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Report

Aurora B and 14-3-3 Coordinately Regulate Clustering of Centralspindlin during Cytokinesis

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Summary

Centralspindlin is essential for the formation of microtubule bundle structures and the equatorial recruitment of factors critical for cytokinesis [1, 2]. Stable accumulation of centralspindlin at the spindle midzone requires its multimerization into clusters [3] and Aurora B kinase activity [4–10], which peaks at the central spindle during anaphase [11, 12]. Although Aurora B phosphorylates centralspindlin directly [13–17], how this regulates centralspindlin localization is unknown. Here we identify a novel regulatory mechanism by which Aurora B enables centralspindlin to accumulate stably at the spindle midzone. We show that 14-3-3 protein binds centralspindlin when the kinesin-6 component MKLP1 is phosphorylated at S710. 14-3-3 prevents centralspindlin from clustering in vitro, and an MKLP1 mutant that is unable to bind 14-3-3 forms aberrant clusters in vivo. Interestingly, 14-3-3 binding is inhibited by phosphorylation of S708, a known Aurora B target site that lies within the motif bound by 14-3-3. S708 phosphorylation is required for MKLP1 to stably localize to the central spindle, but it is dispensable in an MKLP1 mutant that does not bind 14-3-3. We propose that 14-3-3 serves as a global inhibitor of centralspindlin that allows Aurora B to locally activate clustering and the stable accumulation of centralspindlin between segregating chromosomes.

Results and Discussion

Mutation of S710 Suppresses the Cytokinesis Defects Associated with Mutation of S708, a Conserved Aurora B Phosphorylation Site

To investigate the molecular mechanism by which Aurora B regulates centralspindlin localization, we analyzed the role of the most conserved Aurora B target site on centralspindlin (Figure 1A) [15], S708 of MKLP1, in HeLa cells. We performed knockdown and rescue assays and observed cell division via time-lapse imaging (see Figures S1A and S1B available online). After depletion of MKLP1, approximately 50% of cells failed cytokinesis (Figure 1B). This defect was rescued by expression of wild-type GFP-MKLP1, but not by a mutant in which S708 was substituted for alanine (Figure 1B), which is consistent with previous work in *Caenorhabditis elegans* [15]. Forty percent of the GFP-MKLP1-S708A-expressing cells that failed cytokinesis only transiently localized GFP-MKLP1-S708A to the spindle midzone without forming a midbody (Figure 1C, middle), a phenotype similar to the depletion of a chromosomal passenger complex (CPC) component [7]. The remaining 60% formed a midbody that appeared to fragment during late division (Figure 1C, bottom). Importantly, these cytokinesis defects are not due to differences in the level of transgene expression (Figure 1D). Interestingly, sequence alignment indicated that the serine residue 2 amino acids downstream of S708, S710, is also highly evolutionarily conserved (Figure 1A), and a genome-wide phosphoproteome screen has detected phosphorylation of S710 in vivo [18]. Expression of a construct in which S710 was mutated to alanine rescued cytokinesis as efficiently as the wild-type (Figure 1B, GFP-MKLP1-S710A). Remarkably, when S710 was substituted for alanine in addition to S708, the cytokinesis defects associated with mutation of S708 were suppressed (Figure 1B, GFP-MKLP1-S708A/S710A). These data indicate that phosphorylation of S708, a conserved and essential Aurora B target site, is required to antagonize an activity mediated by phosphorylated S710 that prevents MKLP1 from stably accumulating at the spindle midzone and forming a functional midbody.

14-3-3 Protein Binds MKLP1 at a Highly Conserved Motif Centered on Phosphorylated S710

An explanation for this could be that an interaction partner of MKLP1 is bound or released depending on the combinatorial phosphorylation state of S708 and S710. To test this, we performed a pull-down analysis with phosphopeptides covering this region and found a band of approximately 30 kDa specifically bound to S710-monophosphorylated peptide (Figure 2A). Mass spectrometry analysis identified this band to contain 14-3-3 zeta and gamma isoforms. 14-3-3 proteins are ubiquitous and highly abundant phosphoserine- or phosphothreonine-binding proteins involved in multiple signal transduction and cell-cycle events (reviewed in [19]), including cytokinesis [20–22]. Centralspindlin has previously been detected in screens for 14-3-3-interacting proteins [23–25], but neither the sequence recognized by 14-3-3 nor the functional role of this interaction has been addressed. We identified a highly conserved 14-3-3-binding motif encompassing S708 and S710 of MKLP1 (Figure 2B), in which S710 is positioned at the phosphoserine/phosphothreonine essential for 14-3-3 binding. Endogenous 14-3-3 and centralspindlin bound one another in reciprocal coimmunoprecipitation assays (Figures 2C and 2D), and their interaction was sensitive to phosphatase treatment (Figure 2E). This is consistent with the phosphopeptide-binding pattern observed above and the requirement of phosphorylation for mode 1 14-3-3 binding [26]. S710 was essential for fragments of MKLP1 to interact with human 14-3-3 zeta in yeast two-hybrid analyses (Figure 2F; Figure S2), in which endogenous yeast kinases presumably generate the phosphoepitope required for binding [27]. To test whether phosphorylation of S710 is required for 14-3-3 and MKLP1 to interact in vivo, we expressed full-length myc-tagged MKLP1 mutants containing alanine substitutions at S708 and/or S710. Whereas wild-type MKLP1 and MKLP1-S708A both coimmunoprecipitated with 14-3-3, neither MKLP1-S710A nor MKLP1-S708A/S710A did so (Figure 2G). Thus, phosphorylated S710, the function of which is antagonized by phosphorylated S708 (Figure 1), is essential for centralspindlin to bind 14-3-3 (Figure 2).

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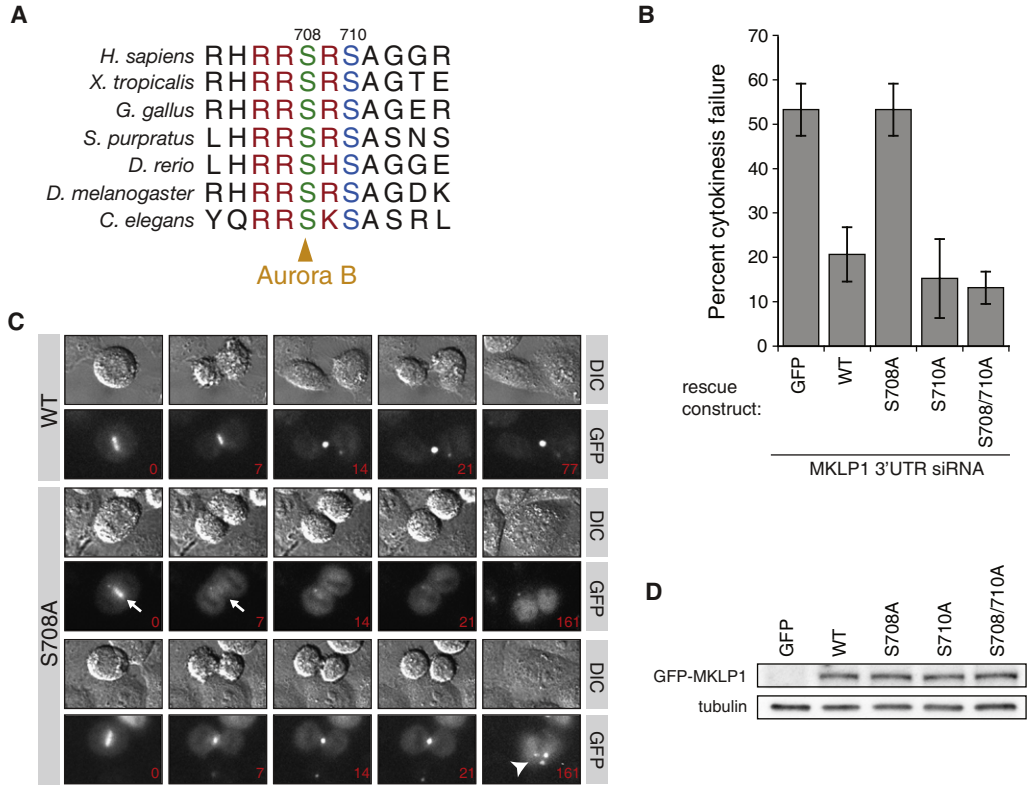

Figure 1. Cytokinesis Defects Caused by Mutation of S708, an Aurora B Phosphorylation Site on MKLP1, Are Suppressed by the Additional Mutation of S710

(A) Sequence alignment indicates that both S708 and S710 of *Homo sapiens* MKLP1 are highly conserved across the animal kingdom.

(B) Quantitation of cytokinesis failures from live cell recordings of HeLa cells treated with MKLP1 small interfering RNA (siRNA) and transfected with GFP vector or the MKLP1 variants as indicated. Data show the percentage of cells that failed cytokinesis averaged from three independent experiments in which at least 85 GFP-MKLP1- or 64 GFP-expressing cells were analyzed (see Figure S1 for details). Error bars indicate the standard deviation.

(C) Stills taken from the films quantitated in (B) showing the localization of wild-type (WT) MKLP1 and MKLP1-S708A. MKLP1-S708A either transiently localized to the central spindle (arrows) or condensed to form a midbody, which then fragmented (arrowhead). Numbers indicate time after anaphase onset (min).

(D) Equal expression of transgenes was confirmed by western blotting whole-cell lysate with anti-GFP or anti-tubulin antibody as indicated.

S708 and S710 Are Differentially Phosphorylated during Cytokinesis

Importantly, 14-3-3 did not bind MKLP1 peptides when S708 was phosphorylated (Figure 2A), perhaps because a phosphate group at this position is incompatible with the architecture of the 14-3-3 peptide-binding pocket [28]. This suggests that the relative levels and subcellular distribution of S708- and S710-phosphorylated MKLP1 determine the temporal and spatial patterns of 14-3-3 binding. To investigate these, we generated three phosphospecific antibodies recognizing S708-monophosphorylated (pS708), S710-monophosphorylated (pS710), or S708- and S710-diphosphorylated (pS708/710) MKLP1. Each of these antibodies is highly specific for the MKLP1 peptide against which it was raised (Figures S3A–S3E). We could detect all three S708- and S710-phosphorylated forms of MKLP1 in HeLa cells released from nocodazole-induced prometaphase arrest (Figure 3A). Both forms containing phosphorylated S708 remained constant until cells completed furrowing (90 to 150 min after release), and then they declined dramatically. This decrease is consistent with previous work on S708 monophosphorylation [15] and a reported global decrease in Aurora B activity after anaphase onset [12]. In contrast, S710 appeared to be constitutively phosphorylated as levels of pS710-MKLP1 followed those of total MKLP1 after release from an S-phase block (Figure S3F) and were relatively unchanged throughout mitosis and cytokinesis, even as cells completed division (Figure 3A). Thus, although all three forms of S708- and S710-phosphorylated MKLP1 coexist during cell division, levels of 14-3-3 binding-competent S710-monophosphorylated MKLP1 and 14-3-3 binding-incompetent S708-phosphorylated MKLP1 are differentially regulated.

To determine whether these phosphorylated forms show different subcellular distributions, we immunostained dividing HeLa cells. pS708-MKLP1 and pS708/710-MKLP1 clearly colocalized with Aurora B accumulated at the central spindle and midbody (Figures 3B and 3C). In contrast, pS710-MKLP1 was not enriched at the central spindle and early midbody (Figure 3B). This distinct subcellular distribution suggests that S710-monophosphorylated centralspindlin is less active than S708-phosphorylated forms, which strongly accumulate at the central spindle. Interestingly, pS710-MKLP1 became enhanced at the late midbody, which is intriguing given a recently proposed abscission checkpoint involving Aurora B [29] and the functional linkage of S710 with S708, an Aurora B target site (Figure 1). Importantly, phosphorylation of S708 at the central spindle required colocalization of MKLP1 with Aurora B because depletion of MKLP2, a mitotic kinesin that is necessary for Aurora B to localize to the spindle midzone [9, 30], abolished pS708/pS710-MKLP1 from this structure (Figure 3C).

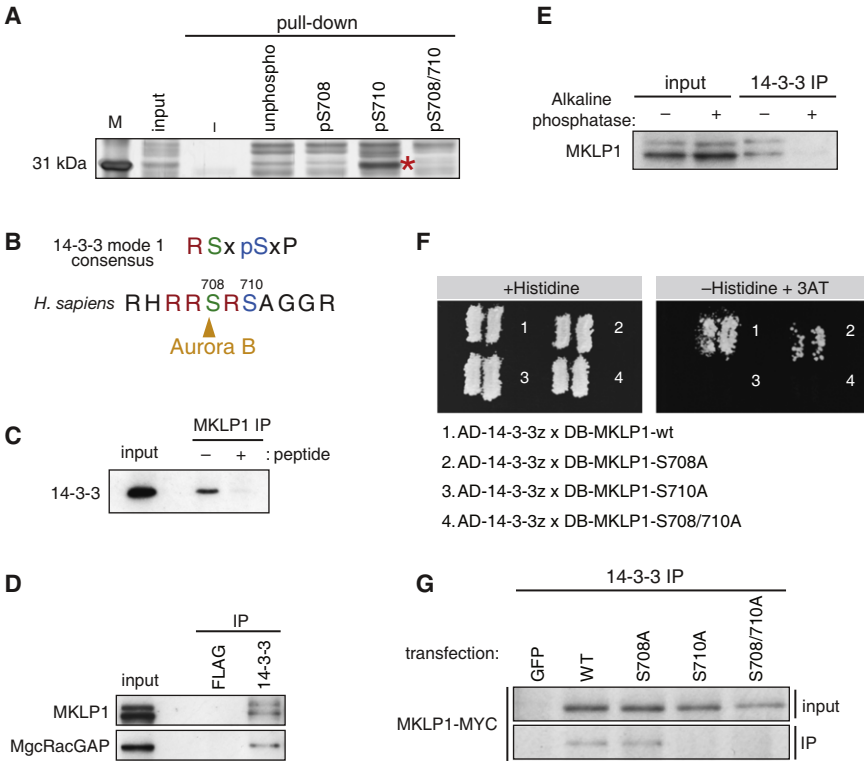

Figure 2. 14-3-3 Protein Binds MKLP1 at a Motif Centered on Phosphorylated S710

(A) MKLP1 peptides phosphorylated as indicated were incubated with mitotic HeLa cell lysate. Bound proteins were separated by SDS-PAGE and visualized by silver stain. An asterisk marks the band identified to contain 14-3-3 zeta and gamma. "Unphospho" denotes unphosphorylated. Input is 10% of the lysate used for each pull-down.

(B) Comparison of the mode 1 14-3-3 binding consensus sequence bound by 14-3-3 with the sequence surrounding S708 and S710 indicates the presence of an evolutionarily conserved 14-3-3 binding site.

(C) MKLP1 was immunoprecipitated from the microtubule-bound fraction of mitotic HeLa cells in the presence or absence of a blocking peptide, and bound proteins were probed for 14-3-3 by western blotting.

(D) 14-3-3 was immunoprecipitated from mitotic HeLa cell extract, and bound proteins were probed for MKLP1 or MgcRacGAP, the nonmotor component of the centralspindlin complex [36], as indicated. The two bands detected for MKLP1 are MKLP1 and its minor splicing variant containing an extra exon [37].

(E) 14-3-3 was immunoprecipitated from mitotic HeLa cell extract in the presence or absence of alkaline phosphatase, and bound proteins were probed for MKLP1.

(F) Yeast two-hybrid analyses were performed with amino acids 690–856 of MKLP1 and full-length human 14-3-3 zeta. Growth on medium lacking histidine and supplemented with 3-aminotriazol (3AT) indicates an interaction between proteins fused to the activation domain (AD) and DNA-binding domain (DB). Alanine substitution at S710 (3 and 4), but not at S708 (2), abolished the interaction between MKLP1 and 14-3-3 (1). See Figure S2 for controls.

(G) HeLa cells were transfected with myc-tagged MKLP1 variants as indicated or GFP as a control. 14-3-3 was immunoprecipitated from mitotic extract, and bound MKLP1 was detected with an anti-myc antibody.

Aurora B Inhibits Binding of 14-3-3 to Centralspindlin

Collectively, these data indicate that pS710-MKLP1 and both forms of S708-phosphorylated MKLP1 coexist during mitosis and cytokinesis but are differentially regulated temporally and spatially depending on the distribution and activity of Aurora B. Importantly, these differences correlate with the ability to bind 14-3-3 (Figure 2A), which suggests that the 14-3-3-MKLP1 interaction is spatially regulated by Aurora B-mediated phosphorylation of S708. To test this, we determined whether levels of S710-monophosphorylated MKLP1, to which 14-3-3 binds, change in the absence of active Aurora B. We fixed and stained cells undergoing cytokinesis after brief treatment with ZM447439, a specific Aurora kinase inhibitor. Whereas Aurora inhibition reduced both S708-phosphorylated forms of MKLP1 at the midbody, pS710-MKLP1 was increased by more than 2-fold (Figure 3D). Similar changes were observed by immunoblotting MKLP1 from Aurora-inhibited cell lysate (Figure 3E). These data strongly suggest that Aurora B phosphorylates S708, but not S710, which seems to be phosphorylated by a constitutively active and ubiquitous kinase (Figures 3A and 3B; Figure S3F). Remarkably, Aurora inhibition also led to an enhancement of the MKLP1-14-3-3 interaction (Figure 3F). From these data, we propose that Aurora B inhibits binding of 14-3-3 to MKLP1 at the central spindle and midbody by phosphorylating S708 and so decreasing the level of S710-monophosphorylated MKLP1, to which 14-3-3 binds. Considering that S708 phosphorylation is dispensable for cytokinesis in the absence of S710 and thus 14-3-3 binding (Figure 1; Figure 2), this model suggests that 14-3-3 inhibits centralspindlin and that the role of S708 phosphorylation is to remove 14-3-3. In the context of a previous study [31], this suggests that phosphorylation of the first serine of the mode 1 motif is a general mechanism by which 14-3-3 binding can be regulated.

14-3-3 Inhibits Clustering of Centralspindlin

What is the centralspindlin function that is inhibited by 14-3-3? For stable localization to the spindle midzone and accumulation into the midbody matrix, centralspindlin heterotetramers must multimerize into clusters of varying size that travel along microtubules and accumulate at plus ends [3]. Aurora B is likely to regulate this process because inactivation [6–8] or mislocalization [10] of the CPC results in the unstable localization of centralspindlin to the spindle midzone, which is also observed in a clustering-defective centralspindlin mutant [3]. Mutation of S708 of MKLP1 in human cells causes a similar phenotype (Figure 1). Therefore, we investigated whether 14-3-3 binding might directly affect the assembly of centralspindlin clusters, which can be detected as a decrease in solubility under physiological conditions. MKLP1-S710A, which cannot bind 14-3-3, was significantly less soluble than wild-type MKLP1 in crude cell lysate (Figure 4A). Moreover, in knockdown and rescue assays (Figure 1), we observed punctate aggregates of strongly expressed GFP-MKLP1-S710A after nuclear envelope breakdown (Figure 4B; 14 of 19 cells), but not of wild-type GFP-MKLP1 expressed at a similar or higher level (1 of 12 cells). These aggregates of MKLP1-S710A formed in the presence of ZM447439 and are therefore assembled independently of Aurora B (data not shown). Thus,

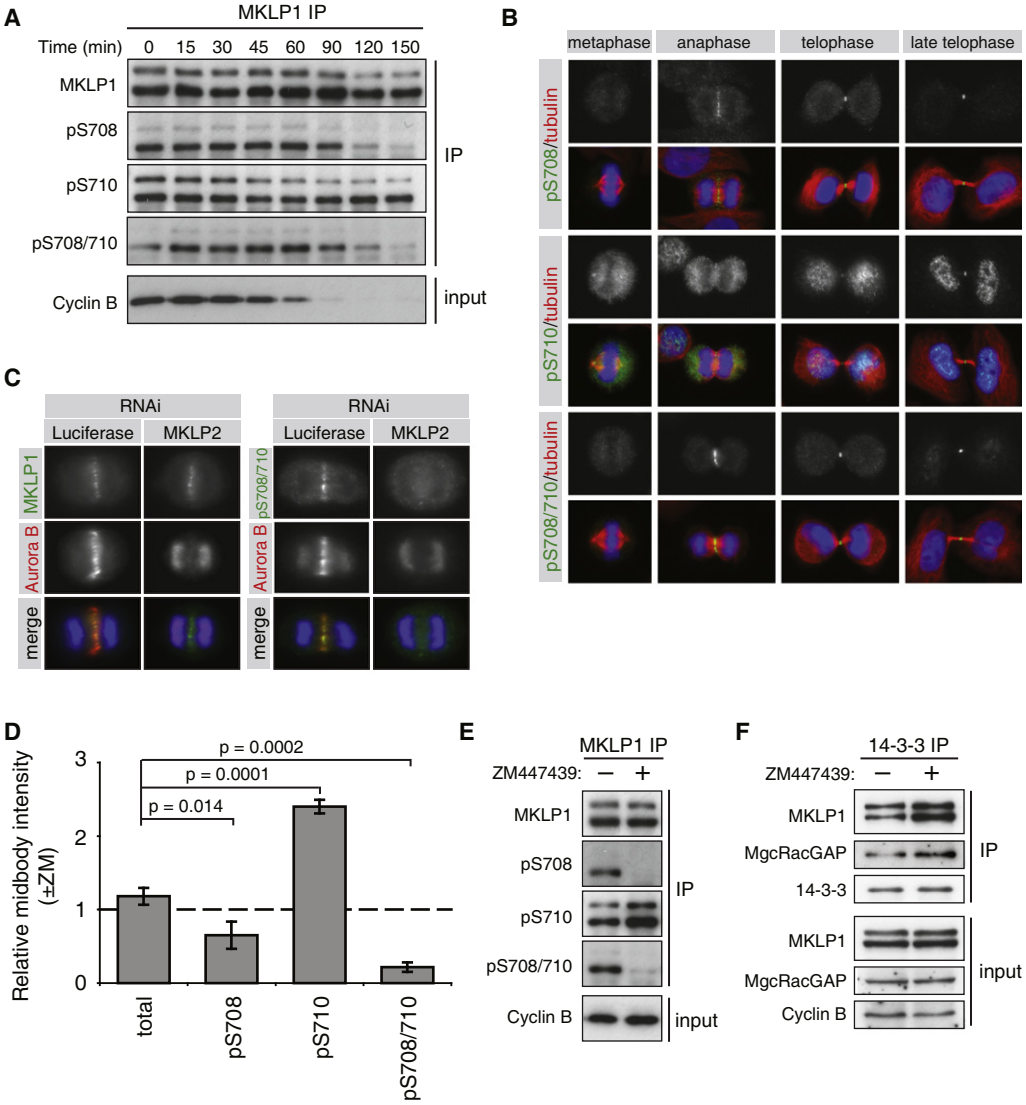

Figure 3. Aurora B Phosphorylates S708 of MKLP1 at the Central Spindle and Midbody and Inhibits the MKLP1-14-3-3 Interaction

(A) Synchronized HeLa cells were released from a nocodazole block to progress through mitosis and were collected at the times shown. MKLP1 was then immunoprecipitated and probed with phosphospecific antibodies as indicated.

(B) HeLa cells were synchronized by single thymidine block and release and then fixed and stained in mitosis and cytokinesis with antibodies to tubulin and phosphorylated MKLP1 as indicated.

(C) HeLa cells were subjected to luciferase or MKLP2 siRNA, synchronized by a single thymidine block and release, and then fixed and stained for Aurora B and total or phosphorylated MKLP1 as indicated.

(D) HeLa cells synchronized by a single thymidine block and release were allowed to progress to cytokinesis, were treated with the Aurora inhibitor ZM447439 (ZM), and were then fixed and stained for total or phosphorylated MKLP1 as indicated. Data show the fold change in midbody intensity in the presence of Aurora inhibitor relative to its absence averaged from three independent experiments, with at least 27 midbodies measured per sample. Error bars show the standard deviation. p values were calculated via Student's t test.

(E) HeLa cells were synchronized by double thymidine block and release and then arrested in mitosis with nocodazole in the presence or absence of the Aurora inhibitor ZM447439. MKLP1 was immunoprecipitated from extract and probed for total or phosphorylated MKLP1.

(F) Cells were synchronized, arrested, and inhibited as in (E). 14-3-3 was then immunoprecipitated from extract and probed for bound MKLP1 or MgcRacGAP as indicated.

14-3-3 binding is necessary to prevent aberrant clustering of centralspindlin.

To determine whether 14-3-3 is sufficient to prevent cluster formation, we purified native centralspindlin competent for binding to 14-3-3 (Figure 4C) from mitotic HeLa cells in the presence of high salt. We induced clustering by diluting this complex to physiological salt in the presence of either 14-3-3 or bovine serum albumin (BSA) as a control. Centralspindlin was rendered almost completely soluble by 14-3-3, whereas it was mostly insoluble and clustered in the presence of BSA (Figure 4D). Importantly, this 14-3-3-bound centralspindlin showed significantly weaker microtubule bundling activity (Figure 4E), consistent with the effect of clustering on the processive motility and microtubule bundling activity of centralspindlin [3]. We conclude that 14-3-3 binding is necessary and sufficient to sequester centralspindlin dispersed throughout the cytoplasm in an inactive, unclustered form.

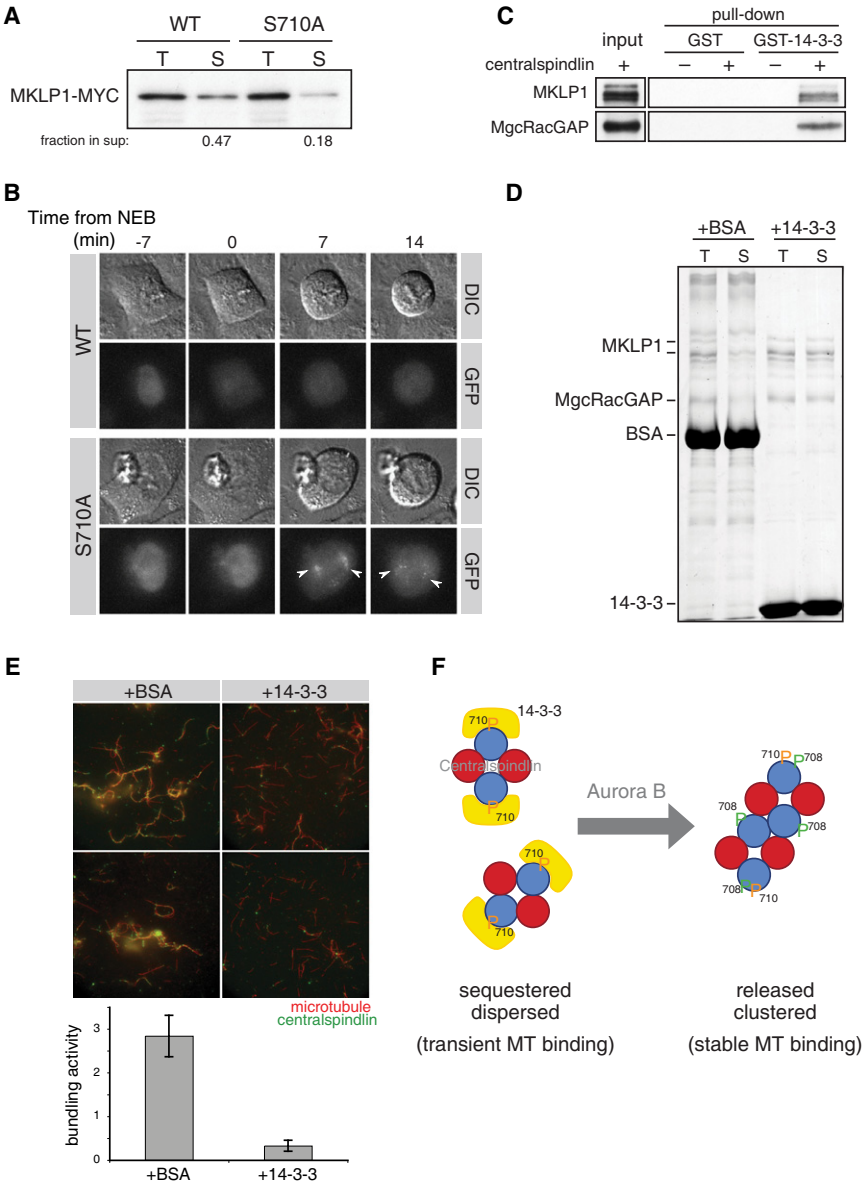

Figure 4. 14-3-3 Regulates the Clustering of Centralspindlin

(A) HeLa cells were transiently transfected with myc-tagged MKLP1 constructs, as indicated, and were arrested in mitosis with nocodazole. The levels of myc-tagged MKLP1 in total (T) extract prepared under physiological concentrations of salt were compared with those that remained in the soluble fraction (S) after centrifugation.

(B) Stills from knockdown and rescue experiments (Figure 1) show aggregates of GFP-MKLP1-S710A (arrowheads) in prometaphase. Numbers indicate the time after nuclear envelope breakdown (min).

(C) GST and GST-14-3-3 were incubated with native centralspindlin that was purified as a soluble heterotetramer in the presence of high salt from nocodazole-arrested HeLa cells treated with ZM447439 (see Experimental Procedures). Bound proteins were probed for MKLP1 and MgcRacGAP by western blotting, as indicated.

(D) Native centralspindlin was purified as in (C). Clustering was then induced by diluting to physiological salt in the presence of recombinant 14-3-3 zeta or bovine serum albumin (BSA) as a control. Following centrifugation, the amount remaining in the soluble fraction (S) was compared with that in the total fraction (T) by Coomassie staining.

(E) Purified GFP-tagged centralspindlin was incubated with 14-3-3 zeta or BSA as in (D) and then mixed with rhodamine- and biotin-labeled microtubules stabilized with taxol. Microtubules were immobilized on avidin-coated coverslips and were observed without fixation. Bundling activity was quantified based on rhodamine fluorescence per unit length of single or bundled microtubules (see Experimental Procedures for details). Error bars indicate the standard error of the mean.

(F) A model for 14-3-3- and Aurora B-regulated clustering of centralspindlin. Whereas 14-3-3-bound centralspindlin is dispersed and only transiently bound to microtubules (MT), removal of 14-3-3 by Aurora B causes centralspindlin to cluster, which in turn enables stable association with and bundling of microtubules at the spindle midzone.

14-3-3 Works as a Global Buffer that Enables Local Activation of a Positive Feedback Loop by Aurora B

Here we have described a novel mechanism in which Aurora B and 14-3-3 coordinately regulate clustering of centralspindlin and control its stable accumulation to the spindle midzone (Figure 4F). This scheme explains our finding that the cytokinesis defects associated with MKLP1-S708A are suppressed when S710 is additionally mutated to alanine (Figure 1). Modulation of oligomeric state is a new mechanism to regulate the activity of kinesin-like motor proteins. Furthermore, regulation of protein multimerization is a novel and potentially widely applicable mode of action by which 14-3-3 can attenuate target protein activity. The molecular mechanism by which 14-3-3 inhibits centralspindlin clustering instead of promoting it by crosslinking MKLP1 dimers will be the focus of future research.

We have previously proposed a model to explain how centralspindlin steeply accumulates to the central spindle, in which clustering plays a critical role [3]. Cluster formation is enhanced when local concentrations of MKLP1 are high. As clustering facilitates the accumulation of MKLP1 to microtubule plus-ends, which in turn increases its local concentration, this gives rise to a positive feedback loop that is maximal at regions of antiparallel microtubule overlap. Although positive feedback enables rapid responses to regulatory switches and sharp spatial macromolecular gradients, it can result in instability and sensitivity to random fluctuations in local protein concentration. The regulatory pathway we have described enables clustering and therefore positive feedback to be globally buffered by 14-3-3 and locally activated by the CPC (Figure 4F). This pathway predicts that the accumulation of centralspindlin should correlate with the position and activity of Aurora B. This is indeed the case: centralspindlin and the CPC colocalize at ectopic Rappaport furrows in fused mammalian cells [32], and, during monopolar cytokinesis, MKLP1 accumulates where the CPC is concentrated, but only when Aurora B is active [33]. We propose that, in concert with temporal regulation by CDK1 kinase [34, 35], the regulation of centralspindlin by Aurora B and 14-3-3 helps to maintain genomic stability by spatially coordinating cytokinesis with chromosome segregation.

Experimental Procedures

Pull-Down Analysis with MKLP1 Phosphopeptides

The peptide sequences used were NAPPIRLRHRRSRSAGDRWVDHKPAS, NAPPIRLRHRRpSRSAGDRWVDHKPAS, NAPPIRLRHRRSRpSAGDRWVDHKPAS, and NAPPIRLRHRRpSRpSAGDRWVDHKPAS, where pS denotes phosphoserine. Peptides were synthesized to greater than 95% purity with a biotin tag conjugated to the N terminus and immobilized on M-280 streptavidin dynabeads (Invitrogen). Nocodazole-arrested HeLa cells were lysed in buffer containing 20 mM PIPES (pH 7), 2 mM EGTA, 2 mM $MgCl_2$, 0.1% (v/v) Triton X-100, 1 mM dithiothreitol (DTT), 10 μg/ml leupeptin, 10 μg/ml pepstatin, 1 mM phenylmethanesulphonylfluoride, 20 mM NaF, 5 μM microcystin, and 150 mM NaCl. Extract was clarified by centrifugation and mixed with peptide-coupled beads for 2 hr with rotation at 4°C. Beads were washed three times with lysis buffer, and bound proteins were run on SDS-PAGE. After silver staining, proteins contained within a band of interest were identified by mass spectroscopy.

In Vitro Assays with Centralspindlin Holocomplex

GFP-FLAG-tagged MKLP1 stably expressed in HeLa cells was purified as described [3]. For the 14-3-3 binding assay, tagged holocomplex was incubated for 2 hr with GST-14-3-3 or GST beads in the buffer described for peptide pull-down analysis. After washing, bound protein was probed for MKLP1 or MgcRacGAP [36] by western blotting as indicated. For solubility analysis, untagged holocomplex was diluted into a reaction mixture finally containing 30 μg/ml centralspindlin, 0.25 mg/ml of either BSA or recombinant human 14-3-3 zeta, 100 mM NaCl, 1.3 mM $MgCl_2$, 1.3 mM EGTA, 0.1 mM EDTA, 0.01% (w/v) Triton X-100, 0.4 mM DTT, 3 μM ATP, 10 mM PIPES, and 5 mM HEPES (pH 7). After incubation at room temperature for 30 min, the sample was centrifuged at 14,000 rpm (k factor ~50) for 30 min at 4°C and analyzed by SDS-PAGE and Coomassie staining. For microtubule bundling assays, GFP-tagged centralspindlin complex affinity-purified with the FLAG tag was diluted (final 2 μg/ml) in the reaction mixture above supplemented with 10 mM DTT and 1 mM ATP and mixed with taxol-stabilized microtubules polymerized in vitro from a mixture of unlabelled or rhodamine-labeled bovine tubulin, as well as biotin tubulin. This was incubated with a coverglass that had been coated sequentially with biotin-labeled BSA and neutravidin (Molecular Probes) and was visualized with fluorescence microscopy. For quantification of microtubule bundling, the integrated intensity of rhodamine fluorescence per unit length of microtubules or microtubule bundles (ρ) was measured for six areas (randomly chosen from a sample containing >450 microtubules or bundles in total) with ImageJ, and the equivalent value for unbundled microtubules (ρ_0) was subtracted. Bundling activity consists of this value normalized to ρ_0, i.e., $(\rho - \rho_0)/\rho_0$, and indicates the average number of microtubules added to a single microtubule by bundling.

For additional experimental procedures, please refer to the Supplemental Experimental Procedures.

Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures and three figures and can be found with this article online at doi:10.1016/j.cub.2010.03.055.

Acknowledgments

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