A conserved role for COMA/CENP-H/I/N kinetochore proteins in the spindle checkpoint

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The COMA/CENP-H/I kinase complex regulates microtubule dynamics at kinetochores. The complex is also required to generate spindle checkpoint signals in both yeast and human cells under conditions where Aurora B activity is compromised. Our data explain why mammalian cells treated with Aurora inhibitors still have a functional spindle assembly checkpoint (SAC), since the checkpoint signals through CENP-H/I/N. The SAC effect from depleting the CENP-H/I/N complex cannot be explained by a weakened SAC signal, and the complex has no role in the SAC response to paclitaxel. We propose a model to explain the differential response of human cells to nocodazole and paclitaxel.

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Improperly attached kinetochores prevent anaphase onset by generating a signal through the spindle assembly checkpoint (SAC) (Nezi and Musacchio 2009). Robust SAC signals can be generated by a single kinase that recruits the SAC proteins Mps1, Mad1, Mad2, BubR1, Bub1, and Bub3, which are modified to generate a signal that can inhibit mitotic progression (Musacchio and Salmon 2007). The KMN complex, composed of Knl1, Mis12, and the Ndc80 kinetochore complex proteins, binds microtubules and is required to recruit SAC proteins to unattached kinetochores (McCleland et al. 2003; Kiyomitsu et al. 2007). It remains unclear how kinetochores coordinate SAC protein recruitment with microtubule attachments to regulate the signal.

The 17-protein constitutive centromere-associated network (CCAN) has multiple functions in kinetochores (Foltz et al. 2006). It recruits CENP-A to centromeres, anchors kinetochores to centromeric nucleosomes, and has a role in chromosome congression (Liu et al. 2003; Carroll et al. 2009, 2010; Gascoigne et al. 2011; Screrpani et al. 2011). Most of the CCAN proteins are conserved in budding yeast, where they are members of the COMA and COMA-associated complexes (De Wulf et al. 2003; Westermann et al. 2003; Meraldi et al. 2006). The CENP-H/I complex is a subassembly of CCAN that includes the CENP-H/I/K proteins and is required to recruit the CENP-O/E/F/Q proteins to kinetochores. The CENP-H/I complex regulates the dynamics of microtubules that are embedded in kinetochores, and CENP-N contacts the CENP-A nucleosome. (Carroll et al. 2009; Amaro et al. 2010)

The SAC signal is generated by kinetochores that lack embedded microtubules or by kinetochores that are not physically separated by pulling forces from the mitotic spindle (Nezi and Musacchio 2009). The latter may be indirect because the Aurora kinases can release kinetochore-bound microtubules (Biggins et al. 2001; Tanaka et al. 2002). Paclitaxel treatment allows kinetochores of human cells to bind microtubules but causes cells to arrest in mitosis in an Aurora B-dependent manner (Ditchfield et al. 2003; Hauf et al. 2003). In contrast, cells lacking Aurora B activity arrest in the presence of microtubule-depolymerizing drugs (Ditchfield et al. 2003; Hauf et al. 2003). Yeast cells lacking cohesion between sister chromatids arrest in mitosis. However, cells lacking cohesion cannot arrest if the Aurora kinase Ipl1 is unable to phosphorylate Mad3, but are able to arrest when microtubules are completely depolymerized (King et al. 2007). In this study, we build on Aurora-dependent and Aurora-independent checkpoint signaling responses in yeast to identify novel SAC proteins. We show that yeast require Chl4, Cct3, and Cct19, members of the COMA-associated kinetochore complex [COMA], to arrest in response to microtubule depolymerization when Mad3 is unphosphorylated by Ipl1. This novel SAC response is evolutionarily conserved. Human cells lacking COMA homologs CENP-H, CENP-I, or CENP-N [hereafter referred to as CENP-H/I/N] arrest in paclitaxel but require full activity of Aurora kinase to arrest in response to microtubule depolymerization by nocodazole. CENP-H/I/N is required to recruit the Mad2 SAC protein to prometaphase kinetochores. We found no role for the CENP-H/I/N in paclitaxel, arguing that the CENP-H/I/N pathway is extinguished upon microtubule binding. We confirmed that Aurora has a SAC role independent of releasing microtubules and propose that the spindle checkpoint has independent branches that converge on cell cycle regulation.

Results and Discussion

Budding yeast depleted of Cdc6 enter mitosis in the absence of DNA replication and arrest cell cycle progression by triggering the SAC (Stern and Murray 2001). Ipl1 must phosphorylate Mad3 on S337 to generate a SAC-dependent arrest in response to the loss of sister chromatid cohesion [King et al. 2007]. mad3-S337A cells [hereafter referred to as mad3*] are unable to arrest as large-budded cells [mitotic] in response to Cdc6 depletion but are able to arrest in response to benomyl, which depolymerizes microtubules (Fig. 1A). This is in contrast to the previous study that required an additional mutation in mad3 for the similar phenotype for reasons that are probably attributable to strain differences (King et al. 2007). The ability to arrest when microtubules were missing but not after Cdc6 depletion suggested that there was an additional component of the SAC in mad3* cells mediating the microtubule-
dependent arrest. To identify this SAC activity, we performed a genome-wide screen using synthetic genetic array (SGA) technology to construct all possible double mutants with the haploid deletion collection [Tong et al. 2001]. We compared the benomyl sensitivity of haploids from the deletion collection to double mutants. We were interested in any mutants where growth in the presence of a sublethal concentration of benomyl was reduced in the double mutant (Fig. 1B). chl4 was benomyl-sensitive as a single mutant but had enhanced sensitivity in the double mutant, similar to mad3Δ (Fig. 1C). The chl4 and mad3Δ cells arrested as large-budded cells, but the double-mutant cells did not and behaved like mad3Δ cells (Fig. 1D). Chl4 is a member of a three-protein subcomplex of the kinetochore (along with Ctf3 and Ctf19) that is part of the larger COMA complex. All three proteins are required for SAC activity, as judged by their requirement to arrest as double-budded cells during growth in benomyl (Fig. 1D). To confirm the requirement for SAC activity, we analyzed the single and double mutants by synchronous growth. Cells were released from α-factor arrest, followed through mitosis by measuring the degradation of Pds1 and DNA content by flow cytometry. Wild-type, chl4, and mad3Δ cells arrested when microtubules were depolymerized by benomyl as Pds1 levels were stabilized (Fig. 2A–C) and the cells arrested with replicated DNA (Fig. 2F,I). In contrast, chl4 mad3Δ cells did not maintain stable Pds1 or replicated DNA (Fig. 2F,I).

We analyzed COMA homologs in human cells to determine whether they have a conserved role in the SAC. We used siRNA conditions that depleted the CENP proteins but did not appreciably affect CENP-A levels. We confirmed an increase in mitotic index after siRNA-mediated depletion of CENP-H, CENP-I, or CENP-N in HeLa cells after 48 h that had previously been attributed to defects in chromosome segregation [Liu et al. 2003; Amaro et al. 2010]. The arrest required Aurora B activity, as 1-h treatment with the Aurora B inhibitor ZM447439 induced mitotic exit and reduced the mitotic index (Fig. 3A). Inhibiting Aurora B causes paclitaxel-arrested cells to exit mitosis after 1 h, while nocodazole-arrested cells remain in mitosis for 6 h [Ditchfield et al. 2003; Hauf et al. 2003]. HeLa cells were treated with siRNA against CENP-I or LacZ for 32 h and incubated with 3.3 μM nocodazole for an additional 16 h with either the inhibitor ZM447439 [Ditchfield et al. 2003; Hauf et al. 2003] or DMSO added for the final hour. LacZ cells treated with ZM447439 remained arrested in nocodazole, while CENP-I-depleted cells exited mitosis (Fig. 3B). Increasing the concentration of ZM447439 caused an increase in the number of CENP-I-depleted cells that exited mitosis but had little effect on control cells (Fig. 3C). Similar results were obtained with the Aurora B inhibitor Hesperadin, whose structure is unrelated to ZM447439 [Fig. 4A]. In addition, A549, U2OS, and 293T cells in nocodazole also exited mitosis after CENP-I depletion and ZM447439 treatment (Supplemental Fig. 2). The phenotype was due to CENP-I depletion, as it was partially rescued with a plasmid containing CENP-I cDNA (Supplemental Fig. 3). CENP-H and CENP-N are also required for this Aurora B-independent pathway of the SAC [Supplemental Fig. 4A]. Our siRNAs did not have off-target effects on the SAC protein Mad2 [Hubner et al. 2010; Westerhout et al. 2010] because the intracellular Mad2 levels remained constant after siRNA treatment (Supplemental Fig. 4A).
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Figure 3. Depletion of CENP-I impairs the ability of ZM447439-treated HeLa cells to arrest in nocodazole. (A) HeLa cells depleted of CENP-H, CENP-I, or CENP-N increased the basal mitotic index in a manner sensitive to Aurora kinase inhibition. Western blots of depletion are shown in Supplemental Figure 5. Even a modest depletion of CENP-H resulted in a significant increase in mitotic index that was susceptible to Aurora kinase inhibition. ( [*] 0.02; [**] 0.005. (B) CENP-I siRNA-treated HeLa cells exit mitosis in the presence of nocodazole after 1-h treatment with ZM447439, while LacZ control cells remain arrested. (C) CENP-I-depleted HeLa cells treated with nocodazole exit mitosis in response to ZM447439 in a dose-dependent manner. (D) Western blots of HeLa whole-cell lysate demonstrating degree of CENP-I depletion.

Figure 4. CENP-I-depleted HeLa cells are sensitive to Aurora B inhibition in the presence of nocodazole but arrest similarly to control cells at low doses of paclitaxel. (A) CENP-I-depleted HeLa cells treated with either Hesperadin or ZM447439 at concentrations that inhibit 50% of Aurora B activity produce similar reductions in mitotic index. Similar numbers of cells arrested in either 3.3 μM or 0.33 μM nocodazole. (B) Anti-tubulin IF of LacZ or CENP-I siRNA-treated HeLa cells following treatment with 0.33 μM or 3.3 μM nocodazole, demonstrating that 3.3 μM nocodazole is required to completely depolymerize cellular tubulin. (C) CENP-I-depleted HeLa cells arrest at concentrations of paclitaxel similar to controls.
>97% depletion of Ndc80 complex activity to abrogate the SAC signal (Meraldi et al. 2004). In contrast, the levels of Mad2 were significantly reduced to 10% of wild-type levels ($P = 0.0031$), and Mad1, which recruits Mad2 to prometaphase kinetochores, was also reduced (Fig. 5), in agreement with a previous report [Liu et al. 2003]. BubR1 was present at prometaphase kinetochores after CENP-I depletion at levels not different from control cells (Fig. 5A,B). The levels of the Aurora B substrate pS7CENP-A were increased almost 300% in CENP-I-depleted cells over controls, even though the amount of substrate was slightly reduced. We did not detect a significant difference in Aurora B levels at these kinetochores, indicating that net Aurora B activity is increased at the kinetochores of CENP-I-depleted cells, consistent with our observation that CENP-I-depleted cells arrest in an Aurora B-dependent manner.

We demonstrated that a subset of kinetochore proteins from the COMA-associated complex in yeast and their homologs, CENP-H/I/N in humans, play a role in both recruiting Mad2 to prometaphase kinetochores and generating the SAC signal. The role of CENP-H/I/N is unique among SAC proteins and is only uncovered when Aurora kinase activity is compromised. We found no role for CENP-H/I/N in a paclitaxel arrest that is dependent on high amounts of Aurora B activity [Ditchfield et al. 2003; Hauf et al. 2003]. Previous studies have struggled with the role of Aurora kinase in the SAC, in part because the enzyme can activate the checkpoint indirectly by generating unattached kinetochores, making it difficult to assign a direct signaling role to the kinase [Pinsky et al. 2006; Santaguida et al. 2011]. Our data support a direct role for Aurora B in checkpoint signaling in cells treated with nocodazole (Santaguida et al. 2011; Saurin et al. 2011). Furthermore, they are the first to explain why cells lacking Aurora B activity in nocodazole can still arrest [Ditchfield et al. 2003; Hauf et al. 2003], since they are still able to generate a signal through the CENP-H/I/N complex. Mad1 and Mad2 recruitment are codependent on three kinetochore complexes. Ndc80 and RZZ complexes are also required for Mad1 and Mad2 recruitment to kinetochores, and both complexes are at kinetochores depleted of CENP-I (Fig. 5; Supplemental Fig. 6; Liu et al. 2003).

We propose a model to explain the differential response of human cells to nocodazole and paclitaxel and the requirement of CENP-H/I/N in the nocodazole response. Mad1/2 recruitment by the three kinetochore complexes RZZ, Ndc80, and CENP-H/I/N is antagonized by kinetochore microtubule attachment [Fig. 5C]. Aurora B has at least two functions in SAC signaling: BubR1 and RZZ recruitment to kinetochores. The latter is also required to recruit Mad2. Paclitaxel only triggers the Aurora B recruitment of BubR1, and the entire RZZ, Ndc80, and CENP-H/I/N branch is extinguished by microtubule attachment. Nocodazole triggers both branches of the SAC pathways. The paclitaxel response requires Mad1 and Mad2, which can be explained by either additional roles for the soluble pools of these proteins or occasional release of paclitaxel-stabilized microtubules from kinetochores [Waters et al. 1998; Musacchio and Salmon 2007].

Since taxanes and vinca alkaloids are effective chemotherapy agents [Matson and Stukenberg 2011], the identification of any new proteins involved in spindle checkpoint signaling provides a promising new avenue for the development of anti-neoplastic therapies. Our findings also suggest that paclitaxel arrests cancer cells in a manner that depends solely on Aurora kinase activity, while microtubule destabilizing agents like nocodazole also arrest cells through the CENP-H/I/N complex. It will be important to determine how this insight can be used to generate more efficacious combination chemotherapeutic strategies.

Materials and methods

Yeast strains and media

Yeast strains are listed in Supplemental Table 1. Ser 337 on Mad3 was mutated (mad3-S337A) with QuickChange [Stratagene] following the manufacturer’s instructions. PDS1-3HA in pVG319 digested with KpnI was integrated at PDS1. GAL-CDC6 was integrated using pG15 [PmlI], and transformants were selected on 1% galactose + 2% raffinose containing SC-Trp. Transformations were as described (Gietz and Schiestl 2007). YM-1, YPD, and SC media were prepared as described [Amberg et al. 2005].

Benomyl sensitivity assay

Cells were grown to saturation in YM-1, and 5 μL of the 10-fold serially diluted aliquots were spotted and incubated for 4 d at 23°C.

Yeast cell cycle experiments

Cells in YM-1 or SC-Leu (OD = ~0.4) were synchronized in G1 with 1:200 dilution of a 10 mM α-factor stock solution in acidic YM-1 + Glu (pH 3.41) as described previously [Yellman and Burke 2004]. Arrested cells were washed three times with water and released into YM-1 medium in the presence or absence of microtubule-depolymerizing drugs. Nocodazole (13μg/mL) was used to induce microtubule depolymerization. Some experiments were with a mixture of 25 μg/mL carbendazim (Sigma) + 10 μg/mL
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benomyl, and either treatment is labeled “Ben.” Samples were collected for immunoblots and FACScan every 20 min. Protein extracts were prepared as described previously [Kushnirov 2000]. Immunoblots were for mouse monoclonal 12CAS and anti-PGK1 [Yellman and Burke 2004]. Samples were fixed with 70% ethanol and prepared for flow cytometry as described previously [Yellman and Burke 2004]. The DNA content of 40,000 cells was determined for each sample.

Yeast budding assay

Cells were spread onto agar Petri plates containing YPD and YPD in 70 μg/mL benomyl in 1% DMSO, and pictures of random fields were taken (ts). The same fields were photographed after 6 and 9 h, and the unbudded cells (to) were categorized into double-budded or multibudded phenotypes.

Genetic screen

The plasmid-borne mad3-S337A allele (marked by LEU2) was crossed into the haploid deletion collection, and double mutants were obtained in a manner analogous to that described previously [Tong et al. 2001]. Diploids were selected and sporulated, and haploids were selected as described [Tong et al. 2001]. The haploid deletion library and the haploid double mutants were individually replica-plated onto agar medium containing YPD 15μg/mL benomyl and compared after 4 d.

Cell culture, siRNA, and drug treatments

HeLa cells were cultured in Dulbecco’s modified Eagle medium (DMEM) and plated at 25% confluency into 12-well plates the evening prior to siRNA transfection. Transfection of siRNA was carried out using RNAiMAX [Invitrogen] as described by the manufacturer. CENP-siRNAs (Dharmacon) were used at 20 nM [sequences in Supplemental Table 2]. LacZ siRNA was the control. A different siRNA against the CENP-I untranslated region was used in the rescue experiment (Qiagen) at 50 nM. Nocodazole was used at 3.3 μM, paclitaxel was used at 1 μM, and treatment periods were 16 h, unless noted otherwise. ZM447439 (Tocris) was used at 70 μM nocodazole. ZM447439 (2 μM) were added to one group for the last hour. The cells were then trypsinized and assayed for mitotic index.

CENP-I rescue experiments

pCS2-CENP-I was constructed using restriction sites Cla1 and Xho1. HeLa cells were plated at 30% and the next morning were transfected with 50 nM siRNA against CENP-I UTR. Medium was changed after 6 h. The cells were transfected with either pCS2-CENP-I or pCS2+ control plasmid after 24-h incubation, the medium was changed after 6 h, and the cells were allowed to incubate for 48 h post-transfection. For the last 16 h, the cells were treated with 3.3 μM nocodazole. ZM447439 (2 μM) was added to one group for the last hour. The cells were then trypsinized and assayed for mitotic index.

Cell fate experiments

HeLa cells were seeded into 1.5 borosilicate chamber slides and treated with 20 nM CENP-I siRNA or Lipofectamine control the following morning. After 6 h, the medium was replaced with DMEM/10% FBS and 15 mM HEPES (pH 7.4). Cells were incubated for 36 h and then placed in 1 μM paclitaxel. The cells were then imaged for 20 h on a DeltaVision microscope [Applied Precision] [Barnhart et al. 2011] using a 40× objective, with bright-field images captured every 10 min. Duration of mitoses and cell fates were scored by analyzing the resulting movies. Cells that remained after imaging were fixed for IF against CENP-I as previously described [Bolton et al. 2002] to assess the degree of depletion.

Statistical methods

For statistical analysis, mean values with standard deviation (SD) are shown in most graphs that were generated from several independently obtained data sets. P-values were obtained from t-tests with paired or unpaired samples.

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Kinetochore and the spindle checkpoint

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