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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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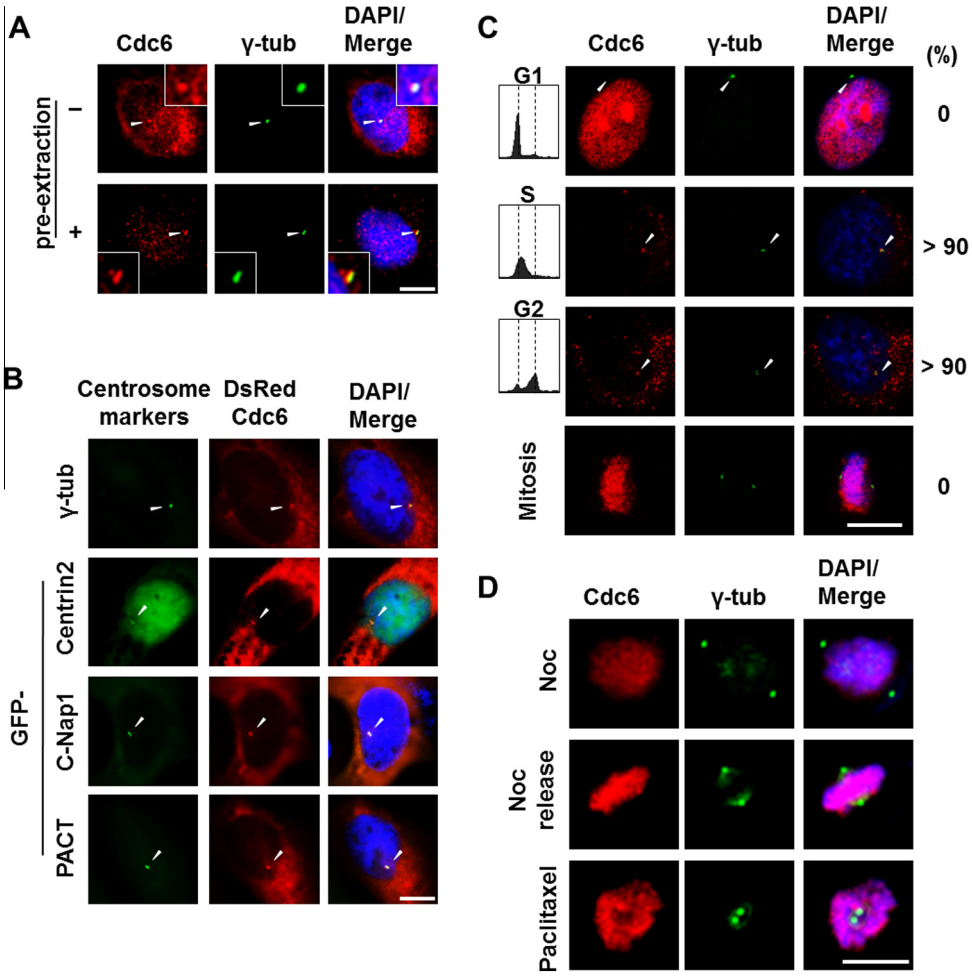

Fig. 1. Cdc6 localizes to S- and G2-phase centrosomes. (A) Immunostaining of U2OS cells for Cdc6 and γ-tubulin (γ-tub) with or without permeabilization in PBST (pre-extraction). Nuclei were counterstained with DAPI. Arrowheads indicate centrosomes. Fields containing centrosomes are shown at higher magnification 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]

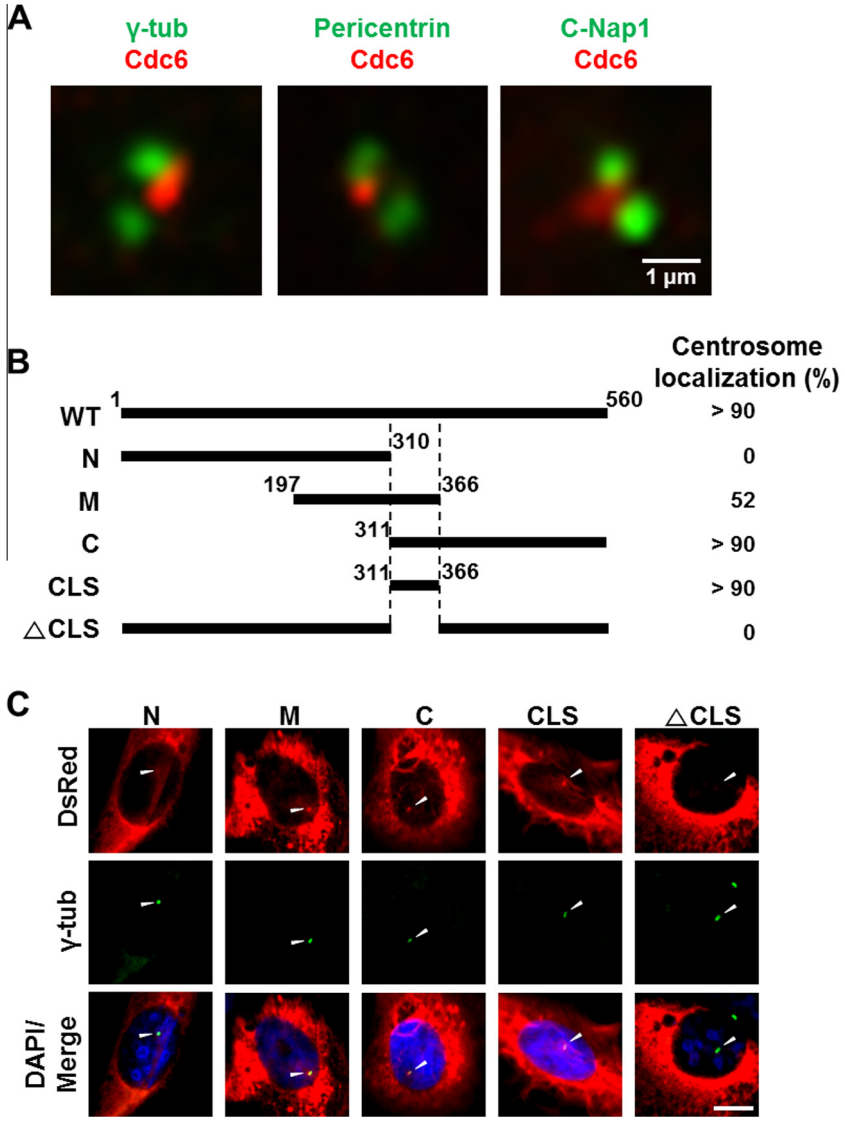

Fig. 2. Identification of a centrosomal localization signal of Cdc6. (A) UsOS cells were immunostained with the indicated antibodies. Scale bar: 1 μm. (B) Schematic representation of deletion mutant constructs used to identify CLS. Numbers represent positions of amino acid residues. The indicated Cdc6 fragments were fused to the C-terminus of vector DsRed-monomer-C1. Centrosomal localization of each fragment was quantified with at least 50 DsRed-positive cells. WT, wild type; N, N-terminal; M, middle; C, C-terminal; ΔCLS, CLS-excised (C) representative fluorescence micrographs of U2OS cells expressing the indicated deletion mutants. Arrowheads indicate centrosomes.

(Fig. 1B). Non-chromatin-bound Cdc6 is exported from the nucleus to the cytoplasm at entry into S phase [7]. The presence of Cdc6 in the cytoplasm and colocalization with other centrosomal proteins suggested that Cdc6 exists in the centrosomes during S and G2 phases of the cell cycle.

The localization of Cdc6 to centrosomes was determined during cell cycle progression in HeLa cells (Fig. 1C). In G1-phase cells, Cdc6 was present in the nucleus, but was not detected in centrosomes. When Cdc6 was present in the cytoplasm, FACS analysis indicated that the cells were in S or G2 phase, as previously reported [7,28,29]. In these cells, Cdc6 was detected in centrosomes. However, Cdc6 was not detected at the spindle poles of mitotic cells, but was associated with chromosomes, as shown previously [30]. Nocodazole or paclitaxel treatment arrests cells at prometaphase [31]. In nocodazole- and paclitaxel-treated cells, Cdc6 was not detected at the spindle pole (Fig. 1D), and metaphase cells, which were released from nocodazole arrest, did not contain Cdc6 at the spindle poles. These results support the idea that Cdc6 localizes to centrosomes during S and G2 phase in a cell cycle-dependent manner.

3.2. Cdc6 centrosome localization signal

Cdc6 localizes to centrosomes of S and G2 phase cells. In S and G2 phase, centrioles are duplicated and maturated [10]. We determined where Cdc6 locates at centrosomes (Fig. 2A). γ-Tubulin and pericentrin are PCM proteins [32]. C-Nap1 is a component of mother centriole and locates at the proximal ends of mother and daughter centrioles [33]. In the enlarged images of centrosomes, Cdc6 (in red) was detected between those proteins, suggesting that Cdc6 locates between the duplicated, pairs of centrioles (Fig. 2A).

To identify the region responsible for localization of Cdc6 to the centrosome, we generated deletion mutants fused to the C-terminus of the DsRed-monomer-C1 expression vector and expressed the proteins in U2OS cells (Fig. 2B and C). Whereas the N-terminal fragment containing amino acid residues 1–310, did not localize to the centrosomes, both the C-terminal fragment, 311–560, and the fragment containing amino acid residues 197–366 did. Fragment 311–366, which overlapped both fragments 311–560 and 197–366, also localized to the centrosomes. Deletion of amino residues 311–366 abolished Cdc6 localization to

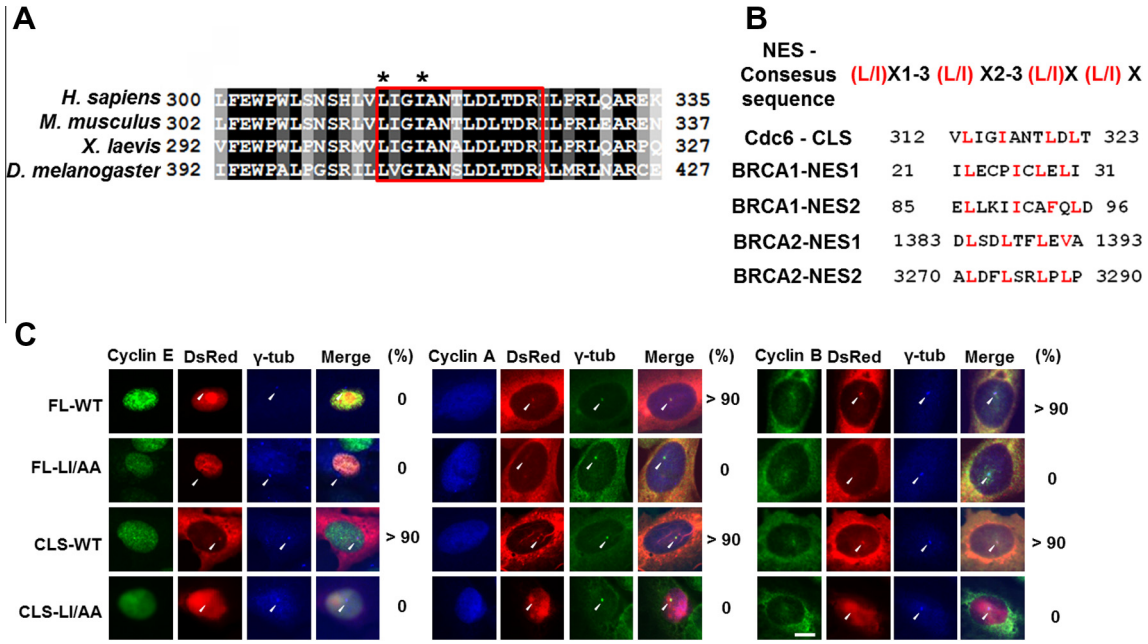

Fig. 3. Cdc6 centrosomal localization signal sequence. (A) Amino acid sequences of human and homologous CLSs. Asterisks indicate amino acids substituted to inactivate CLS. Nuclear export signal (NES) consensus sequences are boxed in red. (B) Comparison of amino acid sequences of Cdc6 CLS and NESs of BRCA1 and BRCA2 [34]. X indicates any amino acid. (C) Asynchronously grown U2OS cells were immunostained with anti-γ-tubulin and anti-cyclin E (monoclonal, mouse), anti-cyclin A (rabbit), or anti-cyclin B (monoclonal, mouse). Representative images were shown of cells expressing full-length (FL) Cdc6 and the CLS fragment. LI/AA indicates mutant carrying substitutions L313A and I316A in the CLS. (%) described percentage of cells containing the indicated protein at centrosomes.

centrosomes. The upstream region of amino acid residue 311 of fragment 197–366 somehow reduced centrosomal localization to 52% relative to the C-terminal or 311–366 fragment with >90%. The ability to localize the DsRed-tagged Cdc6 fragment to centrosomes indicated that a centrosome localization signal (CLS) is contained within amino acid residues 311–366.

The Cdc6 CLS region was conserved in the Cdc6 homologues of other eukaryotes and contained a leucine-rich domain (Fig. 3A). The CLS region contains a putative nuclear export signal (NES), $\Phi X_{1-3}\Phi X_{2-3}\Phi X\Phi X$ (Fig. 3B); Φ indicates a large hydrophobic residue, such as Leu, Ile, Val, Met, and X indicates any amino acid [34]. This leucine-rich domain is found in other proteins, including BRCA1 and BRCA2, that localize to the centrosome (Fig. 3B). NES1 of BRCA1 was shown to participate in centrosomal localization of BRCA1 [35,36], and NES1 and NES2 of BRCA2 are involved in the centrosomal localization of BRCA2 [34].

Cdc6 localized to S and G2 phase centrosomes (Fig. 1). Consistently, DsRed-Cdc6 localized to the centrosomes of cyclin A- and cyclin B-positive cells with >90% frequencies (Fig. 3C). Cyclin A is a S and G2 phase cyclin and cyclin B is a G2 phase cyclin [37]. However, DsRed-CLS was found in the centrosomes of cyclin E-positive cells, implying that the cells are in G1 phase, in addition to cyclin A- and cyclin B-positive cells. No significant signal of DsRed-CLS was detected at mitotic centrosomes. These results suggested that CLS of Cdc6 possesses centrosome localization signal that operates during interphase.

Leu313 and Ile316 of DsRed-Cdc6 were substituted with Ala (L313A/I316A, LI/AA) in DsRed-tagged Cdc6 and Cdc6-CLS fragment. Like full-length wild-type Cdc6, full-length mutant Cdc6 (Cdc6-LI/AA) was present in the cytoplasm, but did not localize to centrosomes (Fig. 3C). In contrast, the mutant CLS fragment (CLS-LI/AA) did not localize to either the cytoplasm or centrosome, but remained in the nucleus. The NESs in other regions of Cdc6 were shown to be responsible for export of Cdc6 to the cytoplasm [8]. Therefore, the Cdc6-ΔCLS and Cdc6-LI/AA mutant proteins, bearing absent and mutated CLS regions, respectively, did localize to the cytoplasm (Figs. 2B and 3C). These results suggest that the Cdc6 CLS functions in centrosomal localization of Cdc6 and not in nuclear export of the full-length protein.

Although Cdc6 participates in pre-RC formation in the nucleus during G1 phase, Cdc6 mRNA and protein levels increase in S phase, with a limited amount of Cdc6 present in G1 phase [20]. Also, non–chromatin-bound Cdc6 is exported from the nucleus in G1/S transition phase. Centrosomal localization of Cdc6, along with the increased protein level and export from the nucleus of Cdc6, in S and G2 phases suggests that Cdc6 plays important functions in the centrosomes.

Cdc6 possesses a CLS sequence that is responsible for centrosomal localization of Cdc6 (Figs. 2 and 3). The Cdc6 CLS contains a leucine-rich domain, which is also preserved in NESs. The Cdc6 CLS fragment was present in both the cytoplasm and centrosomes. On the other hand, mutation of the leucine-rich domain of the Cdc6 CLS fragment resulted in defects in cytoplasmic as well as centrosomal localization, suggesting that this domain functions as a NES as well as a CLS. Cdc6 contains several putative NES sequences, which are responsible for transport of Cdc6 to the cytoplasm from the nucleus [8], and other NESs, distinct from the NES sequence in Cdc6-CLS, have been shown to contribute to the cytoplasmic localization of Cdc6. Therefore, mutation of the leucine-rich sequence of Cdc6-CLS of Cdc6 full-length protein did not affect the localization of the mutant Cdc6 to the cytoplasm; however, the mutant protein did not localize to the centrosome. These results suggest that the centrosomal localization of Cdc6 is carried out specifically by the Cdc6-CLS.

Acknowledgments

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