The Peptidyl-Prolyl Isomerase Pin1 Regulates Cytokinesis through Cep55

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Abstract

Failure of cytokinesis results in tetraploidy and can increase the genomic instability frequently observed in cancer. The peptidyl-prolyl isomerase Pin1, which is deregulated in many tumors, regulates several processes, including cell cycle progression. Here, we show a novel role for Pin1 in cytokinesis. Pin1 knockout mouse embryonic fibroblasts show a cytokinesis delay, and depletion of Pin1 from HeLa cells also causes a cytokinesis defect. Furthermore, we provide evidence that Pin1 localizes to the midbody ring and regulates the final stages of cytokinesis by binding to centrosome protein 55 kDa (Cep55), an essential component of this ring. This interaction induces Polo-like kinase 1–mediated phosphorylation of Cep55, which is critical for the function of Cep55 during cytokinesis. Importantly, Pin1 knockdown does not enhance the cytokinesis defect in Cep55-depleted cells, indicating that Pin1 and Cep55 act in the same pathway. These data are the first evidence that Pin1 regulates cytokinesis and may provide a mechanistic explanation as to how pathologic levels of Pin1 can stimulate tumorigenesis. [Cancer Res 2009;69(16):6651–9]

Introduction

Cytokinesis is the final step of cell division in which the cytoplasms of the two prospective daughter cells are separated (reviewed in ref. 1). It is a multistep process that starts with the ingression of the plasma membrane, mediated by the actomyosin ring, and ends in abscission, which is controlled by the midbody ring (2, 3). Failure of cytokinesis results in tetraploid cells, which display increased chromosomal instability and are more tumorigenic compared with diploid cells (4).

The peptidyl-prolyl isomerase Pin1 is a member of the evolutionary conserved family of peptidyl-prolyl isomerases and regulates a diverse array of cellular processes such as cell cycle progression and cellular stress responses (reviewed in ref. 5). Pin1 contains an NH_2-terminal WW domain that is important for protein-protein interactions and a COOH-terminal isomerase domain that catalyzes the isomerization of substrates between the *cis* and *trans* configuration, thereby inducing a conformational change. Originally, Pin1 was shown to bind to a plethora of mitotic phosphoproteins that are phosphorylated on phosphoserine- and phosphothreonine-proline motifs (6–8). More recently, Pin1 has also been shown to regulate nonmitotic proteins, such as FoxO transcription factors and the tumor suppressor protein p53 (9–11). These findings together led to the notion that Pin1 may be a molecular timer of the cell cycle (12). Not surprisingly therefore, Pin1 expression is abnormal in the majority of tumors (13).

Centrosome protein 55 kDa (Cep55) is a mitotic phosphoprotein that localizes to the centrosome during mitosis and translocates to the midbody ring during cytokinesis (14–16). Cep55 acts during this last stage of cell division by regulating membrane fission and fusion events near the midbody ring to allow abscission (16–18). We previously reported that both overexpression and knockdown of Cep55 cause cytokinesis defects, that is, a delay in completion of abscission (also referred to as midbody arrest) and an increase in the number of multinucleated cells (14). Notably, Cep55 is overexpressed in a large number of tumors (19, 20) and is one of 70 proteins in a chromosomal instability signature (21), suggesting that aberrant expression of Cep55 could contribute to tumorigenesis.

Cep55 is phosphorylated by the mitotic kinase Cdk1 on S425 and S428, both of which are followed by a proline residue (14). These two phosphoserine-proline motifs, which are conserved among mammals, make Cep55 a potential Pin1 substrate. Cep55 is also phosphorylated on S436 by Polo-like kinase 1 (Plk1) in a manner dependent on the phosphorylation of S425 and S428. Notably, phosphorylation of Cep55 on S436 is absolutely required for the function of Cep55 during cytokinesis (14).

Although almost 20 years ago loss of the *Saccharomyces cerevisiae* homologue of Pin1 was shown to result in multibudded yeast (22), a phenomenon that is indicative of a cytokinesis defect, a role for Pin1 in cytokinesis of mammalian cells has not been shown to date. Here, we provide data indicating that Pin1 regulates the final stages of mammalian cytokinesis. Cells lacking Pin1 and cells that have low levels of Pin1 display cytokinesis defects. Moreover, we show that Pin1 localizes to the midbody ring and that it interacts with Cep55 in a manner dependent on phosphorylation of Cep55 on S425 and S428. We show that functional Pin1 is required for subsequent phosphorylation of Cep55 by Plk1 on S436. Finally, we show that the midbody ring component Cep55 is a major substrate of Pin1 for its function during cytokinesis. These findings provide a new mechanism controlling cytokinesis that may have implications for cancer initiation and/or progression.

Materials and Methods

Cell culture, transfection, and treatment. $Pin1^{+/+}$ and $Pin1^{-/-}$ mouse embryonic fibroblasts (MEFs), kindly provided by Dr. G. Del Sal (Laboratorio Nazionale CIB, Trieste, Italy; ref. 10), of (HEK)293T, and HeLa cells were maintained in RPMI 1640 supplemented with penicillin/streptomycin and 10% serum supreme at 37°C in a humidified atmosphere and 5% CO_2. 293T cells were transiently transfected using Lipofectamine 2000 (Invitrogen) in case of small interfering RNA (siRNA) duplexes and using FuGENE6 reagent (Roche) for expression plasmids according to the manufacturer's instructions. HeLa cells were transfected using Lipofectamine 2000. Total amounts of transfected DNA were equalized using pBluescript KSII+.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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doi:10.1158/0008-5472.CAN-09-0825

For mitotic synchronization, cells were treated with nocodazole (Sigma) at 150 ng/mL for 16 h. Synchronization using the double thymidine block was done essentially as described (23).

Plasmids, oligos, and recombinant proteins. pFLAG-CMV2-Cep55, pFLAG-CMV2-Cep55-S425/428A, pFLAG-CMV2-Cep55-S436A (14), pcDNA3.1-FLAG-Pin1, pcDNA3.1-FLAG-Pin1-W34A, pcDNA3.1-FLAG-Pin1-SM, pGEX-GST, pGEX-GST-Pin1, pGEX-GST-Pin1-W34A (9), and EB1-GFP (24) have been described before.

pFLAG-CMV2-Cep55-S425/428E was created by site-directed mutagenesis (forward oligo: 5′-GAAAAAGTTGCCGCCGAACCAAAAGAACCCACTGCTG-CAC-3′). The resulting plasmid was verified by automated sequencing.

pcDNA3.1-FLAG-Pin1-Δ4 (K63A, R68/69A, C113A) was a kind gift of Dr. A.B. Brenkman (Department of Metabolic and Endocrine Diseases and Netherlands Metabolomics Centre, University Medical Center Utrecht, Utrecht, The Netherlands).

siRNA duplexes [nontargeting control, sense sequence: 5′-UUCUCC-GAACGUGUCACGU-dTdT-3′; Cep55: 5′-GGAACAACAGAUGCAGGCAU-GUACU-dTdT-3′; Pin1 (#1): 5′-GCCAUUUGAAGACGCCUCG-dTdT-3′ (9); Pin1 #2: 5′-CUGGCCUCACAGUUCAGCG-dTdT-3′ (25); Plk1: 5′-AAGAU-CACCCUCCUUAAAUAU-dTdT-3′] were purchased from Invitrogen. pSUPER-GFP and pSUPER-Cep55 have been described before (14).

Antibodies. Monoclonal antibodies recognizing the FLAG-M2 epitope, β-actin, α-tubulin, Pin1, or Plk1 were obtained from Sigma (3×), R&D Systems, and Zymed Laboratories, respectively. Polyclonal antibodies recognizing Pin1, cyclin B1, or MKLP2 were purchased from Cell Signaling, Abcam, and Bethyl Laboratories, respectively. The polyclonal antibody against Cep55 has been described before (14). A polyclonal antibody recognizing phosphorylated S436 of Cep55 was raised using the synthetic peptide AALNEphosphoSLVECP containing a COOH-terminal cysteine (Open Biosystems) essentially as described (14).

Western blot analysis. Western blot analysis was done as described before (26). Proteins were visualized using Western Lightning Chemiluminescence Reagent (Perkin-Elmer) and a LAS-3000 imaging system (Fujifilm). For quantification of protein levels, MultiGauge software was used according to the manufacturer (Fujifilm). Actin was used as a loading control.

Cell lysis for Pin1 expression and Cep55 mobility shift. HeLa cells were lysed in universal immunoprecipitation lysis buffer [50 mmol/L Tris (pH 7.4), 0.2% Triton X-100, 0.3% NP40, 150 mmol/L NaCl, 2 mmol/L EDTA, 2 mmol/L EGTA, 25 mmol/L NaF, 25 mmol/L β-glycerolphosphate, 0.1 mmol/L sodium orthovanadate, and protease inhibitor cocktail] and equalized using Bradford reagent and Laemmli sample buffer.

(Co-)immunoprecipitation. HeLa and 293T cells were transfected with the indicated constructs and arrested using nocodazole or a double thymidine block as indicated. Cells were lysed in universal immunoprecipitation or coimmunoprecipitation buffer [in case of Cep55-Pin1 coimmunoprecipitations; 50 mmol/L Tris (pH 8.0), 10% glycerol, 1% NP40, 50 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L $MgCl_2$, 25 mmol/L NaF, 0.1 mmol/L sodium orthovanadate, and protease inhibitor cocktail]. Plk1 or Cep55 was immunoprecipitated from whole-cell lysates using protein A/G agarose beads. Rabbit IgG or beads only were used for control immunoprecipitations. Immunoprecipitation samples were analyzed by Western blot analysis using the indicated antibodies.

Glutathione *S*-transferase pull-downs. Glutathione *S*-transferase (GST) proteins were coupled to glutathione-Sepharose beads in 0.5% NP40, 1 mmol/L EDTA, 20 mmol/L Tris (pH 8.0), 150 mmol/L NaCl, and protease inhibitors. Beads were washed thrice with coimmunoprecipitation buffer. 293T cells transfected with the indicated Cep55 expression constructs were lysed in coimmunoprecipitation buffer [containing 2 mmol/L $MnCl_2$ when λ-phosphatase (New England Biolabs) was used in the experiment (30 min at 30°C)]. Cleared lysates were incubated with immobilized GST proteins for 2 h at 4°C. Beads were washed, boiled in Laemmli sample buffer, and analyzed by Western blotting for the presence of Cep55. Expression of GST-tagged recombinant protein was assessed by Ponceau staining or Western blotting.

Time-lapse microscopy. Phase-contrast time-lapse photographs of $Pin1^{+/+}$ and $Pin1^{-/-}$ MEFs growing at 37°C were obtained at ×40 magnification using an Olympus IX81 inverted microscope.

Fluorescent time-lapse photographs of HeLa cells expressing EB1-GFP and transfected with either control or Pin1 siRNA were obtained at ×40 magnification using the Deltavision Imaging system (Applied Precision) equipped with an Olympus IX71 inverted microscope. Images were acquired with a cooled charge-coupled device camera (CoolSNAP ES2; Photometrics) and SoftWoRx 3.6.1 imaging software (Applied Precision).

Immunofluorescence. HeLa cells were transfected with control, Pin1, Plk1, or Cep55 siRNA duplexes. Forty-eight hours after transfection, cells were fixed using ice-cold methanol (for detection of phospho-S436-Cep55), 2% paraformaldehyde {after preextraction using PHEM-T [60 mmol/L PIPES, 25 mmol/L HEPES (pH 6.9), 10 mmol/L EGTA, 2 mmol/L $MgCl_2$, 0.5% Triton X-100]; for Pin1} or 4% paraformaldehyde (all other cases). Pin1 was stained using an α-Pin1 polyclonal antibody, monoclonal antibody, or anti-FLAG monoclonal antibody as indicated. Cep55, Plk1, the FLAG epitope, MKLP2, and α-tubulin were stained using the respective antibodies. Secondary antibodies were α-mouse-Alexa488, α-rabbit-Alexa488, α-mouse-Alexa594, and α-rabbit-Alexa546. DNA was stained using 4′,6-diamidino-2-phenylindole (DAPI). Fluorescence was captured using the above-mentioned Deltavision Imaging system using an Olympus 60× or 100×/1.40 oil immersion lens.

Flow cytometry. Flow cytometric analysis of HeLa cells using propidium iodide to determine cell cycle distribution was essentially done as described (26).

Statistical analysis. Student's *t* tests were performed using GraphPad statistical analysis and two-tailed *P* values were calculated as indicated.

Results

Pin1 regulates cytokinesis. As mutation of the yeast homologue of Pin1, Ess1, results in multibudded cells, we set out to study a possible role for Pin1 in cytokinesis of mammalian cells. To this end, we used previously described (10) Pin1 knockout MEFs ($Pin1^{-/-}$ MEFs; see Supplementary Fig. S1) and followed the progression of these cells and MEFs expressing Pin1 ($Pin1^{+/+}$) through mitosis and cytokinesis by time-lapse microscopy. $Pin1^{-/-}$ MEFs displayed cleavage furrow ingression and midbody formation; however, these cells took twice as long to complete cytokinesis (from furrow ingression to midbody abscission) compared with $Pin1^{+/+}$ MEFs (Fig. 1*A* and *B*; see Supplementary Movies S1 and S2). Subsequent immunofluorescence analysis of fixed cells stained with anti-α-tubulin antibody revealed an ~2-fold higher number of dividing cells that were undergoing cytokinesis (i.e., in midbody stage) in $Pin1^{-/-}$ MEFs relative to $Pin1^{+/+}$ MEFs (Fig. 1*C*), supporting the above time-lapse results. Moreover, overall, ~7% of the $Pin1^{-/-}$ MEFs displayed midbody arrest and multinucleation, indicative of a cytokinesis defect, compared with ~2.5% for the $Pin1^{+/+}$ MEFs (Fig. 1*D*). Together, these data suggest a role for Pin1 in a late stage of cytokinesis.

As a complementary approach, we used a previously described siRNA duplex against Pin1 (9) to deplete it from HeLa cells (see Supplementary Fig. S2). In accordance with the cytokinesis phenotype of $Pin1^{-/-}$ MEFs, Pin1-depleted cells also displayed a ~2-fold delay in cytokinesis compared with control siRNA–transfected cells (Fig. 2*A* and *B*; see Supplementary Movies S3 and S4). Moreover, Pin1 depletion resulted in an ~3-fold increase in the number of cells with a cytokinesis defect, that is, multinucleated cells or cells arrested at the midbody stage (midbody arrest; Fig. 2*C*). Subsequent analysis of exclusively cytokinetic cells revealed that a majority of Pin1-depleted cells were arrested at the midbody stage, characterized by decondensed chromatin and interphase-like morphology, whereas most of the cytokinetic control siRNA–treated cells displayed condensed chromatin and rounded morphology (Fig. 2*D*; see Supplementary

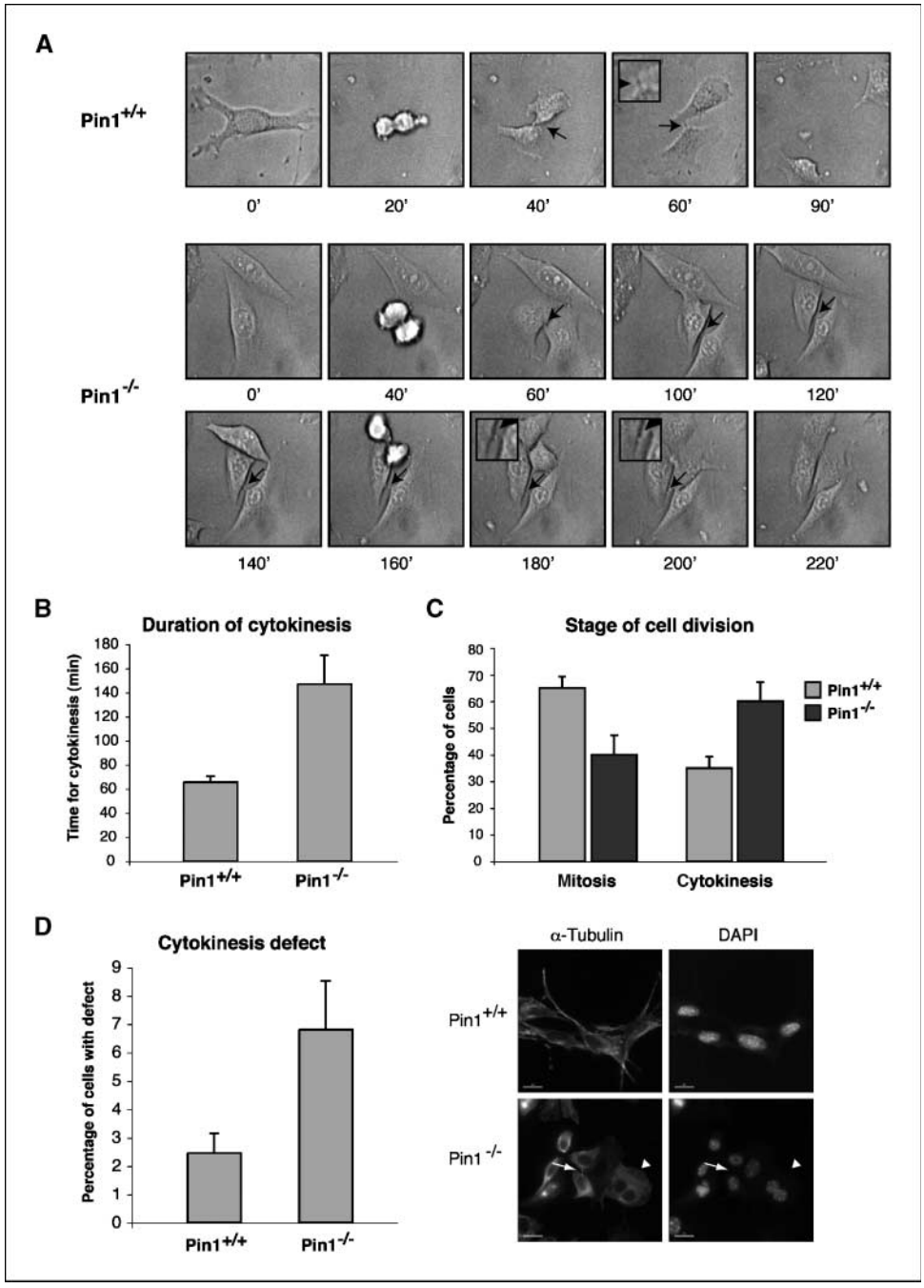

Figure 1. Pin1$^{-/-}$ MEFs show a cytokinesis defect. *A,* phase-contrast time-lapse photographs of asynchronous Pin1$^{+/+}$ and Pin1$^{-/-}$ MEFs were obtained. Frames from representative cells are shown. See also Supplementary Movies S1 and S2. *B,* time-lapse photographs of 60 cells of each cell line were analyzed: the time taken from the appearance of two connected daughter cells to completion of abscission was determined. *Columns,* mean; *bars,* SE. The difference between the two cell lines is statistically significant ($P = 0.0011$). *C,* Pin1$^{+/+}$ and Pin1$^{-/-}$ MEFs were stained for α-tubulin and DNA (DAPI). The percentage of cells in mitosis and cytokinesis was quantified. *Columns,* mean of three experiments (50 cells per experiment); *bars,* SD. The difference between the two cell lines is statistically significant ($P = 0.023$). *D,* MEFs were stained as in *C*. The percentage of cells that showed a cytokinesis defect (*arrowhead*, multinucleation; *arrow,* midbody arrest) in the total cell population was quantified. *Columns,* mean of three experiments (~300 cells in total per sample per experiment); *bars,* SD. The difference between the two conditions is statistically significant ($P = 0.015$). *Bar,* 15 μm.

Fig. S4). Notably, expression of a FLAG-Pin1 construct that is insensitive to the Pin1 siRNA (Pin1-SM) reverted the phenotype induced by the knockdown of Pin1 (Fig. 2*D*). We attribute the phenotype of Pin1-depleted cells to prolonged cytokinesis due to an abscission defect that allows the cells to adopt an interphase (nonrounded) morphology. Importantly, the partial (~50–60%) knockdown of Pin1 we obtained in HeLa cells as visualized by Western blotting and immunofluorescence microscopy (see Supplementary Figs. S2 and S3) was essential to minimize the induction of adverse (pre-)mitotic defects documented previously with profound depletion of Pin1 in these cells (6, 27) and therefore allowed us to follow cells into cytokinesis. At the same time, we feel that this partial knockdown precluded a strong siRNA-induced cytokinesis defect. In agreement with this assumption, a second siRNA against Pin1 (25), which caused a more profound knockdown of Pin1, prevented the majority of cells recovering from a nocodazole-induced mitotic arrest.[1] Nevertheless, when analyzing cells that reached cytokinesis, we observed a cytokinetic phenotype similar to that induced by the first Pin1-specific siRNA duplex (see Supplementary Figs. S4 and S5). Taken together, we conclude that Pin1 plays a role in cytokinesis and we hypothesize that Pin1 acts on a substrate that functions in resolution of the midbody/abscission.

[1] A. van der Horst and K.K. Khanna, unpublished observation.

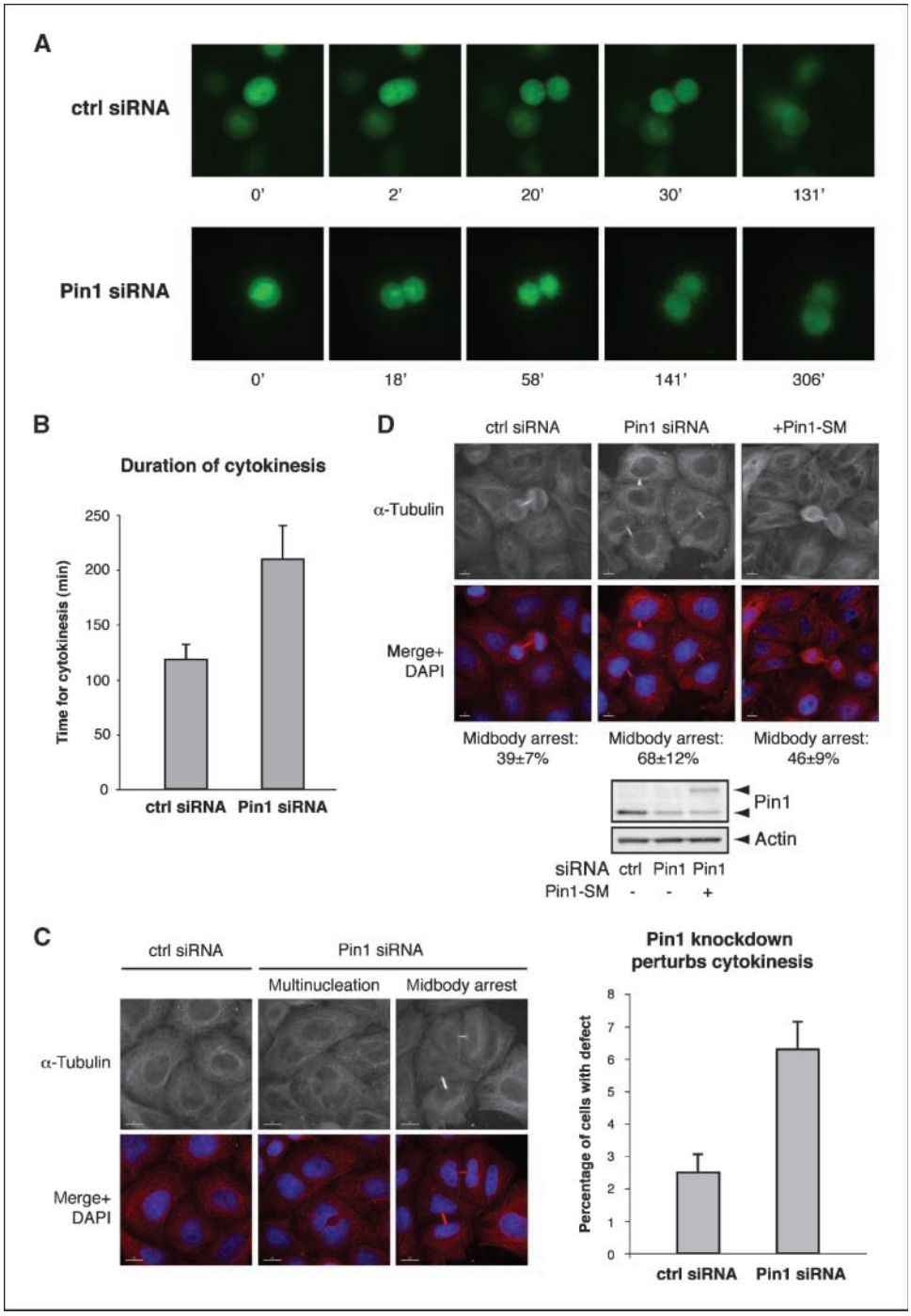

Figure 2. Depletion of Pin1 in HeLa cells causes a cytokinesis defect. *A,* fluorescent time-lapse photographs of HeLa cells expressing EB1-GFP to visualize microtubuli and transfected with control (*ctrl*) or Pin1 siRNA were obtained after release from a nocodazole-induced arrest. Frames from representative cells are shown. See also Supplementary Movies S3 and S4. *B,* time-lapse photographs of 10 cells of each condition were analyzed as in Fig. 1*B*. *Columns,* mean; *bars,* SE. The difference between the two conditions is statistically significant ($P = 0.015$). *C,* HeLa cells were transfected using control or Pin1 siRNA and stained for α-tubulin and DNA (DAPI) 48 h after transfection. The percentage of cells that showed a cytokinesis defect (*middle*, multinucleation; *right*, midbody arrest) relative to the total number of cells was quantified. *Columns,* mean of three experiments (~300 cells in total per sample per experiment); *bars,* SD. The difference between the two conditions is statistically significant ($P = 0.003$). *Bar,* 15 μm. *D,* cells were transfected as indicated (Western blot results show Pin1 expression) and stained for α-tubulin and DNA (DAPI). The percentage of cells displaying midbody arrest (example in *middle*) was quantified relative to the number of cells in cytokinesis. Numbers indicate mean ± SD of at least three experiments (50 cells with a midbody structure per experiment). The differences between Pin1-depleted cells and control cells or those expressing siRNA-insensitive Pin1-SM are statistically significant ($P = 0.0021$ and $P = 0.035$, respectively). *Bar,* 5 μm.

Pin1 interacts with Cep55 *in vivo* and *in vitro*. One candidate protein that is involved in abscission (17, 18) and that contains two potential Pin1 interaction motifs is Cep55 (see Supplementary Fig. S6). We thus analyzed whether Pin1 interacts with Cep55 by performing coimmunoprecipitations in 293T cells overexpressing both proteins. Remarkably, the interaction between Pin1 and Cep55 was detected in mitotic (nocodazole arrested) cells but not in an asynchronous population of cells (Fig. 3*A*). Furthermore, a mutant form of Pin1 that lacks enzymatic activity also interacted with Cep55 with a similar efficiency (see Supplementary Fig. S7), consistent with earlier reports that isomerase-defective Pin1 mutants retain the ability to interact with substrates (28, 29). Importantly, we could also detect an association between endogenous Cep55 and FLAG-Pin1 (Fig. 3*B*). Taken together, these data indicate that Pin1 binds to Cep55 *in vivo* during mitosis.

The putative Pin1 interaction motif of Cep55 consists of phosphorylated S425 and S428, both of which are followed by a proline (14). To confirm the interaction between Pin1 and Cep55 *in vitro* and to determine whether this interaction depends on these mitosis-specific phosphorylation sites in Cep55, we performed GST pull-down experiments. FLAG-tagged wild-type Cep55 in mitotic extracts interacted with GST-Pin1 but not with GST alone or GST-Pin1-W34A (a WW domain mutant that cannot interact with phosphorylated substrates; Fig. 3*C*). Moreover, GST-Pin1 bound only

weakly to the Cep55-S425/428A (serine mutated to alanine) mutant compared with wild-type Cep55 and Cep55-S436A. Notably, the slow-migrating band representing the phosphorylated wild-type Cep55 protein was clearly enriched in the GST-Pin1 pull-downs compared with the fast-migrating band representing Cep55 not phosphorylated on S425 and S428 (compare total lysate and pull-down samples). In agreement with Pin1 interacting with phosphorylated Cep55, phosphatase pretreatment of mitotic extracts lowered the amount of Cep55 that was pulled down with Pin1 *in vitro* (see Supplementary Fig. S8). Together, these data indicate that Pin1 binds to Cep55 during mitosis in a manner dependent on phosphorylation of S425 and S428 in Cep55.

Previous studies clearly showed that Cep55 localizes to centrosomes during mitosis and then translocates to the midbody ring during cytokinesis (14–16). As Pin1 interacts with Cep55 in mitotic cells, we determined whether Pin1 displays a similar localization pattern. To this end, we performed immunofluorescence staining of HeLa cells. In analogy with a previous report (30), we did not observe Pin1 staining at the centrosomes in mitotic cells. However, we found Pin1 staining at the midbody ring during cytokinesis (Fig. 3*D*). This staining was specific as it was undetectable in Pin1-depleted cells. Moreover, FLAG-Pin1 also localized to the midbody ring (see Supplementary Fig. S9), reinforcing our findings that Pin1 is recruited to the midbody ring during cytokinesis. Taken together, these data strongly indicate that Pin1 binds to phosphorylated Cep55 during mitosis and cytokinesis and suggest that these proteins may cooperate to regulate the final stages of cell division.

Pin1 enhances Plk1-mediated phosphorylation of Cep55. The interaction of Pin1 with Cep55 phosphorylated on S425 and S428 shown here (see Fig. 3*C*) and the requirement of these two phosphorylation events for Plk1-dependent phosphorylation of Cep55 on S436, shown by us previously (14), led us to investigate whether Pin1 regulates the phosphorylation of Cep55 on S436. To assess this possibility in detail, we generated a rabbit polyclonal phospho-specific antibody against phosphorylated S436 of Cep55. To check the specificity of this antibody, we probed immunoprecipitates from 293T cells overexpressing wild-type Cep55 or different phosphorylation site mutants with this antibody. Consistent with dependence of S436 phosphorylation on prior phosphorylation on both S425 and S428 (14), the phosphomimic S425/428E mutant was strongly recognized by this phospho-specific antibody, whereas the S425/428A and S436A mutants showed very weak or absent reactivity (Fig. 4*A*). To confirm that Plk1 is the kinase responsible for phosphorylation of Cep55 on S436 (14), we immunoprecipitated FLAG-Cep55 from mitotic HeLa cells with or without specific knockdown of Plk1. Plk1-depleted cells displayed considerably lower levels of S436 phosphorylation compared with control siRNA–treated cells (Fig. 4*B*), reinforcing our previous findings that Plk1 phosphorylates Cep55 on S436 (14).

To analyze the kinetics of phosphorylation of Cep55-S436 during cell cycle progression, Western blotting was performed on lysates from synchronized HeLa cells. Phosphorylated Cep55-S436 was almost undetectable at the G_1-S boundary under thymidine block and at the indicated times up to 8 hours after release. The phosphorylation of Cep55-S436 was maximal 10 hours after release from the block when the cells had entered mitosis (Fig. 4*C*). This phosphorylation but not Cep55 protein levels diminished in the next 4 hours during which cells progressed into the G_1 phase of the cell cycle. These data suggest that phosphorylation of Cep55-S436, but not the protein itself, is lost around completion of cytokinesis. Furthermore, these results imply that Plk1-mediated Cep55-S436 phosphorylation occurs early in mitosis when Cep55 is localized at the centrosome and is still present during execution of cytokinesis when Cep55 is localized at the midbody ring. Indeed, immunofluorescence microscopy analysis with the same antibody revealed a signal on the centrosome from early mitosis to

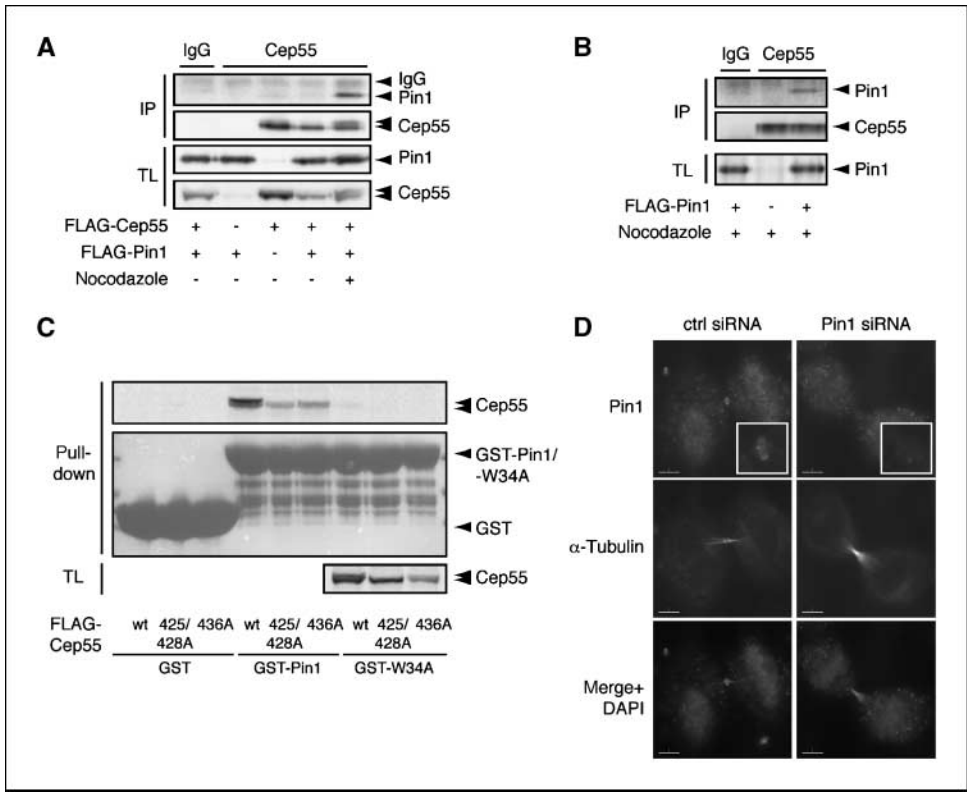

Figure 3. Pin1 interacts with Cep55 during mitosis and localizes to the midbody ring. *A,* 293T cells expressing FLAG-Cep55 and/or FLAG-Pin1 were synchronized by nocodazole treatment. Cep55 was immunoprecipitated using α-Cep55 antibody and immunoprecipitation (*IP*) and total lysate (*TL*) samples were Western blotted using FLAG antibody. *B,* 293T cells expressing FLAG-Pin1 were synchronized using nocodazole. Endogenous Cep55 was immunoprecipitated and Western blots containing immunoprecipitation and total lysate samples were analyzed using FLAG and Cep55 antibodies. *C,* GST, GST-Pin1, and GST-Pin1-W34A were incubated with lysates of nocodazole-arrested 293T cells overexpressing the indicated FLAG-Cep55 expression plasmids. Pull-down and total lysate samples were analyzed for the presence of Cep55 and GST proteins by Western blotting using FLAG antibody and Ponceau staining, respectively. *D,* asynchronous HeLa cells were transfected using control or Pin1 siRNA and stained for Pin1 (polyclonal antibody), α-tubulin, and DNA (DAPI). Representative cells in cytokinesis are shown. *Bar,* 5 μm.

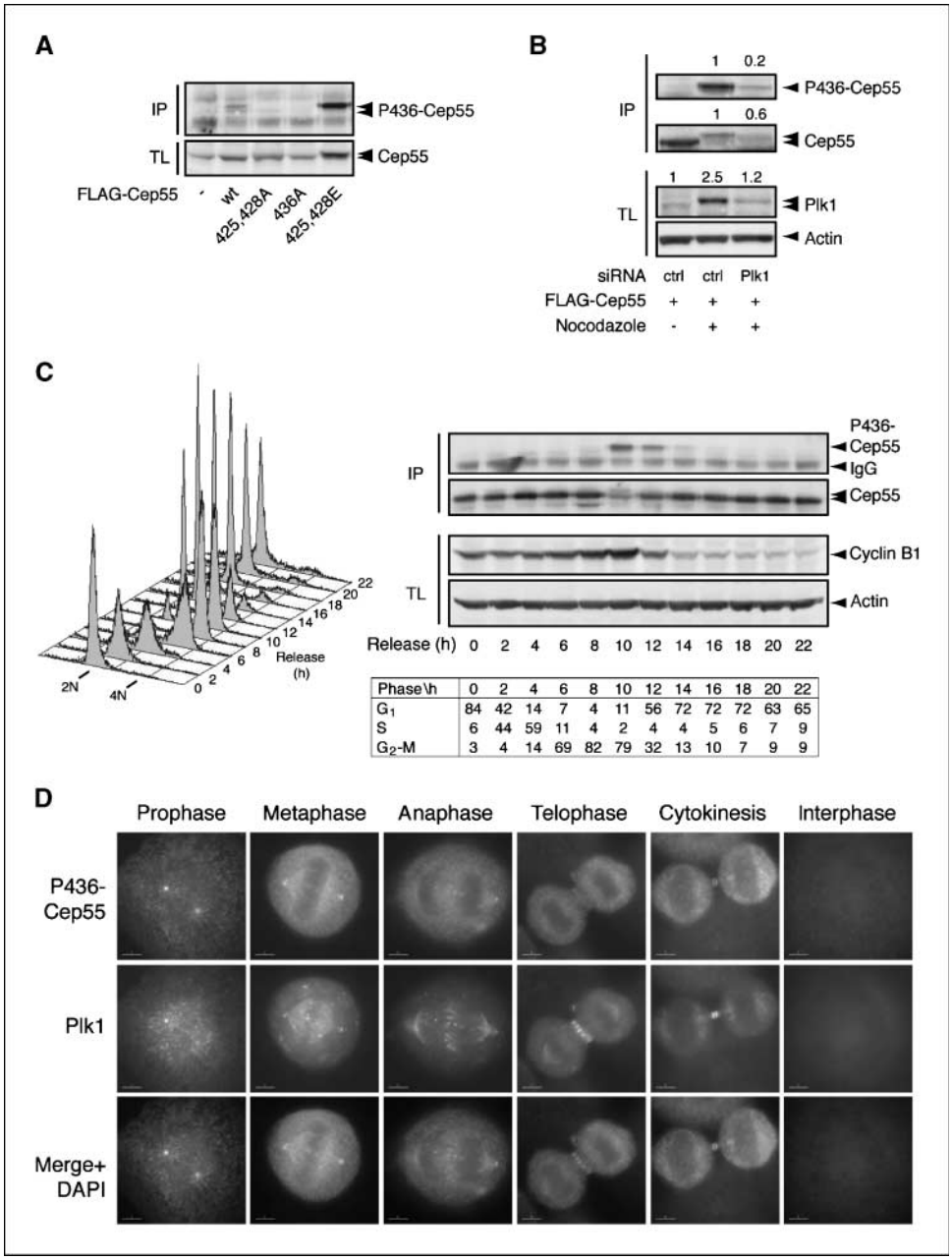

Figure 4. Characterization of a phospho-specific antibody against phosphorylated S436 of Cep55. *A,* Cep55 was immunoprecipitated using FLAG-M2 beads from HeLa cells overexpressing the indicated FLAG-Cep55 constructs. Immunoprecipitation and total lysate samples were analyzed for the presence of phospho-S436-Cep55 (*P436-Cep55*) and Cep55, respectively, by Western blotting. *B,* HeLa cells were transfected with FLAG-Cep55 and control or Plk1 siRNA. Cells were synchronized using nocodazole as indicated. Cep55 immunoprecipitation samples and total lysates were analyzed for the presence of the indicated proteins by Western blotting. *C,* HeLa cells were synchronized by a double thymidine block and released into fresh medium for the indicated time points. *Left,* cell cycle distribution was analyzed by propidium iodide staining and flow cytometry; *right,* FLAG-Cep55 was immunoprecipitated and Western blots containing immunoprecipitation and total lysate samples were probed using phospho-S436-Cep55, Cep55, cyclin B1, and actin antibodies. *D,* asynchronous HeLa cells were stained for phospho-S436-Cep55, Plk1, and DNA (DAPI). Representative examples of cells in different phases of the cell cycle are shown. *Bar,* 5 μm.

anaphase, which then translocated to the midzone and subsequently to the midbody ring during cytokinesis (Fig. 4*D*). Interphase cells as well as mitotic cells depleted of either Cep55 or Plk1 showed negative reactivity for the phospho-S436-Cep55 antibody (see Supplementary Fig. S10), showing that the antibody specifically detects Cep55 phosphorylated on S436 by Plk1 during mitosis and cytokinesis. Because the phosphorylation of Cep55 on S436 does not seem to be important for its localization at the midbody ring as shown previously (14) and in this study (see Supplementary Fig. S11), we propose that this phosphorylation event mediates the recruitment of one or more other proteins involved in the regulation of cytokinesis. We are currently investigating this possibility further.

We next tested whether Pin1 regulates phosphorylation of Cep55 on S436. To this end, FLAG-Cep55 was immunoprecipitated from nocodazole-arrested HeLa cells transfected with Pin1 or control siRNA, and immunoprecipitates were analyzed using the phospho-S436-Cep55 antibody. Depletion of Pin1 resulted in a significant reduction of Cep55-S436 phosphorylation (Fig. 5*A*). As Pin1 can activate Cdk1 under certain circumstances (31, 32), we sought to exclude the possibility that Pin1 regulates Cep55-S436 phosphorylation indirectly by enhancing phosphorylation of S425 and S428. To this end, we compared the mobility shift of Cep55, which is caused by Cep55-S425 and S428 phosphorylation (see Fig. 3*C* and ref. 14), in Pin1-depleted and control cells and this remained unaffected (Fig. 5*B*), indicating that Pin1 acts through a different mechanism. This alternative mechanism does not seem to involve a direct action at the level of Plk1, as Pin1 depletion had no effect on Plk1 expression and its nocodazole-induced mobility shift (Fig. 5*B*). We next studied the possibility that Pin1 regulates binding of Plk1 to Cep55. Notably, expression of wild-type Pin1, but not a Pin1 mutant (W34A) that cannot interact with substrates,

induced the binding between Plk1 and Cep55 (Fig. 5*C*). Moreover, expression of an isomerase mutant form of Pin1 did not enhance the interaction of Plk1 with Cep55 (see Supplementary Fig. S12). These data indicate that both the integrity of the WW domain and the isomerase activity of Pin1 are essential for the Plk1-Cep55 interaction. Taken together, we conclude that Pin1 binds to and likely isomerizes Cep55 phosphorylated on S425 and S428, thereby allowing Plk1 to bind to and phosphorylate Cep55 on S436.

Pin1 acts through Cep55 to regulate cytokinesis. Because Pin1 plays a role in cytokinesis and regulates phosphorylation of Cep55 on S436, which is important for abscission (14), we next investigated whether Pin1 acts through Cep55 in regulating cytokinesis. To this end, we depleted Pin1 and/or Cep55 from HeLa cells (see Fig. 6*C*) and quantified the number of cells that showed a midbody arrest (as in Fig. 2*D*). Knockdown of Pin1 resulted in a ~2-fold increase in the number of cells showing a midbody arrest (Fig. 6*A*), in agreement with observations in Fig. 2*D* and Supplementary Fig. S4. Consistent with our previous report (14), knockdown of Cep55 also increased the number of midbody-arrested cells. Importantly, knockdown of Pin1 in Cep55-depleted cells did not result in a further increase in the number of cells that showed a midbody arrest, indicating that Pin1 acts in the same pathway as Cep55. In addition, when we counted the number of cells that displayed a cytokinesis defect (like in Fig. 2*C*), we observed that knockdown of Pin1 only increased the number of cells with a cytokinesis defect in cells that expressed normal levels of Cep55 (Fig. 6*B*), reinforcing the notion that Cep55 is an important substrate of Pin1 acting in cytokinesis.

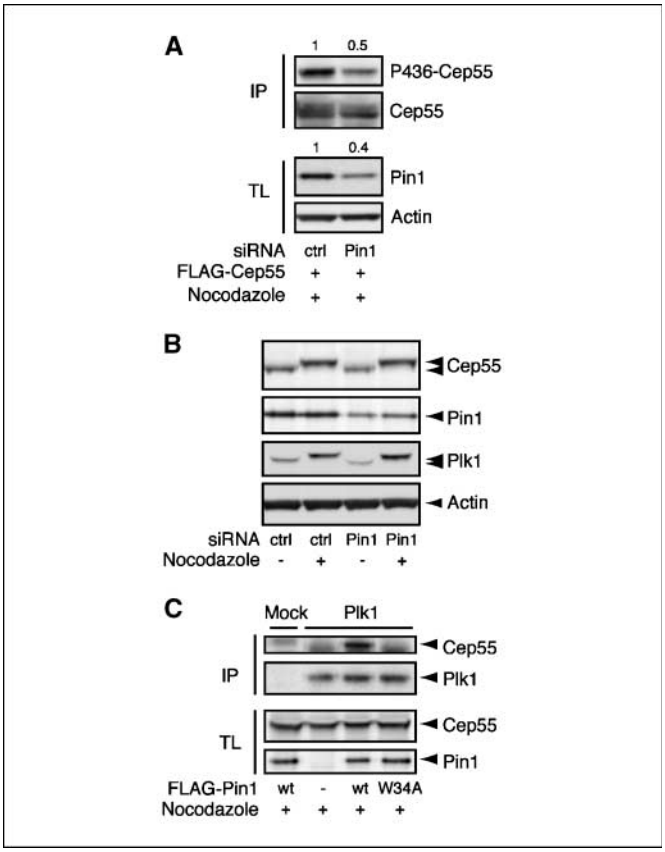

Figure 5. Pin1 induces Plk1-mediated phosphorylation of Cep55. *A,* HeLa cells were transfected with FLAG-Cep55 and control or Pin1 siRNA and synchronized using nocodazole. Immunoprecipitation and total lysate samples were analyzed for the presence of the indicated proteins by Western blotting. *B,* HeLa cells were transfected with control or Pin1 siRNA and synchronized using nocodazole and total lysates were analyzed for a mitosis-specific mobility shift of Cep55 and Plk1 and for Pin1 and Plk1 levels. *C,* Plk1 was immunoprecipitated from nocodazole-arrested HeLa cells transfected with wild-type Pin1 or Pin1-W34A. Immunoprecipitation and total lysate samples were analyzed for Cep55, Pin1, and Plk1 levels.

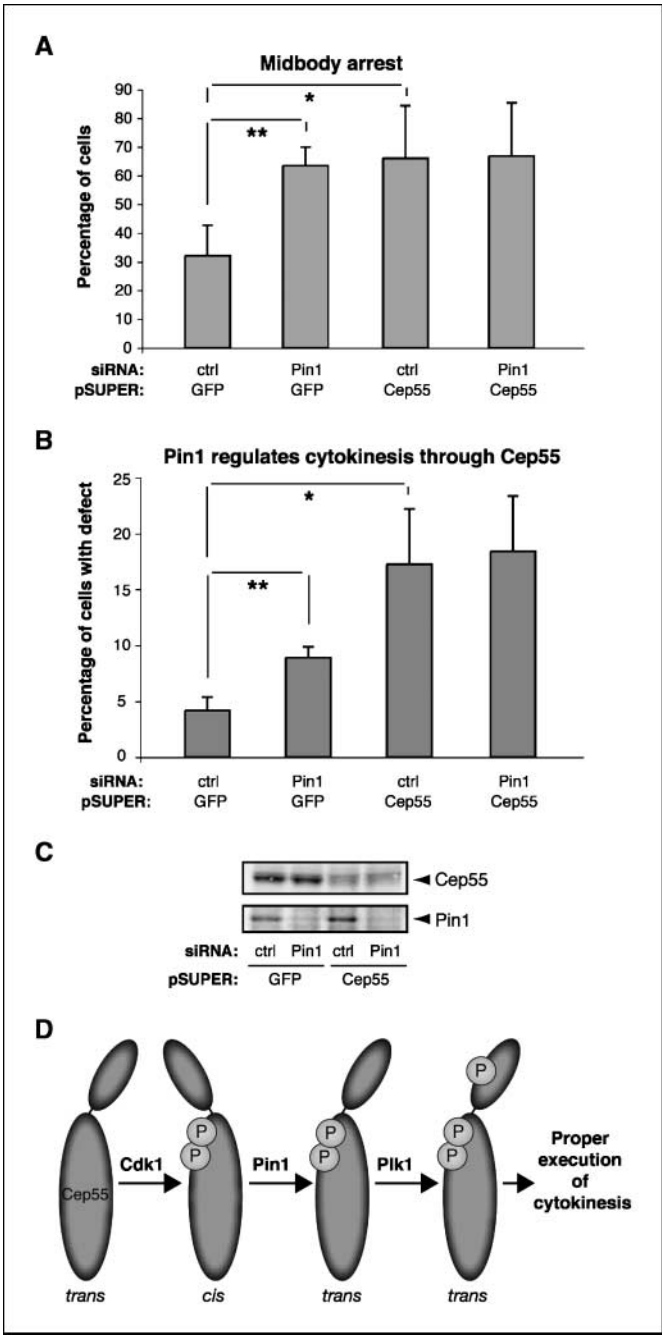

Figure 6. Pin1 acts through Cep55 to regulate cytokinesis. *A,* asynchronous HeLa cells transfected with the indicated combinations of control or Pin1 siRNA and pSUPER plasmid encoding GFP or Cep55-specific siRNA were stained for α-tubulin and DNA (DAPI). Samples were analyzed as in Fig. 2*D*. *Columns,* mean of three experiments (50 cells each); *bars,* SD. Statistical significance is indicated with asterisks: *, $P = 0.05$; **, $P = 0.012$. *B,* experiments were performed as in Fig. 2*C* using the conditions as in *A*. *, $P = 0.012$; **, $P = 0.006$. *C,* representative Western blot results showing the levels of knockdown of Pin1 and Cep55 in the experiments shown in *A* and *B*. *D,* a schematic model illustrating how Pin1 enhances Plk1-mediated phosphorylation of Cep55 on S436, which is essential for timely execution of cytokinesis, by binding to and isomerizing Cep55 when phosphorylated on S425 and S428 by Cdk1.

Discussion

In this study, we show that the peptidyl-prolyl isomerase Pin1 plays a role in cytokinesis. Pin1 localizes to the midbody ring, binds to the midbody ring component Cep55, and regulates the final stages of cytokinesis. Strikingly, cells that have no or low levels of Pin1 display cytokinesis defects that, albeit weaker, are similar to those seen in Cep55-deficient cells (i.e., delayed abscission and failure of cytokinesis resulting in multinucleation). In support of a direct interaction, siRNA-mediated knockdown of Pin1 expression in Cep55-depleted cells does not further enhance the cytokinetic defects of these cells. We further show that two phosphorylated serines (S425 and S428) in Cep55 and the WW domain of Pin1 are required for their interaction. Functional Pin1, but not a binding- or isomerase-defective mutant of Pin1, then enhances binding of Plk1 to Cep55. Consistent with this, in Pin1-deficient cells, Cep55 is hypophosphorylated on the Plk1 site S436, phosphorylation of which is critical for proper execution of cytokinesis.

These findings suggest the following model (Fig. 6*D*): Pin1 binds to Cep55 on mitotic entry when Cep55 is phosphorylated on S425 and S428 by the *trans* conformation-specific and proline-directed kinase Cdk1. This phosphorylation event itself probably induces a conformational change to the *cis* conformation (5, 33). Subsequent binding of Pin1 to Cep55 reverses this effect by inducing *cis/trans* isomerization of Cep55, resulting in enhanced binding of Plk1 to Cep55 to enable Plk1-mediated phosphorylation of Cep55 on S436 and completion of cytokinesis. As Pin1 binding to Cep55 occurs during mitosis and because Pin1, like Cep55, localizes to the midbody ring during cytokinesis, we propose that Pin1 tips the *cis/trans* balance of Cep55 throughout mitosis and cytokinesis toward the conformation that allows Plk1-mediated phosphorylation of Cep55-S436. This conclusion is reinforced by our finding that phosphorylation of S436 is detectable throughout mitosis and cytokinesis.

We noted that the phenotype induced by Pin1 depletion occurs at the same stage of cytokinesis as that caused by knockdown of Cep55 (i.e., at abscission) but that it is weaker. The latter could be explained by the fact that Pin1 is an enzyme whose role is to accelerate the conformational change of its substrates from *cis* to *trans*. This conformational change is most likely only somewhat slower when Pin1 levels are reduced by ~2-fold. So, in many cells, such a depletion of Pin1 may only delay cytokinesis as opposed to blocking it.

Pin1 has been shown to bind to Plk1 directly through its NH_2-terminal WW domain and as such could recruit Plk1 to Cep55 (34–36). However, as Pin1 isomerase mutants are capable of interacting with Cep55 and Plk1 (35, 37) and because such a mutant does not increase binding of Plk1 to Cep55, we conclude that Pin1 enhances the interaction between Cep55 and Plk1 by isomerizing Cep55 and not by recruiting Plk1. Although this is not direct evidence of a catalytic role of Pin1 in promoting a conformational change in Cep55, notably, Pin1 isomerase activity is required to promote Plk1 binding to Cep55.

The Plk1-dependent phosphorylation of S436 in Cep55 is important for normal execution of cytokinesis (14), although the precise role of this phosphorylation event remains unclear at present. We have shown that phosphorylation does not seem to be required for the recruitment of Cep55 to the midbody ring and the structure of this ring is not visibly disrupted in cells expressing Cep55 phosphorylation site mutants (S425/S428A and S436A). We therefore propose that phosphorylation of Cep55 may mediate the interaction of Cep55 with other proteins that play a critical role in regulation of cytokinesis, possibly in membrane fission and/or fusion events involved in abscission. We have tested this possibility by studying the previously described direct interaction between Cep55 and Alix/AIP1 (17, 18). Whereas we could confirm the interaction of Alix to Cep55, we did not observe a difference in binding of Alix to wild-type Cep55 and the phospho-mutant Cep55-S436A (data not shown). We are currently pursuing other possibilities.

Cdk1 and Plk1 phosphorylate a plethora of substrates involved in mitosis and/or cytokinesis. Interestingly, several of these substrates are shared between Cdk1 and Plk1. For example, Cdc25C is phosphorylated by Cdk1 in early mitosis, which enhances subsequent Plk1-mediated phosphorylation that also occurs on mitotic entry (38). Cep55 is similarly regulated by Cdk1 and Plk1. Interestingly, Pin1 also interacts with Cdc25C and regulates its activity through isomerization (31). It would therefore be of particular interest to determine whether Pin1 modifies substrates of Cdk1 other than Cep55 for subsequent phosphorylation by Plk1. Moreover, several proteins that play a role in cytokinesis, such as ECT2, MKLP1, MgcRacGAP, and PRC1 (39–42), have potential Pin1 binding motifs. We thus cannot exclude the possibility that, besides functioning through Cep55, Pin1 might execute its cytokinetic role also via one or more of these proteins.

As Pin1 is deregulated in many cancers (13) and because cytokinesis defects can cause tumor formation (4), our findings may provide a mechanistic link between aberrant expression of Pin1 and cancer. Notably, in most tumors with deregulated Pin1 expression, Pin1 levels are higher compared with normal tissue. Therefore, the findings as reported here could indicate that down-regulation of Pin1 only causes a cytokinesis defect in specific cellular backgrounds. Alternatively, as observed for other proteins involved in cytokinesis such as Cep55, where both overexpression and down-regulation can lead to defective cytokinesis, it is possible that up-regulation of Pin1 also gives rise to a cytokinesis defect. This defect could eventually lead to aneuploidy and aggressive tumor growth. Consistent with this, overexpression of Pin1 has been linked to poor prognosis (43, 44).

In summary, our findings provide mechanistic insight into a previously unappreciated role for Pin1 in cytokinesis. Furthermore, our findings add to the notion that Pin1 might be a molecular timer of the cell cycle by including cytokinesis in the list of cell cycle phases/events that are regulated by Pin1 (12). Strikingly, we show the existence of a Cdk1-Pin1-Plk1 axis in which Pin1 binds to proteins containing Cdk1 phosphorylation sites and promotes their phosphorylation by Plk1. As aberrant cytokinesis seems to be an early event in tumorigenesis, basic knowledge about proteins that play a role in the regulation of cytokinesis may lead to the development of novel cancer therapeutics.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Received 3/5/09; revised 5/29/09; accepted 6/17/09; published OnlineFirst 7/28/09.

Grant support: Netherlands Organization for Scientific Research Rubicon grant (A. van der Horst) and National Health and Medical Research Council, Australia (K.K. Khanna).

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We thank the members of the Khanna lab for helpful discussions; Dr. G. Del Sal for kindly providing the Pin1 MEFs; Drs. A.B. Brenkman and L. Cassimeris for sharing Pin1 and EB1-GFP expression constructs, respectively; Dr. R.H. Medema for critically reading the manuscript; and M. Lorentz for technical assistance.

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