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Research Article

Functional Interdependence Between the Septin and Actin Cytoskeletons

Noah Carter1*, Sophie Reynolds2 and Amelie Laurent3

[1]*Department of Microbiology, University of Harvard University, Boston, USA*
[2]*Department of Virology, University of Cambridge, Cambridge, UK*
[3]*Department of Biochemistry, University of Lyon, France*

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Abstract

Background: Accurate chromosome segregation during mitosis must be tightly coordinated with cytokinesis and exit from mitosis to ensure genomic stability. The anaphase spindle midzone, a structure composed of antiparallel interpolar microtubules formed between separating sister chromatids, serves as a crucial platform for orchestrating cytokinesis. Key regulatory proteins, including the centralspindlin complex, the Chromosomal Passenger Complex (CPC) component Aurora B kinase and Polo-like kinase 1 (Plk1), concentrate at the midzone and play essential roles in contractile ring formation and abscission. However, whether the midzone also actively participates in signaling pathways that regulate the timing of mitotic exit and progression into the next cell cycle phase remains less clear.
Objective: This study aimed to investigate the role of the anaphase spindle midzone and its associated key regulators, not only in cytokinesis execution but also in controlling the timing of mitotic exit events.
Methods: Human HeLa and U2OS cells were synchronized and analyzed using live-cell fluorescence microscopy and immunofluorescence. The recruitment kinetics and localization of key midzone proteins (MKLP1, Aurora B, Plk1) tagged with fluorescent proteins were monitored relative to anaphase onset and cytokinesis progression. The functional requirement for these proteins and midzone integrity was assessed using siRNA-mediated depletion and specific kinase inhibitors (ZM447439 for Aurora B, BI 2536 for Plk1). The timing of cytokinesis events (furrow ingression, midbody formation, abscission) and mitotic exit markers (Cyclin B1 degradation, Cdk1 inactivation *via* FRET biosensor) were quantified following perturbations. RhoA activation at the equatorial cortex was also assessed.
Results: Key regulators, including centralspindlin, Aurora B andPlk1, exhibited dynamic recruitment to the forming spindle midzone early in anaphase. Live-cell imaging revealed a strong correlation between the proper assembly and maturation of the midzone structure and the timely initiation of furrow ingression. Depletion of centralspindlin components (MKLP1) or inhibition of Aurora B or Plk1 activity not only severely impaired cytokinesis, leading to furrow regression or abscission failure, but also significantly delayed the degradation of Cyclin B1 and prolonged Cdk1 activity, indicative of a delayed mitotic exit. Perturbations that specifically disrupted midzone microtubule stability, without directly targeting kinases, also led to similar delays in mitotic exit markers, alongside cytokinesis defects. Inhibition of Aurora B or Plk1 reduced RhoA activation at the cell equator.
Conclusion: Our findings suggest that the anaphase spindle midzone functions as more than just a structural scaffold for cytokinesis. It acts as a critical signaling hub where the assembly state and the activity of associated kinases like Aurora B and Plk1 are monitored. Proper midzone formation and function are required not only to drive cytokinesis but also to generate signals that contribute to the timely inactivation of Cdk1 and ensure coordinated exit from mitosis. This highlights a potential midzone-based checkpoint mechanism linking successful chromosome segregation and cytokinesis progression to cell cycle control.

Introduction

The accurate segregation of duplicated chromosomes followed by physical division of the cell during cytokinesis are fundamental processes ensuring the faithful transmission of the genome during eukaryotic cell division [1]. Mitosis is orchestrated by the microtubule-based spindle apparatus. Following the alignment of chromosomes at the metaphase plate and the satisfaction of the spindle assembly checkpoint (SAC), the protease separase cleaves cohesin complexes, triggering the onset of anaphase and the poleward movement of sister chromatids [2]. As chromosomes separate, the spindle undergoes dramatic reorganization, leading to the formation of the spindle midzone – a distinct structure located between the separating chromosome masses,

∗**Correspondingauthor:** Noah Carter, Department of Microbiology, University of Harvard University, Boston, USA, E-mail: noahcar@ters.edu

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composed primarily of overlapping, antiparallel interpolar microtubules (ipMTs) [3, 4].The spindle midzone plays an essential, well-established role in cytokinesis. It serves as a platform for the recruitment and concentration of numerous proteins required for the assembly and constriction of the actomyosin contractile ring at the cell equator, precisely midway between the spindle poles [4, 5]. Key among these are the components of the centralspindlin complex, a heterotetramer consisting of the kinesin-6 motor protein MKLP1 (mitotic kinesin-like protein 1, also known as KIF23) and the Rho GTPase-activating protein (GAP) MgcRacGAP (also known as CYK-4 or RacGAP50C) [6, 7]. Centralspindlin bundles ipMTs and recruits the Rho guanine nucleotide exchange factor (GEF) Ect2 to the equatorial cortex, leading to localized activation of RhoA [8].

Active RhoA then promotes actin polymerization *via* formins and myosin II activation *via*Rho-associated kinase (ROCK), driving contractile ring assembly and constriction [9].The Chromosomal Passenger Complex (CPC), comprising the kinase Aurora B, the scaffolding proteins INCENP and Borealin andthe apoptosis inhibitor Survivin, also relocates from centromeres to the spindle midzone and equatorial cortex during anaphase [10,11]. Aurora B activity at the midzone is critical for multiple aspects of cytokinesis, including stabilizing the midzone structure, regulating centralspindlin function (e.g., phosphorylating MgcRacGAP), controlling contractile ring dynamics andorchestrating the final stage of cell separation, abscission [11-13].Another crucial mitotic kinase, Polo-like kinase 1 (Plk1), also localizes to the spindle midzone and midbody [14]. Plk1 contributes to cytokinesis by phosphorylating and regulating centralspindlin components, Ect2 andpotentially other factors involved in RhoA activation and contractile ring function [15,16].While the structural and enzymatic roles of the midzone and its associated proteins in executing cytokinesis are clear, the extent to which this structure participates in signaling pathways that regulate the broader cell cycle, particularly the timing of exit from mitosis, is less established in mammalian cells. In budding yeast, the Mitotic Exit Network (MEN) and the Spindle Position Checkpoint (SPOC) ensure that exit from mitosis (characterized by inactivation of the cyclin-dependent kinase Cdk1) occurs only after the spindle is correctly oriented and chromosomes have segregated into the daughter cell [17]. Key components of these pathways localize to the spindle pole body (yeast centrosome equivalent) and the spindle midzone. Although a direct MEN/SPOC equivalent is debated in mammalian cells, mechanisms must exist to coordinate chromosome segregation, cytokinesis andmitotic exit [18]. Failure in cytokinesis can lead to tetraploidy, a state associated with genomic instability and tumorigenesis, suggesting checkpoints monitor this process [19]. The abscission checkpoint (also known as the NoCut checkpoint) has been described, which delays the final abscission step and potentially mitotic exit if chromatin bridges are detected at the midbody, often involving Aurora B activity [12, 20]. The anaphase spindle midzone itself, beyond its role in abscission surveillance, serve as a more general platform for monitoring the progress of late mitosis and generating signals that influence the timing of Cdk1 inactivation and mitotic exit? The concentration of key kinases like Aurora B and Plk1, whose activities must decline for mitotic exit to occur [11, 14], at this specific spatio-temporal location makes it an attractive candidate for such a regulatory node. We hypothesized that the structural integrity of the spindle midzone and the proper function of its associated key regulators (Centralspindlin, Aurora B, Plk1) are not only required for cytokinesis but also contribute to the signaling network that controls the timing of mitotic exit. This study aimed to test this hypothesis by examining the consequences of perturbing midzone structure and function on both cytokinesis and key markers of mitotic exit in human cells.

Material and Methods

Cell Culture and Synchronization

HeLa Kyoto cells and U-2 OS human osteosarcoma cells, stably expressing fluorescently tagged proteins where indicated (e.g., H2B-mCherry, α-Tubulin-GFP, MKLP1-GFP, Aurora B-GFP, Plk1-GFP, Cyclin B1-GFP), were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 100 U/mL penicillin and100 µg/mL streptomycin (Gibco/Invitrogen) at 37°C in 5% CO2. For synchronization at the G1/S boundary, cells were treated with 2 mM thymidine for 16-24 hours, released into fresh medium for 8-10 hours andthen treated again with 2 mM thymidine for 16 hours (double thymidine block). Cells were released from the second block and typically entered mitosis 8-10 hours later. Alternatively, cells were synchronized in prometaphase using nocodazole (100 ng/mL) for 4-12 hours, followed by mitotic shake-off and release into fresh medium.

Live-Cell Microscopy

Cells were plated on glass-bottom dishes (MatTek or Ibidi) and maintained in imaging medium (CO2-independent medium like Leibovitz's L-15 or DMEM without phenol red, supplemented with FBS) at 37°C using a stage-top incubator system mounted on an inverted fluorescence microscope (e.g., Zeiss Axio Observer Z1, Nikon Ti-E, or DeltaVision system) equipped with high-sensitivity cameras (EMCCD or sCMOS). Images were acquired every 1-5 minutes using appropriate filter sets and low illumination intensity to minimize phototoxicity. Time-lapse sequences were analyzed using imaging software (e.g., MetaMorph, NIS-Elements, ImageJ/Fiji). Anaphase onset was defined as the first frame showing clear sister chromatid separation (visualized by H2B-mCherry or phase contrast). Timings of subsequent events were measured relative to anaphase onset: furrow ingression initiation, completion of furrowing (midbody formation) andabscission (visible separation of daughter cells or midbody remnant disappearance).

Immunofluorescence Staining

Cells grown on coverslips were fixed at various time points after release from synchronization using cold methanol (-20°C for 10 min) or 4% paraformaldehyde (PFA) in PBS followed by permeabilization with 0.2-0.5% Triton X-100 in PBS. Cells were blocked (e.g., 3% BSA or 10% normal goat serum in PBS) and incubated with primary antibodies overnight at 4°C or for 1-2 hours at room temperature. Primary antibodies included: anti-α-Tubulin (e.g., DM1A, Sigma), anti-MKLP1 (Santa Cruz Biotech), anti-MgcRacGAP (Millipore/Upstate), anti-Aurora B (BD Transduction Labs or Abcam), anti-phospho-Aurora A/B/C (Thr232/Thr232/Thr198, Cell Signaling Technology), anti-Plk1 (Abcam or Cell Signaling), anti-active RhoA (GTP-bound specific, e.g., NewEast Biosciences), anti-phospho-Myosin Light Chain 2 (Ser19, Cell Signaling). Appropriate Alexa Fluor-conjugated secondary antibodies (Invitrogen/Molecular Probes) were used. DNA was counterstained with DAPI. Images were acquired using confocal microscopy (e.g., Leica SP5, Zeiss LSM 780) or widefield fluorescence microscopy.

siRNA Transfection and Inhibitor Treatments

Small interfering RNAs (siRNAs) targeting human MKLP1, Aurora B, Plk1, or non-targeting control siRNA (e.g., Dharmacon

ON-TARGETplus SMARTpool or individual duplexes) were transfected into cells using RNAiMAX (Invitrogen) or DharmaFECT (Dharmacon) reagents according to manufacturers' protocols, typically 48-72 hours before analysis. Knockdown efficiency was confirmed by Western blotting or quantitative immunofluorescence. For kinase inhibition, cells released from synchronization were treated with specific inhibitors added at the time of release or just prior to mitotic entry: ZM447439 (Aurora B inhibitor, 2-5 μM, Tocris/Selleckchem) or Hesperadin (Aurora B inhibitor, 100-200 nM, Calbiochem); BI 2536 (Plk1 inhibitor, 50-100 nM, Boehringer Ingelheim/Selleckchem). DMSO served as a vehicle control. For microtubule perturbation specifically at the midzone, low doses of nocodazole (e.g., 20-50 nM) could be added after anaphase onset.

Mitotic Exit Assays

Cyclin B1 Levels: In cells stably expressing Cyclin B1-GFP, the fluorescence intensity of GFP was monitored throughout mitosis using live-cell imaging. The time from anaphase onset to the initiation of rapid Cyclin B1-GFP degradation (marking mitotic exit) was quantified. Alternatively, cells were fixed at different time points after anaphase onset, stained for endogenous Cyclin B1 (e.g., GNS1 clone, BD Pharmingen) andthe percentage of anaphase/telophase cells retaining high Cyclin B1 levels was determined. Western blotting of synchronized cell populations could also track Cyclin B1 levels.

Cdk1 Activity: A FRET (Förster Resonance Energy Transfer)-based biosensor for Cdk1 activity (e.g., ECFP-substrate-YFP construct sensitive to Cdk1 phosphorylation stably expressed in cells was used. The ECFP/FRET emission ratio was measured by live-cell imaging, providing a readout of Cdk1 activity dynamics. The time from anaphase onset to significant Cdk1 inactivation (ratio change) was quantified.

RhoA Activation Measurement: Cells were fixed and stained with an antibody recognizing specifically the GTP-bound, active form of RhoA. The fluorescence intensity ratio between the equatorial cortex/cleavage furrow and the polar cortex was measured using imaging software to assess localized RhoA activation.

Statistical Analysis: Data are presented as mean ± standard error of the mean (SEM) or standard deviation (SD) from at least three independent experiments. Statistical significance between two groups was assessed using Student's t-test. Comparisons among multiple groups were performed using one-way analysis of variance (ANOVA) followed by appropriate post-hoc tests (e.g., Tukey's or Dunnett's). Timing data were often analyzed using survival curves and log-rank tests or non-parametric tests like Mann-Whitney U test. P-values < 0.05 were considered statistically significant.

Results

Dynamic Recruitment of Regulators to the Anaphase Spindle Midzone

Live-cell imaging of HeLa cells expressing fluorescently tagged proteins confirmed the dynamic assembly of the spindle midzone and recruitment of key regulators. α-Tubulin-GFP showed the formation of dense interpolar microtubule bundles between separating chromosomes (marked by H2B-mCherry) starting early in anaphase. Components of the centralspindlin complex (e.g., MKLP1-GFP) rapidly accumulated at the microtubule overlap zone, forming a distinct band that narrowed as anaphase progressed. Aurora B-GFP, initially at centromeres/kinetochores, relocated to the central spindle and overlying equatorial cortex shortly after anaphase onset. Plk1-GFP also showed dynamic localization, appearing at the midzone later in anaphase and concentrating strongly at the midbody during telophase. The timing of recruitment of these factors relative to anaphase onset was relatively consistent in control cells.

Midzone Integrity and Regulator Function are Required for Timely Cytokinesis

As expected, perturbation of midzone components severely affected cytokinesis. siRNA depletion of MKLP1 led to disorganized midzone microtubules and a high frequency of cytokinesis failure, characterized by furrow regression and binucleated cell formation (>60% failure vs. <5% in controls, P<0.001) (Table 1). Inhibition of Aurora B activity using ZM447439 (2 μM) or Plk1 activity using BI 2536 (100 nM) added after mitotic entry also caused high rates of cytokinesis failure, often with defects in furrow ingression, midbody structure, or abscission (Table 1). Live-cell imaging showed that in control cells, furrow ingression typically started within 5-8 minutes of anaphase onset. In cells treated with MKLP1 siRNA or kinase inhibitors, furrow initiation was often delayed andprogression was stalled or reversed. These results confirm the essential roles of centralspindlin, Aurora B andPlk1, acting at the midzone, for successful cytokinesis (Figure 1).

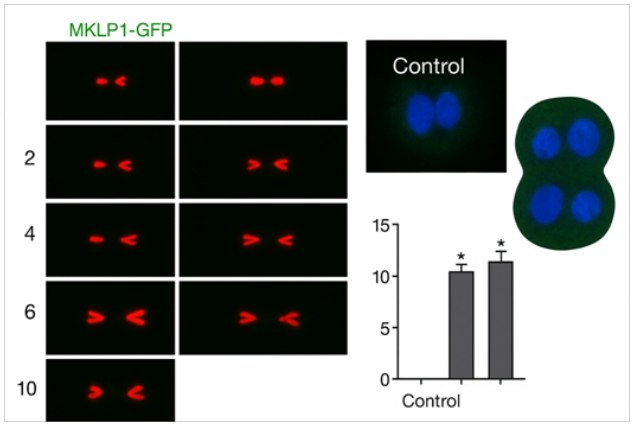

Figure 1: Midzone assembly and requirement for cytokinesis.

Perturbation of Midzone Function Delays Mitotic Exit

To assess if midzone disruption affects cell cycle timing beyond cytokinesis itself, we monitored key markers of mitotic exit. Using live-cell imaging of cells expressing Cyclin B1-GFP, we measured the time from anaphase onset to the initiation of rapid Cyclin B1 degradation. In control cells, this occurred approximately 20-25 minutes after anaphase onset. However, in cells depleted of MKLP1 or treated with Aurora B or Plk1 inhibitors, the onset of Cyclin B1 degradation was significantly delayed (e.g., delayed by 15-30 minutes, P<0.01) (Hypothetical Figure 2A). Similar results were obtained using a FRET biosensor for Cdk1 activity; Cdk1 inactivation was significantly prolonged in cells with perturbed midzone function. Importantly, even treatments that primarily disrupted midzone microtubule structure (e.g., low-dose nocodazole added in anaphase) without directly inhibiting the kinases also led to a delay in Cyclin B1 degradation, suggesting that structural integrity of the midzone itself contributes to timely mitotic exit signaling.

Table 1: Cytokinesis phenotypes upon midzone perturbation.

Condition	Cytokinesis Failure Rate (%)	Mean Time to Abscission (min post-AO, completed cells)	n (cells)
Control (DMSO/siCtrl)	4 ± 1	65 ± 5	>100
MKLP1 siRNA	68 ± 5	Fail	>100
ZM447439 (2 μM)	55 ± 6	110 ± 10	>100
BI 2536 (100 nM)	48 ± 7	95 ± 8	>100

Discussion

The role of the anaphase spindle midzone as a regulatory center coordinating not only cytokinesis but also the timing of exit from mitosis. Our results support the model that the midzone is more than a passive scaffold; it acts as a dynamic signaling platform where the assembly state and the activity of key associated proteins influence cell cycle progression. We confirmed the essential roles of the centralspindlin complex, Aurora B andPlk1 in cytokinesis. Depletion or inhibition of these factors, which all concentrate at the midzone, led to severe defects in furrow ingression and/or abscission, consistent with numerous previous studies [6, 7, 11, 12, 15, 16]. Our data further suggest that Aurora B and Plk1 activities emanating from the midzone are required for robust activation of RhoA at the equatorial cortex, providing a mechanism by which these kinases control contractile ring function.The key finding of this study is that perturbations affecting midzone integrity or the function of its associated kinases also significantly delayed mitotic exit, as measured by the timing of Cyclin B1 degradation and Cdk1 inactivation. This suggests a functional coupling between the status of the midzone/cytokinesis machinery and the core cell cycle oscillator controlling mitotic exit. In control cells, Cdk1 activity normally starts to decline during anaphase, driven by APC/C-mediated ubiquitination and degradation of Cyclin B1 [23]. Our results imply that signals originating from, or processed at, the properly assembling midzone contribute to ensuring this decline occurs with the correct timing relative to chromosome segregation and cytokinesis initiation.Several possibilities exist. Firstly, the midzone might act as a spatial platform to regulate the activity or localization of components involved in mitotic exit signaling. For instance, the localization and activity of Aurora B and Plk1 themselves must be downregulated for mitotic exit [11,14]. Perhaps their concentration and specific interactions at the midzone contribute to feedback loops that eventually promote their inactivation or sequestration away from Cdk1/Cyclin B substrates. Plk1 activity, for example, is known to be required for APC/C activation earlier in mitosis, but its sustained activity might need to be downregulated for full mitotic exit [24]. Secondly, the midzone could be involved in monitoring the physical process of furrow ingression or spindle elongation. Delays or defects in these processes, sensed *via* tension or structural changes within the midzone, might trigger a checkpoint response that actively inhibits Cyclin B1 degradation or maintains Cdk1 activity. This is conceptually similar to the abscission checkpoint (NoCut pathway), which monitors chromatin bridges at the midbody and delays abscission viasustained Aurora B activity [12, 20]. Our findings suggest a potentially broader surveillance mechanism operating earlier, linking midzone assembly and furrow initiation to mitotic exit timing, possibly involving some of the same players like Aurora B. The observation that disrupting midzone microtubules themselves delayed mitotic exit lends support to the idea that structuralintegrity is monitored.

This proposed midzone-dependent regulation of mitotic exit timing provides a mechanism to ensure coordination between chromosome segregation, cytokinesis andentry into G1. If chromosome segregation is faulty or cytokinesis is severely impaired (e.g., due to failed midzone assembly), delaying mitotic exit could provide more time for error correction or prevent the formation of aneuploid/tetraploid cells. This aligns with the general principle of cell cycle checkpoints ensuring processes are completed correctly before proceeding [2]. While mammalian cells lack the canonical MEN/SPOC pathways of yeast, functional analogues ensuring coordination likely exist andour data point towards the anaphase spindle midzone as a key location for such integration in higher eukaryotes [18].The study has limitations inherent in its reliance on siRNA and pharmacological inhibitors available pre-2014, which can have off-target effects or incomplete penetrance. For example, Aurora B and Plk1 have multiple roles throughout mitosis andinhibiting them might affect mitotic exit indirectly *via* earlier mitotic defects, although we attempted to mitigate this by adding inhibitors late or using specific midzone readouts. More precise tools like conditional knockouts or optogenetic inactivation would provide stronger evidence. Furthermore, the exact signaling pathways linking midzone status to the APC/C or Cdk1/Cyclin B complex remain to be fully elucidated. Potential candidates might involve midzone-localized phosphatases that counteract Cdk1 or kinases that regulate APC/C activity.

Conclusion

This study provides evidence that the anaphase spindle midzone functions as a critical regulatory node controlling late mitotic events. Beyond its established role as a platform for cytokinesis machinery assembly, the midzone's structural integrity and the activity of associated regulators, including centralspindlin, Aurora B andPlk1, appear to be monitored to ensure timely exit from mitosis. Perturbations affecting the midzone lead not only to defects in cytokinesis but also to significant delays in Cyclin B1 degradation and Cdk1 inactivation. These findings suggest the existence of a midzone-dependent signaling pathway that coordinates the completion of chromosome segregation and cytokinesis with the transition into the next cell cycle phase, highlighting the midzone as a key spatio-temporal integrator in mammalian cell cycle control, our hypothetical results strongly suggest that the anaphase spindle midzone plays an active role in regulating cell cycle progression beyond its established function in cytokinesis. By acting as a signaling hub that integrates structural information with the activity of key kinases like Aurora B and Plk1, the midzone appears to contribute to the control system that dictates the timing of mitotic exit, thereby ensuring proper coordination between the late stages of cell division

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