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**Functional characterization of hCdc14B phosphatase
and its role during mitosis**

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Hiermit erkläre ich an Eides statt, dass ich die vorliegende Dissertation selbständig und ohne unerlaubte Hilfsmittel durchgeführt habe.

Heidelberg, den

Indra Tumurbaatar

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Abbreviations

aa	Amino acid
app.	approximately
ATP	Adenosine-5'-triphosphate
bp	base pairs
Ci	Curie
C-terminal	Carboxy-terminal
CTP	Cytidintriphosphate
DNA	Deoxyribonucleic acid
E.coli	Escherichia coli
EDTA	Ethylemediaminetetraacetic acid
FACS	Fluorescence activated cell sorter
f.c.	final concentration
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
Fig.	Figure
GTP	Guaninetriphosphate
HAMSF 12	Hams Medium 12
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulphonic acid
IPTG	Isopropyl β -D-thiogalactopyranoside
kDa	Kilo daltons
LB	Luria-bertani broth
MDa	Mega daltons
MOPS	3-(N-Morpholino) propanesulfonic acid
N-terminal	Amino-terminal
nt	nucleotide
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RT	Room temperature
SDS-PAGE	Sodium dodecylsulphate-polyacrylamide gel

	electrophoresis
SSC	Saline-sodium citrate buffer
TBE	Tris-EDTA-borate buffer
Tet	tetracycline
Tris	Tris(hydroxymethyl)-amino-methane
TTP	Tymidinetriphosphate
Tween 20	Polyoxyethylene-sorbitan-monolaurate
UTP	Uridine triphosphate
X-gal	5-bromo-4-chloro-3-indoyl- β -D-galactopyranoside

Zusammenfassung

Der Ablauf des Zellzyklus wird durch die phasen-spezifischen Aktivitäten von Proteinkinasen und Proteinphosphatasen entscheidend gesteuert. Die Cyclin-abhängige Kinase Cdk1/Cyclin B ist insbesondere für den Eintritt in die Mitose und frühe Phasen der Mitose erforderlich. Umgekehrt erfordert der Austritt aus der Mitose eine Inaktivierung dieser mitotischen Kinase, sowie die Dephosphorylierung von Substraten, die durch Cdk1/Cyclin B modifiziert worden waren. Für den Austritt aus der Mitose ist in der Bäckerhefe die nukleoläre Phosphatase Cdc14 (γ Cdc14) essentiell. γ Cdc14 dephosphoryliert zum einen Substratproteine an mitotischen Cdk1 Zielsequenzen, und fördert dadurch den Abbau von Proteinen, die die Mitose steuern und vorantreiben, und inaktiviert zum anderen mitotische Cdk/Cyclin Komplexe. Obwohl Cdc14 in der Bäckerhefe gut charakterisiert ist, ist wenig über die homologe Phosphatase im Mensch bekannt. Die vorliegende Arbeit hatte zum Ziel, eine Funktion von hCdc14B in der Regulation der Mitose in Menschzellen zu untersuchen. Dies wurde mit verschiedenen experimentellen Ansätzen erforscht. Hierzu gehörten die Herstellung transgener menschlicher Zelllinien, RNAi zur Inhibition der Genexpression, verschiedene Protein-Protein-Interaktionsstudien, die Bestimmung der Aktivität von Phosphatasen und Kinasen, die Isolierung and Größenfraktionierung zellulärer Proteinkomplexe, sowie Chromatinimmunpräzitationsversuche (ChIP) und Fluoreszenzmikroskopie.

Es wurden HeLa and U2OS Zelllinien generiert, die Flag-, Flag-HA-, or TAP-hCdc14B Wildtyp bzw. eine katalytisch defekte Punktmutante, hCdc14B^{PD}, exprimieren. Die Analysis von U2OS Zelllinien, die stabil unter der Kontrolle eines mit Tetrazyklin induzierbaren Promotors exprimieren zeigte, dass die Überexpression von hCdc14B Wildtyp den Eintritt in die Mitose verzögert, während durch Überexpression der enzymatisch inaktiven Punktmutante die Mitose verlängert oder der Austritt aus der Mitose verzögert wird. Diese Befunde wurden maßgeblich durch siRNA-Experimente erhärtet, die zeigten, dass die zelluläre Depletion von hCdc14B zu einer Anhäufung von zwei- und mehrkernigen Zellen führt, zu einer Verzögerung des Übertritts von der Metaphase in die Anaphase, sowie, letztendlich, zur Arretierung in der Mitose und zum Zelltod.

Zur Identifizierung der molekularen Mechanismen wurden der Phosphorylierungsgrad und die Aktivität ausgewählter Regulatoren der Mitose untersucht. Dies zeigte, dass hCdc14B die mitotische Phosphatase Cdc25, die sowohl

für die Aktivierung von Cdk1/Cyclin B an der G₂/M-Grenze als auch für den korrekten Ablauf der frühen Mitosestadien erforderlich ist, dephosphoryliert. Die Überexpression von hCdc14B führte zur Hemmung von Cdc25, zur Akkumulierung von Cdk1, die an T14/Y15 inhibitorisch phosphoryliert war und daher zu einer erheblichen Verzögerung der Aktivierung des Cdk1/Cyclin B Komplexes. Andererseits fehlte die zeitgerechte Inaktivierung von Cdk1/Cyclin B in Zellen, in denen hCdc14B durch siRNAs depletiert worden war. Diese Ergebnisse weisen darauf hin, dass hCdc14B die positive Feedback Schleife zwischen Cdc25 und Cdk1/Cyclin B zerstört und so das Durchschreiten der späten Stadien der Mitose vorantreibt.

Chromatinimmunpräzipitationsversuche zeigten, dass hCdc14B bevorzugt mit dem „Intergenen Spacer“ (IGS) der ribosomalen RNA Gene (rDNA) assoziiert ist. Die Binding an die rDNA erfolgt zellzyklus-spezifisch, da hCdc14B nur während der Intrphase, nicht jedoch während der Mitose mit rDNA assoziiert war. Auch durch Fraktionierung von Zellextrakten konnte die zellzyklus-abhängige nachgewiesen werden, dass sich die subzelluläre Lokalisation von hCdc14B während des Zellzyklus ändert. hCdc14B ist in der Interphase an Chromatin gebunden, jedoch von der Prometaphase der Mitose bis zur frühen G₁-Phase löslich.

Die native Größenbestimmung von hCdc14B durch Gelfiltration zeigte, dass hCdc14B in der Interphase in einen hochmolekularen, ca. 500 kDa großen Proteinkomplexes integriert ist. Während der Mitose hingegen liegt hCdc14B größtenteils als Monomer vor, eine Subfraktion von hCdc14B ist jedoch auch mit Proteinkomplexen unterschiedlicher Größen assoziiert. Diese Ergebnisse deuten darauf hin, dass hCdc14B während des Zellzyklus mit verschiedenen Proteinpartnern interagiert.

Um Interaktionspartner und Substrate von hCdc14B zu identifizieren, wurden hCdc14B Proteinkomplexe durch sequentielle Immunpräzipitation aus Menschzelllinien, die Flag-HA-hCdc14B exprimieren, isoliert und assoziierte Proteine durch Massenspektrometrie analysiert. In diesen Untersuchungen wurden APC1, die größte Untereinheit des „Anaphase-Promoting-Complex/Cyclosome“ (APC/C), sowie ELP1, ein RNA Polymerase II-spezifischer Elongationsfaktor, identifiziert. Die Interaktion zwischen hCdc14B und APC/C wurde durch Co-immunoprecipitationsversuche bestätigt. Es konnte darüber hinaus gezeigt werden, dass hCdc14B mit einer bestimmten Form des APC/C Komplexes, nämlich APC/C^{Cdh1},

assoziiert. Dieser Komplex wird während der späten Mitose gebildet, reguliert den Proteasomen-abhängigen Abbau wichtiger mitotischer Regulatoren und ist essentiell für den Austritt aus der Mitose.

Die in dieser Arbeit durchgeführten Studien zeigten zum ersten Mal, dass die Phosphatase hCdc14B einen essentieller Regulator der Mitose darstellt. Es konnten molekulare Mechanismen aufgedeckt werden, die der Regulation der Mitose durch hCdc14B zugrunde liegen.

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Summary

Progression through the cell cycle requires coordinated activity of kinases and phosphatases. Cdk1/cyclin B is a major kinase that promotes entry into and progression through early mitosis. Exit from mitosis requires down-regulation of Cdk1/cyclin B as well as reversal of Cdk1/cyclin B-mediated phosphorylations. In budding yeast, the nucleolar phosphatase Cdc14 (yCdc14) triggers mitotic exit by dephosphorylating mitotic Cdk/cyclin target proteins, promoting degradation of mitotic regulators, and inactivating mitotic Cdk/cyclin complexes. In contrast to yeast, hCdc14B, a human homologue of yCdc14, is poorly characterized. The aim of this study was to investigate a potential regulatory function of hCdc14B in mitosis. To address this issue, a variety of experimental approaches were used, including the generation of transgenic human cell lines, gene silencing by RNAi, protein-interaction assays, determination of phosphatase and kinase activities, purification and size fractionation of cellular protein complexes as well as chromatin immunoprecipitation assay (ChIP) and fluorescence microscopy.

HeLa and U2OS cell lines were established that express Flag-, Flag-HA-, or TAP-tagged wildtype hCdc14B or a catalytically defective mutant, called hCdc14B^{PD}. Analysis of U2OS cell lines that stably express either wildtype hCdc14B or the phosphatase-deficient mutant under the control of a tetracycline-inducible promoter showed that ectopic hCdc14B caused a delay in mitotic entry, while overexpression of the enzymatic inactive form prolonged mitosis and delayed exit from mitosis. These data were supported by siRNA-directed silencing of hCdc14B expression, demonstrating that ablation of hCdc14B leads to accumulation of bi- and multinucleated cells, delay in meta- to anaphase transition and finally to mitotic arrest and cell death.

To dissect the molecular mechanism underlying hCdc14B function, the phosphorylation state and activity of selected key regulators of mitosis were analyzed. These studies revealed that hCdc14B dephosphorylates the mitotic phosphatase Cdc25, which is required for activation of Cdk1/cyclin B at the G₂/M transition and during early stages of mitosis. Overexpression of hCdc14B suppressed Cdc25 activity, leading to accumulation of Cdk1 phosphorylated at T14/Y15 and a substantial delay in Cdk1/cyclin B activation. In contrast, Cdk1/cyclin B activity remained elevated in cells depleted of hCdc14B. The results suggest that hCdc14B interrupts the positive feedback loop between Cdc25 and Cdk1/cyclin B and promotes progression through late mitosis.

ChIP revealed a preferred binding of hCdc14B to the intergenic spacer region of ribosomal RNA genes (rDNA). Binding to rDNA was abrogated during mitosis, indicating that hCdc14B associates with rDNA in a cell cycle-dependent fashion. In support of this, analysis of the subcellular distribution of hCdc14B throughout the cell cycle revealed that hCdc14B is bound to chromatin during interphase and released from chromatin from prometaphase until early G₁-phase.

Size fractionation by gel filtration revealed that hCdc14B is contained in one major protein complex with a molecular weight of 400-600 kDa. During mitosis the majority of hCdc14B was monomeric with a small portion being contained in different protein complexes suggesting that during the cell cycle hCdc14B associates with different interaction partners.

To identify proteins that interact with and are dephosphorylated by hCdc14B, hCdc14B complexes were isolated from human cells that overexpress Flag-HA-tagged hCdc14B by sequential immunopurification, and co-purifying proteins were subjected to mass spectrometry. This analysis identified APC1, the largest subunit of the anaphase-promoting complex/cyclosome (APC/C), and ELP1, an RNA polymerase II specific elongation factor. The interaction between hCdc14B and APC/C was confirmed by co-immunoprecipitation experiments revealing that hCdc14B interacts with a distinct subform of the APC/C complex, APC/C^{Cdh1} that forms during late mitosis and regulates proteasome-dependent degradation of mitotic regulators at the exit from mitosis and during G₁-phase. Studies carried out in the present work provide first evidence that hCdc14B is an essential regulator of late mitotic events and reveal the molecular mechanisms underlying hCdc14B function.

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1 Introduction

1.1 The cell cycle-an overview

The size of a cell population is determined by the rates of three processes occurring in each cell population: division, growth and death. Newly divided cells grow to resume the size typical for a particular cell type. Under appropriate physiological and environmental conditions the genome will be replicated and the cell will divide into two daughter cells. This cycle of growth and division, known as the cell cycle, represents the basic mechanism by which all living organisms propagate. The cell cycle is a highly regulated process. During evolution complex control systems developed that regulate the cell cycle. If any of these control systems fail the cell cycle will continue in an unregulated fashion and may cause cancer.

The eukaryotic cell cycle is divided into 4 phases: G_1 , S, G_2 and M. During S-phase the DNA is replicated. Chromosome segregation and cell division occur during mitosis (M-phase). S- and M-phase are separated by two gap phases: G_1 between M- and S-phase and G_2 between S- and M-phase. The two gap phases provide time for the cell to monitor the external environment for the presence of growth factors or cell density as well as the internal environment for DNA damage or abnormal cellular structure. Under certain conditions cells can exit the cell cycle during the early G_1 -phase to remain in G_0 , e.g. in the absence of growth stimuli or after terminal differentiation.

The G_1 -, S- and G_2 -phases comprise the interphase. In typical mammalian cells the interphase takes about 24 hours, whereas passage through mitosis is accomplished within 1 hour. The M-phase is subdivided into 5 stages: prophase, pro-/metaphase, anaphase, telophase and cytokinesis. During prophase the chromatin becomes highly condensed and the nuclear envelope breaks down. In metaphase sister chromatids align at the equatorial plate of the bipolar spindle. Anaphase starts with separation of the sister chromatids to the two poles of the spindle. The nuclear envelope is rebuilt during telophase and chromosomes start to decondense. Finally, during cytokinesis the mother cell divides into two daughter cells each containing the same genetic information.

The cell cycle is driven by a complex regulatory system that generates waves of cyclin-dependent kinase (Cdk) activities that in turn trigger cell cycle events (Morgan, 1997). Activation and inactivation of phase-specific Cdk complexes is achieved by different mechanisms (Desai et al., 1995; Morgan, 1995; Pavletich, 1999; Obaya and Sedivy, 2002). Activation requires positive phosphorylation and removal of inhibitory

phosphates from Cdks, as well as association of Cdks with the corresponding cyclins in a cell cycle-specific manner. Likewise, inactivation is achieved by different molecular mechanisms including ubiquitin-dependent degradation of cyclins, inhibitory phosphorylation of Cdks and/or association with Cdk inhibitors (CKIs) (Glotzer et al., 1991; Zachariae et al., 1998; Obaya and Sedivy, 2002).

1.2 The Cyclin-dependent kinases (Cdks)

Cdks represent a family of proline-directed serine/threonine protein kinases with a size of appr. 34-40 kDa. For activation, Cdks must associate with their respective cyclins. Cdk/cyclin complexes phosphorylate a large number of substrates. In particular during mitosis, when many aspects of cellular structure and metabolism are altered, Cdks phosphorylate hundreds of proteins. Cdk substrates are phosphorylated at serine (S) or threonine (T) residues followed by a proline residue (P). The optimal target sites have a basic amino acid two positions carboxy-terminal to the phosphorylated residue. The consensus phosphorylation site for Cdks is [S/T]-P-X-[K/R].

Although originally identified as enzymes that control cell cycle events, members of the Cdk family also regulate additional cellular processes. Only four (Cdk1, Cdk2, Cdk4 and Cdk6) out of nine mammalian Cdks are directly involved in the cell cycle regulation. Cdk1 binds to cyclin B forming the M-phase promoting factor, which is the key kinase for mitosis (Hunt, 1989; Takizawa and Morgan, 2000). Cdk2/cyclin E represents the main regulator of the G₁/S transition. Cdk2 binds also to cyclin A at the beginning of S-phase and promotes DNA replication. Cdk4 and Cdk6 associate with cyclin D and are required for G₀/G₁ transition as well as G₁-progression (Lucibello et al., 1993; Matsuoka et al., 1994). Cdk7 contributes indirectly to the cell cycle regulation by activating other Cdks (Makela et al., 1994; Tassan et al., 1995). Besides this function, Cdk7 is a part of the basal transcription factor complex TFIIF being required for RNA polymerase II-dependent transcription (Fisher and Morgan, 1994; Fisher, 2005). Likewise, Cdk8 and Cdk9 are regulators of the RNA polymerase II transcription machinery as components of the mediator and the transcription elongation complex, respectively (Pei et al., 2003). Cdk5 regulates a variety of processes such as neural differentiation, intracellular transport and DNA repair (Dhavan and Tsai, 2001; Smith and Tsai, 2002).

Despite their differences in the mode of function, the structure of Cdks is highly conserved (Fig. 1.1). All Cdks have a common tertiary structure comprising a small N-terminal lobe and a larger C-terminal lobe. The ATP fits snugly in the cleft between the two lobes in such a way that the phosphate is oriented towards the mouth of the cleft. The protein substrate binds at the entrance of the cleft and mainly interacts with the surface of the C-terminal lobe. Residues adjacent to this site catalyze the transfer of the terminal γ -phosphate of ATP to the hydroxyl oxygen in the protein substrate.

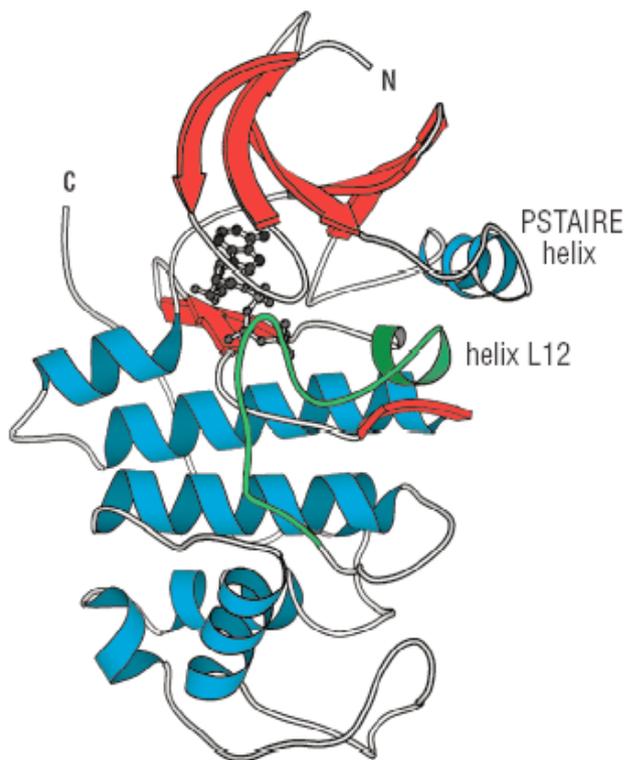


Figure 1.1. Crystal structure of Cdk2. Cdk2 is composed of two lobes. The smaller N-terminal lobe (top) consists of β -sheets and the PSTAIRE helix. The larger C-terminal lobe (bottom) is primarily made of α helices. The ATP is shown as a ball-and-stick model locating deep within the active site cleft between the two lobes. In the cyclin unbound state, the cleft is blocked by the T-loop (green). Upon binding of the cyclin, helix L12 changes its conformation rendering the active site accessible (Morgan, 2007).

1.2.1 The cyclin family

Cyclins are the essential activators of Cdks. More than 18 cyclins have been identified in mammals. They contain a highly conserved cyclin box, which mediates the association with the Cdks and consists of about 100 amino acids that are folded in a five-helix bundle (Hunt, 1991; Brown et al., 1995; Jeffrey et al., 1995; Kim et al., 1996; Morgan, 1997). The most characteristic feature of cyclins is their periodical synthesis and degradation (Evans et al., 1983). Cyclin degradation requires a polyubiquitination at a short sequence motif called destruction box, which contains nine highly conserved

amino acid residues with the consensus sequence -RXALGXIXN- (Glotzer et al., 1991). Polyubiquitination targets the cyclins to proteasome-mediated degradation. Each cyclin binds a defined Cdk, which in turn determines the specificity of the kinase complex. In Table 1.1 cyclins, their binding partners and their functions are summarized.

Cyclins	Associated Cdk	Function
A1	Cdk1, Cdk2	testis differentiation
A2	Cdk1, Cdk2	S-phase entry
B1, B2	Cdk1	G ₂ /M transition
C	Cdk8	transcription regulation
D1, D2, D3	Cdk4, Cdk6	G ₀ /G ₁ transition
E	Cdk2	G ₁ /S transition
F	?	G ₂ /M transition
G1, G2	?	DNA damage response
H	Cdk7	CAK activity, transcription regulation
I	?	?
K	?	Cdk activation, transcription regulation
T1, T2	Cdk9	transcription regulation

Table 1.1. Diversity of cyclins and their functions.

1.2.2 Mechanism of Cdk regulation

Cdks are regulated at multiple levels: association with cyclins, reversible phosphorylation of Cdks, and binding to specific inhibitors (Fig. 1.2.). Studies of the three-dimensional structure of the Cdk2/cyclin A complex revealed the molecular mechanism of Cdk2 activation by cyclin A in detail (De Bondt et al., 1993; Brown et al., 1995). In the absence of the cyclin, the phosphotransfer reaction is severely restrained by two mechanisms. First, the ATP molecule is incorrectly bound to the active site and second, the T-loop or activation-loop blocks the access of the peptide substrate to the active site. Binding of cyclin A displaces the T-loop allowing access of the substrate. In addition, cyclin A directly interacts with the PSTAIRE helix of Cdk2 and moves it toward the cleft causing a reorientation of residues that interact with ATP. Full

activation of the Cdk2/cyclin A complex occurs upon phosphorylation of threonine at position 160 by the Cdk-activating kinase (CAK) resulting in a shift of the T-loop closer to the cyclin.

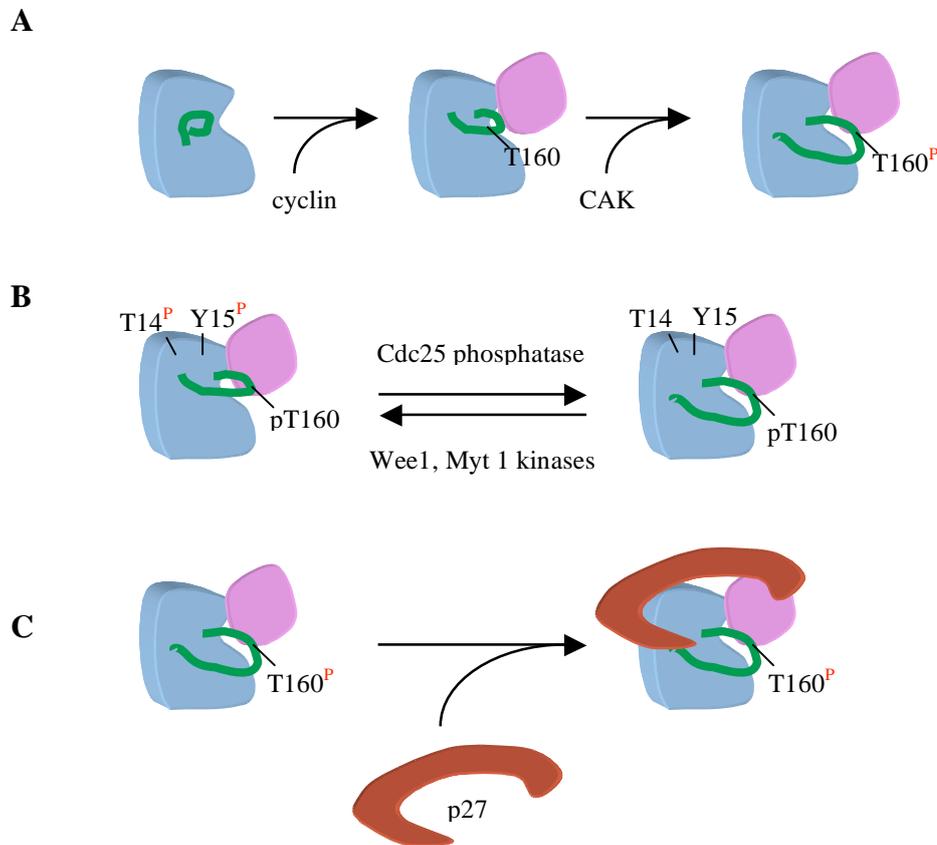


Figure 1.2. Mechanisms of Cdk regulation. **A)** In the absence of cyclin, the active site is blocked by the T-loop. The binding of cyclin to Cdk results in a movement of the T-loop and in partial activation of the Cdk/cyclin complex. Full activation requires the phosphorylation of threonine 160 by CAK, which further changes the relative position of the T-loop, improving the binding of the substrate. **B)** The activity of Cdk/cyclin is turned off, when Wee1 and Myt 1 kinases phosphorylate threonine 14 and tyrosine 15. Removal of these phosphates by the Cdc25 phosphatases results in re-activation of the Cdk/cyclin complex. **C)** The Cdk inhibitor p27/Kip1 binds to both the Cdk and cyclin distorting the active site of the Cdk. It also inserts into the ATP-binding site and inhibits the enzymatic activity.

The activity of Cdk1/2 can be inhibited by phosphorylation at threonine (T14) and tyrosine (Y15) residues by the Myt1 and Wee1 kinases. These phosphorylations block both the phosphate transfer to the bound substrate and the ATP binding (Atherton-Fessler et al., 1993; Booher et al., 1997). The Cdc25 dual-phosphatases remove these inhibitory phosphates thereby activating the Cdk/cyclin complex (Boutros et al., 2006; Trinkle-Mulcahy and Lamond, 2006; Rudolph, 2007).

Besides proteasome-mediated degradation of cyclins, the Cdk/cyclin complexes are also downregulated by binding to Cdk inhibitors (CKI). There are two classes of CKIs in mammalian cells. The Ink4 family includes Ink4a or p16. The ankyrin repeat of p16 competes with cyclin D for binding to the Cdks. The second class of CKIs represents the Cip/Kip family, composed of p21/Cip, p27/Kip1 and p57/Kip2. They share a homologous inhibitory domain and act by binding to the Cdk in a stoichiometric manner. They inhibit all G₁-Cdks with a strong preference for Cdk2 (Gu et al., 1992; Morgan, 1995; Zerfass-Thome et al., 1997). However, these inhibitors are not as specific as p16, since they also play a role in the G₂- to M-phase transition of the cell cycle.

1.3 The Cdc25 phosphatases

Cdc25 phosphatases play a crucial role in activating Cdks. They are dual-specificity phosphatases that can dephosphorylate Cdks at phospho-tyrosine (pY15) as well as phospho-threonine (pT14) thereby activating the Cdks (Dunphy and Kumagai, 1991; Honda et al., 1993; Sebastian et al., 1993). Cdc25 was first identified in the fission yeast as a dose-dependent inducer of mitosis. Yeast cells carrying a temperature-sensitive Cdc25 allele arrest in G₂ at the non-permissive temperature (Russell and Nurse, 1986). Mammalian cells have three isoforms of Cdc25, called Cdc25A, Cdc25B and Cdc25C, which are app. 60% identical in their C-terminal regions, which comprises the catalytical core. The catalytical core contains the CX₅R motif, which is common to protein tyrosine phosphatases. In contrast, sequence identity within the N-terminal regulatory domains is only app. 20%. The N-terminus contains sites for various post-translational modifications such as phosphorylation and ubiquitination, and is involved in interaction with regulatory proteins such as 14-3-3. These modifications and interactions are critical for regulating Cdc25 phosphatases both during the cell cycle and checkpoint responses. Additionally, all Cdc25 proteins have a nuclear localization signal (NLS) and a nuclear export signal (NES) that contribute to the regulation of their cellular localization.

Despite sharing common mechanisms of action, each Cdc25 family member has unique characteristics. Cdc25A regulates both early (G₁/S) and late (G₂/M) cell cycle transitions. Microinjection of α -Cdc25A antibodies leads to arrest in G₁-phase in serum-stimulated cells and overexpression of Cdc25A accelerates S-phase entry (Molinari et al., 2000; Zhao et al., 2002). These results suggested that Cdc25A regulates S-phase entry.

Recent studies showed that Cdc25A protein levels increased from late G₁-phase until mitosis and that its overexpression induced unscheduled mitosis suggesting an additional role of Cdc25A in later cell cycle events. Moreover, siRNA-mediated silencing of Cdc25A caused a decrease in the amount of mitotic cells (Lindqvist et al., 2005).

Cdc25A is an unstable protein, whose cellular level is regulated by balanced de novo synthesis and degradation through the ubiquitin-proteasome pathway (Donzelli et al., 2002). During late G₁-phase, transcription factors like E2F-1 and c-Myc stimulate expression of Cdc25A. In early mitosis, Cdk1/cyclin B phosphorylates Cdc25A on two serine residues, S17 and S115, preventing ubiquitination and thereby stabilizing Cdc25A (Mailand et al., 2002a). In late mitosis, APC/C initiates the degradation of Cdc25A in an ubiquitin-dependent fashion.

Both Cdc25B and Cdc25C are regulators of G₂/M transition. However, it seems that they function in different pathways. The activity of Cdc25B peaks before that of Cdc25C and its overexpression induces premature mitosis more efficiently than overexpression of Cdc25C. It was suggested that Cdc25B initially activates a pool of Cdk1/cyclin B, which is located at the centrosomes and that Cdc25C contributes to activation of the residual pool of Cdk1/cyclin B (Karlsson et al., 1999; Lindqvist et al., 2005). Cdc25B and Cdc25C are regulated in different ways during the cell cycle. During interphase, Cdc25B is sequestered in the cytoplasm by 14-3-3 proteins (Peng et al., 1997; Dalal et al., 1999; Takizawa and Morgan, 2000). In late G₂, Aurora A/Ajuba kinase phosphorylates Cdc25B causing its release from 14-3-3. During mitosis, Cdk1/cyclin B phosphorylates Cdc25B, which activates and stabilizes Cdc25B (Gabielli et al., 1997; Lammer et al., 1998).

Unlike Cdc25B, protein levels of Cdc25C do not change during the cell cycle. Cdc25C is regulated by cellular localization and hyper/hypophosphorylation. Cdc25C is phosphorylated at S216 during interphase and checkpoint activation that promotes its binding to 14-3-3. At the transition to mitosis, pS216 is dephosphorylated and 14-3-3 is released. Cdk1/cyclin B phosphorylates Cdc25C at S214 and blocks the inhibitory phosphorylation at S216 during mitosis. The polo-like kinase Plk1 also positively phosphorylates Cdc25C and contributes to its full activation (Toyoshima-Morimoto et al., 2002).

1.4 Cell cycle transitions

1.4.1 The G₁/S transition

In quiescent cells and during the early G₁-phase the activity of Cdk/cyclin complexes is down-regulated. Rb (retinoblastoma protein) binds to the transcription factor E2F and blocks transcription of G₁/S and S-phase specific cyclins. The presence of growth factors triggers signal transduction via the ERK pathway leading to activation of critical transcription factors, such as c-FOS and AP-1 that stimulate synthesis of cyclin D promoting the assembly of Cdk4/cyclin D or Cdk6/cyclin D (Brown et al., 1998; Albanese et al., 1999). Cdk/cyclin D complexes phosphorylate Rb and de-repress the transcriptional activity of E2F (Nevins et al., 1997; Brehm et al., 1999; Harbour and Dean, 2000; Kaye, 2002) that in turn activates transcription of many genes including cyclin E and c-Myc. Cyclin E binds to Cdk2, c-Myc transactivates cyclin D expression resulting in the accumulation of Cdk4/cyclin D and/or Cdk6/cyclin D complexes. Furthermore, accumulation of Cdk/cyclin D causes redistribution of Cip/Kip from Cdk2/cyclin E leading to its activation. Activated Cdk2/cyclin E hyperphosphorylates Rb triggering release and activation of E2F and, in turn, expression of cyclin A. High levels of cyclin A lead to competition with cyclin E for binding to Cdk2. Cdk2/cyclin A phosphorylates Cdc6, which is required for initiation of DNA replication (Petersen et al., 1999).

1.4.2 The G₂/M transition

Entry into mitosis requires activation of Cdk1/cyclin B by Cdc25 phosphatases at late G₂. Active Cdk1/cyclin B phosphorylates Cdc25 phosphatases and creates a positive feedback loop leading to its rapid activation (Okamoto and Sagata, 2007). Rapid activation of Cdk1/cyclin B is a prerequisite of M-phase entry. Moreover, onset of mitosis requires importin α and importin β -mediated translocation of active Cdk1/cyclin B from the cytoplasm to the nucleus, where it targets hundreds of substrates and promotes progression through the early stages of mitosis (Moore et al., 1999; Takizawa and Morgan, 2000).

Polo-like kinase 1 (Plk1) represents another important regulator of M-phase entry. In mammalian cells, Plk1 protein levels increase as cells approach M-phase, and its activity peaks during mitosis. Plk1 plays an important role in phosphorylation-dependent activation of Cdc25C. Many additional mitotic Plk1 substrates have been identified

including cyclin B, a cohesin subunit of the mitotic spindle, subunits of the APC/C, mammalian kinesin-like protein 1 (MKLP-1), and several kinesin related motor proteins (Nigg, 1998). The variety of its substrates points to the multiple roles of Plk1 in promoting mitosis.

1.5 Mitosis

Early mitosis

Mitosis starts with chromatin condensation in prophase. During prophase Cdk1/cyclin B phosphorylates condensin subunits triggering the assembly of condensin complexes on DNA and chromosome condensation. In addition, histone H1 and H3 are phosphorylated by Cdk1/cyclin B and Aurora kinases. These histone modifications also contribute to chromosome condensation (Nigg, 2001). Also, during prophase the mitotic spindle starts to assemble. During prometaphase Cdk1/cyclin B phosphorylates B-type lamins of the nuclear lamina and initiates breakdown of the nuclear envelope into vesicles (Peter et al., 1990; Ward and Kirschner, 1990). After breakdown of the nuclear envelope (NEB), microtubules attach to the kinetochores that are located at the centromeric regions of the chromosomes, and the mitotic spindle is being stabilized. After each kinetochore has been attached to the microtubules extending from the opposite spindle poles, the sister chromatids are aligned at the equatorial plate of the spindle becoming ready to be separated to the opposing poles of the bioriented spindle.

Late mitosis

The separation of the sister chromatids is triggered by cleavage of cohesin, a complex that holds the sister chromatids together (Uhlmann, 2003; Nasmyth and Haering, 2005). The anaphase-promoting complex/cyclosome APC/C^{Cdc20} initiates degradation of cohesion: APC/C^{Cdc20} ubiquitinates securin, an inhibitor of separase, releasing active separase. Separase cleaves the SCC1 subunit of the cohesion complex and thereby dissolves the complex that kept the sister chromatids together (Nasmyth et al., 2001; Peters, 2006). Besides securin, APC/C^{Cdc20} ubiquitinates cyclin B leading to its degradation. During progression through late mitosis, the amount of Cdc20 declines, whereas Cdh1, another co-activator of APC/C, is up-regulated leading to formation of the APC/C^{Cdh1} complex. APC/C^{Cdh1} targets a variety of substrates for degradation including cyclin B, securin, cyclin A, Plk 1, and Cdc20. During telophase, the

phosphatase PP1 dephosphorylates lamin B initiating polymerization of the nuclear lamina and reassembly of the nuclear membrane, which forms around the separated chromatids (Steen et al., 2000; Steen and Collas, 2001; Steen et al., 2003). Mitosis is successfully completed after cytokinesis, which physically divides the mother cell into two daughter cells by abscission (Field et al., 1999).

Spindle assembly checkpoint

The spindle assembly checkpoint (SAC) prevents chromosome missegregation and aneuploidy ensuring the fidelity of chromosome segregation during mitosis. The SAC negatively regulates the ability of Cdc20 to initiate the APC/C-mediated polyubiquitination of cyclin B and securin, thereby preventing their degradation by the 26S proteasome (Musacchio and Hardwick, 2002; Peters, 2006). The kinetochore is thought to act as a catalytic site for the production of signals that activate the SAC. Remarkably, the presence of a single kinetochore that is not attached to spindle microtubules is sufficient to initiate the SAC. In 1991, two independent yeast screens identified the Mad1-3 (Mitotic-arrest defective) genes and the Bub1, 3 and R1 (Budding inhibited by benzimidazole) genes, mutations of which bypassed the ability of wild-type *S. cerevisiae* cells to arrest in mitosis in the presence of the microtubule polymerization inhibitor benomyl (Hoyt et al., 1991; Li and Murray, 1991). The Mad and Bub proteins are highly conserved among eukaryotes. Mad2 and BubR1 have been shown to interact directly with APC/C^{Cdc20} *in vivo* and to inhibit its ubiquitination activity *in vitro* (Peters, 2006). BubR1 performs its function as a part of the mitotic checkpoint complex (MCC) (Sudakin et al., 2001). The MCC consists of Mad2, BubR1 and Bub3, which sequester Cdc20 to the kinetochore preventing formation of APC/C^{Cdc20}. Additional SAC proteins such as Mad1, Bub1, MPS1 (multipolar spindle-1) and Aurora B are required to amplify the SAC signal and the rate of MCC formation (Abrieu et al., 2001; Ditchfield et al., 2003). SAC is inactivated when each sister kinetochore is attached to the spindle microtubules in a bioriented fashion. In addition to attachment of the microtubules to the kinetochore, tension is important for the SAC inactivation (Nicklas et al., 1995; Li and Nicklas, 1997). It has been shown that the SAC activity is turned on when metaphase HeLa cells are treated with taxol or low doses of vinblastine, which represses kinetochore-microtubule dynamics and reduces the tension (Musacchio and Hardwick, 2002).

1.6 Cdc14 phosphatases

1.6.1 Yeast Cdc14 phosphatase

The dual-specificity phosphatase Cdc14 was first identified in the budding yeast. In *S. cerevisiae*, yCdc14 is part of the nucleolar RENT (REgulator of Nucleolar silencing and Telophase exit) complex. The RENT complex consists of Net1, yCdc14 and the NAD-dependent deacetylase Sir2 (Shou et al., 1999). Net1 binds directly to rDNA and is required for the nucleolar localization of Sir2 and yCdc14 (Straight et al., 1999; Huang and Moazed, 2003). The main function of yCdc14 comprises regulation of exit from mitosis (Visintin et al., 1999). Throughout G₁, S, G₂ and early mitosis yCdc14 is sequestered in the nucleolus by Net1 in an inactive form. yCdc14 is released from the RENT complex during mitosis by two distinct mechanisms acting at different time points during mitosis. The FEAR (Cdc Fourteen Early Anaphase Release) network mediates release of a minor portion of yCdc14 at early anaphase. Recent work has shown that the FEAR-dependent release of yCdc14 is accomplished by mitotic Cdk-mediated phosphorylation of Net1 (Azzam et al., 2004).

Full release of yCdc14 is achieved at late anaphase and depends on activation of the Mitotic Exit Network (MEN) (Jaspersen et al., 1998; Stegmeier et al., 2002). The MEN represents a huge signalling cascade comprising a variety of players, which include the GTPase Tem 1, the protein kinases Cdc15, Dbf2 and Cdc5, Mob1, the GTPase-activation protein Bub2-Bfa1/Byr4, the guanidine-nucleotide exchange factor Lte1, and the scaffold protein Nud 1 (Bardin and Amon, 2001). The MEN ensures that mitotic exit occurs only after sister-chromatid separation is completed and the genetic material has been segregated. It has been suggested that the phosphatase PP2A prevents premature release of Cdc14 from the nucleolus prior to anaphase (Yellman and Burke, 2004; Queralt et al., 2006). Upon release, yCdc14 is involved in regulation of a variety of mitotic events and ensures mitotic exit (Bosl and Li, 2005; Torres-Rosell et al., 2005). yCdc14 activity is essential for antagonizing mitotic Cdk activity during late mitosis. yCdc14 performs this function on different levels. It stabilizes and upregulates transcription of the mitotic Cdk inhibitor Sic1 and dephosphorylates Cdh1/Hct1, an activator of the anaphase promoting complex (APC) leading to APC-dependent mitotic cyclin degradation (Knapp et al., 1996; Toyn et al., 1997; Visintin et al., 1998). Also, yCdc14 directly dephosphorylates substrates, which have been phosphorylated by mitotic Cdks. yCdc14 is required for proper segregation of rDNA and telomeric regions,

being involved in targeting condensin to the rDNA (Sullivan et al., 2004; Wang et al., 2004). Recently it has been shown that yCdc14 positively regulates spindle midzone assembly by desphorylating microtubule-bundling protein Ase1 (Khmelinskii et al., 2007).

A homologue of the *S. cerevisiae* Cdc14 has been identified in *S. pombe*, called Clp1/Flp1 (Cdc14-like protein1) (Cueille et al., 2001; Trautmann et al., 2001). Like yCdc14, Clp1/Flp1 localizes to the nucleolus and the spindle pole body during G₁- and S-phase. Clp1/Flp1 is released during early mitosis, and initially moves to the mitotic spindle and kinetochores. Later, Clp1/Flp1 localizes to the medial ring. It has been suggested that Clp1, together with Aurora kinase, functions in repairing mono-orientation of the sister kinetochores to ensure proper chromosome segregation (Trautmann et al., 2004). The Septic Initiation Network (SIN) keeps Clp1 in the released state during mitosis (Bardin and Amon, 2001). Prior to anaphase, Cdk1 phosphorylates and inhibits the catalytic activity of Clp1. As Cdk1 activity declines during anaphase progression, Clp1 auto-dephosphorylates itself to stimulate its own activity (Wolfe et al., 2006). Upon activation Clp1 targets the mitotic inducer Cdc25, and inactivates and destabilizes Cdc25 (Esteban et al., 2004; Wolfe and Gould, 2004).

1.6.2 Cdc14 in non-mammalian metazoans

Cdc14 is highly conserved in the genomes of all metazoans. However, little is known about the function of Cdc14 in these organisms. *C. elegans* Cdc14 (CeCdc14) localizes to the spindle midzone in anaphase and the midbody during telophase, while during interphase and early mitosis no distinct localization was observed. siRNA mediated depletion of CeCdc14 in embryonic cells blocked cytokinesis leading to multinucleated cells (Gruneberg et al., 2002). Therefore, it was suggested that during embryogenesis CeCdc14 is involved in cytokinesis rather than in mitotic exit. Despite this defect, timing of the cell cycle appeared similar to that in embryos with functional CeCdc14. The functional role of CeCdc14 in adult cells, however, is not yet known.

X. laevis cells have two Cdc14 isoforms, XCdc14A and XCdc14B (Kaiser et al., 2004; Krasinska et al., 2007). Ectopically expressed XCdc14A is centrosomal during interphase and is recruited to the midbody at the end of mitosis. XCdc14A can dephosphorylate Cdc25 in *Xenopus* egg extracts that were induced to enter mitosis. In addition, ectopic expression of XCdc14A inhibited recruitment of SNARE/exocyst

complexes, which are required for abscission (Krasinska et al., 2007). Nothing is known about the physiological role of the second isoform, XCdc14B.

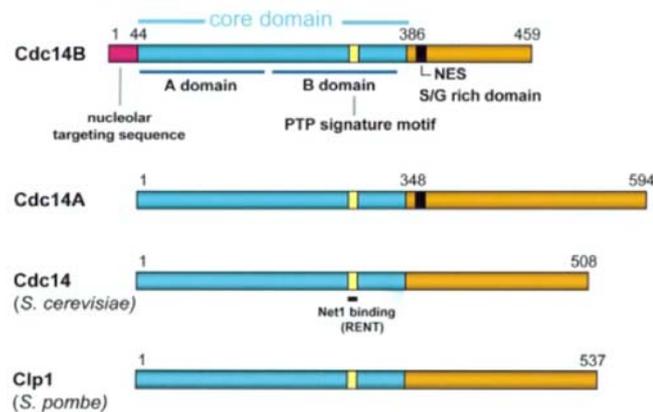
1.6.3 Human Cdc14 phosphatases

In human cells two homologues of yeast Cdc14 have been identified (Li et al., 1997). hCdc14A (623 aa) and hCdc14B (498 aa) are encoded by two different genes and display 50% amino acid sequence identity. The structure of human Cdc14 phosphatases is similar to that of yeast Cdc14 (Fig. 1.3.A). All share a conserved core domain of approx. 350 amino acids containing the protein tyrosine phosphatase (PTP) motif C(X)₅R. A nuclear export signal is embedded in the C-terminal domain that differs in size among the isoforms and orthologues. The N-terminal part of the core domain contributes to substrate specificity and is not required for catalytic activity. hCdc14B has unique extension at the N-terminus that comprises the nucleolar targeting sequence (Kaiser et al., 2002; Mailand et al., 2002b). Both human Cdc14 isoforms can rescue Clp1-deficient fission yeast strains indicating that basal functional properties have been conserved throughout evolution (Vazquez-Novelle et al., 2005). *In vitro* studies have shown that hCdc14 phosphatases exhibit a strong preference for substrates of the proline-directed kinases e.g. proteins phosphorylated at S/T-P sites (Gray et al., 2003).

Though both isoforms target the same proteins *in vitro*, they presumably exert different functions *in vivo*. hCdc14A localizes to the centrosomes and is mostly cytoplasmic (Mailand et al., 2002b). The cytoplasmic localization of hCdc14A is regulated by Crm1-dependent nuclear export, since treatment of cells with the Crm1 inhibitor leptomycin B causes redistribution of hCdc14A. Loss of the nuclear export sequence (NES) results in nuclear retention of hCdc14A and aberrant centrosome duplication suggesting that the nuclear export is a prerequisite for the function of hCdc14A (Bembenek et al., 2005). Overexpression of hCdc14A leads to premature centrosome splitting and erroneous formation of the mitotic spindles. Consistently, down regulation of endogenous hCdc14A by siRNAs resulted in mitotic defects including failure of chromosome segregation, non-productive cytokinesis and multinucleation (Kaiser et al., 2002; Mailand et al., 2002b). Though several *in vitro* targets of hCdc14A have been identified, it is not known, whether those proteins represent physiological targets. hCdc14A interacts *in vitro* with p53 and dephosphorylates the Cdk1/cyclin B-phosphorylation site of p53 (Li et al., 2000). hCdc14A is also able to dephosphorylate INCENP *in vitro* suggesting that it may

regulate the translocation of chromosomal passenger proteins (Bembenek et al., 2001). Moreover, hCdc14A dephosphorylates Cdh1 and activates APC/C^{Cdh1} *in vitro* (Bembenek and Yu, 2001). It was recently shown that hCdc14A dephosphorylates *in vitro* the Cdk1/cyclin B-dependent phosphorylation sites of Cdc25A and targets it for degradation (Esteban et al., 2006).

A



B

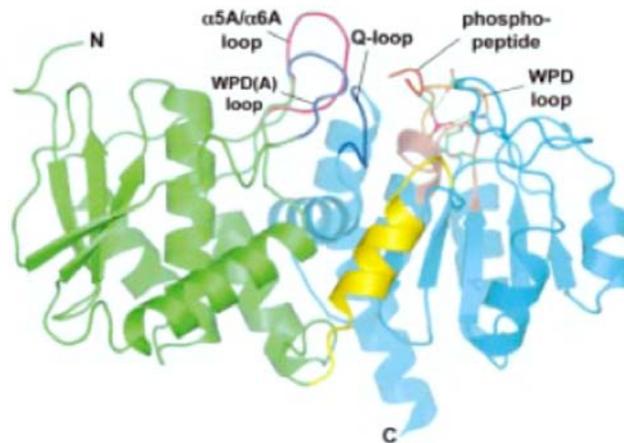


Figure 1.3. Structure of hCdc14B phosphatase. **A)** Structural relationship between Cdc14 phosphatases. The conserved core domains of Cdc14 are shown in blue, the C-terminal domains in yellow. The N-terminal extension of hCdc14B phosphatase that contains the nucleolar targeting sequence is coloured in pink. The positions of the nuclear export signal (NES) are indicated. The protein tyrosine phosphatase (PTP) motif is located in the catalytic site (yellow box). In *S. cerevisiae* Cdc14p the catalytic site also comprises the interaction site for Net1. **B)** Ribbon diagram of the core domain of hCdc14B encompassing amino acid residues 44-386. The A- and B-domains are green and cyan, respectively, and the α -helical inter-domain is yellow. The phosphopeptide substrate is shown as a red coil, and key catalytic loops are labelled (*from* Gray et al., 2003).

In contrast to hCdc14A, hCdc14B is a nucleolar protein. Crystal structure analysis of the central core domain revealed that hCdc14B is composed of two similar sized domains, called A- and B-domain, which are arranged in tandem (Gray et al., 2003). The B-domain contains the PTP motif, which is located within a long groove at the interface between the A- and B-domain. Residues of two loops of the A-domain, the extended WPD (Trp-Pro-Asp)-loop and the $\alpha 5A/\alpha 6A$ loops create one side of the groove. The WPD- and the Q-loop of the B-domain form the opposite face of the groove, whereas the inter-domain linker α -helix is positioned at the entrance of one end of the groove. This region of the α -helix is rich in acidic residues that cluster to generate an acidic groove forming the catalytic site. The catalytic site provides the specificity for pSer and pThr residues followed by a proline residue (Fig. 1.3.B). The function of hCdc14B is barely characterized. A recent study has shown that hCdc14B can bind, bundle and stabilize microtubules *in vitro* independently of its catalytic activity (Cho et al., 2005). hCdc14B that contains mutations in the nucleolar targeting domain accumulated in the cytoplasm and caused bundling and stabilization of microtubules during interphase. Moreover, nucleation of microtubules from the microtubule organization center was delayed in those cells. These observations suggested that hCdc14B may play a role in assembly and disassembly of the mitotic spindle.

1.7 Objectives

Entry into mitosis and exit from mitosis are tightly regulated processes. In *S. cerevisiae*, the phosphatase yCdc14 has been identified as a crucial regulator of mitotic exit that inhibits mitotic Cdk1 activity and reverses mitotic Cdk1-dependent phosphorylations during late mitosis, and thereby triggers mitotic exit (Stegmeier and Amon, 2004). Though the Cdc14 family is highly conserved, the functions of Cdc14 phosphatases in higher eukaryotes are ill-defined. Human cells have two homologues of yCdc14, called hCdc14A and hCdc14B. Previous studies have shown that both human Cdc14 proteins can functionally complement yCdc14 suggesting that hCdc14A/B regulate mitotic events in human cells. In line with this, hCdc14A, which is associated with centrosomes, is required for cytokinesis, correct centrosome duplication and centrosome splitting (Kaiser et al., 2002; Mailand et al., 2002b). In contrast, the function of hCdc14B, which is located in the nucleolus like yCdc14, is largely unknown. Previous work suggested that hCdc14B might be required for progression through mitosis (Tumurbaatar, 2003;

Fritsch, 2004). Based on these preliminary studies, the aim of this project was to investigate the role of hCdc14B in mitotic events. The following experiments should reveal the function of hCdc14B.

- Cell cycle progression should be measured in human cell lines that stably and inducibly overexpress the wildtype form of hCdc14B or a phosphatase-dead mutant, hCdc14B^{PD}. It should be analyzed whether overexpression of the different hCdc14B proteins affects mitotic entry, progression, or exit.
- Knock-down of hCdc14B by siRNA had to be established. Cell cycle progression should be analyzed in cells depleted of hCdc14B by siRNA. In particular, it should be analyzed whether knock-down of hCdc14B leads to defects in cell division, mitotic arrest or cell death.
- The capability of hCdc14B to inactivate mitotic Cdk1/cyclin B had to be tested both *in vitro* and *in vivo*. Given that hCdc14B would inactivate Cdk1/cyclin B it should be investigated whether this is accomplished in a direct or indirect fashion.
- The sub-cellular localization and chromatin association of hCdc14B at different phases of the cell cycle had to be analyzed in fractionated cell extracts. Chromatin immunoprecipitation should reveal whether hCdc14B is bound to distinct regions of the rDNA.
- The native molecular mass of cellular hCdc14B should be analyzed by size exclusion chromatography and it should be determined whether the native size changes during the cell cycle.
- Finally, hCdc14B associated proteins should be isolated to get information about putative target proteins of hCdc14B. hCdc14B complexes had to be purified in large scale to allow subsequent analysis of hCdc14B-associated factors by mass spectrometry.

2. Materials and Methods

2.1 Materials

2.1.1 Standard buffers and solutions

TE buffer	10 mM Tris-HCl, pH 8.0 1 mM EDTA, pH 8.0
TAE buffer	40 mM Tris-acetate, pH 7.8 5 mM Na-acetate 1 mM EDTA, pH 8.0
PBS buffer	137 mM NaCl 2.7 mM KCl 4.3 mM Na ₂ HPO ₄ 1.4 mM KH ₂ PO ₄ , pH 7.3
Laemmli sample buffer (6x)	300 mM Tris-HCl, pH 6.8 30% glycerol (v/v) 12% SDS (w/v) 0.6 mM DTT 0.012% bromphenol blue (w/v)
SDS-PAGE electrophoresis buffer	0.1% SDS (w/v) 25 mM Tris-HCl, pH6.8 250 mM glycine
AM-X buffer	20 mM Tris-HCl, pH 7.9 5 mM MgCl ₂ 0.2 mM EDTA 10% glycerol (v/v) x mM KCl
Ponceau staining solution	0.5% Ponceau S (w/v) 1% acetic acid (v/v)
Coomassie staining solution	0.1% Coomassie Brilliant Blue R 250 (w/v) 45% methanol (v/v) 10% acetic acid (v/v)

IgGs:

Mouse IgG: ChromPure Mouse IgG, whole molecule (11.2 mg/ml), Dianova

Rabbit IgG: ChromPure Rabbit IgG, whole molecule (10.1 mg/ml), Dianova

2.1.3 shRNAs and siRNAs

shRNA	shRNA DNA-oligonucleotide sequence
sh-651 sense	5'-GATCCCTTGGATAATACCAGACCGATTCAAGAGATCGGTCTG GTATTATCCAATTTTTGGAAA-3'
sh-651 antisense	5'-AGCTTTTCCAAAAATTGGATAATACCAGACCGATCTCTTGAAT CGGTCTGGTATTATCCAAGG-3'
sh-510 sense	5'-GATCCCAGGAAAGTTGCAATTTCTACATTCAAGAGATGTAGAAA TTGCAACTTCCTTTTTGGAAA-3'
sh-510 antisense	5'-AGCTTTTCCAAAAAGGAAAGTTGCAATTTCTACATCTCTTGAAT GTAGAAATTGCAACTCCGG-3'
sh-640 sense	5'-GATCCCAGGAGATTTAAATTGGATAATTCAAGAGATTATCCAAT TTAAATCTCCTTTTTGGAAA-3'
sh-640 antisense	5'-AGCTTTTCCAAAAAGGAGATTTAAATTGGATAATCTCTTGAAT TATCCAATTTAAATCTCCGG-3'
sh-892 sense	5'-GATCCCATTCCTAGATATCTGTGAATTCAAGAGATTCACAGAT ATCTAGGAATTTTTGGAAA-3'
sh-892 antisense	5'-AGCTTTTCCAAAAATTCCTAGATATCTGTGAATCTCTTGAATT CACAGATATCTAGGAATGG-3'
sh-GFP sense	5'-GATCCCAGGCTACGTCCAGGAGCGCACCTTCAAGAGAGGTGCG CTCCTGGACGTAGCCTTTTTGGAAA-3'
sh-GFP antisense	5'-AGCTTTTCCAAAAAGGCTACGTCCAGGAGCGCACCTCTCTTGA AGGTGCGCTCCTGGACGTAGCCGG-3'

shRNA targeting sequences are highlighted in **bold**. Nucleotide positions of the target sequences on the coding region of the mRNA are shown in Appendix 1.

siRNA	sense sequence	antisense sequence
hCdc14B(#1)	GGGUGCCAUUGCAGUACAUTT	AUGUACUGCAAUGGCACCCTT
hCdc14B(#2)	GAAGCAGCAUAUAGAAUAUTT	AUAUUCUAUAUGCUGCUUCTT
hCdc14B(#3)	GAGCAGCCUUCUCCAAACUTT	AGUUUGGAGAAGGCUGCUUCTT
GFP	GGCUACGUCCAGGAGCGCACCTT	GGUGCGCUCCUGGACGUAGCCTT
hCdc14B510	GGAAGUUGCAAUUUCUACATT	UGUAGAAAUUGCAACUUCCTT
hCdc14B640	GGAGAUUUAAAUUGGAUAATT	UUAUCCAAUUAAAUCUCCTT
non-targeting siRNA pool 1	Dharmacon, sequences are not available	
Cdc14B Smart pool	Dharmacon, sequences are not available	

Nucleotide positions of the siRNA target sequences on the coding region of mRNA are shown in Appendix 2.

2.1.4 Plasmids

Plasmid name	Source
pRc/CMV-Flag-hCdc14B	C. Eckert
pRc/CMV-Flag-hCdc14B(C326S/A328L) ¹	S. Fritsch
pGEX-hCdc14B	R. Voit
pGEX2T-hCdc14B ^{PD}	This work
pX-HA-Cdc25A	A kind gift from I. Hoffmann
pcDNA3-HA-Cdc25B3	A kind gift from I. Hoffmann
pCMX-GFP-Cdc25C	A kind gift from I. Hoffmann
pGEX2T-GST-Cdc25A	A kind gift from I. Hoffmann
pGEX4T3-GST-Cdc5B2	A kind gift from I. Hoffmann
pGEX-2T'6/hsCdc25C	A kind gift from I. Hoffmann
pTβ-Flag-hCdc14B	R. Voit
pTβ-Flag-hCdc14B(C326S/A328L) ²	S. Fritsch
pXCdc2/hs	P. Jansen-Dürr
pCMX-Cyclin B	R. Voit
pcDNA4/TO-Flag-Cdc14B ^{WT}	S. Fritsch
pcDNA4/TO-Flag-Cdc14B(C326S/A328L) ³	S. Fritsch
pcDNA4/TO-Flag-HA-hCdc14B ^{WT}	This work
pcDNA4/TO-Flag-HA-hCdc14B ^{PD}	This work
pcDNA4/TO-TAP-hCdc14B ^{PD}	This work
pZOME-1-N-hCdc14B ^{WT}	This work
pZOME-1-N-hCdc14B ^{PD}	This work
pTER ⁺	A kind gift from M. van de Wetering
pTER-hCdc14B-sh407 ⁴	This work
pTER-hCdc14B-sh510	This work
pTER-hCdc14B-sh640	This work
pTER-hCdc14B-sh892	This work

¹ This construct was renamed pRc/CMV-Flag-hCdc14B^{PD}.

² This construct was renamed pTβ-Flag-hCdc14B^{PD}.

³ This construct was renamed pcDNA4/TO-Flag-hCdc14B^{PD}.

⁴ This construct was renamed pTER-hCdc14B-sh651.

pTER-GFP-shRNA	This work
pMSCV/puro-U6-shCdc14B-64	This work
pMSCV/puro-U6-shCdc14B-2311	This work

2.2 Methods

2.2.1 DNA methods

2.2.1.1 Standard procedures

Preparation of chemically competent bacteria, transformation, amplification of plasmid DNA in bacteria, purification, concentration determination, restriction enzyme digestion, ligation of DNA fragments, analysis of DNA on agarose and polyacrylamide gels, and amplification of DNA by PCR were performed according to standard protocols (Sambrook et al., 1989).

2.2.1.2 Construction of shRNA expression plasmids

The plasmid pTER⁺ that contains the human RNA polymerase III-dependent H1 promoter was used to clone DNA oligonucleotides that comprised sequences complementary to 19-21 nt long stretches of the hCdc14B mRNA.

Designing shRNA oligonucleotides

The shRNA target sequences were selected according to criteria described by Reynolds et al. (2004). For cloning into the vector pTER⁺ (van de Wetering et al., 2003) DNA oligonucleotides were synthesized that correspond to shRNA target sequences, hairpin loop sequences, and 5'- and 3'-flanking sequences as follows:

Sense shRNA DNA-oligo: 5'-GATCCC-19 nt sense target sequence-TTCAAGAGA-19 nt antisense target sequence-TTTTTGGAAA-3'

Antisense shRNA DNA-oligo: 5'-AGCTTTCCAAAAA-19 nt sense target sequence-TCTCTTGAA-19 nt antisense target sequence-GG-3'

Preparation of pTER⁺ plasmid for cloning

The pTER⁺ plasmid DNA was digested with Hind III and Bgl II, dephosphorylated, and purified by agarose gel electrophoresis and gel extraction using the Qiaquick Gel

Extraction Kit (Qiagen). The concentration of the extracted DNA was measured by spectrophotometer at 260 nm.

Annealing and cloning of shRNA DNA-oligonucleotides

shRNA DNA-oligos were dissolved in H₂O at a concentration of 100 pmol/μl. The annealing reaction contained

- 2 μl of sense shRNA DNA-oligonucleotides
- 2 μl of antisense shRNA DNA-oligonucleotides
- 46 μl of annealing buffer (10 mM potassium acetate, 30 mM Hepes-KOH, pH 7.4, 2 mM magnesium acetate)

The reaction was incubated at 95°C for 10 min, and slowly cooled to RT. 25 μl of the annealed oligos were phosphorylated by T4 DNA polynucleotide kinase (NEB) in a total volume of 50 μl. The oligos were ligated without further purification overnight at 16°C with the linearized pTER plasmid as follows:

- 5 μl of annealing reaction
- 1.5 μl of T4 DNA Ligase buffer (10x, NEB)
- 0.3 μl of T4 DNA Ligase (400 U/μl, NEB)
- 1 μl 10 mM ATP
- 300 ng of linearized vector DNA
- ddH₂O ad 15 μl.

Cloning was performed using *E. coli* DH5α cells and insertion of the DNA-oligonucleotides into the vector was verified by analytical restriction enzyme digestion with Hind III and Bgl II.

2.2.1.3 5'-end labeling of oligonucleotides with γ -³²P-ATP

20 pmol of oligonucleotides were incubated at 37°C for 1 h with 2 μl of 10x T4 DNA polynucleotide kinase buffer (NEB), 5 U of T4 DNA polynucleotide kinase (NEB), and 5 μl of γ -³²P-ATP (10 mCi/ml, Perkin Elmer). To remove unincorporated radioactive nucleotides the reaction was purified using the Nucleotide Removal kit (Qiagen). Radiolabeled oligonucleotides were eluted with 100 μl of H₂O.

2.2.1.4 Sequencing of DNA

Plasmid DNA was sequenced using the Thermosequenase cycle sequencing kit (USB). For each reaction 20 pmol of sequence specific primer was radiolabeled at the 5' end as described in 2.2.1.3. 1 μ l of this reaction was directly used for the following sequencing reaction without any further purification:

Radiolabeled primer	1 μ l
10 x Thermosequenase buffer	2 μ l
Thermosequenase (4 U/ μ l)	1 μ l
DNA	500 ng
ddH ₂ O ad 17.5 μ l	

4 μ l of this mixture were added to PCR tubes containing 4 μ l of ddATP, ddTTP, ddCTP or ddGTP (each consisting of 150 μ M dNTPs and 1.5 μ M of the corresponding ddNTP). The conditions of the PCR were as follows: initial denaturation at 94°C for 2 min followed by 40 cycles each consisting of 30 sec at 94°C, 30 sec at 52°C and 1 min at 72°C. The reaction was stopped by adding 3 μ l of Stop solution (400 mM NH₄Acetate, 0.4 % SDS, 0.2 mg/ml yeast tRNA). After denaturation for 3 min at 95°C, 3 μ l of each sample were analyzed on 6% (w/v) PAA/8 M urea denaturing gel. The radioactive signals were visualized using a PhosphorImager (FUJIFILM-BAS 1800 II).

2.2.2 Biochemical methods

2.2.2.1 Determination of protein concentration and SDS-polyacrylamide gel electrophoresis

Protein concentration was determined using the colorimetric assay described by Bradford (1976). SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the Bio-Rad Mini protein gel system. Separating and stacking gels were prepared according to Sambrook *et al.* (1989). Protein samples were mixed with Laemmli sample buffer, denatured at 95°C for 5 min and directly loaded onto the gels. Electrophoresis was performed at 180 V. The molecular weight of proteins was estimated by running pre-stained (Dual colour standards, Bio-Rad) or non-stained (Unstained protein standard, Bio-Rad) marker proteins.

2.2.2.2 Staining of proteins gels

Coomassie staining

Proteins were separated by SDS-PAGE, and protein gels were incubated in coomassie staining solution (0.1% (w/v) coomassie brilliant blue R 250, 45% (v/v) methanol, 10% (v/v) acetic acid) with gentle agitation at RT for 15-20 min. To remove unspecific staining, gels were incubated in destaining solution (10% (v/v) methanol, 10% (v/v) acetic acid) overnight at RT.

Silver staining

Prior to staining, protein gels were incubated at RT overnight in fixation solution containing 40% (v/v) ethanol and 10% (v/v) acetic acid. Gels were washed twice for 20 min each with 30% (v/v) ethanol, and soaked in 0.02% (w/v) sodium thiosulfate solution for 1 min. After rinsing with water three times each for 20 sec, gels were stained in 0.2% (w/v) silver nitrate solution for 1-2 h at RT, and washed with water as described above. Next gels were incubated in developing solution (3% (w/v) sodium carbonate, 0.05% (v/v) formaldehyde and 0.0004% (w/v) sodium thiosulfate) for 5-10 min, washed in water for 5 min, and incubated in 0.5% (v/v) glycerin for 5 min to stop the developing reaction. Finally, gels were washed in water for 30 min and stored in hot-seal bags until protein bands were cut for mass spectrometry analysis.

2.2.2.3 Western blot analysis

Proteins were separated by SDS-PAGE and transferred to nitrocellulose filters (Protran, Schleicher&Schuell) using the Bio-Rad "Trans-Blot Semi Dry Apparatus" for 45 min at 14 V. For transfer, gels were sandwiched between gel-sized-Whatman 3 MM papers soaked in anode or cathode buffers as follows:

- 6 pieces of Whatman paper soaked in anode buffer I (300 mM Tris-HCl, pH 10.4)
- 3 pieces of Whatman paper soaked in anode buffer II (25 mM Tris-HCl, pH 10.4)
- nitrocellulose membrane soaked in ddH₂O
- SDS-polyacrylamide gel
- 3 pieces of Whatman paper soaked in cathode buffer (25 mM Tris-HCl, pH 9.4, 40 mM 6-aminohexan acid)

After transfer, membranes were incubated in blocking solution (PBS/milk: 1xPBS containing 5% (w/v) dried milk, and 0.2% (v/v) Tween-20) for 1 h at RT. Primary antibodies were diluted in PBS/milk and incubated with membrane-bound proteins

either for 1 h at RT or overnight at 4°C. Filters were washed in PBS/milk for 1 h at RT and then incubated for 1 h at RT with the appropriate secondary antibody coupled to horseradish peroxidase (diluted in PBS/milk). Filters were washed twice with PBS/milk, twice with PBS, and protein-antibody complexes were detected using Enhanced Chemi-Luminescence (ECL, Amersham Pharmacia Biotech) according to the manufacturer's instructions.

2.2.2.4 Extraction of hCdc14B for Western blot analysis

3×10^5 cells were lysed in 150 μ l of buffer AM-300 containing 0.5% (v/v) NP-40, 2 mM $MgCl_2$, 0.5 mM PMSF, and protease inhibitor (Complete, Roche) or in Laemmli sample buffer containing 2 mM $MgCl_2$, 0.5 mM PMSF, and protease inhibitor Complete (Roche). Samples were sonicated for 5 min using a Bioruptor (Diagonade, setting: high, 15 sec on, 15 sec off), treated with 12.5 U of Benzonase (Merck) for 15 min at room temperature and clarified by centrifugation at 13000 rpm for 10 min. 30 μ g of the lysates were separated on 10% SDS-PAA gels and analyzed by Western blotting using α -Cdc14B antibodies (Zymed).

2.2.2.5 Expression of recombinant proteins in *E.coli*

The *E.coli* strain BL21-DE3 codon plus was transformed with the expression plasmid and cultured in 50 ml of LB medium with the appropriate antibiotic overnight at 37°C. The overnight culture was diluted 1:100 in 800 ml of LB medium containing the antibiotic and 1% of glucose, and cultured at 37°C until cell density reached OD_{600} of 0.2. To induce expression of the recombinant protein 1 mM IPTG (f.c.) was added and the culture was incubated overnight at 16°C.

2.2.2.6 Purification of GST-tagged proteins

Cells from an 800 ml bacterial culture were harvested and washed with PBS. The cell pellet was resuspended in lysis buffer (20 mM Hepes, pH 8.0, 100 mM NaCl, 1 mM EDTA pH 8.0, 2 mg/ml lysozyme) containing 0.5% (v/v) NP-40 and protease inhibitors, incubated on ice for 10 min and sonicated (6 times, each 30 sec, amplitude 30%, Branson sonifier). The cell extracts were clarified by centrifugation at 13000 rpm for 30 min at 4°C, and incubated at overnight 4°C with 200 μ l of Glutathione-Sepharose (GT-Sepharose, Amersham). Beads were washed 4 times in buffer AM-200 containing 0.2%

NP-40, and finally resuspended in washing buffer (1:2 ratio of beads and washing buffer). 5 μ l of the 50% slurry was separated by SDS-PAGE, and bead-bound proteins were stained with coomassie to determine the amount of immobilized proteins. Beads were either stored at 4°C or bead-bound proteins were eluted. For elution, beads were washed in 50 mM Tris-HCl, pH 8.0 containing protease inhibitors, before incubation with 400 μ l elution buffer (50 mM Tris-HCl, pH 8.0, 20 mM glutathione, protease inhibitors) at 4°C for 1 h. Elution was repeated twice, and 7 μ l of each eluate were analyzed by SDS-PAGE and coomassie staining. The eluted fractions were dialyzed against buffer AM-100/20% glycerol and stored at -80°C.

2.2.2.7 *In-vitro* translation

In-vitro translation was carried out using the ‘TNT coupled reticulocyte lysate system’ (Promega) and ³⁵S-labeled methionine. The translated proteins were analyzed by SDS-PAGE and visualized using a PhosphorImager (FUJIFILM-BAS 1800 II).

2.2.2.8 GST pull-down assay

GST-tagged proteins or GST were immobilized on GT-Sepharose, and incubated for 4 h at 4°C with 20 μ l of rabbit reticulocyte lysates containing the ³⁵S-labeled proteins and 80 μ l of buffer AM-120 supplemented with 0.2% (v/v) NP-40 and protease inhibitors. Beads were washed 3 times with buffer AM-120/0.2% (v/v) NP-40. Bead-bound proteins were separated by SDS-PAGE and radiolabeled proteins were detected using a PhosphorImager.

2.2.2.9 Preparation of nuclear extracts from HeLa cells

1x10⁷ HeLa cells were harvested, washed in PBS, and resuspended in 500 μ l of hypotonic buffer (10 mM Hepes, pH 8.0, 10 mM KCl, 1.5 mM MgCl₂, 0.5 mM DTT, complete protease inhibitor cocktail). After incubation on ice for 10 min, cells were dounce homogenized (30 strokes, tight pestle) and nuclei were collected by centrifugation at 4000 rpm for 5 min at 4°C. The nuclei were resuspended in 1 ml of buffer S1 (0.25 mM sucrose, 10 mM MgCl₂, protease inhibitors), layered on top of a 1 ml cushion of buffer S2 (0.35 mM sucrose, 0.5 mM MgCl₂, protease inhibitors) and centrifuged at 2500 rpm for 5 min at 4°C. The nuclei were lysed in 150 μ l of buffer AM-400 supplemented with 1% (v/v) NP-40 and 50 U of DNase I and incubated for 30

min at 4°C. After sonication for 10 min with the Bioruptor (setting: high, 30 sec on/off), the sample was clarified by centrifugation at 13000 rpm for 10 min at 4°C, and the supernatant was transferred to a fresh eppendorf tube. The chromatin pellet was resuspended in 150 µl of buffer AM-0 and treated as described above. After centrifugation, both supernatants were combined, 300 µl of buffer AM-0 were added to dilute the salt concentration of the extract to 100 mM, and the extract was stored at -80°C.

2.2.2.10 Gel filtration of nuclear extracts

600 µl of HeLa nuclear extract (4 mg/ml) were loaded onto a Superose 6 prep grade column (Amersham, column volume 50 ml) equilibrated with buffer AM-100. Chromatography was performed at a flow rate of 0.5 ml/min. Fractions of 500 µl were collected, proteins of the individual fractions precipitated with TCA and analyzed by Western blotting. For calibration of the column, thyroglobulin (670 kDa), apoferritin (440 kDa), alcohol dehydrogenase (150 kDa) and BSA (66 kDa) were used.

2.2.2.11 Cellular fractionation

1×10^6 of HeLa cells were washed twice in PBS. The cell pellet was resuspended in 100 µl of buffer A (10 mM Hepes, pH 8.0, 10 mM KCl, 1.5 mM MgCl₂, 0.34 M Sucrose, 10% glycerol, 1 mM DTT, 0.1% Triton X-100 and protease inhibitors), incubated on ice for 5 min, and the nuclei were collected by low speed centrifugation (4000 rpm for 5 min at 4°C). The supernatant (cytosolic fraction) was further clarified by centrifugation at 13000 rpm for 15 min at 4°C. The nuclei were washed once in buffer A, and then resuspended in 100 µl of buffer B (10 mM Hepes, pH 8.0, 150 mM NaCl, 3 mM EDTA, pH 8.0, 0.2 mM EGTA, pH 8.0, 1 mM DTT, 0.1 mM sodium vanadate and protease inhibitors), passed 10 times through a 22-gauge needle, incubated on ice for 30 min, and centrifuged at 3400 rpm and 4°C for 4 min. The soluble nucleoplasmic fraction was transferred to a fresh eppendorf tube. The pellet, which contains the chromatin-associated fraction was washed once in buffer B, resuspended in 100 µl of Laemmli sample buffer supplemented with 2 mM MgCl₂, and sonicated for 5 min using the Bioruptor (settings: high, 15 sec on/off). To release chromatin-bound proteins, 12 U of Benzonase were added and the sample was incubated for 15 min at RT. The reaction

was stopped by addition of 10 mM EDTA and the sample centrifuged at 13000 rpm for 10 min at RT. Fractions were analyzed by Western blotting.

2.2.2.12 Chromatin immunoprecipitation

Protein-DNA complexes of 2×10^7 cells were crosslinked in the presence of 1% formaldehyde for 10-15 min at RT. Adding 0.125 M glycine stopped the crosslinking. Cells were harvested, rinsed twice in PBS, resuspended in 300 μ l of hypotonic buffer (5 mM Hepes pH 8.0, 85 mM KCl, 0.5 % (v/v) NP-40, protease inhibitors), incubated on ice for 10 min, and collected by centrifugation at 5000 rpm for 5 min at 4°C. Pellets were resuspended in 500 μ l of nuclei lysis buffer (1% (w/v) SDS, 10 mM EDTA, 50 mM Tris-HCl pH 8.1, protease inhibitors), incubated on ice for 10 min, sonicated (Branson; 7 times, each 90 sec, amplitude constant), and microcentrifuged. The chromatin solution was precleared by incubating with 20 μ l of packed protein A-Agarose (500 μ l of packed beads were blocked with 500 μ g of *E.coli* DNA and 500 μ g of bovine serum albumin) for 30 min at 4°C. Precleared chromatin from 0.5×10^7 cells was incubated with 5 μ g of antibodies against hCdc14B, hUBF or rabbit IgG at 4°C overnight. 20% of the precleared chromatin was saved as total input chromatin and was processed with the eluted immunoprecipitates at the de-crosslinking step. Protein-DNA-antibody complexes were incubated with 20 μ l of packed to protein A-beads at 4°C for 1 h, and unspecifically bound proteins were removed by washing once with 1 ml of low salt buffer (0.1% (w/v) SDS, 1% (v/v) Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.0, 150 mM NaCl), once with high salt buffer (500 mM NaCl in the same buffer as above), once with LiCl buffer (10 mM Tris-HCl pH 8.0, 250 mM LiCl, 1% (v/v) NP-40, 1% (w/v) deoxycholic acid, 1 mM EDTA), and twice with TE buffer each for 10 min at RT. Protein-DNA crosslinks were reversed in the presence of 200 mM NaCl at 65°C for 4-5 h. To remove RNA 10 μ g of RNase A were added during de-crosslinking. Samples were precipitated at -20°C overnight by adding 2.5 vol. of 96% ethanol and pelleted by microcentrifugation. Pellets were resuspended in proteinase K buffer, incubated with 30 μ g of proteinase K at 45°C for 2 h, extracted once with phenol-chloroform-isoamyl alcohol (25:24:1), once with chloroform-isoamyl alcohol (24:1), and DNA was precipitated with 1/10 volume of 3 M NaOAc (pH 5.3), 5 μ g of glycogen, and 2.5 vol. of 96% ethanol at -80°C for 1 h. DNA was collected by microcentrifugation, resuspended in 30 μ l of H₂O, and analyzed by PCR. PCR was carried out with 0.5 and 2

μl of the co-immunoprecipitated DNA samples or from a 1:300 dilution of the total input sample in the presence of α -³²P-dCTP. The conditions for PCR were as follows: initial denaturation at 94°C for 5 min followed by 28 cycles each consisting of 30 sec at 92°C, 30 sec at 52°C, and 30 sec at 72°C. PCR products were run on 8% (w/v) PAA gels and visualized by PhosphorImager.

2.2.2.13 Immunopurification of Flag-hCdc14B

Flag-hCdc14B was either transiently overexpressed in U2OS cells or stably overexpressed in U2OS or HeLa S3 cells. 1×10^6 cells were lysed in 200 μl of buffer A (50 mM Tris-HCl, pH 7.5, 400 mM NaCl, 0.2% (v/v) Triton X-100, 1 mM DTT, 2 mM MgCl₂ and protease inhibitors), incubated for 30 min at 4°C and sonicated for 5 min (Bioruptor, settings: high 15 sec on/off). 50 U of Benzonase were added for 30 min at 4°C, the lysate was sonicated as above, and centrifuged at 13000 rpm and 4°C for 20 min. The supernatant was transferred to a fresh eppendorf tube and diluted with 1 volume of buffer AM-0 containing 10 mM EDTA. For immunoprecipitation, 300 μg of the lysate were incubated at 4°C for 3-4 h with 7 μl of packed α -Flag agarose beads (M2-beads, Sigma). The beads were washed three times with buffer AM-200 substituted with 0.1% (v/v) Triton X-100, and once with phosphatase assay buffer.

2.2.2.14 Tandem affinity purification of hCdc14B complexes

U2OS cells stably expressing TAP-hCdc14B^{PD} in a tetracycline-inducible manner were grown on fifteen 15 cm dishes. At a cell density of 70%, expression of TAP-hCdc14B^{PD} was induced overnight by addition of 2 μg/ml doxycycline. Cells were washed two times with PBS, harvested in 4.5 ml of buffer A (10 mM Hepes, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT and protease inhibitors), and incubated for 15 min at 4°C. The cell pellet was collected by centrifugation at 5000 rpm for 5 min, resuspended in 2 ml of buffer B (20 mM Hepes, pH 7.9, 420 mM NaCl, 1.5 mM MgCl₂, 25% glycerol, 0.2 mM EDTA and protease inhibitors), dounce homogenized with 30 strokes (tight pestle) and incubated for 30 min at 4°C. The lysate was clarified by centrifugation at 13000 rpm for 30 min at 4°C and dialyzed against buffer AM-100. Batch purification via the IgG binding domain of the tagged proteins was performed using 300 μl of IgG-Sepharose 6 resin (Amersham) equilibrated in buffer AM-100. Precipitation was performed at 4°C for 6 h and beads were washed three times in TEV-cleavage buffer

(25 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% (v/v) NP-40, 0.5 mM EDTA, 1 mM DTT). For TEV-cleavage, beads were rotated overnight at 4°C in TEV-cleavage buffer containing 70 U of TEV-protease (Invitrogen). The supernatant from the TEV-cleavage reaction was collected, substituted with 2 mM (f.c.) CaCl₂ diluted with 3 volumes of calmodulin binding buffer (25 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM Mg-acetate, 1 mM imidazole, 2 mM CaCl₂, 10 mM β-mercaptoethanol, 0.1% (v/v) NP-40, protease inhibitors), and incubated with 100 μl of packed calmodulin beads (Stratagene) for 4 h at 4°C. The beads were washed three times in the calmodulin binding buffer and eluted three times with calmodulin elution buffer (25 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM Mg-Acetate, 1 mM imidazole, 10 mM EGTA, 10 mM β-mercaptoethanol, 0.02% (v/v) NP-40 and protease inhibitors). The eluted fractions were analyzed by Western blotting.

3.2.2.15 Isolation of hCdc14B complexes by sequential immunoprecipitation

Fifteen 15 cm dishes of 293T cells were transfected with Flag-HA-hCdc14B^{WT} or Flag-HA-hCdc14B^{PD} expression plasmids (3 μg of expression plasmid per dish). 48 h after transfection cells were harvested and nuclear extracts were prepared as described in 2.2.2.9. For the first immunoprecipitation, nuclear extracts were incubated overnight at 4°C with 200 μl of packed M2-beads. The beads were washed four times in buffer AM-200 containing 0.2% NP-40, and the bead-bound proteins were eluted three times at 4°C for 1 h with 500 μl of buffer AM-250 containing 0.2% NP-40 and 0.2 mg/ml Flag-peptide (DYKDDDDK). The eluates were combined, diluted with 1 volume of buffer AM-0 containing 0.2% NP-40, and incubated overnight at 4°C with 150 μl of packed α-HA agarose beads (HA-beads, Sigma). Beads were washed 6 times in buffer AM-200 containing 0.2% NP-40. Bead-bound proteins were eluted three times for 1 h at RT each with 400 μl of buffer AM-400 containing 0.2 % NP-40, complete protease inhibitor cocktail and 0.2 mg/ml HA peptide (YPYDVPDYA). Aliquots of the eluted fractions were analyzed by Western blotting, and the residual material was precipitated with TCA and subjected to SDS-PAGE on 4-15% (w/v) Tris-HCl gels (Bio-Rad). Proteins were detected by silver staining and analyzed by mass spectrometry.

3.2.2.16 Co-immunoprecipitation of hCdc14B and its interacting proteins

Flag-hCdc14B was transiently overexpressed either in U2OS or HEK-293T cells. 3×10^6 cells were harvested and lysed in 200 μ l of buffer AM-120 containing 0.5% NP-40, 10 mM MgCl₂, 20 μ g of DNase I, 10 μ g of RNase A and 25 U of Benzonase. Lysates were sonicated for 5 min with a Bioruptor (Diagonade, setting: high, 15 sec on, 15 sec off), and clarified by centrifugation at 13000 rpm for 20 min at 4°C. The lysates were incubated overnight at 4°C with 10 μ l of packed M2-beads. Beads were washed twice with buffer AM-120 containing 0.2% NP-40, and twice with buffer AM-100 substituted with 0.2 % NP-40. Bead-bound proteins were analyzed by Western blotting using α -hCdc14B, α -HA and α -GFP antibodies.

3.2.2.17 Determination of phosphatase activity

pNPP (p-Nitrophenyl Phosphate) assay

1-10 μ l of soluble bead-bound GST-hCdc14B^{WT} or GST-hCdc14B^{PD}, or 1 to 0.01 U of calf intestine alkaline phosphatase (Roche) were incubated for 30 min at 37°C in phosphatase assay buffer (50 mM Tris, pH 6.8, 2 mM EDTA, 5 mM DTT, 0.01 % Triton X-100, 2% glycerol, 150 mM NaCl containing 50 mM pNPP (Calbiochem)). The final volume of the reaction was 50 μ l. The reactions were stopped by addition of 950 μ l of 0.25 M NaOH, and the yellow, reaction product was measured by spectrophotometer at $\lambda=405$ nm.

OMFP assay

Expression of Flag-hCdc14B^{WT} in HeLa-Flag-hCdc14B^{WT} cells, lysate preparation and binding to M2-agarose was done as described (2.2.2.13). 1.6 mg of cell lysate were incubated with 5 μ l of packed M2-beads for 2-3 h at 4°C. 4 μ l of packed M2-Flag-hCdc14B beads were incubated in 30 μ l of phosphatase assay buffer (see above) containing 0.7 mM OMFP (Sigma) at 37°C for 30 min. The reaction was stopped by addition of 70 μ l of 0.2 M NaOH and transferred to a 96-well microtiter plate, and the absorbance of the hydrolysis product OMF was measured at $\lambda=477$ nm using an ELISA reader.

2.2.2.18 *In vitro* dephosphorylation of GST-Cdc25 by hCdc14B

GST-tagged Cdc25A, B and C were expressed in *E.coli* and immobilized on GT-beads as described in 2.2.2.6. The GT-beads were incubated with nuclear extract from mitotic HeLa cells for 30 min at 30°C in kinase buffer (50 mM Tris, pH 7.5, 10 mM MgCl₂, 1 mM EGTA, 1 mM DTT and 10 mM ATP) containing 5 μCi γ-³²P-ATP. Beads were then washed three times with 1 ml of phosphatase assay buffer (see 2.2.2.17) containing 14 mM DMAP (Sigma). GST-hCdc14B^{WT} or GST-hCdc14B^{PD} was added and the samples were incubated in phosphatase assay buffer for 40 min at 37°C. The reactions were stopped by adding Laemmli sample buffer and boiled for 5 min. The samples were resolved by SDS-PAGE, and radiolabeled proteins were visualized by PhosphoImager.

2.2.2.19 Immunoprecipitation of Cdk1/cyclin B from HeLa cells

3x10⁵ HeLa cells were harvested, washed in PBS, and resuspended in 200 μl of lysis buffer (50 mM Tris-HCl, pH 7.4, 250 mM NaCl, 0.1 % Triton X-100, 50 mM NaF, 5 mM EDTA, 1 mM DTT) containing protease inhibitors. After incubation for 30 min at 4°C, the lysate was sonicated for 5 min with a Bioruptor (setting: high, 15 sec on/off) and centrifuged at 13000 rpm for 10 min at 4°C. The protein concentration of the lysate was determined by Bradford assay. 350 μg of the lysate were mixed with 10 μl of mouse IgG conjugated Dynabeads (DynaL Biotech) or α-cyclin B antibody pre-bound to Dynabeads. After incubation for 2-3 h at 4°C, Dynabeads were washed three times in lysis buffer, and the precipitated Cdk1/cyclin B activity was measured using the *in vitro* peptide-based assay (see 2.2.2.21).

2.2.2.20 Purification of Cdk1/cyclin B from Sf9 cells

1.5x10⁸ Sf9 cells were co-infected with baculoviruses encoding myc-cyclin B and HA-Cdk1. 2 days after infection cells were harvested, washed in PBS and lysed in 12 ml of lysis buffer (80 mM β-glycerophosphate, 15 mM EGTA, 10 mM MgCl₂, 1 mM DTT) containing protease inhibitors. After sonication (Branson; output 30%, four cycles: each 15 sec), the lysate was incubated for 15 min on ice, and clarified by centrifugation at 55000 rpm (Beckman TLA 100.4 rotor) for 30 min at 4°C. The lysate was fractionated by ammoniumsulfate precipitation (45%). Precipitated proteins were collected by centrifugation at 30000 rpm for 30 min at 4°C in (Beckman TLA 100.4 rotor), resuspended in 500 μl of Buffer B (5 mM β-glycerophosphate, 1 mM EGTA, 1,5 mM MgCl₂, 1 mM DTT, and protease inhibitors), loaded onto a NAP-5 column equilibrated

with Buffer B, and eluted with Buffer B. In total, 8 eluates of each 500 μ l were collected and the protein concentration was determined by Bradford assay. The peak protein fractions were combined and loaded onto a DEAE-sepharose column (column volume: 7 ml) equilibrated with 10 volumes of Buffer B and with 10 volumes of Buffer C (5 mM β -glycerophosphate, 1 mM EGTA, 1.5 mM $MgCl_2$, 1 mM DTT, 35 mM NaCl, and protease inhibitors). After loading of the samples, the column was washed with 5 volumes of Buffer C, and proteins were eluted with 10 volumes of Buffer B containing 200 mM NaCl. In total 16 fractions of 300 μ l were collected and stored at $-80^\circ C$ after addition of glycerol (ad 20%). Fractions were analyzed by Western blotting for the presence of cyclin B and Cdk1, and Cdk1/cyclin B activity was determined using the peptide-based assay (see 3.2.2.21).

2.2.2.21 Cdk1/cyclin B kinase assay

Purified Cdk1/cyclin B complexes or partially purified fractions containing Cdk1/cyclin B were incubated with 10 μ g of Cdk1-specific substrate peptide (PKTPKKAKKL) and 2 μ Ci γ - ^{32}P -ATP in kinase buffer (50 mM Tris, pH 7.5, 10 mM $MgCl_2$, 1 mM EGTA, 1 mM dithiothreitol, 10 μ M ATP) in a total volume of 10 μ l for 30 min at $30^\circ C$. To monitor the autophosphorylation activity, incubation was done in the absence of the substrate peptide. The reactions were stopped by adding 10 μ l of 30% acetic acid and spotted onto 2x2 cm pieces of Whatman P81 phosphocellulose paper. Filters were washed four times in 15% acetic acid, dried and incorporation of ^{32}P into the peptides was measured by scintillation counting.

2.2.3 Cell culture techniques

2.2.3.1 Cell culture and maintenance of cell lines

U2OS, HEK-293T and HeLa cells were grown in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen) supplemented with heat inactivated 10% FCS, 4 mM N-acetyl-L-glutamine, 63 mg/l penicillin, 100 mg/l streptomycin (Lenaris) and 1 mM sodiumpyruvate (Biochrom). Cells stably expressing recombinant hCdc14B in a tetracycline-inducible manner were cultured in DMEM supplemented with 10% TET-system approved FCS (Clontech), 125 μ g/ml zeocin (Invitrogen) and 2.5 μ g/ml blasticidin HCl (Invitrogen). HeLa 'Kyoto' cells expressing H2B-GFP were cultured in

DMEM/10% FCS containing 500 µg/ml G418. Cells were trypsinized and subcultivated 2-3 times a week by 1:4-1:6 ratios.

Sf9 (*Spodoptera frugifera*) cells were cultured in suspension in TC-100 medium (Cambrex) containing 10% FCS, 4 mM N-acetyl-L-glutamine, 63 mg/l penicillin, 100 mg/l streptomycin at 27°C. The cell density was kept between 5×10^5 and 1.2×10^6 cells/ml.

2.2.3.2 Synchronization of culture cells

Exponentially growing U2OS and HeLa cells were synchronized at different phases of the cell cycle phase as follows:

Double thymidine block: Cells were treated with 2 mM thymidine for 19 h, released into fresh medium for 12 h, and again treated with 2 mM thymidine for 17 h to arrest at G₁/S. For release from the G₁/S arrest, the cells were extensively washed with PBS before adding fresh medium. At various time points after release the cells were harvested and the cell cycle profiles were analyzed by flow cytometry.

Nocodazole arrest: Cells were treated with 40 ng/ml of nocodazole for 20 h. The mitotically arrested cells were collected by mitotic shake-off. For release, the mitotic cells were extensively washed with PBS before seeding in fresh medium. Cells were harvested at various time points after release, and the cell cycle profiles were determined by flow cytometry.

2.2.3.3 Flow cytometry analysis

1×10^6 cells were trypsinized, washed twice with cold PBS and resuspended in 1 ml of cold PBS. For fixation, 3 volumes of ice-cold 96% ethanol were added drop-wise and cells were incubated overnight at 4°C. The fixed cells were washed with cold PBS/0.01% FCS, resuspended in 100 µl of PBS and passed through a 21-gauge needle into a FACS analysis tube containing 1 ml of FACS staining solution (0.1% sodium citrate, 0.1% NP-40, 50 µg/ml of propidium iodide (Sigma), 50 µg/ml RNase A). The suspension was incubated in the dark at 4°C for at least 1 h. The probes were analyzed using the Becton Dickinson FACScan and the software Cell Quest. Quantitation was performed with the software ModFit LT.

2.2.3.4 TUNEL assay

The TUNEL assay was performed to detect apoptosis-dependent DNA breaks using the In Situ Cell Death Detection Kit, TMR red (Roche) according to the manufacturer's protocol.

2.2.3.5 Transfection of plasmid DNA

Plasmid transfections were performed according to the calcium phosphate precipitation method of Graham and van der Eb (1973). 24 h before transfection, cells were plated onto 10 cm plates at a density of 30%. 4 h prior to transfection fresh DMEM/HAMSF12 medium supplemented with 10% FCS was added to the cells. The transfection mixture (final volume 1 ml) contained 20 µg of DNA, 50 µl of 2.5 M CaCl₂ and 430 µl of 1/10 TE (1 mM Tris-HCl, pH 8.0 and 0.1 mM EDTA) that had been mixed with 500 µl of 2xHBS buffer (280 mM NaCl, 50 mM Hepes, 1.5 mM Na₂HPO₄, pH 7.09) in a drop-wise manner. After incubation for 20-30 min at RT the transfection mixture was added to the cells. 16 h post-transfection the medium was replaced with fresh DMEM/10% FCS.

2.2.3.6 Establishment of tetracycline-inducible stable cell lines

U2OS cells that express recombinant hCdc14B in a tet-inducible manner, were generated by transfection of pcDNA4/TO-Flag-hCdc14B^{WT}, pcDNA4/TO-Flag-hCdc14B^{PD} or pcDNA4/TO-TAP-hCdc14B^{PD} expression plasmids together with the pcDNA6/TR (Invitrogen) plasmid at DNA ratio of 6:1. Clones were selected in the presence of 250 µg/ml zeocin (Invitrogen) and 5 µg/ml blasticidin HCl (Invitrogen), individual clones were picked and grown in DMEM supplemented with 10% TET-system approved FCS (Clontech) and the selection markers. To induce expression of the recombinant proteins, 2 µg/ml of doxycycline (Sigma) were added to the culture medium.

To generate tet-inducible HeLa cells, transfections were done as described above. Positive clones were selected in tetracycline-free medium supplemented with 100 µg/ml zeocin and 1 µg/ml blasticidin HCl. Individual cell clones were further cultured in DMEM containing 50 µg/ml zeocin and 0.5 µg/ml blasticidin HCl. To induce expression of the recombinant hCdc14B proteins, 2 µg/ml of doxycycline were added to the culture medium.

2.2.3.7 Transfection of siRNA

siRNAs were introduced into human cells using Lipofectamine 2000 and a reverse transfection procedure. The following protocol is optimized for transfection of 1.4×10^5 HeLa cells seeded in 12-well dishes. 6 μ l of 20 μ M siRNA were spotted onto a 12-well plate and mixed with 120 μ l of unsupplemented DMEM. 3 μ l of Lipofectamine 2000 (Invitrogen) were diluted by gently pipetting with 120 μ l of unsupplemented DMEM in an eppendorf tube and incubated at RT for 15 min. The diluted Lipofectamine 2000 was added to the siRNAs and gently mixed. After an incubation at RT for 20-30 min, 760 μ l of the cell suspension (1.4×10^5 cells) was added to the siRNA:Lipofectamine 2000 mixture and incubated at 37°C for 9-14 h. Fresh DMEM containing 10% FCS, glutamine and sodiumpyruvate, but no antibiotics replaced the medium. 24 h later cells were trypsinized and transfected with siRNA as described above. 72-120 h after the first transfection the cells were harvested and analyzed by Western Blotting.

2.2.3.8 Infection of Sf9 cells with baculoviruses

3×10^7 Sf9 cells were seeded on a 15 cm dish. After cells had attached, the medium was removed and about 5 ml of amplified-baculovirus stock was added onto the cells. After incubation on a rocking platform for 1.5 h at RT, 20 ml of supplemented TC-100 medium was added, the cells were incubated in a wet chamber at 27°C and harvested 2 days after infection.

3. Results

3.1 Functional analysis of hCdc14B during the cell cycle

In the budding yeast, the phosphatase yCdc14 is required for mitotic exit (Stegmeier and Amon, 2004). Interestingly, both human homologues, hCdc14A and B, can functionally complement yeast strains, in which the yCdc14 gene has been deleted (Vazquez-Novelle et al., 2005). This suggests that essential functions of the Cdc14 phosphatase family have been conserved among eukaryotes and that Cdc14 is also required for mitotic events in human cells. Consistent with this, previous studies have pointed to a role of hCdc14A in cytokinesis and centrosome splitting (Kaiser et al., 2002; Mailand et al., 2002b). Much less is known about the second isoform, hCdc14B.

Time-lapse fluorescent microscopy has shown that U2OS cells transiently overexpressing GFP-tagged wildtype hCdc14B (hCdc14B^{WT}) failed to divide and died when hCdc14B^{WT} was overexpressed for more than 72 h (Tumurbaatar, 2003). Subsequent studies revealed that the mitotic index of U2OS cells expressing GFP-hCdc14B^{WT} was decreased to 1.75% in comparison to 4% in cells expressing GFP. In contrast, overexpression of a GFP-tagged, phosphatase-dead mutant of hCdc14B, termed hCdc14B^{PD}, raised the mitotic index from 4% to 11% (Fritsch, 2003). hCdc14B^{PD} carries two point mutations within the catalytic site at residues C324 and A326 that are essential for the phosphatase activity of hCdc14B (Taylor et al., 1997) (Fig. 3.1.A).

3.1.1 Generation of U2OS cells stably overexpressing Flag-hCdc14B^{WT} or Flag-hCdc14B^{PD}

As a first approach for deciphering the functional role of hCdc14B in cell cycle progression, U2OS cell lines were established that stably overexpress hCdc14B^{WT} or hCdc14B^{PD}. Previous studies in our group have shown that constitutive overexpression of hCdc14B in different human and mouse cells, such as HeLa, U2OS, HEK-293T, or NIH-3T3, is lethal (Tumurbaatar, 2003; Fritsch, 2003). Therefore, stable expression of ectopic hCdc14B was performed under the control of a tetracycline-inducible promoter. U2OS cells were co-transfected with plasmids that encode the tet-repressor (pcDNA6/TR) and plasmids that contain the tet-operator, a CMV-promoter and cDNAs for either Flag-tagged hCdc14B^{WT} (pcDNA4/TO-Flag-hCdc14B^{WT}) or the phosphatase-dead mutant hCdc14B^{PD} (pcDNA4/TO-Flag-hCdc14B^{PD}). Co-transfected cells were

selected in the presence of zeocin (for pcDNA4/TO-Flag-hCdc14B plasmid) and blasticidin HCl (for pcDNA6/TR plasmid), individual clones were isolated and cultured in tetracycline-free medium supplemented with the selection markers. Expression of the recombinant hCdc14B proteins was induced by adding the tetracycline derivative doxycycline at a final concentration of 2 $\mu\text{g/ml}$ to the culture medium for 4-16 h. The level of the recombinant hCdc14B proteins was monitored by Western blotting. As shown in Fig. 3.1.B, Flag-hCdc14B proteins were expressed within 4 h after the addition of doxycycline, and were not detected in non-induced cells. For subsequent functional analysis, the clones 6-WT and 7-WT that express Flag-hCdc14B^{WT}, and clones 1-PD, 4-PD and 12-PD that express Flag-hCdc14B^{PD} were chosen.

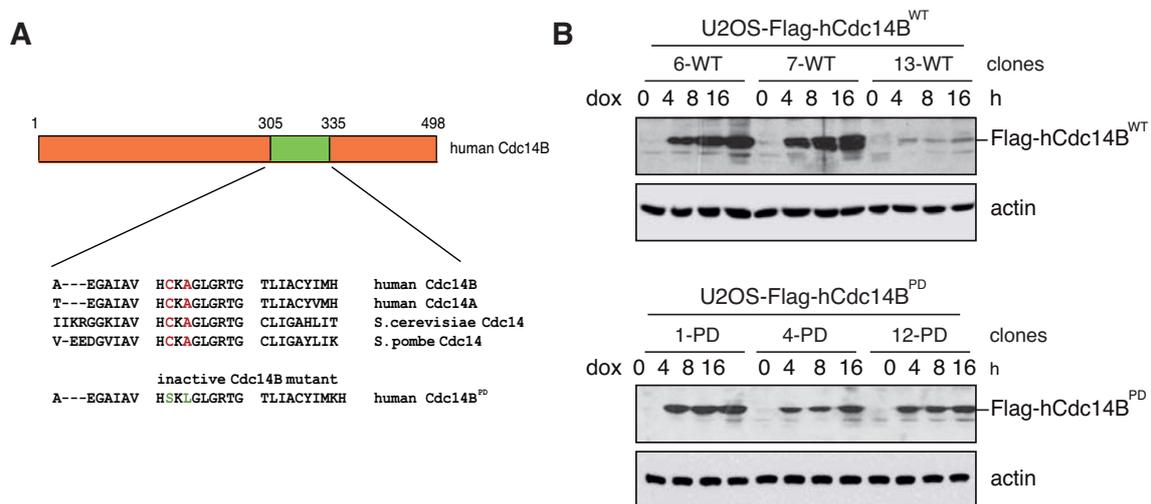


Figure 3.1. Tetracycline-induced expression of Flag-hCdc14B proteins in different clones of U2OS-Flag-hCdc14B^{WT} and U2OS-Flag-hCdc14B^{PD} cell lines. **A)** Amino acid sequence comparison of the catalytic site in different Cdc14 orthologues. The localization of the catalytic site of hCdc14B is presented in green (top). The conserved C324 and A326 residues are highlighted in red. The two amino acid residues exchanged in hCdc14B^{PD} are shown in green (bottom). **B)** Induction of Flag-hCdc14B expression by doxycycline. Individual clones of stably transfected U2OS cells were incubated with doxycycline (dox, 2 $\mu\text{g/ml}$) for the indicated times (h) and Flag-hCdc14B proteins were detected on immunoblots using α -hCdc14B antibodies. The Western blots were reprobbed with antibodies specific to actin to monitor equal protein loading.

3.1.3 Cell cycle progression is altered in U2OS cells overexpressing Flag-hCdc14B^{WT} or Flag-hCdc14B^{PD}

To analyze cell cycle progression, U2OS-Flag-hCdc14B^{WT} and U2OS-Flag-hCdc14B^{PD} and parental U2OS cells were arrested at G₁/S by a double thymidine block and released into the cell cycle. Expression of Flag-hCdc14B was induced in the presence of 2 $\mu\text{g/ml}$

of doxycycline 4 h before release from G₁/S (Fig. 3.2.A). To monitor progression through G₂-, M-, and the next G₁-phase, cells were harvested at different time points after release and analyzed by flow cytometry (Fig. 3.2.B). At least two different clones of each cell line that express Flag-hCdc14B^{WT} or Flag-hCdc14B^{PD} were used in these studies. Cell clones that express similar amounts of the same recombinant hCdc14B had the same phenotype (data not shown). Therefore, results from cell clones 6-WT expressing Flag-hCdc14B^{WT}, and 1-PD expressing Flag-hCdc14B^{PD} are shown, representatively.

As shown in Fig. 3.2.B, accumulation of cell population with 4N DNA content, and thus migration to G₂/M occurred at the same time in U2OS-Flag-hCdc14B^{WT}, U2OS-Flag-hCdc14B^{PD} and parental cells, e.g. 12 h after release. 16 h after release from G₁/S, all of the parental cells had entered the G₁- and S-phase of the next cell cycle indicated by accumulation of the 2N DNA containing cell population and the absence of cells with 4N DNA content. Interestingly, at the same time point 27% of the U2OS-Flag-hCdc14B^{WT} cells and 14% of the U2OS-Flag-hCdc14B^{PD} cells were contained in G₂/M as indicated by the 4N DNA content. This suggests that overexpression of Flag-hCdc14B^{WT} and Flag-hCdc14B^{PD} prolonged either the G₂-phase and/or the M-phase.

3.1.3 Overexpression of hCdc14B^{WT} and hCdc14B^{PD} delays mitotic entry and progression through mitosis

To distinguish, whether the G₂- or the M-phase was affected by overexpression of hCdc14B, immunoblotting analysis was performed using antibodies against pY15-Cdk1. Cdk1 is phosphorylated at T14/Y15 from G₁- until late G₂-phase and thereby inactivated. At late G₂-phase Cdc25 phosphatases remove these inhibitory phosphates and thereby activate Cdk1/cyclin B (Nilsson and Hoffmann, 2000). In addition, level of histone H3-pSer10 was monitored on immunoblots. The phosphorylation of histone H3 at Ser10 starts at onset of mitosis, reaches the highest level at metaphase and declines during late mitosis (Nowak and Corces, 2004).

U2OS-Flag-hCdc14B^{WT} and U2OS-Flag-hCdc14B^{PD} cells were synchronized and Flag-hCdc14B proteins were expressed as described above. At different time points after release cells were harvested, lysed and analyzed by immunoblotting (Fig. 3.2.C). Y15-Cdk1 was phosphorylated and histone H3-Ser10 was unphosphorylated at G₁/S (0 h, lanes 1, 7 and 13). 12 h after release, the parental cells had entered mitosis as

indicated by the decline of pY15-Cdk1 and the presence of H3-pSer10 (lane 2). 15 h after release H3-pSer10 was almost completely lost, and at 18 h phosphorylation of Y15-Cdk1 increased again, demonstrating that cells had completed mitosis and progressed through the next cell cycle. This is consistent with the results from FACS analysis demonstrating that 18 h after release, parental cells had reached the next G₁- and S-phase (Fig. 3.2.B).

Pronounced differences were observed in the kinetics of phosphorylation/dephosphorylation of Y15-Cdk1 and H3-Ser10 in U2OS-Flag-hCdc14B^{WT} and U2OS-Flag-hCdc14B^{PD} cells. In U2OS-Flag-hCdc14B^{WT} cells, dephosphorylation of Y15-Cdk1 was prolonged until 14 h after release from G₁/S (lane 9) suggesting that activation of Cdk1/cyclin B, and hence entry into mitosis was impaired. This is supported by the delayed increase of H3-pSer10, which peaks at 14 h (lane 9). The decrease of H3-pSer10 levels at later time points indicates that cells successfully progressed through mitosis and exited mitosis without any further delay (lanes 10-12).

Notably, U2OS-Flag-hCdc14B^{PD} cells showed a different phenotype. These cells entered mitosis at the same time as parental cells according to the decline of pY15-Cdk1 and the appearance of H3-pSer10 at 12 h after release from the thymidine-block. However, phosphorylation of H3-Ser10 persisted until 18 h after release (lanes 14-18) indicating that progression through mitosis was prolonged. In support of this, no re-phosphorylation of Y15-Cdk1 was observed at later time points (lanes 14-18).

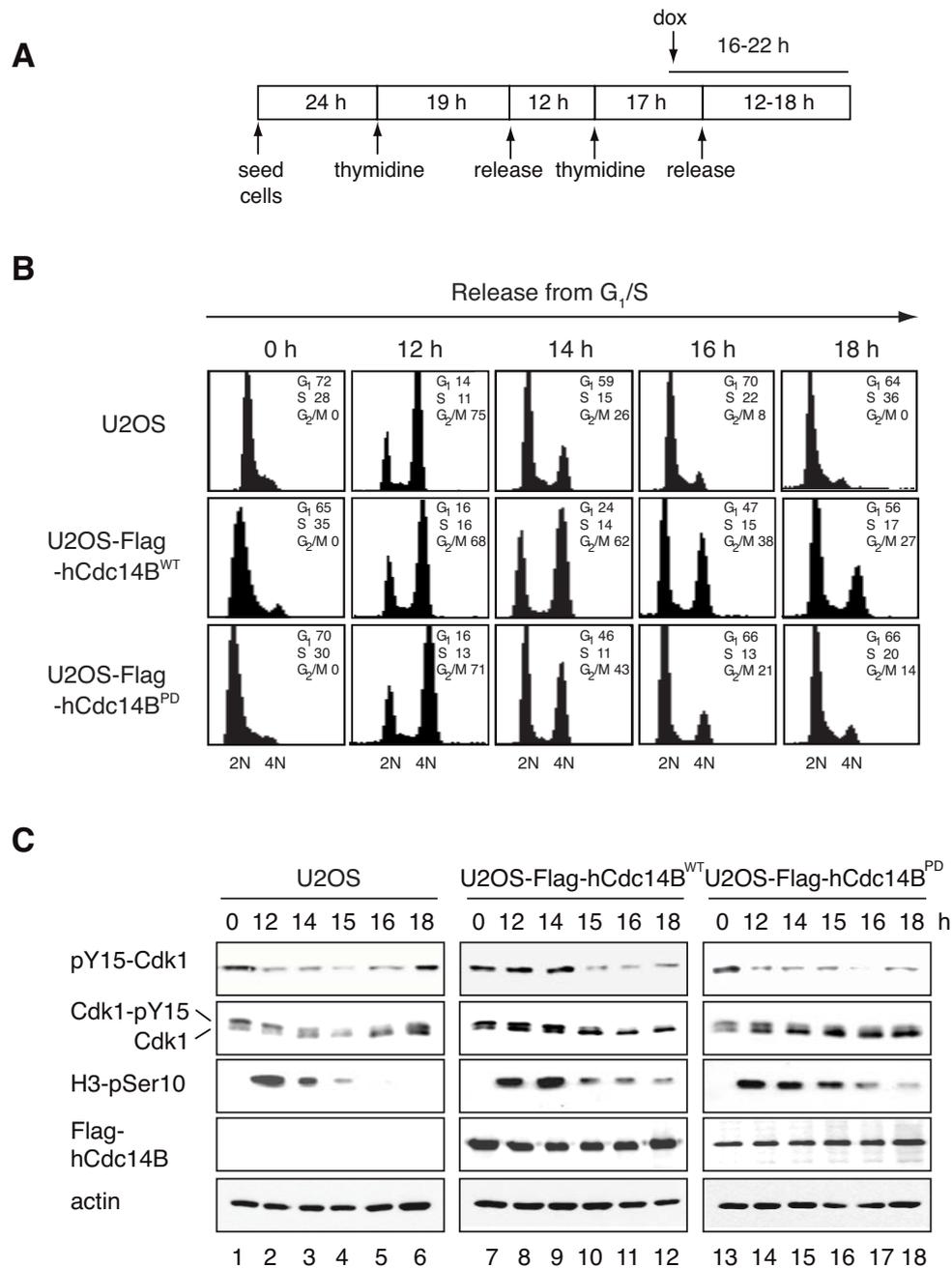


Figure 3.2. Overexpression of hCdc14B alters mitotic entry and progression. **A)** A schematic presentation of the protocol used for cell cycle synchronization and induction of Flag-hCdc14B expression. Parental U2OS cells, U2OS-Flag-hCdc14B^{WT} (clone 6-WT) and U2OS-Flag-hCdc14B^{PD} (clone 1-PD) cells were synchronized at G₁/S by double thymidine-block, and Flag-hCdc14B expression was induced with doxycycline (2 μg/ml). Cells were released for the indicated time points, harvested and subjected to FACS analysis (**B**) and immunoblotting (**C**). **B)** FACS analysis of U2OS, U2OS-Flag-hCdc14B^{WT} or U2OS-Flag-hCdc14B^{PD} cells. The percentage of cells at different phases of the cell cycle is indicated. **C)** Overexpression of Flag-hCdc14^{WT} delays mitotic entry, overexpression of Flag-hCdc14^{PD} prolongs mitosis. Cell lysates were analyzed by immunoblotting with antibodies against pY15-Cdk1 (G₂-phase marker), Cdk1, H3-pSer10 (M-phase marker), the Flag epitope and actin. The upper bands in the α-Cdk1 blot represent pY15-Cdk1.

3.1.4 Cdk1/cyclin B activity is altered in U2OS cells overexpressing Flag-hCdc14B^{WT} or Flag-hCdc14B^{PD}

The different kinetics of Cdk1-Y15 phosphorylation/dephosphorylation in U2OS-Flag-hCdc14B^{WT} and U2OS-Flag-hCdc14B^{PD} cells suggested that ectopic hCdc14B affects Cdk1/cyclin B activity. To test this, the kinase activity was determined in both kinds of cells that had been synchronized, treated with doxycycline and released into the cell cycle as described in 3.1.2. Cdk1/cyclin B complexes were immunoprecipitated using α -cyclin B antibodies coupled to magnetic beads (anti-mouse Dynabeads), and immunoprecipitates were analyzed for Cdk1/cyclin B activity using a peptide-based kinase assay. Indeed, in U2OS-Flag-hCdc14B^{WT} cells Cdk1/cyclin B activity peaked 3 h later (15 h time point) than in the parental cells (12 h time point) (Fig. 3.3.). This is consistent with the high level of pY15-Cdk1 until 14 h after release from G₁/S in these cells (see Fig. 3.2.C). On the other hand, in U2OS-Flag-hCdc14B^{PD} cells Cdk1/cyclin B was most active 12 h after release, e.g. the same time point as in the parental cells, but Cdk1/cyclin B activity declined more slowly. These results suggest that hCdc14B regulates Cdk1/cyclin B activity.

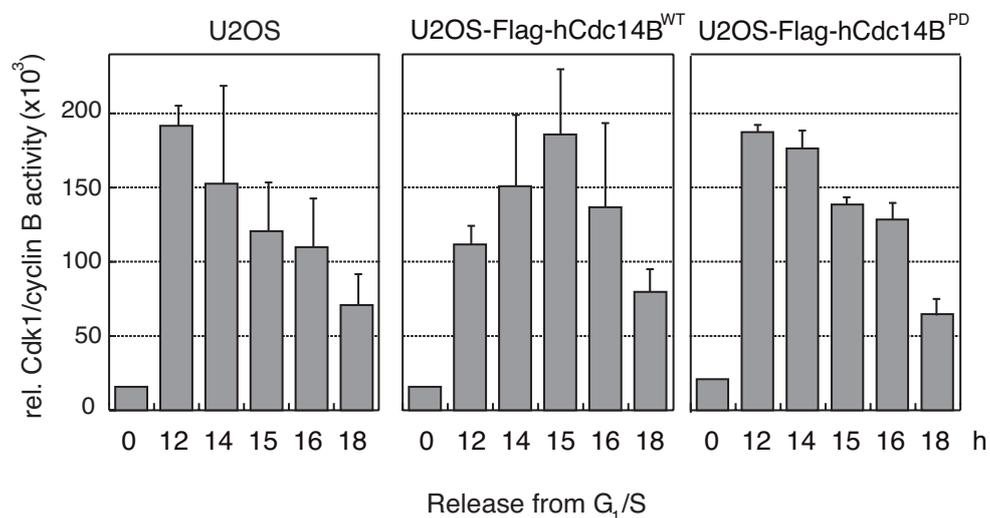


Figure 3.3. Overexpression of hCdc14B affects Cdk1/cyclin B activity. U2OS, U2OS-Flag-hCdc14B^{WT} and U2OS-Flag-hCdc14B^{PD} cells were synchronized at G₁/S by a double thymidine-block and expression of Flag-hCdc14B proteins was induced. At different time points after release from G₁/S, cells were harvested, Cdk1/cyclin B was immunoprecipitated by α -cyclin B antibodies, and the immunoprecipitated kinase activity was assayed *in vitro* using Cdk1-specific peptides and γ ³²P-ATP. The bar diagram shows results from two independent experiments. Values were normalized to the activity of Cdk1/cyclin B in G₁/S cells.

3.1.5 Establishment of conditions for hCdc14B knock-down by RNAi

To confirm the putative role of hCdc14B in mitosis, cell cycle studies were performed with cells depleted of hCdc14B. To knock-down cellular hCdc14B, first, a plasmid-based RNAi approach was used. I designed four short hairpin RNAs (shRNAs) directed against 19-nt long sequences of different regions of the hCdc14B mRNA (nt positions 510, 640, 651 and 892 of the coding region, Appendix 1). These shRNAs were obtained as ds-DNA oligonucleotides containing sense and antisense sequences of the target region separated by a hairpin loop, and were cloned into the expression plasmid pTER⁺ downstream of the human Pol III-dependent H1 promoter. Upon transfection the recombinant plasmids express 63 nt long shRNAs, which are converted into 19 nt short interference dsRNAs (siRNAs) by the cellular RISC complex.

hCdc14B-shRNAs were first tested for knock-down efficiency of hCdc14B in HEK-293T cells that had been co-transfected with GFP-hCdc14B and pTER-hCdc14B-sh510, -sh640, -sh651, or -sh892 plasmids. 48 h post-transfection the amount of GFP-hCdc14B was analyzed on Western blots. In cells co-transfected with pTER-hCdc14B-sh510 and pTER-hCdc14B-sh640 plasmids the level of GFP-hCdc14B was reduced by 80-100% compared to cells transfected with the empty pTER⁺ plasmid (Fig. 3.4.A). This indicates that hCdc14B-shRNAs expressed by these plasmids silenced expression of the GFP-hCdc14B proteins. The pTER-hCdc14B-sh510 and -sh640 plasmids were further tested for their ability to knock-down endogenous hCdc14B in U2OS cells. Despite numerous efforts, no knock-down of the endogenous hCdc14B was observed (Fig. 3.4.B).

As an alternative approach, hCdc14B-siRNAs were used to knock-down cellular hCdc14B. A pool of three siRNAs specific to hCdc14B was designed and obtained from Dharmacon. For control, a pool of three irrelevant siRNAs (non-targeting siRNA pool 1, Dharmacon) was used. HeLa cells were transfected with hCdc14B specific or control siRNAs and the amount of hCdc14B was analyzed by Western blotting. However, the reduction of the hCdc14B level was too low for further functional analysis, being only 20-30% less than in control cells (Fig. 3.4.C).

Finally, three siRNAs termed hCdc14B-siRNA #1, 2 and 3, were designed and obtained from Eurogentec (Appendix 2). These siRNAs were used to transfect HeLa “Kyoto” cells that express GFP-tagged histone H2B. As control, GFP-siRNA and the non-targeting siRNA pool 1 (Dharmacon) were used. As shown in Fig. 3.4.D, the most efficient and reproducible knock-down of endogenous hCdc14B was achieved by

transfecting ‘Kyoto’ cells twice with hCdc14B-siRNAs #2 and #3 using Lipofectamine 2000 and a reverse transfection protocol. These siRNAs specifically knocked-down hCdc14B, since the cellular level of hCdc14A or actin was not affected (Fig. 3.4.D, bottom panels).

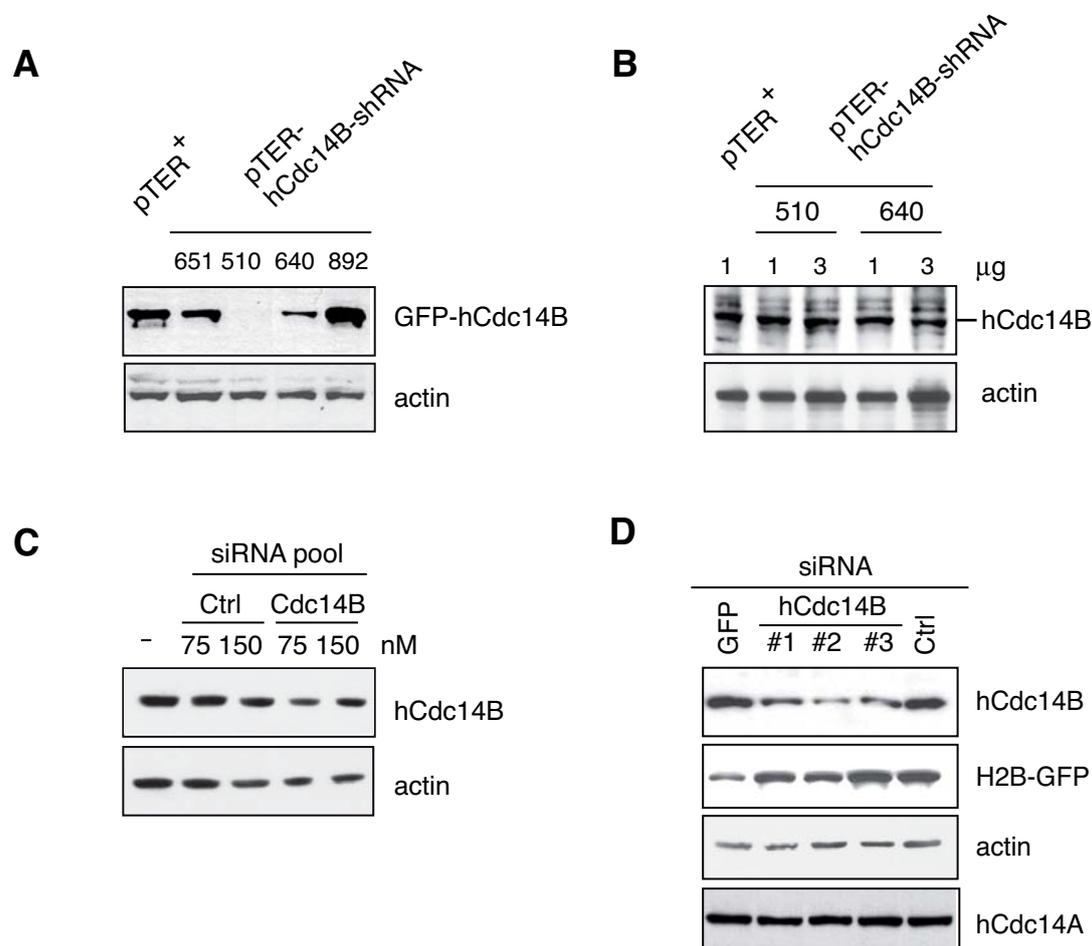


Figure 3.4. Knock-down of hCdc14B by RNAi. **A)** Knock-down of hCdc14B in HEK-293T cells by shRNA expression plasmids. HEK-293T cells were seeded in 6-well plates and co-transfected with 100 ng of pEGFP-hCdc14B expression plasmid and 1 μ g of either empty pTER⁺ or pTER-hCdc14B-shRNA expression plasmids targeting the regions around nt position 510, 640, 651 and 892 of the coding region of hCdc14B mRNA (for details, see Appendix 1). 48 h after transfection cells were harvested, and cell lysates were probed by Western blotting for hCdc14B and actin. **B)** Knock-down of endogenous hCdc14B in U2OS cells by pTER-hCdc14B-sh510/-sh610. U2OS cells were seeded on 6-well plates and transfected with increasing amounts of pTER-hCdc14B-sh510 or pTER⁺-hCdc14B-sh640 plasmids. As control, U2OS cells were transfected with the pTER⁺ plasmid. 72 h after transfection cells were harvested and analyzed by Western blotting as in (A). **C)** Knock-down of hCdc14B in HeLa cells by siRNA pool from Dharmacon. HeLa cells were transfected with 75 and 150 nM of either non-targeting (Ctrl) or hCdc14B-specific siRNAs. 72 h after transfection cells were harvested and the level of endogenous hCdc14B was analyzed as in (A). **D)** Knock-down of hCdc14B in HeLa cells by three distinct hCdc14B-siRNAs from Eurogentec. HeLa ‘Kyoto’ cells were transfected twice with 120 nM of siRNAs that target GFP or hCdc14B (#1, 2, 3), or with a pool of non-targeting siRNAs. 96 h after the first transfection cells were harvested and analyzed by immunoblotting with the indicated antibodies.

3.1.6 Depletion of hCdc14B leads to mitotic arrest

To study cell cycle progression in cells depleted of hCdc14B, “Kyoto” cells were transfected with hCdc14B-siRNAs or non-targeting control siRNAs, synchronized at G₁/S by double thymidine block, and released for 10-15 h (Fig. 3.5.A). The effect of hCdc14B depletion on cell cycle progression could be studied only in synchronized cells because many of the cells productively transfected with hCdc14B-siRNAs were lost due to cell death in the asynchronous cell population. At different time points after release from G₁/S, entry and progression through mitosis were monitored by fluorescence microscopy of live cells, and the mitotic index was determined (Fig. 3.5.B and C). Cells depleted of hCdc14B, entered mitosis 12 h after release, e.g. at the same time as control cells. However, cells treated with hCdc14B siRNAs stayed in mitosis as indicated by the high mitotic index of 42.8% 16 h after release from G₁/S. Control cells, on the other hand, progressed through mitosis into next G₁, since the mitotic index declined to 15% (Fig. 3.5.C). Fluorescence microscopy of GFP-tagged histone H2B revealed that a considerable amount of cells that were depleted of hCdc14B was arrested in metaphase of mitosis (Fig. 3.5.B). This result strongly suggests that hCdc14B is required for mitotic progression beyond metaphase.

3.1.7 Cdk1/cyclin B activity is elevated upon RNAi-mediated depletion of hCdc14B

Given that overexpression of Flag-hCdc14B^{WT} delayed activation of Cdk1/cyclin B, and overexpression of Flag-hCdc14B^{PD} delayed inactivation of Cdk1/cyclin B, I investigated whether depletion of hCdc14B would up-regulate Cdk1/cyclin B activity. For this, HeLa ‘Kyoto’ cells were transfected with siRNAs targeting hCdc14B or GFP and arrested at G₁/S. 13, 14 and 15 h after release from G₁/S, e.g. at time points when mock-transfected cells had progressed through mitosis (see Fig. 3.5.B and C), cells were harvested, Cdk1/cyclin B was immunoprecipitated and the kinase activity measured using the Cdk1 peptide-based assay. Consistent with the elevated Cdk1/cyclin B activity in Flag-hCdc14B^{PD} expressing cells, cellular depletion of hCdc14B caused a two-fold increase of Cdk1/cyclin B activity in comparison to control cells that were transfected with GFP-siRNAs (Fig. 3.6., 13 h time point). Moreover, in control cells, Cdk1/cyclin B activity dropped between 13 and 15 h after release as cells progressed through M- into G₁-phase of the next cell cycle. In contrast, in cells depleted of hCdc14B, Cdk1/cyclin B

activity dropped slowly being 4-fold higher than in control cells 15 h after release. This suggests that hCdc14B is required for inactivation of Cdk1/cyclin B and thereby regulates progression through mitosis.

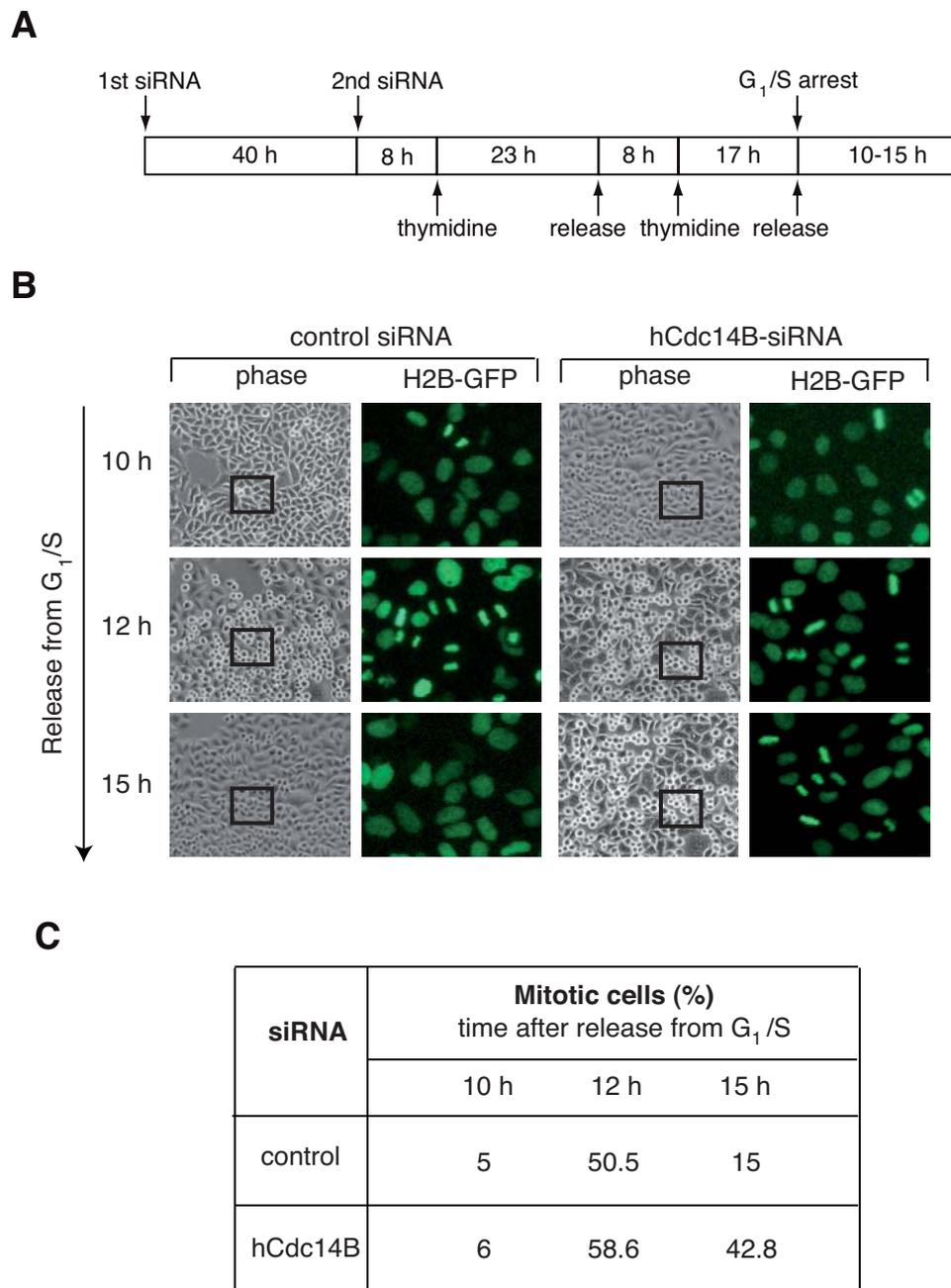


Figure 3.5. Depletion of hCdc14B leads to mitotic arrest. **A)** A schematic presentation of the protocol used for siRNA transfection and cell cycle synchronization. HeLa “Kyoto” cells were transfected twice with 120 nmol of non-targeting control siRNA or hCdc14B specific siRNA #3, synchronized at G₁/S by a double thymidine-block, released into fresh medium, and analyzed at different time points after release. **B)** Depletion of hCdc14B leads to mitotic arrest. Phase contrast (magnification x100) and GFP-fluorescence (magnification x200) images were taken from live cells at 10, 12 and 15 h post release from G₁/S arrest. Progression through mitosis was determined by chromatin condensation that was visualized by histone H2B-GFP fluorescence. The GFP-fluorescence images shown represent a magnification of the

regions marked by the rectangles in the phase contrast images. C) Determination of the mitotic index. Numbers are derived from two independent experiments and from counting 300 cells.

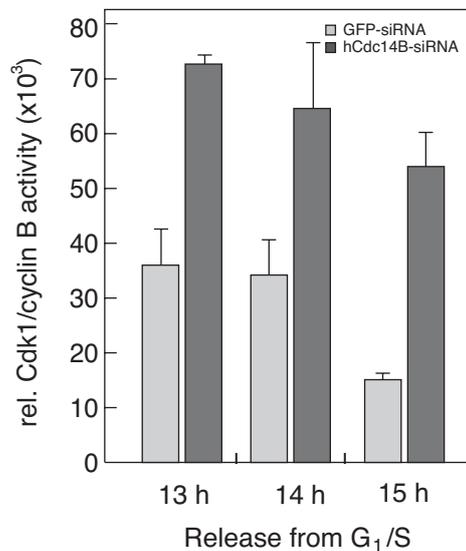


Figure 3.6. Knock-down of hCdc14B elevates Cdk1/cyclin B activity. HeLa ‘Kyoto’ cells were transfected with GFP- or hCdc14B-siRNAs (#2), arrested at G₁/S, and released for 13, 14 or 15 h. Cdk1/cyclin B was precipitated from 150 µg of cell lysate using α -cyclin B antibodies bound to Dynabeads. The kinase activity was measured in a Cdk1 peptide-based assay. The bar diagrams represent the results from two independent experiments.

3.1.8 Cyclin B is stabilized in cell depleted of hCdc14B

The activity of Cdk1 is regulated by different mechanisms including inhibitory phosphorylation at T14/Y15, activating phosphorylation at T161, and association with cyclin A or B (Morgan, 1995). Cyclin B levels increase up to late G₂-phase. As cells progress through late mitosis, cyclin B is ubiquitinated and degraded. Knowing that Cdk1/cyclin B activity is elevated in cells depleted of hCdc14B, I asked whether this reflects inhibition of proteasome-dependent degradation and stabilization of cyclin B. To test this, the level of cyclin B was analyzed by Western blotting using synchronized HeLa ‘Kyoto’ cells that were transfected with hCdc14B or GFP specific siRNAs as described before (see Fig. 3.5.A). As shown in Fig. 3.7., in cells depleted of hCdc14B the amount of cyclin B was significantly elevated (lane 2 and 3) compared to untransfected cells (lane 4) and cells treated with the GFP-siRNA (lane 1), which did not affect the cellular level of hCdc14B. This indicates that hCdc14B is required for cyclin B degradation during late mitosis.

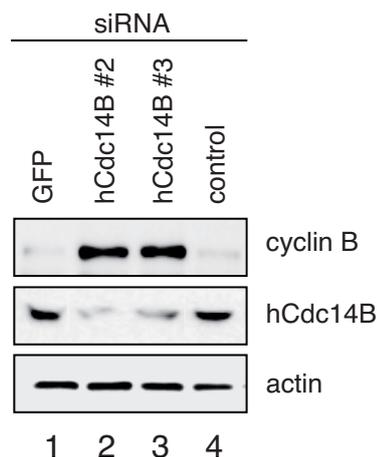


Figure. 3.7. Cyclin B is stabilized in cells depleted of hCdc14B. HeLa 'Kyoto' cells were transfected twice with 120 nM GFP-, hCdc14B (#2)- and (#3)-siRNAs or left untransfected (control). Cells were synchronized at G₁/S by double thymidine-block and released. 16 h after release cells were harvested and cell lysates were analyzed by Western blotting using antibodies against cyclin B and hCdc14B. To control equal protein loading the blot was reprobbed to actin.

3.1.8 Cells depleted of hCdc14B undergo cell death

The previous experiments have indicated that depletion of hCdc14B leads to accumulation of mitotic cells (see Fig. 3.5.B and C). To investigate whether those cells would re-enter the cell cycle with delay or die, cell viability was determined by TUNEL assays. HeLa 'Kyoto' cells transfected with non-targeting siRNAs or hCdc14B-siRNAs, were synchronized at G₁/S, released for 24 h and analyzed by fluorescence microscopy. As shown in Fig. 3.8.A, the majority of cells transfected with hCdc14B-siRNAs stained positive in the TUNEL assay that detects dead cells with fragmented chromatin. This demonstrates that prolonged depletion of hCdc14B leads to cell death. Moreover, analysis of the cells by phase contrast microscopy revealed a significant, 10-fold accumulation of large cells with two or more nuclei in comparison to control cells (Fig. 3.8.B). This indicates that correct cell cycle progression requires a distinct amount of hCdc14B phosphatase and that proper cell division is impaired beyond a critical threshold of hCdc14B.

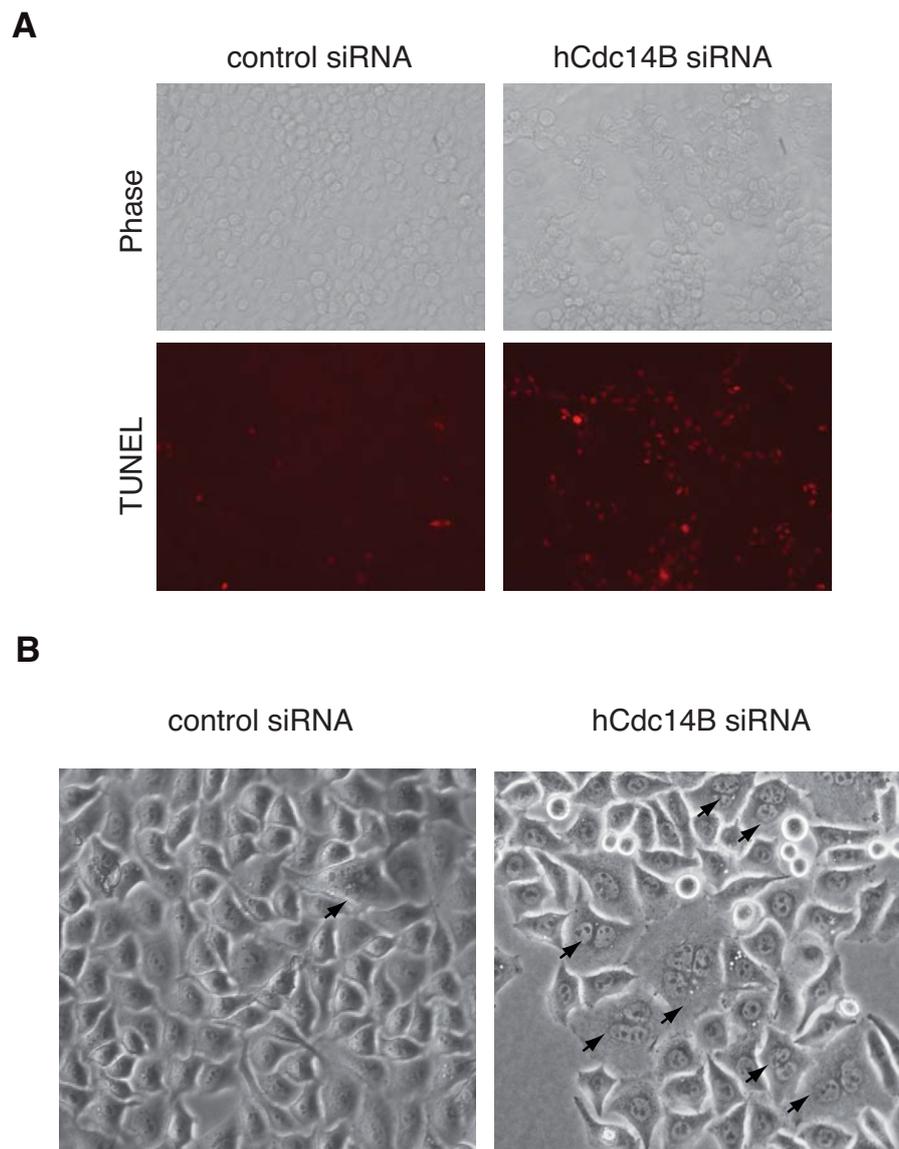


Figure 3.8. Depletion of hCdc14B causes cell death and accumulation of multinucleated cells. A) TUNEL assay of cells depleted of hCdc14B. HeLa ‘Kyoto’ cells were transfected twice with 120 nmol of non-targeting control siRNA or hCdc14B specific siRNA #3, synchronized at G₁/S by a double thymidine-block, and released into fresh medium. 24 h after release cells were fixed, and analyzed by TUNEL assay using the In Situ Cell Death Detection Kit (Roche). TMR-dUTP labeled DNA fragments were visualized using fluorescent microscopy (magnification x100). **B)** Multinucleated cells accumulate upon depletion of hCdc14B. HeLa ‘Kyoto’ cells were transfected with non-targeting siRNAs or hCdc14B-siRNA #3 and synchronized as in (A). 24 h after release, phase contrast images were taken from live cells (magnification x400).

3.2 hCdc14B down-regulates Cdk1/cyclin B activity via Cdc25

3.2.1 hCdc14B inactivates Cdk1/cyclin B indirectly

Given that Cdk1 activation is delayed in cells overexpressing Flag-hCdc14B^{WT} and that Cdk1/cyclin B activity is elevated in cells depleted of hCdc14B, I asked whether hCdc14B directly down-regulates Cdk1/cyclin B. To test this, Sf9 cells were co-infected with baculoviruses encoding His-Cdk1 and myc-cyclin B. Cdk1/cyclin B complexes were purified by ammonium sulfate precipitation, ion exchange chromatography and finally immobilized onto anti-cyclin B antibody-coupled Dynabeads (Heix et al., 1998). Bead-bound Cdk1/cyclin B complexes were incubated with increasing amounts of GST-hCdc14B^{WT}, before the kinase activity was determined. As shown in Fig. 3.9., pre-incubation of Cdk1/cyclin B with active GST-hCdc14B did not affect the kinase activity in the peptide-based assay. This indicates that hCdc14B-mediated inhibition of Cdk1/cyclin B is not direct and suggests that hCdc14B inactivates an upstream regulator of Cdk1/cyclin B.

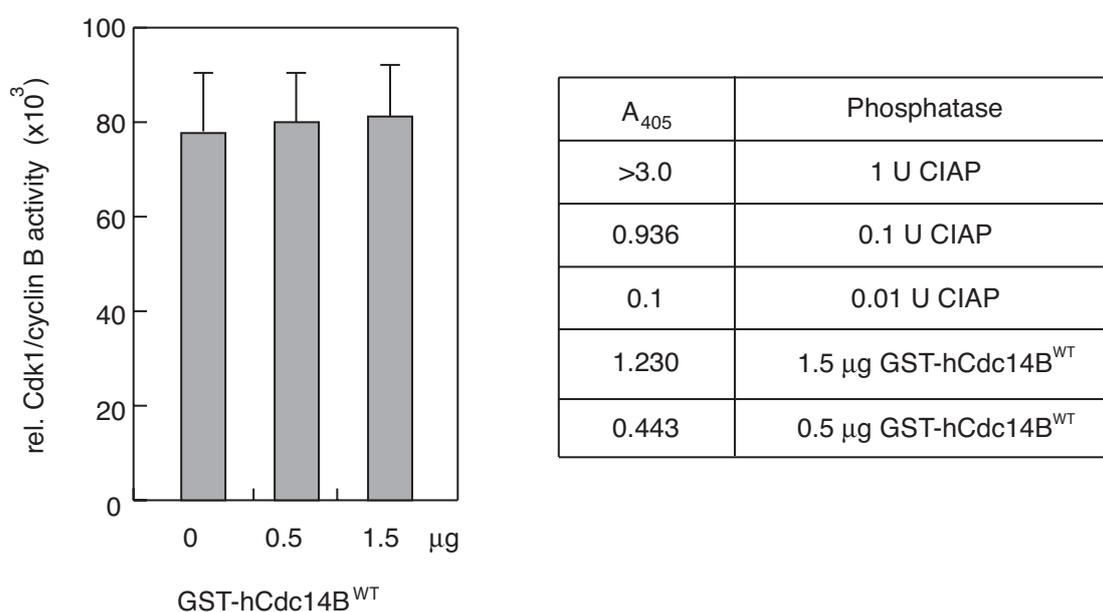


Figure 3.9. hCdc14B down-regulates Cdk1/cyclin B activity indirectly. Recombinant Cdk1/cyclin B complexes were expressed and purified from Sf9 cells, and immobilized onto anti-cyclin B antibody-coupled Dynabeads. Bead-bound Cdk1/cyclin B was incubated with increasing amounts of GST-hCdc14B for 30 min at 30°C before the kinase activity was assayed using Cdk1-specific peptides and $\gamma^{32}\text{P}$ -ATP. The bar diagram represents results from three independent experiments. The activity of GST-hCdc14B^{WT} was measured using the pNPP-based phosphatase assay. Calf intestine alkaline phosphatase (CIAP, Roche) was used as a standard in the phosphatase assay.

3.2.2 hCdc14B targets Cdc25 phosphatases

In cycling cells, activation of Cdk1/cyclin B at G₂/M requires dephosphorylation of pT14/pY15-Cdk1 by the Cdc25 family of phosphatases. Mammalian cells have three isoforms of Cdc25 named Cdc25A, B and C, all of which can activate Cdk1/cyclin B. Cdk1/cyclin B contributes to its own up-regulation by phosphorylating and activating Cdc25 phosphatases, thereby creating a positive feedback loop (Donzelli and Draetta, 2003). Therefore, it is conceivable that hCdc14B dephosphorylates Cdc25, and thereby disrupts the positive feedback loop between Cdc25 and Cdk1/cyclin B and promotes inactivation of Cdk1.

3.2.2.1 hCdc14B dephosphorylates Cdc25 phosphatases *in vitro*

To test this hypothesis, *in vitro* dephosphorylation experiments were performed. Bacterially expressed GST-Cdc25 proteins were immobilized on GT-Sepharose, and phosphorylated using cell extracts prepared from mitotic HeLa cells and $\gamma^{32}\text{P}$ -ATP. After removal of the mitotic extract, ^{32}P -labeled GST-Cdc25 proteins were incubated with either GST-hCdc14B^{WT} or GST-hCdc14B^{PD} for 45 min at 37°C. The samples were subjected to SDS-PAGE, and ^{32}P -labeled Cdc25 was visualized by a PhosphorImager. As shown in Fig. 3.10.A (^{32}P panels), GST-hCdc14B^{WT}, but neither the same activity of alkaline phosphatase (lanes 8-10) nor the same amount of GST-hCdc14B^{PD} (lanes 5-7) dephosphorylated all three isoforms of Cdc25. To prove that hCdc14B removes the phosphates from the Cdk1/cyclin B target sites of Cdc25, bead-bound GST-Cdc25 was phosphorylated using Cdk1/cyclin B complexes purified from Sf9 cells and $\gamma^{32}\text{P}$ -ATP, before incubation with GST-hCdc14B^{WT} or GST-hCdc14B^{PD}. Again, GST-hCdc14B^{WT} but not GST-hCdc14B^{PD} dephosphorylated GST-Cdc25 (Fig. 3.10.B, compare lanes 2 and 3 in ^{32}P panels).

These results are further supported by GST pull-down protein interaction experiments using *in vitro* translated, ^{35}S -labeled Flag-hCdc14B^{WT} and GST-Cdc25A, B, and C bound to GT-Sepharose. Recombinant Flag-hCdc14B^{WT} was retained by all three Cdc25 isoforms indicating that hCdc14B and Cdc25 directly interact with each other (Fig. 3.10.C).

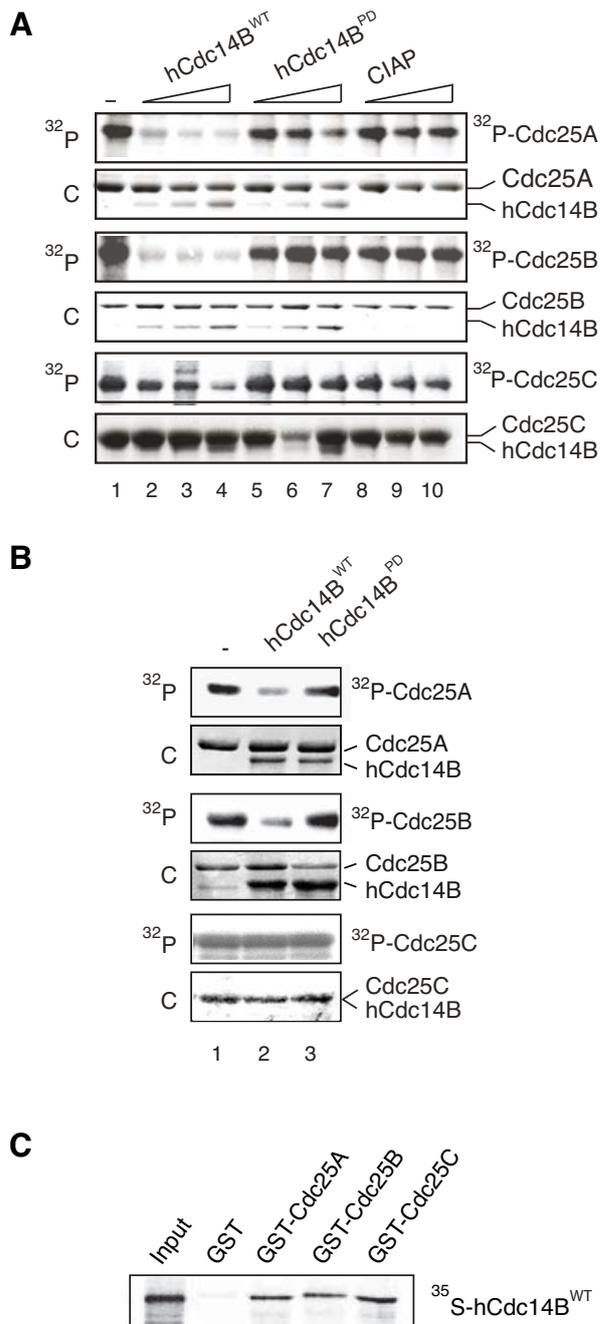


Figure 3.10. hCdc14B dephosphorylates and interacts with Cdc25 phosphatases *in vitro*. **A)** *In vitro* dephosphorylation of GST-Cdc25 by hCdc14B. GST-tagged Cdc25 proteins were immobilized on GT-Sepharose and phosphorylated *in vitro* using extracts from mitotic HeLa cells and $\gamma^{32}\text{P}$ -ATP. After removal of the mitotic extract, beads were incubated for 45 min at 37°C with 0.025, 0.05, and 0.125 units of calf intestine alkaline phosphatase (CIAP), with the corresponding activity of GST-hCdc14B^{WT} or with an amount of GST-hCdc14B^{PD} corresponding to the protein amount of GST-hCdc14B^{WT} as indicated. Samples were separated by 8% SDS-PAGE, stained with Coomassie Blue (C panels) and ^{32}P -labeled Cdc25 (^{32}P panels) was detected by a PhosphorImager. **B)** hCdc14B dephosphorylates Cdk1/cyclin B-target sites of GST-Cdc25. GT-bead bound GST-Cdc25 was phosphorylated using Cdk1/cyclin B expressed and purified from Sf9 cells and $\gamma^{32}\text{P}$ -ATP. ^{32}P -GST-Cdc25 proteins were dephosphorylated by hCdc14B and analyzed as described in (A). **C)** hCdc14B interacts with all three isoforms of Cdc25 *in vitro*. GST or GST-Cdc25A, B, and C were incubated for 4 h at 4°C with ^{35}S -labeled, *in vitro* translated Flag-hCdc14B^{WT}. Bead-bound proteins were separated by SDS-PAGE, and radioactive signals visualized using a PhosphorImager. The input lane contains 10% of the rabbit reticulocyte lysate.

3.2.2.2 hCdc14B dephosphorylates Cdc25 phosphatases *in vivo*

Having shown that hCdc14B dephosphorylates Cdc25, I next investigated whether hCdc14B can also dephosphorylate Cdc25 *in vivo*. HA-Cdc25A, HA-Cdc25B or GFP-Cdc25C was co-expressed with Flag-hCdc14B^{WT} or Flag-hCdc14B^{PD} in HEK-293T cells. 24 h after transfection, cells were synchronized in mitosis by addition of nocodazole for 20 h, harvested, and phosphorylation of Cdc25 was monitored on immunoblots. After co-expression of Flag-hCdc14B^{WT}, but not Flag-hCdc14B^{PD}, the low mobility, hyperphosphorylated forms of all Cdc25 phosphatases disappeared, and

only the hypophosphorylated forms that have a higher mobility on SDS-PAA gels were clearly detected (Fig. 3.11.A). This indicates that excess amounts of phosphatase-active hCdc14B led to dephosphorylation of all three isoforms of Cdc25 in mitotic cells. To strengthen this result, co-immunoprecipitation experiments were performed using lysates from U2OS cells that co-express Flag-hCdc14B^{PD} and HA-Cdc25A, HA-Cdc25B or GFP-Cdc25C. Flag-hCdc14B^{PD} was precipitated using anti Flag M2-beads, and bead-bound proteins were analyzed on Western blots. As shown in Fig. 3.11.B, Flag-hCdc14B^{PD} interacted preferentially with Cdc25B, and albeit less efficient, with Cdc25C and Cdc25A. These results are in accordance with the *in vitro* studies showing that recombinant hCdc14B interacts with and dephosphorylates Cdc25 in a direct manner. This proposes that hCdc14B antagonizes Cdk1/cyclin B-dependent phosphorylations on Cdc25 phosphatases, and thereby disrupts the positive feedback loop that ensures high Cdk1 activity during early stages of mitosis.

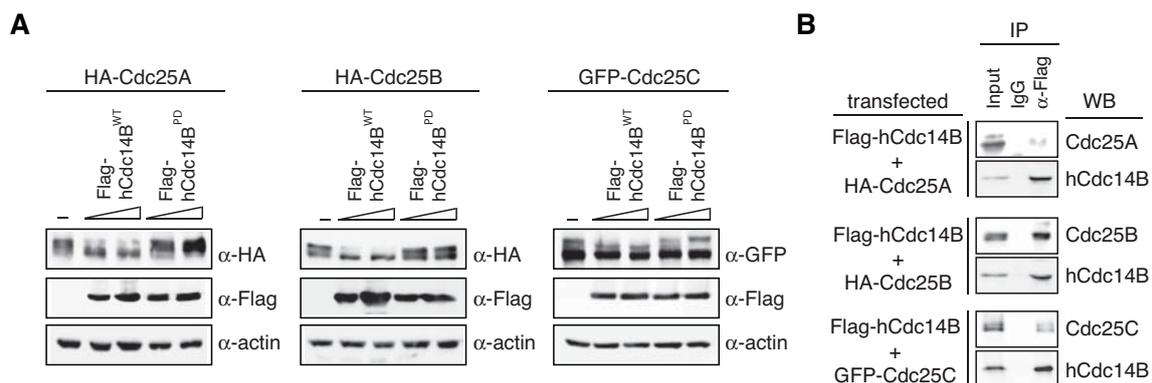


Figure 3.11. hCdc14B dephosphorylates and interacts with Cdc25 phosphatases *in vivo*. **A**) hCdc14B dephosphorylates Cdc25 *in vivo*. 1×10^6 HEK-293T cells were transfected with 2.5 μ g of plasmids encoding HA-Cdc25A, HA-Cdc25B or GFP-Cdc25C together with plasmids encoding Flag-hCdc14B^{WT} or Flag-hCdc14B^{PD} (1 and 2 μ g). 24 h after transfection, cells were treated with nocodazole for 20 h. Cell lysates were analyzed by Western blotting using α -HA or α -GFP antibodies to detect Cdc25 proteins, and α -Flag antibodies to visualize ectopic hCdc14B. Lower mobility bands in the HA-Cdc25A, -Cdc25B, and GFP-Cdc25C blots represent the hyperphosphorylated forms of Cdc25. Equal protein loading was monitored using α -actin antibodies. **B**) hCdc14B interacts with Cdc25 *in vivo*. U2OS cells were co-transfected with expression plasmids for HA-Cdc25A/B, or GFP-Cdc25C and Flag-hCdc14B^{PD} as indicated. 44 h post transfection, Flag-hCdc14B was immunoprecipitated and co-precipitation of HA-Cdc25A and B and GFP-Cdc25C was analyzed on Western blots. To monitor IP efficiency, 30% of the immunoprecipitate were probed with α -Cdc14B antibodies and compared to 10% of input levels.

3.3 Regulation of hCdc14B during the cell cycle

3.3.1 Expression level of hCdc14B during the cell cycle

This work has established an important role of hCdc14B activity during mitosis. It is conceivable that hCdc14B is tightly regulated during the cell cycle to prevent unscheduled activation and dephosphorylation of targets. To investigate whether the cellular level of hCdc14B fluctuates during the cell cycle, HeLa cells were synchronized at G₁/S by a double-thymidine block, or at prometaphase by nocodazole. Mitotic cells were collected by mitotic shake-off, seeded into fresh medium and harvested at different time points after release from the nocodazole-arrest. Determination of the amount of hCdc14B by immunoblotting with antibodies specific to hCdc14B indicated that the cellular level of hCdc14B did not change during the phases of the cell cycle tested (Fig. 3.12.).

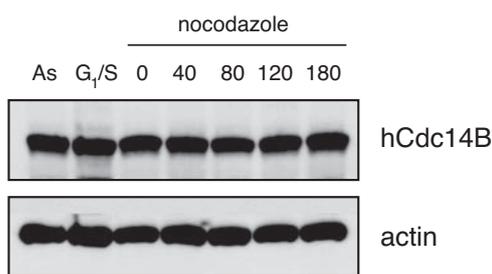


Figure 3.12. The cellular level of hCdc14B at different phases of the cell cycle. HeLa cells were arrested at G₁/S by double-thymidine block or synchronized in mitosis by nocodazole treatment, released for indicated time points and harvested. The cell lysates were separated by SDS-PAGE and hCdc14B was detected using α -Cdc14B antibodies. As a loading control, α -actin antibody was used.

3.3.2 Determination of hCdc14B phosphatase activity during the cell cycle

3.3.2.1 Establishment of HeLa cells stably expressing Flag-hCdc14B^{WT}

To examine, whether the enzymatic activity of hCdc14B fluctuates during the cell cycle, hCdc14B was precipitated from lysates of cells synchronized at different phases of the cell cycle and assayed for phosphatase activity *in vitro*. Because the α -Cdc14B antibody poorly precipitates endogenous hCdc14B under non-denaturing conditions, I had to express recombinant Flag-tagged hCdc14B at a moderate level and to perform immunoprecipitation with M2-beads. Since the U2OS-Flag-hCdc14B^{WT} cell lines used in this study expressed Flag-hCdc14B^{WT} at a high level, new cell lines were to be generated that express the recombinant hCdc14B at low level. Therefore, HeLa cells that stably express Flag-hCdc14B^{WT} in a tet-inducible manner were established. Individual clones of this cell line were isolated and tet-inducible expression of Flag-

hCdc14B^{WT} was analyzed on Western blots. Only one cell clone, 17, expressed Flag-hCdc14B^{WT} at a level comparable to the level of endogenous hCdc14B (Fig. 3.13.A and B). This clone was used for further functional studies.

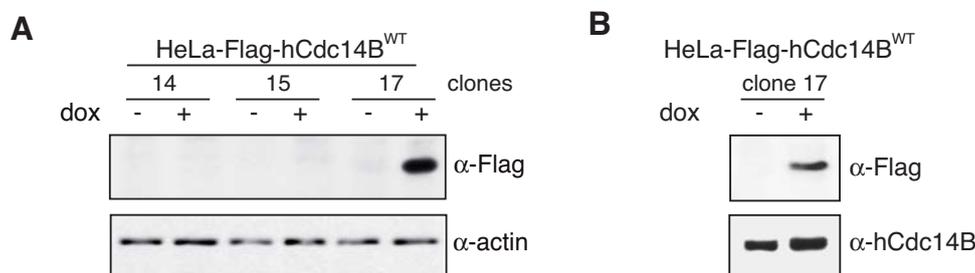


Figure 3.13. Tetracycline-induced expression of Flag-hCdc14B^{WT} in different HeLa-Flag-hCdc14^{WT} cell clones. **A)** Analysis of Flag-hCdc14B^{WT} expression in different HeLa cell clones. Individual cell clones were incubated with doxycycline (dox, 2 μg/ml) for 16 h and Flag-hCdc14B^{WT} expression levels were monitored on immunoblots using α-Flag antibodies. The Western blot was reprobbed with antibodies specific to actin to monitor equal protein loading. **B)** Analysis of the expression level of Flag-hCdc14B^{WT} in clone 17 of HeLa-Flag-hCdc14B^{WT}. Flag-hCdc14B^{WT} expression was induced as described in (A) and the cell lysate was analyzed by Western blotting using α-Flag and α-Cdc14B antibodies.

3.3.2.2 Phosphatase activity of hCdc14B from HeLa cells expressing

Flag-Cdc14^{WT}

Expression of Flag-hCdc14B^{WT} in HeLa-Flag-hCdc14B^{WT} cells (clone 17) and synchronization at G₁/S was performed in the presence of doxycycline and thymidine for 25 h, respectively. For mitotic arrest, cells were cultured in the presence of doxycycline and nocodazole for 20 h, mitotic cells were collected by shake-off, and released by transfer to fresh medium for 1 h. Synchronized cells were harvested, one half of the sample was processed for FACS analysis to monitor efficiency of synchronization, the second half was used to assay hCdc14B activity. As shown in Fig. 3.14.A, after treatment with thymidine the majority of cells had DNA content between 2N and 4N indicating that cells were arrested in S-phase. Cells treated with nocodazole had 4N DNA content demonstrating mitotic arrest. 1 h after release from nocodazole arrest, the G₂/M population was reduced and cells with 2N DNA content increased indicating completion of mitosis and entry into the next G₁-phase.

Flag-hCdc14B^{WT} was immunoprecipitated from whole cell lysates and assayed for phosphatase activity. As shown in Fig. 3.14.B, hardly any changes of hCdc14B activity were observed under these experimental conditions. This confirms results

obtained by Kaiser et al. (2002), who precipitated endogenous hCdc14B from cells synchronized at different phases of the cell cycle, and tested its ability to dephosphorylate *in vitro* ^{32}P -labeled Cdh1. In their assay, hCdc14B activity did not change throughout the cell cycle either.

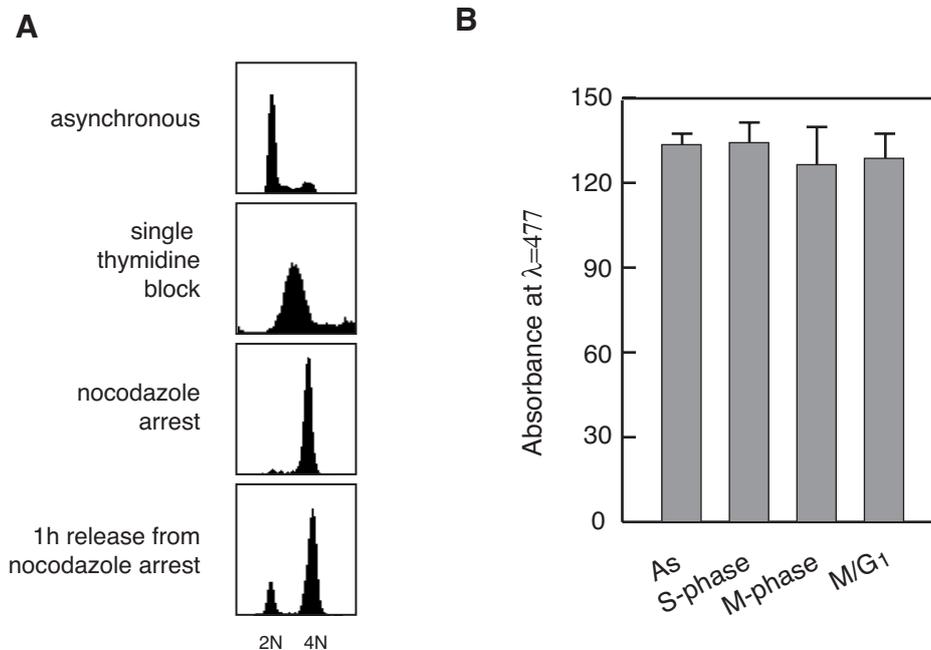


Figure 3.14. The activity of hCdc14B does not change during the cell cycle. **A)** FACS analysis of synchronized HeLa cells stably expressing Flag-hCdc14B^{WT}. HeLa-Flag-hCdc14B^{WT} cells were cultured in the presence of 2 $\mu\text{g}/\text{ml}$ doxycycline to induce expression of Flag-hCdc14B^{WT} and 2 mM thymidine for 25 h (S-phase arrest) or 40 ng/ml nocodazole for 16 h (mitotic arrest). Mitotic cells were collected by shake-off and released for 1 h. Cells were harvested and analyzed by FACS. **B)** Activity of Flag-hCdc14B^{WT} in synchronized cells. Flag-hCdc14B^{WT} was immunoprecipitated from lysates of synchronized cells using M2-beads. Activity of bead-bound Flag-hCdc14B^{WT} was determined by measuring hydrolysis of OMFP. Absorbance of OMF, the product of OMFP hydrolysis, was measured at $\lambda=477$ nm using an ELISA reader. Data were normalized to the amount of Flag-hCdc14B^{WT} in the input according to quantitative Western blotting.

3.3.3 Nucleolar sequestration of hCdc14B fluctuates during the cell cycle

3.3.3.1 hCdc14B is associated with rDNA during interphase but not during mitosis

Though previous immunofluorescence studies have shown that endogenous and recombinant hCdc14B localize to nucleoli during interphase (Kaiser et al., 2002; Mailand et al., 2002b), it is not known whether nucleolar sequestration involves binding to the repetitive rDNA elements, which represent a main component of nucleoli. To elucidate whether hCdc14B is associated with rDNA, chromatin immunoprecipitation

(ChIP) was used. A single rDNA repeat has a length of app. 43 kb and consists of the rDNA promoter, the transcribed region, and the intergenic spacer (IGS) that separates individual rDNA repeats. After crosslinking, protein-DNA complexes were precipitated with antibodies against hCdc14B, hUBF or rabbit IgGs, and co-immunoprecipitated DNA was amplified in a semi-quantitative manner using primers specific to different regions of rDNA repeat, e.g. sequences of the 5'-IGS which are located proximal to the promoter (A), the promoter region (B), the 3' end of the 28S rRNA transcribed region (C) and the 3'-IGS (D) (Fig. 3.15.A). As shown in Fig. 3.15.B, hCdc14B was associated with the intergenic sequences of the rDNA, but not with the promoter or the transcribed region (lanes 5 and 6). Remarkably, rDNA occupancy was only observed in asynchronously growing cells, but not in nocodazole-treated cells that are arrested at prometaphase of mitosis. This suggests that hCdc14B is associated with rDNA during interphase being released not later than prometaphase. Consistent with previous reports (Mais et al., 2005), the RNA polymerase I specific transcription factor hUBF was mainly detected at the promoter, promoter-proximal 5' upstream regions, and the transcribed regions of rDNA throughout the cell cycle (Fig. 3.15.B, lanes 7 and 8).

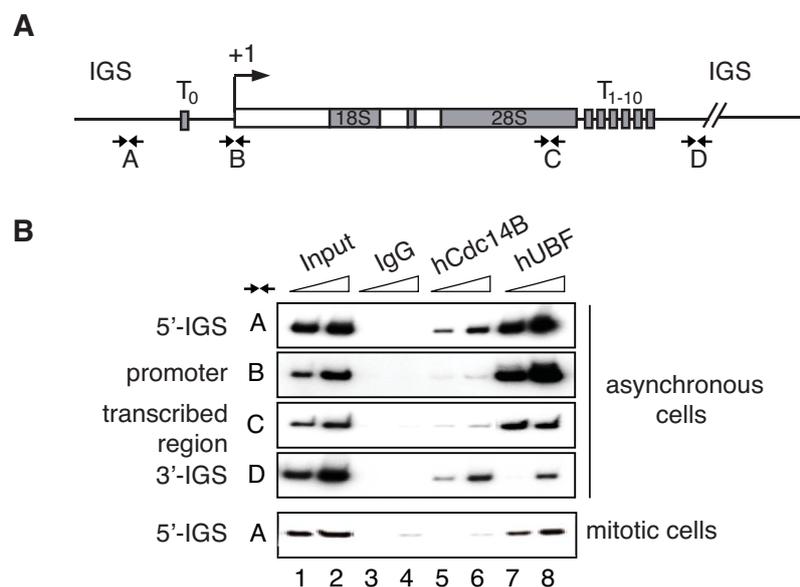


Figure 3.15. hCdc14B is associated with rDNA. **A**) A scheme depicting the organisation of the human rDNA repeat and the positions of the PCR primers relative to the transcription start site (arrow). **B**) ChIP analysis using asynchronous or nocodazole-treated HeLa cells. Protein-DNA complexes were cross-linked by 1% formaldehyde and precipitated using antibodies against hCdc14B and hUBF or rabbit IgGs as indicated. 0.5 and 2 μ l of the precipitated DNA was amplified by PCR using α -³²P-dCTP and primers that amplify the following regions of the human rDNA: -1017/-924 (A), -48/+32 (B), +12855/+12970 (C), +27365/+27475 (D). PCR products were separated by PAA-gel electrophoresis and the radioactive signals visualized by a PhosphorImager. The input lanes contain PCR products obtained from amplification of 0.05% and 0.2% of the total non-precipitated input chromatin.

3.3.3.2 hCdc14B is released from chromatin during mitosis

Knowing that binding of hCdc14B to rDNA chromatin is lost during mitosis, I next investigated the timing of release and reassociation of hCdc14B to chromatin during mitosis and at G₁. To address this, extracts from synchronized HeLa cells were fractionated to yield cytoplasmic, nucleoplasmic and chromatin fractions (Watrin et al., 2006), and probed for hCdc14B.

HeLa “Kyoto” cells were either synchronized at G₁/S by double thymidine-block and released for 6 h into S-/G₂-phase, or at prometaphase by nocodazole and released into late stages of mitosis and the next G₁-phase. To monitor cell cycle progression, FACS analysis, fluorescence microscopy, and immunoblotting analysis of cyclin A, cyclin E and H3-pSer10 were performed. FACS analysis showed that 6 h after release from G₁/S, cells had passed through S-phase as indicated by the accumulation of the 4N DNA content cells (Fig. 3.16.A). Consistent with this, cyclin E levels dropped and cyclin A levels increased, whereas H3-pSer10 was not detected indicating that cells had not yet entered mitosis (Fig. 3.16.B). Western blotting analysis of the cytoplasmic, nucleoplasmic and chromatin fractions showed that at G₁/S-, S- and G₂-phase, the majority of hCdc14B was contained in the chromatin fraction demonstrating that hCdc14B is tightly bound to rDNA during interphase (Fig. 3.16.C, lower panels). In contrast, hCdc14A was exclusively detected in the cytoplasmic fraction and absent from the nucleoplasmic and chromatin fractions (Fig. 3.16.C, upper panels). This is in agreement with the centrosomal localization of hCdc14A (Kaiser et al., 2002; Mailand et al., 2002b). Synchronization at prometaphase by nocodazole and progression through mitosis and the next G₁-phase upon release from the nocodazole-arrest was monitored by live cell fluorescence microscopy of histone H2B-GFP (Fig. 3.16.D) and by Western blot analysis of cyclin A, cyclin E and H3-pSer10 (Fig. 3.16.E). 40 min after release, the majority of cells had reached metaphase as indicated by the fully condensed chromatin that was located at the cellular equator (Fig. 3.16.D, second panel). 80 min after release, sister chromatids had separated to the two poles indicative of anaphase/telophase (Fig. 3.16.D, third panel). Cells exited mitosis and entered G₁-phase of the next cell cycle within 120 min after release, as indicated by the decondensation of chromatin, dephosphorylation of Ser10 on histone H3 and drop of the cyclin A level (Fig. 3.16.D, fourth panel and 3.16.E). In agreement with results from ChIP analysis, only trace amounts of hCdc14B were contained in the chromatin fraction during prometaphase, whereas the majority of hCdc14B was soluble (Fig. 3.16.F, lower panels). Enrichment

of hCdc14B in the soluble fraction was observed until anaphase/telophase. At later time points, e.g. 120 min and beyond, hCdc14B had re-associated with chromatin (Fig. 3.16.F, lower panels). Taken together, these results demonstrate that hCdc14B is tethered to rDNA chromatin during interphase, released from chromatin from prometaphase until telophase, and rebound to chromatin in early G₁-phase (Fig. 3.16.C and F, lower panels). This suggests that nucleolar sequestration and release of hCdc14B is linked to the physiological role of hCdc14B during mitosis. hCdc14A, on the other hand, is never associated with chromatin and mainly contained in the cytoplasmic fraction throughout the cell cycle (Fig. 3.16.C and F, upper panels), indicating that the localization of both human Cdc14 phosphatases is regulated in an isoform-specific manner.

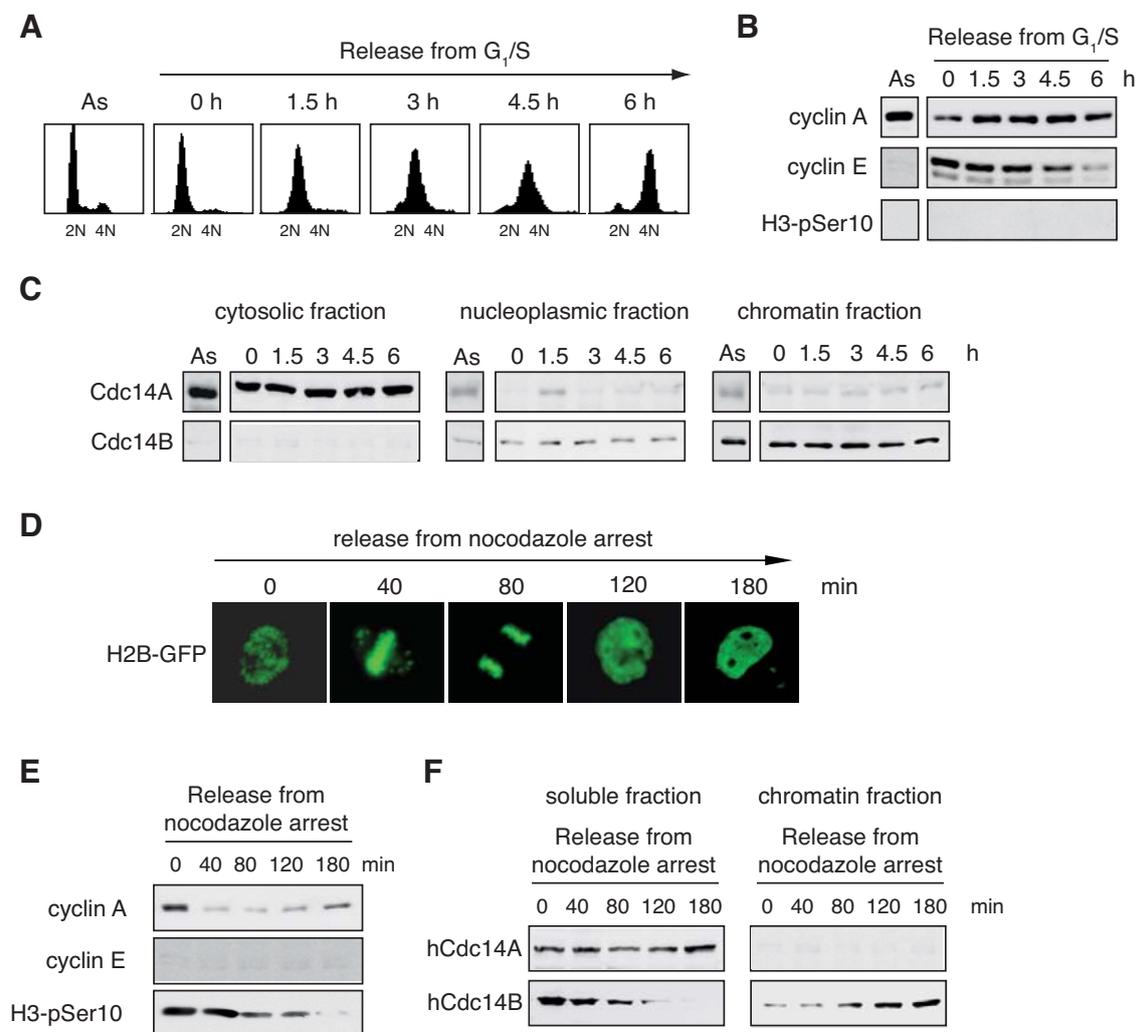


Figure. 3.16. Cellular distribution of hCdc14B during the cell cycle. **A)** HeLa 'Kyoto' cells expressing histone H2B-GFP were arrested at G₁/S by double thymidine-block, released for the indicated times and analyzed by FACS. **B)** Western blot analysis of cells synchronized and released from G₁/S using

antibodies against cyclin A, E and H3-pSer10. **C)** hCdc14B is retained in the chromatin fraction during interphase. Cells were synchronized and released from G₁/S as described in (A), and harvested at the indicated time points. Cell lysates were separated into cytosolic, nucleoplasmic and chromatin fractions, and analyzed on Western blots using antibodies against hCdc14A and B. **D)** Fluorescence microscopy of H2B-GFP labeled chromatin in HeLa 'Kyoto' cells arrested by nocodazole in pro-metaphase and released for the indicated time (magnification x400). **E)** Western blot analysis of HeLa 'Kyoto' cells arrested by nocodazole and released for the indicated time. The cell lysate was probed to antibodies against cyclin A, E and H3-pSer10. **F)** hCdc14B is released from chromatin during prometaphase until anaphase/telophase. HeLa 'Kyoto' cells synchronized as described in (D) were fractionated into soluble and chromatin fractions. Western blot analysis was performed as indicated in (C).

3.3.4 Determination of the native size of hCdc14B

In yeast, Cdc14p is a subunit of the 280 kDa RENT complex, which contains Net1 (190 kDa) and the histone deacetylase Sir2 (60 kDa), and tethers yCdc14 to the rDNA (Shou et al., 1999; Visintin et al., 1999). Like yCdc14, hCdc14B is bound to rDNA in human cells and released during mitosis from nucleoli. It was tempting to speculate that also hCdc14B is tethered to rDNA via a multisubunit complex similar to RENT. hCdc14B has a molecular weight of 54 kDa, but its native size is unknown.

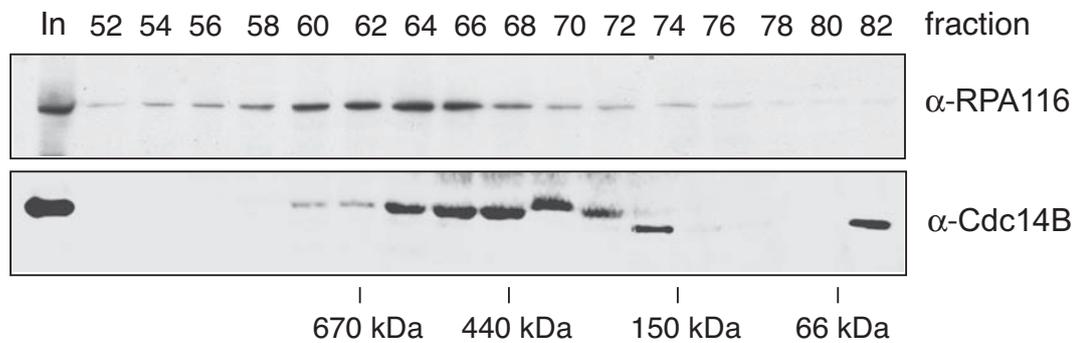
To determine the native size of hCdc14B, nuclear extracts prepared from HeLa cells were fractionated by gel filtration on a Superose 6 column that was calibrated with the following molecular size standards: thyroglobulin (670 kDa) eluting at fraction 62, apoferritin (443 kDa) eluting at fraction 68, alcohol dehydrogenase (150 kDa) eluting at fraction 76, and bovine serum albumin (66 kDa) eluting at fraction 81. Fractions ranging from 52-82 were collected, the proteins of each fraction were precipitated with TCA and analyzed on Western blots using antibodies against hCdc14B and the RNA polymerase I subunit RPA116 (Fig. 3.17.). RNA polymerase I has a native size of ~600 kDa (Carles and Riva, 1998). In agreement with this, RPA116 was enriched in fractions 60-66 (Fig. 3.17.A, upper panel). hCdc14B eluted with two peaks comprising fractions 60-74 and fraction 82 (Fig. 3.17.A, lower panel). The first hCdc14B protein peak corresponds to a molecular size of 400-600 kDa. The second peak contains most likely monomeric hCdc14B, since its calculated size is smaller than 66 kDa. This indicates that the majority of cellular hCdc14B is bound within a high molecular weight complex.

The native size of hCdc14B was strikingly different in mitotic HeLa cells (Fig. 3.17.B, lower panel). The bulk of hCdc14B eluted in fractions 76-80 corresponding to a molecular weight of app. 50-100 kDa. Besides this, a part of hCdc14B was also contained in high molecular weight fractions having apparent molecular masses of about 500 kDa (fractions 66-68), and bigger than 1 MDa (fraction 52). The elution

behaviour of RPA116 was not altered (Fig. 3.17.B, upper panel). These results suggest that during mitosis, hCdc14B is either monomeric or associated with cellular complexes of different molecular masses rather than with one distinct complex.

A

Extract from non-synchronized HeLa cells



B

Extract from mitotic HeLa cells

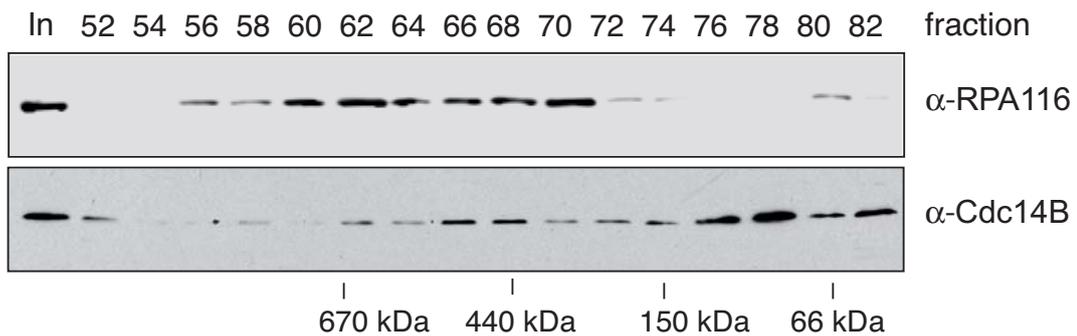


Figure 3.17. Fractionation of hCdc14B on a Superose 6 sizing column. 2.4 mg of nuclear extract from non-synchronized (**A**) or whole cell extracts from mitotic cells (**B**) were fractionated on a Superose 6 column. Fractions from 52 to 82 were collected, and proteins of each fraction were precipitated with TCA. Samples of every second fraction were analyzed on Western blots using α -RPA116 and α -hCdc14B antibodies. 5% of input were loaded in separate lanes. The elution of the molecular weight standards is indicated at the bottom of the blots.

3.3.5 hCdc14B is phosphorylated by Cdk1/cyclin B

ChIP and analysis of biochemically fractionated cells showed that hCdc14B is released from nucleoli between prometaphase of mitosis and early G_1 -phase. However, the mechanism underlying the release of hCdc14B is unknown. In *S. cerevisiae* Net1 binds to the catalytic core of yCdc14 and sequesters it to the nucleolus. In early anaphase Cdk1 phosphorylates Net1, which induces release of Cdc14p and thereby activates yCdc14 (Pereira et al., 2002; Yoshida and Toh-e, 2002). In *S. pombe*, nucleolar release

of Cdc14/Clp1 occurs during early mitosis (Cueille et al., 2001; Trautmann et al., 2001 and 2004). Upon initial release, Clp1 is phosphorylated and inhibited by mitotic Cdk/cyclin complexes. It has been suggested that Clp1 autocatalytically reverses its phosphorylation and restores full activity as Cdk1 activity decreases during anaphase progression (Wolfe et al., 2006).

hCdc14B	5	SERRSSWAAAPPCSRRCSSSTSPGVKKIRSSSTQDDPRRRDPQDDVYLD----ITDRLCFAI
hCdc14A	1	-----MAAESGELIGACEFMKDRLYFAT
yCdc14 (S.c.)	1	-----MRRSVYLDNTIEFLRGRVYLG
Clp1 (S.p.)	1	-----MDYQDDGLGEMIEFLEDKLYYTS
hCdc14B	61	LYSRP--KSASNVHYFSIDNELEYENFYADFGPLNLMVYRYCCKINKKLSITMLR--K
hCdc14A	24	LRNRP--KSTVNTHYFSIDEELVYENFYADFGPLNLMVYRYCCKLNKKSISLSR--K
yCdc14 (S.c.)	23	YDYTP--EDTDELVFFTVEDAFYNSFHLDFGPMNIGHLYRFAVIFHEILNDPENAN--K
Clp1 (S.p.)	24	LSQPPKAELYPHMHFFTTIDDELIYNPFYHDFGPLNVSHLIRFAVIVHGIMGKHRQAKKSK
hCdc14B	117	KIVHFTGSDQRKQANAFLVGCYMVVYLGRPEEAYRILIFG-ETSYIPFRDAAYGSCNF
hCdc14A	80	KIVHYTCFDQRKRANAFLIGAYAVIYLKKTPEEAYRALLSGSNPPYLPFRDASFGNCTY
yCdc14 (S.c.)	79	AVVFYSSASTRQRANAACMLCCYMLVQAWTTPHQLVQPLAQV-DPFMPFRDAGYSNADF
Clp1 (S.p.)	84	AIVLYSSTDTRLRANAACLLACYMVLVQNWPPHLALAPLAQA-EPPFLGFRDAGYAVSDY
hCdc14B	176	YITLLDCFHAVKKAMQYGLNFSNLFNDEYEHYKAENGLNWIIPDRFIAFCGPHS---
hCdc14A	140	NLTILDCLQGIRKGLQHGFFDFETFDVDEYEHYERVENGFNWIIPGKFLAFSGPH---
yCdc14 (S.c.)	138	EITIQDVVYGVWRAKEKGLIDLHSFNLESYKEYEHVEFGDFNVLTLP-DFIAFASPDQEDHP
Clp1 (S.p.)	143	YITIQDCVYGLWRARESSILNIRNIDVHDYETERYVENGFNWIISP-KFIAFASPIQAGW
hCdc14B	233	--RARLESYHQHSPETYIQYFKHNHVTTIIRLNKRMYDAKRFDTAGFDHDLFFADGST
hCdc14A	197	--KSKIENGYPLHAPEAYFPYFKHNVTAVVRLNKKIYEAKRFDTAGFEHYDLFFIDGST
yCdc14 (S.c.)	197	KGYLATKSSHLNQPFKSVLNFFANNNVQLVRLNSHLYNKKHFEDIGIQHLDLIFEDGTC
Clp1 (S.p.)	202	N-HASTRPPKLPQPPFAIVLDYFVANKVKLIVRLNGLYDKKTFENVGIRHKEMYFEDGTV
hCdc14B	291	PTDAIVKEFLDICENAEG---AIAVHCKAGLGRGTGLIACYIMKHYRMTAAETIAWVRIC
hCdc14A	255	PSDNIVRRFLNICENTEG---AIAVHCKAGLGRGTGLIACYVMKHYRFTHAEIIAWIRIC
yCdc14 (S.c.)	257	PDLISIVKNFVGAETIIKRGKGIKVHCKAGLGRGTGLIAGHLIYTYGFTANECIGFLRFI
Clp1 (S.p.)	261	PELSLVKEFIDLTEEVEE-DGVIAVHCKAGLGRGTGLIAGAYLIYKHCFTANEVIAYMRIM
hCdc14B	348	RPGSVIGPQQQFLVMKQTNLWLEGDYFRQKLGQENGQHRAAFSKLLSGVDDISING---
hCdc14A	312	RPGSIIIGPQQHFLEEKQASLWVQGDIFRSKLNRPSSSEG--SINKILSGLDDMSIGGNLS
yCdc14 (S.c.)	317	RPGMVVGPQQHWLYLHQVDFR---EWKYTTTRISLKPSEAIGGLYPLISLEEYRLQKPKLK
Clp1 (S.p.)	320	RPGMVVGPQQHWLHINQVHFR---AYFYEKAMGRAIQQAT-AAEPLATPPRHPLN-----
hCdc14B	405	-VENQDQOPEPEPYSDD--DEINGVTQGDRLRALKSRRQSKTN-----
hCdc14A	370	KTQNMERFGEDNLEDDDVEMKNGITQGDKLRALKSQRQPRTSPSCAFRRSDDTKGHPRAVS
yCdc14 (S.c.)	374	DDKRVAQNNI EGE LRDLTMTPPS--NGHGALSARNSSQPSTANN-----
Clp1 (S.p.)	371	ATNGTSQSNISTPLPEPTPGQPRKVS GHNPPSARRLPSASSVKF-----
hCdc14B	444	-AIPLTVILQSSVQSCKTSEPNISGSAGITKRTRTSASRKSSVKSLSISR-----
hCdc14A	430	QFRLSSSLQGSVAVTLKTSKMALSPSATAKRINRTSLSSGATVRSFSINSRLASSLGNLN
yCdc14 (S.c.)	416	-GSNSFKSSAVPQTSPPGQPRKQNGSNTIEDINNNRNPTSHANRKVVIESNNSDDESMQD
Clp1 (S.p.)	415	-NEKLKNASKQSIQENKASYSSYEDSEIQNDDETRTVGTPPETISVVRLRRSSSQSNIE
hCdc14B		-----
hCdc14A	490	AATDDPENKKTSSSSKAGFTASPTNLLNGSSQPTRNYPELNNQYNRSSNSNGGNLNS
yCdc14 (S.c.)	475	-----TNGTSNHYPKVSRRKNDISSASSSRMEDN
Clp1 (S.p.)	474	-----PNGVR--SPTSSPTGSPIRRRTSGNRWSS-
hCdc14B	493	-----TKTVLR-----
hCdc14A	550	PPGPHSAKTEEHTTILRPSYTLGSSSSARFLRSRIPSLQSEYVHY---
yCdc14 (S.c.)	504	EPSATNINNAADDTILRQLLPKNRRVTSGRRTSAAGGIRKISGSIKK
Clp1 (S.p.)	500	--GSSHKSKSAQRSVSMSSLNN---TSNGRVAKPKPSKRLIS-----

Figure 3.18. Putative Cdk1/cyclin B phosphorylation sites of hCdc14B. NetPhos 2.0 analysis of putative Cdk1/cyclin B target sites in hCdc14B. The candidate phosphorylation sites are highlighted in red and the target sites conserved in other Cdc14 orthologues are boxed in grey.

Whether Cdk1/cyclin B targets hCdc14B is not known. The hCdc14B polypeptide chain contains four sites, which perfectly correspond to the minimal Cdk consensus phosphorylation site Ser/Thr-Pro (Fig. 3.18.). Three of these sites T147, S244 and T290, are located in the catalytic core domain and one site, S25, is located in the amino-terminal nucleolar localization domain (see also Fig. 1.3.A). To test whether hCdc14B is phosphorylated by Cdk1/cyclin B, GST-hCdc14B^{WT} and GST-hCdc14B^{PD} were incubated with $\gamma^{32}\text{P}$ -ATP and recombinant Cdk1/cyclin B purified from Sf9 cells. Purified Flag-hUBF1 was used as a positive control for Cdk1/cyclin B-specific phosphorylation. The RNA polymerase I transcription factor UBF is phosphorylated by Cdk1/cyclin B during mitosis (Klein and Grummt, 1999). Both GST-hCdc14B^{WT} and GST-hCdc14B^{PD} were phosphorylated by Cdk1/cyclin B like hUBF (Fig. 3.19.A). This shows that hCdc14B serves as a substrate for Cdk1/cyclin B *in vitro*. To further support this, I tested in GST pull-down assays whether hCdc14B and Cdk1/cyclin B interact with each other. For this, GST-hCdc14B^{WT} was incubated with *in vitro* translated, ³⁵S-labeled Cdk1 or ³⁵S-labeled cyclin B. As shown in Fig. 3.19.B, GST-hCdc14B^{WT} retained Cdk1 but not cyclin B. Taken together, these data provide the first evidence that hCdc14B is a target for Cdk1/cyclin B. Future studies have to address the functional implications of Cdk1/cyclin B-dependent phosphorylation of hCdc14B *in vivo*.

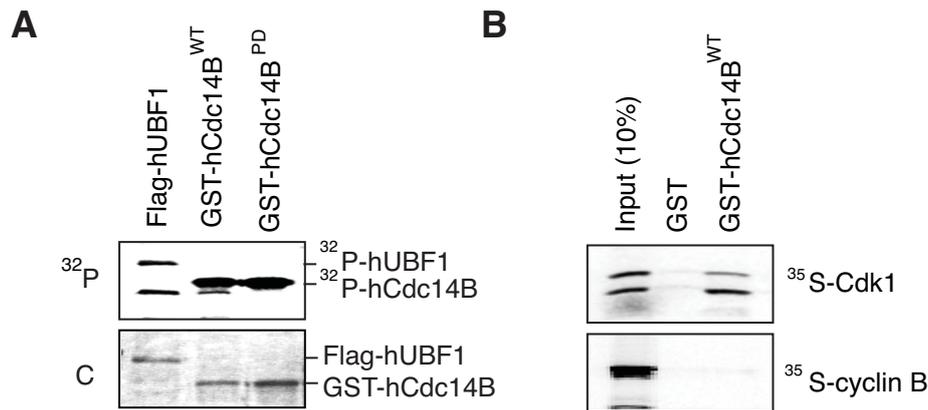


Figure 3.19. Cdk1/cyclin B phosphorylates hCdc14B *in vitro*. **A)** *In vitro* phosphorylation of GST-hCdc14B by Cdk1/cyclin B. Flag-hUBF1, GST-hCdc14B^{WT} and GST-hCdc14B^{PD} were phosphorylated *in vitro* using Cdk1/cyclin B and γ ³²P-ATP. After incubation for 30 min at 30°C, the reaction was stopped by addition of SDS-sample buffer. The samples were separated by 10% SDS-PAGE, stained with Coomassie Blue (C panel), and ³²P-labeled hUBF1 and GST-hCdc14B were visualized by PhosphorImager (³²P panel). **B)** GST-hCdc14B directly interacts with Cdk1. GST and GST-hCdc14B^{WT} were immobilized on GT-beads and incubated with ³⁵S-labeled Cdk1 (upper panel) or cyclin B (lower panel) for 4 h at 4°C. Bead-bound proteins were separated by SDS-PAGE, and radioactive signals visualized using a PhosphorImager. The input lanes contain 10% of the lysate. The lower bands in the ³⁵S-Cdk1 gel contain a degradation form of ³⁵S-Cdk1.

3.4 Identification of novel hCdc14B substrates

To gain further insight into the function of hCdc14B, it is important to identify proteins that interact with hCdc14B and that either serve as substrates of the phosphatase or mediate nucleolar sequestration of hCdc14B. For this, hCdc14B complexes should be isolated and components of the complex identified by mass spectrometry. As a first approach, tandem affinity purification (TAP) was chosen, since this method has been already successfully applied by several groups for isolation of protein complexes from eukaryotic cells (Gavin et al., 2002; Bertwistle et al., 2004). The classical TAP-tag consists of the IgG binding domain of protein A, followed by a protease cleavage site for the tobacco etch virus (TEV) protease, and the calmodulin binding domain (Fig. 3.20.A). The protein of interest is expressed as a fusion protein with the TAP-tag being positioned either at the amino- or the carboxy-terminus. For isolation, the fusion protein is first bound to IgG-Sepharose, and released from the beads by digestion with TEV protease. Then, the supernatant is bound to calmodulin beads, and the protein is eluted in the presence of EGTA (Fig. 3.20.B).

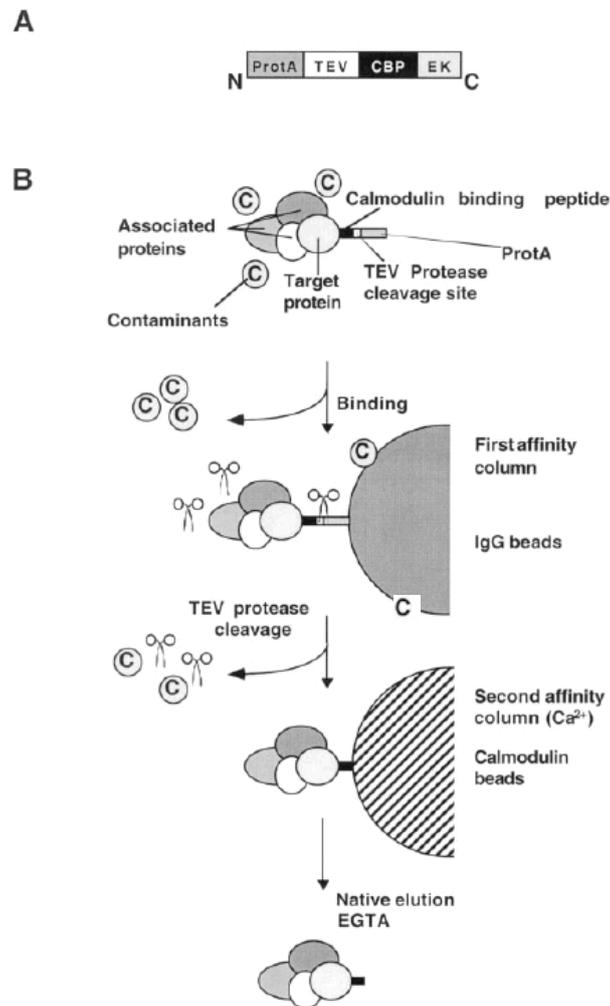


Figure 3.20. Strategy of TAP-tag purification. **A)** Components of the TAP-tag. ProtA: IgG-binding domain of protein A; TEV: a protease cleavage of TEV protease; CBP: calmodulin binding domain. **B)** Overview of the sequential steps for TAP-tag purification. First, the recombinant protein is bound to IgG-Sepharose via the IgG domain of protein A. After washing the resin, the IgG-Sepharose is treated with TEV-protease to release the recombinant protein that is next bound to the calmodulin binding resin in the presence of 2 mM CaCl_2 via the calmodulin binding domain present in the TAP-tag. Calmodulin beads are washed and the recombinant protein along with its interaction partners is eluted with a buffer containing 10 mM EGTA (Puig et al., 2001).

3.4.1 TAP-tag purification of the hCdc14B complex

3.4.1.1 Generation of U2OS cells stably expressing TAP-hCdc14B^{PD}

Since catalytically inactive enzymes often show a higher binding affinity towards their substrates, the cDNA of the phosphatase-dead mutant hCdc14B^{PD} was cloned into pcDNA4/TO-TAP. The recombinant plasmid pcDNA4/TO-TAP-hCdc14B^{PD} encodes N-terminally TAP-tagged hCdc14B^{PD} under the control of a tet-inducible promoter. U2OS cells were co-transfected with the pcDNA/6TR plasmid encoding the tetracycline-repressor and pcDNA4/TO-TAP-hCdc14B^{PD} or pcDNA4/TO-TAP. Co-

transfected cells were selected with zeocin and blasticidin HCl, individual clones were isolated, and cultured as described in 3.1.1. U2OS-TAP cells and four clones of U2OS-TAP-hCdc14B^{PD} cells were cultured for 16 h in medium containing 2 µg/ml of doxycycline to induce expression of the TAP tag and TAP-hCdc14B^{PD}, respectively, and the expression of the recombinant TAP-hCdc14B^{PD} was analyzed by Western blotting. In the presence of doxycycline, all four clones of U2OS-TAP-hCdc14B^{PD} cells expressed TAP-hCdc14B^{PD}, which has a molecular weight of app. 75 kDa (TAP tag: 20 kDa + hCdc14B: 54 kDa) (Fig. 3.21.B, lanes 2, 4, 6, 8). TAP-hCdc14B^{PD} was hardly detected in uninduced cells (lanes 1, 3, 5, 7), and absent from cells expressing the TAP-tag alone (Fig. 3.21.A). Since the expression level of TAP-hCdc14B^{PD} was lowest in clone 4, this cell line was used for large-scale expression and purification of TAP-tagged hCdc14B^{PD} complexes.

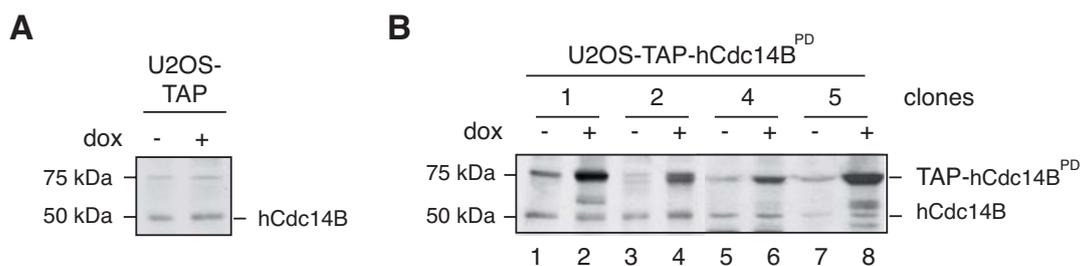


Figure 3.21. Western blot analysis of U2OS-TAP and U2OS-TAP-hCdc14B^{PD} cell lines. U2OS-TAP cells (**A**) and individual clones of U2OS-Tap-hCdc14B^{PD} cells (**B**) were cultured in the absence (lanes 1, 3, 5, 7) or the presence of doxycycline (lanes 2, 4, 6, 8). The recombinant and the endogenous hCdc14B proteins were detected on Western blots using antibodies specific to hCdc14B. Positions of molecular weight standards are indicated.

3.4.1.2 TAP-tag purification of hCdc14B complexes from U2OS-TAP-hCdc14B^{PD} cells

Nuclear extracts were prepared from 1×10^8 U2OS-TAP and U2OS-TAP-hCdc14B^{PD} cells that were cultured in the presence of doxycycline for 16 h. The TAP purification was performed as shown in Fig. 3.20.B. The efficiency of each purification step was monitored by analyzing 10% of a given sample on Western blots using α -hCdc14B antibodies. As shown in Fig. 3.22.A, the binding of the fusion protein to IgG-Sepharose was highly efficient (lanes 3-6). However, elution efficiency upon TEV cleavage was low (lanes 7-10). Incubation with the TEV protease resulted in fusion proteins devoid of

the IgG domain with a molecular weight of app. 60 kDa. Only one-third of the total bound proteins was released from the IgG-Sepharose as judged by comparison of the amount of the 60 kDa, released hCdc14B^{PD} with the amount of recombinant hCdc14B^{PD} retained in the bead (lanes 8 and 10). Recovery of the protein was also low during the next purification steps. In total, only app. 7% of the recombinant hCdc14B^{PD} present in the nuclear extracts were finally eluted from calmodulin beads (compare lane 2 and 18). The eluted proteins were precipitated with TCA, separated by SDS-PAGE and visualized by silver staining. As shown in Fig. 3.22.B, there was no difference between the proteins retrieved from control cells and cells expressing TAP-hCdc14B^{PD} even though the recombinant hCdc14B was clearly visible. This suggests that TAP-hCdc14B^{PD} associated proteins were either lost during the purification or that the efficiency of TAP purification was too low to allow detection of interacting proteins.

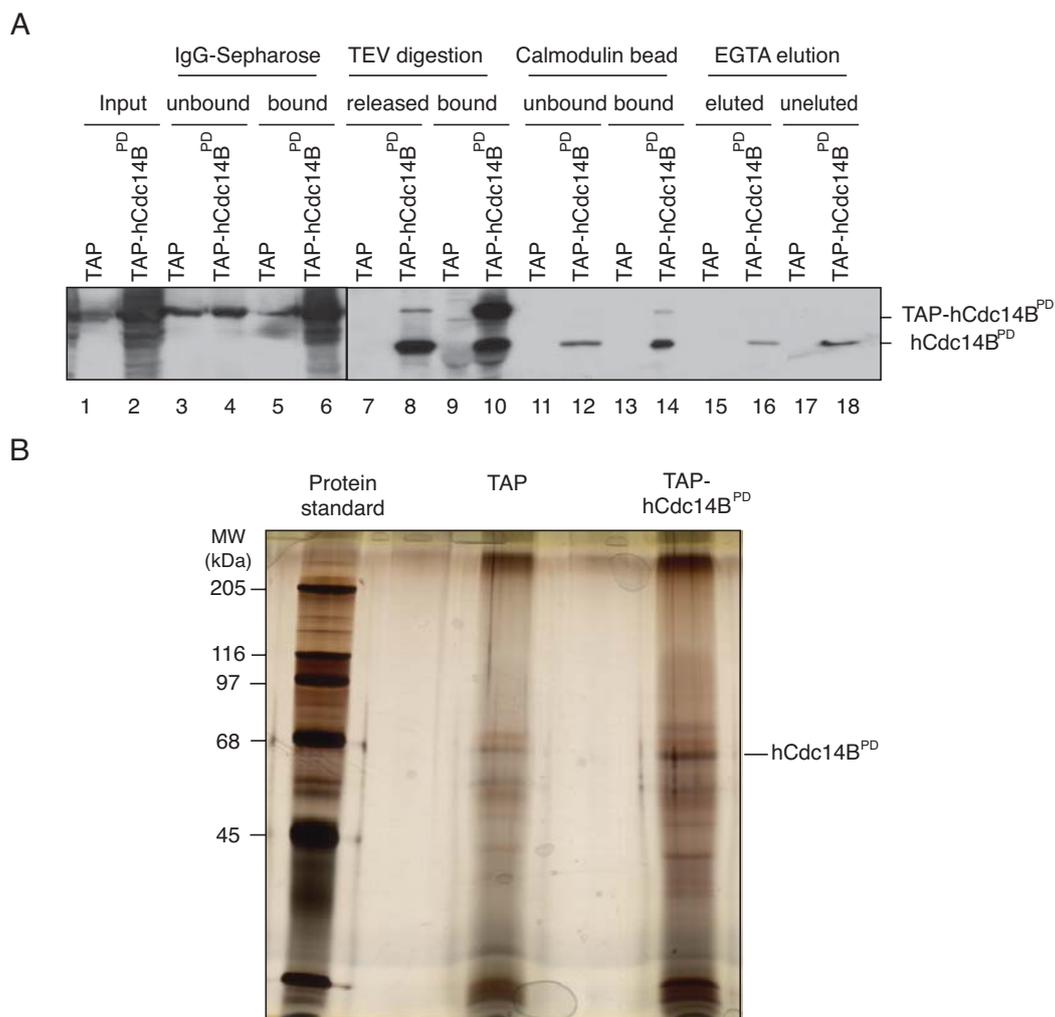


Figure 3.22. TAP-tag purification of TAP-hCdc14B^{PD}. A) Control of efficiency of the different purification steps by Western Blot analysis. TAP-hCdc14B^{PD} was expressed in U2OS-TAP-hCdc14B^{PD}

cells by culturing in the presence of doxycycline (2 µg/ml) for 16 h. As a control U2OS-TAP cells were used. 10% of the input and 10% of the fractions from each purification steps were analyzed on Western Blot using antibodies specific to hCdc14B. The positions of hCdc14B^{PD} containing the full-length TAP-tag (75 kDa) and cleaved TAP-tag (60 kDa) are indicated. **B**) Silver staining of TAP-tag purified proteins. The eluates from calmodulin beads were precipitated with TCA, separated by SDS-PAGE and stained with silver. As a protein size marker Broad Range SDS-PAGE standards (BioRad) were used. Each marker protein band contains 100 ng.

3.4.2 Isolation of hCdc14B complexes by double immunoprecipitation

3.4.2.1 Isolation of hCdc14B complex from HeLa cells stably expressing Flag-HA-hCdc14B^{PD}

Since no hCdc14B complexes could be retrieved by tandem affinity purification, I next tried to purify hCdc14B complexes by double immunoprecipitation using hCdc14B proteins fused to Flag- and HA-tags. First, the hCdc14B^{PD} cDNA was cloned into a vector containing the tet-operator, a CMV-promoter, followed by sequences encoding the Flag- and the HA-tag. The obtained plasmid, pcDNA4/TO-Flag-HA-hCdc14B^{PD} was used to generate Tet-inducible HeLa-Flag-HA-hCdc14B^{PD} cells. Induction of expression of Flag-HA-hCdc14B^{PD} was analyzed in different individual clones by adding 2 µg/ml doxycycline to the culture medium for 16 h. Out of six clones tested, only clones 13 and 16 expressed Flag-HA-hCdc14B^{PD} in a tet-inducible manner (Fig. 3.23.A). The expression level of Flag-HA-hCdc14B^{PD} in clone 13 was app. 50% of the level of the endogenous protein as shown by Western blot analysis (Fig. 3.23.B). This clone was chosen for large-scale purification.

Nuclear extracts were prepared from 1.5×10^8 HeLa-Flag-HA-hCdc14B^{PD} cells cultured in medium containing doxycycline. The recombinant protein was isolated using the sequential immunoprecipitation protocol depicted in Fig. 3.23.C. The efficiency of each purification step was monitored by analyzing 10% of obtained samples on Western blots with α -hCdc14B antibodies. As shown in Fig. 3.23.D, the purification efficiency was so low that Flag-HA-hCdc14B^{PD} was not detectable in the eluates from the α -HA-beads. This is probably due to the low expression level of the recombinant protein in the HeLa-Flag-HA-hCdc14B^{PD} cells.

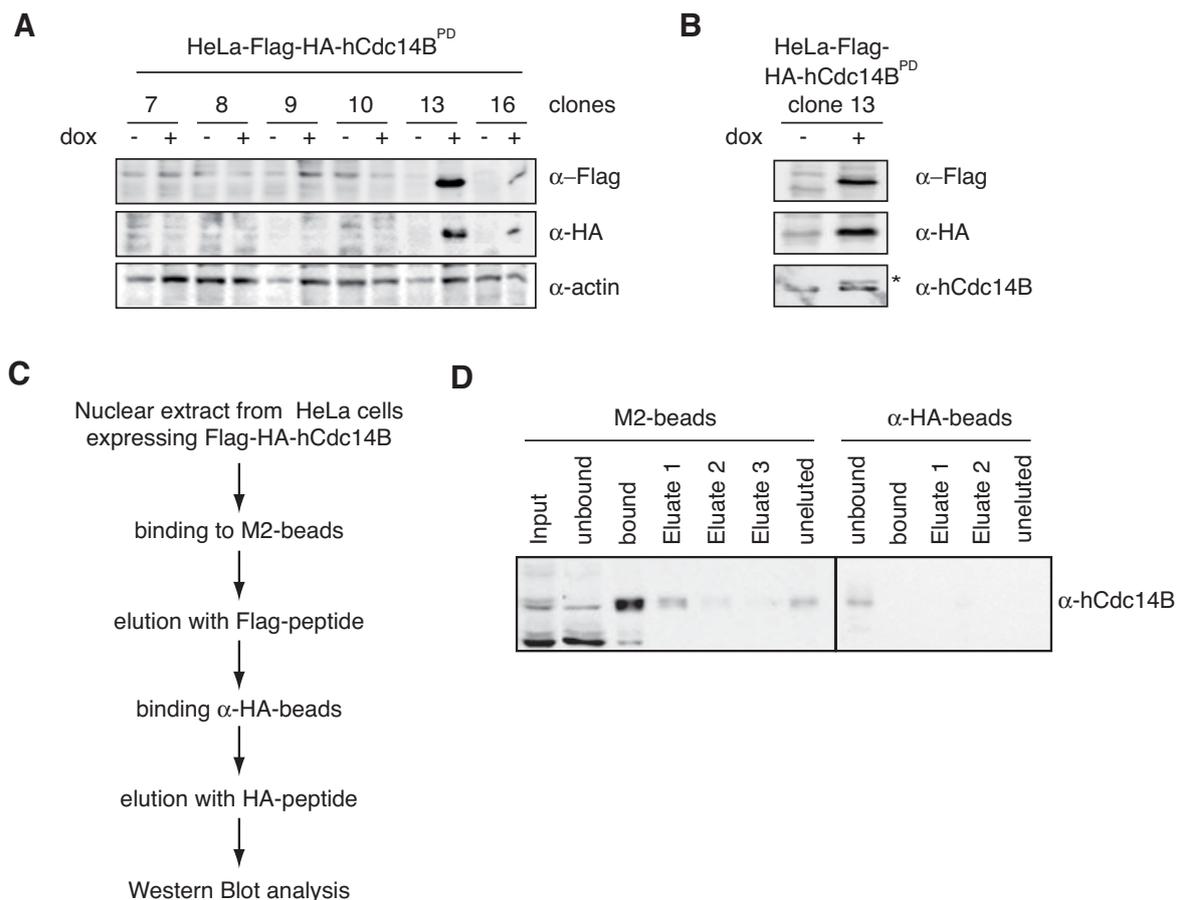


Figure 3.23. Double immunoprecipitation of Flag-HA-hCdc14B^{PD} from HeLa-Flag-HA-hCdc14B^{PD} cells. **A)** Expression of Flag-HA-hCdc14B^{PD} in individual HeLa cell clones. HeLa cells stably co-transfected with pcDNA4/TO-Flag-HA-hCdc14B^{PD} and pcDNA6TR plasmids were selected in zeocin (125 µg/ml) and blasticidin HCl (2 µg/ml) containing medium. Individual clones were isolated and incubated in presence of 2 µg/ml dox for 16 h, and the expression of the recombinant protein was analyzed on Western Blots using antibodies against the Flag-epitope, the HA-epitope and actin. **B)** Flag-HA-hCdc14B^{PD} expression level in clone 13 of HeLa-Flag-HA-hCdc14B^{PD} cells. The recombinant protein was expressed as described in (A) and analyzed on Western blots using antibodies against the Flag-epitope, the HA-epitope and hCdc14B. The recombinant hCdc14B is marked with asterisk (*) in the hCdc14B blot. **C)** Schematic overview of the double immunoprecipitation protocol. Double-tagged hCdc14B is precipitated using M2-beads from nuclear extracts of HeLa cells expressing Flag-HA-hCdc14B^{PD}. After washing, the bead-bound proteins are eluted with the Flag peptide and bound to α-HA-beads. After the final elution from α-HA-beads with the HA peptide, the eluted proteins are analyzed on Western Blot. **D)** Analysis of double-immunoprecipitation efficiency of the recombinant hCdc14B. HeLa-Flag-HA-hCdc14B^{PD} cells cultured for three days in the presence of doxycycline were harvested and nuclear extract was prepared. The recombinant protein was isolated by double immunoprecipitation, and 10% of the input and 10% of the fractions from each purification steps were analyzed on Western Blot using antibodies specific to hCdc14B.

3.4.2.2 Isolation of hCdc14B complexes from HEK-293T cells expressing Flag-HA-hCdc14B^{PD}

As a third approach for isolation of hCdc14B complexes, Flag-HA-hCdc14B^{PD} was transiently overexpressed in HEK-293T cells. 48 h after transfection with the expression

plasmid pcDNA4/TO-Flag-HA-hCdc14B^{PD}, the cells were harvested, and nuclear extract was prepared. Double immunoprecipitation was performed as depicted in Fig. 3.23.C. Western blot analysis showed that sufficient amounts of Flag-HA-hCdc14B^{PD} were retrieved at each step of purification (Fig. 3.24.A). The final eluates were pooled, the proteins were precipitated with TCA, separated by SDS-PAGE on a 4-15% PAA gel and stained with silver. As shown in Fig. 3.24.B, at least 10 distinct proteins could be visualized, which co-eluted with Flag-HA-hCdc14B^{PD} and were absent in the eluates from mock transfected cells. Gel slices containing these proteins were digested with trypsin, and subjected to mass spectrometry. The peptide masses obtained were further analyzed using the Mascot search engine to identify the respective proteins. In total, 4 protein bands could be assigned to specific proteins. These were APC1 (MW: 215 kDa) the biggest subunit of the anaphase promoting complex/cyclosome (APC/C), the RNA polymerase II elongation factor Elp1 (MW: 128 kDa), the heat shock protein hsp70 (MW: 70 kDa), and hCdc14B itself (Fig. 3.25.).

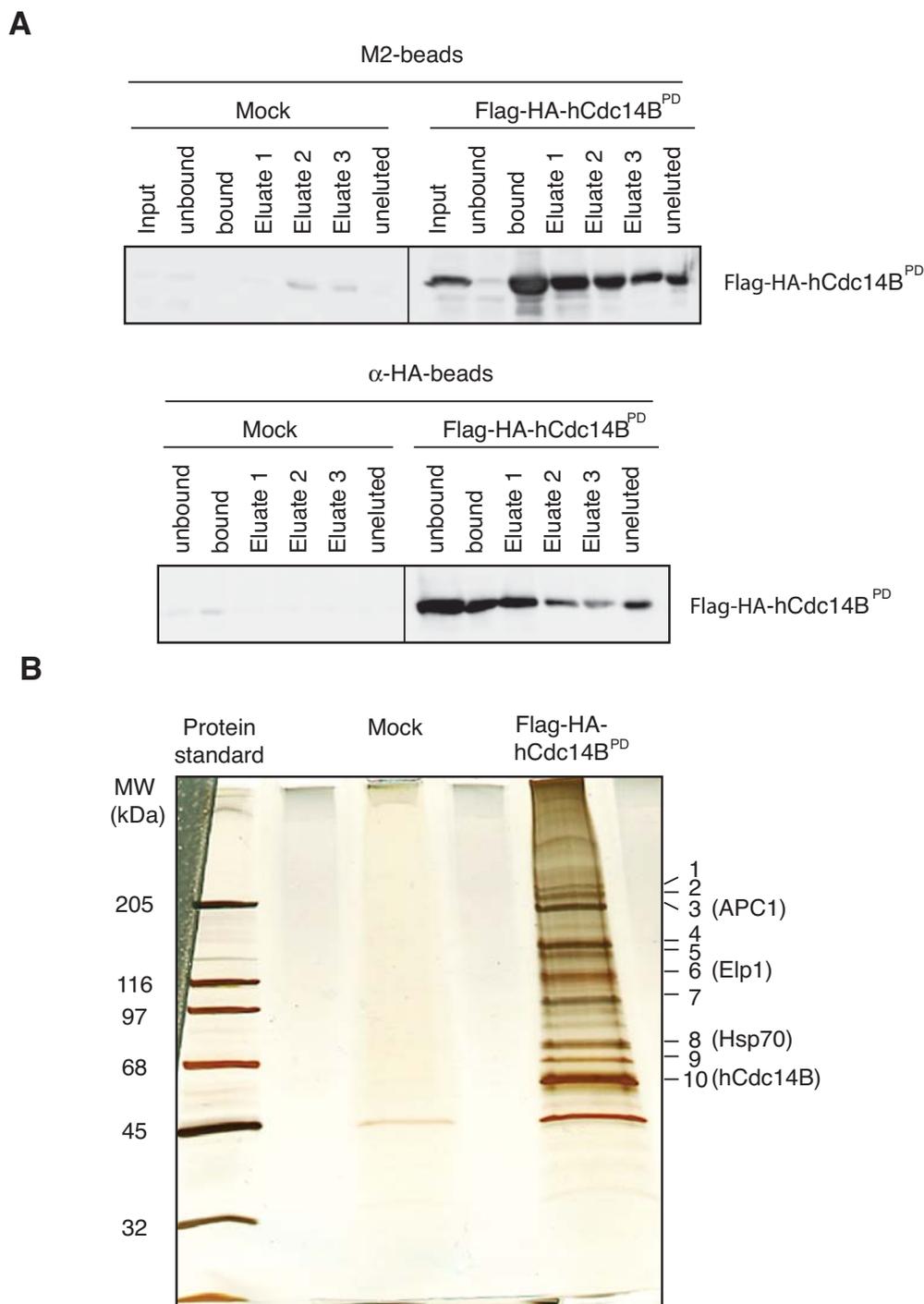


Figure 3.24. Isolation of hCdc14B containing complex by double immunoprecipitation. **A)** Western Blot analysis of Flag-HA-hCdc14B^{PD} isolation. HEK-293T cells were transfected with Flag-HA-hCdc14B^{PD} expression plasmid. 60 h post-transfection cells were harvested and Flag-HA-hCdc14B^{PD} along with interaction partners was isolated according to double-immunoprecipitation protocol represented in Fig. 3.23.C. 10% of samples obtained from each purification steps were analyzed on Western Blot using α -hCdc14B antibodies. **B)** Silver staining of proteins co-purified with Flag-HA-hCdc14B^{PD}. The proteins eluted from α -HA-beads were precipitated with TCA, separated by SDS-PAGE and protein gels were silver stained. As a protein size marker, unstained Broad Range SDS-PAGE standards (Bio-Rad) were used. Each marker protein marker band represents 100 ng. The proteins identified by mass spectrometry are indicated behind each corresponding protein bands.

APC1 subunit of APC/C complex

1 MSNFYEERTT MIAARDLQEEF VPFGRDHCKH HPNALNLQLR QLPASELWS SDGAAGLVGS
 61 LQEVTIHEKQ KESWQLRKGV SEIGEDVDYD EELYVAGNMV IWSKGSKSQA LAVYKAFQTV
 121 SPVQQALWCD FIISQDKSEK AYSSNEVEKC ICILQSSCIN MHSIEGKDYI ASLPFQVANV
 181 WPTKYGLLFE RSASSHEVPP GSPREPLPTM FSMLHPLDEI TPLVCKSGSL FGSSRVQYV
 241 DHAMKIVFLN TDPISIVMTYD AVQNVHSVWT LRRVKSEEN VVLKFSEQGG TPQNVATSSS
 301 LTAHLRSLSK GDSPVTSFPQ NYSSIHQSQR STSSPSLHSR SPSISNMAAL SRAHSPALGV
 361 HSFSGVQRFN ISSHNQSPKR HSISHSPNSN SNGSFLAPET EPIVPELCLD HLWTETITNI
 421 REKNSQASKV FITSDLGQK FLCFLVESQL QLRCVKFQES NDKTQLIFGS VTNIPAKDAA
 481 PVEKIDTMLV LEGSGNLVLY TGVVVRGKVF IPGLPAPSLT MSNTMPRPST PLDGVSTPKP
 541 LSKLLGSLDE VVLLSPVPEL RDSSKLHDSL YNEDCTFQQL GTYIHSIRDP VHNRTLELS
 601 NGSMVRITIP EIATSELVQT CLQAIKFILP KEIAVQMLVK WYNVHSAPGG PSYHSEWNLF
 661 VTCLMNMGMGY NTDRLAWTRN FDFEGSLSPV IAPKKARPSE TGSDDDWEYL LNSDYHQNVE
 721 SHLLNRSCL SPSEASQMKD EDFSQNLSD SSTLLFTHIP AIFFFVLHLY EELKLNLMG
 781 EGICSLVELL VQLARDLKL PYVDHYRDY PTLVTRTTGQV CTIDPGQTGF MHHPSFFTSE
 841 PPSIYQWVSS CLKGEGMPPY PYLPGICERS RLVVLSIALY ILGDESLVSD ESSQYLTRIT
 901 IAPQKLQVEQ EENRFSFRHS TSVSSLAERL VVWMTNVGFT LRDLETLPFG IALPIRDAIY
 961 HCREQPASDW PEAVCLLIGR QDLSKQACEG NLPKGSVLS SDVPSGTEE EEDDGMNDMN
 1021 HEVMSLIWSE DLRVQDVRR LQSAHPVRVN VVQYPELSDH EFIEEKENRL LQLCQRTMAL
 1081 PVGRGMFTLF SYHPVTEPL PIPKLNLTGR APPRNTTVDL NSGNIDVPPN MTSWASFHNG
 1141 VAAGLKIAPA SQIDSAWIVY NPKKHAELAN EYAGFLMALG LNHGLTKLAT LNIHDYLTKG
 1201 HEMTSIGLLL GVSAAKLGTM DMSITRLLSI HIPALLPPTS TELDVPHNVQ VAAVVGIGLV
 1261 YOGTAHRHTA EVLLAEIGRP PGPMEYCTD RESYSLAAGL ALGMVCLGHG SNLIGMSDLN
 1321 VPEQLYQYMV GGHRRFQTM HREKHKSPSY QIKEGDTINV DVTCPGATLA LAMIYKTN
 1381 RSIADWLRAP DTMYLDFVK PEFLLLRDLA RCLILWDDIL PMSKWDVSNV PQIIRENSIS
 1441 LSEIELPCE DLNLETLSQA HVYIIAGACL SLGFRFAGSE NLSAFNCLHK FAKDFMITYLS
 1501 APNASVTGPH NLETCLSVVL LSLAMVMGAS GNLKVLQLCR FLHMKGTGEM NYGFHLAHHM
 1561 ALGLLFLGGG RYSLSTSNSS IAALLCALYP HFPASTDNR YHLQALRHLV VLAAEPRLLV
 1621 PVDVDTNTPC YALLEVITYK TQWYEQTKEE LMAPTLLPEL HLLKQIKVKG PRYWELLIDL
 1681 SKGTQHLKSI LSKDGVLYVK LRAQQLSYKE DPMGWQSLLA QTVANRNSEA RAFKPETISA
 1741 FTSDPALLSF AEYFCKPTVN MGQKQEILD FSSVLYECVT QETPEMLPAY IAMDQAIRRL
 1801 GRREMSETSE LWQIKLVLEF FSSRSQHERL QNHPKRGLFM NSEFLPVVKC TIDNTLDQWL
 1861 QVGGDMCVHA YLSGQPLEES QLSMLACFLV YHSVPAPQHL PPIGLEGSTS FAELLFKFKQ
 1921 LKMPVRALLR LAPLLLGNPQ PMVM

Elongation complex protein 1-Elp1

1 MRNLKLFRTL EFRDIQPGN PQCFSLRTEQ GTVLIGSEHG LIEVDPVSRE VKNEVSLVAE
 61 GFLPEDGSGR IVGVQDLLDQ ESVCVATASG DVILCSLSTQ QLECVGSVAS GISVMSWSPD
 121 QELVLLATGQ QTLIMTKDF EPILEQQIHQ DDFGESKFIT VGWGRKETQF HGSEGRQAAF
 181 QMQMHESALP WDDHRPQVTW RGDGQFFAVS VVCPETGARK VRVWNRFPAL QSTSEPVAGL
 241 GPALAWKPSG SLIASTQDKP NQQDIVFFEK NGLLHGHTL PFLKDEVKVN DLLWNADSSV
 301 LAVRLEDLQR EKSSIPKTCV QLWTVGNVHW YLKQSLSFST CGKSKIVSLM WDPVTPYRLH
 361 VLCQGWYLYA YDWHWTDRS VGDNSSLN VAVIDGNRVL VTVFRQTVVP PPMCTYQLLF
 421 PHPVNQVTF L AHPQKSNDLA VLDASNQISV YKCGDCPSAD PTVKLGAVGG SGFKVCLRTP
 481 HLEKRYKIQF ENNEDQVNP LKLGLLTWIE EDVFLAVSHS EFSRPSVIHH LTAASSEMDE
 541 EHGQNVSS AAVDGVII SL CCNSKTKSVV LQLADGQIFK YLWESPLAI KPWNSSGFP
 601 VRFYPCTQT ELAMIGEEEC VLGLTDRCRF FINDIEVASN ITSFVYDEF LLLTTHSHTC
 661 QCFCLRDASF KTLQAGLSSN HVSHGEVLRK VERGSRI TV VPQDTKLVLQ MPRGNLEVH
 721 HRALVLAQIR KWLDKLMFKE AFECMRKLRI NLNPIYDHP KVFLLGNVETF IKQIDSVNHI
 781 NLFFTELKEE DVTKTMY PAP VTSSVYLSRD PDGNKIDLVC DAMRAVME SI NPHKYCLSIL
 841 TSHVKTTPPE LEIVLQVHE LQGNAPSDPD AVSAAEALKY LLHLVDVNEL YDHSGLTYDF
 901 DLVLMVAEKS QKDPKEYLPE LNTLTKMETN YQRFITDKYL KRYEKAIGHL SKCGPEYFPE
 961 CLNLIKDKNL YNEALKLYSP SSQQYQDISI AYGEHLMQEH MYEPAGLMFA RCGAHEKALS
 1021 AFLTCGNWKQ ALCVAAQLNF TKDQLVGLGR TLAGKLVEQR KHIDAAMVLE ESAQDYEEAV
 1081 LLLLEGAWE EALRLVYKYN RLDIIETNVK PSILEAQKNY MAFLDSQTAT FSRHKKRLLV
 1141 VRELKEQAQQ AGLDDEVPHG QESDLFSETS SVVSGSEMSG KYSHSNSRIS ARSSKNRRKA
 1201 ERKKHSLKEG SPLEDLALLE ALSEVVQNT E NLKDEVYHIL KVLFLFEFDE QGRELQKAFE
 1261 DTLQLMERSL PEIWTLYQQ NSATPVLGPN STANSIMASY QQQKTSVPVL DAELFIPPKI
 1321 NRRTQWKLSL LD

Heat shock protein Hsp70

1 MAKAAAIGID LGTTYSCVGV FQHGKVEIIA NDQGNRTTPS YVAFTDTERL IGDAAKNQVA

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61 LNPQNTVFDA KRLIGRKFGD PVVQSDMKHW PFQVINDGDK PKVQVSYKGD TKAFYPPEEIS
121 SMVLTKMKEI AEAYLGYPVT NAVITVPAYF NDSQRQATKD AGVIAGLNVL RIINEPTAAA
181 IAYGLDRTGK GERNVLIFDL GGGTFDVSIL TIDDGIFEVK ATAGDTHLGG EDFDNRLVNH
241 FVEEFKRKHK KDISQNKRAV RRLRTACERA KRTLSSSTQA SLEIDSLFEG IDFYTSITRA
301 RFEELCSDLF RSTLEPVEKA LRDAKLDKAQ IHDLVLVGGS TRIPKVQKLL QDFFNGRDLN
361 KSINPDEAVA YGAAVQAAIL MGDKSENVQD LLLLDVAPLS LGLETAGGVM TALIKRNSTI
421 PTKQTQIFTT YSDNQPGVLI QVYEGERAMT KDNLLGRFE LSGIPPAPRG VPQIEVTFDI
481 DANGILNVTA TDKSTGKANK ITITNDKGR LSKKEIERMVQ EAEKYKAEDE VQRERVSANK
541 ALESYAFNMK SAVEDEGLKG KISEADKKKV LDKCQEVISW LDANTLAEKD EFEHKRKELE
601 QVCNPIISGL YQGAGGPGPG GFQAQGPCKG SSGSPTIEEV D

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hCdc14B

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1 MKRKSERRSS WAAAPCSRR CSSTSPGVKK IRSSTQDPR RRDPQDDVYL DITDRLCFAI
61 LYSRPKSASN VHYFSIDNEL EYENFYADFG PLNLAMVYRY CCKINKKLS ITMLRKKIVH
121 FTGSDQRKQA NAAFLVGCYM VIYLGRTPEE AYRILIFGET SYIPFRDAAY GSCNFYITLL
181 DCFHAVKKAM QYGFLNFNSF NLDEYEHYK AENGLNWII PDRFIAFCGP HSRARLESGY
241 HQHSPEYIQ YFKNHNVTTI IRLNKRMIDA KRFTDAGFDH HDLFFADGST PTDAIVKEFL
301 DICENAEGAI AVHCKAGLGR TGTLIACYIM KHYRMTAET IAWVRICRPG SVIGPQQOFL
361 VMKQTNLWLE GDYFRQKLKG QENGQHRAAF SKLLSGVDDI SINGVENQDQ QEPEPYSDDD
421 EINGVTQDR LRALKSRRQS KTNAIPLTVI LQSSVQSCKT SEPNISGSAG ITKRTTRSAS
481 RKSSVKSLSI SRTKTVLR

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Figure 3.25. Identification of APC1, Elp1, Hsp70 and hCdc14B by mass spectrometry. Protein bands were cut out from the silver stained gel, the gel slices were digested with trypsin, the peptides extracted from the gel and analyzed by mass spectrometry. Data were analyzed using the Mascot database search engine. Matched peptides are shown in red.

3.4.3 hCdc14B interacts with APC/C

The anaphase promoting complex/cyclosome (APC/C) is a multisubunit ubiquitin ligase that consists of 12 subunits in mammalian cells. Upon binding of the co-activators Cdc20 or Cdh1, APC/C catalyzes the ubiquitination of specific substrates such as securin, cyclin A, cyclin B, Plk1, Cdc20 and UbcH10, thereby targeting them for degradation by the 26S proteasome (reviewed in Peters, 2006). APC/C triggers the transition from metaphase to anaphase by initiating the degradation of securin thereby activating separase, which in turn cleaves cohesin that holds the sister chromatids together. APC/C also regulates late mitotic events and promotes mitotic exit by targeting cyclin A, B, Plk1, Cdc20 to proteasome-mediated degradation (Shteinberg et al., 1999; Rudner and Murray, 2000).

Mass spectrometry analysis of proteins co-purifying with Flag-HA-hCdc14B^{PD} identified APC1, a subunit of APC/C. To confirm this result, I performed immunoprecipitation experiments using lysates from HEK-293T cells that were transfected with the pcDNA4/TO-Flag-hCdc14B^{PD} expression plasmid. Flag-hCdc14B^{PD} was precipitated using M2-beads and co-precipitation of APC1 was detected on Western blots using antibodies specific to APC1. Indeed, APC1 was co-precipitated by M2-beads, but not by mouse IgG and protein A-agarose (Fig. 3.26.A)

indicating that hCdc14B and APC1 interact with each other. To further test whether hCdc14B interacts with the APC/C complex, HEK-293T cells were either mock transfected or transfected with the Flag-HA-hCdc14B^{PD} expression plasmids. 48 h post transfection, Flag-HA-hCdc14B^{PD} was isolated by double immunoprecipitation using M2-beads and α -HA-beads. Proteins co-precipitating with Flag-HA-hCdc14B^{PD} were separated by SDS-PAGE and analyzed on Western blots using antibodies against the APC/C subunits APC1 and Cdc27, and the co-activators Cdc20 and Cdh1. As shown in Fig 3.26.B, besides APC1, hCdc14B immunoprecipitates also contained Cdc27 (APC3), the third largest subunit of APC/C suggesting that hCdc14B associates with the intact APC/C. Moreover, the co-activator Cdh1, but not Cdc20 was detected suggesting that hCdc14B specifically interacts with APC/C^{Cdh1}, which assembles during late mitosis.

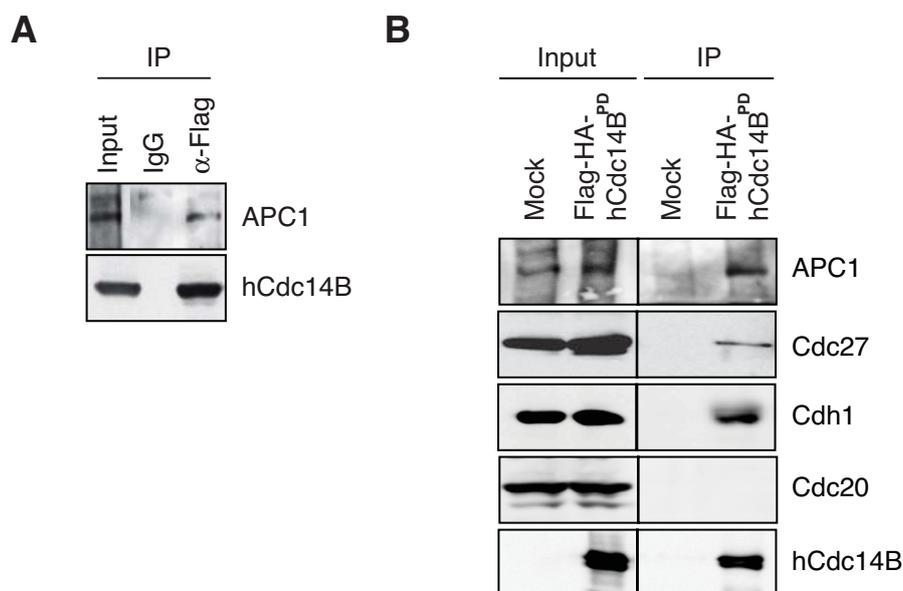


Figure 3.26. hCdc14B interacts with APC/C. **A)** Co-immunoprecipitation of hCdc14B and APC1. HEK-293T cells were transfected with the pcDNA4/TO-Flag-hCdc14B^{PD} expression plasmid. 48 h post transfection cells were harvested and Flag-hCdc14B^{PD} was precipitated using α -Flag M2 beads. Precipitated proteins were analyzed on Western blots using α -hCdc14B and α -APC1 antibodies. **B)** hCdc14B interacts with APC/C^{Cdh1}. HEK-293T cells were transfected and harvested as in (A). Flag-HA-hCdc14B^{PD} was isolated by double precipitation and co-precipitated proteins were analyzed on immunoblots using antibodies against APC1, Cdc27, Cdc20 and Cdh1. Input lanes contain 10% of the lysates used for precipitation.

4 Discussion

Exit from mitosis and cytokinesis are highly regulated to ensure faithful cell division. Yeast Cdc14 (yCdc14) phosphatase is a key player that regulates mitotic exit by antagonizing mitotic phosphorylations of Cdk1 target proteins and promoting degradation of mitotic regulators (reviewed in Stegmeier and Amon, 2004). Clp1/Flp1, a homologue of the yCdc14 in *S. pombe*, is not essential for mitotic exit but is required for cytokinesis. It has been shown that *clp1/flp1*^{-/-} cells exhibit defects in chromosome segregation and cytokinesis (Trautmann et al, 2001; 2004). Unlike yeast, human cells have two Cdc14 phosphatases, hCdc14A and B. hCdc14A is involved in centrosome regulation and cytokinesis (Kaiser et al., 2002; Mailand et al., 2002b). The present work has demonstrated that hCdc14B regulates mitotic progression. This conclusion is supported by following results: (i) overexpression of wildtype hCdc14B delays mitotic entry; (ii) overexpression of phosphatase deficient hCdc14B delays mitotic progression; (iii) depletion of hCdc14B results in mitotic arrest.

hCdc14B promotes progression to late mitosis

The main finding of this thesis was that hCdc14B is required for proper mitotic progression. Overexpression of wildtype hCdc14B prevents timely activation of Cdk1/cyclin B, whereas overexpression of phosphatase-deficient hCdc14B delayed Cdk1/cyclin B inactivation. This strongly suggests that hCdc14B down-regulates Cdk1/cyclin B. It is well established that in budding yeast yCdc14 regulates mitotic exit by inhibiting Cdk1 activity. However, the mitotic events regulated by hCdc14B and yCdc14 do not seem to be identical. Cells carrying a temperature-sensitive *CDC14* mutation arrest in late anaphase with 2N DNA content and an elongated mitotic spindle when cultured at the non-permissive temperature (Visintin et al., 1998). In contrast, cells depleted of hCdc14B show a complex phenotype including arrest in mitosis, stabilization of cyclin B, elevated Cdk1/cyclin B activity, formation of bi- and multinucleated cells and cell death. Fluorescence microscopy revealed that significant number of cells had condensed chromatin that was arranged at the equatorial plate indicating arrest in metaphase. This suggests that transition from metaphase to anaphase was defective in these cells. Metaphase-anaphase transition occurs when separase cleaves the cohesion complex that holds the sister chromatids together. Separase is activated at the end of metaphase when APC/C^{Cdc20} ubiquitinates securin, an inhibitor of

separate, and targets it for degradation. The spindle assembly checkpoint (SAC) regulates metaphase-anaphase transition (reviewed in Musacchio and Hardwick, 2002). It is possible that hCdc14B depletion activated the SAC and thereby prevented metaphase-anaphase transition.

Progressive loss of Cdk1/cyclin B activity is essential for successful completion of mitosis (reviewed in Pines et al., 2006). Down-regulation of Cdk1/cyclin B starts during metaphase-anaphase transition by APC/C^{Cdc20}-mediated degradation of cyclin B (Clute and Pines, 1999). It is possible that in cells depleted of hCdc14B SAC activation led to stabilization of cyclin B and elevation of Cdk1/cyclin B activity. When cells were treated with siRNAs specific for hCdc14B for a prolonged period of time most of the cells died, presumably due to irreversible arrest in mitosis. Moreover, bi- and multinucleated cells accumulated. There are two possible mechanisms, which led to multinucleation: (i) Cells may have bypassed SAC-mediated arrest by mitotic slippage that allows exit from mitosis without cell division. During mitotic slippage nuclear envelopes form around random groups of chromosomes as they decondense producing multinuclei. Cells bypass SAC due to slow but continuous degradation of cyclin B that ultimately drives the cell out of mitosis (Brito and Rieder, 2006). It has been suggested that inhibition of transcription during mitosis is sufficient to trigger mitotic exit (reviewed in Blagasklonny, 2007); (ii) Chromosome segregation and cytokinesis may be defective in these cells. This would implicate that hCdc14B is involved in cytokinesis like hCdc14A. However, hCdc14A and B must act on different events targeting different substrates because endogenous hCdc14A, which was not affected by hCdc14B-siRNAs, could not rescue depletion of hCdc14B. This points to non-redundant functions of human Cdc14 phosphatases.

Nucleolar sequestration of hCdc14B

Examination of the cellular localization of hCdc14B by indirect immunofluorescence has revealed that hCdc14B localizes to nucleoli in interphase cells (Kaiser et al., 2002; Cho et al., 2005). In the present work I show by CHIP that hCdc14B binds to the intergenic spacer of rDNA but not to the promoter and transcribed regions. Likewise, in budding yeast yCdc14 is enriched in the centre of the non-transcribed spacer 1 (NTS1) and absent from the promoter and transcribed regions of rDNA (Stegmeier et al., 2004). During late mitosis, yCdc14 is transiently released from rDNA by two signalling networks. The FEAR network initiates release of yCdc14 during early anaphase, the

MEN network accomplishes full release of yCdc14 during late mitosis and maintains it in the released state until exit from mitosis (Jaspersen et al., 1998; Stegmeier et al., 2002; Azzam et al., 2004). yCdc14 itself is a component of MEN, which dephosphorylates and thereby activates several phosphoproteins of the MEN network (Pereira et al., 2002). Similar to yCdc14, hCdc14B is released from rDNA during mitosis as shown by ChIP analysis and chromatin association (Fig. 3.15. and 3.16.). Currently nothing is known about the regulatory networks leading to the temporal release of human Cdc14B and presumably to its temporal activation. In higher eukaryotes, the existence of a network analogous to the FEAR network has not been demonstrated yet. On the other hand, various components of the MEN network are conserved in mammals including Dbf2, Mob1, Nud1, Bub2, Cdc5 and finally Cdc14 itself. Interestingly, it has been shown that several of these proteins function in cytokinesis rather than in Cdk1/cyclin B inactivation in higher eukaryotes. In this regard, also the role of Cdc14 appears to be conserved through evolution, as inactivation of Cdc14 in *S. pombe* and *C. elegans*, and of Cdc14A in humans leads to defects in cytokinesis (Cueille et al., 2001; Trautmann et al., 2001; Gruneberg et al., 2002; Mailand et al., 2002b). RNAi experiment using hCdc14B siRNA resulted in formation of bi- or multinucleated cells suggesting that hCdc14B is also involved in faithful completion of cytokinesis.

The cellular hCdc14B complex

The protein complex responsible for the nucleolar localization of yCdc14 has been identified as the RENT complex (Shou et al., 1999). RENT consists of Net1, yCdc14 and the NAD-dependent deacetylase Sir2 (Shou *et al.*, 1999). Net1 tethers yCdc14 to rDNA by tightly binding to the core domain of yCdc14, where the phosphatase motif is located (Shou et al., 1999; Traverso et al., 2001). In addition to its function in RENT, Net1 regulates RNA polymerase I transcription by directly binding to the polymerase and stimulating its transcriptional activity (Shou et al., 2001). Net1 is also required for nucleolar stabilization (Shou et al., 2001). The nucleolar protein Fob1 recruits RENT to rDNA (Huang and Moazed, 2003, Stegmeier et al., 2004). Fob1 seems to be a negative regulator of the FEAR network and prevents release of yCdc14 prior to anaphase (Stegmeier et al., 2004). It is not known whether a protein complex similar to RENT exists in human cells. A Net1 homologue has not been identified in other eukaryotes including fission yeast.

This work has shown by gel filtration that hCdc14B is organized in a complex of a molecular weight of 400-600 kDa during interphase. It is likely that this protein complex mediates sequestration of hCdc14B to rDNA. In order to characterize this complex, TAP-tagged or double-tagged hCdc14B was expressed in human cells and isolated by TAP-tag purification or sequential immunoprecipitation. Unfortunately, mass spectrometry analysis of co-purifying proteins did not identify any known nucleolar protein. One reason why this approach failed might be that the nucleolar hCdc14B complex was not sufficiently extracted from rDNA during extract preparation. In future, to circumvent this problem nucleolar extract of cells expressing recombinant hCdc14B should be used.

yCdc14 is released during early mitosis by Cdk1-dependent phosphorylation of Net1 and during late mitosis by Cdc5-dependent phosphorylation of Net1. hCdc14B is also regulated by release during mitosis. As revealed by gel filtration of extracts prepared from mitotic cells the majority of hCdc14B is monomeric during mitosis. This is consistent with the idea that hCdc14B is released from rDNA during mitosis and becomes able to target its substrates. A small portion of hCdc14B was contained in protein complexes of different higher molecular weights suggesting that during mitosis hCdc14B transiently interacts with different protein complexes. Interestingly, previous analysis of the native size of hCdc14A has shown that this isoform is contained in a cellular complex of a size of 500 kDa throughout the cell cycle (Bembenek and Yu, 2001). This suggests that hCdc14A and hCdc14B are associated with distinct complexes and target different proteins.

Cdc25 is a physiological target of hCdc14B

Several previous reports have shown that *in vitro* both hCdc14 isoforms can dephosphorylate a number of proteins at their Cdk1/cyclin B targets sites including p53 (Li et al., 2000), cyclin E (Furstenthal et al., 2001), Cdh1 (Bembenek and Yu, 2001) and INCENP (Gruneberg et al., 2004). Structural analysis of hCdc14B demonstrated that the hydrophobic pocket at the catalytic site of hCdc14B has a clear specificity for a phospho-Ser/phospho-Thr-Pro sequence motif (Gray et al., 2003), e.g. sites modified by Cdk1 and MAP kinases. Therefore, it is not surprising that *in vitro* hCdc14A and B preferentially dephosphorylate proteins modified by proline-directed kinases. So far, the *in vivo* targets of hCdc14B had not been identified. Cho et al. (2005) have shown that hCdc14B interacts with and stabilizes microtubules. However, this microtubule-

stabilizing function of hCdc14B was independent of its phosphatase activity.

This work has identified Cdc25 as a physiological substrate of hCdc14B. hCdc14B interacts with all three human Cdc25 isoforms and dephosphorylates them at Cdk1/cyclin B target sites both *in vitro* and *in vivo*. Members of the Cdc25 phosphatase family have been well characterized as inducers of mitosis. Cdc25 dephosphorylates Cdk1 at pT14/pY15, thereby activates Cdk1/cyclin B and promotes mitotic entry (Dunphy and Kumagai, 1991; Honda et al., 1993; Sebastian et al., 1993). Active Cdk1/cyclin B phosphorylates Cdc25 and increases its activity and stability, and triggers its nuclear translocation (reviewed in Trinkle-Mulcahy and Lamond, 2006; Boutros et al., 2006). During late mitosis, the positive phosphorylations of Cdc25 must be removed to down-regulate Cdc25. This in turn down-regulates activity of Cdk1/cyclin B and leads to accumulation of inactivate Cdk1/cyclin B. Two lines of experimental evidence suggest that hCdc14B is the cellular phosphatase that inactivates Cdc25 during late mitosis and thereby acts as a negative regulator of Cdk1/cyclin B. Overexpression of hCdc14B prevented timely dephosphorylation of Cdk1-pY15 presumably by counteracting phosphorylation-dependent activation of Cdc25 at G₂/M, and delays mitotic entry. Similarly, ablation of Cdc25B by RNAi has been shown to lead to high levels of inactive Cdk1/cyclin B complexes and prolonged G₂-phase (Lindqvist et al., 2005). On the other hand, overexpression of the phosphatase-deficient hCdc14B mutant prolonged mitosis, probably because dephosphorylation-dependent inactivation of Cdc25 was impaired and Cdk1 remained active. These findings fit to our working hypothesis that hCdc14B regulates progression through mitosis by inactivating Cdk1/cyclin B. Although all three isoforms of human Cdc25 activate Cdk1/cyclin B, they have apparently distinct roles in regulating cell cycle transitions. Results from RNAi studies implicate Cdc25B and C in the regulation of the G₂/M transition (Karlsson et al., 1999; Lindqvist et al., 2005). Cdc25A appears to play a more general role, regulating both early (G₁/S) and late (G₂/M) cell cycle transitions (Molinari et al., 2000; Zhou et al., 2002; Lindqvist et al., 2005). Phosphorylation-dependent activation of Cdc25B increases at the end of G₂-phase, whereas Cdc25C activity peaks later (Lammer et al., 1998). Co-immunoprecipitation experiments showed that hCdc14B interacts with a preference for Cdc25B and Cdc25C than Cdc25A. This could suggest that Cdc25B and C are better substrates of hCdc14B. Strikingly, recent studies pointed to a similar regulation mechanism in *S. pombe*. It has been shown that the fission yeast Clp1/Flp1 down-regulates Cdk1 activity in late mitosis by dephosphorylating Cdc25

(Wolfe and Gould, 2004). This suggests that inactivation of Cdk1 and Cdc25 by Clp1p/hCdc14B is a highly conserved mechanism restricting activity of the mitotic inducers to early M-phase.

hCdc14B interacts with the anaphase-promoting complex/cyclosome (APC/C)

To decipher the cellular function of hCdc14B, hCdc14B interacting proteins were isolated by sequential co-immunoprecipitation from cells that express double-tagged hCdc14B, and subjected to mass spectrometry. This analysis led to the identification of APC1, the biggest subunit of APC/C. Further biochemical analysis showed that antibodies against overexpressed hCdc14B co-precipitated APC1, and the APC/C subunits Cdc27 and Cdh1. Cdh1 is a co-activator of APC/C. The finding that Cdh1, but not the second co-activator, Cdc20, was contained in the complex interacting with hCdc14B strongly suggests a selective association of hCdc14B with APC/C^{Cdh1}. Cdh1 is phosphorylated during the S-, G₂- and early M-phase by Cdk2/cyclin A and Cdk1/cyclin B at multiple sites, which prevents association with APC/C (Lukas et al, 1999; Listovsky et al, 2000). During anaphase, Cdh1 must be dephosphorylated to allow association with APC/C and its activation (reviewed in Peters, 2006). APC/C^{Cdh1} ubiquitinates different mitotic regulators including cyclin B, cyclin A, securin, and Plk1 and targets them for proteasome-dependent degradation during late mitosis and G₁-phase. As shown in this work, cyclin B levels were highly elevated in cells depleted of hCdc14B by RNAi. This suggests that hCdc14B activity is required for degradation of cyclin B. Therefore, it is likely that hCdc14B is the cellular phosphatase, which dephosphorylates and activates Cdh1 at the cyclin-dependent phosphorylation sites and triggers formation of APC/C^{Cdh1}. Consistent with this idea yCdc14 dephosphorylates Cdh1 and activates APC/C^{Cdh1} in *S. cerevisiae* (Visintin et al., 1998). Besides cyclin B, APC/C^{Cdh1} targets a variety of additional mitotic regulators for degradation. Future studies have to address, whether also the stability of those proteins is altered in hCdc14B-depleted cells. Alternatively, it may be possible that APC/C^{Cdh1} interacts with hCdc14B to target it for degradation. However, this is very unlikely, since the level of hCdc14B does not fluctuate during the cell cycle (Kaiser et al., 2002; this work). Though previous studies have shown that hCdc14A is able to dephosphorylate Cdh1 *in vitro* and stimulate activity of APC/C^{Cdh1} *in vitro* (Bembenek and Yu, 2001), there is no evidence for the functional importance *in vivo*.

Putative role of hCdc14B in mitotic regulation of transcription

In higher eukaryotes, transcription is switched off at the onset of mitosis and resumes rapidly upon exit from mitosis. Mitotic repression of RNA polymerase II (Pol II) transcription is achieved by inhibition of transcription initiation as well as elongation. During mitosis, the Pol II transcription initiation factor TFIID is phosphorylated and its activity is repressed (Segil et al., 1996). Moreover, the elongating Pol II complex is released from DNA suggesting that components required for transcription elongation are mitotically inactivated (Parsons 1997; Jiang et al., 2004). Elp1, a Pol II elongation factor was identified to co-purify with hCdc14B. Therefore, it is tempting to speculate that hCdc14B interacts with the Pol II elongation complex and enables Pol II-dependent transcription elongation. This has to be addressed in future studies.

Recent unpublished work of our lab has demonstrated that hCdc14B activates mitotically repressed RNA polymerase I (Pol I) transcription. hCdc14B specifically dephosphorylates TAF₁110, a subunit of Pol I transcription initiation factor SL1. hCdc14B targets phospho-Thr852 of TAF₁110, the site that is phosphorylated by Cdk1/cyclin B and restores Pol I transcription.

It is not known whether hCdc14B is involved in RNA polymerase III transcription. Pol III transcription inhibition is achieved by mitotic phosphorylation of Pol III transcription initiation factor TFIIB (Gottesfeld et al., 1994; Fairley et al., 2003). It would be interesting to study whether hCdc14B reverses mitotic phosphorylation of TFIIB and activates Pol III transcription initiation, too. Thus, hCdc14B could be the main activator of transcription at M/G₁ transition.

Cdk1/cyclin B targets hCdc14B

It is not known whether mechanisms besides nucleolar sequestration regulate hCdc14B. An intriguing possibility is that Cdk1/cyclin B phosphorylation of hCdc14B is important for its regulation. The hCdc14B polypeptide chain contains four sites, which perfectly correspond to the minimal Cdk consensus phosphorylation site Ser/Thr-Pro. The findings that Cdk1 interacts with hCdc14B *in vitro* and Cdk1/cyclin B phosphorylates hCdc14B *in vitro* argue for the regulation of hCdc14B by Cdk1/cyclin B. Three of the putative Cdk1/cyclin B phosphorylation sites of hCdc14B are located in the catalytic core domain. Thus, Cdk1/cyclin B might phosphorylate the catalytic domain of hCdc14B and alter its activity. Studies from fission yeast showed that Cdk1 phosphorylates and inactivates Clp1/Flp1 during early anaphase (Wolfe et al., 2006).

However, no significant alterations in the phosphatase activity of hCdc14B during the cell cycle were observed. In these assays overexpressed recombinant hCdc14B was used to measure the phosphatase activity. Whether this reflects the activity of endogenous hCdc14B is not clear. It is also possible that during the immunoprecipitation procedure important protein modifications or protein-protein interactions were lost. Another possibility is that hCdc14B activity indeed does not fluctuate throughout cell cycle and nucleolar sequestration is the only and efficient mechanism of hCdc14B regulation. The molecular mechanism underlying the release of hCdc14B from nucleoli is not identified. It might involve hCdc14B phosphorylation by Cdk1/cyclin B. One putative Cdk target site of hCdc14B is S25, which is located at the N-terminal domain of hCdc14B that is required for nucleolar localization (Kaiser et al., 2002). Cdk1/cyclin B might phosphorylate the nucleolar targeting domain of hCdc14B and thereby trigger release of hCdc14B during prometaphase. To test the regulation of hCdc14B by Cdk1/cyclin B, the putative Cdk1/cyclin B target sites should be mutated and biochemical properties of mutant hCdc14B must be analyzed.

Model for hCdc14B function

Based on the results produced during this work the following model for hCdc14B function is proposed (Fig. 4.1.). During interphase hCdc14B is sequestered to nucleoli by rDNA association and inactivated. At late G₂-phase Cdc25 activates Cdk1/cyclin B by removing inhibitory phosphatases from T14/Y15 of Cdk1. In turn, Cdk1/cyclin B phosphorylates and activates Cdc25, thereby initiating a feedback activation loop to ensure rapid G₂/M-phase transition. Cdk1/cyclin B phosphorylates a large number of proteins and regulates early mitotic events. During late mitosis hCdc14B is activated upon release from rDNA and down-regulates Cdk1/cyclin B by two pathways. In one pathway, it targets Cdc25 removing Cdk1/cyclin B-mediated phosphorylations and thereby disrupts the positive feedback between Cdk1/cyclin B and Cdc25. This leads to accumulation of inactive Cdk1/cyclin B phosphorylated at T14/Y15 of Cdk1. In the other pathway, hCdc14B activates APC/C^{Cdh1}, that ubiquitinates cyclin B targeting it to proteasome-dependent degradation and disassembles Cdk1/cyclin B complex. In addition, hCdc14B might reverse mitotic repression of transcription to restore transcriptional activity upon mitotic exit.

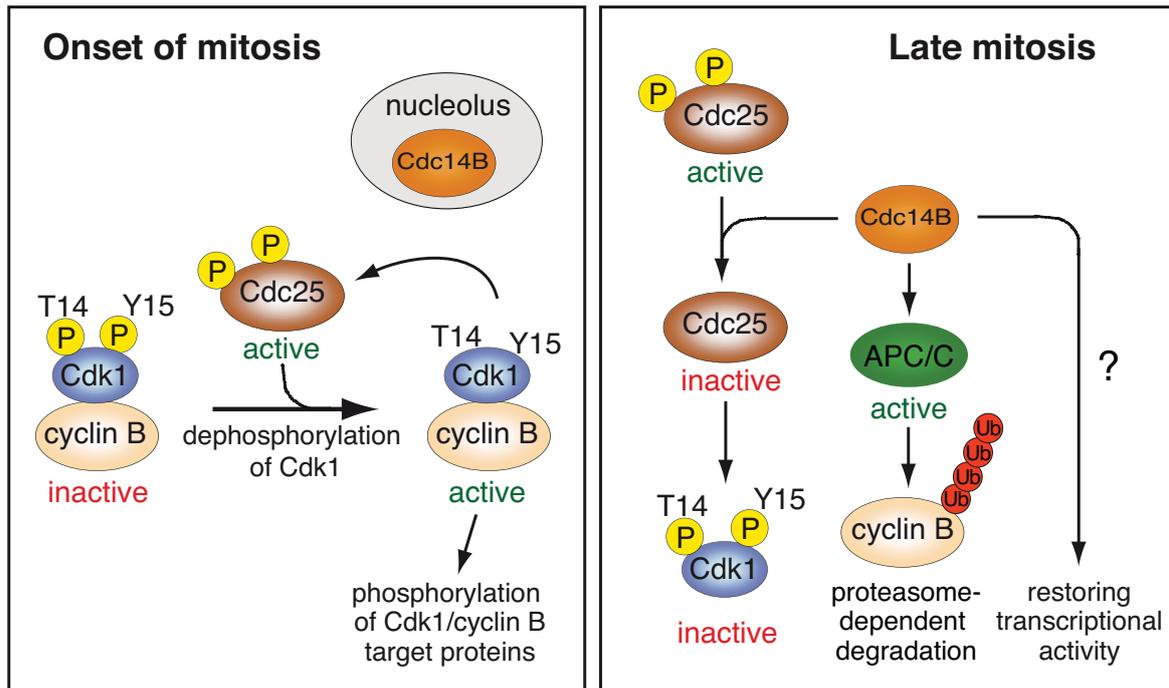


Figure 4.1. A model for hCdc14B function. At G₂/M, hCdc14B is sequestered within the nucleolus. Entry into mitosis requires activation of Cdk1 by Cdc25-mediated dephosphorylation. In turn, Cdk1/cyclin B phosphorylates and activates Cdc25, initiating a positive feedback loop. Cdk1/cyclin B regulates early mitotic events by phosphorylating a large number of proteins. Upon release from rDNA hCdc14B inactivates Cdc25 and activates APC/C^{Cdh1}, thereby down-regulating Cdk1 and promoting exit from mitosis. In addition, hCdc14B may reverse mitotic repression of transcription and restore cellular transcription.

6 Reference

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6 Appendix

6.1 Position of hCdc14B-shRNA target regions

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1   ATGAAGCGGA AAAGCGAGCG GCGGTCGAGC TGGGCCGCCG CGCCCCCTG CTGCGGGCGC
61  TGCTCGTCGA CCTCGCCGGG TGTGAAGAAG ATCCGCAGCT CCACGCAGCA AGACCCGCGC
121 CGCCGGGACC CCCAGGACGA CGTGTACCTG GACATCACCG ATCGCCTTTG TTTTGCCATT
181 CTCTACAGCA GACCAAAGAG TGCATCAAAT GTACATTATT TCAGCATAGA TAATGAACTT
241 GAATATGAGA ACTTCTACGC AGATTTTGGG CCACTCAATC TGGCAATGGT TTACAGATAT
301 TGTGCAAGA TCAATAAGAA ATTAAAGTCC ATTACAATGT TAAGGAAGAA AATTGTTTCAT
361 TTTACTGGCT CTGATCAGAG AAAACAAGCA AATGCTGCCT TCCTTGTTGG ATGCTACATG
421 GTTATATATT TGGGGAGAAC CCCAGAAGAA GCATATAGAA TATTAATCTT TGGAGAGACA
481 TCCTATATTC CTTTCAGAGA TGCTGCCTAT GGAAGTTGCA ATTCTACAT TACTTCTT
541 GACTGTTTTT ATGCAGTAAA GAAGGCAATG CAGTATGGCT TCCTTAATTT CAACTCATTT
601 AACCTTGATG AATATGAACA CTATGAAAAA GCAGAAAATG GAGATTTAAA TTGGATAATA
661 CCAGACCGAT TTATTGCCTT CTGTGGACCT CATTC AAGAG CCAGACTTGA AAGTGTTTAC
721 CACCAACATT CTCCTGAGAC TTATATTCAA TATTTTAAGA ATCACAATGT TACTACCATT
781 ATTCGTCTGA ATAAAAGGAT GTATGATGCC AAACGCTTTA CGGATGCTGG CTTGATCAC
841 CATGATCTTT TCTTTGCGGA TGGCAGCACC CCTACTGATG CCATTGTCAA AGAATTCCTA
901 GATATCTGTG AAAATGCTGA GGGTGCCATT GCAGTACATT GCAAAGCTGG CCTTGGTCGC
961 ACGGGCACTC TGATAGCCTG CTACATCATG AAGCATTACA GGATGACAGC AGCCGAGACC
1021 ATTGCGTG GG TCAGGATCTG CAGACCTGGC TCGGTGATTG GGCCTCAGCA GCAGTTTTTG
1081 GTGATGAAGC AAACCAACCT CTGGCTGGAA GGGGACTATT TTCGTCAGAA GTTAAAGGGG
1141 CAGGAGAATG GACAACACAG AGCAGCCTTC TCCAAACTTC TCTCTGGCGT TGATGACATT
1201 TCCATAAATG GGGTCGAGAA TCAAGATCAG CAAGAACCCG AACCGTACAG TGATGATGAC
1261 GAAATCAATG GAGTGACACA AGGTGATAGA CTTCGGGCCT TGAAAAGCAG AAGACAATCC
1321 AAAACAAACG CTATTCCTCT CACTCTCTCC ATTTC AAGGA CTAAAACAGT CTTGCGTTAA

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Coding region of hCdc14B mRNA. hCdc14B-shRNA target regions are highlighted in **Bold**. The target regions of hCdc14B-sh640 and -sh651 partially overlap. The overlapping sequence is shown in **blue** color.

6.2 Position of hCdc14B-siRNA target regions

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1   CACGGAACAG CCCTCCTGGG GTCCCCACGA GCCGCGTCCT GCTGTGCCCC GGCGCCTACG
61  CAGCAGCGGC CGCGGCCGCG GTGGGCACGC ACGGTTACCC CGGGCAGCTC CGGCCGCCAG
121 CTGCAGCCCC GTCGCCTCGG CCGCGCCAGC CGGCTGCGGG CACCTGGGGG CGGGCTGGGG
181 GCGCCGGCCG CGGCAGGAGG CGCTGTAGCG AGGGCTGCGG CGCCGGTCCT GCGCGGCCCG
241 CGGGAGGCAG CGGGGCAGGC GCTGTGGGCC GGGCTCCTCC TCCGGCTCCT GCGCGACCCG
301 CTCCC GCCG GCTCTGCCGG CGCCGCCGT CCCC GCAGCG CCGCTCTGCG CCCGCCGCC
361 CGAGCGCCCG CGCGGGGCTG GCGGGAGCCT CGCGGGGCGC GCGGGGCGGC GGGGCCATGG
421 TCGTGGCCCC CTGACGGGCC GCGGCCGCTT CCATGAAGCG GAAAAGCGAG CGGCGGTCTGA
481 GCTGGGCCGC CGCGCCCCCG TGCTCGCGGC GCTGCTCGTC GACCTCGCCG GGTGTGAAGA
541 AGATCCGCAG CTCACGCAG CAAGACCCGC GCCGCCGGA CCCCAGGAC GACGTGTACC
601 TGGACATCAC CGATCGCCTT TGTTTTGCCA TTCTCTACAG CAGACCAAAG AGTGCATCAA
661 ATGTACATTA TTTCAGCATA GATAATGAAC TTGAATATGA GAACTTCTAC GCAGATTTTG
721 GACCACTCAA TCTGGCAATG GTTACAGAT ATTGTTGCAA GATCAATAAG AAATTAAGT
781 CCATTACAAT GTTAAGGAAG AAAATTGTTT ATTTTACTGG CTCTGATCAG AGAAAACAAG
841 CAAATGCTGC CTTCCTTGTT GGATGCTACA TGTTTATATA TTGGGGGAGA ACCCCAGGAAG
siRNA #2
901 AAGCATATAG AATATTAATC TTTGGAGAGA CATCTATAT TCCTTTCAGA GATGCTGCCT
961 ATGGAAGTTG CAATTTCTAC ATTACACTTC TTGACTGTTT TCATGCAGTA AAGAAGGCAA
1021 TGCAGTATGG CTTCCTTAAT TTCAACTCAT TTAACCTTGA TGAATATGAA CACTATGAAA
1081 AAGCAGAAAA TGGAGATTTA AATTGGATAA TACCAGACCG ATTTATTGCC TTCTGTGGAC
1141 CTCATTCAAG AGCCAGACTT GAAAGTGGTT ACCACCAACA TTCTCCTGAG ACTTATATTC
1201 AATATTTTAA GAATCACAAT GTTACTACCA TTATTCGTCT GAATAAAAGG ATGTATGATG
1261 CCAAACGCTT TACGGATGCT GGCTTCGATC ACCATGATCT TTTCTTTGCG GATGGCAGCA
1321 CCCCTACTGA TGCCATTGTC AAAGAATTCC TAGATATCTG TGAAAATGCT GAGGGTGCCCA
siRNA #1
1381 TTGCAGTACA TTGCAAAGCT GGCCTTGGTG GCACGGGCAC TCTGATAGCC TGCTACATCA
1441 TGAAGCATTG CAGGATGACA GCAGCCGAGA CCATTGCGTG GGTGAGGATC TGCAGACCTG
1501 GCTCGGTGAT TGGCCCTCAG CAGCAGTTTT TGGTGATGAA GCAAACCAAC CTCTGGCTGG
siRNA
1561 AAGGGGACTA TTTTCGTCAG AAGTTAAAGG GGCAGGAGAA TGGACAACAC AGAGCAGCCT
#3
1621 TCTCCAAACT TCTCTCTGCG GTTGTATGACA TTCCATAAAA TGGGGTTCGAG AATCAAGATC
1681 AGCAAGAACC CGAACCGTAC AGTGATGATG ACGAAATCAA TGGAGTGACA CAAGGTGATA
1741 GACTTCGGGC CTTGAAAAGC AGAAGACAAT CAAAAACAAA CGCTATTCTT CTCACAGTAA
1801 TTCTTCAATC CAGTGTTCAG AGCTGTAAAA CATCTGAACC TAACATTTCT GGCAGTGCAG
1861 GCATTACTAA AAGAACCACC AGATCTGCTT CAAGGAAAAG CAGTGTAAAG AGTCTCTCCA
1921 TTTCAAGGAC TAAAACAGTC TTGCGTTAAG TAAAAACCTG TGACCAGAGC TGAAGGAAGA

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A part hCdc14B mRNA sequence from nt 1-1980. Target sequences of hCdc14B-siRNAs are highlighted in **Bold**.