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Cep55, a Microtubule-bundling Protein, Associates with Centralspindlin to Control the Midbody Integrity and Cell Abscission during Cytokinesis[D] [V]

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We report here an efficient functional genomic analysis by combining information on the gene expression profiling, cellular localization, and loss-of-function studies. Through this analysis, we identified Cep55 as a regulator required for the completion of cytokinesis. We found that Cep55 localizes to the mitotic spindle during prometaphase and metaphase and to the spindle midzone and the midbody during anaphase and cytokinesis. At the terminal stage of cytokinesis, Cep55 is required for the midbody structure and for the completion of cytokinesis. In Cep55-knockdown cells, the Flemming body is absent, and the structural and regulatory components of the midbody are either absent or mislocalized. Cep55 also facilitates the membrane fusion at the terminal stage of cytokinesis by controlling the localization of endobrevin, a v-SNARE required for cell abscission. Biochemically, Cep55 is a microtubule-associated protein that efficiently bundles microtubules. Cep55 directly binds to MKLP1 in vitro and associates with the MKLP1-MgcRacGAP centralspindlin complex in vivo. Cep55 is under the control of centralspindlin, as knockdown of centralspindlin abolished the localization of Cep55 to the spindle midzone. Our study defines a cellular mechanism that links centralspindlin to Cep55, which, in turn, controls the midbody structure and membrane fusion at the terminal stage of cytokinesis.

INTRODUCTION

Cytokinesis, the division of a cell, involves the selection of the cleavage site, the assembly of the contractile ring, the ingression of the cleavage furrow, and finally the abscission of the cell (Glotzer, 2001). The initial events of cytokinesis are mediated by the concerted actions of the central spindle/spindle midzone and the contractile ring. At the molecular level, a key regulator is the centralspindlin complex, consisting of the mitotic kinesin MKLP1 and the GTPase-activating protein MgcRacGAP (Mishima *et al.*, 2002). At anaphase, centralspindlin, localized to the spindle midzone, interacts with and possibly activates the guanine nucleotide exchange factor, ECT2, which, in turn, activates the small GTPase RhoA to determine the positioning of the cleavage site and to induce the assembly and ingression of the contractile ring (Kamijo *et al.*, 2005; Yuce *et al.*, 2005; Zhao and Fang, 2005b). Other regulatory and structural proteins also accumulate and function at the midzone; these include mitotic kinases Aurora B (Terada *et al.*, 1998) and Plk1 (Golsteyn *et al.*, 1995; Lane and Nigg, 1996), a mitotic kinesin MKLP2 (Hill *et al.*, 2000; Fontijn *et al.*, 2001), and a microtubule-associated protein PRC1 (Jiang *et al.*, 1998; Mollinari *et al.*, 2002). Phosphorylation of MKLP1 (Guse *et al.*, 2005; Gruneberg *et al.*, 2006) and MgcRacGAP (Minoshima *et al.*, 2003) by Aurora B is necessary for the completion of cytokinesis, whereas phosphorylation of MKLP1 (Liu *et al.*, 2004) and MKLP2 (Neef *et al.*, 2003) by Plk1 also contributes to the regulation of cytokinesis. On the other hand, localization of Aurora B and Plk1 to the spindle midzone requires MKLP2 (Neef *et al.*, 2003; Gruneberg *et al.*, 2004).

The terminal stage of cytokinesis involves cell abscission to generate two separate daughter cells (Glotzer, 2001). Although it has been established that both the midbody structure and membrane fusion are essential for cell abscission (Albertson *et al.*, 2005; Otegui *et al.*, 2005), the biochemical machinery and the cellular processes for abscission remain ill-defined. Several proteins have been shown to function at this terminal stage. PRC1, a microtubule-bundling protein (Mollinari *et al.*, 2002, 2005), MKLP1, a subunit of the centralspindlin complex (Matuliene and Kuriyama, 2002), and annexin 11, a calcium-dependent phospholipid-binding protein (Tomas *et al.*, 2004), are all required for the integrity of the midbody structure and for the completion of cytokinesis. On the other hand, both t-SNARE syntaxin 2 and v-SNARE endobrevin localize to the midbody and play an essential role at the terminal stage of cytokinesis (Low *et al.*, 2003). Centriolin, a coiled-coil protein required for membrane fusion and cell abscission, anchors the membrane-vesicle-tethering exocyst complex at the midbody and controls the localization of syntaxin 2 and endobrevin during cytokinesis (Gromley *et al.*, 2003, 2005). Thus, separation of two daughter cells requires membrane trafficking and fusion (Finger and White, 2002; Strickland and Burgess, 2004; Albertson *et al.*, 2005). To understand the molecular mechanism of cytokinesis, we initiated a functional genomic screen for novel regulators of cytokinesis and identified Cep55 as a protein that controls the terminal stage of cytokinesis. During the preparation of this article, Cep55 was also reported as a centrosomal protein required for cytokinesis, although the

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Abbreviations used: siRNA, small interfering RNA; DIC, differential interference contrast.

mechanism of its function was not clear (Fabbro *et al.*, 2005). We reported here the molecular and cellular pathway in which Cep55 acts. We found that Cep55 is required for the establishment and proper function of the midbody structure. In addition, Cep55 facilitates membrane fusion at the terminal stage of cytokinesis. Interestingly, Cep55 directly interacts with MKLP1 and its cellular localization is under the control of centralspindlin. Our study provides a molecular and cellular mechanism that controls the terminal stage of cytokinesis.

MATERIALS AND METHODS

Gene Expression Analyses

Whitfield *et al.* (2002) performed microarray analysis on genome-wide gene expression across the cell cycle in HeLa cells and identified 566 genes transcriptionally induced in G2 or in G2/M. Segal *et al.* (2004) analyzed gene coexpression profile among 1975 published microarrays derived from 22 different types of tumors . Based on statistic analyses of coexpression profiling, they organized human genes into different functional modules, each of which corresponds to a set of genes that act in concert to carry out a specific physiological function. Of 577 functional modules identified, two modules function in the cell cycle regulation and consist of genes that covary (co-up-regulate or co-down-regulate) with known cell cycle regulators (Segal *et al.*, 2004). Our current analyses were based on these two previous studies.

Among 566 G2- or G2/M-induced genes reported by Whitfield *et al.* (2002), we initially analyzed the following 30 novel genes that have the best induction profile at G2/M: C10orf3 (Cep55), HURP, DKFZp762E1312, FLJ11029, FLJ11252, KIAA0042, ARL6IP, E2-EPF, LRRC17, MELK, MAPK13, PEPP2, HAN11, C9orf100, C9orf140, GAS2L3, FLJ40629, FLJ22624, FLJ20333, FLJ13354, FLJ20364, FLJ20510, FLJ20699, FLJ23293, FLJ21480, KIAA0952, GTSE1, LPP, MDS025, and PMSCL1. We found that the first 12 of the above listed 30 genes were also present in the cell cycle modules reported by Segal *et al.* (2004). Therefore, we focused our functional analysis on these 12 genes and found that several of them, including C10orf3 (Cep55), DKFZp762E1312, HURP, and LRRC17, were associated with microtubules in vivo during mitosis. At the functional level, we found that HURP is required for efficient capture of kinetochores by the mitotic spindle during mitosis (Wong and Fang, 2006) and reported here that C10orf3 is required for cell abscission at the terminal stage of cytokinesis.

Cell Culture and Cell Synchronization

HeLa cells (ATCC, Manassas, VA) were maintained in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Invitrogen) and 2 mM L-glutamine (Invitrogen). HeLa S3 cells were grown in suspension and synchronized by thymidine-nocodazole treatments as previously described (Fang *et al.*, 1998).

Recombinant Proteins and Antibodies

Recombinant GST-Cep55 was expressed in Sf9 cells using the Bac-to-Bac system (Invitrogen) according to the manufacturer's instructions. Anti-Cep55 antibodies were generated against full-length human Cep55 and affinity-purified. Anti-centriolin and MKLP2 antibodies were generated against aa 820–994 in centriolin and aa 530–664 in MKLP2, respectively, and the specificity of these two antibodies was confirmed in siRNA-mediated knockdown experiments assayed by both Western blotting and immunofluorescence staining (unpublished data). Anti-MgcRacGAP and anti-anillin antibodies were described previously (Zhao and Fang, 2005a, 2005b). Anti-MKLP1 monoclonal antibody (mAb) were a gift from Dr. Ryoko Kuriyama, anti-syntaxin 2 and endobrevin antibodies from Dr. Thomas Weimbs, and anti-sec3 and sec8 antibodies from Dr. Charles Yeaman. The following antibodies were from commercial sources: anti-FLAG antibody (Sigma, St. Louis, MO), anti-Aurora B antibody (BD Transduction Laboratories, Lexington, KY), sheep anti-β-tubulin (Cytoskeleton, Denver, CO), goat anti-MgcRacGAP (Ab-Cam, Cambridge, MA), and anti-cyclin B1, anti-ECT2, anti-MKLP1, anti-Plk1, anti-PRC1, and anti-p38MAPK antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Anti-β-tubulin E7 mAb was obtained from the Developmental Studies Hybridoma Bank.

Transfection and RNA Interference

DNA transfection was carried out with Lipofectamine 2000 (Invitrogen). RNA interference (RNAi) of ECT2, MgcRacGAP, MKLP1, MKLP2, and Anillin was performed as described previously (Zhao and Fang, 2005a, 2005b). SMARTpool siRNA oligonucleotides against Cep55 were ordered from Dharmacon (Boulder, CO). Three of four oligonucleotides gave efficient knockdowns, and all three gave identical phenotypes. The target sequences are (in the sense orientation) as follows: 5′-GGAGAAGAAUGCUUAUCAAUU-3′ (sequence A), 5′-GAAGAGAAUGAUAUUGCUAUU-3′ (sequence B), and 5′-GCGAUCUGCUUGUCCAUGUUU-3′ (sequence C). si*CONTROL* NonTargeting siRNA no. 2 (Dharmacon) was used as a control oligonucleotide. RNAs were transfected into HeLa cells using DharmaFECT 1 reagent (Dharmacon).

Immunoprecipitation

Rabbit antibodies against Cep55, MKLP1, and MgcRacGAP were coupled to Affi-Prep Protein A beads (Bio-Rad, Richmond, CA) at a concentration of 0.3 mg/ml. HeLa S3 cell pellets collected at various cell cycle stages were lyzed in the NP-40 lysis buffer (50 mM Tris-HCl, pH 8.0, 140 mM NaCl, 1% NP-40, 10% glycerol, 0.1 mM EDTA, 1 mM DTT, 0.5 μM microcystin, 10 μg/ml each of leupeptin, pepstatin, and chymostatin). Lysates were centrifuged and incubated with protein A beads coupled with preimmune rabbit IgG at 4°C for 1 h. The precleared lysates were then incubated with protein A beads coupled with anti-Cep55, MKLP1, or MgcRacGAP antibodies at 4°C overnight. Beads were recovered by centrifugation, washed five times with the lysis buffer in the presence of 500 mM NaCl and twice with the lysis buffer, resuspended in SDS sample buffer, heated at 95°C, analyzed by SDS-PAGE, and immunoblotted with appropriate antibodies.

In Vitro Binding Assays

MKLP1, MgcRacGAP, Cep55, and fragments thereof were synthesized by in vitro transcription and translation (TNT) systems in reticulocyte lysates (Promega, Madison, WI). The in vitro–translated proteins (20 μl total volume) were then incubated together and subjected to immunoprecipitaions to assay for protein–protein interactions. The immunoprecipitaion reactions were incubated at room temperature for 2 h and washed as described in *Immunoprecipitation*.

For glutathione pulldown experiments, the in vitro–translated proteins (20 μl total volume) were incubated with purified recombinant GST-Cep55 for 1 h at room temperature, and the reactions were then incubated with glutathione beads for 1 h at room temperature. Glutathione beads were washed as described above.

Microtubule Pelleting and Bundling Assays

Assembly-competent α/β-tubulin was isolated as described (Hyman *et al.*, 1991). Recombinant GST-Cep55 at a final concentration of 0.3 μM was added to the reaction mix containing 2 mM GTP, 10 μg/ml each of leupeptin, pepstatin, and chymostatin, 20 μM taxol, and 2 μM taxol-stabilized microtubules in 1× BRB80 buffer (80 mM PIPES, pH 6.8, 1 mM $MgCl_2$, 1 mM EGTA). The reaction was incubated at room temperature for 30 min and pelleted through a 140-μl 40% glycerol cushion containing 20 μM taxol and protease inhibitors in 1× BRB80 buffer at 100,000 × *g* for 20 min at 30°C. Pellets were washed three times with 1× BRB80 buffer and analyzed by SDS-PAGE, followed by Western blotting.

The microtubule-bundling assay was performed with modifications from a published protocol (Mishima *et al.*, 2002). Recombinant proteins was mixed with taxol-stabilized microtubules (final concentration 2 μM) in 1× BRB80, 1 mM DTT, 2 mM ATP, and 150 mM KCl in 10 μl reactions and incubated for 15 min at room temperature. Samples were then fixed with 100 μl of 1× BRB80 containing 1% glutaraldehyde at room temperature for 5 min before being overlaid onto a 5 ml of cushion (1× BRB80, 25% glycerol) and spun onto coverslips at 18,000 rpm for 1 h in a Beckman SW41Ti rotor at 22°C. After the spin, the coverslips were fixed with −20°C methanol for 10 min, rehydrated, quenched with $NaBH_4$ in phosphate-buffered saline (PBS), and stained with the antibodies indicated.

Immunofluorescence and Time-Lapse Microscopy

To observe the spindle staining of Cep55, cells were pre-extracted with PHEM-T (60 mM Pipes, 25 mM HEPES, 10 mM EGTA, 2 mM $MgC1_2$, pH 6.9, 0.5% Triton X-100) at room temperature for 5 s before paraformaldehyde was added to 1.6% final concentration. Fixation was performed at room temperature for 15 min. For all the other immunofluorescence staining, cells were fixed in methanol at −20°C. After fixation, cells were further permeabilized and blocked with PBS-BT (1× PBS, 0.1% Triton X-100, 3% bovine serum albumin) at room temperature for 30 min. Coverslips were subsequently incubated in primary and secondary antibodies diluted in PBS-BT. Images were acquired with Openlab 4.0.2 (Improvision, Lexington, MA) under a Zeiss Axiovert 200M microscope (Thornwood, NY) using 63× or 100× oil immersion lenses. Deconvolved images were obtained using AutoDeblur v9.1 and AutoVisualizer v9.1 (AutoQuant Imaging, Watervliet, NY).

For time-lapse microscopy, HeLa cells were cultured in Leibovitz's L-15 medium (Invitrogen) supplemented with 10% FBS (Invitrogen) and 2 mM L-glutamine (Invitrogen). Cells were placed in a 37°C heated microscope chamber and observed under differential interference contrast (DIC) on a Zeiss Axiovert 200M microscope with a 20× lens. Images were acquired every three minutes with Openlab 4.0.2 software (Improvision).

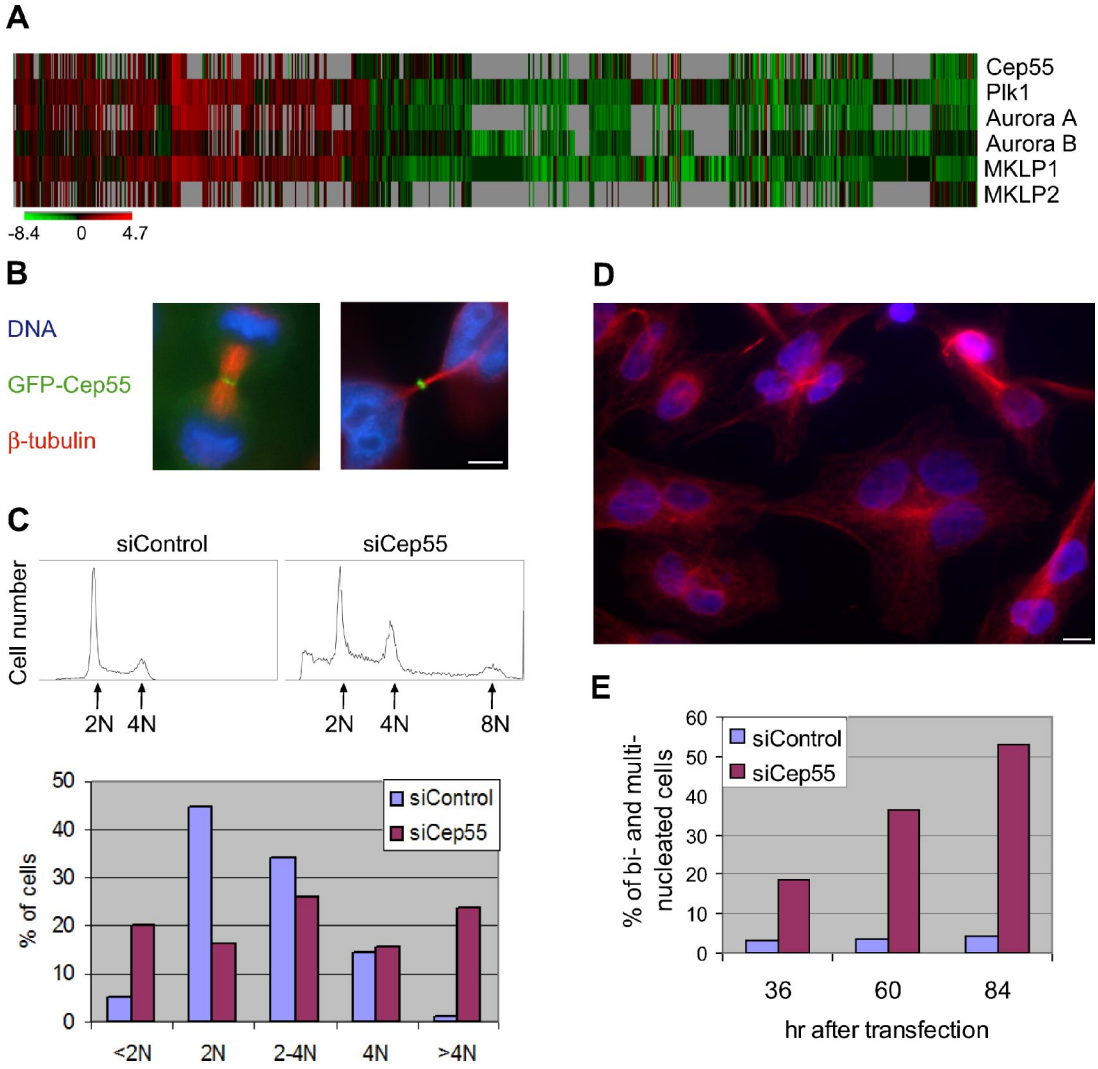


Figure 1. Cep55 controls cytokinesis. (A) Coexpression of Cep55 with Plk1, Aurora A, Aurora B, MKLP1, and MKLP2 in 944 tumor microarrays. The expression data/profiling were extracted from microarray analyses reported by Segal *et al.* (2004). The change in expression of each gene in a given array is relative to the average expression of the gene across all arrays. Each column represents one tumor microarray experiment (944 total) and gray pixels represent missing values. (B) HeLa cells were transfected with GFP-Cep55, fixed at 30 h after transfection, and immunostained with anti-β-tubulin (red) antibody. DNA, blue. Bar, 5 μm. (C) HeLa cells were transfected with either control siRNA (siControl) or siRNA against Cep55 (siCep55) for 60 h. Cell cycle profile was determined by FACS analysis and quantified by FlowJo. (D) A representative image of Cep55-depleted cells at 36 h after transfection. DNA, blue; β-tubulin, red. Bar, 10 μm. (E) Quantification of bi-/multinucleated cells at the indicated times after transfection of a siRNA against Cep55. More than 300 cells were counted for each time point.

RESULTS

A Functional Genomic Analysis Identified Cep55 as a Gene Required for Cytokinesis

To identify novel regulators of cytokinesis, we selected candidate genes based on their gene expression profiles and then assayed their involvement in cytokinesis functionally. Our genomic analysis was based on two predictions. First, we predicted that genes induced in G2 or G2/M likely function in mitosis and cytokinesis. We expected that only a subset, but not all, of these genes acts in mitosis and cytokinesis and therefore needed another criterion to select functionally important ones. Second, we predicted that expression of genes in the core mitosis and cytokinesis machinery tends to covary during tumorigenesis, as these regulators should function as one module during tumor proliferation. It has been reported that 566 genes in human genome are induced in G2 or G2/M in HeLa cells (Whitfield *et al.*, 2002). Thus, we searched in published microarray databases (Rhodes *et al.*, 2004; Segal *et al.*, 2004) for novel genes in this group that covary (coinduce or corepress) in hundreds of tumor tissues with known regulators of cytokinesis, such as Plk1, Aurora A, Aurora B, MPLK1, and MPLK2.

Our initial prove-of-principle test focused on 30 novel genes with the best induction profiles in G2/M (see *Materials and Methods* for the list of genes). Twelve of these genes also satisfied the coexpression criterion (Segal *et al.*, 2004). The function of these 12 candidates was further examined in the following two assays. First, candidate proteins fused to GFP were expressed in HeLa cells and proteins localized to cytokinesis structures, such as the contractile ring, the spindle midzone, and the midbody, were selected for further analysis. Second, expression of endogenous genes in HeLa cells

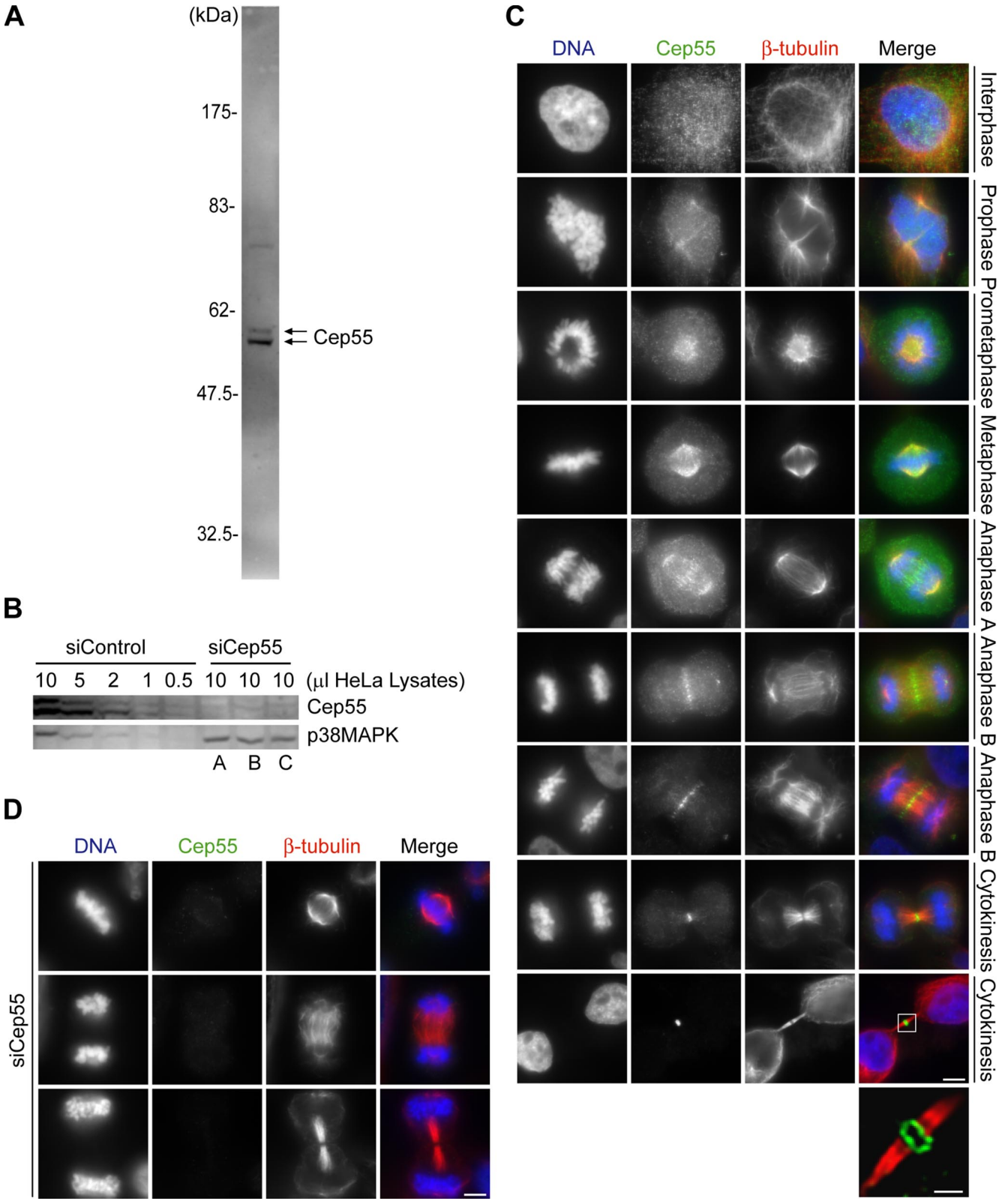


Figure 2. Cep55 associates with the spindle during mitosis and with the spindle midzone/midbody during cytokinesis. (A) HeLa lysates were analyzed with the affinity-purified antibody against Cep55 by Western blotting. (B) Western blot analysis of lysates from Cep55- and control-knockdown cells to determine the knockdown efficiency. p38MAPK was used as a loading control. Three sequences of siRNAs against Cep55 (A–C) were analyzed, and different amounts of lysates from control-knockdown cells (volume indicated) were loaded to quantify the degree of Cep55 knockdown. (C) HeLa cells were immunostained for Cep55 (green) and β-tubulin (red). DNA, blue. A deconvolved image of a single focal plane from the boxed midbody is shown at the bottom. Scale bar, 5 μm, except for the deconvolved image (1 μm). (D) The specificity of Cep55 immunofluorescence staining. HeLa cells were transfected with siCep55 and immunostained for Cep55 (green) and β-tubulin (red). Bar, 5 μm.

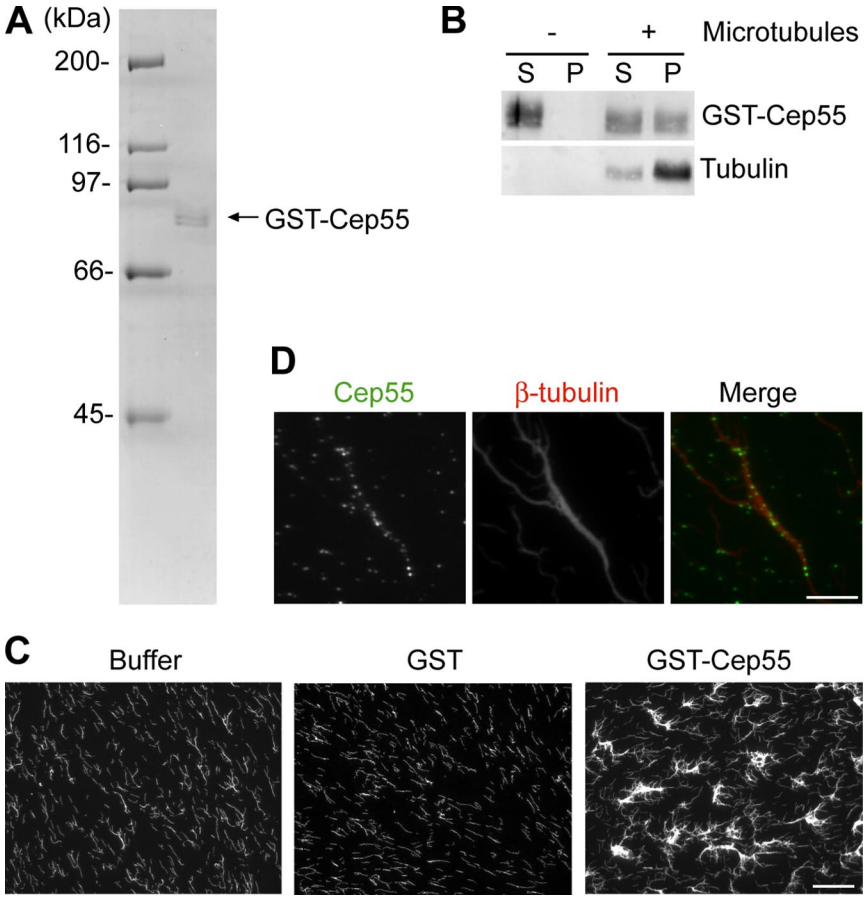


Figure 3. Cep55 is a microtubule-associated protein with an in vitro bundling activity. (A) Purified recombinant GST-Cep55. Purity of recombinant GST-Cep55 was analyzed by SDS-PAGE and stained by Coomassie blue. (B) Pelleting assay for microtubule-associated proteins. GST-Cep55 (0.3 μM) was incubated with taxol-stabilized microtubules (2 μM) and then pelleted through a 40% glycerol cushion. The amounts of GST-Cep55 and tubulin in supernatant (S) and pellet (P) were analyzed by SDS-PAGE and Western blotting. (C and D) GST-Cep55 (0.5 μM) was incubated with taxol-stabilized microtubules, fixed, and sedimented onto coverslips. Immunofluorescence staining of tubulin was shown in C, and staining of tubulin (red) and Cep55 (green) were shown in D. In C, the exposure time for the buffer and GST controls was twice as long as that for the image of GST-Cep55–incubated sample. Scale bar, 20 μm for C and 5 μm for D.

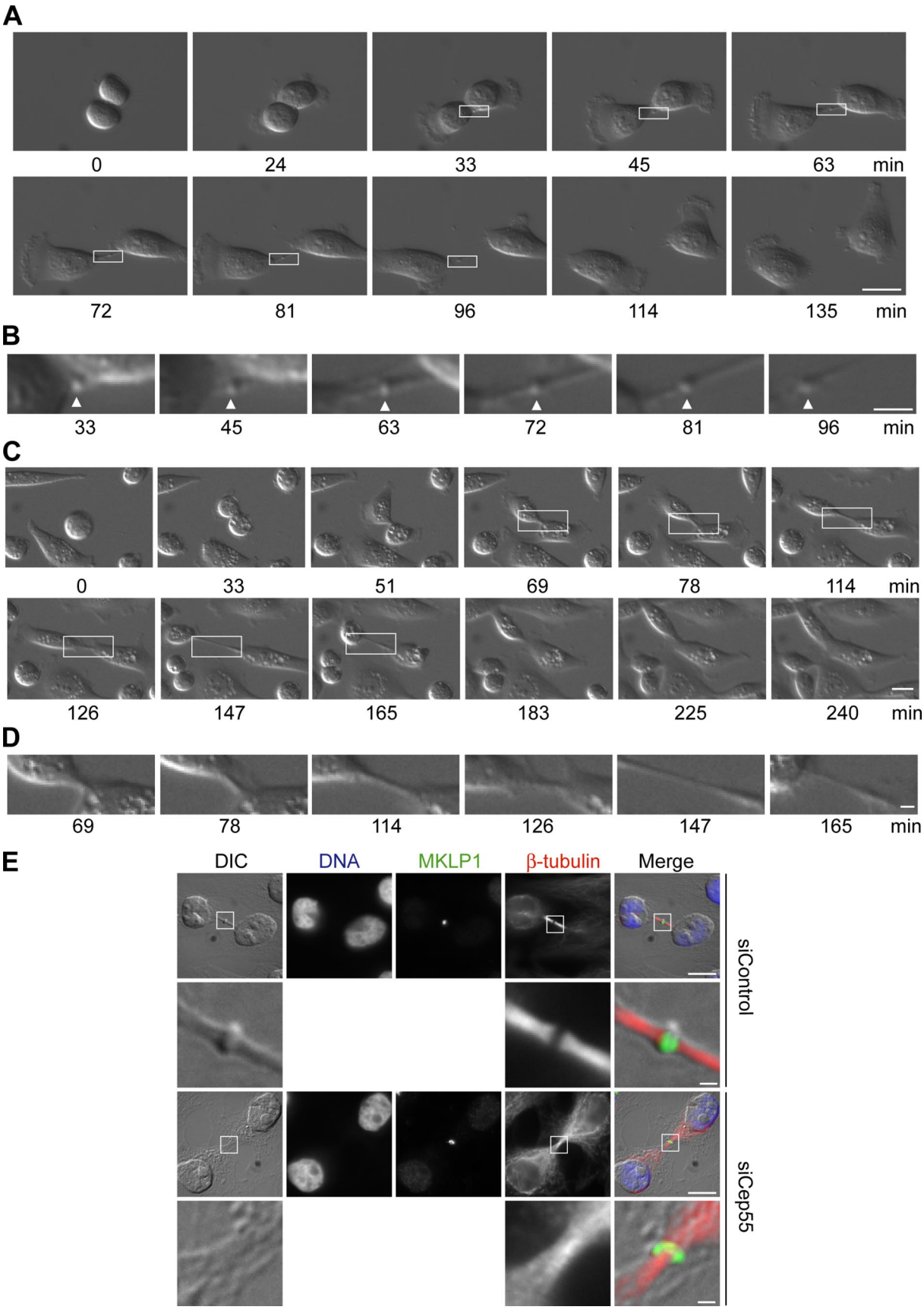
was knocked down by gene-specific small interfering RNAs (siRNAs) and the presence of binucleated and multinucleated cells were determined by fluorescence-activated cell sorter (FACS) and by immunofluorescence staining. These analyses led to the identification of C10orf3, a protein initially reported for its overexpression in colon cancer (Sakai *et al.*, 2005), as a regulator required for cytokinesis. C10orf3 was induced transcriptionally in G2/M (Whitfield *et al.*, 2002). Furthermore, its expression covaried with those of known regulators of cytokinesis in 944 tumor tissues (Figure 1A), based on our analysis of data reported by Segal *et al.* (2004). While this manuscript was in preparation, independent studies showed that C10orf3 (also known as FLJ10540) is a centrosomal protein required for cytokinesis (Fabbro *et al.*, 2005; Martinez-Garay *et al.*, 2006). We will refer C10orf3 as Cep55 in this article. We found that GFP-Cep55 localized to the spindle midzone and the midbody (Figure 1B). FACS analysis indicated that knockdown of Cep55 gave rise to 25% of cells with more than 4 N DNA content compared with 1% in control-knockdown cells (Figure 1C). We also showed that Cep55 is required for cytokinesis: at 84 h after transfection more than 50% of knockdown cells were bi- and multinucleated, whereas <4% control cells were bi- and multinucleated (Figures 1, D and E).

Cep55 Localizes to the Mitotic Spindle, the Spindle Midzone and the Midbody

We next raised an anti-Cep55 antibody and determined the localization of endogenous Cep55. Our antibody recognized a doublet about 55 kDa in lysates of asynchronous HeLa cells (Figure 2A). The specificity of this recognition was confirmed, because the levels of the doublet were reduced by at least 95% in Cep55-knockdown cells using three different siRNA sequences (Figure 2B). Immunofluorescence staining of the endogenous protein indicated that Cep55 existed as punctates in interphase cells. During mitosis, Cep55 localized to the spindle pole regions at late prophase and to the mitotic spindle at prometaphase and metaphase (Figure 2C). At anaphase A, Cep55 accumulated at the spindle midzone region with some signals remained on the mitotic spindle. During cytokinesis, Cep55 localized at the spindle midzone and then at the midbody (Figure 2C). Deconvolved image showed that Cep55 exhibited a ring-like structure at the midbody (Figure 2C). All the localizations of Cep55 observed here were specific because Cep55 signals were abolished in knockdown cells (Figure 2D and unpublished data). Our analysis of Cep55 localization is in sharp contrast to that reported by Fabbro *et al.* (2005), because localization of Cep55 on the spindle at prometaphase and metaphase was not observed in their report. On the other hand, we did not detect centrosomal staining of Cep55 in interphase cells over a wide range of our antibody concentrations under several different fixation methods (Figure 2C; unpublished data). These discrepancies are likely antibody-specific and deserve further investigation with additional specific antibodies in the future.

Cep55 Is a Microtubule-associated Protein with In Vitro Bundling Activity

As Cep55 colocalized with the mitotic spindle and the spindle midzone (Figure 2), we investigated whether Cep55 is a microtubule-associated protein. We expressed and purified


A
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E
DIC
DNA
MKLP1
β-tubulin
Merge
siControl
siCep55

Figure 4.

recombinant GST-Cep55 from Sf9 cells to homogeneity (Figure 3A). Quantitative Western blot analysis using purified GST-Cep55 as a standard indicated that the concentration of endogenous Cep55 in HeLa cells was 0.15 μM in asynchronous cells and 0.5 μM in mitotic cells (unpublished data). This concentration range was used in in vitro assays. GST or GST-Cep55 (0.3 μM) was incubated with taxol-stabilized microtubules polymerized from purified α/β-tubulins, and microtubules were then pelleted and analyzed for the presence of associated Cep55. Purified GST-Cep55 (Figure 3B), but not GST (unpublished data), directly associated with microtubules in vitro. Furthermore, recombinant Cep55 has microtubule-bundling activity. Taxol-stabilized microtubules were incubated with GST-Cep55 at its physiological concentrations found in mitotic cells (0.5 μM). Microtubules were then purified onto coverslips by centrifugation through a glycerol cushion and analyzed by immunofluorescence staining of tubulin and Cep55. The presence of Cep55 led to a high degree of microtubule bundling (Figure 3C), and Cep55 colocalized along the highly bundled microtubules as individual dots (Figure 3D). As a control, incubation of microtubules with GST did not alter microtubule morphology (Figure 3C). Quantification of Figure 3C indicated that in the presence of GST-Cep55 there was a 25-fold increase in the number of microtubule bundles, if the microtubule bundle was defined as an object with the tubulin fluorescence intensity five times greater than that of a single microtubule.

Cep55 Is Required for the Completion of Cytokinesis

We next investigated the cellular function of Cep55 during cytokinesis. HeLa cells with knockdown of Cep55 were imaged in time lapse under DIC, starting from 30 h after transfection. In control-knockdown cells, the bulge in the midbody (Flemming body; Paweletz, 1967) was morphologically visible as soon as the ingression of the cleavage furrow was complete and as the two daughter cells moved away from each other (Figures 4, A and B, and Supplementary Movie S1). The Flemming body persisted during and even after cell abscission. However, in Cep55-knockdown cells, a morphological Flemming body never formed throughout the whole duration of the terminal stage. The two daughter cells remained interconnected for extended time and then fused back to become a binucleated cell (Figure 4, C and D, and Supplementary Movie S2). We also observed Cep55-knockdown cells in which two daughter cells, without an apparent Flemming body, were connected by an intercellular bridge throughout the 7-h duration of time-lapse microscopy (unpublished data). Such a connection between two daughters can be quite stable, as we observed that two mitotic cells interconnected from the previous cell cycle undergone chromosome separation and cytokinesis to generate four interconnected interphase cells (unpublished data). Among cells undergoing anaphase and cytokinesis, 91% of Cep55-knockdown cells (n = 80), but only 17% of control cells (n = 120), had two daughter cells interconnected for greater than 7 h during time-lapse imaging. Consistent with this, 83% of control cells, but only 6% of Cep55-knockdown cells, underwent successful cytokinesis and completed cell abscission during the duration of time-lapse imaging. Thus, the failure to complete cytokinesis is specific to Cep55-knockdown cells, and the main defect in knockdown cells is the disruption of the midbody structure.

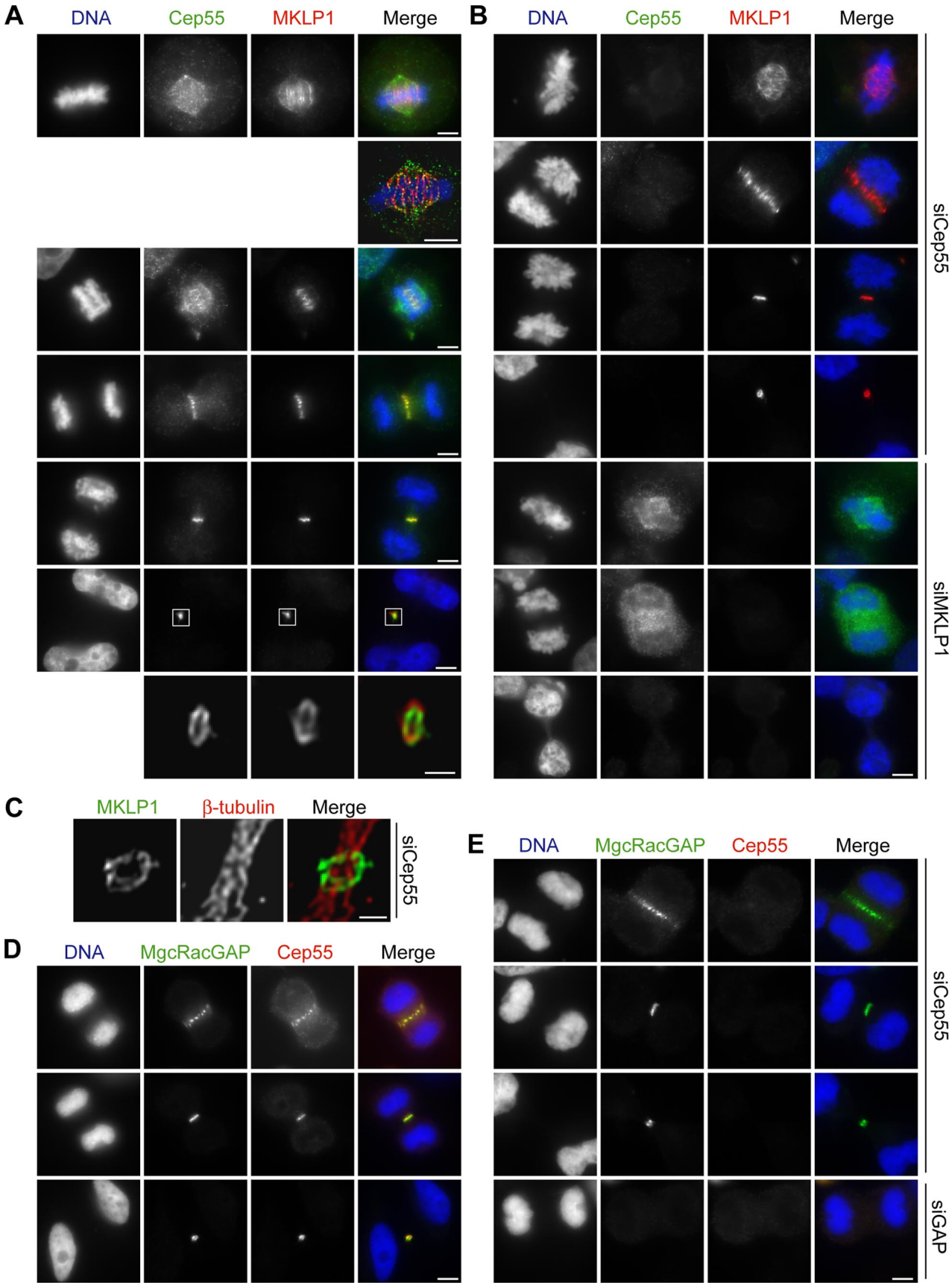
The disruption of the midbody structure in Cep55-knockdown cells at the terminal stage of cytokinesis was confirmed on fixed samples by high-resolution DIC imaging (Figure 4E). In these cells, not only the morphological Flemming body was disrupted, but also the organization of the midbody microtubules was altered. In control-knockdown cells, microtubule staining stopped at the dense Flemming body, and a gap existed in the microtubule staining (Matuliene and Kuriyama, 2002). However, in Cep55-knockdown cells, microtubules were continuous throughout the intercellular bridges (Figure 4E). Cep55 is required for the proper midbody structure, because we never observed a gap in the microtubule staining in cells with efficient knockdown of Cep55 (n = 17). Although the midbody structure is disrupted morphologically in Cep55-knockdown cells, a residual structure may still exist, based on the presence of the MKLP1 ring structure (Figure 4E). This phenotype is specific, as all three siRNAs targeted against Cep55 generated the same results. Statistic analysis of cells at 36 h after transfection indicated that 72% of knockdown cells (n = 114) had microtubules running across the midbody at the terminal stage of cytokinesis. The precise structural defects of the Flemming body in Cep55-knockdown cells await for the high-resolution electron microscopic analysis.

Centralspindlin Controls the Localization of Cep55 during Anaphase and Cytokinesis

Next, we set out to investigate what controls the function of Cep55. As the motor activity of MKLP1 has been shown to be required for the formation of the Flemming body in CHO cells (Matuliene and Kuriyama, 2002), we first examined the functional relationship between Cep55 and MKLP1. Interestingly, MKLP1, when analyzed with previously characterized anti-MKLP1 antibodies (Kuriyama *et al.*, 2002), colocalized with Cep55 at the spindle midzone and midbody during cytokinesis (Figure 5A). Deconvolved images indicated that Cep55 and MKLP1 both formed ring structures that partially overlapped at the midbody (Figure 5A). However, Cep55 and MKLP1 had different staining patterns at prometaphase and metaphase. Although MKLP1 colocalized with the spindle in regions proximal to chromosomes, Cep55 was more concentrated toward the spindle poles (Figure 5A and unpublished data). At anaphase A, all MKLP1 signals were on the central spindle, whereas Cep55 localized to both mitotic spindle and the central spindle (Figure 5A).

We then determined whether the localization of Cep55 and MKLP1 is interdependent during cytokinesis and found that the localization of MKLP1 was not affected by knockdown of Cep55 in mitosis and during cytokinesis (n = 32 cells; Figure 5B). Deconvolved images indicated that the ring structure of MKLP1 at the midbody was still intact with the loss of Cep55 (Figure 5C). However, knockdown of MKLP1 abolished the localization of Cep55 at the spindle midzone and the midbody in 34 of 34 cells analyzed (Figure 5B). At anaphase, Cep55 was dispersed in the region of central spindle and failed to concentrate at spindle midzone in

Figure 4 (facing page). The midbody structure was disrupted and cytokinesis failed at the terminal stage in Cep55-knockdown cells. (A–D) Selected frames from time-lapse movies of HeLa cells transfected with control siRNA (A and B and Supplementary Movie S1) or siRNA against Cep55 (C and D and Supplementary Movie S2). DIC images were recorded every 3 min starting from 30 h after transfection. To determine the presence of the Flemming body (arrowhead in B), enlarged images of the boxed areas in A and C are shown in B and D, respectively. Scale bar, 20 μm for A and C, and 4 μm for B and D. (E) HeLa cells were transfected with indicated siRNAs and immunostained for MKLP1 (green) and β-tubulin (red). Boxed regions of the midbody are shown with a higher magnification. Scale bar, 10 μm, except for the enlarged midbody images (1 μm).


A
DNA
Cep55
MKLP1
Merge
B
DNA
Cep55
MKLP1
Merge
siCep55
siMKLP1
C
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β-tubulin
Merge
siCep55
D
DNA
MgcRacGAP
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E
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MgcRacGAP
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Figure 5.

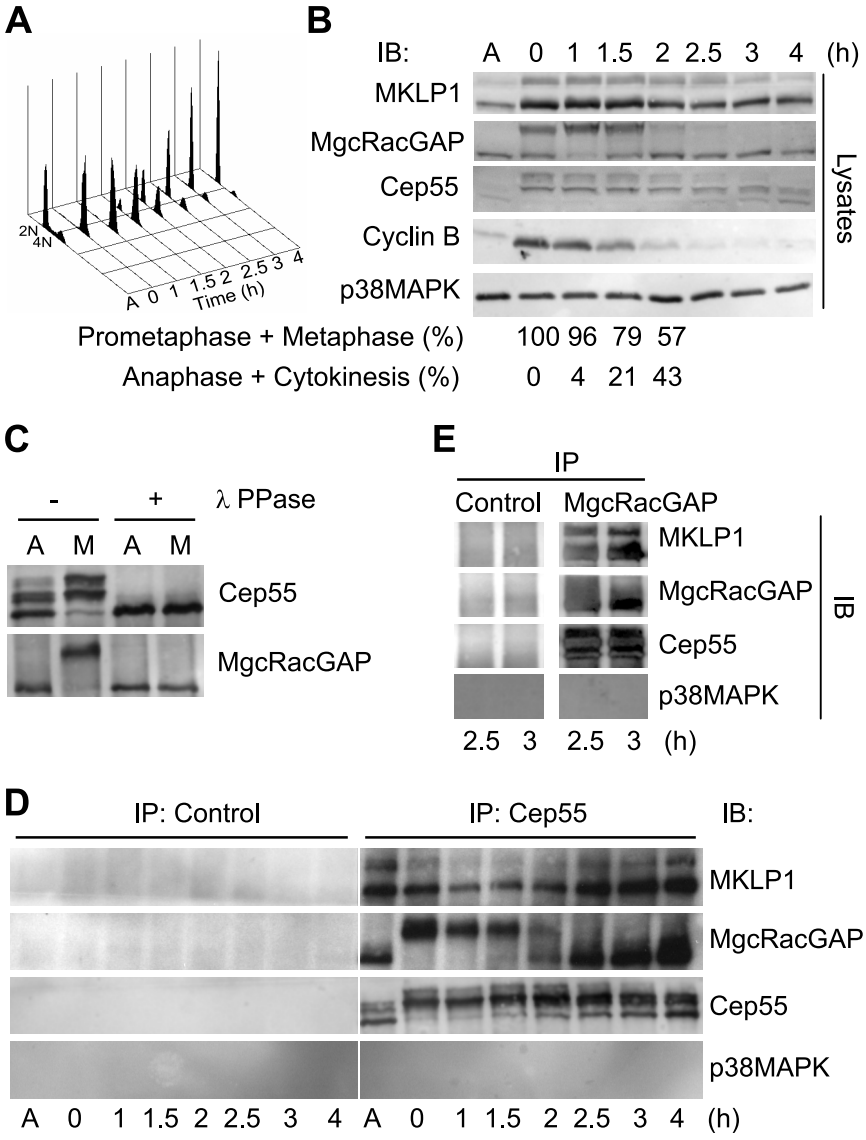

Figure 6. Cep55 associates with centralspindlin in vivo. HeLa S3 cells were released from a thymidine-nocodazole block and collected at the times indicated. The release time was indicated in each panel in hours (h). Cell cycle profile was determined by FACS analysis (A). Lysates of synchronized HeLa S3 cells were either immunoblotted (IB; B and C) with indicated antibodies or immunoprecipitated (IP; D–E) with control or anti-Cep55 antibodies (D)/anti-MgcRacGAP antibodies (E) followed by immunoblotting (IB). The percentage of pre- and postanaphase cells was quantified for the selected time points based on immunofluorescence staining and indicated in B. In C, cell lysates were incubated with or without λ phosphatase (PPase) before immunoblotting to determine the phosphorylation states of Cep55 and MgcRacGAP. A, asynchronous cells; M, nocodazole-arrested mitotic cells.

MKLP1-knockdown cells (n = 17), although the protein was still enriched between the separating chromosomes (Figure 5B). Thus, MKLP1 is required for anchoring Cep55 at the spindle midzone. As expected, the localization of Cep55 at metaphase was not affected by the loss of MKLP1 (n = 14 cells).

MKPL1 forms a centralspindlin complex with MgcRacGAP (Mishima *et al.*, 2002) and the localization of MgcRacGAP and MKLP1 is interdependent (Yuce *et al.*, 2005; Zhao and Fang, 2005b). As expected, Cep55 also colocalized with MgcRacGAP during cytokinesis (Figure 5D) and the localization of Cep55 in anaphase and cytokinesis cells was dependent on MgcRacGAP (Figure 5E). Thus, the centralspindlin complex controls the localization Cep55 during cytokinesis. This regulation is unique to the centralspindlin complex, because knockdown of other regulators of early cytokinesis, such as ECT2, anillin and MKLP2, did not affect the localization of Cep55 (unpublished data).

Figure 5 (facing page). Centralspindlin controls the localization of Cep55 at anaphase and during cytokinesis. (A and D) HeLa cells were immunostained for Cep55, MKLP1, and MgcRacGAP. The color of the antigen was labeled in each panel. Deconvolved images of single focal planes from a metaphase cell and from the boxed midbody region were also shown in A. Scale bar, 5 μm, except for the deconvolved images of the midbody (1 μm). (B, C, and E) HeLa cells were transfected with indicated siRNAs, fixed at 36 h after transfection, and immunostained for antigens indicated. The color of the antigen was labeled in each panel. siGAP, siRNA against MgcRacGAP. Scale bar, 5 μm, except for the deconvolved images of the midbody (1 μm) shown in C.

Cep55 Forms a Complex with Centralspindlin during Cytokinesis

We next investigated whether Cep55 is regulated biochemically by centralspindlin during cytokinesis. HeLa S3 cells were synchronized by a thymidine-nocodazole block and then released from prometaphase into G1 (Fang *et al.*, 1998). The cell cycle profile of the time points was determined by FACS (Figure 6A) and by immunofluorescence staining of DNA and of spindle morphology (unpublished data). Western blot analysis indicated that, in contrast to cyclin B, the

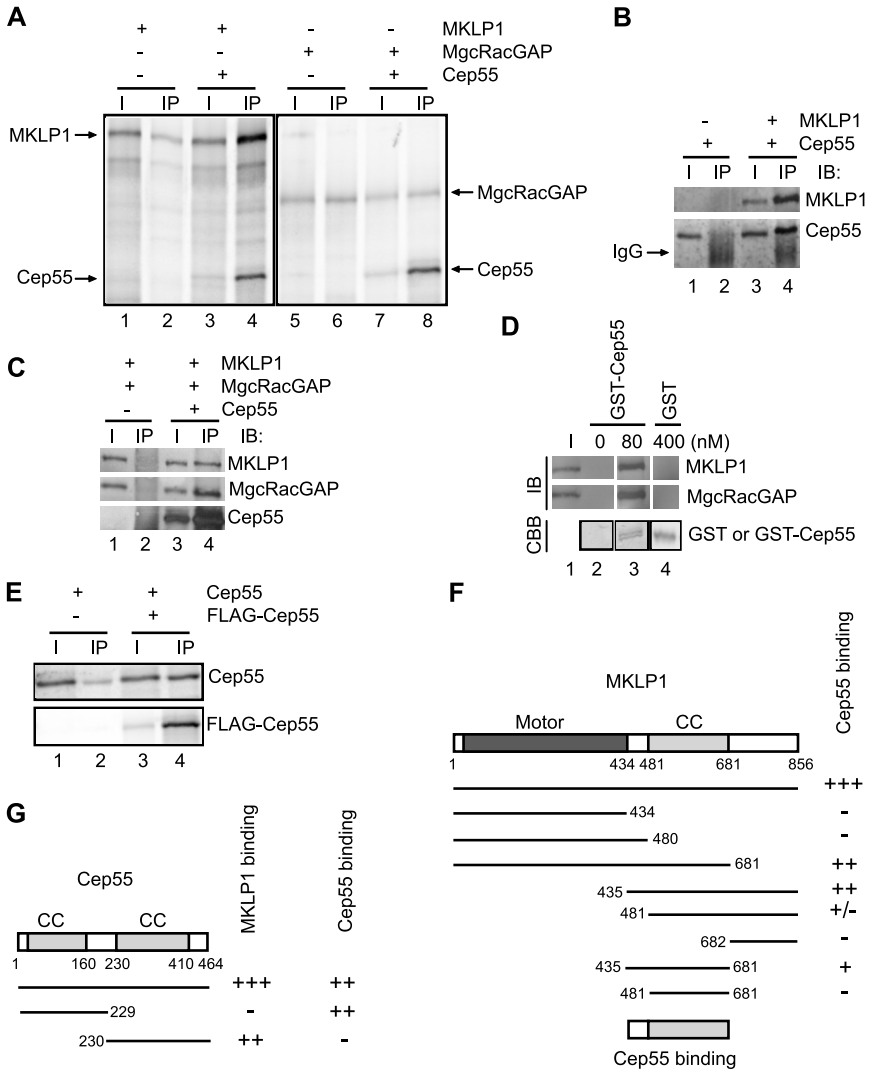

Figure 7. Cep55 binds to MKLP1 and self-associates through two independent domains. (A–D) Association of Cep55 with MKLP1 and MgcRacGAP. (A) ^{35}S-labeled MKLP1 or MgcRacGAP were synthesized in vitro with or without Cep55 and then subject to immunoprecipitation (IP) with anti-Cep55 antibodies. The immune-complexes were analyzed by SDS-PAGE and detected by PhosphoImager. (B) Cep55 was synthesized in vitro with or without MKLP1 and then subject to immunoprecipitation (IP) with anti-MKLP1 antibodies followed by immunoblotting (IB). IgG heavy chain was indicated. (C) MKLP1 and MgcRacGAP were synthesized in vitro with or without Cep55 and then subject to immunoprecipitation (IP) with anti-Cep55 antibodies followed by immunoblotting (IB). (D) MKLP1 and MgcRacGAP were synthesized in vitro and incubated with indicated concentrations of purified recombinant GST-Cep55 or GST. The complexes were then captured on glutathione beads and subjected to immunoblotting (IB) or Coomassie Brilliant Blue (CBB) staining. I (lane 1), 5% input. In panels B–D, bindings were analyzed by Western blots since the degradation products of in vitro–translated MKLP1 interferes with the signals of MgcRacGAP and Cep55, which have molecular weights smaller than that of MKLP1. (E) Cep55 was synthesized in the presence of ^{35}S-Met, and FLAG-Cep55 was synthesized in the presence of nonradioactive Met in vitro. Cep55 and Flag-Cep55 were then incubated together and subjected to immunoprecipitation (IP) with an anti-FLAG antibody. Cep55 was detected by PhosphoImager and Flag-Cep55 by Western blotting. In A–C and E, I, 5% input; IP, 100% immunoprecipitates. (F and G) Summary of Cep55 and MKLP1 deletion mutants analyzed in binding assays. The degrees of binding were quantified using the ratio of specific IP against nonspecific IP and assigned as follows: +++, >10; ++, 5–10; +, 2–5; +/−, 1–2; −, 1.

levels of Cep55, MgcRacGAP, and MKLP1 were constant from mitosis to early G1 (Figure 6B). Both Cep55 and MgcRacGAP exist as multiple forms in the cell cycle, and the slower migrating bands of Cep55 and MgcRacGAP in mitosis correspond to the hyperphosphorylated forms, because the treatment of cell lysates by λ phosphatase resulted in the downshift of these bands (Figure 6C). Interestingly, the slower migrating bands of Cep55 in asynchronous cells were also downshifted with the treatment of λ phosphatase, indicating that Cep55 is also phosphorylated in interphase cells. Next, Cep55 was immunoprecipitated from samples released from the nocodazole arrest with an anti-Cep55 antibody and the immune-complexes were analyzed by blotting with anti-Cep55, MgcRacGAP, and MKLP1 antibodies. Although there was no specific signal immunoprecipitated with control IgG, both MgcRacGAP and MKLP1, but not p38MAPK, were coprecipitated with Cep55 (Figure 6D). This interaction was also confirmed by immunoprecipitating MgcRacGAP, followed by Western blotting of Cep55 (Figure 6E). Although the association between Cep55 and centralspindlin was also detected in asynchronous cells, the association was stronger in mitosis (cf. the first vs. second lanes in Cep55 IP; Figure 6D). Interestingly, as cells were released from the nocodazole arrest, this association first decreased at prometaphase and metaphase (cf. 0 h vs. 1–1.5 h after release) and then increased when cells passed through anaphase and cytokinesis (cf. 1–1.5 h vs. later time points). Significantly, the complex of Cep55 and centralspindlin peaked when the majority of cells had undergone cytokinesis and exited into G1 (cells collected 2.5–4 h after release; Figure 6D). Thus, centralspindlin associates with Cep55 in vivo, and the peak kinetics for the formation of this complex coincides with cytokinesis, consistent with the fact that centralspindlin controls the localization of Cep55 during cytokinesis.

Cep55 Directly Associates with MKLP1 and Itself In Vitro

We next determined the biochemical basis of the interaction between Cep55 and centralspindlin. Cep55 was synthesized together with either MKLP1 or MgcRacGAP by in vitro–coupled transcription and translation in reticulocyte lysates and then was subjected to immunoprecipitation with the anti-Cep55 antibodies. MKLP1, but not MgcRacGAP, was specifically precipitated with the anti-Cep55 antibody in a Cep55-dependent manner (Figure 7A, lane 4 vs. 2 for MKLP1 and lane 8 vs. 6 for MgcRacGAP). Similarly, Cep55 was specifically precipitated with the anti-MKLP1 antibody in the presence, but not in the absence, of MKLP1 (Figure 7B, lane 4 vs. 2). We then determined whether MKLP1, MgcRacGAP and Cep55 can form a ternary complex. Cep55 was

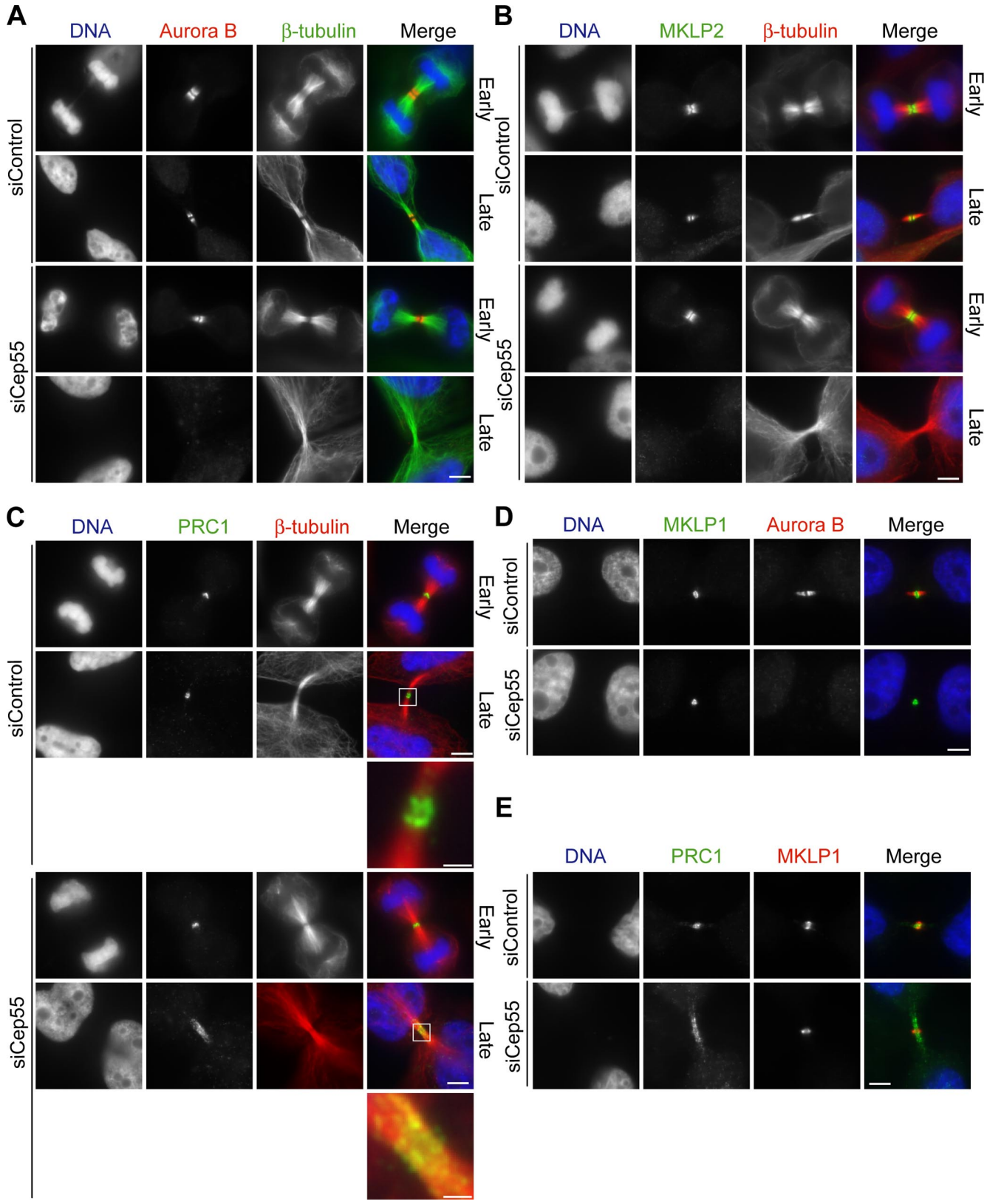

Figure 8. Cep55 controls the midbody structure. HeLa cells were transfected with indicated siRNAs, fixed at 36 h after transfection and immunostained for antigens indicated. The color of the antigen was labeled in each panel. Boxed regions of the midbody were shown at a higher magnification in C. "Early" and "Late" represent early and late cytokinesis as determined by the degree of DNA de-condensation and by cell morphology. Scale bar, 5 μm, except for the boxed images (1 μm).

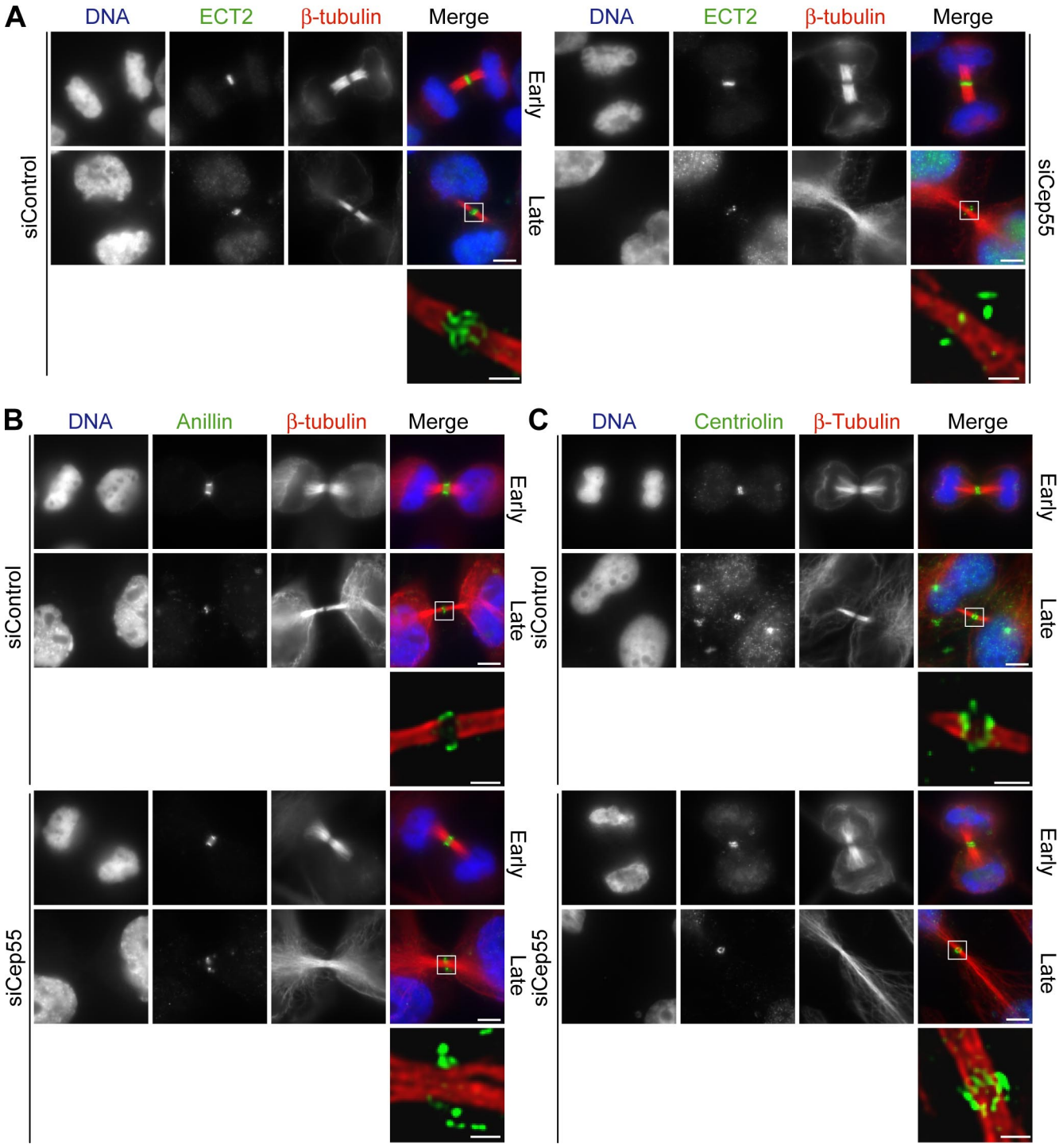

Figure 9. The effect of Cep55 on the localization of ECT2 (A), anillin (B), and centriolin (C). HeLa cells were transfected with indicated siRNAs, fixed at 36 h after transfection, and immunostained for antigens indicated. The color of the antigen was labeled in each panel. Deconvolved images of single focal planes from boxed regions were also shown at a higher magnification. "Early" and "Late" represent early and late cytokinesis as determined by the degree of DNA de-condensation and by cell morphology. Scale bar, 5 μm, except for the boxed images (1 μm).

synthesized in vitro together with MKLP1 and MgcRacGAP and then subjected to immunoprecipitation with the anti-Cep55 antibodies. Both MKLP1 and MgcRacGAP were specifically precipitated with the anti-Cep55 antibody in a Cep55-dependent manner (Figure 7C, lane 4 vs. 2), suggesting that Cep55 interacts with the centralspindlin complex in vitro. This observation was further confirmed by incubating in vitro–translated MKLP1 and MgcRacGAP with either purified recombinant GST-Cep55 or recombinant GST followed by glutathione beads pulldown. GST-Cep55, but not GST, efficiently pulled down both MKLP1 and MgcRacGAP (Figure 7D).

Cep55 contains extensive coiled-coil structure, which tends to dimerize or oligomerize. Thus, we investigated whether Cep55 self-associates. When Cep55 and FLAG-Cep55 were translated separately and then incubated together in vitro, untagged Cep55 coprecipitated with an anti-FLAG antibody, indicating that Cep55 self-associates (Figure 7E, lane 4 vs. 2).

Using the binding assays described above, we determined the domain structure of Cep55 and MKLP1. A region (247 amino acids) C-terminal to the motor domain in MKLP1 is required for binding to Cep55 (Figure 7F), and the same region is also required for MKLP1 dimerization and for its binding to MgcRacGAP (Mishima *et al.*, 2002), suggesting a complex regulatory function in this region of MKLP1, which is currently under investigation. On the other hand, the N-terminal half of Cep55 is responsible for the self-association, and the C-terminal half is required for its binding to MKLP1 (Figure 7G). When fused to GFP and expressed at very low levels, the C-terminal half of Cep55 localized to the midbody, whereas the N-terminal half did not (Figure S1), consistent with the fact that localization of Cep55 is dependent on its interaction with MKLP1. Analysis, in overexpression experiments, on the function of these Cep55 fragments in cytokinesis was not informative because of the insolubility of these fragments in vivo (unpublished data).

Cep55 Is Required for Midbody Structure

The midbody structure was disrupted morphologically in Cep55-knockdown cells. To understand the cellular consequence of this defect, we next analyzed the localization of selected cytokinesis regulators in Cep55-knockdown cells. Using specific antibodies previously characterized by us and others (Ban *et al.*, 2004; Gruneberg *et al.*, 2004; Wong and Fang, 2005; Zhao and Fang, 2005a, 2005b), we examined proteins associated with the midbody microtubules, such as PRC1, Aurora B, Plk1, MKLP2, ECT2, and centriolin, as well as proteins associated with the cleavage furrow structure, such as anillin. In our analyses here, we focused on Cep55-knockdown cells in which the midbody structure was disrupted, because these cells represent the strongest knockdown of Cep55. We found that the localization patterns for all the proteins examined were not affected during furrow ingression in Cep55-knockdown cells (Figures 8 and 9, unpublished data for Plk1), indicating that Cep55 does not regulate early events in cytokinesis. However, Cep55 controls the terminal stage of cytokinesis. At this stage, Aurora B, MKLP2, and Plk1 were all absent from the midbody microtubules in Cep55-knockdown cells (n = 27, 23, and 18, respectively; Figure 8, A and B; data for Plk1 is not published). On the other hand, in Cep55-knockdown cells, PRC1 became dispersed along the midbody microtubules (n = 16 cells), in sharp contrast to its localization in the Flemming body in control cells (Figure 8C). Interestingly, in cells at late stage of cytokinesis in which Aurora B was absent from the midbody and PRC1 became dispersed around the midbody microtubules, the localization of MKLP1 remained intact (Figure 8, D and E), confirming that centralspindlin is not affected by the loss of Cep55 at late stage of cytokinesis.

In control cells, ECT2 was localized around the midbody at late stage cytokinesis (Figure 9A). However, with the knockdown of Cep55, ECT2 signals were reduced to a few dots around the midbody microtubules, possibly associated with or underneath the membrane (n = 19 cells; Figure 9A). Interestingly, we found that the midbody localization of anillin, a contractile ring component required to maintain the localization of RhoA and active myosin during furrow ingression (Straight *et al.*, 2005; Zhao and Fang, 2005a), was not affected by the knockdown of Cep55 (n = 26 cells; Figure 9B). Furthermore, the midbody localization of centriolin, another key regulator for cell abscission (Gromley *et al.*, 2003, 2005), was not affected by the knockdown of Cep55 (n = 19 cells; Figure 9C). We concluded that Cep55 controls

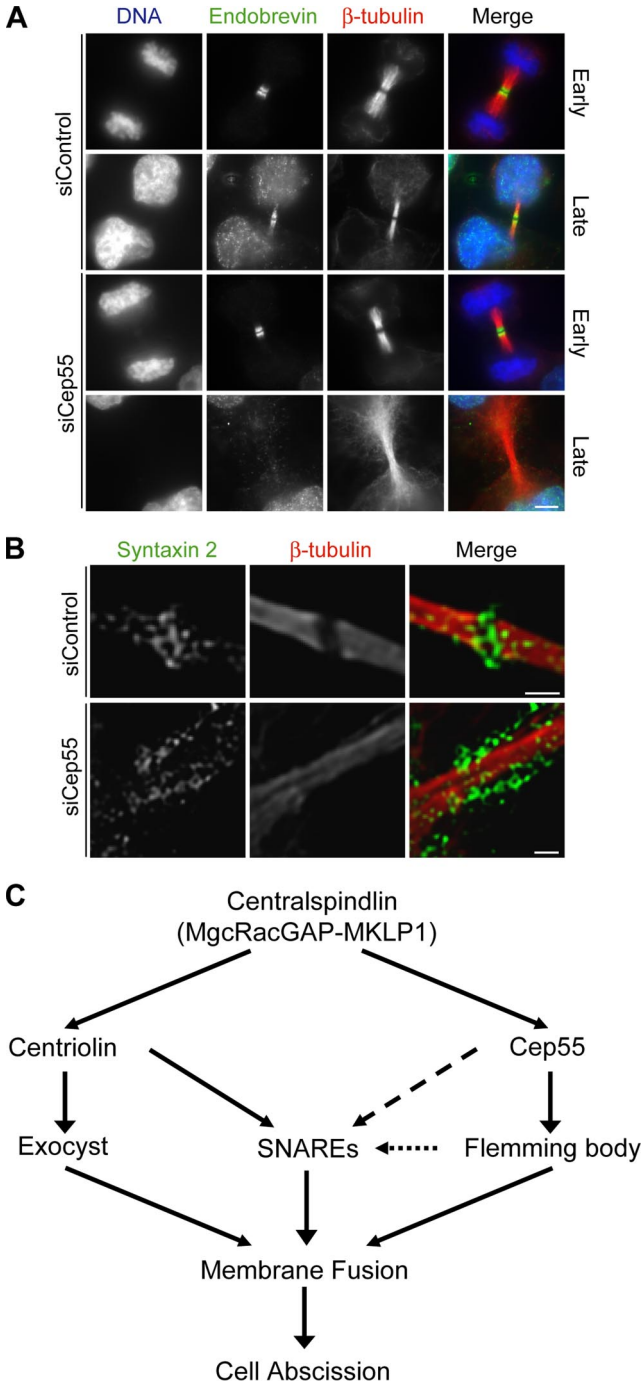

Figure 10. Cep55 controls the localization of SNAREs at the midbody. (A and B) HeLa cells were transfected with indicated siRNAs, fixed at 36 h after transfection, and immunostained for antigens indicated. The color of the antigen was labeled in each panel. Deconvolved images of single focal planes in the midbody regions were shown in B. "Early" and "Late" represent early and late cytokinesis as determined by the degree of DNA de-condensation and by cell morphology. Scale bar, 5 μm in A and 1 μm in B. (C) A model for Cep55 function at the terminal stage of cytokinesis.

the morphology of the midbody and organizes the localization of cytokinesis regulators at the terminal stage of cytokinesis.

Cep55 Controls the Localization of Endobrevin

Completion of cytokinesis requires active vesicle transport and membrane fusion during cell abscission (Finger and White, 2002; Strickland and Burgess, 2004; Albertson *et al.,* 2005). We next examined whether Cep55 also controls vesicle trafficking and fusion events at the terminal stage of cytokinesis. The t-SNARE syntaxin 2 and the v-SNARE endobrevin/VAMP8 localize specifically to the midbody during cytokinesis, and overexpression of dominant-negative mutants of these two proteins does not affect the initiation of cytokinesis or furrow ingression, but prevents abscission in mammalian cells (Low *et al.,* 2003). The intriguing similarities in loss-of-function phenotypes between SNAREs and Cep55 prompt us to determine the relationship between Cep55 and SNAREs, using anti-syntaxin 2 and anti-endobrevin antibodies whose specificities have been characterized and documented previously (Low *et al.,* 2003). Although the localization of endobrevin during furrow ingression was not affected ("Early" in Figure 10A), its midbody staining was absent at the terminal stage in Cep55-knockdown cells (n = 15) in which the Flemming body was absent ("Late" in Figure 10A). At the terminal stage, syntaxin 2 only existed at the intercellular bridge in areas outside the microtubules in Cep55-knockdown cells (n = 21; Figure 10B), but was localized to the Flemming body and along the midbody microtubules in control cells (Figure 10B; Low *et al.,* 2003; Gromley *et al.,* 2005). Because the docking of t-SNARE and v-SNARE is required for the membrane fusion and cell abscission, the lack of endobrevin at the midbody is likely to contribute to a failure of abscission in Cep55-knockdown cells, although we do not know whether this effect is a direct function of Cep55 or not.

DISCUSSION

We report here an efficient functional genomic screen for regulators of cytokinesis. We identified novel cell cycle proteins based on their covariation with known cell cycle regulators in tumor tissues and defined their functional specificity to mitosis and cytokinesis based on their induction in G2 and G2/M. Candidate genes were then analyzed for their cellular localizations and their loss-of-function phenotypes. Our initial analysis of 5% of G2- and G2/M-induced genes (30 analyzed of total 566 genes) identified one novel regulator of cytokinesis, and our ongoing functional genomic screen is expected to yield additional regulators of mitosis and cytokinesis.

Through this targeted screen, we identified Cep55 as a protein required for cell abscission and reported here the biochemical and cellular mechanism of its function. Although Cep55 was recently reported as a protein linked to cytokinesis, the mechanism of its function remained unknown (Fabbro *et al.,* 2005). We found that Cep55 directly binds to microtubules in vitro and has a strong microtubule-bundling activity at its physiological concentration (Figure 3). Cep55 associates in vivo with the mitotic spindle before anaphase and with the spindle midzone and the midbody during cytokinesis (Figure 2). The localization of Cep55 during cytokinesis is under the control of centralspindlin, and Cep55 directly interacts with MKLP1 (Figures 5–7 and 10C). Cep55 controls the terminal stage of cytokinesis. In Cep55-knockdown cells, cleavage furrow specification and ingression proceed normally, but the cell abscission fails to occur. At the cellular level, the morphological Flemming body is disrupted in Cep55-knockdown cells (Figure 4), and several regulatory and structural components of the midbody, such as Aurora B, Plk1, MKLP2, PRC1, and ECT2, are either missing or mislocalized at the terminal stage of cytokinesis (Figures 8 and 9). Furthermore, knockdown of Cep55 abolishes the localization of v-SNARE at the midbody (Figure 10), indicating a defect in vesicle–membrane/vesicle–vesicle fusion required for cell abscission, although it is not clear whether this defect results from a direct function of Cep55 in membrane trafficking/fusion or is simply an indirect consequence of disrupting the midbody structure. We conclude that Cep55 is a key regulator of cytokinesis essential for the midbody structure and for vesicle trafficking and fusion (Figure 10C).

The Microtubule-bundling Protein Cep55 Controls the Midbody Structure

One of the primary defects in Cep55-knockdown cells is the disruption of the midbody structure. Similar defect has been reported in cells overexpressing an ATP-binding mutant of MKLP1 as well as in MKLP1-knockdown cells (Matuliene and Kuriyama, 2002). Indeed, we found that MKLP1 controls the localization of Cep55, indicating that Cep55 is a direct downstream target of MKLP1 in assembling the midbody structure (Figure 10C). As Cep55 associates with MKLP1-MgcRacGAP in vivo and both Cep55 and MKLP1-MgcRacGAP have microtubule-bundling activities in vitro, we speculate that these proteins may act synergistically during cytokinesis, a hypothesis currently under test. Another protein required for the midbody structure and for the completion of cytokinesis is annexin 11, a MKLP1-interacting protein that also localizes at the midbody (Tomas *et al.,* 2004). Interestingly, MKLP1 is no longer at the Flemming body in annexin 11-knockdown cells, suggesting that annexin 11 acts to recruit or to maintain MKLP1 at the Flemming body.

Cep55 is required for correct localization of Aurora B, MKLP2, PRC1, and ECT2 at the terminal stage of cytokinesis (Figures 8 and 9). During cytokinesis, MKLP2 associates with and translocates Aurora B from centromeres to the spindle midzone (Gruneberg *et al.,* 2004). In Cep55-knockdown cells, both MKLP2 and Aurora B are absent from the midbody. Because Aurora B is required for the completion of cytokinesis by phosphorylating both MKLP1 (Guse *et al.,* 2005) and MgcRacGAP (Minoshima *et al.,* 2003), the lack of Aurora B at the midbody in Cep55-knockdown cells underscores the importance of the regulatory function of Cep55. On the other hand, PRC1 is required for the formation and maintenance of the central spindle structure, and the cell abscission is disrupted in PRC1-knockdown cells (Mollinari *et al.,* 2005). Thus, mislocalization of PRC1 in Cep55-knockdown cells may also directly contribute to the failure of cell abscission in these cells. The requirement of Cep55 for the localization of MKLP2, Aurora B and PRC1 is likely to be structural, because Cep55 does not directly interact with any of these proteins during cytokinesis in our immunoprecipitation experiments (unpublished data).

We also found that Cep55 controls the proper localization of ECT2, a guanine nucleotide exchange factor required for the assembly of contractile ring and for the initiation of cytokinesis (Somers and Saint, 2003; Kamijo *et al.,* 2005; Yuce *et al.,* 2005; Zhao and Fang, 2005b). In addition, we found that Cep55 specifically associated with ECT2 in vivo at the terminal stage of cytokinesis in our immunoprecipitation assays (unpublished data), although this interaction could be indirectly mediated through centralspindlin because both

ECT2 and Cep55 biochemically associate with centralspindlin during cytokinesis (Figure 6; Zhao and Fang, 2005a, 2005b). These findings suggest a potential function of ECT2 at the terminal stage of cytokinesis. Consistent with this prediction, overexpression of a N-terminal fragment of ECT2, which mediates the localization of ECT2 to the midbody, induces cytokinesis failure due to the lack of cell abscission (unpublished data; Tatsumoto *et al.*, 1999).

Although we cannot completely exclude the possibility that the disruption of the midbody in Cep55-knockdown cells results from a degeneration of this structure due to the prolonged arrest at the terminal stage of cytokinesis and that changes in localization patterns of the above mentioned regulators of cytokinesis are the consequence of, but not the cause for, failed cytokinesis, this is very unlikely for the following two reasons. First, time-lapse microscopy indicated that the morphologically distinct Flemming body was never formed at the terminal stage of cytokinesis in Cep55-knockdown cells (Figure 4A). Second, the Flemming body was never detected morphologically in cells with efficient knockdown of Cep55 (Figure 4B). Both experiments indicate that Cep55 is required for the establishment of the proper midbody structure in the first place. However, Cep55 only controls certain aspects of the midbody structure and the lack of Cep55 does not cause a global degeneration of the midbody, because MKLP1 (Figures 5 and 8), centriolin, and anillin (Figure 9) all remain correctly localized in Cep55-knockdown cells.

We speculate that the microtubule-bundling activity of Cep55 contributes to its function at the midbody. Given that Cep55 associates with centralspindlin which by itself has microtubule-bundling activity, it will be interesting to determine whether Cep55 and centralspindlin act cooperatively in promoting microtubule bundling.

Two Pathways Regulate Membrane Fusion at the Terminal Stage of Cytokinesis

Disruption of the midbody structure also leads to a defect in the membrane trafficking and fusion. The loss of Cep55 results in the absence of v-SNARE endobrevin from the midbody (Figure 10). Furthermore, t-SNARE syntaxin 2 localizes to a broader region around the midbody microtubules in Cep55-knockdown cells, whereas in control cells syntaxin 2 is concentrated in the midbody matrix. Because docking of v-SNARE with t-SNARE is required for the membrane fusion during cell abscission (Low *et al.*, 2003), the lack of v-SNARE at the midbody in Cep55-knockdown cells indicated that Cep55 also facilitates the membrane trafficking and fusion (Figure 10C), although it is also possible that the effect of Cep55 on membrane fusion results indirectly from the lack of the midbody structure.

Another cytokinesis regulator essential for membrane trafficking and fusion is centriolin, which, under the control of centralspindlin, recruits and anchors the vesicle-membrane-tethering complex, exocysts, to the midbody (Gromley *et al.*, 2005). Similarly, the localization of the t-SNARE and v-SNARE at the midbody is also dependent on centriolin (Gromley *et al.*, 2005). We found that centriolin and exocyst components, such as sec3 and sec8, remained correctly localized at the midbody in Cep55-knockdown cells (Figure 9; unpublished data), indicating that centriolin does not act downstream from Cep55. Given that Aurora B is still present at the midbody in centriolin-knockdown cells (Gromley *et al.*, 2005), but is absent in Cep55-knockdown cells (Figure 8), we speculate that Cep55 and centriolin function in two parallel pathways, both of which are downstream from centralspindlin and both of which are required for cell abscission (Figure 10C). Endocytosis-based membrane trafficking and fusion are also required for cytokinesis, because the recycling endosomes are essential for the completion of cytokinesis (Riggs *et al.*, 2003). Indeed, several studies have linked the endocytic proteins to cell abscission (Thompson *et al.*, 2002; Fielding *et al.*, 2005; Monzo *et al.*, 2005; Wilson *et al.*, 2005). In the future, it will be interesting to investigate the role of centriolin and Cep55 on the endosomal pathway at the terminal stage of cytokinesis.

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