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Exclusion of c-Abl from the Nucleus Restrains the p73 Tumor Suppression Function*

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The p73α protein is a functional homolog of the p53 tumor suppressor. Although the *TP53* gene is frequently mutated in human cancers, the *TP73* gene is rarely inactivated. We have found that p73α is highly expressed in a significant fraction of anaplastic thyroid cancer, whereas it is not detectable in normal thyroid epithelial cells or in papillary and follicular thyroid cancer cells. Interestingly, the tumor suppression function of p73α is actively restrained in anaplastic thyroid cancer cells. We have also found that c-Abl tyrosine kinase, an activator of p73, is excluded from the nucleus of p73α-positive thyroid cancer cells; whereas c-Abl undergoes nuclear-cytoplasmic shuttling in normal thyroid and p73-negative thyroid cancer cells. We constructed an AblNuk-FK506-binding protein (FKBP) fusion protein to enforce the nuclear accumulation of an inducible Abl kinase. Activation of this nuclear AblNuk-FKBP by dimerization with AP20187 in anaplastic thyroid cancer cells increased the levels of p73α and p21Cip1 and caused p73-dependent apoptosis. These results suggest subcellular segregation of c-Abl from p73 to be a strategy for disrupting the tumor suppression function of p73α.

The inactivation of tumor suppressors through genetic and/or epigenetic mechanisms is important to cancer development. For example, the tumor suppressor p53 is inactivated in human cancer by mutation of its gene or, alternatively, by the increased expression of its inhibitor, mouse double minute 2 (1). The p53 tumor suppressor belongs to a family of transcription factors with related function. This family includes three members, p53, p63, and p73 (2, 3). The *TP73* gene was cloned as a probable suppressor of neuroblastoma (4). The ectopic expression of p73 can stimulate p53-regulated genes such as *p21Cip1*[1] and *Bax* to cause growth arrest and apoptosis (5). In response to DNA damage, p73 is activated to induce apoptosis (6–8). The p73 function is also required for E2F1-dependent apoptosis in fibroblasts and activated T-cells (9, 10). Although p73-knockout cells exhibit apoptosis defects, the p73-knockout mice are not prone to spontaneous tumor development (11). Instead, the p73-knockout mice are runt and exhibit developmental defects, which might interfere with tumor formation. Moreover, p73 and p63 have redundant function in DNA damage-induced apoptosis (12). Thus, the knockout of p73 alone might not be sufficient to generate spontaneous tumors. The p73/p63 double knockout causes embryonic lethality, precluding the analysis of tumor development in these mice (12). Although the tumor suppression function of p73 has not been demonstrated in the mice, there is no question that p73 can inhibit cell proliferation and promote apoptosis.

The *TP73* gene encodes several alternatively spliced variants, including the ΔN-p73 (11, 13, 14). The ΔN-p73 is transcribed from an internal promoter in intron 3 and lacks the N-terminal transactivation domain that is required for p73 to induce apoptosis (11, 13, 14). The ΔN-p73 can interfere with the function of p53 and p73, and its expression has been implicated in tumor development (5, 15, 16). In sporadic human cancer cells, the *TP73* gene is seldom mutated (5). Suppression of *TP73* gene expression by hypermethylation has been described in neuroblastoma and leukemia cells (17–19). However, *TP73* expression is observed in breast, lung, bladder, and liver cancers (20–25). This raises the question of whether alternative mechanisms other than the repression of *TP73* expression are used by cancer cells to control the tumor suppression function of p73.

Previous studies (6–8, 26) have established c-Abl to be an obligatory activator of p73 in genotoxic response. The murine c-*Abl* gene was identified as the cellular homologue of the v-*Abl* oncogene of the Abelson murine leukemia virus (27). The oncogenic potential of human c-*ABL* is demonstrated by *BCR-ABL* in human chronic myelogenous leukemia (28). The c-*Abl* gene encodes a non-receptor tyrosine kinase that can shuttle between the cytoplasmic and the nuclear compartments (29). In the cytoplasm, c-Abl interacts with actin (30–32) and regulates the F-actin dynamics in response to extracellular signals (33, 34). In the nucleus, c-Abl interacts with retinoblastoma 1 (35–37), ataxia telangiectasia mutated (38, 39), p73 (6–8),

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[1] The abbreviations used are: p21Cip1, cyclin-dependent kinase inhibitor 1A (p21); c-Abl, normal cellular homologue of the Abelson murine leukemia oncogene; AblNuk, a constitutively nuclear Abl mutant; FKBPv, an engineered AP20187-binding domain that is different from the natural FKBP; AP20187, a synthetic chemical dimerizer inducing homodimerization of Fv-domain containing fusion proteins; LMB, leptomycin B; NLS, nuclear localization sequence; GFP, green fluorescent protein; FKBP, FK506-binding protein; MEF, mouse embryo fibroblasts; HA, hemagglutinin; RT, reverse transcription.

breast cancer 1 (40), and RNA polymerase II (41–43). Activation of the nuclear c-Abl tyrosine kinase by DNA damage occurs in S-phase cells (44) through ataxia telangiectasia mutation (38, 39), and can lead to the induction of apoptosis (45). That the nuclear c-Abl can stimulate apoptosis is best illustrated by the ability of BCR-ABL kinase to kill cells when this oncogenic protein is trapped in the nucleus (46).

In this study, we have found that the p73α protein is expressed in cells derived from anaplastic thyroid cancer, which is the most malignant form of thyroid carcinoma. In these p73α-expressing cancer cells, c-Abl is excluded from the nucleus. These results show that the apoptotic function of the c-Abl/p73 pathway can be controlled by the subcellular segregation of these two proteins and suggest an alternative mechanism to inactivate the p73 tumor suppression function.

EXPERIMENTAL PROCEDURES

Cells—Human thyroid papillary cancer cell lines CA 300 and CA 301 (established in our laboratory), follicular cancer cell lines FRO and WRO (provided by A. Fusco, Naples, Italy), anaplastic cancer cell lines KAK and ARO (provided by A. Fusco, Naples, Italy), and normal thyroid primary cultures were grown in RPMI 1640/10% fetal bovine serum. C643 anaplastic cell line (provided by H. Heldin, Uppsala, Sweden) was grown in minimum Eagle's medium/10% fetal bovine serum. Saos-2 cells were cultured in Dulbecco's modified Eagle's medium/10% fetal bovine serum. p53(−/−), Abl(−/−), or p73(−/−) mouse embryo fibroblasts (MEFs) were routinely cultured in Dulbecco's modified Eagle's medium/10% fetal bovine serum. All transfections were performed with FuGENE 6 (Roche Applied Science).

Immunoprecipitation and Immunoblotting—Cell lysates were prepared in radioimmune precipitation assay buffer (150 mM NaCl, 10 mM Tris, pH 7.2, 0.1% SDS, 1.0% Triton X-100, 1% deoxycholate, 5 mM EDTA). For immunoprecpitations, 1 mg of total protein was used. Immunoprecipitates were fractionated on SDS-PAGE and then transferred to polyvinylidene difluoride, which were immunoblotted with primary antibodies and horseradish peroxidase-conjugated secondary antibodies and visualized by chemiluminescence. The following antibodies were used: anti-p73 clones 429 and 1288 from Imgenex, clone ER-15 from Neomarker; anti-Abl 8E9 (BD Biosciences); anti-actin (Sigma) anti-phosphotyrosine 4G10 (USB); and anti-tubulin, anti-p21Cip1, anti-p53, and anti-histone H2B were from Santa Cruz Biotechnology Inc.

Immunofluorescence—Cells were fixed in 4% formaldehyde, permeabilized with phosphate-buffered saline/0.1% Triton X-100, blocked with phosphate-buffered saline/10% normal goat serum, and incubated with primary antibodies (anti-HA, anti-p21Cip1, or anti-Abl) for 1 h. Cells were then incubated with Cy3 or fluorescein isothiocyanate-conjugated secondary antibodies for 1–2 h. To visualize actin stress fibers, cells were incubated with Alexa Fluor 488-conjugated phalloidin for an additional 30 min. Cells were finally counterstained with Hoechst to visualize the nuclei. Deconvolution microscopy was performed with a Delta Vision System.

Transcript Analysis by RT-PCR—Total RNAs were prepared from cultured cells using Trizol (Invitrogen). RT-PCR was performed with a One-Step RT-PCR kit (Invitrogen) using forward primer 5′-CGGGACGGACGCCGATG-3′ and reverse primer 5′-CTTGGCGATCTGGCAGTAG-3′ annealing to human TP73 exon 1 and 5, respectively.

Subcellular Fractionation—Cells were incubated with or without 10 nM leptomycin B (LMB) overnight. Cell pellets for fractionation were resuspended in hypotonic buffer (10 mM Tris, pH 8.0, 10 mM KCl, 2 mM phenylmethylsulfonyl fluoride plus protease inhibitor mixture) to allow cell swelling for 2 min at 4 °C. Then, Nonidet P-40 was added to a final concentration of 0.4%. Samples were centrifuged at 2,000 rpm for 5 min at 4 °C and the supernatants collected as the cytoplasmic fractions. Pellets containing cell nuclei were washed once with hypotonic buffer and then extracted with high salt lysis Buffer (50 mM Tris pH 8.0, 5 mM EDTA, pH 8.0, 1% Nonidet P-40, 1% sodium deoxycolate, 0.025% SDS, 400 mM NaCl, 2 mM phenylmethylsulfonyl fluoride plus protease inhibitor mixture). Equal amount of proteins was loaded onto a 7.5% SDS-acrylamide gel, transferred onto polyvinylidene difluoride membranes, and blotted with anti-Abl, anti-tubulin, and anti-histone-2B antibodies.

Plasmid Construction—To construct AblNuk, Abl nuclear export signal was inactivated by a point mutation as described in Ref. 47. Fv, a modified version of the FK506 binding domain of FK506-binding protein (FKBP) was derived from pC4M-Fv2E vector provided by ARIAD Inc. Two copies of Fv were fused in-frame to the C terminus of c-Abl. An HA tag was placed in-frame at the end of the last copy of Fv. Two complementary oligonucleotides containing three contiguous copies of

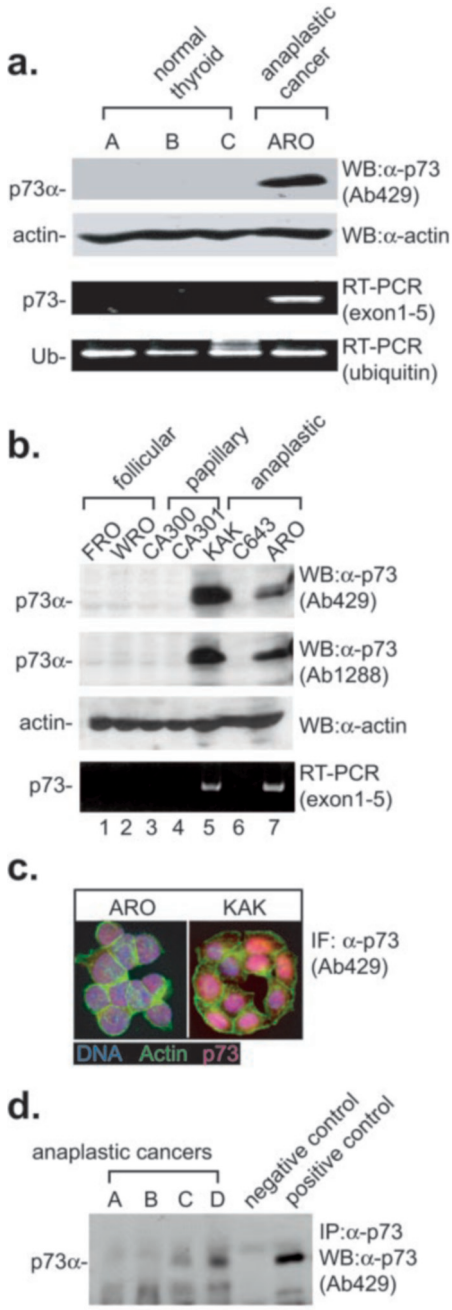


FIG. 1. **Expression of p73 in normal and cancerous thyroid cells.** *a*, total extracts of normal thyroid epithelial cells from primary culture (*A–C*) and anaplastic thyroid cancer cells ARO were immunoblotted with an anti-p73 or an anti-actin (*top two panels*). Total RNA was prepared from these cells and subjected to RT-PCR with primers that amplify exons 1–5 of human *TP73* gene or ubiquitin gene (*bottom two panels*). *b*, total extracts from follicular (*FRO* and *WRO*), papillary (*CA300* and *CA301*), and anaplastic (*KAK*, *C643*, and *ARO*) thyroid cancer cell lines were immunoblotted with two different anti-p73. The same blot was probed with an anti-actin. RT-PCR was performed on total RNA with primers that amplify exons 1–5 of the human *TP73* gene. *c*, anaplastic thyroid cancer cells (ARO and KAK) were fixed and stained with anti-p73 followed by Cy3 (*red*) conjugate secondary antibody. Cells were also stained with fluorescein isothiocyanate-phalloidin to label F-Actin (*green*) and Hoechst dye to label DNA (*blue*). *d*, protein extracts from tissue specimens of four human anaplastic thyroid tumors were immunoprecipitated with anti-p73 polyclonal antibody (G. Sun and J. Y. J. Wang, unpublished observations) and immunoblotted with anti-p73 monoclonal. ARO cell lysate was used as positive control, and CA301 cell lysate as negative control.

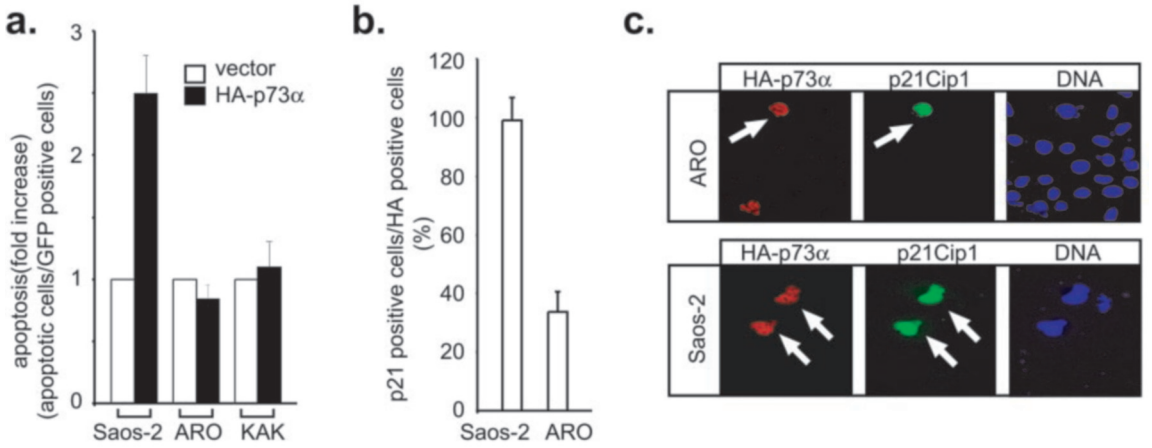


FIG. 2. **Tumor supression function of p73α is repressed in ARO and KAK cells.** *a*, the indicated cells were transfected with a plasmid expressing HA-tagged full-length p73α. An H2B-GFP-encoding plasmid was co-transfected to aid the identification of transfected cells. The percentage of apoptotic cells (with fragmented nuclei) among GFP-positive cells was scored as a measure of apoptosis. *b*, the indicated cells transfected with HA-p73α were fixed and stained with an anti-HA (*red*) and anti-p21Cip1 (*green*) and Hoechst (*blue*). The *bar graph* represents the percentage of p21-positive cells among HA-positive cells. *c*, representative images for *b*.

SV40 NLS (PKKKRAKV) were annealed and digested with *Sal*I. The digested oligonucleotides were then cloned in-frame to the *Sal*I site of mouse c-*Abl* cDNA.

Apoptosis Assay in Transiently Transfected Cells—Cells were seeded onto coverslips and either transfected with empty vector or with the indicated constructs. A plasmid expresing H2B-GFP was included in all transfection mixtures to mark the transfected cells. The transfected cells were either left untreated (−) or treated (+) with 50 nM chemical dimerizer AP20187 (ARIAD Inc., Cambridge, MA) for up to 24 h. Cells were then fixed and stained with Hoechst 33342. Apoptotic cells were scored based upon their fragmented nuclei and condensed chromosomes. Percentages of apoptotic cells among GFP-positive cell population were scored.

Stable Expression of AblNuk-FKBP and Treatment with Dimerizer—ARO and KAK cells were infected with vesicular stomatitis virus G protein (VSV-G)-pseudotyped retrovius expressing the pMSCV-vector or AblNuk-FKBP. Infected cultures were expanded without antibiotics selection (see "Results"). Immunofluorescence using anti-HA showed between 40–50% of cells in the infected cultures to express AblNuk-FKBP. Mock and AblNuk-FKBP-infected cultures were seeded onto cover slips and treated with 50 nM dimerizer AP20187 (ARIAD Inc.) for 24 h. Cells were then fixed and stained with monoclonal anti-HA antibody (BabCO, Richmond, CA), then with Hoechst 33342. Anti-HA-positive nuclei were counted, and the percent of HA (+) nuclei was determined for each treatment condition.

RESULTS

Expression of p73 in Anaplastic Thyroid Cancer Cells—We performed RT-PCR using primers derived from exon 1 and exon 5 of the *TP73* gene to detect the expression of transactivation-positive p73 (Fig. 1*a*). This RT-PCR strategy did not detect any signal in normal thyroid cells (Fig. 1*a*, *panels A–C*) or in follicular or papillary thyroid cancer cells (Fig. 1*b*, *lanes 1–4*). By contrast, in two of three anaplastic thyroid cancer cell lines, RT-PCR detected a strong p73 signal (Fig. 1, *a*, *ARO*, and *b*, *lanes 5* and *7*). The authenticity of the RT-PCR products was confirmed by nucleotide sequencing (not shown).

Expression of the p73 protein was confirmed with three monoclonal anti-p73 antibodies, directed at the transactivating domain (clone 429), the DNA binding domain (clone 1288), and the C-terminal region of the α/β isoforms (clone ER15) (not shown) (48). Reactivity of a 73 kDa protein band with these three antibodies plus size comparison with recombinant p73α, p73β, p73γ, and p73δ proteins established that p73α is expressed in KAK and ARO cells (Fig. 1, *a* and *b* and data not shown). The p73α protein was not detected in primary thyroid epithelial cells (Fig. 1*a*, *panels A–C*) or in follicular and papillary thyroid cancer cells (Fig. 1*b*, *lanes 1–4*). Indirect immunofluorescence staining with anti-p73 showed it to be localized in the nucleus of ARO and KAK cells (Fig. 1*c*). We also found p73α to be expressed in two of four anaplastic cancer tissues examined (Fig. 1*d*). These results showed that p73α is up-regulated in a significant fraction of anaplastic thyroid cancer.

Tumor Suppression Function of p73α Is Repressed in Anaplastic Thyroid Cancer Cells—To determine whether the anaplastic thyroid cancer cells have developed strategies to restrain the tumor suppression function of p73α, we tested if these cells can respond to ectopically expressed p73α (HA-p73α). As a control, we also expressed HA-p73α in Saos-2, an osteosarcoma cell line that does not express p73 (not shown). Consistent with previous results (49), HA-p73α caused apoptosis in Saos-2 cells (Fig. 2*a*). However, HA-p73α did not induce apoptosis in ARO or KAK cells (Fig. 2*a*). We also found that HA-p73α caused the cleavage of pro-caspase 3 in Saos-2 and CA301 cells, but not in ARO or KAK cells (not shown). The ectopic expression of HA-p73α induced the expression of p21Cip1 in every transfected Saos-2 cell (Fig. 2, *b* and *c*). By contrast, induction of p21Cip1 was observed in only 30% of ARO cells transfected with HA-p73α (Fig. 2, *b* and *c*). These results suggest that the function of p73α in transactivating p21Cip1 and in inducing apoptosis is compromised in the ARO and KAK cells.

Reduced Nuclear Entry of c-Abl in Anaplastic Thyroid Cancer Cells—Previous studies (7, 8) have shown that the apoptosis function of p73 is compromised in cells derived from the *Abl*-knockout mice. We therefore examined the expression, the tyrosine kinase activity, and the subcellular distribution of c-ABL in thyroid cells. The c-ABL protein is expressed at similar levels in primary thyroid epithelial cells and thyroid cancer cells (Fig. 3*a*). Immune complex kinase assays showed that c-ABL isolated from the different thyroid cancer cells to contain similar levels of kinase activity (not shown). Indirect immunofluorescence staining of primary thyroid cells and thyroid cancer cells revealed a predominantly cytoplasmic distribution of the c-ABL protein (Fig. 3*b*, *−LMB panels*). The c-ABL protein contains nuclear localization and nuclear export signals (29, 47). We have previously shown that the nuclear export of Abl can be inhibited by Leptomycin B (LMB) (29, 46), which inactivates the nuclear export protein Exportin-1. Treatment with LMB led to the nuclear accumulation of c-ABL in primary thyroid cells and WRO cells derived from a follicular thyroid carcinoma (Fig. 3*b*, *+LMB panels*). These results showed that c-ABL enters the nucleus of thyroid cells, and its predominant cytoplasmic localization is due to nuclear export. Interestingly,

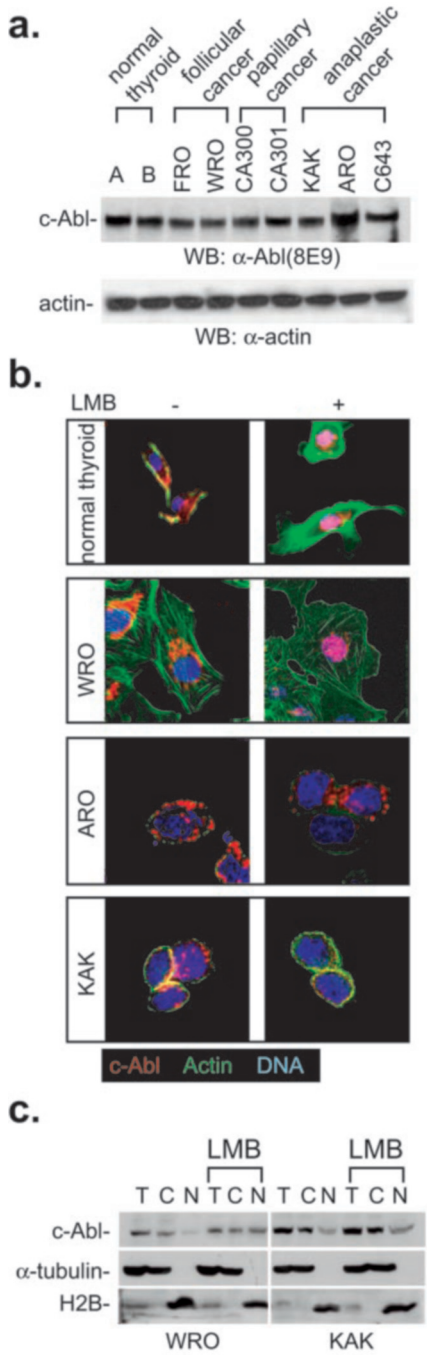


FIG. 3. **Reduced c-ABL nuclear entry in ARO and KAK cells.** *a*, total lysates prepared from normal thyroid epithelia (*A* and *B*), follicular (*FRO* and *WRO*), papillary (*CA300* and *CA301*), and anaplastic (*KAK*, *ARO*, and *C643*) thyroid cancer cells were immunoblotted with anti-Abl and an anti-actin. *b*, normal thyroid epithelial cells, follicular (*WRO*), and anaplastic (*ARO* and *KAK*) thyroid cancer cells were either left untreated (−) or treated with 10 nM LMB (+) for 12 h before being fixed and stained with anti-Abl (*red*), phalloidin (*green*), and Hoechst (*blue*). The images were captured with a deconvolution microscope. *c*, follicular (*WRO*) and anaplastic (*KAK*) thyroid cancer cells were either left untreated or treated with 10 nM LMB for 12 h before fractionation. Equal amounts of proteins in total lysates (*T*), nuclear (*N*), and cytoplasmic (*C*) extracts were immunoblotted with anti-Abl, anti-tubulin, or anti-histone H2B antibodies.

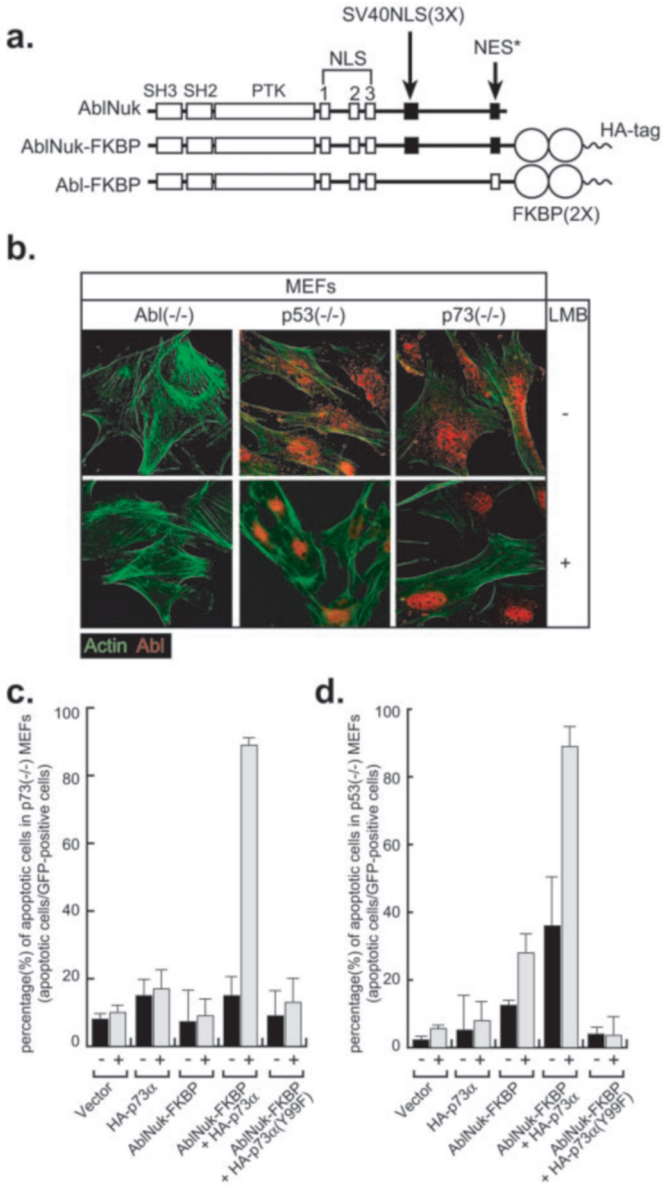


FIG. 4. **Interdependence of activated nuclear Abl tyrosine kinase and p73 in the induction of apoptosis.** *a*, *diagram* of AblNuk, AblNuk-FKBP, and Abl-FKBP. To render Abl constitutively nuclear, its nuclear export sequence was inactivated with a single point mutation (*NES**, *filled box*), whereas three copies of the canonical SV40 nuclear localization signals (*SV40NLS(3×)*) were inserted in-frame downstream of the native NLSs of c-Abl. To conditionally activate c-Abl tyrosine kinase through dimerization, two copies of FKBPv were fused to the C terminus of c-Abl, followed by an HA epitope. *b*, c-Abl subcellular localization in p73(−/−) MEFs and p53(−/−) MEFs. p73(−/−) or p53(−/−) MEFs were either left untreated (−) or treated with 10 nM LMB for 12 h before the cells were fixed and stained with anti-Abl mAb (*red*). The cells were then counterstained with fluorescein isothiocyanate-phalloidin to visualize F-actin (*green*). c-Abl(−/−) MEFs were used as specificity control for the staining with anti-Abl antibody. *c*, Abl-induced apoptosis requires p73. p73(−/−) MEFs were transfected with either HA-p73α, AblNuk-FKBP, both AblNuk-FKBP and HA-p73α, or both AblNuk-FKBP and HA-p73α(Y99F) expression plasmids as indicated *underneath* the *bar graph*. H2B-GFP construct was included in all transfections to identify transfected cells. The transfected cells were either left untreated (−) or treated (+) with chemical dimerizer (AP20187, 50 nM) for 24 h. The percentages of apoptotic cells among the GFP-positive cell population were counted and plotted as shown. *d*, Abl/p73 apoptotic pathway is p53-independent. Same as in *c* except p53(−/−) MEFs were used.

c-ABL did not accumulate in the nucleus following LMB treatment of ARO and KAK cells (Fig. 3*b*, *+LMB panels*). Thus, the rate of ABL nuclear import was negligible in ARO and KAK cells.

The immunofluorescence results were confirmed by cell fractionation experiments (Fig. 3*c*). When normalized to histone H2B, it was estimated that only 1–5% of the total ABL was present in the nuclear fraction of WRO and KAK cells prior to

LMB treatment (Fig. 3*c*). With WRO cells, the amount of ABL in the nuclear fraction was significantly increased after LMB treatment. With KAK cells, however, LMB treatment did not alter the low level of ABL in the nuclear fraction. The restriction on ABL nuclear import was similarly observed by fractionation experiments with ARO cells (not shown). The mechanism underlying the reduced nuclear import of ABL in KAK and ARO cells is presently unknown.

Enforced Nuclear Entry with AblNuk—To overcome the restriction of nuclear import, we engineered "AblNuk" by inserting three tandem copies of the SV40 NLS in the C-terminal region of mouse c-Abl. We also inactivated the nuclear export signal to prevent export (Fig. 4*a*). In transient transfection experiments, we demonstrated that AblNuk, but not Abl, accumulated in the nucleus of ARO and KAK cells (not shown). Thus, the SV40 NLS is functional in these cancer cells. These results also suggested that the three NLS of ABL are specifically inactivated in ARO and KAK cells.

Dimerization of AblNuk-FKBP Induces Apoptosis in p53(−/−) but Not p73(−/−) MEFs—We developed a way to conditionally activate the kinase activity of AblNuk. Previous studies (50, 51) have shown that oligomerization of the Abl protein can activate its kinase activity, observed with BCR-ABL. The FK506-binding protein, FKBP, has been engineered as an inducible dimerization domain (52). An N-terminal fusion of Abl with FKBP has been shown to cause the inducible dimerization and activation of Abl kinase (53). We used a modified FKBPv domain (Ariad Inc.) to engineer AblNuk-FKBP so that we could use a synthetic dimerizer (AP20187) that does not interact with the abundant endogenous FKBP protein (54). Two copies of the FKBPv domain, tagged with the HA-epitope, were fused in-frame with Abl or AblNuk at the C terminus (Fig. 4*a*). In transient co-transfection experiments with AblNuk-FKBP and HA-p73α, we observed an increase in p73 tyrosine phosphorylation following the addition of AP20187 (not shown). Thus, AblNuk-FKBP retains its ability to interact and phosphorylate p73α.

We then expressed AblNuk-FKBP in p73(−/−) and p53(−/−) MEFs and determined its ability to induce apoptosis in these cells. The endogenous Abl protein was mostly cytoplasmic in these MEFs, but it accumulated in the nucleus following treatment with LMB (Fig. 4*b*). Hence, nuclear import of endogenous c-Abl was not blocked in these MEFs. The AblNuk-FKBP protein was constitutively localized to the nucleus of transfected MEFs, as expected (not shown). Expression of AblNuk-FKBP did not induce apoptosis of p73(−/−) MEFs, either in the absence or the presence of the dimerizer (Fig. 4*c*). Transfection with HA-p73α did not cause a significant death response in the p73(−/−) MEFs. However, co-expression of HA-p73α with AblNuk-FKBP caused dimerizer-dependent apoptosis in p73(−/−) MEFs (Fig. 4*c*). To further demonstrate the interdependence between c-Abl tyrosine kinase and p73 in inducing apoptosis, we tested the HA-p73α(Y99F) mutant lacking the Abl phosphorylation site (6). In p73(−/−) MEFs, co-expression of AblNuk-FKBP with HA-p73α(Y99F) did not induce apoptosis with or without dimerizer (Fig. 4*c*). These results showed that dimerization of AblNuk-FKBP causes p73-dependent apoptosis. When expressed in p53(−/−) MEFs, AblNuk-FKBP induced apoptosis in the presence of dimerizer (Fig. 4*d*). Co-expression of HA-p73α with AblNuk-FKBP in p53(−/−) MEFs caused apoptosis in the absence of the dimerizer most likely due to the combination of increased p73α and dimerizer-independent Abl kinase activity, but apoptosis was significantly increased following dimerization (Fig. 4*d*). In p53(−/−) MEFs, HA-p73α(Y99F) suppressed apoptosis induced by AblNuk-FKBP (Fig. 4*d*), suggesting a possible dominant negative effect

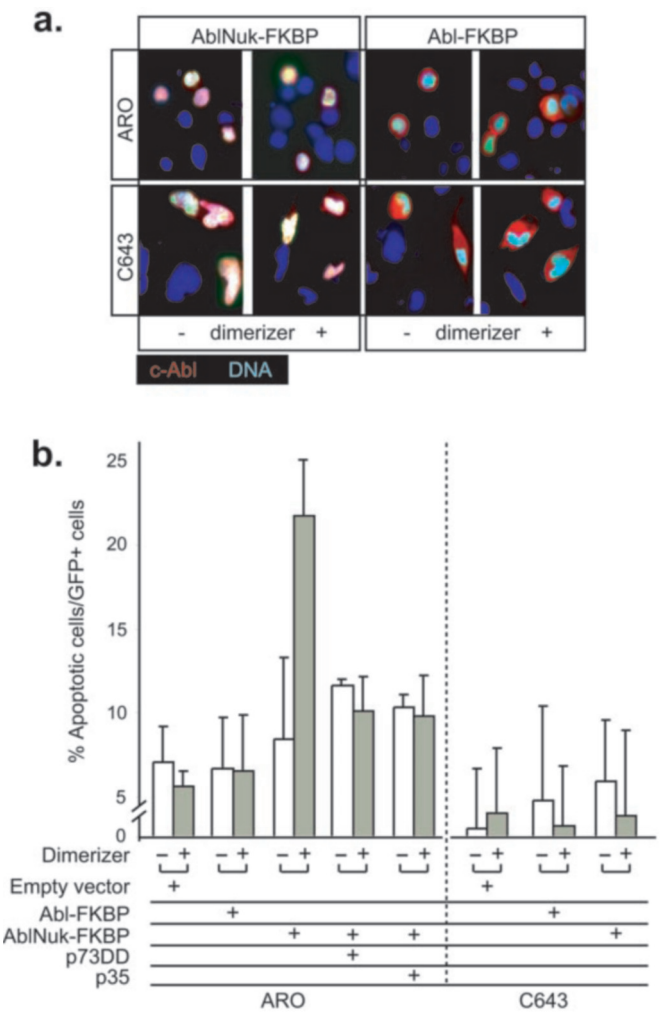
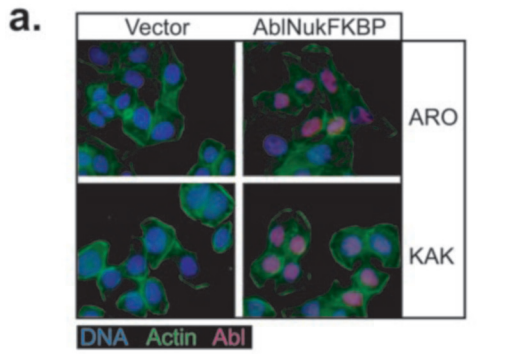
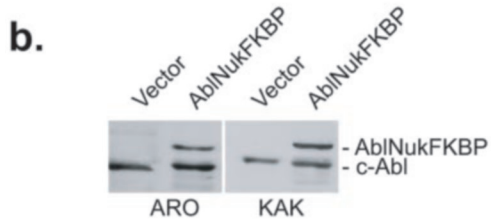


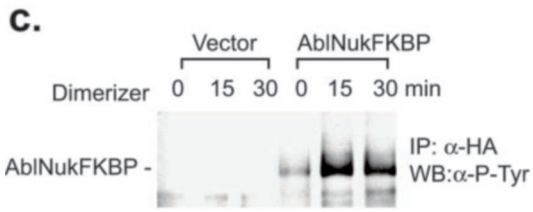
FIG. 5. **Transient expression and dimerization of AblNuk-FKBP activates p73-dependent apoptosis in anaplastic thyroid cancer cells.** *a*, AblNuk-FKBP and Abl-FKBP were transiently expressed in anaplastic thyroid cancer cells (*ARO* and *C643*), which were either left untreated (−) or treated (+) post-transfection with 50 nM chemical dimerizer (AP20187) for 24 h. Their subcellular localization was visualized by immunofluoresence staining with anti-HA (*red*) and Hoechst (*blue*). *b*, anaplastic thyroid cancer cells, ARO (p73-positive) and C643 (p73-negative) were transiently transfected with Abl-FKBP or AblNuk-FKBP. p73DD, a dominant negative mutant of p73 (10) or the baculovirus p35 (a pan-caspase inhibitor) were included in the transfection to suppress the activity of endogenous p73 or caspase, respectively. A GFP-H2B-encoding plasmid was included in all conditions to aid the indentification of transfected cells. The cells were either left untreated (−) or treated (+) post-transfection with 50 nM chemical dimerizer (AP20187) for 24 h before they were fixed and stained with Hoechst. The percentages of cells with fragmented nuclei among the GFP-positive population were scored as a measure of apoptosis.

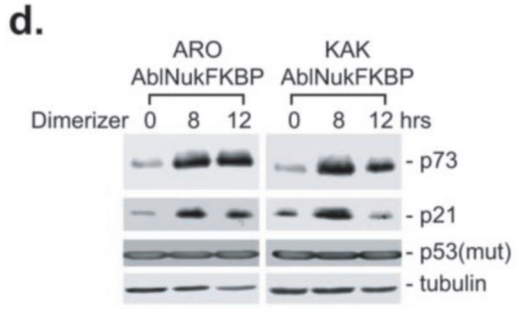
of p73α(Y99F) on the Abl/p73-dependent apoptosis. These results established that AblNuk-FKBP, when activated by dimerization, caused p73-dependent apoptosis that required tyrosine phosphorylation of the p73 protein.

Transient Expression of AblNuk-FKBP Induces Apoptosis of p73α-positive Thyroid Cancer Cells upon Dimerization—When transiently expressed, AblNuk-FKPB preferentially localized to the nucleus, whereas Abl-FKBP localized mainly to the cytoplasm of ARO (p73-positive) and C643 (p73-negative) anaplastic thyroid cancer cells (Fig. 5*a*). The subcellular distribution of these fusion proteins was not altered by dimerizer treatment (Fig. 5*a*). We then examined whether the enforced activation of AblNuk-FKBP could stimulate apoptosis by counting transfected cells (marked with a co-transfected GFP-his-









e.

Cells	Dimerizer(nM)	(%)HA(+) nuclei
ARO AblNukFKBP	0	157/412 (38%)
	50	171/398 (43%)
KAK AblNukFKBP	0	156/375 (42%)
	50	178/369 (48%)

FIG. 6. **Dimerization of stably expressed AblNuk-FKBP induces p73α and p21Cip1 in anaplastic thyroid cancer cells.** *a*, anaplastic thyroid cancer cells, ARO and KAK were infected with retrovirus encoding AblNuk-FKBP or non-encoding control virus (Mock). Infected cultures were expanded without selection. Cells were then fixed and stained with anti-HA antibody (*red*), phalloidin (*green*), and Hoechst (*blue*) to examine the subcellular localization of AblNuk-FKBP. *b*, total lysates of ARO and KAK cells either mock-infected or infected with AblNuk-FKBP retrovirus (as described in *a*) were immunoblotted with anti-Abl monoclonal antibody. Endogenous c-ABL and AblNuk-FKBP are indicated on the blots. *c*, the mock or AblNuk-FKBP infected KAK cells were treated with 50 nM AP20187 for the indicated periods of time. AblNukFKBP was immunoprecipitated with anti-HA antibody and immunoblotted with anti-phosphotyrosine antibody 4G10 to examine its autophosphorylation. *d*, AblNukFKBP-expressing ARO and KAK cells were treated with 50 nM AP20187 for the indicated periods of time. Total cell lysates were then immunoblotted with an anti-p73 to examine the expression level of the endogenous p73α protein. The same blot was also probed with anti-p21Cip1, anti-p53, or anti-tubulin antibody. *e*, ARO and KAK cells infected with AblNuk-FKBP retrovirus and expanded without selection were treated with 50 nM chemical dimerizer for 24 h. The AblNuk-FKBP-expressing cells were revealed by staining with an anti-HA antibody. The percentage of HA-positive cells among the total cell population was determined. The actual numbers of cells counted per experiment are shown.

tone H2B fusion protein) with condensed chromatin typical of apoptotic cell death. The basal level of apoptosis (~7%) in vector-transfected cultures was not altered by the addition of dimerizer (Fig. 5*b*). The transient expression of Abl-FKBP, which localizes to the cytoplasm, did not increase apoptosis either with or without the dimerizer (Fig. 5*b*). Transient expression of AblNuk-FKBP did not increase apoptosis. However, addition of AP20187 to AblNuk-FKBP transfected cultures resulted in a 3-fold increase in apoptosis (Fig. 5*b*). Co-expression of a dominant negative p73DD fragment (9) diminished the apoptosis response to dimerized AblNuk-FKBP. Co-expression of the baculovirus p35 protein, a potent inhibitor of caspases (55), also reduced this apoptotic response (Fig. 5*b*). Dimerizer did not cause an increase in apoptosis when it was added to C643 cells (p73-negative) transfected with Abl-FKBP or AblNuk-FKBP (Fig. 5*b*). These results showed that dimerization of AblNuk-FKBP, under conditions of transient transfection, could cause p73 and caspase-dependent chromatin condensation in anaplastic thyroid cancer cells.

Activation of Stably Expressed AblNuk-FKBP Induces p73α in Thyroid Cancer Cells—We also stably expressed AblNuk-FKBP in ARO and KAK cells through retroviral-mediated gene transfer (Fig. 6*a*). Initially, we selected for the infected cells by hygromycin resistance and found AblNuk-FKBP localized mostly to the cytoplasm of cells that survived the antibiotics selection. Thus, a selective pressure exists to exclude the stably expressed AblNuk-FKBP from the nucleus. To avoid this selective pressure, we infected ARO and KAK cells and expanded the infected populations without selection. Under this condition, we observed the exclusive nuclear localization of AblNuk-FKBP in the infected cells, which represented approximately half of the population (Fig. 6*a*). The AblNuk-FKBP was expressed at a level that was comparable with the endogenous ABL protein (Fig. 6*b*, *KAK*). Because only 40–50% of the cells were infected (Fig. 6*e*), the level of AblNuk-FKBP could be 2-fold higher than that of the endogenous ABL on a per cell basis. With the ARO cells, AblNuk-FKBP was likely to be at the same level as the endogenous ABL on a per cell basis (Fig. 6*b*, *ARO*). A low level of phosphotyrosine (Ptyr) was detected on AblNuk-FKBP in the absence of dimerizer (Fig. 6*c*, *lane 4*). Addition of AP20187 caused an increase in Ptyr of AblNuk-FKBP as early as 15 min (Fig. 6*c*). Importantly, AP20187 caused an increase in the levels of p73α, and a concomitant increase of p21Cip1 without altering the levels of p53 (Fig. 6*d*). Again, the actual increase in p73α and p21Cip1 on a per cell basis could be higher than depicted by the immunoblots, because only 40–50% of the cells expressed AblNuk-FKBP. The *in vivo* half-life of AP20187 is estimated to be around 12 h (not shown). Therefore, the increase in p73α and p21Cip1 peaked around 4–8 h after the addition of AP20187 and declined thereafter. Despite the increase in p73 and the induction of p21Cip1, treatment with AP20187 did not inhibit DNA synthesis (not shown), nor did it cause a preferential loss of AblNuk-FKBP-positive cells (Fig. 6*e*). Thus, stably expressed AblNuk-FKBP can induce p73 and p21Cip1, but it is not sufficient to trigger growth arrest or apoptosis. In other experiments with repeated addition of AP20187 for 48 h, we still did not observe the induction of apoptosis. Retrovirus-mediated AblNuk-FKBP protein expression was significantly lower than the level that

can be achieved by plasmid-mediated transient expression. Therefore, our failure to demonstrate an effect of stably expressed AblNuk-FKBP on thyroid cancer cell apoptosis may be ascribed to the insufficient levels of its expression.

DISCUSSION

In summary, this study has uncovered the up-regulation of p73α and the repression of its tumor suppression function in anaplastic thyroid cancer cells. We have found that c-ABL tyrosine kinase, a known activator of p73, is excluded from the nucleus of p73-expressing thyroid cancer cells. The subcellular segregation of c-ABL and p73 contributes to the repression of p73α function. This is because AblNuk-FKBP, which accumulates in the nucleus, can induce p73-dependent apoptosis when it is overproduced in ARO and KAK cells. With stably expressed AblNuk-FKBP, its dimerization increased the levels of p73α and p21Cip1, without causing growth arrest or apoptosis. These observations suggested that additional mechanisms may exist in anaplastic thyroid cancer cells to restrain the function of p73α. Previous studies have identified several other upstream regulators of p73, including the p300 acteyltransferase (26), the p38 mitogen-activated protein kinase (56) and the mismatch repair protein postmeiotic segregation increased 2 (PMS2) (57). It will be interesting to examine their interactions with p73α in anapalstic thyroid cancers.

The up-regulation of p73α in a malignant form of thyroid cancer is unexpected and appears to be at odds with its proposed function in tumor suppression. Our finding that ARO and KAK cells have developed strategies to accommodate the up-regulation of p73α is also perplexing. These observations imply that p73α may confer some advantage to these cancer cells. Whether the up-regulation of p73α, combined with the nuclear exclusion of c-Abl, can contribute to the development of anaplastic thyroid cancer cells will await further investigation.

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REFERENCES

1. Michael, D., and Oren, M. (2002) *Curr. Opin. Genet. Dev.* **12,** 53–59
2. Irwin, M. S., and Kaelin, W. G. (2001) *Cell Growth & Differ.* **12,** 337–349
3. Yang, A., Kaghad, M., Caput, D., and McKeon, F. (2002) *Trends Genet.* **18,** 90–95
4. Kaghad, M., Bonnet, H., Yang, A., Creancier, L., Biscan, J. C., Valent, A., Minty, A., Chalon, P., Lelias, J. M., Dumont, X., Ferrara, P., McKeon, F., and Caput, D. (1997) *Cell* **90,** 809–819
5. Melino, G., De Laurenzi, V., and Vousden, K. H. (2002) *Nat. Rev. Cancer* **2,** 605–615
6. Yuan, Z. M., Shioya, H., Ishiko, T., Sun, X., Gu, J., Huang, Y. Y., Lu, H., Kharbanda, S., Weichselbaum, R., and Kufe, D. (1999) *Nature* **399,** 814–817
7. Agami, R., Blandino, G., Oren, M., and Shaul, Y. (1999) *Nature* **399,** 809–813
8. Gong, J. G., Costanzo, A., Yang, H. Q., Melino, G., Kaelin, W. G., Jr., Levrero, M., and Wang, J. Y. J. (1999) *Nature* **399,** 806–809
9. Irwin, M., Marin, M. C., Phillips, A. C., Seelan, R. S., Smith, D. I., Liu, W., Flores, E. R., Tsai, K. Y., Jacks, T., Vousden, K. H., and Kaelin, W. G., Jr. (2000) *Nature* **407,** 645–648
10. Lissy, N. A., Davis, P. K., Irwin, M., Kaelin, W. G., and Dowdy, S. F. (2000) *Nature* **407,** 642–645
11. Yang, A., Walker, N., Bronson, R., Kaghad, M., Oosterwegel, M., Bonnin, J., Vagner, C., Bonnet, H., Dikkes, P., Sharpe, A., McKeon, F., and Caput, D. (2000) *Nature* **404,** 99–103
12. Flores, E. R., Tsai, K. Y., Crowley, D., Sengupta, S., Yang, A., McKeon, F., and Jacks, T. (2002) *Nature* **416,** 560–564
13. Pozniak, C. D., Radinovic, S., Yang, A., McKeon, F., Kaplan, D. R., and Miller, F. D. (2000) *Science* **289,** 304–306
14. Grob, T. J., Novak, U., Maisse, C., Barcaroli, D., Luthi, A. U., Pirnia, F., Hugli, B., Graber, H. U., De Laurenzi, V., Fey, M. F., Melino, G., and Tobler, A. (2001) *Cell Death & Differ.* **8,** 1213–1223
15. Douc-Rasy, S., Barrois, M., Echeynne, M., Kaghad, M., Blanc, E., Raguenez, G., Goldschneider, D., Terrier-Lacombe, M. J., Hartmann, O., Moll, U., Caput, D., and Benard, J. (2002) *Am. J. Pathol.* **160,** 631–639
16. Stiewe, T., and Putzer, B. M. (2002) *Cell Death & Differ.* **9,** 237–245
17. Liu, M., Taketani, T., Li, R., Takita, J., Taki, T., Yang, H. W., Kawaguchi, H., Ida, K., Matsuo, Y., and Hayashi, Y. (2001) *Leuk. Res.* **25,** 441–447
18. Kawano, S., Miller, C. W., Gombart, A. F., Bartram, C. R., Matsuo, Y., Asou, H., Sakashita, A., Said, J., Tatsumi, E., and Koeffler, H. P. (1999) *Blood* **94,** 1113–1120
19. Dong, S., Pang, J. C., Hu, J., Zhou, L. F., and Ng, H. K. (2002) *Int. J. Cancer* **98,** 370–375
20. Nomoto, S., Haruki, N., Kondo, M., Konishi, H., and Takahashi, T. (1998) *Cancer Res.* **58,** 1380–1383
21. Mai, M., Yokomizo, A., Qian, C., Yang, P., Tindall, D. J., Smith, D. I., and Liu, W. (1998) *Cancer Res.* **58,** 2347–2349
22. Mihara, M., Nimura, Y., Ichimiya, S., Sakiyama, S., Kajikawa, S., Adachi, W., Amano, J., and Nakagawara, A. (1999) *Br. J. Cancer* **79,** 164–167
23. Han, S., Semba, S., Abe, T., Makino, N., Furukawa, T., Fukushige, S., Takahashi, H., Sakurada, A., Sato, M., Shiiba, K., Matsuno, S., Nimura, Y., Nakagawara, A., and Horii, A. (1999) *Eur. J. Surg. Oncol.* **25,** 194–198
24. Zaika, A. I., Kovalev, S., Marchenko, N. D., and Moll, U. M. (1999) *Cancer Res.* **59,** 3257–3263
25. Shishikura, T., Ichimiya, S., Ozaki, T., Nimura, Y., Kageyama, H., Nakamura, Y., Sakiyama, S., Miyauchi, M., Yamamoto, N., Suzuki, M., Nakajima, N., and Nakagawara, A. (1999) *Int. J. Cancer* **84,** 321–325
26. Costanzo, A., Merlo, P., Pediconi, N., Fulco, M., Sartorelli, V., Cole, P. A., Fontemaggi, G., Fanciulli, M., Schiltz, L., Blandino, G., Balsano, C., and Levrero, M. (2002) *Mol. Cell* **9,** 175–186
27. Wang, J. Y. J., Ledley, F., Goff, S., Lee, R., Groner, Y., and Baltimore, D. (1984) *Cell* **36,** 349–356
28. Shtivelman, E., Lifshitz, B., Gale, R. P., Roe, B. A., and Canaani, E. (1986) *Cell* **47,** 277–284
29. Taagepera, S., McDonald, D., Loeb, J. E., Whitaker, L. L., McElroy, A. K., Wang, J. Y. J., and Hope, T. J. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 7457–7462
30. McWhirter, J. R., and Wang, J. Y. J. (1993) *EMBO J.* **12,** 1533–1546
31. McWhirter, J. R., and Wang, J. Y. J. (1991) *Mol. Cell. Biol.* **11,** 1553–1565
32. van Etten, R. A., Jackson, P. K., Baltimore, D., Sanders, M. C., Matsudaira, P. T., and Janmey, P. A. (1994) *J. Cell Biol.* **124,** 325–340
33. Woodring, P. J., Litwack, E. D., O'Leary, D. D., Lucero, G. R., Wang, J. Y. J., and Hunter, T. (2002) *J. Cell Biol.* **156,** 879–892
34. Plattner, R., Kadlec, L., DeMali, K. A., Kazlauskas, A., and Pendergast, A. M. (1999) *Genes Dev.* **13,** 2400–2411
35. Welch, P. J., and Wang, J. Y. J. (1993) *Cell* **75,** 779–790
36. Welch, P. J., and Wang, J. Y. J. (1995) *Genes Dev.* **9,** 31–46
37. Welch, P. J., and Wang, J. Y. J. (1995) *Mol. Cell. Biol.* **15,** 5542–5551
38. Baskaran, R., Wood, L. D., Whitaker, L. L., Canman, C. E., Morgan, S. E., Xu, Y., Barlow, C., Baltimore, D., Wynshaw-Boris, A., Kastan, M. B., and Wang, J. Y. J. (1997) *Nature* **387,** 516–519
39. Shafman, T., Khanna, K. K., Kedar, P., Spring, K., Kozlov, S., Yen, T., Hobson, K., Gatei, M., Zhang, N., Watters, D., Egerton, M., Shiloh, Y., Kharbanda, S., Kufe, D., and Lavin, M. F. (1997) *Nature* **387,** 520–523
40. Foray, N., Marot, D., Randrianarison, V., Venezia, N. D., Picard, D., Perricaudet, M., Favaudon, V., and Jeggo, P. (2002) *Mol. Cell. Biol.* **22,** 4020–4032
41. Baskaran, R., Dahmus, M. E., and Wang, J. Y. J. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 11167–11171
42. Baskaran, R., Chiang, G. G., and Wang, J. Y. J. (1996) *Mol. Cell. Biol.* **16,** 3361–3369
43. Baskaran, R., Escobar, S. R., and Wang, J. Y. J. (1999) *Cell Growth & Differ.* **10,** 387–396
44. Liu, Z. G., Baskaran, R., Lea-Chou, E. T., Wood, L. D., Chen, Y., Karin, M., and Wang, J. Y. J. (1996) *Nature* **384,** 273–276
45. Wang, J. Y. J. (2000) *Oncogene* **19,** 5643–5650
46. Vigneri, P., and Wang, J. Y. J. (2001) *Nat. Med.* **7,** 228–234
47. Wen, S. T., Jackson, P. K., and van Etten, R. A. (1996) *EMBO J.* **15,** 1583–1595
48. Marin, M. C., Jost, C. A., Irwin, M. S., DeCaprio, J. A., Caput, D., and Kaelin, W. G., Jr. (1998) *Mol. Cell. Biol.* **18,** 6316–6324
49. Jost, C. A., Marin, M. C., and Kaelin, W. G., Jr. (1997) *Nature* **389,** 191–194
50. McWhirter, J. R., and Wang, J. Y. J. (1997) *Oncogene* **15,** 1625–1634
51. McWhirter, J. R., Galasso, D. L., and Wang, J. Y. J. (1993) *Mol. Cell. Biol.* **13,** 7587–7595
52. Klemm, J. D., Schreiber, S. L., and Crabtree, G. R. (1998) *Annu. Rev. Immunol.* **16,** 569–592
53. Smith, K. M., and van Etten, R. A. (2001) *J. Biol. Chem.* **276,** 24372–24379
54. Clackson, T., Yang, W., Rozamus, L. W., Hatada, M., Amara, J. F., Rollins, C. T., Stevenson, L. F., Magari, S. R., Wood, S. A., Courage, N. L., Lu, X., Cerasoli, F., Jr., Gilman, M., and Holt, D. A. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 10437–10442
55. Clem, R. J. (2001) *Cell Death & Differ.* **8,** 137–143
56. Sanchez-Prieto, R., Sanchez-Arevalo, V. J., Servitja, J. M., and Gutkind, J. S. (2002) *Oncogene* **21,** 974–979
57. Shimodaira, H., Yoshioka-Yamashita, A., Kolodner, R. D., and Wang, J. Y. J. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 2420–2425