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Cdc6 knockdown inhibits human neuroblastoma cell proliferation

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Abstract The cell division controller Cdc6 plays a central role in the initiation of DNA replication. It was found that elevated levels of Cdc6 were present in many of human cancer cells, and the accumulation of Cdc6 is required for cell proliferation. In this study, we have investigated the control of Cdc6 expression and its effect on cell proliferation and death in human neuroblastoma cells. Elevated levels of Cdc6 are found in the LA-N-2, CHLA255, and other cell lines that grow fast. Cdc6 knockdown via a Cdc6 short hairpin RNA lentivirus causes the accumulation of sub-G1 populations with the decrease of S contents in the LA-N-2 and CHLA255 cells. Expression profile from the selected genes shows the reduction of cyclin E, cyclin A, and Cdc25C, with a boosted increase of the CDK inhibitor $p27^{Kip1}$, indicating the suppression of tumor cell proliferation. Further, Cdc6 knockdown causes the increase of pro-apoptotic Bax accompanied with the decrease of anti-apoptotic Bcl-2, resulting in the increased cell death. Furthermore, Cdc6 knockdown causes a sharp reduction of tumor suppressor protein p53, and Cdc6 overexpression renders a boosted p53 expression; and this regulation is at p53 posttranscriptional level. Our study indicates that human Cdc6 functions in several pathways to control the cell proliferation and the cell death.

Keywords Cdc6 · shRNA · Neuroblastoma

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Introduction

Cdc6 is essential for assembling pre-replicative complex (pre-RC), and plays a role in the initiation of DNA replication [1–4]. In proliferating cells Cdc6 is subject to modification during cell-cycle progression. Cdc6 is targeted to ubiquitin-mediated proteolysis by the anaphase promoting complex (APC) in G_1 [5]. There are three serine residues at the amino terminal domain (NTD) of human Cdc6, and they are phosphorylated by cyclin-dependent kinases (CDKs) [6]. The recent finding that the NTD phosphorylation by CDKs protects Cdc6 from the proteolysis underscores Cdc6 modification for S phase entry [2]. More specifically, Cdc6 is phosphorylated by cyclin E-cdk2 kinase and stabilized. These phoshorylation modifications are required to ensure that DNA is replicated only once per S phase [2]. Human Cdc6 is also involved in G2/M phase checkpoint as overexpression of Cdc6 in G2 blocks HeLa cell into mitosis [7]. These studies suggest that in addition to its essential role in the initiation of DNA replication, human Cdc6 also functions in G2/M phase checkpoint, a step to ensure genome integrity.

In human cells, the levels of Cdc6 change markedly in response to various stresses. High level of Cdc6 has been found to promote the heterochromatinization of the p14 regulatory domain (RD^{p14}), and has been associated with the oncogenic activities in human cancers [8]. DNA re-replication has been found in those Cdc6 and Cdt1 overexpressing normal or tumor cells with the activation of p53, and p53 activation prevents this unusual DNA replication [9]. However, expression of Cdc6 alone ectopically confers a very limited re-replication or no re-replication in HeLa and other tumor cells in which p53 is defective [5, 9]. More recently, p53 downregulates Cdc6 in response to DNA damage, linking Cdc6 directly to p53-mediated

replication stress response [10]. Cdc6 downregulation is followed by the stabilization of p53 in the cells with DNA damage, while the S54 phosphorylation by CDK confers Cdc6 resistant to the p53-dependent proteolysis [10]. 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 [10].

p53 plays a central role in suppressing tumor growth through induction of apoptosis. p53 functions either through transcriptional activation of pro-apoptotic protein genes [11], or through its direct activation of mitochondrial outer membrane permeabilization (MOMP) [12–14], which requires functional Bcl-2 family proteins to mediate cell death reactions. The Bcl-2 family proteins play different roles in cell fate: the pro-apoptotic proteins promote programmed cell death, while the anti-apoptotic ones cell survival (for review, see [15, 16]). Both Bax and Bak are multiple *B*cl-2 *h*omology (BH)-domain apoptotic effecters. Bax/Bak can be activated by Bim/Bad, or Bid. Both Bim and Bad are BH3-only pro-apoptotic Bcl-2 members [17, 18]. Bim is a transcriptional target of p53, and functions to activate Bax/Bak, and inhibit Bcl-2 or Bcl-x by associating with these pro-survival proteins. Loss of p53 inactivates Bim and Bax directly or indirectly [12], and frees Bcl-2 protein [13, 14], a consequence of cell survival and cancer progression.

Mutations of p53 gene were found in about 50% of human tumors [19], and the tumor cells are defective in DNA damage checkpoints with compromised cell-cycle arrest and decreased apoptosis [20, 21]. Cdc6 could be more stable in tumor cells, due to p53 deficiency and due to strong Cdc6 phosphorylation by activated cyclin E-cdk2 kinase [2, 10, 20, 21]. Therefore, Cdc6 may act as a powerful engine to drive tumor cell growth, while the cyclin E-cdk2 kinase fuels this unlimited cell proliferation. As Cdc6 knockdown causes the loss of proliferative capability in those cells with p53 deficiency [10], it is interesting to determine whether the suppression of Cdc6 would stop the unlimited proliferation of human cancer cells. We report, here, the inhibition of Cdc6 expression in human neuroblastoma cells in an effort for cancer suppression. Cdc6 expression is determined in several neuroblastoma cell lines, and elevated levels of Cdc6 have been found in most of them. Cdc6 knockdown via a Cdc6 short hairpin RNA (shRNA) lentivirus infection causes a significant increase of sub-G1 population with the reduction of S populations. The inhibition of Cdc6 expression results in the accumulation of $p27^{Kip1}$, and the reduction of cyclin E and cyclin A, indicating a strong inhibition of S phase progression. Moreover, Cdc6 knockdown causes the increase of pro-apoptotic Bcl-2 family proteins, leading to an increased cell death. Furthermore, Cdc6 knockdown results in a reduction of p53, and Cdc6 overexpression renders a boosted p53 expression. Since many kind of human cancer cells bear mutated p53, or non-functional p53, inhibition of Cdc6 expression independent of p53 levels may be more potential in cancer suppression.

Materials and methods

Cell lines and culture

PA317 retrovirus packaging cell line was obtained from the CHLA Gene Therapy Laboratory, and the cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin at 37°C in a 5% CO_2 incubator. Human embryonic kidney 293T cell was obtained from Dr. Samson A. Chow of University of California at Los Angeles (UCLA), and was cultured in the same condition as that for growing PA317 cells. Human MCF7 breast carcinoma cells were maintained in RPMI 1640 medium plus 10% fetal bovine serum. Human neuroblastoma cell lines were maintained, as described [22].

Oligonucleotides

A hairpin structure of Cdc6 DNA fragment contains oligonucleotide sequences of OD6Ai-5 (64 mer): *GAT CCCC***AGG CACTTGCTAC CAGCAA** TTCAAGAGA *TTGCTGGTAG CAAGTGCCTT TTTTGGAAA*. Its complementary oligonucleotide sequence of OD6Ai-3 (64 mer) is: *AGCTTTTCCA AAAA***AGGCAC TTGCTACCAG CAA** TCTCTTGAA *TTGCTGGTAG CAAGTGCCTG GG*. The nucleotides in bold are derived from Cdc6 cDNA. The two oligonucleotides were annealed and subcloned into pBlueScript.

Retroviral vector construction, viral stock preparation, and virus infection

Human Cdc6 cDNA (1.6 kb) and human p53 cDNA (1.2 kb cDNA) were cloned into pRetro-On vector (Clontech Laboratories, Inc.) at Not I and BamH I sites. Calcium phosphate precipitation was performed for plasmid DNA transfection of PA317 cells. The transfected cells were selected on puromycin (3 μg/ml final concentration) for 7 days. Viral medium were collected, and passed through a 0.45 μM filter. The titer was determined by infecting NIH3T3 cells, and the viral stock was stored at −80°C.

The day before infection, LA-N-2 or CHLA255 cells were seeded at 0.5×10^5 cells per 25-cm^2 flask. The cell was infected with the retrovirus (2×10^6 CFU) by incubating the cell with the virus medium at 37°C for 12–24 h. After infection, the cells were rinsed with fresh medium and grown with or without adding doxcycline (final concentration 1 μg/ml) for 2 days. The cells were harvested for further analysis.

Human immunodeficiency virus type 1 (HIV-1) derived lentivirus vector, viral stock preparation, and infection

Plasmid pNL4-3 was obtained from Dr. Samson A. Chow of UCLA. The pTHTN was derived from pNL4-3 by deleting a Nde I and Bgl II fragment containing the envelop gene of the HIV-1 genome. pTHTD6Ai was constructed by inserting a Mlu I-Xho I fragment containing a 1.4-kb promoter of human telomerase reverse transcriptase (*hTERT*) and the oligonucleotides OD6Ai for human Cdc6 shRNA at the Mlu I and Xho I sites in the pTHTN vector, as described previously [23]. Briefly, HIV-1 virus was generated by plasmid co-transfection of 293T cells. The day before transfection, 2.0×10^6 293T cells were seeded in 75-cm^2 tissue culture flasks. Calcium phosphate precipitation was performed with 10 μg of HIV-1 plasmid DNA and 2 μg of a pCMV-VSV/G expression construct (for expressing the protein G of vesicular stomatitis virus, VSV-G). Supernatants containing viral particles were harvested 36–60 h after transfection. The virus-containing medium was passed through a 0.45 μm pore size filter and stored at −80°C. The titer of viral particles was determined using an HIV-1 p24 ELISA assay kit (Coulter Inc., Miami, Florida).

LA-N-2 or CHLA255 cells were seeded at 0.5×10^6 cells per 75-cm^2 flask. The cells were incubated with THTD6Ai or THTP at 1.0 multiplicity of infection (m.o.i.) per cell. The infection was allowed for 12–16 h, and the cells were rinsed three times with 1 ml of fresh medium. Two days post-infection, the cell was harvested for further analysis.

Western blot

About 20 μg of protein in nuclear extracts or cytoplasmic extracts were mixed with 10 μl of SDS loading buffer and boiled for 10 min. Proteins were separated on a 12.5 % SDS-polyacrylamide gel, and were transferred to an Immobilon-P PVDF membrane (Millipore). The blot was probed with a mouse monoclonal antibody against human Cdc6 (clone DCS-180, Sigma), or p53 (clone 80, BD transduction Laboratories). The protein–antibody reaction signal was detected with the Lumi-Light Western Blotting Substrate (Roche). The anti-HA antibody was from Roche Applied Science (clone 12CA5), anti-β actin antibody was from Sigma (AC-15). The anti-Bax (clone 3), anti-Bcl-2 (clone 7) antibodies were from BD transduction Laboratories. The anti-Bim polyclonal antibody was from Calbiochem. The results of Cdc6 expression were scanned in a densitometry and the data were processed with software of Sigma plot.

The Phospho-Erk1/2 pathway sample kit and the p38 MAP kinase assay kit were from Cell Signaling Technology. The recombinant GST-ATF2 (obtained from Dr. Roger J. Davis of University of Massachusetts Medical School) was used as substrate in the p38 MAP immunoprecipitation and immuno-kinase assay following the manufactory's instruction.

Annexin V or propidium iodide (PI) staining, and flow cytometry analysis

An Annexin V-PE apoptosis detection kit (BD Pharmingen) was used for staining LA-N-2 or CHLA255 cells infected with the Cdc6 shRNA lentivirus following the manufactory's protocol. PI staining was conducted by first fixing the cell in cold 80% ethanol on ice overnight. The cell was then resuspended in a PI master mixture containing 40 μg/ml of propidium iodide, 5 μg/ml of RNase A (DNase free) in 1× PBS in 1×10^6 cells/ml. The staining was allowed at 37°C for 1 h before processed for flow cytometry analysis. All samples were analyzed with a BD LSR II (BD Biosciences, San Jose, CA, USA), or a Beckman Coulter Epics Elite (Miami, FL, USA). Fluorescence emission was detected at 610 ± 10 nm for PI staining. For Annexin V staining, fluorescence emission was detected at 575 ± 13 for Annexin V-PE, and 660 ± 10 for 7-AAD.

Results

Detecting Cdc6 expression in human neuroblastoma cell lines and Cdc6 knockdown

Expression of Cdc6 was examined in various neuroblastoma cell lines derived from different tumor samples. Cdc6 protein could be detected in 11 out of 14 cell lines with high levels of the Cdc6 protein found in LHN, LA-N-2, and BE1, while moderate levels of Cdc6 in CHLA15, CHLA255, CHLA20, SHN, and CHLA42 (Fig. 1a). Low levels of Cdc6 were in LA-N-1, LA-N-5, and CHLA134, with no Cdc6 protein detected in KANR, CHLA79, and CHLA90 (Fig. 1a). Tumor cells with elevated levels of Cdc6 grew fast (like LA-N-2 and CHLA255, etc.), while

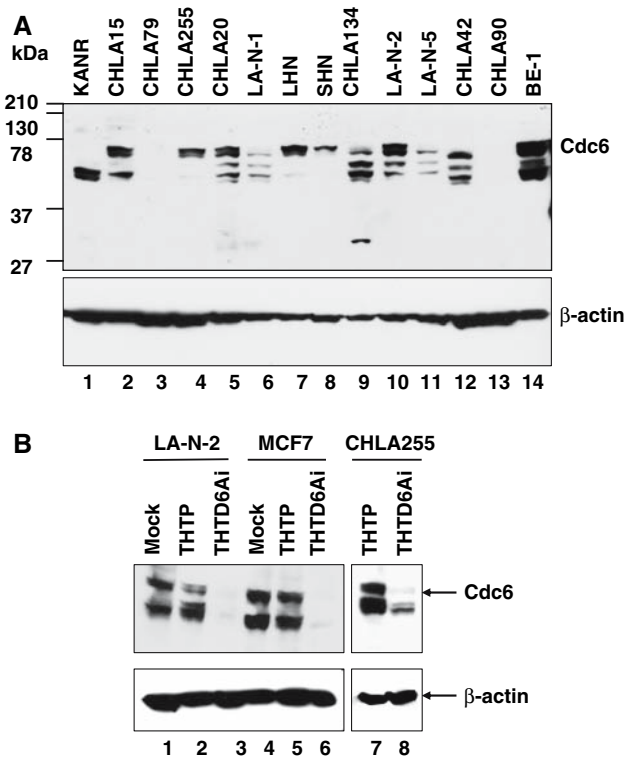

Fig. 1 Cdc6 expression in human neuroblastoma cell lines. (**a**) Nuclear extracts (20 μg) of different tumor cell lines were used in the Western blot, probed with the anti-Cdc6 monoclonal antibody. The blot was also probed with antibody against *β*-actin. (**b**) Inhibition Cdc6 expression with the Cdc6 shRNA virus. About 0.3×10^6 LA-N-2 (lane 1–3), CHLA255 (lane 7 and 8), and human breast carcinoma MCF7 cells (lane 4–6) were infected with the Cdc6 shRNA lentivirus (THTD6Ai), or the control virus for scramble sequence (THTP) at m.o.i. of 1.0. Cell extracts were prepared 48 h post infection and 20 μg of each sample were used in the Western blotting

those with undetectable Cdc6 expression grew very poor (such as KANR) (data not shown). In order to suppress endogenous Cdc6 expression, the fast growing LA-N-2 and CHLA255 cells were chosen and infected with the Cdc6 shRNA lentivirus (THTD6Ai) [23]. The cells were also infected with the lentivirus vector expressing a scramble sequence (THTP). The infection was verified by detecting HIV-1 reverse transcriptase (RT) (data not show). The Cdc6 shRNA virus infection caused *CDC6* mRNA degradation, and the levels of Cdc6 were decreased sharply. The percentages of inhibition of Cdc6 expression were obtained by scanning the Western blot films, and then processed the signals in a densitometry with software of Sigma plot. Two major bands were detected by using monoclonal antibody against Cdc6 (Sigma, Clone DSC-180). Cdc6 is a short half-life protein, i.e., it is synthesized, phosphorylated, and then degraded in each cell-cycle. Presumably, the top ones on the blots were functional (intact and/or phosphorylated) forms, and the lower bands were probably degraded products of Cdc6. Two days post infection, less than 2% Cdc6 could be detected in both LA-N-2 and CHLA255 cells (Fig. 1b). Moreover, levels of Cdc6 in human breast carcinoma MCF7 cells were also decreased with the Cdc6 shRNA virus infection. The knockdown cut the Cdc6 protein levels in MCF7 cells by 98% (Fig. 1b). The control virus infection, however, could not cause Cdc6 decrease (Fig. 1b, THTP); therefore, the Cdc6 shRNA virus specific inhibited endogenous Cdc6 in human cancer cells.

Accumulation of sub-G1 population and increased cell death with Cdc6 knockdown

Since Cdc6 is essential for the S phase entry, inhibiting Cdc6 expression may disturb the cell-cycle progression. The cell-cycle profile of the parental LA-N-2 cells showed some 10% sub-G1 populations, and these tumor cells died constantly in tissue culture (data not shown). The lentivirus infection, whether it was THTD6Ai or THTP, caused a temporary increase of G0/G1 contents, along with the decrease of S phase contents (Fig. 2a). The control virus infection revealed no harm on the cell-cycle progression, though it indeed caused somewhat delay. The Cdc6 shRNA virus infection, however, caused the deleterious effect on the cell-cycle progression. The cell-cycle profile showed a sharp increase of sub-G1 populations by as much as 22% (average 14% in LA-N-2 cells), together with a considerable reduction of S contents (Fig. 2a, b). The accumulation of sub-G1 populations and the reduction of S contents indicated the suppression of cell-cycle progression. It would be followed by programmed cell death. We determined early stage of this result using Annexin V-PE staining. Less than 5% of mock infected and around 10% of the control virus-infected LA-N-2 cells were stained positive. However, 48 hours post Cdc6 shRNA virus infection, the Annexin V-PE positive-staining populations was increased to 31%, indicating the significant cell death (Fig. 2c). Consistent with the above observations, we found the cleavage of Poly (ADP-ribose) polymerase (PARP) from ~116 to 50 kDa or less in Cdc6 depleted LA-N-2 cells (data not shown).

Cdc6 knockdown inhibits p53 expression in tumor cells

The results that Cdc6 knockdown-enhanced tumor cell death would be the activation of p53-mediated apoptosis pathway. We first addressed whether Cdc6 had any effect on p53 expression by determining p53 protein levels in neuroblastoma cells. The p53 was presented in non-stressed LA-N-2 cells (Fig. 3a, lane 1), and was functional for

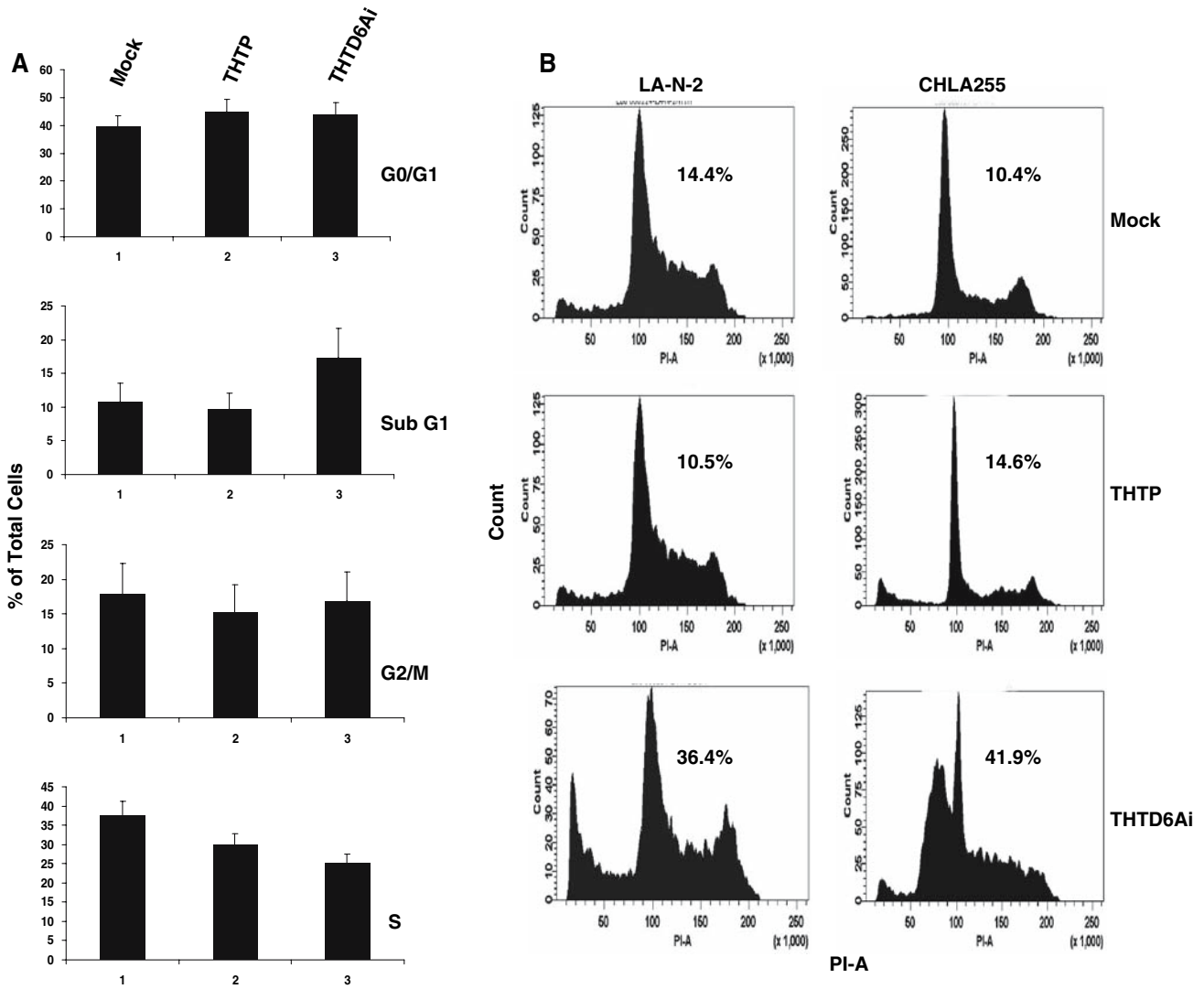

Fig. 2 Determining cell-cycle progression and cell death. (**a**) Comparison of different stages of the cell-cycle. LA-N-2 cells were infected with the Cdc6 shRNA lentivirus (THTD6Ai), or the control virus with scramble sequence (THTP), or mock infected. About 0.5×10^6 cells were harvested 48 hours post infection, and stained with propidium iodide (PI) for flow cytometry analysis. The data presented were the average values from four separated experiments with the standard deviation between 5% and 15%. (**b**) Flow cytometric courses of PI staining LA-N-2 cells and CHLA255 cells. The sub-G1 contents were indicated as percentage number in the panel. (**c**) Annexin V-PE and 7-ADD double staining (right panel). LA-N-2 cells were infected with THTD6Ai, or THTP, or mock infected. The cell was harvested 48 h post infection, and around 0.2×10^6 cells were used for staining. Single Annexin V-PE or 7-AAD staining (left panel) shows as control

transcription activation of its target Bim (see below). We found that Cdc6 knockdown could not stimulate p53 expression, instead p53 was diminished in the LA-N-2 cells (Fig. 3a, lane 3). In contrast, there was no reduction, but a moderate increase of p53 in the control virus infected tumor cells (Fig. 3a, lane 2). These experiments indicated that Cdc6 knockdown caused a downregulation of p53 in LA-N-2 tumor cells.

If Cdc6 functions to regulate p53 expression in tumor cells, overexpression of Cdc6 would cause p53 upregulated. In order to further demonstrate that Cdc6 regulated p53 protein expression, we prepared a retrovirus for expressing Cdc6, which could be induced by doxycycline. The LA-N-2 cells were infected with the Cdc6 retrovirus. With the addition of doxycycline, higher levels of Cdc6 were evident (Fig. 3b). Cdc6 overexpression rendered a boosted p53 expression, particularly in the slow-moving form of p53 (Fig. 3b). The upregulation of p53 was also tested by plasmid DNA transfection in 293T and MCF7 cells. Upon doxycycline induction, Cdc6 was expressed ectopically, and increased p53 was detected in the transfected cells (Fig. 3b, lane 4 and 6). These experiments indicated that Cdc6 specifically regulated the levels of p53 in neuroblastoma cells.

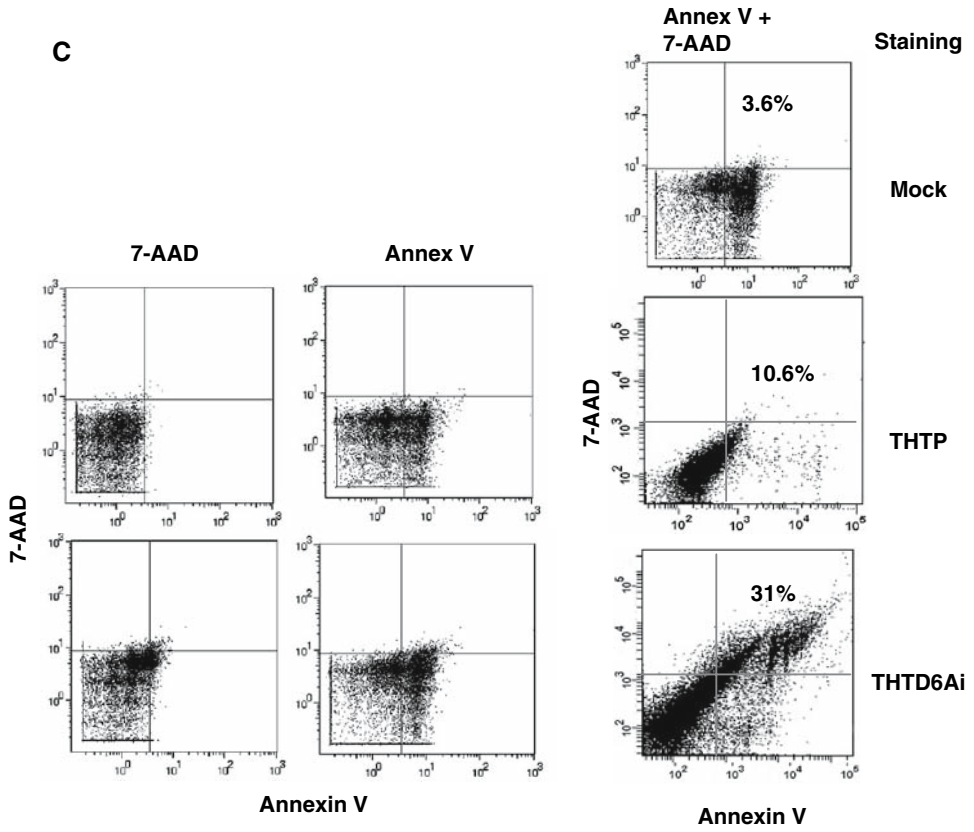

Fig. 2 continued

Since, Bim was a target in p53 mediated transcriptional activation, we tested whether Cdc6 knockdown affected this regulation by determining Bim expression in LA-N-2 cells. In the non-stressed LA-N-2 cells, baseline level of Bim could be easily detected (Fig. 3c, lane 1), and Cdc6 knockdown could not cause any significant change (Fig. 3c, lane 2). The parental LA-N-2 and the Cdc6 knockdown cells were then infected with a p53/HA retrovirus. The doxycycline induction for expressing p53/HA caused a boosted production of Bim in the parental LA-N-2 cells (Fig. 3c, lane3), indicating that p53 was functional in transcriptional activation its target gene in the tumor cells. However, the effect of p53/HA on Bim expression was dimmed in the Cdc6 knockdown cells (Fig. 3c, lane 5). It was unlikely that the detected Bim was from p53/HA transcriptional activation because the same level of Bim was presented in the Cdc6 knockdown cells that were not induced with doxycycline (Fig. 3c, lane 4). Thus, the Cdc6 knockdown not only caused a reduction of p53 expression, but also blocked p53-dependent transcriptional activation.

Real time PCR was performed to determine the abundance of p53 mRNA in the Cdc6 expressing and Cdc6 knockdown LA-N-2 cells. Under non-stressed condition, the copy numbers of p53 mRNA were relatively same with threshold cycle (C_t) value at 26.34 for LA-N-2, 23.58 for LA-N-2/THTP, and 25.59 for LA-N-2/THTD6Ai, though lower than the normal control from human blood cells (C_t value: 22.62). There was no significant difference of p53 mRNA abundance among these cells. This experiment indicated that Cdc6 regulated p53 expression not at transcriptional level; presumably, at posttranscriptional level.

Selected Gene expression profile in Cdc6 knockdown tumor cells points to the suppression of cell proliferation

The decreased S phase contents and the accumulation of sub-G1 populations by Cdc6 knockdown indicated the inhibition of tumor cell-cycle progression. To detail this cell event, we first investigated the variation of levels of cell-cycle regulators. LA-N-2 cells expressed elevated levels of cyclin A and cyclin E, which were required for the activation cdk2 kinase, and for the cell proliferation. Infection of the tumor cells with the control virus did not make any significant changes in cyclin A or cyclin E expression. Infection with the Cdc6 shRNA virus, however, caused the sharp reduction of cyclin E and cyclin A (Fig. 4a). Moreover, Cdc25C protein level was also decreased upon Cdc6 knockdown (Fig. 4a). Cdc25C or

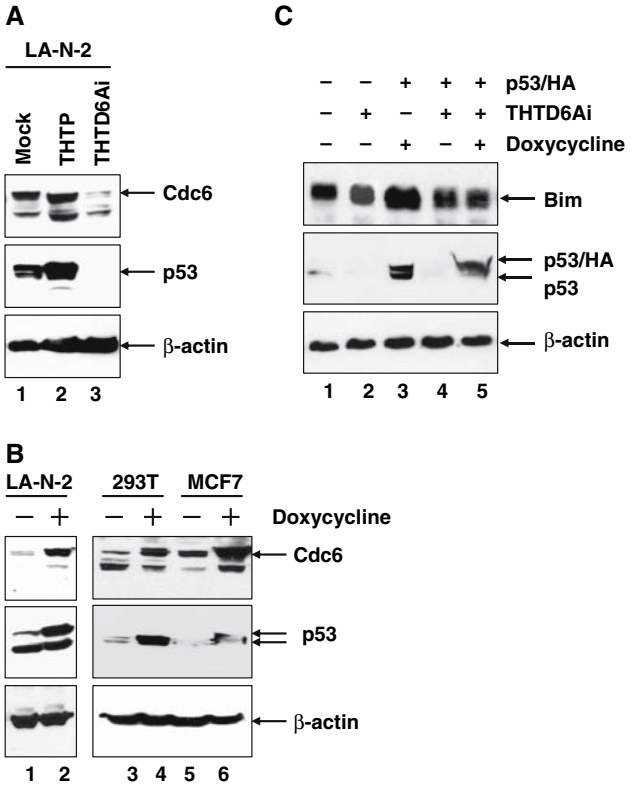

Fig. 3 Cdc6 controls p53 expression. (**a**) About 20 μg of nuclear extracts (for probing Cdc6) or cytoplasmic extracts (for probing p53) prepared from the Cdc6 knockdown cells or from control cells were used in the Western blotting. (**b**) LA-N-2 cells were infected with a Cdc6 inducible retrovirus, while 293T and MCF7 cells were transfected with the Cdc6 retrovirus plasmid DNA. Two days after infection or transfection, doxycycline was added to a final concentration of 1 μg/ml for another 2 days. The cell extracts were prepared and used in the Western blotting. (**c**) LA-N-2 cells were first infected with the Cdc6 shRNA to knockdown endogenous Cdc6, the cell was then infected with a retrovirus for expressing doxycycline-inducible p53/HA. After doxycycline induction, the cell extracts were prepared. Cell extracts were also prepared from different control cultures. About 20 μg of the cytoplasmic extracts from each sample were used in the Western blotting

Cdc25B activated cyclin A/cdk2 or cyclin B1/cdk1 by dephosphorylation of the CDKs for G2/M phase entry [24, 25]. The Cdc6 knockdown, therefore, inhibited the cell-cycle progression by suppressing the expression of cell-cycle-promoting cyclins and regulators.

On the other hand, the CDK inhibitor $p27^{Kip1}$ or $p21^{Cip1}$ could be more stable as the decreased cyclin E and cyclin A weakened the phosphorylation of these CDK inhibitors, as well as pRB [26]. If this is true, the levels of $p27^{Kip1}$ or $p21^{Cip1}$ will be higher due to the accumulation of CDK inhibitors. We determined the levels of $p27^{Kip1}$ or $p21^{Cip1}$ in the Cdc6 knockdown tumor cells. Baseline level of $p27^{Kip1}$ was presented in LA-N-2 cells, and infection with the control virus had no effect on the protein level. However, $p27^{Kip1}$ was markedly increased 48 h post infection with the Cdc6 shRNA virus infection (Fig. 4b). In contrast to $p27^{Kip1}$, relatively high levels of $p21^{Cip1}$ were presented in the LA-N-2 cells. Infection with the control virus caused a significant $p21^{Cip1}$ decrease. However, infection with the Cdc6 shRNA virus could not augment further for $p21^{Cip1}$ reduction (Fig. 4b). These results indicated that Cdc6 knockdown resulted in the accumulation of $p27^{Kip1}$, but not $p21^{Cip1}$ in LA-N-2 cells. The level of $p21^{Cip1}$ was unexpectedly reduced, indicating that the S phase inhibition upon Cdc6 knockdown was independent on the $p21^{Cip1}$ activation.

Though it caused the p53 downregulation, the Cdc6 knockdown enhanced the death of tumor cells. In order to understand the cause of increased cell death, the tumor cells were analyzed for the alteration of different kinds of

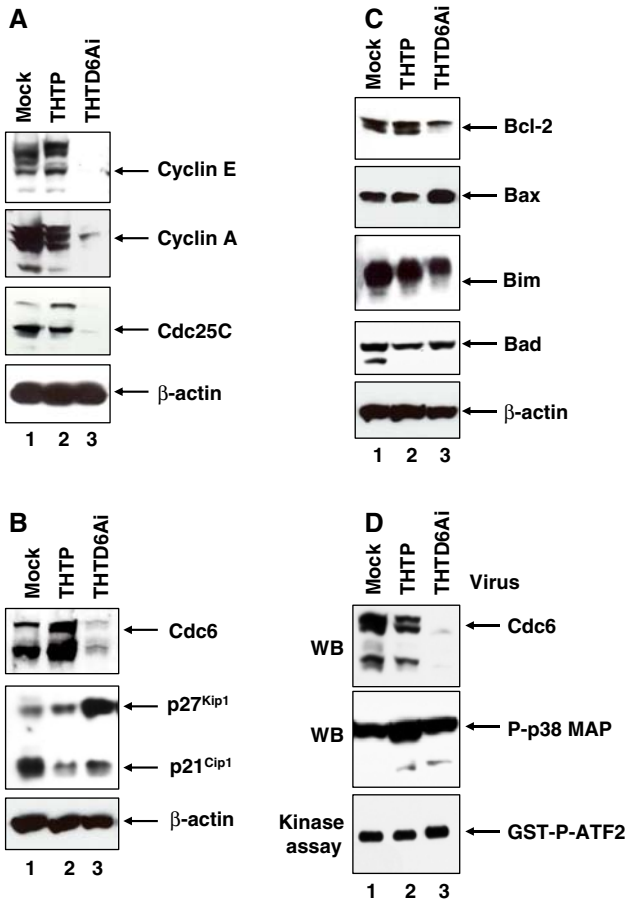

Fig. 4 Expression profiles in the Cdc6 knockdown cells. (**a**–**c**) About 20 μg of cytoplasmic extracts from each sample were used in the Western blot, probed with the indicated protein antibodies. The signal on the blots were scanned and quantitated with a densitometry. (**d**) About 200 μg of total cell extracts were immunoprecipitated with the anti-phospho-p38 MAP monoclonal antibody. The immunoprecipitates on the beads were used in the kinase reaction assay using the recombinant GST-ATF2 as substrate. The kinase activity was determined in Western blotting using the monoclonal antibody against the phosphorylated ATF2

anti-apoptotic and pro-apoptotic Bcl-2 family proteins. While Cdc6 knockdown caused downregulation of Bcl-2, increased levels of Bax were evident, though it had no effect on Bim and Bad (Fig. 4c). Neither Bcl-2, nor Bim/Bad, nor Bax did show a clear change as the cell was infected with the control virus (Fig. 4c). These experiments ruled out the involvement of HIV-1 viral proteins in the increased cell death. Therefore, Cdc6 knockdown caused the increased cell death by activating pro-apoptotic Bcl-2 members, while inhibiting the anti-apoptotic ones.

Bim function could be restrained due to the phosphorylation of Bim by extracellular signal-regulated kinase (Erk), and the phosphorylated Bim was incapable of associating with Bax/Bak [18]. In all of our experiments, no phosphorylation of Erk were observed in both the Cdc6 knockdown cells and in the control cells (data not shown). It was unlikely that Bim was activated by the suppression of Erk phosphorylation. Moreover, phosphorylated p38 MAP kinase was indeed detected in non-stressed LA-N-2 cells, and there was no significant change of p38 MAP phosphorylation upon the Cdc6 shRNA lentivirus or the control virus infection (Fig. 4d). These results ruled out the involvement of p38 MAP kinase in activation of pro-apoptotic Bcl-2 members.

Discussion

In this study we have found that Cdc6 controls cell proliferation and death in human neuroblastoma cells. Inhibition of Cdc6 expression via the Cdc6 shRNA virus infection suppresses tumor cell proliferation. We have demonstrated that Cdc6 knockdown causes the accumulation of sub-G1 populations with the decreased S contents. We have also shown that the selected gene expression profile in the tumor cell with Cdc6 knockdown is highlighted with the decreased levels of proteins for proliferation, while the increased levels of those for anti-proliferation and cell death. Also, we have found that Cdc6 alters p53 expression. Cdc6 knockdown causes a sharp reduction of p53, and p53-mediated Bim expression is inhibited as well. It is interesting to note that the fate of tumor cells with Cdc6 knockdown resembles the process of p53-mediated tumor suppression. As the function of p53 has been lost or inactivated in most of human cancers, suppression of Cdc6 could be an alternative approach to inhibit cancer cell proliferation. Our results show that the diminution of p53 upon Cdc6 knockdown suppresses cell proliferation accompanied with the increased cell death, therefore, reducing the malignacy of the tumor cells. At present time we do not have any evidence that p53 is a wild-type version in the LA-N-2 cells, though it is functional to activate Bim. Since this version of p53 has not been able to inhibit malignant development, it would be no harm to remove it from the cell.

The suppression of cancer cell proliferation by Cdc6 knockdown can be validated from three lines of our experiments. First, the decreased levels of cyclin E and cyclin A cause the cancer cell in replication stress. Overexpression of cyclin E, and cyclin A, and other cyclins is a common feature in human cancer cells, and most human cancer cells rely heavily on cyclin E or cyclin A activities to promote S phase progression [20, 27]. High level of cyclin E predicts a poor prognosis in many breast cancer patients [28, 29]. Therefore, downregulation of cyclin E and cyclin A upon Cdc6 knockdown would benefit the cancer treatment. Second, the accumulation of $p27^{Kip1}$ contributes the suppression of cancer cell proliferation. Since cyclin E and cyclin A are decreased, the phosphorylation of $p27^{Kip1}$ by cyclin E-cdk2 or cyclin A-cdk2 is inhibited, which blocks $p27^{Kip1}$ degradation [26]. The accumulation of $p27^{Kip1}$ may not be the direct effect of Cdc6 knockdown, it is more likely through tumor-suppressor-retinoblastoma protein pRB [30]. pRB binds amino-terminal end of S-phase kinase-associated protein 2 (Skp2), this helps to release $p27^{Kip1}$ from the interaction with Skp2 [31]. Skp2 is a major E3 ligase for $p27^{Kip1}$ ubiquitination. It is possible that Cdc6 knockdown causes the downregulation of Skp2, weakening the Skp2 mediated proteolysis of $p27^{Kip1}$. Support for this prediction, a recent report has found that inhibition of Skp2 expression blocks the ubiquitination-mediated proteolysis of $p27^{Kip1}$, leading to the accumulation of the CDK inhibitor [32]. Moreover, inhibition of Skp2 expression causes a significant accumulation of sub-G1 populations accompanied with the reduction of cyclin E and the increased apoptosis of human lung carcinoma cells [32]. Third, the activation of pro-apoptotic Bcl-2 members switches on the death machine in the cancer cells. The downregulation of anti-apoptotic Bcl-2 and the upregulation of pro-apoptotic Bax highlight the impact of Cdc6 knockdown on the life of cancer cells. The lack of p53 due to the Cdc6 knockdown could add some roadblock on cell death pathway since p53 plays a critical role in the activation of pro-apoptotic Bim and Bax, and some other Bcl-2 members, such as p53-upregulated modifier of apoptosis (PUMA) [12–14]. However, the loss of p53 is compensated by the reduction of Bcl-2 and increase of Bax, that activates the MOMP. Although the level of Bim is unchanged upon Cdc6 knockdown, the reduction of Bcl-2 breaks the balance and allows Bim to activate its downstream apoptotic effectors on the mitochondrial membrane. The activation of this mitochondrial pathway will ultimately bring the cancer cell to die [33]. Further studies regarding the Cdc6 knockdown and its biological effect would benefit us very much in understating human Cdc6 function and cancer suppression.

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References

1. Greenlee RT, Murray T, Bolden S, Wingo PA (2000) Cancer statistics, 2000. CA Cancer J Clin 50:7–33
2. Mailand N, Diffley JF (2005) CDKs promote DNA replication origin licensing in human cells by protecting Cdc6 from APC/C-dependent proteolysis. Cell 122:915–926
3. Randell JC, Bowers JL, Rodriguez HK, Bell SP (2006) Sequential ATP hydrolysis by Cdc6 and ORC directs loading of the Mcm2-7 helicase. Mol Cell 21:29–39
4. Takeda DY, Shibata Y, Shibata Y, Parvin JD, Dutta A (2005) Recruitment of ORC or CDC6 to DNA is sufficient to create an artificial origin of replication in mammalian cells. Genes Dev 19:2827–2836
5. Petersen BO, Wagener C, Marinoni F, Kramer ER, Melixetian M, Denchi EL, Gieffers C, Matteucci C, Peters JM, Helin K (2000) Cell cycle- and cell growth-regulated proteolysis of mammalian CDC6 is dependent on APC-CDH1. Genes Dev 14:2330–2343
6. Jiang W, Wells NJ, Hunter T (1999) Multistep regulation of DNA replication by Cdk phosphorylation of HsCdc6. Proc Natl Acad Sci USA 96:6193–6198
7. Clay-Farrace L, Pelizon C, Santamaria D, Pines J, Laskey RA (2003) Human replication protein Cdc6 prevents mitosis through a checkpoint mechanism that implicates Chk1. EMBO J 22: 704–712
8. Gonzalez S, Klatt P, Delgado S, Conde E, Lopez-Rios F, Sanchez-Cespedes M, Mendez J, Antequera F, Serrano M (2006) Oncogenic activity of Cdc6 through repression of the INK4/ARF locus. Nature 440:702–706
9. Vaziri C, Saxena S, Jeon Y, Lee C, Murata K, Machida Y, Wagle N, Hwang DS, Dutta A (2003) A p53-dependent checkpoint pathway prevents rereplication. Mol Cell 11:997–1008
10. Duursma A, Agami R (2005) p53-dependent regulation of Cdc6 protein stability controls cellular proliferation. Mol Cell Biol 25:6937–6947
11. Johnstone RW, Ruefli AA, Lowe SW (2002) Apoptosis: a link between cancer genetics and chemotherapy. Cell 108:153–164
12. Chipuk JE, Kuwana T, Bouchier-Hayes L, Droin NM, Newmeyer DD, Schuler M, Green DR (2004) Direct activation of Bax by p53 mediates mitochondrial membrane permeabilization and apoptosis. Science 303:1010–1014
13. Chipuk JE, Bouchier-Hayes L, Kuwana T, Newmeyer DD, Green DR (2005) PUMA couples the nuclear and cytoplasmic proapoptotic function of p53. Science 309:1732–1735
14. Mihara M, Erster S, Zaika A, Petrenko O, Chittenden T, Pancoska P, Moll UM (2003) p53 has a direct apoptogenic role at the mitochondria. Mol Cell 11:577–590
15. Adams JM, Cory S (1998) The Bcl-2 protein family: arbiters of cell survival. Science 281:1322–1326
16. Huang DC, Strasser A (2000) BH3-only proteins-essential initiators of apoptotic cell death. Cell 103:839–842
17. Cartron PF, Gallenne T, Bougras G, Gautier F, Manero F, Vusio P, Meflah K, Vallette FM, Juin P (2004) The first alpha helix of Bax plays a necessary role in its ligand-induced activation by the BH3-only proteins Bid and PUMA. Mol Cell 16:807–818
18. Harada H, Quearry B, Ruiz-Vela A, Korsmeyer SJ (2004) Survival factor-induced extracellular signal-regulated kinase phosphorylates BIM, inhibiting its association with BAX and proapoptotic activity. Proc Natl Acad Sci USA 101:15313–15317
19. Vogelstein B, Lane D, Levine AJ (2000) Surfing the p53 network. Nature 408:307–310
20. Bartkova J, Horejsi Z, Koed K, Kramer A, Tort F, Zieger K, Guldberg P, Sehested M, Nesland JM, Lukas C, Orntoft T, Lukas J, Bartek J (2001) DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis. Nature 434:864–870
21. Gorgoulis VG, Vassiliou LV, Karakaidos P, Zacharatos P, Kotsinas A, Liloglou T, Venere M, Ditullio RA Jr, Kastrinakis NG, Levy B, Kletsas D, Yoneta A, Herlyn M, Kittas C, Halazonetis TD (2005) Activation of the DNA damage checkpoint and genomic instability in human precancerous lesions. Nature 434:907–913
22. Keshelava N, Zuo JJ, Chen P, Waidyaratne SN, Luna MC, Gomer CJ, Triche TJ, Reynolds CP (2001) Loss of p53 function confers high-level multidrug resistance in neuroblastoma cell lines. Cancer Res 61:6185–6193
23. Feng L, Yee JK, Huang SH, Wu LT, Jong AY (2006) Down-regulation of human Cdc6 protein using a lentivirus RNA interference expression vector. Methods Mol Biol 342:287–293
24. Millar JB, Blevitt J, Gerace L, Sadhu K, Featherstone C, Russell P (1991) p55CDC25 is a nuclear protein required for the initiation of mitosis in human cells. Proc Natl Acad Sci USA 88:10500–10504
25. Sadhu K, Reed SI, Richardson H, Russell P (1990) Human homolog of fission yeast cdc25 mitotic inducer is predominantly expressed in G2. Proc Natl Acad Sci USA 87:5139–5143
26. Sherr CJ, Roberts JM (1999) CDK inhibitors: positive and negative regulators of G1-phase progression. Genes Dev 13:1501–1512
27. Hunter T, Pines J (1991) Cyclins and cancer. Cell 66:1071–1074
28. Keyomarsi K, Herliczek TW (1997) The role of cyclin E in cell proliferation, development and cancer. Prog Cell Cycle Res 3:171–191
29. Porter PL, Malone KE, Heagerty PJ, Alexander GM, Gatti LA, Firpo EJ, Daling JR, Roberts JM (1997) Expression of cell-cycle regulators p27Kip1 and cyclin E, alone and in combination, correlate with survival in young breast cancer patients. Nat Med 3:222–225
30. Weinberg RA (1995) The retinoblastoma protein and cell cycle control. Cell 81:323–330
31. Ji P, Jiang H, Rekhtman K, Bloom J, Ichetovkin M, Pagano M, Zhu L (2004) An Rb-Skp2-p27 pathway mediates acute cell cycle inhibition by Rb and is retained in a partial-penetrance Rb mutant. Mol Cell 16:47–58
32. Jiang F, Caraway NP, Li R, Katz RL (2005) RNA silencing of S-phase kinase-interacting protein 2 inhibits proliferation and centrosome amplification in lung cancer cells. Oncogene 24:3409–3418
33. Spierings D, McStay G, Saleh M, Bender C, Chipuk J, Maurer U, Green DR (2005) Connected to death: the (unexpurgated) mitochondrial pathway of apoptosis. Science 310:66–67