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VentX *trans*-activates p53 and p16^{ink4a} to regulate cellular senescence

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Abstract

Cell senescence is a process of irreversible arrest of cell proliferation and plays an important role in tumor suppression. Recent studies showed that Wnt inhibition is a trigger of cellular senescence. Using methods of reverse genetics, we recently identified VentX, a human homologue of the vertebrate Xenopus Vent family of homeobox genes, as a novel Wnt repressor and a putative tumor suppressor in lymphocytic leukemia. Here we show that VentX is a direct transcriptional activator of p53-p21 and p16^{ink4a}-Rb tumor suppression pathways. Ectopic expression of VentX in cancer cells caused an irreversible cell cycle arrest with a typical senescence-like phenotype. Conversely, inhibition of VentX expression by RNA interference ameliorated chemotherapeutic agent induced senescence in lymphocytic leukemia cells. The results of our study further reveal the mechanisms underlying the tumor suppression function of VentX and suggest a role for VentX as a potential target in cancer prevention and treatment.

Introduction

Canonical Wnt/β-catenin signaling plays essential roles in cell fate determination during early embryogenesis and is required to maintain stem cell pools of adult tissues (1-3). Gain of function mutations of the Wnt signaling pathway have been implicated in both solid tumors, such as colorectal cancers, and hematological malignancies, such as myeloid and lymphocytic leukemia (4-6).

The high-mobility group (HMG) box-containing transcriptional factors lymphoid-enhancing factor/T-cell factors (LEF/TCFs) are the nuclear transcriptional mediators of canonical Wnt/β-catenin signaling (7,8). Using methods of reverse genetics, we recently identified VentX, a human homologue of the Xenopus homeobox transcriptional factor Xom, as a novel LEF1/TCF-associated Wnt repressor and a putative tumor suppressor (9,10). Consistent with its role as a negative regulator of Wnt signaling, a recent cancer genome study showed that the VentX gene locus is frequently deleted in cancers, such as colorectal cancer and melanoma, in which aberrant Wnt signaling is implicated (11). Moreover, gene expression profiles from published databases suggest that VentX expression is downregulated in lymphocytic leukemia (12,13).

In parallel with its prominent role in development, recent studies suggest a critical role for Wnt signaling in cellular senescence, an irreversible process of cell proliferation arrest (14). Initially described as a cellular mechanism underlying physiological aging of fibroblasts, cellular senescence is being recognized to play critical roles in tumor suppression (15-17). Similar to primary fibroblasts, tumor cells also retain the ability to undergo senescence in response to genetic manipulation or treatment with chemotherapeutic drugs (18-21). Senescent cells

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display positive staining for senescence-associated-β-galactosidase (SA-β-gal) and form senescence-associated heterochromatic foci (SAHF) (22,23). It has been shown that downregulation of Wnt signaling triggers the formation of the SAHF and onset of cellular senescence (14).

The p53-p21 and Rb-p16^{ink4a} are two critical tumor suppression pathways implicated in cellular senescence (16,17). *P53* is a well-established tumor suppressor gene and exerts its function in part by transcriptional activation of p21, an inhibitor of cyclin-dependent kinases (CDKs) (24). Rb exerts its function by binding to the E2F family of transcriptional factors and inhibiting the downstream transcriptional cascades required for cell cycle entry (25). The inhibitory effects of Rb on E2F are abolished through phosphorylation of Rb by cyclin/CDK complexes which, in turn, are inhibited by p16^{ink4a} and p15^{ink4b} (26). Clinical genetics studies have showed that silencing of the p53-p21 pathway occurs in approximately 50% of cases of ALL. Likewise, deletion or epigenetic silencing of p16^{ink4a} and p15^{ink4b} occurs frequently in ALL (27-29).

VentX is a novel Wnt repressor implicated in the pathogenesis of lymphocytic leukemia (9). To explore the mechanisms underlying VentX tumor suppression function, we screened for VentX effects on the expression of a panel of key regulators of cell proliferation. Here, we report that VentX is a direct transcriptional activator of the p53-p21 and Rb-p16^{ink4a} tumor suppressor pathways. We found that VentX expression induces a senescence phenotype in several tumor cell lines and that downregulation of VentX expression by RNA interference is associated with reduced senescence and increased resistance of leukemia cells to chemotherapeutic agents. Our data suggest a potential role for VentX as a novel therapeutic target in cancer treatment.

Experimental procedures

Cell Culture

Human embryonic kidney cell line 293 (HEK293), human cervical cancer cell line HeLa, human osteosarcoma U2OS cell line and human primary fibroblasts IMR90 were cultured in DMEM medium supplemented with 10% FBS and 1% antibiotics. The human lymphoblastic acute leukemia cell line Nalm16 was maintained in RPMI1640 medium. Primary CD19^{+} B lymphocytes were purified from peripheral blood mononuclear cells (PBMC) by positive selection using a magnetic cell separator (MACS, Miltenyi Biotec; Auburn, CA). The lymphocytes were seeded at a density of 10^6/ml in RPMI 1640 medium supplemented with 10% fetal bovine serum.

Western blot

Cells were lysed in solution A (50 mM Tris-HCl, pH 7.8, 420 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 0.34 M sucrose, 10% glycerol, 1 mM Na_3VO_4, 10 mM NaF and *β*-glycerophosphate, 1 mM PMSF, and protease inhibitor cocktail). Lysates were cleared by centrifugation and protein concentration was determined by the Bradford assay (Bio-Rad; Hercules, CA). Proteins resolved by SDS-PAGE were transferred onto PVDF membrane, and were detected with optimal dilutions of primary antibodies, followed by horseradish peroxidase-linked secondary antibodies. The blots were developed by enhanced chemiluminescence (Amersham; Piscataway, NJ). Primary antibodies used were from Cell Signaling (Danvers, MA) except anti-VentX sera, generated in our lab.

Luciferase reporter assay

The luciferase reporters of p53 (-2.4kb) and p16^{ink4a} (-869bp) promoters have been described previously (30,31). The p21 luciferase reporter is a generous gift of Dr. W Gu as described (32). Cells seeded in 24-well plates were transfected with 500ng reporter plasmid and increasing amount of pcDNA-VentX plasmid. Total plasmid transfected was brought to the same amount by addition of empty pcDNA vector. 10ng Renilla luciferase plasmid was included for each transfection to normalize reporter activity. Cells were harvested at 48 hrs after transfection and analyzed with Dual-Luciferase Reporter Assay System (Promega; Madison, WI).

ChIP assay

Cells were cultured in 6 cm dishes overnight and then transfected with pCS2 plasmids encoding myc-VentX or myc-tag. Forty-eight hours post-transfection, the chromatin immunoprecipitation (ChIP) assay was performed

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with a kit from Upstate Biotechnology (Billerica, MA) following manufacturer's instructions. The p53 promoter sequence was amplified with specific primers: 5'-CCTGACTCTGCACCCTCCTC-3' and 5'-CGAGGCTCCTGGCACAAAGC-3'; and the $p16^{ink4a}$ promoter sequence was amplified with primers 5'-TAGCTTAGGATGTGTGGCAC-3' and 5'-TCCTTCACACTTCTCACATT-3'. The following primers were used to amplify the constant region of immunoglobulin M heavy chain gene (Cμ): 5'-AACCCTTTTCCCCCTCGTCT-3' and 5'- AGCACCTGTGAGGTGGCTGC-3'. The promoter region of GAPDH was amplified with the following primers: 5'-TACTAGCGGTTTTACGGGCG-3' and 5'-TCGAACAGGAGGAGCAGAGAGCGA-3'. The primers 5'- GAGACCGGCGGATCAAATGTC-3' and 5'- CACCATGCCCAGCTAATTTTTG-3' were used to amplify a 100bp sequence at 3.7 kb upstream of the potential binding site of VentX on p53 promoter, which serves as another negative control. PCR products were separated on 8% polyacrylamide gel.

Generation of stable cell lines expressing VentX under control of tetracycline (Tet)-inducible promoter

U2OS cell line expressing Tet-inducible VentX was generated through transfection of a TetR-expressing U2OS cell line (a gift of Dr. Yao, Department of Surgery, Brigham and Women's Hospital, Boston) with pcDNA^{TM}4/TO-VentX plasmid. Positively transfected cells were selected with 2μg/ml Zeocin (Invitrogen). Single colonies were selected-out by limiting dilution. IMR90 cell lines expressing GFP.VentX (or GFP as control) were generated through co-transduction of pRetroX-GFP-VentX (or pRetroX-GFP) and pRetroX-Tet-On advanced retroviruses (Retro-X™ Tet-On Advanced Expression System, Clontech; Mountain View, CA). GFP positive cells were sorted by FACS after a brief incubation with Tet (24 hrs) and maintained in the absence of Tet.

RNA interference

Small hairpin RNA (shRNA) retroviral vectors targeting VentX (pRS vectors) with a puromycin selection marker were purchased from Origene (Rockville, MD) and described in our previous publication (9). Stable knockdown of VentX expression in Nalm16 cells was described as previously (9). Transient knockdown of VentX in primary $CD19^+$ B lymphocytes was achieved through electroporation of duplex RNA oligonucleotides (siRNA) with a kit from Lonza (Allendale, NJ) following the manufacturer's instructions. Retroviral shRNA against human $p16^{ink4a}$ is a kind gift of Dr. Lowe (Cold Spring Harbor Laboratory). pMKO.1-shp53 and pBabe-SV40 large T (SV40-LT) antigen retroviral vectors were obtained from Addgene.

RT-PCR

Total RNA was isolated by the TRIzol method, and same amount of RNA was used for first-strand cDNA synthesis with SuperSript First-Strand Synthesis System (Invitrogen) according to the manufacturer's protocol. The primers 5'-AAGGCAATTAGGCGCTGCTT-3' and 5'-ACAGAACAACTGAGTCCTCCA-3' were used for conventional PCR of VentX. For quantitative PCR, the following primers were used. VentX: 5'-CCGTCAGCATCAAGGAGG-3' and 5'-CTGGACCTCTGAGAGCTGC-3'; p53: 5'-TCTACCTCCCGCCATAAAA-3' and 5'-CTCCTCCCCACAACAAAAC-3'; p21: 5'-AAACTTTGGAGTCCCCTCAC-3' and 5'-AAAGGCTCAACACTGAGACG-3'; $p16^{ink4a}$: 5'-CTTCCCCCACTACCGTAAAT-3' and 5'-TGCTCACTCCAGAAAACTCC-3'. We performed quantitative PCR with SYBR Green on a LightCycler® 480 system (480 Real-Time PCR System; Roche, Indianapolis, IN).

β-galactosidase staining

Senescence of U2OS, Nalm16 and IMR90 cells was evaluated by a SA-β-galactosidase staining kit from Cell Signaling. Cell counts were made on five random fields of at least 200 cells.

Cell viability assay

Nalm 16 cells were subjected for the following treatments: 800nM All-*trans*-Retinoic Acid (ATRA) for 10 days, 100ng/ml Doxorubicin (Doxo) for 4 days, 5μM 5-fluorouracil (5-FU) for 3 days or 1μM camptothecin (CPT) for 2 days. Stable knockdown of VentX in Nalm 16 cells and the MTS assay of cells viability were performed as described previously (9).

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FACS analysis of Ki67

Nalm16 cells were treated with 800nM ATRA for 5 days, 100ng/ml Doxo for 2 days or mock treated. Cells were then harvested for intracellular staining of PE-conjugated Ki67 antibody with protocol provided by the manufacturer (eBiosceince, San Diego, CA) and analyzed with FACScan flow cytometer (BD Bioscience) using FlowJo software.

Results

Identification of p53, p21 and $p16^{ink4a}$ as transcriptional targets of VentX

Our recent study showed that VentX is a negative regulator of cell proliferation. Mechanistically, we found that VentX exerts its function on cell proliferation at least in part through inhibition of Wnt signaling and its downstream targets, such as Cyclin D1 (9). Given the broad implications of Wnt signaling in cell proliferation, we sought to further determine the molecular pathways underlying VentX-induced cell growth arrest through a targeted screen of cell cycle regulators, using a transient transfection assay. HEK293 cells were transfected with expression constructs encoding VentX or vector control. At 48 hrs after transfection, total cell lysates were harvested and the expression levels of a panel of cell cycle regulatory proteins were determined by Western blot analysis. As shown in Fig. 1, we found that VentX expression led to a significant increase in the protein levels of p53 and two CDK inhibitors: p21 and $p16^{ink4a}$ (Fig.1). Consistent with our previous finding, the Cyclin D1 protein level was downregulated upon the expression of VentX (9). No obvious expression change was detected for other tested proteins (Fig. 1).

VentX trans-activates p53/p21 pathway

P53 and p21 are key regulators of cell proliferation and are broadly implicated in tumorigenesis. To corroborate our screening findings, we used U2OS cells to further explore the effects of VentX on the expression of p53 and p21. Plasmid encoding VentX or vector control was transiently transfected into U2OS cells. At 48 hrs after transfection, total proteins were extracted and the expression levels of p53 and p21 were determined by Western blot analysis. Again, we observed an increase in the protein levels of p53 and p21 in U2OS cells upon VentX expression (Fig.2 A left panel). The effect of VentX on the protein level of p53 was also observed in HeLa cells (data not shown). Collectively, these data suggest that the p53-p21 pathway is a downstream target of VentX. Either increased transcription or reduced proteolysis may account for the effects of VentX on p53 protein level. To elaborate the mechanism of VentX action, we tested whether VentX affects the stability of p53. U2OS cells were treated with cycloheximide (CHX) after transfection of VentX or control plasmid. As shown in Fig.2 A (right panel), we did not observe significant alteration on the kinetics of p53 protein degradation upon transfection of VentX, suggesting that VentX does not exert significant effects on p53 stability. Next, we explored the effects of VentX on transcriptional activation of the p53-p21 pathway. As shown in Fig.2 B (left panel), in comparison with the vector control, VentX expression led to increased p53 and p21 mRNA levels in U2OS cells. In addition to its effects on p53 in U2OS cells, VentX also up-regulated p53 mRNA level in Sw480 cells, which harbor a mutant p53 protein (Fig.2 B right panel) (33). These data further suggest that p53 is a transcriptional target of VentX. Consistently, we showed that ectopic expression of VentX trans-activated the human *p53* and *p21* promoter-luciferase reporters in a dose-dependent manner (Fig.3). Human *p53* promoter region contains several putative homeodomain core binding sequences (ATTA) (Fig.2 G, and (31)). Chromosome immunoprecipitation (ChIP) assay therefore was performed to examine a potential direct interaction between VentX and the *p53* promoter (Fig.2 G). As shown in Fig.2 F, the ChIP assay revealed specific binding of VentX to the *p53* promoter. As negative controls, VentX was found not to bind to the region 3.7kb upstream of the VentX binding site on *p53* promoter. Further, VentX did not bind to the constant region of immunoglobulin M heavy chain gene (Cμ) (9) or the *GAPDH* promoter.

VentX trans-activates $p16^{ink4a}$/Rb pathway

The $p16^{ink4a}$-Rb pathway has been implicated in the pathogenesis of a variety of cancers including lymphocytic leukemia (29). Previous studies showed that $p16^{ink4a}$ inhibits

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phosphorylation of Rb by CDKs (26). Our recent study indicated that VentX is a putative tumor suppressor in lymphocytic leukemia (9). The finding that VentX up-regulates the expression of $p16^{ink4a}$ (Fig.1) prompted us to test the hypothesis that VentX also exerts its function by transactivation of the $p16^{ink4a}$-Rb tumor suppressor pathway. HeLa cells, in which the $p16^{ink4a}$-Rb pathway is functional (34), were employed to test this hypothesis. As shown in Fig.2 D (left panel), with increased expression of VentX, there was a corresponding increase of $p16^{ink4a}$ protein expression. In comparison to the elevated expression of $p16^{ink4a}$, no substantial change of Rb protein level was observed. However, when the level of phosphorylated Rb was examined with specific antibody, we found that VentX expression is associated with significant reduction of the hyperphosphorylated form of Rb (Fig.2 D, left panel). Our findings support the hypothesis that VentX promotes the expression of $p16^{ink4a}$, which, in turn, inhibits the phosphorylation of Rb by CDKs (35). The potential transcriptional activation of $p16^{ink4a}$ by VentX was suggested by the increased $p16^{ink4a}$ mRNA levels upon ectopic expression of VentX (Fig.2 D, right panel). Using *p16^{ink4a}* promoter-luciferase reporter assay, we further demonstrated that VentX promoted *p16^{ink4a}* transactivation in a dose dependent manner (Fig.2 E). The human p16 promoter also contains putative homeodomain binding sites (Fig.2 G). Consistently, the ChIP assay demonstrated specific binding of VentX to the *p16^{ink4a}* promoter (Fig.2 F).

Knockdown of VentX is associated with reduced p53-p21 and $p16^{ink4a}$ expression in primary lymphocytes

VentX is expressed predominately in hematopoietic cells. Expression analysis of VentX in peripheral blood leukocytes shows that VentX is expressed in both myeloid and lymphoid lineages (9). To determine whether VentX regulates p53, p21 and $p16^{ink4a}$ expression under physiological condition in primary hematopoietic cells, we examined the effects of VentX knockdown in primary B cells. As shown in Fig.3 A, VentX expression in isolated primary B cells was efficiently downregulated by electroporation of VentX siRNA. Correspondingly, with the downregulation of VentX, there was significant decreases of p53, p21 and $p16^{ink4a}$ expression at both mRNA and protein levels (Fig.3 B and C). These data suggest that VentX plays a critical physiological role in regulating the expression of p53, p21 and $p16^{ink4a}$ in primary lymphocytes.

VentX induces irreversible cell growth arrest and a senescence phenotype

Originally described during normal aging of fibroblasts, senescence pathways have been shown to play critical roles in preventing tumorigenesis (17,36). Previous investigations showed that the p53-p21 and $p16^{ink4a}$-Rb tumor suppressor pathways are critical mediators of cellular senescence following various stimuli (16,17). Therefore, the findings that VentX activates both pathways in transient transfection studies prompted us to investigate whether VentX is able to exert a tumor suppression function by inducing cellular senescence in tumor cells. To address this question, we generated a stable U2OS cell line, U2OS/$VentX^{Tet}$, in which the expression of VentX is under control of a tetracycline (Tet)-inducible promoter. As shown in Fig.4 A, U2OS/$VentX^{Tet}$ cells express VentX in a Tet dependent manner. Upon addition of Tet, there was a strong induction of p53 and p21 expression in U2OS/$VentX^{Tet}$ cells (Fig.4 A). Consistent with its role as an inhibitor of cell proliferation, induction of VentX expression is associated with significant inhibition of U2OS/$VentX^{Tet}$ cell growth (Fig.4 B). Interestingly, VentX expression caused a striking morphological change with U2OS/$VentX^{Tet}$ cells appeared to be enlarged and flattened. These cells displayed positive staining for SA-β-gal, a characteristic marker of cellular senescence (Fig.4 C). Because irreversible growth arrest is a key feature of cellular senescence, we examined whether the growth inhibitory effect of VentX was irreversible. As shown in Fig.4 D, when Tet was removed from the culture medium after 3 days of exposure, the expression of VentX and p53 gradually decreased (Fig.4 D, left panel). However, the U2OS/$VentX^{Tet}$ cells remained positive for SA-β-gal activity (Fig.4 D, middle panel); Moreover, the growth arrest of the U2OS/$VentX^{Tet}$ cells continued (Fig.4 D, right panel). In addition to U2OS cells, stable VentX expression also provoked a senescent phenotype in HeLa cells (unpublished data).

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We next explored the potential dependence of VentX-induced cellular senescence on the p53 and $p16^{ink4a}$ pathways. For this purpose, we employed IMR90 primary human fibroblasts, which possess intact p53-p21 and $p16^{ink4a}$-Rb pathways and have been widely used as a model to study cellular senescence (37,38). As shown in Fig.4 E, inducible expression of VentX caused a typical senescence phenotype in early passage IMR90 cells as indicated by enlarged and flattened cell morphology, positive staining for SA-β-gal activity (Fig.4 E, left panel, control). First, p53 and $p16^{ink4a}$ shRNAs were used to down-regulate the expression p53 and $p16^{ink4a}$ and the efficacy of the p53 and $p16^{ink4a}$ shRNA was confirmed by western blot analysis (data not shown). We found that, while suppression of p53 or $p16^{ink4a}$ individually exerted little effects on VentX-induced senescence, the combined suppression of both p53 and p16 significantly ameliorated the senescence phenotype induced by VentX (Fig.4 E). Previous studies have showed that SV40 large T antigen (SV40 LT) inhibits the function of both p53 and $p16^{ink4a}$ pathways through direct binding to p53 and Rb (39). Consistently, we found that ectopic expression of SV40LT substantially ameliorated the cellular senescence induced by VentX (Fig.4 E). Collectively, our data suggest that VentX induces cellular senescence, at least in part, through p53/$p16^{ink4a}$ mediated mechanisms.

Knockdown of VentX results in amelioration of senescence and enhanced chemotherapeutic resistance in leukemic cells

Chemotherapy remains as a main therapeutic modality for leukemia patients. Chemotherapeutic drugs such as doxorubicin (Doxo) and *all-trans* retinoic acid (ATRA) have been show to induce premature senescence in tumor cells (19,40,41). Our previous study showed that lymphoblastic leukemia cells Nalm16 express a low level of VentX (9). Using the Nalm16 model, we found that VentX expression can be induced in Nalm16 cells by various chemotherapeutic agents (Fig.5 A). To determine whether VentX plays a role in mediating senescence of Nalm16 cells induced by chemotherapeutic agents, we downregulated VentX expression in Nalm16 cells with specific shRNA (Fig.5 B, left panel). We found that Nalm16 cells transfected with shVentX became more resistant to chemotherapeutic-agents induced growth inhibition (Fig.5 C) and showed enhanced cellular viability (Fig.5 D). Supporting a role for VentX in mediating chemotherapeutic agent-induced cellular senescence, Nalm16 cells transfected with shVentX displayed significantly lower SA-β-gal staining upon exposure to chemotherapeutic agents in comparison with the control cells (Fig.5 E-F). The shVentX transfected Nalm16 cells also showed stronger Ki67 staining, a cell proliferation marker (42) (Fig.5 G). In addition, down-regulation of VentX diminished the DNA damage agent camptothecin (CPT)-induced up-regulation of p53 (Fig.5 B, right panel), suggesting that VentX may also be involved in the DNA damage response.

Discussion

VentX is a human homeobox gene that shares strong homology to the Xenopus Vent gene family (9,43). Besides its ventralizing activity in the zebrafish model, little is known about the function of VentX (43). Our recent investigation found that VentX is a negative regulator of cell proliferation and a LEF/TCF-associated Wnt repressor (9). The present study was undertaken to further dissect the mechanisms of the VentX tumor suppression function. We screened a variety of proteins involved in cell cycle regulation, and identified novel downstream targets of VentX in the cell cycle regulatory machinery. We demonstrated that VentX was able to activate two cyclin-dependent kinase inhibitors: p21 and $p16^{ink4a}$. When ectopically expressed, VentX up-regulated p53-p21 and $p16^{ink4a}$ protein level in multiple cell lines. In contrast, knockdown of VentX in primary B cells led to reduced expression of p53-p21 and $p16^{ink4a}$ (Fig.3). Combined with other biochemical analyses (Luciferase reporter and ChIP, as shown in Fig.2), we conclude that VentX is a direct transcriptional activator of p53-p21 and $p16^{ink4a}$.

Our recent finding that VentX is an inhibitor of oncogenic Wnt/β-catenin signaling, and is downregulated in blood samples of chronic lymphoblastic leukemia patients, led us to propose that VentX is a putative tumor suppressor (9). Here, we found that VentX is a potent activator of p53/p21 and $p16^{ink4a}$/Rb tumor suppressor

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pathways, and is also downregulated in acute lymphoblastic leukemia samples (unpublished data). The current study further showed that VentX expression can be induced upon chemotherapeutic treatments. Consistent with a role as a tumor suppressor, we demonstrated that knockdown of VentX expression in lymphoblastic leukemic Nalm16 cells led to enhanced resistance of these cells to cytotoxic effects of chemotherapeutic agents. Thus, data from this and our previous studies strongly support the conclusion that VentX is a novel tumor suppressor and a cancer therapeutic target. Recently, Rawat et al. reported that knockdown of VentX in human acute myeloid leukemia cell lines inhibited their proliferative potential. It was suggested that specific functions of VentX may depend on cellular context (44).

VentX expression induced pronounced senescence phenotypes in several tumor cell lines and primary fibroblasts and downregulation of VentX was associated with a reduction in chemotherapeutic drugs-induced senescence in lymphoblastic leukemia cells. Our study indicated that p53/p21 and $p16^{ink4a}$/Rb, previously defined as key regulators of cellular senescence (16,17), may be important mediators of VentX induced senescence (Fig.4 E). Our findings are consistent with previous findings that Wnt inhibition activates cellular senescence pathway (14). Therefore, VentX appears to provide a link between Wnt inhibition and activation of p53 and $p16^{ink}$ senescence pathways. It should be noted, however, in contrast to oncogene-induced senescence, which is usually accompanied by mitogenic effects and activation of the DNA damage response (DDR) (37,45,46), VentX-induced senescence triggered immediate growth inhibition without evidence of DDR (lack of phospho-Chk1 Ser317/345 and Chk2 Thr68, unpublished data).

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Figure Legends

Figure 1. Effect of VentX transfection on the expression of cell cycle regulatory proteins. HEK293 cells grown in 6cm dishes were transfected with 5.0 μg pcDNA-VentX or empty pcDNA vector plasmid. At 48 hrs after transfection, whole cell lysates were prepared for western blot analysis with the indicated antibodies.

Figure 2. VentX trans-activates p53-p21 and $p16^{ink4a}$-Rb pathway. (A) Left panel: U2OS cells were transfected with pcDNA-VentX or empty pcDNA vector plasmid. At 48 hrs after transfection, whole cell lysates were prepared for western blot analysis of p53 and p21 expression levels. Right panel: U2OS cells were transfected as above. At 48 hrs after transfection, cells were treated with cycloheximide (CHX). Total cell lysates were harvested at indicated time points after CHX treatment and p53 protein level was determined by western blot analysis. (B). U2OS cells or Sw480 cells were transfected as above, and total RNA was isolated by Trizol method. The mRNA levels of p53 and p21 were determined by quantitative PCR for U2OS cells (left panel) and p53 mRNA level was determined for Sw480 cells (right panel). (C) Luciferase assays on extracts of U2OS cells transfected with pcDNA-VentX plasmid plus human *p53* (left panel) or *p21* (right panel) promoter luciferase reporters. The relative fold increase in luciferase activity compared with cells transfected with pcDNA empty vector was determined. Results show mean of standard error (SEM) from triplicate samples. (D) HeLa cells were transfected with empty pcDNA vector plasmid or increasing amount of pcDNA-VentX plasmid. At 48 hrs after transfection, whole cell lysates were prepared for western blot analysis of $p16^{ink4a}$ and Rb level (left panel). Total RNA was isolated by

the Trizol method and mRNA level of $p16^{ink4a}$ was determined by quantitative PCR (right panel). (E) Luciferase assays on extracts of HeLa cells transfected with pcDNA-VentX plasmid plus human *p16*^{ink4a} promoter luciferase reporter. The relative fold increase in luciferase activity compared with cells transfected with pcDNA empty vector was determined. Results show SEM of triplicates. (F) ChIP analysis showing association of VentX with *p53* and *p16*^{ink4a} promoter regions but not with the region 3.7kb upstream of the VentX binding site on *p53* promoter, immunoglobulin Cμ region and *GAPDH* promoter region. Increasing amount of input or precipitated DNA were used as templates for PCR analysis. (G) Schematic diagrams depict the putative homeodomain binding sites and the position of primers used for ChIP assay on the promoter regions of p53 an $p16^{ink4a}$. The primers amplify the regions that encompass the most proximal potential homeodomain binding sequences to the transcription start sites.

Figure 3. Knockdown of VentX in primary B cells is associated with decreased p53-p21 and $p16^{ink4a}$ expression. Human primary B cells isolated from three different donors were electroporated with VentX siRNA or control siRNA as described in Experimental procedures. Cells were then cultured in the presence of CD40 ligand and IL4 for 3 days. (A) mRNA level of VentX was determined by reverse transcription and Q-PCR. (B) mRNA level of p53, p21 and $p16^{ink4a}$ was determined by Q-PCR. (C). Whole cell lysates were prepared from B cells and used to probe protein levels of p53, p21 and $p16^{ink4a}$ with corresponding antibodies.

Figure 4. Stable expression of VentX induces senescence in tumor cells. (A) U2OS cells stably expressing VentX under the control of tetracycline (U2OS/$VentX^{Tet}$) were treated with 1μg/ml Tet for 48 hrs. The p53 and p21 levels in the total cell lysates were detected by western blot analysis. (B) U2OS/$VentX^{Tet}$ cells were plated at the density of 2×10^4 cells/well in 6-well plate. Cells were then treated or not treated with Tet for 5 days. Left panel: Crystal violet staining of U2OS cells at day 3 and day 5; Right panel: Proliferation curve of U2OS cells. (C) Staining for SA-β-gal activity at 5 days after Tet exposure. Left panel: Two representative images for senescent U2OS cells (+Tet) are shown; Right panel: Quantification of SA-β-gal positivity. Error bars represent the SEM of three experiments. (D) U2OS/$VentX^{Tet}$ cells were cultured in the presence of 1μg/ml Tet continuously for 7 days, or for 3 days followed by 4 days culture without Tet. Left panel: whole cell lysates were prepared at day 1, 3, 5 and 7 in the presence of Tet (lane 2-5) or at day 1, 2, 3 and 4 after Tet removal (lane 6-9). Lane 1 is the cell lysates from U2OS/$VentX^{Tet}$ cells cultured in absence of Tet. Western blots were performed with indicated antibodies. Middle panel: SA-β-gal staining of U2OS cells. Right panel: growth curves of U2OS/$VentX^{Tet}$ cells cultured in the absence of Tet (no Tet), in the presence of Tet for consecutive 7 days (with Tet), or for 3 days followed by 4 days culture without Tet (Tet removed at day 3). (E) Early passage of Tet-inducible IMR90 cell lines stably expressing GFP or GFP.VentX were generated as described in experimental procedures. These cell lines were then infected with indicated retroviruses followed by a 3-days antibiotic selection. Uninfected cells were used as control. Tet was then added into cultures to induce VentX expression and cellular senescence was analyzed at 6 days after Tet addition. Left panel: A representative image of SA-β-gal staining of IMR90 cells. Right panel: Quantification of SA-β-gal positivity.

Figure 5. Knockdown of VentX leads to reduced senescence and enhanced chemotherapeutic resistance in leukemia cells. (A) Nalm 16 lymphoblastic leukemia cells were subjected for the following treatments: 800nM All-*trans*-Retinoic Acid (ATRA) for 10 days (fresh ATRA was added every 3 days), 100ng/ml Doxorubicin (Doxo) for 4 days, 5μM 5-fluorouracil (5-FU) for 3 days. The mRNA level of VentX was determined by conventional RT-PCR (upper panel) and quantitative RT-PCR (lower panel). (B) Left panel: RT-PCR showing knockdown of VentX by small hairpin RNA (shRNA) and western blot showing downregulation of p53 by VentX knockdown in Nalm16 cells. Right panel: Nalm16 cells were treated with 1μM camptothecin (CPT) for 2 days or mock treated. The mRNA and protein levels of p53 were determined by RT-PCR and western blot, respectively. (C) Effect of VentX knockdown on the

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growth curve of Nalm 16 cells after chemotherapeutic treatments. 0.5M Nalm 16 cells were treated with 800nM ATRA for 6 days, 100ng/ml Doxo for 4 days or mock treated. Viable cells were counted every day and plotted. (D) 1.0M Nalm 16 cells were subjected for the following treatments: 800nM ATRA for 10 days, 100ng/ml Doxo for 4 days, and 5μM 5-FU for 3 days. The viability of Nalm16 cells (control and VentX knockdown) after drug treatments were determined as described in experimental procedures. (E) A representative image of SA-β-gal staining in control versus VentX suppressed Nalm16 cells after treatments with ATRA for 10 days or Doxo for 4 days. (F) Quantitative analysis of SA-β-gal positivity in control versus VentX suppressed Nalm16 cells at different time points after drug treatments. (G) Ki67 staining in control versus VentX suppressed Nalm16 cells after treatments with ATRA for 5 days or Doxo for 2 days.

References

1. Espada, J., Calvo, M. B., Diaz-Prado, S., and Medina, V. (2009) *Clin Transl Oncol* **11**, 411-427
2. Reya, T., and Clevers, H. (2005) *Nature* **434**, 843-850
3. Zhao, C., Blum, J., Chen, A., Kwon, H. Y., Jung, S. H., Cook, J. M., Lagoo, A., and Reya, T. (2007) *Cancer Cell* **12**, 528-541
4. Simon, M., Grandage, V. L., Linch, D. C., and Khwaja, A. (2005) *Oncogene* **24**, 2410-2420
5. Ysebaert, L., Chicanne, G., Demur, C., De Toni, F., Prade-Houdellier, N., Ruidavets, J. B., Mansat-De Mas, V., Rigal-Huguet, F., Laurent, G., Payrastre, B., Manenti, S., and Racaud-Sultan, C. (2006) *Leukemia* **20**, 1211-1216
6. Lu, D., Zhao, Y., Tawatao, R., Cottam, H. B., Sen, M., Leoni, L. M., Kipps, T. J., Corr, M., and Carson, D. A. (2004) *Proc Natl Acad Sci U S A* **101**, 3118-3123
7. Behrens, J., von Kries, J. P., Kuhl, M., Bruhn, L., Wedlich, D., Grosschedl, R., and Birchmeier, W. (1996) *Nature* **382**, 638-642
8. Huber, O., Korn, R., McLaughlin, J., Ohsugi, M., Herrmann, B. G., and Kemler, R. (1996) *Mech Dev* **59**, 3-10
9. Gao, H., Le, Y., Wu, X., Silberstein, L. E., Giese, R. W., and Zhu, Z. (2010) *Cancer Res* **70**, 202-211
10. Gao, H., Wu, B., Giese, R., and Zhu, Z. (2007) *Cell Res* **17**, 345-356
11. Bignell, G. R., Greenman, C. D., Davies, H., Butler, A. P., Edkins, S., Andrews, J. M., Buck, G., Chen, L., Beare, D., Latimer, C., Widaa, S., Hinton, J., Fahey, C., Fu, B., Swamy, S., Dalgliesh, G. L., Teh, B. T., Deloukas, P., Yang, F., Campbell, P. J., Futreal, P. A., and Stratton, M. R. (2010) *Nature* **463**, 893-898
12. Basso, K., Margolin, A. A., Stolovitzky, G., Klein, U., Dalla-Favera, R., and Califano, A. (2005) *Nat Genet* **37**, 382-390
13. Choi, Y. L., Tsukasaki, K., O'Neill, M. C., Yamada, Y., Onimaru, Y., Matsumoto, K., Ohashi, J., Yamashita, Y., Tsutsumi, S., Kaneda, R., Takada, S., Aburatani, H., Kamihira, S., Nakamura, T., Tomonaga, M., and Mano, H. (2007) *Oncogene* **26**, 1245-1255
14. Ye, X., Zerlanko, B., Kennedy, A., Banumathy, G., Zhang, R., and Adams, P. D. (2007) *Mol Cell* **27**, 183-196
15. Hayflick, L., and Moorhead, P. S. (1961) *Exp Cell Res* **25**, 585-621
16. Campisi, J., and d'Adda di Fagagna, F. (2007) *Nat Rev Mol Cell Biol* **8**, 729-740
17. Collado, M., Blasco, M. A., and Serrano, M. (2007) *Cell* **130**, 223-233
18. te Poele, R. H., Okorokov, A. L., Jardine, L., Cummings, J., and Joel, S. P. (2002) *Cancer Res* **62**, 1876-1883
19. Roninson, I. B. (2003) *Cancer Res* **63**, 2705-2715
20. Schmitt, C. A., Fridman, J. S., Yang, M., Lee, S., Baranov, E., Hoffman, R. M., and Lowe, S. W. (2002) *Cell* **109**, 335-346
21. Kahlem, P., Dorken, B., and Schmitt, C. A. (2004) *J Clin Invest* **113**, 169-174
22. Funayama, R., and Ishikawa, F. (2007) *Chromosoma* **116**, 431-440

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23. Narita, M., Nunez, S., Heard, E., Narita, M., Lin, A. W., Hearn, S. A., Spector, D. L., Hannon, G. J., and Lowe, S. W. (2003) *Cell* **113**, 703-716
24. Abbas, T., and Dutta, A. (2009) *Nat Rev Cancer* **9**, 400-414
25. Polager, S., and Ginsberg, D. (2009) *Nat Rev Cancer* **9**, 738-748
26. Sherr, C. J., and McCormick, F. (2002) *Cancer Cell* **2**, 103-112
27. Pui, C. H., Relling, M. V., and Downing, J. R. (2004) *N Engl J Med* **350**, 1535-1548
28. Agirre, X., Novo, F. J., Calasanz, M. J., Larrayoz, M. J., Lahortiga, I., Valganon, M., Garcia-Delgado, M., and Vizmanos, J. L. (2003) *Mol Carcinog* **38**, 201-208
29. Omura-Minamisawa, M., Diccianni, M. B., Batova, A., Chang, R. C., Bridgeman, L. J., Yu, J., Pullen, J., Bowman, W. P., and Yu, A. L. (2000) *Clin Cancer Res* **6**, 1219-1228
30. Hara, E., Smith, R., Parry, D., Tahara, H., Stone, S., and Peters, G. (1996) *Mol Cell Biol* **16**, 859-867
31. Raman, V., Martensen, S. A., Reisman, D., Evron, E., Odenwald, W. F., Jaffee, E., Marks, J., and Sukumar, S. (2000) *Nature* **405**, 974-978
32. Luo, J., Su, F., Chen, D., Shiloh, A., and Gu, W. (2000) *Nature* **408**, 377-381
33. Rochette, P. J., Bastien, N., Lavoie, J., Guerin, S. L., and Drouin, R. (2005) *J Mol Biol* **352**, 44-57
34. Goodwin, E. C., and DiMaio, D. (2000) *Proc Natl Acad Sci U S A* **97**, 12513-12518
35. Knudsen, E. S., and Wang, J. Y. (1997) *Mol Cell Biol* **17**, 5771-5783
36. Prieur, A., and Peeper, D. S. (2008) *Curr Opin Cell Biol* **20**, 150-155
37. Lin, A. W., Barradas, M., Stone, J. C., van Aelst, L., Serrano, M., and Lowe, S. W. (1998) *Genes Dev* **12**, 3008-3019
38. Serrano, M., Lin, A. W., McCurrach, M. E., Beach, D., and Lowe, S. W. (1997) *Cell* **88**, 593-602
39. Ahuja, D., Saenz-Robles, M. T., and Pipas, J. M. (2005) *Oncogene* **24**, 7729-7745
40. Roninson, I. B., and Dokmanovic, M. (2003) *J Cell Biochem* **88**, 83-94
41. Chen, Y., Dokmanovic, M., Stein, W. D., Ardecky, R. J., and Roninson, I. B. (2006) *Cancer Res* **66**, 8749-8761
42. Scholzen, T., and Gerdes, J. (2000) *J Cell Physiol* **182**, 311-322
43. Moretti, P. A., Davidson, A. J., Baker, E., Lilley, B., Zon, L. I., and D'Andrea, R. J. (2001) *Genomics* **76**, 21-29
44. Rawat, V. P., Arseni, N., Ahmed, F., Mulaw, M. A., Thoene, S., Heilmeier, B., Sadlon, T., D'Andrea, R. J., Hiddemann, W., Bohlander, S. K., Buske, C., and Feuring-Buske, M. (2010) *Proc Natl Acad Sci U S A* **107**, 16946-16951
45. Di Micco, R., Fumagalli, M., Cicalese, A., Piccinin, S., Gasparini, P., Luise, C., Schurra, C., Garre, M., Nuciforo, P. G., Bensimon, A., Maestro, R., Pelicci, P. G., and d'Adda di Fagagna, F. (2006) *Nature* **444**, 638-642
46. Hemann, M. T., and Narita, M. (2007) *Genes Dev* **21**, 1-5

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Fig.1

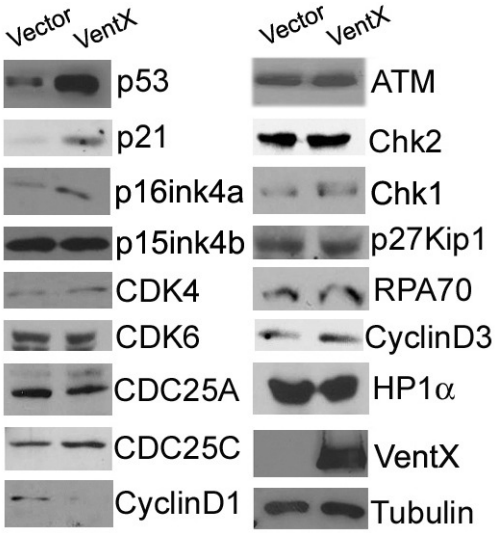

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Fig.2

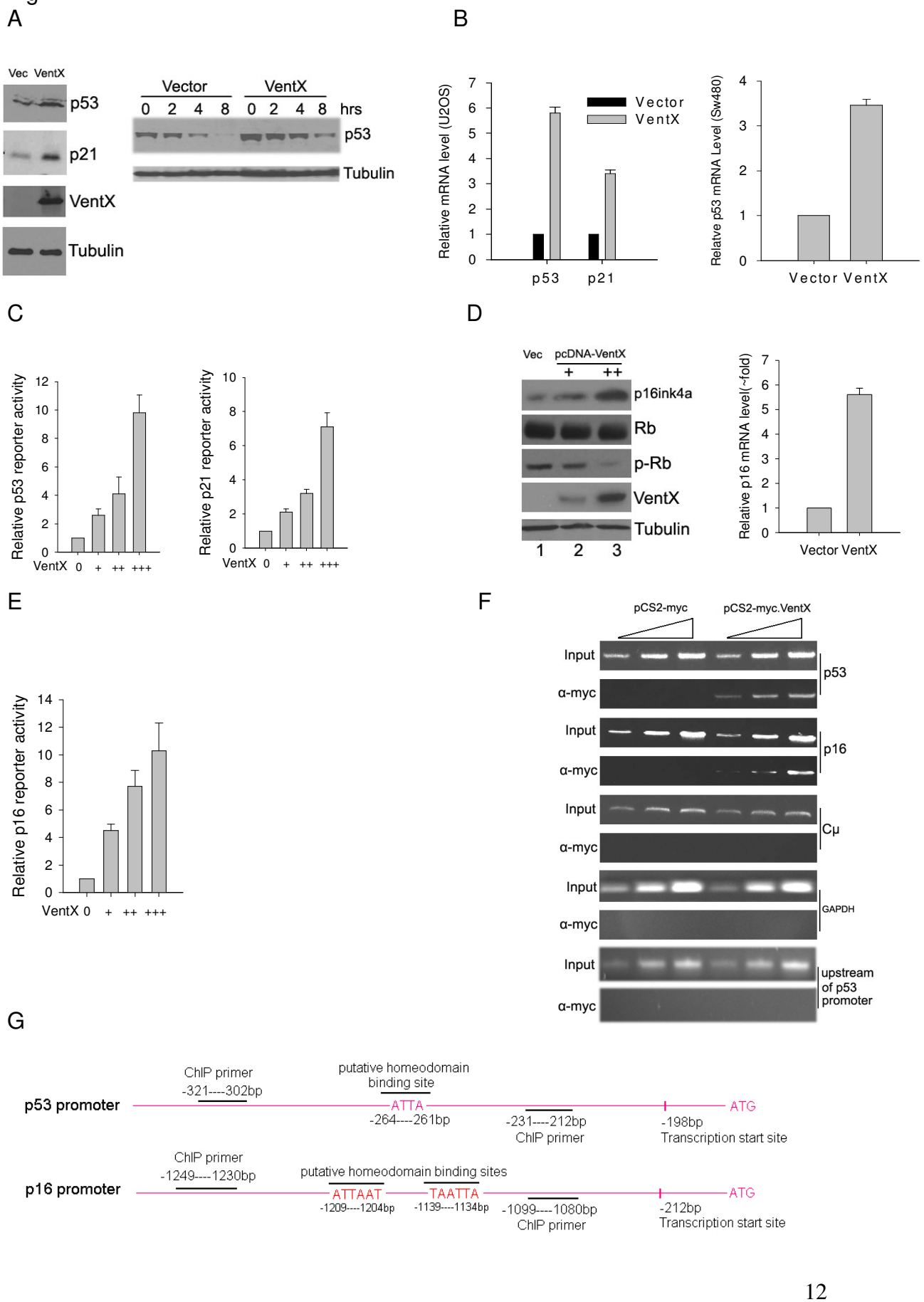

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Fig.3

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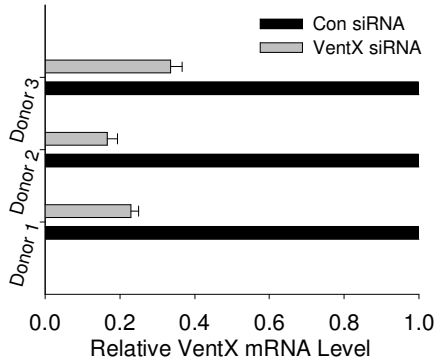

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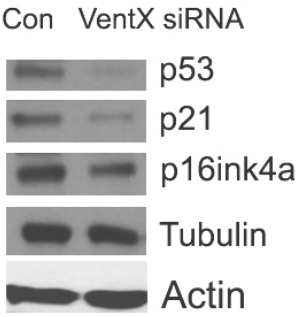

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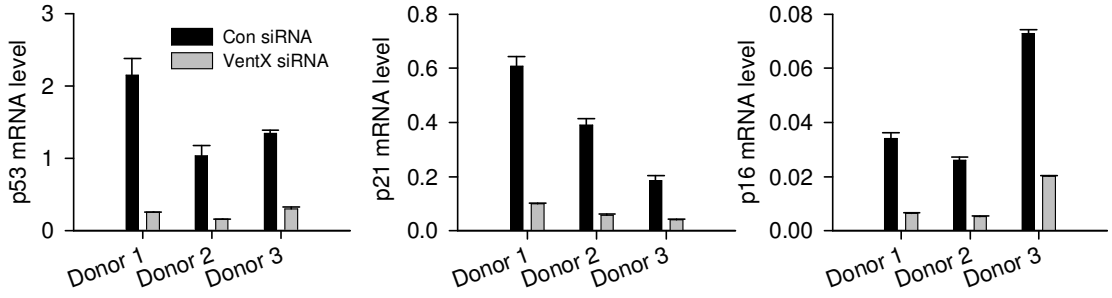

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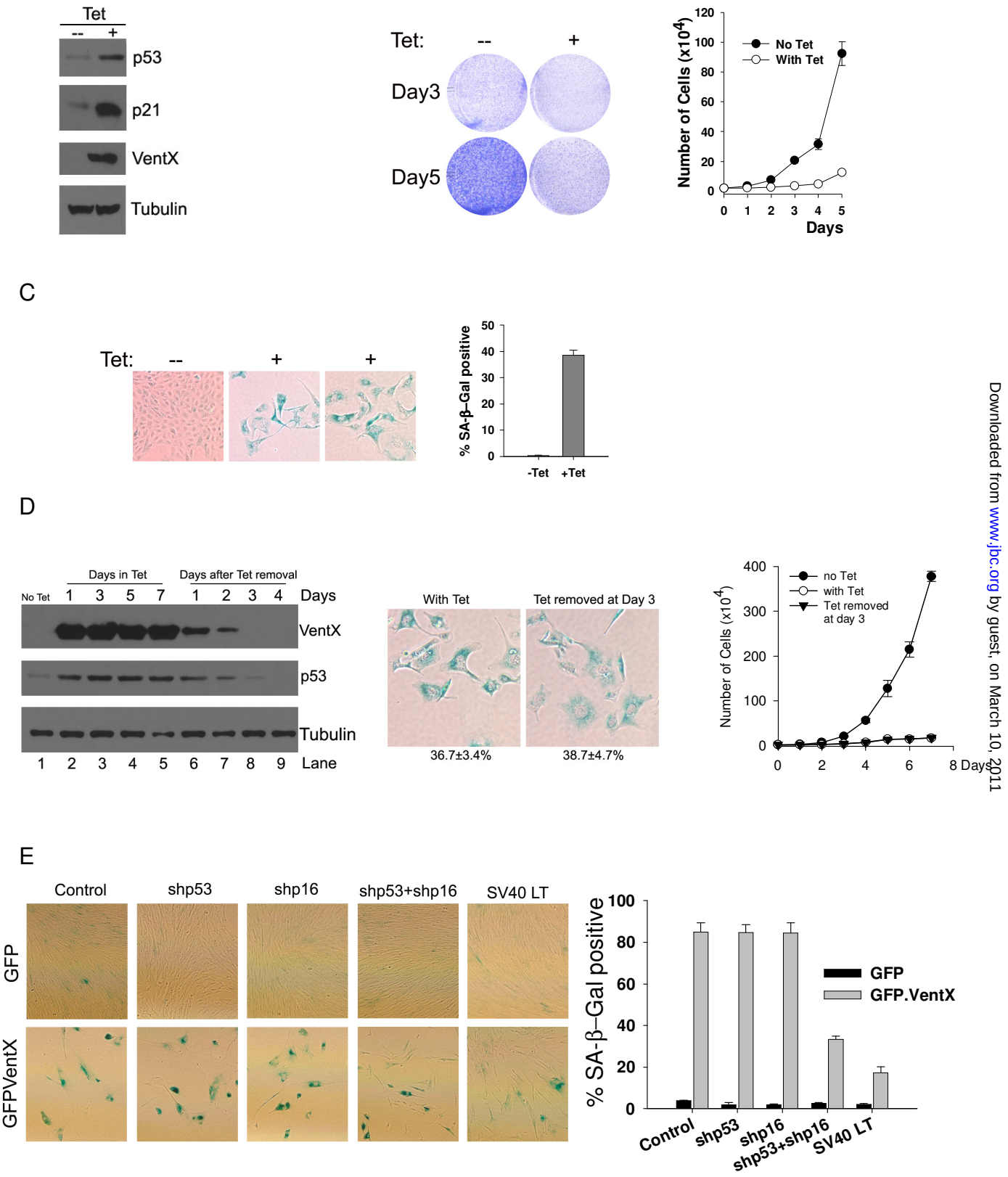

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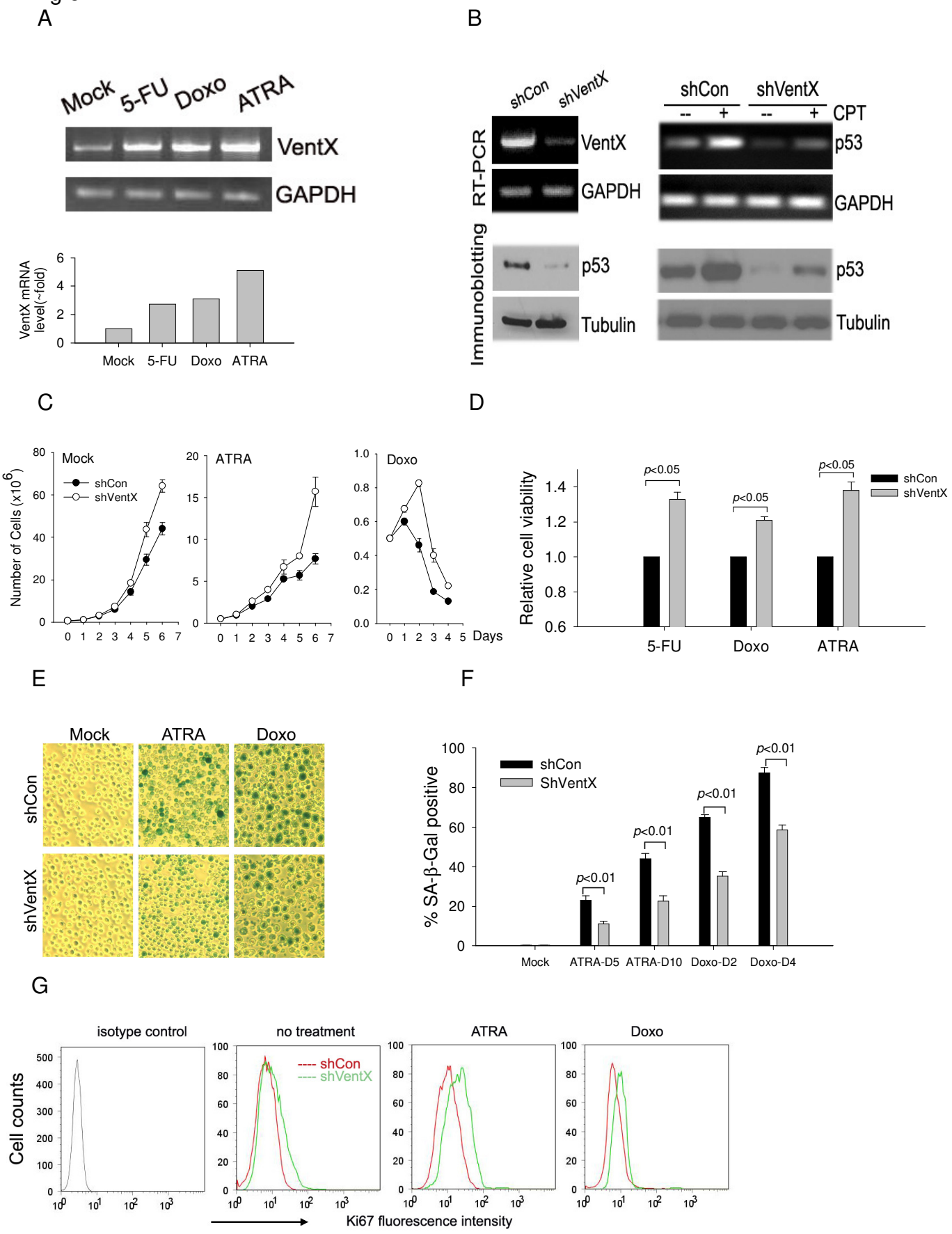
Fig.5



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