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REPORT

Mitotic phosphatase activity is required for MCC maintenance during the spindle checkpoint

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ABSTRACT

The spindle checkpoint prevents activation of the anaphase-promoting complex (APC/C) until all chromosomes are correctly attached to the mitotic spindle. Early in mitosis, the mitotic checkpoint complex (MCC) inactivates the APC/C by binding the APC/C activating protein CDC20 until the chromosomes are properly aligned and attached to the mitotic spindle, at which point MCC disassembly releases CDC20 to activate the APC/C. Once the APC/C is activated, it targets cyclin B and securin for degradation, and the cell progresses into anaphase. While phosphorylation is known to drive many of the events during the checkpoint, the precise molecular mechanisms regulating spindle checkpoint maintenance and inactivation are still poorly understood. We sought to determine the role of mitotic phosphatases during the spindle checkpoint. To address this question, we treated spindle checkpoint-arrested cells with various phosphatase inhibitors and examined the effect on the MCC and APC/C activation. Using this approach we found that 2 phosphatase inhibitors, calyculin A and okadaic acid (1 μM), caused MCC dissociation and APC/C activation leading to cyclin A and B degradation in spindle checkpoint-arrested cells. Although the cells were able to degrade cyclin B, they did not exit mitosis as evidenced by high levels of Cdk1 substrate phosphorylation and chromosome condensation. Our results provide the first evidence that phosphatases are essential for maintenance of the MCC during operation of the spindle checkpoint.

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APC/C; mitosis; mitotic checkpoint complex; phosphatases; spindle checkpoint

Introduction

The spindle checkpoint is activated in early mitosis to protect chromosomal integrity by preventing entry into anaphase until all chromosomes are aligned and properly attached to the mitotic spindle. Defects in the spindle checkpoint may lead to missegragated chromosomes and aneuploidy, which could contribute to tumorigenesis.[1] Therefore the spindle checkpoint is essential for maintaining genomic stability.[2-4]

The spindle checkpoint prevents cells from progressing into anaphase by inactivating the anaphase-promoting complex/cyclosome (APC/C), an E3 ubiquitin ligase required for mitotic progression.[5] The primary inhibitor of the APC/C during checkpoint operation is the mitotic checkpoint complex (MCC), which is composed of the APC/C coactivator CDC20 in complex with Mad2, BubR1, and Bub3.[6,7] Although sub-complexes of Mad2-CDC20 and BubR1-Bub3-CDC20 have some inhibitory effect on the APC/C, the complete MCC is a much stronger inhibitor.[6,8-10] Once all chromosomes are properly engaged by the spindle, with one sister chromatid attached to microtubules from one pole and the other sister chromatid to microtubules from the opposite pole, such that tension is applied at the kinetochores (bi-orientation), the checkpoint is satisfied and the MCC releases CDC20, which can in turn activate the APC/C.[11-13] The activated APC/C then targets cyclin B and securin for degradation thereby leading to Cdk1 inactivation and sister chromatid segregation, respectively.[3] APC/C^{CDC20} is also responsible for cyclin A degradation, which occurs slightly earlier in mitosis than cyclin B degradation due in part to its high affinity for CDC20.[14]

Kinetochores lacking tension or attachment to the mitotic spindle form a platform for MCC formation.[3] This process occurs via sequential recruitment of MCC components to the kinetochore, and while there are still many unknowns regarding the molecular mechanisms regulating MCC assembly, it is well established that phosphorylation is essential for both MCC assembly and checkpoint activation.[3,15] One of the key mediators of MCC formation is the kinase MPS1, which is required for kinetochore localization of all known spindle checkpoint components.[3,16-20] Importantly, inhibition of MPS1 leads to disassembly of the MCC and a decrease in cyclin B and securin levels, indicating that the APC/C has been activated.[20] MPS1 phosphorylation of the kinetochore protein KNL1 forms a docking site for Bub1 and Bub3, which in turn recruit BubR1 and a heterodimer of Mad1 and

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Mad2.[21,22] The Mad1-Mad2 complex recruits an additional Mad2 to the kinetochore where it undergoes a conformational change and binds CDC20.[4,23] The Mad2-CDC20 complex then binds BubR1-Bub3 to form the functional MCC, which diffuses away from the kinetochore to inhibit the APC/C.[3,15] Aurora B kinase also contributes to the kinetochore recruitment of several essential checkpoint proteins.[24] Additionally, Aurora B indirectly promotes spindle checkpoint maintenance by destabilizing improperly attached microtubules at the kinetochore.[24-28] Another important kinase for checkpoint signaling is Plk1, which was recently shown to enhance MPS1 activity and the localization of MCC components to kinetochores.[29]

As the MPS1, Aurora B, and Plk1 kinases all promote spindle checkpoint activation, it is unsurprising that protein phosphatases have been implicated in checkpoint silencing. The two main phosphatases known to be involved in checkpoint silencing are PP1 and PP2A-B56. These phosphatases exist in both negative and positive feedback loops with the above mentioned kinases to allow for robust checkpoint activation and also rapid inactivation and dissociation of the MCC upon proper microtubule attachment.[30-33] MPS1 phosphorylation of KNL1 recruits PP2A to kinetochores through its interaction with BubR1.[18,30,34,35] PP1 is also recruited to KNL1, but its binding is inhibited early in prometaphase by strong Aurora B phosphorylation of KNL1.[32] Interestingly, BubR1-associated PP2A-B56 opposes Aurora B phosphorylation of KNL1 thereby promoting PP1 recruitment.[30,31] In addition to PP2A-B56, PP1 has also been shown to oppose Aurora B at the kinetochore, thereby stabilizing kinetochore-microtubule attachments and promoting checkpoint silencing.[30,32,36-38] PP1 and PP2A-B56 have also both been implicated in dephosphorylating MPS1 phosphorylation sites on KNL1, which in turn dissociates PP2A-B56 and the MCC components from the kinetochore.[30,33] Taken together, these findings all indicate that PP1 and PP2A-B56 are essential for spindle checkpoint silencing and MCC disassembly. However, in examining the involvement of phosphatases in the spindle checkpoint, we found that their role is more complex: using 2 phosphoprotein phosphatase (PPP) inhibitors, calyculin A and okadaic acid, we demonstrate that a PPP family Ser/Thr phosphatase is required during the spindle checkpoint to prevent MCC disassembly and APC/C activation.

Results

Calyculin A and okadaic acid can override the spindle checkpoint to activate the APC/C

To test whether protein phosphatases are involved in APC/C inhibition during the spindle checkpoint, we treated synchronized nocodazole-arrested cells with a panel of phosphatase inhibitors and then measured cyclin B levels in cell lysates (Fig. 1A). Cells treated with okadaic acid (0.1 μM), fostriecin, or the calcineurin inhibitors FK506 and cyclosporin A retained high levels of cyclin B, indicating the APC/C was still being inactivated by the spindle checkpoint. However, in cells treated with the phosphatase inhibitors calyculin A or a high concentration of okadaic acid (1 μM), cyclin B levels were dramatically reduced, indicating that the APC/C had been activated as a result of phosphatase inhibition. We also measured cyclin A levels in these cells and observed that levels were high in nocodazole-arrested cells but were reduced following calyculin A or okadaic acid (1 μM) treatment similar to cyclin B. Furthermore, calyculin A treatment accelerated cyclin B degradation during mitotic exit as compared to control cells or cells treated with fostriecin or cyclosporin A (Fig. 1B). As calyculin A and okadaic acid are both inhibitors of the PPP family of Ser/Thr phosphatases, we deduced that a PPP phosphatase must be required during the spindle checkpoint to prevent cyclin A and B degradation.

During normal mitotic exit, cyclin A and B are both degraded via the proteasome after being targeted by the APC/C. To determine whether the calyculin A-induced decrease in cyclin levels was also mediated by the proteasome, synchronized nocodazole-arrested cells were treated with the proteasome inhibitor MG132 prior to treatment with the phosphatase inhibitors. Pre-treatment with MG132 rescued cyclin A and B levels in okadaic acid and calyculin A-treated cells, confirming that okadaic acid and calyculin A induce proteasomal degradation of cyclin A and B (Fig. 1C and 1D). Furthermore, directly inhibiting the APC/C with the small molecule inhibitor proTAME or depleting CDC20 using siRNA also restored cyclin B levels following calyculin A treatment (Fig. 1E and 1F). Therefore, calyculin A-induced cyclin degradation appears to occur via the pathway normally activated upon mitotic exit and is dependent on both the APC/C^{CDC20} and the proteasome. Taken together, these results demonstrate that a PPP phosphatase is necessary for APC/C inactivation during the spindle checkpoint.

Phosphatase activity is required for MCC maintenance during the spindle checkpoint

As the MCC is the major inhibitor of the APC/C during the spindle checkpoint, we hypothesized that calyculin A and okadaic acid (1 μM) may induce MCC disassembly. This dissociation would free CDC20 to activate the APC/C such that it could ubiquitinate cyclin A and B to target them for degradation. To test this hypothesis, endogenous CDC20 was immunoprecipitated from synchronized taxol-arrested mitotic cells treated with our panel of phosphatase inhibitors. In control cells, CDC20 co-immunoprecipitated with the other components of the MCC (Mad2, BubR1, and Bub3), but the binding between CDC20 and the other MCC components was greatly reduced in calyculin A and okadaic acid (1 μM) treated cells (Fig. 2A). Similar results were found when this experiment was repeated in cells arrested by nocodazole (Figure S1). Additionally, CDC20 still disassociated from the MCC in the presence of MG132, which rescued cyclin A and B degradation (Fig. 2B). This result indicates that calyculin A and okadaic acid-induced MCC disassembly is upstream of the proteasomal degradation of cyclin A and B.

To investigate the timing of MCC dissociation with respect to cyclin A and B degradation, nocodazole-arrested cells were treated with calyculin A for varying amounts of time, followed by CDC20 immunoprecipitation and immunoblot analysis of the MCC components and cyclin A and B. CDC20 interaction with BubR1 and Bub3 began to weaken after just 15 minutes of calyculin A treatment while binding with Mad2 was noticeably decreased by 30 minutes

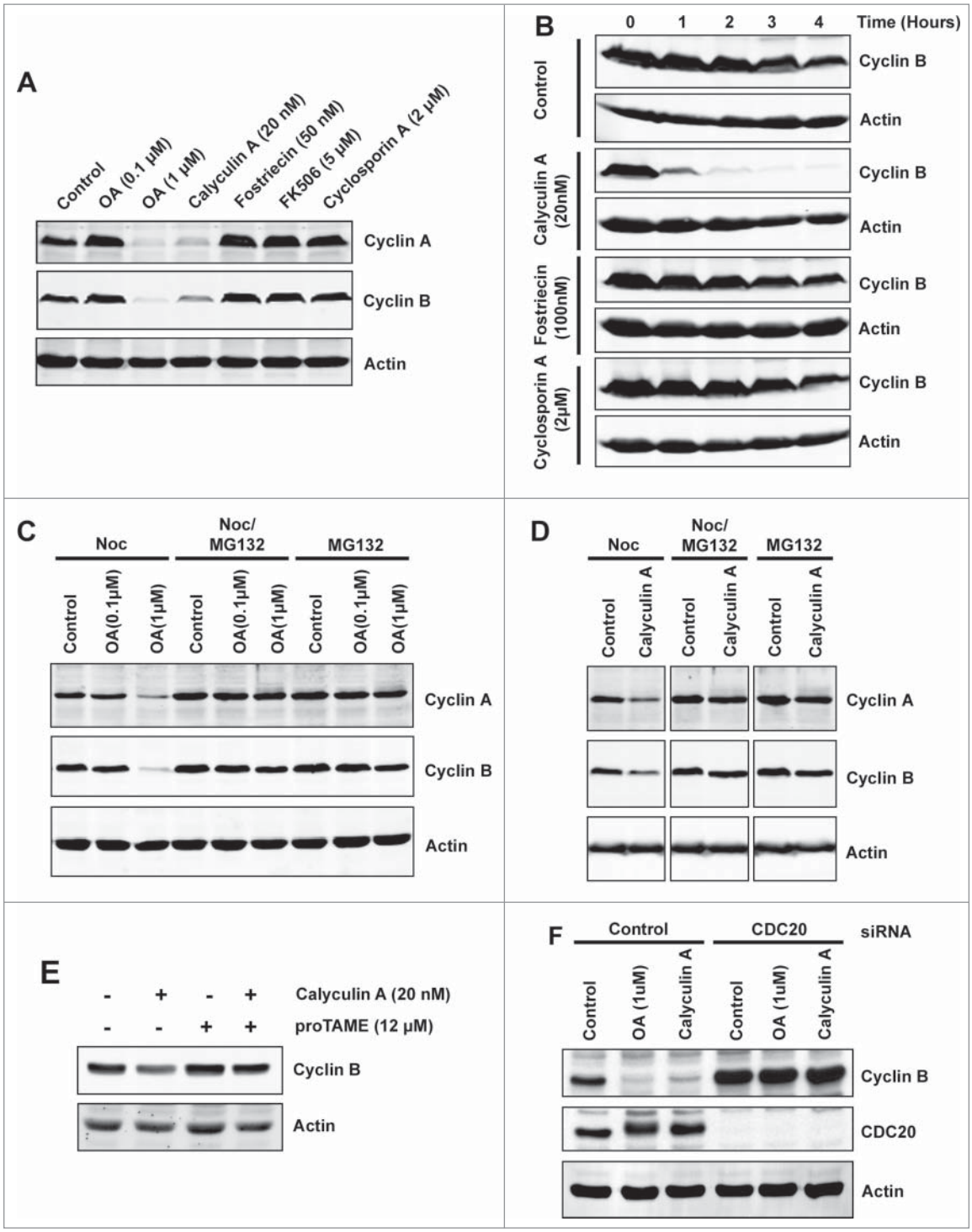

Figure 1. Calyculin A and okadaic acid (1 μM) induce cyclin degradation in spindle checkpoint-arrested cells via the APC/C^{CDC20}. (A) Synchronized nocodazole-arrested HeLa Tet-Off cells were treated with the indicated phosphatase inhibitors for 2 hours. Protein levels were detected by immunoblotting with the indicated antibodies. (B) Synchronized nocodazole-arrested HeLa Tet-Off cells were treated with the indicated phosphatase inhibitors for 30 minutes prior to washing out nocodazole. Fresh media containing the phosphatase inhibitors was then added, and cells were harvested at indicated time points post nocodazole release. Protein levels were detected by immunoblotting with the indicated antibodies. (C and D) Synchronized HeLa Tet-Off cells were treated with nocodazole for 16 hours and then treated with MG132 (20 μM) for 1 additional hour before the indicated phosphatase inhibitors were added for 2 hours. Protein levels were detected by immunoblotting with the indicated antibodies. (E) Synchronized nocodazole-arrested HeLa Tet-Off cells were pretreated with proTAME (12 μM) for 1 hour and then treated with calyculin A (20 nM) for 2 hours. Protein levels were detected by immunoblotting with the indicated antibodies. (F) HeLa Tet-Off cells were transfected with CDC20 or control siRNA, synchronized with a double thymidine block, and released into nocodazole. Cells were then treated with the indicated inhibitors for 2 hours. Protein levels were detected by immunoblotting with the indicated antibodies. OA: okadaic acid; Noc: nocodazole.

and almost completely abolished by 60 minutes (Fig. 2C). Strikingly, the degradation of cyclin A and B began to occur shortly after CDC20 lost its interaction with the MCC components. These data further support our hypothesis that calyculin A-induced cyclin degradation is the result of MCC dissociation and subsequent APC/C activation.

In carrying out these experiments, we observed a dramatic SDS-PAGE mobility shift of both CDC20 and BubR1 after

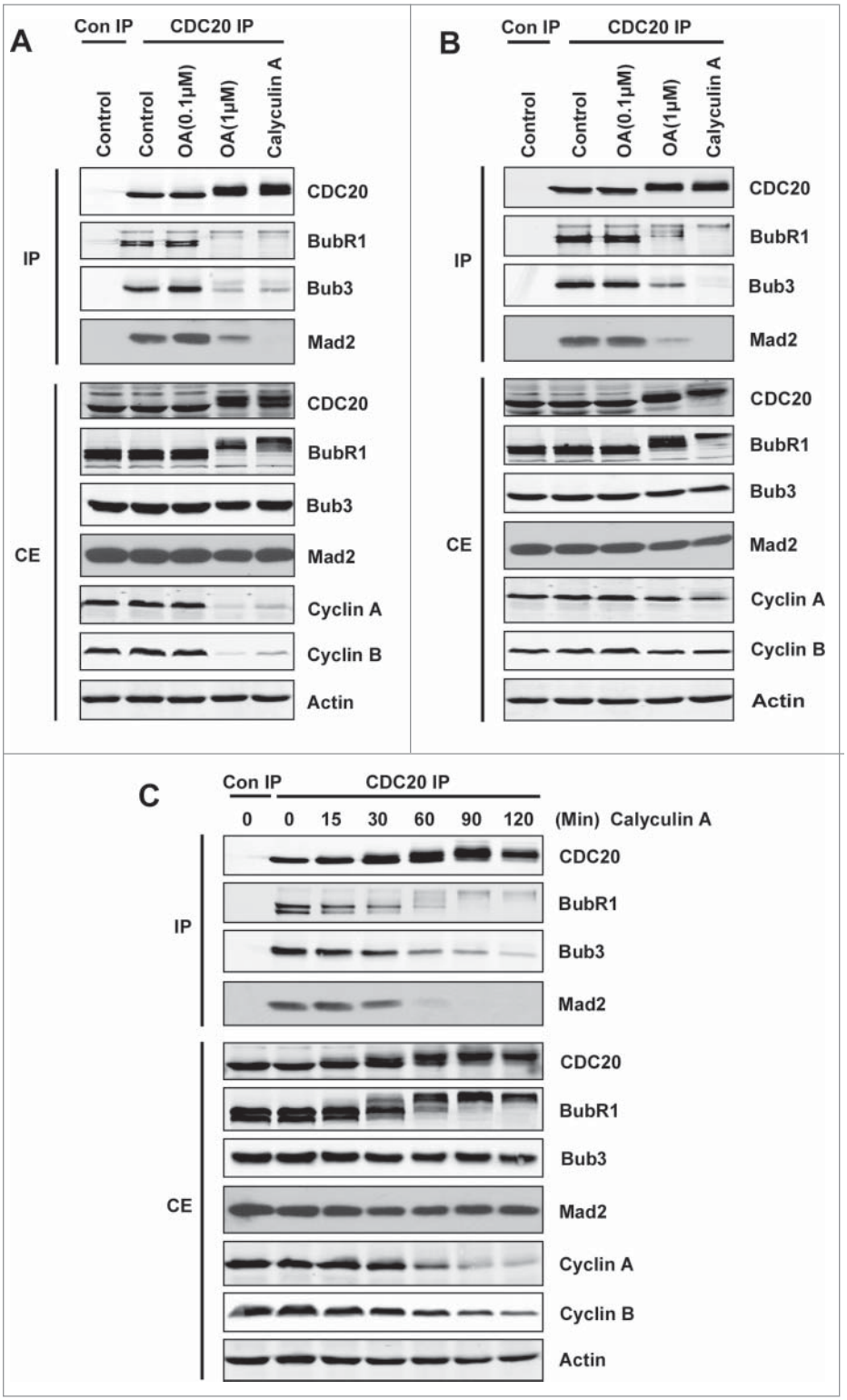

Figure 2. Calyculin A and okadaic acid (1 μM) induce MCC dissociation during the spindle checkpoint. (A) Synchronized taxol-arrested HeLa Tet-Off cells were treated for 2 hours with the indicated inhibitors before cells were harvested and lysed. CDC20 was immunoprecipitated from lysates, and protein levels were detected by immunoblotting with the indicated antibodies. (B) Synchronized nocodazole-arrested cells were pre-treated with MG132 (20 μM) for 1 hour prior to phosphatase inhibitor treatment. CDC20 was immunoprecipitated from lysates, and protein levels were detected by immunoblotting with the indicated antibodies. (C) Synchronized nocodazole-arrested HeLa Tet-Off cells were treated with calyculin A (20 nM) and harvested at the indicated time points. CDC20 was immunoprecipitated from lysates, and protein levels were detected by immunoblotting with the indicated antibodies. Con IP: IgG immunoprecipitation; OA: okadaic acid; IP: immunoprecipitation; CE: cell extract.

treating with calyculin A. We verified these 2 proteins were hyperphosphorylated following calyculin A treatment by treating cell lysates with lambda phosphatase, which caused both CDC20 and BubR1 to downshift to their normal point of migration in the gel (Figure S2A and S2B). We speculated that the increased phosphorylation of CDC20 or BubR1 may be responsible for the MCC disassembly following calyculin A treatment. To test this hypothesis, we performed mass

spectrometry analysis of overexpressed CDC20 and BubR1 in nocodazole-arrested 293T cells and identified several new phosphorylation sites on both proteins in calyculin A-treated samples versus control samples (Figure S2C). Non-phosphorylatable mutants of CDC20 and BubR1, in which the identified serine and threonine sites were mutated to alanine, were transfected into cells to determine the importance of these phosphorylation events for calyculin A-induced cyclin degradation during the spindle checkpoint. However, no significant differences in cyclin levels were observed between cells expressing wild type protein and those expressing mutant proteins (Figure S2D and S2E). This result suggests that hyperphoshporylation of BubR1 and CDC20 may not be responsible for the MCC inactivation induced by calyculin A. However, it should be noted that these mutations did not completely abolish the observed electrophoretic shift of BuBR1 and CDC20 proteins following calyculin A treatment, so it remains possible that unidentified phosphorylation sites on these proteins are responsible for the observed effects of calyculin A.

Calyculin A and okadaic acid-induced APC/C activation does not lead to mitotic exit

We have shown that treating mitotic cells with calyculin A and okadaic acid (1 μM) causes cyclin B degradation. Degradation of cyclin B leads to Cdk1 inactivation, which is typically followed by dephosphorylation of Cdk1 mitotic substrates and rapid exit from mitosis. However, as phosphatases (notably PP1 and PP2A) are necessary for Cdk1 substrate dephosphorylation at mitotic exit, inhibiting PP1 and PP2A with calyculin A or okadaic acid prevents cells from exiting mitosis even though cyclin B has been degraded. To demonstrate that calyculin A and okadaic acid treated cells remain in mitosis, cell lysates from inhibitor-treated, nocodazole-arrested cells were immunoblotted with the MPM2 antibody to determine Cdk1 substrate phosphorylation. As predicated, the MPM2 signal was present in all conditions, indicating that the cells remained arrested in mitosis (Fig. 3A). The MPM2 signal was greatly enhanced in cells treated with calyculin A or okadaic acid (1 μM), despite the fact that cyclin A and B were degraded, highlighting the necessity of phosphatases for mitotic exit. As a positive control for mitotic exit, we treated cells with the Cdk1 inhibitor roscovitine, which induced robust Cdk1 substrate dephosphorylation (Fig. 3B). We also visualized chromosome condensation using chromosome spreading to further assess the mitotic state of the cells treated with phosphatase inhibitors. Using this technique, we could clearly see condensed chromosomes in the nocodazole-arrested cells treated with calyculin A and okadaic acid (1 μM) further confirming the cells remained in mitosis (Fig. 3C). Taken together, our data demonstrate that while calyculin A and okadaic acid (1 μM) lead to MCC disassembly and APC/C activation, the cells are not able to fully exit mitosis.

Lastly, in an effort to identify the specific phosphatase(s) required for MCC maintenance during the spindle checkpoint, we used siRNA or shRNA to knockdown the PPP family phosphatases that are inhibited by calyculin A and okadaic acid (PP1, PP2A, PP4, PP5, and PP6)[39] and have a potential role in the spindle checkpoint, and then measured cyclin levels in the nocodazole-arrested cells. Low concentrations of okadaic acid (0.1 μM) are known to inhibit PP2A and PP4 (and likely PP6 based on sequence similarity to PP2A and PP4 [39]), but as we did not observe any changes in cyclin A or B levels at low okadaic acid concentrations, we hypothesized that the phosphatase involved in MCC maintenance during the spindle checkpoint would likely be PP1, which is only inhibited by higher concentrations of okadaic acid.[39] PP5′s okadaic acid sensitivity is in between PP1 and PP2A, but its sensitivity to calyculin A is several fold higher than PP1 and PP2A,[39] which further supports our hypothesis that PP1 is the relevant phosphatase for MCC maintenance. However, none of the PPP phosphatase siRNAs or shRNAs decreased cyclin B levels in synchronized nocodazole-arrested cells (Figure S3A–H). We actually observed increased levels of cyclin A and B when knocking down each of the 3 isoforms of PP1 (α, β, and γ), indicating that PP1 knockdown cells may have arrested in mitosis prior to nocodazole treatment. To test if there is redundancy between the 3 PP1 isoforms, all 3 isoforms were knocked down together, but cyclin A and B levels were still slightly elevated in the nocodazole-arrested cells (Figure S3D).

To further investigate which phosphatase is responsible for MCC maintenance during the spindle checkpoint, we sought to identify MCC interacting phosphatases using mass spectrometry. Myc-tagged Mad2 and BubR1 were transfected into cells and then immunoprecipitated from nocodazole-arrested cell lysates following DSP crosslinking to stabilize protein interactions. Mass spectrometry was then used to identify the MCC-binding proteins. CDC20 and Bub3 were co-immunoprecipitated with both proteins, confirming that our overexpressed proteins were binding with other MCC components. Thirteen phosphatases and phosphatase regulatory subunits were identified by mass spectrometry, including PP1α, PP2Ac, PP5, one PP1 regulatory subunit, and 3 PP2A regulatory subunits (Table S1). We also identified PP2c-gamma, but we did not investigate this phosphatase because it is not inhibited by calyculin A. As we already tested the knockdown effect of PP1, PP2A, and PP5, we used siRNA to test the effect of knocking down the remaining phosphatases and regulatory subunits. However, there were no detectable differences in cyclin A or B levels during the spindle checkpoint following knockdown of each of these phosphatases (Figure S4A and S4B). It is possible that residual levels of phosphatase activity following knockdown were responsible for persistence of the MCC or that multiple phosphatases contribute to this activity. Nonetheless, our results clearly indicate that phosphatases, as well as kinases, are required for maintenance of the MCC during spindle checkpoint function.

Discussion

In previous reports examining the phosphoregulation of spindle checkpoint signaling, phosphatases have solely been implicated in activities promoting checkpoint silencing (e.g., microtubule-kinetochore stabilization and dephosphorylation of MPS1 targets), while the counteracting kinases have clearly been shown to be critical for checkpoint activation and

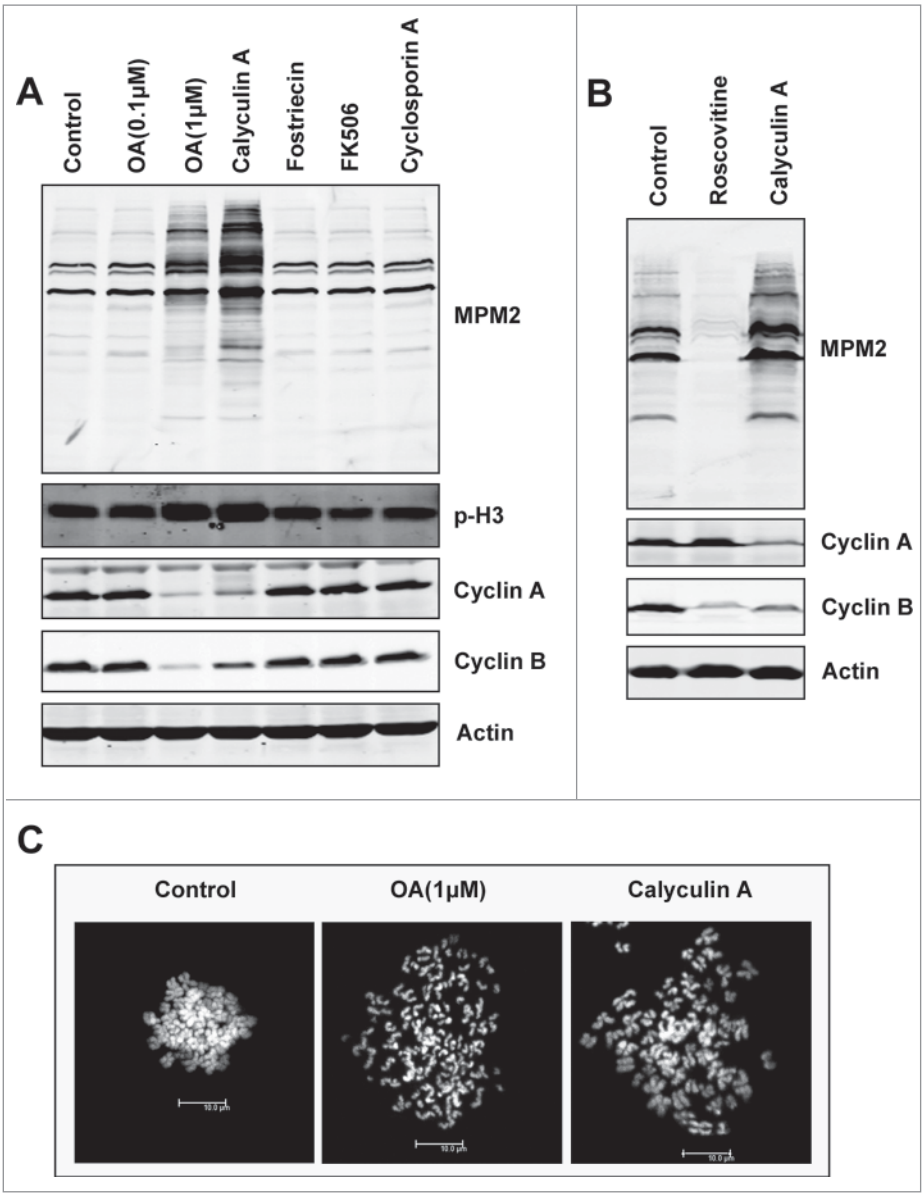

Figure 3. Calyculin A and okadaic acid (1 μM) do not induce mitotic exit despite APC/C activation. (A and B) Synchronized nocodazole-arrested HeLa Tet-Off cells were treated for 2 hours with the indicated inhibitors. Protein levels were detected by immunoblotting with the indicated antibodies. (C) Synchronized nocodazole-arrested HeLa Tet-Off cells were treated for 2 hours with the indicated inhibitors. Cells were then harvested and chromosome spreads were performed. OA: okadaic acid; p-H3: phospho-histone H3 (Ser10).

maintenance.[3,15] Using phosphatase inhibitors in cells arrested at the spindle checkpoint, we specifically explored the role of phosphatases during the checkpoint as opposed to entry or exit. Here we have demonstrated a novel role for phosphatases in maintaining the MCC during the spindle checkpoint. These findings also reveal that an as yet unknown kinase may be required for dissolving the MCC once the spindle checkpoint has been satisfied. This apparent role reversal of kinases and phosphatases highlights the complexity of spindle checkpoint signaling.

Our results clearly demonstrate that phosphorylation of an unknown target leads to MCC disassembly; therefore, we attempted to identify the relevant phosphatase substrate(s) that becomes phosphorylated and drives MCC dissociation following calyculin A and okadaic acid (1 μM) treatment. Two attractive potential substrates were the MCC components CDC20 and BubR1 because phosphorylation of both proteins is significantly increased following calyculin A treatment. However, despite identifying a number calyculin A-induced phosphorylation sites on these 2 proteins by mass spectrometry and performing mutational analysis of these sites, we were unable to observe any reversal of calyculin A's ability to cause cyclin degradation during the spindle checkpoint. Nevertheless, we cannot rule out these 2 proteins as relevant phosphatase targets during the checkpoint because it is possible there are other calyculin A-induced phosphorylation sites that were not identified by mass spectrometry but that promote MCC disassembly. It is also possible that phosphorylation of multiple substrates contributes to MCC dissociation following calyculin A treatment.

Lastly, we attempted to identify the relevant phosphatase required for MCC maintenance by using siRNA or shRNA to

systematically deplete cells of the PPP family phosphatases that are inhibited by calyculin A and okadaic acid. Based on the phosphatases known to be inhibited by 1 μM okadaic acid and calyculin A but not 0.1 μM okadaic acid or fostriecin, we speculated that PP1 would likely be the responsible phosphatase. Given that PP1 is required for checkpoint silencing, the notion that it may also be required for checkpoint maintenance may seem contradictory. However, PP1 does not appear to be heavily recruited to kinetochores until metaphase, where it promotes stable kinetochore-microtubule attachments.[32] An interesting possibility to consider is that during prometaphase when the spindle checkpoint is highly active, a pool of PP1 not localized at the kinetochores may be responsible for maintaining the MCC in an intact complex so it can effectively inhibit the APC/C. However, we did not detect any decreases in cyclin A or B levels following knockdown of any of the phosphatases, including all 3 isoforms of PP1 in tandem, in nocodazole-arrested cells. It is possible there is redundancy among the phosphatases and that calyculin A and okadaic acid cause such a dramatic decrease in cyclin A and B levels because they inhibit multiple phosphatases at once. Future studies investigating the precise phosphatase(s) responsible for MCC maintenance along with the specific targets of the phosphatase will be important to improve our understanding of MCC maintenance and spindle checkpoint signaling.

Materials and methods

Cell culture

HeLa Tet-Off cells were a kind gift from Donald McDonald (Duke University, Durham, NC). All cultures were maintained in Dulbecco's modified Eagle medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) at 37°C and 5% CO_2. HeLa Tet-Off cells were single or double thymidine blocked using 2.5 mM thymidine (Sigma), washed twice with PBS, and released into medium containing 100 ng/mL nocodazole (Calbiochem) or 100 nM taxol (LC Laboratories) for 16 hours to arrest cells at the spindle checkpoint. Phosphatase inhibitors were then added for 2 hours before collection. In certain experiments, MG132 or the APC/C inhibitor proTAME was added for one hour prior to phosphatase inhibitor treatment. For mitotic exit assays, nocodazole or taxol-arrested cells were pre-treated for 30 minutes with inhibitor, washed twice with PBS, and re-plated in media containing an inhibitor or DMSO. The following inhibitors were used: okadaic acid (0.1 μM or 1 μM, Enzo), fostriecin (10 μM, Enzo), FK506 (10 μM, Enzo), cyclosporin A (10 μM, Enzo), calyculin A (20 nM, Calbiochem), MG132 (20 μM, Enzo), proTAME (12 μM, Boston Biochem), and roscovitine (10 μM, Calbiochem).

siRNA and shRNA

siRNAs were synthesized by Sigma or Invitrogen and transfected using Lipofectamine RNAiMax (Invitrogen) per the manufacturer's instructions immediately prior to synchronization. Luciferase or AllStars Negative Control (Qiagen, 1027281) siRNA were used as controls. esiRNAs (Sigma) were used to knockdown 6 phosphatases identified by mass spectrometry: DUSP6 (EHU123191), NUDT5 (EHU013191), PNKP (EHU132321), PPP1R12A (EHU072171), PPA1 (EHU109021), and DCTPP1 (EHU042641). Sigma's pLKO.1 lentiviral shRNA constructs were used for PP4 and PP6, and the control pLKO.1 shRNA construct is from Addgene (1864).

The following siRNA sequences were used:
Luciferase: UCGAAGUAUUCCGCGUACG
CDC20: CGGAAGACCUGCCGUUACA
PP1α: SIHP0511-250PMOL, Sigma
PP1β-1: AAAUGCGAUUGAUGCUAGC
PP1β-2: AUUCAGUCCACCAUACUGG
PP1γ: SIHP0518-250PMOL, Sigma
PP2A: CAACGUGCAAGAGGUUCGAUGUCCA
PP5-1: AACAUAUUCGAGCUCAACGGU
PP5-2: CUCAACAUAUUCGAGCUCA
The following shRNAs were used:
PP4-1: TRCN0000002760
PP4-2: TRCN0000002761
PP4-3:TRCN0000002762
PP6-1: TRCN0000002764
PP6-2: TRCN0000002765
PP6-3: TRCN0000002766

Plasmids and transfections

Stratagene's site-directed mutagenesis kit was used to mutate serine and threonine residues to alanine in pCS2-HA-CDC20 and pcDNA3-Myc-BubR1 per the manufacturer's instructions. pCS2-HA-CDC20, pcDNA3-Myc-BubR1, and pCS2-Myc-Mad2 plasmids were transfected into 293T cells using X-tremeGENE 9 DNA Transfection Reagent (Roche) per the manufacturer's instructions 48 hours before collection.

Antibodies, immunoblotting, and Co-Immunoprecipitation

The following antibodies were used for immunoprecipitations and immunoblotting: anti-cyclin A (sc-751), anti-cyclin B (sc-254), anti-actin (sc-1616-R), anti-CDC20 (Abcam, ab26483), anti-CDC20 (sc-5296, used for immunoprecipitation), anti-Mad2 (sc-6329), anti-BubR1 (Abcam, ab54894), anti-Bub3 (BD Transduction Lab, 611730), anti-PP1α (sc-6104), anti-PP1β (Abcam, ab53315), anti-PP1γ (sc-6108), anti-PP2Ac (Millipore, 05-421), anti-PP5 (BD transduction Lab, 611020), anti-vinculin (Sigma, V9131), anti-myc (sc-789), anti-HA (sc-805), anti-MPM2 (Millipore, 05-368), and anti-phospho-histone H3 (Ser10) (Cell Signaling, 9701). The following secondary antibodies were used: Alexa Fluor 680 goat anti-rabbit (Life Technologies, A21076), IRDye 800CW goat anti-mouse (LI-COR, 926-32210), and IRDye 800CW donkey anti-goat (LI-COR, 926-32214). Co-immunoprecipitation was carried out as previously described.[40] Briefly, mitotic cells were lysed in Co-IP buffer (150 mM NaCl, 20 mM HEPES pH 7.4, 0.5% Triton X-100, 1.5 mM $MgCl_2$, 2 mM EGTA, 5 ug/mL aprotinin, 5 ug/mL leupeptin) at 4°C for 30 minutes, followed by centrifugation at 16,000 xg for 15 minutes. Lysates containing equal amounts of protein were incubated with CDC20 antibody for 4 hours at 4°C. Proteins were collected by adding Protein G Sepharose beads for another 1 hour at 4°C. The beads were washed 3 times

with Co-IP buffer + 150 mM NaCl, boiled in SDS sample buffer, and analyzed by SDS-PAGE and immunoblotting.

To identify calyculin A-induced phosphorylation sites on CDC20 and BUBR1, pCS2-HA-CDC20 and pcDNA3-Myc-BubR1 were transfected into 293T cells, which were then arrested with nocodazole for 16 hours followed by DMSO or Calyculin A (20 nM) treatment for 2 hours. Cells were harvested 48 hours post transfection and immunoprecipitated using HA Epitope Tag Antibody Agarose Conjugate (Pierce, 26181) or Anti-c-Myc Agarose Affinity Gel (Sigma, A7470). After washing beads 3 times with Co-IP + 150 mM NaCl, proteins were eluted in SDS sample buffer, separated by SDS-PAGE, and analyzed by mass spectrometry.

To identify MCC binding proteins, pCS2-Myc-Mad2 and pcDNA3-Myc-BubR1 were transfected into 293T cells, which were then arrested with nocodazole and harvested 48 hours post transfection. DSP crosslinking was performed as previously described,[41] and Myc-Mad2 and Myc-BubR1 were co-immunoprecipitated using Anti-c-Myc Agarose Affinity Gel (Sigma, A7470). Beads were washed 3 times with Co-IP + 150 mM NaCl, and proteins were eluted with 0.1 M Ammonium Hydroxide, which was neutralized with 1 N Acetic Acid before samples were analyzed by mass spectrometry.

qPCR

cDNA was generated using BioRad's iScript cDNA Synthesis Kit (170-8891). qPCR was performed using BioRad's iQ SYBR Green Supermix (170-8882). All samples were amplified in triplicate. mRNA levels were determined relative to GAPDH using the $2^{-\Delta\Delta Ct}$ method.

The following primers were used:

PP4: Forward 5- ATCAAGGAGAGCGAAGTCAAG −3; Reverse 5- CCTACTCTGAACAGCTCTTTGAG −3

PP6: Forward 5- CCTGAAGGTGAGCCCTATTTG −3; Reverse 5- ACAAACGTAGTCACATAGCCG −3

GAPDH: Forward 5- ACATCGCTCAGACACCATG −3; Reverse 5- ATGACAAGCTTCCCGTTCTC −3

GAPDH (#2): Forward 5- CTCCTGTTCGACAGTCAGCC −3; Reverse 5- ACCAAATCCGTTGACTCCGAC −3

PPP1R12A: Forward 5- GAGACGGACCTCGAGCCT −3; Reverse 5- CATCAATGCAAGCCTGGTGC −3

DUSP9: Forward 5- TTCCGGTGGCGTTAGGCTG −3; Reverse 5- GATCGGCTCCCTACACGCTG −3

PNKP: Forward 5- ACCGGTTTCGAGAGATGACG −3; Reverse 5- TCGAACTGCTTCCTGTAGCC −3

PPA1: Forward: 5- TGGAACTATGGTGCCATCCC −3; Reverse 5- CACCTCTTGCACATACCTTGC −3

DCTPP1: Forward 5- AGCTGGCAGAACTCTTTCAGTG −3; Reverse 5- AGGACGTCACTAAGCTCCTCT −3

NUDT5: Forward 5- TCTCCAGCGGTCTGTATGGA −3; Reverse 5- CTCTCCATCCCCTGGCTTTG −3

Chromosome spread

Mitotic cells were swelled in a prewarmed hypotonic solution containing 75 mM KCl for 20 min at 37°C. Cells were adjusted to a density of 2×10^5/ml and fixed with methanol:acetic acid (v/v = 3 :1) for 15 min at room temperature. Cells were dropped onto microscope slides, dried at room temperature and stained with Hoechst 33258 (Molecular Probes) for 5 min. The slides were washed, sealed, and viewed using a using a Leica SP5 confocal scanning microscope.

In vitro lambda phosphatase assay

To assess the phosphorylation status of CDC20 and BubR1, whole cell lysates or immunoprecipitated endogenous proteins were incubated with 1μl lambda phosphatase (New England Biolabs) for 1 hour at 30°C.

Abbreviations

APC/C	anaphase-promoting complex/cyclosome
BubR1	budding uninhibited by benzimidazole-related
Bub1	budding uninhibited by benzimidazole 1
Bub3	budding uninhibited by benzimidazole 3
CDC20	cell division cycle 20
Cdk1	cyclin dependent kinase 1
KNL1	kinetochore null protein 1
Mad1	mitotic arrest-deficient 1
Mad2	mitotic arrest-deficient 2
MCC	mitotic checkpoint complex
MPS1	monopolar spindle 1 kinase
OA	okadaic acid; Plk1: polo-like kinase 1
PPP	phosphoprotein phosphatase.

Disclosure of Potential Conflict of Interest

No potential conflicts of interest were disclosed.

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