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A Novel Protein Phosphatase 1-Dependent Spindle Checkpoint Silencing Mechanism

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Summary

The spindle checkpoint is a surveillance system acting in mitosis to delay anaphase onset until all chromosomes are properly attached to the mitotic spindle [1, 2]. When the checkpoint is activated, the Mad2 and Mad3 proteins directly bind and inhibit Cdc20, which is an essential activator of an E3 ubiquitin ligase known as the anaphase-promoting complex (APC) [3]. When the checkpoint is satisfied, Cdc20-APC is activated and polyubiquitinates securin and cyclin B, leading to the dissolution of sister chromatid cohesion and mitotic progression. Several protein kinases play critical roles in spindle checkpoint signaling, but the mechanism (or mechanisms) by which they inhibit mitotic progression remains unclear [4]. Furthermore, it is not known whether their activity needs to be reversed by protein phosphatases before anaphase onset can occur. Here we employ fission yeast to show that Aurora (Ark1) kinase activity is directly required to maintain spindle checkpoint arrest, even in the presence of many unattached kinetochores. Upon Ark1 inhibition, checkpoint complexes are disassembled and cyclin B is rapidly degraded. Importantly, checkpoint silencing and cyclin B degradation require the kinetochore-localized isoform of protein phosphatase 1 (PP1\textsuperscript{Dis2}). We propose that PP1\textsuperscript{Dis2}-mediated dephosphorylation of checkpoint components forms a novel spindle checkpoint silencing mechanism.

Results

Aurora Kinase Activity Is Required for the Maintenance of Spindle Checkpoint Arrest in the Absence of Microtubules

The role of Aurora kinases in checkpoint signaling remains controversial [5, 6]. Budding yeast and mammalian studies suggest that Aurora B is only required for checkpoint delays where kinetochores lack tension as a result of inappropriate attachments [7–9]. These studies argue that Aurora B has an indirect role in the spindle checkpoint. That role is to recognize inappropriately attached kinetochores, disrupt microtubule binding, and thereby produce unattached kinetochores [10] that can be recognized by the Mad/Bub proteins, leading to Cdc20 inhibition. However, two reports have argued that Aurora B activity is also required to respond to unattached kinetochores in Xenopus [11] and fission yeast [12]. In addition, we have previously shown that Aurora-dependent phosphorylation of budding yeast Mad3p is required for the checkpoint response to lack of tension at microtubule-bound kinetochores [6]. These findings argue that Aurora kinase has one or more direct roles to play in spindle checkpoint signaling in addition to the production of unattached kinetochores.

PP1 Phosphatase Is Necessary for Checkpoint Silencing

Fission yeast has only one Aurora kinase (Ark1), and this kinase is known to carry out the Aurora B role of correcting inappropriate kinetochore-microtubule attachments [13]. It was previously argued by means of ark1 promoter shutoff and conditional survivin alleles that Aurora function is required to respond to unattached kinetochores [12]. Because these experiments took several hours to inactivate Ark1 kinase, we wanted to confirm the result. A conditional, ATP analog-sensitive allele of Aurora kinase (ark1-as3) was recently described in fission yeast [13]. With this strain, we can activate the spindle checkpoint and then inhibit Aurora kinase activity simply by adding the ATP analog (1NMPP1) to cells. Microtubule depolymerization via the cold-sensitive tubulin mutant nda3-KM311 [14] results in a robust, spindle-checkpoint-dependent mitotic arrest, because all kinetochores are unattached [15, 16]. nda3-arrested cells accumulate high levels of cyclin B (Cdc13) on spindle pole bodies (SPBs) (see Figure 1). To directly test whether Aurora kinase activity is required to maintain spindle checkpoint arrest in fission yeast, we made movies of arrested nda3ark1-as3 cells, added 1NMPP1, and followed cyclin B levels by microscopy. The SPB-enriched nuclear Cdc13-GFP signal decreased within 15 min of Aurora inhibition (Figure 1A), and many cells decondensed their chromosomes and separated (Figure 1B). We quantitated the fraction of arrested and escaped cells (Figure 1C) in a 60 min time course, during which cells were maintained at 18°C with no microtubules and unattached kinetochores (Figure 1D). In fission yeast, Mad2p and Mad3p bind to the anaphase-promoting complex (APC) upon checkpoint arrest [17], and the escape from mitotic arrest upon Ark1 inhibition correlated well with the loss of Mad3p and Mad2p-APC binding (Figures 1E and 1F). Note that in this assay, the nda3 cells were maintained at 18°C throughout, spindles did not form, and all kinetochores remained unattached (Figure 1D). Thus, we directly assayed the ability of cells to degrade cyclin B after Ark1 inhibition independently of its role in kinetochore-microtubule attachment and error correction processes. A similar result was obtained when an experiment was performed at 32°C in the presence of the microtubule-depolymerizing drug carbendazim, ruling out the possibility that cyclin B degradation upon Ark1 inhibition is a side effect of growing cells at 18°C (see Figure S1 available online). We conclude that Ark1 activity is required to maintain spindle checkpoint arrest, even in the presence of many unattached kinetochores.

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Figure 1. Ark1 Activity Is Required to Maintain the Mitotic Checkpoint Complex/Anaphase-Promoting Complex and Cell-Cycle Arrest

(A) In the presence of the ATP analog 1NMPP1, Ark1 is the only kinase directly inhibited in ark1-as3 cells [13]. The spindle checkpoint was activated via the cold-sensitive tubulin mutant nda3-KM311. After 6 hr at the restrictive temperature, 80%–90% of cells were arrested with high levels of cyclin B on spindle pole bodies (SPBs), and all kinetochores were unattached. At this point, DMSO (control) or 1NMPP1 was added to the culture to inhibit Ark1, and the levels of cyclin B were monitored by live-cell imaging. In the presence of 1NMPP1, cyclin B levels dropped rapidly, demonstrating that Ark1 activity is required to maintain spindle checkpoint arrest in response to unattached kinetochores. The time after DMSO or 1NMPP1 addition is indicated in minutes.

(B) Cells were fixed 40 min after addition of 1NMPP1. Most cells escaped arrest and underwent cytokinesis (arrows).

(C) Top: after addition of 1NMPP1, a few cells remain arrested with high levels of cyclin B on SPBs (cell 1), whereas most escaped the arrest and precociously degraded cyclin B (cell 2). Bottom: the number of “escaped” cells was quantified in a 60 min time course. Data are derived from four independent experiments; error bars represent standard deviation.

(D) 1NMPP1 addition and Ark1 inhibition have no effect on microtubule depolymerization in the nda3KM311 mutant. Anti-tubulin immunofluorescence confirms that at 18°C, all microtubules are depolymerized, whereas spindles are formed 15 min after release at 32°C.

(E) Mitotic checkpoint complex/anaphase-promoting complex (MCC–APC) complexes are disassembled following Aurora inhibition with 1NMPP1. Lid1-TAP ark1-as3 Mad2-GFP Mad3-GFP nda3KM311 cells were arrested in mitosis (6 hr at 18°C), and the culture was divided before DMSO or 5 μM 1NMPP1 addition. Lid1p was immunoprecipitated from samples collected every 10 min and immunoblotted for associated Mad2-GFP and Mad3-GFP.

(F) Model of MCC–APC binding.
checkpoint arrest (nda3)

% of cells with high levels of cyclin B on SPBs

3 hr 4 hr 5 hr 6 hr 7 hr 8 hr

time after temp shift to 18°C

checkpoint silencing

% of escaped cells

0 20 40 60 80 100 120

time after 1NMPP1 addition (min)

nda3 release assay

% of metaphase cells

0 5 10 15 20 25 30

time after nda3 release (min)

nda3 Kar1-as3

% of anaphase cells

0 5 10 15 20 25 30

time after nda3 release (min)

It has proven difficult to isolate mutants and devise assays that specifically address the mechanism (or mechanisms) of checkpoint silencing. This is due to the intimate link between biorientation and checkpoint signaling: an apparent failure to silence the checkpoint could be caused by prolonged checkpoint activation if defective kinetochore-microtubule interactions persist. The nda3Kar1-as3 “silencing” assay described above (Figure 1) is independent of chromosome attachment and biorientation and is thus specific for checkpoint silencing and APC activation. We used this assay to screen fission yeast mutants and thereby identify factors that act downstream of Ark1 during checkpoint silencing. Silencing mutants should fail to degrade cyclin B upon ark1-as3 inhibition. In particular, we tested whether protein phosphatase activity was necessary to silence the checkpoint. PP1 phosphatase has been argued to oppose Aurora kinase in a number of systems [22–24]. Fission yeast expresses two PP1 phosphatases, PP1Dis2 and PP1Sds21 [25], and PP1Dis2 has been localized to kinetochores (26) and data not shown). To analyze the role of PP1, we constructed dis2Δnda3 and sds21Δnda3 strains. These phosphatase mutants undergo spindle checkpoint arrest efficiently (Figure 2A). Strikingly, addition of the Ark1 inhibitor 1NMPP1 to dis2Δnda3ark1-as3-arrested cells failed to result in cyclin B degradation, even after 2 hr (Figure 2B). This silencing defect was highly specific: lack of the other PP1 phosphatase (PP1Sds21) or of two other centromeric phosphatases (PP2APart and the Cdc14 homolog Flp1p/Clp1p) did not delay cyclin B degradation upon Aurora inhibition (Figure 2B and data not shown). This assay provides direct evidence for a role for PP1Dis2 in spindle checkpoint silencing.

To look for a silencing defect under more physiological conditions, we analyzed the recovery of dis2Δ cells from an nda3 block, where checkpoint silencing takes place after spindle reassembly and chromosome attachment. When nda3 cells were shifted back to their permissive temperature (32°C), they efficiently assembled a spindle and typically entered anaphase within 10–20 min (Figure 1D). We compared nda3 and dis2Δnda3 cell populations and observed a clear delay in anaphase onset following spindle reassembly in dis2Δnda3 (Figures 2C and 2D). Again, this phenotype was specific to the loss of PP1Dis2, because sds21Δnda3 mutants released with wild-type kinetics (Figures 2C and 2D). This demonstrates that, in addition to inhibition of Ark1 kinase activity, the action of kinetochore-localized PP1Dis2 is needed for efficient spindle checkpoint silencing.

Discussion

This study has two important findings highlighting the critical balance between Aurora kinase and PP1 activities in the regulation of anaphase onset:

1. Ark1 activity is required to maintain a spindle checkpoint arrest, even in the presence of many unattached kinetochores (see Supplemental Discussion on Aurora kinases and checkpoint signaling). This suggests that Ark1 has important Mad/Bub checkpoint substrates, which is consistent with our previously published budding yeast work [6].

2. We describe a novel role for kinetochore-localized protein phosphatase 1, PP1Dis2, in spindle checkpoint...
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Figure 3. Silencing Model

Top: at unattached kinetochores, centromeric chromatin is not under tension, and centro-
more-localized Ark1 kinase (the green cloud of activity) is in close proximity to kinetochore-
localizd checkpoint components and can phosphorylate them efficiently, leading to effective
APC inhibition.

Bottom: stable attachments are formed after bio-
rientation and lead to inter- and intrakinetochore
stretching. This displaces Ark1 from kinetochore
substrates and reduces its ability to phosphory-
late checkpoint components, which can in turn
be dephosphorylated by PP1^{Dis2} (orange clouds
of activity), leading to checkpoint silencing.

We propose the following simple model to explain how PP1
activity toward the kinetochore is regulated by kinetochore
attachment to microtubules (Figure 3). Upon chromosome
biorientation, inter- and intrakinetochore stretching spatially
separates Aurora B kinase from outer kinetochore substrates,
such as Ndc80 and MCAK, and thereby stabilizes kinetochore-
microtubule attachments [27–30]. In the same way, this
stretching could separate Aurora B from relevant checkpoint
targets, such as BubR1/Mad3 [6], whose modification is impor-
tant for spindle checkpoint arrest (Figure 3). Physical separa-
tion of Aurora B from kinetochore substrates is a key silencing component
and thereby induce anaphase onset [19, 20,
and it is not yet clear how p31^{comet} function is regulated by
kinetochore attachment. Cdc20 ubiquitylation might lead to
disruption of Mad2/BubR1 binding and thereby relieve check-
point inhibition [21]. However, a nonubiquitylatable Cdc20
mutant where all lysine residues had been replaced with argi-
nine was recently shown to have little difficulty in exiting
spindle checkpoint arrests [36]. In vertebrates, CENP-E-
BubR1 interactions have been proposed to silence the check-
point upon chromosome attachment [37], but yeast and C. ele-
gans lack a CENP-E homolog, and their Mad3 lacks a C-
terminal kinase domain. Finally, in budding yeast it has been
argued that Mps1 kinase is degraded to prevent checkpoint
signaling in anaphase [38], but this has yet to be confirmed in
other systems. Although there could be several ways to turn
off the spindle checkpoint, we propose that PP1 plays an
important role in several of these silencing mechanisms. Our
findings on the role of PP1 in spindle checkpoint silencing are
strongly supported by a study of the budding yeast PP1
homolog Glc7 appearing in this issue of Current Biology [39].

Conclusions

We have developed a novel assay completely independent of
kinetochore-microtubule attachment to dissect spindle check-
point silencing mechanisms acting downstream of Aurora
kinase inhibition. This has enabled us to identify PP1^{Dis2} as
a key silencing component. A more in-depth analysis of the
silencing mechanism (or mechanisms) and how it is coordi-
nated with biorientation will be important for a full under-
standing of mitotic regulation and may also be relevant to
human disease. Indeed, the mitotic exit mechanisms utilized
by cells, particularly during clinical treatment of cancers with
antimicrotubule drugs such as taxanes, may in part determine
their fate and thereby the effectiveness of therapies [40, 41].

Experimental Procedures

Yeast Strains

A complete list of all of the strains used in this study is given in Table S1.

silencing. We propose that PP1^{Dis2} has one or more
Mad/Bub protein substrates and do not rule out
Cdc20-APC as another important target that needs to
be dephosphorylated before the checkpoint is silenced
and the APC activated. In a separate study, we have
shown that APC activation upon Ark1 inhibition requires
the Bub3 checkpoint component (unpublished data).
Mad3/BubR1, Bub3, and Bub1 are all found at kineto-
chores later in mitosis than Mad1 and Mad2, which are
no longer recruited to attached kinetochores. There-
fore, we believe that the Bub3p binding partners
(Bub1p and Mad3p) are both strong candidates for
important Aurora and PP1^{Dis2} substrates. To date, we
have mapped >70 phosphorylation sites on these two
proteins, and their functional dissection is a major
goal of ongoing studies.

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their fate and thereby the effectiveness of therapies [40, 41].

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Microscopy

Microscopy was performed as described previously [16] with an Intelligent Imaging Innovations (3i) Marianas microscope, CoolSnap CCD, and SlideBook software (3i). For live-cell imaging in Figure 1A, mid-log arl1-as3 cdc13-GFP nda3-KM311 cells were first arrested in early mitosis in liquid cultures by shifting the temperature to 18°C for 6 hr. Precleared dimethyl sulfoxide (DMSO) or 5 μM 1NMPP1 was added to the culture, and cells were immediately mounted on precooled (18°C) slides and coverslips and imaged on a prewarmed (18°C) microscope stage. Images were acquired every minute. At least ten movies were acquired for each condition.

Checkpoint Silencing Assay

Mid-log arl1-as3 cdc13-GFP nda3-KM311 cells were arrested in early mitosis in liquid cultures by shifting to 18°C for 6 hr. The culture was divided in two, and DMSO or 5 μM 1NMPP1 was added. Cells (300 μl) were fixed every 10 min by mixing with 1 ml 100% methanol precooled to −80°C and once with phosphate-buffered saline containing 0.02% Tween 20.

Mitotic Checkpoint Complex/Anaphase-Promoting Complex Interaction

Cells expressing TAP-tagged Lid1 (Apc4) and Mad2p and Mad3p tagged with GFP from their endogenous loci were presynchronized either in G2 via the cdc25-22 mutation (Figure 3) or in early mitosis via the nda3-KM311 mutation (Figure 1). Proteins were extracted in lysis buffer (50 mM HEPES [pH 7.6], 75 mM KCl, 1 mM MgCl2, 1 mM EGTA, 0.1% Triton, 1 mM sodium vanadate, microcystin, leupeptin, pepstatin, chymostatin, and Pefabloc) from ≈2 × 10^6 cells as described previously [42]. Extracts were incubated for 30 min with IgG-coupled Dynabeads, which bind to Lid1-TAP. The immunoprecipitated complexes were washed three times with lysis buffer and once with phosphate-buffered saline containing 0.02% Tween 20. Immunoprecipitated complexes were analyzed by immunoblot with a sheep anti-GFP antibody.

nda3-KM311 Release Assay

Mid-log nda3-KM311 cells were arrested in early mitosis in liquid cultures by shifting the temperature to 18°C for 6 hr. Cells were then filtered on a DuraPore filter (Millipore) and released from the filter into prewarmed medium (32°C) by shaking. At each time point following release at 32°C, cells (2 ml) were fixed by mixing with 20 ml 100% methanol precooled to −80°C. Cells were then processed for immunofluorescence with an anti-tubulin antibody (TAT1, a kind gift of K. Gull, University of Oxford).

Supplemental Data

Supplemental Data include Supplemental Discussion, one table, and one figure and can be found with this article online at http://www.cell.com/current-biology/supplemental/S0960-9822(09)01196-8.

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