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Cdc6 Knockdown Renders $p16^{INK4a}$ Re-Activation, Leading to Senescence Human Breast Carcinoma Cells

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1. Introduction

DNA replication is initiated by assembling pre-replicative complex (pre-RC), and DNA replicative protein Cdc6 plays a central role in the initiation of DNA replication. As cells enter G1 from mitosis, Cdc6, together with Cdt1 are recruited to the replicating origins bound with six components of origin recognition complex (ORC) (Dutta and Bell, 1997; Méndez and Stillman, 2000). The pre-RC is formed by loading minichromosome maintenance proteins (MCMs) along with other replicating proteins onto origins, and Cdc6 controls the loading. The stability of Cdc6 during G1 is a key step to regulate pre-RC assembly. Cdc6 is targeted to ubiquitin-mediated proteolysis by the anaphase promoting complex (APC) in G1. The cyclin-dependent kinase (CDK), specifically, cyclin E-Cdk2 kinase is responsible for phosphorylation of human Cdc6, and Cdc6 is stabilized with its amino-terminal end phosphorylated (Mailand and Diffley, 2005). The Cdc6 phosphorylation by CDKs ensures that origin is fired at S phase, and DNA is replicated only once per S phase. Human Cdc6 is also involved in G2/M phase checkpoint as overexpression of Cdc6 in G2 blocks HeLa cell into mitosis (Clay-Farrace et al., 2003). More recently, human Cdc6 shows to play a role in late S phase and S-G2/M transition in controlling cell cycle progression (Lau et al., 2006). These studies suggest that human Cdc6 functions beyond its essential role in the initiation of DNA replication.

Senescence is an irreversible cell event with permanent G1 arrest. Senescent normal somatic cells show features such as the permanent arrest of proliferation, repression of telomerase reverse transcriptase (TERT) expression, and expression of senescence associated-β-galactosidase (SA-β-Gal). The lack of TERT activities has been observed in most human somatic cells and is linking to replicative senescence (Campisi, 2001). Replicative senescence occurs with functional p53 and $p16^{INK4a}$–Rb pathway (Campisi, 2001; Beauséjour et al., 2003). Accumulated evidence shows that tumor cells experience p53 mutation, $p16^{INK4a}$ or Rb inactivation or deletion, accompanied with the re-expression of TERT. Evidence shows that senescence normal fibroblasts undergo the formation of senescence-associated heterochromatic foci (SAHF), and tri- or di-methylation of lysine 9 on histone H3 (H3K9me3/me2) has been found in the SAHF (Narita et al., 2003, 2006).

High level of Cdc6 has been associated with the oncogenic activities in human cancers, and there is a reciprocal association in protein levels between Cdc6 and $p16^{INK4a}$ (Gonzalez et al., 2006). Genetically, human Cdc6 suppresses $p16^{INK4a}$ functions. Cdc6 shows dual effects on the *INK4/ARF* locus: it associates with the replication origin on chromatin together with ORCs for loading MCM proteins, and it binds a region called regulatory domain ($RD^{INK4/ARF}$) in the putative replication origin. This association results in transcriptional repression of the three genes encoding for $p15^{INK4b}$, $p16^{INK4a}$, and $p14^{ARF}$, leading to heterochromatinization of the *INK4/ARF* locus (Gonzalez et al., 2006). The gene products from the *INK4/ARF* locus are thought as barries to immortalization and oncogenesis. $p16^{INK4a}$ inhibits D-type cyclins-Cdk4/Cdk6 kinases, which activates $p16^{INK4a}$-Rb pathway in controlling cell cycle progression.

2. Cdc6 is a decisive factor in promoting cell proliferation

Cdc6 is essential in the initiation of DNA replication. Human Cdc6 function is of importance in cell cycle progression and cell proliferation. In human cells, the levels of Cdc6 change markedly in response to various stresses. DNA re-replication has been found in those Cdc6 and Cdt1 overexpressing normal or tumor cells. Cdc6 could be more stable in tumor cells, due to p53 deficiency, and due to strong Cdc6 phosphorylation by activated cyclin E-Cdk2 kinase (Bartkova et al., 2005; Duursma and Agami, 2005; Gorgoulis et al., 2005; Mailand and Diffley, 2005).

2.1 Oncogenic Cdc6 is involved in replicative stress response

Recently, a report that p53 downregulates Cdc6 in response to DNA damage links Cdc6 directly to p53 mediated replication stress response (Duursma and Agami, 2005). The p53-mediated Cdc6 downregulation takes place without causing detectable cell cycle re-distribution. The inhibition of endogenous Cdc6 via Cdc6 knockdown, however, results in an increased G1 content with decreased S fraction in cells with p53 deficiency (Duursma and Agami, 2005).

Human Cdc6 has been involved in cancer cell growth, while the depletion of Cdc6 induces apoptosis in several cancer cell lines, but not in normal cells. The siRNA or gene transduction vectors producing short hairpin RNA (shRNA) mediated Cdc6 depletion occurs specifically without disturbing other replicative proteins, such as Orc2, Cdt1, and MCM7 (Lau et al., 2006).

Here, we report the reactivation of $p16^{INK4a}$-Rb pathway and senescence human breast carcinoma MCF7 cells with Cdc6 knockdown.

2.1.1 Overexpression of Cdc6 in human cancer cell lines

We have extended our research to explore human Cdc6 functions. We first examined Cdc6 overexpression in several human cancer cell lines including neuroblastoma LA-N-2 and CHLA255 cells, MCF7 cells, and HeLa cells. Cells were transfected with tetracycline-inducible Cdc6 expressing vector, or infected with a retrovirus for expressing Cdc6. Our results showed that Cdc6 overexpression in some cancer cell lines could not induce significant Chk1 or Chk2 phosphorylation. Cdc6 overexpression resulted in, however, p53 upregulation. At the present moment, we haven't observed senescence these cancer cells by overexpressing Cdc6. Instead, DNA re-replication and DNA damages caused by Cdc6

overexpression makes DNA replication checkpoints compromised, which might be related to unlimited cancer cell proliferation.

2.1.2 Generating a human Immunodeficiency virus type 1 (HIV-1) derived lentivirus expressing *CDC6* short hairpin RNA (shRNA)

We have constructed an HIV-1 derivative vector (THTD) for producing *CDC6* shRNA. The details of the lentivirus vector are published. A control lentivirus for producing a scramble sequence (THTP) is also generated. Infection of THTD in various kinds of human cell lines causes the depletion of Cdc6. The inhibition of endogenous Cdc6 expression is over 98% (Figure 1., lane 3, 6, and 8). However, infection of THTP shows no change of Cdc6 expression (Figure 1., lane 2, 5, and 7). The recombinant lentivirus, therefore, becomes a powerful vector for us to study Cdc6 function.

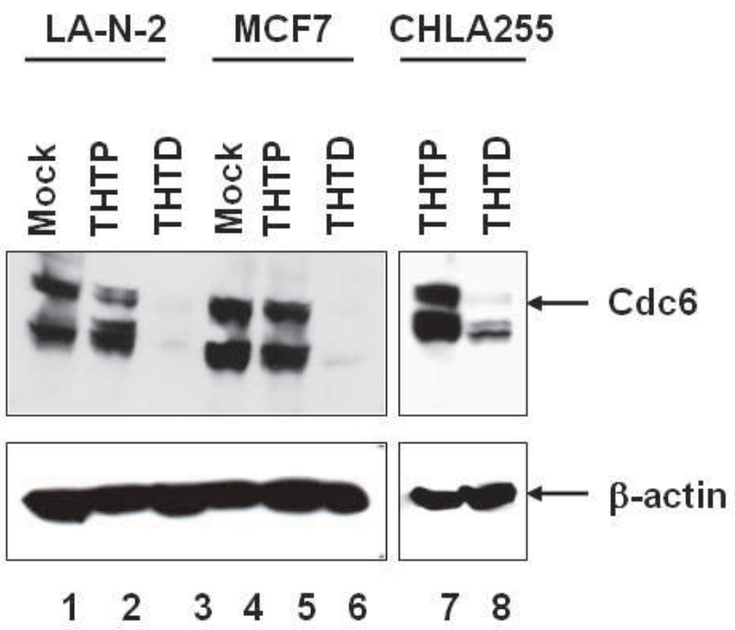


Fig. 1. Inhibition of Cdc6 expression via recombinant lentivirus producing *CDC6* shRNA .

LA-N-2 cells, CHLA-255 cells, and MCF7 cells were infected with THTD for Cdc6 knockdown (lane 3, 6 and 8), or infected with THTP (lane 2, 5 and 7), or mock infected (lane 1 and 4) at m.o.i. of 1.0. Two days postinfection, the cell extracts were prepared. About 20 μg proteins in extracts from each sample were assayed in Western blots probed with indicated antibodies.

2.1.3 Cdc6 knockdown induces apoptosis of human neuroblastoma cells

Elevated levels of Cdc6 were found in the LA-N-2, CHLA255, and other human neuroblastoma cell lines that grew fast. Cdc6 knockdown via THTD infection caused the accumulation of sub G1 populations with declined S contents in the LA-N-2 and CHLA255 cells. The cells were stained positive with Annex V and 7-AAD by 31% (Figure 2.). Expression profile from the selected genes showed the reduction of cell cycle progression proteins such as cyclin E, cyclin A, and Cdc25C with a boosted increase of CDK inhibitor

p27Kip1, indicating the suppression of tumor cell proliferation. Further, Cdc6 knockdown caused the increase of pro-apoptotic Bax accompanied with the decrease of anti-apoptotic Bcl-2, resulting in massive cell death. Our study indicates that human Cdc6 functions in several pathways to control cell proliferation and the cell death.

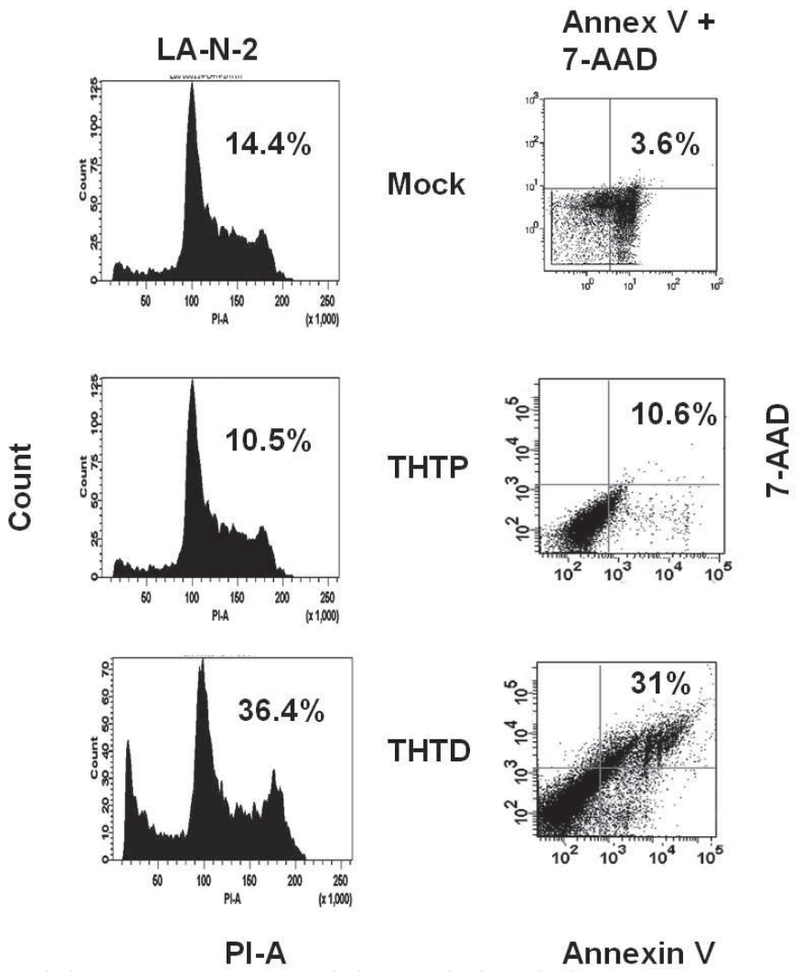


Fig. 2. Cdc6 knockdown induces apoptosis of human neuroblastoma cells.

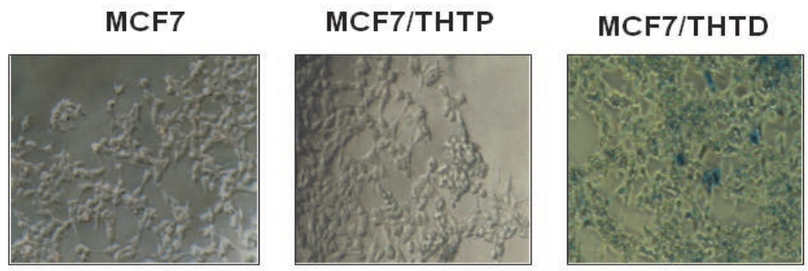
LA-N-2 cells were infected with the *CDC6* shRNA lentivirus (THTD), or the control virus with scramble sequence (THTP), or mock infected. Left panel: About 0.5x10^6 cells were harvested 48 hours post infection, and stained with propidium iodide (PI) for flow cytometry analysis. The data presented were the average values from 4 separated experiments with the standard deviation between 5-15%. Right panel: The cells were harvested 48 hours post infection, and about 0.2x10^6 cells were used for Annexin V-PE and 7-ADD double staining. The flow cytometry analysis was performed for the stained cells. Fluorescence emission was detected at 575±13 for Annexin V-PE, and 660±10for 7-AAD.

2.1.4 Depletion of Cdc6 renders senescence MCF7 cancer cells

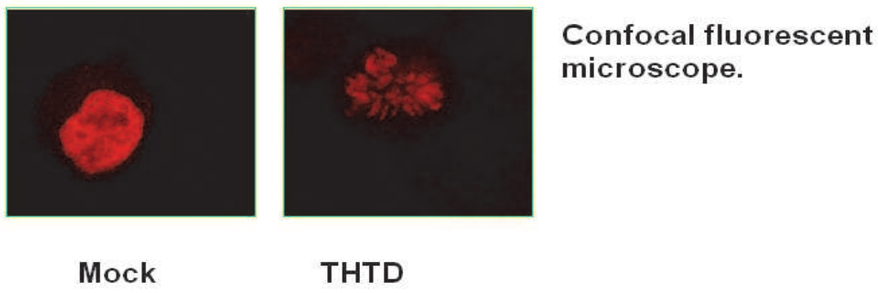
We have found apoptosis of human neuroblastoma LA-N-2 and CHLA255 cells with Cdc6 knockdown. However, Cdc6 knockdown could not confer the programmed cell death in

MCF7 cells. Instead, Cdc6 knockdown induced the cancer cell senescence. Cell cycle profile showed that Cdc6 knockdown caused G1 contents increased by 30% along with declined S populations. At the same condition analyzed, MCF7 cells infected with the control virus showed no decline of S populations, neither the increase of the G1 contents. Brd (U) incorporation was performed to determine the cell proliferation status. Fluorescent signals from incorporated Brd (U) in DNA in the nucleus could be observed in both mock and THTP infected MCF7 cells, but not in the Cdc6 knockdown cells infected with THTD, indicating no DNA replication in the Cdc6 depleted cells.

The Cdc6 depleted MCF7 cells were subjected to SA-β-Gal staining 48 hours postinfection. While the mock and the control virus infected MCF7 showed a background staining, the Cdc6 depleted cancer cells displayed positive staining for SA-β-Gal, a hall marker for senescence (Figure 3A.). Because senescence cells undergo heterochromatin formation, a



(A)



(B)

Fig. 3. Knockdown Cdc6 renders senescence human breast carcinoma MCF7 cells. (A). Senescence-associated β-galactosidae (SA-β-Gal) staining: MCF7 cells ($2.0x10^4$/well) were grown in a six-well plate, infected with THTD, THTP, and mock infected at an m.o.i. of 1.0. Two days postinfection, the cell was washed in 2 ml 1x PBS, fixed in 2 ml of 3% formaldehyde, washed, and incubated for 24 h at 37°C with a solution containing 1 mg/ml of 5-bromo-4-chloro-3-indolyl B-D-galactoside (X-Gal), 40 mM citric acid/sodium phosphate at pH 6.0, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, and 2 mM $MgCl_2$. After washing, the cell staining was viewed with a fluorescent microscope, and photographed. (B). Anti-H3K9me2 indirect immunofluorescent staining. MCF7 cells were infected with THTD at m.o.i. of 1.0. Two days postinfection, the cell was fixed and stained with the anti-H3K9me2 antibody. The slides were then stained with a goat anti rabbit Ig G conjugated with rhodamine red, and then with DAPI. The fluorescent-stained cells were visualized with a fluorescent microscope and its image processing system. The RGB color model of the cell images was applied by using Adobe Photoshop program.

distinct chromatin structure known as SAHF with the accumulation of H3K9me3/me2 would be observed in the nuclei of senescent cells (Narita et al., 2003, 2006). To demonstrate this cellular event, the Cdc6 knockdown cells along with the mock infected MCF7 cells were stained with an antibody against methylated lysine 9 on histone H3 (H3K9me2). Indirect-immunofluorescent staining revealed an overall elevated positive staining for H3K9me2 in the nuclei of the Cdc6 knockdown MCF7 cells in comparison of those mock infected cells. By conducting confocal fluorescent microscope, a grainy-structured nucleus with the accumulated H3K9me2 was evident, a characteristic change for the complete proliferation arrest. This change was in contrasting to the mock infected cells in which a uniformly distributed H3K9me2 staining was observed in the nucleus of mock-infected cell (Figure 3B.).

2.2 Human Cdc6 stimulates D-type cyclins-Cdk4 kinases by suppressing $p16^{ink4a}$ Cdk inhibitory activity

In MCF7 cells, Cdk4 kinase activity is high as hyper Rb-C phosphorylation is observed, even if elevated levels of $p16^{INK4a}$ is presented. One reason is that $p16^{INK4a}$ CDK inhibitory activity has been repressed. We have found that this repression is relieved when Cdc6 is knockdown.

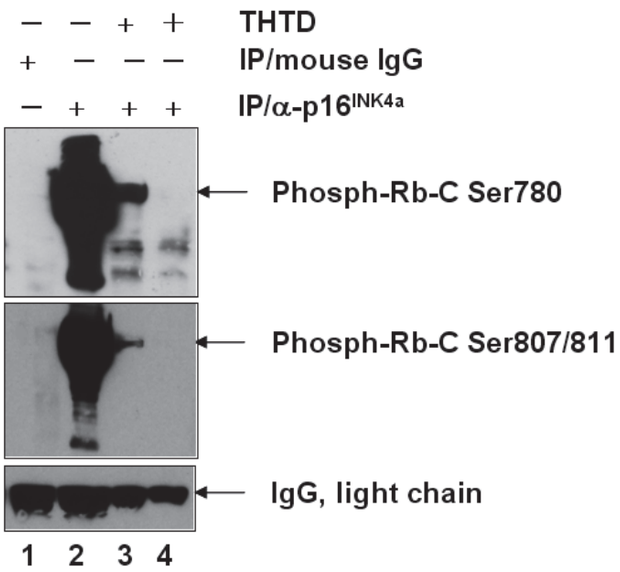
2.2.1 Cdc6 is involved in Rb-C phosphorylation

The cell cycle progression in MCF7 cells was not inhibited even in the presence of $p16^{INK4a}$. We reasoned that Cdc6 might play a role in inactivating $p16^{INK4a}$. To test this speculation, a nonradioactive immunoprecipitation (IP)-kinase assay was carried out. Because $p16^{INK4a}$ associates with D-type cyclins-Cdk4/Cdk6 complexes, and because phosphorylation of Rb C-terminal domain is catalyzed by D-type cyclins-Cdk4/Cdk6 kinases (Harbour et al., 1999), the Rb phosphorylation was determined using anti-Phospho Rb S780 and anti-Phospho Rb S807/811 specific antibodies. $p16^{INK4a}$ IP from MCF7 cells phosphorylated the Rb-C in vitro (Figure. 4., lane 2), while IP with mouse IgG could not (Figure. 4., lane 1). When Cdc6 was depleted via *CDC6* shRNA lentivirus (THTD) infection, the Rb-C phosphorylation was inhibited (Figure. 4., lane 3), and no kinase activity was detected with transgene p53 expression in the THTD-infected cells (lane 4). We determined the Cdc6 inhibitory effect on $p16^{INK4a}$ activity using $p16^{INK4a}$ IP dilutions in the kinase reactions. We found that $p16^{INK4a}$ CDK inhibitory activities were at least suppressed 25 times in the presence of Cdc6. In other words, at least 25 times suppression of Rb phosphorylation was archived upon Cdc6 knockdown.

2.2.2 Cdc6 is found in Cdk4 complex and is required for Rb-C phosphorylation

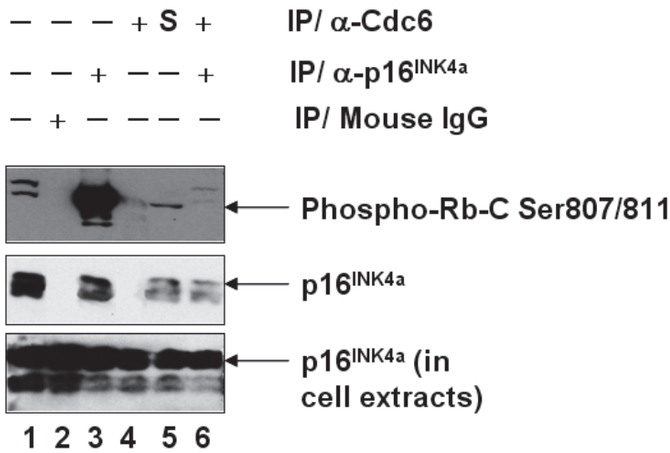
The result that $p16^{INK4a}$ IP from the Cdc6 depleted MCF7 cells showed very low levels of Rb-C phosphorylation (Figure 4.) indicates that Cdc6 might be required for Rb phosphorylation. To test this prediction, Cdc6 was first depleted from whole cell extracts prepared from MCF7 cells in vitro by using anti-Cdc6 antibody (Immunodepletion). The Cdc6 immunodepleted extracts were then subjected to kinase reactions.

Crude extracts turned Rb-C phosphorylated at very low level, while Cdc6 IP could not confer the phosphorylation, nor did the Cdc6 immunodepletion (Figure 5., lanes 4 and 5). In contrast to the direct $p16^{INK4a}$ immunoprecipitation, the Cdc6 depletion followed by $p16^{INK4a}$ IP showed no Rb-C phosphorylation (Figure 5., lane 6). Cdc6, therefore, was required for the Rb-C phosphorylation.



MCF7 cells were infected with THTD for Cdc6 knockdown, or mock infected at m.o.i. of 1.0. Two days postinfection, the cell extracts were prepared. The whole cell extracts (~1000 μg of protein) were mixed with the anti-p16^{INK4a} monoclonal antibody. Immunoprecipitation (IP) proceeded at 4°C overnight. In vitro kinase reactions were set up with one quarter of IP beads mixed with Rb-C substrate and ATP, and incubated at 37°C for 30 minutes according to the manufactory's instruction. The specific Rb-C phosphorylations were determined in Western blot with the anti-Phospho Ser780 antibody (top panel), or anti-Phospho Ser807/811 antibody (middle panel). The mouse IgG signals were presented as IP controls (bottom panel).

Fig. 4. Knockdown Cdc6 causes sharp decline of Rb-C phosphorylation.



MCF7 cell extracts were mixed with the anti-Cdc6 monoclonal antibody, and the IP proceeded at 4°C overnight. In Lane 1 are cell extracts only. Top panel: samples for Rb-C phosphorylation were assayed (S: the supernatants of Cdc6 IP). Middle panel: immunoblotting (IB) of p16^{INK4a} with different IPs, or immunodepletion (S). Bottom panel: p16^{INK4a} immunoblotting with extracts from each sample.

Fig. 5. Cdc6 is required for Rb-C phosphorylation.

It was interesting to note the presence of large amounts of Cdk4, but no $p16^{INK4a}$ in Cdc6 IP. Cdc6, therefore, forms complex with Cdk4. In the Cdc6 knockdown MCF7 cells, however, immunoblotting showed a sharp decline of Cdk4 contents in the Cdc6 IP in which 16^{INK4a} could be detected.

2.2.3 Cdc6 actively represses $p16^{INK4a}$ CDK inhibitory functions

In our experimental system, Cdk-4 IP could not confer the Rb-C phosphorylation (Figure 6., lane 7) due to Cdk-4 antibody used had neutralization effect on Cdk-4 kinase activity. In order to study the effect of Cdc6 on stimulating D-type-cyclins-Cdk4 kinase activity, a hemagglutinin (HA)-tagged human Cdk4 (Cdk4-HA) was expressed in MCF7 cells. Ectopic Cdk4 expression induced high levels of $p16^{INK4a}$ whether Cdc6 was presented or depleted. The Cdk4-HA was immunoprecipitated with anti-HA-agarose. The presence of Cdk4-HA was confirmed in Western blot. In contrast to the Cdk4 IP prepared from the parental MCF7 cells, the anti-HA-agarose IP contained $p16^{INK4a}$, indicating that Cdk4-HA was bound with the CDK inhibitor $p16^{INK4a}$ even in the presence of endogenous Cdc6. With $p16^{INK4a}$ associated, the Cdk4-HA IP, however, rendered phosphorylation of Rb-C, though the kinase activity was lower in comparison of that from the $p16^{INK4a}$ IP (Figure. 6., lane 2, 3 and 4).

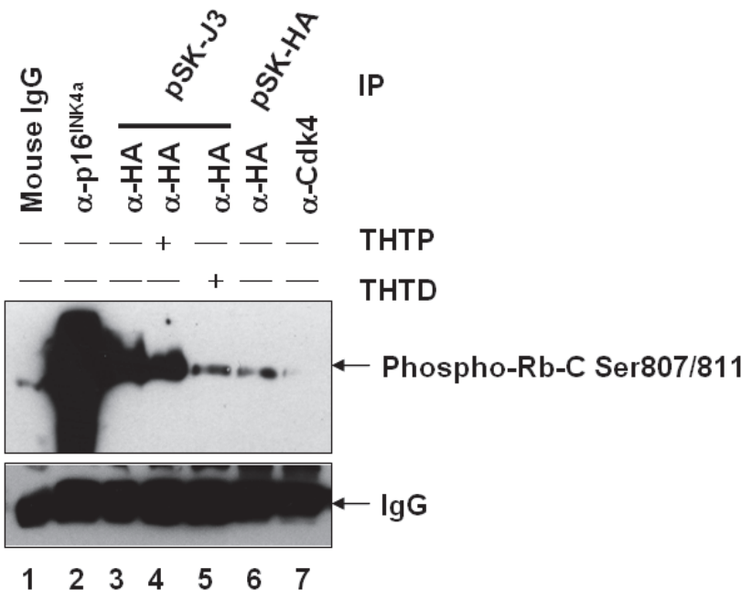


Fig. 6. Cdc6 suppresses $p16^{INK4a}$ CDK inhibitory activities even $p16^{INK4a}$ is bound with Cdk4.

MCF7 cells were infected with THTD or THTP. Two days postinfection, the cells were then transfected with pSK-J3 (for Cdk4-HA) or pSK-HA (HA-tag control). Cell extracts were prepared, and were subjected to IP with different antibodies. Top panel: each IP sample was added into the kinase reactions, and the in vitro Rb-C phosphorylation was performed. The phosphorylated Rb-C was examined in Western blot with the anti-Phospho Ser807/811 antibody. Bottom panel: mouse IgG was presented as the loading control.

The ectopic Cdk4-HA could contribute to the Rb-C phosphorylation without Cdc6 stimulation given the $p16^{INK4a}$ CDK inhibitory activity is bypassed upon Cdk4 overexpression. To test this possibility, Cdc6 was first knockdown with THTD lentivirus

producing *CDC6* shRNA in MCF7 cells. The cell was then transfected with the Cdk4-HA expressing plasmid pSK-J3. As Cdc6 was knockdown, Rb-C phosphorylation by Cdk4-HA kinases precipitated with anti-HA-agarose, if any, was to a basal level (Figure. 6., lane 5), indicating Cdc6 is required for stimulating the D-type cyclins-Cdk4 kinases. These results indicated that the effect of Cdc6 was dominated over $p16^{INK4a}$ even if Cdk4 was associated with $p16^{INK4a}$, and the CDK inhibitory activity had been suppressed, rendering the Rb hyperphosphorylation by D-type cyclins-Cdk4 kinases.

2.3 The significance of inhibiting $p16^{INK4a}$-Rb pathway by Cdc6

Our studies address the mechanisms of cell proliferation and senescence. The approach that we have adopted and the results we have accomplished are novel in that we don't try to artificially restore the function of $p16^{INK4a}$-Rb pathway by overexpressing either p16INK4a, or pRb protein. Rather, we biologically remove a specific blocker, Cdc6, from the cancer cell. We have found that Cdc6 forms complex with Cdk4, and this association activates D-type cyclins-Cdk4 kinases in Rb-C phosphorylation. Moreover, Cdc6 stimulates Rb-C phsophorylation by D-type cyclins-Cdk4 kinases even in the presence of high levels of $p16^{INK4a}$. Further, Cdc6 is required for Cdk4 kinase activity because ectopic Cdk4 expression can not augment phosphorylation of Rb-C when Cdc6 is depleted (Figure 6.). The effect of Cdc6 and Cdk4 interaction is overwhelm that a dramatic decline of association between $p16^{INK4a}$ and Cdk4 takes place, indicating that Cdc6 could block or interfere $p16^{INK4a}$ associating with Cdk4. Cdc6 has been shown in the interaction with cyclin B, which is involved in S phase entry (Dutta and Bell, 1997; Méndez and Stillman, 2000), or with cyclin E-Cdk2 complex (Mailand and Diffley, 2005), which controls the initiation of DNA replication. In all of these cases Cdc6 is the target of CDK kinase reactions. Different from these interactions, the association with Cdk4 enables Cdc6 actively suppressing $p16^{INK4a}$ CDK inhibitory activity, which is necessary for cell proliferation.

The most striking feature of oncogene-induced senescence is through p53 pathway (Hemann and Narita, 2007; Yaswen and Campisi, 2007). However, there is always exception, and $p16^{INK4a}$-Rb network exists as secondary senescence pathway (Sedivy, 2007). $p16^{INK4a}$ is responsible for telomerase-independent senescence induced in various cellular stresses. $p16^{INK4a}$ inhibits D-type cyclins-Cdk4/Cdk6, which activates Rb pathway, and functional $p16^{INK4a}$-Rb pathway is involved in cellular senescence (Beauséjour et al., 2003; Narita et al., 2003, 2006). The key issue is that the $p16^{INK4a}$-Rb pathway has been inactivated in most human cancers. Cdc6 has been shown to suppress $p16^{INK4a}$ expression via chromatin epigenetic modifications (Gonzalez et al., 2006). Our study demonstrates that human Cdc6 suppresses $p16^{INK4a}$ CDK inhibitory activity, and $p16^{INK4a}$-Rb pathway is inactivated in MCF7 cells and perhaps in many different kinds of human cancers. Elevated levels of Cdc6 expression have been evident in many human cancers. Cdc6 overexpression induces senescence of several human cancer cell lines with double-stranded DNA breaks (Bartcova et al., 2006). The Cdc6-induced senescence human cancer cells are associated with the ATM-Chk2 DNA damage checkpoint control, and the depletion of ATM kinase, or its substrate p53, suppresses the senescence (Bartcova et al., 2006). Unlike ATM-Chk2/p53 pathway, which can be spontaneously activated in response to replicative stresses (Bartkova et al., 2005, 2006), and which is potential for the emergence of p53-resistant tumors, the reactivation of $p16^{INK4a}$-Rb pathway needs Cdc6 depletion accompanied with the formation of SAHF inside the cancer cells.

Functional p16^{INK4a}-Rb pathway is essential for the SAHF formation (Narita et al., 2003). The production of H3K9me3/me2 catalyzed by histone methyltransferase SUV39H1 is an important step of heterochromatinization, and is regulated by p16^{INK4a}-Rb pathway. Recently, a non-histone protein, high mobility group A (HMGA), has been identified as an essential component of SAHF. Moreover, HMGA proteins promote complete proliferation arrest, and p16^{INK4a} has been shown to coordinate with HMGA to induce senescence normal fibroblasts (Narita et al., 2006). HMGA protein functions by interacting with the minor groove of AT-rich DNA sequences. The association of HMGA protein with chromatin can be displaced by DNA-binding drug distamycin A, which binds the minor groove of AT-rich DNA (Narita et al., 2006).

In late M phase, accumulated human Cdc6 associates with chromatin for pre-RC assembly (Dutta and Bell, 1997; Méndez and Stillman, 2000). Cdc6 could block Rb in preventing heterochromatinization since Cdc6 knockdown induces hypophosphorylated Rb, which is the activated form of tumor suppressor, and which promotes the formation of SAHF (Narita et al., 2006) (Figure 3.). We have shown that budding yeast Cdc6 interats with DNA non-specifically with preference to A/T-rich tracks. It is likely that human Cdc6 interacts DNA directly since this protein shares identical amino acid residuals with the budding yeast *Sccharomyces cerevisiae* Cdc6 within highly conserved domains. The positive results could support one of this protein's important biological functions in promoting cell proliferation.

Future work will focus on demonstrating the competition of chromatin association between Cdc6 and HMGA proteins. By interacting with the minor groove of the AT-rich DNA sequences, Cdc6 would compete with HMGA proteins in associating with chromatin. It is essential that the association of HMGA proteins with DNA for the complete proliferation arrest, that is the formation of SAHF (Narita et al., 2006). In the parental MCF7 cells, Cdc6 is expected to prevent the heterochromatin formation by blocking and displacing HMGA proteins in binding to DNA. In contrast, in the Cdc6 depleted cells, no chromatin-bound Cdc6 will be expected. Moreover, the Cdc6 depletion reactivates p16^{INK4a}-Rb pathway, this will stimulate the HMGA proteins binding to DNA constitutively and promotes SAHF formation.

3. Conclusion

We would draw some conclusions from these studies: Human Cdc6 is required for phosphorylation of cell-cycle controller and tumor suppressor Rb protein, which is of important for cell proliferation. Cdc6 functions to associate with D-type cyclins-Cdk4 and suppresses p16^{INK4a}-Rb pathway, which otherwise controls cell cycle progression and prevents cell from unlimited proliferation. In many human cancers, p16^{INK4a}-Rb pathway has been inactivated. Cdc6 plays a role in inactivating biological functions of p16^{INK4a}-Rb pathway to promote cancer cell proliferation. Cdc6 depletion via gene transduction vector producing shRNA renders reactivation of p16^{INK4a}-Rb pathway and leads the cancer cells to replicative senescence.

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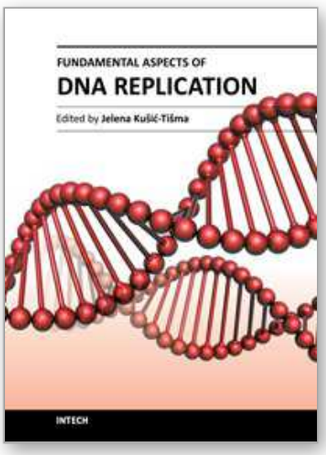

Fundamental Aspects of DNA Replication

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DNA replication, the process of copying one double stranded DNA molecule to produce two identical copies, is at the heart of cell proliferation. This book highlights new insights into the replication process in eukaryotes, from the assembly of pre-replication complex and features of DNA replication origins, through polymerization mechanisms, to propagation of epigenetic states. It also covers cell cycle control of replication initiation and includes the latest on mechanisms of replication in prokaryotes. The association between genome replication and transcription is also addressed. We hope that readers will find this book interesting, helpful and inspiring.

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