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Cdk1/Erk2- and Plk1-Dependent Phosphorylation of a Centrosome Protein, Cep55, Is Required for Its Recruitment to Midbody and Cytokinesis

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Summary

Centrosomes in mammalian cells have recently been implicated in cytokinesis; however, their role in this process is poorly defined. Here, we describe a human coiled-coil protein, Cep55 (*ce*ntrosome *p*rotein *55* kDa), that localizes to the mother centriole during interphase. Despite its association with γ-TuRC anchoring proteins CG-NAP and Kendrin, Cep55 is not required for microtubule nucleation. Upon mitotic entry, centrosome dissociation of Cep55 is triggered by Erk2/Cdk1-dependent phosphorylation at S425 and S428. Furthermore, Cep55 locates to the midbody and plays a role in cytokinesis, as its depletion by siRNA results in failure of this process. S425/428 phosphorylation is required for interaction with Plk1, enabling phosphorylation of Cep55 at S436. Cells expressing phosphorylation-deficient mutant forms of Cep55 undergo cytokinesis failure. These results highlight the centrosome as a site to organize phosphorylation of Cep55, enabling it to relocate to the midbody to function in mitotic exit and cytokinesis.

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Introduction

The centrosome is the principle microtubule organizing center of the mammalian cell, consisting of a pair of barrel-shaped microtubule assemblies which are nonidentical and are described as the mother and daughter centrioles (Nigg, 2002). They (mainly the mother) are surrounded by pericentriolar material (PCM), which consists of a matrix of predominantly coiled-coil proteins. PCM is the main site for nucleation of cytoplasmic microtubules and microtubules to form the meiotic and mitotic spindles. The centrosome is structurally and functionally regulated in a cell cycle-dependent manner to form a bipolar spindle to ensure the proper segregation of replicated chromosomes into two daughter cells (Blagden and Glover, 2003). Defects in the number, structure, and function of centrosomes can generate mono- or multipolar mitotic spindles and cytokinesis defects resulting in aneuploidy and chromosome instability, which are common characteristics of tumor cells. Therefore, it is not surprising that these centrosome abnormalities are frequently found in tumors and are usually associated with high cytological grade (Pihan et al., 2003). Thus, it is critical to understand regulators of the centrosome cycle because it must be carefully coordinated with the cell cycle to complete cell division precisely.

Microtubule nucleation by the PCM requires the conserved complex, γ-tubulin ring complex (γ-TuRC), in metazoan organisms (Schiebel, 2000). In yeast, the large coiled-coil spindle pole body (SPB) protein Spc110p anchors γ-tubulin ring complex, providing sites for microtubule nucleation. In mammalian cells, Kendrin, like its yeast homolog Spc110p (Flory et al., 2000), complexes with CG-NAP to provide a structural scaffold for γ-TuRC (Takahashi et al., 2002). Nlp has also been implicated in γ-TuRC anchorage (Casenghi et al., 2003). CG-NAP and Kendrin associate with several protein kinases and phosphatases (Diviani et al., 2000; Takahashi et al., 1999, 2000), suggesting that microtubule nucleation may be regulated through phosphorylation of CG-NAP/Kendrin complexes or associated proteins which remain to be defined.

In recent years, several studies have provided a link between centrosomes and cytokinesis. Acentrosomal cells have been shown to form mitotic spindles and progress through mitosis but fail to complete cytokinesis (Hinchcliffe et al., 2001; Khodjakov and Rieder, 2001). The molecular understanding of centrosome function in cytokinesis is only beginning to emerge. During cytokinesis, the mother centriole has been shown to transiently reposition to the midbody correlating with bridge narrowing and microtubule depolymerization, while movement away from the midbody correlates with cell

cleavage (Piel et al., 2001). It is proposed that the mother centriole regulates an as yet unidentified pathway anchored at the centrosome that is analogous to the mitotic exit pathways in budding yeast called the "mitotic exit network," which is anchored at the SPB and controls mitotic exit and cytokinesis (McCollum and Gould, 2001). The siRNA silencing of a recently identified mother centriole component, centriolin, produces cytokinesis failure (Gromley et al., 2003), suggesting that it is a component of this pathway in mammalian cells. However, additional components and pathways that control cytokinesis will need to be identified to understand the precise role of centrosomes in this process.

Here, we report the molecular characterization of a coiled-coil protein called Cep55. Cep55 localizes to the centrosome of interphase cells and to the midbody during cytokinesis. Characterization of Cep55-depleted cells reveals that Cep55 participates in membrane abscission to form two daughter cells. Furthermore, Cdk1, Erk2, and Plk1 cooperate in the mitotic phosphorylation of Cep55, and this modification is required for its correct mitotic localization and cytokinesis function to maintain genomic stability.

Results

Identification of a Mother Centriole- and Midbody-Associated Protein, Cep55

Using a bioinformatics screen (see Supplemental Data), we identified a protein, encoded by EST FLJ10540, referred to as Cep55 (*c*entrosome *p*rotein *55* kDa). Cep55 encodes a cDNA that is transcribed from chromosome 10q23.33, consisting of nine exons, of which exon 1 is noncoding. This cDNA encodes a protein of 464 amino acids with three centrally located coiled-coil domains (residues 57–355; Figure 1A), a motif found in several centrosome proteins. BLAST analysis revealed that this protein shares 21% identity/39% similarity with the yeast SPB proteins Nuf1 in *Saccharomyces cerevisiae* and Spc110p in *Schizosaccharomyces pombe*. Beyond these matches, however, only weak similarity with other coiled-coil proteins could be identified. Northern blot analysis revealed that Cep55 is highly expressed in testis and thymus (Figure 1B).

The putative yeast homologs of Cep55, Nuf1, and Spc110p locate to the yeast SPB (Kilmartin et al., 1993; Mirzayan et al., 1992). Therefore, we investigated whether Cep55 could locate to the centrosome (equivalent organelle to SPB in mammals) by assessing the localization of green fluorescent protein (GFP)-tagged Cep55 in mammalian cells. GFP-Cep55 localized to centrosomes as demonstrated by its colocalization with the centriole marker GT335, which recognizes polyglutamylated tubulin (Figure 1C). Interestingly, GFP-Cep55 appeared to preferentially localize to one of the two GT335 centriole spots, suggesting that Cep55 may preferentially associate with one of the two centrioles. This staining is reminiscent of centriolin (Gromley et al., 2003) and ninein (Mogensen et al., 2000), whereby electron microscopy has localized these proteins to the mother centriole. Colocalization of GFP-Cep55 with centriolin indicated that Cep55 is a mother centriole protein (Figure 1D). In addition, microscopy analysis revealed that only a small portion of GFP-Cep55 localized to the mother centriole, whereas the majority localized to the PCM (Figure 1C, lower panel). Endogenous Cep55 was also shown to localize to the centrosome by using anti-Cep55 antibodies as indicated by its colocalization with γ-tubulin (Figure 1E). Furthermore, centrosome localization of endogenous Cep55 was shown to be independent of microtubules because it remained associated with the centrosome when microtubules were depolymerized (Figure 1E). The centrosome localization of both endogenous and GFP-Cep55 was confirmed in other cells lines, MCF-7 and 293T (data not shown). Consistent with this, proteome analysis identified Cep55 in crude centrosome preparations from human lymphoblastic KE-37 cells (Andersen et al., 2003).

In contrast to most centrosome proteins, such as γ-tubulin, which are recruited to the centrosome late in G2 to prepare for microtubule nucleation (Blagden and Glover, 2003), a detailed analysis of Cep55 localization revealed that Cep55 loses affinity for the centrosome at the onset of prophase and diffuses throughout the cell, coinciding with centrosome separation and chromatin condensation (Figure 1F). This lack of centrosome staining during mitosis is unlikely to be due to epitope masking, as similar observations were made in cells expressing Flag-Cep55 and GFP-Cep55. Furthermore, during anaphase and telophase, GFP-Cep55 diffusely labels the midzone and then concentrates at the midbody during cytokinesis (Figure 1F, lower panel). Endogenous Cep55 was also observed at the midbody (Figure 1F). These findings indicate that Cep55 localizes to the mother centriole of interphase cells and is recruited to the midbody during cytokinesis.

Localization of Cep55 to the Centrosome and Midbody Is Mediated by Its C-Terminus

To assess the region of Cep55 that is important for targeting it to the centrosome and midbody of cells, we transiently expressed GFP-Cep55 deletion proteins in U2OS cells that lacked regions either N-terminal (57–464) or C-terminal (1–355) to the coiled-coil domains, or both (57–355) (Figure 2A). Like wild-type GFP-Cep55, the N-terminal deletion mutant, 57–464, localized to the interphase centrosome and to the midbody of U2OS cells (Figure 2B). In contrast, GFP-Cep55 proteins 1–355 and 57–355, which lacked the C-terminal residues 355–464, lost the ability to locate to these intracellular domains (Figure 2B), indicating that residues 355–464 target Cep55 to the centrosome and midbody. Indeed, analysis of ectopically expressed GFP-Cep55 (355–464) in U2OS cells demonstrated that this is the case (Figure 2B). Similar results were obtained in HeLa and 293T cells (data not shown), indicating that the C-terminal residues 355–464 target Cep55 to the interphase centrosome and to the midbody of cells undergoing cytokinesis.

Cep55 Is Phosphorylated on the C-Terminal Residues S425 and S428 upon Mitotic Entry

We next tested whether the changes in localization of Cep55 during mitosis are caused by its phosphorylation. HeLa cells were synchronized at G_1, S phase, G_2,

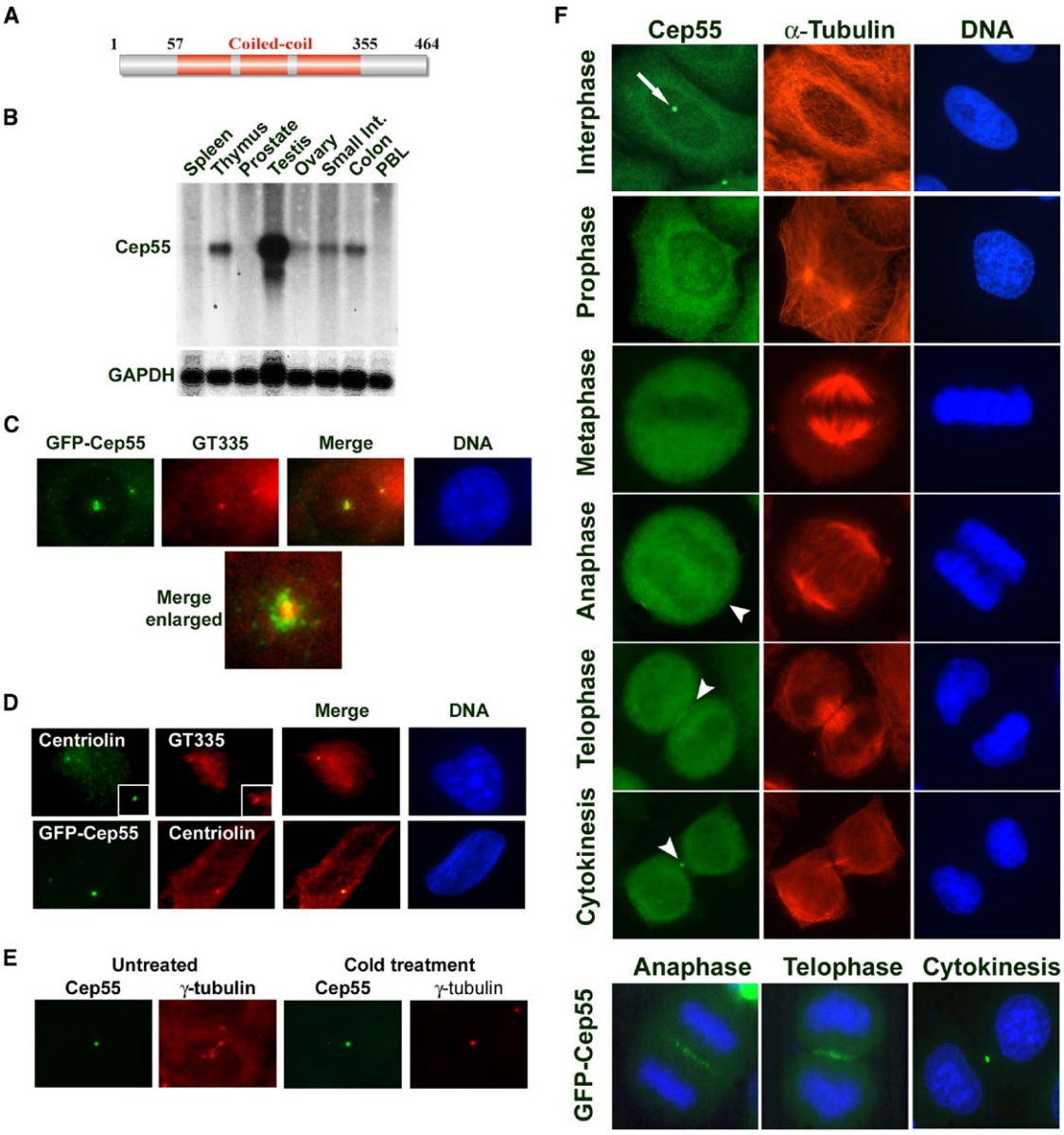

Figure 1. Cep55 Is a Coiled-Coil Protein that Localizes to the Mother Centriole and the Midbody

(A) Schematic representation of Cep55 indicating the location of the coiled-coil domains.

(B) Expression of Cep55 in the indicated tissues was assessed by Northern blotting.

(C) Representative microscopy images of HeLa cells transfected with GFP-Cep55 costained with antibodies directed against the centriole marker GT335 (red). In the lower panel, an enlargement of the merged image is displayed demonstrating that GFP-Cep55 localizes to the PCM of centrosomes.

(D) HeLa cells coimmunostained with anti-centriolin (green) and GT335 (red) antibodies illustrate that centriolin preferentially localizes to the mother centriole (upper panels), as previously described (Gromley et al., 2003). A representative U2OS cell illustrating colocalization of GFP-Cep55 and centriolin antibody (red) at the mother centriole is shown (lower panels).

(E) U2OS cells were immunostained with anti-Cep55 and anti-γ-tubulin antibodies before and after depolymerization of microtubules by incubation on ice for 30 min.

(F) Asynchronously growing HeLa cells were stained for endogenous Cep55 (green) and α-tubulin (red). Representative cells from different stages of the cell cycle are shown. Midzone/midbody localization of GFP-Cep55 ectopically expressed in HeLa cells is shown below. DNA is shown in blue.

and M phase by a double-thymidine release assay and, interestingly, Cep55 was detected as a doublet in mitotic cells compared to interphase cells (Figure 3A). The slower migrating Cep55 form was confirmed to be mitosis specific, as it was predominant in HeLa cells that had been synchronized in prometaphase of mitosis by treatment with the microtubule depolymerizing drug nocodazole (Figure 3B). Lambda phosphatase (λ-PPase)

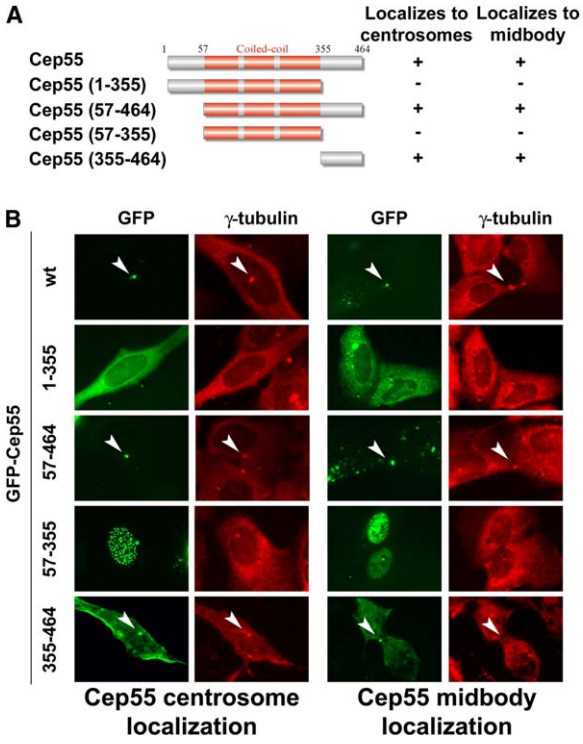

Figure 2. The C-Terminal Residues, 355–464, Target Cep55 to the Centrosome and Midbody

(A) Schematic representation of full-length Cep55 and the indicated Cep55 deletion mutants. A summary of the ability of these Cep55 proteins to localize to the centrosome and midbody is shown.

(B) Representative microscopy images of the centrosome and midbody localization of the GFP-Cep55 mutants described in (A) in U2OS cells. γ-tubulin is shown in red.

treatment of mitotic lysates caused Cep55 to shift to the faster migrating form, indicating that this mitosis-specific form is phosphorylated (Figure 3C). Furthermore, treatment of asynchronous cells with the serine/threonine phosphatase inhibitor okadaic acid caused Cep55 to shift to the slower migrating form, mimicking mitotic phosphorylation (Figure 3C). These findings provide evidence that Cep55 is phosphorylated specifically during mitosis.

To define the region and specific residues of Cep55 that are phosphorylated during mitosis, Flag-Cep55 full-length and deletion mutants were assessed for their phosphorylation status following nocodazole treatment. Mitotic phosphorylation of Flag-Cep55 was not affected in the protein that lacked the N-terminus, 57–464 (Figure 3D). In contrast, the Flag-Cep55 proteins (1–355 and 57–355), which lacked the C-terminus (355–464), were not phosphorylated following nocodazole treatment, suggesting that Cep55 is phosphorylated on a residue(s) located within 355–464 during mitosis. Consistent with these data, Flag-Cep55(1–355) did not incorporate ^{32}P-orthophosphate compared to full-length Flag-Cep55 in an in vivo labeling experiment (Figure 3E). Phosphoamino acid analysis of Flag-Cep55 revealed that it is phosphorylated exclusively on serine residues during mitosis (data not shown), and sequence analysis of Cep55 residues 355–464 identified four serine residues which could be phosphorylated based on their conservation between human, mouse, and rat (Figure 3F). These four sites were S396, S425, S428, and S436 and each was systematically mutated to alanine in Flag-Cep55 to generate four mutant constructs. Mutation of S425 and S428 to alanine partially reduced Flag-Cep55 phosphorylation in mitotic cells (Figure 3G), whereas S396 and S436 had no effect (data not shown). Mutagenesis of both sites (S425/428A) completely abolished Flag-Cep55 phosphorylation (Figure 3G) and incorporation of ^{32}P-orthophosphate into Flag-Cep55 during mitosis (Figure 3E). These residues were confirmed to be in vivo phosphorylation sites by mass spectrometry (see Supplemental Data). Mass spectrometry also identified S436 as an in vivo phosphorylation site and strongly suggested that there is a hierarchy of phosphorylation, where both S425 and S428 are phosphorylated prior to S436 phosphorylation to produce three in vivo phosphorylation sites on Cep55.

Cep55 Residues S425 and S428 Are Phosphorylated by Erk2 and Cdk1 upon Mitotic Entry

The Cep55 mitosis-specific phosphorylation sites, S425 and S428, are followed by a proline at the 1+ position. Two serine/proline-directed kinases implicated in mitotic regulation are Cdk1 and Erk2, suggesting that Cep55 may be a substrate of one or both of these kinases. Overexpression of constitutively active forms of Cdk1 (AF) and/or Erk2 (LA), but not their kinase dead forms, caused a phosphorylation-induced mobility shift in Flag-Cep55 (Figure 4A). However, overexpression of Cdk1-AF did not retard the mobility of the entire pool of Flag-Cep55 (Figure 4A). In contrast, constitutively active Erk2 and Cdk1 were not able to shift the phosphorylation mutant S425/428A (Figure 4B). The involvement of Cdk1 and Erk2 were confirmed by using their specific inhibitors, roscovitine and U0126, respectively, as both inhibitors impaired the nocodazole-induced phosphorylation of Cep55 (Figure 4C). However, it is important to note that the Erk2 inhibitor was better than the Cdk inhibitor in blocking phosphorylation of Cep55 (Figure 4C). Next, we asked whether Erk2 and/or Cdk1 phosphorylate S425 and S428 directly in vitro. Indeed, kinase assays demonstrated Cep55 is a substrate of both kinases; however, Cdk1/cyclin B1 was inefficient at mediating the phosphorylation of Cep55 compared to the autophosphorylation of cyclin B1. In contrast, undetectable/minimal phosphorylation of the double mutant S425/428A was observed (Figures 4D and 4E), indicating that both kinases can mediate phosphorylation of Cep55 at the same sites.

Phosphorylation of Cep55 at S425/428 Enhances Plk1 Binding and Phosphorylation at S436

Recent studies have suggested that proline-directed kinases, such as Cdk1 and Erk2, cooperate with the Polo-like kinase (Plk1) to phosphorylate several proteins, including Cdc25C (Elia et al., 2003) and cyclin B1 (Yuan et al., 2002). The polo-box domain of Plk1 is a phosphopeptide binding motif that binds phosphorylation sites generated by Cdk1 and Erk2, enabling Plk1 to mediate the phosphorylation of the target protein at

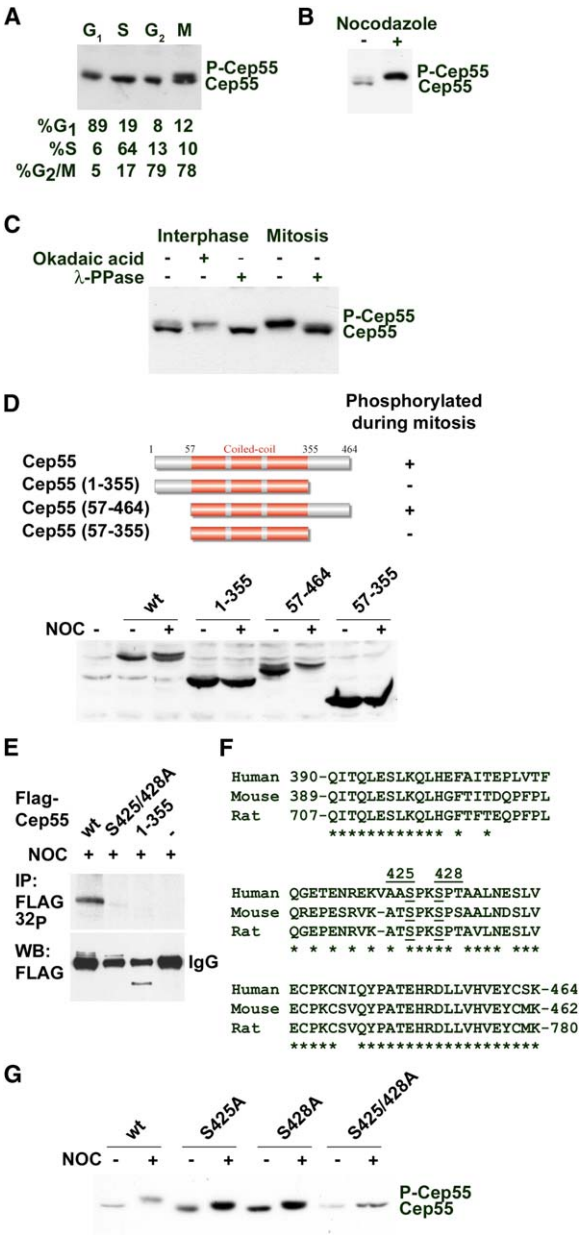

Figure 3. Cep55 Is Phosphorylated during Mitosis at S425 and S428

(A) Immunoblot analysis of Cep55 in HeLa cells synchronized at the indicated cell cycle phases.

(B) Cell lysates (30 μg) from asynchronous and mitotic cells (nocodazole-treated) were immunoblotted with an anti-Cep55 antibody.

(C) Lysates were prepared as described in (B) and treated with λ-PPase. Cellular lysates were also prepared from HeLa cells treated with the phosphatase inhibitor okadaic acid. Lysates (30 μg) were immunoblotted with an anti-Cep55 antibody.

(D) Schematic illustration of wild-type Cep55 and the indicated Cep55 deletion mutants. A summary of the phosphorylation status of these Flag-Cep55 proteins during mitosis is shown (upper panel). The indicated Flag-Cep55 deletion mutants were transfected into HeLa cells and assessed for their phosphorylation status, indicated by a mobility shift, following nocodazole treatment by immunoblotting lysates (30 μg) with an anti-Flag antibody (lower panel).

(E) Labeling of Flag vector, pFlag-Cep55, pFlag-Cep55-S425/428A, and pFlag-Cep55 (1–355) with ^{32}P in mitotic HEK293 cells. Autoradiography and immunoblot of the Flag-Cep55 proteins are shown in the upper and lower panels, respectively.

(F) Comparison of the C-terminal sequence of Cep55 orthologs. Conserved residues are indicated with asterisks and the two mitosis-specific phosphorylation sites, S425 and S428, are underlined.

(G) Lysates from asynchronous and nocodazole-treated HeLa cells ectopically expressing wild-type or the indicated pFlag-Cep55 phosphomutant were immunoblotted with an anti-Flag antibody. S425/428A mutation completely abolishes Flag-Cep55 nocodazole-induced mobility shift.

additional sites (Barr et al., 2004). Several lines of evidence suggest that Cep55 is phosphorylated by these kinases in this manner: (1) Cep55 is phosphorylated at S436, which is a Plk1 consensus site and (2) S436 phosphorylation requires prior phosphorylation at S425/428 by Cdk1/Erk2 (see Supplemental Data). Consistent with our hypothesis, overexpression of Plk1, but not another mitotic kinase, Aurora A caused a phosphorylation-induced mobility shift of wild-type Flag-Cep55, analogous to nocodazole treatment (Figure 4F). In contrast, Plk1 was not able to mediate the phosphorylation of Flag-Cep55-S425/428A (Figure 4F), suggesting that Plk1-dependent phosphorylation of Cep55 requires prior phosphorylation at S425 and S428 by Erk2 and/or Cdk1. Next, we asked whether Cep55 interacts with Plk1 and whether this interaction occurs in a phosphorylation-dependent manner. Remarkably, wild-type but not the phosphorylation mutant form (S425/428A) of Flag-Cep55 coimmunoprecipitates with wild-type Myc-Plk1 when coexpressed in HeLa cells (Figure 4G), again suggesting that the association requires Cep55 phosphorylation. Furthermore, we could detect an association between endogenous Plk1 and the phosphorylated form of Cep55 in mitotic cells, but not in asynchronous cells, by coimmunoprecipitation experiments (Figure 4H), indicating that this interaction occurs under physiological conditions. We next asked whether Plk1 can directly phosphorylate Cep55 at S436. In vitro Plk1 kinase assays illustrated that Cep55 is a Plk1 substrate and mutagenesis of S436 to alanine revealed that Plk1 specifically phosphorylates this residue, but not another site within the C-terminus of Cep55, which conforms to the Plk1 consensus, S396 (Figure 4I). Taken together, we provide evidence that Cdk1, Erk2, and Plk1 cooperate to phosphorylate Cep55 during mitosis.

Mutation of Cep55 Phosphorylation Sites S425 and S428 Prevents Its Dissociation from the Centrosome at the G_2/M Boundary

Cep55 loses affinity for the centrosome at the onset of mitosis (Figure 1) and this correlates with its phosphorylation (Figure 3), suggesting that phosphorylation of Cep55 may induce its centrosome dissociation at the G_2/M boundary. Both wild-type Flag-Cep55 and the Plk1-dependent phosphorylation mutant, Flag-Cep55-S436A, did not label the mitotic centrosomes, but diffusely labeled the cytoplasm (Figure 4J), indicating that phosphorylation at S436 is not responsible for mediating Cep55 centrosome dissociation upon mitotic entry. In contrast, the phosphorylation mutant, S425/428A, remained associated with the centrosome as cells progressed through mitosis (Figure 4J), indicating that

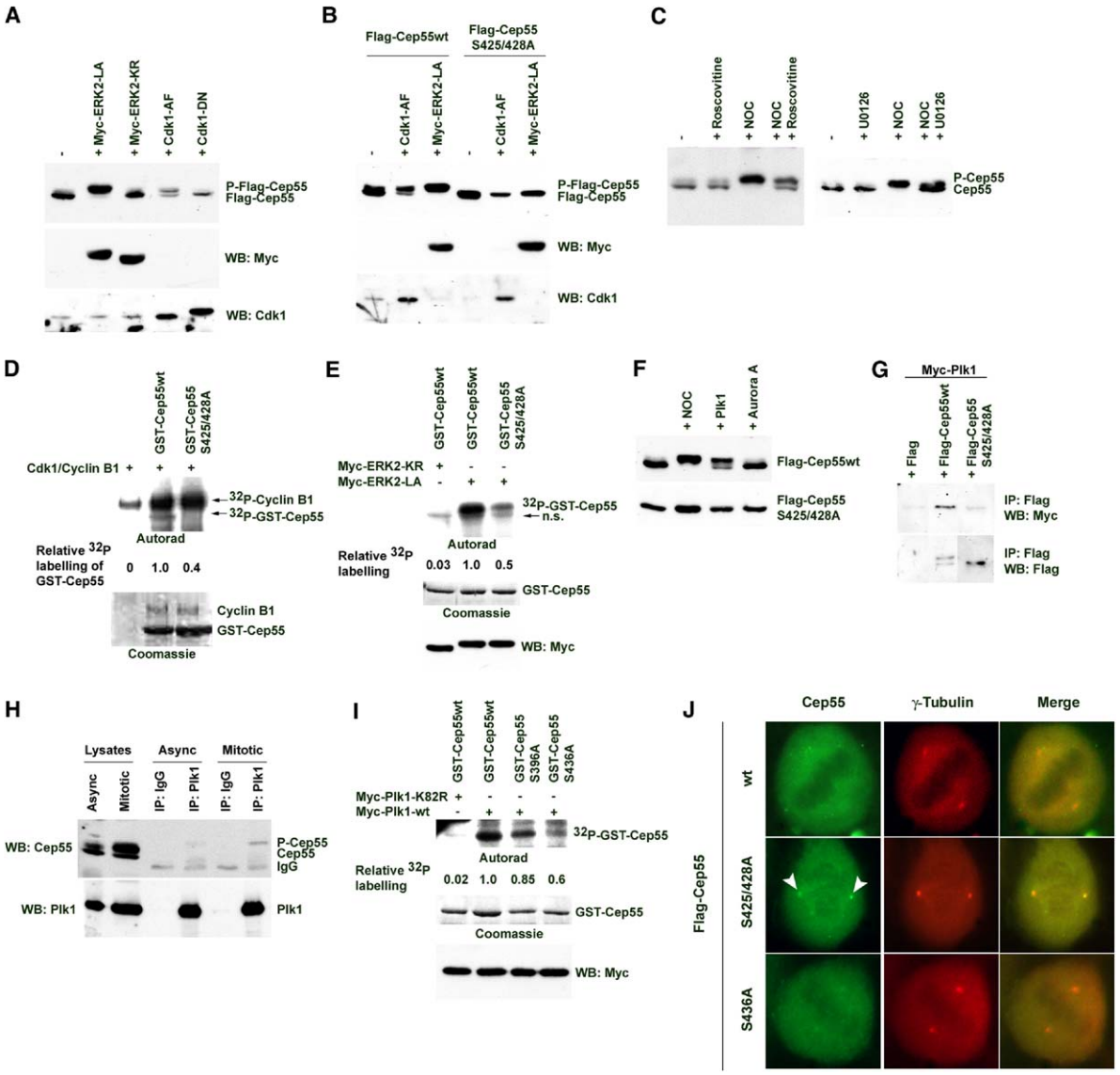

Figure 4. Cdk1/Cyclin B1, Erk2, and Plk1 Kinases Contribute to Cep55 Mitotic Phosphorylation

(A) 293T cells were transfected with pFlag-Cep55 alone or in combination with constitutively active Erk2 (LA) or Cdk1 (AF) or with kinase dead Erk2 (KR) or Cdk1 (DN). At 48 hr posttransfection, lysates were prepared and immunoblotted with anti-Flag, anti-Myc, and anti-Cdk1 antibodies.

(B) Lysates prepared from 293T cells ectopically expressing wild-type and phosphomutant (S425/428A) Flag-Cep55 either alone or with Erk2-LA or Cdk1-AF were immunoblotted with anti-Flag, anti-Myc, and anti-Cdk1 antibodies.

(C) HeLa cells were treated with the Cdk1 inhibitor roscovitine, or the Erk2 inhibitor U0126, or in combination with nocodazole as described in Experimental Procedures. Lysates were immunoblotted with anti-Cep55 antibodies.

(D) In vitro Cdk1/cyclin B1 kinase assay. Bacterially produced recombinant Cdk1/cyclin B1 kinase was incubated with the indicated GST-Cep55 protein in a kinase buffer containing ^{32}P-ATP. Phosphorylated proteins were detected by autoradiography.

(E) In vitro Erk2 kinase assay. Lysates from HeLa cells ectopically expressing kinase dead (KR) or constitutively active (LA) Myc-Erk2 were immunoprecipitated with an anti-Myc antibody and then incubated with the indicated GST-Cep55 protein in kinase buffer containing ^{32}P-ATP. Phosphorylated proteins were detected by autoradiography.

(F) Lysates prepared from 293T cells ectopically expressing wild-type and phosphomutant (S425/428A) Flag-Cep55 either alone or with Myc-Plk1 or Flag-Aurora A were immunoblotted with anti-Flag antibodies.

(G) HeLa cells were transfected with Myc-Plk1 and Flag vector or the indicated Flag-Cep55 construct. Prepared lysates were immunoprecipitated with an anti-Flag antibody and then immunoblotted with anti-Myc or anti-Flag antibodies.

(H) Lysates prepared from asynchronous and mitotic HeLa cells were immunoprecipitated with either a nonspecific IgG or anti-Plk1 antibody and then immunoblotted with anti-Cep55 and anti-Plk1 antibodies.

(I) In vitro Plk1 kinase assay. Lysates prepared from HeLa cells ectopically expressing wild-type or kinase dead Myc-Plk1 (K82R) were immunoprecipitated with an anti-Myc antibody and then incubated with the indicated GST-Cep55 protein in kinase buffer containing ^{32}P-ATP. Phosphorylated proteins were detected by autoradiography.

(J) HeLa cells ectopically expressing wild-type or phosphorylation mutant forms of Flag-Cep55 were costained with anti-Cep55 and anti-γ-tubulin antibodies. Immunofluorescence microscopy of cells in metaphase illustrates that unlike wild-type Flag-Cep55 and the Plk1-dependent phosphorylation mutant S436A, the Erk2/Cdk1-dependent phosphorylation site mutant S425/428A labels the centrosome during metaphase as indicated by its colocalization with γ-tubulin.

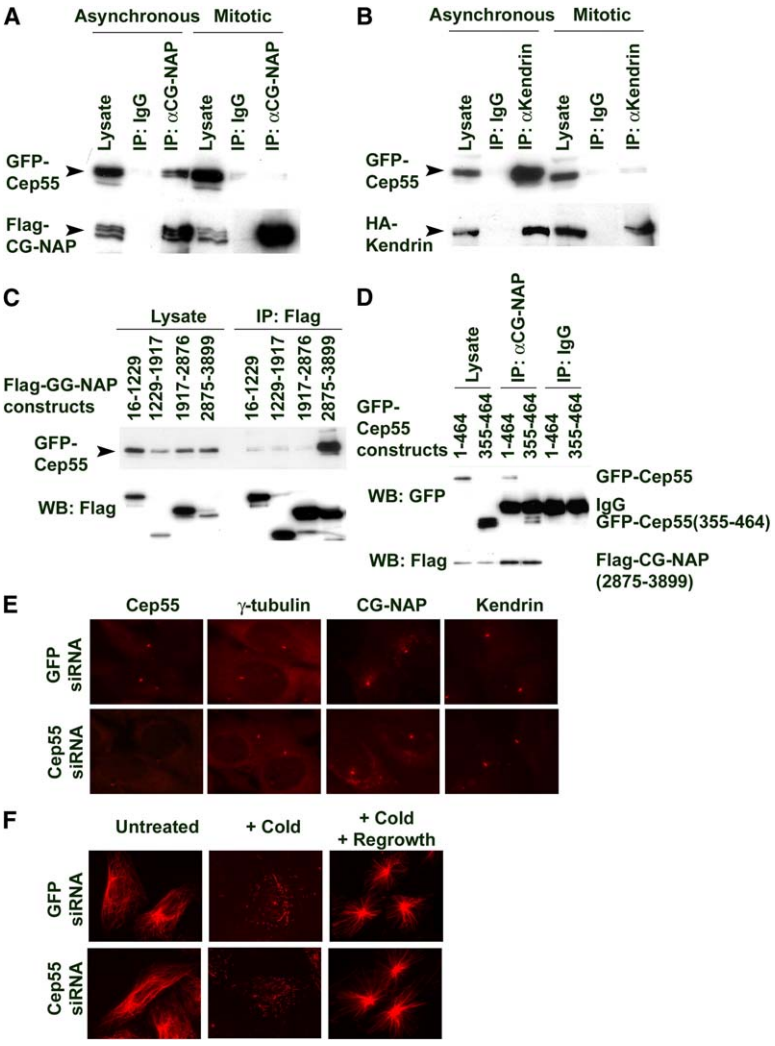

Figure 5. Cep55 Complexes with the Centrosome Proteins CG-NAP and Kendrin, but Is Not Required for Microtubule Nucleation

(A and B) Lysates from asynchronous and mitotic Cos-7 cells ectopically expressing either (A) GFP-Cep55 and Flag-CG-NAP or (B) GFP-Cep55 and HA-Kendrin were immunoprecipitated with an anti-CG-NAP antibody or an anti-Kendrin antibody, respectively, and then immunoblotted with anti-GFP antibody.

(C) GFP-Cep55 and the indicated Flag-CG-NAP deletion mutants were coexpressed in Cos-7 cells. Prepared lysates were immunoprecipitated with an anti-Flag antibody and then immunoblotted with an anti-GFP antibody.

(D) Cos-7 cells were transfected with either full-length GFP-Cep55 or GFP-Cep55(355–464) and Flag-CG-NAP(2875–3899). Prepared lysates were immunoprecipitated with an anti-Flag antibody and then immunoblotted with an anti-GFP antibody.

(E) Immunofluorescence microscopy of Cep55-stained HeLa cells transfected with the indicated siRNA demonstrates that Cep55 expression is depleted by its specific siRNA and not by the control (GFP siRNA). These cells were also stained for γ-tubulin, CG-NAP, and Kendrin to analyze their centrosome localization.

(F) U2OS cells were transfected with GFP or Cep55 siRNA and subjected to an in vivo microtubule nucleation assay at 72 hr post-transfection. Microtubules were visualized with α-tubulin.

phosphorylation at S425 and S428 is required for its dissociation from the centrosome at the G_2/M boundary.

Cep55 Associates with the Centrosome Proteins CG-NAP and Kendrin, but Is Not Required for Microtubule Nucleation

To assign Cep55 a function, we assessed the ability of Cep55 to associate with the centrosome proteins CG-NAP and Kendrin, which are involved in microtubule nucleation in mammalian cells (Takahashi et al., 2002), the best characterized function of centrosomes. GFP-Cep55 coimmunoprecipitated with Flag-CG-NAP and HA-Kendrin in asynchronous Cos-7 cells (Figures 5A and 5B). In contrast, consistent with our data indicating that Cep55 dissociates from the centrosome upon mitotic entry, GFP-Cep55 did not coimmunoprecipitate with Flag-CG-NAP or HA-Kendrin in mitotic cells (Figures 5A and 5B). We investigated this association further by defining the region of CG-NAP and Cep55 that interacts with the other. Coimmunoprecipitation experiments revealed that these proteins associate via their C-termini (Figures 5C and 5D), which interestingly is the centrosome targeting domain of each protein (Figure 2; Takahashi et al., 2002).

We next investigated whether Cep55 is required for microtubule nucleation because it complexes with CG-NAP and Kendrin. Although this interaction dissociates and Cep55 disappears from the centrosome upon mitotic entry, suggesting that Cep55 might not participate directly in this function, the centrosome displacement, however, of other proteins such as Nlp have been shown to be required for microtubule nucleation by allowing the establishment of a mitotic scaffold in order to enhance microtubule nucleation activity (Casenghi et al., 2003). To analyze the requirement of Cep55 for microtubule nucleation, we utilized siRNA technology to deplete Cep55 from cells. Importantly, at 72 hr post-transfection, Cep55 siRNA specifically depleted Cep55 expression by >90%, whereas the GFP siRNA control had no effect (Figure 5E; and shown by Western blotting; Figure 6A). Immunofluorescence microscopy also revealed that Cep55 depletion does not affect centrosome localization of γ-tubulin and the γ-TuRC anchoring proteins CG-NAP and Kendrin (Figure 5E). By using an in vivo microtubule regrowth assay to assess microtu-

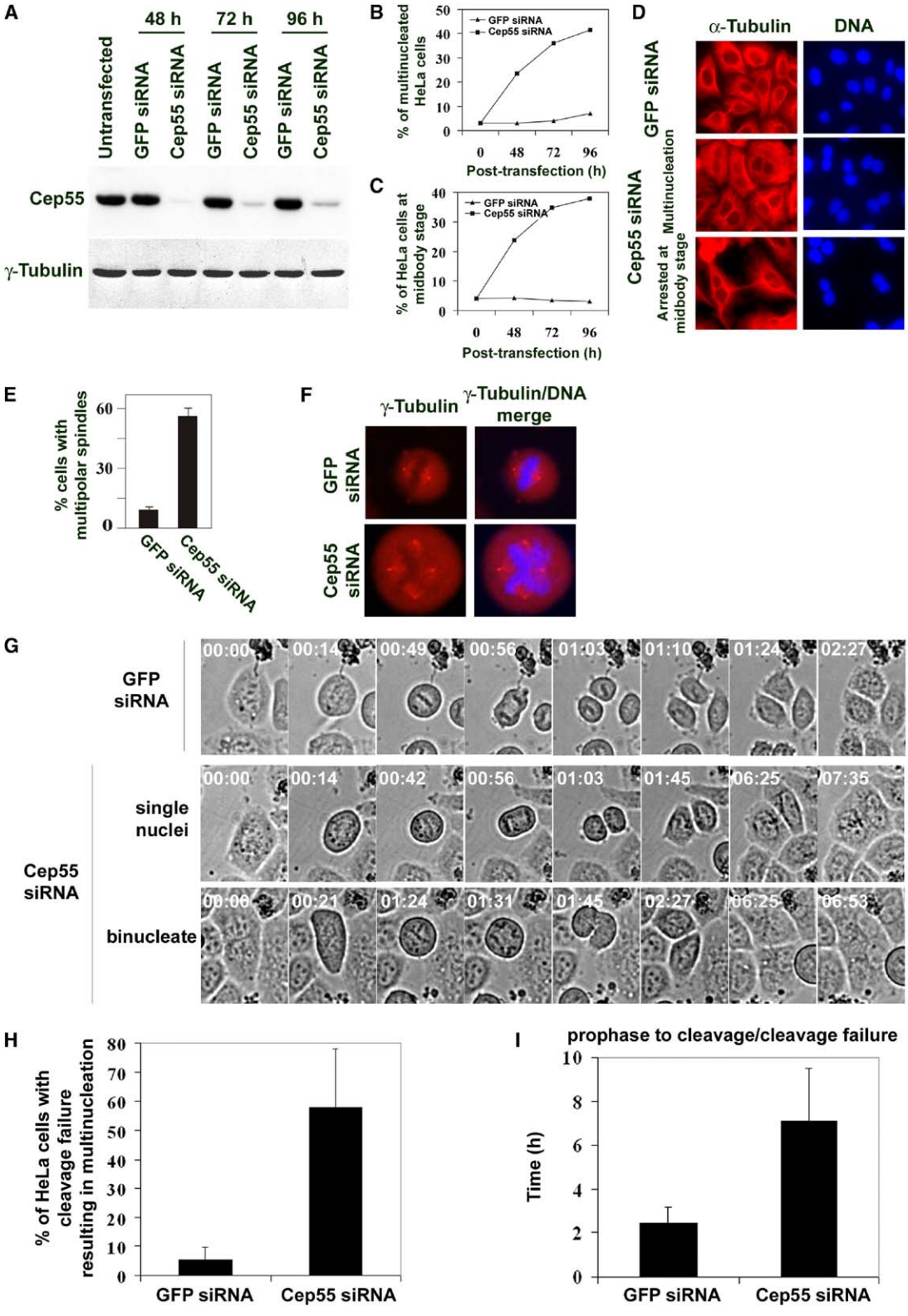

Figure 6. Cep55 Depletion Causes Cytokinesis Failure

(A) Immunoblot of Cep55 in GFP siRNA and Cep55 siRNA transfected HeLa cells at 48, 72, and 96 hr posttransfection. γ-tubulin expression was used as a loading control.

(B and C) At the indicated times following transfection, GFP and Cep55 siRNA transfected cells were immunostained with anti-α-tubulin antibodies. Per sample >200 cells were scored for the presence of ≥2 nuclei/cell (multinucleation; [B]) or for cells that were arrested at the midbody stage (C).

bule nucleation, we demonstrate that Cep55 is not required for microtubule nucleation, because microtubule asters were visible upon regrowth to the same extent in Cep55-depleted cells as in control cells (GFP siRNA; Figure 5F).

siRNA-Mediated Depletion of Cep55 Induces Cellular Phenotypes Consistent with Cytokinesis Failure

To assign a function to Cep55, we sought to characterize the cellular phenotype of Cep55-depleted cells using siRNA. At all time points analyzed after transfection (48, 72, and 96 hr), Cep55 expression was reduced markedly by Cep55 siRNA, but not by GFP siRNA (Figure 6A). Immunofluorescence microscopy analysis revealed that depletion of Cep55 produces phenotypes consistent with cytokinesis failure, that is, multinucleated cells (Figures 6B and 6D) and cells arrested at the midbody stage (late telophase; Figures 6C and 6D). Importantly, no significant cytokinesis defects were observed in control cells. Moreover, an increasing number of Cep55-depleted cells showed cytokinesis defects with time. Aberrant mitotic spindles (>2 mitotic spindle poles) were also frequently observed during metaphase of cells depleted of Cep55 (57%), compared to control cells (10%) (Figures 6E and 6F). These were not the result of loss of centriole cohesion or hyperamplification of centrosomes in Cep55-depleted cells (data not shown); instead, this defect arises as a consequence of cytokinesis failure, as most Cep55-depleted cells with multipolar spindles were aneuploid (i.e., binucleate).

To thoroughly characterize the mechanism of cytokinesis failure, we subjected live HeLa cells transfected with Cep55 siRNA to time-lapse microscopy analysis for 18 hr beginning at 30 hr posttransfection (see Movies S1–S3). The majority of control cells (GFP siRNA) that entered mitosis carried out normal cell cleavage to form two daughter cells with normal kinetics (2 hr 25 min ± 6 min; Figure 6G). Cep55-depleted cells also progressed through mitosis from prophase to telophase with normal kinetics; however, the majority of Cep55-depleted cells failed to complete cytokinesis (57.8% ± 20.1%; Figures 6G and 6H). Consistent with observations of fixed cells, these live cells remained in a post-telophase position, connected by a thin cytoplasmic bridge for a prolonged period of time, increasing their mitotic period by up to 7 hr prior to regression of the cleavage furrow, resulting in a binucleate cell (Figures 6G and 6I). These binucleate cells did not have difficulty entering the next cell division, but again failed to cleave for a prolonged period, resulting in multinucleation (four nuclei/cell; Figure 6G). Again, consistent with our observations of fixed cells, only binucleate cells produced multipolar spindles during metaphase (Figure 6G), indicating that this phenotype is a result of cytokinesis failure. These observations collectively indicate that Cep55 is not required for normal mitotic progression but is essential for successful completion of cytokinesis.

Ectopic Expression of the Cep55 Phosphorylation Mutants, S425/428A and S436A, Induces Cytokinesis Failure

Several lines of evidence have implicated Plk1 in the regulation of cytokinesis (Mundt et al., 1997). In this report, we demonstrate that Cep55 associates with and is phosphorylated by Plk1 during mitosis and that Cep55 itself is required for the completion of cytokinesis, suggesting that these proteins may cooperate to regulate this final stage of cell division. In support of this idea, we demonstrate that endogenous Cep55 and Plk1 colocalize at the midbody during cytokinesis (Figure 7A). We next aimed to understand the relationship between Plk1-dependent phosphorylation of Cep55 and function. In contrast to untransfected control cells (<5%), two or more nuclei were observed in >15% of HeLa cells expressing ectopic Flag-Cep55 and in ~30% of HeLa cells ectopically expressing either of the Flag-Cep55 phosphorylation mutants S425/428A or S436A (Figures 7B and 7C). Furthermore, a significant proportion of cells expressing Flag-Cep55-S425/428A or Flag-Cep55-S436A were arrested at the midbody stage compared to control cells (Figures 7B and 7C). Immunofluorescence microscopy revealed that mutagenesis of these phosphorylation sites does not affect targeting of Cep55 to this intracellular domain (Figure 7C), ruling out the possibility that these cytokinesis defects are due to an indirect effect caused by mislocalization. Thus, we demonstrate that Cep55 phosphorylation at S425, S428, and S436 is required for its function during the final stages of cell division to complete cytokinesis successfully.

Discussion

The molecular mechanisms of cytokinesis in animal cells are less well known compared to yeast cell division, particularly the signaling pathways that result in and regulate mitotic exit and cytokinesis. In this study, we have identified a protein, Cep55, that is required for the successful completion of cytokinesis in mammalian

(D) Representative images of GFP and Cep55 siRNA transfected cells from the data described in (B) and (C) at 72 hr posttransfection. α-tubulin is shown in red. DNA is shown in blue.
(E) The number of mitotic cells with multipolar spindles was compared in Cep55-depleted cells to control cells at 72 hr posttransfection by immunostaining for γ-tubulin. The graph illustrates mean ±SD from two independent experiments. More than 50 mitotic cells were scored per sample.
(F) Representative images of the data described in (E). γ-tubulin is shown in red. DNA is shown in blue.
(G) Representative time-lapse images of a control cell proceeding through mitosis is shown (upper panel). In the lower panels, representative time-lapse images of Cep55-depleted cells with either one or two nuclei proceeding through mitosis are shown.
(H) Graph of time-lapse microscopy data shows the percentage of GFP (n = 287) and Cep55 siRNA (n = 167) transfected cells that proceeded through mitosis but failed to cleave, resulting in multinucleation.
(I) Graph illustrates the average time taken for GFP (n = 63) and Cep55 siRNA (n = 38) transfected HeLa cells to proceed from prophase to cell cleavage and prophase to cell cleavage failure, respectively.

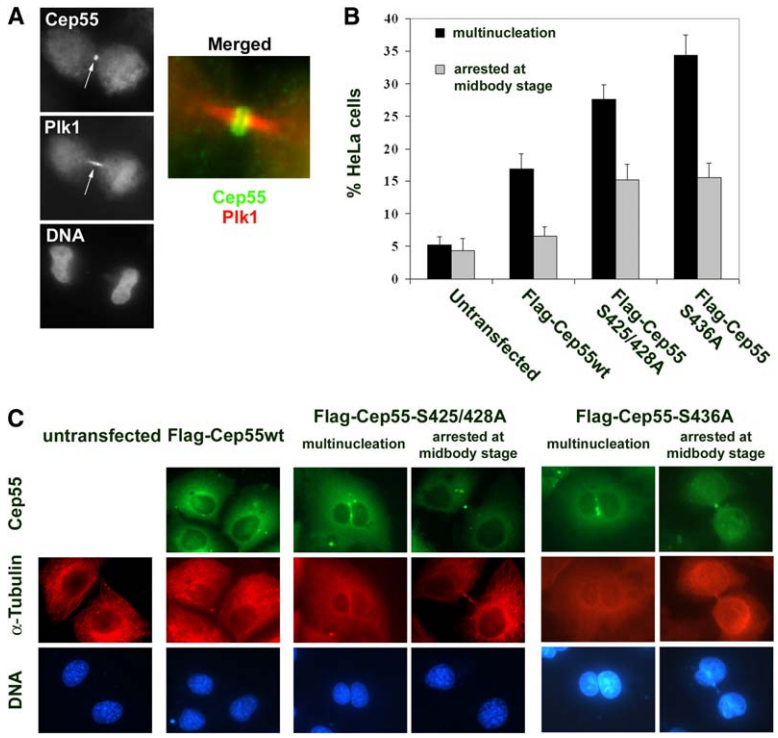

Figure 7. Ectopic Expression of Cep55 Phosphorylation Mutants Causes Cytokinesis Failure

(A) Representative immunofluorescence microscopy images of HeLa cells costained with anti-Cep55 and anti-Plk1 antibodies illustrating midbody colocalization.

(B) At 72 hr posttransfection, HeLa cells ectopically expressing wild-type Flag-Cep55 or the Flag-Cep55 phosphorylation mutants S425/428 and S436A were costained with anti-Cep55 and anti-α-tubulin antibodies. Transfected cells were scored for the indicated cytokinesis defects. The graph shows the mean percentage of cells with these cytokinesis defects ±SD from two independent experiments.

(C) Representative images of the data shown in (B).

cells as cell membrane abscission fails in cells depleted of Cep55. Interestingly, the cytokinesis defects produced by depletion of Cep55 are identical to the phenotypes produced by acentrosomal cells (Hinchcliffe et al., 2001; Khodjakov and Rieder, 2001). We also demonstrate that Cep55 is a centrosome protein and thus our data further enhance the importance of centrosomes in the final stages of cell division. Interestingly, we illustrate that Cep55 does not remain associated with the centrosome during mitosis, and its displacement from the centrosome is triggered by its phosphorylation. Therefore, we propose a model whereby the centrosome acts as a regulatory site to organize phosphorylation of Cep55 upon mitotic entry. This phosphorylated Cep55 is then able to locate to the midbody during the final stages of cell division to function in the signal transduction pathway(s) that results in mitotic exit and cytokinesis. Therefore, we propose that dephosphorylation of Cep55 may allow it to relocate to the centrosome upon entry into G1.

Fluctuations in the activity of Cdks drive cells to progress through mitosis. Specifically, mitotic entry is promoted by elevated activity of Cdk1 when complexed with cyclin B1 (Pines, 1995), while the exit from mitosis requires the inactivation of Cdk1 (Zachariae and Nasmyth, 1999) and the dephosphorylation of at least a subset of Cdk1 substrates (Visintin et al., 1998). We demonstrate that Cep55 is phosphorylated at S425 and S428 by Cdk1/cyclin B1 upon mitotic entry, which is when this kinase is found at the centrosome. Another kinase, Erk2, has recently been shown to also localize to the mitotic centrosomes (Lou et al., 2004). We illustrate that S425 and S428 can also be phosphorylated by Erk2 upon mitotic entry. In addition to Cdk1 and Erk2, we demonstrate that Cep55 is also phosphorylated by Plk1 and this phosphorylation event is dependent on prior phosphorylation by Cdk1 and/or Erk2 at S425 and S428. Plk1 locates to the midbody and its role during cytokinesis has been demonstrated in yeast, *Drosophila*, and mammals. Our data strongly indicate that Cep55 and Plk1 colocalize at the midbody and that Plk1-dependent phosphorylation of Cep55 is required for completion of cytokinesis. In addition, the Plk1-dependent phosphorylation mutant S436A causes cytokinesis failure to the same extent as the Cdk1/Erk2-dependent mutant S425/428A, indicating that phosphorylation at S436 is absolutely required for the function of Cep55 during cytokinesis, whereas phosphorylation at S425 and S428 is not required for Cep55 cytokinesis function directly but is essential for Plk1-dependent phosphorylation at S436. These findings indicate that Cep55 and Plk1 may cooperate at the midbody to coordinate mitotic exit and cytokinesis. Thus, we propose that unphosphorylatable Cep55 may fail to bind or generate the required signal to downstream components of the mitotic exit pathway.

Cep55 was found to localize to the centrosome via its C-terminus and this same region was shown to associate in vivo with the centrosome proteins CG-NAP and Kendrin. In contrast to CG-NAP and Kendrin, which gain affinity for the centrosome to participate in γ-TuRC anchorage (Takahashi et al., 2002), we show that Cep55 loses affinity for the centrosome upon mitotic entry coinciding with its dissociation from CG-NAP and Kendrin. Thus, it is not surprising that we find no requirement of Cep55 for microtubule nucleation. Loss of Cep55 from the centrosome coincides with its phosphorylation at the C-terminal residues S425 and S428.

It is plausible to suggest that phosphorylation of Cep55 by either Erk2 or Cdk1 causes a conformational change in the protein causing it to lose affinity for CG-NAP and Kendrin, consequently becoming displaced from the centrosome at the G_2/M boundary. This in turn may enable S436 of Cep55 to be accessible to Plk1 for phosphorylation. We hypothesize that the displacement of Cep55 from the centrosome enables CG-NAP and Kendrin to strongly anchor themselves to the centrosome by binding calmodulin (Takahashi et al., 2002). Consistent with this idea, Cep55 and calmodulin bind the same region of CG-NAP, and the CG-NAP/Kendrin/calmodulin interaction is thought to occur only during mitosis, which is when calmodulin is observed at the centrosome (Li et al., 1999).

Increased centrosome number and abnormal centrosome morphology have been described in many human tumors (D'Assoro et al., 2002). Therefore, it is not surprising that abnormal expression of many centrosome proteins, such as Aurora A kinase (Ewart-Toland et al., 2003), has been linked with different stages of cancer progression, particularly high cytological grade. Centrosomes act as dominant microtubule nucleating sites, and when multiple centrosomes are present they give rise to multiple spindle poles during metaphase, which in turn leads to aberrant divisions and increased genetic instability (Doxsey, 2002). We show that Cep55-depleted cells undergo cytokinesis failure inducing aneuploidy and multiple spindle poles. This event could generate genomic instability and thus facilitate the loss of tumor suppressor genes and activation of oncogenes. Furthermore, the overexpression of Cep55 also induces aneuploidy, albeit to a lesser extent than Cep55 depletion. Nevertheless, these data suggest that the expression of Cep55 must be tightly regulated to ensure that the final stages of cell division occur correctly. Therefore, it will be interesting to determine whether the expression and phosphorylation of Cep55 is altered in cancer cells.

Experimental Procedures

Cell Culture and Transfection

293T, Cos-7, and HeLa cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum and grown at 37°C in a humidified 5% CO_2 atmosphere. For immunoblotting experiments, cells were seeded at 50%–60% confluence/10 cm^2 dish and transfected with 7.5 μg of plasmid DNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Alternatively, cells (5×10^6) were transfected by electroporation with 7.5 μg of plasmid DNA, and then seeded onto 10 cm^2 dishes. For immunofluorescence experiments, cells were seeded onto coverslips and transfected at 50%–60% confluence with 1.5 μg of plasmid DNA with Lipofectamine 2000. Where indicated, to inhibit phosphatase activity, asynchronous cells were treated with the serine/threonine phosphatase inhibitor okadaic acid (10 μM) for 16 hr.

Cell Synchronization and Flow Cytometry

Cells were synchronized by double-thymidine block and collected at the G_1/S border (0 hr), S phase (4 hr), G_2 phase (8 hr), and mitosis (10 hr). At each time point, cells were processed for immunoblotting and for FACS analysis as previously described (Fabbro et al., 2004). For mitotic synchronization of cells, cells were treated with 0.5 μg/ml nocodazole for 16 hr. Mitotic arrested cells were collected by "mitotic shake-off." To inhibit Cdk1 activity, after nocodazole treatment (16 hr), roscovitine (55 μM) was added for 4 hr. To inhibit Erk2 activity, cells were treated with 0.5 μg/ml nocodazole and 10 μM U0126 for 16 hr. To ensure a mitotic arrest, these cells were also treated with 10 μg/ml ALLN, a proteasome inhibitor, because it has been suggested that cells can escape nocodazole-induced arrest in the presence of U0126 (Chung and Chen, 2003).

Immunoprecipitation and Immunoblotting

Cellular extracts were prepared as described previously (Fabbro et al., 2004). Where indicated, cell lysates were treated with lambda phosphatase (λ-PPase; New England Biolabs, Ipswich, MA) for 30 min at 30°C. For Plk1/Cep55 coimmunoprecipitation experiments, protein samples precleared with protein G beads were incubated with the required antibody for 2 hr. Immune complexes were collected with protein G-Sepharose beads, washed twice with lysis buffer, and then fractionated by SDS-PAGE for immunoblot analysis. For CG-NAP/Kendrin/Cep55, coimmunoprecipitation experiments were performed as described previously (Takahashi et al., 2002). The following primary antibodies were used for immunoblotting: anti-Cep55, anti-γ-tubulin (T5192; Sigma, St. Louis, MO), anti-Flag M2 (Sigma), anti-Myc (Cell Signaling, Beverly, MA), anti-GFP (Molecular Probes, Eugene, OR), anti-Plk1 (37-7000; Zymed, San Francisco, CA), and anti-Cdk1 (De Souza et al., 2000). Primary antibody bound to the indicated protein was detected by incubation with a horseradish peroxidase-conjugated secondary antibody (Sigma). Blotted proteins were visualized using the ECL detection system (Amersham, Piscataway, NJ).

Immunofluorescence and Confocal Microscopy

Cells were fixed in ice-cold methanol for 10 min at –20°C, washed three times with PBS, and then blocked in 3% bovine serum albumin/PBS for 45 min before the required primary antibody was applied. The following antibodies were used: anti-Cep55, anti-α-tubulin (clone DM1A; Sigma), anti-γ-tubulin (GTU88; Sigma), GT335, anti-CG-NAP, and anti-Kendrin (Takahashi et al., 2002), anti-Plk1 (37-7000; Zymed), and anti-centriolin (Gromley et al., 2003). Cells were then washed with PBS and incubated with Alexa 488- or Alexa 546-conjugated secondary antibody (Molecular Probes). Cell nuclei were counterstained with Hoechst 33285 (Sigma). After extensive washing, coverslips were mounted onto glass microscope slides with Mowiol and cells were viewed with a fluorescence microscope (Zeiss, Jena, Germany). Fluorescence and differential interference contrast (DIC) images were captured and processed using a Leica (Bernsheim, Germany) DMIRB/E confocal microscope.

In Vivo Microtubule Nucleation Assay

Cells were placed on ice for 30 min to depolymerize microtubules. The cold medium was aspirated and medium prewarmed to 37°C was added for 2 min. Cells were washed twice with PBS, and then permeabilized in 80 mM K-PIPES (pH 6.8), 1 mM EGTA, 1 mM $MgCl_2$, 0.1% Triton X-100 for 45 s. Cells were washed three times with PBS and then fixed in ice-cold methanol for 10 min at –20°C before being processed for immunofluorescence microscopy to visualize microtubules by staining for α-tubulin.

Supplemental Data

Plasmid construction, Northern blotting, in vitro kinase assays, in vivo 32P labeling, phosphoamino acid analysis, mass spectrometry, and time-lapse microscopy are available at http://www.developmentalcell.com/cgi/content/full/9/4/477/DC1/.

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