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RESEARCH ARTICLE

DNA replication initiator Cdc6 also regulates ribosomal DNA transcription initiation

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ABSTRACT

RNA-polymerase-I-dependent ribosomal DNA (rDNA) transcription is fundamental to rRNA processing, ribosome assembly and protein synthesis. However, how this process is initiated during the cell cycle is not fully understood. By performing a proteomic analysis of transcription factors that bind RNA polymerase I during rDNA transcription initiation, we identified that the DNA replication initiator Cdc6 interacts with RNA polymerase I and its co-factors, and promotes rDNA transcription in G1 phase in an ATPase-activity-dependent manner. We further showed that Cdc6 is targeted to the nucleolus during late mitosis and G1 phase in a manner that is dependent on B23 (also known as nucleophosmin, NPM1), and preferentially binds to the rDNA promoter through its ATP-binding domain. Overexpression of Cdc6 increases rDNA transcription, whereas knockdown of Cdc6 results in a decreased association of both RNA polymerase I and the RNA polymerase I transcription factor RRN3 with rDNA, and a reduction of rDNA transcription. Furthermore, depletion of Cdc6 impairs the interaction between RRN3 and RNA polymerase I. Taken together, our data demonstrate that Cdc6 also serves as a regulator of rDNA transcription initiation, and indicate a mechanism by which initiation of rDNA transcription and DNA replication can be coordinated in cells.

KEY WORDS: Cdc6, RNA polymerase I, RRN3, Cell cycle, Nucleophosmin, rDNA transcription

INTRODUCTION

RNA polymerase I (Pol I)-dependent ribosomal DNA (rDNA) transcription, which is fundamental to ribosome biogenesis (Russell and Zomerdijk, 2005), begins with the recruitment of Pol I into a pre-initiation complex (PIC) by at least three Pol I transcription factors, including the upstream binding factor (UBF, also know as UBTF), the promoter-selectivity factor complex (SL1) and RRN3 at the rRNA gene promoter (Bell et al., 1988; Comai et al., 1992, 1994; Zomerdijk et al., 1994). UBF binds to an upstream control element and the core promoter at the rRNA gene loci. It then induces a loop structure that brings the upstream control element and the core promoter of the rRNA gene into close proximity and facilitates the recruitment of SL1 and Pol I (Bazett-Jones et al., 1994; Bell and Dutta, 2002; Jantzen et al., 1992). SL1 is a protein complex that contains the TATA-binding protein (TBP) and Pol-I-specific TBP-associated factors (Comai et al., 1992, 1994; Gorski et al., 2007; Heix et al., 1997; Zomerdijk et al., 1994). SL1 therefore recognizes the Pol-I-specific promoter and confers the selectivity of the PIC for rDNA transcription. For the assembly of PIC, SL1 recruits Pol I to the promoter through interaction with RRN3. RRN3 interacts with and converts Pol I into a transcriptionally active form that can initiate rDNA transcription (Miller et al., 2001; Yuan et al., 2002).

rDNA transcription is regulated by the cell cycle in mammals; rDNA transcription is shut down in mitosis, gradually recovers in G1 phase, and peaks in S and G2 phases. The known mechanisms of mitotic silencing, and G1 phase reactivation of rDNA transcription, are controlled by protein phosphorylation of SL1 and UBF by Cdk1 (Heix et al., 1998; Voit and Grummt, 2001; Voit et al., 1999). RNA Pol I, as well as UBF, is thought to remain associated with nucleolus organizer regions (NORs) throughout mitosis (Gilbert et al., 1995; Scheer and Rose, 1984; Weisenberger and Scheer, 1995); however, a study using quantitative 4D microscopy and live-cell imaging is in opposition to this prevailing model, and found that multiple RNA polymerase I subunits transiently leave the NORs during metaphase, whereas UBF remains associated with NORs throughout mitosis (Leung et al., 2004). However, how rDNA transcription is initiated after the mitotic inhibition is still unknown.

Cdc6 has been extensively studied for its crucial role in the initiation of DNA replication. DNA replication begins with the sequential recruitment of the pre-replication complex components to DNA replication origins (Bell and Dutta, 2002; Donovan et al., 1997; Liang and Stillman, 1997; Yan et al., 1998). Cdc6 functions in this complex to recruit the DNA helicase [comprising six Mcm subunits (Mcm2–Mcm7, hereafter Mcm2-7)] to the replication origins, which are initially recognized by the origin recognition complex (ORC) proteins (Donovan et al., 1997; Evrin et al., 2009; Mendez and Stillman, 2000; Saha et al., 1998). Cdc6 is a member of the ubiquitous ATPase associated with various cellular activities (AAA^+ ATPase) family, in which both the Walker A and B motifs constitute a purine nucleoside triphosphate active-site important for its enzymatic function. The Walker A motif binds the triphosphate moiety of the ATP molecule, whereas the Walker B motif coordinates with Mg^{2+} to hydrolyze ATP. In chromatin fractionation experiments, a mutant of Cdc6 Walker A motif abrogates its chromatin binding, whereas a Walker B motif mutant deficient in ATP hydrolysis fails to recruit DNA helicase Mcm2-7 to the chromatin (Cook et al., 2002; Frolova et al., 2002; Herbig et al., 1999; Weinreich et al., 1999). Overall, Cdc6 is reported to initiate DNA replication through its ATPase activity.

In this work, we demonstrate that Cdc6 has an additional role within the nucleolus during late mitosis and G1 phase. Specifically, Cdc6 binds to the rDNA promoter and coding regions of rRNA genes, and promotes rDNA transcription initiation by facilitating RRN3-mediated Pol I recruitment. Taken together, our data reveal that Cdc6 is a regulator of not only DNA replication initiation but also rDNA transcription in eukaryotic cells.

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RESULTS

A fraction of Cdc6 localizes in the nucleolus

To explore the mechanism of rDNA transcription initiation in cells, we first investigated the protein interactions of the Pol I complex. HeLa cells expressing the PIC component RRN3 or Pol I were synchronized to early G1 phase. Afterwards, RRN3 or Pol I was immunoprecipitated using specific antibodies. A proteomic analysis of the immunoprecipitates revealed that Cdc6 interacted with both RRN3 and Pol I (Tables S1 and S2). Next, we performed co-immunoprecipitation experiments using antibodies against RRN3 or RPA194 (also known as POLR1A; the large subunit of Pol I) and confirmed that Cdc6 binds both RRN3 and Pol I (Fig. 1A; Fig. S1G).

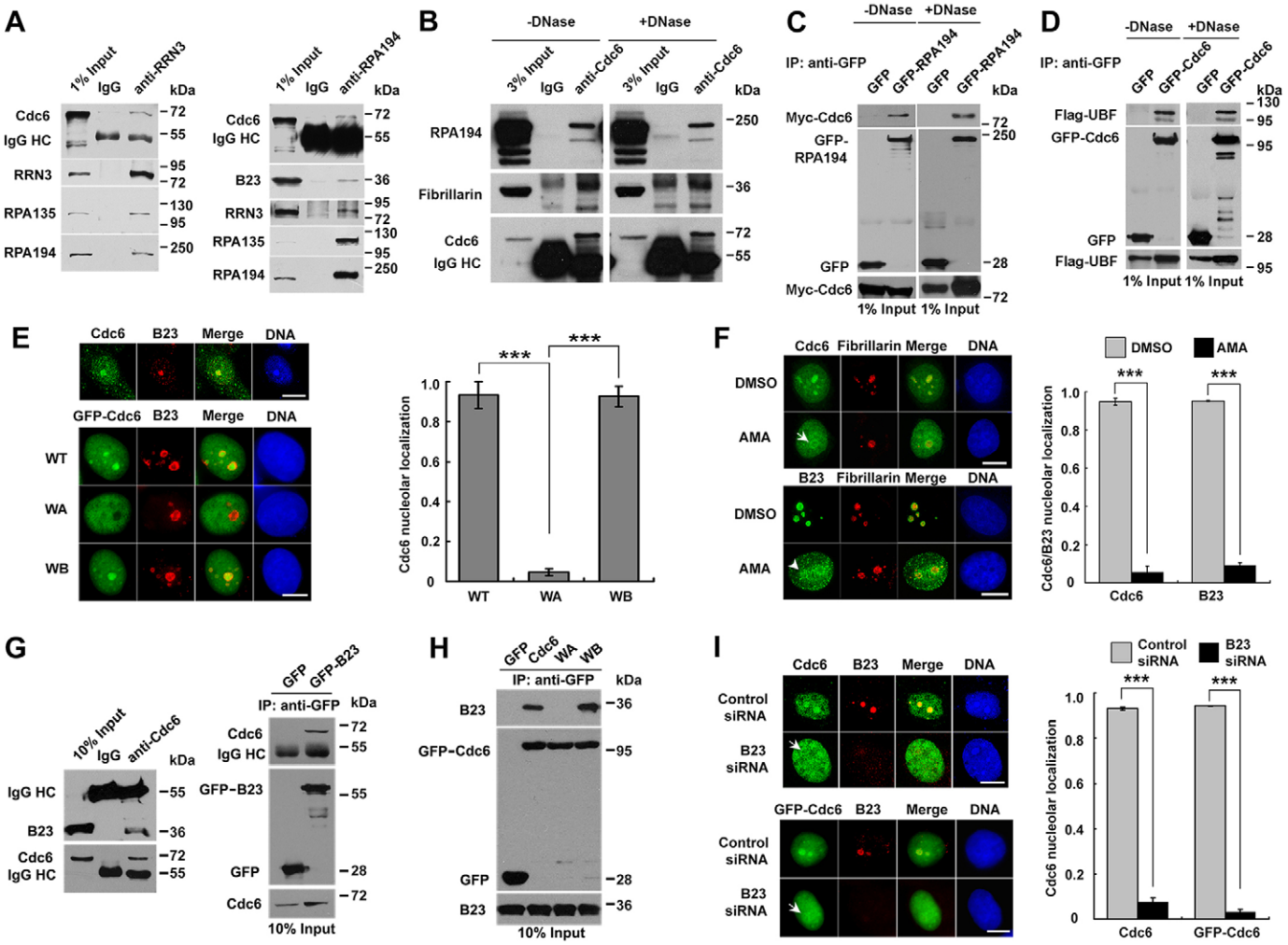

Fig. 1. Cdc6 localizes in the nucleolus in a B23-dependent manner. (A) Cdc6 interacts with RRN3 and RPA194. HEK 293T cell nuclear extracts were immunoprecipitated with anti-RRN3 or -RPA194 antibody and analyzed by western blotting. The input (1%) and control immunoprecipitations (IgG) are also shown. (B,C) DNase treatment does not affect the interaction between Cdc6 and RPA194. (B) HEK 293T cell nuclear extracts before or after DNase treatment were immunoprecipitated with anti-Cdc6 antibody and analyzed by western blotting. The input (3%) and control immunoprecipitations (IgG) are also shown. (C) Nuclear extracts from GFP–RPA194 and Myc–Cdc6 co-transfected HEK 293T cells treated with DNase or left untreated were immunoprecipitated (IP) with anti-GFP antibody followed by western blotting. (D) DNase treatment does not affect the interaction between Cdc6 and UBF. Nuclear extracts from GFP–Cdc6 and Flag–UBF co-transfected HEK 293T cells before or after DNase treatment were immunoprecipitated with anti-GFP antibody followed by western blotting. (E) WT or WB mutant Cdc6, but not the WA mutant localizes in the nucleolus. Representative confocal fluorescence images of HeLa cells stained for endogenous Cdc6 and B23 (upper panel). Representative fluorescence images of HeLa cells transfected with GFP-tagged WT, WA or WB Cdc6 and stained for B23 (lower panel). Quantification (mean±s.d.) of Cdc6 nucleolar localization was from three independent experiments (right panel). (F) Inhibiting the ATP level by antimycin A (AMA) treatment decreases Cdc6 nucleolus localization. Representative fluorescence images of HeLa cells treated with 5 µg/ml AMA or DMSO for 1.5 h and stained for Cdc6 or B23, and fibrillarin. Note that the nucleolar localizations of Cdc6 (arrow) and B23 (arrowhead) decreased upon AMA treatment. Quantification (mean±s.d.) of Cdc6 or B23 nucleolar localization was from three independent experiments (right panel). (G) Cdc6 interacts with B23. Whole-cell extracts from HeLa cells were immunoprecipitated with anti-Cdc6 antibody and analyzed by western blotting for B23 and Cdc6 (left panel). Extracts from GFP–B23-transfected HeLa cells were immunoprecipitated with anti-GFP antibody and analyzed by western blotting for Cdc6 and GFP–B23 (right panel). The input (3%) and control immunoprecipitations (IgG) are also shown. (H) The ATP-binding domain of Cdc6 is required for Cdc6 and B23 interaction. Whole-cell extracts from HeLa cells transfected with GFP and GFP-tagged WT, WA or WB Cdc6 were immunoprecipitated with anti-GFP antibody and analyzed by western blotting for B23 and GFP–Cdc6. (I) Cdc6 nucleolar localization is dependent on B23. Representative fluorescence images of HeLa cells transfected with control siRNA or B23 siRNA and stained for Cdc6 and B23 (upper panel) or HeLa cells co-transfected with GFP–Cdc6 and control siRNA or B23 siRNA and stained for B23 (lower panel). Quantification (mean±s.d.) of Cdc6 or GFP–Cdc6 nucleolar localization was from three independent experiments (right panel). Note that the endogenous or exogenous nucleolar Cdc6 was decreased in B23 siRNA transfected cells (arrows). ***P<0.001 (Student's t-test). DNA in E, F and I was stained with DAPI. Scale bars: 10 µm.

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Treatment of the cell lysates with DNase before immunoprecipitation did not abrogate this interaction, indicating that Cdc6 is a part of the Pol I complex but this interaction is not mediated by DNA (Fig. 1B,C). We further confirmed that Cdc6 also interacted with UBF but that another nucleolar protein, fibrillarin, does not (Fig. 1B,D), suggesting the interaction between Cdc6 and Pol I complex proteins is specific. Immunofluorescence labeling revealed that Cdc6 was enriched in the nucleolus and colocalized with B23 (also known as NPM1), a nucleolar marker (Fig. 1E; Fig. S1A,B). GFP-tagged Cdc6 was overexpressed and also localized to the nucleolus, confirming this localization (Fig. 1E). These results, taken together, indicate that a fraction of Cdc6 is a component of the PIC complex.

To investigate whether the ATP binding and hydrolysis domains of Cdc6 are essential for its nucleolar localization, we mutated exogenous Cdc6 K208 (within the Walker A motif) to a glutamate residue (K208E, denoted WA mutant) to abolish its ATP-binding activity, and in a separate construct mutated E285 (within the Walker B motif) to a glycine residue (E285G, denoted WB mutant) to abolish its ability to hydrolyze ATP (Herbig et al., 1999) (Fig. S1C). Upon expressing these mutants in HeLa cells, we observed the WA mutant to be present in the nucleus, but conspicuously absent from the nucleolus (Fig. 1E). In contrast, the WB mutant showed clear nuclear and nucleolar localization, like the wild-type (WT) (Fig. 1E). These results were further confirmed by immunofluorescence labeling of the cells expressing Myc-tagged WT, WA mutant and WB mutant Cdc6 (Fig. S1D) and by live-cell imaging of the cells expressing GFP-tagged WT, WA mutant and WB mutant Cdc6 (Fig. S1E). We observed identical patterns of nuclear localization to those in human cells when expressing *Xenopus* WT, WA mutant and WB mutant Cdc6 in *Xenopus* tissue culture cells (Fig. S1F). To further assess whether ATP is required for the nucleolar localization of Cdc6, we treated HeLa cells with 5 µg/ml antimycin A (AMA), an inhibitor of mitochondrial electron transfer, for 1.5 h to decrease the cellular ATP levels. Under these conditions we examined endogenous Cdc6 localization using immunofluorescence. B23 was used as a positive control because it is known to have an ATP-dependent nucleolar localization (Choi et al., 2008). Using this procedure, we observed that endogenous Cdc6, as well as the control B23, were restricted to a ring-like shape surrounding the nucleolus and were not present in the center region (Fig. 1F). Therefore, this mutational analysis unambiguously demonstrates that the localization of Cdc6 in the nucleolus is dependent on its ATP-binding domain.

The nucleolus localization of Cdc6 is dependent on B23

Next, we investigated how Cdc6 is localized to the nucleolus. B23 is a nucleolar shuttle protein (Yun et al., 2003) and targets proteins such as ARF (encoded by *CDKN2A*) and Rb (also known as RB1) to the nucleolus (Korgaonkar et al., 2005; Takemura et al., 2002). As both B23 and Cdc6 nucleolar localization require ATP (Fig. 1F), we tested whether the nucleolar localization of Cdc6 is dependent on B23. First, using co-immunoprecipitation studies, we showed that Cdc6 interacted with B23 (Fig. 1G). Because the ATP-binding domain of Cdc6 was required for its nucleolar localization, we further tested whether the interaction between Cdc6 and B23 is dependent on the ATP-binding domain of Cdc6. Only the WT and WB mutant Cdc6, but not the WA mutant, interacted with B23 (Fig. 1H), indicating that the ATP-binding domain is required for an interaction to occur. Next, we examined the localization of Cdc6 in B23-knockdown cells and found that both endogenous and exogenous Cdc6 were absent from the nucleolus (Fig. 1I). These data, taken together, demonstrate that Cdc6 localizes to the nucleolus in a B23-dependent manner.

Cdc6 is localized to nucleolus only in late mitosis and G1 phases

To investigate whether the nucleolar localization of Cdc6 is dependent on the cell cycle, we performed time-lapse microscopy of living HeLa cells expressing GFP-tagged WT, WA mutant or WB mutant Cdc6. In late mitosis and G1 phase, WT, WA mutant and WB mutant Cdc6 were localized to the nucleus; during S phase, WT, WA mutant and WB mutant Cdc6 were found in the cytoplasm (Fig. 2A; Movies 1–4). To assess precisely when Cdc6 is recruited to the nucleolus in late mitosis, we compared the timing of nucleolar accumulation of GFP-tagged Cdc6, mCherry-tagged B23 or GFP-tagged RPA194 in living HeLa cells progressing through the cell cycle. As shown in Fig. 2B,C and Movies 5–10, B23 and RPA194 were in the nucleolus throughout interphase. In late mitosis and early G1 phase, Cdc6 was recruited to the nucleus and enriched in the nucleolus almost at the same time as B23 and RPA194. To confirm the cellular location of Cdc6 during the cell cycle, we also observed a whole cell cycle of GFP-tagged Cdc6-expressing cells progressing through G1, S, G2, mitosis and the following G1 phase. This revealed that Cdc6 was found in the cytoplasm after G1 phase, and relocated into the nucleus and nucleolus again in late mitosis and the next G1 phase (Fig. 2D; Fig. S2A). Taken together, these results indicate that Cdc6 is recruited to the nucleolus at late mitosis and G1 phase and removed after G1 phase, and furthermore that Cdc6 might function in the nucleolus only during G1 phase.

Cdc6 associates with rDNA promoter and coding region

To corroborate the result from the live-cell imaging showing that Cdc6 and Pol I colocalize in the nucleolus in G1 phase, we synchronized HeLa cells into early, middle and late G1 phase and performed immunofluorescence labeling using antibodies against Cdc6 and Pol I to reveal the endogenous proteins (Fig. 3A,B; Fig. S2B). We found that during the progression from early to late G1 phase, the nucleolar Cdc6 constantly colocalized with Pol I, although the nucleoplasmic Cdc6 increased and the nucleolar Cdc6 decreased in late G1 (Fig. 3B). By performing confocal microscopy and line-scan bisecting analysis, we also found that Cdc6 and Pol I colocalized in the nucleolus (Fig. S2C).

To further examine whether Cdc6 colocalizes with rDNA transcription sites, we performed a 5-ethynyluridine incorporation experiment, which allows the visualization of rDNA transcription sites by tracking the nucleolar incorporation of 5-ethynyluridine. 5-ethynyluridine is a uridine analog that is incorporated into the newly transcribed RNA *in vivo* (Jao and Salic, 2008). We found that nucleolar Cdc6 colocalized with the incorporated nucleolar 5-ethynyluridine, indicating that this fraction of Cdc6 is situated on rDNA transcription sites (Fig. S2D), and probably associates with rDNA.

To investigate the possibility of whether Cdc6 associates with rDNA, we performed chromatin immunoprecipitation (ChIP) assays using specific antibodies against Cdc6 and RPA194, and then primers that amplify the human rDNA promoter, the pre-rRNA coding region [5′-external transcribed spacer (ETS), 18S, and 28S rRNA coding regions] and the intergenic spacer (IGS) region (Fig. 3C). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter was used as a negative binding control. The results showed that a fraction of Cdc6 was associated with the rDNA promoter (H42.9 and P region, Fig. 3C) and the coding region (H1, H4 and H7.9 region, Fig. 3C) but not the intergenic spacer region (H13 region, Fig. 3C), and that the occupancy pattern of the Cdc6 on rDNA was very similar to that of Pol I (Fig. 3D). The occupancy of Pol I on the rDNA in our assay is consistent with previous reports (O'Sullivan et al., 2002). To assess the region of DNA that was associated with Cdc6 exactly, the

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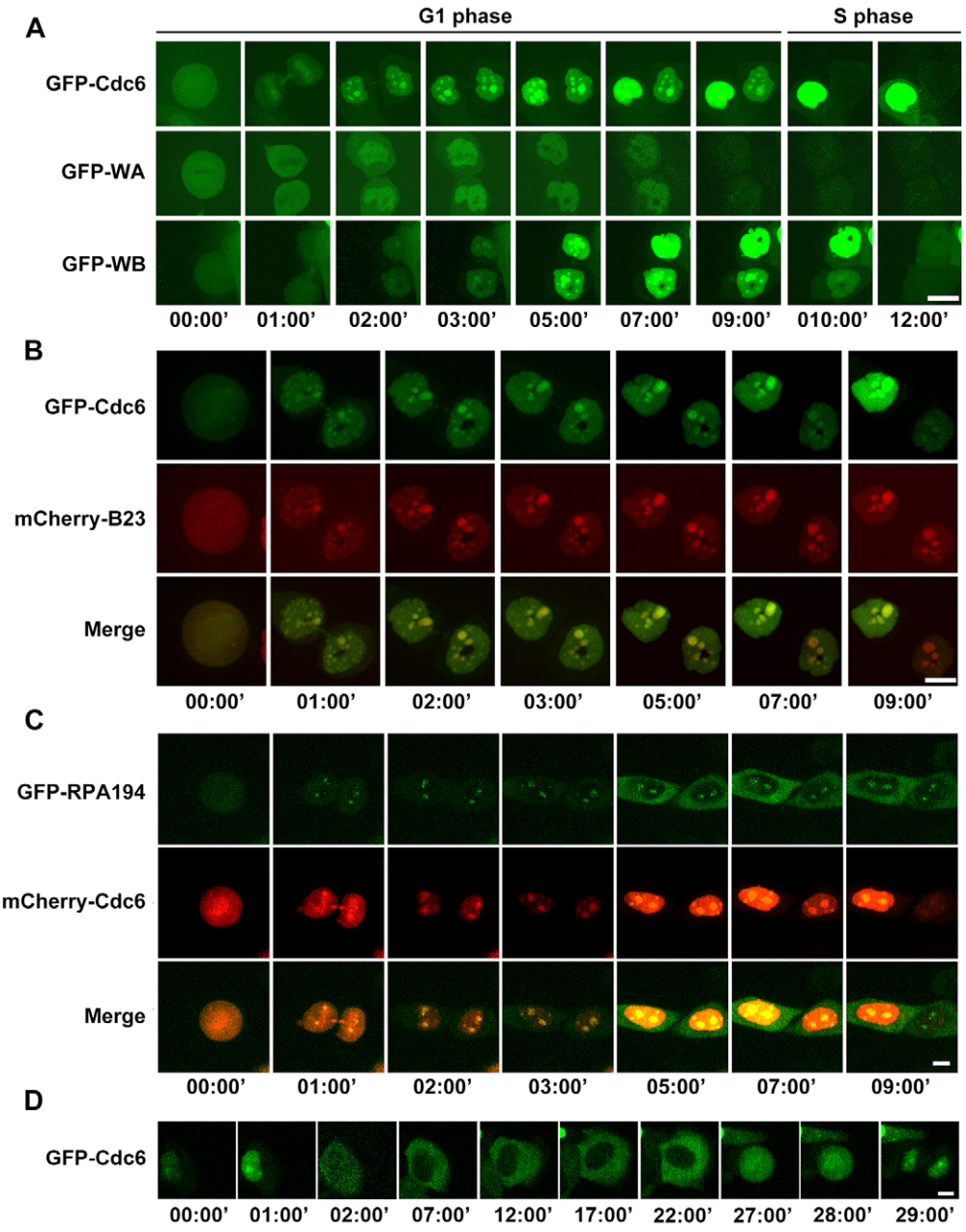

Fig. 2. Cdc6 is localized to nucleolus only in late mitosis and G1 phases. (A) WT, WA mutant or WB mutant Cdc6 localizes in the nucleus in G1 phase, and in the cytoplasm in S phase. Time-lapse live-cell imaging of HeLa cells transfected with GFP-tagged WT, WA mutant or WB mutant Cdc6. (B) Cdc6 is recruited to nucleolus at the same time after mitosis as B23. Time-lapse live-cell imaging of HeLa cells co-transfected with GFP–Cdc6 and mCherry–B23. (C) Cdc6 is recruited to nucleolus at the same time after mitosis as RPA194. Time-lapse live-cell imaging of HeLa cells co-transfected with mCherry–Cdc6 and GFP–RPA194. (D) Cellular location of Cdc6 during a complete cell cycle. Time-lapse live-cell imaging of HeLa cells transfected with GFP–Cdc6 progressing from being nucleolar localized G1 phase to the next G1 phase. Scale bars: 10 µm.

immunoprecipitated DNA was subjected to quantitative reverse transcriptase PCR (qRT-PCR) (Fig. 3E). The results showed that there was significantly more Cdc6 associated with the promoter (H42.9 and P) and coding regions (H1, H4 and H7.9) (Fig. 3E, right panel) than with the H18 region, similar to the regions of Pol I association (Fig. 3E, left panel). Depletion of Cdc6 by Cdc6 siRNA-1 or Cdc6 siRNA-2 significantly abolished the Cdc6 association with the promoter and coding regions, indicating the Cdc6 association is specific (Fig. 3E, right panel).

To determine whether the ATP-binding and hydrolysis domains of Cdc6 are required for its association with rDNA, we expressed GFP-tagged WT, WA mutant and WB mutant Cdc6 in HEK 293T cells to detect the rDNA binding of these proteins using ChIP assays. Similar to endogenous Cdc6, the exogenous WT Cdc6 associated mainly with the rDNA promoter region and extended weakly into the coding region (Fig. 3F). In contrast, the association of the Cdc6 WA mutant with the rDNA was completely abolished (Fig. 3F). Compared to the WT Cdc6, the association of the WB mutant with the rDNA promoter region and the coding region was stronger (Fig. 3F), suggesting that the ATP hydrolysis activity might contribute to the dissociation of Cdc6 from the rDNA. The primer amplifying $RD^{INK4/ARF}$ (also known as *CDKN2A*) was used as a positive technical control in the ChIP assays (Fig. 3F) because it has been reported that Cdc6 binds to the regulatory domain of the INK4/ARF locus in performing its oncogenic activity, and this binding is abolished when the WA or WB motif is mutated (Gonzalez et al., 2006). qRT-PCR was also performed to analyze the ChIP results and results were consistent with the ethidium-bromide-stained agarose gels analysis in Fig. 3F (Fig. 3G). The WT and WB mutant Cdc6, but not the WA mutant, were capable of binding to rDNA regions (Fig. 3G), confirming that the rDNA binding of Cdc6 was regulated by the ATP-binding ability of Cdc6. Taken together, we conclude that Cdc6 is associated with the rDNA promoter and the coding region in an ATP-binding-dependent manner.

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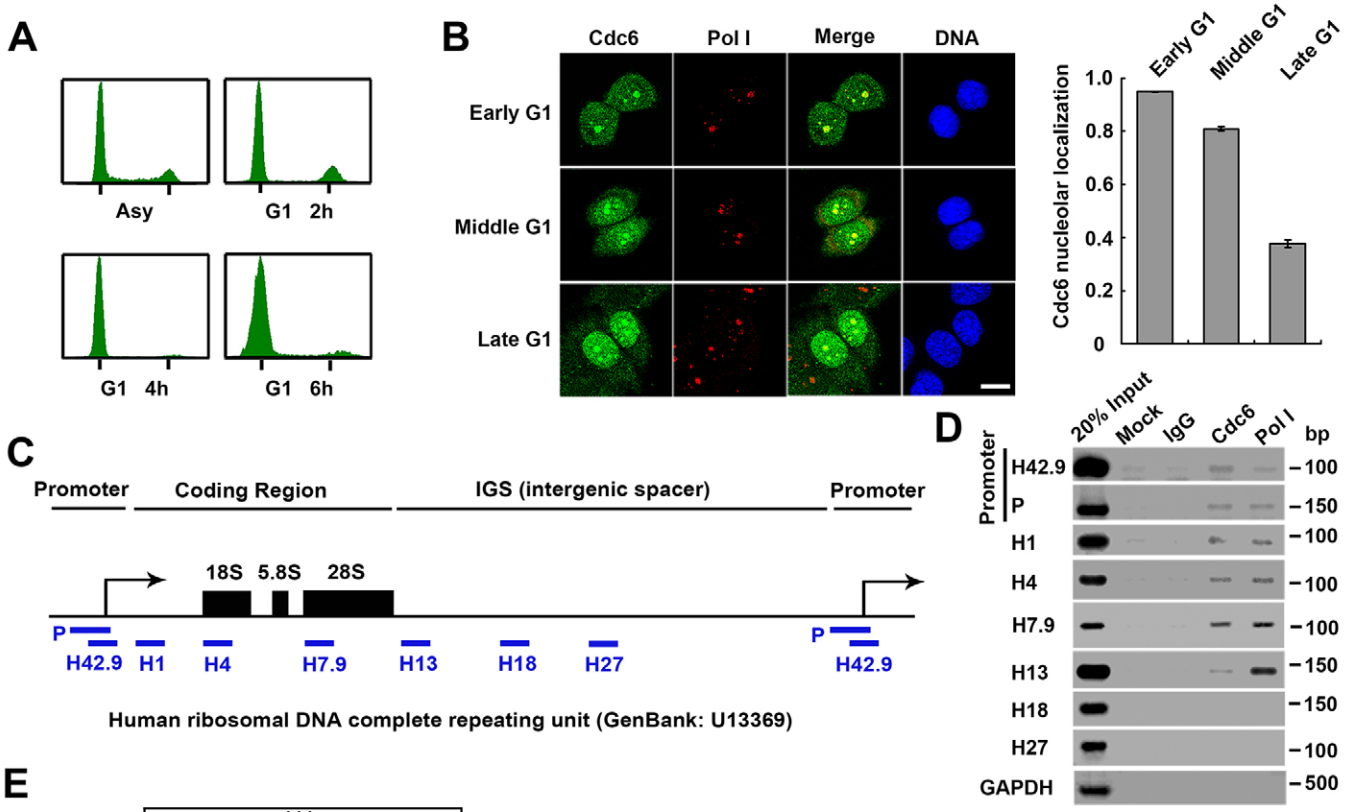

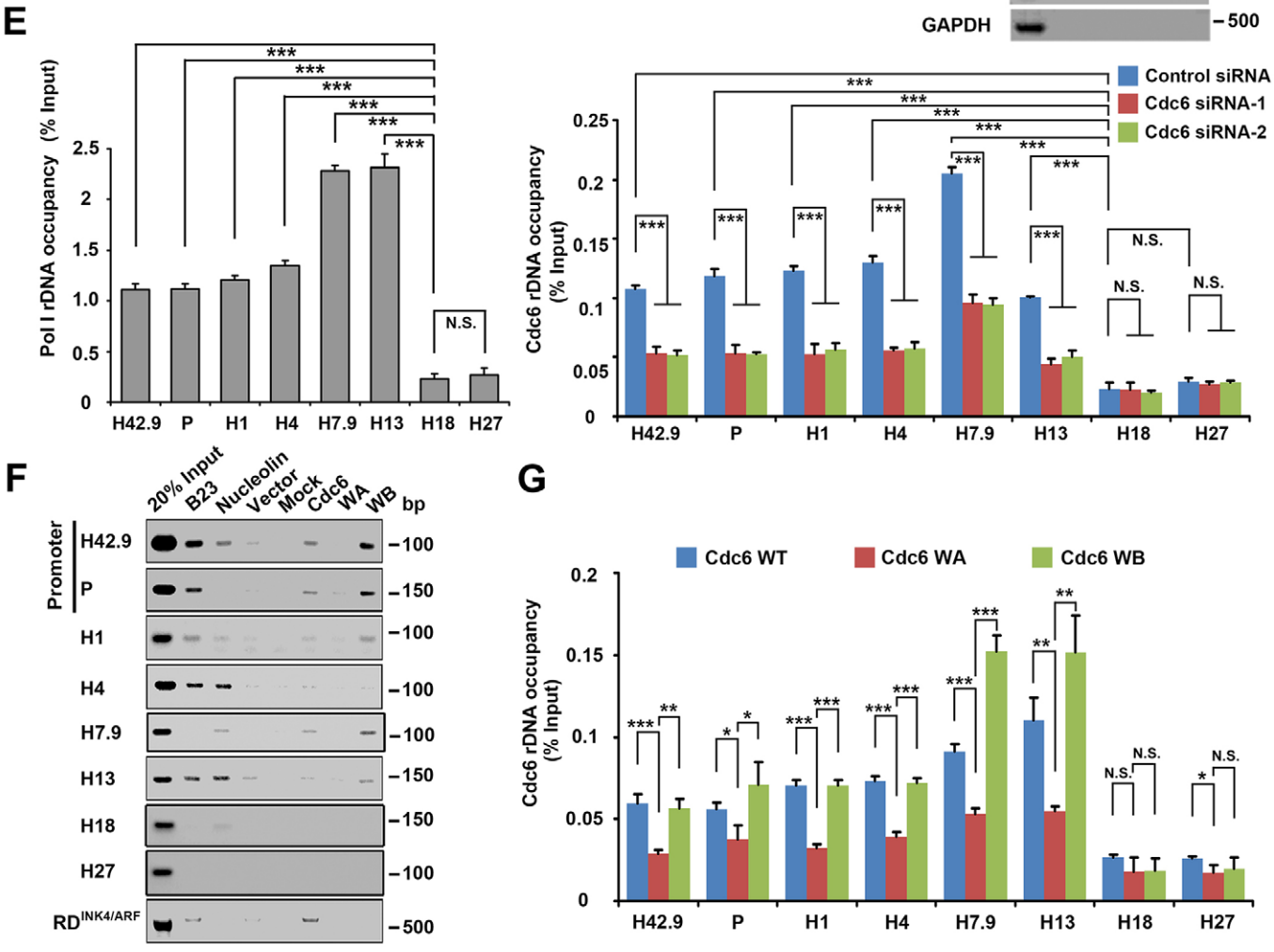

Fig. 3. See next page for legend.

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Fig. 3. Cdc6 associates with rDNA promoter and coding regions in an ATP-binding-domain-dependent manner. (A,B) Cdc6 colocalizes with Pol I in the nucleolus in G1 phase. HeLa cells were synchronized to early, middle and late G1 as described in the Materials and Methods section. (A) Flow cytometry analysis for synchronization efficiency. Asy, asynchronous culture. (B) Immunofluorescence analysis of endogenous Cdc6 and RPA194 (left panel). Quantification of Cdc6 nucleolar localization (mean±s.d.) from three independent experiments (right panel). Scale bar: 10 µm. (C) Schematic representation of a human rDNA repeat unit. Primers used in the ChIP assay are indicated by blue lines. (D,E) Cdc6 associates with the rDNA promoter and coding regions. (D) Representative ethidium-bromide-stained agarose gel images of the amplified rDNA promoter, coding and IGS regions that had been immunoprecipitated by anti-Cdc6 or anti-Pol-I antibodies in ChIP assays. (E) As in D, except that the ChIP performed with anti-Pol I antibody was analyzed by qRT-PCR (left panel), and the ChIP performed with anti-Cdc6 antibody was undertaken in extracts from HEK 293T cells transfected with control siRNA, Cdc6 siRNA-1, or Cdc6 siRNA-2 and analyzed by qRT-PCR (right panel). Results are mean±s.d. for three independent experiments. (F,G) The ATP-binding domain is required for Cdc6 association with rDNA. (F) Representative ethidium-bromide-stained agarose gel images of the amplified rDNA promoter, coding, IGS and $RD^{INK4/ARF}$ regions immunoprecipitated by anti-GFP antibody in ChIP assays from HEK 293T cells transfected with GFP-tagged WT (Cdc6 lane), WA or WB Cdc6. (G) As in F, except that ChIP was analyzed by qRT-PCR. Results are mean±s.d. for three independent experiments. *P<0.05; **P<0.01; ***P<0.001; N.S., not significant (Student's t-test).

Cdc6 promotes rDNA transcription initiation in G1 phase

To understand why Cdc6 binds rDNA in the nucleolus, we tested whether its nucleolar localization is related to rDNA transcription. Given that a low dose of actinomycin D (ActD) specifically represses Pol I, whereas a high dose represses both Pol I and Pol II (Schöfer et al., 1996), we treated HeLa cells with ActD and performed 5-ethynyluridine incorporation experiments to reveal RNA transcription. We found that a low dose (100 nM for 1 h) of ActD specifically inhibited Pol-I-dependent transcription, as shown by the absence of nucleolar incorporation of 5-ethynyluridine (Fig. S3A). When a high dose (2 µM for 1 h) of ActD was applied, both Pol-I-dependent transcription in the nucleolus and Pol-II-dependent transcription in the nucleus were inhibited (Fig. S3A). Therefore, we treated the cells with a low dose (100 nM for 1 h) of ActD to specifically inhibit Pol-I-dependent transcription and immunostained the cells for Cdc6. We observed that Cdc6 was expelled from the central nucleolar region to a ring-like shape surrounding the nucleolus (Fig. S3B). These results indicate that the nucleolar Cdc6 is linked to rDNA transcription. We also treated HeLa cells with 5,6-dichlorobenzimidazole 1-β-D-ribofuranoside (DRB), a direct inhibitor of Pol II (Zandomeni et al., 1986). In this negative control experiment, we found that the nucleolar localization of Cdc6 was not affected (Fig. S3C).

To explore whether Cdc6 regulates rDNA transcription *in vivo*, we assessed rDNA transcription by measuring the levels of pre-rRNA synthesis with qRT-PCR. Compared to the vector-transfected control cells, the 45S pre-rRNA synthesis in the cells expressing exogenous Cdc6 was increased (Fig. 4A), suggesting that the expression of exogenous Cdc6 activated rDNA transcription. We also assessed cells expressing exogenous WA or WB mutant Cdc6 and found that both mutants were deficient in activating rDNA transcription (Fig. 4A). Therefore, Cdc6 promotes rDNA transcription in an ATP-binding- and hydrolysis-dependent manner.

To confirm the role of Cdc6 in rDNA transcription, we depleted Cdc6 by performing RNA interference (RNAi) with two different small interfering RNAs (siRNAs) targeting different regions of the Cdc6 gene. The results showed that both siRNAs caused Cdc6 depletion and significantly decreased the 45S pre-rRNA synthesis (Fig. 4B,C; Fig. S2E). Through a timecourse 5-ethynyluridine incorporation assay followed by immunofluorescence labeling, we also revealed that Cdc6 depletion by use of siRNA significantly reduced and delayed 5-ethynyluridine incorporation into the nucleolus (Fig. 4D,E). This result confirms that rDNA transcription is repressed upon Cdc6 knockdown.

As Cdc6 specifically localizes to the nucleolus in late mitosis and G1 phase (Fig. 2), we propose that the nucleolar Cdc6 only functions in rDNA transcription during this period. To test this possibility, we depleted Cdc6 in differently synchronized cells by a novel strategy as described in the Materials and Methods section. In the 'Cdc6 depletion after synchronization' strategy, we first synchronized cells to the G1/S transition and then depleted Cdc6, by use of siRNA, in the presence of thymidine to keep the cells at the G1/S transition; then we released the cells to S, G2 or the next G1 phase, and collected the synchronized cells 72 h after siRNA transfection. The synchronization efficiency of the analyzed cells was assessed by flow cytometry analysis (Fig. 5A,B). The Cdc6 or Orc2 depletion efficiency is shown in Fig. S4A. The qRT-PCR results showed that rDNA transcription was disrupted only in the cells during the next G1 phase, but not the initial S and G2 phases (Fig. 5A). This result strongly indicates that Cdc6 activates rDNA transcription only in G1 phase, and depletion of Cdc6 in S and G2 phase does not affect rDNA transcription. In the 'Cdc6 depletion before synchronization' strategy (Fig. 5B), we first depleted Cdc6, by use of siRNA, and afterwards synchronized the cells at G1, S or G2 phase. Strikingly different from the results shown in Fig. 5A, rDNA transcription was depressed in all synchronized cells during G1, S and G2 phase in this protocol (Fig. 5B). This result strongly indicates that, owing to the Cdc6 depletion before the cell synchronization, all the cells that progressed from the Cdc6-depleted G1 stage were defective in rDNA transcription (Fig. 5B). This novel set of experiments, taken together, demonstrates that Cdc6 activates rDNA transcription in G1 but not S and G2 phases.

To test for possible indirect effects to rDNA transcription caused by DNA replication defects upon expression of Cdc6 siRNA, we also depleted another pre-replication complex component, Orc2, as a control. The results showed that the rDNA transcription levels were decreased by Cdc6 depletion but not Orc2 depletion (Fig. 5A,B). In addition, rDNA transcription was not disrupted in Cdc6-depleted S and G2 cells (Fig. 5A). These results demonstrate that the regulation of rDNA transcription by the nucleolar Cdc6 is independent of its function in DNA replication initiation and also is not due to cell cycle phase interruption. Taken together, these data demonstrate that Cdc6 positively regulates rDNA transcription in G1 phase.

Cdc6 promotes rDNA transcription initiation by facilitating the recruitment of RRN3 and Pol I to the rDNA promoter

We then investigated whether or not Cdc6 is involved in the assembly of Pol I transcriptional machinery on the rDNA promoter to initiate rDNA transcription. Cdc6 binds RRN3, Pol I and UBF in the PIC (Fig. 1). As the assembly of PIC recruits Pol I to the rDNA promoter (Grummt, 2010), we tested whether Cdc6 regulates Pol I recruitment to the rDNA promoter. Using ChIP assays followed by qRT-PCR, we analyzed the level to which the rDNA promoter region associated with PIC components. The promoter occupancy of Pol I and RRN3, but not of SL1 and UBF, was markedly reduced in Cdc6-depleted cells (Fig. 6A). We also confirmed that the reduction of the promoter occupancy of Pol I and RRN3 was not due to the protein level change (Fig. 6B). To further explore how Cdc6 depletion disrupted Pol I recruitment to the rDNA promoter, we

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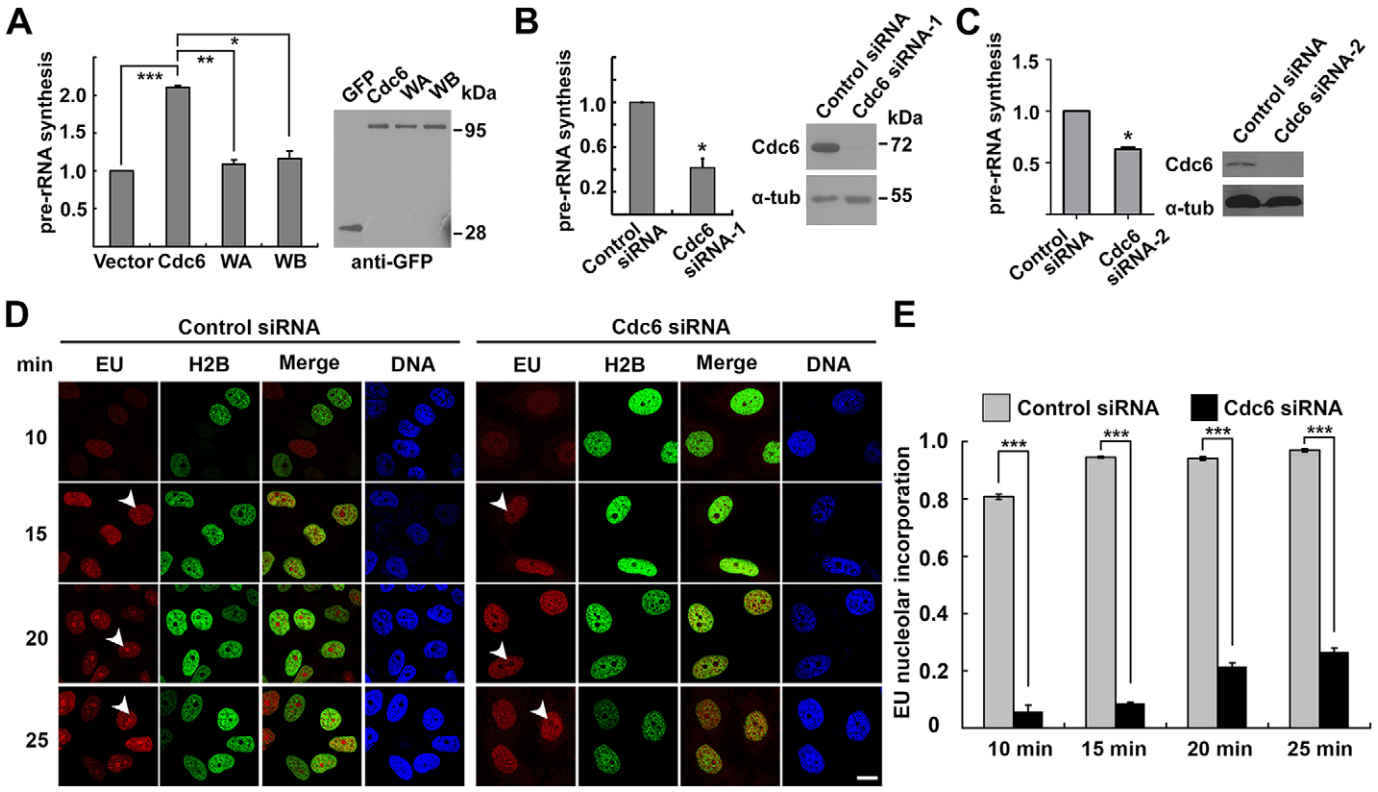

Fig. 4. Cdc6 promotes rDNA transcription initiation in an ATP-binding and hydrolysis-dependent manner. (A) WT Cdc6, but not the WA mutant or WB mutant, promotes rDNA transcription initiation. qRT-PCR of pre-rRNA from HEK 293T cells transfected with GFP (vector) or GFP-tagged WT, WA or WB Cdc6 (left panel; results are mean±s.d. for three independent experiments). The expression levels of the constructs were analyzed by western blotting with anti-GFP antibody (right panel). (B,C) Cdc6 depletion decreases rDNA transcription initiation. qRT-PCR of pre-rRNA from HEK 293T cells transfected with control siRNA, Cdc6 siRNA-1 or Cdc6 siRNA-2 (left panels). Results are mean±s.d. for three independent experiments. The depletion efficiency of Cdc6 was analyzed by western blotting for Cdc6 and α-tubulin (right panels). (D) Cdc6 depletion delays 5-ethynyluridine (EU) incorporation in the nucleolus. Representative confocal immunofluorescence images of 5-ethynyluridine incorporation in HeLa cells co-transfected with control siRNA or Cdc6 siRNA and GFP–H2B constructs (serving as a transfection-positive marker) at a ratio of 20 to 1 (siRNA to GFP–H2B). Note that rDNA transcription in the nucleolus visualized by 5-ethynyluridine incorporation (arrowheads) was delayed in Cdc6-siRNA-transfected cells. Scale bar: 10 µm. (E) Quantification (mean±s.d.) of 5-ethynyluridine nucleolar incorporation was from three independent experiments as shown in D. *P<0.05, **P<0.01, ***P<0.001 (Student's t-test).

characterized the interactions of PIC components in Cdc6-depleted cells and found that Cdc6 depletion significantly impaired the interaction between RRN3 and Pol I (Fig. 6C,D; Fig. S4B). Furthermore, immunofluorescence results revealed that the nucleolar Pol I, but not UBF, level in Cdc6-depleted cells was significantly reduced (Fig. 6E). Therefore, these data indicate that Cdc6 positively regulates rDNA transcription by facilitating the recruitment of RRN3 and Pol I to the rDNA promoter region.

DISCUSSION

This work reveals a new role for Cdc6 in rDNA transcription in addition to its role in DNA replication initiation. It has been established that Cdc6 regulates pre-replication complex assembly mainly by recruiting the Mcm2-7 helicase to DNA replication origins, licensing replication initiation (Donovan et al., 1997; Liang and Stillman, 1997; Yan et al., 1998). It is also worth mentioning that two studies have revealed that Cdc6 represses E-cadherin locus or INK4/ARF locus transcription by binding to the promoter of these genes, inspiring the notion that Cdc6 also functions in transcription (Gonzalez et al., 2006; Sideridou et al., 2011). In this work, we found that Cdc6 localizes in the nucleolus in cycling cells at the end of mitosis and early G1 phase. This duration of Cdc6 nucleolus localization correlates with the timing of rDNA transcription machinery assembly and rDNA transcription activation. Therefore, we further assessed and demonstrated that Cdc6 activates rDNA transcription in G1 phase by binding with rDNA genes and PIC components in order to stabilize RRN3-mediated Pol I recruitment to the rDNA promoter region. It is known that rDNA transcription is triggered by reactivation of the rDNA transcription machinery in early G1 phase after the PIC assembly during mitotic exit (Voit and Grummt, 2001; Voit et al., 1999). Here, we show that DNA replication initiator Cdc6 is a new positive regulator for rDNA transcription initiation, which suggests an intriguing mechanism for the coordination of DNA replication and rDNA transcription initiation mediated by Cdc6.

It is known that the ATP loading to Cdc6 facilitates its binding to the DNA replication origin during initiation of DNA replication, and the ATP hydrolysis on the Cdc6 molecule promotes the recruitment of Mcm2-7 to the pre-replication complex. We find that the ATPase activity of Cdc6 is required during rDNA transcription initiation as it is in DNA replication initiation. We show that the ATP binding to Cdc6 is required for its association with the rDNA promoter region, and the hydrolysis of ATP on Cdc6 is required for rDNA transcription initiation. Thus, the biological functions of Cdc6 in DNA replication and rDNA transcription initiations are distinct, whereas ATPase activity is essential for both functions. We can speculate that ATP binding to Cdc6 helps to locate it to the

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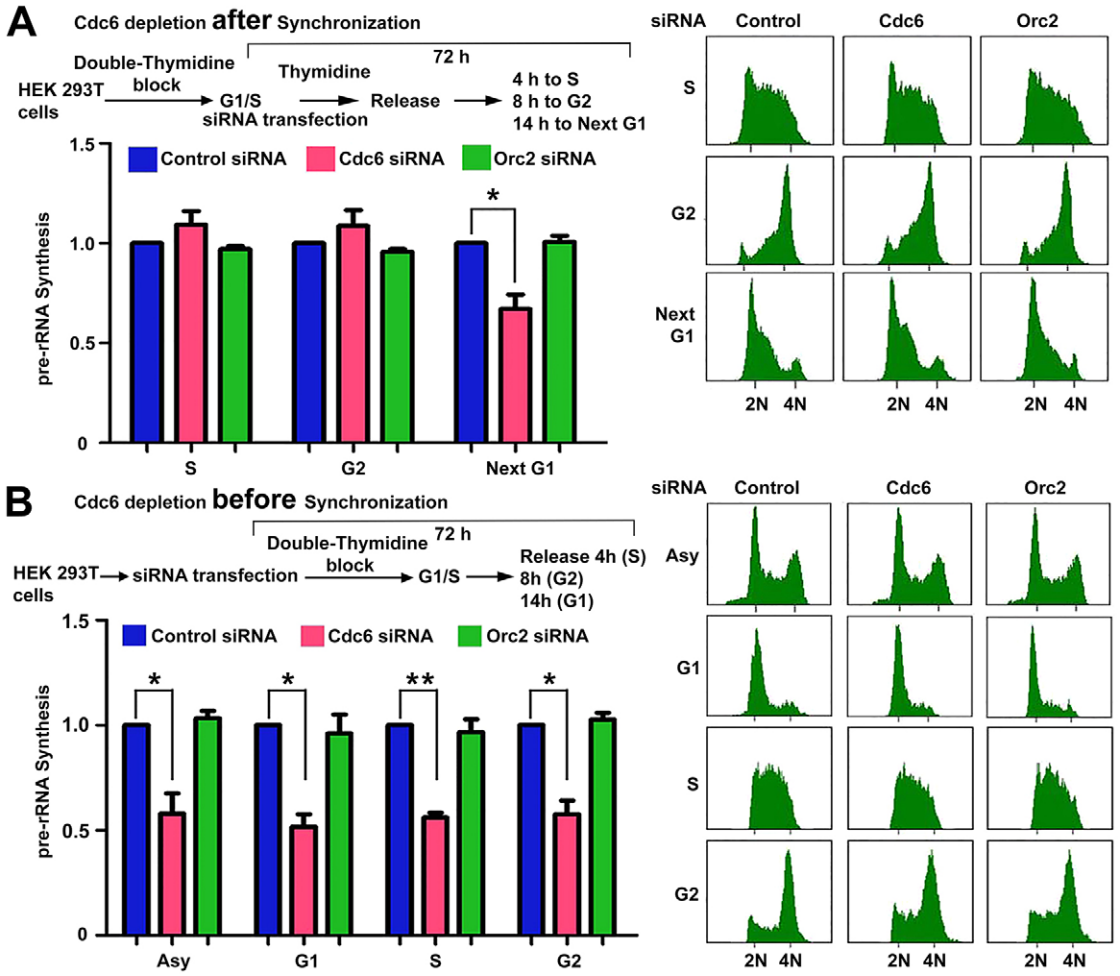

Fig. 5. Cdc6 promotes rDNA transcription initiation in G1 phase. (A) rDNA transcription is disrupted in G1 phase cells but not in S or G2 phase cells as determined by a 'Cdc6 depletion after synchronization' protocol. qRT-PCR of pre-rRNA from HEK 293T cells synchronized to S, G2, or the next G1 phase by double-thymidine block, and transfected with control, Cdc6 or Orc2 siRNA in the presence of thymidine (left panel). Results are mean±s.d. for three independent experiments. Flow cytometry analysis for synchronization efficiency (right panel). (B) rDNA transcription is depressed in all synchronized cells of G1, S or G2 phase as determined by a 'Cdc6 depletion before synchronization' protocol. qRT-PCR of pre-rRNA from HEK 293T cells transfected with the control, Cdc6 or Orc2 siRNA, and then synchronized to G1, S, and G2 or left asynchronized (Asy) (left panel). Results are mean±s.d. for three independent experiments. Flow cytometry analysis for synchronization efficiency (right panel). *P<0.05, **P<0.01 (Student's t-test).

specific sites of DNA, and the ATP hydrolysis functions in the recruitment of downstream factors.

Despite Cdc6 ATPase activity being required for both rDNA transcription and DNA replication, Cdc6 function in rDNA transcription is independent of its role in DNA replication for two reasons. First, Cdc6 binding to rDNA is not due to rDNA replication. As a DNA replication initiator, Cdc6 binds to the replication origin to recruit DNA helicase. Despite this fact, it does not move along the DNA with the replication forks, and is removed from the origins after DNA replication origin firing to prevent multiple DNA replications during one cell cycle. If Cdc6 is detectable in replicating rDNA, this might be due to its binding only to the rDNA replication origins. rDNA replication origins are reportedly located within the IGS region upstream of the rDNA transcription start site (Gögel et al., 1996) but not in other rDNA regions. The present work shows that Cdc6 does not associate with the rDNA IGS region where the rDNA replication origin is located, but binds to the promoter and coding regions with a similar occupancy pattern as Pol I (Fig. 3D,E). This indicates that the Cdc6 function in rDNA transcription is not a secondary effect of rDNA replication. In addition, it has been reported that rDNA transcription sites and rDNA replication sites occur simultaneously at the periphery of the nucleolus during early S phase (Dimitrova, 2011). Our results show that Cdc6 is located within the nucleolus and extensively colocalizes with Pol I in G1 phase, indicating that the nucleolar localization of Cdc6 observed here is not responsible for rDNA replication. Therefore, Cdc6 associates with rDNA in the nucleolus independently of its role in DNA replication.

Based on our present data and previous reports, we propose a working model that describes the role of Cdc6 in rDNA transcription (Fig. 7). During rDNA transcription initiation, B23 associates with rRNA genes in a UBF-dependent manner (Hisaoka et al., 2010) and also recruits Cdc6 to the rDNA promoter in a manner that is dependent on the ATP-binding domain of Cdc6. Through hydrolyzing ATP, Cdc6 promotes RRN3-mediated Pol I recruitment onto the rDNA promoter region to initiate rDNA transcription. After Cdc6 activates rDNA transcription in G1 phase, Cdc6 is removed from the nucleolus and is not required for rDNA transcription in S and G2 phase. The dual roles of Cdc6 in DNA replication initiation, as reported previously, and rDNA

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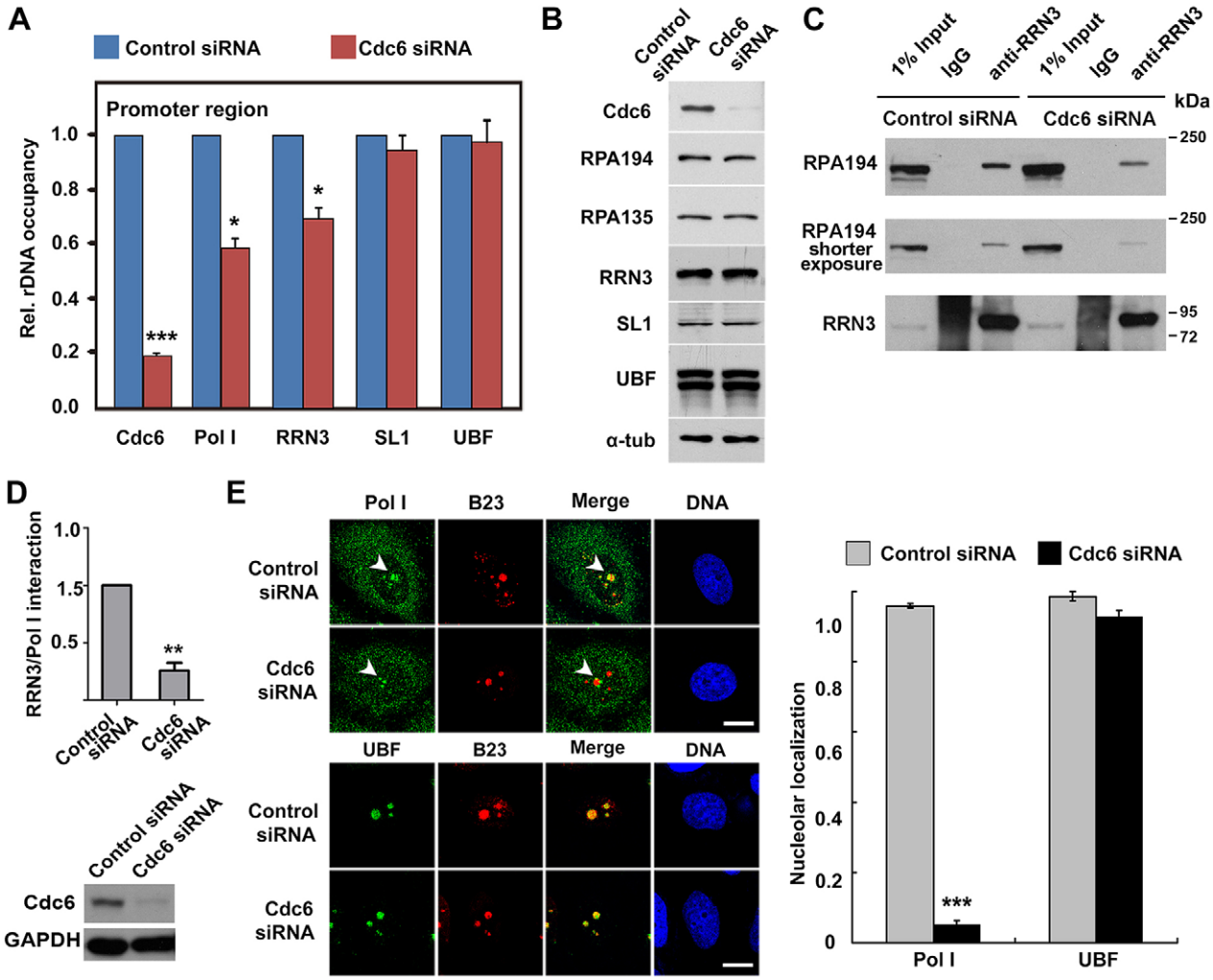

Fig. 6. Cdc6 promotes RRN3-mediated Pol I recruitment to the rDNA promoter region. (A) The rDNA promoter occupancy of Pol I or RRN3 is reduced in Cdc6-depleted cells. qRT-PCR analysis of the amplified rDNA promoter region immunoprecipitated by antibodies against Cdc6, Pol I, RRN3, SL1 or UBF in ChIP experiments from HEK 293T cells transfected with control siRNA or Cdc6 siRNA. Results are mean±s.d. for three independent experiments. (B) Cdc6 siRNA does not affect the protein level of the Pol I transcription machinery components as assessed by western blotting. (C) The interaction between RRN3 and RPA194 is impaired by Cdc6 depletion. Nuclear extracts of HEK 293T cells transfected with control siRNA or Cdc6 siRNA were immunoprecipitated with anti-RRN3 antibody and western blotted for RPA194 and RRN3. The input (1%) and control immunoprecipitations (IgG) are also shown. (D) Quantification of the relative RRN3–Pol-I interaction fold change, normalized to the immunoprecipitated RRN3 level and the input (upper panel). Results are mean±s.d. from three independent experiments as shown in C. The Cdc6 depletion efficiency for experiments in C as assessed by western blotting for Cdc6 and GAPDH (lower panel). (E) Representative confocal fluorescence images of HeLa cells transfected with control siRNA or Cdc6 siRNA and stained with Pol I, UBF or B23 (left panel). Note that Pol I nucleolar localization is decreased in Cdc6-siRNA-transfected cells (arrowheads). Scale bars: 10 µm. Quantification (mean±s.d.) of cells with normal nucleolar localization of Pol I or UBF from three independent experiments (right panel). *P<0.05, **P<0.01, ***P<0.001 (Student's t-test).

transcription, as we identified in this work, indicate a possible intriguing coordination mechanism between DNA replication and rDNA transcription that is mediated by Cdc6 or other DNA replication initiators.

Similar coordination between DNA replication and rDNA biogenesis by DNA replication initiators has been reported in yeast where the nucleolar-complex-associated protein Noc3p (Zhang et al., 2002) and Ipi3p proteins (Huo et al., 2012) play dual rules in DNA replication and ribosome biogenesis. Noc3p, a nucleolar protein that is required for pre-ribosome transport, interacts with MCM and Orc2, and is essential for Cdc6 and MCM chromatin association and DNA replication initiation (Zhang et al., 2002). The Rix1 complex (Ipi1p–Ipi2p–Ipi3p), which plays a role in ribosome biogenesis, has also been identified as a pre-replication complex component (Huo et al., 2012). Combining these previous reports and the present work, it is an intriguing hypothesis that DNA replication initiators (Cdc6 for rDNA transcription, Ipi1-3 in pre-rRNA processing and Noc3 for the pre-ribosome, and perhaps others) form a network to coordinate DNA replication and ribosome biogenesis.

MATERIALS AND METHODS

Plasmids, antibodies, chemicals and RNAi

Human Cdc6 was cloned from a cDNA library by RT-PCR, and inserted into pEGFP-C3 vector using primers 5′-TCACTCGAGATGCCTCAAACCC-3′ (sense, Xho I site underlined) and 5′-ACCGGTACCAAAGGCAATCCAGT-3′ (antisense, Kpn I site underlined). The Cdc6 WA mutant (K208E) and Cdc6 WB mutant (E285G) were generated by site-directed mutagenesis using complementary primers of 5′-TGGAACTGGAGAAACTGCCT-3′ (WA) and 5′-TGGTATTGGACGGGATGGAT-3′ (WB) (the mutated codon is underlined) and then inserted into pEGFP-C3 vectors.

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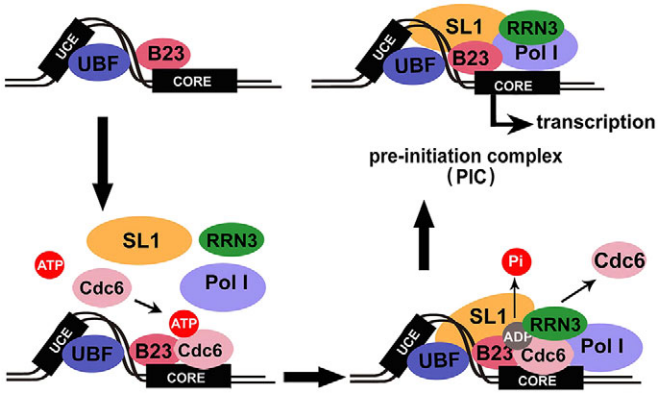

Fig. 7. A proposed model for the role of Cdc6 in rDNA transcription. Cdc6 positively regulates rDNA transcription by promoting the recruitment of the RRN3–Pol-I complex to the promoter region of the rRNA genes. During rDNA transcription initiation after mitosis, B23 associates with the rRNA gene in a manner that is dependent on UBF. B23 at the rDNA promoter region targets Cdc6 to the promoter in a manner that is dependent on the ATP-binding domain of Cdc6. Through hydrolyzing the ATP, Cdc6 promotes the RRN3-mediated recruitment of Pol I to the rDNA promoter region to initiate rDNA transcription. After Cdc6 activates rDNA transcription in G1 phase, Cdc6 is removed from the nucleolus and is not required for rDNA transcription in S and G2 phase. The dual roles of Cdc6 in DNA replication initiation, as reported previously, and rDNA transcription, as we identified in this work, indicate a possible intriguing cooperation mechanism between DNA replication and rDNA transcription by Cdc6 or other DNA replication initiators.

siRNA-resistant mutants of Cdc6 WT, WA mutant or WB mutant were cloned using WT, WA or WB Cdc6 as templates and generated by using complementary primers of 5′-TGTTGAATTTCCCGCCTTATACCAGA-3′ and 5′-TTCTGGTATAAGGCGGGAAATTCA-3′ (altered nucleotides are underlined) and then inserted into pEGFP-C3 vectors. Human B23 was cloned from a cDNA library by RT-PCR, and inserted into pCMV-Myc vector using primers 5′-TTTGAATTCATGGAAGATTCGATGGACAT-GGACA-3′ (sense, EcoR I site underlined) and 5′-TTTGTCGACTCAAT-GCGCTTTTTCTATACTTGCT-3′ (antisense, Sal I site underlined). *Xenopus* Cdc6 cDNA was cloned from a cDNA library by RT-PCR, and then inserted into pEGFP-C2 using primers 5′-TTAGAATTCATGCCAA-GCACCAGG-3′ (sense, EcoR I site underlined) and 5′-CGGGTCGACT-TAAATCCCTGAA-3′ (antisense, Sal I site underlined). The *Xenopus* Cdc6 WA mutant (K202E) and WB mutant (E277G) were generated by PCR site-directed mutagenesis.

The following antibodies were used: polyclonal antibodies against GFP (1:1000 dilution for western blot, Fu et al., 2009), B23 (1:1000 dilution for western blot; 1:100 dilution for immunofluorescence, ab37659, Abcam), fibrillarin (1:100 dilution for immunofluorescence, ab5821, Abcam), UBF (1 to 1000 dilution for western blot; 1:100 dilution for immunofluorescence, sc-9131, Santa Cruz Biotechnology), RPA135 (1:1000 dilution for western blot, sc-17913x, Santa Cruz Biotechnology) and RRN3 (1:1000 dilution for western blot, ab79375, Abcam); mouse monoclonal antibodies against Cdc6 (1:1000 dilution for western blot; 1:100 dilution for immunofluorescence, sc-9964, Santa Cruz Biotechnology), Cdc6 (1:100 dilution for immunofluorescence, sc-13136, Santa Cruz Biotechnology), B23 (1:1000 dilution for western blot; 1:100 dilution for immunofluorescence, B0556, Sigma), RPA194 (1:1000 dilution for western blot; 1:100 dilution for immunofluorescence, sc-48385, Santa Cruz Biotechnology) and α-tubulin (1:1000 dilution for western blot, T6074, Sigma). Anti-RPA194 (1:100 dilution for ChIP, sc-48385, Santa Cruz Biotechnology) antibody and ChIP grade mouse monoclonal antibody against Cdc6 (1:1000 dilution for ChIP, sc-9964x, Santa Cruz Biotechnology) were used for ChIP assays. Rabbit polyclonal antibody against nucleolin (1:100 dilution for immunofluorescence, N2662, Sigma) for immunofluorescence analysis of nucleolin. The dilutions of the commercial antibodies were performed according to the manufacturer's protocol.

For the 5-ethynyluridine incorporation assay, 1 mM 5-ethynyluridine (E10345, Invitrogen) was incubated with cells for 10, 15, 20 or 25 min before fixation. Then the fixed cells were stained with a fluorescent Alexa-Fluor-594-conjugated azide (A10270, Invitrogen) to detect incorporated 5-ethynyluridine by click chemistry as previously described (Jao and Salic, 2008). For AMA treatment to decrease cellular ATP levels, cells were treated with 5 μg/ml AMA (A8674, Sigma) for 1.5 h and then released to fresh culture medium for another 12 h before fixation, as previously described (Choi et al., 2008).

Cdc6 siRNA-1 (5′-AACUUCCCACCUUAUACCAGA-3′) (Mailand and Diffley, 2005), Cdc6 siRNA-2 (5′-AAGAAUCUGCAUGUGUGAG-AC-3′), Orc2 siRNA (5′-UGCUCCUCUCAUGUGGGAU-3′), B23 siRNA (5′-AGUGGAAGCCAAAUUCAUC-3′) or a non-specific negative control siRNA (5′-UUCUCCGAACGUGUCACGUTT-3′) was synthesized by GenePharma. HeLa cells were transfected using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. 5 μl 20 μM siRNA and 5 μl Lipofectamine 2000 were each added to 250 μl DMEM and mixed. After 20 min, the mixture was added to cells with 2 ml medium. The medium was replaced with fresh medium 24 h later. At 72 h after siRNA transfection, cells were collected and subjected to western blotting.

Cell culture, transfection and cell cycle synchronization

HEK 293T and HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM, GIBCO) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Plasmid transfections were performed at 40% cell confluency using Lipofectamine 2000 (Invitrogen). 2 μg plasmid and 5 μl Lipofectamine were added to 250 μl DMEM, respectively, and were mixed after 20 min. The mixtures were incubated with cells for 4 h, and then replaced with fresh medium.

For synchronization at different stages in G1 phase, mitotic cells were gently shaken from culture dishes and cultured on coverslips for 2, 4 and 6 h to collect early, middle and late G1 cells, respectively. Cells synchronized at the G1/S transition were obtained by a double-thymidine block. Briefly, cells were treated with 2.5 mM thymidine (Sigma) for 16 h, and then released for 12 h with fresh medium. Then, the cells were treated with 2.5 mM thymidine a second time for 16 h to acquire cells at the G1/S transition. After the double-thymidine block, the cells were released in fresh medium for 4 h to S phase, 8 h to G2 phase and 14 h to the next G1 phase cells.

In the 'Cdc6 depletion after synchronization' method, HeLa cells were synchronized to the G1/S transition by double-thymidine block and then transfected with Cdc6 siRNA in the presence of thymidine. The Cdc6-siRNA-transfected G1/S cells were kept in the thymidine medium for 68 h after siRNA transfection to keep the cells at the G1/S transition, and then were released to fresh medium for 4 h to S phase, or kept in the thymidine medium for 64 h after siRNA transfection, and then were released to fresh medium for 8 h to G2 phase, or kept in the thymidine medium for 58 h after siRNA transfection, and then were released to fresh medium for 14 h to the next G1 phase. Therefore, cells in S, G2 or the next G1 phase were collected 72 h after Cdc6 siRNA transfection. In this way, Cdc6 is specifically depleted in S, G2, or the next G1 phase.

In the 'Cdc6 depletion before synchronization' method, HeLa cells were transfected with Cdc6 siRNA. At 24 h after Cdc6 siRNA transfection, cells were treated with double-thymidine block (2.5 mM thymidine for 16 h, release for 12 h with fresh medium, and 2.5 mM thymidine a second time for 16 h) and released to fresh medium for 4 h to S phase. Alternatively, at 20 h after Cdc6 siRNA transfection, cells were treated with a double-thymidine block and released to fresh medium for 8 h to G2 phase, or at 14 h after Cdc6 siRNA transfection, cells were treated with double-thymidine block and released to fresh medium for 14 h to the next G1 phase. Therefore, cells in S, G2, or the next G1 phase were collected 72 h after Cdc6 siRNA transfection. In this way, Cdc6 is depleted in asynchronized cells and afterwards Cdc6-depleted cells were synchronized to S, G2, or the next G1 phase.

For ActD treatment to inhibit Pol I or Pol II transcription, HeLa cells were treated with 100 nM or 2 nM ActD (A9415, Sigma) for 1 h. For DRB treatment to inhibit Pol II transcription, HeLa cells were treated with 50 μM DRB (D1916, Sigma) for 1.5 h.

Co-immunoprecipitation

2 μg antibody or IgG was incubated with 25 μl protein-A-conjugated Sepharose beads (Amersham) in 500 μl phosphate-buffered saline (PBS)

for 2 h at 4°C with agitation. Cells were concurrently lysed in immunoprecipitation buffer [50 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4 and 1 mM PMSF, 0.5% NP-40 and protease inhibitors (Roche)] for 15 min on ice and cleared by centrifugation at 12,000 ***g*** for 15 min. The antibody-conjugated protein A beads were added to the lysate and incubated for 2 h at 4°C. After extensive washing with the immunoprecipitation buffer, the beads were analyzed by SDS-PAGE and western blotting. For immunoprecipitation from the nuclear extracts, 1 mg of HEK 293T cell nuclear extract was treated with 10 U DNase I (Roche) for 10 min at room temperature or left untreated, and incubated with 2 µg antibody in 500 µl immunoprecipitation buffer (20 mM Tris-HCl, pH 7.5, 325 mM NaCl, 2 mM EDTA and 2 mM EGTA, 1% Triton-X-100 and protease inhibitors) overnight at 4°C. 40 µl Protein-A- and -G-conjugated Sepharose beads (Amersham) (2:1) were added to the nuclear extracts and incubated for 4 h at 4°C, washed extensively, and analyzed by SDS-PAGE and western blotting.

Immunofluorescence microscopy

For immunofluorescence, HeLa cells were fixed with −20°C precooled methanol (100%) for 5 min and processed for immunofluorescence microscopy with the indicated antibodies. Images were taken with a Zeiss Axiovert 200M microscope with a Zeiss MRM CCD camera and analyzed using Axiovision image acquisition software. Alternatively, images were taken with a confocal microscope Zeiss LSM 710NLO, and analyzed by Zen image acquisition software, or with an UltraView VoX spinning disc confocal microscope (PerkinElmer Inc., USA) for live-cell imaging. To quantify the percentage of cells with Cdc6 nucleolar localization, 200 cells were counted in each experiment. The results are presented as the mean±s.d. from three independent experiments. Statistical significance was assessed by a two-tailed Student's *t*-test and is presented as $*P<0.05$, $**P<0.01$ and $***P<0.001$.

Quantitative reverse transcriptase PCR

Total RNA was extracted and 1 µg RNA was reverse transcribed using M-MLV reverse transcription kit (Invitrogen). qRT-PCR was performed with SYBR Green mix (Takara) on a LightCycler II Thermal Cycler (Roche). The synthesis of 45S rRNA was detected using primers extending from +307 to +325 (5′-GTCAGGCGTTCTCGTCTC-3′) and from +442 to +424 (5′-GCACGACGTCACCACATC-3′) of the 45S rRNA gene. 45S rRNA gene synthesis was normalized to GAPDH synthesis. The quantitative PCR results were analyzed by LightCycler software version 3 which was purchased with the LightCycler II Thermal Cycler. The results are presented as the mean±s.d. from three independent experiments. Statistical significance was assessed by a two-tailed Student's *t*-test and is presented as $*P<0.05$, $**P<0.01$ and $***P<0.001$.

Chromatin immunoprecipitation

ChIP experiments were performed as described previously (Xie et al., 2012). Briefly, 2×10^8 HEK 293T cells with 75% confluency were fixed for 15 min with 1% formaldehyde, suspended in 200 µl of 1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1, and sonicated to yield 0.5–1 kb DNA fragments. 25 µg chromatin was pre-cleared with protein-A/G–agarose in the presence of 20 mg/ml sonicated salmon sperm DNA for 1 h at 4°C and immunoprecipitated overnight with 4 µg antibodies. After elution from protein-A/G–agarose and reversion of crosslinks by heating for 6 h at 65°C, 3% of purified DNA was amplified for 30 cycles (30 s at 95°C, 40 s at 55°C and 40 s at 72°C) in the presence of 1.5 mM $MgCl_2$, 200 µM dNTPs and 10 pmol primers. PCR products were visualized on ethidium-bromide-stained agarose gels in Fig. 3D,F or analyzed by qRT-PCR in Figs 3E,G and 6A. For primers extending the promoter regions of rRNA genes, two sets of primers extending different parts of rDNA promoter regions were used. 'H42.9' was used to extend from −57 to +33 (sense, 5′-CCCGGGGGA-GGTATATCTTT-3′; antisense, 5′-CCAACCTCTCCGACGACA-3′) as reported previously (O'Sullivan et al., 2002); 'P' was used to extend from −126 to +20 (sense, 5′-GTTTTTGGGGACAGGTGT-3′; antisense, 5′-CCAGAGGACAGCGTGTCAGCA-3′) (Ayrault et al., 2006). $RD^{INK/ARF}$ primer sequences were: sense, 5′-GAGATTGAGAGTGGCAACCT-3′; antisense, 5′-CATGGTGGTGGCACTTCCAC-3′ as reported previously (Gonzalez et al., 2006). *GAPDH* primer sequences were: sense, 5′-TCC-ACCACCCTGTTGCTGTA-3′ and antisense, 5′-ACCACAGTCCATGC-CATCAC-3′ (Frescas et al., 2007). Other primers for human rRNA genes were as reported previously (O'Sullivan et al., 2002). In detail, H1 was used to extend 952-1030 (sense, 952-972; anti-sense, 1010-1030). H4 was used to extend 3990-4092 (sense, 3990-4010; anti-sense, 4072-4092). H7.9 was used to extend 8204-8300 (sense, 8204-8224; anti-sense, 8280-8300). H13 was used to extend 12855-12970 (sense, 12855-12875; anti-sense, 12950-12970). H18 was used to extend 18155-18280 (sense, 18155-18175; anti-sense, 18260-18280). H27 was used to extend 27366-27477 (sense, 27366-27386; anti-sense, 27457-27477). The sequence coordinates are derived from GenBank accession no. U13369.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

C.M.Z., Q.J., S.J.H., X.W.X. and W.T. conceived and designed the study; S.J.H., X.W.X., G.P.W., G.L.L. and W.B.X. performed the experiments; C.M.Z., Q.J., S.J.H., X.W.X. and H.Y.Z. wrote the manuscript.

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Supplementary information

Supplementary information available online at
http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.178723/-/DC1

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