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Aberrant expression of Cks1 and Cks2 contributes to prostate tumorigenesis by promoting proliferation and inhibiting programmed cell death

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Summary

The mammalian Cks family consists of two well-conserved small proteins, Cks1 and Cks2. Cks1 has been shown to promote cell cycle progression by triggering degradation of $p27^{kip1}$. The function of Cks2 in somatic mammalian cells is not well understood although it is required for the first metaphase/anaphase transition during the meiosis. Emerging evidence shows that elevated expression of Cks1 and Cks2 is often found in a variety of tumors, and is correlated with poor survival rate of the patients. Here we demonstrated that expression of Cks1 and Cks2 were elevated in prostate tumors of human and animal models, as well as prostatic cancer cell lines. Forced expression of Cks1 and Cks2 in benign prostate tumor epithelial cells promoted cell population growth. Knockdown of Cks1 expression in malignant prostate tumor cells inhibited proliferation, anchorage-independent growth, and migration activities, whereas knockdown of Cks2 expression induced programmed cell death and inhibited the tumorigenicity. Collectively, the data suggest that elevated expression of Cks1 contributes to the tumorigenicity of prostate tumor cells by promoting cell growth and elevated expression of Cks2 protects the cells from apoptosis. Thus, the finding suggests a novel therapeutic strategy for prostatic cancer based on inhibiting Cks1 and Cks2 activity.

Introduction

The human Cks (also designated as CksHs) family consists of two well-conserved members, Cks1 and Cks2, both of which are identified based on the sequence homology to yeast suc1 and Cks1 (Cdc28 kinase subunit 1) that are essential for cell cycle control [1-3]. Emerging evidence shows that the two Cks members in mammalian cells have distinct regulatory function from the yeast counterparts. Cks1 is required for SCF^{Skp2}-mediated ubiquitination and degradation of $p27^{kip1}$, which is essential for the G1/S transition during the cell cycle [4, 5]. Although function of Cks2 in the cell cycle is not clear, expression of both Cks1 and Cks2 has been shown oscillates during the cell cycle and is positively related to cell proliferation [6]. Recently, Cks2 has been shown essential for the first metaphase/anaphase transition of mammalian meiosis [7].

Numerous reports demonstrate that expression of Cks2 is frequently elevated in tumors of different tissue origins, including nasopharyngeal carcinoma, melanocytic tumors, Wilms

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tumor, breast, bladder, cervical, esophageal, lymphoid, and metastatic colon cancer 6, 8-14. In addition, expression of Cks2 is downregulated by p53, a tumor suppressor, at the transcription and the protein levels 15. Similarly, elevated expression of Cks1 has been found in tumors from a variety of tissue origins, and is correlated with poor survival rate of oral squamous cell carcinoma 16-23. Knockdown of Cks1 inhibits growth and tumorigenicity of oral squamous cells 23. Consistent with the finding that Cks1 is a negative regulator of a cell cycle control protein, $p27^{kip1}$, elevated expression of Cks1 is found coincident with the reduction of $p27^{kip1}$ proteins in tumor cells. Expression of $p27^{kip1}$ is often aberrantly reduced in cancer cells, including prostate cancer cells. We recently reported that the fibroblast growth factor (FGF) signaling axis directly regulates activity of Cks1 during the G1/S transition in the cell cycle through FGF receptor substrate 2α (FRS2α), a proximal FGF receptor-interactive adaptor protein of the FGF receptor tyrosine kinase, which connects multiple downstream signaling molecules to the FGF receptor tyrosine kinase 24.

The prostate is an accessory organ of the male reproductive system, which consists of epithelial and stromal compartments separated by basement membranes. Cancers arising from the prostate epithelium are the most commonly diagnosed cancer and the second most common cause of cancer death in American males. In America alone, about 230 thousand new cases and 30 thousand deaths are expected every year. To date, whether the Cks family is overexpressed in prostate tumor, and if yes, whether aberrantly expressed Cks contributes to prostate tumor initiation and progression remain to be characterized. Recently, it has been shown that Cks1 expression is associated with the aggressive behavior of prostate cancer 25. In addition, treating LNCaP prostate cancer cells with an herbal mixture, PC-SPES, inhibits cell proliferation and reduces expression of Cks2 26.

To determine whether overexpression of the Cks family was associated with prostate tumorigenesis, we analyzed the expression pattern of Cks1 and Cks2 in the Dunning prostate tumor model of rats, the TRAMP prostate cancer model of mice, human prostate cancer cell lines, and human prostate cancer samples. The results showed that in comparison with normal prostate tissues that only weakly express Cks1 and Cks2, all tested human, rat, and mouse prostate tumor tissues and cells exhibited elevated expression of Cks1 and Cks2. Forced expression of Cks1 and Cks2 by transfection in benign prostate tumor cells somewhat promoted cell population increases. Consistently, knockdown of Cks1 and Cks2 expression by shRNA in malignant AT3 cells inhibited cell population growth. In addition, knockdown of Cks1 expression also inhibited anchorage-independent growth, and migration activities, and knockdown of Cks2 expression induced the cells to undergo massive program cell death, especially when the cells were maintained in suboptimal growth condition. The results suggested that elevated expressed Cks1 may contribute to prostate tumorigenesis by promoting proliferation, anchorage-independent growth, and migration of the cells, and Cks2 by protecting cells from undergoing programmed cell death. The finding also provides new hints for cancer therapeutic strategies based on inhibition of the Cks activity.

Experimental Procedures

Animals

All animals were housed in the Program of Animal Resources at the Institute of Biosciences and Technology and were handled in accordance with the principles and procedures of the *Guide for the Care and Use of Laboratory Animals*. All experimental procedures were approved by the Institutional Animal Care and Use Committee. Genotypes of the mice were determined by PCR analyses as described 27, 28. For tumorigenesis, 1×10^6 cells were implanted subcutaneously in 0.1 ml MEM medium. The mice were observed carefully, and the palpable tumors were recorded daily. Tumors were harvested and histologically

characterized as described 27, 28. Tissues were fixed with 4% paraformaldehyde paraffin embedded. In situ hybridization was carried out with paraffin-embedded sections as described 29.

cDNA constructions

Full-length cDNAs for mouse Cks1 and Cks2 were RT-PCR amplified from mouse embryo RNA pools with primers mcks1-u: CTCCTGCAGAGCGATCATGTCGCACAAACA and mcks1-d: CTTCTGCAGCTTCATTTCTTTGGTTTCTTG, and mcks2-u: TTTCTGCAGCGCGCCGGCAGGATGGCC and mcks2-d: AGATAAGCTTCATTTTTGTTGTTCTTTTGG, respectively. The cDNAs were cloned into the pBluescript SK vector for sequence verification and pEGFP-N3 vector for expression in mammalian cells 24.

The cDNA for rat Cks1-shRNA was constructed by annealing the primers pRcks1-t: GATCCGTCAGAAGCATGTGAAATCTTCAAGAGAGATTTCACATGCTTCTGACTTTTTTACGCGTG and pRcks1-b: AATTCACGCGTAAAAAAGTCAGAAGCATGTGAAATCTCTCTTGAAGATTTCACATGCTTCTGACG; rat Cks2-shRNA by pRcks2-t: GATCCGCCAGAACCGCATATTCTTTTCAAGAGAAAGAATATGCGGTTCTGGCTTTTTTACGCGTG and pRcks2-b: AATTCACGCGTAAAAAAGCCAGAACCGCATATTCTTTCTCTTGAAAAGAATATGCGGTTCTGGCG; mouse Cks1 shRNA by pMcks1-t: GATCCGGTCCACTATATGATCCATTTCAAGAGAATGGATCATATAGTGGACCTTTTTTACGCGTG and pMcks1-b: AATTCACGCGTAAAAAAGGTCCACTATATGATCCATTCTCTTGAAATGGATCATATAGTGGACCG and mouse Cks2 shRNA by pMcks2-t: GATCCGTCCAACAGAGTCTAGGATTTCAAGAGAATCCTAGACTCTGTTGGACTTTTTTACGCGTG, and pMcks2-b: AATTCACGCGTAAAAAAGTCCAACAGAGTCTAGGATTCTCTTGAAATCCTAGACTCTGTTGGACG. The annealed oligonucleotides were cloned to the pSIREN-RetroQ-Tet vectors (Clontech, Mountain View, CA) and the sequences were verified.

Gene expression analyses

Expression of Cks1 and Cks2 was analyzed by RT-PCR and RNase protection assay (RPA). Total RNA was extracted from tissues or cells with RNAzol (Ambion, San Antonio, TX). Reverse transcriptions were carried out with 1 μg RNA and 2.5 μg random hexamers with SuperScript reverse transcription kit (Invitrogen, Carlsbad, CA). PCR was carried out for Cks1 and Cks2 with the indicated primers for 30 cycles at 94 °C, 55 °C, and 72 °C for 1 min each using the Taq DNA Polymerase (Promega, Madison, WI). Primers for rat Cks1 are rcks1-u: CTGGATCCGCAAATTCACACCATCCCTG and rcks1-d: GTGATATCCTGATAGCGTGACCGTGTTG, and rat Cks2 are rcks2-u: CGTTTCCTTGTCCCGGTTT and rcks2-d: TATGCGGTTCTGGCTCATGG, respectively. Primers for human Cks1 are hcks1-u: CTCCTGCAGAGCGATCATGTCGCACAAACA and hcks1-d: CTTCTGCAGCTTCATTTCTTTGGTTTCTTG. Primers for human Cks2 are hcks2-u: TTTCTGCAGCGCGCCGGCAGGATGGCC and hcks2-d: AGATAAGCTTCATTTTTGTTGTTCTTTTGG. The RT-PCR products were verified in sizes on a 2% agarose gel. For RPA, the RNA probe was transcribed and radiolabeled with α-[^{32}P]UTP from the Cks1 and Cks2 cDNA templates with the Maxiscript Kit (Ambion, San Antonio, TX) 30. The labeled antisense RNA probe was hybridized with 25 μg of total RNA at 68 °C for 11 minutes followed by incubation with RNase A1/T1 at 37 °C for 30 minutes. Protected fragments were separated on 5 percent polyacrylamide sequencing gels and the protected fragments were visualized by autoradiography.

Cell culture

The cells were maintained in RD media (50% RPMI, 50% DMEM) containing 5% fetal bovine serum (FBS). For proliferation assays, 10^3 cells per well were inoculated to 24-well plates containing 1 ml of RD medium with 5%, or indicated concentrations of FBS. The cell numbers were counted daily. For soft agar growth assays, 1000 cells per well were mixed with 0.6% top agar at a ratio of 1:1 and inoculated to 6-well plates containing 1% base agar. The final serum concentration in the top and base agar was 5%. The cells were cultured for 3-4 weeks until the colonies were visible. The colonies were stained with Crystal Purple for observation and quantification. Data are mean and standard deviation of three separate samples.

To establish stable cell lines expressing the tetracycline-controlled transcriptional suppressor (tTS), cells (5×10^5) were transfected with the ptTS-Neo Vector (Clontech, Mountain View, CA) and selected for G418 resistant. The healthy colonies with the highest tetracycline inducible expression of the pSIREN-RetroQ-Tet-Luc reporter were picked for further transfection with pSIREN-RetroQ-Tet-Cks1/Cks2-shRNA plasmids. After being selectively cultured in hygromycin (200 μg/ml) media, the colonies were screening for the best knockdown efficiency induced by doxycycline at a concentration of μg/ml. RT-PCR analyses were employed for assessing the knockdown efficiency. For overexpression analyses, the stable transfectants were pooled to avoid clonal deviations. RT-PCR analyses were used to confirm expression of Cks1 and Cks2.

Cell migration assay

Prior to the experiments, the cells were cultured in the presence doxycycline for over 48 hours to induce expression of shRNAs where indicated. The cells (2.5×10^4) in 0.5 ml of serum-free RD medium were inoculated to gelatin-coated 6.5 mm Millicell inserts of 8 μm pore size (COSTA Corning NY) placed in 12-well culture plates, which contain 10% FBS RD medium with or without doxycycline. After incubation at 37 °C for 12 hours, the cells migrated to the outer surface of the membrane were fixed, stained with hematoxylin, and counted under a microscope.

Apoptosis analysis

Cells (3×10^5) cultured in 6-well dishes in 5% FBS/RD medium were treated with doxycycline for the indicated days to induce shRNA expression. The cells were then stained with the APOPercentage™ dye (Biocolor Ltd, Newtonabbey, Northern Ireland) for 30 minutes. The stained cells were visualized by microscopy, and the positive cells were quantified. For Annexin V staining, the cells ($1\text{-}5 \times10^5$) were harvested 48 hours after the transfection and suspended in 500 μl binding buffer containing 1% Annexin V-Cy3. The cells were replated on 6-well dishes and the Annexin V-Cy3 stained cells were observed under a fluorescent microscope.

Western blotting

The cells (2×10^5) in 6-well plates were cultured in 5% FBS for 24 hours and the expression of shRNA was induced for 48 hours. The cells were then lysed with 0.5% Triton-PBS. Aliquots containing 10 μg of protein were subjected to SDS-polyacrylamide gel electrophoreses (SDS-PAGE) on 3 gels, and were electroblotted to nylon membranes for Western blot analyses with the indicated antibodies. For Akt and Bcl-xl staining, the membrane was cut into two pieces for staining with anti-Akt and anti-Bcl-xl antibodies individually. The other 2 membranes were stained with anti-Bad and anti-β-actin, respectively. The experiments were repeated for 3 times and the representative results were showed. The specifically bound antibodies were detected with the ECL-plus

chemiluminescent detection reagents. Anti-$p27^{kip1}$, anti-Bcl-xl, anti-Bad, and anti-β-actin antibodies was purchased from Santa Cruz Biotechnology Inc., (Santa Cruz, CA).

Results

Expression of Cks1 and Cks2 is elevated in prostate cancer

To determine whether expression of Cks1 and Cks2 was elevated in prostate tumors, we employed the RPA to quantitatively analyze expression of Cks1 and Cks2 mRNA in rat prostates and the Dunning3327 (DT3) rat prostate tumors, which consist of androgen-dependent benign DT tumors and androgen-independent malignant AT tumors [31, 32]. The results showed that Cks1 was weakly expressed in normal rat prostates and the benign DT3 tumors; the expression was apparently stronger in the malignant AT tumors than in normal prostates and benign prostate tumors (Fig. 1Aa). The Cks2 expression in normal rat prostates was below the detection limit (Fig.1Aa). Strong Cks2 expression was evident in benign DT3 and malignant AT tumors, as well as in cell lines derived from both tumors, including the DTE epithelial and DTS stromal cells derived from benign DT tumors, and AT3 cells from the malignant AT tumors (Fig. 1Aa). To determine whether Cks1 and Cks2 were overexpressed in other prostate tumors, RT-PCR (30 cycles) was employed to assess Cks1 and Cks2 expression in the TRAMP autochthonous mouse prostate tumor model [28]. Expression of Cks1 and Cks2 was apparent in TRAMP tumor tissues and the C2 cells derived from TRAMP tumors. Under the same condition, Cks expression was undetectable in adult mouse prostates (Fig. 1Ab).

To investigate whether aberrant expression of Cks1 and Cks2 was associated with human prostate cancers, RT-PCR analyses were employed to assess the expression of Cks1 and Cks2 in human prostate cell lines, including nontumorigenic prostate epithelial cell line PNTIA, low tumorigenic LNCaP, and highly tumorigenic DU-145 and PC3 cells (Fig 1Ac). The results showed that expression of Cks1 and Cks2 mRNA was evident in all tested cell lines, including the T antigen-immortalized human prostate epithelial cells isolated from the normal prostate tissues adjacent to prostate tumors.

Furthermore, real-time RT-PCR analyses of cDNAs prepared from human prostate cancer and its peripheral normal tissues showed that a significant number of the tested tumors expressed Cks2 at a higher level than did the controls. Overall, relative to normal peripheral tissues, elevated expression of Cks2 was apparent in the tumor tissues ($p<0.01$), whereas the difference in Cks1 expression was not statistically significant between the tumor and normal peripheral zone tissues (Fig. 1B and data not shown). In situ hybridization also demonstrated that expression of Cks2 was evident in lesion foci with high-grade prostate intraepithelial neoplasia (PIN) in human prostate (Fig. 1Ca), whereas no Cks2 expression was detectable in the epithelium with normal tissue histology (Fig. 1Cb). To further investigate whether aberrant expression of Cks was associated with prostate tumorigenesis, prostate tissues were harvested from TRAMP mice and wildtype littermates at the age of 4-8 weeks. Tissue sections with relatively normal tissue structures, low to medium grade PIN, high grade PIN, and adenocarcinomas were selected for in situ hybridization analyses (Fig. 1D). The results confirmed that Cks1 and Cks2 expression in normal prostate was under the detection limit (panels a,b). The expression of Cks was also very low in foci with low grade PIN lesions (data not shown), whereas the expression was apparent in tissues with lesions of high grade PIN (panels c and d, arrows), and adenocarcinomas (panels e and f, arrows).

Knockdown of Cks1 and Cks2 expression inhibits population growth of prostate cancer cells

To study the role of aberrantly expressed Cks1 and Cks2 in prostatic cancer cells, expression of Cks1 and Cks2 were knocked down in malignant AT3 cells by stably transfected with cDNAs encoding Cks1- or Cks2-shRNA cloned in a doxycycline-inducible vector. Real-time RT-PCR analyses revealed that expression of Cks1 and Cks2 in the cells was reduced to about 25% and 35% of the original levels in the presence of doxycycline inducers. To determine whether knockdown of Cks1 or Cks2 expression inhibited cell growth, population curves of the cells cultured in normal media containing 5% FBS were measured with and without the induction of Cks1- and Cks2-shRNA expression. The results showed that Cks1 knockdown (Fig. 2A) reduced the population growth rate of the cells. Under the same condition, doxycycline treatment did not affect the growth rate of cells expressing stochastic shRNA and Cks2 shRNA (Fig. 2A and data not shown). Yet, when the cells were cultured in suboptimal media, including low serum concentration (data not shown) or containing 50% medium conditioned by AT3 cells (Fig. 2A), the population doubling rate was apparently reduced by expression of Cks2-shRNA. Western analyses demonstrated that the protein abundance of $p27^{kip1}$ was elevated in Cks1, but not Cks2, knockdown cells (Fig. 2B), which was consistent with the finding that Cks1 triggers degradation of $p27^{kip1}$. The results suggest Cks2 may be critical for cells to grow or survive in non-optimal conditions. Comparable to the cells expressing GFP alone or untransfected cells, DTE cells expressing either Cks1- or Cks2-GFP fusion proteins exhibited an accelerated population growth activity (Fig. 2C). Interestingly, only the cells overexpressing Cks1, but not Cks2, had reduced $p27^{kip1}$ abundance, which is a negative regulator of cell cycle progression (Fig. 2D). The results suggest that overexpressed Cks1 accelerates the population growth by directly promoting $p27^{kip1}$ degradation and Cks2 by other mechanisms.

Knockdown of expression of Cks2 induces programmed cell death

To further investigate the role of elevated expressed Cks1 or Cks2 in prostate tumor cells, the colony formation activity of AT3 cells stably transfected with the inducible shRNA constructs were assessed. It was apparent that the colony numbers were reduced by knockdown of Cks1 (Fig 3A), suggesting that elevated expression of Cks1 in malignant AT3 cells contributed to the anchorage-independent growth activity. In contrast, knockdown of Cks2 expression only slightly reduced the colony number, suggestive that Cks2 might not be essential for the anchorage-independent growth activity. Furthermore, cell migration assay showed that knocking down Cks1, but not Cks2, expression in AT3 cells significantly reduced cell migration activity (Fig. 3B). Separated experiments revealed that force expression of Cks1 and Cks2 to benign DTE cells did not affect the anchorage-independent growth and cell migration activities (data not shown).

Although knockdown of Cks2 expression did not severely inhibit migration and anchorage-independent growth in AT3 cells, it induced dramatic cell morphology change within 12 hours after the induction especially when the cells were cultured in a suboptimal culture medium that contained 50% of fresh medium and 50% of conditioned media. Most cells became rounded and loosely attached to the culture surface (Fig. 3C,D), suggestive of programmed cell death. To test this possibility, the cells cultured in suboptimal conditions were stained with the APOPercentage™ dye 12-72 hours after the induction with doxycycline, which stained the apoptotic cells that lost the membrane potential. The results showed that knockdown of Cks2 expression induced significant cell death within 12 hours (Fig. 4A). In contrast, knockdown of Cks1 expression did not induce apoptosis under the same condition. Consistently, knockdown of Cks2 expression increased the caspase 3 activity by at least 2 folds (Fig. 4B). To further confirm this, Western blot analyses were carried out to assess the abundance of apoptosis markers at the protein levels. It was

apparent that knockdown of Cks2 expression increased Bad and somewhat decreased Bcl-xl and Akt expression at the protein levels (Fig. 4C).

In order to determine whether knockdown of Cks2 expression also induced apoptosis in other prostate cancer cells, Cks2 shRNA was transiently expressed in mouse prostate tumor cells (C2) derived from TRAMP tumors 28. To monitor the transfected cells, the GFP was coexpressed in the cells. Since about 85% of the transfectants are cotransfected with more than one vector, the majority of the GFP expressing cells would also express Cks2 shRNA. If knocking down Cks2 expression induced apoptosis in C2 cells, the majority of GFP positive cells would undergo apoptosis. The cells were stained with Cy3 conjugated Annexin V that recognized phosphatidylserine exposed only in apoptotic cell surface. Fluorescent microscopy analyses showed that, indeed, at 48 hours after the transfection, about 80% of GFP-positive cells were stained with Annexin V-Cy3, suggestive of the programmed cell death (Fig. 5). It is noticeable that not every GFP positive cells underwent apoptosis, and not every apoptotic cells expressed GFP, which reflects the fact that not every GFP positive cells also expressed Cks2 shRNA, and vice versa. In contrast, coexpression of stochastic shRNA and GFP or GFP alone rarely induced apoptosis in cells (Fig. 5). Similarly, knockdown of Cks2 expression also induced apoptosis in human DU145 cells (data not shown). Thus, the results further suggest a role of aberrantly expressed Cks2 in protecting prostate cancer cells from undergoing apoptosis.

Knockdown of Cks2 expression inhibits tumorigenicity of prostate cancer cells

The data that elevated Cks2 expression protected the cells from undergoing programmed cell death prompted us to investigate whether knockdown of Cks2 expression inhibited tumorigenicity of the cells. TRAMP C2 cells stably transfected with the inducible Cks2-shRNA construct were implanted to synergetic C57 mice as described 33. Doxycycline was administrated through drinking water to induce expression of Cks2-shRNA. As expected, the control cells gave rise to tumors within 2-3 weeks and caused the death of host animals by the time of 30-45 days. In sharp contrast, only 14% animals implanted with the cells expressing Cks2-shRNA died by the time of 55 days (Fig. 6A). The results were confirmed by separated experiments with another individual clone. It is noticeable that the Cks2-shRNA transfectants were less tumorigenic than the control even in the absence of doxycycline inducers. RT-PCR analyses revealed that expression of Cks2 in the two individual transfectants was somewhat lower than the cells expressing stochastic shRNA even in the absence of doxycycline (Fig. 6B), suggestive of leaky expression of Cks2 shRNA. Thus, the reduction in Cks2 expression may inhibit tumorigenesis of the cells in the absence of doxycycline inducer.

Discussion

Here we demonstrate that expression of Cks1 and Cks2 was elevated in prostate tumors, including the Dunning prostate tumors in rat, the TRAMP tumors in mouse, and more importantly, in human prostate tumors and cell lines. Forced expression of Cks1 and Cks2 in benign prostate tumor epithelial cells accelerated cell growth; knockdown of Cks1 and Cks2 malignant prostate tumor cells inhibited cell growth, anchorage-independent growth, and migration activity. In addition, knockdown of Cks2 expression induced apoptosis in vitro and compromised tumorigenic activity of the cells in vivo. The results suggest that elevated expression of Cks1 may contribute to tumorigenicity of prostate tumor cells by promoting cell growth and elevated expression of Cks2 by protecting the cells from undergoing apoptosis.

The function of the Cks family appears to be complicated. In yeast, both Cks1 and Cks2 bind to Cdk1 at a high affinity and function as a suppressor of Cdk1 mutants 1, 3, 34.

Despite the high sequence homology between the two members, whether Cks1 and Cks2 in mammalian cells elicit redundant or synergistic activities is not well understood although one report demonstrates that Cks1 compensates for Cks2 loss of function in the germ line 7. In mammalian cells, Cks1 induces an allosteric change in Skp2, which increases its affinity for phosphorylated $p27^{kip1}$ and triggers ubiquitination of $p27^{kip1}$, resulting in a rapid proteasome-mediated degradation of $p27^{kip1}$ during G1/S transition in the cell cycle. To date, decreases in $p27^{kip1}$ expression or mutations of $p27^{kip1}$ has been reported in tumors of many tissue origins, including prostate cancer 35, 36. Here we showed that forced expression of Cks1 reduced $p27^{kip1}$ in the cells and accelerated cell growth, while knockdown Cks1 expression in malignant AT3 cells inhibited proliferation and increased $p27^{kip1}$ abundance (Fig. 2).

In addition to being a mitotic inhibitor, $p27^{kip1}$ also has cell cycle-independent function, including regulating cell migration. However, $p27^{kip1}$ has been shown both promoting and inhibiting cell migration 37-42. Recently, it has been showed that the binding of $p27^{kip1}$ to the microtubule-destabilizing protein stathmin regulates the activity of $p27^{kip1}$ to repress cell migration. Upregulation of $p27^{kip1}$ or downregulation of stathmin expression results in inhibition of cell motility that is essential for invasion and metastasis of tumors 43, 44. Our data showed that knockdown of Cks1 expression increased $p27^{kip1}$ abundance in the cells and inhibited cell migration, which was consistent with the finding that $p27^{kip1}$ inhibits migration and invasion of tumor cells. In addition, inhibition of $p27^{kip1}$ in LNCaP cells significantly increases colony formation in soft agar, suggesting that $p27^{kip1}$ inhibits anchorage-independent growth activity of prostate cancer cells 45. Consistently, our data showed that knockdown of Cks1 expression increased abundance of $p27^{kip1}$ and inhibited colony formation activity of the malignant AT3 cells. The result further suggests that overexpression of Cks1 might contribute to the tumorigenicity through promoting anchorage-independent growth activity.

In contrast to Cks1, knockdown of Cks2 expression in AT3 cells did not increase $p27^{kip1}$ abundance. Instead, it slightly reduced the abundance of $p27^{kip1}$. It is possible that the reduction of $p27^{kip1}$ abundance in AT3 cells was a result of cell death caused by knockdown of Cks2 expression (Fig. 2). Further experiments are needed to address the issue. Similarly, forced expression of Cks2 did not reduce $p27^{kip1}$ albeit it accelerated DTE cell growth (Fig. 2). Therefore, although changes in Cks2 expression affect cell growth and has been shown to oscillate during the cell cycle 6, it is likely that Cks2 may not directly control the cell cycle progression. Indeed, expression of Cks2 in DU145, AT3, and TRAMP C2 cells was not reduced when the cells were cultured in serum-free medium for 48 hours (Supplemental Fig. 1), in which condition, the cell proliferation was relatively lower than in full culture medium. It is possible that changes in Cks2 expression may impact cell growth by preventing apoptosis. Both cell growth and apoptosis assays showed that knockdown of Cks2 expression in malignant AT3 cells had a more potent impact in cells maintained in non-optimal culture conditions than in those maintained in optimal condition. This may explain why the leaky expression of Cks2 shRNA had limited effects on stock cell cultures that were maintained in the optimal conditions, yet, had significant effects on in vivo tumorigenesis analyses where the cells were implanted subcutaneously and did not have optimal supplies of oxygen and nutrition.

Together with the fact that *Cks2* null mice did not have apparent defect in all tissues except the testes 7, our data here imply that Cks2 may not be essential for growth, differentiation, and maintenance of normal prostatic cells, yet, is essential for protecting the cells from undergoing apoptosis in harsh growth conditions, such as deficient in nutritional or oxygen supplies. Thus, inhibition of Cks2 activity may be of therapeutic potential for tumor treatment, and promotion of Cks2 activity may be useful for preventing cells to undergo

apoptosis due to insufficient oxygen or nutrition supplies, such as in stroke, heart attacks, or other injury. In addition, although having distinct roles, the two Cks members may synergistically contributed to prostate tumorigenesis by promoting cell proliferation and preventing apoptosis.

In summary, here we report that aberrant expression of Cks1 in prostate tumor cells promoted tumorigenicity by promoting proliferation, anchorage-independent growth, and cellular migration activity, while aberrant expression of Cks2 may promote the tumorigenicity by protecting the cells from apoptosis. Together with the reports that Cks1 and Cks2 are not essential for somatic cell growth and tissue homeostasis, the results here suggest a novel strategy for prostatic cancer treatment without affecting normal somatic cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Hayles J, Beach D, Durkacz B, Nurse P. The fission yeast cell cycle control gene cdc2: isolation of a sequence suc1 that suppresses cdc2 mutant function. Mol Gen Genet. 1986; 202:291–3. [PubMed: 3010051]
2. Richardson HE, Stueland CS, Thomas J, Russell P, Reed SI. Human cDNAs encoding homologs of the small p34Cdc28/Cdc2-associated protein of Saccharomyces cerevisiae and Schizosaccharomyces pombe. Genes Dev. 1990; 4:1332–44. [PubMed: 2227411]
3. Hadwiger JA, Wittenberg C, Mendenhall MD, Reed SI. The Saccharomyces cerevisiae CKS1 gene, a homolog of the Schizosaccharomyces pombe suc1+ gene, encodes a subunit of the Cdc28 protein kinase complex. Mol Cell Biol. 1989; 9:2034–41. [PubMed: 2664468]
4. Spruck C, Strohmaier H, Watson M, Smith AP, Ryan A, Krek TW, Reed SI. A CDK-independent function of mammalian Cks1: targeting of SCF(Skp2) to the CDK inhibitor p27Kip1. Mol Cell. 2001; 7:639–50. [PubMed: 11463388]
5. Ganoth D, Bornstein G, Ko TK, Larsen B, Tyers M, Pagano M, Hershko A. The cell-cycle regulatory protein Cks1 is required for SCF(Skp2)-mediated ubiquitinylation of p27. Nat Cell Biol. 2001; 3:321–4. [PubMed: 11231585]
6. Urbanowicz-Kachnowicz I, Baghdassarian N, Nakache C, Gracia D, Mekki Y, Bryon PA, Ffrench M. ckshs expression is linked to cell proliferation in normal and malignant human lymphoid cells. Int J Cancer. 1999; 82:98–104. [PubMed: 10360827]
7. Spruck CH, de Miguel MP, Smith AP, Ryan A, Stein P, Schultz RM, Lincoln AJ, Donovan PJ, Reed SI. Requirement of Cks2 for the first metaphase/anaphase transition of mammalian meiosis. Science. 2003; 300:647–50. [PubMed: 12714746]
8. Kawakami K, Enokida H, Tachiwada T, Gotanda T, Tsuneyoshi K, Kubo H, Nishiyama K, Takiguchi M, Nakagawa M, Seki N. Identification of differentially expressed genes in human bladder cancer through genome-wide gene expression profiling. Oncol Rep. 2006; 16:521–31. [PubMed: 16865252]
9. Wong YF, Cheung TH, Tsao GS, Lo KW, Yim SF, Wang VW, Heung MM, Chan SC, Chan LK, Ho TW, Wong KW, Li C, et al. Genome-wide gene expression profiling of cervical cancer in Hong Kong women by oligonucleotide microarray. Int J Cancer. 2006; 118:2461–9. [PubMed: 16353136]

10. Chow LS, Lam CW, Chan SY, Tsao SW, To KF, Tong SF, Hung WK, Dammann R, Huang DP, Lo KW. Identification of RASSF1A modulated genes in nasopharyngeal carcinoma. Oncogene. 2006; 25:310–6. [PubMed: 16116475]
11. de Wit NJ, Rijntjes J, Diepstra JH, van Kuppevelt TH, Weidle UH, Ruiter DJ, van Muijen GN. Analysis of differential gene expression in human melanocytic tumour lesions by custom made oligonucleotide arrays. Br J Cancer. 2005; 92:2249–61. [PubMed: 15900300]
12. Zirn B, Samans B, Spangenberg C, Graf N, Eilers M, Gessler M. All-trans retinoic acid treatment of Wilms tumor cells reverses expression of genes associated with high risk and relapse in vivo. Oncogene. 2005; 24:5246–51. [PubMed: 15897880]
13. Lin CY, Strom A, Kong SL, Kietz S, Thomsen JS, Tee JB, Vega VB, Miller LD, Smeds J, Bergh J, Gustafsson JA, Liu ET. Inhibitory effects of estrogen receptor beta on specific hormone responsive gene expression and association with disease outcome in primary breast cancer. Breast Cancer Res. 2007; 9:R25. [PubMed: 17428314]
14. Uchikado Y, Inoue H, Haraguchi N, Mimori K, Natsugoe S, Okumura H, Aikou T, Mori M. Gene expression profiling of lymph node metastasis by oligomicroarray analysis using laser microdissection in esophageal squamous cell carcinoma. Int J Oncol. 2006; 29:1337–47. [PubMed: 17088971]
15. Rother K, Dengl M, Lorenz J, Tschop K, Kirschner R, Mossner J, Engeland K. Gene expression of cyclin-dependent kinase subunit Cks2 is repressed by the tumor suppressor p53 but not by the related proteins p63 or p73. FEBS Lett. 2007; 581:1166–72. [PubMed: 17336302]
16. Kudo Y, Kitajima S, Ogawa I, Miyauchi M, Takata T. Down-regulation of Cdk inhibitor p27 in oral squamous cell carcinoma. Oral Oncol. 2005; 41:105–16. [PubMed: 15695111]
17. Ouellet V, Provencher DM, Maugard CM, Le Page C, Ren F, Lussier C, Novak J, Ge B, Hudson TJ, Tonin PN, Mes-Masson AM. Discrimination between serous low malignant potential and invasive epithelial ovarian tumors using molecular profiling. Oncogene. 2005; 24:4672–87. [PubMed: 15940270]
18. Shapira M, Ben-Izhak O, Linn S, Futerman B, Minkov I, Hershko DD. The prognostic impact of the ubiquitin ligase subunits Skp2 and Cks1 in colorectal carcinoma. Cancer. 2005; 103:1336–46. [PubMed: 15717322]
19. Slotky M, Shapira M, Ben-Izhak O, Linn S, Futerman B, Tsalic M, Hershko DD. The expression of the ubiquitin ligase subunit Cks1 in human breast cancer. Breast Cancer Res. 2005; 7:R737–44. [PubMed: 16168119]
20. Ouellet V, Guyot MC, Le Page C, Filali-Mouhim A, Lussier C, Tonin PN, Provencher DM, Mes-Masson AM. Tissue array analysis of expression microarray candidates identifies markers associated with tumor grade and outcome in serous epithelial ovarian cancer. Int J Cancer. 2006; 119:599–607. [PubMed: 16572426]
21. Li SH, Li CF, Sung MT, Eng HL, Hsiung CY, Huang WW, Lin CN, Yu SC, Huang HY. Skp2 is an independent prognosticator of gallbladder carcinoma among p27(Kip1)-interacting cell cycle regulators: an immunohistochemical study of 62 cases by tissue microarray. Mod Pathol. 2007; 20:497–507. [PubMed: 17384652]
22. Hershko DD, Shapira M. Prognostic role of p27Kip1 deregulation in colorectal cancer. Cancer. 2006; 107:668–75. [PubMed: 16826582]
23. Kitajima S, Kudo Y, Ogawa I, Bashir T, Kitagawa M, Miyauchi M, Pagano M, Takata T. Role of Cks1 overexpression in oral squamous cell carcinomas: cooperation with Skp2 in promoting p27 degradation. Am J Pathol. 2004; 165:2147–55. [PubMed: 15579456]
24. Zhang Y, Lin Y, Bowles C, Wang F. Direct Cell Cycle Regulation by the Fibroblast Growth Factor Receptor (FGFR) Kinase through Phosphorylation-dependent Release of Cks1 from FGFR Substrate 2. J Biol Chem. 2004; 279:55348–54. [PubMed: 15513912]
25. Shapira M, Ben-Izhak O, Slotky M, Goldin O, Lahav-Baratz S, Hershko DD. Expression of the ubiquitin ligase subunit cyclin kinase subunit 1 and its relationship to S-phase kinase protein 2 and p27Kip1 in prostate cancer. J Urol. 2006; 176:2285–9. [PubMed: 17070313]
26. Lu X, Guo J, Hsieh TC. PC-SPES inhibits cell proliferation by modulating p21, cyclins D, E and B and multiple cell cycle-related genes in prostate cancer cells. Cell Cycle. 2003; 2:59–63. [PubMed: 12695690]

27. Jin C, McKeehan K, Guo W, Jauma S, Ittmann MM, Foster B, Greenberg NM, McKeehan WL, Wang F. Cooperation between ectopic FGFR1 and depression of FGFR2 in induction of prostatic intraepithelial neoplasia in the mouse prostate. Cancer Res. 2003; 63:8784–90. [PubMed: 14695195]
28. Kaplan-Lefko PJ, Chen TM, Ittmann MM, Barrios RJ, Ayala GE, Huss WJ, Maddison LA, Foster BA, Greenberg NM. Pathobiology of autochthonous prostate cancer in a pre-clinical transgenic mouse model. Prostate. 2003; 55:219–37. [PubMed: 12692788]
29. Lin Y, Liu G, Zhang Y, Hu YP, Yu K, Lin C, McKeehan K, Xuan JW, Ornitz DM, Shen MM, Greenberg N, McKeehan WL, et al. Fibroblast growth factor receptor 2 tyrosine kinase is required for prostatic morphogenesis and the acquisition of strict androgen dependency for adult tissue homeostasis. Development. 2007; 134:723–34. [PubMed: 17215304]
30. Wu X, Jin C, Wang F, Yu C, McKeehan WL. Stromal cell heterogeneity in fibroblast growth factor-mediated stromal-epithelial cell cross-talk in premalignant prostate tumors. Cancer Res. 2003; 63:4936–44. [PubMed: 12941818]
31. McKeehan WL, Wang F, Kan M. The heparan sulfate-fibroblast growth factor family: diversity of structure and function. Prog Nucleic Acid Res Mol Biol. 1998; 59:135–76. [PubMed: 9427842]
32. Feng S, Wang F, Matsubara A, Kan M, McKeehan WL. Fibroblast growth factor receptor 2 limits and receptor 1 accelerates tumorigenicity of prostate epithelial cells. Cancer Res. 1997; 57:5369–78. [PubMed: 9393762]
33. Foster BA, Gingrich JR, Kwon ED, Madias C, Greenberg NM. Characterization of prostatic epithelial cell lines derived from transgenic adenocarcinoma of the mouse prostate (TRAMP) model. Cancer Res. 1997; 57:3325–30. [PubMed: 9269988]
34. Brizuela L, Draetta G, Beach D. p13suc1 acts in the fission yeast cell division cycle as a component of the p34cdc2 protein kinase. Embo J. 1987; 6:3507–14. [PubMed: 3322810]
35. Bloom J, Pagano M. Deregulated degradation of the cdk inhibitor p27 and malignant transformation. Semin Cancer Biol. 2003; 13:41–7. [PubMed: 12507555]
36. Philipp-Staheli J, Payne SR, Kemp CJ. p27(Kip1): regulation and function of a haploinsufficient tumor suppressor and its misregulation in cancer. Exp Cell Res. 2001; 264:148–68. [PubMed: 11237531]
37. Daniel C, Pippin J, Shankland SJ, Hugo C. The rapamycin derivative RAD inhibits mesangial cell migration through the CDK-inhibitor p27KIP1. Lab Invest. 2004; 84:588–96. [PubMed: 15064772]
38. Itoh Y, Masuyama N, Nakayama K, Nakayama KI, Gotoh Y. The cyclin-dependent kinase inhibitors p57 and p27 regulate neuronal migration in the developing mouse neocortex. J Biol Chem. 2007; 282:390–6. [PubMed: 17092932]
39. Li Z, Jiao X, Wang C, Ju X, Lu Y, Yuan L, Lisanti MP, Katiyar S, Pestell RG. Cyclin D1 induction of cellular migration requires p27(KIP1). Cancer Res. 2006; 66:9986–94. [PubMed: 17047061]
40. Nguyen L, Besson A, Heng JI, Schuurmans C, Teboul L, Parras C, Philpott A, Roberts JM, Guillemot F. p27kip1 independently promotes neuronal differentiation and migration in the cerebral cortex. Genes Dev. 2006; 20:1511–24. [PubMed: 16705040]
41. Kawauchi T, Chihama K, Nabeshima Y, Hoshino M. Cdk5 phosphorylates and stabilizes p27kip1 contributing to actin organization and cortical neuronal migration. Nat Cell Biol. 2006; 8:17–26. [PubMed: 16341208]
42. Besson A, Gurian-West M, Schmidt A, Hall A, Roberts JM. p27Kip1 modulates cell migration through the regulation of RhoA activation. Genes Dev. 2004; 18:862–76. [PubMed: 15078817]
43. Condeelis J, Segall JE. Intravital imaging of cell movement in tumours. Nat Rev Cancer. 2003; 3:921–30. [PubMed: 14737122]
44. Baldassarre G, Belletti B, Nicoloso MS, Schiappacassi M, Vecchione A, Spessotto P, Morrione A, Canzonieri V, Colombatti A. p27(Kip1)-stathmin interaction influences sarcoma cell migration and invasion. Cancer Cell. 2005; 7:51–63. [PubMed: 15652749]
45. Galardi S, Mercatelli N, Giorda E, Massalini S, Frajese GV, Ciafre SA, Farace MG. miR-221 and miR-222 expression affects the proliferation potential of human prostate carcinoma cell lines by targeting p27Kip1. J Biol Chem. 2007; 282:23716–24. [PubMed: 17569667]

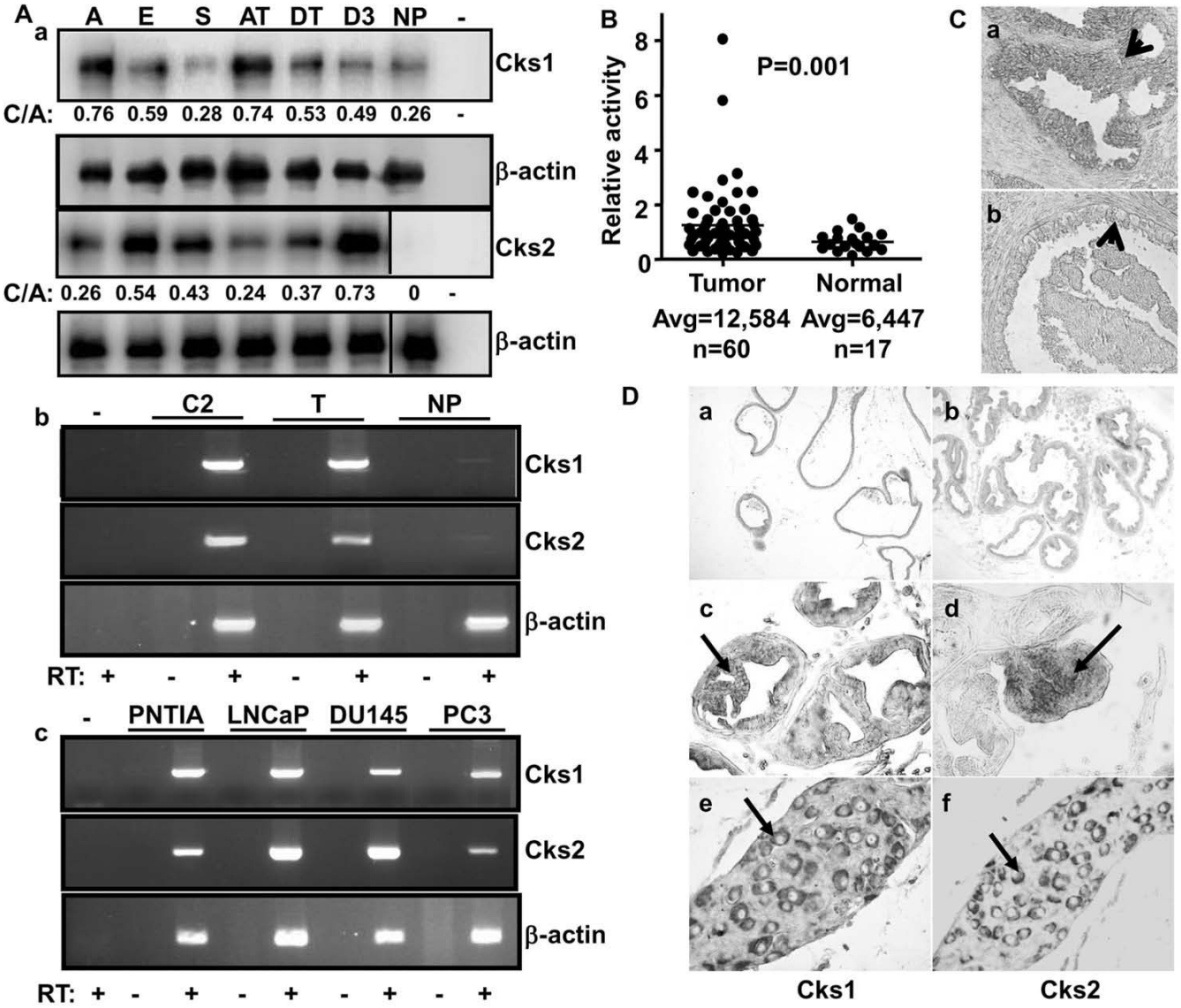

Fig. 1. Overexpression of Cks1 and Cks2 in prostate tumors

A. Total RNA samples were extracted from the indicated tumors and cells. Expression of Cks1 and Cks2 was assessed with RNase protection assays (a) or RT-PCR (b,c). β-actin was used as a loading control. Vertical lines indicate that the lane orders had been rearranged for better illustrations. Specific bands were quantified using the Image J software, and the values were expressed as abundances relative to β-actin (C/A). **B**. Total RNA samples were prepared from human prostate tumors (Tumor) and the adjacent normal tissues (Normal). The expression of Cks2 was assessed with real-time RT-PCR. Data were normalized to β-actin and were expressed as ratios of Cks2/β-actin × 10^3. **C**. Human prostate sections with tumor (a) and normal (b) histological structures were prepared for in situ hybridization with probes specific for human Cks2. Note that Cks2 expression was apparent in tumor foci (a), but absent in normal section (b). Arrow heads indicate the epithelium. **D**. Prostate tissue sections were prepared from TRAMP and control mice at the age of 6 weeks, and expression of Cks1 (a, c, and e) and Cks2 (b, d, and f) was assessed by in situ hybridization. a, b, areas with relatively normal prostate tissue structures; c, d, areas with high-grade PIN lesions; e

and f, areas with carcinoma lesions. Arrows indicate Cks expression in lesion foci. A, AT3 cells; E, DTE cells; S, DTS cells; AT, AT tumor; DT, tumors derived from DTE cells; D3, DT3 tumors; NP, normal prostates; C2, TRAMP C2 cells; T, TRAMP tumors; RT, reverse transcription; −, negative control without RNA samples.

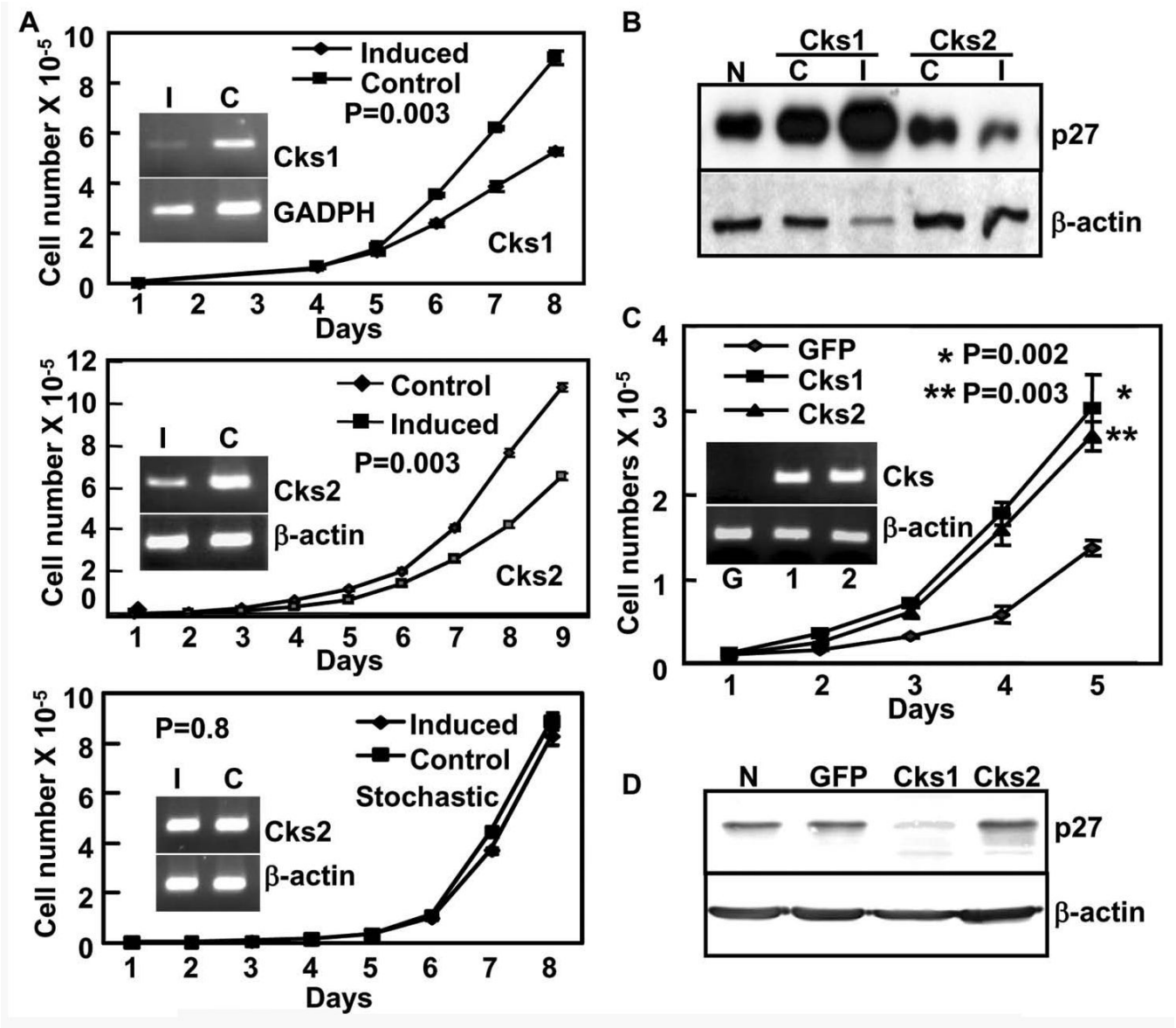

Fig. 2. Aberrantly expressed Cks1 and Cks2 contribute to prostate cancer cell growth

A. AT3 cells stably transfected with Cks1-, Cks2-, and stochastic-shRNA were inoculated in 24-well dishes with or without the doxycycline induction and the cell numbers were determined daily. Note that Cks2 shRNA-expressing cells were cultured in 50% conditioned medium as described in Methods. The data are means±sd of triplicate samples. **B**. Lysates were prepared from the transfected AT3 cells with or without doxycycline induction were Western blotted with anti-$p27^{kip1}$ or anti-β-actin antibodies. The specifically bound antibodies were visualized with ECL-Plus chemoluminescent reagents. **C.** Pool of DTE cells stably expressing Cks1-GFP and Cks2-GFP fusion proteins, or GFP alone, were cultured in 24-well plates in 2.5% fetal bovine serum and the cells numbers were determined daily. The data are means±sd of triplicate samples. **D.** Lysates prepared from the transfected DTE cells with or without doxycycline induction were Western blotted with anti-$p27^{kip1}$ or anti-β-actin antibodies. The specifically bound fractions were visualized with alkaline phosphatase staining. Inserts, RT-PCR analyses of Cks expression. β-actin was used as a loading control. I, induced with doxycycline; C, without doxycycline induction; N, untransfected cells.

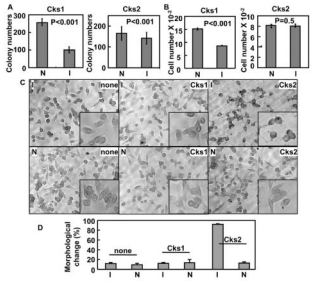

Fig. 3. Knockdown of Cks1 and Cks2 expression has different impacts on malignant AT3 cells

A. Stable Cks1 and Cks2 shRNA transfectants of AT3 cells were inoculated in soft agar plates with or without doxycycline induction as indicated. Colony numbers were scored 10 days after the inoculation and expressed as means±sd of duplicate samples. **B.** The same cells were cultured in Transwell inserts. Only the medium in outer chambers contained 10% FBS. After incubation at 37 °C for 12 hours, the numbers of cells migrating across the membranes were determined. Data are means±sd of triplicate samples. **C.** The same cells cultured in 6-well plates were treated with doxycycline to induce shRNA expression. Cell morphological changes were documented at 48 hours after the induction by staining with 0.5% Methylene Green. D. Statistical analyses of morphological changed cells induced by Cks2 knockdown. Data are means±sd of triplicate samples. N, not induced; I, induced.

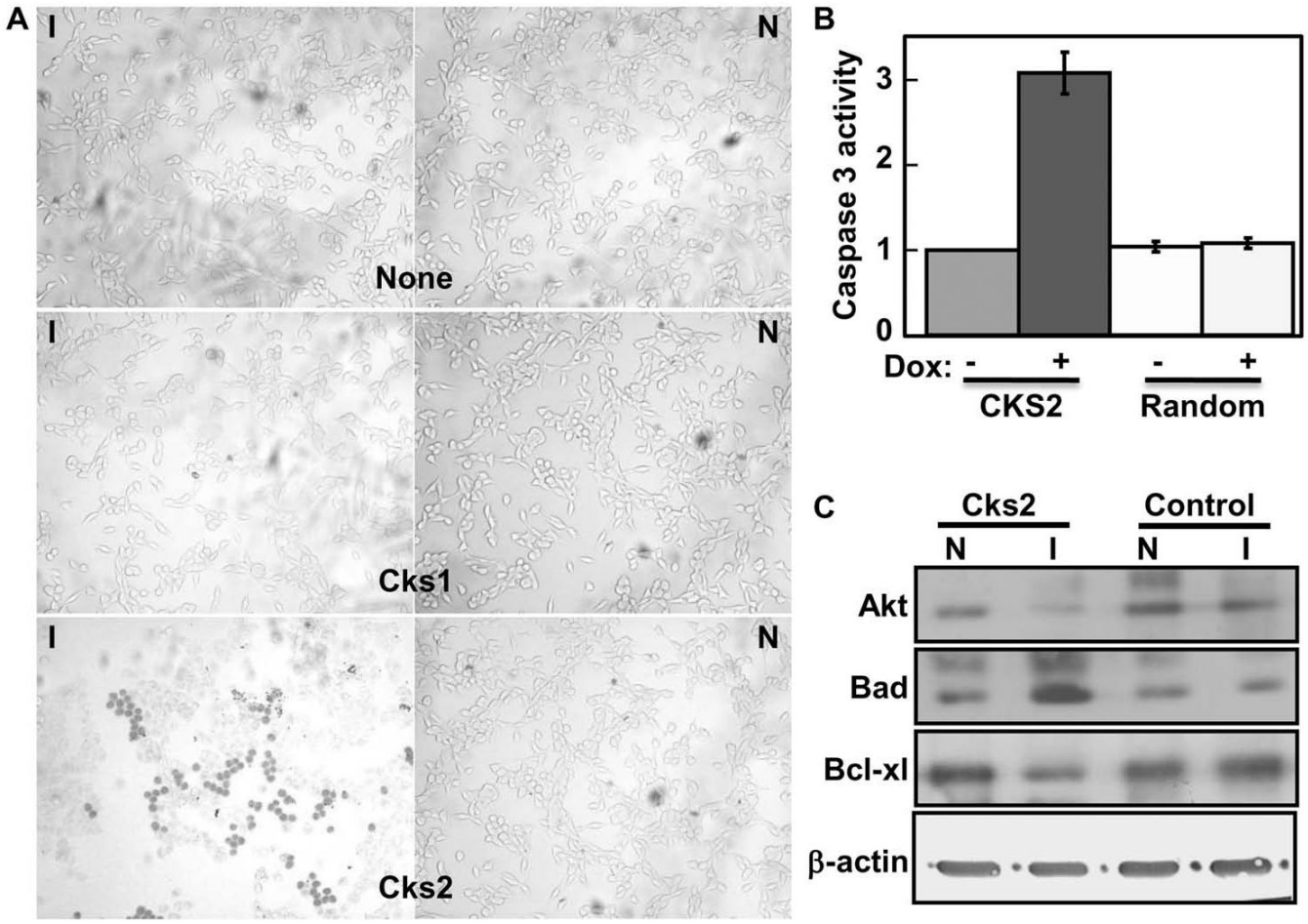

Fig. 4. Knockdown of Cks2 expression in AT3 cells induces apoptosis

A. Stable Cks1- and Cks2-shRNA transfected AT3 cells were cultured in 6-well dishes. Expression of the shRNAs was induced by doxycycline induction. The apoptotic cells were stained with ApoPcentage dye and were visualized by microscopy. **B&C.** The same cells were cultured in 15-cm dishes, and the expression of shRNAs was induced with doxycycline for 48 hours. The caspase 3 activity was quantitated as described in the Methods (B). Data are expressed as fold of increase from non-induced cells and are means±sd of triplicate samples. Expression of Bad, Bcl-xl, and Akt was assessed with Western blot analyzed (C). β-actin was used as loading controls. I, induced; N, not induced.

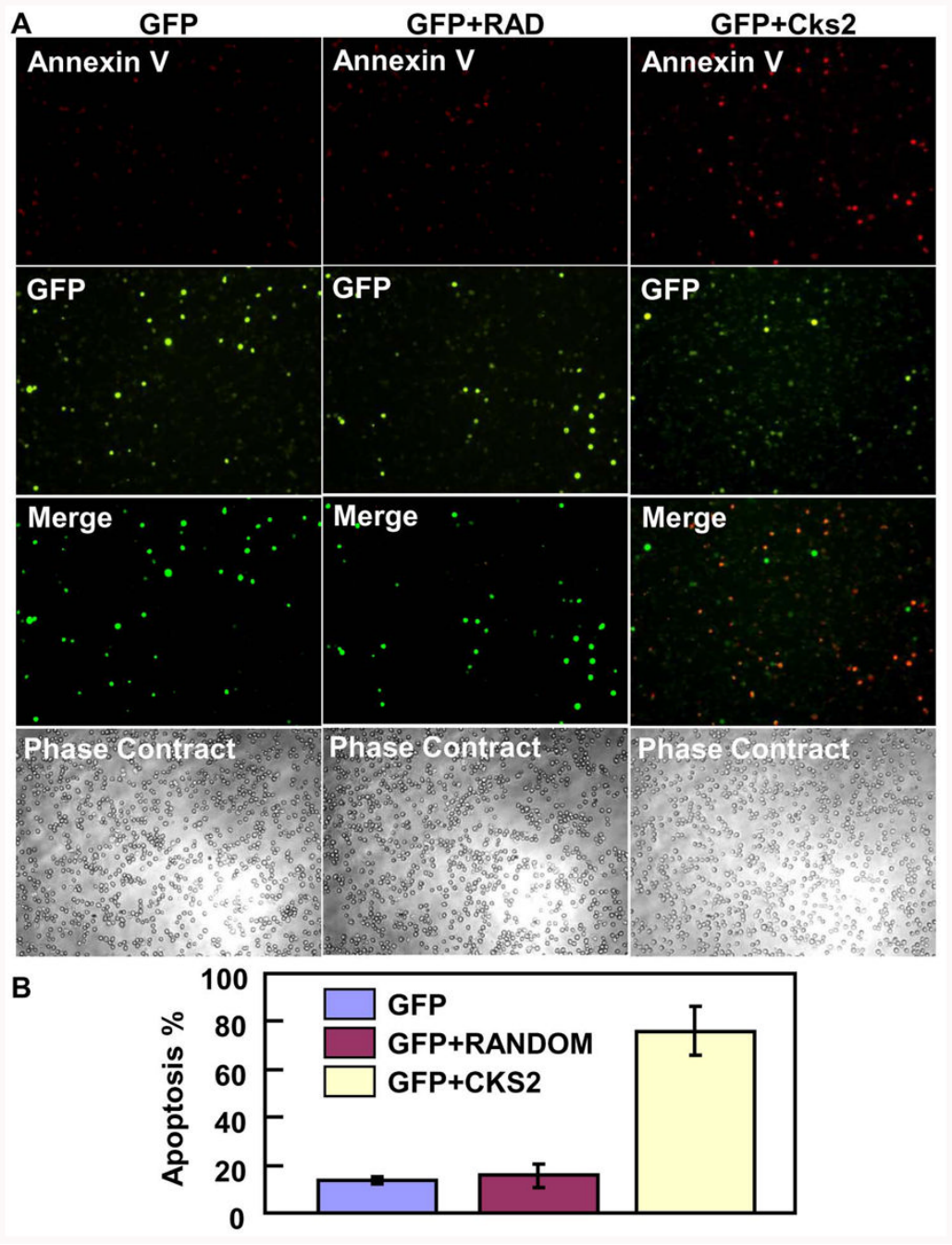

Fig. 5. Knockdown of Cks2 expression in TRAMP C2 cells induces programmed cell death

A. Mouse Cks2-shRNA and GFP were coexpressed in TRAMP C2 cells by transient transfection. The apoptotic cells were stained with Cy3-conjugated Annexin V at day 2 after the transfection. The GFP expression and Annexin V-Cy3 stained cells were visualized by fluorescent microscopy and the cell morphology by phase contrast microscopy. **B**. The GFP- and Annexin V/GFP-positive cells were quantified. The data are the percent of Annexin V/GFP double positive cells in total GFP-positive pool and are means±sd of triplicate samples.

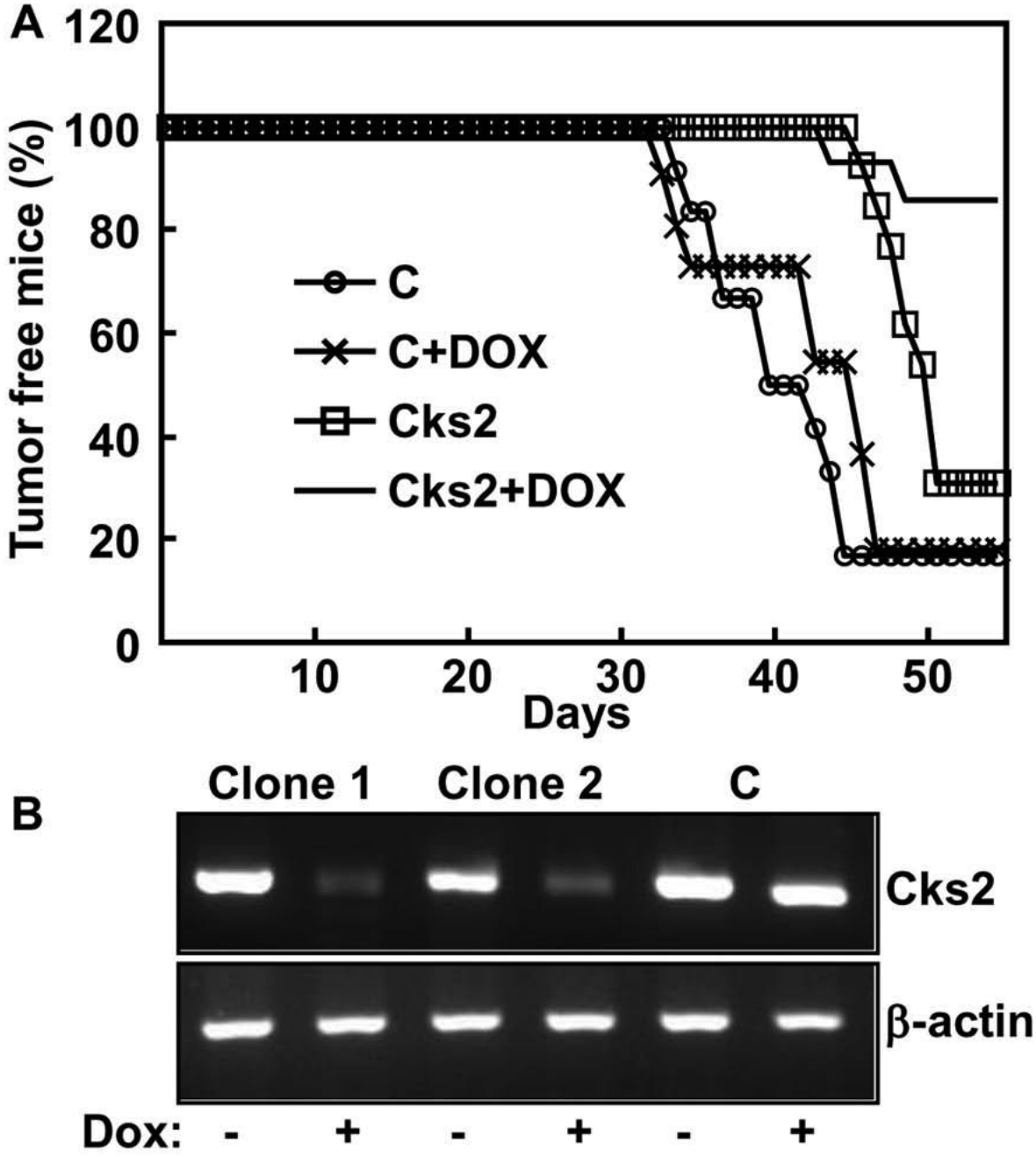

Fig. 6. Knockdown of Cks2 expression inhibits tumorigenicity of TRAMP tumor cells

A. Stable transfectants of TRAMP C2 cells (1×10^6) carrying inducible expressed Cks2-shRNA or control shRNA sequences were implanted subcutaneously to BL6/C57 mice as described 33. Expression of shRNA was induced with doxycycline in drinking water where indicated. Mice were observed every day and palpable tumors were recorded. The percentage of tumor-free mice was scored once a day. The y-axis is the number of tumor-free mice at the respective age, as a percentage of all mice in each group. B. Expression of Cks2 in 2 individual stable Cks2-shRNA transfected clones was assessed with RT-PCR. β-actin was used as loading controls. C, control cells carrying stochastic shRNA sequence; Cks2, cells carrying Cks2-shRNA; DOX, doxycycline.