



The Replication Protein Cdc6 Suppresses Centrosome Over-Duplication in a Manner Independent of Its ATPase Activity

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The Cdc6 protein is essential for the initiation of chromosomal replication and functions as a licensing factor to maintain chromosome integrity. During the S and G2 phases of the cell cycle, Cdc6 has been found to inhibit the recruitment of pericentriolar material (PCM) proteins to the centrosome and to suppress centrosome over-duplication. In this report, we analyzed the correlation between these two functions of Cdc6 at the centrosome. Cdc6 depletion increased the population of cells showing centrosome over-duplication and premature centrosome separation; Cdc6 expression reversed these changes. Deletion and fusion experiments revealed that the 18 amino acid residues (197-214) of Cdc6, which were fused to the Cdc6-centrosomal localization signal, suppressed centrosome over-duplication and premature centrosome separation. Cdc6 mutant proteins that showed defective ATP binding or hydrolysis did not exhibit a significant difference in suppressing centrosome over-duplication, compared to the wild type protein. In contrast to the Cdc6-mediated inhibition of PCM protein recruitment to the centrosome, the independence of Cdc6 on its ATPase activity for suppressing centrosome over-duplication, along with the difference between the Cdc6 protein regions participating in the two functions, suggested that Cdc6 controls centrosome duplication in a manner independent of its recruitment of PCM proteins to the centrosome.

Keywords: ATPase, Cdc6, cell cycle, centrosome, DNA replication

INTRODUCTION

The centrosome functions as the major microtubule-organizing center for microtubule synthesis and plays important roles in development, tumorigenesis, and signal transduction (Franker and Hoogenraad, 2013; Tanaka and Desai, 2008; Watanabe et al., 2005). Cells in the G1-phase of cell cycle possess a centrosome containing a pair of orthogonal centrioles, surrounded by pericentriolar material (PCM) (Nigg and Stearns, 2011). Centrosome duplication occurs during S and G2 phases. The duplicated centrosomes separate and migrate to opposite poles during the prophase of the mitotic phase, where they function as mitotic spindle poles for chromosome segregation. They are then segregated with the duplicated chromosomes into each daughter cell during mitosis. This centrosome duplication cycle is regulated by CDKs (cyclin-dependent kinases) such that they are coordinated with the cell cycle progression. Failure to control the centrosome duplication and function can lead to multipolar spindles, aneuploidy, asymmetric cell division, and cell polarity disruption (Vitre and Cleveland, 2012).

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Centrosome duplication is coordinated with chromosome replication during cell cycle progression by control of the common CDKs (Lacey et al., 1999; Nam and van Deursen, 2014; Tsou and Stearns, 2006; Vitre and Cleveland, 2012). Both these processes occur once during the cell cycle, and the duplicated centrosomes and chromosomes are equally segregated into daughter cells during mitosis. These similarities suggest that common factors, in addition to the CDKs, are involved in both duplications. Origin recognition complex (ORC), Cdc6, Cdt1, and MCM2-7 helicase complex proteins form and/or mediate the formation of the pre-replicative complex (pre-RC) on the replication origin during the G1 phase for the initiation of chromosomal replication (Hook et al., 2007). The pre-RC plays a critical role in the chromosome origin firing, which occurs once per cell cycle to maintain the chromosome integrity. Subunits of the ORC (Hemerly et al., 2009; Prasanth et al., 2004; Stuermer et al., 2007) and MCM2-7 helicase complex (Ferguson and Maller, 2008; Ferguson et al., 2010), Cdc6 (Lee et al., 2017) and geminin, which control Cdt1 functions (Lu et al., 2009), exist in the centrosome. Depletion of these ORC and MCM2-7 helicase subunits and geminin causes centrosome over-duplication, while over-expression of those proteins reverses the over-duplication and reduces hydroxyurea (HU)-induced centrosome over-duplication (or amplification). These results suggested that the proteins that form and control the pre-RC contribute to the link between cell cycle progression and the duplication of chromosome and centrosome.

Cdc6 is an essential component of pre-RC and functions as a licensing factor along with Cdt1 (Jiang et al., 1999; Paolinelli et al., 2009; Petersen et al., 1999). Cdc6 belongs to the AAA⁺ family of ATPases that contain the Walker A (P-loop) and Walker B motifs required for ATP binding and hydrolysis, respectively (Herbig et al., 1999; Koonin, 1993; Zwerschke et al., 1994). The ATPase activity of Cdc6 is essential for the initiation of chromosomal replication. Phosphorylation of non-chromatin bound Cdc6 by cyclin A/Cdk2 allows the translocation of Cdc6 from the nucleus to the cytoplasm during the S phase (Petersen et al., 1999). The level of Cdc6 is minimal in the G1 phase and its mRNA and protein levels begin to increase at the G1/S transition phase (Petersen et al., 2000). These results suggested the non-chromatin-related functions of Cdc6 during the S and G2 phases.

Cdc6 localizes to the centrosome during the S and G2 phases of the cell cycle (Kim et al., 2015). Recently, two reports on the functions of centrosomal Cdc6 were published. It was reported that centrosomal Cdc6 inhibits recruitment of PCM proteins such as γ -tubulin, pericentrin, CDK5RAP2, and Cep192 to the centrosome to control microtubule nucleation (Lee et al., 2017). In addition, the interaction of Cdc6 with Sas-6, which is regulated by Plk4 phosphorylation on Cdc6, suppresses the centrosome over-duplication (Xu et al., 2017). These two reports showed that depletion of Cdc6 increases the amount of PCM proteins at the centrosome (Lee et al., 2017) and promotes centrosome over-duplication (Xu et al., 2017). In this study, we aimed to analyze the link between the increase in PCM proteins and centrosome over-duplication.

MATERIALS AND METHODS

DNA, short interfering RNA (siRNA), and transfection

Open reading frames of Cdc6 derivatives were cloned into the vector pDsRed-Monomer-C1 (Clontech), unless indicated otherwise. DNA constructs were transfected into cells using Lipofectamine 2000 transfection reagent (Invitrogen). The following siRNA oligonucleotides were purchased from Samchully Pharmaceutical: control GL3 siRNA, 5'-CUUACGC UGAGUACUUCGATT-3', and Cdc6 siRNA, 5'-UAAGCCGGA UUCUGCAAGA-3' (Lee et al., 2017). These siRNA oligonucleotides were transfected into cells using Oligofectamine (Invitrogen).

Cell culture, treatment, and cell line construction

U2OS human bone osteosarcoma cells and U2OS Tet-On cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% antibiotics (penicillin/streptomycin). For centrosome over-duplication, the U2OS cells were treated with 4 mM hydroxyurea for 72 h. Cdc6 derivative-inducible U2OS Tet-On stable cell lines and their culture conditions have been previously described (Lee et al., 2017).

Immunofluorescence microscopy

Cells grown on coverslips were fixed with 4% paraformaldehyde for 15 min and then treated with cold methanol for 10 min. The cells were then permeabilized by incubation with phosphate-buffered saline (PBS) containing 0.1% Triton X-100 (PBST) for 15 min. After a 30-min incubation in a blocking solution (PBS containing 3% bovine serum albumin [BSA] and 0.1% Triton X-100), the cells were immunostained with monoclonal anti-Cdc6 (Abcam), monoclonal anti- γ -tubulin (Sigma), polyclonal anti- γ -tubulin (Sigma), monoclonal anti-cyclin E (Santa Cruz), polyclonal anti-cyclin A (Santa Cruz), or monoclonal anti-cyclin B (Santa Cruz) antibodies. The anti-CP110 antibodies have been previously described (Chang et al., 2010). The cells were washed thrice with PBST, incubated with Cy3- or FITC-conjugated anti-rabbit or anti-mouse secondary antibodies, washed thrice with PBST again, and then mounted on glass slides in a mounting medium (Biomeda Corp.), which contained 1 μ g/ml 4',6-diamidino-2-phenylindole DAPI (Vectashield). The cells were observed using an Olympus BX51 microscope.

Fluorescence-activated cell sorting (FACS) analysis

Cells were trypsinized and fixed in 70% ethanol at 4°C and then incubated in 0.05% Nonidet P-40, 10 μ g/ml RNase A, and 50 μ g/ml propidium iodide for 1 h. For FACS analysis of BrdU staining, the cells were incubated for 15 min in the presence of 10 μ M BrdU. The harvested cells were fixed in 70% ethanol at 4°C and denatured in 2 N HCl and 0.5% Triton X-100 for 1 h. The cells were then neutralized with 0.1 M sodium borate (pH 8.5) and incubated with monoclonal rat anti-BrdU antibodies (Abcam) and FITC-conjugated anti-rat secondary antibodies in PBS containing 1% BSA and 0.5% Tween-20 for 1 h. The cells were then stained with propidium iodide and analyzed using the FACS Calibur instrument (BD Biosciences).

Statistical analysis

Results from different groups were compared using the two-tailed Student's *t*-test and Prism software (GraphPad). Differences with *p*-value < 0.05 were considered statistically significant. At least three independent experiments were performed for statistical analyses.

RESULTS

Cdc6 depletion promotes centrosome over-duplication and premature centrosome separation

Depletion of Cdc6 protein for 24 h using Cdc6-specific siRNA (siCdc6) increased the recruitment of PCM proteins to the centrosome, thus increasing microtubule nucleation (Lee

et al., 2017). When the Cdc6 depletion was prolonged to 72 h in U2OS cells, we observed an increased number of interphase cells with more than two centrosomes ($\geq 3C$) or two prematurely separated centrosomes (2C-S) (Figs. 1A-1C). Centrosomes were detected by immunostaining of γ -tubulin with anti- γ -tubulin antibody (Stearns et al., 1991). The majority of control siRNA GL3-treated cells showed two adjacent γ -tubulin spots (2C-L), indicating two centrosomes were linked to each other and not yet separated (Hut et al., 2003; Loffler et al., 2013). In contrast, after Cdc6 depletion, a significant number of cells with more than two γ -tubulin spots ($\geq 3C$) were observed; these cells made up over 25% of the cells after 72 h of Cdc6-specific-siRNA treatment (Figs. 1B and 1C). In addition, although the duplicated centrosomes

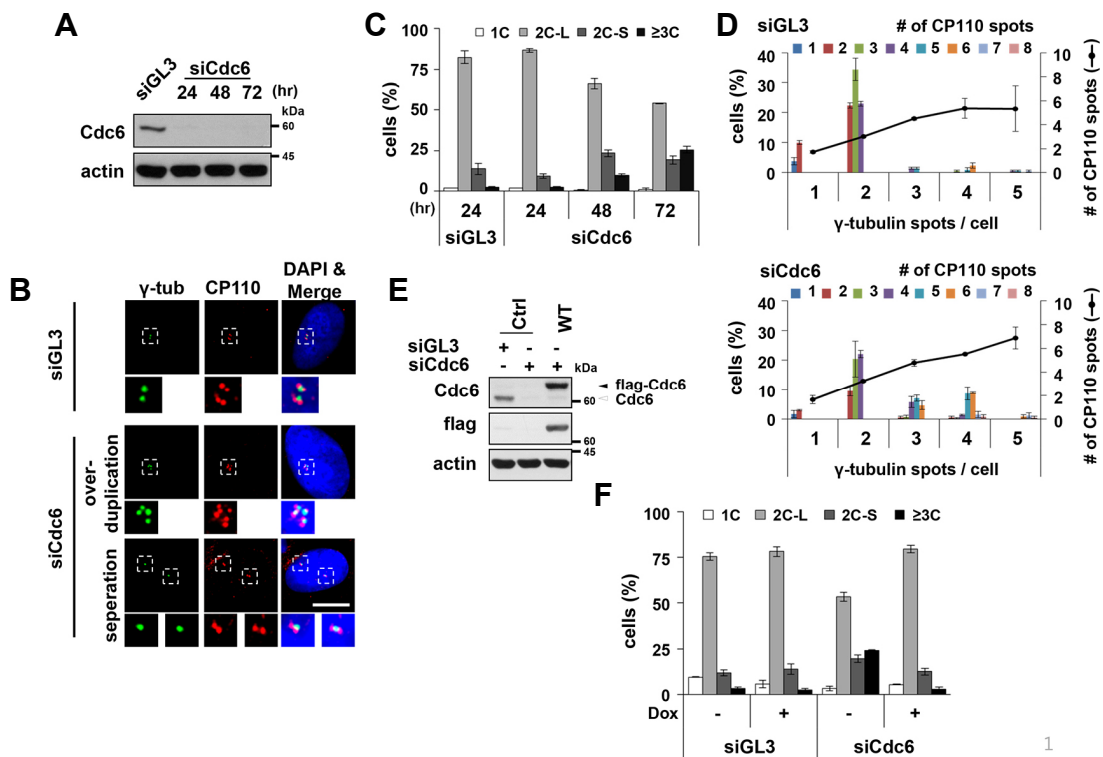


Fig. 1. Centrosome over-duplication and premature centrosome separation in Cdc6-depleted cells. (A) U2OS cells transfected with the Cdc6-specific siRNA (siCdc6) or control GL3-siRNA (siGL3) were analyzed by immunoblotting with indicated antibodies at indicated times after transfection. (B) U2OS cells transfected with siGL3 (*upper panel*) or siCdc6 (*lower panel*) for 72 h were co-immunostained with antibodies specific to γ -tubulin (green) and CP110 (red); representative images are displayed. DNA was stained with DAPI. Fields containing centrosomes are shown at higher magnification in insets. Scale bar = 10 μ m. (C) Number of γ -tubulin spots per cell was determined at the indicated time points after siRNA transfection and described in terms of the ratios over total cell number. 1C, one γ -tubulin spot; 2C-L, two linked γ -tubulin spots; 2C-S, two separated γ -tubulin spots; $\geq 3C$, more than two γ -tubulin spots. (D) After Cdc6-depletion for 72 h, the cells were co-immunostained with antibodies specific to CP110 and γ -tubulin. Centriole amplification was determined by observing CP110 spots, while centrosome number was determined by observing γ -tubulin spots. The number of γ -tubulin spots per cell is presented on the *x*-axis. The ratios of cells containing the indicated number of CP110 spots (top of the figure) and the average number of CP110 spots to the number of γ -tubulin spots are presented on the left and right of the *y*-axis, respectively. (E) Cdc6-siRNA-resistant FLAG-Cdc6-inducible U2OS Tet-On cells were transfected with the indicated siRNA for 72 h and doxycycline (2 mg/ml) was added to the culture medium for 48 h, prior to collecting the cells. The indicated proteins were detected by immunoblotting. Ctrl, U2OS Tet-On control cells; WT, FLAG-Cdc6-inducible U2OS Tet-On cells. (F) Cells from (E) were immunostained with anti- γ -tubulin antibody, and the number of γ -tubulin spots per cell was counted, as described in (C). Dox, doxycycline. Values represent mean \pm standard deviation. At least 100 cells/experimental group were analyzed in three independent experiments.

during S and G2 phases separated and migrated to opposite poles in the prophase (Nigg and Stearns, 2011), almost 25% of the Cdc6-depleted cells in the interphase exhibited premature centrosome separation; in contrast, about 10% of the control cells showed premature centrosome separation.

CP110 is known to localize at the distal end of the centriole (Chen et al., 2002); one or two CP110 spots were observed adjacent to each γ -tubulin spot of the control siRNA-treated cells (Figs. 1B and 1D). Majority of the Cdc6-depleted cells containing three centrosomes showed four to six CP110 spots, while those containing four centrosomes showed five or six CP110 spots. If centriole fragmentation caused centrosome amplification, each resultant centrosome would contain one or no CP110 spot (Chen et al., 2002; Schmidt et al., 2009). Similar to the control cells, an increase in the number of CP110 spots was observed along with an increase in the number of γ -tubulin spots in the Cdc6-depleted cells. This implied that centrosome over-duplication, rather than centriole fragmentation, was responsible for the increased number of centrosomes.

We also examined whether the expression of Cdc6 could suppress the centrosome over-duplication induced by Cdc6 depletion (Figs. 1E and 1F). The U2OS Tet-On cell line can stably induce FLAG-Cdc6 protein, which shows resistance to Cdc6-specific siRNA upon the addition of doxycycline (Lee et al., 2017). While inducing FLAG-Cdc6, the cells were transfected with the indicated siRNA. Induction of FLAG-Cdc6 in the Cdc6-depleted cells reduced the ratio of the number cells containing ≥ 3 centrosomes and showing premature

centrosome separation to that of control siRNA-treated cells. This reduction induced by Cdc6 suggested that it might be involved in the suppression of centrosome over-duplication and premature centrosome separation. This reduction of over-duplicated centrosomes by the induced Cdc6 was consistent with a previous report that the over-expression of Cdc6 suppresses centrosome over-duplication (Xu et al., 2017).

Centrosome over-duplication by Cdc6 depletion occurs in a manner independent of cell cycle arrest in a specific phase

Because Cdc6 is an essential component of the pre-RC for the initiation of chromosome replication (Fernandez-Cid et al., 2013; Randell et al., 2006), its depletion decreased DNA synthesis, as determined by BrdU incorporation followed by fluorescence-activated cell sorting (FACS) analysis, and increased the number of cells in the S and G2/M phase, caused by cell cycle arrest (Fig. 2A)(Lau et al., 2006). Prolonged cell cycle arrest in the G2 phase often induces centrosome over-duplication (Dodson et al., 2004). Arrest at the S phase induced by hydroxyurea (HU) also results in centrosome amplification in certain cell lines such as U2OS and CHO cells (Kuriyama et al., 2007; Mikule et al., 2007). Therefore, we examined whether the centrosome over-duplication induced by Cdc6 depletion was due to cell cycle arrest in the S and/or G2 phases (Figs. 2B-2D). Cyclin E-positive cells are in the G1 phase, cyclin A-positive interphase cells in the S or G2 phase, and cyclin B-positive interphase cells in the G2

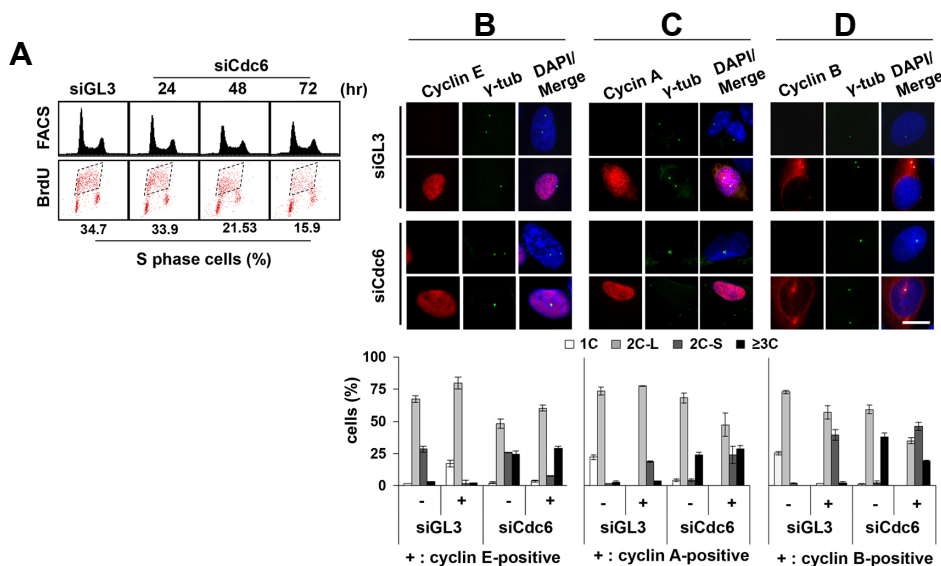


Fig. 2. Depletion of Cdc6 induces centrosome over-duplication in a manner independent of cell cycle arrest. (A) FACS analysis was performed with propidium iodide (upper panel) and BrdU (lower panel) staining of U2OS cells transfected with siRNA for the indicated time. Replicating S-phase cells are indicated in the dashed boxes. The proportion of replicating S-phase cells is shown below the FACS profiles. (B-D) Cells transfected with siRNA for 72 h were immunostained with anti-cyclin E (B), anti-cyclin A (C), or anti-cyclin B (D) and anti- γ -tubulin antibodies. The ratios of cells containing the indicated number of γ -tubulin spots (bottom panel) were determined as described in Figs. 1C and 1D. Values represent means \pm standard deviations from at least 100 cells in each of the three independent experiments. siGL3, control siRNA GL3; siCdc6, Cdc6-specific siRNA; 1C, one γ -tubulin spot; 2C-L, two linked γ -tubulin spots; 2C-S, two separated γ -tubulin spots; $\geq 3C$, more than two γ -tubulin spots.

phase (Moore, 2013). Because mitotic cells were eliminated upon scoring, the cyclin E-negative cells were in the S or G2 phase, cyclin A-negative cells in the G1 phase, and cyclin B-negative cells in the G1 or S phase. Consequently, each siRNA-treated cyclin E-positive cell exhibited centrosome statuses similar to those of the cyclin A-negative cells.

Over 25% of the Cdc6-depleted cyclin E-positive cells contained ≥ 3 centrosomes ($\geq 3C$), which was comparable to the Cdc6-depleted cyclin A-positive cells. Centrosome duplication during the S and G2 phases (Nigg and Stearns, 2011) and the absence of Cdc6 in the G1 phase centrosomes (Kim et al., 2015; Lee et al., 2017) suggested that the over-duplicated centrosomes in the G1 phase were produced in previous phases of the cell cycle and transmitted to the G1 phase. In addition, almost 25% of the Cdc6-depleted cyclin E-negative cells contained ≥ 3 centrosomes, similar to the Cdc6-depleted cyclin A-negative cells (Fig. 2B). These similarities in the number of cells showing centrosome over-duplication indicated that centrosome over-duplication occurs regardless of cell cycle arrest in a specific phase.

The number of cells with prematurely separated centrosomes (2C-S) was lesser among Cdc6-depleted cyclin E-positive or cyclin A-negative cells in the G1 phase than among Cdc6-depleted cyclin E-negative or cyclin A-positive cells in the S or G2 phase (Figs. 2B and 2C). Almost 50% of the Cdc6-depleted cyclin B-positive interphase cells in the G2 phase possessed prematurely separated centrosomes, in contrast to only 2.1% of the Cdc6-depleted cyclin B-negative cells in the G1 or S phase (Fig. 2D). This result implied that Cdc6 depletion facilitated the premature separation of duplicated centrosomes in the G2 phase.

Amino acid residues 197-214 of Cdc6 participates in the suppression of centrosome over-duplication

Treatment of certain cell lines such as U2OS and CHO with HU could induce centrosome over-duplication/amplification (Kuriyama et al., 2007; Mikule et al., 2007). Cell cycle arrest in the S phase induced by HU decouples the centrosome duplication cycle from the cell cycle, leading to centrosome over-duplication. Based on the observation of centrosome

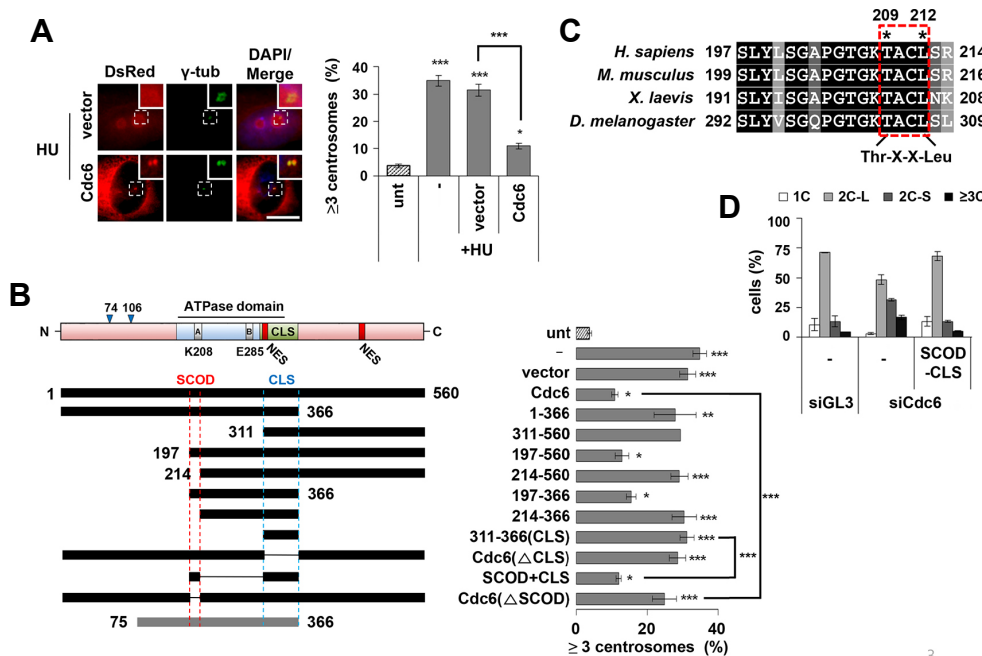


Fig. 3. Over-expression of Cdc6 suppresses HU-induced centrosome over-duplication. (A) U2OS cells transfected with empty or DsRed-Cdc6-expressing vectors were treated with HU (4 mM) for 72 h. Centrosomes were immunostained with anti- γ -tubulin (green) antibody, and DsRed-Cdc6 (red) was detected based on the fluorescence of DsRed. Representative fluorescence images (*left panel*) and the ratio of cells containing more than two γ -tubulin spots per cell (*right panel*) are displayed. Fields containing centrosomes are shown at higher magnifications in insets. Scale bar = 10 μ m. unt, HU-untreated; -, un-transfected; vector, empty vector; Cdc6, DsRed-Cdc6-expressing vector. (B) Schematic structure of Cdc6 domains and motifs, as shown in a previous publication (Lee et al., 2017), are displayed (*top*). Numbers represent the amino acid residues. Ser residues 74 and 106, phosphorylation sites by CDKs; A, Walker A motif; B, Walker B motif; CLS, centrosome localization signal; NES, nuclear export signal. Amino acid residues 75-366, which control the recruitment of PCM proteins to the centrosome, are shown at the bottom. The indicated Cdc6 fragments, fused to the C-terminus of DsRed-monomer-C1 vector, were transfected and treated with HU. The ratio of cells containing more than two γ -tubulin spots per cell was determined and is shown (*right panel*). At least 50 DsRed-positive cells transfected with each construct were scored. unt, HU-untreated; -, un-transfected; vector, empty vector; Δ , deleted; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. (C) Comparison of amino acid sequences of human and homologous SCOD. A putative Thr-X-X-Leu motif, which is bound to the FHA domain, is boxed. (D) The Cdc6-depleted U2OS cells were transfected with the Cdc6(SCOD+CLS) construct (shown in Fig. 3B) and the centrosomes were analyzed (as described in Figs. 1B and 1C).

over-duplication due to Cdc6 depletion (Fig. 1), we investigated whether the over-expression of Cdc6 suppressed the HU-induced centrosome over-duplication. Addition of HU (4 mM) into the U2OS cell culture for 72 h amplified centrosomes, and 34.8% of the treated cells showed ≥ 3 centrosomes per cell, while only 3.4% of the untreated cells did (Fig. 3A). Although transfection of the HU-treated cells with vector DNA did not significantly affect the ratio of cells with ≥ 3 centrosomes, transfection of DsRed-tagged Cdc6-expressing DNA construct decreased the percentage of these cells to 10.5%. This reduction of centrosome over-duplication by Cdc6 over-expression suggested that Cdc6 could suppress HU-induced centrosome over-duplication.

To determine the region of the Cdc6 protein involved in suppressing centrosome over-duplication, HU-treated cells were transfected with deletion constructs expressing DsRed-tagged Cdc6 fragments (Fig. 3B). The fragment containing amino acid residues 197-366 of Cdc6, namely Cdc6(197-366), was found to suppress HU-induced centrosome over-duplication. However, further deletions led to the loss of this suppressing activity. Cdc6(197-366) contained Cdc6-CLS (the centrosomal localization signal of Cdc6; amino acid residues 311-366), which is responsible for the centrosomal localization of Cdc6 (Kim et al., 2015; Lee et al., 2017). Deletion of Cdc6-CLS from the full-length Cdc6, Cdc6(Δ CLS), did not exhibit the suppressing ability. These results suggested that centrosomal localization of Cdc6 was necessary for the suppression of centrosome over-duplication.

The deletion of amino acid residues 197-214 from Cdc6 fragments 197-366, 197-560, or the full-length Cdc6 removed its ability to suppress HU-induced centrosome over-duplication (Fig. 3B). In addition, the fusion of amino acid residues 197-214 to Cdc6-CLS suppressed the centrosome over-duplication as much as the wild-type Cdc6. Therefore, we found that the amino acid residues 197-214 were responsible for the suppression of centrosome over-duplication and was named Cdc6-SCOD (the region required to suppress centrosome over-duplication). The 18 amino acid sequence of Cdc6-SCOD was highly conserved in the Cdc6 homologues (Fig. 3C). Cdc6-SCOD contains a putative fork-head-associated (FHA) domain binding consensus sequence, Thr/Tyr-x-x-Leu; the phosphorylation of this Thr/Tyr is required to bind to FHA domain-containing proteins such as Chk2, Nbs1, Mdc1, Chfr, kinesins, and transcription factors (Jungmichel and Stucki, 2010; Li et al., 2002; Mahajan et al., 2008; Stavridi et al., 2002; Westerholm-Parvinen et al., 2000; Zhao et al., 2002). The interaction of FHA-domain-containing proteins with their partners containing the Thr/Tyr-x-x-Leu motif controls the participating pathways.

Expression of Cdc6 (SCOD + CLS), in which Cdc6-SCOD was fused with Cdc6-CLS (Fig. 3B), in the Cdc6-depleted cells reduced the number of centrosomes and premature centrosome separation to the levels similar to those observed for the control siRNA-treated cells (Fig. 3D). These results supported that Cdc6-SCOD participated in the suppression of centrosome over-duplication.

ATPase activity of Cdc6 is dispensable for its ability to suppress centrosome over-duplication

The necessity of Cdc6-CLS for the suppression of HU-induced centrosome over-duplication suggested that the localization of Cdc6 at the centrosome is also necessary for the suppression of centrosome over-duplication (Fig. 3B). The over-expression of Cdc6 Δ cy mutant, which has defective centrosomal localization, fails to suppress centrosome over-duplication in Cdc6-depleted cells, but the over-expression of Cdc6 Δ cy-Plk4^{CTS}, in which Plk4^{CTS} allows the attached protein to localize to the centrosome, suppresses the centrosome over-duplication (Xu et al., 2017). We also found that Cdc6 required centrosomal localization to suppress centrosome over-duplication. The U2OS Tet-On stable cell line, which induces Cdc6 siRNA-resistant FLAG-Cdc6 wild type or FLAG-Cdc6(LI/AA) mutant protein (Lee et al., 2017) upon addition of doxycycline, was treated with HU or Cdc6-specific siRNA after inducing the corresponding protein (Figs. 4A-4C). Cdc6(LI/AA), in which Leu-313 and Ile-316 in the Cdc6-CLS region are substituted with Ala, cannot localize to the centrosome (Kim et al., 2015; Lee et al., 2017). The induced wild-type protein suppressed both Cdc6 depletion-induced (Fig. 4B) and HU-induced centrosome over-duplication (Fig. 4C). In contrast, Cdc6(LI/AA) failed to significantly suppress either HU-induced or Cdc6 depletion-induced centrosome over-duplication. These inability of centrosome localization-defective Cdc6(LI/AA) indicated that the suppression of centrosome over-duplication was dependent on the localization of Cdc6 to the centrosome.

Cdc6-SCOD (amino acid residues 197-214), which suppressed the HU-induced centrosome over-duplication, consisted of 18 amino acid residues of the ATPase domain (amino acid residues 180-330) that does not display the ATPase activity (Fig. 3B)(Herbig et al., 1999). To verify the involvement of Cdc6 ATPase activity in the suppression of centrosome over-duplication, we used the Cdc6(75-366) fragment, which contained the intact ATPase domain (including the Walker A and B motifs) (Fig. 3B)(Lee et al., 2017) and was defective in DNA replication (Perkins and Diffley, 1998; Weinreich et al., 1999), to reduce or avoid the possibility of wild type and mutant Cdc6 proteins acting differently on chromosome replication followed by alternating cell cycle progression, thus affecting centrosome over-duplication. The U2OS Tet-On stable cell line induces Cdc6 siRNA-resistant FLAG-Cdc6(75-366) wild type (WT), Walker A (K208A) containing a substitution of Lys-208 with Ala, or Walker B (E285G) containing a substitution of Glu-285 with Gly. The Cdc6(75-366) wild-type protein inhibits the recruitment of PCM proteins to the centrosome, while Walker A and B mutant proteins do not (Lee et al., 2017). Both these mutant proteins possess negligible *in vitro* ATPase activity. The FLAG-Cdc6(75-366) proteins were induced, whereas the endogenous Cdc6 protein was depleted (Figs. 4D-4F). The induced wild type and Walker A (K208A) and B (E285G) mutant proteins suppressed the increase in the ratio of cells containing Cdc6 depletion-induced centrosome over-duplication, although the difference was not statistically significant (Fig. 4E). In addition, these three proteins also suppressed the increase in the ratio of cells with HU-induced centrosome over-duplication (Fig. 4F). These similarities between the wild type and Walker A and B mutant proteins,

together with the result that Cdc6-SCOD comprised 18 amino acid residues of ATPase domain (Fig. 3B), suggested that Cdc6-dependent suppression of centrosome over-duplication does not require ATPase activity, ATP binding and hydrolysis, of the Cdc6 protein.

DISCUSSION

The amino acid residues 75-366 of Cdc6 contain the ATPase domain (Fig. 3B). Introduction of mutations in the Walker A (K208A) or Walker B (E286G) motif of Cdc6(75-366) abolishes the *in vitro* ATPase activity as well as the inhibitory activity for recruitment of PCM proteins to the centrosome, indicating that the inhibitory activity requires the ATP binding and hydrolysis functions of Cdc6 ATPase (Lee et al., 2017). In contrast, Cdc6(75-366) wild type, and the Walker A (K208A) and Walker B (E286G) mutant proteins exhibited no significant differences in the suppression of centrosome over-duplication induced by Cdc6 depletion or HU treatment (Fig. 4). This similarity between the wild type and mutant proteins implied that the suppression of centrosome over-duplication does not require the ATP binding or ATP hydrolysis activity of Cdc6. This dispensability of ATP binding and hydrolysis activi-

ty for the suppression of centrosome over-duplication was supported by the observation that Cdc6-SCOD, which suppressed HU-derived centrosome over-duplication, contained the 18 amino acid residues (residues 197-214; Fig. 3B).

During Cdc6 depletion, the increased amount of PCM proteins at the centrosome was observed at 24 h after the depletion (Lee et al., 2017); the accumulation of over-duplicated centrosomes was first observed at 48 h and was significant at 72 h after the depletion (Fig. 1C). This increase in the number of centrosomes might have been triggered by the increase in PCM proteins at the centrosomes. However, certain protein regions (amino acid residues 75-196 and 215-310) and ATPase activity of Cdc6, which were necessary for the inhibition of the recruitment of PCM proteins, were not required for the suppression of centrosome over-duplication (Figs. 3 and 4). These results suggested that Cdc6 suppressed centrosome over-duplication in a manner independent of its function in inhibiting the recruitment of PCM proteins. Thus, we proposed that Cdc6 has two independent functions in the centrosomes at the S and G2 phases of the cell cycle: inhibiting the recruitment of PCM proteins to the centrosome and suppressing centrosome over-duplication (Fig. 5)(Lee et al., 2017).

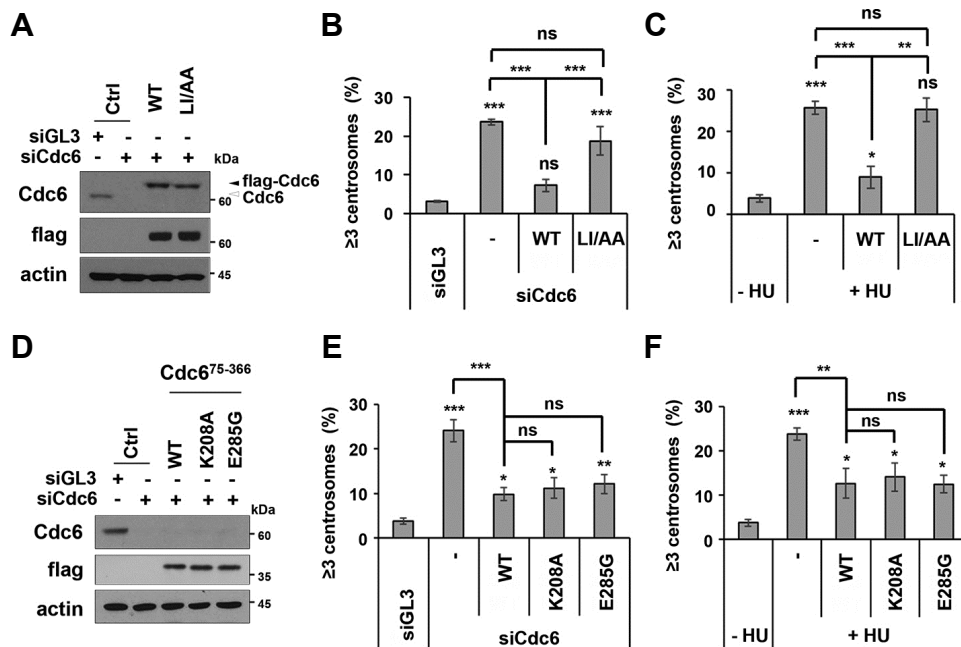


Fig. 4. ATPase activity of Cdc6 is not essential for the suppression of centrosome over-duplication. (A-C) U2OS Tet-On cells expressing Cdc6-siRNA resistant FLAG-Cdc6 wild type or FLAG-Cdc6 (LI/AA) were induced by adding 2 μ g/ml of doxycycline. (A) The cells were treated with the indicated siRNA, and the depletion and induction of the indicated proteins were detected by immunoblotting. Seventy-two hours after Cdc6 depletion (B) or HU-treatment (C), centrosomes were immunostained with the anti- γ -tubulin antibody. The percentage of cells with more than two γ -tubulin spots (≥ 3 C) per cell is shown in bar graphs. (D-F) Experiments on U2OS Tet-On cells expressing Cdc6-siRNA resistant FLAG-Cdc6(75-366) derivatives were performed as described in (A-C). Depletion or induction of Cdc6 was analyzed by immunoblotting in (D). Seventy-two hours after Cdc6 depletion (E) or HU-treatment (F), over-duplicated centrosomes were quantified. Tet-Ctrl, control U2OS Tet-On cells; WT, FLAG-Cdc6 full-length wild-type; LI/AA, FLAG-Cdc6(LI/AA); Ctrl, U2OS Tet-On control cells; WT, FLAG-Cdc6(75-366) wild-type; K208A, FLAG-Cdc6(75-366)(K208A); E285G, FLAG-Cdc6(75-366)(E285G). Values represent means \pm SDs of at least 50 cells from three independent experiments. One-way ANOVA and Tukey's post-test were used for statistical analyses. ns, not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

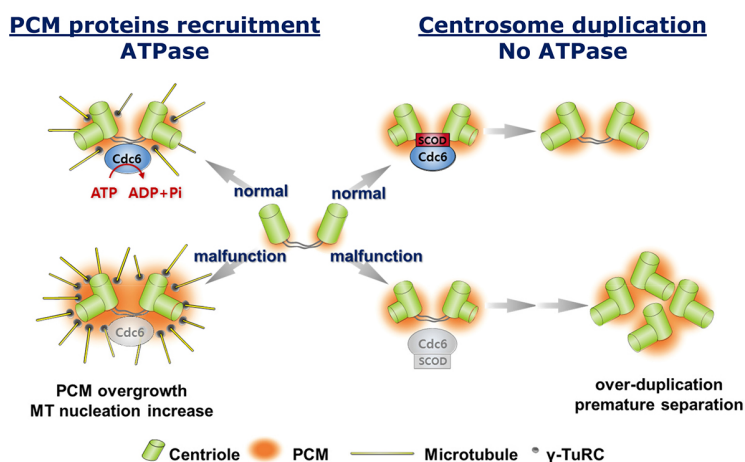


Fig. 5. Cdc6 controls centrosome duplication, independent of the recruitment of PCM proteins in the centrosome.

Both ATP binding and hydrolysis activities, which require the Walker A and Walker B motifs, respectively, of Cdc6 have been demonstrated to be essential for diverse cellular functions of Cdc6, including the formation of pre-RC (pre-replicative complex) for the initiation of chromosomal DNA replication (Donovan et al., 1997; Herbig et al., 1999; Randell et al., 2006; Weinreich et al., 1999), the removal of p21 and p27 from Cdk2 for the activation of Cdk2 (Kan et al., 2008; Uranbileg et al., 2012), and the inhibition of the apoptosome assembly necessary for cell death (Niimi et al., 2012). The ATPase activity of Cdc6 in the protein complexes is known to modulate the functions of complex-forming proteins (Donovan et al., 1997; Herbig et al., 1999; Kan et al., 2008; Niimi et al., 2012; Randell et al., 2006; Uranbileg et al., 2012; Weinreich et al., 1999). Furthermore, the ATP binding and hydrolysis activities of Cdc6 are required for the regulation of the recruitment of PCM proteins to the centrosome, which controls microtubule nucleation (Lee et al., 2017). However, the ATPase activity of Cdc6 was not essential for suppressing the centrosome over-duplication (Fig. 4); Cdc6-SCOD (amino acid residues 197-214), which suppressed centrosome over-duplication, possessed a small portion of the ATPase domain (amino acid residues 180-330) of Cdc6 (Fig. 3B). Thus, Cdc6-SCOD, which comprises 18 amino acid residues that are highly conserved in eukaryotes, may interact with a protein involved in centrosome duplication to regulate it.

Recently, it was reported that the phosphorylation of Cdc6 on Ser-30 and Thr-527 by Plk4 could regulate the interaction of Cdc6 with Sas-6 to control centrosome duplication (Xu et al., 2017). This paper demonstrated that Cdc6 depletion promoted centrosome over-duplication, and Cdc6 over-expression suppressed Cdc6 depletion-induced and HU-induced centrosome over-duplication. Furthermore, the Cdc6 Walker B (E286G) mutant containing a substitution of Glu-286 with Gly localized to the centrosome, while the Cdc6 Walker A (K208E) mutant containing a substitution of Lys-208 with Glu did not; this suggested that ATP binding to Cdc6 was necessary for its centrosomal localization. The over-expression of the Walker A or B mutant proteins did not suppress HU-induced centrosome over-duplication, sug-

gesting that the suppression of centrosome over-duplication by Cdc6 is dependent on its ATPase activity. In contrast to these results, when the Cdc6 Walker A (K208A) mutant was over-expressed or induced in the U2OS Tet-On cell line, it localized to the centrosome as efficiently as the Cdc6 Walker B (E285G) mutant and wild type proteins (Lee et al., 2017). Both mutant proteins were defective in *in vitro* ATPase activity. The induced Walker A (K208A) and Walker B (E285G) mutant proteins suppressed Cdc6 depletion-induced or HU-induced centrosome over-duplication in a manner similar to the wild type protein (Figs. 4E and 4F). The Cdc6(75-366) protein, which does not contain the Plk4 phosphorylation sites Ser-30 and Thr-527, induced from Cdc6-depleted U2OS Tet-On cell-line showed similar number of centrosomes as shown by the full-length protein (Figs. 4B and 4E). In addition, the Cdc6 fragments that did not contain Ser-30 and Thr-527, including the Cdc6 fragment 197-214 (Cdc6-SCOD) attached to Cdc6-CLS, efficiently suppressed centrosome over-duplication (Figs. 3B and 3D). It remains to be elucidated whether the difference between the use of full-length Cdc6 protein (Xu et al., 2017) and the fragment 75-366 (Figs. 3 and 4) (Lee et al., 2017) was responsible for such discrepancies.

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