Inhibition of CCAAT/Enhancer Binding Protein Family DNA Binding in Mouse Epidermis Prevents and Regresses Papillomas

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Abstract

The CCAAT/enhancer binding proteins (C/EBP) are a family of B-ZIP DNA binding proteins that act as transcription factors to regulate growth and differentiation of many cell types, including keratinocytes. To examine the consequences of inhibiting the C/EBP family of transcription factors in skin, we generated transgenic mice that use the tetracycline system to conditionally express A-C/EBP, a dominant negative that inhibits the DNA binding of C/EBP family members. We expressed A-C/EBP in the basal layer of the skin epidermis during a two-step skin carcinogenesis protocol. A-C/EBP expression caused hyperplasia of the basal epidermis and increased apoptosis in the suprabasal epidermis. The mice developed fewer papillomas and had systemic hair loss. A-C/EBP expression caused C/EBPβ protein to disappear whereas C/EBPα, p53, Bax, and caspase-3 protein levels were dramatically up-regulated in the suprabasal layer. Primary keratinocytes recapitulate the A-C/EBP induction of cell growth and increase in p53 protein. A-C/EBP expression after papilloma development caused the papillomas to regress with an associated increase in apoptosis and up-regulation of p53 protein. Furthermore, A-C/EBP–expressing mice heterozygous for p53 were more susceptible to papilloma formation, suggesting that the suppression of papilloma formation has a p53-dependent mechanism. These results implicate DNA binding of C/EBP family members as a potential molecular therapeutic target. [Cancer Res 2007;67(4):1867–76]

Introduction

The CCAAT/enhancer binding protein (C/EBP) family of basic leucine zipper (B-ZIP) transcription factors are involved in many aspects of cell growth and differentiation in a variety of cell types (1–3). The six C/EBP family members are similar in the B-ZIP domain and can homodimerize and/or heterodimerize with each other to bind specific DNA sequences (4–9). Despite the high degree of homology within the B-ZIP domain, each of the C/EBP family members has a unique phenotype when deleted in mice (10, 11).

The two most studied family members are C/EBPα and C/EBPβ, which have contrasting roles in cell growth, differentiation, and oncogenesis. Elegant studies in adipocytes show that during differentiation in a cell culture model, there is a cascade of temporal changes in the levels of individuals C/EBP members. C/EBPδ and C/EBPβ expression precedes expression of C/EBPα (12–14) with the latter inducing expression of fat-specific genes (15). A similar sequential use of the C/EBP family members is observed in the epidermis (16–21). C/EBPβ is expressed in the undifferentiated growing basal epidermis whereas C/EBPα is expressed in the more quiescent differentiating suprabasal epidermis (21).

Contrasting roles for C/EBPα and C/EBPβ in oncogenesis have also been observed. These roles are consistent with C/EBPα functioning in cellular differentiation and C/EBPβ in cell growth regulation. Inactivation of C/EBPα prevents differentiation and it is mutated in many patients with acute myeloid leukemia (22). In contrast, C/EBPβ is implicated in supporting tumor formation in several cell types. For example, human C/EBPβ expression is increased in primary breast tumors, breast cancer cell lines (23), and colorectal tumors (24) and is associated with ovarian tumor progression (25). In mice, C/EBPβ has been shown to be required for chemically induced skin papilloma formation (26).

To investigate the function of the C/EBP proteins in the skin, several groups have studied null mice for individual C/EBP family members. A germ line disruption of the *C/EBPβ* gene in C57BL/6 mice produced no reported phenotypes in the skin (27), although it did lead to the development of lymphocyte abnormalities in aged mice. However, when the *C/EBPβ* null mice were backcrossed into a FVB/N background, they had a scruffy coat phenotype and were slightly smaller than controls (20). Mice with the conditional deletion of *C/EBPβ* in the epidermis are also resistant to carcinogen-induced skin tumorigenesis (28).

Whereas C/EBPβ has been implicated in carcinogenesis, the molecular basis of this activity remains unknown. To evaluate if inhibition of DNA binding is effective at mimicking the effects observed in the *C/EBPβ* null mice, we have expressed in mouse basal epidermis a dominant negative that inhibits DNA binding of C/EBP family members. The C/EBP dominant negative, termed A-C/EBP (29), consists of the C/EBPα leucine zipper dimerization domain and an acidic region that replaces the basic region that is critical for DNA binding. A-C/EBP heterodimerizes with C/EBP family members via the leucine zipper region, and the designed acidic extension forms a coiled coil structure with the basic region of the C/EBP B-ZIP domain to stabilize the heterodimer and block DNA binding (30).

We have generated a transgenic mouse line that expresses the A-C/EBP dominant negative gene (30) under the regulated control of the tetracycline operon (*TetO*) promoter and examined the effect of its expression in a skin carcinogenesis protocol. A-C/EBP is expressed by the tetracycline transactivator (tTA) in the skin basal epidermis using the keratin 5 (*K5*) promoter (31). Transgenic mice were subjected to a two-stage chemical carcinogenesis protocol. It was seen that A-C/EBP expression prevented papilloma formation. Furthermore, A-C/EBP expression following

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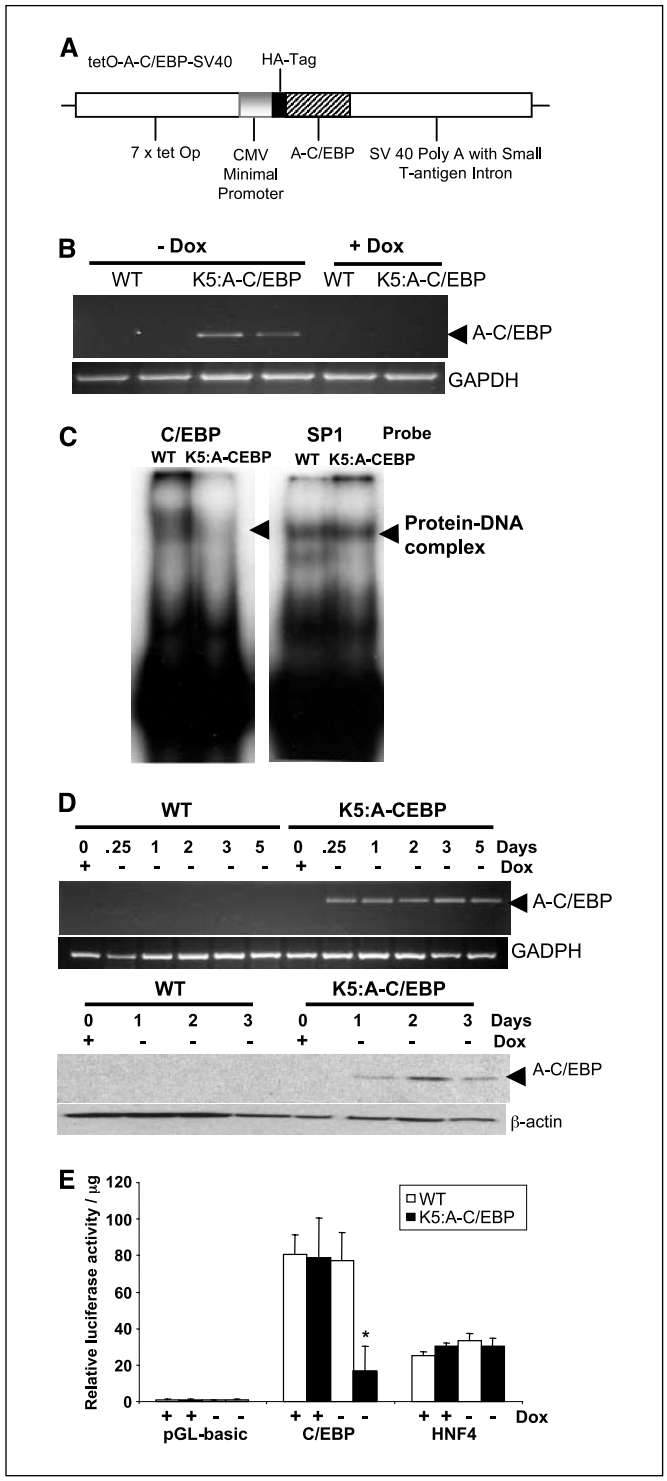

Figure 1. Doxycycline-dependent expression of A-C/EBP in mouse skin. *A,* schematic of TetO-A-C/EBP construct as described in Materials and Methods. *B,* genotype and doxycycline-dependent transcription of A-C/EBP transcript in skin detected by RT-PCR. *C,* EMSA using nuclear extracts from WT and A-C/EBP skin bound to radiolabeled C/EBP or SP1 consensus oligonucleotides (*arrow*). *D,* genotype and doxycycline-dependent transcription and translation of A-C/EBP in primary newborn pup keratinocytes assayed by RT-PCR (*top*) and Western blot analysis (*bottom*). *E,* doxycycline-dependent A-C/EBP expression specifically blocks a C/EBP responsive luciferase reporter but not a HNF4 reporter in primary keratinocytes. Luciferase activity is expressed as fluorescent units per microgram of protein. *Columns,* mean of triplicate dishes per treatment; *bars,* SD. *, $P < 0.01$, WT versus A-C/EBP keratinocytes in the absence of doxycycline.

papilloma formation causes papilloma regression. Because the mouse skin model reflects cancer development in several human target sites, these data indicate that inhibiting the DNA binding of C/EBP family members may be a molecular target for clinical intervention.

Materials and Methods

Plasmid construction of A-C/EBP and generation of TetO-A-C/EBP transgenic mice. A transgenic mouse line in the FVB 129P3/J background was generated with a construct consisting of the Tet-operator DNA binding sequence, the *A-C/EBP* dominant negative cDNA (29), and human growth hormone (hGH) polyadenylation signal (ref. 32; Fig. 1*A*). These mice were crossed to transgenic mice containing the tTA protein under control of the *K5* promoter (31). Transgenic mice were identified using specific PCR primers for each transgene. All experiments with mice were conducted under NIH-approved protocols. The following primers were used: tTA 5′ primer, 5′-AACAACCCGTAAACTCGCCC-3′; 3′ primer, 5′-GCAACCTAAAGTAAAATGCCCCAC-3′. These primers produce a 300-bp product. TetO-A-C/EBP 5′ primer, 5′-CCACGCTGTTTTGACCTCCATAG-3′; 3′ primer 5′-ATTCCACCACTGCTCCCATTC-3′. These primers produce a 600-bp product. Amplification of PCR products for each gene was done by denaturation at 94°C for 5 min and then 30 cycles of amplification using the following conditions: 94°C for 45 s, 60°C for 45 s, and 72°C for 45 s, followed by 7-min extension at 72°C.

The *TetO-A-C/EBP* transgenic line could not be made homozygous. *K5:A-C/EBP* mice were crossed with $p53^{+/-}$ mice (33) to generate *K5:A-C/EBP:*$p53^{+/-}$ mice and subsequent offspring were identified by PCR using primers 5′-GTGTTTCATTAGTTCCCCACCTTGAC-3′ and 5′-GGAGGCTGCCAGTCCTAAC-3′ for wild-type (*WT*) *p53* allele. PCR primers used to detect p53 knockout allele were 5′-GTGGGAGGGACAAAAGTTCGAG-3′ and 5′-AGCCCTGGCGCTCGATGT-3′.

Reporter assay. Primary keratinocytes were isolated from *WT* or *K5:A-C/EBP* newborn littermates (1 day old) as described (32). At 70% confluence, keratinocytes were transfected using Lipofectin reagent (Invitrogen Life Technologies, Carlsbad, CA) with 1.0 μg of the empty vector control or the C/EBP (17) and hepatocyte nuclear factor 4 (HNF4)–dependent luciferase reporters (34). After 6 h, cultures were grown in low-calcium Eagle's MEM containing 8% chelex-treated fetal bovine serum with or without doxycycline (2 ng/mL). Forty-eight hours later, cells were harvested and luciferase activity was determined. Statistical analysis was done using Student's *t* test.

Isolation of RNA and reverse transcription-PCR. Primary keratinocytes and skin tissues were harvested from the back of 7,12-dimethylbenz(*a*)anthracene (DMBA)/12-*O*-tetradecanoylphorbol-13-acetate (TPA)–treated *WT* or *K5:A-C/EBP* mice and equivalent *WT* mice and were snap frozen in liquid nitrogen. Frozen skin sections were homogenized in TRIzol (Life Technologies). DNA-free total RNA was collected by ethanol precipitation and treated for 1 h at 37°C with 100 units of DNase I (Roche, Indianapolis, IN). RNA was further purified through RNeasy columns (Qiagen, Valencia, CA) and examined for degradation in 1% agarose gels. Reverse transcription was done at 70°C for 3 min and 42°C for 60 min followed by 10-min denaturation at 92°C in a total volume of 20 μL of reaction mixture using a reverse transcription-PCR (RT-PCR) kit (Ambion, Austin, TX). Amplification of RT-PCR products was done by denaturation at 94°C for 5 min and then 27 cycles of amplification at 94°C for 45 s, 60°C for 45 s, and 72°C for 30 s, followed by 7-min extension at 72°C (Perkin-Elmer PCR system 9700). The A-C/EBP transcript was identified using primers specific for the transgene (5′ primer in the four-heptad coding sequences, 5′-TGGTGGACAGCAAATGGGTC-3′; 3′ primer in SV40 polyadenylate sequences, 5′-AATGTTGAGAGTCAGCAGTAGCCTC-3′) to detect the final 600-bp band.

Electrophoretic mobility shift assay. The nuclear extracts were prepared from *WT* and *K5:A-C/EBP* mouse skin treated with DMBA/TPA. For electrophoretic mobility shift assay (EMSA), 4 μg of nuclear extracts were incubated with labeled oligonucleotide probe containing a C/EBP or a SP1 binding site in a reaction buffer containing 20 mmol/L HEPES (pH 7.9), 60 mmol/L NaCl, 5 mmol/L $MgCl_2$, 1 mmol/L DTT, 17% glycerol.

DNA-protein complexes were separated by electrophoresis on 7% native polyacrylamide gels in 0.25× Tris-borate EDTA, dried, and exposed to film. The sequences of the double-stranded oligonucleotides, with the consensus binding site in bold, are as follows: 5′-GTCAGTCAG**ATTGCGCAAT**ATCGGTCAG (for C/EBP), 5′-GTCAGTCA**GGGGGGCGGGG**CATCGGTCAG (for SP1).

Skin carcinogenesis experiments. For mouse skin tumor initiation, mice at 8 weeks of age were topically treated with a single dose of DMBA (100 μg/200 μL in acetone) applied on shaved dorsal skin (35, 36). For 20 weeks after initiation, TPA (5 μg/200 μL in acetone) was applied twice weekly to the skin. Papilloma multiplicity was recorded weekly. Study groups included equal numbers of male and female mice for all groups. In Fig. 2*C*, animal numbers were *WT* [+doxycycline (n = 20), −doxycycline (n = 24)], *K5* [+doxycycline (n = 11), −doxycycline (n = 12)], and *K5:A-C/EBP* [+doxycycline (n = 10), −doxycycline (n = 14)]. For *A-C/EBP* and *p53* crosses, animal numbers were *WT:p53*$^{+/+}$ [−doxycycline (n = 6)], *WT:p53*$^{+/-}$ [−doxycycline (n = 8)], *K5:A-C/EBP:p53*$^{+/+}$ [−doxycycline (n = 6)], and *K5:A-C/EBP:p53*$^{+/-}$ [−doxycycline (n = 19)] (Supplementary Fig. S1).

A-C/EBP expression was induced in *K5:A-C/EBP* mice at three different times. First, *K5:A-C/EBP* mice were grown in the absence of doxycycline to constitutively express A-C/EBP. Second, mating cages were grown with doxycycline and pups were weaned at 3 weeks to a doxycycline-free food. These mice were used for the DMBA/TPA experiment. Third, mice were weaned to doxycycline and the DMBA/TPA experiment was done in the presence of doxycycline and mice were switched to doxycycline-free food after papillomas developed at 28 weeks to examine possible papilloma regression. Doxycycline was administered in the food at a concentration of 200 mg/kg (Bioserv, Baltimore, MD). In Fig. 5*A*, animal numbers were *WT* (n = 6) and *K5:A-C/EBP* (n = 8).

Immunoblot analysis. Protein extracts were made from whole skin or primary keratinocytes using radioimmunoprecipitation assay (RIPA) buffer containing 20 mmol/L Tris-Cl (pH 7.5), 137 mmol/L NaCl, 10% glycerol, 1% NP40, 0.1% SDS, 0.5% sodium deoxycholate, 5 mmol/L EDTA, 2 μg/mL aprotinin, 1 mmol/L phenylmethylsulfonyl fluoride, and 20 μmol/L leupeptin. Proteins were quantified using the Bradford method (Bio-Rad, Hercules, CA) and separated on 12% SDS-PAGE. p53 monoclonal antibody (mAb) was used at 1:1,000 (Oncogene, San Diego, CA and Santa Cruz Biotechnology, Santa Cruz, CA), C/EBPα mAb at 1:500, C/EBPβ mAb at 1:1,000 (Santa Cruz Biotechnology), Bax polyclonal antibody at 1:1,000 (Santa Cruz Biotechnology), caspase polyclonal antibody at 1:1,000 (Santa Cruz Biotechnology), and Ø10 mAb at 1:1,000 (Novagen, San Diego, CA) to detect A-C/EBP protein. The enhanced chemiluminescence (GE Healthcare, Piscataway, NJ) detection system was used.

Skin histology and measurement of cell proliferation. Skin tissues were fixed in 10% neutral buffered formalin overnight and transferred to 95% ethanol and embedded in paraffin. Six-micrometer sections were cut and stained with H&E. To label the rapidly dividing cells, mice were sacrificed 60 min after a single i.p. injection of a sterile solution of bromodeoxyuridine (BrdUrd; Sigma, St. Louis, MO; 10 mg/mL in PBS, 50 mg/kg of body weight). Skin sections were treated for 30 min at 37 °C with 2 N HCl in PBS containing 0.5% Triton X-100. They were rinsed in sodium tetraborate buffer (0.1 mol/L, pH 8.5) and processed for immunohistochemistry with an anti-BrdUrd antibody (DAKO Diagnostics, Carpinteria, CA).

Immunohistochemistry and apoptosis assay. Skins of *WT* and *K5:A-C/EBP* mice were excised at various times after DMBA/TPA treatment and fixed in 10% formalin solution (Sigma-Aldrich), paraffin embedded, sectioned, and stained with H&E. Serial sections were incubated with C/EBPα mAb at 1:100. Immunoreactivity was detected using the avidin-biotin complex method and 3,3′-diaminobenzidine kits from Vector Laboratories (Burlingame, CA). To detect the differentiation and the p53 level in the epidermis, FITC-conjugated K5 antibody, keratin 10 (K10) antibody (Covance, Richmond, CA), and rabbit anti-p53 antibody (Novocastra Laboratories, Newcastle upon Tyne, United Kingdom) were incubated and, subsequently, Cy3-conjugated donkey secondary antibody (Jackson Immunologicals, West Grove, PA) was applied. Slides were stained with 4′,6-diamidino-2-phenylindole for visualization of nuclei before fluorescence microscopy. Terminal deoxynucleotidyltransferase–mediated dUTP-biotin nick end labeling (TUNEL) assays were done in formalin-fixed, paraffin-embedded skin sections using *in situ* cell death detection kit (Roche Diagnostics). TUNEL-positive epidermal keratinocytes were visualized by fluorescence microscopy. To examine the immune response in the skin, CD3

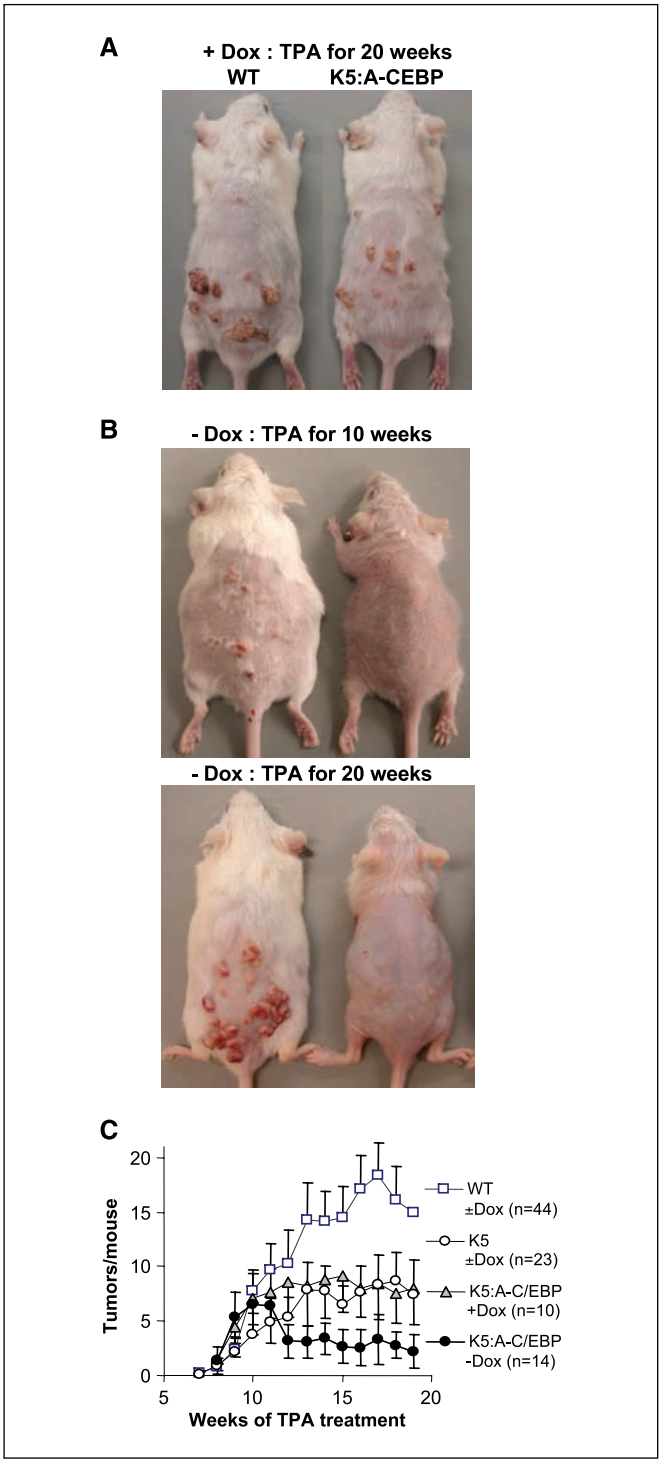

Figure 2. A-C/EBP expression in skin causes resistance to carcinogen-induced skin tumorigenesis. *A,* in the presence of doxycycline (*Dox*), there is no difference of papilloma formation between WT and K5:A-C/EBP mice. *B,* A-C/EBP–expressing mice show both hair loss and papilloma resistance at 10 wk (*top*) and 20 wk (*bottom*). *C,* plot of papilloma number following a single DMBA application followed by 20 wk of TPA for four groups: WT mice, K5:A-C/EBP (+doxycycline) mice, K5:A-C/EBP (−doxycycline) mice, and K5 mice. The numbers in parentheses are the number of animals in each group.

to monitor T lymphocytes and CD45R polyclonal antibody to monitor B lymphocytes were used. These experiments were conducted by Pathology and Histotechnology Laboratory in Frederick, National Cancer Institute (Frederick, MD).

Cell viability assay and colony formation assay. Primary keratinocyte cultures were switched to a medium without doxycycline and cell viability was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay using the CellTiter96 cell proliferation assay kit (Promega, Madison, WI). The results represent the mean of three independent experiments. For colony formation assay, the cells were stained with 0.1% crystal violet to assay colony formation after 1 week.

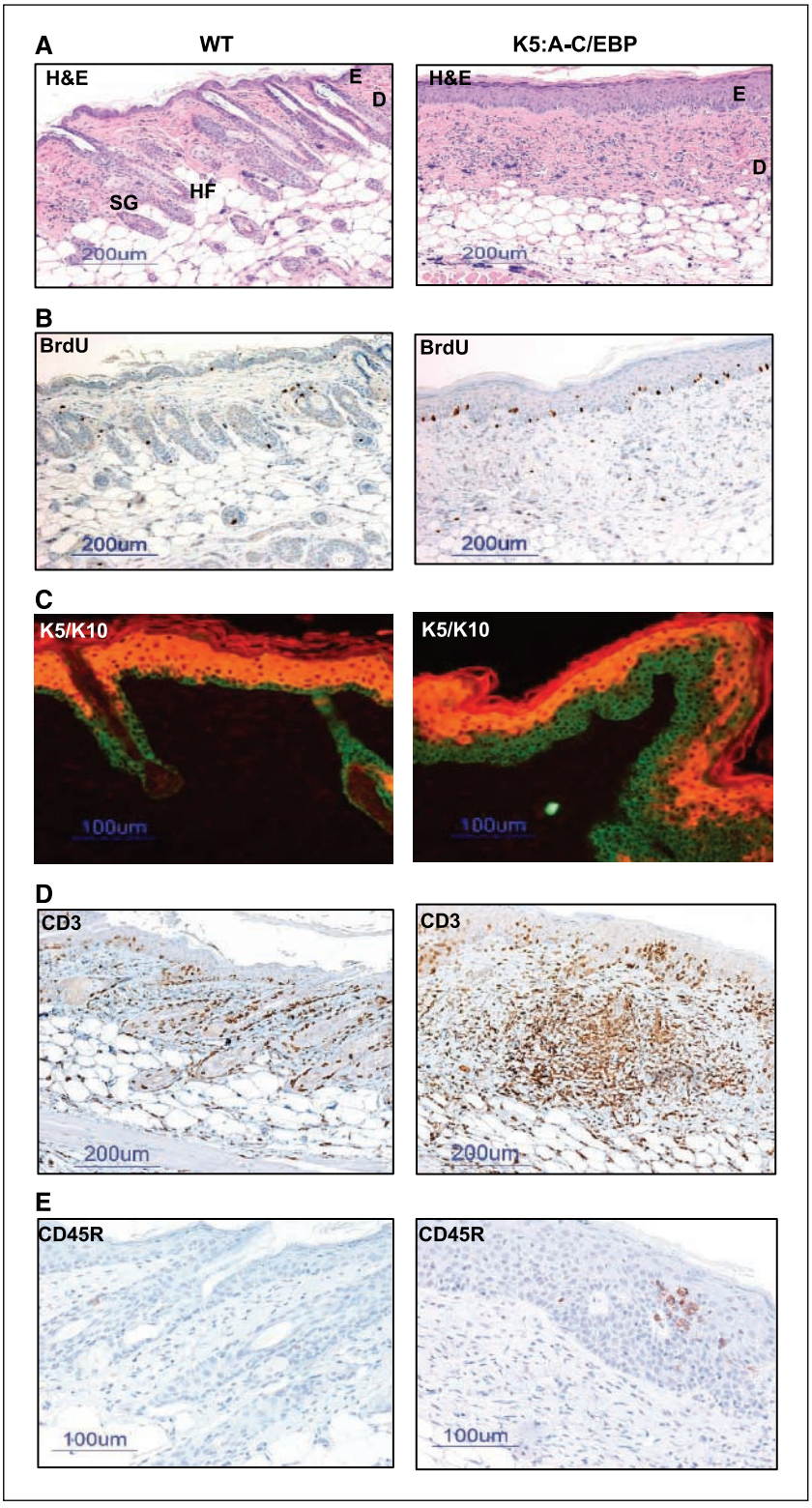

Figure 3. Changes in skin of A-C/EBP–expressing mice after 20 wk of TPA promotion. *A,* H&E staining of WT and A-C/EBP mice shows hyperplasia of epidermis, hair loss, and dermal infiltration (×100). *B,* BrdUrd (*BrdU*) staining shows high numbers of proliferating cell in the basal layer of the epidermis in A-C/EBP mice (×100). *C,* immunohistochemistry of the basal marker K5 (*green*) and the suprabasal marker K10 (*red*). Note the expansion of the basal layer marker K5 in K5:A-C/EBP mice (×200). *D,* immunohistochemistry of CD3 to monitor the T lymphocytes (×100); *E,* immunohistochemistry of CD45R to monitor the B lymphocytes (×200). *E,* epidermis; *HF,* hair follicle; *D,* dermis; *SG,* sebaceous gland.

Small interfering RNA and adenovirus infections. Small interfering RNAs (siRNA) specifically targeting *C/EBPα* or *C/EBPβ* (Dharmacon, Lafayette, CO) were transfected into primary keratinocytes using the DharmaFECT 1 reagent according to the manufacturer's instructions (Dharmacon). After 3-day incubation, cells were harvested with RIPA buffer. A-C/EBP protein was introduced into primary keratinocytes using an adenoviral construct driven by a cytomegalovirus promoter and empty adenovirus was used as a control (37). The cells were infected for 60 min in low-calcium medium with a multiplicity of infection (MOI) of 10 or 50 viral particles per cell and 2.5 μg/mL polybrene (Sigma-Aldrich) to enhance uptake. Cells were harvested 2 days after the infection. For measuring p53-dependent luciferase activity, WT keratinocytes were transfected with 1.0 μg of the p53-dependent luciferase reporter. After 24 h, cells were infected with 10 viral particles per cell of empty, p53, GCN4, A-VBP, and A-C/EBP adenovirus (37). Following 24-h incubation, cells were harvested and luciferase activity was determined.

Results

Regulated expression of A-C/EBP in the skin basal epidermis. We have used the two-transgene tetracycline system (38, 39) to regulate expression of A-C/EBP, a dominant negative that inhibits the DNA binding of C/EBP family members (30). We generated a transgenic mouse (*TetO-A-C/EBP*) that expresses A-C/EBP under the promoter control of seven tTA DNA binding elements (Fig. 1*A*). To express A-C/EBP in the skin, *TetO-A-C/EBP* mice were crossed with a second transgenic mouse (*K5-tTA*) that expresses the tTA using the K5 promoter (31) that is expressed in the basal epidermis. *K5-tTA:TetO-A-C/EBP* double transgenic mice (herein referred to as *K5:A-C/EBP*) were weaned at the expected Mendelian frequencies (Supplementary Table) independent of the presence of doxycycline, a tetracycline analogue, in the food of the mating cage, which regulates A-C/EBP expression. Examination of adult skin by RT-PCR indicates that A-C/EBP is tightly regulated and expression only occurs in *K5:A-C/EBP* mice in the absence of doxycycline (Fig. 1*B*). A-C/EBP expression inhibited the DNA binding of skin nuclear extracts from *K5:A-C/EBP* mice to a C/EBP consensus oligonucleotide, compared with *WT* nuclear extracts, suggesting that A-C/EBP expression was inhibiting the expected molecular targets (Fig. 1*C*). In contrast, the binding to a SP1 containing oligonucleotide was not changed.

A-C/EBP expression specifically inhibits a C/EBP transcriptional reporter. We examined whether A-C/EBP expression in primary keratinocytes from *K5:A-C/EBP* pups could inhibit C/EBP-specific reporter activity. A-C/EBP mRNA was not detected from cultures continually grown with doxycycline. However, following doxycycline removal, A-C/EBP mRNA and protein could be detected within 24 h (Fig. 1*D*). Primary *WT* or *K5:A-C/EBP* keratinocytes were transfected with three luciferase reporter constructs: (*a*) a negative control containing a promoterless luciferase reporter, (*b*) a C/EBP-dependent myelomonocytic growth factor promoter that contains two C/EBP-binding sites (17), and (*c*) a HNF4-responsive promoter (34). In the presence of doxycycline when A-C/EBP is not expressed, there was no difference in the activities of the three reporters transfected into either *WT* or *K5:A-C/EBP* keratinocytes. However, in the absence of doxycycline, which induces A-C/EBP expression, the luciferase activity of the C/EBP reporter was decreased 4-fold in transgenic primary keratinocytes whereas the other two reporters were unchanged, indicating that A-C/EBP was specifically inhibiting C/EBP binding sites in transfected reporters (Fig. 1*E*).

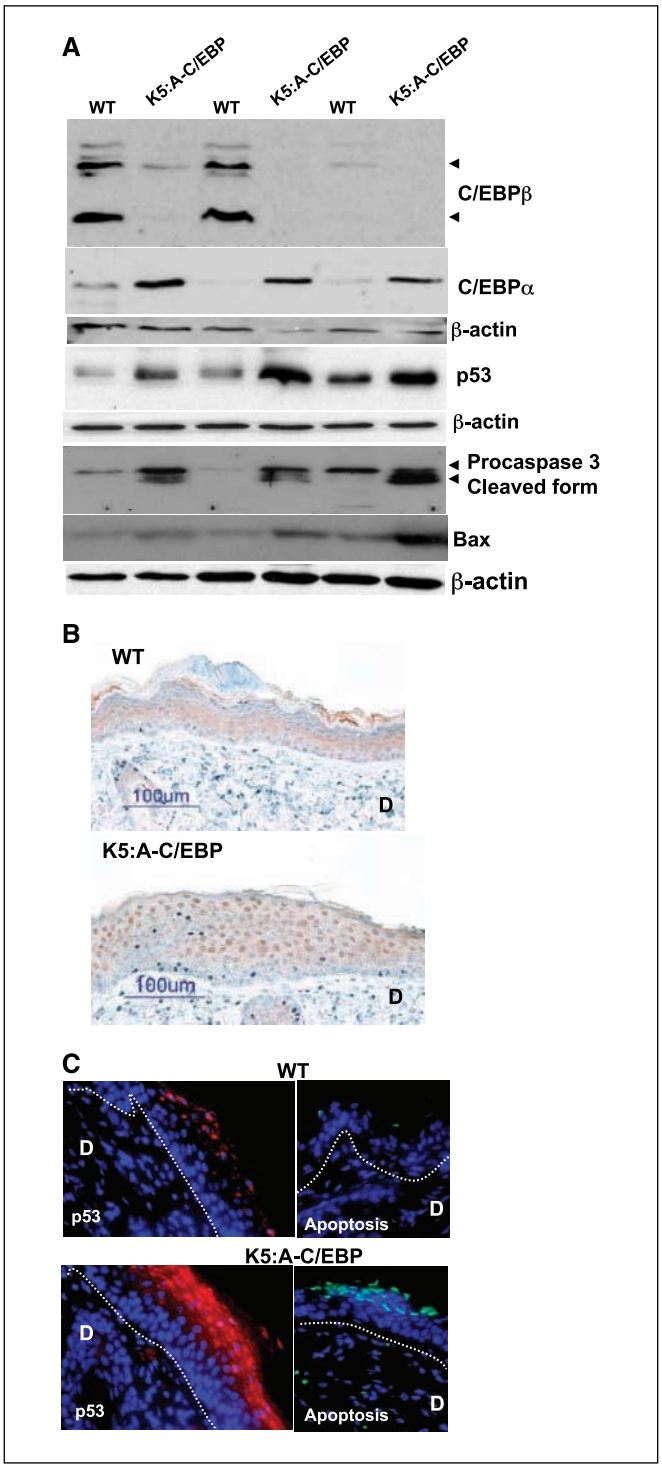

Figure 4. Changes of C/EBP family, p53, and proapoptotic proteins in skin of A-C/EBP–expressing mice treated with TPA. *A,* Western blot analysis of C/EBPβ, C/EBPα, p53, caspase-3, and Bax protein expression in WT and K5:A-C/EBP mice at 20 wk following DMBA initiation and TPA promotion. *B,* immunohistochemistry of C/EBPα in WT and K5:A-C/EBP skin (×200). *C,* immunohistochemistry of p53 and TUNEL analysis in WT and K5:A-C/EBP mice. *Dotted line,* dermal-epidermal border (×400).

A-C/EBP expression suppresses papilloma formation. We examined the effect of A-C/EBP expression on papilloma formation in a two-stage skin carcinogenesis experiment (35, 36). Mating cages containing heterozygous *K5* and *A-C/EBP* mice were fed food

containing 200 mg/kg doxycycline; litters were weaned at 3 weeks to food without doxycycline; and skin carcinogenesis was initiated with a single dose of DMBA at week 8 followed by twice weekly applications of TPA for 20 weeks. When mice were switched to food without doxycycline at 3 weeks to induce A-C/EBP expression in *K5:A-C/EBP* mice, papilloma numbers did not change in *WT* and *K5* mice. In contrast, *K5:A-C/EBP* mice produced few small papillomas (Fig. 2*B* and *C*). For example, *WT* mice developed an average of 13 to 15 squamous papillomas per mouse whereas *K5:A-C/EBP* mice only developed an average of two to three small squamous papillomas per mouse. *K5* mice with or without doxycycline and *K5:A-C/EBP* mice in the presence of doxycycline develop intermediate numbers of papillomas between *WT* mice and *K5:A-C/EBP* mice expressing A-C/EBP (Fig. 2*A* and *C*).

The tTA driven by the *K5* promoter partially impairs papilloma development, independent of the presence of doxycycline in the food. Previous work has reported that tTA expression in the heart causes complex changes in physiology and gene expression (40). Our data show that tTA expression in the skin does not cause any dramatic phenotypes as has been reported earlier but does slightly reduce papilloma number and size. However, this result is minor compared with the effect of A-C/EBP expression on papilloma number and size.

An additional phenotype of *K5:A-C/EBP* mice during the two-stage carcinogenesis experiment was systemic hair loss starting at week 10, resulting in near complete hair loss after 20 weeks of TPA treatment. If A-C/EBP expression is suppressed after 20 weeks of TPA treatment, the hair loss could not be reversed even after 3 months (data not shown).

A-C/EBP expression causes epidermal hyperplasia and immune infiltration. We examined the histology of the dorsal skin of *WT* and *K5:A-C/EBP* mice 20 weeks after DMBA initiation and TPA promotion. As previously reported, mild epidermal hyperplasia in the epidermis is observed in WT mice (ref. 36; Fig. 3*A*). However, *K5:A-C/EBP* mice expressing A-C/EBP have marked hyperplasia in the epidermis and the absence of hair follicles. Cell proliferation in the skin, as measured by BrdUrd-positive cells, increased >4-fold only in the basal epidermal layer in *K5:A-C/EBP* mice expressing A-C/EBP (Fig. 3*B*).

We analyzed expression of several markers of skin differentiation to determine which part of the epidermis is hyperplastic in *K5:A-C/EBP* mice. Immunofluorescent staining with antibodies to K5 that is typically expressed in the basal layer identified a broader range of staining in *K5:A-C/EBP* mice (Fig. 3*C*). We found no changes in K10 (Fig. 3*C*) or K1 (data not shown) staining in *K5:A-C/EBP* mice. These data suggest that the differentiation of the epidermis is delayed. We also observed a massive increase in infiltration that was mainly lymphocytes in *K5:A-C/EBP* mice (Fig. 3*D* and *E*).

K5:A-C/EBP mice that were not exposed to doxycycline *in utero* produce a hair loss phenotype and infiltrate similar to what is observed if the mice express A-C/EBP at weaning and are included in a carcinogenesis experiment.

A-C/EBP expression suppresses C/EBPβ and increases C/EBPα protein. We examined whether A-C/EBP expression influenced the protein levels of C/EBPα and C/EBPβ at 20 weeks after DMBA initiation and TPA promotion. In WT mice, C/EBPβ was observed, but in A-C/EBP–expressing mice, C/EBPβ protein was undetectable (Fig. 4*A*). In WT mice, C/EBPα was observed, but in A-C/EBP–expressing mice, C/EBPα protein levels increased (Fig. 4*A*). Immunohistochemistry for C/EBPα protein indicates that it is abundantly detected in the suprabasal layer (Fig. 4*B*), whereas the expression is minimal in *WT* epidermis. These results are similar to those in *C/EBPβ* knockout mice where C/EBPα protein is up-regulated in the suprabasal epidermis (20).

A-C/EBP expression up-regulates p53 protein and increases apoptosis. The p53 tumor suppressor protein is known to induce C/EBPα in keratinocytes following DNA damage (41). Therefore, we examined the p53 protein levels in the skin of *K5:A-C/EBP* mice as a potential cause of the up-regulation of C/EBPα. p53 protein was up-regulated in the skin of A-C/EBP–expressing mice (Fig. 4*A*). n addition, Bax and caspase-3 proteins, which are known pro-apoptotic markers, also increased in *K5:A-C/EBP* mice (Fig. 4*A*), suggesting that A-C/EBP expression resulted in the induction of proteins involved in apoptosis.

Immunohistochemistry of skin indicates that p53 protein is mainly expressed in the suprabasal epidermis in *WT* mice. In *K5:A-C/EBP* mice, p53 protein expression is increased in a similar pattern to the C/EBPα protein (Fig. 4*C*). Analysis of apoptosis using the TUNEL assay indicates that the A-C/EBP–expressing skin, where expression of p53 has been induced, has more apoptotic cells, primarily in the suprabasal epidermis than *WT* skin (Fig. 4*C*).

A-C/EBP expression causes papillomas to regress. We examined whether A-C/EBP expression following papilloma formation could cause tumor regression. Papillomas were produced in *K5:A-C/EBP* mice by continuously feeding them food containing doxycycline during the carcinogenesis experiment to suppress A-C/EBP expression (Fig. 2*A*). After 20 weeks of TPA treatment following initiation with DMBA, *K5:A-C/EBP* mice had papillomas. Doxycycline was then withdrawn from the food to allow A-C/EBP expression and papillomas started to regress within 2 weeks and most regressed completely by 4 weeks, but some papillomas did not regress (Fig. 5*A* and *B*).

We examined p53 protein levels and apoptosis by TUNEL staining in both the skin and papillomas of *K5:A-C/EBP* mice where A-C/EBP expression is induced after papilloma formation. Immunohistochemistry shows that p53 protein and apoptosis are induced in the suprabasal epidermis similarly to *K5:A-C/EBP* mice expressing A-C/EBP during the carcinogenesis experiment (Fig. 5*C*). In papillomas, both p53 protein and apoptosis are also massively increased throughout the regressing tumor mass, compared with WT mice.

A-C/EBP–mediated papilloma suppression is dependent on p53 protein. To evaluate if p53 is required to prevent papilloma formation in A-C/EBP–expressing mice, *K5:A-C/EBP* mice were generated that had only one copy of the *p53* locus. Mice with two deleted copies of *p53* were not examined because they died due to tumor burden unrelated to the carcinogenesis experiment (42). In *K5:A-C/EBP:p53*$^{+/-}$ mice with only one copy of *p53*, A-C/EBP expression was less effective at preventing papilloma formation (Supplementary Fig. S1). These data suggest that the two copies of *p53* are needed to produce the complete A-C/EBP phenotype. The *p53*$^{+/-}$ mice were in a C57BL/6 background, which produces fewer papillomas than the FVB background of both the K5 and A-C/EBP mice (43).

Expression of A-C/EBP in primary keratinocytes induces proliferation and p53 expression. A-C/EBP expression in the basal epidermis increases cell proliferation in the basal epidermis and p53 expression in the suprabasal epidermis. We are able to recapitulate these observations in primary keratinocyte cultures from *K5:A-C/EBP* newborn pups. Seven days after A-C/EBP expression, *K5:A-C/EBP* keratinocytes have increased cell growth (Fig. 6*A*) and cell density (Fig. 6*B*) compared with *WT*

keratinocytes. Keratinocytes expressing A-C/EBP protein showed p53 protein up-regulation with an accompanying decrease in C/EBPβ protein (Fig. 6*C*).

To verify A-C/EBP protein as a p53 protein inducer, *WT* keratinocytes were infected with adenovirus encoding A-C/EBP (37). In a dose-dependent manner, A-C/EBP adenovirus–infected keratinocytes increased p53 protein levels compared with empty adenovirus–infected keratinocytes (Fig. 6*D*). In addition, when *WT* keratinocytes were transfected with p53-responsive luciferase plasmid with a variety of adenoviruses, luciferase activity was dramatically increased only in A-C/EBP adenovirus–infected keratinocytes (Fig. 6*E*). These results with primary keratinocytes were consistent with skin results that A-C/EBP expression not only promoted cell proliferation but also up-regulated p53 expression.

To evaluate which of the C/EBP family members may be producing the observed phenotype caused by A-C/EBP expression, we used siRNA-mediated knockdown of either *C/EBPα* or *C/EBPβ*. C/EBPα and C/EBPβ proteins were greatly reduced within 3 days of transfection with C/EBPα and C/EBPβ siRNAs, respectively. Unlike A-C/EBP–expressing keratinocytes, neither siRNA against C/EBPβ nor siRNA against C/EBPα caused an induction of p53 protein in adherent keratinocytes. However, p53 protein significantly increased in floating keratinocytes transfected with C/EBPβ siRNA, suggesting that A-C/EBP may be partially acting via C/EBPβ inhibition (Fig. 6*F*).

Discussion

We describe a new transgenic mouse that reversibly expresses A-C/EBP, a dominant negative to the C/EBP family of B-ZIP transcription factors (30), in response to the tTA. Expression of A-C/EBP in the basal epidermis of adult skin results in a mild hair loss phenotype. During a two-stage chemical carcinogenesis experiment, A-C/EBP expression caused irreversible alopecia and severe epidermal hyperplasia and prevented papilloma formation. When A-C/EBP expression is induced after papilloma formation, papillomas regressed with an accumulation of p53 protein and apoptosis. The functional significance of p53 protein induction was examined by expressing A-C/EBP in the epidermis in mice with single copy of p53 gene. These mice produce more papillomas, showing the importance of p53 induction by A-C/EBP expression in the prevention of papilloma formation. These data imply that DNA binding of C/EBP family members is a potential molecular target for both tumor prevention and regression.

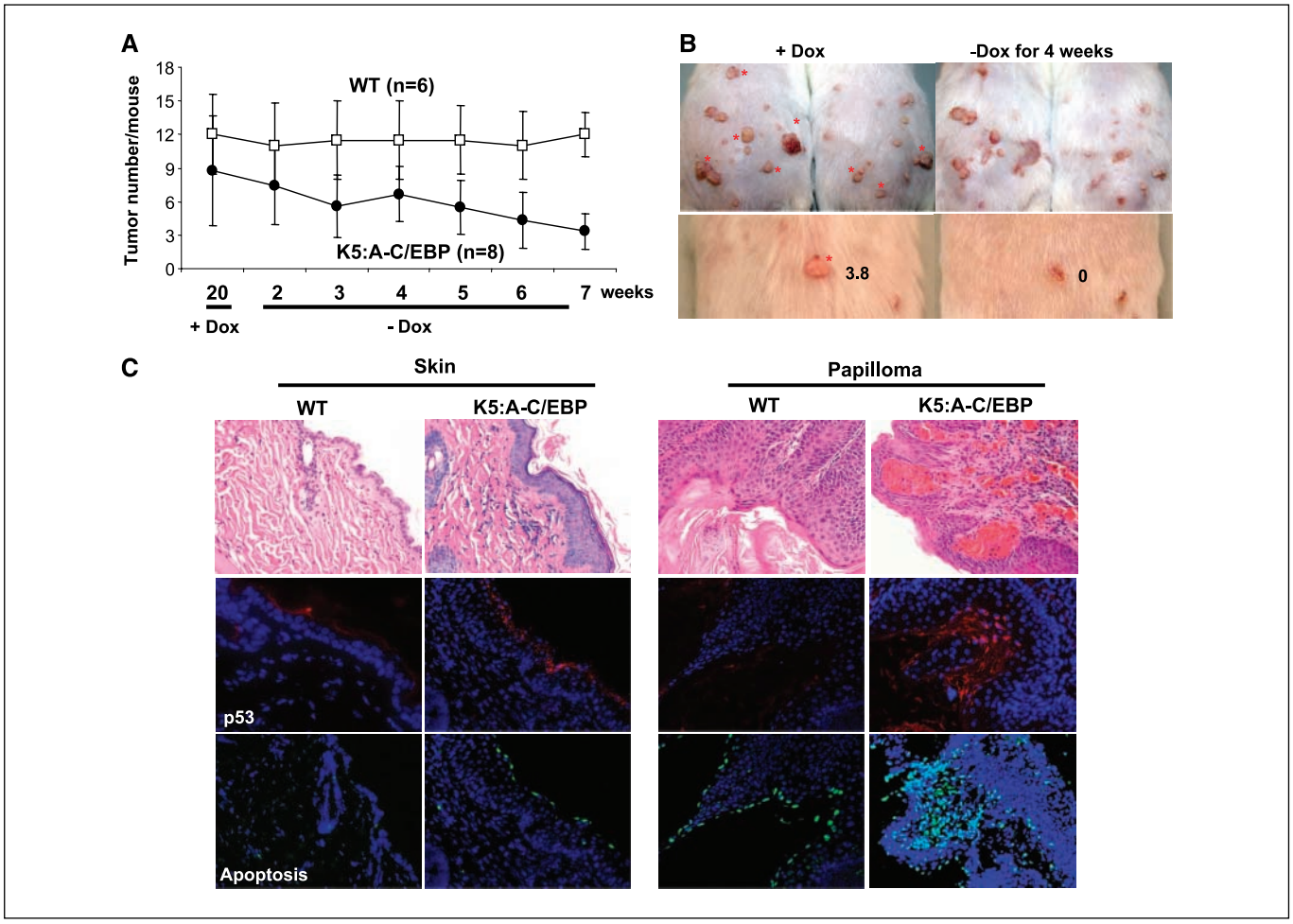

Figure 5. Papilloma regression after A-C/EBP expression accompanied by an increase in p53 expression and apoptosis. *A,* plot of papilloma number after A-C/EBP expression. *B,* K5:A-C/EBP mice were fed doxycycline during the skin carcinogenesis experiment and they developed papillomas. After 20 wk, A-C/EBP expression was induced by doxycycline withdrawal and papillomas regressed, leaving a bald scar on the skin. *Asterisks,* papillomas that regressed; the number represents papilloma size in millimeter. *C,* H&E staining, p53 expression, and TUNEL assay of four samples (WT skin, K5:A-C/EBP skin, WT papilloma, and K5:A-C/EBP papilloma) 4 wk after doxycycline withdrawal (×200).

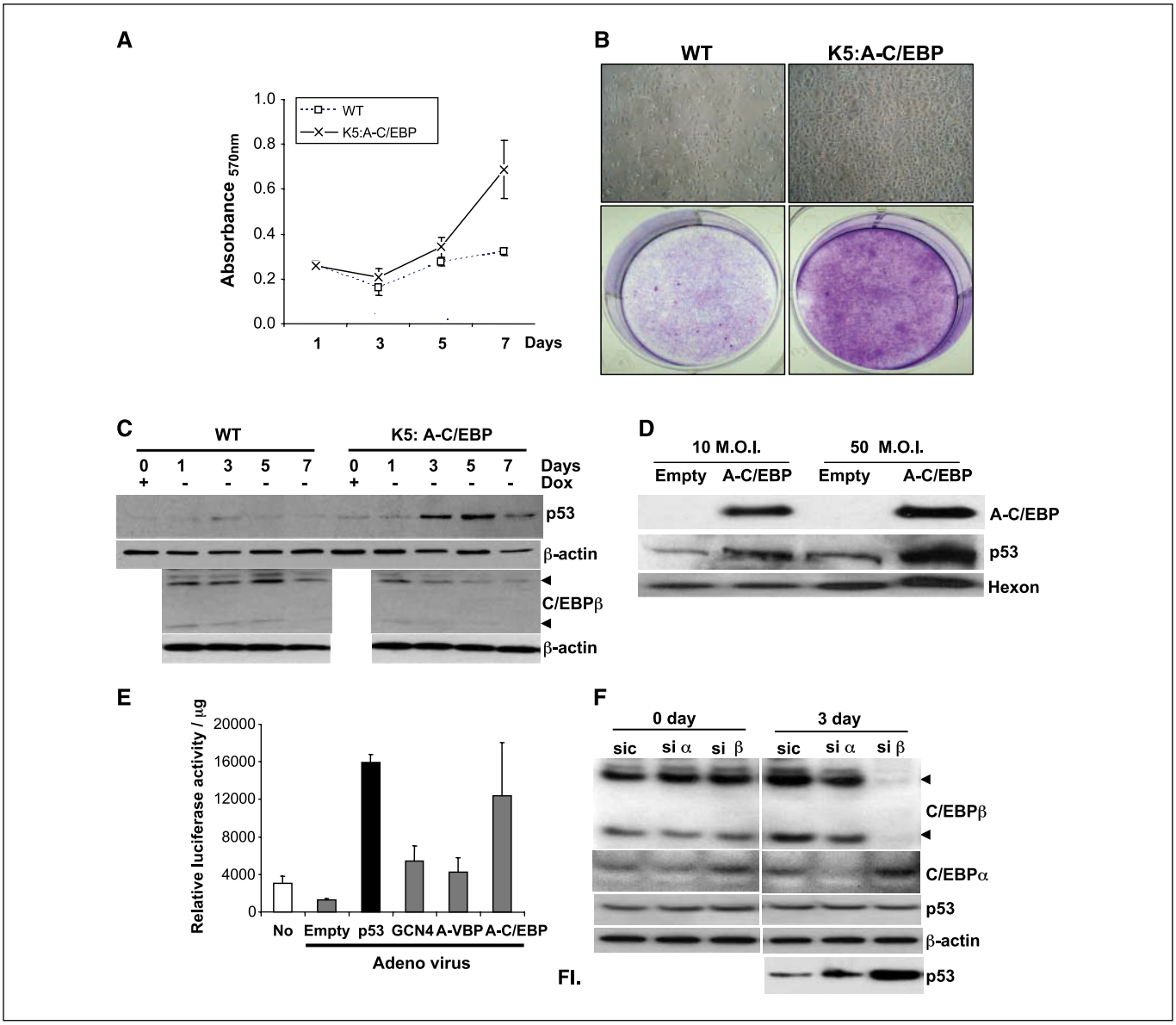

Figure 6. Increased proliferation rate and p53 expression in primary keratinocytes expressing A-C/EBP protein. *A,* cell proliferation rate increased in A-C/EBP–expressing keratinocytes. Equal numbers of primary keratinocytes were plated and switched to medium without doxycycline. Cell proliferation was determined by MTT assays in a time course manner. *Points,* mean of triplicate dishes per treatment; *bars,* SD. *B,* cell density (*top*) and colony formation assay (*bottom*) in WT and K5:A-C/EBP keratinocytes for 7 d. Cell populations were monitored by phase-contrast microscopy. *C,* time-dependent expression levels of C/EBPβ and p53 proteins in WT and K5:A-C/EBP keratinocytes by Western blot analysis. *D,* expression level of p53 protein after adenoviral expression of A-C/EBP. WT keratinocytes were infected for 2 d at MOIs of 10 and 50 for Western blot analysis. The viral coat protein Hexon was used as an internal control. *E,* p53-responsive luciferase activity was enhanced in primary keratinocytes infected with A-C/EBP adenovirus, not with other adenoviruses. Adenovirus encoding p53 was used as a positive control. Luciferase activity is expressed as fluorescent units per microgram of protein. *Columns,* mean of triplicate dishes per treatment; *bars,* SD. *No,* basal activity of p53-responsive luciferase in cells. *F,* expression of C/EBPα, C/EBPβ, and p53 proteins at 3 d after transfection with C/EBPα siRNA or C/EBPβ siRNA by Western blot analysis. *sic,* control siRNA; *siα,* C/EBPα siRNA; *siβ,* C/EBPβ siRNA; *Fl.,* floating cells. Otherwise, adherent cells were used.

Mice expressing A-C/EBP in the basal epidermis mimic some aspects of the *C/EBPβ* knockout mice (26) and the conditional deletion of *C/EBPβ* in the epidermis (28). Each of these mouse models is refractory to papilloma formation. However, several differences exist between these mice. The A-C/EBP–expressing mice have an infiltrate and alopecia that is exacerbated following the two-stage chemical carcinogenesis experiment, which is not reported in the *C/EBPβ* knockout mice (20, 26). The alopecia is irreversible because suppression of A-C/EBP expression after hair loss does not result in new hair growth. Consistent with the hair loss is a complete loss of hair follicle structures. Furthermore, there is extensive hyperplasia in the basal layer of the epidermis and infiltration of lymphocytes in the A-C/EBP–expressing mice, which are not reported in the *C/EBPβ* knockout mice, suggesting that A-C/EBP expression is inhibiting additional C/EBP family members in the basal epidermis to produce these phenotypes. It will be interesting to determine if *C/EBPβ* deletion following papilloma formation will be able to cause papillomas to regress to more accurately understand the mechanism of A-C/EBP function.

At the molecular level, several aspects of the *K5:A-C/EBP* mice are similar to the *C/EBPβ* knockout mice (20). C/EBPβ protein is absent in the skin of both mice. This decrease of C/EBPβ expression was also detected in primary keratinocytes produced from *K5:A-C/EBP* pups expressing A-C/EBP protein (Fig. 6*C*). In addition, C/EBPα protein is induced in the suprabasal epidermis in both the A-C/EBP–expressing mice and the C/EBPβ knockout mice. An enigmatic aspect of both mouse models is that deletion of C/EBPβ that is abundant in the basal epidermis or expression of A-C/EBP in the basal epidermis causes up-regulation of C/EBPα in the suprabasal epidermis. Although the mechanism of C/EBPα induction remains obscure, it has been suggested that AP-2 proteins that are C/EBPα repressors and regulated by C/EBPβ may be involved (21). A difference between the *K5:A-C/EBP* and *C/EBPβ* knockout mice is the increase of K10 in the suprabasal layer of *C/EBPβ* knockout mice (21), which is not observed in *K5:A-C/EBP* mice (Fig. 3).

Mice constitutively expressing A-C/EBP from the *K5* promoter, which is active *in utero*, are born at the expected Mendelian frequencies and show hyperplastic skin and increased dermal infiltrate during the first or second month (data not shown), which is similar to the phenotype we observe in the adult following the DMBA/TPA treatment. Induction of A-C/EBP in 1-month-old mice also caused mild alopecia, epidermal hyperplasia, and p53 induction after 6 months, all of which are less severe than in mice following the two-stage chemical carcinogenesis protocol.

We are able to recapitulate the increase in cell proliferation in the basal epidermis and p53 expression observed in the suprabasal epidermis in A-C/EBP–expressing mice with primary keratinocytes expressing A-C/EBP. A-C/EBP expression does not affect the level of p53 mRNA (Supplementary Fig. S2) but does affect both p53 protein level (Fig. 6*C*) and stability (Supplementary Fig. S3) after several days in culture. It has been reported that C/EBPβ and p53 can physically interact (44), suggesting that A-C/EBP may contribute to the stability of p53 protein by affecting the interaction between C/EBPβ and p53.

A-C/EBP expression induces apoptosis in both the suprabasal epidermis and papillomas without causing a dramatic skin phenotype while causing papillomas to regress. We propose that this difference between the skin and papillomas is that papillomas have mutations in H-Ras and that A-C/EBP induction of p53 protein is potentiated in H-Ras–mutated cells.

An advantage of the tetracycline-regulated system is the ability to regulate transgene expression. This allowed us to produce papillomas and then express A-C/EBP resulting in p53 protein induction, apoptosis, and papilloma regression. These results validate the DNA binding of C/EBP family members as a potential molecular target for therapeutic intervention. Small molecules that specifically inhibit C/EBP family members have not been identified but this work supports efforts to identify such compounds (45).

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References

1. Lekstrom-Himes J, Xanthopoulos KG. Biological role of the CCAAT/enhancer-binding protein family of transcription factors. J Biol Chem 1998;273:28545–8.
2. Freytag SO, Paielli DL, Gilbert JD. Ectopic expression of the CCAAT/enhancer-binding protein α promotes the adipogenic program in a variety of mouse fibroblastic cells. Genes Dev 1994;8:1654–63.
3. Buck M, Poli V, Hunter T, Chojkier M. C/EBPβ phosphorylation by RSK creates a functional XEXD caspase inhibitory box critical for cell survival. Mol Cell 2001;8:807–16.
4. Akira S, Isshiki H, Sugita T, et al. A nuclear factor for IL-6 expression (NF-IL6) is a member of a C/EBP family. EMBO J 1990;9:1897–906.
5. Osada S, Yamamoto H, Nishihara T, Imagawa M. DNA binding specificity of the CCAAT/enhancer-binding protein transcription factor family. J Biol Chem 1996; 271:3891–6.
6. Ubeda M, Wang XZ, Zinszner H, Wu I, Habener JF, Ron D. Stress-induced binding of the transcriptional factor CHOP to a novel DNA control element. Mol Cell Biol 1996;16:1479–89.
7. Ryden TA, Beemon K. Avian retroviral long terminal repeats bind CCAAT/enhancer-binding protein. Mol Cell Biol 1989;9:1155–64.
8. Moll JR, Acharya A, Gal J, Mir AA, Vinson C. Magnesium is required for specific DNA binding of the CREB B-ZIP domain. Nucleic Acids Res 2002;30:1240–6.
9. Newman JR, Keating AE. Comprehensive identification of human bZIP interactions with coiled-coil arrays. Science 2003;300:2097–101.
10. Linhart HG, Ishimura-Oka K, DeMayo F, et al. C/EBPα is required for differentiation of white, but not brown, adipose tissue. Proc Natl Acad Sci U S A 2001;98: 12532–7.
11. Tanaka T, Yoshida N, Kishimoto T, Akira S. Defective adipocyte differentiation in mice lacking the C/EBPβ and/or C/EBPδ gene. EMBO J 1997;16:7432–43.
12. Cao Z, Umek RM, McKnight SL. Regulated expression of three C/EBP isoforms during adipose conversion of 3T3-1 cells. Genes Dev 1991;5:1538–52.
13. MacDougald OA, Lane MD. Transcriptional regulation of gene expression during adipocyte differentiation. Annu Rev Biochem 1995;64:345–73.
14. Yeh WC, Cao Z, Classon M, McKnight SL. Cascade regulation of terminal adipocyte differentiation by three members of the C/EBP family of leucine zipper proteins. Genes Dev 1995;9:168–81.
15. Friedman AD, Landschulz WH, McKnight SL. CCAAT/enhancer binding protein activates the promoter of the serum albumin gene in cultured hepatoma cells. Genes Dev 1989;3:1314–22.
16. Bull JJ, Muller-Rover S, Chronnell CM, Paus R, Philpott MP, McKay IA. Contrasting expression patterns of CCAAT/enhancer-binding protein transcription factors in the hair follicle and at different stages of the hair growth cycle. J Invest Dermatol 2002;118:17–24.
17. Zhu S, Oh HS, Shim M, Sterneck E, Johnson PF, Smart RC. C/EBPβ modulates the early events of keratinocyte differentiation involving growth arrest and keratin 1 and keratin 10 expression. Mol Cell Biol 1999;19:7181–90.
18. Oh HS, Smart RC. Expression of CCAAT/enhancer binding proteins (C/EBP) is associated with squamous differentiation in epidermis and isolated primary keratinocytes and is altered in skin neoplasms. J Invest Dermatol 1998;110:939–45.
19. Swart GW, van Groningen JJ, van Ruissen F, Bergers M, Schalkwijk J. Transcription factor C/EBPα: novel sites of expression and cloning of the human gene. Biol Chem 1997;378:373–9.
20. Maytin EV, Lin JC, Krishnamurthy R, et al. Keratin 10 gene expression during differentiation of mouse epidermis requires transcription factors C/EBP and AP-2. Dev Biol 1999;216:164–81.
21. Maytin EV, Habener JF. Transcription factors C/EBPα, C/EBPβ, and CHOP (Gadd153) expressed during the differentiation program of keratinocytes *in vitro* and *in vivo*. J Invest Dermatol 1998;110:238–46.
22. Tenen DG. Disruption of differentiation in human cancer: AML shows the way. Nat Rev Cancer 2003;3: 89–101.
23. Bundy LM, Sealy L. CCAAT/enhancer binding protein β (C/EBPβ)-2 transforms normal mammary epithelial cells and induces epithelial to mesenchymal transition in culture. Oncogene 2003;22:869–83.
24. Rask K, Thorn M, Ponten F, et al. Increased expression of the transcription factors CCAAT-enhancer binding protein-β (C/EBβ) and C/EBζ (CHOP) correlate with invasiveness of human colorectal cancer. Int J Cancer 2000;86:337–43.
25. Sundfeldt K, Ivarsson K, Carlsson M, et al. The expression of CCAAT/enhancer binding protein (C/EBP) in the human ovary *in vivo*: specific increase in C/EBPβ during epithelial tumour progression. Br J Cancer 1999; 79:1240–8.
26. Zhu S, Yoon K, Sterneck E, Johnson PF, Smart RC. CCAAT/enhancer binding protein-β is a mediator of keratinocyte survival and skin tumorigenesis involving oncogenic Ras signaling. Proc Natl Acad Sci U S A 2002; 99:207–12.
27. Screpanti I, Romani L, Musiani P, et al. Lymphoproliferative disorder and imbalanced T-helper response in C/EBPβ-deficient mice. EMBO J 1995;14:1932–41.
28. Sterneck E, Zhu S, Ramirez A, Jorcano JL, Smart RC. Conditional ablation of C/EBPβ demonstrates its keratinocyte-specific requirement for cell survival and mouse skin tumorigenesis. Oncogene 2006;25: 1272–6.
29. Krylov D, Olive M, Vinson C. Extending dimerization

interfaces: the bZIP basic region can form a coiled coil. EMBO J 1995;14:5329–37.
30. Vinson C, Myakishev M, Acharya A, Mir AA, Moll JR, Bonovich M. Classification of human B-ZIP proteins based on dimerization properties. Mol Cell Biol 2002;22:6321–35.
31. Diamond I, Owolabi T, Marco M, Lam C, Glick A. Conditional gene expression in the epidermis of transgenic mice using the tetracycline-regulated transactivators tTA and rTA linked to the keratin 5 promoter. J Invest Dermatol 2000;115:788–94.
32. Gerdes MJ, Myakishev M, Frost NA, et al. Activator protein-1 activity regulates epithelial tumor cell identity. Cancer Res 2006;66:7578–88.
33. Donehower LA, Harvey M, Slagle BL, et al. Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. Nature 1992;356:215–21.
34. Inoue Y, Hayhurst GP, Inoue J, Mori M, Gonzalez FJ. Defective ureagenesis in mice carrying a liver-specific disruption of hepatocyte nuclear factor 4α (HNF4α). HNF4α regulates ornithine transcarbamylase *in vivo*. J Biol Chem 2002;277:25257–65.
35. Yuspa SH, Dlugosz AA, Cheng CK, et al. Role of oncogenes and tumor suppressor genes in multistage carcinogenesis. J Invest Dermatol 1994;103:90–5S.
36. Yuspa SH, Dlugosz AA, Denning MF, Glick AB. Multistage carcinogenesis in the skin. J Investig Dermatol Symp Proc 1996;1:147–50.
37. Bonovich M, Olive M, Reed E, O'Connell B, Vinson C. Adenoviral delivery of A-FOS, an AP-1 dominant negative, selectively inhibits drug resistance in two human cancer cell lines. Cancer Gene Ther 2002;9:62–70.
38. Gossen M, Bujard H. Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. Proc Natl Acad Sci U S A 1992;89:5547–51.
39. Gossen M, Freundlieb S, Bender G, Muller G, Hillen W, Bujard H. Transcriptional activation by tetracyclines in mammalian cells. Science 1995;268:1766–9.
40. Turnbull L, Zhou HZ, Swigart PM, et al. Sustained preconditioning induced by cardiac transgenesis with the tetracycline transactivator. Am J Physiol Heart Circ Physiol 2006;290:H1103–9.
41. Yoon K, Smart RC. C/EBPα is a DNA damage-inducible p53-regulated mediator of the G_1 checkpoint in keratinocytes. Mol Cell Biol 2004;24:10650–60.
42. Harvey M, McArthur MJ, Montgomery CA, Jr., Butel JS, Bradley A, Donehower LA. Spontaneous and carcinogen-induced tumorigenesis in p53-deficient mice. Nat Genet 1993;5:225–9.
43. Woodworth CD, Michael E, Smith L, et al. Strain-dependent differences in malignant conversion of mouse skin tumors is an inherent property of the epidermal keratinocyte. Carcinogenesis 2004;25:1771–8.
44. Schneider-Merck T, Pohnke Y, Kempf R, Christian M, Brosens JJ, Gellersen B. Physical interaction and mutual transrepression between CCAAT/enhancer-binding protein β and the p53 tumor suppressor. J Biol Chem 2006;281:269–78.
45. Rishi V, Potter T, Laudeman J, et al. A high-throughput fluorescence-anisotropy screen that identifies small molecule inhibitors of the DNA binding of B-ZIP transcription factors. Anal Biochem 2005;340:259–71.