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Spatio-temporal regulation of the human licensing factor Cdc6 in replication and mitosis

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Abbreviations: APC, anaphase promoting complex; FRAP, fluorescence recovery after photobleaching; MCM, minichromosome maintenance complex; ORC, origin recognition complex; ori, origin of replication; PCNA, proliferating cell nuclear antigen; preRC, pre-replicative complex; STR, small tandem repeat

To maintain genome stability, the thousands of replication origins of mammalian genomes must only initiate replication once per cell cycle. This is achieved by a strict temporal separation of ongoing replication in S phase, and the formation of pre-replicative complexes in the preceding G1 phase, which "licenses" each origin competent for replication. The contribution of the loading factor Cdc6 to the timing of the licensing process remained however elusive due to seemingly contradictory findings concerning stabilization, degradation and nuclear export of Cdc6. Using fluorescently tagged Cdc6 (Cdc6-YFP) expressed in living cycling cells, we demonstrate here that Cdc6-YFP is stable and chromatin-associated during mitosis and G1 phase. It undergoes rapid proteasomal degradation during S phase initiation followed by active export to the cytosol during S and G2 phases. Biochemical fractionation abolishes this nuclear exclusion, causing aberrant chromatin association of Cdc6-YFP and, likely, endogenous Cdc6, too. In addition, we demonstrate association of Cdc6 with centrosomes in late G2 and during mitosis. These results show that multiple Cdc6-regulatory mechanisms coexist but are tightly controlled in a cell cycle-specific manner.

Introduction

Due to their size, mammalian genomes are replicated from thousands of independent origins of replication. To maintain genome stability, it is mandatory that each origin initiates replication only once per cell cycle. This is accomplished by a strict temporal separation of the formation of pre-replicative complexes (preRCs), which "license" each origin competent for replication in G1 phase, and the actual initiation of replication at the beginning of S phase. Origin licensing in G1 takes place in a dynamic and sequential manner:[1,2] The origin recognition complex (ORC) binds first to origins of replication, and origin-bound ORC then recruits the loading factor Cdc6. The ORC/Cdc6 complex is required for the stable loading of a preformed complex consisting of the second loading factor Cdt1 and the minichromosome maintenance (MCM2–7) complex. The cooperative loading of 2 MCM2–7 complexes results after ATP-dependent dissociation of ORC, Cdc6 and Cdt1[3] in the stable association of MCM2–7 double hexamers.[4] In S phase, the MCM complex provides the helicase activity required for DNA strand separation during DNA replication.

PreRC assembly is strictly regulated by a combination of overlapping mechanisms targeting individual preRC factors.[5-7] On the one hand, this regulation needs to allow sufficient preRCs to be assembled for a complete replication of the genome. On the other hand, it is as important to prevent further rounds of replication initiated after the beginning of S phase because re-replication of a previously fired origin would lead to the duplication of a chromosome segment and thus to genome instability. This regulation is mainly achieved by the modulation of specific preRC components via the interplay between cell cycle-specific phosphorylation, dephosphorylation and proteasomal degradation events.[7] For example, loading factor Cdt1 is phosphorylated by Cyclin A/Cdk2 during S phase, which targets it for ubiquitination by E3 ubiquitin ligases and subsequent proteasomal degradation. Its activity is additionally repressed from S to M phase by the presence of the Cdt1 inhibitor geminin.

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The cell cycle-dependent regulation of the Cdt1-interacting partner Cdc6 is less clear. It is well established that the stability of Cdc6 protein is regulated by phosphorylation at 3 canonical CDK sites within its N-terminal domain,[8,9] which depends on acetylation by the acetyltransferase GCN5 at specific residues nearby.[10] In quiescent mammalian cells the anaphase promoting complex (APC) E3 ubiquitin ligase provides for constant proteasomal degradation of Cdc6. Upon re-entry into the cell cycle, degradation in G1 phase is prevented by Cyclin E/Cdk2-dependent phosphorylation of Cdc6.[11] The same mechanism protects Cdc6 during G1 phase in proliferating cells,[12] and it has been proposed that degradation of Cdc6 takes place after replication licensing in early G1.[13,14] However, regulation of Cdc6 and Cdk2 appear to be interdependent. Activation of CyclinE/Cdk2 is required for entrance into S phase and initiation of DNA synthesis, but this activation does not take place when Cdc6 is missing.[15,16]

Phosphorylation also regulates the cellular localization of Cdc6. It has been demonstrated that phosphorylation of Cdc6 by Cyclin A/Cdk2 translocates the protein to the cytoplasm during S phase,[9,10,17-19] and some authors suggested that Cdc6 might first be exported to the cytoplasm, and then degraded in an APC-dependent manner.[20,21] This interpretation is however challenged by reports demonstrating that at least a fraction of Cdc6 protein remains nuclear and chromatin-bound even in S-phase.[13,20,22-25] The reason for this is currently believed to lie in a discrepancy between endogenous and exogenously expressed Cdc6. Investigations favoring the nuclear export model utilized transient heterologous overexpression of tagged Cdc6, which lead to the assumption that simply the excess and thus freely diffusible fraction of exogenous Cdc6 is exported, thereby masking the remaining nuclear pool of endogenous Cdc6.[5,6,20,26,27] This interpretation however was in turn challenged by one investigation that detected by immunstaining both endogenous and exogenously overexpressed Cdc6 either in the nucleus or in the cytoplasm in non-synchronized cells.[10]

An additional and so far ill-understood feature of human Cdc6 is its importance for mitotic cell division. It is shown that transient over-expression of Cdc6 blocks progression into mitosis by activation of a G2/M checkpoint.[28] In contrast, RNAi-mediated depletion of Cdc6 in HeLa cells did not affect the timely onset of mitosis, but the cells failed to complete cell division and underwent apoptotic cell death likely due to an abnormal mitotic spindle formation and misalignment of chromosomes.[21] Thus, Cdc6 could play a role in cell division other than control of replication and surveillance of cell division.

To gain further insight into these questions, we stably expressed Cdc6 fused to yellow fluorescent protein (YFP), and monitored Cdc6-YFP protein in its native environment in living human cells at precisely defined cell cycle phases. Coexpression of proliferating cell nuclear antigen (PCNA), an established marker of S-phase progression,[29] fused to cyan fluorescent protein (CFP) was used to identify different cell cycle stages in interphase. We demonstrate the exact chronology of cell cycle-specific origin licensing, Cdc6 degradation and nuclear export, and we show that Cdc6 interacts with centrosomes during mitotic cell division.

Results

Normal cell cycle-dependent regulation of recombinant Cdc6-YFP

To monitor Cdc6 in actively dividing cells, we stably expressed YFP- or GFP-fused Cdc6 in 3 different cell lines to exclude the possibility that results are based on a cell type-specific regulation of Cdc6. We selected clones from (i) the human fibrosarcoma cell line HT-1080, (ii) the adenovirus type 5-transformed human primary embryonal kidney cell line HEK293, and (iii) the newly established keratinocyte cell line HaSK-pw (Human adult Spontaneous Keratinocytes-p53 wild type) which spontaneously immortalized from primary skin keratinocytes by telomerase expression (Personal communication P. Boucamp, German Cancer Research Center, Heidelberg, Germany).

For the initial characterization of heterologous Cdc6 expression we used the C-terminal fusion Cdc6-YFP expressed in HT-1080 cells, but all findings regarding regulation and subcellular distribution were corroborated later on in the other cell lines and in addition by expressing an N-terminal fusion of Cdc6 to GFP as well (see below). Six puromycin-resistant clones (C1–C6) were selected in HT-1080 cells. To enable simultaneous microscopic monitoring of the various stages of S-phase, we additionally generated cell clone C/P, which stably coexpresses Cdc6-YFP and CFP-PCNA. Immunoblotting revealed expression levels of Cdc6-YFP ranging from about 1-fold (clones C1 and C3) to 10-fold the amount (C6) of endogenous Cdc6 (**Fig. 1A**). Clone C/P expressed a 27-fold excess of Cdc6-YFP over endogenous Cdc6 (rightmost lane in **Fig. 1A**, and lanes 5 and 6 in **Fig. 1B**), and similar amounts of Cdc6-YFP and CFP-PCNA are expressed (lane 2 in **Fig. 1B**). Interestingly, heterologous expression of CFP-PCNA apparently resulted in a down-regulation of endogenous PCNA yielding an unaltered net level of the protein. The amount of endogenous Cdc6, on the other hand, was unaltered in all transfected and untransfected HT-1080 cells (**Fig. 1A-B**).

We next addressed the concern that either the high quantity or the YFP-tag might perturb the regulation of Cdc6-YFP with respect to cell cycle-dependent protein stability. Previous reports demonstrated that Cdc6 levels vary as cells traverse through or re-enter a mitotic cell cycle. We find a comparable cell cycle-specific regulation of Cdc6 in HT-1080 cells used here. **Figure 1C** shows that untransfected HT-1080 cells (*left panel*) enriched in M phase by nocodazole blockade contained a significant amount of Cdc6 (*0h*). After release from the block cells entered G1 and proceeded to S phase within 6 hours. Similar to a previous report[14] this was accompanied by a decrease of Cdc6 protein after 1 to 2 hours and its reappearance after 6 hours. We analyzed the clone expressing the highest level of Cdc6-YFP (clone C/P) in the same way, and find that in clone C/P (**Fig. 1C**, *right panel*) both endogenous Cdc6 and overexpressed Cdc6-YFP were degraded and resynthesized in the same time frame as in untransfected HT-1080 cells. This suggests that both endogenous and YFP-tagged Cdc6 underlie the same mechanisms of degradation and re-synthesis in cycling cells. Degradation is shown to also take place in an APC-dependent manner when cells have exited the cell cycle into G0 phase. When cells are stimulated to re-enter the cell cycle, Cdc6 is

protected from destruction by phosphorylation at 3 canonical CDK sites in its N-terminal domain at Serine positions 54, 74 and 106. Since this takes place several hours after stimulation, it was concluded that Cdc6 stabilization takes place during S phase.[9-11,18] We wanted to test whether this regulation applies to YFP-tagged Cdc6, too. When untransfected HT-1080 cells (**Fig. 1D**, *top*) were enriched in G0 by serum starvation (*0h*), Cdc6 was not detectable on the Cdc6-specific immunoblot. After serum stimulation, cells proceeded into S phase (*16 h – 20 h*), and Cdc6 was expressed again. Use of phosphorylation-specific antibodies confirmed that this was accompanied by phosphorylation of Ser-54 and Ser-106. Analysis of clone C2 revealed the same regulation pattern for endogenous and YFP-tagged Cdc6 (**Fig. 1D**, *bottom*). These data indicate that heterologously expressed Cdc6-YFP is targeted to cell cycle-dependent degradation in same way as endogenous Cdc6, and that the important regulatory phosphorylation of the N-terminal domain is not impeded by fusion of YFP to the C-terminus of Cdc6 protein.

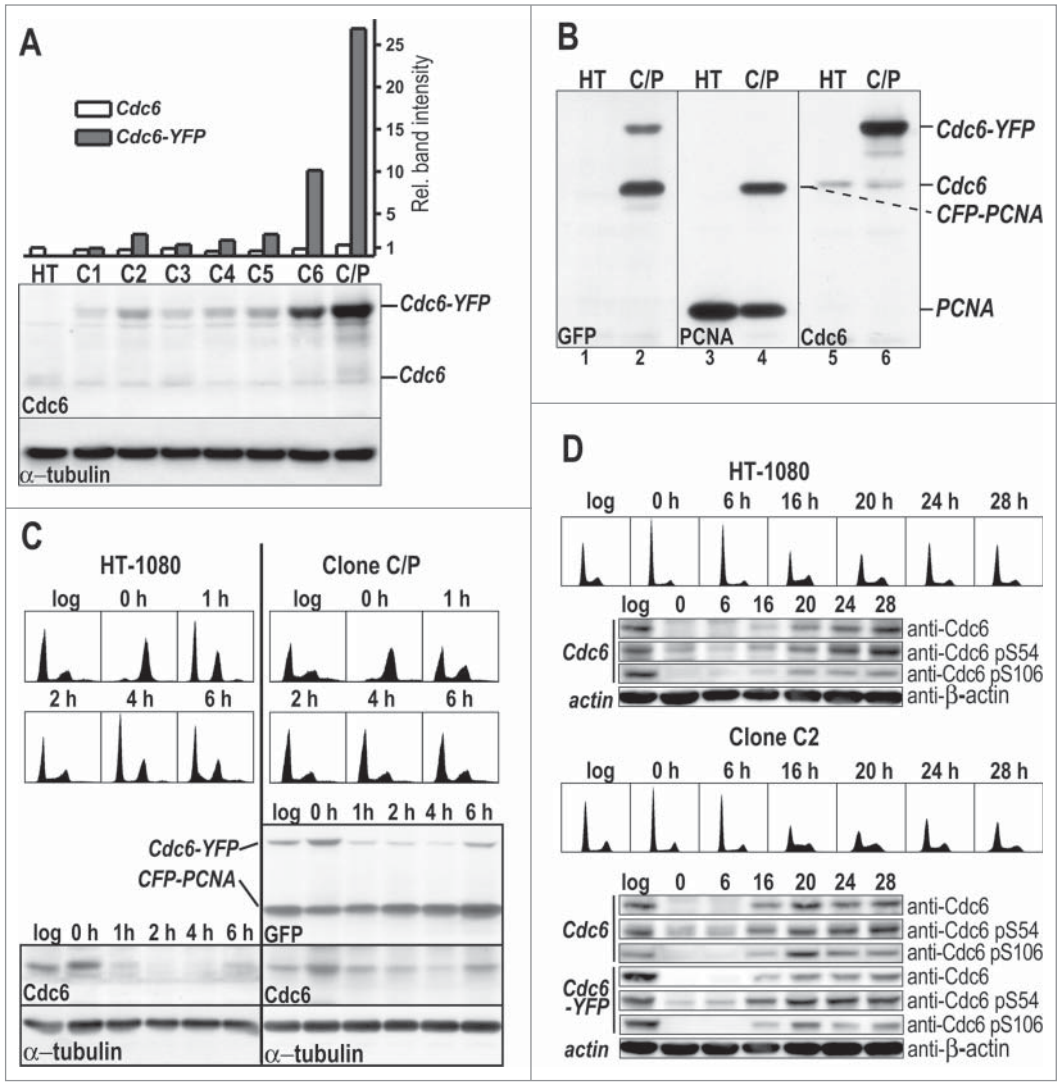

Figure 1. Expression, regulation and functional testing of Cdc6-YFP. (**A**) Total cell lysates of untransfected HT-1080 cells (*HT*), cell clones expressing varying levels of Cdc6-YFP alone (*C1-C6*), and a cell clone coexpressing CFP-PCNA and Cdc6-YFP (*C/P*) were subjected to Western blotting using specific antibodies for human Cdc6 (*top*) or α-tubulin (*bottom*), the latter serving as loading control. Positions of endogenous and YFP-fused Cdc6 are indicated. The diagram above the blots shows relative band intensities of Cdc6 and Cdc6-YFP in the respective clones. All bars are normalized to the intensity of the Cdc6 band in untransfected HT-1080 cells arbitrarily set to 1. (**B**) Further Western blot analysis of untransfected HT-1080 cells (*HT*) and the clone coexpressing CFP-PCNA and Cdc6-YFP (*C/P*) using anti-GFP, -PCNA, and -Cdc6 antibodies, respectively. The positions of endogenous and C/YFP-fused proteins are indicated. (**C**) Cultures of untransfected HT-1080 cells and cells of clone C/P were synchronized in mitosis by a nocodazole block followed by reseeding in fresh medium. At the indicated time points after nocodazole withdrawal, samples were harvested for flow-cytometric determination of their cell cycle distribution (*top*), and immunoblotting (*bottom*). *log*: Asynchronous, logarithmically growing cultures. Western blots of both untransfected cells (*left*) and cells of clone C/P (*right*) were probed with anti-Cdc6 and anti-α-tubulin antibodies. Extracts from clone C/P were in addition probed with anti-GFP antibody. (**D**) Untransfected HT-1080 cells and cells of clone C2 were blocked in G0 by serum starvation, harvested at the indicated time points after serum stimulation, and analyzed by flow cytometry (*upper panels*) and immunoblotting (*lower panels*). Asynchronous, logarithmically growing cultures were also analyzed (*log*). The blots show the levels of Cdc6, Cdc6-YFP and β-actin as indicated on the left side. Cdc6 was detected with antibodies specific for Cdc6, or Cdc6 phosphorylated at Serine 54 and Serine 106, respectively.

Degradation and nuclear export of Cdc6 are temporally separated events

To visualize the consequences of the observed destruction and reappearance of Cdc6-YFP as cells proceed from metaphase through interphase (**Fig. 1C**), we imaged the protein over time in living cells. **Figure 2A** shows as an example clone C1 expressing relatively low levels of Cdc6-YFP. In metaphase (**Fig. 3A**, *0 min*), Cdc6-YFP was associated almost exclusively with condensed chromosomes. Association persisted from anaphase (*15 min*) to telophase (*30 min*), at which time point the nuclear membrane reforms. Consequently, Cdc6-YFP was nuclear

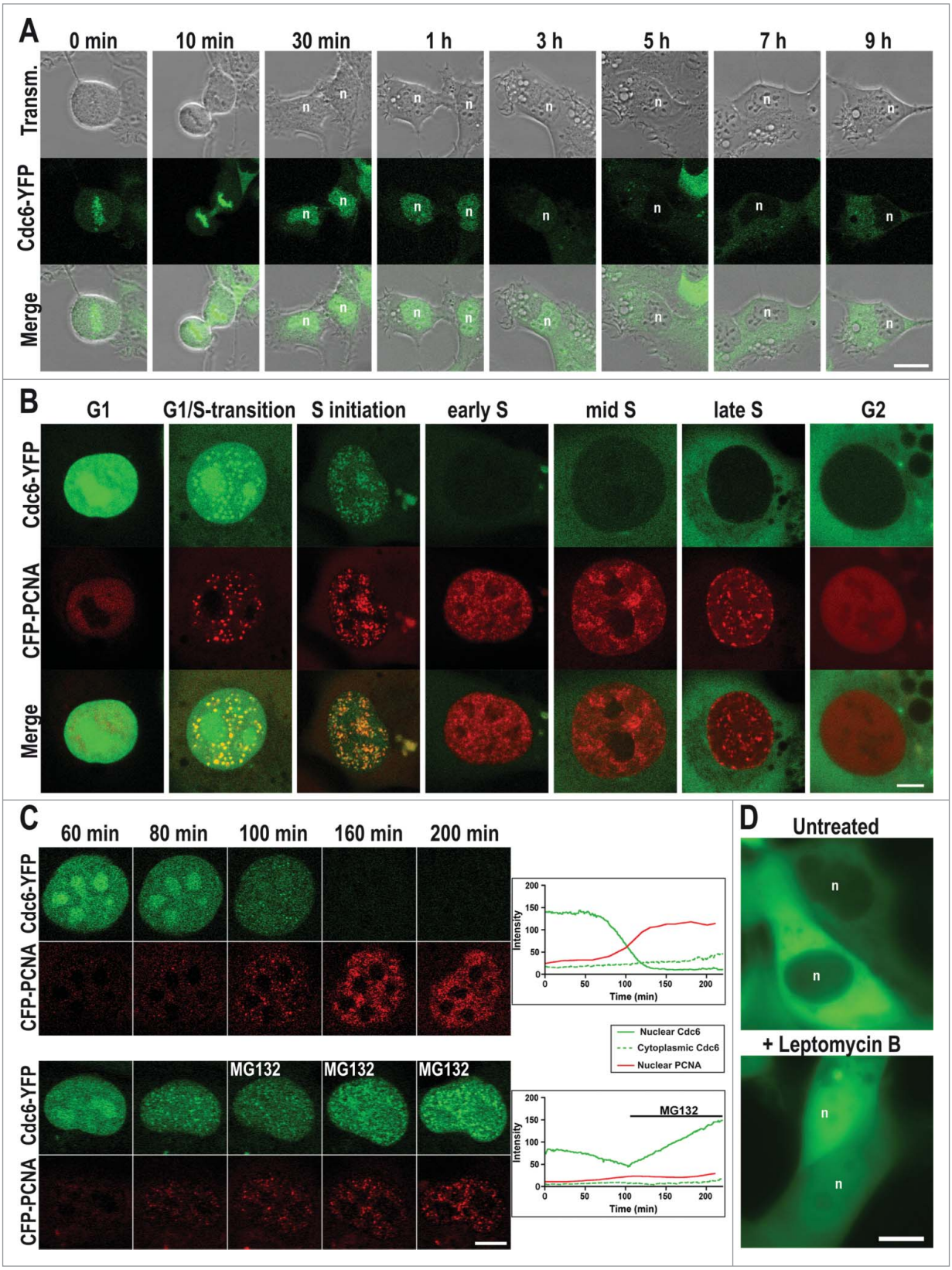

Figure 2. For figure legend, see page 1708.

during the following G1 phase (*1 h*). After three hours, however, the fluorescent signal disappeared, likely due to proteasomal destruction during G1. After five hours, Cdc6-YFP-specific fluorescence reappeared and gradually increased in intensity during the following hours, but this time it was localized in the cytoplasm. All other HT-1080 cell clones displayed the same time-dependent changes in the sub-cellular distribution of Cdc6-YFP, even those with strongly elevated Cdc6-YFP expression levels (not shown). The same was true for Cdc6-GFP in HaSK-pw and HEK293 cells (Supplemental **Figure S1 B**). Similarly, the N-terminal fusion protein GFP-Cdc6 stably expressed in several clones of HT-1080 and HaSK-pw cells was chromosomally localized in mitosis, resided afterwards in the nucleus for up to one hour, disappeared, and reappeared in the cytosol after several hours (Supplemental **Figure S1 C**). It is worth noting, however, that binding to mitotic chromosomes of N-terminally fused GFP-Cdc6 was not as complete in both cell lines; a substantial fraction resided in the cytoplasm during mitosis. Since this was the only difference to all other cells expressing the C-terminal fusion protein, we assume that chromosomal binding in mitosis and time-dependent redistribution in the following cell cycle phases reflect a general regulation of Cdc6 in cycling cells. Taken together, these observations indicate that the reported proteasomal degradation of Cdc6[13,14] and its active export from the nucleus[9,17-19] do not exclude each other but rather are events temporally separated from each other in the course of interphase.

To determine in greater detail the cellular distribution of Cdc6-YFP with respect to cell cycle phases G1, S, and G2, we monitored clone C/P coexpressing Cdc6-YFP and CFP-PCNA. We first confirmed that CFP-PCNA expressed in HT-1080 conform to established distribution changes as cells proceed through interphase[29,30] (Supplemental **Figure S2**). We then acquired numerous high-resolution confocal images of single cells from clone C/P. **Figure 3B** shows a set of representative images. In G1, Cdc6-YFP was exclusively nuclear and enriched to some extent in the nucleoli, whereas fluorescence of CFP-PCNA was weak, also nuclear, but mostly excluded from nucleoli. When cells proceeded from G1 to S phase, CFP-PCNA accumulated in so-called replication foci[29,30] (**Fig. 2B**, *second column*). Surprisingly, Cdc6-YFP as well concentrated in each of these foci, but this association of Cdc6-YFP with replicating chromatin did not persist during the S phase. Cdc6-YFP concentration decreased in the course of S phase initiation (*third column*) and was entirely lost from the cells at early stages of DNA replication, which are characterized by an accumulation of CFP-PCNA in numerous small foci (*fourth column*).

We investigated the destruction of Cdc6-YFP at the G1 to S phase transition in greater detail. Imaging of single cells during S phase initiation revealed that the first appearance of CFP-PCNA-labeled replication foci directly accompanies the onset of Cdc6-YFP degradation (**Fig. 2C**). Quantification of fluorescence intensities (**Fig. 2C**, *right panel*) corroborated the exact concurrence of Cdc6-YFP degradation with the increase of nuclear CFP-PCNA, a hallmark of the beginning of S phase. Direct evidence that Cdc6-YFP destruction was due to proteasomal degradation comes from an experiment where the specific proteasome inhibitor MG132 was added to the culture medium at a time point, at which Cdc6 degradation had just started (**Fig. 2C**, *bottom row*). Cdc6-YFP degradation was prevented in the presence of MG132, and Cdc6-YFP signal intensity rose again as a consequence of ongoing protein expression. Of interest, adding MG132 to the medium when cells were at the G1/S restriction point also prevented the increase of CFP-PCNA protein, which suggests that inhibition of the proteasome prevented further progression into S phase. Further experiments, where MG132 incubation times were extended to up to 5 hours revealed that this block of cell cycle progression was irreversible. Cdc6 continued to accumulate in the nucleus, and the return to an even distribution of CFP-PCNA demonstrated that cells did not progress into S phase (data not shown). It must be pointed out that the proteasome serves for numerous functions during the cell cycle, and our observation that inhibiting the proteasome leads to a block of S phase entry and progression is likely the result of interference with a number of different regulatory processes. Hence we cannot deduce from our experiments whether Cdc6 biology is involved at all.

Figure 2B (*early to late S*) further shows that during the course of S phase the Cdc6-YFP protein level gradually increased again and peaked in G2 phase. In contrast to G1 phase, the majority of the protein was now detected in the cytoplasm with no or very little YFP fluorescence in the nucleus. It is important to note that here we monitor the total cellular pool of Cdc6-YFP. It is therefore possible that a minor fraction of the protein remains chromatin-bound to serve important nuclear functions during S phase. In fact, close inspection of mid-S phase cells of the high expressing clone C/P in **Figure 2B** is suggestive of a faint nuclear Cdc6-YFP signal.

It has been suggested that cytoplasmic localization is due to an active nuclear export mediated by the nuclear transport receptor Crm1.[9] Also in our hands exclusive cytosolic localization of Cdc6-YFP during late stages of the cell cycle could as well be

Figure 2 (See previous page). Localization and stability of Cdc6-YFP changes over time. (**A**) Selected confocal images from a series of consecutive images taken every 15 min for a period of 9 hours of a single cell of clone C1. The suboptimal image quality is due to the low Cdc6-YFP expression level of this clone and the need for short exposure times to reduce irradiation-mediated cell damage. *n* marks the position of the nucleus. Bar, 10 μm. (**B**) Distribution of Cdc6-YFP at distinct stages of interphase: High resolution confocal images of different cells of clone C/P coexpressing Cdc6-YFP (pseudo-colored green) and CFP-PCNA (red) at selected cell cycle phases. Bar, 5 μm. (**C**) Confocal images of single cells of clone C/P starting at G1 were taken every minute. The upper row shows a cell left untreated as it proceeds into S phase, whereas the cell shown in the lower row was exposed to the proteasome inhibitor MG132 (100 μM) at the onset of S phase. The right panel shows a plot of fluorescence intensities of nuclear and cytoplasmic Cdc6-YFP, and of nuclear CFP-PCNA as they change over the time period of image acquisition. Bar, 5 μm. (**D**) Epifluorescent images of cells in S/G2 phase of clone C1 either left untreated (*top*) or treated for 2 h with 40 nM Leptomycin B. *n*, nucleus. Bar, 10 μm.

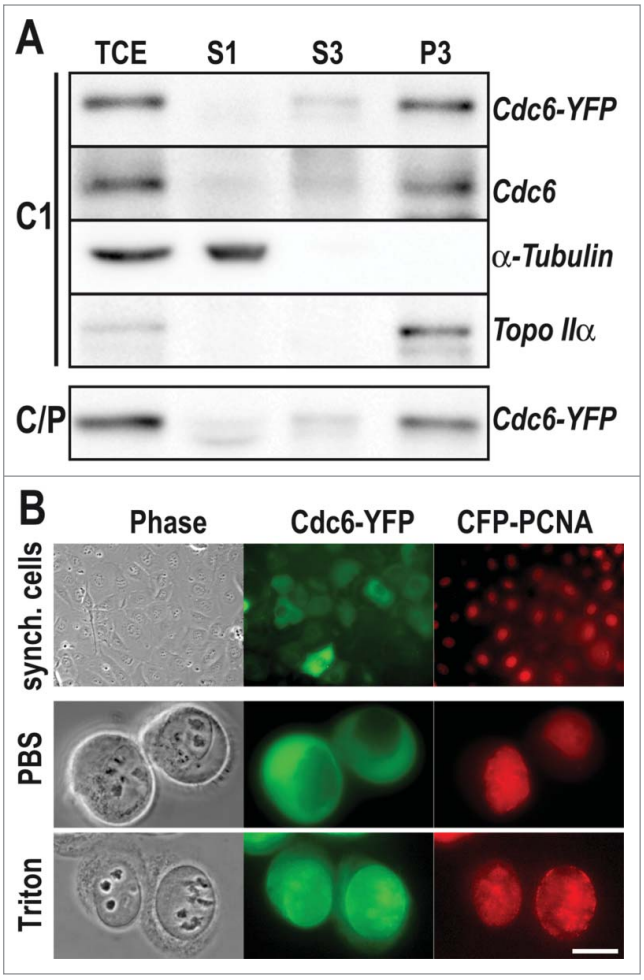

Figure 3. Cdc6-YFP resides in fractionated chromatin preparations of S-phase cells. Clones C1 and C/P were synchronized in early S phase by a double thymidine block, and released from the block for 4 h. Efficient synchronization in S/G2 was confirmed by flow cytometry (not shown). Fluoresence-microscopic visual inspection revealed more than 95% cells with cytoplasmic Cdc6-YFP. (**A**) Synchronized cell clones were subjected to the biochemical fractionation protocol for chromatin isolation as described.[13] Cells were harvested and suspended on ice in the hypotonic buffer A, and lysed by addition of the non-ionic detergent Triton X 100. Nuclei were separated from the cytosolic supernatant (*S1*) by centrifugation, lysed in no-salt buffer, and solubilized nuclear proteins (*S3*) were separated by centrifugation from the chromatin-enriched fraction (*P3*). Presence of Cdc6-YFP and endogenous Cdc6 in the total cell extract (*TCE*), cytoplasmic fraction (*S1*), soluble nuclear fraction (*S3*), or the chromatin-enriched fraction (*P3*) was determined by Western blotting. Probing against cytoplasmic α-tubulin and nuclear topoisomerase IIα confirmed the efficiency of the biochemical fractionation procedure. The lowest panel shows the result for overexpressed Cdc6-YFP from clone C/P. (**B**) Clone C/P was synchronized in early S phase by a double thymidine block, released from the block for 4 h to yield a culture of predominantly S-phase cells, and images of the cells and corresponding fluorescence were acquired in the culture flask using a 10x/0,25 NA objective (*upper panel*). After trypsin harvest, an aliquot was resuspended in PBS and the same image set was taken from cells on a glass slide using a 63x/1.4 NA oil immersion objective (*middle panel*). Another aliquot was suspended in buffer A, Triton X 100 was added, and cells were again inspected by epifluorescence microscopy (*lower panel*). Note that all cells displayed the same distribution of Cdc6-YFP and CFP-PCNA as in the representative examples shown. Bar, 10 μm.

reverted into a more nuclear localization when cells were treated with the specific Crm1 inhibitor Leptomycin B (**Fig. 2D**).

In an attempt to verify that the features of Cdc6-YFP distribution described above resemble the behavior of endogenous Cdc6, we tried to determine the subcellular localization of Cdc6 by immunostaining of untransfected HT-1080 cells with Cdc6-specfic antibodies. We first applied different fixation protocols on Cdc6-YFP-expressing cells, and learned that subsequent fixation in frozen Methanol and Acetone preserved Cdc6-YFP distribution best (Supplemental **Figure S3 A**). Applying this protocol on native HT-1080 cells revealed that endogenous Cdc6 is detected on mitotic chromosomes, that in some cells it resides in the nucleus and concentrates in nucleoli, and that Cdc6 is mostly cytoplasmic to a varying extent in many other cells (Supplemental **Figure S3 B**). This suggests that endogenous Cdc6 as well adopts most of the cell cycle-dependent, subcellular distribution patterns of Cdc6-YFP.

When heterologously expressed, tagged Cdc6 is imaged by fluorescence microscopy, the protein is consistently detected in the cytoplasm of cells in S/G2 phase (Refs.9, 17-19, 31 and this report). However, when endogenous Cdc6 was investigated after biochemical fractionation of nucleus and cytoplasm of S/G2 phase cells, a significant percentage of Cdc6 was detected in the nuclear fraction as analyzed by Western blotting.[9,13,20,23-25] Thus, we wanted to analyze how stably expressed exogenous Cdc6-YFP behaves in a biochemical cell fractionation and chromatin extraction procedure. Cells of a low and a high expressing clone (C1 and C/P) were synchronized in early S phase by a double thymidine block, and then released from the block for 4 hours to yield a culture of predominantly S-phase cells. These cells were harvested and chromatin was extracted as described.[13] **Figure 3A** demonstrates that, also in our hands, endogenous Cdc6 was significantly enriched in fraction P3 representing proteins bound to nuclear chromatin in S phase-cells, and the same was true for YFP-fused Cdc6 in both clones. This indicates that the extent of overexpression had no impact on the result. However, since chromatin association of Cdc6 in S phase does not fit to our observations in living cells (see also **Figs. 2A and B**) where Cdc6-YFP was mostly excluded from the nuclei, we examined the fractionation procedure by fluorescence microscopy. Imaging of an S phase-synchronized culture (**Fig. 3B**, *upper panel*) verified that almost all cells displayed the expected localization of Cdc6-YFP in the cytoplasm. This was also the case after trypsin harvest and re-suspending the cells in isotonic buffer (*middle panel*). However, at the first step of the fractionation procedure – lysis of cellular and nuclear membranes by the non-ionic detergent Triton X100 - normally cytosolic S-phase Cdc6-YFP was found to be nuclear (*lower panel*). This observation and the fact that Cdc6-YFP remained chromatin-bound during all subsequent extraction steps (**Fig. 3A**) suggests that Cdc6 retains a strong chromatin affinity despite its localization in the cytosol of S-/G2 phase cells. It also indicates that the assumed difference between endogenous and heterologous Cdc6 was mainly due to different methods of detection. Thus, endogenous Cdc6 may undergo the same fate during extraction as Cdc6-YFP and may be exported to the cytoplasm as well in unperturbed S-phase cells. Nevertheless,

it must be noted that some experimental approaches applied the established chromatin fractionation procedure,[13] and detected some Cdc6 in the cytoplasmic fraction.[10,20] The reason for such different experimental outcomes is unclear, but these examples show that differential extraction can successfully be applied to substantiate, for example, observations on the impact of post-translational modifications on the subcellular distribution of Cdc6.[10]

Association of Cdc6-YFP with centrosomes and during mitosis

In **Figure 2B** (*rightmost image*) a point-shaped structure of high fluorescence intensity of Cdc6-YFP close to the nucleus stands out. We observed this in all low and high expressing cell clones, when Cdc6-YFP was enriched at the end of G2. We assumed that it could reflect an association of Cdc6 with the centrosome. Immunohistochemical detection of the centrosomal marker γ-tubulin confirmed that the punctual enriched subpopulation of Cdc6-YFP indeed co-localized with the centrosome (**Fig. 4A**). To exclude that this enrichment was an artifact of Cdc6-YFP expression or cell line-specific, we co-immunostained endogenous Cdc6 and γ-tubulin in non-transfected HT-1080 cells and in primary non-transformed MRC-5 cells (**Fig. 4B**). The images in **Figure 4B** show representative examples of cells displaying co-

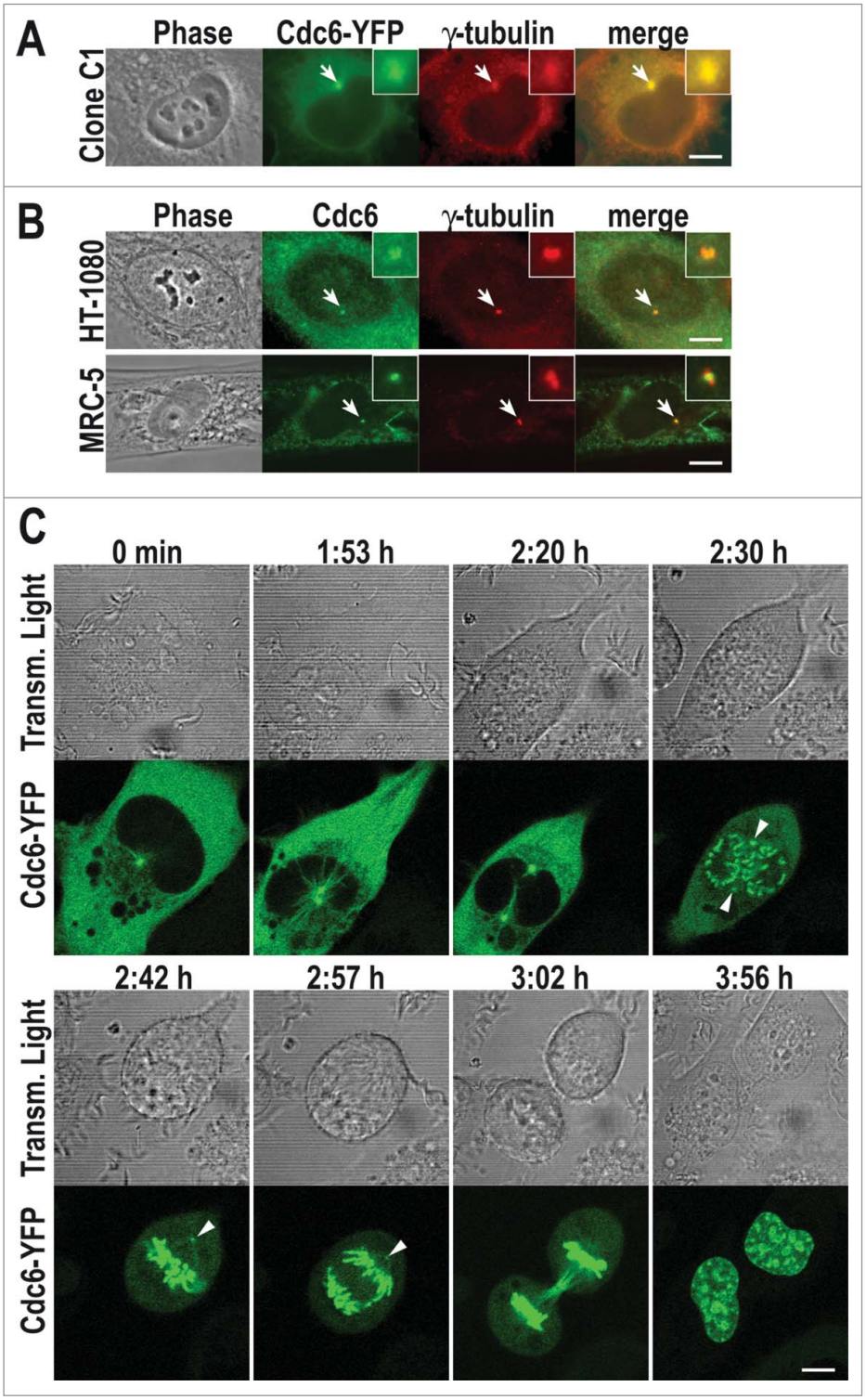

Figure 4. Distribution of Cdc6-YFP during late G2 and M phase. (**A**) The punctual accumulation of Cdc6-YFP co-localizes with the centrosomal marker γ-tubulin. The images show a representative cell of clone C1 expressing low levels of Cdc6-YFP (*green*) that was fixed and stained with antibodies recognizing endogenous γ-tubulin (*red*). All G2 cells in a specimen, which displayed the punctual Cdc6-YFP accumulation, showed co-localization with γ-tubulin, and the same result was obtained with high expressing clones C6 and C/P (not shown). *Left*: Phase contrast image; *Right*: Merge of the 2 fluorescent images. (**B**) Co-localization of endogenous Cdc6 with γ-tubulin. The pictures show a phase contrast image of untransfected HT-1080 (*top row*) and MRC-5 cells (*bottom row*), which were co-immunstained with antibodies against endogenous Cdc6 (*green*) and endogenous γ-tubulin. The rightmost image is a merge of the latter 2. (**C**) Clone C6 expressing high levels of Cdc6-YFP was cultured under a confocal microscope, and images of a single cell were taken either every minute or, during fast mitotic events, every 30 seconds. Selected images of the transmitted light and YFP fluorescence are shown. The whole sequence of events can be viewed in the supplemented time-lapse movie. Acquisition of yellow fluorescence was intentionally overexposed to reveal the weak centrosome staining (*arrowheads*) from pro- to anaphase. Bars, 5 μm.

localization of endogenous Cdc6 and centrosomal γ-tubulin. In about 4% of all HT-1080 cells and 1% of the slower growing MRC-5 cells we detected co-localization of Cdc6 and γ-tubulin. When both cell lines were arrested in late G2 by treating growing cultures with the CDK inhibitor RO-3306, co-localization of Cdc6 and γ-tubulin was detectable in almost all cells of both cell lines (not shown). These data indicate that endogenous Cdc6 as well associates with the centrosome in late G2. In addition, we detected centrosomal staining also in HEK 293 and HaKS-pw cells in mitosis and G2 phase, and with N-terminal GFP-Cdc6 fusions as well (Supplemental **Figure S4**).

We next investigated in greater detail the behavior of the protein in cells progressing from G2 through mitosis. Time-lapsed imaging by confocal microscopy (**Fig. 4C**; Supplemental movie) revealed that the prominent centrosomal association of Cdc6-YFP persisted during prophase when the duplicated centrosomes separate and move to opposite sides of the nucleus (**Fig. 4C**, 1:53 – 2:20 h). Association of Cdc6-YFP was not restricted to centrosomes but extended markedly to the microtubules extending from them. When the 2 centrosomes reached opposing poles of the nucleus, the nuclear envelope broke down at prometaphase (*2:30 h*), which led to an immediate relocation of Cdc6-YFP from the cytosol to the condensing chromatin. Within minutes almost all Cdc6-YFP became chromosome-bound and remained so until G1 phase. Noteworthy, a clearly detectable fraction continued to associate with centrosomes and microtubules until anaphase (*2:57 h*), albeit to a much reduced extent. In telophase (*3:02 h*), however, centrosomal Cdc6-YFP was no more detectable. Instead, the appearance of YFP-labeled fibrous structures adjacent to the forming cleavage furrow of the daughter cells, which is typical for overlap microtubules at this point of cytokinesis, indicates that Cdc6-YFP remained associated with microtubules until cells were finally separated. Finally, in late telophase/G1 (*3:56 h*), when the nuclear envelope had reformed, Cdc6-YFP was exclusively nuclear and no residual staining at centrosomes or in the cytoplasm was detectable anymore. Importantly, neither the immunostainings shown **Figure 4B**, nor those applied on G2-arrested cells did indicate any association of endogenous Cdc6 with microtubules in fixed cells. Thus we cannot draw a definite conclusion from our observations in living cells whether or not Cdc6 binds to microtubules during mitosis.

In summary, our findings demonstrate a direct interaction of Cdc6-YFP with centrosomes in G2 and throughout mitosis, and they define the time point, at which Cdc6-YFP switches back from its cytosolic to a chromosome-associated state, to the mitotic breakdown of the nuclear envelope.

Immobilization of Cdc6-YFP in telophase

We show here that Cdc6-YFP is chromatin-associated from prophase until the beginning of S-phase. It is however currently not well defined when precisely during this time licensing takes place. A well established concept of nuclear architecture postulates that binding of mobile proteins to immobile components of the nucleus (e.g. genomic DNA) retards their overall mobility, which can be assessed by measuring the fluorescence recovery after photobleaching (FRAP) of GFP chimera of such proteins.[32] In FRAP experiments the fluorescence of GFP-labeled proteins is bleached by a brief laser pulse in a defined area in the nucleus, and then exchange rates of bleached GFP chimera with the surrounding pool of fluorescent chimera are measured by consecutive imaging of the nucleus. These exchange rates are taken as a measure of the overall mobility of the protein in a particular subcellular region. We reasoned that photobleaching could be suitable to narrow down the time point of replication licensing more precisely in living cells, since the mobility of Cdc6 should decrease during MCM loading onto chromatin due to the

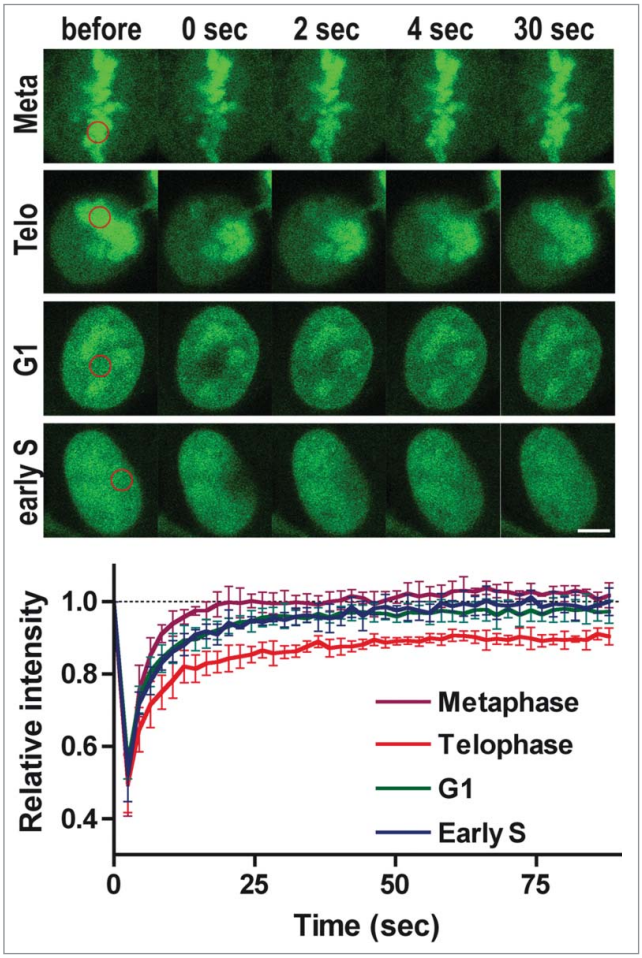

Figure 5. Immobilization of Cdc6-YFP in telophase. Images of cells at selected cell cycle phases expressing low levels of Cdc6-YFP (clone C1) were taken before and every 2 seconds after YFP was bleached in the indicated areas (*red circles*). Fluorescence intensities in the bleached regions were measured and expressed as the relative recovery over time in the plot below. Data of individual cells were normalized to a pre-bleach fluorescence level of 1. Error bars: Standard error of the mean (SEM) of at least 6 independent FRAP curves. To estimate statistical significant differences between FRAP recovery curves, we applied unpaired t tests to sample means from all individual time points. The values of the telophase curve differed from all other curves with a probability of $P < 0{,}0001$. Differences between metaphase, G1-, or early S phase were not significant with the exception of the first 20 seconds FRAP recovery on metaphase chromosomes which differed from the other 2 curves with mean probabilities of $p = 0{,}0109$ (Meta- vs. G1 phase) and $p = 0{,}0335$ (Meta- vs. early S-phase). Bar, 5 μm.

interaction of Cdc6 with chromatin-bound ORC. **Figure 5** summarizes the results of FRAP measurements of the mobility of Cdc6-YFP in metaphase, telophase, G1 phase, and at the onset of S-phase. We found that fluorescence recovery in the bleached area (due to constant exchange with surrounding unbleached molecules) was fastest in metaphase chromosomes indicating a short residence time of Cdc6-YFP on chromosomes. In comparison, recovery was somewhat retarded in G1 and early S phase, but in all 3 cases the fluorescence intensity rapidly returned to its initial value within 20 – 40 seconds. Only in telophase, recovery was incomplete. About 10% of Cdc6-YFP did not exchange with the remaining protein pool within 90 seconds, which implies that in telophase a substantial fraction of Cdc6-YFP stably associated with genomic DNA over an extended period of time. We interpret this significant retardation as the major time frame at which Cdc6-YFP is mostly involved in the licensing process, which does not exclude, however, that Cdc6-aided licensing takes place at the other phases, albeit to a likely reduced extent.

Discussion

We present here a detailed analysis of the intracellular localization and regulation of fluorescently labeled Cdc6 during the entire cell cycle. We find that degradation and nuclear export of Cdc6 are temporally separated events. Cdc6 protein present in the cell nucleus at the onset of S phase is subjected to complete proteasomal degradation, whereas Cdc6 protein synthesized from then on until the next cell division is excluded from the nucleus by continuous Crm1-dependent export. Thus, degradation and nuclear export regulate the nuclear availability of Cdc6 independently of each other and at different cell cycle stages. We further show for the first time that Cdc6 co-localizes with centrosomes before and during mitosis, which suggests a second, replication-independent function of Cdc6 in the light of reported mitotic malfunctions in the absence of Cdc6.[21]

The life cell imaging of labeled Cdc6 reveals that the protein has access to chromatin from mitosis to early S phase. The FRAP technique allowed detection of distinct mobility changes of Cdc6-YFP during this time. Since it is an established view that the mobility of nuclear chromatin-binding proteins is determined by their retention time on the relatively immobile chromosomal DNA,[32] we interpret the distinct decrease in mobility of Cdc6-YFP in telophase, as compared to the other cell cycle phases, as evidence that Cdc6 interacts with chromatin more often and/or longer during this phase. It is likely that the immobilization of Cdc6-YFP in telophase reflects the time frame at which most replication origins are licensed, since the second loading factor Cdt1,[33] the origin recognition complex ORC,[34] and human MCM proteins[13,24] are also shown to associate with chromatin mainly at the M/G1 transition. Of interest, it was recently shown that loading of the first MCM2–7 hexamer onto DNA occurrs within seconds, whereas the subsequent formation of a MCM2–7 double hexamer is slow and takes several minutes.[35] Consistently, we show here that about 10% of Cdc6-YFP was immobilized on chromatin for more than a minute during telophase suggesting that its engagement in loading MCM2–7 onto replication origins is a time-consuming process.

A major motivation of this work was to address the ongoing discussion whether Cdc6 is degraded, exported to the cytoplasm, or remains nuclear during S phase (see for example Ref. 36). With respect to a potential degradation, earlier work did not detect fluctuations of Cdc6 protein levels during the cell cycle.[9,17] Later on, however, it was shown that Cdc6 is targeted to proteasomal degradation when cells have exited the cell cycle into G0 phase. After re-entry into the cell cycle, Cdc6 is protected from destruction by phosphorylation probably during S phase.[9-11,18] To our knowledge, only one work investigated Cdc6 stability in cycling cells and showed that Cdc6 levels decline rapidly after releasing cells from a mitotic block. This was interpreted as Cdc6 degradation in G1.[14] Here we reproduce this data (**Fig. 1C**), and we specify the time point of proteasomal destruction precisely to the very beginning of DNA replication at the end of G1.

The probably biggest uncertainty concerned the question whether vertebrate Cdc6 during S phase is exported from the nucleus or whether a substantial fraction remains nuclear. This debate obviously stems from different experimental systems for the detection of endogenous versus 'ectopically' expressed, tagged Cdc6.[7,27,36] Here, we demonstrate that cytoplasmic YFP-tagged Cdc6 binds to chromatin preparations of S phase cells in the same way as endogenous Cdc6, and we provide evidence that this binding takes place during the preparation procedure when the nuclear membrane is lysed by detergents. The fact that the protein remains stably bound to chromatin during subsequent extraction procedures further implies that cytoplasmic Cdc6 retains a high affinity for chromatin. This interpretation is supported by the observation that, later on in mitosis, Cdc6-YFP relocates immediately to chromosomes as soon as the barrier of the nuclear envelope breaks down (**Fig. 4C**). On the basis of these data, we would like to suggest that in unperturbed S phase cells the majority of Cdc6 (exogenous and endogenous) is absent from the nucleus, although it binds to chromatin when given a chance (e.g., by inhibition of nuclear export or disruption of the nuclear envelope) due to an unaltered chromatin affinity of cytosolic Cdc6.

These findings raise the question why Cdc6 is kept in an active form in the cytoplasm by an energy consuming mechanism instead of being depleted from the cell like its binding partner Cdt1. Possibly Cdc6 serves function(s) between S- and M phase unrelated to origin licensing suggested for example by our observation that the protein localizes to centrosomes. Of interest, Cdc6 protein and the subunits of the Origin Recognition Complex, ORC are structurally related and belong to the AAA+ ATPase family. It has been shown that ORC subunits Orc1 – 5 co-localize with centrosomes as well.[37] Orc2 even resembles Cdc6 in that it also binds to the mitotic spindle apparatus.[38] Si-RNA-mediated depletion experiments further revealed a role for Orc1 in preventing centrosome reduplication in a single cell division cycle[39] and depletion Orc2 resulted in part in mitotic defects.[38] These data suggest that binding of ORC subunits to the centrosome plays a functional role during mitotic cell division, and possibly the same is true for cytoplasmic centrosome-

bound Cdc6, since RNAi-mediated ablation of Cdc6 resulted in abnormal spindle formation and chromosomal misalignment.[21]

Another putative reason for the continued presence of functional Cdc6 in the cytosol of S- and G2 phase cells could be its involvement in DNA surveillance. Evidence exists that Cdc6 is involved in the surveillance of the replication process during S phase via the checkpoint kinase Chk1,[28,40] and it was also shown, that Cdc6 is degraded in response to induced DNA damage during all cell cycle phases.[41] If Cdc6 plays a role in these processes it must either remain available in the cell nucleus during S phase at low levels, or it relocates to the cell nucleus in response to signals induced by stalled replication forks or extrinsic DNA damage. Future work is required to examine these aspects of Cdc6 regulation and their functional consequences.

In synopsis with the current knowledge about replication licensing and Cdc6 regulation[7,27,36] we suggest the following chronology of regulatory events affecting Cdc6 (**Fig. 6**): Upon breakdown of the nuclear envelope in prophase, Cdc6 gains access to condensing chromosomes. Given the high chromatin affinity of Cdc6, a dynamic binding equilibrium establishes throughout mitosis, where most of the protein is chromosome bound while a minor fraction binds to centromers. The phosphorylation status of Cdc6 during this period is currently not clear. In the subsequent G1 phase, however, Cdc6 is acetylated by GCN5[10] and subsequently phosphorylated by Cyclin E/Cdk2, which protects it from ubiquitinylation by APC^{Cdh1} and subsequent degradation.[11,12] Cdc6 thus stabilized remains in the cell nucleus from telophase to the beginning of S phase. During this time it participates in loading MCM2–7 onto origins of replication with the major licensing activity taking place at telophase. Upon initiation of replication in early S phase, Cdc6 colocalizes with early, PCNA-labeled sites of replication, and is concurrently degraded by the proteasome. Loss of protection from the APC^{Cdh1} (or other ubiquitin ligases) could involve dephosphorylation of Cdc6 – an activity that could be provided by protein phosphatase PP2A, which targets to Cdc6 and is critical for proper progression from G1- to S phase.[42] As cells proceed through S phase, Cyclin A/Cdk2-dependent phosphorylation of newly synthesized Cdc6 then results in its protection from proteasomal degradation and export to the cytoplasm by Crm1.[9,10,17-19,36] Finally, exported Cdc6 binds to centrosomes in late G2 and throughout mitosis, where it may carry out a functional role in regulating proper chromosomal alignment.[21]

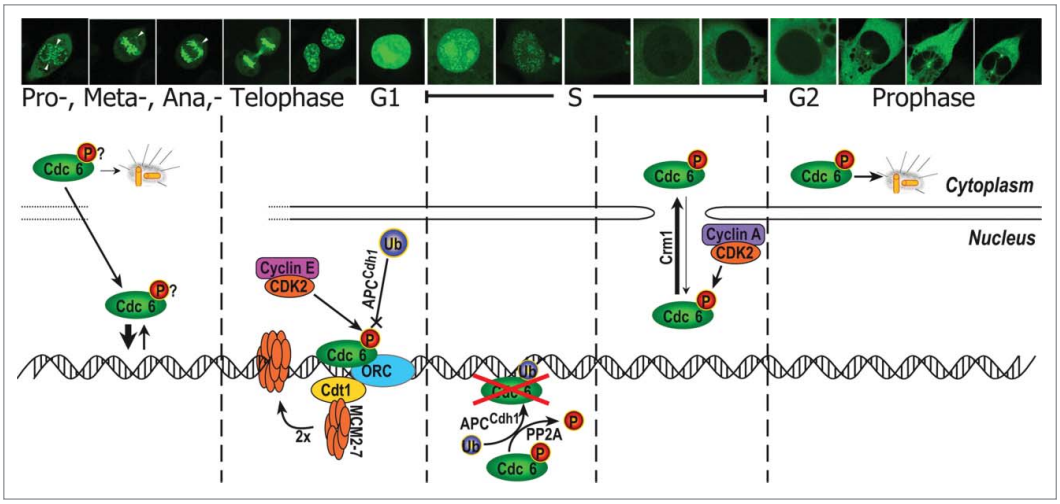

Figure 6. Summary of the temporal order of regulatory events affecting Cdc6 localization and stability. The upper row is a compilation of the subcellular Cdc6 localization during the cell cycle as presented in **Figures 2 and 4**. Below is a sketch of the proposed regulatory mechanisms taking place in the respective cell cycle phase. See text for details.

Materials and Methods

Plasmid Construction

The cDNA of human Cdc6 was amplified from p-hsCDC6-EGFP-N3 kindly provided by F. Grummt, Würzburg, and PCNA was amplified by RT-PCR from untransformed human MRC-5 lung cells. The PCR primers were designed to provide appropriate restriction sites for cloning Cdc6 and PCNA in frame to GFP, YFP, and CFP, respectively in vectors conferring bicistronic expression of the gene of interest and the selection marker on one mRNA.[43] In these vectors, bicistronic expression of tagged Cdc6 was linked to Puromycin-N-acetyltransferease, and CFP-PCNA to Hygromycin B phosphotransferase. The expression vectors were finally sequenced confirming that sequences of cloned cDNAs and adjacent vector sequences were as expected.

Cell culture

HT-1080 cells (DSMZ no.: *ACC 315*, obtained from the Leibnitz Institute DSMZ, German Collection of Microorganisms and Cell Cultures) and HEK293 cells (*ACC 305*) were grown at 37°C in a humidified atmosphere of 10% CO_2 in DMEM with Glutamax-I (Invitrogen) supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. The untransformed MRC-5 cell strain (from European Collection of Cell Cultures ECACC) was maintained in the same medium supplemented 20% FCS. HaSK-pw keratinocytes (provided by Petra Boukamp, German Cancer Research Center, Heidelberg, Germany) were maintained in FAD II medium, a 3:1 mixture of DMEM and Ham's F12 *medium supplemented with* 10% FBS, 24 ng/ml Adenin, 1 ng/ml hEGF, 0.4 μg/ml hydrocortisone, and 5 μg/ml insuline. The HaSK-pw cell line was authenticated by short tandem repeat (STR)

profiling confirming its uniqueness (Supplemental Material S5). A detailed description of the cell line will be published elsewhere.

Cells were transfected using effectene (Qiagen). Stable transgenic cell clones were selected 48 h after transfection and maintained in medium containing either 0.4 μg/ml puromycin or, for generation of HT-1080 clones coexpressing Cdc6-YFP and YFP-PCNA, 100 μg/ml hygromycin in addition.

For synchronization in pro-/metaphase, HT-1080 cells were grown for 12 h in medium with 40 ng/ml nocodazole. Mitotic cells were tapped off, reseeded in fresh medium and samples were collected at indicated time points. To synchronize cells in G0 phase, semi-confluent cells were kept in serum-free medium for 3 days, and stimulated by reseeding in fresh medium with 10% FBS. To synchronize cells in G2 phase, cells were incubated for 24 h in the presence of 10 μM RO-3306 (SML0659, Sigma-Aldrich). Cell cycle position was confirmed by staining with propidium iodide and flow cytometry (FACSCalibur, BD bioscience).

Microscopy

Confocal imaging was done with a Zeiss LSM 510 META inverted confocal laser-scanning microscope equipped with a 40x/1.3 NA Plan-NeoFluar® oil immersion objective and a ZEISS Incubator XL To maintain 37°C during live cell imaging. Cells were cultured under the microscope in CO_2-independent medium (Invitrogen).

For FRAP experiments, single optical sections were acquired with 8x zoom. One image was acquired, followed by bleaching of a circular area at 20 mW nominal laser power with 15 iterations without scanning. Further imaging scans were then collected at 2 s time intervals at a laser power attenuated to 0.1% of the bleach intensity. For quantification, fluorescence intensities of the entire cell nucleus and the bleached region were measured at each time point. The total fluorescence intensity in the cell decreased as a result of the bleach pulse itself (~17%) and the following imaging scans (~6%). Therefore, FRAP recovery curves were generated by calculating the relative intensity of the bleached area I_{rel} as described:[32] $I_{rel} = \frac{T_0 I_t}{T_t I_0}$ with T_0, total cellular intensity in the prebleach image; T_t, total cellular intensity at time point t; I_0, intensity in the bleached region in the prebleach image; I_t, intensity in the bleached region at time point t. This way, fluorescence recovery curves in the bleached area are corrected for the inherent loss of fluorescent intensity, and the initial value in this region is set to 1. Bleaching of mitotic cells was performed in the presence of 0.1 μM Taxol to reduce movements of the chromosomes.

Epifluorescent images were acquired with a Zeiss Axiovert 100 inverted light microscope equipped with an on-stage heating chamber, an objective heater (both from Bioptechs) for the 63x/1.4 NA oil immersion objective, a CCD camera (SPOT-RT-SE18 Monochrome, Diagnostic instruments, MI, USA) and specific filter sets (Chroma Tech.). Images were acquired using Meta Morph acquisition software (V6.3r6, Molecular devices).

Western blots and immunocytochemistry

Antibodies: Cdc6: sc-9964; Cdc6 pSer54: sc-12920-R; Cdc6 pSer106: sc-12922-R; PCNA: sc-56 (Santa Cruz Biotechnology, Inc..); α-tubulin: B-5–1–2; γ-tubulin: T-6557; β-actin: A5316 (Sigma); GFP: JL8 (Clontech). Secondary antibodies: Cy2™-Cy3™-conjugated goat anti-mouse (Jackson), PO-conjugated sheep anti-mouse (Amersham).

For immunofluorescence staining in **Figure 4**, cells were grown on microscopic coverslips, washed in PBS, fixed in 4% formaldehyde in PBS for 15 min at 37°C. All subsequent steps were carried out at ambient temperature. After permeabilization with 0.5% Triton X-100 in PBS for 10 min, cells were blocked for 1 h in PBS/2% BSA/5% goat serum and then incubated for 1 h with primary antibodies in the same solution. After washing, the bound primary antibody was counterstained by incubation for 1 h with Cy2™- and/or Cy3™-conjugated goat anti-mouse secondary antibodies. For immunofluorescence staining in the Supplemental **Figure S3**, cells were fixed for 6 min in Methanol at −20°C followed by 3 min Acetone at −20°C. Cells were blocked for 2 h in PBS/5% goat serum and then incubated overnight with the rabbit polyclonal anti-Cdc6 antibody (sc-8341, Santa Cruz), in the same solution at 4°C. After washing with PBS/0.2% goat serum, cells were incubated for 1 h at 37°C with Cy2™- or Cy3™-conjugated goat anti-mouse secondary antibodies.

Chromatin fractionation

Chromatin was isolated as described:[13] 2.5×10^7 cells were harvested, and 2 aliquots ($3{,}5 \times 10^6$ cells) were saved for later Western and FACS-analyses. The remainder was spun down and resuspended (4×10^7 cells/ml) in ice-cold buffer A (10 mM Hepes pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.34 M sucrose, 10% glycerol, 1 mM DTT, 5 mg/ml aprotinin, 5 mg/ml leupeptin, 0.5 mg/ml pepstatin, 0.1 mM PMSF). All subsequent steps were carried out at 4°C. 0.1% Triton X-100 was added, and the cells were incubated for 5 min. Nuclei were collected by low-speed centrifugation (4 min, 1,300 x g) to yield pellet P1 and the supernatant S1. Nuclei were washed once in buffer A, and then lysed in buffer B (3 mM EDTA, 0.2 mM EGTA, 1 mM DTT, protease inhibitors as described above). Insoluble chromatin was separated from the supernatant S3 by centrifugation (4 min, 1,700 x g), washed once in buffer B, and centrifuged again to yield the final chromatin pellet P3. The final pellet was resuspended in Western lysis buffer, and subjected to Western blotting together with equivalent amounts of untreated cells and supernatants S1 and S3.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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