Cdc14B depletion leads to centriole amplification, and its overexpression prevents unscheduled centriole duplication

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Introduction

The centrosome is the major microtubule organization center of animal cells, where it nucleates interphase microtubules responsible for cell polarity and organizes mitotic spindles for the bipolar separation of sister chromatids (Meraldi and Nigg, 2002). Numerical and structural centrosome aberrations have been implicated in almost all types of cancer cells (Saunders, 2005). Despite the apparent link between centrosome amplification and cancer, research is still in the early stages of understanding the molecular mechanisms of centrosome regulation and its deregulation in human diseases. Centrosome aberrations have been implicated in the generation of multipolar mitotic spindles and chromosomal instability during tumor development and progression (Saunders, 2005). The proline-directed phosphatase Cdc14 has been extensively studied in budding yeast where, from G1 until early anaphase, Cdc14 is sequestered in an inactive state in the nucleolus (Shou et al., 1999; Visintin et al., 1999). Cdc14 is activated after release from the nucleolus and functions primarily to inactivate Cdk activity at the end of mitosis, thereby allowing reentry into G1 phase (Stegmeier and Amon, 2004). Cells that lack Cdc14 are unable to exit from mitosis, with defects in both the movement of chromosomes to the spindle poles and the elongation of anaphase spindles (Higuchi and Uhlmann, 2005). Human cells possess two Cdc14 paralogues, Cdc14A and Cdc14B, identified based on their sequence similarity to the budding yeast Cdc14 (Li et al., 1997). Studies suggest that Cdc14A and Cdc14B may be deregulated in various cancer cells including mantle cell lymphoma, breast cancer, prostate cancer, and acute myeloid leukemia (Wong et al., 1999; Martinez et al., 2003; Ashida et al., 2004; Yu et al., 2004; Neben et al., 2005; Rubio-Moscardo et al., 2005). Recent findings indicate that Cdc14A and Cdc14B may be involved in distinctive cellular functions. Although Cdc14A regulates centrosome separation and cytokinesis (Kaiser et al., 2002; Mailand et al., 2002), Cdc14B may participate in the maintenance of nuclear structure (Nalepa and Harper, 2004), regulation of mitotic exit through SIRT2 (Dryden et al., 2003), microtubule stability (Cho et al., 2005), and G1 length through Skp2 (Rodier et al., 2008). Interestingly, previous studies, including ours, have shown that a fraction of endogenous and GFP-tagged Cdc14B associated with centrosomes (Nalepa and Harper, 2004; Cho et al., 2005). Yet, the functional significance of Cdc14B in centrosome cycle regulation has never been explored. Here, we investigate the functional relationship...
between Cdc14B and centriole number control in human cells using both RNA interference and a drug-induced centriole overduplication system.

Results

Cdc14B centrosomal retention requires Cdc14B phosphatase activity

Cdc14B has been shown to associate with distinctive cellular structures such as the nucleolus, nuclear filament, centrosome, and the spindle midzone and midbody (Kaiser et al., 2002; Mailand et al., 2002; Nalepa and Harper, 2004; Cho et al., 2005), and to undergo active nucleocytoplasmic translocation (Bembenek et al., 2005). To explore the functional significance of centrosomal Cdc14B, we first examined whether centrosomal localization of Cdc14B was cell cycle–dependent using a chicken anti-Cdc14B antibody. After preextraction and fixation of U2OS cells, immunofluorescence analysis revealed that Cdc14B partially colocalized with centrin, a marker that stains at the distal lumen of centrioles, in addition to the nucleolar, intracellular bridge (Fig. 1 A) and midzone (not depicted) localization previously characterized using other anti-Cdc14B antibodies (Kaiser et al., 2002; Mailand et al., 2002; Nalepa and Harper, 2004; Cho et al., 2005). Depending on the centriole configuration during different stages of the cell cycle (Tsou and Stearns, 2006a; Nigg, 2007), Cdc14B associates with each of the disengaged centrioles in G1 and late M phase but only one of the engaged centrioles in S, G2, and early M phase (Fig. 1 A).

Cdc14B-GFP also showed a similar cell cycle–dependent centriole localization pattern (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200710127/DC1). We believe that the reason we and others previously did not observe centriole–associated Cdc14B with the rabbit anti-Cdc14B antibodies (Kaiser et al., 2002; Cho et al., 2005) was that these antibodies might react with epitopes that were not exposed at centrioles under the extraction/fixation conditions. In fact, Nalepa and Harper (2004) found that the centrosomal Cdc14B only became visible after Triton X-100 extraction before fixation using another rabbit anti-Cdc14B antibody. To independently evaluate the centrosomal association of Cdc14B, we purified centrosome fractions from asynchronized HeLa cells by discontinuous sucrose gradient centrifugation according to a published protocol (Moudjou and Bornens, 1998). Proteins in each fraction were resolved by SDS-PAGE and analyzed by Western blotting. At the expected ~60% sucrose density, fractions 14–17 contained the most abundant amounts of γ-tubulin, an archetypal centrosomal marker. In parallel, Cdc14B was highly enriched in those γ-tubulin–containing centrosome fractions (Fig. 1 B, top). Moreover, when a purified centrosomal fraction (fraction 15) was subject to immunostaining, we found that Cdc14B but not nucleolin, a noncentrosomal protein, stained positive at the purified centrosomes (Fig. 1 B, bottom). Together, these data suggest that a fraction of Cdc14B phosphatase specifically associates with centrioles.

To investigate whether Cdc14B catalytic activity is required for Cdc14B centrosome localization, we assessed the centrosomal localization of a catalytic “dead” Cdc14B C314S mutant (Cho et al., 2005). As shown in Fig. 2 (A and B), Cdc14B C314S–GFP localized in nuclei/nucleoli (Cho et al., 2005) and was absent from the centrosomes in virtually all the interphase cells examined. In contrast, wild-type Cdc14B-GFP (Cdc14B WT–GFP) was found to associate with centrosomes in 75% of GFP–positive interphase cells (Fig. 2 B). Although these findings suggest that catalytic activity is essential for Cdc14B centrosomal localization, its perturbation may also inhibit Cdc14B nucleocytoplasmic shuttling ability, which indirectly prevents Cdc14B from reaching centrosomes in interphase cells where the nuclear envelope is still intact. To exclude this possibility, we studied the localization of Cdc14B C314S–GFP at the centrosomes of mitotic cells where the nuclear envelopes have disassembled. As shown in Fig. 2, no Cdc14B C314S–GFP was found to associate...
its nucleocytoplasmic shuttling activity and thus supports the possibility that Cdc14B centrosomal localization requires its catalytic activity. We previously demonstrated that the cytoplasmic translocation/microtubule bundling ability of Cdc14B(C314S) was impaired but restored when an additional mutation (KKIR29-32AAIA, Cdc14B(KKIR) of a potential NLS was introduced to the Cdc14B(C314S) mutant (KKIR29-32AAIA+C314S, Cdc14B(KKIR)). Interestingly, introduction of this mutation also restored Cdc14B(C314S) localization to centrosomes (Fig. 2). Because breakdown of the nuclear/cytoplasmic barrier in mitosis did not restore Cdc14B(C314S) to centrosomes (Fig. 2), this finding indicates that the additional KKIR mutation not only helps Cdc14B(C314S) regain cytoplasmic translocation but also acts as a gain-of-function mutant that facilitates Cdc14B(C314S) to re-associate with centrosomes.

Ablation of Cdc14B leads to centriole amplification

To determine whether Cdc14B plays a role in centrosome cycle regulation, we took a Cdc14B knockdown approach. We first attempted to establish stable cell lines in which endogenous Cdc14B could be conditionally knocked down. In search of siRNA oligos to deplete the endogenous Cdc14B, we found that an oligo corresponding to nucleotide position 1,234–1,254 relative to the Cdc14B start codon was most effective in ablating Cdc14B expression. We cloned this siRNA oligo into a pSuperior-neo-GFP vector and the resulting plasmid was transfected into TREx HeLa cells to obtain Cdc14B knockdown stable clones. Western blot analysis showed a reduction of endogenous Cdc14B expression in two of the representative clones (clones No. 2 and 3) in the presence of tetracycline (Fig. 3 A). Consistently, real-time quantitative RT-PCR showed that the level of Cdc14B mRNA level was also decreased in these two clones (unpublished data). The same siRNA could also knock down Cdc14B-GFP expression in U2OS Tet-On cells, confirming the target specificity of the Cdc14B siRNA (Fig. 3 A). Moreover, immunostaining with the anti-Cdc14B antibody revealed that centrosomal Cdc14B expression was reduced in Cdc14BsiRNA cells (Fig. S2 A, available at http://www.jcb.org/cgi/content/full/jcb.200710127/DC1), though the efficiency of knockdown varied in each cell. Careful examination of those Cdc14B knockdown clones revealed a centriole amplification phenotype (Fig. 3, B and C). In comparison with the parental TREx HeLa and clone No. 1, which did not exhibit any reduction of Cdc14B expression (Fig. 3 A), clones No. 2 and 3 showed two- to threefold increases in the number of cells with more than four centrioles (Fig. 3, B and C) judged by immunostaining with an anti-centrin antibody. The centriole number usually ranged from 5 to 20 with the majority around six to eight centrioles per cell. Similar results were obtained (Fig. S2 B) when γ-tubulin was used as a centrosome marker (Fig. S2 C). To ascertain the specificity of Cdc14B knockdown phenotype, we tested a Cdc14B siRNA oligo pool (SMARTpool containing four different Cdc14B siRNA oligos) and a small hairpin RNA (shRNA) designated as pSuperCdc14BshRNA-E9, each targeting different regions of Cdc14B mRNA sequences in transient and stable transfection studies, respectively. Western blot analysis confirmed that both the
To determine if the observed supernumerary centrioles may derive from centrosome fragmentation, we carefully evaluated the $\alpha$-tubulin–decorated centrosomes in HeLa cells transiently transfected with the Cdc14BsiRNA SMARTpool. We found that a majority of the supernumerary centrosomes were clustered together and similar in size (Fig. 3 E). All the supernumerary centrosomes ($n = 105$) contained centrin-labeled centrioles and many of the centrioles were in pairs. These data suggest that the supernumerary centrosomes may not arise from centrosome fragmentation.

Examination of TREx-HeLa Cdc14BsiRNA stable cells revealed a slight increase in the number of polyploid cells, such as multinucleate and macronuclear cells (Fig. S3 A, available at http://www.jcb.org/cgi/content/full/jcb.200710127/DC1). The siRNA pool and the shRNA specifically knocked down Cdc14B but not its Cdc14A paralogue in HeLa cells (Fig. 3 D, top). Moreover, knockdown of Cdc14B by both the siRNA pool and shRNA led to a significant accumulation of cells with more than four centrioles in HeLa cells (Fig. 3 D, bottom). Although HeLa cells have been widely used for centrosome studies, it is a tumor cell line. To examine the effect of Cdc14B on centrosome cycle control in normal cells, we transfected normal human fibroblast BJ and MRC-5 cells with the Cdc14B siRNA SMARTpool. As shown in Fig. 4, the centriole amplification phenotype was faithfully reproduced in the normal fibroblast cells depleted of Cdc14B. These results demonstrate that depletion of Cdc14B leads to centrosome amplification in both normal and transformed cells.

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whether localization of Cdc14B at centrioles conveys a critical function in centriole duplication. The number of centrin-labeled centrioles was counted in the inducible Cdc14B WT-GFP cells after cultivation in the presence of HU without DOX induction, and HU + DOX for 72 h. As expected, HU treatment led to centriole amplification in uninduced Cdc14B WT-GFP cells (Fig. 5 A, bottom) or mock-transfected U2OS Tet-On cells (Fig. 5 B). Remarkably, this centriole amplification phenotype was significantly attenuated in cells where Cdc14B WT-GFP was at centrioles but not in cells where Cdc14B WT-GFP was not at centrioles (Fig. 5 A). This finding was confirmed in the same sets of cells treated with HU + DOX for 72 h using /H9253-tubulin as a centrosome marker (Fig. S4, A and B, available at http://www.jcb.org/cgi/content/full/jcb.200710127/DC1). Similar and yet more dramatic results were obtained in transient transfection experiments in which cells transfected with DOX-inducible Cdc14B WT-GFP exhibited an even more significant reduction of HU-induced centrosome amplification in comparison with the mock-transfected cells (Fig. 5 B). Together, these results suggest that Cdc14B overexpression suppresses abnormal centriole amplification, and this inhibitory function requires the presence of Cdc14B WT-GFP at centrioles in the HU-induced centriole overduplication system. Fluorescence-activated cell sorting analysis revealed that induction of Cdc14B-GFP did not perturb cell cycle progression at the expression level set by the DOX-inducible promoter (Fig. 5 C), which suggests that the inhibition of centriole amplification in HU-arrested cells was not the result of a potential G1 cell cycle arrest.

Macronuclear and multinucleate cells were determined by visual judgment of the sizes (nuclear diameter and area) of DAPI-stained nuclei and the number of nuclei per cell, respectively. It is important to note that among the TREx-HeLa Cdc14BsiRNA stable cells with centriole amplifications, only ~0.2 (n = 300) were polyploid. Using the same criteria, we were unable to detect any significant increase of polyploid cells in HeLa cells transiently transfected with Cdc14BsiRNA SMARTpool (Fig. S3 B). In line with this finding, flow cytometry analysis did not reveal any significant increase of polyploidy in those Cdc14B knockout cells (Fig. S3 C). Moreover, none of the Cdc14B-depleted BJ (0%; n = 115) or MRC-5 (0%; n = 74) cells with more than four centrioles were polyploid. Thus, the centriole amplification phenotype in Cdc14B knockout cells may not be the product of aborted cell division, and the slight increase in the number of polyploid cells in the TREx-HeLa Cdc14BsiRNA stable clones may have evolved during the course of stable clone selection.

**Ectopic expression of centriole-associated Cdc14B inhibits centriole overduplication in prolonged S phase-arrested cells**

In certain transformed cells, such as U2OS cells, prolonged S phase arrest by hydroxyurea (HU) causes multiple rounds of centriole duplication in the absence of DNA replication and mitotic division (Balczon et al., 1995; Chang et al., 2003). We therefore used this well-established centriole overduplication system to directly evaluate whether Cdc14B plays a role in the regulation of centriole duplication. For this purpose, we first tested if Cdc14B overexpression could inhibit HU-induced centriole overduplication in the stable doxycycline (DOX)-inducible Cdc14B WT-GFP U2OS Tet-On cells. Based on the localization of Cdc14B WT-GFP at centrioles (Fig. 2), the Cdc14B WT-GFP-positive cells can be divided into two groups: Cdc14B WT-GFP at centrioles and not at centrioles. This provides an ideal system to directly examine whether localization of Cdc14B at centrioles conveys a critical function in centriole duplication. The number of centrin-labeled centrioles was counted in the inducible Cdc14B WT-GFP cells after cultivation in the presence of HU without DOX induction, and HU + DOX for 72 h. As expected, HU treatment led to centriole amplification in uninduced Cdc14B WT-GFP cells (Fig. 5 A, bottom) or mock-transfected U2OS Tet-On cells (Fig. 5 B). Remarkably, this centriole amplification phenotype was significantly attenuated in cells where Cdc14B WT-GFP was at centrioles but not in cells where Cdc14B WT-GFP was not at centrioles (Fig. 5 A). This finding was confirmed in the same sets of cells treated with HU + DOX for 72 h using γ-tubulin as a centrosome marker (Fig. S4, A and B, available at http://www.jcb.org/cgi/content/full/jcb.200710127/DC1). Similar and yet more dramatic results were obtained in transient transfection experiments in which cells transfected with DOX-inducible Cdc14B WT-GFP exhibited an even more significant reduction of HU-induced centrosome amplification in comparison with the mock-transfected cells (Fig. 5 B).

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**Cdc14B phosphatase activity is essential to prevent centriole overduplication in prolonged S phase-arrested cells**

To investigate whether inhibition of centriole overduplication in HU-arrested U2OS cells requires the presence of active Cdc14B at centrioles, we took advantage of and tested the centriole-bound
inhibition of centriole duplication and that docking a catalytically inactive Cdc14B-GFP to centrioles is not sufficient to prevent centriole overduplication.

Inhibition of Z-L 3 VS–induced centriole overduplication requires Cdc14B phosphatase activity

It has been documented that treatment of U2OS cells with a proteasome inhibitor Z-L 3 VS causes multiple daughter centriole growth from a single mother centriole template (Duensing et al., 2007). This aberrant daughter centriole overduplication requires cyclin E/Cdk2 and Plk4, the known positive regulators of centriole duplication (Nigg, 2007). We thus used the catalytic dead Cdc14B K & C-GFP mutant as described in Fig. 2. When the Cdc14B K & C-GFP cells were exposed to HU, the DOX-induced Cdc14B K & C-GFP was unable to block centriole overduplication, whereas the centriole-associated catalytic “active” Cdc14B KKIR mutant (Fig. 2) was as potent as its wild-type counterpart in inhibition of centriole overduplication (Fig. 5 A). Similar results were obtained when the HU-treated cells were examined by γ-tubulin staining (red). Representative centrosome amplification was detected in mock-transfected cells after HU treatment but not in pBI-tet-Cdc14B WT-GFP transfected cells where Cdc14B WT-GFP (green) associated with centrosomes. Insets show magnified images of centrosomes. DAPI (blue), DNA. Bar, 5 μm. [bottom] The percentage of cells with the indicated centrosome numbers was calculated from the experiments shown in the top panel. Centrosomes were counted in both mock and Cdc14B-GFP–transfected cells (Cdc14B-GFP–positive at centrosomes). All the data are shown as the means ± SD of three independent experiments. At least 500 cells were counted in each experiment. [C] Representative fluorescence-activated cell sorting profile on cell cycle distribution of DOX-inducible Cdc14B-GFP U2OS Tet-On stable clones. Cells were cultivated in the presence or absence of 4 μg/ml DOX for 72 h. Positions of cells with 2 N and 4 N DNA contents are labeled with arrowheads.
Z-L3VS–induced centriole duplication system to evaluate the possibility of Cdc14B as a counterbalancing enzyme of cyclin E/Cdk2 and/or Plk4 in centriole duplication control. In the absence of Cdc14BWT-GFP induction (−DOX), treatment of U2OS Tet-On–Cdc14BWT-GFP cells with Z-L3VS evoked an aberrant centriole overgrowth, whereas in the presence of Cdc14BWT-GFP induction (+DOX), centriole-bound Cdc14BWT-GFP significantly attenuated the centriole overduplication phenotype (Fig. 6). Moreover, similar to our observation in the HU experiment (Figs. 5 and S4, A and B), the centriole-bound catalytic dead Cdc14BΔCas mutant failed to prevent Z-L3VS–induced centriole overduplication (Fig. 6), indicating that this inhibition requires Cdc14B catalytic activity. Although additional experiments are required, our study supports the possibility that Cdc14B may counterbalance centrosomal kinases required for centriole overduplication in the Z-L3VS induction system.

Discussion

The findings that depletion of Cdc14B leads to centriole amplification and that overexpression of Cdc14B inhibits HU- and Z-L3VS–induced centriole overduplication imply that Cdc14B may play a pivotal role in the regulation of centriole duplication cycle. Because catalytic activity of Cdc14B is required to harness centriole overduplication in both HU- and Z-L3VS experimental systems, Cdc14B may exert its effect through modulating the phosphorylation status of its substrates, in particular those involved in the control of centrosome duplication. It has been well documented that the activities of centrosome-associated protein kinases, such as Cdk2/cyclin E/A, Plk2, Plk4, calcium-calmodulin kinase II, MpsI, and Zyg-1, are required for centriole duplication in various species and experimental settings including the HU- and Z-L3VS–induced centriole overduplication systems (Matsumoto et al., 1999; Meraldi et al., 1999; Fisk and Winey, 2001; O’Connell et al., 2001; Matsumoto and Maller, 2002; Fisk et al., 2003; Warnke et al., 2004; Habedanck et al., 2005; Tsou and Stearns, 2006b; Duensing et al., 2007; Kleylein-Sohn et al., 2007), which suggests that phosphorylation plays an important role for the precise reproduction of centrosomes during the cell cycle (Tsou and Stearns, 2006a; Nigg, 2007). Phosphorylation of centrosome-associated proteins such as nucleophosmin/B23, CP110, and Mps1 has been implicated in centrosome duplication control. In particular, phosphorylation of B23 Thr199 by Cdk2/cyclin E and of B23 Thr95 by an unknown kinase dissociates B23 from centrosomes, which in turn allows centrosome duplication or reduplication to occur (Okuda et al., 2000; Budhu and Wang, 2005). Likewise, depletion of CP110 abolishes centrosome reduplication in S phase–arrested cells (Chen et al., 2002) and suppresses Plk4–induced procentriole reduplication (Habedanck et al., 2005), whereas inactivation of Cdk2 activity abolishes Mps1-dependent centrosome duplication (Fisk and Winey, 2001). Based on our observation in this study, it is possible that Cdc14B may regulate the centriole duplication cycle by modulating the phosphorylation status of centrosomal proteins such as B23, CP110, and Mps1.

A counterbalance of kinase and phosphatase activities involving Nek2 and PP1α has been proposed for governing centrosome splitting (Meraldi and Nigg, 2001). A similar mechanism may prevail in the regulation of centrosome duplication (Nigg, 2007). To our knowledge, Cdc14B is the only phosphatase identified as a negative regulator of centrosome duplication, though a recent study shows that another phosphatase, Cdc25B, also regulates centrosome duplication, albeit as a positive regulator (Boutros et al., 2007). It is possible that Cdc14B may act to keep the centrosomes in check before and/or after centrosome duplication by opposing the activities of Cdk2 and other related kinases required for the initiation of centrosome duplication. It has been demonstrated that mammalian Cdc14s preferentially dephosphorylate Ser/Thr-proline–directed sites of Cdk-phosphorylated substrates (Li et al., 2000; Kaiser et al., 2002), Cdk2/cyclin E and A are involved in the initiation of centrosome duplication in cycling cells (Hinchcliffe et al., 1999; Meraldi et al., 1999). In this context, Cdc14B may oppose cyclin A/Cdk2 activity to prevent centrosome reduplication because centrosome duplication terminates at the end of S phase and cyclin A/Cdk2 is still active throughout S phase and early mitosis (Pagano et al., 1992). Cdc14B may also stabilize Cdk inhibitor 1/Kip1 (Saito et al., 2004) by promoting dephosphorylation–dependent Skp2 degradation (Rodier et al., 2008), which in turn inhibits Cdk2/cyclin E activity in G1 to prevent premature centriole duplication. Recent studies suggest that Plk4 plays an important role in triggering procentriole assembly (Kleylein-Sohn et al., 2007) and, thus, uncontrolled activation may impose risks of abnormal procentriole growth (Habedanck et al., 2005). Under this circumstance, Plk4 activity may call for scrupulous counterbalance by Cdc14B-like phosphatases, though centrosomal substrates of Plk4 and Cdc14B remain unknown and the consensus phosphorylation motif of Plk4 substrates (Leung et al., 2007) may not conform to a typical Ser/Thr-proline motif of Cdc14B substrates.
Cdc14B has been implicated in the regulation of nuclear structure maintenance (Nalepa and Harper, 2004), microtubule dynamics (Cho et al., 2005), mitotic exit control (Dryden et al., 2003), and G1 progression (Rodier et al., 2008). The abnormal centrosome amplification observed in Cdc14B-depleted cells may arise from the combinatorial events involving failures in nuclear structure, cell, and centrosome cycle regulation. However, because active Cdc14B can directly inhibit unscheduled centriole overduplication, and Cdc14B depletion leads to centriole amplification in the absence of obvious polyplody in normal human fibroblast cells, it is possible that one of Cdc14B functions is to serve as a centrosomal regulatory protein, and compromising centrosomal regulatory function of Cdc14B may account for at least part of the observed centrosome amplification phenotype. Because it is frequently down-regulated in tumor cells (Wong et al., 1999; Martinez et al., 2003; Ashida et al., 2004; Yu et al., 2004; Neben et al., 2005; Rubio-Moscoso et al., 2005), Cdc14B may function as a tumor suppressor to maintain the fidelity of the centrosome duplication cycle and genomic stability in human cells.

Materials and methods

Cell culture, transfection, drug treatment, and cell cycle analysis

U2OS Tet-On (BD Biosciences), Hela, and TREx Hela (Invitrogen) were cultured as described previously (Cho et al., 2005). Normal human fibroblast BJ and MRC-5 cells were obtained from the American Type Culture Collection and maintained in Dulbecco’s modified Eagle's medium supplied with 10% fetal bovine serum, 50 U/ml penicillin, and 50 μg/ml streptomycin. To establish DOX-inducible Cdc14B-GFP stable clones, the pBNetCdc14B WT, pBNetCdc14B G38T, pBNetCdc14B C314S, or pBNetCdc14B ECC plasmid (Cho et al., 2005) was cotransfected with pBabe-puro into U2OS Tet-On cells harboring endogenous Cdc14B by FuGene6 (Roche). Stable clones were obtained after selection with 500 μg/ml G418 and 2 μg/ml puromycin in the absence of DOX. For the prolonged S phase arrest experiment, the Cdc14B-GFP stable clones were treated with or without 4 μg/ml DOX, 2 mM HU (Sigma-Aldrich) alone, or 2 mM HU and 4 μg/ml DOX together for 72 h. For the Z-L-VS experiment, Cdc14B-GFP stable clones were treated with 1 μM of Z-L-VS (BIOMOL International, L.P.) in the presence or absence of 4 μg/ml DOX for 48 h. For cell cycle analysis, cells were stained with a buffer containing 1 μg/ml propidium iodide, 0.6% NP-40, 0.1% sodium citrate, and 20 μg/ml RNase A and analyzed by a fluorescence-activated cell sorter (Guava Technologies, Inc.). The percent-age of cells in difference phases of the cell cycle was determined using ModFit LT software (Verity Software House).

siRNA and shRNA experiments

Cdc14B protein was depleted using the following three approaches. First, siRNA oligos that target the sequences 5'-GAACCGAGACCGTACGCT-3' (1,234–1,254, relative to the start codon of Cdc14B) were annealed and cloned into the HindIII and BglII sites of pSuper-neo-GFP vector (Oligo-engine). Tetracycline-inducible cell lines expressing pSuperiorCdc14B siRNA were generated by cotransfection with pBabe-puro into T-REx Hela cells that contain Tet repressors. Stable clones were established by selection in a growth medium containing 100 μg/ml G418 and 1 μg/ml puromycin in the absence of tetracycline. Second, a shRNA expression vector carrying a Cdc14B shRNA oligo (989–1,017, relative to Cdc14B start codon) was obtained from Open Biosystems and subcloned into the pSuper-neo-GFP vector (Oligo-engine), which was designated as pSuper-Cdc14B shRNA-E9. Stable transfectants were obtained by cotransfection with pBabe-puro into Hela cells followed by selection with 800 μg/ml G418 and 1 μg/ml puromycin. Third, Cdc14B siRNA SMARTpool (targeting four different regions of Cdc14B mRNA sequences) and a siGLO control oligo were obtained from the siGENOME collection (Thermo Fisher Scientific) and transfected into Hela, BJ, and MRC-5 cells using siPORT NeoFX reagent (Ambion) according to the manufacturer’s instructions followed by a 66-h incubation. Cell lysates were then prepared and subjected to SDS-PAGE. Western blots were performed using anti-Cdc14A (Invitrogen), -Cdc14B (Invitrogen), -GFP (BD Biosciences), and -β-tubulin (Cytoskeleton, Inc.) antibodies.

Immunofluorescence

For indirect immunofluorescence, cells were grown on glass coverslips and fixed with either paraformaldehyde or cold 100% methanol. The cells were then permeabilized with PBS/0.5% Triton X-100 for 10 min followed by blocking with PBS/1% BSA for 30 min. Centrosomes or centrioles were visualized by immunostaining with antibodies against γ-tubulin (GTU-88 [Sigma-Aldrich] and C-20 [Santa Cruz Biotechnology, Inc.], per- centered) or 53BP1 (C-31) as a gift from J. Salisbury, Mayo Clinic Foundation, Rochester, MN). For visualization of centrosome-associated endogenous Cdc14B, cells were treated with or without 10 μg/ml nocodazole (for better exposure of centrosomal Cdc14B) for 2 h, briefly extracted with 0.5% Triton X-100 on ice, fixed with cold 100% methanol, and immunostained with chicken anti-Cdc14B antibody (GenWay Biotech, Inc.). In our hands, methanol fixation preserved GFP signals and, thus, anti-GFP immunostaining was not used to visualize Cdc14B-GFP fusion proteins in the fixed cells. Secondary antibodies including Alexa Fluor 488 and 594 donkey anti-mouse and goat anti-rabbit, and anti–chicken IgY antibodies were obtained from Invitrogen. DNA was counterstained with DAPI. Cells were visualized with a 100× Plan Neofluar objective (1.30 oil; =:0.17; Carl Zeiss, Inc.) under an epifu- rescence microscope (Axioskop 2; Carl Zeiss Inc.). Images were acquired with a charge-coupled device camera (AxioCam HRC; Carl Zeiss, Inc.) controlled by OpenLab software (version 3.5; PerkinElmer). For confocal microscopy, images were captured with an HCK-FL/APO 63 X 1.32 oil objective (Leica) under a SP2 laser scanning confocal microscope (Leica) equipped with confocal software (Leica version 2.0; Wetzlar). The coverslips were mounted using PermaFlour Mountant medium (Thermo Fisher Scientific) and imaging was performed at room temperature. Image processing was performed using Photoshop CS (8.0).

Centrosome fractionation experiments

Centrosomes were prepared from exponentially growing HeLa cells according to a previously published procedure (Moudou and Bornens, 1998). In brief, a total of 6 × 10^6 cells were treated with 1 μg/ml cytochalasin D and 2.2 μM nocodazole. Cells were lysed in a buffer containing 1 M Hepes, pH 7.2, 0.5% NP-40, 0.5 mM MgCl₂, 0.1% β-mercaptoethanol, and protease inhibitor cocktail (Roche) and centrifuged at 2,500 g. The resulting supernatant was filtered, incubated with 2 μl/ml DNase I, and loaded over a 60% sucrose cushion for centrifugation at 10,000 g with a SW28 rotor (Beckman Coulter). Concentrated centrosomes were centrifuged again over a discontinuous gradient containing 70, 50, and 40% sucrose solutions at 75,000 g. A total of 32 fractions were collected from the bottom of the tube. Each fraction was separated by SDS-PAGE. Centrosome-enriched fractions were determined by immunoblotting with anti–γ-tubulin antibody, and the presence of Cdc14B was judged by immunoblotting with anti-Cdc14B antibody (Invitrogen). For immunofluorescence analysis of isolated centrosomes, each 10 μl of fraction 15 was diluted into 4 ml of 10 mM Pipes buffer, pH 7.2, and transferred into a 38.5-m1 ultracentrifuge tube (Beckman Coulter) with a specially designed adaptor to fit to a 15-mm round coverslip. The samples were then subjected to centrifugation at 20,000 g (10,000 rpm) for 20 min with a SW28 rotor followed by fixation in methanol at –20°C for 10 min and immunostaining with antibodies against centrin, Cdc14B (GenWay Biotech, Inc.), and nucleolin (4E2; Research Diagnostics, Inc.), respectively. Finally, coverslips were placed in pure ethanol for 2 min at room temperature, air-dried, and mounted with a drop of PermaFlour (Thermo Fisher Scientific) on a microscope slide. Images were captured as described in the preceding paragraph.

Online supplemental material

Fig. S1 shows the specificities of Cdc14B and γ-tubulin antibodies, and centrosome amplification in Cdc14B knockdown cells. Fig. S2 shows polyclonal antibodies in Cdc14BsiRNA stable clones. Fig. S4 shows that Cdc14B catalytic activity is required to prevent HU-induced centrosome overduplication. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200710127/DC1.

We thank Dr. Jeffrey Salisbury for kindly providing the anticentrin antibody, Ying Huang for technical support, and Richard Giannone for critical reading of the manuscript.

Y. Wang acknowledges the support of the Laboratory Directed Research and Development Program and the Seed Money Fund of Oak Ridge National Laboratory, the Office of Biological and Environmental Research at the U.S. Department of Energy (under contract DE-AC05-00OR22725), with UT-Battelle, the Battelle Memorial Institute (under contract DE-AC05-00OR22725), and the U.S. Department of Energy (grant DE-EE08904). Y. Liu acknowledges the
support of the Intramural Research Program of the National Institutes of Health/ National Institute on Aging.

Submitted: 18 October 2007
Accepted: 3 April 2008

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