these proteins to influence the SAC is becoming more accepted30. In Mad1s case, certain amino terminal truncation mutations that disrupt its ability to bind NPCs lead to constitutive kinetochore association suggesting that NPC binding may negatively regulate Mad1s kinetochore association by sequestering it away from binding sites at these locations34. Moreover, loss of Nup53, a nucleoporin that directly physically interacts with Mad1, causes delayed release from the mitotic checkpoint suggesting an inhibitory role for this protein in modulating the SAC34.

Activation of the SAC triggers Mad1 movement off of NPCs where it targets to binding sites at unattached kinetochores33-35. While most SAC proteins dynamically turnover at unattached kinetochores36, with the exception stably bound residents like Bub1, Mad1s movement is unique in that it rapidly traverses between NPCs and kinetochores in a cyclical fashion during SAC arrest (Fig.6)33,37. Intriguingly, movement between these intranuclear locations is facilitated primarily by the nuclear export karyopherin Xpo1p. Regulation of this intranuclear targeting event is analogous to export of macromolecules in that Xpo1s interaction with Mad1 is dependent upon an NES near Mad1s carboxy terminus33. Disruption to the NES results in a striking reduction of Mad1 at kinetochores.

While dynamic intranuclear movement of Mad1 between NPCs and kinetochores is important for SAC signaling, recent work has led to the discovery of an alternate function for this cycling event. During nocodazole induced metaphase arrest, transport of the import karyopherin Kap121 significantly attenuated under these conditions38. Inhibition of kap121-mediated transport is facilitated by alterations to the molecular binding arrangements within the Nup53-containing sub-complex (Fig.7)38. These conclusions were derived from a series immunoprecipitation experiments from yeast cell extracts demonstrating that Nup53 is released from its neighbor Nup170 to establish a newly formed interaction with Nic96 during nocodazole arrest. Such binding alterations lead to the exposure of a Kap121-specific NLS sequence positioned in the carboxy terminus of Nup53 that functions to bind any Kap121 molecules moving through central channel38. Through an as of yet unresolved mechanism, Mad1s interaction with the NPCs during SAC arrest somehow mediates these molecular rearrangements as loss of Mad1, or its ability to target/turnover at kinetochores inactivates the transport inhibitory pathway.

Coincidental with the activation of the signaling activities of Mad1 during SAC arrest is its hyperphosphorylation. It is widely suggested that hyperphosphorylation of Mad1 is important for its SAC signaling function from yeast to vertebrates39,40. This is highlighted by the observation that hyperphosphorylation of Mad1 is undetectable in asynchronously cycling cells in yeast39. It is not until the spindle is disrupted with microtubule depolymerizing drugs that Mad1 hyperphosphorylation becomes observable39,41. In mammalian cells, Mad1 hyperphosphorylation occurs during early metaphase when the mitotic checkpoint is activated and is no longer observable upon anaphase onset when the SAC is silenced42.

Furthermore, genetic studies in budding yeast demonstrate that mutations that abrogate SAC signaling, including Mad2 and Bub1 deletion mutations, accompanies the loss of the ability of Mad1 to become hyperphosphorylated43. Likewise, in mammalian cells, reducing Mad1 phosphorylation by inhibiting or depleting polo like kinase-1 (Plk1) results in the loss of SAC signaling and reduces Mad1 kinetochore targeting42. Taken together, these data imply that hyperphosphorylation of Mad1 is likely an important step towards activating checkpoint signaling.

Biochemical studies in budding yeast led to the discovery that one of the kinases responsible for phosphorylating Mad1 during SAC arrest is the essential dual-specific kinase Mps1, whose catalytic activity is essential for SAC signaling44. From these studies, it was revealed that Mps1 overexpression induces phosphorylation of Mad1 and causes a constitutive SAC arrest44. In mammalian cells, inhibition of the catalytic activity of Mps1 blocks Mad1 kinetochore targeting and abolishes mitotic checkpoint signaling thus suggesting a conserved role for this kinase in the regulation of functional activity of Mad145.

Although these studies have made significant contributions to understanding the context and basic requirements for Mad1 phosphorylation, the functional significance of such modifications has never been directly tested. It is tempting to speculate that various different phosphorylations of Mad1 could contribute to its ability to carry out its diverse signaling functions in the context of its kinetochore targeting ability, mitotic checkpoint signaling, and the kap121-import inhibitory pathway.

***Formulation of hypothesis.*** The observations above have lead us to propose that SAC induced phosphorylation of Mad1 is pivotal to the activation of its diverse signaling functions. We envisage that phosphorylation of Mad1 induces electrostatic alterations or conformational changes that potentiate the formation of interactions to positively promote its signaling activities while perhaps, simultaneously disrupting interactions that could negatively regulate these activities.

Based on a variety of previous works, we explicitly propose that phosphorylation of Mad1 has at least a three-fold impact on its physical interactions to promote its signaling functions. First, upon engagement of the SAC, we predict that efficient kinetochore targeting of Mad1 requires release from binding sites at NPCs. We predict that certain phospho-modifications could serve to repel potentially inhibitory interactions with certain NPC components to promote its release from these structures. Second, as mentioned above, facilitated targeting of Mad1 to kinetochores and dynamic intranuclear movement between these structures and NPCs requires an interaction with the exportin Xpo133. We therefore predict that the interaction between Mad1 and Xpo1 is fostered by certain phosphorylation events to Mad1. Third, Mad1 forms a tight complex with other components of the SAC signaling machinery, namely Bub1 and Bub3, whose regulated formation is a vital requirement for the SAC. Loss of Mps1 activity, and thus loss of Mad1 phosphorylation, results in the inability of Mad1 to associate with the Bub1-Bub3 complex46. With this observation, we predict that phosphorylation of Mad1 is likely required for the binding of Mad1 with the Bub1-Bub3 complex. Taken together, we propose that phosphorylation of Mad1 activates its signaling activities through altering its physical interactions.

Consistent with the idea that phosphorylation fosters the formation of interactions to positively regulate the signaling activities of Mad1, we propose that phosphatase driven reversal of these post-translational modifications serves as a means to counterbalance these effects. Specifically, we predict that the kinetochore bound phosphatase Glc7, whose phosphatase activity is required for SAC exit, has a role in dephosphorylating Mad1 to counter its signaling activities.

Using budding yeast as a model, we have outlined a broad series of experiments to examine the functional consequences of Mad1 phosphorylation on its signaling activities. Mad1 has similar functional roles and signaling activities in mammalian cells24,25. Therefore, the knowledge gained from the proposed studies in yeast should be directly applicable to understanding the signaling functions of the mammalian ortholog. Additionally, the wide availability of genetic tools for budding yeast makes this model organism optimal for the proposed studies. As misregulation of Mad1 expression is linked to human disease like cancer47, a greater understanding of how Mad1 signaling functions are regulated in yeast could potentially yield new therapeutic strategies for various human cancers.

**Specific Aim 1:Towards identification of physiological phosphorylation sites in Mad1 and examining their contribution to Mad1 function.** *The primary tenet of this proposal is to define the functional consequences of Mad1 phosphorylation on its ability to carry out its signaling functions. Expanding our understanding of how phosphorylation affects Mad1 function requires a thorough, in-depth investigation into the identification of amino acid residues within Mad1 that are modified by phosphorylation during SAC arrest. The identification of phosphorylation sites followed by mutational analysis using site-directed mutagenesis will allow for the development of models of how phosphorylation specifically contributes to controlling Mad1 function.*

*As outlined above, Mad1 has signaling functions that are critical to both the mitotic checkpoint and the Kap121-import inhibitory pathway. Therefore, the successive series of experiments proposed in this aim are designed to explore how phosphorylation of Mad1 influences its ability to carry out both its roles in the mitotic checkpoint and the Kap121p-import inhibitory pathway. First (****I.1****), we will attempt to identify the serine/threonine residues within Mad1 that are modified by phosphorylation during SAC arrest. Second (****I.2****), we will assess the functional impacts of mutating the identified phosphorylation sites in Mad1 on its ability to carry out its signaling functions in vivo.*

**I.1) Mapping phosphorylated amino-acid residues in Mad1p.** Phosphorylation prediction analysis of Mad1 reveals an abundance of putative phosphorylation sites distributed throughout the entirety of the protein including a total of twenty-five predicted serine residues and a single threonine residue. The identity of the amino-acid residues that are phosphorylated *in vivo* and the functional significance of each site remains to be determined. Therefore, to begin to map physiologically relevant phosphorylation sites within Mad1, IgG-Sepharose chromatography will be used to immunopurify epitope tagged Mad1-pA from yeast cell extracts acquired from asynchronous and metaphase arrested cell populations. Metaphase arrest will be achieved with the microtubule depolymerizing drug nocodazole to induce the SAC and drive Mad1 hyperphosphorylation. To ensure that the SAC is engaged in these cells, and thus Mad1 is hyperphosphorylated, we will acquire a sample of cells from this population for western blot analysis to look for a shift in gel-mobility and loss of shift when incubated in the presence of lambda phosphatase. Next, immunopurified Mad1-pA, from both cell populations, will be subjected to in gel digestion with trypsin and phosphopeptides will be analyzed with liquid chromatography electrospray ionizing tandem mass spectrometry (LC-ESI MS/MS) for precise identification of phosphorylated amino-acid residues. As Mad1 is phosphorylated at a basal level in cycling cells, we will perform a comparative analysis to determine which sites in Mad1 are differentially phosphorylated in SAC-arrested versus asynchronous cell populations to help guide the analysis in the subsequent aim. However, one potential pitfall of this approach is the possibility that such a comparative analysis may not sufficiently narrow down the number of phosphorylation sites if the number of residues modified during SAC arrest is significantly higher compared to cycling conditions. Should this be the case, we will determine whether those SAC-dependent phosphorylation sites identified by tandem mass spectrometry are highly conserved by performing a sequence alignment with Mad1 sequences from other fungi and higher eukaryotes. This method could potentially provide an additional filter to narrow down phosphorylation sites to analyze in the subsequent aim.

An alternative approach we could also employ to identify important phospho-sites is through the use of a chemical genetics approach to specifically inhibit Mps1 catalytic activity to identify those sites within Mad1 that are phosphorylated by Mps1. The *mps1-as1* allele contains an ATP binding pocket mutation that allows for the encoded protein to be specifically inhibited by bulky ATP analogs like 1-NM-PP148. Using the *mps1-as1* mutation could allow us to potentially identify residues in Mad1 where phosphorylation decreases due to Mps1 inhibition. To accomplish this, cells harboring the *mps1-as1* mutation will be treated with nocodazole following incubation with the inhibitor 1-NM-PP1. In parallel, *mps1-as1* cells will be treated with nocodazole in the absence of the inhibitor. Next, Mad1-pA will be immunopurified from cell lysates acquired from both cell populations using IgG-Sepharose chromatography. Immunopurified Mad1 will then be digested with proteases and subjected to LC-ESI-MS/MS to identify phospho-peptides from both cell populations. A comparative analysis will then be conducted to identify those sites in Mad1 where phosphorylation is decreased following inhibition of Mps1. This approach should allow us to isolate those residues that are specifically modified by Mps1 during SAC arrest and likely reflect those sites that are important for the signaling functions of Mad1.

**I.2)Towards understanding the relationship between of Mad1 phosphorylation and its signaling activities.** To initiate our evaluation on how phosphorylation of Mad1 impacts its signaling functions *in vivo*, we will utilize point mutagenesis to disrupt specific phosphorylation sites identified by mass spectrometry in aim I.1. The first part of our analysis will be geared towards identifying those phospho-sites that are important for the spindle assembly checkpoint. To accomplish this, we will utilize oligonucleotide-directed mutagenesis to construct various mutant forms of Mad1 where phosphorylated serine/threonine residues (identified by MS in aim I) are changed to alanine (*mad1A*). Each alanine mutant constructed, under the control of the endogenous *MAD1* promoter, will be introduced into otherwise wildtype strains lacking endogenous *MAD1* (*mad1Δ*). Strains harboring these mutations will then be tested for benomyl sensitivity to assess if the corresponding mutations convey a SAC defect. We predict that if phosphorylation of Mad1 is important for its SAC signaling function, then disrupting various phospho-sites should compromise the SAC.

Next, we will investigate the functional impacts of disrupting phosphorylation sites on the ability of Mad1 to dynamically target onto unattached kinetochores during SAC arrest. We consider the possibility that the number of phosphorylation sites identified by mass spectrometry could potentially be high making analysis of all possible mutant combinations infeasible. Should this be the case, we will focus our attention to sites identified in the carboxy-terminus of Mad1, as this is the region most critical for its kinetochore targeting capabilities34. Therefore, to initiate this analysis, we will insert an eGFP tag at the C-terminus of each of the selected *mad1A* mutant proteins generated above. Because the aforementioned point mutants could abolish SAC activity, each *mad1A-*GFP mutant will be introduced into a strain lacking the non-essential APC component Cdc26. This additional genetic manipulation is required, as cells lacking a functional SAC are rendered insensitive to spindle poisons thereby disabling metaphase arrest when the mitotic spindle is disrupted. At 37° C, *cdc26Δ* mutants arrest in metaphase in a SAC independent manner49 and will thus enable us to synchronize checkpoint defective cells in metaphase. Finally, visualization of Mad1 movement to kinetochores will require removal of the nucleoporin Nup60 (*nup60Δ*). Removal of Nup60 liberates the Mlp proteins (One of Mad1s binding sites at NPCs) from the nuclear periphery causing them to concentrate in a single intranuclear focus that retains Mad1 binding 34. This focus can easily be distinguished from kinetochores allowing for the visualization of Mad1 movement off these structures and onto unattached kinetochores(Fig.5)34.

Following *cdc26-*dependant metaphase arrest, we will disrupt the mitotic spindle with the addition of nocodazole. Next, we will evaluate the efficiency with which each point mutant targets onto unattached kinetochores by quantifying the florescence intensity of kinetochore associated Mad1-GFP relative to the appropriate controls. Kinetochore associated Mad1-GFP will be identifiable by comparing its localization pattern relative to Mtw1-RFP. We predict that if phosphorylation is required for kinetochore targeting of Mad1, substituting phospho-sites with alanine should block the ability of Mad1 to move from NPCs onto unattached kinetochores upon SAC engagement.

Next, as the ability of Mad1 to target onto unattached kinetochores is required for activation of the import inhibitory pathway, we will examine how each phospho-site mutant generated above impacts the regulation of this pathway. To test this, plasmid copies of the Kap121 import reporter Pho4-NLS-GFP will be transformed into cells harboring various *mad1A* mutations. Briefly, Pho4-NLS-GFP is a nuclear transport reporter comprised solely of NLS sequence derived from *PHO4* fused to three tandem GFP molecules50. Nuclear accumulation is of this reporter robust in asynchronously cell populations and is observably attenuated in nocodazole-arrested cells and is thus a metric for the activation of the Kap121-import inhibitory pathway38. Metaphase arrest will be achieved with the use of the *cdc26-*deletion mutation and nocodazole will subsequently be added to cultures as described above. Florescence microscopy will then be utilized to analyze the subcellular distribution of the import reporter. Overtly, we predict that any Mad1 phospho-site mutation that compromises its kinetochore association will also result in the inability to activate the Kap121 import inhibitory pathway. We will also perform immunoprecipitation studies using IgG-Sepharose affinity purification to isolate Nup53-pA from yeast cell extracts isolated from cells containing the above Mad1 phospho-site mutations. As alterations to molecular binding arrangement within the Nup53 sub-complex is key to import inhibition38 (Fig.7), we anticipate that those mutations which do not result in inhibition of nuclear accumulation of Pho4-NLS-GFP will also fail to induce the molecular rearrangements within the Nup53 sub-complex.

To further evaluate the function of Mad1 phosphorylation, we will analyze the effects of simulating constitutive phosphorylation by mutating identified phospho-sites to aspartate. In terms of SAC function, we predict that phospho-mimetic mutations at critical sites could either enhance SAC activity or cause aberrant activation of the SAC leading to constitutive checkpoint arrest. To examine these possibilities, phospho-sites identified in the above mutational analysis as being important for SAC activity will be mutated to aspartate (*mad1D)*. If certain phospho-mimetic mutations enhance SAC activity, we anticipate that cells harboring these mutations will be more adapt to handling disruptions to microtubule dynamics making them more resistant to growth on benomyl. These experiments will be confirmatory to the experiments proposed above, as this assay should provide us with additive information on which sites are most critical for SAC function.

If phosphorylation is important for Mad1 kinetochore targeting, then perhaps certain phopho-mimetic mutations should enhance or cause constitutive recruitment of Mad1 to kinetochores. To test this, we will insert an eGFP tag at the C-terminus of various *madD* mutant proteins in strains already containing the *nup60Δ* and *MTW1-RFP* manipulations. Next, cells harboring these mutations will be arrested in metaphase with nocodazole and the efficiency with which each point mutant targets to kinetochores will be quantified as described above. Perhaps certain phospho-mimetic mutations might induce constitutive Mad1 kinetochore association in the absence of disruptions to kinetochore-microtubule interactions. If this was observed to be the case, such results would parallel observations made when the SAC kinase Mps1 is overproduced, which causes robust Mad1 kinetochore accumulation while microtubule occupancy at kinetochores is retained. Isolation of a Mad1 phospho-mimetic mutant that is constitutively kinetochore associated would allow us to assess whether both phosphorylation of Mad1 and kinetochore recruitment is sufficient for activation of the SAC. If such a mutant fails to activate the SAC, this would suggest that events downstream from Mad1 phosphorylation and kinetochore recruitment are required for activation checkpoint signaling and could potentially provide insight into the hierarchy of events required for activation of SAC signaling. Ultimately, the goal of these experiments is to test the prediction that phosphorylation of Mad1 is required for its kinetochore targeting activity.

**Specific Aim 2: To examine how Mad1 phosphorylation regulates its physical associations with components of the spindle assembly checkpoint and nuclear transport machinery.** *Phosphorylation can regulate protein function by inducing conformational change to secondary structure or by altering electrostatic interactions as a means to enhance or repel physical interactions. With this stated, we estimate that SAC dependant phosphorylation of Mad1 directs its activity through abetting the formation new interactions while potentially disrupting others. Specifically, we envision that Mps1 dependent phosphorylation of Mad1 following SAC arrest fosters the formation of new interactions to facilitate its signaling activities while disrupting protein interactions that may serve to negatively regulate these activities.*

*To test this hypothesis, we have outlined a series of experiments to examine how SAC dependent phospho-modifications to Mad1 alter its physical interaction network. Specifically, in the following aims, we will investigate how phospho-modifications to Mad1 affect its interactions with components of nuclear pore complexes (****II.1****), spindle assembly checkpoint signaling machinery (****II.2****), and the nuclear export karyopherin Xpo1 (****II.3****). Should the results from the proposed experiments demonstrate a role for phosphorylation in the regulation of the evaluated interactions, it will demonstrate an important mechanistic link between these modifications and how Mad1 function is regulated.*

**II.1) Analyzing how Mad1 phosphorylation affects its association with nuclear pore complex components.** Previously published observationssuggest that NPCs act as sequestration sites for Mad1 as a means to prevent inexpedient association with kinetochores. This hypothesis was developed on the premise of two observations. First, certain Mad1 truncation mutants that fail to bind to NPCs have a greater propensity to associate with kinetochores. This is exemplified by a truncation mutant lacking its amino terminal half, *mad1475-749*, which fails to bind NPCs and is otherwise constitutively associated with kinetochores34. Second, cells lacking Nup53, a nucleoporin that directly physically interacts with Mad1, remain arrested in metaphase for significantly longer periods of time in the presence of nocodazole indicating exit from the SAC is delayed34. These observations, along with the benomyl resistance phenotype of both the *nup53*Δ and *mad1475-749* mutants suggest that NPCs have a role in negatively regulating the SAC, possibly by sequestering Mad1 away from binding sites at kinetochores.

From these data, we predict that upon activation of the SAC, phospho-modifications to Mad1 potentially alter its interactions with binding components at NPCs to promote its release from these structures as a step towards facilitating its kinetochore association. More specifically, we propose that phospho-modifications to the amino-terminus of Mad1, the region of Mad1 required for NPC binding, is what drives release from these structures. Several pieces of data support this hypothesis. For instance, data acquired from *in vitro* phosphorylation studies using purified Mps1 and an amino terminal fragment of Mad1 (*mad1 20-380*) demonstrates the existence of several Mps1 phosphorylation sites in the N-terminus of Mad144. Also, the localization of Mad1 at the nuclear periphery is significantly reduced in cells overproducing Mps1 demonstrating that elevated kinase activity is directly correlated with reduced binding of Mad1 to NPCs (Fig.8).

To test this idea, *in vitro* binding experiments will be performed using a GST-tagged version of the mad1 amino-terminal truncation derivative, *mad11-325.* This portion of Mad1 contains two coiled-coil domains that are necessary its NPCs association34. GST-*mad11-325* will be expressed and purified from *E.coli* lysates and bound to glutathione-Sepharose beads. Next, 6His-Mps1 and kinase dead 6His-*mps1*KD will be purified and subsequently added to a reaction with GST-*mad11-325* and ATP to induce substrate phosphorylation. Following termination of the reaction, excess Mps1 will be removed with the addition of Ni-nitriloacetic acid beads and both forms of GST-*mad11-325* will be incubated with purified, recombinant TAP-Nup53. Following binding, quantitative immuno-blotting will be performed to determine the levels of TAP-tagged Nup53 bound to *mad11-325* under each condition using the appropriate antibodies. If phosphorylation of the N-terminal portion of Mad1 serves to disrupt its interaction with NPCs, then we would predict that the phosphorylated form of *mad11-325* would show quantifiably reduced binding to Nup53 *in vitro.* Since Mad1 is known to interact with the Mlp proteins, we will also conduct these experiments in parallel with TAP-Mlp1 and TAP-Mlp2.

To further the above analysis, we will attempt to identify the amino acid residues within amino-terminus of Mad1 that are modified by Mps1 through utilization of *in vitro* phosphorylation followed by LC-MS/MS. *In vitro* phosphorylation of Mad1 will be performed as described above. Each relevant N-terminal phospho-site identified by MS will be mutagenized to aspartate and the corresponding mutations will be individually engineered into a *MAD1-GFP* gene fusion construct. If our prediction is correct, mutants harboring amino-terminal phospho-mimetic mutations should appear to be reduced at the nuclear periphery with perhaps with visibly higher nucleoplasmic levels. To further these data, we will also conduct immunoprecipitation experiments with each mutant generated above to determine whether Mad1s physical interactions with Nup53, and perhaps with Mlp1/Mlp2, are altered. Using monoclonal anti-GFP antibodies, we can utilize the strains created above to isolate GFP tagged versions of each mad1 point mutant from cell lysates and probe for the presence of specific nucleoporins using specific antibodies. Taken together, these experiments will test the prediction that phosphorylation of Mad1 modulates its physical interactions with its binding partners at NPCs.

**II.2) Analyzing how phosphorylation affects the association of Mad1 with components of the spindle assembly checkpoint.** Mad1 physically interacts with a variety of SAC signaling components including Mad2, Bub1, and Bub346,51. Mad1 and Mad2 form a highly stable interaction that is irrespective of cell cycle position while the association of Mad1 with Bub1 and Bub3 is cell cycle dependent. Whilst the latter interaction is significantly enhanced upon SAC activation, disrupting the ability of Mad1 to associate with either of these protein complexes is detrimental to the SAC. The physical association of Mad1 with Mad2 does not vary with the phosphorylation state of Mad1 as demonstrated by the fact that Mad2 is competent to bind all phospho-isoforms of Mad143. However, the association of Mad1 with the Bub1-Bub3 complex depends upon Mps1 activity. Since neither Bub1 nor Bub3 are known substrates of Mps1 catalytic activity in yeast, we predict that Mps1 dependant phosphorylation of Mad1 regulates Mad1 binding to the Bub1-Bub3 complex during SAC arrest.

To test this hypothesis, *in vitro* binding assays will be performed using recombinant forms of these various proteins. First, we will purify pre-assembled GST-Bub1-Bub3 complexes from *E.coli* extracts using glutathione-Sepharose affinity purification. Next, purified TAP-Mad1 bound to IgG beads will be incubated with recombinant 6His-Mps1 and 6His-*mps1-*KD in the presence of ATP to induce phosphorylation of Mad1. As described in aim II.1, excess Mps1 will be removed from bead bound TAP-Mad1 through the addition Ni-nitriloacetic acid beads. Both purified forms of Mad1 will then be incubated with preformed GST-Bub1-Bub3 complex bound to beads as bait. The ability of either form of Mad1 to enter into complex with Bub1-Bub3 will be determined by immunoblotting. This experiment will directly test the prediction that Mps1 dependant phosphorylation of Mad1 regulates its ability to associate with the Bub1-Bub3 complex.

Should Mad1 phosphorylation be pre-requisite for the formation of this complex, we will further dissect which phospho-sites are important for this interaction. To accomplish this, we will utilize point mutagenesis to disrupt those Mps1 specific phospho-sites identified in aim II.1 to alanine. Each mutation will be engineered into a TAP-Mad1 fusion construct for expression and purification from *E.coli* cell lysates. Purified, recombinant mutant forms of Mad1 will then be incubated with Mps1, in the presence of ATP, and subsequently with purified bead bound GST-Bub1-Bub3 as described above. This assay should allow for the identification of phospho-sites within Mad1 required for this interaction.

**II.3) Analyzing how phosphorylation affects the association of Mad1 with the nuclear export karyopherin Xpo1p.**

Interactions between a karyopherin and its respective cargo can often be controlled through post-translational modifications as a means to regulate nuclear transport. Specifically, post-translational modifications to cargo molecules can either facilitate or hinder an interaction with its respective karyopherin. With this stated, we predict that the ability of Mad1 to interact with Xpo1 is, in part, regulated by the phophorylation state of Mad1. We suspect this to be the case for two reasons. First, the ability of Xpo1 to interact with certain cargos often depends upon the phosphorylation state of the cargo, as exemplified by its interaction with the metabolic enzyme Hxk252, whose nuclear export depends upon phosphorylation of a critical serine residue. Second, Mad1s phosphorylation is temporally coincidental with its interaction with Xpo1.

Therefore, to explore this hypothesis, a series of *in vitro* binding assays will be performed. Since Ran-GTP is required for the formation of exportin-cargo complexes, TAP-Ran will be expressed and purified from *E.coli* extracts. Next, equal amounts of purified Ran will be pre-loaded with either guanosine di- or tri-phosphate to form both GDP and GTP bound forms of the protein. Either form of Ran will be incubated with purified GST-Xpo1 bound to glutathione-Sepharose beads to assemble a Xpo1/Ran complex. Next, i*n vitro* phosphorylation of purified Mad1, via Mps1, will be performed as described above to isolate both phosphorylated and unphosphorylated forms of the protein. Equal amounts of purified Mad1 will then be incubated with the GST-Xpo1/Ran complex and binding interactions will then be assessed by immunoblotting. Should our predictions be correct, the interaction between Mad1 and Xpo1 should only be detectable when Mad1 is phosphorylated. We will also perform analogous *in vitro* pull down experiments using purified GST-Xpo1 bound to beads and whole cell extracts isolated from asynchronous and nocodazole arrested cell populations. This approach will provide another way to examine whether SAC induced phospho-modifications to Mad1 are required for its interaction with Xpo1.

The primary goal of the aforementioned experiments is to simply determine whether Mps1 mediated phosphorylation of Mad1 is required for Xpo1s association with Mad1. These experiments could potentially provide valuable mechanistic insight into how Xpo1s interaction with Mad1 is governed upon induction of the SAC. Moreover, these experiments should provide a higher resolution view of how Mps1 dependent phosphorylation influences the SAC and Mad1 kinetochore targeting dynamics.

**Specific Aim 3: Towards understanding the mechanism by which Mad1 release from kinetochores is facilitated: A role for the kinetochore associated phosphatase Glc7p.**

*As kinase activity is an essential requirement for activation of the mitotic checkpoint, an accumulating body of data has uncovered a role for phosphatases in the regulation of the SAC. Specifically, new evidence demonstrates that Protein Phosphatase-1 (PP1), or Glc7 in yeast, facilitates escape from the mitotic checkpoint by reversing phosphorylation events induced by the essential SAC kinases Ipl1 and Mps1*53*-*54*. Similar observations have been made in vertebrate cells as PP1 has a role in regulating the localization of CENP-E by reversing Aurora-B induced phospho-modifications to CENP-E*55*. An emerging theme from these observations is the idea that mitotic checkpoint signaling is in part regulated by a balancing act between kinase and phosphatase activity.*

*Based on this concept, we propose that opposing kinase and phosphatase activity controls the signaling activities of Mad1 during SAC arrest. Explicitly, we predict that cycling of Mad1 between NPCs and kinetochores is a two-step reaction regulated by spatially separate phosphorylation and dephosphorylation events. We propose that the front end of the cycling reaction (NPCs to kinetochores) involves Mps1 dependent phosphorylation of Mad1 triggering its release from NPCs and targeting it to kinetochores while the backside of the reaction involves dephosphorylation of Mad1 at kinetochores facilitating kinetochore release for reassociation with NPCs. This hypothesis was developed from preliminary data demonstrating that Glc7 inactivation following SAC arrest alters the ability of Mad1 to re-associate with NPCs following its kinetochore association (Fig.9). Therefore, we surmise that during activation of the SAC, kinase/phosphatase activity is precisely balanced in such a way that Mad1 kinetochore targeting on rates are equivocal to off targeting rates.*

*To explore these possibilities, the experiments proposed in this aim will establish whether phopshorylated Mad1 is a direct substrate of Glc7 phosphatase activity as outlined in aim (****III.1****). Second, we will further explore how Mad1 signaling activity is potentially modulated through Glc7 mediated phosphatase activity in aim (****III.2****).*

**III.1) Towards detection of Glc7 mediated phosphatase activity against Mad1 *in vivo* and *in vitro*.** To examine whether phospho-Mad1 is a target of Glc7 phosphatase activity, we will perform an *in vitro* phosphorylation assay using recombinant forms of 6His-Mps1 and GST-Mad1 as described in aim II. In this *in vitro* reaction, we will utilize γP32-ATP to incorporate radiolabelled phosphates into Mad1 for quantification of phosphorylation levels at a subsequent step. Next, radiolabelled phospho-Mad1 will be incubated with purified recombinant TAP-Glc7. As a control, we will pre-incubate a fraction of purified TAP-Glc7 with microcystin, a known potent phosphatase inhibitor55. Following the reaction, we will quantify Mad1 phosphorylation levels using autoradiography. If our prediction is correct, the results from this experiment will provide us with data to demonstrating that Mad1 is a substrate of Glc7 phosphatase activity *in* *vitro.*

Next, *in vivo,* we will assess how Glc7 overproduction impacts Mad1 phosphorylation following activation of the SAC. To test this, plasmid copies of Glc7, under the control of the *GAL1-10* promoter, will be transformed into a *cdc26Δ* strain. Utilization of the *cdc26Δ* mutation will allow cells to maintain arrest in metaphase as Glc7 overproduction induces release from SAC arrest. Cells will be grown in raffinose media is required to keep the *GAL1-10* promoter repressed. Following metaphase synchronization at 37° C, nocodazole will be added cultures to activate the SAC to induce Mad1 hyperphosphorylation. Following SAC activation, Glc7 overproduction will be induced with the addition of galactose to cultures to activate the *GAL* promoter. As a control, parallel experiments will be conducted with the catalytically inactive version of Glc7, *glc7*H65K56*.* SDS-PAGE and western analysis with anti-Mad1 antibodies will be performed to assess changes in Mad1 phosphorylation status. These experiments will test the prediction that phospho-Mad1 is a direct substrate of Glc7 phosphatase activity.

**III.2) Analysis of how Glc7 phosphatase activity influences Mad1 kinetochore dynamics.** As highlighted above,we speculate that the opposing activities of Mps1 kinase and Glc7 phosphatase control the dynamic intranuclear signaling activity of Mad1 during SAC arrest. To investigate this hypothesis, we will C-terminally tag endogenous Mad1 with eGFP in a strain harboring the temperature sensitive (*ts*) *glc7-10* allele56 along with the *nup60Δ* and Mtw1-RFP manipulations. Cells harboring these mutations will be grown at 37 °C to inactivate *glc7-10* upon which nocodazole will be added to engage the SAC. Since our preliminary data suggests that NPC re-association is impaired under these conditions (Fig.8), we will conduct FRAP studies to determine if the observed phenomenon is the result of reduced turnover at kinetochores. Therefore, the kinetochore associated pool of Mad1 will be bleached with full intensity 488-nm light and recovery will be quantified by measuring florescence intensity of KT associated Mad1-GFP in comparison to an appropriate wildtype control. If our prediction is correct, we surmise that after inhibition of Glc7 activity, the rate of Mad1 turnover at kinetochores could be significantly compromised. This experiment will test the prediction that Glc7 is required for dynamic turnover of Mad1 at kinetochores.

In a parallel set of experiments, we will examine how overproduction of Glc7 impacts the kinetochore dynamics of Mad1 during SAC arrest. To begin this analysis, plasmid copies of the Glc7 driven by the *Gal1-10* promoter will be transformed into cells containing both the *MAD1-GFP* gene fusion and the *cdc26Δ* mutation. Metaphase arrest will then be induced by shifting cells to 37° C upon which nocodazole will be added to activate the SAC to allow for recruitment of Mad1 onto unattached kinetochores. Following synchronization, galactose will be added to cultures to drive Glc7 overproduction. Again, the *cdc26Δ-*dependantmetaphase arrest is required to ensure cells stay metaphase arrested following Glc7 overproduction. If our hypothesis is correct, we predict that elevated levels of phosphatase activity following SAC activation should result in quantitatively lower levels of Mad1 at kinetochores accompanied by higher levels of Mad1 at NPCs.

**Concluding Remarks.** We have proposed a broad set of experiments to investigate how phosphorylation of Mad1 contributes to the regulation of its known functions. The results acquired from these experiments will contribute to greater understanding of how Mad1 signaling activities are modulated as a result of such post-translation modifications and will potentially promote future, more indepth studies on this topic. Importantly, as the activity of Mad1 is indispensible to regulatory mechanisms that contribute to the maintenance of genomic integrity, and thus cellular vitality, understanding its regulation could become key in the context of understanding disease processes such as cancer.

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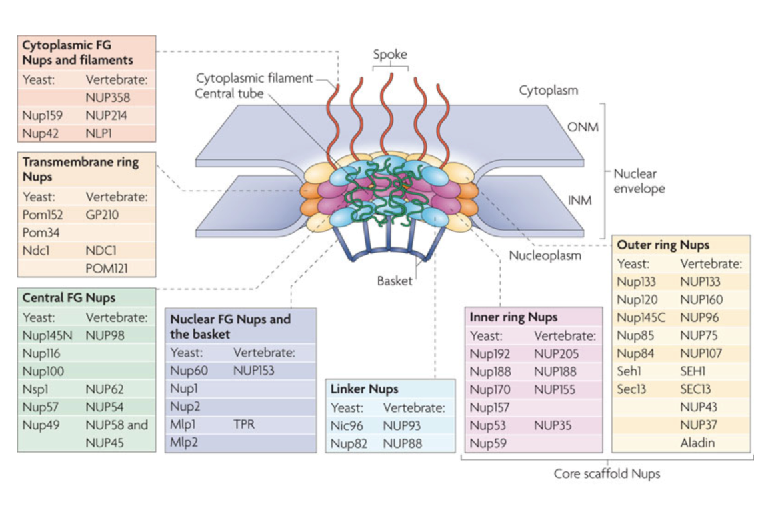
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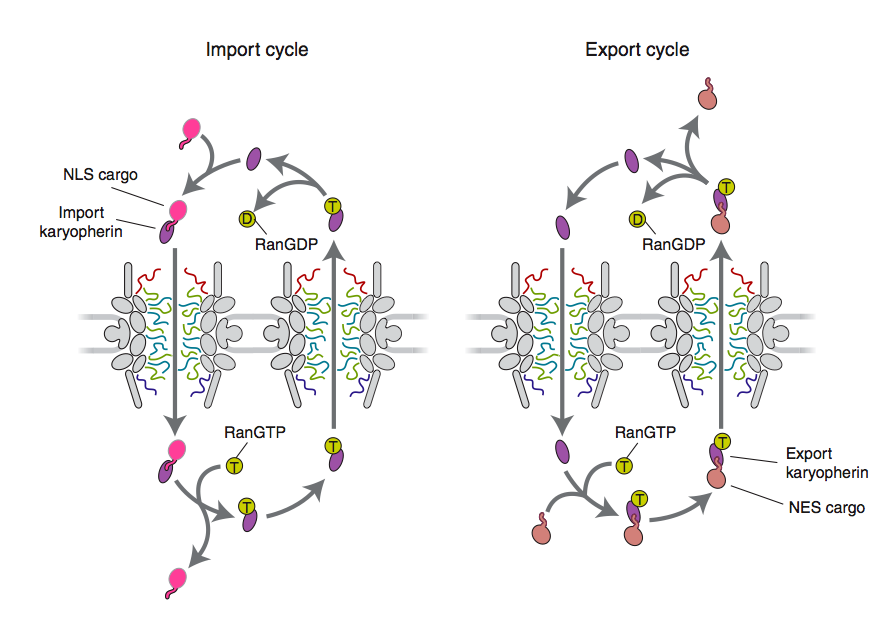
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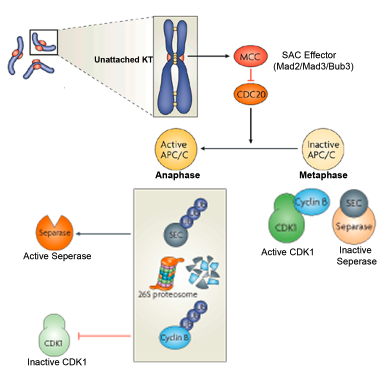
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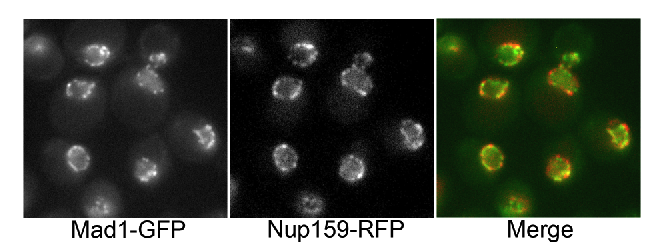
**Figure 1.** *Nuclear Pore Complex.* Nuclear pore complexes are large macromolecular assemblies comprised of eight radially organized interconnecting spokes encircling a central channel through which bidirectional transport occurs. The outer and inner nuclear envelope joins together to form a void where the NPC resides. All the major structural elements are depicted. The core structural scaffold of the NPC forms linkages to the nuclear envelope (NE) via a transmembrane ring composed of integral membrane proteins. Phenylalanine-Glycine (FG) nucleoporins line the central conduit and are connected to linker nuceloporins that serve as the core attachment sites for FG nups. Peripheral emanations extending off of the NPC core structure are filamentous fibril-like proteins that constitute the nuclear basket on the nucleoplasmic side and spokes on the cytoplasmic side. All yeast and vertebrate nucleoporins that constitute each substructure are listed in the colored boxes. (Figure adapted from Strambio-De-Castillia et. al *Nat Rev Cell Biol 11, 490-501*)



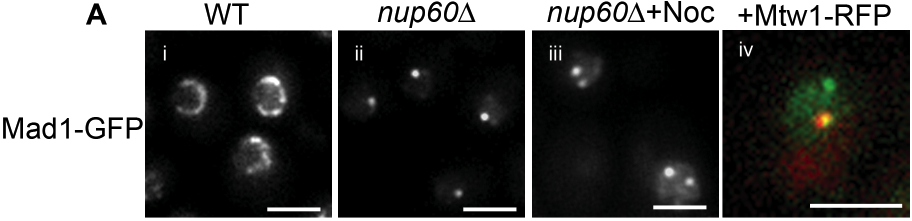
**Figure 2.** *The Nuclear Transport Cycle.* Import of macromolecular cargos is initiated by karyopherin recognizing of cargos bearing a nuclear localization signal (NLS). Importin-cargo complex formation occurs in the cytoplasm where Ran-GTP concentrations are low. Once the import complex translocates through the NPC, high concentrations of Ran-GTP cause dissociation of the Kap-cargo complex thereby terminating the import cycle. Conversely, nuclear export is initiated in the nucleoplasm with the formation of a hetero-trimeric complex between an export karyopherin, its cognate NES containing cargo, and Ran in the GTP bound form. Following translocation into the cytoplasm, Ran -GTP is hydrolyzed to Ran-GDP through the activity of Ran-GAP thereby causing export complex dissociation. (Figure adapted from Wente and Rout *Cold Spring Harb Perspect Biol* 10, 1101)



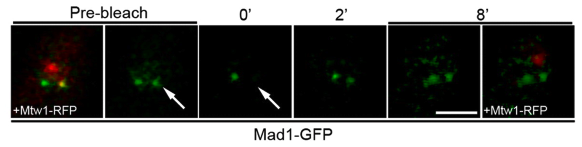
**Figure 3.** *Relationship between SAC signaling apparatus and the cell cycle machinery.* Upon activation of the spindle assembly checkpoint, unattached kinetochores (depicted in red) contribute to the formation of the ‘SAC effector’ known as the mitotic checkpoint complex (MCC). The MCC is comprised of the SAC signaling components Mad2/Mad3/Bub3 that form a complex with the APC co-activator Cdc20 during checkpoint arrest. Interaction between components of the MCC and Cdc20 render the APC inactive thus blocking its ubiquitin ligase activity. This ultimately prevents activation of the protease Seperase, whose activity is required for cleavage of sister chromatid cohesin at chromosomes, an event which facilitates the onset of anaphase. It is not until the SAC is satisfied by proper kinetochore-microtubule attachments that inhibition of the APC by the MCC is relieved. (Figure adapted from Musacchio and Salmon *Nat Rev Cell Biol* 8, 379-393)



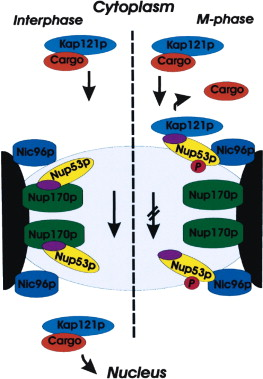
**Figure 4.** *Subcellular distribution of Mad1*. Mad1-GFP localizes to nuclear pore complexes and is highlighted by its peripheral punctate localization pattern at the nuclear periphery. The localization of Mad1-GFP is compared relative to Nup159-mRFP.

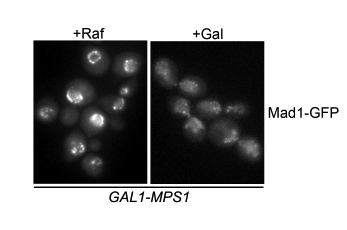


**Figure 5.** *Deletion of Nup60 results in release of Mad1 from the nuclear periphery.* In wildtype cells, Mad1 is associated with NPCs (i). The ability of Mad1 to associate with NPCs is dependent on its interaction with the proteins that make up the nuclear basket known as Mlp1 and Mlp2. Deletion of Nup60 (*nup60Δ*) causes the release of Mlp1/Mlp2from NPCs where they concentrate in a single intranuclear focus that still retains Mad1 binding (ii). Treatment of *nup60Δ* cells with nocodazole induces the recruitment of Mad1 onto unattached kinetochores (iii and iv). (Figure adapted from Scott et. al *JCB* 184, 21-29)

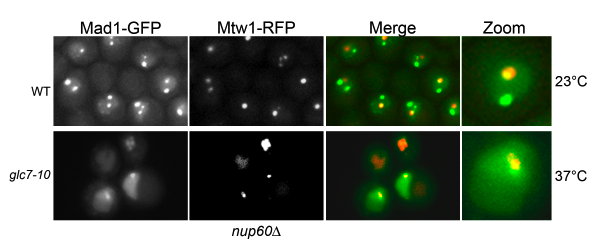
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**Figure 6.** *Mad1 dynamically cycles between NPCs and unattached Kinetochores during SAC arrest.* Fluorescence recovery after photobleaching (FRAP) data demonstrating that kinetochore associated Mad1 recovers at a rapid rate following being bleached with full intensity 488-nm light. Fluorescence recovery at kinetochores accompanied by concomitant decay in fluorescence intensity at the Mlp foci reflecting dynamic exchange of Mad1-GFP between these two intranuclear sites. This experiment was conducted in a strain lacking Nup60 (*nup60Δ).* Also, the inner kinetochore component Mtw1 is tagged with mRFP in order to visualize the location of kinetochore bound Mad1. (Figure adapted from Scott et. al *Mol Biol Cell* 16, 4362-74)

**Figure 7.** *A model for the binding alterations within the Nup53-containing subcomplex that drive Kap121-import inhibition*. During unperturbed cellular division, Nup53 directly interacts with Nup170 and this interaction serves to mask a Kap121-binding domain located in the C-terminal region of Nup53. Under these conditions, Kap121 can efficiently translocate through the central conduit and into the nucleoplasm. However, during nocodazole-induced metaphase arrest, by an as of yet undefined molecular mechanism, binding alterations occur within this subcomplex inducing the release of Nup53 from Nup170. This leads to the exposure of the Kap binding domain in Nup53 allowing for Kap121 to establish a high affinity interaction with Nup53 thereby impeding its entry into the nucleus. (Figure adapted from Makhnevych et. al *Cell* 115, 813-23)

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**Figure 8.** *Mps1 overproduction drives Mad1 off of the nuclear periphery.* Under cycling conditions, Mad1 distinctly localizes to the nuclear periphery in a punctate like pattern indicative of its NPC association. Upon overproduction of the kinase Mps1, Mad1 fails to robustly concentrate at the nuclear periphery and instead redistributes to kinetochores and the nucleoplasm. (Figure adapted from Cairo *et.al* to be submitted)

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**Figure 9.** *Inactivation of the Glc7 during SAC arrest results in the inability of Mad1 to re-associate with the intranuclear Mlps.* In otherwise wildtype cells lacking Nup60p (*nup60Δ*)*,* SAC activation causes Mad1-GFP to be visible at two intranuclear foci, one overlapping with the kinetochore marker Mtw1-RFP, and the other that is associated with an intranuclear Mlp body. Inactivation of Glc7 (*glc7-10)* at 37° C during SAC arrest does not impair Mad1 kinetochore association, however there is an observable loss of association of Mad1-GFP at the intranuclear Mlp foci. (Figure adapted from Cairo *et.al* to be submitted)

**Table1.***Table of Abbreviations.*

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| **Abbreviations** |  |
| **NPC** | Nuclear Pore Complex |
| **NE** | Nuclear envelope |
| **KAP** | Karyopherin |
| **PP1** | Protein Phosphatase-1 or Glc7 |
| **SAC** | Spindle Assembly Checkpoint aka Mitotic Checkpoint |
| **FRAP** | Fluorescence Recovery After Photobleaching |
| **KT** | Kinetochore |
| **TS** | Temperature Sensitive |
| **NUP** | Nucleoporin |