Inhibition of CREB Function in Mouse Epidermis Reduces Papilloma Formation

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Abstract
We used a double transgenic tetracycline system to conditionally express A-CREB, a dominant negative protein that prevents the DNA binding and function of cAMP-responsive element binding protein (CREB) family members, in mouse basal epidermis using the keratin 5 promoter. There was no phenotype in the adult. However, following a 7,12-dimethylbenz(a)anthracene (DMBA)/phorbol-12-myristate-13-acetate two-stage skin carcinogenesis experiment, A-CREB-expressing epidermis develop 5-fold fewer papillomas than wild-type controls. However, A-CREB expression one month after DMBA treatment does not prevent papilloma formation, suggesting that CREB functions at an early stage of papilloma formation. Oncogenic H-Ras genes with A→T mutations in codon 61 were found in wild-type skin but not in A-CREB-expressing skin 2 days after DMBA treatment, suggesting that A-CREB either prevents DMBA mutagenesis or kills oncogenic H-Ras cells. In primary keratinocyte cultures, A-CREB expression induced apoptosis of v-RasH1b-infected cells and suppressed the expression of cell cycle proteins cyclin B1 and cyclin D1. These results suggest that inhibiting CREB function is a valuable cancer prevention strategy. (Mol Cancer Res 2009;7(5):654–64)

Introduction
The mammalian genome contains three genes that encode cAMP-responsive element binding protein (CREB)-related transcription factors: CREB, CREM, and activating transcription factor 1 (ATF-1). They have similar B-ZIP domains and each has many alternative splicing forms. Homodimerization and heterodimerization between CREB, CREM, and ATF-1 (1) potentially produce a large number of transcriptional complexes (2, 3). These proteins bind the consensus CRE sequence (TGACGTCA; refs. 4, 5) that is preferentially localized in the proximal promoters of human (6) and mouse (7) genes. Mice lacking CREB die immediately after birth from respiratory distress (8). Mice lacking CREM have arrested spermatogenesis (9) whereas inactivation of the ATF-1 gene does not cause any obvious phenotypic abnormalities (10). Knockout mice lacking two of the three CREB isoforms, α and Δ, develop normally (11) but CREM mRNA and protein levels are elevated in all tissues examined, suggesting that CREB family members may compensate for each other (12). Classically, CREB is thought to become activated when phosphorylated at Ser133 by a variety of kinases including extracellular signal–regulated kinase 1/2, p38, and protein kinase A (3, 13). Inhibition of these kinases affects cell viability through CREB-dependent mechanisms (14-16).

Overexpression of CREB protein is observed in some cancers (17-19). For example, in the majority of patients with acute myelogenous leukemia, CREB protein is increased in the bone marrow and this overexpression is associated with a poor prognosis (20). Similarly, in breast cancer patients, the level of CREB protein is elevated and is associated with poor prognosis and metastatic disease (21). CREB has also been reported to control hepatocellular carcinoma growth by supporting angiogenesis and rendering the cells resistant to apoptosis (19). CREB regulates the transcription of many cancer-related genes involved in cell survival and antiapoptotic response [BCL-2 (22, 23), c-IAP-2 (15), c-Fos (24), IRS2 (25), cyclin D1 (16, 26, 27), cyclin D2 (28)] and angiogenesis (VEGF; ref. 29).

The role of CREB family members in carcinogenesis is best exemplified by the chromosomal translocation [t(12;22)(q13; q12)] found recurrently in malignant melanoma of soft parts that produces a chimeric protein containing the B-ZIP domain of ATF-1 and the Ewing’s sarcoma oncogene product (EWS), an RNA-binding protein (30). The EWS-ATF-1 fusion enhances expression of numerous CREB target genes, independent of protein kinase A (30, 31). Disrupting EWS-ATF-1 activity seems sufficient to both block proliferation and promote apoptosis in these cells (30).

To examine the role of CREB family members in gene expression, we previously designed a dominant negative protein termed A-CREB that inhibits the DNA binding of CREB family members (1, 32). A-CREB has been used by many investigators to examine the function of CREB family members in a variety of biological contexts. In tissue culture, A-CREB can suppress BCL-2 expression, which regulates survival of...
neurons after withdrawal of nerve growth factor (22). In transgenic animals, A-CREB expression in pancreatic β-cells causes β-cell apoptosis, resulting in diabetes (25). Furthermore, transgenic animals expressing A-CREB in skeletal myocytes develop a dystrophic phenotype (33).

The role of CREB in skin papilloma formation has not been investigated, although CREB regulates expression of transcription factors participating in skin papilloma formation, including CCAAT/enhancer binding protein (C/EBP)-β (34, 35) and activator protein 1 (36-38). We hypothesized that because CREB regulates many genes critical for tumor formation, using A-CREB in keratinocytes to suppress CREB family function will inhibit formation of skin tumors. To test this hypothesis, we developed a transgenic mouse that expresses A-CREB in a tetracycline-dependent manner and crossed these mice to mice expressing the tetracycline transactivator in basal keratinocytes of the skin epithelium. We report that compared with wild-type (WT) controls, A-CREB expression in the epidermis of mouse reduces 5-fold the number of papillomas formed during a two-stage chemical carcinogenesis experiment.

Results
Reversible Expression of A-CREB in Mouse Epidermis
We reversibly expressed the dominant negative protein A-CREB that inhibits the DNA binding activity of the CREB family of B-ZIP transcription factors in mouse epidermis. A-CREB contains the CREB leucine zipper dimerization domain fused to a designed NH2-terminal acidic extension that replaces the DNA-binding region (32). A-CREB heterodimerizes with CREB family members via the leucine zipper, and then the acidic extension dimerizes with the DNA-binding region, essentially zipperring the leucine zipper up into the basic region. The CREB|A-CREB heterodimer does not bind DNA, which, in turn, abolishes CREB transcriptional activity (32). We used the double transgenic tetracycline regulated system (39) to express A-CREB in the epidermis (Fig. 1A). One transgene, K5-tTA (referred to as K5), uses the bovine keratin 5 promoter that is active in basal keratinocytes and outer root sheath cells of hair follicles (40). K5-tTA expresses the tetracycline transactivator protein, which, in the absence of tetracycline, binds to tetracycline transactivator DNA binding sequences found in the second transgene to activate transcription. The second transgene, tetO containing seven DNA binding sites for the tTA protein, induces expression of the A-CREB gene. In the absence of Dox, tTA binds to DNA and induces expression of the A-CREB transgene. The presence of Dox prevents DNA binding of the tetracycline transactivating protein, which suppresses A-CREB mRNA expression.

B. Reverse transcription-PCR showing reversible A-CREB mRNA expression in skin of double transgenic K5:A-CREB mice. The presence of Dox in food prevents A-CREB expression; however, within 4 d of switching adult mice to food without Dox, A-CREB mRNA expression is observed. When mice were switched back to food containing Dox, no A-CREB mRNA expression was detected within 3 d. A-CREB reduces CREB protein levels and CREB DNA binding in primary newborn mouse keratinocytes. C. A-CREB protein expression in K5:A-CREB keratinocytes 1 d after Dox withdrawal was detected using an antibody against the CREB leucine zipper region. D. CREB protein and mRNA abundance after 2 d of A-CREB expression with both protein and mRNA loading controls. E. Chromatin immunoprecipitation of CREB protein from K5 and K5:A-CREB keratinocytes showing that A-CREB expression prevents CREB binding to the McKusick-Kaufman syndrome (MKKS) protein promoter. Primers specific to GAPDH coding region are used to detect nonspecific binding.
A-CREB (referred to as A-CREB), contains a promoter with seven tetracycline transactivator DNA binding sequences and an open reading frame for A-CREB. The presence of doxycycline (Dox; a tetracycline analogue) inhibits tTA DNA binding, resulting in suppression of A-CREB transcription.

Heterozygous mice carrying the K5-tTA transgene were crossed with heterozygous mice carrying the A-CREB transgene to produce double transgenic mice (K5-tTA:tetO A-CREB, referred to as K5:A-CREB) that were born at the expected Mendelian frequencies. K5:A-CREB mice did not produce any obvious phenotype throughout their life span but expressed the A-CREB mRNA in a doxycycline-regulated and reversible manner (Fig. 1B). In the presence of Dox, A-CREB mRNA expression was undetectable. Within 4 days of switching mice to food without Dox, A-CREB mRNA expression was observed. When mice were switched back to food containing Dox, A-CREB mRNA expression became undetectable within 3 days (Fig. 1B).

In Primary Keratinocytes, the DNA Binding of CREB Is Suppressed by A-CREB

We evaluated if primary newborn keratinocytes could express A-CREB in a Dox-regulated manner and inhibit CREB DNA binding. In primary K5:A-CREB mouse keratinocytes, 1 day after Dox withdrawal, A-CREB protein was detectable (Fig. 1C). The level of endogenous CREB protein decreased after 2 days of A-CREB expression (Fig. 1C and D). The level of CREB mRNA remained the same after 2 days of A-CREB expression, arguing that the decrease in CREB protein level is a posttranscriptional effect (Fig. 1D).

To evaluate the function of A-CREB, we determined by chromatin immunoprecipitation if binding of CREB to DNA is inhibited by A-CREB. DNA binding of CREB to the promoter region of the target gene McKusick-Kaufman syndrome protein (MKKS), a centrosomal component required for cytokinesis, was decreased following A-CREB expression, indicating that A-CREB prevented CREB binding in vivo (Fig. 1E).

A-CREB Expression Inhibits 7,12-Dimethylbenz(a)anthracene/Phorbol-12-Myristate-13-Acetate Induction of CREB Protein

We observed that A-CREB expression induces reduction of CREB protein level in primary mouse keratinocytes, suggesting that A-CREB suppresses CREB function not only through inhibition of DNA binding but also through reduction in protein level. To evaluate if a similar effect is observed in vivo, we checked CREB protein level in the skin of WT and A-CREB–expressing mice after 7,12-dimethylbenz(a)anthracene (DMBA) and during multiple phorbol-12-myristate-13-acetate (TPA) applications. Wild-type skin had slightly elevated CREB protein level 2 days after DMBA application, which became more than 10 times higher in treated skin 1 day after TPA application and remained elevated during subsequent TPA treatments. A-CREB expression, however, prevented this DMBA/TPA–induced increase in CREB protein level (Fig. 2).

A-CREB Suppresses Papilloma Formation at an Early Stage of Carcinogenesis

To test if A-CREB altered papilloma formation, we treated single and double transgenic mice in the absence of Dox for 1 month, once with DMBA (100 μg) and twice weekly with 5 μg TPA for 18 weeks (Fig. 3A–C; ref. 35). After 12 weeks of promotion, WT mice formed tumors but A-CREB mice did not (Fig. 3A, left). After 15 weeks, the difference became more dramatic (Fig. 3A, right). Papillomas arose earlier in WT and K5 mice and K5:A-CREB mice on Dox compared with K5:A-CREB mice without Dox (Fig. 3A and B). By 12 to 15 weeks of promotion, there was 100% incidence of papillomas in all groups except for K5:A-CREB mice without Dox, which had only 25% incidence, and this increased to only 50% incidence by 18 weeks. Similarly, Fig. 3B (right) shows that at 12 weeks, WT mice had 15 papillomas per mouse and K5 and K5:A-CREB mice with Dox had 7 to 8 papillomas per mouse, suggesting a K5tTA strain–dependent effect on tumor formation; however, K5:A-CREB mice with Dox had 1 to 2 papillomas per mouse. Histologic examination of tumors from each group confirmed them as papillomas (data not shown). These results indicate that A-CREB expression in the epidermis suppresses papilloma formation in comparison with K5 mice or K5:A-CREB mice on Dox.

Although the mice containing only the K5 transgene had half the papillomas of WT mice (Fig. 3B, right), the presence of Dox did not affect either the percentage of mice with one or more papillomas or the number of papillomas per mouse over time (Fig. 3C). This indicates that the suppression of papilloma formation is independent of DNA binding of the tetracycline transactivator expressed by the K5 promoter. In addition, the incidence of papillomas in the wild-type or single transgenic A-CREB mice was indistinguishable (data not shown), indicating that the A-CREB transgenic mice in the absence of the tetracycline transactivator are similar to the wild-type mice.

To determine when papilloma formation was effected by A-CREB expression, A-CREB was expressed 4 weeks after the initial TPA treatment, and these mice developed as many papillomas as K5:A-CREB mice that were continuously kept on Dox and hence never expressed A-CREB (Fig. 3D), suggesting...
that inhibition of CREB DNA binding can influence the early stages of papilloma formation.

**A-CREB Expression Induces Apoptosis after DMBA Treatment and Decreases the Fraction of Cells in S Phase of the Cell Cycle in Epidermis after TPA Treatment**

In other mouse models, resistance to DMBA/TPA–induced skin tumor formation has been attributed to the increased apoptosis after DMBA treatment and suppressed proliferation after TPA treatment (34, 41). We evaluated if CREB plays a role in the DMBA-induced apoptosis and TPA-induced proliferation using A-CREB–expressing mice. H&E staining of untreated adult skin expressing A-CREB in the epidermis did not reveal any differences between WT, K5, or A-CREB–expressing mice (data not shown). We found apoptotic cells in hair follicles of WT and A-CREB–expressing epidermis after DMBA treatment (Fig. 4A). Forty-eight hours after DMBA treatment, the fraction of terminal deoxyribonucleotidyl transferase–mediated dUTP nick end labeling (TUNEL)–positive apoptotic cells was increased in K5:A-CREB relative to the WT animals (Fig. 4B). In contrast, the fraction of cells in S phase of the cell cycle was the same in WT and K5:A-CREB epidermis (Fig. 4C).

When animals were treated with DMBA followed by a single TPA application a week later, the fraction of keratinocytes in S phase of the cell cycle was the same in WT and K5:A-CREB epidermis (Fig. 4C).

FIGURE 3. K5:A-CREB mice are resistant to papilloma formation. **A.** Left, images of WT, K5, and K5:A-CREB (−Dox) mice after 13 wk of DMBA/TPA treatment; right, images of WT and K5:A-CREB (−Dox) mice after 15 wk of DMBA/TPA treatment. **B.** Papilloma incidence (left) and papilloma number per mouse (right) for WT (n = 44), K5 (n = 23), K5:A-CREB +Dox (n = 19), and K5:A-CREB −Dox (n = 35) mice during 18 wk of TPA treatment. **C.** Papilloma incidence (left) and papilloma number per mouse (right) for mice containing only the K5 transgene with and without Dox: K5 +Dox, n = 10; K5 −Dox, n = 13. **D.** A-CREB expression suppresses papilloma formation in the early stage of carcinogenesis. Papilloma number for K5:A-CREB +Dox mice (n = 15) and K5:A-CREB mice in which A-CREB expression is induced 4 wk after the initiation of TPA treatment (n = 6). Bars, 95% confidence interval for mean values calculated using EXEL function "CONFIDENCE."
epidermis occurred only in the context of DMBA/TPA treatment (Fig. 5D).

A-CREB Blocks Oncogenic H-Ras Mutations after DMBA Treatment

A hallmark of DMBA/TPA–induced skin papillomas in the mouse is the occurrence of H-Ras mutations in more than 90% of papillomas (37). To evaluate if A-CREB expression influences the growth of cells with an oncogenic Ras mutation in the epidermis, we used PCR specific for A→T Ras mutation (adapted from ref. 42) in the WT and K5:A-CREB skin without Dox collected during different stages of carcinogenesis experiment (Fig. 6). Untreated skin DNA was used as a negative control, and papilloma DNA was used as a positive control (Fig. 6). Two days after DMBA treatment, we were able to detect the oncogenic H-Ras mutation in WT skin but not in A-CREB–expressing skin. Similar results were obtained at later time points. However, in one A-CREB–expressing skin sample (32 days after the DMBA treatment), oncogenic H-Ras was detected, suggesting that a papilloma may be growing, which is consistent with the low incidence of papillomas in A-CREB–expressing mice (Fig. 3A).

The Growth of v-RasHa–Transformed Primary Mouse Keratinocytes Is Suppressed by A-CREB Expression

H-Ras mutation occurs in a very small fraction of keratinocytes (43). To study the effect of A-CREB on cells with H-Ras mutation, we used primary keratinocytes infected with mouse sarcoma retrovirus expressing v-Ras Ha and measured cell growth 24 and 48 hours after infection. We found that without infection, A-CREB influences keratinocyte morphology and growth. Keratinocytes grew to confluence but did not become as densely packed as WT or K5 cells during further growth (Fig. 7A and C). Both wild-type and K5-containing keratinocytes became hyperproliferative after infection, whereas A-CREB–expressing keratinocytes decreased in number (Fig. 7A and B). K5 cells became confluent 48 hours after infection (Fig. 7C, top right) whereas the number of K5:A-CREB keratinocytes decreased (Fig. 7C, bottom right). We found apoptotic cells in A-CREB–expressing keratinocytes 1 day after v-RasHa infection (Fig. 7D, bottom right). Apoptosis was detectable in K5:A-CREB–expressing keratinocytes without v-RasHa infection, but the fraction of apoptotic cells and the intensity of staining were low (Fig. 7D, bottom left). We did not find apoptosis with or without v-RasHa infection when A-CREB was not expressed.

The changes in cell growth of A-CREB–expressing keratinocytes following v-RasHa infection changed the cell cycle profile (Fig. 8A). Mock-infected cells (WT and K5:A-CREB + Dox) had similar cell cycle profiles with only ~12% of cells in S phase. Mock-infected K5:A-CREB −Dox cells progress slowly through the cell cycle with a lower fraction of cells in S phase (8%) and a higher fraction of cells in G2–M phase, which is consistent with measurements of viability (Fig. 8A). After v-RasHa infection, the fraction of cells in S phase increased for all of the samples except the one expressing A-CREB, showing that CREB function is critical for v-RasHa induction of cell growth. In A-CREB–expressing keratinocytes, the fraction of cells in S phase was half that of K5:A-CREB (+Dox) cells with an increased fraction of cells in the G2–M phase. Consistent with cell cycle profile changes, we found that 2 days after v-RasHa infection, the levels of cyclin D1, cyclin B1, and c-Myc increased in

FIGURE 4. Increase in apoptosis in A-CREB–expressing epidermis 48 h after DMBA treatment. A. Methylene blue and TUNEL staining of skin from WT and A-CREB–expressing epidermis 48 h after DMBA treatment. Because TUNEL-positive cells were detected exclusively in hair follicles, the average number of apoptotic cells per follicle was calculated for each animal. B. Number of apoptotic cells per hair follicle in WT and K5 mice grouped together and K5:A-CREB mice 24 and 48 h after DMBA treatment. The total numbers of hair follicles counted for each group are 561 for WT at 24 h, 877 of WT at 48 h, 342 for K5:A-CREB at 24 h, and 493 for K5:A-CREB at 48 h. The number of mice in each group is shown. Forty-eight hours after DMBA treatment, apoptosis in skin of K5:A-CREB mice was increased in comparison with WT (P < 0.01). Bars, 95% confidence interval. C. Quantification of BrdUrd immunohistochemistry to determine fraction of cells in the epidermis of WT, K5, and K5:A-CREB mice that are in S phase of the cell cycle 24 and 48 h after DMBA treatment.

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WT and K5 but not in the A-CREB–expressing keratinocytes (Fig. 8B). These data support the hypothesis that CREB is important for growth and viability of keratinocytes with an oncogenic H-Ras mutation.

Discussion

We describe a new transgenic mouse line that expresses A-CREB, a dominant negative protein that inhibits the DNA binding of the CREB family of B-ZIP transcription factors, under the promoter control of seven tetracycline binding sites. We have expressed A-CREB in the basal epidermis where the keratin 5 promoter activates the expression of the tetracycline transactivator. CREB protein binds the CRE DNA sequence that is preferentially localized in proximal promoters (6, 44). When A-CREB is expressed in the basal epidermis, no phenotype is observed in the skin of the adult mice, suggesting that CREB is not essential for epidermal cell survival. However, during a two-stage chemical carcinogenesis experiment, A-CREB expression caused hair loss and an 80% reduction in papilloma formation. These data support other reports that have implicated CREB in cancer (17-19, 21). Reduction of papilloma formation in A-CREB–expressing mice indicates that CREB DNA binding is a potential molecular target for cancer prevention. Small molecules that inhibit CREB binding to DNA have recently been described and may be interesting candidates for pharmacologic evaluation (45).

FIGURE 5. A-CREB suppresses TPA-induced proliferation. Histology of the skin of WT, K5, and K5:A-CREB mice 24 h after the first TPA treatment, 1 wk after the DMBA during the carcinogenesis experiment. A, Methylene blue staining and BrdUrd (BrdU) immunohistochemistry of dorsal skin. B, Quantification of TUNEL staining of dorsal skin. Because TUNEL-positive cells were detected exclusively in hair follicles, the average number of apoptotic cells per follicle was calculated for each mouse. Bars, 95% confidence interval. C, Fraction of cells in the epidermis that are in S phase of the cell cycle (BrdUrd positive) for the experimental conditions described in A. Results are averaged from four WT, three K5, and six K5:A-CREB mice. The P value was calculated using the Ftest EXCEL function. D, H&E staining of dorsal skin from adult WT, K5, and K5:A-CREB mice after 6 wk of DMBA/TPA treatment. Note hyperplasia in all samples and hair follicle loss in only the K5:A-CREB skin.

FIGURE 6. A-CREB blocks oncogenic H-Ras mutations after DMBA treatment. Oncogenic H-Ras mutation (codon 61, A→T) was detected in mouse skin DNA using mutation-specific PCR at different time points during the skin carcinogenesis experiment. For each time point, a wild-type mouse and an A-CREB–expressing mouse was examined. The number of days since either a single DMBA treatment or biweekly TPA treatments is presented. GAPDH PCR is used as a loading control. DNA from untreated skin was used as a negative control (first two lanes) and papilloma DNA was used as a positive control (last lane). Note the absence of oncogenic H-Ras in A-CREB–expressing samples except at the 25-d time point of TPA application (see text for details).
oncogenic H-Ras mutation are depleted in A-CREB–expressing skin 2 days after DMBA treatment. This could reflect either that DMBA is less mutagenic because the enzymes responsible for DMBA activation, such as cytochrome P450 (47), are less active so that fewer cells acquire the oncogenic H-Ras mutation or that oncogenic H-Ras–containing cells need CREB function for viability. The observation that A-CREB expression reduces the growth and induces apoptosis of the v-RasHa–transformed primary keratinocytes suggests the later possibility. The increase in apoptosis following DMBA treatment in A-CREB–expressing hair follicles might reflect the loss of initiated cells carrying H-Ras mutation. Significant hair loss observed in the A-CREB–expressing mice during promotion phase might also contribute to the loss of initiated cells and reduced tumor formation in the A-CREB–expressing epidermis.

A-CREB expression 4 weeks after papilloma initiation by DMBA treatment did not prevent papilloma formation, suggesting that CREB function is critical for the initial stages of papilloma formation. In contrast, the family of C/EBP transcription factors seems to be critical for the late stage of tumor formation. When C/EBP dominant negative (A-C/EBP) is expressed in skin, the number of tumors declines in A-C/EBP–expressing mice starting from the same number of tumors in WT, K5, and K5:A-C/EBP mice at 10 weeks (35). Moreover, tumor regression is observed when A-C/EBP is induced after 20 weeks of TPA application (35). Alternatively, when the A-FOS dominant negative is expressed in the epidermis, the number of tumors does not change, but tumors change their identity from squamous papillomas to sebaceous adenomas (37). Collectively, these data suggest that the activity of different transcription factors is crucial during different stages of tumor development. CREB is essential for the initial stages of tumor formation; C/EBP is essential for growth of established tumors; and activator protein 1 is essential for maintenance of tumor identity.

The more than 10-fold increase in CREB protein level after TPA treatment of WT skin suggests a role for CREB in the response of mouse skin to TPA. A-CREB prevents the TPA-induced increase of CREB protein level in skin and reduces CREB protein level in primary keratinocytes. Although the exact mechanism of reduction of CREB protein in A-CREB–expressing keratinocytes is not known, our data indicate that this is a posttranscriptional phenomenon. A similar effect has been described in 293 human embryonic kidney cells (48) where dominant negative A-CREB induces degradation of...
A-CREB inhibits cell cycle progression of primary mouse newborn keratinocytes transformed with v-RasHa retrovirus. A. Cell cycle profile at 3.5 d of the four newborn mouse keratinocytes samples (WT, K5, K5:A-CREB +Dox, and K5:A-CREB ‐Dox) treated with either mock or v-RasHa infection as described in Fig. 7. Forty-eight hours after infection, cells were pulse-labeled with BrdUrd for 2 h before collection, stained with anti-BrdUrd antibody and propidium iodine, and subjected to fluorescence-activated cell sorting analysis. The percentages of cells in G1 phase (bottom left), S phase (top), and G2-M phase (bottom right) of the cell cycle are shown. Values are averages of two experiments. Note that A-CREB expression prevents the v-RasHa‐induced increase in the fraction of cells in S phase. B. Induction of the cell cycle proteins by v-RasHa is suppressed in the A-CREB‐expressing keratinocytes.
**Tissue Histology, Cell Proliferation, and Apoptosis**

Skin or tumor tissues were fixed in 10% NBF overnight and transferred to 95% ethanol and embedded in paraffin. Six-micrometer sections were cut and stained with H&E. To quantify the fraction of cells in S phase of the cell cycle, mice were sacrificed 60 min after a single i.p. injection of a sterile solution of bromodeoxyuridine (BrdUrd) (Sigma; 10 mg/mL in PBS, 50 mg/kg of body weight). Skin sections were treated for 30 min at 37°C with 2N HCl in PBS containing 0.5% Triton X-100, rinsed in sodium tetraborate buffer (0.1 mol/L, pH 8.5), and processed for immunohistochemistry with an anti-BrdUrd antibody. TUNEL was done with an ApopTag In Situ Apoptosis Detection Kit (Chemicon) according to the manufacturer’s specification.

**Cultures of Primary Newborn Keratinocytes**

Primary mouse keratinocytes were isolated from newborn transgenic K5:A-CREB mice and wild-type littermate epidermis (53). Primary keratinocytes were seeded at a density of 5 × 10^5 per 10-cm dish or equivalent density in Ca^2+- and Mg^2+-free EMEM (Cambrex Bio Science Walkersville, Inc.) supplemented with 8% Chex (Bio-Rad), treated fetal bovine serum (Atlanta Biologicals, Inc.), 0.2 mmol/L Ca^2+, and 2 mmol/L Dox. After 24 h, cultures were washed twice with PBS and switched to the same medium with 0.05 mmol/L Ca^2+ without Dox to select for basal cells and induce A-CREB expression. Thirty hours later, cells were infected with a replication-defective retrovirus containing the v-Ras^Hta^ gene (43) in medium containing 4 μg/mL polybrene and used for subsequent experiments. Cell viability was determined by colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Promega) assay before and at 24 and 48 h after v-Ras^Hta^ infection.

**Western Blot Analysis**

Protein extracts were isolated from whole skin using radioimmunoprecipitation assay lysis buffer containing 0.1% SDS, 1% NP40, 0.5% Na-deoxycholate, phosphatase, and protease inhibitors and sonicated for 30 s at lowest power. Lysates were run on 12% SDS-PAGE, transferred to polyvinylidene fluoride membrane, and probed with CREB C-21 (Santa Cruz) polyclonal antibodies that react with the leucine zipper of CREB, CREM, ATF-1, and A-CREB. Other antibodies were cyclin D1 (NeoMarkers), cyclin B1, c-Myc (Santa Cruz), CREB (Zumed), cleaved caspase-3, cleaved PARP (Cell Signaling Technology), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Abcam). Horseradish peroxidase–conjugated secondary antibodies in combination with ECL+ (Amersham) detection system were used according to the manufacturer’s protocol.

**mRNA Isolation and Reverse Transcription-PCR**

RNA from dorsal skin or keratinocytes was extracted using TRIzol reagent (Invitrogen), purified, and treated with DNase using Quiagen RNaseasy kit according to the manufacturer’s instructions. Reverse transcription was done using 1 μg of total RNA, oligo dT, and Invitrogen Superscript II reverse transcriptase. PCR ReadyMix from Sigma was used for PCR. Primers sequences were, for GAPDH, 5′-TGTTCCCTACCCC-CAATGTGT-3′ and 5′-CCCTGTGCTGTAGCCGTAT-3′; for MKKS, 5′-ATCAGTGCTACGTAACGATGTGA-3′ and 5′-ACCAGTGAAAGCACCCTTAT-3′; and for CREB, 5′-ACCAAGCAGTGGAGGATGCT-3′ and 5′-ATGGCAATG-TACTGCCACT-3′.

**Cell Cycle Analysis**

Cells were incubated with 10 μmol/L BrdUrd for 2 h before the cells were harvested. Cells were trypsinized, pelleted, resuspended in 100 μL of cold PBS, and fixed in 70% ethanol. Cells were treated with 2 N HCl/Triton X-100 to denature DNA, followed by neutralization with sodium borate. Cells were pelleted, resuspended in 0.5% Tween 20, 1% bovine serum albumin in PBS with FITC-tagged anti-BrdUrd antibody (1:50; Becton Dickinson) and 0.5 mg of RNase A/mL, and incubated at 4°C overnight. Cells were pelleted and resuspended in PBS containing 10 μg propidium iodide/mL and subjected to fluorescence-activated cell sorting analysis. Data were collected and presented on a scatter plot with BrdUrd incorporation on the x axis and DNA content in the cells (propidium iodide intensity) on the y axis.

**Apoptosis Analysis**

Apoptosis was measured using the In situ Cell Death Detection Kit from Roche according to the manufacturer’s instruction. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole, and microscopic images were taken using the same exposure.

**Determination of Oncogenic H-Ras Mutation in Epidermis of DMBA-Treated Skin**

The method for determination of codon 61 A→T mutation in H-Ras gene was adapted from ref. 42. DNA was extracted from 25 mg of skin using Quiagen DNAeasy Tissue Kit according to the manufacturer’s instructions. Sixty nanograms of DNA were used in a 10-μL PCR reaction containing 1× GeneAmp PCR buffer, 2 mmol/L MgCl_2, 250 μmol/L deoxynucleotide triphosphate, and 15 units of AmpliTaq Gold DNA polymerase (Applied Biosystems). PCR conditions were 94°C for 7 min; 94°C for 30 s, 62°C for 30 s, and 72°C for 30 s and for a total of 36 cycles; final hold at 72°C for 5 min. PCR products were separated by agarose gel electrophoresis stained with ethidium bromide and visualized under UV light.

**Chromatin Immunoprecipitation**

Chromatin immunoprecipitation was done using equal concentrations of antibodies against CREB from Santa Cruz and Upstate. The chromatin immunoprecipitation protocol was from P. Farnham. For immunoprecipitation, protein G-agarose beads were used (Invitrogen).

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4 http://genomics.ucdavis.edu/farnham/protocol.html
Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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