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Cdc6 localizes to S- and G2-phase centrosomes in a cell cycle-dependent manner

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ABSTRACT

The Cdc6 protein has been primarily investigated as a component of the pre-replicative complex for the initiation of chromosome replication, which contributes to maintenance of chromosomal integrity. Here, we show that Cdc6 localized to the centrosomes during S and G2 phases of the cell cycle. The centrosomal localization was mediated by Cdc6 amino acid residues 311–366, which are conserved within other Cdc6 homologues and contains a putative nuclear export signal. Deletions or substitutions of the amino acid residues did not allow the proteins to localize to centrosomes. In contrast, DsRed tag fused to the amino acid residues localized to centrosomes. These results indicated that a centrosome localization signal is contained within amino acid residues 311–366. The cell cycle-dependent centrosomal localization of Cdc6 in S and G2 phases suggest a novel function of Cdc6 in centrosomes.
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1. Introduction

For the initiation of chromosome replication in eukaryotes, binding of the origin recognition complex (ORC) to the replication origins is followed by association of Cdc6 and Cdt1, recruiting the helicase MCM2-7 complex to the origins, which results in the formation of the pre-replicative complex (pre-RC), in the G1 phase of the cell cycle [1]. Formation of the pre-RC in G1 is critical to ensure that chromosomal replication occurs only once in each cell cycle [2]. Cdc6, which is highly conserved within metazoans, interacts with DNA through the winged helix domain in its C-terminal region. Also, Cdc6 contains ATP-binding and hydrolytic activities, which are required for formation of the pre-RC [3,4]. After Cdc6 participates in pre-RC formation in the nucleus during G1 phase, non-chromatin-bound Cdc6 translocates to the cytoplasm at the G1/S-phase transition [5]. These subcellular localizations through cell cycle progression are controlled by nuclear localization sequences (NLSs), nuclear export signals (NESs), and post-translational modifications, such as acetylation and phosphorylation [6–8].

The centrosome functions as microtubule-organizing center (MTOC) for microtubule formation [9]. Centrosomes consist of a pair of centrioles surrounded by pericentriolar material (PCM), which is composed of a meshwork of proteins. The two centrioles are distinguished as mother and daughter centrioles. The mother centriole possesses appendages at its distal end, where γ-TuRCs are anchored for microtubule formation. Microtubules are also nucleated at the γ-TuRC in the PCM. During interphase the centrosome is closely associated with the nucleus and is duplicated; the two centrosomes then separate and migrate to the poles to function as spindle poles for chromosome segregation during mitosis.

Centrosome duplication and chromosomal replication during the cell cycle share similarities in the following aspects. Both duplications take place in interphase in a cell cycle-dependent manner; these cell cycle-dependent duplication processes are commonly regulated by cyclins and cyclin-dependent kinases; and the duplicated centrosomes and chromosomes are equally segregated into daughter cells [10–13]. Furthermore, the pre-RC forming and controlling proteins such as ORC subunits [14–16], MCM2-7 subunits [17,18], and geminin [19] also exist in centrosomes to maintain centrosome integrity.

Although Cdc6 functions in pre-RC formation in G1 phase, anaphase-promoting complex (APC)-CDH1 degrades Cdc6 in early G1, and Cdc6 mRNA and protein levels begin to increase in S phase [20]. Also, non-chromatin-bound Cdc6 is exported to the cytoplasm in S phase [4,21]. This increase in expression level and export of Cdc6 to the cytoplasm in S and G2 phases suggests that Cdc6 may have another function in addition to its role as a component of the pre-RC. In this report, we demonstrate that Cdc6 localized to the centrosome in S and G2 phases in a cell cycle-dependent manner.

Abbreviations: CLS, centrosome localization signal; LI/AA, L313A/I316A; MTOC, microtubule-organizing center; MAP, microtubule-associated protein; NES, nuclear export signal; PCM, pericentriolar material; pre-RC, pre-replicative complex.

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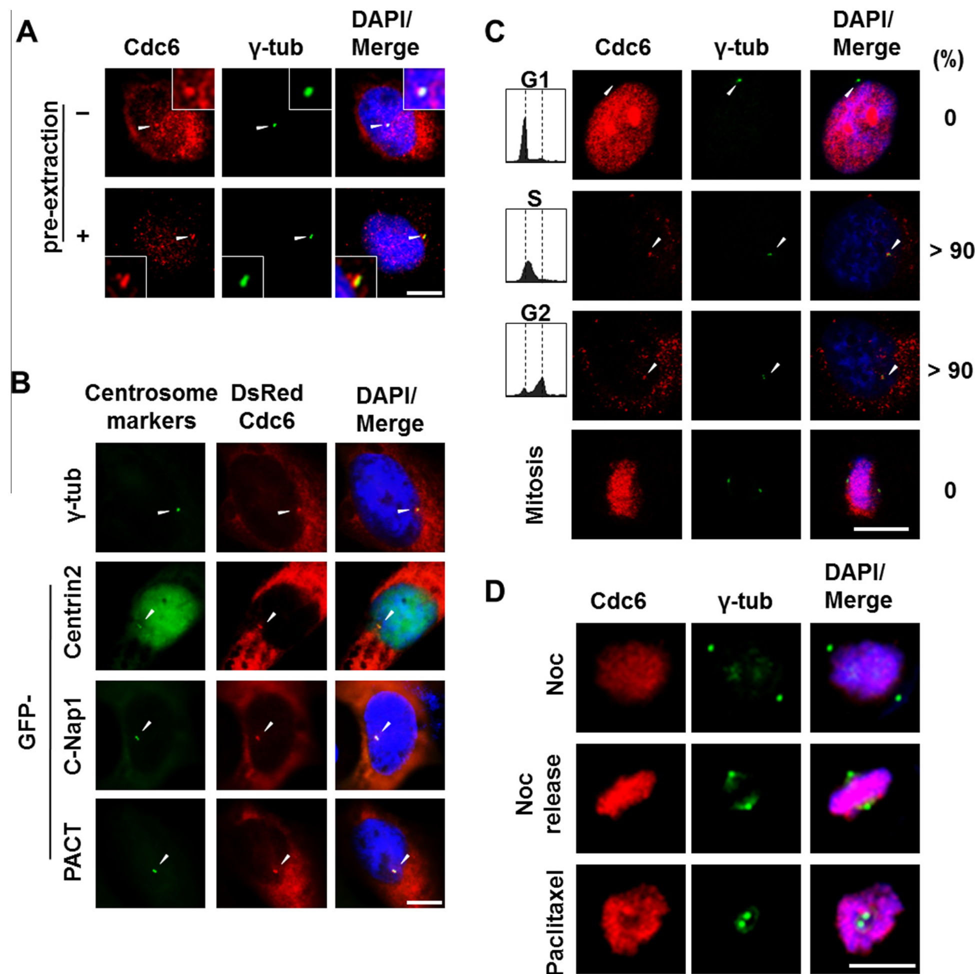

Fig. 1. Cdc6 localizes to S- and G2-phase centrosomes. (A) Immunostaining of U2OS cells for Cdc6 and γ-tubulin (γ-tub) with or without permeabilization in PBST (pre-extraction). Nuclei were counterstained with DAPI. Arrowheads indicate centrosomes. Fields containing centrosomes are shown at higher magnification in insets. (B) U2OS cells were cotransfected with DNA constructs encoding DsRed-Cdc6 and the indicated GFP-tagged centrosomal markers. Arrowheads indicate centrosomes. Nuclei were counterstained with DAPI. (C) HeLa cells were synchronized by double-thymidine block and release. Cell cycle progression was analyzed by FACS analysis. (%) described percentage of cells exhibiting co-localization of Cdc6 with γ-tubulin. (D) Localization of Cdc6 and γ-tubulin in U2OS arrested with paclitaxel or nocodazole (Noc) and released from nocodazole arrest. Scale bar: 10 μm.

2. Materials and methods

2.1. Cell culture

U2OS human bone osteosarcoma cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics (penicillin/streptomycin).

2.2. Immunofluorescence microscopy

Cells grown on coverslips were fixed with 4% paraformaldehyde for 15 min, followed by treatment with cold methanol for 10 min. Cells were permeabilized by incubation with phosphate-buffered saline (PBS) containing 0.1% Triton X-100 (PBST) for 15 min. After a 30-min incubation in blocking solution (PBS containing 3% bovine serum albumin [BSA] and 0.1% Triton X-100), cells were immunostained with monoclonal anti-Cdc6 antibody (Abcam), anti-pericentrin [22], anti-cyclin E (Santa Cruz), anti-cyclin A (Santa Cruz), anti-cyclin B (Santa Cruz), and anti-γ-tubulin (Sigma). Anti-C-Nap1 antibodies [23] were previously described. Cells were washed three times with PBST, incubated with Cy3- or FITC-conjugated anti-rabbit or anti-mouse secondary antibody, washed three times with PBST, and then mounted on glass slides with mounting media (Biomeda Corp.) containing 1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI, Vectashield). Cells were viewed using an Olympus BX51 microscope.

3. Results and discussion

3.1. Cdc6 localizes to the centrosomes of S- and G2-phase cells

A subpopulation of Cdc6 has been reported to exist at the centrosomes and spindle poles of mitotic cells [24,25]. In contrast, Cdc6 was also observed at the centrosomes of interphase cells [24]. To clarify the difference in the centrosomal localization of Cdc6 during cell cycle progression, the centrosomal localization of Cdc6 was assessed by immunofluorescence analysis of U2OS cells using anti-Cdc6 monoclonal antibody (Fig. 1A). The morphology of the DAPI-stained nuclei indicated that the cells were in interphase. Colocalization of Cdc6 and γ-tubulin, which is a centrosomal protein [26], implied that Cdc6 localized to interphase centrosomes. Even after extraction of cells with 0.1% Triton X-100 prior to immunostaining, Cdc6 was detected in the centrosomes. Transiently expressed DsRed-tagged Cdc6 (DsRed-Cdc6) also colocalized with γ-tubulin, as well as with the GFP-tagged centrosomal proteins centrin2, C-Nap1, and the PACT domain of AKAP450 [27]

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