ABSTRACT

YOON, KYUNGSIL. CCAAT/enhancer binding protein-alpha (C/EBPα) is a DNA-damage inducible p53-regulated mediator of the G1 checkpoint. (Under the direction of Dr. Robert C. Smart).

CCAAT/enhancer binding proteins (C/EBPs) are members of basic leucine zipper class of transcription factors, and C/EBPα and C/EBPβ are abundantly expressed in epidermal keratinocytes. While the function of C/EBPα in skin has not been characterized, C/EBPβ is involved in the regulation of the early stages of squamous differentiation and also plays a critical role in Ras-mediated mouse skin tumorigenesis and keratinocyte survival. C/EBPα has been shown to play an important role in cell cycle arrest and/or differentiation in various cell types, and forced expression of C/EBPα blocks keratinocyte proliferation suggesting a cell cycle regulatory function of C/EBPα in keratinocytes. Skin is constantly challenged by environmental stressors including sunlight, and there is a strong association between human skin cancers and exposure to UVB radiation. The ability of cells to respond to DNA damage is essential to ensure the integrity of the genome. In response to UV-induced DNA damage, cells activate cell cycle checkpoints that arrest cell cycle progression allowing time for DNA repair, or if the damage is too extensive, initiate apoptosis. Failure to engage the proper DNA damage response results in the accumulation of mutations in critical genes, contributing to the development of cancer.

We report that UVB radiation is a potent inducer of C/EBPα in human and mouse keratinocytes as well as in mouse skin in vivo. UVB irradiation of keratinocytes resulted in the transcriptional upregulation of C/EBPα mRNA producing >70 fold increase in C/EBPα protein. Treatment of keratinocytes with other DNA damaging agents such as MNNG, etoposide and bleomycin also induced C/EBPα, suggesting that the induction of C/EBPα is a general DNA damage response in keratinocytes. The UVB-induction of C/EBPα was temporarily accompanied by UVB-induced p53 accumulation implying a possible relationship between these two proteins. Caffeine, an inhibitor of ATM and ATR kinases, inhibited UVB-induction of C/EBPα as well as p53 increase, and C/EBPα promoter was activated by p53 and UVB irradiation. Exposure of p53-deficient or mutant p53 containing keratinocytes to UVB failed to induce C/EBPα demonstrating that p53 is required for UVB-induction of C/EBPα. Enforced expression of C/EBPα in keratinocytes inhibited cell proliferation suggesting C/EBPα negatively regulates cell growth. UVB induced a more rapid growth arrest in keratinocytes overexpressing C/EBPα. Furthermore, UVB irradiation of C/EBPα knockdown keratinocytes displayed a greatly attenuated DNA damage G1 checkpoint and this was associated with increased sensitivity to UVB-induced apoptosis. UVB irradiation of primary keratinocytes also enhanced squamous differentiation as determined by the increased number of unattached differentated keratinocytes which expressed increased levels of involucrin and loricrin, two markers of squamous differentiation. Overexpression of C/EBPα in keratinocytes also induced involucrin, whose promoter contains C/EBP binding sites.

Taken together, our results identify C/EBPα as a novel p53-regulated DNA damage-inducible gene that has a critical function in the DNA damage G1 checkpoint

response in keratinocytes. Our results also suggest that C/EBPα, in response to UVB treatment, may enhance the differentiation of keratinocytes.

CCAAT/ENHANCER BINDING PROTEIN-ALPHA (C/EBPα) IS A DNA-DAMAGE INDUCIBLE P53-REGULATED MEDIATOR OF THE G1 CHECKPOINT

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DEDICATION

I dedicate this dissertation to my beautiful daughter, Hyeeun Cho.

BIOGRAPHY

Kyungsil Yoon was born on September 27, 1971 in Daegu, the Republic of Korea. She grew up in Busan, a southeast coastal city in Korea until she graduated from high school. Kyungsil enrolled in SungKyunKwan University, Seoul, Korea in 1991 and received a Bachelor of Science degree in Pharmacy in 1995. She began working as a licensed Pharmacist in 1995 as a member of the Drug information Team at the Choongwae Pharma Corporation, Seoul, Korea.

Kyungsil joined the graduated school in Toxicology program at Texas A&M University in 1998. In 1999, she transferred to the department of Toxicology at North Carolina State University and continued working toward a Ph.D. in Toxicology under the direction of Dr. Robert C. Smart.

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General Introduction

1. Epidermis

The skin forms the continuous outer surface of the body interfacing between the organism and external environment. It provides protection against ultraviolet light (UV), chemical and mechanical insults, infection and dehydration. It is a sensory organ with metabolic functions and regulates body temperature. The skin consists of epidermis which is a stratified squamous epithelium that renews itself continuously, dermis which supports and nourishes the epidermis, hypodermis which contains adipose tissue, and epidermal appendages such as hair follicles, sweat glands and sebaceous glands (1).

The epidermis at the surface of skin is mostly composed of keratinocytes that undergo a highly coordinated program of sequential changes in gene expression during differentiation. Keratinocytes progress from proliferating basal cells through morphologically distinct spinous and granular cells, ending in the nonviable, flattened, enucleated stratum corneum, which is sloughed off from the surface and continually replaced by inner cells differentiating outward (2). Basal keratinocytes are attached to the basement membrane and have the capacity for DNA synthesis and mitosis. These cells produce keratin 5 (K5) and keratin 14 (K14), which build intracellular cytoskeletal filaments. When basal keratinocytes are triggered to terminal differentiation, they become postmitotic and migrate upward to suprabasal spinous layer (3, 4). Withdrawal from the cell cycle seems to be a prerequisite for terminal differentiation (2). Then the expression of K5 and K14 is suppressed, and cells express new keratins, cytokeratin 1 (K1) and cytokeratin 10 (K10), and involucrin which is a glutamine and lysine rich

envelop protein deposited on the inner surface of the plasma membrane (2-4). As spinous keratinocytes are further differentiated into granular keratinocytes, they stop generating keratins, and produce assembly proteins, loricrin and filaggrin, major components of the cornified envelope (5, 6). As differentiating cells become permeable, a calcium influx activates epidermal transglutaminase I which covalently crosslinks the envelop proteins such as loricrin, involucrin, and cornifin-α/SPRR1, resulting in the formation of the stratum corneum which is characterized by the loss of intracellular organelles and programmed cell death of mature squames (7, 8).

The epidermis contains a series of the independent epidermal proliferative unit (EPU). Each EPU contains basal keratinocytes covered over by suprabasal layers which are characterized by regularly spaced columns of three flattened differentiated keratinocytes. At the center of basal cells in each unit, there is a cell representing the slow cycling stem cell which has unlimited life span (9). It has been suggested that a two-compartment proliferative system is comprised of a single stem cell and several secondary proliferative cells, transient amplifying cells, which are the progeny of the stem cell and whose divisions amplify the mitosis of the stem cell to some extent. These transient amplifying cells have limited life span and after a few divisions become postmitotic cells which ultimately commit to differentiation (10). The basal cell layer consists of stem cells, transient amplifying cells and postmitotic cells. The epithelial stem cells are believed to be cellular targets for chemical carcinogenesis in skin, which occur in both the interfollicular and follicular epithelia (11, 12).

To maintain epidermal homeostasis growth and terminal differentiation of the keratinocytes should be balanced. If proliferation is predominant, hyperproliferative

disorders of the skin can result, such as psoriasis and basal- or squamous-cell carcinomas. Conversely, premature differentiation can yield a thin epidermis as seen in aged skin (13). Stimulators of cell growth include epidermal growth factor (EGF) (14), transforming growth factor α (TGF- α) (15), keratinocyte growth factor (KGF) (16) and cytokines such as IL-6 (17) and IL-1α (18, 19). EGF and TGF-α stimulate EGF receptors (EGFRs), a member of receptor tyrosine kinases, located on the surface of basal keratinocytes (20), and epidermal keratinocytes can autoregulate their own growth via TGF-α production (21). TGF-β is a negative regulator of keratinocyte growth and also secreted from keratinocytes (22). In addition, it has been shown that TGF-β_1 induces its own expression in mouse keratinocytes (23). Epidermis has a gradient of extracellular and intracellular Ca^{2+} concentration in vivo which is low in the basal cell layer and high in the granular cell layer (24). Mouse keratinocytes actively proliferate, when they are cultured in medium containing 0.05 mM calcium. Upon a switch to high (1.2 mM) calcium, terminal differentiation is induced (25).

The balance between keratinocytes proliferation and differentiation is maintained in part by orchestrating a transcriptional program that determines temporally and spatially distinct epidermal cells (13). The expression patterns of structural genes in the epidermis, as discussed earlier, are well known, however, little is known about how those gene expressions are regulated during terminal differentiation at the transcriptional level. Transcriptional control is achieved by specific transcription factors that interact with regulatory sequences in the promoter or enhancer region of target genes, which results in increased or decreased transcription of those genes (26). Transcription factors belonging to the CCAAT/enhancer binding protein (C/EBP) family have been shown to play a role

in regulating the epidermal differentiation program as well as differentiation of various other cell types.

2. C/EBPs

CCAAT/Enhancer Binding Proteins (C/EBPs) are members of basic leucine zipper (bZIP) class of transcription factors. The bZIP class of transcription factors is characterized by a leucine zipper region and a basic DNA binding domain (27). The leucine zipper region consists of a heptad repeat of leucine within a 35 amino acid sequence, which is proposed to form an amphipathic α-helix with a leucine residue every seventh position or two turns of helix. The leucine zipper chains extending from one α-helix interdigitate with those from a α-helix of another polypeptide, facilitating dimerization (28). Other members of bZIP class of transcription factors include Fos and Jun proteins bound to AP1 (activator protein 1) sites, the CREB/ATF (cAMP responsive element binding protein/activating transcription factor) proteins bound to cAMP responsive element (CRE) and the PAR (proline- and acidic amino acid-rich) domain proteins bound to PAR sites are also members of bZIP class.

1) Structure of C/EBPs

The genes for six C/EBP members, C/EBPα (C/EBP) (29-32), C/EBPβ (NF-IL6, IL-6DBP, LAP, CRP2, NF-M, AGP/EBP) (30, 31, 33, 34), C/EBPγ (Ig/EBP-1) (35), C/EBPδ (NF-IL6β, CRP3) (30, 31), C/EBPε (CRP-1) (31), and C/EBPζ (CHOP10, GADD153) (36, 37) have been identified. C/EBPα (30), C/EBPβ (34), C/EBPδ (30) and C/EBPγ (37) genes are intronless, and C/EBPε (38) and C/EBPζ (39) contain two and four exons, respectively. Various size of polypeptides are produced for C/EBPα (42 kDa

and 30 kDa) or C/EBPβ (38 kDa, 35 kDa (LAP, liver-enriched activating protein) and 20 kDa (LIP, liver-enriched inhibitory protein)) by use of different translation initiation sites due to a leaky ribosome scanning, or regulated proteolysis (40-42).

C/EBP isoforms have high sequence homology in the C-terminal domain (>90%) which consists of basic DNA binding domain and leucine zipper region (43). Homodimers and heterodimers between C/EBP isoforms are formed (31, 35), and dimer formation is a prerequisite for DNA binding (44). C/EBPs recognize and bind to specific DNA sequences, RTTGCGYAAT, where R is A or G, and Y is C or T, in the promoters, although substantial variations have been shown (45). C/EBPζ can dimerize with other members of the family through its intact leucine zipper, but dimers do not bind C/EBP sites but bind to a different DNA sequence in the promoter due to variant basic region of C/EBPζ (37, 46). Therefore, C/EBPζ can act both as an inhibitor of C/EBP and as an activator of other genes. In addition to interaction within the C/EBP family, dimerization with other bZIP proteins has been reported. Dimerization of C/EBPβ with Fos or Jun results in binding away from C/EBP sites towards AP1 sites (47) and dimerization of C/EBPα or C/EBPβ with C/ATF towards CRE sites (48). C/EBP proteins also interact with transcription factors without bZIP region. A direct protein-protein interaction of C/EBPα, C/EBPβ or C/EBPε, and Rel-domain proteins such as nuclear factor kappaB (NF-κB) p50, p65 or RelA has been described, and this interaction depends on a functional leucine zipper and Rel-domain (49). The C/EBP-NF-κB crosstalk results in inhibition of promoters with κB enhancer motifs and synergistic stimulation of promoters with C/EBP binding sites or the serum response elements (SREs), as in the c-fos promoter (49). In the IL-6 and IL-8 promoters which contain both a C/EBP and a NF-κB

sites, NF-κB and C/EBPβ synergize to activate transcription (50). C/EBPβ and ligand-bound glucocorticoid receptor (GR) synergistically stimulate transcription of the α_1-acid glycoprotein, which depends on an intact GR-DNA binding domain and the leucine zipper of C/EBPβ (51).

The N-terminal, transactivation region of C/EBPs is divergent (<20% sequence homology) with exception of short subregions that are conserved in most members (43). The conserved regions represent the activation domains that interact with components of the basal transcription machinery and stimulate target gene transcription (52, 53). C/EBPγ and LIP, truncated form of C/EBPβ, lack a transactivation region and act as dominant negative inhibitors of C/EBP function by forming inactive heterodimers with other members (42, 54). The N-termini of C/EBPα, C/EBPβ and C/EBPε proteins also contain repression/attenuator regions (52, 55, 56).

2) Tissue expression

C/EBPα is expressed in liver, fat, lung, peripheral leukocytes, epidermis, adrenal gland, intestine and skeletal muscle (30-32, 57, 58), and C/EBPα mRNA is expressed at high levels in terminally differentiated cells in the skin, liver and adipose tissue (30, 31, 57, 58). Expression of C/EBPβ is high in the liver, intestine, lung, adipose tissue, spleen, kidney, myelomonocytic cells, and epidermis (30, 31, 33, 34, 58), and C/EBPδ is constitutively expressed in the adipose tissue, lung and intestine (30, 31). Whereas C/EBPγ and C/EBPζ are expressed ubiquitously (35, 37), C/EBPε mRNA and protein are restricted to myeloid and lymphoid cells (38). For some C/EBP members the expression of protein does not associated with that of mRNA indicating post-transcriptional

regulation, and expression profile is also regulated by a variety of extracellular signals (31, 34, 58).

2) Function of the C/EBP family

- **Cellular differentiation**

C/EBP family plays an important role in cellular differentiation in adipocytes, myeloid cells, hepatocytes, mammary epithelial cells, ovarian luteal cells, keratinocytes, neuronal cells and intestinal epithelial cells (59-61). C/EBP binding sites are present in the promoter regions of numerous genes which are required for the proper maturation and function of each cell type (62-64).

Differentiation of growth arrested preadipocytes is initiated by the addition of a cAMP elevating agent (methylisobutylxanthine), a glucocorticoid (dexamethasone), insulin and fetal bovine serum (FBS). The preadipocytes reenter the cell cycle synchronously and undergo two rounds of mitosis upon the treatment. C/EBPβ and C/EBPδ mRNA are induced during mitotic clonal expansion in response to cAMP elevating agents and glucocorticoid, respectively (30). Then the preadipocytes exit the cell cycle and begin to express C/EBPα, which is followed by induction of adipocyte-specific markers (62, 65). C/EBPβ and C/EBPδ are thought to induce C/EBPα expression through C/EBP binding sites in the proximal promoter of the mouse C/EBPα gene. Embryonic fibroblasts lacking both C/EBPβ and C/EBPδ cannot initiate the differentiation program induced by hormonal stimulation (66) and C/EBPα-deficient mice display reduced lipid accumulation in the adipose tissue (67).

C/EBPα, C/EBPβ, C/EBPδ and C/EBPε are expressed in myeloid cells with a unique expression profile during myeloid cell differentiation (68, 69). C/EBPα-deficient

mice lack mature neutrophils, which is correlated with a lack of receptors for granulocyte colony stimulating factor (G-CSF) or interleukin-6 (IL-6), and forced expression of these receptors in C/EBPα-deficient cells restores granulopoiesis (70, 71). C/EBPβ has been indicated to have a role in the differentiation of macrophage. C/EBPβ-deficient mice are susceptible to infections, and display impaired nitric oxide production and low IL-12 levels (72, 73).

C/EBPs are expressed in a differentiation-associated manner in skin. The expression of C/EBPα and C/EBPβ is upregulated during differentiation induced by high calcium (58, 74). C/EBPβ-deficient mice display a mild epidermal hyperplasia and decreased expression of K1 and K10 (61). In cultured cells, C/EBPα and C/EBPβ have been shown to activate K10 promoter via C/EBP binding sites (75). In addition, C/EBPα transcription factor activity is required for the basal promoter activity and TPA induction of the involucrin gene (76)

C/EBPα-deficient mice have deranged liver acinus structure, similar to proliferative hepatocellular carcinoma (77). C/EBPβ has been shown to have an important role in female reproduction from knockout studies. Female C/EBPβ-deficient mice are infertile due to a lack of the corpora lutea in the ovaries. C/EBPβ is needed for periovulatory granulose cell differentiation in response to lutenizing hormone (78). C/EBPβ is also required for differentiation of mammary epithelial cells and the expression of milk protein genes as well as for the proper proliferation and morphogenic responses during mammary gland development (79, 80).

- **Cell growth and survival**

C/EBPα is expressed in high levels in terminally differentiated cells with exception in the myeloid cells and downregulated during proliferation following partial hepatectomy, suggesting an antiproliferative function of C/EBPα (57, 68). When overexpressed in cultured cells, C/EBPα inhibits cell proliferation (57, 81). Hepatocytes and alveolar type II cells of the C/EBPα-deficient mice display increased proliferation (77).

C/EBPα has been shown to interact with various proteins associated with the cell cycle regulation, implying multiple mechanisms of C/EBPα-induced growth arrest. C/EBPα upregulates p21 by activating p21 gene expression and stabilizing protein, and cooperates with p21 by protein-protein interaction to inhibit cyclin dependent kinase 2 (Cdk2) activity (82, 83). C/EBPα has been shown to interact with a Rb family protein, p107 and disrupt S phase-promoting p107/E2F complex (84, 85) and C/EBPα also represses E2F-dependent transcription (86, 87). Moreover, C/EBPα has been shown to block cell cycle progression independent of its DNA binding and transcriptional activity through the formation of an inhibitory complex with cyclin dependent kinase 4 (Cdk4) and Cdk2 which prevents cyclin binding (88). Interaction between C/EBPα and Cdk4 also leads to a proteosomal degradation of Cdk4. More recently the anti-proliferative activity of C/EBPα has been shown to require a SWI/SNF chromatin remodeling complex supporting a transcriptional basis for its anti-proliferative activity (89).
The importance of C/EBPα in the regulation of growth arrest and differentiation is highlighted by recent studies in which C/EBPα is implicated as a human tumor suppressor gene. In human acute myeloid leukemia (AML), C/EBPα is inactivated by

dominant negative mutation or through its association with oncoprotein AML-1-ETO. The inactivation of C/EBPα is thought to result in a differentiation block of the granulocytic blasts (90-92). C/EBPα expression is reduced in hepatocellular carcinomas (93, 94), lung cancer and lung cancer cell lines (95) supporting a possible tumor suppressor function in these organs. Moreover, the induction of C/EBPα in lung carcinoma cells leads to proliferation arrest (95). C/EBPα protein levels are greatly diminished or undetectable in mouse skin squamous cell carcinomas (SCCs) (58), and forced expression of C/EBPα in SCC cells resulted in inhibition of cell proliferation (M Shim and RC Smart, unpublished results), suggesting that C/EBPα is a negative regulator of keratinocyte proliferation and may have a tumor suppressor function in SCC development in skin.

C/EBPβ has been suggested to increase proliferation. Increased proliferation after partial hepatectomy is associated with increased level of C/EBPβ, but hepatocytes of C/EBPβ-deficient mice do not proliferate during this process (96). It has been shown that phosphorylation of Ser105 (rat) or Thr217 (mouse) of C/EBPβ by ribosomal protein S-6 kinase (RSK) is required for hepatocytes proliferation induced by TGF-α (97).

Carbon tetrachloride, a hepatotoxin, is shown to activate RSK, subsequently phosphorylating C/EBPβ on Thr217. In the hepatic stellate cells of C/EBPβ-deficient or C/EBPβ-Ala217 mutant transgenic mice, carbon tetrachloride induces apoptosis. Both phosphoThr217-C/EBPβ and the phosphorylation mimic Glu217-C/EBPβ, but not Ala217-C/EBPβ are shown to be associated with procaspases1 and 8 (98).

C/EBPβ-deficient mice are completely refractory to skin tumorigenesis induced by a variety of carcinogens and carcinogenesis protocols. C/EBPβ-deficient v-Ha-ras transgenic mice display significant reduction in skin tumorigenesis compared to C/EBPβ-wild type v-Ha-ras transgenic mice, which links the proto-oncogene Ras and C/EBPβ. It has been shown that oncogenic Ras stimulates C/EBPβ transcription activity in mouse keratinocytes and C/EBPβ also participates in oncogenic Ras-induced transformation of NIH3T3 cells. In addition, C/EBPβ-null mice treated with a single dose of 7,12-dimethylbenz[a]anthracene (DMBA) exhibit a 17-fold increase in the number of basal apoptotic keratinocytes compared with treated wild-type mice, indicating that C/EBPβ functions as a survival factor in DMBA/Ras-induced tumorigenesis (99, appendix).

Among C/EBP family members, C/EBPδ and C/EBPζ (C/EBP homologous protein (CHOP), growth arrest- and DNA damage-inducible gene 153 (GADD153)) has been suggested to be involved in cellular stress responses. C/EBPδ has been shown to be upregulated transcriptionally by serum and growth factor withdrawal or contact inhibition and play a role on the regulation of G0 growth arrest and apoptosis in mouse mammary epithelial cells (100, 101).

Treatment of various genotoxic agents, growth arrest induced by nutrient deprivation or contact inhibition and endoplasmic reticulum (ER) stresses have been reported to induce C/EBPζ (102, 103). Its association with G1-S arrest or apoptosis has been suggested. ER overload in pancreatic beta cells causes ER stress and leads to apoptosis via C/EBPζ induction, which results in diabetes. C/EBPζ-deficient mice are defective in ER stress induced apoptosis in pancreatic beta cells (104, 105). Most of human myxoid and round cell liposarcomas are associated with chromosomal

translocations leading a fusion protein, consisting of an N-terminus from the FUS or TLS gene and a C-terminus from C/EBPζ gene. Compared to wild type C/EBPζ, this fusion protein fails to cause growth arrest (106, 107).

- **Inflammation and immune response**

Inflammatory stimuli induce the expression of C/EBPβ and C/EBPδ mRNA in hepatocytes and macrophages, whereas C/EBPα mRNA expression is inhibited (108, 109). C/EBPβ has been first recognized as a transcription factor regulating gene transcription in response to IL-1 and IL-6 in hepatocytes (110). C/EBP binding sites have been identified in the promoter regions of genes involved in the inflammatory response, such as those for cytokines, cytokine receptors and acute-phase proteins (57, 62, 70, 71, 109). C/EBPβ-deficient mice display impaired expression of serum amyloid A and P proteins, α_1-acid glycoprotein, complement c3 component and tumor necrosis factor-α (TNF-α) (72, 73, 109).

C/EBPε-deficient mice display slow migration of neutrophils to an inflammatory lesion with a reduced bactericidal response (111). In the macrophages of C/EBPε-deficient mice, genes involved in the immune and inflammatory responses are downregulated (112). C/EBPγ-deficient mice display attenuated natural killer cell cytotoxicity and interferon-γ (INF-γ) (113).

- **Metabolism/memory**

C/EBPα-deficient mice die before or soon after birth due to severe hypoglycemia with very low levels of glycogen in the liver. It results from altered hepatic glucose and glycogen metabolism including reduced glycogen synthase and decreased levels of gluconeogenic enzymes. In addition, C/EBPα-deficient mice also have high blood

ammonia concentrations compared to wild type mice, resulting from a lack of expression of ornithine-cycle enzymes (114). C/EBPβ-deficient mice also display fasting hypoglycemia, with defects in hepatic glucose production and decreased blood lipid levels (115).

Inducers of memory process, cAMP and Ca^{2+}, enhance the expression and DNA binding activities of C/EBPβ and C/EBPδ in hippocampal neurons (116). C/EBPβ has been shown to play an important role in long-term memory process (117). C/EBPδ-deficient mice show an increased contextual fear response, implicating a role of C/EBPδ in learning and memory (118).

4) Regulation of C/EBP

- **Transcriptional regulation**

Mouse C/EBPα proximal promoter has been characterized and shown that it contains potential binding sites for C/EBP, Sp1, nuclear factor (NF)-1, NF-Y, upstream stimulating factor (USF), basic transcription element-binding protein and NF-κB. The C/EBPα promoter can be autoactivated by C/EBPs via C/EBP binding site (119, 120). The human C/EBPα promoter does not contain C/EBP binding sites, however, it can still be autoregulated indirectly by C/EBPα which stimulates the DNA binding activity of USF (121). c-Myc suppresses C/EBPα gene transcription through interaction with the core promoter region (122).

The promoter of C/EBPβ genes have been characterized and shown to be autoregulated (123). CREB also regulates C/EBPβ expression by interacting with two CRE-like sequences near the TATA box in hepatocytes (124). These sequences also mediate IL6-induction of C/EBPβ transcription during acute phase response (APR)

through a pathway which involves IL-6-mediated tethering of signal transducer and activator of transcription-3 (STAT-3) to the promoter (125).

The transcription of the C/EBPδ is induced in growth arrested mammary epithelial cells and in the involuting mammary gland through the activation of STAT-3 and subsequent binding to the promoter (126, 127). The STAT binding site is also needed for the IL-6-induction of C/EBPδ mRNA during APR in hepatocytes (128). The autoregulation of rat C/EBPδ requires C/EBP binding sites at the 3' ends (129), whereas 5' ends of the mouse C/EBPδ gene are enough for autoactivation (130).

The AP-1 site is essential for the C/EBPζ induction in response to growth arrest and DNA damage. Treatment of cells with oxidant or UVC has been shown to stimulate the binding of the c-Jun/c-Fos dimer to the AP-1 sites (131). However, the promoter is activated strongly in transfected cells, but only a modest increase in endogenous gene expression has been observed. There are some conflicting results in the induction of the endogenous C/EBPζ mRNA and protein especially with UV treatment (102, 132, 133). The ER stress response element in C/EBPζ promoter is composed of two overlapping regions which NF-Y interacts with and mediates constitutive and ER stress inducible activation of gene transcription (134). Inhibition of protein synthesis resulting from amino acid depletion can also induce C/EBPζ expression. It is mediated through mammalian target of rapamycin (mTOR) and the phosphoinositide 3-kinase (PI3K) signaling pathways that converge on the amino acid responsive element which ATF-2 interact with (135).

- **Posttranslational modification**

C/EBPβ has a folded conformation and is normally repressed by negative regulatory regions masking its DNA binding and activation domains, and repression is relieved by phosphorylation of the negative regulatory regions (53, 56). C/EBPβ is activated by phosphorylation of Thr235 located in N-terminal close to the DNA binding domain by a Ras/mitogen-activated protein kinase (MAPK) pathway (136), Ser105 in activation domain by protein kinase C (PKC) (137) and Ser276 by Ca^{2+}/calmodulin-dependent protein kinase (138). Insulin inhibits C/EBPβ trasnactivation through PI3K/Akt (PKB, protein kinase B) pathway by disrupting interactions between p300 and C/EBPβ (139). Phosphorylation between the region Ser173 and Ser223, and Ser240 by cAMP-dependent protein kinase (PKA), and Ser240 phophorylation by PKC decrease C/EBPβ DNA binding affinity (140). C/EBPβ has been shown to be translocated into the nucleus through activation of the PKA by forskolin in rat PC12 cells and thus activate transcription of c-fos gene (141). C/EBPβ is also translocated into nucleus upon antioxidant treatment through a PKA-dependent phosphorylation of Ser299 in colorectal cancer cells (142).

PKC has been shown to phosphorylate Ser248, Ser277 and Ser299 of C/EBPα in vitro, resulting in the attenuation of DNA binding affinity (143). In contrast, overexpression of the novel PKC isoforms, PKCδ and PKCη in keratinocytes increases C/EBPα protein, DNA binding of C/EBPα to the promoter of human involucrin (hINV) and hINV gene transcription, by stimulating p38δ MAPK. Novel PKC- or C/EBPα-mediated increase in hINV promoter activity is blocked by expression of a dominant negative form of Ras, MEKK1, p38 or C/EBPα (144). In addition, Ras enhances C/EBPα-mediated trasnactivation of granulocyte colony stimulating factor receptor (G-

CSFR) promoter without changing C/EBPα DNA binding. C/EBPα mutant S248A as well as PKC inhibitor abrogates Ras-mediated C/EBPα transactivation, and inhibits the ability of C/EBPα to induce granulocytic differentiation (145). C/EBPα is phosphorylated on at least six sites in differentiated adipocytes, and insulin treatment mediates dephosphorylation of Thr222 and Thr226 by inactivating glycogen synthase kinase 3 (GSK3) (146).

In response to cellular stress, p38 MAPK pathway is activated and phosphorylates Ser71 and Ser81 of C/EBPζ, which leads to increased transcriptional activity (102).

3. DNA damage response

Maintenance of DNA integrity is vital for the cells and organisms, but is a challenging process considering that cells are constantly assaulted by endogenous metabolic byproducts and exogenous factors which alter DNA structure and encoded message. Aberrant structures of DNA cause mutations, and DNA damage needs to be repaired to ensure complete and accurate DNA replication and transcription. Accumulation of mutations leads to genomic instability of cells and eventually carcinogenesis (147, 148). As the primary defense mechanisms against DNA damage, cells activate cell cycle checkpoints and DNA repair system. Apoptosis is an additional mechanism to eliminate cells with extensive damage (149).

1) DNA damage

Under physiological condition, spontaneous depurination from the sugar phosphate backbone and deamination of cytosine occur. DNA modification from endogenous sources includes oxidative DNA damage mediated by hydroxyradicals which

are byproducts from aerobic metabolism, inflammation process or biotransformation by cytochrome P450 and other enzymes, and alkylation by endogenous alkylating agents (150).

The environmental exposure to alkylating agents such as N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), methylmethane sulfonate (MMS), nitrosoamines and notrosoureas causes DNA methylation. Bulky chemical adducts on DNA are induced by polycyclic aromatic hydrocarbons such as benzo[a]pyrene, DMBA and aflatoxins, aromatic amines from cigarette smoking, and UV light. Crosslinking agents such as nitrogen mustards (cyclophosphamide, chlroambucil), cisplatin and UV radiation, and ionizing radiation (IR) such as gamma-ray and X-ray result in DNA-DNA and DNA-protein crosslinking, and single and double strand DNA breaks, respectively (149).

2) DNA repair

DNA alkylation can be repaired by single step direct reversion by O^6-methylguanine–DNA methyltransferase (MGMT) or AlkB homologue 2 (ABH2) and ABH3 which recognize 1-meA and 3-meC, respectively (151, 152).

Base modifications are mainly cleared by the excision repair which includes base excision repair (BER) and nucleotide excision repair (NER). Relatively minor modification to the bases of DNA can be removed by BER, such as alkylation or oxidization of DNA bases arising either spontaneously within the cell or from exposure to exogenous agents including IR and long wave UV light (153).

Bulky DNA adducts from exposure to carcinogens like aflatoxin, benzo[a]pyrene and DMBA, UV light-induced (6-4) photoproducts and cyclobutane pyrimidine dimers, and intrastrand crosslinks are repaired by nucleotide excision repair (NER) (154). Cells

defective in NER are characterized by UV-hypersensitive disorders such as xeroderma pigmentosum (XP) and Cockayne's syndrome (CS) (155). NER is executed through two distinct pathways, global genomic repair (GGR) and transcription-coupled repair (TCR). GGR removes DNA lesions from the non-transcribed region of the genome and the non-transcribed strand of transcribed regions. (6-4) photoproducts which distort the DNA more than pyrimidine dimers are removed rapidly and predominantly by GGR. Pyrimidine dimers are repaired more efficiently by TCR from the transcribed strand of expressed genes (156).

The mismatch repair (MMR) is responsible for removal of base mismatches caused by spontaneous base deamination, oxidation, methylation and replication errors (157). MMR also binds to various chemically induced DNA lesions such as alkylation-induced O^6-methylguanine paired with cytosine or thymine (158), intrastrand crosslinks generated by cisplatin (159), thymine- or uracil-containing UV light photoproducts paired with mismatched bases (160).

DNA strand breaks are repaired by error-free homologous recombination (HR) or error-prone but quick, non-homologous end-joining (NHEJ). NHEJ pathway predominates in mammalian cells and occurs mainly in G0/G1 phase of cell cycle, whereas HR occurs during the late S and G2 phases (161). A heterodimeric complex consisting of Ku70 and Ku80 binds to damaged DNA and associates with the catalytic subunit of DNA dependent protein kinase (DNA–PKcs) to form the active DNA–PK holoenzyme (162). DNA–PK activates XRCC4–ligase IV, which seals the breaks (163).

HR is mediated by the Rad51-associated proteins that include other Rad proteins and BRCA2 (164). Following DSBs, the MRE11-RAD50-NBS1 (MRN) complex forms

prominent foci and carries out the initial processing of the DSB ends. The foci also include RAD51 and BRCA1 (165).

3) Damage sensor

Although different types of DNA damage are repaired by different repair enzymes, they trigger common signal transduction pathways mediating DNA damage response. One feature of DNA damage response is DNA damage checkpoint activation which delays cell cycle progression until the repair is completed successfully. The central regulators of the DNA damage response pathway are ataxia-telangiectasia mutated (ATM) and ataxia-telangiectasia mutated and Rad3 related (ATR). ATM and ATR belong to PI3K-like protein kinase (PIKK) family which also includes mTOR and DNA-PK. Whereas ATM responds exclusively to DSBs, ATR responds to various types of DNA damage and has a role in genome surveillance during DNA replication (166). The way DNA damage triggers signal transduction pathway to cause DNA damage response has been proposed (167).

After DNA damage is detected by lesion specific repair system, DNA damage becomes reversed instantly if the lesion is easy to repair. Some DNA lesions persist depending on the nature of the lesion and its genomic location, and then ATR and ATR-interacting protein (ATRIP) are recruited to the sites of DNA damage (168) possibly by lesion-specific repair proteins. ATR now phosphorylates proximal target proteins such as histone H2A and ATRIP and may facilitate DNA repair by changing local chromatin structures (169). If DNA repair still cannot be completed, putative damage sensors, Rad17-relplication factor C (Rad17-RFC) complex and the 9-1-1 (Rad9, Rad1 and Hus1) complexes which are also recruited to the damage sites, allow ATR-dependent activation

of the Chk1 (170). These events would trigger the global DNA damage response, including cell cycle arrest, possibly further chromatin modulation and the upregulation of the repair capacity of the cell, all of which may combine to facilitate repair of lesions. As an exception, IR- or radiomimetic drug-induced DSBs, because of the highly deleterious nature of DSBs, seem to be directly recognized by ATM (171). Recently it has been suggested that ATM activation results from changes in the chromatin structure which lead intermolecular autophosphorylation and inactive dimer or multimer dissociation of ATM exposing the kinase domain (172).

In mammalian cells, ATM and ATR, RAD17, RAD1, RAD9 and HUS1 are thought to be involved in the DNA damage recognition and signal initiation of the checkpoint. ATM-deficiency leads to increased radiosensitivity and failure in checkpoint activation including loss of the damage-induced G1 checkpoint, radioresistant DNA synthesis (RDS) and erroneous mitotic entry (173). ATR protein plays a critical role in development as ATR gene inactivation leads to early embryonic lethality in mice (174). Overexpression of kinase-inactive ATR which has a dominant negative effect, leads to increased radiosensitivity and defective activation of cell cycle checkpoints by DNA damaging agents including UV and IR (175, 176). ATR needs an accessory protein, ATRIP which regulates the expression of ATR. ATRIP is one of the immediate substrates of ATR, and both are localized to intranuclear foci after DNA damage (168). ATR/ATRIP can localize to the site of damage in the absence of other sensor proteins indicating that the ATR is involved in the early step of damage detection (177). Consistently, it has been shown that purified ATR binds to UV-damaged DNA with higher affinity than undamaged DNA (178).

Rad17-RFC, in which Rad17 displaces the large RFC140 subunit and interacts with four replication factor C (RFC) subunits (Rfc36, Rfc37, Rfc38, Rfc40), forms a complex with the 9-1-1 heterotrimer. This complex resembles the RFC/proliferating cell nuclear antigen (PCNA) clamp loader/sliding clamp complex of the replication machinery (179, 180). The Rad17-RFC complex purified from Hela cells exhibits a binding to primed DNA, and Rad17 has been shown to be necessary for the recruitment of the 9-1-1 complex after DNA damage (177).

BRCA1, the familial breast and ovarian cancer gene, seems to function as an adaptor of checkpoint activation by localizing substrates for ATM and ATR kinases perhaps linking cell cycle arrest to DNA damage repair. BRCA1 is phosphorylated by ATM and ATR after DNA damage (181, 182), and this process is required to activate downstream kinases, Chk2 and Chk1 to induce cell cycle arrest for DNA repair (183, 184). In BRCA1 mutant cells, Chk1 kinase activity and G2/M arrest appear to be compromised in response to IR (183). BRCA1 is associated large protein complexes, BRCA1-associated genome surveillance complex (BASC) that contain DSB-repair and mismatch-repair enzymes with ATM and thus affects HR, MMR and TCR (185-187).

After IR exposure, 53BP1 is phosphorylated by ATM in wild type cells, and presumably by ATR in ATM-deficient cells. 53BP1 rapidly localizes to sites of DNA damage induced by IR and other DNA damaging agents (188) and is also found to interact with checkpoint and repair proteins suggesting its involvement in the recruitment of downstream targets for the checkpoint kinases, similar to BRCA1 (189, 190).

4) Transducer kinases

Signals originated from ATM and ATR are received by two Ser/Thr kinases, Chk2 and Chk1, respectively. Phosphorylation by ATM/ATR activates the protein kinase activity of Chk1/Chk2 which subsequently phosphorylates downstream effectors. Chk1 and Chk2, as well as other ATM and ATR substrates including BRCA1 and 53BP, have Ser-Gln/Thr-Gln (SQ/TQ) motifs which are consensus phosphorylation sites for ATM and ATR (191).

It has been shown that phosphorylation of Ser 317 and Ser345 of Chk1 by replication arrest or various types of DNA damage by UV, IR, hydroxyurea and topoisomerase inhibitors significantly increases the Chk1 kinase activity (192-196). Overexpression of dominant-negative mutant form of ATR inhibits UV- and hydroxyurea-induced Ser345 phosphorylation while that of wild type ATR enhances phosphorylation. Chk1 mutants containing alanine in place of Ser317 and/or Ser345 are poorly phosphorylated by ATR in vitro and poorly activated to phosphorylate Cdc25C, compared to wild type in response to replication arrest or genotoxic stress in vivo (197).

Full activation of Chk2 needs sequential phosphorylation in an N-terminal forkhead-associated (FHA) domain. Phosphorylation of Thr68 initiates the conformational changes in the kinase domain and makes Thr383 and Thr387 accessible to oligomerization and autophosphorylation (198). In response to IR, ATM is a major kinase phosphorylating Thr68 of Chk2 (199). Upon activation, Chk2 phosphorylates a number of effector proteins including p53, Cdc25A and Cdc25C which activate G1, S and G2/M checkpoints, respectively.

5) Cell cycle checkpoint activation

- **G1 checkpoint**

Mutant cells lacking wild type p53 are defective in the delay of G1/S-phase transition after DNA damage (200). p53 prevents the G1-S phase transition through transcriptional upregulation of the CDK inhibitor p21 (CIP1/WAF1/CDKN1A). p21 binds and inactivates cyclin D/Cdk4,6 and cyclin E/Cdk2 complexes resulting in pRB hypophosphorylation and cell cycle arrest in G1(201).

IR-activated ATM kinase phosphorylates p53 on Ser15 (202, 203), and this phosphorylation contributes to enhance the activity of p53 as a transcription factor by recruitment of coactivators (204, 205). ATM phosphorylates MDM2 on Ser395 to prevent nuclear export of p53-MDM2 complex and further degradation of p53 (206). Chk2 phosphorylates p53 on Ser20 upon activation by ATM-mediated phosphorylation. This phosphorylation has been shown to interfere interaction of p53 with MDM2 which acts as an ubiquitin ligase in p53 ubiquitination to mediate proteosomal degradation (207).

ATR also contributes to the Ser15 phosphorylation of p53 as a maintenance kinase after IR (208). In AT cells exposed to UV radiation, phosphorylation of p53 on Ser15 is similar to that in normal cells (209), but is strongly reduced in fibroblasts overexpressing kinase inactive ATR (208). Taken together, ATR phosphorylates p53 in Ser15, either directly or maybe indirectly through Chk1 to induce DNA damage checkpoint response independent of ATM.

- **S phase checkpoint**

Repair of damaged DNA in S phase is the final defense to eliminate DNA lesions before the damage is converted into inheritable mutations. In the S phase, DNA lesion will be encountered by the replication machinery and may stall the replication fork.

Mechanisms for the attenuation of the S phase in response to DNA damage include ATM-Chk2-Cdc25A, ATR-Chk1-Cdc25A, ATR-NBS and ATM-NBS/SMC1 pathways. One of the phenotypes of AT cells is RDS demonstrating a defect in S phase checkpoint (210). In response to IR, normal cells induce a rapid and reversible decrease in DNA synthesis resulting from the decrease in the rates of replication origin firing and DNA strand elongation.

Exposure to IR during S phase activates ATM-Chk2 pathway which now induces degradation of Cdc25A. Cdc25A is a protein tyrosine phosphatase that dephosphorylates Tyr15 of Cdk2 and activates cyclin A-Cdk2 complex which is needed for S phase progression (211). Chk2 phosphorylation of Cdc25A on Ser123 targets Cdc25A for ubiquitin-dependent degradation. Then cyclin A-Cdk2 activation is inhibited resulting in the lack of the firing of early origin of replication in S phase (212). Mutant Chk2 associated with a variant form of Li-Fraumeni syndrome fails to bind and phosphorylate Cdc25A, implying that genetic lesions in the RDS lead genomic instability and cancer development (211, 213). Human fibroblasts overexpressing kinase inactive ATR also induces RDS by exposure to IR (175). Chk1 is another kinase phosphorylating Cdc25A on Ser123 and other residues upon activation (194).

Another participant in the DNA damage S phase checkpoint is NBS1, which is mutated in the human chromosomal instability disorder, Nijmegen breakage syndrome (NBS) (214). NBS1 mutant cells display RDS similar to AT cells when exposed to IR, suggesting that NBS1 has a role in the S phase checkpoint. IR treatment of cells induces formation the nuclear foci including MRN complex in close proximity to DSBs (215). ATM has been reported to phosphorylate three Ser residues of NBS1 and Ser→Ala

mutants at any sites fail to complement the S-phase checkpoint defect of NBS cells (216, 217).

The structure maintenance of chromosomes 1 (SMC1) protein has been shown to have a role in an ATM-NBS1-dependent S phase checkpoint. SMC1 is phosphorylated by ATM upon IR treatment and the phosphorylation of NBS1 by ATM is required for the phosphorylation of SMC1. Overexpression of a SMC1 mutant of phosphorylation sites impairs the S phase checkpoint (218). In addition, same sites are phosphorylated with treatment of UV and hydroxyurea, suggesting SMC1/NBS1 pathway may have a role in the S phase checkpoint involving ATR (219).

Whereas BRCA1 phosphorylation induced by IR is strongly dependent on ATM, that induced by UV or HU is heavily dependent on ATR. ATM also indirectly activates BRCA1 through the Chk2. Chk2 induced phosphorylation of forced-expressed BRCA1 is required for the protection of BRCA1-deficient breast cancer cells from radiosensitivity (184).

- **G2/M phase checkpoint**

Phosphorylation of Cdc2 at Thr161 by CDK-activating kinase (CAK) is required to activate Cdc2 (220). During G2 phase of cell cycle, Cdc2/cyclin B complex is kept inactive by Tyr15 and Thr14 phosphorylation of Cdc2 by Wee1 and Myt1 kinases, respectively (221, 222). When mitosis starts, both residues are dephosphorylated by the phosphatase Cdc25, which activates the Cdc2/cyclin B complex for mitotic entry in the nucleus (223, 225). Therefore, Cdc25 would be the primary target for controlling G2/M checkpoint.

Chk1 has been shown to phosphorylate mitotic promoting phosphatase, Cdc25, which allows inhibitory binding of Cdc2 to 14-3-3σ and exclusion from nucleus (192, 224). Chk2 also phosphorylates Ser216 on Cdc25C in vitro (226). In ATR-deficient cells exposed to IR, there is erroneous mitotic entry (168), and in addition, Chk1-deficient embryonic stem cells show a substantial reduction in G2 arrest in response to IR (193), demonstrating the critical role of ATR-Chk1 in the G2/M checkpoint. When AT cells are exposed to IR during G1 or S phase, they are effectively arrested in G2 phase before the initiation of mitosis. However, AT cells are not arrested in G2 phase if they are exposed to IR in G2 phase (227). In Chk2-deficient embryonic stem cells, a defect in the maintenance of the G2 arrest has been observed (228). These observations suggest that ATM-Chk2 play a secondary role in G2 checkpoint activation when cells are exposed to IR during G1 or S phase, and when DNA is damaged in G2 phase, ATM is critical for G2 checkpoint response.

Several transcription targets of p53, including p21, 14-3-3σ, and GADD45, also can inhibit Cdc2. p21 has been shown to participate in the G2 checkpoint by inhibiting Cdc2 activity after IR (229). 14-3-3 is induced by IR in the presence of wild type p53, causing cells to arrest in G2 (230). Adriamycin-treated 14-3-3 deficient cells undergo mitotic catastrophe characterized with a disassembled nuclear membrane and fragmented chromatin as a result of entering mitosis before completing DNA synthesis. This event is preceded by entry of Cdc2/cyclin B1 into the nucleus, whereas the complex remains in the cytoplasm in wild type cells treated with adriamycin (231). GADD45-deficient lymphocytes fail to induce partial inhibition of mitotic entry by UV or MMS, whereas GADD45-deficiency does not affect the G2 arrest in response to IR. It suggests a role of

GADD45 in G2/M checkpoint downstream of ATR pathway and a multiplicity of G2/M checkpoint in response to specific types of DNA damage (232). Immunoprecipitation of GADD45 shows that GADD45 interacts with Cdc2 but not with cyclin B1, and addition of GADD45 releases Cdc2 from the Cdc2/cyclin B complex, suggesting that GADD45 inhibits Cdc2 by blocking interaction of Cdc2 with cyclin B (233). In addition, Reprimo, a cytoplasmic protein has been found to be induced by IR in a p53-dependent manner. Overexpression of Reprimo induces cell cycle arrest in G2/M with inactive Cdc2 and cytoplasmic cyclin B, suggesting its role in p53-dependent G2 arrest (234). Furthermore, It has been shown that IR reduces Cyclin B1 and Cdc2 mRNA level and activation or overexpression of p53 represses gene transcription of both Cyclin B1 and Cdc2 (235, 236).

6) Apoptosis

Some DNA lesions are extremely cytotoxic if not repaired, whereas others are mutagenic. The basic cellular defense response is to repair the damage, but when the type and amount of damage overwhelm the checkpoint-repair machinery, programmed cell death (apoptosis) is initiated instead. The mechanism of the choice between survival with growth arrest and apoptosis and the mechanism by which the DNA damage recognition and repair/checkpoint machinery induce the apoptotic events are not entirely clear. However, they are highly dependent upon cell types and circumstances such as the presence of survival factor or oncogene activation (237). Characteristic morphological changes include cell rounding, plasma membrane blebbing, condensation of the nuclear DNA (pyknotic nuclei) without disassembly of the nuclear envelope, eosinophilic cytoplasm and internucleosomal DNA cleavage (238). p53, in addition to play a role in

DNA damage induced checkpoint activation, serves as another important linkage between DNA damage and induction of apoptosis.

Proapoptotic Bcl-2 family member, Bax, and death receptor, Fas/APO1 have been discovered as mediators of p53-induced apoptosis, demonstrating that p53 activates two major apoptotic pathways (239, 240).

Proapoptotic members of Bcl-2 family, Bax and BH3-only members including Puma (241), Noxa (242) and Bid (243), all have consensus p53 response elements in their promoter and can be induced by p53. p53 directly suppresses the antiapoptotic Bcl-2 gene expression (244), and therefore, shifts the balance toward cell death. As a result, cytochrome c is released from the mitochondria subsequently activating apoptosis protease-activating factor 1 (Apaf-1) which activates caspase 9, an initiator caspase. Then effector caspases, caspase 3, 6 and 7 are activated to conduct their role as cysteine aspartyl proteases in the nucleus and cytoplasm (245). p53 can also transactivate Apaf-1 gene (246).

p53 induces the expression of death receptors, Fas (CD96/APO1) (239) and DR5/KILLER (247), and Fas ligand (FasL) (248). Death receptors including tumor necrosis factor receptor 1(TNFR1) share a common structure, intracellular death domain. When the ligand (FasL) binds to its receptor (Fas), the receptor trimerizes and the death domain recruits the adaptor protein, FADD (Fas-associated death domain) which in turn mediates the activation of caspase 8, an initiator caspase (249). Fas/FasL can mediate DNA-damage induced apoptosis in a p53 dependent or independent fashion (250, 251), and vice versa (252).

In addition to the activation of the apoptosis pathways, p53 represses survival pathways. A phosphatase, PTEN, a negative regulator of the PI3K/Akt pathway is induced by p53 (253).

p53 protein has been shown to be localized to mitochondria at the onset of p53-dependent apoptosis, but not during the p53-mediated cell cycle arrest. The accumulation of p53 to mitochondria precedes cytochrome c release and procaspase 3 activation. (254).

4. Skin cancer and UVB signaling pathways

1) Skin cancer

UV radiation in sunlight is a major etiologic factor in skin cancer. The UV part of sunlight is divided as UVA (315-400mm), UVB (280-315nm) and UVC (200-280nm). UVA is a relatively weak carcinogen and generates reactive oxygen species which result in oxidative damage including the formation of 8-hydroxyguanine, protein-DNA cross-linking, base loss, and strand breaks (255, 256). UVB and UVC wavelength are directly absorbed by DNA and result in DNA photoproducts, cyclobutane pyrimidine dimer and pyrimidine-pyrimidone (6-4) photoproduct, usually where two pyrimidines are adjacent. They also induce oxidative DNA damage (257). However, UVC is completely absorbed by the ozone layer of the atmosphere.

UVB radiation is primarily responsible for approximately one million nonmelanoma skin cancer (squamous cell carcinoma (SCC) and basal cell carcinoma (BCC)) cases in the U.S. each year and this incidence is equivalent to the annual incidence of all other human malignancies combined in the U.S. (258). BCCs and SCCs account for approximately 80% and 16% of all skin cancers, respectively, and both are

derived from basal layer of the epidermis of the skin. BCCs are growing slowly and rarely metastasize, but some SCCs are highly invasive and metastatic (259, 260).

UVB radiation is a complete carcinogen functioning as initiation, promotion and progression factors in mouse skin models (261). UV-induced DNA photoproducts cause signature mutations after replication, if not repaired. About 70% of the UV-induced mutations are cytosine (C) to thymidine (T) and 10% of them are CC to TT (262). If UV radiation causes mutations in critical genes in cell growth and differentiation, such as *p53* or *ras*, it will be an important mechanism for UV-induced tumor initiation (263, 264). Over 90% of the SCCs, 50% of the BCCs, and many cases of actinic keratosis (AK) which is benign or premalignant lesion, have mutations in the *p53* tumor suppressor gene, and those mutations are UV signature mutations (265).

The initiated cells could undergo clonal expansion during tumor promotion to result in a benign papilloma or an AK under the repeated exposure to UV radiation (238). Initiated cells with p53 mutation will be more resistant to apoptosis and have defects in cell cycle checkpoint activation induced by UVB than normal epidermal cells, and thus UV radiation could stimulate the proliferation of the initiated cells (promotion) and accumulate other genetic changes (progression).

Tumor promotion by UV radiation seems to be mediated by altering signaling pathways that result in changes in gene expression, especially involved in cell proliferation and apoptosis (266). Transcription factor complexes, AP-1 and NF-κB, p53 and cyclooxygenase-2 (COX2) are implicated in mediating the UV response (267).

UV-induced signal transduction pathways are suggested to be mediated primarily through signaling cascades involving MAPKs and phosphoinositide 3-kinase (PI3K).

2) UVB signaling pathways

MAPKs

MAPKs, including the ERKs, the c-Jun NH2-terminal kinases (JNKs) and the p38 kinases (268, 269), are important in the regulation of complex cellular functions such as proliferation, differentiation and apoptosis as well as development and inflammation (270). Depending upon cell types and dose, UVB alters gene expression to induce various UV response by stimulating JNKs, p38 kinases or ERKs (271-273).

- **UV-induced gene expression**

AP-1

It has been shown that the dominant-negative mutant transgene of c-jun inhibits UVB-induced AP-1 activation in the epidermis and inhibits UVB-induced skin tumor development, suggesting that UVB induction of AP-1 activity in keratinocytes may have a role in cell proliferation (274, 275). AP-1 complex consists of Jun (c-Jun, JunB and JunD) and Fos (c-Fos, FosB, Fra1and Fra2) proteins by forming either Jun-Jun homodimers or Jun-Fos heterodimers. In human keratinocytes, UVB irradiation has been found to cause c-Fos-JunD heterodimers to bind to the AP-1 element in target gene promoters (276).

c-Fos is an immediate early-response gene that is expressed at a low level constitutively and can be induced by various stimuli. In human keratinocytes, UVB-irradiation significantly increases c-Fos transcription and protein expression. At the same time, UVB significantly activates p38 and ERK (277). Treatment with a p38 inhibitor, SB202190, almost completely abolishes UV-induced c-Fos gene expression and protein synthesis, whereas ERK inhibition partially decreases UVB-induced c-Fos expression.

The p38 inhibitor also inhibits UVB-induced AP-1 trasnactivation and DNA binding (278). c-Fos AP-1 site (FAP1) and CRE are identified as important cis elements for the UVB-induction of the c-Fos promoter, and phosphorylated CREB have been shown to bind to those sites (279).

UV induces c-Jun gene transcription mediated through AP-1 binding sites by c-Jun-ATF-2 dimers (280, 281).

COX2

Treatment with a selective inhibitor of COX2 has been shown to reduce UV-induced skin cancer in mice suggesting an important role of COX2 in UV-induced skin carcinogenesis (282). COX2 has been shown to be increased after UVB irradiation in human keratinocytes (283). Human SCC tissues show increased COX2 protein levels compared with normal skin, and overexpression of COX2 antisense in human skin cancer cells suppresses COX2 protein expression and cell growth (284). UVB irradiation of HaCaT cells activates p38 and increases COX2 expression at the mRNA and protein levels, and the increase in COX2 mRNA in response to UVB is inhibited by a p38 inhibitor (285). From the studies with human COX2 promoter, binding of CREB and ATF1 to CRE site in the COX2 promoter has been shown to be required for the induction of basal and UVB-induced COX2 transcription. CREB-ATF-1 has been also shown to be phosphorylated on Ser133 of CREB upon UVB treatment, and the p38 inhibitor decreases phosphorylation of Ser133 and suppresses COX-2 promoter activity (286). Taken together, signaling pathway for UVB induction of human COX-2 involves activation of p38, subsequent phosphorylation of CREB-ATF-1, and activation of the COX-2 promoter through enhanced binding of phosphorylated CREB-ATF-1.

Histone H3

The rapid phosphorylation of histone H3 occurs concomitantly with immediate early gene induction in response to a variety of stimuli, and this event is involved in immediate early gene expression, chromatin remodeling and chromosome condensation during mitosis (287). UV irradiation of cells stimulates phosphorylation of histone H3 at Ser10 through ERKs and p38 kinase, and at Ser28 mediated by MSK1 as a downstream of p38 and ERKs (288, 289).

- **Apoptosis**

UVB irradiation can stabilize p53 by phosphorylation at Ser20 with disruption with MDM2 interaction. Activated JNKs phosphorylates Ser20 of p53, and JNK-induced Ser20 phophorylation is associated with p53-dependent transcriptional activation and apoptosis (290). It has been shown that fibroblasts with targeted disruptions of the functional JNKs are protected against UV-stimulated apoptosis and the absence of JNK causes a failure in cytochrome c release (291).

UVB also stimulates phosphorylation of Ser112 of BAD by JNK1 as well as p90 ribosomal S6 kinase 2 (RSK2) and mitogen- and stress-activate protein kinase 1 (MSK1), downstream kinases of ERKs and p38 kinase in vivo and in vitro (292). BAD is a proapoptotic factor in Bcl-2 family, and phosphorylation at Ser112 and 136 have been shown to result in its dissociation from another Bcl-2 family member, Bcl-$X_{L.}$ Then Bcl-X_L is free to protect cells from apoptosis (293). p38 activation by released cytokines in response to UV irradiation has been shown to regulate the prolonged activation of Akt cell survival pathway (294).

Taken together, MAPKs pathway is likely to play an important role in balancing UV-induced apoptotic signal in the context of severity of damage, condition and cell types.

PI3K-AKT

PI3K is a heterodimer that is composed of a catalytic subunit (p110) and a regulatory subunit (p85) and regulates of cell proliferation and cell survival in response to UVB (295). UV-induced activation of EGFR has been shown to mediate survival function through PI3K-Akt pathway in human keratinocytes. UVB also stimulates phosphorylation of RSK and Bad at Ser112, downstream effectors of Akt, and inhibitors of EGFR and PI3K block UV-induced BAD phosphorylation (296). Insulin-like growth factor 1 (IGF-1) has been found to protect fibroblast from apoptosis induced by UVB irradiation. Cells expressing kinase-inactive IGF receptor (IGFR) or treated with wortmanin, a PI3K inhibitor, are sensitized to UV-induced apoptosis. In addition, overexpression of active PI3K or membrane-targeted Akt is sufficient to protect cells, demonstrating the critical antiapoptotic role of IGFR-PI3K-Akt pathway in UVB-induced apoptosis (297).

UVB also activates PI3K in human keratinocytes in culture, causing phosphorylation of Akt, and GSK-3β phosphorylation on Ser9 which inactivates GSK-3β. Treatment with a PI3K inhibitor decreases c-Fos and Cox2 promoter activity and protein expression, and treatment of a GSK3β inhibitor increases both promoter activity and protein expression. Forced expression of dominant negative Akt or wild type GSK-3β inhibits UVB-induced c-Fos and Cox-2 promoter activity (298, 299).

WNT/GSK3

GSK3 is also a component of WNT signaling pathway, playing a major role in the regulation of the proto-oncogene, β-catenin. GSK3 is active in unstimulated cells and targets β-catenin for proteosomal degradation following phosphorylation. Activation of WNT signaling results in the inhibition of GSK3 activity and the accumulation of β-catenin and subsequent activation of the TCF/LEF transcription factor complex that can activate or repress gene expression (300). Alternatively, WNT can regulate gene expression through GSK3-dependent but β-catenin-independent mechanism as shown in the activation of COX-2 and repression of periostin gene expression (301).

It has been shown that DNA damage inhibits WNT signaling in a p53-dependent manner, resulting in the derepression of GSK3 and the downregulation of LEF/TCF transcription activity. Genotoxic stress such as UVB or adriamycin treatment activates p53 and subsequently induces Siah-1 and Dkk-1. Siah-1 or Dkk-1 inhibits WNT/β-catenin signaling by enhancing the degradation of β-catenin or by functioning as WNT antagonist by interfering coreceptor Lrp-6, respectively (302-304).

In the other hand, DNA damage which activates p53 is found to activate nuclear GSK3β. This activation occurred by direct binding of GSK3β to p53 in the nucleus and this complex regulates p53-mediated cellular response to DNA damage such as increases in p21 levels and caspase 3 activity (305).

5. Research hypothesis

C/EBPα plays an important role in the regulation of mitotic growth arrest associated with terminal differentiation in various cell types. C/EBPα is highly expressed in skin, especially in the suprabasal keratinocytes, but its function and regulation is poorly characterized. Forced expression of C/EBPα blocks keratinocyte proliferation suggesting that C/EBPα has a role in regulation of cell cycle in keratinocytes.

In response to DNA damage, cells activate cell cycle checkpoints that prevent replication of damaged DNA allowing time for DNA repair, and when the damage is too extensive, apoptotic pathways are engaged.

Considering the high potential of the skin for exposure to extrinsic factor, it is plausible that C/EBPα in skin expands its role into the cell cycle checkpoint activation in response to DNA damage induced by environmental stressors such as UVB, a main cause of skin cancer.

We hypothesize that UVB-induced DNA damage regulates C/EBPα expression and/or activity and C/EBPα has a role in the UVB-induced DNA damage response.

The research was focused on 1) the inducibility of C/EBPα expression or activity by UVB or DNA damaging agents, 2) signaling pathway through which UVB regulates C/EBPα induction or activity, and 3) the biological role of C/EBPα in DNA damage response induced by UVB in keratinocyte model.

MANUSCRIPT

C/EBPα is a DNA-Damage Inducible p53-Regulated Mediator of the G_1 Checkpoint in Keratinocytes

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ABSTRACT

The bZIP transcription factor, C/EBPα is abundantly expressed in keratinocytes of the skin, however its function in skin is poorly characterized. UVB radiation is responsible for the majority of human skin cancers. In response to UVB-induced DNA damage, keratinocytes activate cell cycle checkpoints that arrest cell cycle progression and prevent replication of damaged DNA allowing time for DNA repair. We report here that UVB radiation is a potent inducer of C/EBPα in human and mouse keratinocytes as well as in mouse skin in vivo. UVB irradiation of keratinocytes resulted in the transcriptional up-regulation of C/EBPα mRNA producing >70 fold increase in C/EBPα protein. MNNG, etoposide and bleomycin also induced C/EBPα. UVB-induced C/EBPα was accompanied by an increase in p53 protein and caffeine, an inhibitor of ATM/ATR kinases, inhibited UVB induced increases in both C/EBPα and p53. UVB irradiation of p53 null or mutant p53 containing keratinocytes failed to induce C/EBPα. UVB irradiation of C/EBPα knockdown keratinocytes displayed a greatly diminished DNA damage G1 checkpoint and this was associated with increased sensitivity to UVB-induced apoptosis. Our results uncover a novel role for C/EBPα as a p53-regulated DNA damage inducible gene that has a critical function in the DNA damage G_1 checkpoint response in keratinocytes.

INTRODUCTION

CCAAT/enhancer binding protein-α (C/EBPα) is a member of the basic leucine zipper (bZIP) class of transcription factors (For review, see (40). C/EBPα plays a critical role in the regulation of differentiation in myeloid cells (39, 57), preadipocytes (30, 53) and hepatocytes (12). In these cell types, C/EBPα is involved in the regulation of mitotic growth arrest associated with terminal differentiation as well as the expression of genes associated with the differentiated phenotype. C/EBPα is also expressed in skin (27, 33, 47), intestine (1), adrenal gland (4) and ovary (37), however, its function in these tissues is poorly characterized. While the function of C/EBPα in skin has not been characterized, C/EBPβ is involved in the regulation of the early stages of squamous differentiation (61). C/EBPβ also plays a critical role in Ras-mediated mouse skin tumorigenesis and keratinocyte survival. Unlike C/EBPβ, C/EBPα does not cooperate with Ras to induce transformation of NIH3T3 cells (62). However, forced expression of C/EBPα blocks epidermal keratinocyte proliferation suggesting a cell cycle regulatory function for C/EBPα in skin (61).

The ability of cells to respond to DNA damage is essential to ensure the integrity of the genome. With regard to environmental DNA damaging agents, sun or ultraviolet (UV) exposed areas of human skin represent a major site of DNA damage. UV radiation induces cyclobutane pyrimidine dimers, 6-4 photoproducts, cytosine photohydrates, DNA strand breaks, and DNA crosslinks (For reviews, see (6, 11). UVB radiation is responsible for 1 million nonmelanoma skin cancer cases/year in the U.S. accounting for 40% of all new cancer cases diagnosed each year in the U.S. (29). In response to UV-induced DNA damage, keratinocytes activate cell cycle checkpoints (28, 32, 54) that

arrest cell cycle progression and prevent replication of damaged DNA allowing time for DNA repair, or if the damage is too extensive, initiate apoptosis (63). Failure to repair UV-induced DNA damage in keratinocytes is linked to the development of nonmelanoma skin cancer (7). While the expression of C/EBPα and its anti-proliferative function are regulated during development and differentiation, its anti-proliferative function is not thought to be involved in DNA damage response network. However, in view of the high potential of the skin for exposure to extrinsic factors, such as UVB, that induce DNA damage, it is possible that one function of C/EBPα in skin is to participate in mitotic growth arrest in response to environmental stressors such as UVB-induced DNA damage. The results presented in this study are the first to identify a role for C/EBPα in the cellular response to DNA damage induced by extrinsic DNA damaging agents. We demonstrate that C/EBPα is a p53-regulated DNA-damage inducible gene in keratinocytes and that it is an important link between UVB-induced DNA damage and cell cycle arrest in epidermal keratinocytes.

MATERIALS and METHODS

Cell lines and cell culture

BALB/MK2 keratinocytes (B. Weissman, UNC) were cultured in Ca^{2+} free Eagle's minimal essential medium, EMEM (Biowhittaker) supplemented with 8 % chelex-treated fetal bovine serum (FBS, Invitrogen), 4 ng/ml hEGF (Invitrogen), and 0.05 mM calcium. Mouse primary keratinocytes were isolated from the epidermis of newborn mice by overnight trypsin flotation at 4 °C (21). Isolated keratinocytes were plated in Ca^{2+}-free EMEM supplemented with 10% unchelaxed FBS and 10 ng/ml hEGF. Four hours later, cultures were washed with Mg^{2+}- and Ca^{2+}-free phosphate buffered saline (PBS) and refed with Ca^{2+}-free EMEM supplemented with 4% chelex-treated FBS, 10 ng/ml hEGF, 100 U/ml penicillin, 100 μg/ml of streptomycin, 250 ng/ml amphotericin B [Fungizone] and 0.05 mM calcium. NHEK were purchased from Cambrex (Walkerville) and cultured in KGM (Cambrex). HaCaT cells were cultured in DMEM (Sigma) supplemented with 10 % FBS, 100 U/ml penicillin, 100 μg/ml streptomycin. NIH3T3 cells and NRK were cultured in DMEM supplemented with 10 % calf serum. HepG2 cells were MEM supplemented with 10 % FBS, 1.0 mM sodium pyruvate and 0.1 mM nonessential amino acids.

Animals

CD-1 (Charles River) and C57BL/6 and 129/SV mixed strain mice were used for in vivo UVB irradiation. Wild type and p53 null newborn mice were provided by J. French (NIEHS). Primers and PCR conditions were published previously (22). p53-deficient male mice were mated with heterozygous female mice to produce p53-deficient pups. C57BL/6 mice were mated to generate control subjects.

UVB irradiation and chemical treatment

The UVB lamp (Model EB 280C, Spectronics) emits wavelengths between 280-320 nm with a spectrum peak at 312 nm. The light intensity of the lamp was measured by IL1700 Research Radiometer (International Light) equipped with SED 240 sensor. UVB lamp was positioned 15 cm above the cells or mice. For cells in culture, medium was removed, and cells were washed with PBS and irradiated in the presence of PBS for the amount of corresponding time to the indicated UVB dose. After irradiation, PBS was removed and replaced with the specified medium. MNNG (Aldrich), etoposide (Sigma), actinomycin D (Sigma) were dissolved in DMSO. Bleomycin A_2 (Calbiochem), caffeine (Sigma) was dissolved in water. For mouse studies, the hair of the dorsal skin of 6-7 week old female mice was clipped, and two days later, mice were individually irradiated with UVB.

Preparation of cell lysates

Nuclear extracts were prepared as previously described (43). For the preparation of whole cell lysates, cells were washed with cold PBS, harvested by scraping, and collected by centrifugation. Cells were placed in hypotonic buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1X protease inhibitor cocktail (Roche), 1 mM sodium orthovanadate with 0.6% Nonidet P-40) and lysed by sonication, and then one-tenth volume of 5 M NaCl was added. The cell lysates were vortexed, incubated for 15 min on ice, and centrifuged. Supernatants were stored at –80 °C until use. Protein concentration was determined using Bio-Rad protein assay reagent.

Western blot analysis

Equal amounts of protein were precipitated by adding equal volume of 20% trichloroacetic acid and washed with cold acetone. Protein samples were dissolved in SDS sample buffer, boiled and separated by SDS-PAGE. The separated proteins were transferred to an Immobilon-P membrane (Millipore). Following incubation in blocking buffer, the membranes were probed with rabbit polyclonal IgG raised against C/EBPα, C/EBPβ, p53 or p21 (Santa Cruz). The membranes were washed and then probed with a horseradish peroxidase-linked secondary antibody (Amersham). Detection was made with an enhanced chemiluminescence reagent (Amersham) followed by exposure of the membrane to film. Membranes were stained with coomasie blue to confirm equal protein loading. Band intensity was measured by densitometry.

EMSA and supershift

C/EBP consensus oligonucleotides (Santa Cruz) were labeled with [γ-^{32}P] ATP by kinase reaction using T4 polynucleotide kinase. Radiolabeled oligonucleotides were incubated with 4 μg of nuclear extract at room temperature in binding buffer containing poly[dI-dC]. For supershift, samples were further incubated with anti-C/EBPα antibody (Santa Cruz). DNA-protein complexes were loaded onto 4% polyacrylamide gels and run in 0.25X Tris borate EDTA buffer. The gel was transferred to Whatman paper, dried and exposed to film.

Northern blot analysis

Total RNA was isolated from UVB-irradiated or non-irradiated BALB/MK2 cells in culture using acid guanidium thiocyanate-phenol-chloroform extraction. C/EBPα cDNA was labeled with [α-^{32}P] dCTP using Ready-To-Go labeling beads (Amersham). RNA was electrophoresed on agarose-formaldehyde gel, transferred to zeta-probe GT

membrane (BioRad), and UV cross-linked. Membranes were incubated at 65 °C in hybridization buffer (0.25 M Na_2HPO_4, pH 7.2, 7% SDS) overnight, washed, exposed to film at –80 °C and developed.

Construction of C/EBPα promoter-reporter and luciferase assay

The region between +62 to -2808 (2.8 Kb), +62 to -613 (0.6 Kb) and +62 to -517 (0.5 Kb) of the C/EBPα promoter (P. Johnson, NCI) were amplified by PCR. Potential p53 binding sequences in C/EBPα promoter were determined by a consensus binding site for p53 published previously (14) and by using p53 Scanner program from the Ohio State University, Human Cancer Genetics Bioinformatics group. Each amplified product was inserted into the promoterless and enhancerless reporter, pGL3-basic using KpnI and XhoI sites. Colonies were screened by restriction enzyme analysis and 2 positive colonies from 2.8 Kb were verified by DNA sequencing. BALB/MK2 keratinocytes were plated and 24 hours later transfected with pGL3-basic or C/EBPα promoter-reporter along with pCMV-p53 expression or pCMV control vector using Tfx-10 (Promega) following manufacturer's protocol. Cells were harvested 24 hrs after transfection, and the luciferase activity was determined using the luciferase assay kit (Promega).

Immunohistochemistry

Normal and irradiated mouse skins were fixed in 10% neutral buffered formalin phosphate and embedded in paraffin. Tissue sections (5 μm) were subjected to antigen retrieval followed by treatment with 0.1% H_2O_2 and blocking with normal goat serum. Sections were incubated with the anti-C/EBPα antibody for overnight and a biotinylated goat anti-rabbit IgG for 30 min. Detection was made with ABC kit (Vector Laboratories)

and 3,3'-diaminobenzidine (BioGenex) as the chromagen following manufacturer's protocol.

Transfection and colony formation assay

BALB/MK2 keratinocytes were plated and 24 hrs later transfected with 2 μg pcDNA3 or pcDNA3-C/EBPα using lipofectamine (Invitrogen) in serum-free media according to the manufacturer's protocol. Two days later the cultures were split (1:3), and selection media containing 300 μg/ml G418 (Sigma) was added 24 hr after replating. On days 3, 5, and 7 after G418 selection, the number of colonies and the number of cells per colony in 30 random grid squares were counted.

Retroviral infection and thymidine incorporation assay

ϕNX cells were grown in DMEM supplemented with 10 % FBS and transfected with 15 μg pWZL or pWZL-C/EBPα (P. Johnson, NCI) by calcium phosphate precipitation method. C/EBPα nucleotide sequences were confirmed by DNA sequencing. Twenty four hours after transfection the culture medium was replaced with keratinocyte medium. The next day, medium containing virus was collected, filtered, transferred onto the keratinocytes for infection with 5 μg/ml polybrene (Sigma). Fresh keratinocyte medium was added to the packaging cells. Infection procedure was repeated two more times at 4 hr intervals. Infected keratinocytes were selected with 100 μg/ml hygromycin (Roche) for 2 weeks and colonies were pooled. Cells were pulse-labeled with [^{3}H-methyl] thymidine (3 μCi/ml) for 1 hr before collection. Cells were collected by trypsinization, resuspended in 1 mM EDTA buffer and sonicated, and aliquots were collected onto glass fiber filters and the filters were placed in liquid scintillation cocktail and subjected to scintillation counting. For DNA quantitation, an aliquot of each sample and 5 μl of 0.1 mg/ml Hoechst 33258 solution were mixed in 1 ml

of 0.01 M Tris (pH 7.0)-0.1 M NaCl-0.01 M EDTA buffer. The fluorescent units were determined with a fluorometer (excitation at 365 nm and emission at 450 nm).

Generation of C/EBPα knockdown BALB/MK2 keratinocytes

The siRNA target sequence for C/EBPα, 5'-907AAAGCCAAACAACGCAACGTG-3' was selected following manufacturer's protocol and target sequence was analyzed by BLAST search to check significant sequence homology with other genes. Two DNA oligonucleotides were designed and cloned into the pSilencer 2.3-U6 hygro vector (Ambion) following manufacturer's protocol. BALB/MK2 keratinocytes were transfected with these constructs as well as empty vector, selected with hygromycin and colonies were ring cloned. Colonies were expanded and screened for C/EBPα expression after UVB irradiation.

5-Bromo-2'-deoxyuridine (BrdU) labeling and FACS analysis

For G1 checkpoint, C/EBPα knockdown keratinocytes or control keratinocytes were synchronized by serum and hEGF starvation (0.5 % chelexed FBS) at ~25 % confluence for 24 hr and then released into the cell cycle by the addition of complete medium. Six hours later cells were irradiated with 5 mJ/cm^2 UVB or not irradiated and harvested at 18 and 24 hr post release into cell cycle. Cells were incubated with 10 μM BrdU for 2 hours before harvesting the cells at each time point. Cells were trypsinized, pelleted, resuspended in 100 μl cold PBS and fixed in 70 % ethanol. Cells were treated with 2N HCl/Triton x-100 to denature DNA followed by neutralization with $Na_2B_4O_7$. Cells were pelleted and resuspended in a 0.5% Tween 20/1%BSA/PBS with anti-BrdU-FITC antibody (1:50, Becton Dickinson) and 0.5 mg/ml RNase and incubated at 4 °C overnight. Cells were pelleted and resuspended in PBS containing 5 mg/ml propidium iodide (PI) and subjected to FACS

analysis. Data were collected and presented on a scatter plot with BrdU intensity on the y-axis and PI intensity on the x-axis.

Caspase 3/7 assay

C/EBPα knockdown keratinocytes or control keratinocytes were plated in fluorescence readable 96 well plates and grown in keratinocyte medium. The Apo-ONE Homogeneous Caspase-3/7 Assay (Promega) was used to measure apoptosis following manufacturer's protocol. Total cell number was analyzed using the CytoTox 96 Non-Radioactive Cytotoxicity Assay kit (Promega) following manufacturer's protocol.

RESULTS

We examined the effect of UVB treatment on C/EBPα expression in BALB/MK2 keratinocytes. BALB/MK2 keratinocytes, a nontransformed immortalized mouse cell line, were irradiated with a single dose of UVB (10 mJ/cm^2). As shown in Figure 1A, UVB produced a very large increase in C/EBPα protein levels. At 6 hours post-UVB treatment, there was approximately a 10 fold increase in C/EBPα protein and by 18 hours, C/EBPα protein was increased >70 fold. Elevated levels of C/EBPα protein (2-3 fold) could be detected as early as 3 hrs post-UVB treatment (data not shown). By 48 hrs post-UVB treatment, C/EBPα protein levels began to return to baseline levels (Figure 1A). These results demonstrate that UVB radiation is a potent and rapid inducer of the transient expression of C/EBPα protein in BALB/MK2 keratinocytes. While UVB is a potent inducer of C/EBPα, it had a comparatively much smaller effect on C/EBPβ protein levels (Figure 1A). C/EBPδ (34) and C/EBPζ (CHOP/GADD 153) (15, 18, 59) can be induced by various cellular stressors, however, neither C/EBPδ protein or C/EBPζ protein were induced by UVB at the doses and time points we examined for C/EBPα (data not shown). Electrophoretic mobility shift assay (EMSA) analysis utilizing a C/EBP consensus sequence demonstrated UVB treatment also produced a large increase in DNA binding activity (Fig 1B). Supershift EMSA analysis with antibodies to C/EBPα revealed that the great majority of the UVB-induced increase in DNA binding was due to C/EBPα binding (Fig 1C). Therefore, the increase in UVB-induced C/EBPα protein levels is accompanied by a concomitant increase in C/EBPα DNA binding. As shown in Figure 1D, UVB induced C/EBPα in a dose-dependent manner. To determine whether the UVB induction of C/EBPα protein is a result of increased mRNA levels, we isolated

total RNA from UVB treated BALB/MK2 keratinocytes and conducted Northern analysis. As shown in Figure 1E, UVB treatment produced a large increase in C/EBPα mRNA levels. Actinomycin D treatment completely blocked the UVB-induction of C/EBPα protein levels (Figure 1F). Actinomycin D also blocked UVB induced increase in C/EBPα mRNA as determined by quantitative RT-PCR (data not shown). Collectively these data indicate that UVB is a potent inducer of C/EBPα at the transcriptional level.

As shown in Figure 2A, UVB induced C/EBPα protein levels in human primary keratinocytes (NHEK) as well as in mouse primary keratinocytes. UVB also induced C/EBPα in the epidermis of skin of mice treated in vivo with UVB (Figure 2A). To determine whether other types of DNA damage/DNA damaging agents could also induce C/EBPα, we treated BALB/MK2 keratinocytes with: N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), a carcinogen that methylates DNA; etoposide, an inhibitor of topoisomerase II that produces double strand DNA breaks; and bleomycin, an antineoplastic drug that produces both single and double strand breaks. As shown in Figure 2B, all three DNA damaging agents are potent inducers of C/EBPα protein expression. While human and mouse keratinocytes responded to UVB with the robust induction of C/EBPα, treatment of NIH3T3 fibroblasts, human hepatoma cells (HepG2), and normal rat kidney fibroblasts (NRK) with various doses of UVB did not induce C/EBPα (Figure 2C). UVB treatment did, however, increase p53 protein levels indicating that these cells are capable of responding to UVB (Figure 2C). Similar to the UVB results, treatment of NIH3T3, HepG2 and NRK cells with MNNG or etoposide did not induce C/EBPα (data not shown). These results indicate UVB-induction of C/EBPα is cell type specific.

As shown in Figure 3A, UVB treatment of BALB/MK2 keratinocytes induced the accumulation of p53 protein as well as p21, a p53 inducible protein. The kinetics of the increase in C/EBPα protein was similar to p53 accumulation suggesting a possible relationship between the two proteins (Figure 3A). To begin to determine if p53 is involved in UVB-induction of C/EBPα we first examined the effect of caffeine (42), a pharmacological inhibitor of ataxia-telangiectasia mutated (ATM) and ataxia-telangiectasia mutated and Rad3-related (ATR) on UVB-induction of C/EBPα and p53 accumulation. ATR is activated by UVB-induced DNA damage and subsequently phosphorylates p53, contributing to the activation (9, 13) and accumulation of p53 protein (45). As shown in Figure 3B, treatment of BALB/MK2 keratinocytes with caffeine blocked UVB-induced p53 accumulation as well as C/EBPα induction suggesting UVB-induced C/EBPα is ATM/ATR and/or p53 dependent. To determine whether the UVB induction of C/EBPα is p53 dependent, we examined the effects of UVB in HaCaT cells, a human keratinocyte cell line that contains inactive mutant p53 (25). While UVB is an effective inducer of C/EBPα in normal human epidermal keratinocytes (NHEK), UVB did not induce C/EBPα or p21 in HaCaT cells indicating that functional p53 is required for C/EBPα induction (Figure 3C). HaCaT cells displayed elevated levels of p53 in the absence of UVB treatment as well as a lack of increase in p21, both of which are indicative of the presence of inactive mutant p53. To provide additional direct evidence that p53 is required for UVB-induced C/EBPα expression, we examined the effects of UVB in p53 nullizygous and wild type primary mouse keratinocytes. In primary mouse keratinocytes, maximal UVB-induction of C/EBPα was achieved 6 hr after irradiation (Fig 2A). As shown in Figure 3D, UVB failed to induce

C/EBPα in p53-nullizygous mouse keratinocytes. Similar results were obtained at 12 and 18 hours post UVB and at a lower dose (5mJ/cm^2) (data not shown). These results demonstrate that p53 is required for UVB-induction of C/EBPα in human and mouse keratinocytes. We also observed that p53 is also required for C/EBPα induction in keratinocytes treated with other DNA damaging agents including MNNG, etoposide and bleomycin (data not shown). To begin to determine whether p53 regulates C/EBPα through a direct or indirect mechanism we examined the effect of p53 expression on the reporter activity of a C/EBPα promoter-reporter construct that contained the 2.8 kb of the mouse C/EBPα proximal promoter. As shown in Figure 3E, p53 stimulated C/EBPα promoter reporter activity. Analysis of the 2.8 kb C/EBPα promoter revealed a p53 consensus site 5'-GGcCTAGTCC(cagctttta)AcACAAGTCT-3' at -551~ -579 bp. To determine whether p53 regulates the C/EBPα promoter through this site we constructed two promoter reporter constructs, one containing the proximal 0.6 Kb of the C/EBPα promoter which included putative p53 site and the other containing 0.5 Kb of the proximal promoter which lacked the putative p53 site. As shown in Figure 3E, p53 stimulated the C/EBPα promoter-reporter containing the proximal 0.6 Kb, however, the deletion of the putative p53 site did not result in decreased p53 regulated reporter activity. Further analysis of the 0.5 Kb promoter did not reveal any p53 binding sites suggesting p53 may regulate C/EBPα through an indirect mechanism.

The epidermis is primarily composed of basal and suprabasal keratinocytes. Basal keratinocytes are undifferentiated and capable of mitosis while suprabasal keratinocytes are post-mitotic and have entered the squamous differentiation program. Knowing the cellular location of the UVB-induced increase in C/EBPα within the

epidermis could provide insight into the function of C/EBPα in the epidermal UVB response. Immunohistochemical staining for C/EBPα in UVB-irradiated mouse skin revealed increased staining of C/EBPα in the nucleus and cytoplasm of both the suprabasal and basal keratinocytes of the epidermis (Figure 4A). Of particular significance, was the observation that UVB produced a 3-4 fold increase in the number of basal keratinocytes expressing C/EBPα (see Figure 4B). Thus, UVB treatment not only increases the level of C/EBPα in keratinocytes previously expressing C/EBPα, but it induces the expression of C/EBPα in keratinocytes in which C/EBPα was previously not expressed or was undetectable. The UVB-induced increase of C/EBPα in the basal keratinocytes, which represent the proliferating compartment of the epidermis, suggests that C/EBPα may have a role in UVB-induced inhibition of keratinocyte proliferation. In agreement with earlier studies (61), forced expression of pcDNA3-C/EBPα in BALB/MK2 keratinocytes inhibited keratinocyte proliferation as indicated by the decrease in the number of cells/colony and the total number of colonies. As shown in Figure 5A, the number of cells/colony changed little over time in the C/EBPα transfected cells while the number of cells/colony in the control cells transfected with the empty vector increased over 4 fold (Figure 5A). C/EBPα also produced approximately a 75% decrease in the total number of colonies (data not shown).

Transient transfection of BALB/MK2 cells with pcDNA3-C/EBPα produces supra-physiologic levels of C/EBPα and transfection efficiency is less than 5%. In order to further study the effect of C/EBPα on keratinocyte proliferation we infected BALB/MK2 keratinocytes with a retrovirus containing C/EBPα and selected the cells

with hygromycin. Cells infected with the C/EBPα containing retrovirus displayed elevated levels of C/EBPα that were comparable to the UVB-induced C/EBPα levels (Insert in Fig 5B). These elevated C/EBPα levels are at least an order of magnitude less than the cells transfected with pcDNA3-C/EBPα (data not shown). Unexpectedly, the keratinocytes infected with the retrovirus containing C/EBPα continued to proliferate similar to the control cells infected with empty virus as determined by ^{3}H-thymidine incorporation into DNA and FACS analysis (data not shown). While overexpression of C/EBPα inhibits cell proliferation in many cells, this appears to be dependent on the degree of overexpression as well as the cell type (16, 26, 44). When human epidermal growth factor (hEGF) was removed from the culture medium, cells infected with the C/EBPα containing retrovirus displayed greater than 80-90% decrease in ^{3}H-thymidine incorporation into DNA at 24-48 hrs compared to control cells infected with empty virus (Fig 5B). Conditioned medium from control cells did not alter the growth inhibitory response of the C/EBPα expressing cells nor did conditioned medium from the C/EBPα expressing cells induce growth inhibition in the control cells (data not shown) indicating that the differential growth response of C/EBPα overexpressing cells was not due to altered production of autocrine factors present in the medium. These results suggest that hEGF in the culture medium antagonizes the anti-proliferative function of C/EBPα under unstressed conditions.

We examined the effect of UVB on cell proliferation in these keratinocytes infected with either a retrovirus containing C/EBPα or an empty retrovirus. Since C/EBPα is rapidly induced by UVB, we examined proliferation at early time points post-UVB before substantial levels of C/EBPα were induced in the control cells. UVB

inhibited cell proliferation in both C/EBPα overexpressing and control cells, however, cells overexpressing C/EBPα displayed approximately 50% greater inhibition in cell proliferation as determined by ^{3}H-thymidine incorporation into DNA at 2 hours post-UVB (Figure 5C). These results indicate that C/EBPα contributes to growth arrest in UVB treated keratinocytes. Later time points up to 24 hours did not show significant differences in ^{3}H-thymidine incorporation into DNA in cells infected with the retrovirus containing C/EBPα compared to cells infected with empty retrovirus. This lack of effect at the later time points could be due to the induction of endogenous C/EBPα in the control cells, which complicates the interpretation of these C/EBPα overexpression studies.

In order to better define the role of C/EBPα in UVB-induced growth arrest we utilized small-interference RNA (siRNA)-mediated knockdown of C/EBPα. C/EBPα-siRNA blocked C/EBPα induction approximately by 80% (Figure 6B). To determine whether C/EBPα has a role in the UVB-induced DNA damage G_1 checkpoint, keratinocytes were synchronized by serum and hEGF starvation, released from starvation, irradiated with UVB or not 6 hrs after release from starvation and pulsed with BrdU 2 hours prior to collection. At 6 hours after the release from serum starvation $\leq$ 1% of the untreated control cells and C/EBPα knockdown cells were in S-phase (Figure 6A). Similar results were observed at 8 hrs post release (data not shown). As shown in Figure 6A, at 18 hrs post release, 13.5 % and 30.7% of the unirradiated control and C/EBPα knockdown cells progressed into S-phase, respectively. In contrast, 1.4±0.26% and 10.3±0.33% of the irradiated control and C/EBPα knockdown cells progressed into S-phase. Therefore, UVB inhibited by ~90% the entry of control cells into S-phase,

however, entry into S-phase was only inhibited by 67% in the C/EBPα knockdown cells (Figure 6A). Similar results were obtained with another independently isolated C/EBPα knockdown clone (data not shown) and with UVB irradiation 4 hrs after release into the cell cycle (data not shown). By 36 hours post UVB irradiation, recovery into S-phase progression was detected in control cells (data not shown). These results demonstrate that the UVB-irradiated control cells have an effective G_1 checkpoint while C/EBPα knockdown cells have a greatly attenuated G_1 checkpoint in response to UVB-induced DNA damage. We also conducted a BrdU pulse-chase experiment in asynchronously growing control and C/EBPα knockdown keratinocytes. Both UVB-irradiated control and C/EBPα knockdown cells arrested in S-phase at 6 and 12 hours post-UVB to a similar degree suggesting that C/EBPα does not involved in the S-phase checkpoint (data not shown). Attenuation of DNA damage checkpoints, including G_1 checkpoint can result in increased apoptosis (5, 36). As shown in Figure 6C C/EBPα knockdown keratinocytes display an increased sensitivity to UVB-induced apoptosis.

DISCUSSION

We have demonstrated that C/EBPα is a p53-regulated DNA damage inducible gene that has a role in the G_1 checkpoint in epidermal keratinocytes. Our study is the first to characterize a function for C/EBPα in skin and it uncovers a previously unidentified role for C/EBPα in the cellular DNA damage response network as well as a novel regulation of C/EBPα expression involving p53. Previous studies have demonstrated an important role for C/EBPα in growth arrest that is associated with differentiation and development. Our study expands the anti-proliferative function of C/EBPα to now include growth arrest associated with DNA damage mediated by extrinsic factors. We observed that a variety of agents including UVB, MNNG, etoposide and bleomycin which produce different types of DNA damage, all induce C/EBPα in keratinocytes through a p53 dependent pathway. Thus, the induction of C/EBPα appears to be a general DNA damage response in keratinocytes. In contrast, UVB-irradiation, MNNG or etoposide treatment of HepG2, NRK or NIH3T3 cells did not result in the induction of C/EBPα, suggesting that the induction of C/EBPα by DNA damage is cell type specific. Epidermal keratinocytes constitute the outer epithelial surface of the body and as such these cells are exposed to a variety of extrinsic factors that have the potential to damage DNA. Accordingly, it is possible that keratinocytes have evolved additional mechanisms to deal with DNA damage in order to ensure the integrity of the genome. It will be of interest to examine whether DNA damage induces C/EBPα in other types of epithelial cells that are exposed to extrinsic DNA damaging agents, such as those cells that line the gastrointestinal, urinary and respiratory tracts.

UVB treatment results in the posttranslational modification of the p53 protein and involves phosphorylation of multiple serine/threonine sites and acetylation sites located in the carboxy terminus of p53 (41). These complex and multiple modifications are important in stabilizing the p53 protein, enhancing the transcription activity of p53 and may be involved in the determination of promoter specificity (2). ATR is activated by UV-induced DNA damage and phosphorylates serine 15 of p53 (corresponding to serine 18 of mouse p53) (48). The phosphorylation of serine 15 has been reported to enhance p53 transcriptional activity through a mechanism involving acetylation (9) and to disrupt mdm2/p53 interaction resulting in increased level of p53 (45). Our results showing that caffeine, a known ATM/ATR inhibitor, blocks UVB induction of C/EBPα and p53 accumulation supports a role for ATR as one of the upstream kinases of p53 in the induction of C/EBPα. In addition, our observations that etoposide induces C/EBPα through a p53 dependent pathway suggests ATM may also participate in the induction of C/EBPα upstream of p53 in keratinocytes.

Multiple mechanisms can converge on DNA damage-induced cell cycle checkpoints. For example, p53-regulated proteins, p21, 14-3-3σ and GADD45 have roles in the G_2/M checkpoint (8, 58). In contrast to the G_2/M checkpoint in which numerous p53 regulated proteins have a role, p21 is considered to be the sole p53-dependent mediator of the G_1 DNA damage checkpoint (55). However, we observed that C/EBPα knockdown keratinocytes have an attenuated G_1 checkpoint following UV-induced DNA damage indicating C/EBPα is a novel p53-regulated mediator of the G_1 checkpoint in keratinocytes. Thus, we have identified C/EBPα as a second p53 regulated gene that participates in the G_1 checkpoint. The anti-proliferative function of C/EBPα is

multifaceted and involves the upregulation and activation of p21 (20, 51), interaction with Rb family proteins (49, 50) and repression of E2F (38, 46). Moreover, C/EBPα has been shown to block cell cycle progression independent of its DNA binding and transcriptional activity through the formation of an inhibitory complex with cdk4 and cdk2 (56). More recently the anti-proliferative activity of C/EBPα was shown to require a SWI/SNF chromatin remodeling complex supporting a transcriptional basis for its anti-proliferative activity (31). All of these mechanisms are consistent with our observation that C/EBPα has a role in the DNA damage induced G_1 checkpoint and indicate that C/EBPα is regulating the G_1 checkpoint through multiple mechanisms. However, it is unlikely that one of the mechanisms involves p21 as we observed that p21 levels were not decreased in UVB-treated C/EBPα knockdown cells (data not shown). Moreover, C/EBPα can inhibit cell cycle progression in p21-deficient cells (30) and p21 levels are not altered in livers of mice that are C/EBPα-deficient (24).

The importance of C/EBPα in the regulation of growth arrest and differentiation is highlighted by recent studies in which C/EBPα was implicated as a human tumor suppressor gene. In human acute myeloid leukemia (AML), C/EBPα is inactivated by dominant negative mutation or through its association with oncoprotein AML-1-ETO (35, 60). The inactivation of C/EBPα is thought to result in a differentiation block of the granulocytic blasts. C/EBPα expression is reduced in hepatocellular carcinomas (52), lung cancer and lung cancer cell lines (19) supporting a possible tumor suppressor function in these organs. We have observed that C/EBPα protein levels are greatly diminished or undetectable in mouse skin squamous cell carcinomas (SCCs) (33) and mouse skin SCC cell lines and re-introduction of C/EBPα in the SCC cells inhibits cell

proliferation (M Shim and RC Smart, unpublished results). Taken together, our results suggest that the loss of C/EBPα expression may contribute to the altered growth characteristics of skin SCCs.

In terms of human skin cancer, more than 90% of SCC and 50% of basal cell carcinomas (BCCs) contain UV-signature mutations (C→T, CC→TT) in the p53 gene (17, 63). Clusters of mutant p53 containing pre-neoplastic keratinocytes have been identified in sun exposed areas of human skin (23) and UVB-treated skin of mice (3). It has been proposed that additional multiple exposures to UVB allow these "initiated" cells to clonally expand because they are resistant to UVB-induced apoptosis (63). At the same time, UVB exposure of these mutant p53 keratinocytes would not result in p21 and C/EBPα induction, and as such, the DNA damage G1 checkpoint would be compromised allowing for the accumulation of additional critical mutations that contribute to SCC and BCC. Therefore, the loss of function of C/EBPα in the presence of UVB-induced DNA damage may contribute to the development of SCCs and BCCs that contain p53 mutations. It will also be of interest to examine C/EBPα for inactivating or dominant negative mutations in SCCs and BCCs that do not contain p53 mutations.

Our results demonstrate that p53 is required for C/EBPα induction by MNNG, etoposide, bleomycin and UVB keratinocytes and that p53 can stimulate the C/EBPα proximal promoter. Our results utilizing the 2.8, 0.6 and 0.5 kb portions of the C/EBPα proximal promoter suggest that the regulation of the C/EBPα promoter by p53 may be through an indirect mechanism. Contained within the 0.5 kb of the C/EBPα proximal promoter are potential binding sites for C/EBP, SP-1, NF-1, NF-Y and NF-kB. The C/EBPα promoter can be auto-regulated by C/EBPα to induce the activity of the C/EBPα

promoter (10). We have observed the C/EBPα proximal promoter is auto-stimulated by C/EBPα expression in BALB/MK2 keratinocytes. Auto-regulation of transcription factor expression is a mechanism used by cells to respond rapidly to a changing cellular environment, thus it is conceivable that once C/EBPα is up-regulated through the p53 dependent mechanism, C/EBPα then contributes to the up-regulation of its own expression. While further studies are required to determine how p53 regulates C/EBPα and the role of C/EBPα auto-regulation, our study uncovers a previously unidentified role for C/EBPα as a p53-regulated gene that links DNA damage to cell cycle arrest in keratinocytes. Our results suggest that the loss of UVB-induced C/EBPα expression may contribute to UVB-induced skin carcinogenesis.

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REFERENCES

1. **Antonson, P., and K. G. Xanthopoulos.** 1995. Molecular cloning, sequence, and expression patterns of the human gene encoding CCAAT/enhancer binding protein alpha (C/EBP alpha). Biochem Biophys Res Commun **215:**106-13.
2. **Appella, E., and C. W. Anderson.** 2001. Post-translational modifications and activation of p53 by genotoxic stresses. Eur J Biochem **268:**2764-72.
3. **Berg, R. J., H. J. van Kranen, H. G. Rebel, A. de Vries, W. A. van Vloten, C. F. Van Kreijl, J. C. van der Leun, and F. R. de Gruijl.** 1996. Early p53 alterations in mouse skin carcinogenesis by UVB radiation: immunohistochemical detection of mutant p53 protein in clusters of preneoplastic epidermal cells. Proc Natl Acad Sci U S A **93:**274-8.
4. **Birkenmeier, E. H., B. Gwynn, S. Howard, J. Jerry, J. I. Gordon, W. H. Landschulz, and S. L. McKinght.** 1989. Tissue-specific expression, developmental regulation, and genetic mapping of the gene encoding CCAAT/enhancer binding protein. Genes & Dev **3:**1146-1156.
5. **Bissonnette, N., and D. J. Hunting.** 1998. p21-induced cycle arrest in G1 protects cells from apoptosis induced by UV-irradiation or RNA polymerase II blockage. Oncogene **16:**3461-9.
6. **Brash, D. E.** 1997. Sunlight and the onset of skin cancer. Trends Genet **13:**410-4.
7. **Brash, D. E., J. A. Rudolph, J. A. Simon, A. Lin, G. J. McKenna, H. P. Baden, A. J. Halperin, and J. Ponten.** 1991. A role for sunlight in skin cancer: UV-induced p53 mutations in squamous cell carcinoma. Proc Natl Acad Sci U S A **88:**10124-8.

8. **Chan, T. A., P. M. Hwang, H. Hermeking, K. W. Kinzler, and B. Vogelstein.** 2000. Cooperative effects of genes controlling the G(2)/M checkpoint. Genes Dev **14:**1584-8.

9. **Chao, C., M. Hergenhahn, M. D. Kaeser, Z. Wu, S. Saito, R. Iggo, M. Hollstein, E. Appella, and Y. Xu.** 2003. Cell type- and promoter-specific roles of Ser18 phosphorylation in regulating p53 responses. J Biol Chem **278:**41028-33.

10. **Christy, R. J., K. H. Kaestner, D. E. Geiman, and M. D. Lane.** 1991. CCAAT/enhancer binding protein gene promoter: binding of nuclear factors during differentiation of 3T3-L1 preadipocytes. Proc Natl Acad Sci U S A **88:**2593-7.

11. **de Gruijl, F. R., H. J. van Kranen, and L. H. Mullenders.** 2001. UV-induced DNA damage, repair, mutations and oncogenic pathways in skin cancer. J Photochem Photobiol B **63:**19-27.

12. **Diehl, A. M., D. C. Johns, S. Yang, H. Lin, M. Yin, L. A. Matelis, and J. H. Lawrence.** 1996. Adenovirus-mediated transfer of CCAAT/enhancer-binding protein-alpha identifies a dominant antiproliferative role for this isoform in hepatocytes. J Biol Chem **271:**7343-50.

13. **Dumaz, N., and D. W. Meek.** 1999. Serine15 phosphorylation stimulates p53 transactivation but does not directly influence interaction with HDM2. Embo J **18:**7002-10.

14. **el-Deiry, W. S., S. E. Kern, J. A. Pietenpol, K. W. Kinzler, and B. Vogelstein.** 1992. Definition of a consensus binding site for p53. Nat Genet **1:**45-9.

15. **Fornace, A. J., Jr., D. W. Nebert, M. C. Hollander, J. D. Luethy, M. Papathanasiou, J. Fargnoli, and N. J. Holbrook.** 1989. Mammalian genes coordinately regulated by growth arrest signals and DNA-damaging agents. Mol Cell Biol **9:**4196-203.

16. **Freytag, S. O., D. L. Paielli, and J. D. Gilbert.** 1994. Ectopic expression of the CCAAT/enhancer-binding protein a promotes the adipogenic program in a variety of mouse fibroblastic cells. Genes & Dev **8:**1654-1663.

17. **Gailani, M. R., D. J. Leffell, A. Ziegler, E. G. Gross, D. E. Brash, and A. E. Bale.** 1996. Relationship between sunlight exposure and a key genetic alteration in basal cell carcinoma. J Natl Cancer Inst **88:**349-54.

18. **Garmyn, M., M. Yaar, N. Holbrook, and B. A. Gilchrest.** 1991. Immediate and delayed molecular response of human keratinocytes to solar-simulated irradiation. Lab Invest **65:**471-8.

19. **Halmos, B., C. S. Huettner, O. Kocher, K. Ferenczi, D. D. Karp, and D. G. Tenen.** 2002. Down-regulation and antiproliferative role of C/EBPalpha in lung cancer. Cancer Res **62:**528-34.

20. **Harris, T. E., J. H. Albrecht, M. Nakanishi, and G. J. Darlington.** 2001. CCAAT/enhancer-binding protein-alpha cooperates with p21 to inhibit cyclin-dependent kinase-2 activity and induces growth arrest independent of DNA binding. J Biol Chem **276:**29200-9.

21. **Hennings, H., D. Michael, C. Cheng, P. Steinert, K. Holbrook, and S. H. Yuspa.** 1980. Calcium regulation of growth and differentiation of mouse epidermal cells in culture. Cell **19:**245-254.

22. **Hulla, J. E., J. E. French, and J. K. Dunnick.** 2001. Chromosome 11 allelotypes reflect a mechanism of chemical carcinogenesis in heterozygous p53-deficient mice. Carcinogenesis **22:**89-98.

23. **Jonason, A. S., S. Kunala, G. J. Price, R. J. Restifo, H. M. Spinelli, J. A. Persing, D. J. Leffell, R. E. Tarone, and D. E. Brash.** 1996. Frequent clones of p53 keratinocytes in normal human skin. Proc. Natl. Acad. Sci. USA **93:**14025-14020.

24. **Lee, Y. H., B. Sauer, P. F. Johnson, and F. J. Gonzalez.** 1997. Disruption of the c/ebp alpha gene in adult mouse liver. Mol Cell Biol **17:**6014-22.

25. **Lehman, T. A., R. Modali, P. Boukamp, J. Stanek, W. P. Bennett, J. A. Welsh, R. A. Metcalf, M. R. Stampfer, N. Fusenig, E. M. Rogan, and et al.** 1993. p53 mutations in human immortalized epithelial cell lines. Carcinogenesis **14:**833-9.

26. **Lin, F. T., and M. D. Lane.** 1994. CCAAT/enhancer binding protein alpha is sufficient to initiate the 3T3-L1 adipocyte differentiation program. Proc Natl Acad Sci U S A **91:**8757-61.

27. **Maytin, E. V., and J. F. Habener.** 1998. J. Invest. Dermatol. **110:**238-246.

28. **Medrano, E. E., S. Im, F. Yang, and Z. A. Abdel-Malek.** 1995. Ultraviolet B light induces G1 arrest in human melanocytes by prolonged inhibition of retinoblastoma protein phosphorylation associated with long-term expression of the p21Waf-1/SDI-1/Cip-1 protein. Cancer Res **55:**4047-52.

29. **Miller, D. L., and M. A. Weinstock.** 1994. Nonmelanoma skin cancer in the United States: incidence. J Am Acad Dermatol **30:**774-8.

30. **Muller, C., M. Alunni-Fabbroni, E. Kowenz-Leutz, X. Mo, M. Tommasino, and A. Leutz.** 1999. Separation of C/EBPalpha-mediated proliferation arrest and differentiation pathways. Proc Natl Acad Sci U S A **96:**7276-81.

31. **Muller, C., C. F. Calkhoven, X. Sha, and A. Leutz.** 2003. C/EBPalpha requires a SWI/SNF complex for proliferation arrest. J Biol Chem.

32. **Neades, R., L. Cox, and J. C. Pelling.** 1998. S-phase arrest in mouse keratinocytes exposed to multiple doses of ultraviolet B/A radiation. Mol Carcinog **23:**159-67.

33. **Oh, H.-S., and R. C. Smart.** 1998. Expression of CCAAT/enhancer binding protein (C/EBP) is associated with squamous differentiation in epidermis and isolated primary keratinocytes and is altered in skin neoplasms. J. Invest. Dermatol. **110:**939-945.

34. **O'Rourke, J., R. Yuan, and J. DeWille.** 1997. CCAAT/enhancer-binding protein-delta (C/EBP-delta) is induced in growth-arrested mouse mammary epithelial cells. J Biol Chem **272:**6291-6.

35. **Pabst, T., B. U. Mueller, P. Zhang, H. S. Radomska, S. Narravula, S. Schnittger, G. Behre, W. Hiddemann, and D. G. Tenen.** 2001. Dominant-negative mutations of C/EBPA, encoding CCAAT/enhancer binding protein-alpha, in acute myeloid leukemia. Nature Genetics **27:**263-270.

36. **Park, D. S., E. J. Morris, L. A. Greene, and H. M. Geller.** 1997. G1/S cell cycle blockers and inhibitors of cyclin-dependent kinases suppress camptothecin-induced neuronal apoptosis. J Neurosci **17:**1256-70.

37. **Piontkewitz, Y., S. Enerback, and L. Hedin.** 1993. Expression and hormonal regulation of the CCAAT enhancer binding protein-alpha during differentiation of rat ovarian follicles. Endocrinology **133:**2327-33.

38. **Porse, B. T., T. A. Pedersen, X. Xu, B. Lindberg, U. M. Wewer, L. Friis-Hansen, and C. Nerlov.** 2001. E2F repression by C/EBPalpha is required for adipogenesis and granulopoiesis in vivo. Cell **107:**247-58.

39. **Radomska, H. S., C. S. Huettner, P. Zhang, T. Cheng, D. T. Scadden, and D. G. Tenen.** 1998. CCAAT/enhancer binding protein alpha is a regulatory switch sufficient for induction of granulocytic development from bipotential myeloid progenitors. Mol Cell Biol **18:**4301-14.

40. **Ramji, D. P., and P. Foka.** 2002. CCAAT/enhancer-binding proteins: structure, function and regulation. Biochem J **365:**561-75.

41. **Saito, S., H. Yamaguchi, Y. Higashimoto, C. Chao, Y. Xu, A. J. Fornace, Jr., E. Appella, and C. W. Anderson.** 2003. Phosphorylation site interdependence of human p53 post-translational modifications in response to stress. J Biol Chem **278:**37536-44.

42. **Sarkaria, J. N., E. C. Busby, R. S. Tibbetts, P. Roos, Y. Taya, L. M. Karnitz, and R. T. Abraham.** 1999. Inhibition of ATM and ATR kinase activities by the radiosensitizing agent, caffeine. Cancer Res **59:**4375-82.

43. **Schreiber, E., P. Matthias, M. M. Muller, and W. Schaffner.** 1989. Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. Nucleic Acids Res **17:**6419.

44. **Scott, L. M., C. I. Civin, P. Rorth, and A. D. Friedman.** 1992. A novel temporal expression pattern of three C/EBP family members in differentiating myelomonocytic cells. Blood **80:**1725-35.

45. **Shieh, S. Y., M. Ikeda, Y. Taya, and C. Prives.** 1997. DNA damage-induced phosphorylation of p53 alleviates inhibition by MDM2. Cell **91:**325-34.

46. **Slomiany, B. A., K. L. D'Arigo, M. M. Kelly, and D. T. Kurtz.** 2000. C/EBPalpha inhibits cell growth via direct repression of E2F-DP-mediated transcription. Mol Cell Biol **20:**5986-97.

47. **Swart, G. W. M., J. J. M. v. Groningen, F. v. Ruissen, M. Bergers, and J. Schalkwilk.** 1997. Transcription factor C/EBPalpha: Novel sites of expression and cloning of the human gene. Biol. Chem **378:**373-379.

48. **Tibbetts, R. S., K. M. Brumbaugh, J. M. Williams, J. N. Sarkaria, W. A. Cliby, S. Y. Shieh, Y. Taya, C. Prives, and R. T. Abraham.** 1999. A role for ATR in the DNA damage-induced phosphorylation of p53. Genes Dev **13:**152-7.

49. **Timchenko, N. A., M. Wilde, and G. J. Darlington.** 1999. C/EBPalpha regulates formation of S-phase-specific E2F-p107 complexes in livers of newborn mice. Mol. Cell. Biol **19:**2936-2945.

50. **Timchenko, N. A., M. Wilde, P. Iakova, J. H. Albrecht, and G. J. Darlington.** 1999. E2F/107 and E2F/p130 complexes are regulated by C/EBPalpha in 3T3-L1 adipocytes. Nucleic Acids Res **27:**3621-3630.

51. **Timchenko, N. A., M. Wilde, M. Nakanishi, J. R. Smith, and G. J. Darlington.** 1996. CCAAT/enhancer-binding protein alpha (C/EBPalpha) inhibits

cell proliferation through the p21 (WAF-1/CIP-1/SDI-1) protein. Genes & Dev **10:**804-815.

52. **Tomizawa, M., Y. Q. Wang, M. Ebara, H. Saisho, K. Watanabe, A. Nakagawara, and M. Tagawa.** 2002. Decreased expression of the CCAAT/enhancer binding protein alpha gene involved in hepatocyte proliferation in human hepatocellular carcinomas. Int J Mol Med **9:**597-600.

53. **Umek, R. M., A. D. Friedman, and S. L. McKnight.** 1991. CCAAT-enhancer binding protein: A component of a differentiation switch. Science **251:**288-292.

54. **Vincent, F., G. Deplanque, J. Ceraline, B. Duclos, and J. P. Bergerat.** 1999. p53-independent regulation of cyclin B1 in normal human fibroblasts during UV-induced G2-arrest. Biol Cell **91:**665-74.

55. **Waldman, T., K. W. Kinzler, and B. Vogelstein.** 1995. p21 is necessary for the p53-mediated G1 arrest in human cancer cells. Cancer Res **55:**5187-90.

56. **Wang, H., P. Iakova, M. Wilde, A. Welm, T. Goode, W. J. Roesler, and N. A. Timchenko.** 2001. C/EBPalpha arrests cell proliferation through direct inhibition of Cdk2 and Cdk4. Mol Cell **8:**817-28.

57. **Wang, X., E. Scott, C. L. Sawyers, and A. D. Friedman.** 1999. C/EBPalpha bypasses granulocyte colony-stimulating factor signals to rapidly induce PU.1 gene expression, stimulate granulocytic differentiation, and limit proliferation in 32D cl3 myeloblasts. Blood **94:**560-71.

58. **Wang, X. W., Q. Zhan, J. D. Coursen, M. A. Khan, H. U. Kontny, L. Yu, M. C. Hollander, P. M. O'Connor, A. J. Fornace, Jr., and C. C. Harris.** 1999.

GADD45 induction of a G2/M cell cycle checkpoint. Proc Natl Acad Sci U S A **96:**3706-11.

59. **Wang, X. Z., B. Lawson, J. W. Brewer, H. Zinszner, A. Sanjay, L. J. Mi, R. Boorstein, G. Kreibich, L. M. Hendershot, and D. Ron.** 1996. Signals from the stressed endoplasmic reticulum induce C/EBP-homologous protein (CHOP/GADD153). Mol Cell Biol **16:**4273-80.

60. **Westendorf, J. J., C. M. Yamamoto, N. Lenny, J. R. Downing, M. E. Selsted, and S. W. Hiebert.** 1998. The t(8:21) fusion protein, AML-1-ETO, associated with C/EBP-alpha, inhibits C/EBP-alpha-dependent transcription and blocks granulocytic differentiation. Molecular and Cellular Biology **18:**322-333.

61. **Zhu, S., H. S. Oh, M. Shim, E. Sterneck, P. F. Johnson, and R. C. Smart.** 1999. C/EBPβ modulates the early events of keratinocyte differentiation involving growth arrest and keratin 1 and keratin 10 expression. Mol Cell Biol **19:**7181-7190.

62. **Zhu, S., K. Yoon, E. Sterneck, P. F. Johnson, and R. C. Smart.** 2002. CCAAT/enhancer binding protein-beta is a mediator of keratinocyte survival and skin tumorigenesis involving oncogenic Ras signaling. Proc Natl Acad Sci U S A **99:**207-12.

63. **Ziegler, A., A. S. Jonason, D. J. Leffell, J. A. Simon, H. W. Sharma, J. Kimmelman, L. Remington, T. Jacks, and D. E. Brash.** 1994. Sunburn and p53 in the onset of skin cancer. Nature **372:**773-6.

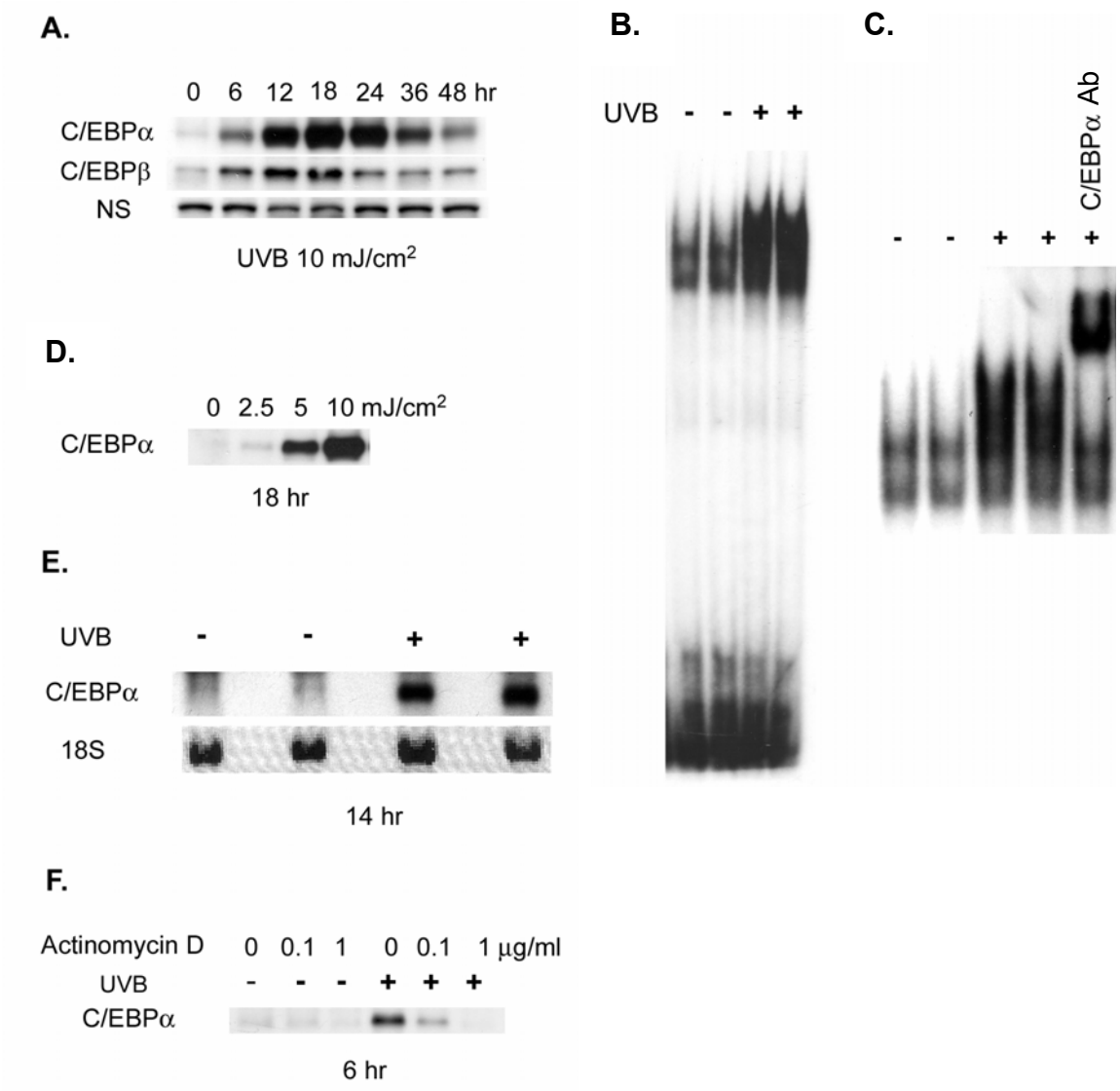


Fig.1 UVB is a potent inducer of C/EBPα.

(A) BALB/MK2 cells were irradiated with 10 mJ/cm^2 UVB and harvested at the various time points. Whole cell lysates were prepared and equal amounts of protein were subjected to immunoblot analysis with rabbit polyclonal anti-C/EBPα or C/EBPβ antibody. Non-specific bands (NS) are shown to confirm equal loading. (B) BALB/MK2

cells were irradiated with 10 mJ/cm^2 UVB and 18 hrs later nuclear extracts were prepared and EMSA and (C) supershift with anti-C/EBPα antibody were conducted. (D) BALB/MK2 cells were irradiated with various doses of UVB and 18 hrs later cells lysates were prepared and Western blot analysis using anti-C/EBPα antibody was conducted. (E) Total RNA was isolated from 10 mJ/cm^2 UVB irradiated BALB/MK2 cells 14 hrs after irradiation. Northern blot analysis for C/EBPα was conducted. 18S RNA is shown for equal loading. (F) BALB/MK2 cells were preincubated with actinomycin D for 30 min before UVB irradiation and incubated further for 6 hrs. Cell lysates were prepared and Western blot analysis was conducted for C/EBPα.

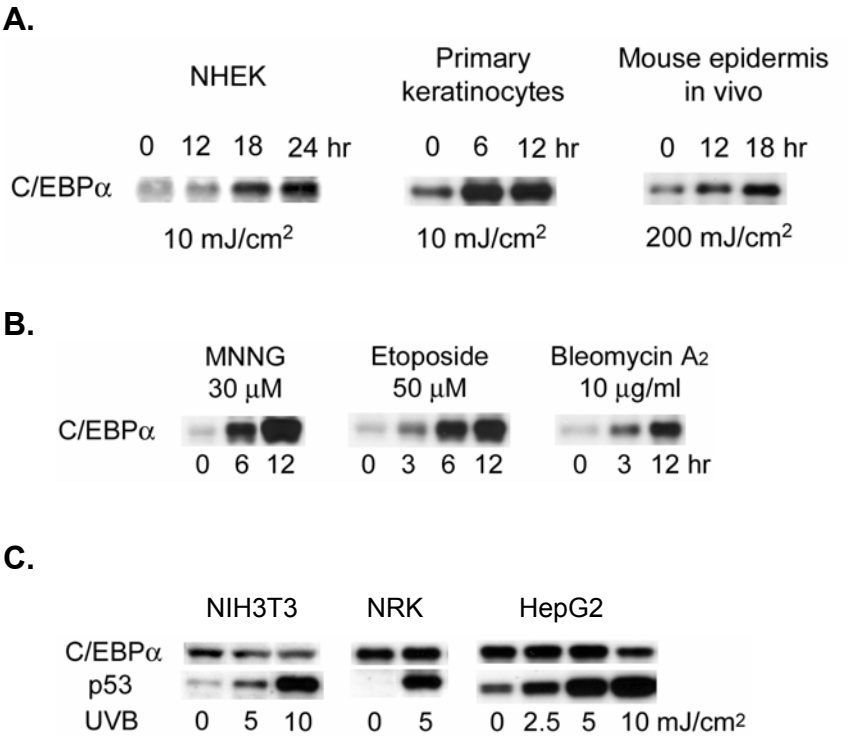
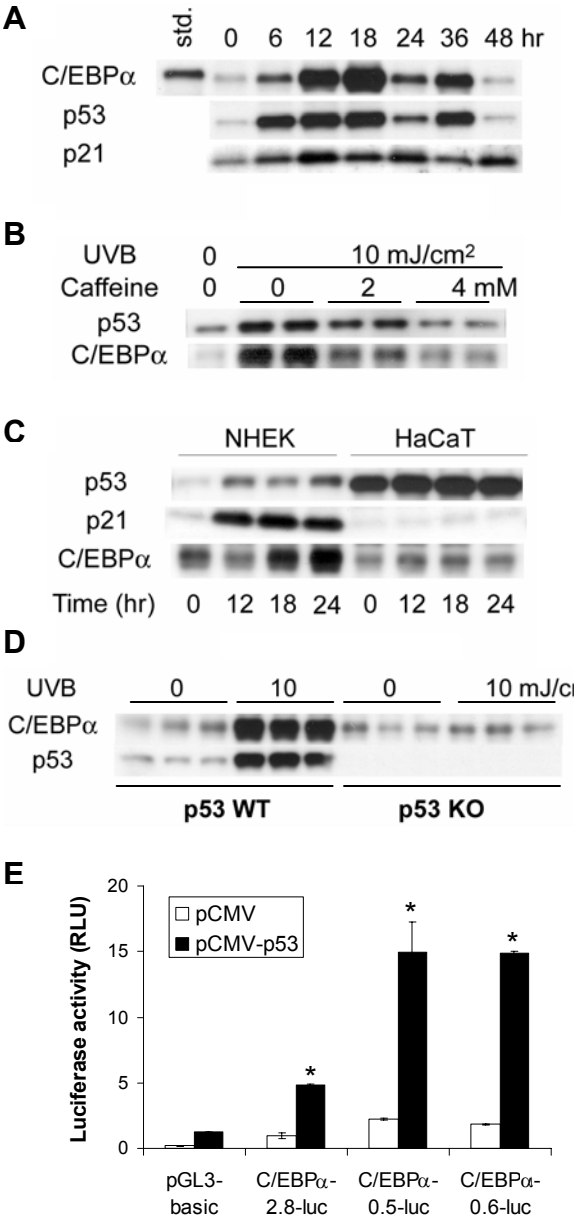


Fig.2 UVB induces C/EBPα in human and mouse primary keratinocytes and mouse skin in vivo and induction of C/EBPα is a general DNA damage response.

(A) NHEK, primary mouse keratinocytes or 8 week old mice were irradiated with indicated doses of UVB. Keratinocytes were collected at the indicated time points, cell lysates were prepared and Western blot analysis was conducted with anti-C/EBPα antibody. (B) BALB/MK2 cells were treated with MNNG, etoposide or bleomycin A_2 for the indicated times and harvested. Cell lysates were prepared and Western blot analysis was conducted for C/EBPα. (C) NIH3T3, NRK or HepG2 cells were irradiated with indicated doses of UVB. Cells were collected 12 hr after irradiation, cell lysates were prepared and Western blot was conducted with anti-C/EBPα or anti-p53 antibody.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p53
C/EBPα
C
NHEK
HaCaT
p53
p21
C/EBPα
Time (hr)
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12
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10 mJ/cm2
C/EBPα
p53
p53 WT
p53 KO
E
Luciferase activity (RLU)
20
15
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5
0
pCMV
pCMV-p53
*
*
*
pGL3-basic
C/EBPα-2.8-luc
C/EBPα-0.5-luc
C/EBPα-0.6-luc

Fig. 3 UVB induction of C/EBPα requires p53.

(A) Time-course of C/EBPα protein, p53 and p21 protein induction after UVB irradiation of BALB/MK2 cells (B) BALB/MK2 cells were pretreated with the indicated concentrations of caffeine for 1 hr before UVB irradiation. Cells were irradiated with UVB, incubated for 12 hr in the presence of caffeine, and collected. Cell lysates were prepared and Western blot analysis was conducted using anti-p53 or -C/EBPα antibody. (C) NHEK or HaCaT cells were irradiated with UVB. At the indicated time points, nuclear extracts were prepared and Western blot analysis was conducted for p53, p21 and C/EBPα protein. (D) Primary keratinocytes were isolated from wild type or p53-null newborn mice. Three plates of each genotype were irradiated with 10 mJ/cm^2 of UVB and three plates of each genotype were not. Whole cell lysates were prepared 6 hr after irradiation and Western blot analysis was conducted using anti-C/EBPα or anti-p53 antibody. (E) BALB/MK2 cells were transfected with C/EBPα promoter-luciferase construct along with p53 expression vector as indicated. Luciferase assay was done in triplicate. For all C/EBPα-luc constructs, another C/EBPα promoter-reporter construct prepared from an independent clone was tested and showed similar results. *, Significantly different from pGL3 basic transfected with pCMV-p53 determined by Student's t test ($p<0.05$)

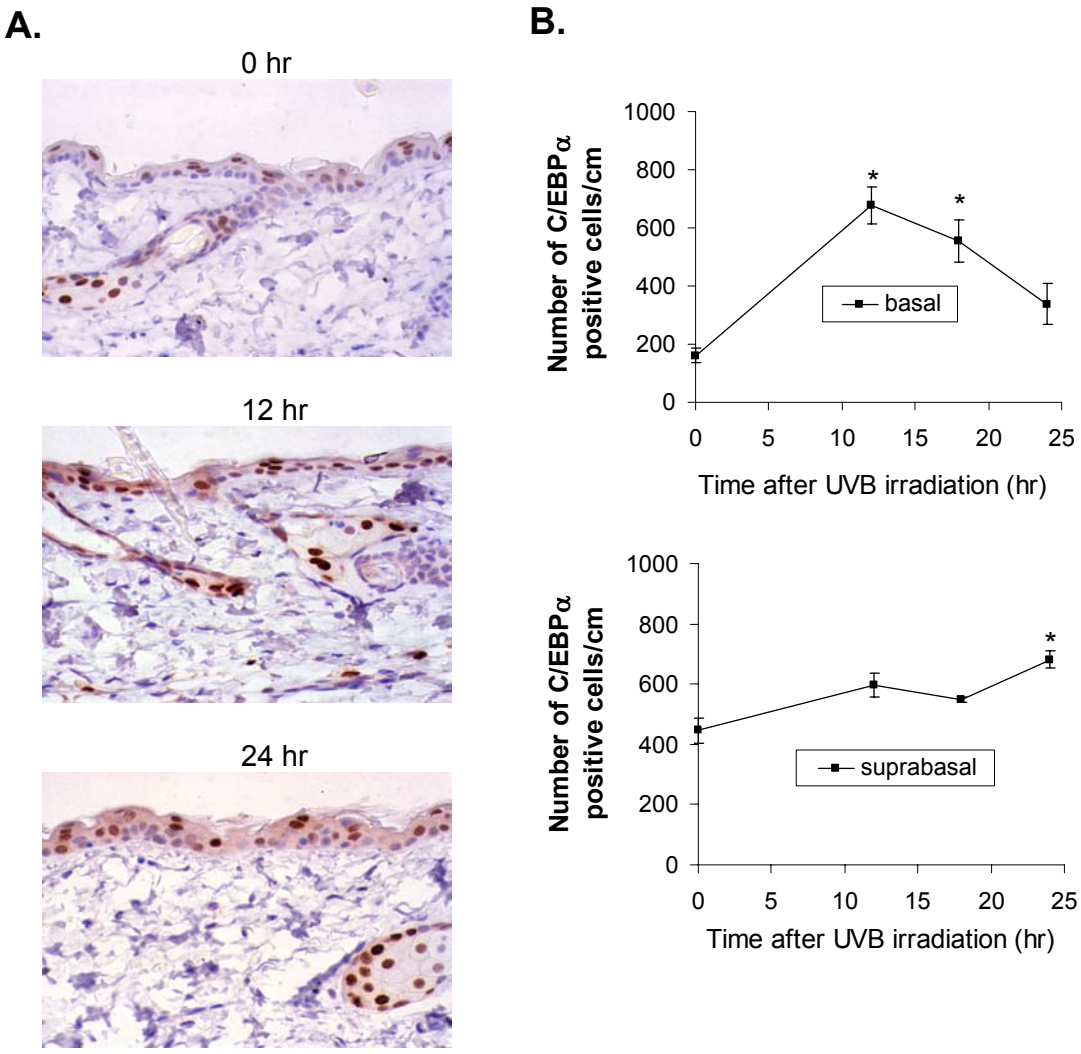

Fig. 4 C/EBPα protein is induced in the basal and suprabasal epidermal keratinocytes irradiated with UVB in vivo.

(A) Dorsal area hair of 7 week-old CD-1 mice was clipped and irradiated with 100 mJ/cm^2 UVB. At the indicated times after UVB irradiation, skins were collected, processed and embedded in paraffin. Tissue sections were stained with anti-C/EBPα antibody, and basal and suprabasal interfollicular keratinocytes with positive C/EBPα staining were scored separately and presented in B. Photographs were taken at

×100 magnifications. (B) Data are expressed as the mean±S.E. of three mice. Three sections/each mouse were processed. *, Significantly different from untreated group as determined by Student's t test ($p < 0.05$).

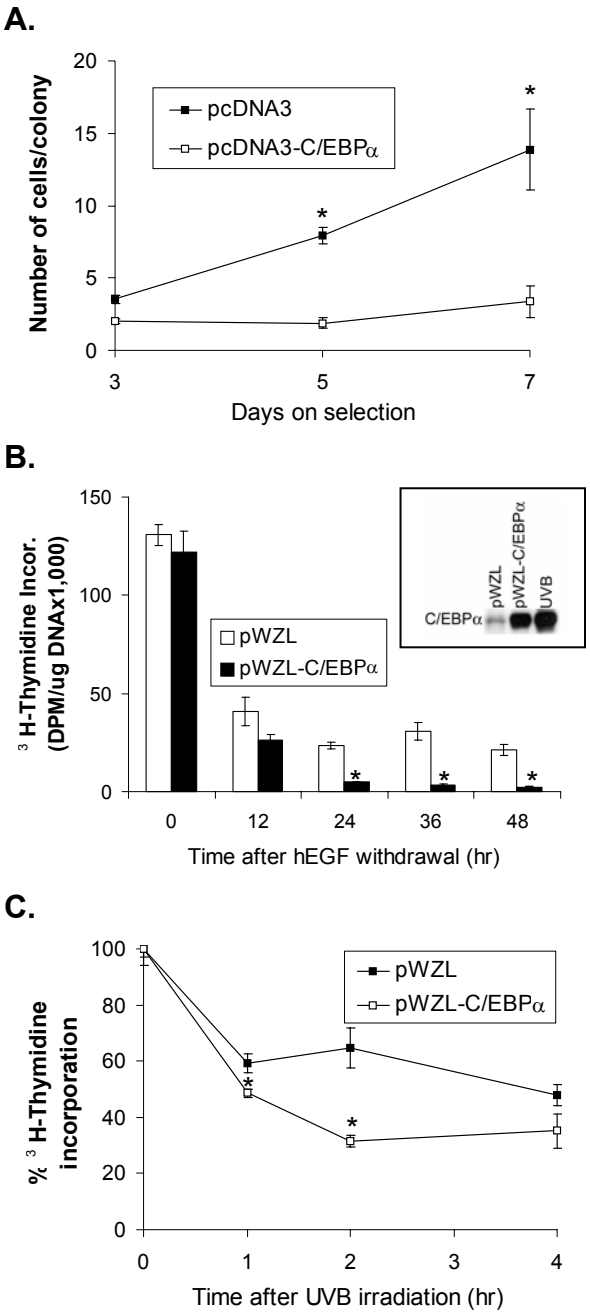

Fig.5 Enforced expression of C/EBPα inhibits proliferation of keratinocytes.

(A) BALB/MK2 cells were transfected with pcDNA3 or pcDNA3-C/EBPα and subcultured in the presence of 300 μg/ml G418. The number of cells/colony was counted

on days 3, 5, 7 after G418 selection. (B) hEGF was withdrawn from BALB/MK2-pWZL or BALB/MK2-pWZL-C/EBPα cells. Cells were pulse-labeled with [^{3}H-methyl] thymidine for 1 hr before each time point indicated and [^{3}H-methyl] thymidine incorporation into DNA was determined. The box shows the C/EBPα protein levels in BALB/MK2-pWZL, BALB/MK2-pWZL-C/EBPα, and irradiated BALB/MK2 cells with 10 mJ/cm^2 UVB at 18 hr time point. (C) BALB/MK2-pWZL or BALB/MK2-pWZL-C/EBPα cells were irradiated with 5 mJ/cm^2 UVB and pulse-labeled with [^{3}H-methyl] thymidine for 1 hr before each time point indicated and incorporated [^{3}H-methyl] thymidine into DNA was determined. Data are expressed as the mean±S.D. of a representative experiment done in triplicate. *, Significantly different between BALB/MK2-pWZL and BALB/MK2-pWZL-C/EBPα as determined by Student's t test ($p< 0.05$).

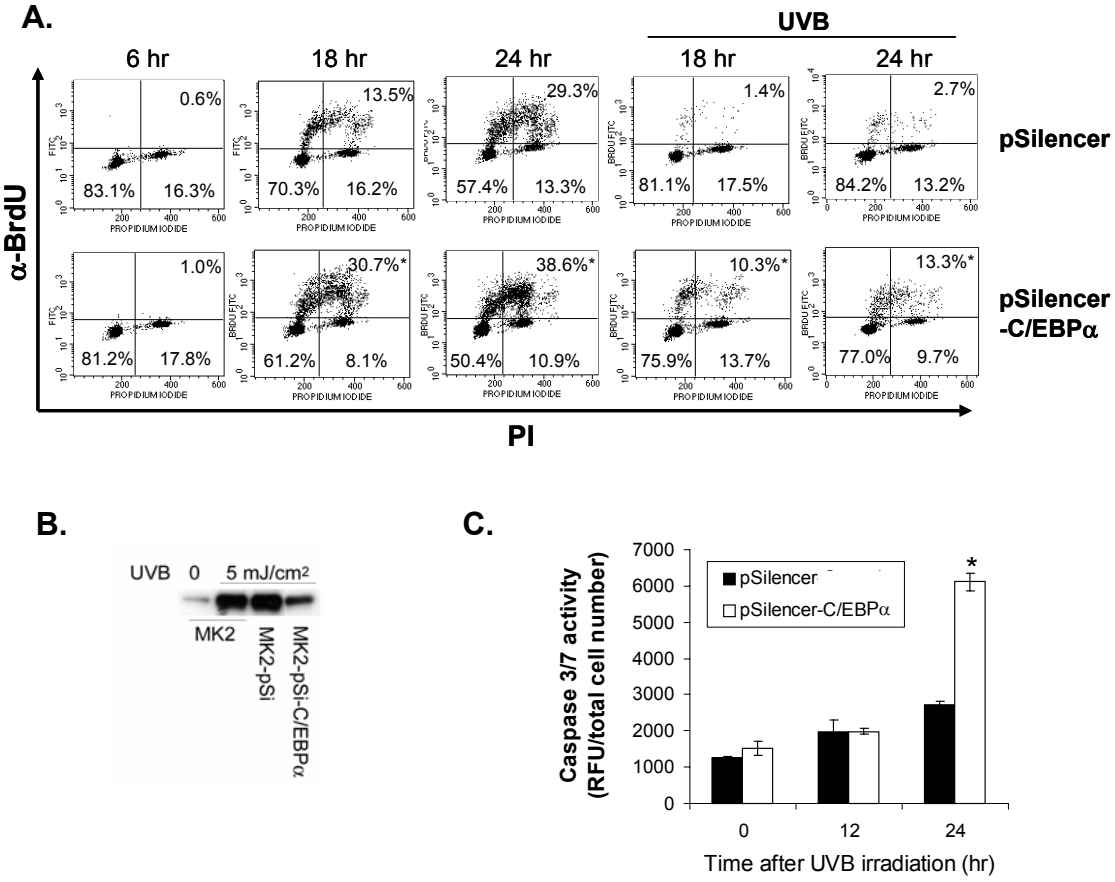

Fig. 6 UVB irradiation of C/EBPα knockdown keratinocytes displays an attenuated DNA damage G1 checkpoint.

(A) C/EBPα knockdown keratinocytes or control keratinocytes were synchronized by starvation as described and either irradiated or not 6 hr after release into the cell cycle. Cells were pulse-labeled with BrdU 2 hrs before collection, stained with anti-BrdU antibody and PI and subjected to FACS analysis at the different time points indicated after release. Percentage of cells in G_1 (lower left), S (upper) and G_2/M (lower right) phases of cell cycle is shown. Values reported are from duplicates for non-irradiated samples (variance < 20%) and triplicates for irradiated samples (STD < 20%). (B) shows

the C/EBPα expression level after indicated treatments. (C) C/EBPα knockdown keratinocytes or control keratinocytes were grown in regular medium and irradiated with 10 mJ/cm^2 UVB. At the indicated time points, caspase 3/7 activity was measured and corrected by total cell number. *, Significantly different between control and C/EBPα knockdown keratinocytes as determined by Student's t test ($p < 0.05$).

General Discussion

UVB signaling to upregulation of C/EBPα

Our results demonstrate that p53 is required for C/EBPα induction by UVB, MNNG, etoposide and bleomycin and that an ATM/ATR inhibitor, caffeine blocks UVB induction of C/EBPα, suggesting that C/EBPα is induced by pathways involving ATR as well ATM. We have identified that putative p53 consensus sites in the proximal and distal C/EBPα promoter and found that forced expression of p53 can stimulate C/EBPα proximal promoters. Additional work will be required to determine if C/EBPα induction is a direct effect of p53 on the C/EBPα promoter or an indirect effect, and if the identified sites are bona fide p53 binding sites that are essential for p53-mediated induction of C/EBPα. The C/EBPα proximal promoter-reporter that we constructed displayed background noise which is likely due to the presence of p53 and C/EBP binding sites in the empty luciferase construct. This noise complicates the use of this construct to identify the role of p53 in the expression of C/EBPα. Characterization of C/EBPα promoter in another construct, along with gel shift or chromatin immunoprecipitation (ChIP) assay using identified, putative p53 consensus sites will provide better understanding in the p53-mediated C/EBPα gene regulation.

In addition to putative p53 sites, the 0.5 kb upstream of C/EBPα proximal promoter contains potential binding sites for C/EBP, SP-1, NF-1, NF-Y and NF-κB, and C/EBPα promoter can be auto-regulated by C/EBPs (119). We observed that the C/EBPα promoter was activated by forced expression of C/EBPα or C/EBPβ in mouse

keratinocytes (data not shown), and this may explain the huge induction of C/EBPα (>70 fold) by UVB irradiation. That is, C/EBPα is initially induced by p53 upon DNA damage and then C/EBPα induction is potentiated by positive regulation of C/EBPα through the activation of its own promoter at the C/EBP binding site.

As mentioned in the introduction, UVB can also activate MAPK pathways and stimulate EGFR signaling. UVB activation of one or more of these pathways may be also responsible for the upregulation of C/EBPα. As shown in Figure 1, the UVB-induction of C/EBPα was not affected by treatment of pharmacological inhibitors of EGFR (PD153035), JNK (JNK inhibitor II), or MEK1/2 (PD98059). However, induction of C/EBPα by UVB was completely blocked by lithium chloride, a GSK inhibitor. Two additional GSK3 inhibitors, SB216763 or SB415286 also inhibited the UVB induction of C/EBPα (Figure 1). These results suggest that in addition to p53, UVB induction of C/EBPα requires catalytically active GSK3.

Studies in 3T3-L1 preadipocytes demonstrated that forced expression of WNT or stabilized forms of β-catenin, or treatment of GSK inhibitors, blocks C/EBPα mRNA and protein expression, and adipocyte differentiation (306). If the WNT signaling pathway suppresses C/EBPα gene expression in keratinocytes, UVB-induced repression of WNT signaling pathway could result in derepression of C/EBPα expression. However, we found that cytosolic β-catenin levels were not affected by UVB irradiation, and that UVB irradiation or forced expression of p53 did not inhibit TCF/LEF transcription activity, suggesting that UVB does not inhibit WNT signaling pathway in our system. It is still possible that UVB induces an active, nuclear GSK3/p53 complex which regulates p53-

mediated target gene expression (305), and the complex functions in the upregulation of C/EBPα.

In addition, the treatment with a PKC or a p38 inhibitor partially inhibited UVB-induction of C/EBPα, and at the same time, C/EBPα showed a faster migration in the SDS-PAGE gel with a p38 inhibitor. This is consistent with the finding that novel PKC isoforms increase C/EBPα levels and activate its transcriptional activity on hINV promoter by activating a p38δ in human keratinocytes (144).

While we observed that UVB treatment of NIH3T3, NRK and HepG2 cells did not result in the induction of C/EBPα, we did observe that UVB treatment of mouse dermal primary fibroblasts did result in the induction of C/EBPα. As shown in Figure 2B, UVB produced a very large increase in C/EBPα protein levels in fibroblasts. In contrast to the keratinocytes, UVB induced C/EBPα protein in p53-deficient primary dermal fibroblasts, indicating that C/EBPα induction by UVB in dermal fibroblasts is a p53-independent event. Thus, not only is the UVB induction of C/EBPα cell type specific but UVB induction of C/EBPα can occur through different signaling pathways that can be dependent or independent of p53.

After UV-induced DNA damage, ATR and downstream kinase Chk1 phosphorylate various substrates to induce DNA damage response. Potential ATR or Chk1 phosphorylation sites in C/EBPα protein were evaluated, but C/EBPα does not have either ATR or Chk1 consensus phosphorylation sites. In contrast, C/EBPβ has a potential Chk1 phophorylation site at Ser-276, **LSR**EL**S**^{276}, and phosphorylation of this residue by calcium/calmodulin-dependnet kinase has been reported to stimulate C/EBPβ transcriptional activity (138, 307). C/EBPβ can induce C/EBPα expression through a

C/EBP binding site in the C/EBPα promoter. UVB may regulate C/EBPα expression via ATR-Chk1-C/EBPβ pathway in mouse dermal fibroblasts.

C/EBPα functions in keratinocytes

Our study showed that C/EBPα is involved in the DNA damage response and G1 checkpoint in keratinocytes. C/EBPα has been shown to interact with various proteins associated with the cell cycle regulation including p21, Rb family members, E2F, Cdk2 and Cdk4. As mentioned earlier, p21 does not seem to be involved in the C/EBPα-induced G1 checkpoint activation. C/EBP has been shown to interact directly with p107, Cdk2 and Cdk4, and interfere G1-S progression. To understand the mechanism of C/EBPα-induced G1 arrest, it will be necessary to determine whether UVB induces the interaction of C/EBPα with one or more of the above target proteins and if C/EBPα binding inhibits kinase activity of Cdk2 or Cdk4. Our preliminary results suggested that Cdk2 does not interact with C/EBPα after UVB irradiation in keratinocytes, however these results are preliminary and should be confirmed. C/EBPα also has been shown to repress E2F-dependent transcription. The E2F responsive promoter-reporter in combination with C/EBPα or UVB could address the involvement of E2F in the DNA damage-induced cell cycle arrest function of C/EBPα.

C/EBPα plays a role in cellular processes including energy metabolism and growth arrest coupled with differentiation in various tissues, either by transcriptional regulation of downstream genes or by protein-protein interaction with proteins involved in the processes. Epidermis is mainly composed of keratinocytes which differentiate from proliferating basal cells through suprabasal cells, ending in the cornified envelope

which will eventually be shed from the skin. The immunostaining of mouse epidermis showed that C/EBPα is weekly expressed in nucleus of some basal keratinocytes and is expressed at high level in suprabasal keratinocytes. C/EBPα expression was exclusively confined to the nuclei of a three-cell cluster of suprabasal keratinocytes and these clusters were repeated at regular interval throughout the epidermis. This characteristic pattern of C/EBPα expression is consistent with suprabasal column of the EPU (9), implicating the role of C/EBPα in growth arrest coupled with differentiation in keratinocytes.

BALB/MK2 keratinocytes infected with a retrovirus carrying C/EBPα displayed increased expression of involucrin, a squamous differentiation marker (Figure 3C). It is consistent with previous finding that C/EBPα increases hINV promoter activity in human keratinocytes (76). However, increased expression of involucrin was not accompanied by changes in keratinocyte proliferation or morphology.

Considering that differentiation is a postmitotic event characterized by a highly coordinated program of sequential changes in gene expression, primary keratinocytes are more suitable model than immortalized keratinocytes to study differentiation. In low calcium medium, attached keratinocytes resemble the basal keratinocytes of the epidermis. When attached keratinocytes terminally differentiate, they spontaneously detach from the plate. Therefore, two distinct populations of keratinocytes, the detached terminally differentiated cells and the attached undifferentiated cells can be evaluated. As shown in Figure 3A, UVB produced an increase in the number of floating cells and these cells expressed elevated levels of the squamous differentiation markers, involucrin and loricrin (Figure 3B). The fact that the floating cells demonstrated elevated levels of involucrin and loricrin, indicates that the cell floating was related to the differentiation.

Attached keratinocytes also induced differentiation markers, although to a lesser extent. These results suggest that UVB induces differentiation in the certain populations of primary keratinocytes, which are likely to be postmitotic transient amplifying cells and cells that have already entered the differentiation program. It should be further determined if C/EBPα is involved in UVB-induced upregulation of the differentiation markers and the increase in the number of floating, differentiated cells. To answer these questions, studies of C/EBPα-deficient primary keratinocytes or C/EBPα-deficient epidermis in vivo will be a valuable approach.

Some proteins such as p21, MyoD and Rb involved in the regulation of cell cycle progression have multifaceted roles including differentiation (308). For example, p21 has a role in the inhibition of cell cycle progression (309) associated with differentiation and senescence (310, 311) as well as in mediating growth arrest induced by DNA damage (312-314). It is possible that C/EBPα has multiple functions in epidermal keratinocytes. One of the functions may be to maintain epidermal homeostasis by inducing growth arrest that precedes stratified squamous differentiation and participating in the regulation of differentiation. In view of the high potential of the skin for exposure to environmental stressors and the self-renewing characteristic of skin being replaced constantly, the other function is to participate in growth arrest in proliferating keratinocytes to inhibit replication of damaged DNA, and to accelerate differentiation in postmitotic keratinocytes, in the presence of DNA damage. Apoptosis will be another option to remove severely damaged cells from the epidermis. It has been reported that UVB induces differentiation markers in human epidermis in vivo (315), and induces proliferation after initial growth arrest in mouse skin (316).

The previous finding that C/EBPα protein levels are greatly diminished or undetectable in mouse skin SCCs (58) along with the important role of C/EBPα in the maintenance of epidermal integrity under the normal and stressed condition suggests a tumor suppressor function of C/EBPα in skin. C/EBPα could be an intriguing target for the treatment of nonmelanoma skin cancer in the future, although the detailed signaling pathway of C/EBPα regulation after DNA damage and the mechanism of induction of cell cycle arrest remain to be determined.

In summary, this study demonstrated that C/EBPα is induced extensively in response to various types of DNA damage in p53 dependent pathway in keratinocytes, and that C/EBPα has an important role in the DNA damage G1 checkpoint response. Our results also suggest that C/EBPα, in response to UVB treatment, may enhance the differentiation of keratinocytes.

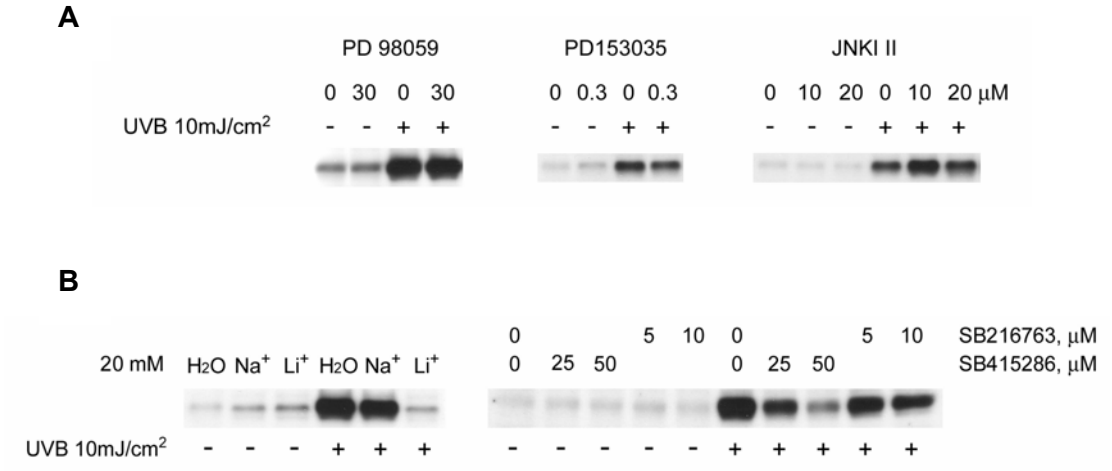

Figure 1. GSK3 inhibitors block UVB-induction of C/EBPα protein.

BALB/MK2 cells were pretreated with each inhibitor for 1hr, and then irradiated with UVB at the dose of 10 mJ/cm^2. Cell lysates were prepared after 12 hr further incubation in the presence of indicated dose of each inhibitor and western blot analysis was conducted for C/EBPα.

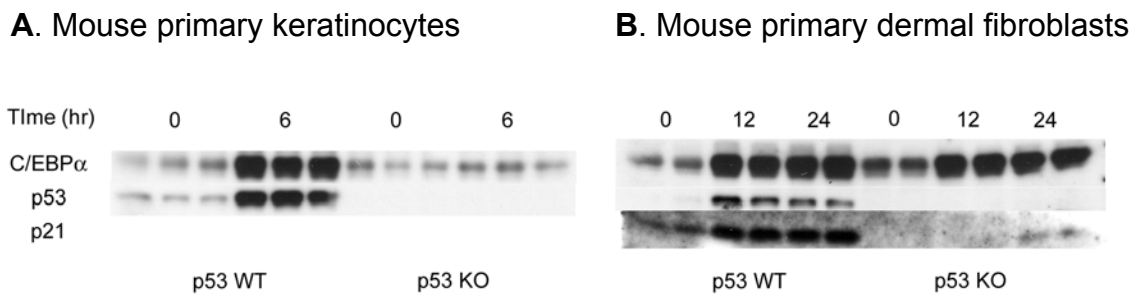

Figure 2. UVB induces C/EBPα in a p53–independent manner in mouse primary dermal fibroblasts. Mouse primary keratinocytes or dermal fibroblasts were isolated from wild type or p53-null newborn mice. Cells were irradiated with 10 mJ/cm^2 of UVB. Whole cell lysates were prepared at the indicated time after irradiation and Western blot analysis was conducted with anti-C/EBPα, anti-p53 or anti-p21 antibody.

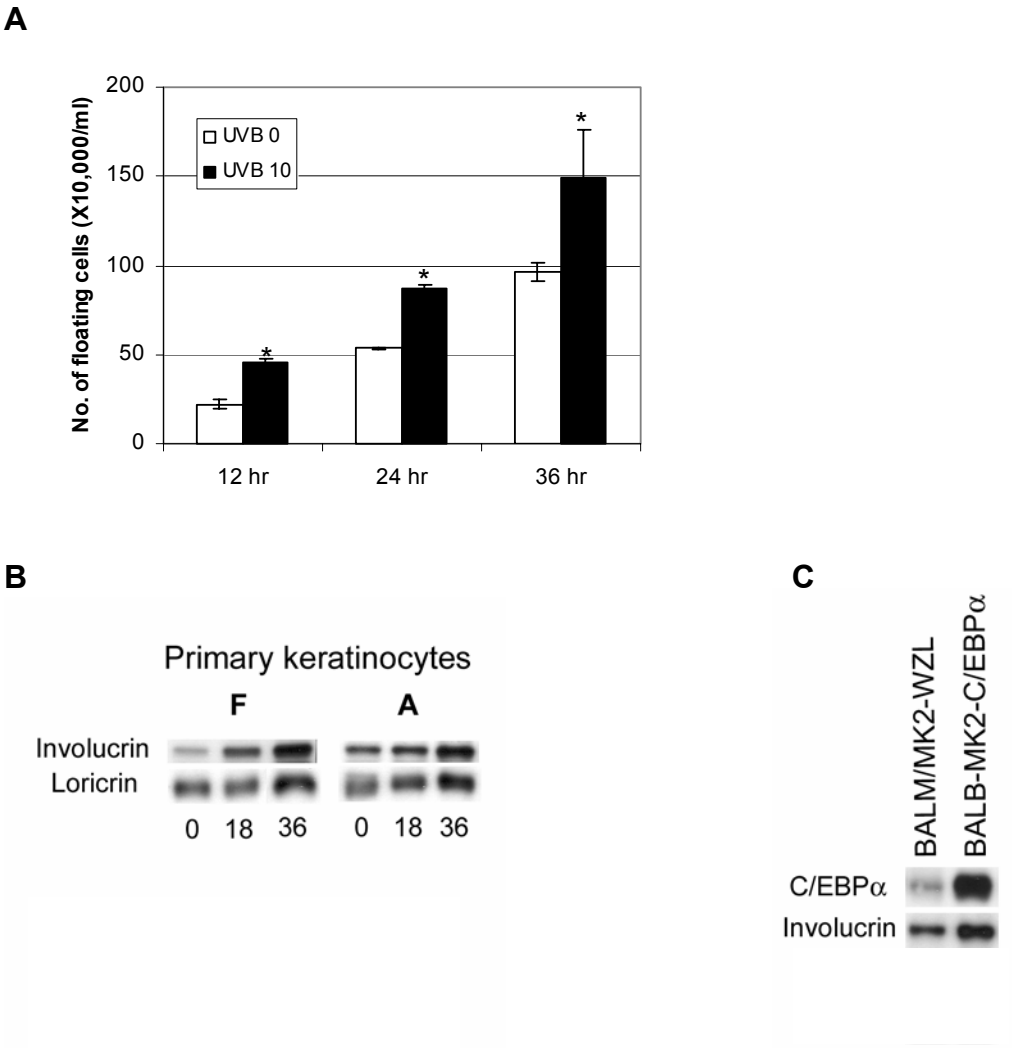

Figure 3. UVB induces differentiation in primary keratinocytes.

(A) UVB produced an increase in the number of floating cells in primary keratinocytes. Mouse primary keratinocytes were irradiated with 10 mJ/cm^2 of UVB, and 12, 24, and 36 hr after irradiation, medium was collected and centrifuged, and cell number was counted. *, Significantly different from untreated control determined by student t-test ($p<0.5$).

(B) Eighteen or thirty six hours after 10 mJ/cm^2 UVB irradiation, floating cells (F) and adherent cells (A) were collected separately. Cell lysates were prepared with sample buffer and Western analysis was conducted for involucrin and loricrin. (C) Cell lysates were prepared in sample buffer from BALB/MK2 cells which were infected with pWZL

or pWZL-C/EBPα and selected with hygromycin. Western analysis was conducted probing with anti-C/EBPα or anti-involucrin antibody.

General References

1. Kanitakis, J. Anatomy, histology and immunohistochemistry of normal human skin. Eur J Dermatol, *12:* 390-399; quiz 400-391, 2002.
2. Fuchs, E. Epidermal differentiation: the bare essentials. J. Cell. Biol., *111:* 2807-2814, 1990.
3. Roop, D. R., Hawley-Nelson, P., Cheng, C. K., and Yuspa., S. H. Keratin gene expression in mouse epidermis and cultured epidermal cells. Proc. Natl. Acad. Sci. USA, *80:* 716-720, 1983.
4. Fuchs, E. and Green, H. Changes in keratin gene expression during terminal differentiation of the keratinocyte. Cell, *19:* 1033-1042, 1980.
5. Fleckman, P., Dale, B. A., and Holbrook, K. A. Profilaggrin, a high-molecular-weight precursor of filaggrin in human epidermis and cultured keratinocytes. J Invest Dermatol, *85:* 507-512, 1985.
6. Mehrel, T., Hohl, D., Rothnagel, J. A., Longley, M. A., Bunoman, D., Cheng, C., Lichti, U., Bisher, M. E., Steven, A. C., Steiner, P. M., Yuspa, S. H., and Roop, D. R. Identification of a major keratinocyte cell envelope protein, loricrin. Cell, *61:* 1103-1112, 1990.
7. Simon, M. and Green, H. Participation of membrane-associated proteins in the formation of the cross-linked envelope of the keratinocyte cell. Cell, *36:* 827-834, 1984.
8. Thacher, S. M. and Rice, R. H. Keratinocyte-specific transglutaminase of cultured human epidermal cells: relation to cross-linked envelope formation and terminal differentiation. Cell, *40:* 685-695, 1985.
9. Potten, C. S. The epidermal proliferative unit: the possible role of the central basal cell. Cell tissue Kinet, *7:* 77-88, 1974.
10. Morris, R. J., Fischer, S. M., and Slaga, T. J. Evidence that the centrally amd peropherally located cells in the murine epidermal proliferative unit are two distict cell populations. J Invest Dermatol, *84:* 277-281, 1985.
11. Cotsarelis, G., Cheng, S. Z., Dong, G., Sun, T. T., and Lavker, R. M. Existence of slow-cycling limbal epithelial basal cells that can be preferentially stimulated to proliferate: implications on epithelial stem cells. Cell, *57:* 201-209, 1989.
12. Cotsarelis, G., Sun, T. T., and Lavker, R. M. Label-retaining cells reside in the bulge area of pilosebaceous unit: implications for follicular stem cells, hair cycle, and skin carcinogenesis. Cell, *61:* 1329-1337, 1990.
13. Fuchs, E. and Raghavan, S. Getting under the skin of epidermal morphogenesis. Nat Rev Genet, *3:* 199-209, 2002.
14. Rheinwald, J. G. Serial cultivation of normal human epidermal keratinocytes. Methods Cell Biol, *21A:* 229-254, 1980.
15. Vassar, R. and Fuchs, E. Transgenic mice provide new insights into the role of TGF-alpha during epidermal development and differentiation. Genes Dev, *5:* 714-727, 1991.
16. Finch, P. W., Rubin, J. S., Miki, T., Ron, D., and Aaronson, S. A. Human KGF is FGF-related with properties of a paracrine effector of epithelial cell growth. Science, *245:* 752-755, 1989.

17. Grossman, R. M., Krueger, J., Yourish, D., Granelli-Piperno, A., Murphy, D. P., May, L. T., Kupper, T. S., Sehgal, P. B., and Gottlieb, A. B. Interleukin 6 is expressed in high levels in psoriatic skin and stimulates proliferation of cultured human keratinocytes. Proc Natl Acad Sci U S A, *86:* 6367-6371, 1989.
18. Kupper, T. S., Lee, F., Birchall, N., Clark, S., and Dower, S. Interleukin 1 binds to specific receptors on human keratinocytes and induces granulocyte macrophage colony-stimulating factor mRNA and protein. A potential autocrine role for interleukin 1 in epidermis. J Clin Invest, *82:* 1787-1792, 1988.
19. Ansel, J. C., Luger, T. A., Lowry, D., Perry, P., Roop, D. R., and Mountz, J. D. The expression and modulation of IL-1 alpha in murine keratinocytes. J Immunol, *140:* 2274-2278, 1988.
20. Green, M. R., Basketter, D. A., Couchman, J. R., and Rees, D. A. Distribution and number of epidermal growth factor receptors in skin is related to epithelial cell growth. Dev Biol, *100:* 506-512, 1983.
21. Coffey, R. J., Jr., Derynck, R., Wilcox, J. N., Bringman, T. S., Goustin, A. S., Moses, H. L., and Pittelkow, M. R. Production and auto-induction of transforming growth factor-alpha in human keratinocytes. Nature, *328:* 817-820, 1987.
22. Shipley, G. D., Pittelkow, M. R., Wille, J. J., Jr., Scott, R. E., and Moses, H. L. Reversible inhibition of normal human prokeratinocyte proliferation by type beta transforming growth factor-growth inhibitor in serum-free medium. Cancer Res, *46:* 2068-2071, 1986.
23. Bascom, C. C., Wolfshohl, J. R., Coffey, R. J., Jr., Madisen, L., Webb, N. R., Purchio, A. R., Derynck, R., and Moses, H. L. Complex regulation of transforming growth factor beta 1, beta 2, and beta 3 mRNA expression in mouse fibroblasts and keratinocytes by transforming growth factors beta 1 and beta 2. Mol Cell Biol, *9:* 5508-5515, 1989.
24. Menon, G. K., Grayson, S., and Elias, P. M. Ionic calcium reservoirs in mammalian epidermis: ultrastructural localization by ion-capture cytochemistry. J Invest Dermatol, *84:* 508-512, 1985.
25. Hennings, H., Michael, D., Cheng, C., Steinert, P., Holbrook, K., and Yuspa, S. H. Calcium regulation of growth and differentiation of mouse epidermal cells in culture. Cell, *19:* 245-254, 1980.
26. Khoury, G. and Gruss, P. Enhancer elements. Cell, *33:* 313-314, 1983.
27. Hurst, H. C. Transcription factors 1: bZIP proteins. Protein Profile, *2:* 101-168, 1995.
28. Landschulz, W. H., Johnson, P. F., and McKnight, S. L. The leucine zipper: A hypothetical structure common to a new class of DNA binding proteins. Science, *240:* 1759-1764, 1988.
29. Landschulz, W. H., Johnson, P. F., Adashi, E. Y., Graves, B. J., and McKnight, S. L. Isolation of a recombinant copy of the gene encoding C/EBP. Genes Dev, *2:* 786-800, 1988.
30. Cao, Z., Umek, R. M., and McKnight, S. L. Regulated expression of three C/EBP isoforms during adipose conversion of 3T3-L1 cells. Genes Dev., *5:* 1538-1552, 1991.

31. Williams, S. C., Cantwell, C. A., and Johnson., P. F. A family of C/EBP-related proteins capable of forming covalently linked leucine zipper dimers in vitro. Genes Dev, *5:* 1553-1567, 1991.
32. Antonson, P. and Xanthopoulos, K. G. Molecular cloning, sequence, and expression patterns of the human gene encoding CCAAT/enhancer binding protein alpha (C/EBP alpha). Biochem Biophys Res Commun, *215:* 106-113, 1995.
33. Akira, S., Issiki, H., Sugita, T., Tanabe, O., Kinoshita, S., Nishio, Y., Nakajima, T., Hirano, T., and Kishimoto, T. A nuclear factor for IL-6 expression (NF-IL6) is a member of a C/EBP family. EMBO. J., *9:* 1897-1906, 1990.
34. Descombes, P., Chojkier, M., Lichisteiner, S., Falvey, E., and Schibler, U. LAP, a novel member of the C/EBP gene family, encodes a liver-enriched transcriptional activator protein. Genes & Dev, *4:* 1541-1551, 1990.
35. Roman, C., Platero, J. S., Shuman, J., and Calame, K. Ig/EBP-1: a ubiquitously expressed immunoglobulin enhancer binding protein that is similar to C/EBP and heterodimerizes with C/EBP. Genes Dev, *4:* 1404-1415, 1990.
36. Luethy, J. D., Fargnoli, J., Park, J. S., Fornace, A. J., Jr., and Holbrook, N. J. Isolation and characterization of the hamster gadd153 gene. Activation of promoter activity by agents that damage DNA. J Biol Chem, *265:* 16521-16526, 1990.
37. Ron, D. and Habener, J. F. CHOP, a novel developmentally regulated nuclear protein that dimerizes with transcription factors C/EBP and LAP and functions as a dominant-negative inhibitor of gene transcription. Genes Dev, *6:* 439-453, 1992.
38. Chumakov, A. M., Grillier, I., Chumakova, E., Chih, D., Slater, J., and Koeffler, H. P. Cloning of the novel human myeloid-cell-specific C/EBP-epsilon transcription factor. Mol Cell Biol, *17:* 1375-1386, 1997.
39. Park, J. S., Luethy, J. D., Wang, M. G., Fargnoli, J., Fornace, A. J., Jr., McBride, O. W., and Holbrook, N. J. Isolation, characterization and chromosomal localization of the human GADD153 gene. Gene, *116:* 259-267, 1992.
40. Welm, A. L., Timchenko, N. A., and Darlington, G. J. C/EBPalpha regulates generation of C/EBPbeta isoforms through activation of specific proteolytic cleavage. Mol Cell Biol, *19:* 1695-1704, 1999.
41. Lin, F. T., MacDougald, O. A., Diehl, A. M., and Lane, M. D. A 30-kDa alternative translation product of the CCAAT/enhancer binding protein alpha message: transcriptional activator lacking antimitotic activity. Proc Natl Acad Sci U S A, *90:* 9606-9610, 1993.
42. Descombes, P. and Schibler, U. A liver-enriched transcriptional activator, LAP, and a transcriptional inhibitor protein, LIP, are translated from the same mRNA. Cell, *67:* 569-579, 1991.
43. Ramji, D. P. and Foka, P. CCAAT/enhancer-binding proteins: structure, function and regulation. Biochem J, *365:* 561-575, 2002.
44. Vinson, C. R., Sigler, P. B., and McKnight, S. L. Scissors-Grip model for DNA recognition by a family of leucie zipper proteins. Science, *246:* 911-916, 1989.
45. Osada, S., Yamamoto, H., Nishihara, T., and Imagawa, M. DNA binding specificity of the CCAAT/enhancer-binding protein transcription factor family. J Biol Chem, *271:* 3891-3896, 1996.

46. Ubeda, M., Wang, X. Z., Zinszner, H., Wu, I., Habener, J. F., and Ron, D. Stress-induced binding of the transcriptional factor CHOP to a novel DNA control element. Mol Cell Biol, *16:* 1479-1489, 1996.
47. Hsu, W., Kerppola, T. K., Chen, P. L., Curran, T., and Chen-Kiang, S. Fos and Jun repress transcription activation by NF-IL6 through association at the basic zipper region. Mol. Cell. Biol., *14:* 268-276, 1994.
48. Vallejo, M., Ron, D., Miller, C. P., and Habener, J. F. C/ATF, a member of the activating transcription factor family of DNA-binding proteins, dimerizes with CAAT/enhancer-binding proteins and directs their binding to cAMP response elements. Proc Natl Acad Sci U S A, *90:* 4679-4683, 1993.
49. Stein, B., Cogsell, P. C., and A.S. Baldwin, J. Functional and physical association between NF-kB and C/EBP family members; a Rel domain-b-ZIP interaction. Molecular and Cellular Biology, *13:* 3964-3974, 1993.
50. Stein, B. and Baldwin, A. S., Jr. Distinct mechanisms for regulation of the interleukin-8 gene involve synergism and cooperativity between C/EBP and NF-kappa B. Mol Cell Biol, *13:* 7191-7198, 1993.
51. Nishio, Y., Isshiki, H., Kishimoto, T., and Akira, S. A nuclear factor for interleukin-6 expression (NF-IL6) and the glucocorticoid receptor synergistically activate transcription of the rat alpha 1-acid glycoprotein gene via direct protein-protein interaction. Mol Cell Biol, *13:* 1854-1862, 1993.
52. Williamson, E. A., Xu, H. N., Gombart, A. F., Verbeek, W., Chumakov, A. M., Friedman, A. D., and Koeffler, H. P. Identification of transcriptional activation and repression domains in human CCAAT/enhancer-binding protein epsilon. J Biol Chem, *273:* 14796-14804, 1998.
53. Williams, S. C., Baer, M., Dillner, A. J., and Johnson, P. F. CRP2 (C/EBP beta) contains a bipartite regulatory domain that controls transcriptional activation, DNA binding and cell specificity. Embo J, *14:* 3170-3183, 1995.
54. Cooper, C., Henderson, A., Artandi, S., Avitahl, N., and Calame, K. Ig/EBP (C/EBP gamma) is a transdominant negative inhibitor of C/EBP family transcriptional activators. Nucleic Acids Res, *23:* 4371-4377, 1995.
55. Pei, D. Q. and Shih, C. H. An "attenuator domain" is sandwiched by two distinct transactivation domains in the transcription factor C/EBP. Mol Cell Biol, *11:* 1480-1487, 1991.
56. Kowenz-Leutz, E., Twamley, G., Ansieau, S., and Leutz, A. Novel mechanism of C/EBPb(NF-M) transcriptional control: activation through derepression. Genes and Development, *8:* 2781-2791, 1994.
57. Lekstrom-Himes, J. and Xanthopoulos, K. G. Biological role of the CCAAT/enhancer-binding protein family of transcription factors. J Biol Chem, *273:* 28545-28548, 1998.
58. Oh, H.-S. and Smart, R. C. Expression of CCAAT/enhancer binding protein (C/EBP) is associated with squamous differentiation in epidermis and isolated primary keratinocytes and is altered in skin neoplasms. J. Invest. Dermatol., *110:* 939-945, 1998.
59. Chandrasekaran, C. and Gordon, J. I. Cell lineage-specific and differentiation-dependent patterns of CCAAT/enhancer binding protein alpha expression in the

gut epithelium of normal and transgenic mice. Proc Natl Acad Sci U S A, *90:* 8871-8875, 1993.
60. Cortes-Canteli, M., Pignatelli, M., Santos, A., and Perez-Castillo, A. CCAAT/enhancer-binding protein beta plays a regulatory role in differentiation and apoptosis of neuroblastoma cells. J Biol Chem, *277:* 5460-5467, 2002.
61. Zhu, S., Oh, H. S., Shim, M., Sterneck, E., Johnson, P. F., and Smart, R. C. C/EBPβ modulates the early events of keratinocyte differentiation involving growth arrest and keratin 1 and keratin 10 expression. Mol Cell Biol, *19:* 7181-7190, 1999.
62. Wedel, A. and Ziegler-Heitbrock., H. W. L. The C/EBP family of transcription factors. Immunobiol, *193:* 171-185, 1995.
63. Ray, A. and Ray, B. K. Serum amyloid A gene expression under acute-phase conditions involves participation of inducible C/EBP-beta and C/EBP-delta and their activation by phosphorylation. Mol Cell Biol, *14:* 4324-4332, 1994.
64. Nerlov, C. and Ziff, E. B. Three levels of functional interaction determine the activity of CCAAT/enhancer binding protein-alpha on the serum albumin promoter. Genes Dev, *8:* 350-362, 1994.
65. Darlington, G. J., Ross, S. E., and MacDougald, O. A. The role of C/EBP genes in adipocyte differentiation. Journal of Biological Chemistry, *273:* 30057-30060, 1998.
66. Tanaka, T., Yoshida, N., Kishimoto, T., and Akira, S. Defective adipocyte differentiation in mice lacking the C/EBPbeta and/or C/EBPdelta gene. Embo J, *16:* 7432-7443, 1997.
67. Wang, N. D., Finegold, M. J., Bradley, A., Ou, C. N., Abdelsayed, S. V., Wilde, M. D., Taylor, L. R., Wilson, D. R., and Darlington, G. J. Impaired energy homeostasis in C/EBP alpha knockout mice. Science, *269:* 1108-1112, 1995.
68. Scott, L. M., Civin, C. I., Rorth, P., and Friedman, A. D. A novel temporal expression pattern of three C/EBP family members in differentiating myelomonocytic cells. Blood, *80:* 1725-1735, 1992.
69. Natsuka, S., Akira, S., Nishio, Y., Hashimoto, S., Sugita, T., Isshiki, H., and Kishimoto, T. Macrophage differentiation specific expression of NF-IL6, a transcription factor for IL-6. Blood, *79:* 460-466, 1992.
70. Zhang, D. E., Zhang, P., Wang, N. D., Hetherington, C. J., Darlington, G. J., and Tenen, D. G. Absence of granulocyte colony-stimulating factor signaling and neutrophil development in CCAAT enhancer binding protein alpha-deficient mice. Proc Natl Acad Sci U S A, *94:* 569-574, 1997.
71. Zhang, P., Iwama, A., Datta, M. W., Darlington, G. J., Link, D. C., and Tenen, D. G. Upregulation of interleukin 6 and granulocyte colony-stimulating factor receptors by transcription factor CCAAT enhancer binding protein alpha (C/EBP alpha) is critical for granulopoiesis. J Exp Med, *188:* 1173-1184, 1998.
72. Tanaka, T., Akira, S., Yoshida, K., Umemoto, M., Yoneda, Y., Shirafuji, N., Fujiwara, H., Suematsu, S., Yoshida, N., and Kishimoto, T. Targeted disruption of the NF-IL6 gene discloses its essential role in bacteria killing and tumor cytotoxicity by macrophages. Cell, *80:* 353-361, 1995.
73. Screpanti, I., Romani, L., Musiani, P., Modesti, A., Fattori, E., Lazzaro, D., Sellitto, C., Scarpa, S., Bellavia, D., Lattanzio, G., and et al. Lymphoproliferative

disorder and imbalanced T-helper response in C/EBP beta-deficient mice. Embo J, *14:* 1932-1941, 1995.

74. Maytin, E. V. and Habener, J. F. Transcription factors C/EBPα, C/EBPβ, and CHOP (Gadd153) expressed during the differentiation program of keratinocytes in vivo and in vitro. J. Invest. Dermatol., *110:* 238-246, 1998.
75. Maytin, E. V., Lin, J. C., Krishnamurthy, R., Batchvarova, N., Ron, D., Mitchell, P. J., and Habener, J. F. Keratin 10 gene expression during differentiation of mouse epidermis requires transcription factors C/EBP and AP-2. Dev Biol, *216:* 164-181, 1999.
76. Agarwal, C., Efimova, T., Welter, J. F., Crish, J. F., and Eckert, R. L. CCAAT/enhancer-binding proteins. A role in regulation of human involucrin promoter response to phorbol ester. J Biol Chem, *274:* 6190-6194, 1999.
77. Flodby, P., Barlow, C., Kylefjord, H., Ahrlund-Richter, L., and Xanthopoulos, K. G. Increased hepatic cell proliferation and lung abnormalities in mice deficient in CCAAT/enhancer binding protein alpha. J Biol Chem, *271:* 24753-24760, 1996.
78. Sterneck, E., Tessarollo, L., and Johnson., P. F. An essential role for C/EBPβ in female reproduction. Genes Dev, *11:* 2153-2162, 1997.
79. Seagroves, T. N., Krnacik, S., Raught, B., Gay, J., Burgess-Beusse, B., Darlington, G. J., and Rosen, J. M. C/EBPβ, but not C/EBPα, is essential for ductal morphogenesis, lobuloaveolar proliferation, and functional differentiation in the mouse mammary gland. Genes Dev., *12:* 1917-1928, 1998.
80. Robinson, G. W., Johnson, P. F., Hennighausen, L., and Sterneck, E. The C/EBP-β transcription factor regulates epithelial cell proliferation and differentiation in the mammary gland. Genes Dev., *12:* 1907-1916, 1998.
81. Hendricks-Taylor, L. R. and Darlington, G. J. Inhibition of cell proliferation by C/EBP alpha occurs in many cell types, does not require the presence of p53 or Rb, and is not affected by large T-antigen. Nucleic Acids Res, *23:* 4726-4733, 1995.
82. Harris, T. E., Albrecht, J. H., Nakanishi, M., and Darlington, G. J. CCAAT/enhancer-binding protein-alpha cooperates with p21 to inhibit cyclin-dependent kinase-2 activity and induces growth arrest independent of DNA binding. J Biol Chem, *276:* 29200-29209, 2001.
83. Timchenko, N. A., Wilde, M., Nakanishi, M., Smith, J. R., and Darlington, G. J. CCAAT/enhancer-binding protein alpha (C/EBPalpha) inhibits cell proliferation through the p21 (WAF-1/CIP-1/SDI-1) protein. Genes & Dev, *10:* 804-815, 1996.
84. Timchenko, N. A., Wilde, M., Iakova, P., Albrecht, J. H., and Darlington, G. J. E2F/107 and E2F/p130 complexes are regulated by C/EBPalpha in 3T3-L1 adipocytes. Nucleic Acids Res, *27:* 3621-3630, 1999.
85. Timchenko, N. A., Wilde, M., and Darlington, G. J. C/EBPalpha regulates formation of S-phase-specific E2F-p107 complexes in livers of newborn mice. Mol. Cell. Biol, *19:* 2936-2945, 1999.
86. Slomiany, B. A., D'Arigo, K. L., Kelly, M. M., and Kurtz, D. T. C/EBPalpha inhibits cell growth via direct repression of E2F-DP-mediated transcription. Mol Cell Biol, *20:* 5986-5997, 2000.

87. Porse, B. T., Pedersen, T. A., Xu, X., Lindberg, B., Wewer, U. M., Friis-Hansen, L., and Nerlov, C. E2F repression by C/EBPalpha is required for adipogenesis and granulopoiesis in vivo. Cell, *107:* 247-258, 2001.
88. Wang, H., Iakova, P., Wilde, M., Welm, A., Goode, T., Roesler, W. J., and Timchenko, N. A. C/EBPalpha arrests cell proliferation through direct inhibition of Cdk2 and Cdk4. Mol Cell, *8:* 817-828, 2001.
89. Muller, C., Calkhoven, C. F., Sha, X., and Leutz, A. C/EBPalpha requires a SWI/SNF complex for proliferation arrest. J Biol Chem, 2003.
90. Westendorf, J. J., Yamamoto, C. M., Lenny, N., Downing, J. R., Selsted, M. E., and Hiebert, S. W. The t(8:21) fusion protein, AML-1-ETO, associated with C/EBP-alpha, inhibits C/EBP-alpha-dependent transcription and blocks granulocytic differentiation. Molecular and Cellular Biology, *18:* 322-333, 1998.
91. Pabst, T., Mueller, B. U., Zhang, P., Radomska, H. S., Narravula, S., Schnittger, S., Behre, G., Hiddemann, W., and Tenen, D. G. Dominant-negative mutations of C/EBPA, encoding CCAAT/enhancer binding protein-alpha, in acute myeloid leukemia. Nature Genetics, *27:* 263-270, 2001.
92. Pabst, T., Muller, B. U., Harakawa, N., Schoch, C., Haferlach, T., Behre, G., Hiddemann, W., Zhang, D. E., and Tenen, D. G. AML1-ETO downregulates the granulocytic differentiation factor C/EBPalpha in t(8;21) myeloid leukemia. Nature Medicine, *7:* 444451, 2001.
93. Xu, L., Hui, L., Wang, S., Gong, J., Jin, Y., Wang, Y., Ji, Y., Wu, X., Han, Z., and Hu, G. Expression profiling suggested a regulatory role of liver-enriched transcription factors in human hepatocellular carcinoma. Cancer Res, *61:* 3176-3181, 2001.
94. Tomizawa, M., Wang, Y. Q., Ebara, M., Saisho, H., Watanabe, K., Nakagawara, A., and Tagawa, M. Decreased expression of the CCAAT/enhancer binding protein alpha gene involved in hepatocyte proliferation in human hepatocellular carcinomas. Int J Mol Med, *9:* 597-600, 2002.
95. Halmos, B., Huettner, C. S., Kocher, O., Ferenczi, K., Karp, D. D., and Tenen, D. G. Down-regulation and antiproliferative role of C/EBPalpha in lung cancer. Cancer Res, *62:* 528-534, 2002.
96. Greenbaum, L. E., Li, W., Cressman, D. E., Peng, Y., Ciliberto, G., Poli, V., and Taub, R. CCAAT enhancer- binding protein beta is required for normal hepatocyte proliferation in mice after partial hepatectomy. J Clin Invest, *102:* 996-1007, 1998.
97. Buck, M., Poli, V., van der Geer, P., Chojkier, M., and Hunter, T. Phosphorylation of rat serine 105 or mouse threonine 217 in C/EBP beta is required for hepatocyte proliferation induced by TGF alpha. Mol Cell, *4:* 1087-1092, 1999.
98. Buck, M., Poli, V., Hunter, T., and Chojkier, M. C/EBPbeta phosphorylation by RSK creates a functional XEXD caspase inhibitory box critical for cell survival. Mol Cell, *8:* 807-816, 2001.
99. Zhu, S., Yoon, K., Sterneck, E., Johnson, P. F., and Smart, R. C. CCAAT/enhancer binding protein-beta is a mediator of keratinocyte survival and skin tumorigenesis involving oncogenic Ras signaling. Proc Natl Acad Sci U S A, *99:* 207-212, 2002.

100. O'Rourke, J., Yuan, R., and DeWille, J. CCAAT/enhancer-binding protein-delta (C/EBP-delta) is induced in growth-arrested mouse mammary epithelial cells. J Biol Chem, *272:* 6291-6296, 1997.
101. O'Rourke, J. P., Newbound, G. C., Hutt, J. A., and DeWille, J. CCAAT/enhancer-binding protein delta regulates mammary epithelial cell G0 growth arrest and apoptosis. J Biol Chem, *274:* 16582-16589, 1999.
102. Wang, X. Z., Lawson, B., Brewer, J. W., Zinszner, H., Sanjay, A., Mi, L. J., Boorstein, R., Kreibich, G., Hendershot, L. M., and Ron, D. Signals from the stressed endoplasmic reticulum induce C/EBP-homologous protein (CHOP/GADD153). Mol Cell Biol, *16:* 4273-4280, 1996.
103. Fornace, A. J., Jr., Nebert, D. W., Hollander, M. C., Luethy, J. D., Papathanasiou, M., Fargnoli, J., and Holbrook, N. J. Mammalian genes coordinately regulated by growth arrest signals and DNA-damaging agents. Mol Cell Biol, *9:* 4196-4203, 1989.
104. Oyadomari, S., Koizumi, A., Takeda, K., Gotoh, T., Akira, S., Araki, E., and Mori, M. Targeted disruption of the Chop gene delays endoplasmic reticulum stress-mediated diabetes. J Clin Invest, *109:* 525-532, 2002.
105. Oyadomari, S., Takeda, K., Takiguchi, M., Gotoh, T., Matsumoto, M., Wada, I., Akira, S., Araki, E., and Mori, M. Nitric oxide-induced apoptosis in pancreatic beta cells is mediated by the endoplasmic reticulum stress pathway. Proc Natl Acad Sci U S A, *98:* 10845-10850, 2001.
106. Rabbitts, T. H., Forster, A., Larson, R., and Nathan, P. Fusion of the dominant negative transcription regulator CHOP with a novel gene FUS by translocation t(12;16) in malignant liposarcoma. Nat Genet, *4:* 175-180, 1993.
107. Barone, M. V., Crozat, A., Tabaee, A., Philipson, L., and Ron, D. CHOP (GADD153) and its oncogenic variant, TLS-CHOP, have opposing effects on the induction of G1/S arrest. Genes Dev, *8:* 453-464, 1994.
108. Alam, T., An, M. R., and Papaconstantinou, J. Differential expression of three C/EBP isoforms in multiple tissues during the acute phase response. J Biol Chem, *267:* 5021-5024, 1992.
109. Poli, V. The role of C/EBP isoforms in the control of inflammatory and native immunity functions. J Biol Chem, *273:* 29279-29282, 1998.
110. Poli, V., Mancini, F. P., and Cortese, R. IL-6DBP, a nuclear protein involved in interleukin-6 signal transduction, defines a new family of leucine zipper proteins related to C/EBP. Cell, *63:* 643-653, 1990.
111. Lekstrom-Himes, J. and Xanthopoulos, K. G. CCAAT/enhancer binding protein epsilon is critical for effective neutrophil-mediated response to inflammatory challenge. Blood, *93:* 3096-3105, 1999.
112. Tavor, S., Vuong, P. T., Park, D. J., Gombart, A. F., Cohen, A. H., and Koeffler, H. P. Macrophage functional maturation and cytokine production are impaired in C/EBP epsilon-deficient mice. Blood, *99:* 1794-1801, 2002.
113. Kaisho, T., Tsutsui, H., Tanaka, T., Tsujimura, T., Takeda, K., Kawai, T., Yoshida, N., Nakanishi, K., and Akira, S. Impairment of natural killer cytotoxic activity and interferon gamma production in CCAAT/enhancer binding protein gamma-deficient mice. J Exp Med, *190:* 1573-1582, 1999.

114. Kimura, T., Christoffels, V. M., Chowdhury, S., Iwase, K., Matsuzaki, H., Mori, M., Lamers, W. H., Darlington, G. J., and Takiguchi, M. Hypoglycemia-associated hyperammonemia caused by impaired expression of ornithine cycle enzyme genes in C/EBPalpha knockout mice. J Biol Chem, *273:* 27505-27510, 1998.
115. Liu, S., Croniger, C., Arizmendi, C., Harada-Shiba, M., Ren, J., Poli, V., Hanson, R. W., and Friedman, J. E. Hypoglycemia and impaired hepatic glucose production in mice with a deletion of the C/EBPbeta gene. J Clin Invest, *103:* 207-213, 1999.
116. Yukawa, K., Tanaka, T., Tsuji, S., and Akira, S. Expressions of CCAAT/Enhancer-binding proteins beta and delta and their activities are intensified by cAMP signaling as well as Ca2+/calmodulin kinases activation in hippocampal neurons. J Biol Chem, *273:* 31345-31351, 1998.
117. Taubenfeld, S. M., Wiig, K. A., Monti, B., Dolan, B., Pollonini, G., and Alberini, C. M. Fornix-dependent induction of hippocampal CCAAT enhancer-binding protein [beta] and [delta] Co-localizes with phosphorylated cAMP response element-binding protein and accompanies long-term memory consolidation. J Neurosci, *21:* 84-91, 2001.
118. Sterneck, E., Paylor, R., Jackson-Lewis, V., Libbey, M., Przedborski, S., Tessarollo, L., Crawley, J. N., and Johnson, P. F. Selectively enhanced contextual fear conditioning in mice lacking the transcriptional regulator CCAAT/enhancer binding protein delta. Proc Natl Acad Sci U S A, *95:* 10908-10913, 1998.
119. Christy, R. J., Kaestner, K. H., Geiman, D. E., and Lane, M. D. CCAAT/enhancer binding protein gene promoter: binding of nuclear factors during differentiation of 3T3-L1 preadipocytes. Proc Natl Acad Sci U S A, *88:* 2593-2597, 1991.
120. Legraverend, C., Antonson, P., Flodby, P., and Xanthopoulos, K. G. High level activity of the mouse CCAAT/enhancer binding protein (C/EBP alpha) gene promoter involves autoregulation and several ubiquitous transcription factors. Nucleic Acids Res, *21:* 1735-1742, 1993.
121. Timchenko, N., Wilson, D. R., Taylor, L. R., Abdelsayed, S., Wilde, M., Sawadogo, M., and Darlington, G. J. Autoregulation of the human C/EBP alpha gene by stimulation of upstream stimulatory factor binding. Mol Cell Biol, *15:* 1192-1202, 1995.
122. Antonson, P., Pray, M. G., Jacobsson, A., and Xanthopoulos, K. G. Myc inhibits CCAAT/enhancer-binding protein alpha-gene expression in HIB-1B hibernoma cells through interactions with the core promoter region. Eur J Biochem, *232:* 397-403, 1995.
123. Chang, C. J., Shen, B. J., and Lee, S. C. Autoregulated induction of the acute-phase response transcription factor gene, agp/ebp. DNA Cell Biol, *14:* 529-537, 1995.
124. Niehof, M., Manns, M. P., and Trautwein, C. CREB controls LAP/C/EBP beta transcription. Mol Cell Biol, *17:* 3600-3613, 1997.
125. Niehof, M., Streetz, K., Rakemann, T., Bischoff, S. C., Manns, M. P., Horn, F., and Trautwein, C. Interleukin-6-induced tethering of STAT3 to the LAP/C/EBPbeta promoter suggests a new mechanism of transcriptional regulation by STAT3. J Biol Chem, *276:* 9016-9027, 2001.

126. Hutt, J. A., O'Rourke, J. P., and DeWille, J. Signal transducer and activator of transcription 3 activates CCAAT enhancer-binding protein delta gene transcription in G0 growth-arrested mouse mammary epithelial cells and in involuting mouse mammary gland. J Biol Chem, *275:* 29123-29131, 2000.
127. Sabatakos, G., Davies, G. E., Grosse, M., Cryer, A., and Ramji, D. P. Expression of the genes encoding CCAAT-enhancer binding protein isoforms in the mouse mammary gland during lactation and involution. Biochem J, *334 (Pt 1):* 205-210, 1998.
128. Yamada, T., Tobita, K., Osada, S., Nishihara, T., and Imagawa, M. CCAAT/enhancer-binding protein delta gene expression is mediated by APRF/STAT3. J Biochem (Tokyo), *121:* 731-738, 1997.
129. Yamada, T., Tsuchiya, T., Osada, S., Nishihara, T., and Imagawa, M. CCAAT/enhancer-binding protein delta gene expression is mediated by autoregulation through downstream binding sites. Biochem Biophys Res Commun, *242:* 88-92, 1998.
130. O'Rourke, J. P., Hutt, J. A., and DeWille, J. Transcriptional regulation of C/EBPdelta in G(0) growth-arrested mouse mammary epithelial cells. Biochem Biophys Res Commun, *262:* 696-701, 1999.
131. Guyton, K. Z., Xu, Q., and Holbrook, N. J. Induction of the mammalian stress response gene GADD153 by oxidative stress: role of AP-1 element. Biochem J, *314 (Pt 2):* 547-554, 1996.
132. Schmitt-Ney, M. and Habener, J. F. CHOP/GADD153 gene expression response to cellular stresses inhibited by prior exposure to ultraviolet light wavelength band C (UVC). Inhibitory sequence mediating the UVC response localized to exon 1. J Biol Chem, *275:* 40839-40845, 2000.
133. Garmyn, M., Yaar, M., Holbrook, N., and Gilchrest, B. A. Immediate and delayed molecular response of human keratinocytes to solar-simulated irradiation. Lab Invest, *65:* 471-478, 1991.
134. Ubeda, M. and Habener, J. F. CHOP gene expression in response to endoplasmic-reticular stress requires NFY interaction with different domains of a conserved DNA-binding element. Nucleic Acids Res, *28:* 4987-4997, 2000.
135. Fafournoux, P., Bruhat, A., and Jousse, C. Amino acid regulation of gene expression. Biochem J, *351:* 1-12, 2000.
136. Nakajima, T., Kinoshita, S., Sasagawa, T., Sasaki, K., Naruto, M., Kishimoto, T., and Akira, S. Phosphorylation at threonine-235 by a ras-dependent mitogen-activated protein kinase cascade is essential for transcription factor NF-IL6. Proc Natl Acad Sci U S A, *90:* 2207-2211, 1993.
137. Trautwein, C., Caelles, C., van der Geer, P., Hunter, T., Karin, M., and Chojkier, M. Transactivation by NF-IL6/LAP is enhanced by phosphorylation of its activation domain. Nature, *364:* 544-547, 1993.
138. Wegner, M., Cao, Z., and Rosenfeld, M. G. Calcium-regulated phosphorylation within the leucine zipper of C/EBPβ. Science, *236:* 370-373, 1992.
139. Guo, S., Cichy, S. B., He, X., Yang, Q., Ragland, M., Ghosh, A. K., Johnson, P. F., and Unterman, T. G. Insulin suppresses transactivation by CAAT/enhancer-binding proteins beta (C/EBPbeta). Signaling to p300/CREB-binding protein by

protein kinase B disrupts interaction with the major activation domain of C/EBPbeta. J Biol Chem, *276:* 8516-8523, 2001.
140. Trautwein, C., van der Geer, P., Karin, M., Hunter, T., and Chojkier, M. Protein kinase A and C site-specific phosphorylations of LAP (NF-IL6) modulate its binding affinity to DNA recognition elements. J Clin Invest, *93:* 2554-2561, 1994.
141. Metz, R. and Ziff, E. cAMP stimulates the C/EBP-related transcription factor rNFIL-6 to trans-locate to the nucleus and induce c-fos transcription. Genes Dev, *5:* 1754-1766, 1991.
142. Chinery, R., Brockman, J. A., Peeler, M. O., Shyer, S. Y., Beauchamp, R. D., and Coffey., R. J. Antioxidants enhance the cytotoxicity of chemotherapeutic agents in colorectal cancer: a p53-independent induction of p21 WAF1/CIPI via C/EBPb. Nat. Med., *11:* 1233-1241, 1997.
143. Mahoney, C. W., Shuman, J., McKnight, S. L., Chen, H. C., and Huang, K. P. Phosphorylation of CCAAT-enhancer binding protein by protein kinase C attenuates site-selective DNA binding. J Biol Chem, *267:* 19396-19403, 1992.
144. Efimova, T., Deucher, A., Kuroki, T., Ohba, M., and Eckert, R. L. Novel protein kinase C isoforms regulate human keratinocyte differentiation by activating a p38 delta mitogen-activated protein kinase cascade that targets CCAAT/enhancer-binding protein alpha. J Biol Chem, *277:* 31753-31760, 2002.
145. Behre, G., Singh, S. M., Liu, H., Bortolin, L. T., Christopeit, M., Radomska, H. S., Rangatia, J., Hiddemann, W., Friedman, A. D., and Tenen, D. G. Ras signaling enhances the activity of C/EBP alpha to induce granulocytic differentiation by phosphorylation of serine 248. J Biol Chem, *277:* 26293-26299, 2002.
146. Ross, S. E., Erickson, R. L., Hemati, N., and MacDougald, O. A. Glycogen synthase kinase 3 is an insulin-regulated C/EBPalpha kinase. Mol Cell Biol, *19:* 8433-8441, 1999.
147. Kinzler, K. W. and Vogelstein, B. Cancer-susceptibility genes. Gatekeepers and caretakers. Nature, *386:* 761, 763, 1997.
148. Hanahan, D. and Weinberg, R. A. The hallmarks of cancer. Cell, *100:* 57-70, 2000.
149. Cline, S. D. and Hanawalt, P. C. Who's on first in the cellular response to DNA damage? Nat Rev Mol Cell Biol, *4:* 361-372, 2003.
150. Lindahl, T. Instability and decay of the primary structure of DNA. Nature, *362:* 709-715, 1993.
151. Tano, K., Shiota, S., Collier, J., Foote, R. S., and Mitra, S. Isolation and structural characterization of a cDNA clone encoding the human DNA repair protein for O6-alkylguanine. Proc Natl Acad Sci U S A, *87:* 686-690, 1990.
152. Duncan, T., Trewick, S. C., Koivisto, P., Bates, P. A., Lindahl, T., and Sedgwick, B. Reversal of DNA alkylation damage by two human dioxygenases. Proc Natl Acad Sci U S A, *99:* 16660-16665, 2002.
153. Christmann, M., Tomicic, M. T., Roos, W. P., and Kaina, B. Mechanisms of human DNA repair: an update. Toxicology, *193:* 3-34, 2003.
154. Friedberg, E. C. How nucleotide excision repair protects against cancer. Nat Rev Cancer, *1:* 22-33, 2001.

155. Vermeulen, W., de Boer, J., Citterio, E., van Gool, A. J., van der Horst, G. T., Jaspers, N. G., de Laat, W. L., Sijbers, A. M., van der Spek, P. J., Sugasawa, K., Weeda, G., Winkler, G. S., Bootsma, D., Egly, J. M., and Hoeijmakers, J. H. Mammalian nucleotide excision repair and syndromes. Biochem Soc Trans, *25:* 309-315, 1997.
156. Mullenders, L. H. and Berneburg, M. Photoimmunology and nucleotide excision repair: impact of transcription coupled and global genome excision repair. J Photochem Photobiol B, *65:* 97-100, 2001.
157. Umar, A. and Kunkel, T. A. DNA-replication fidelity, mismatch repair and genome instability in cancer cells. Eur J Biochem, *238:* 297-307, 1996.
158. Duckett, D. R., Drummond, J. T., Murchie, A. I., Reardon, J. T., Sancar, A., Lilley, D. M., and Modrich, P. Human MutSalpha recognizes damaged DNA base pairs containing O6-methylguanine, O4-methylthymine, or the cisplatin-d(GpG) adduct. Proc Natl Acad Sci U S A, *93:* 6443-6447, 1996.
159. Mello, J. A., Acharya, S., Fishel, R., and Essigmann, J. M. The mismatch-repair protein hMSH2 binds selectively to DNA adducts of the anticancer drug cisplatin. Chem Biol, *3:* 579-589, 1996.
160. Wang, H., Lawrence, C. W., Li, G. M., and Hays, J. B. Specific binding of human MSH2.MSH6 mismatch-repair protein heterodimers to DNA incorporating thymine- or uracil-containing UV light photoproducts opposite mismatched bases. J Biol Chem, *274:* 16894-16900, 1999.
161. Takata, M., Sasaki, M. S., Sonoda, E., Morrison, C., Hashimoto, M., Utsumi, H., Yamaguchi-Iwai, Y., Shinohara, A., and Takeda, S. Homologous recombination and non-homologous end-joining pathways of DNA double-strand break repair have overlapping roles in the maintenance of chromosomal integrity in vertebrate cells. Embo J, *17:* 5497-5508, 1998.
162. Smith, G. C. and Jackson, S. P. The DNA-dependent protein kinase. Genes Dev, *13:* 916-934, 1999.
163. Leber, R., Wise, T. W., Mizuta, R., and Meek, K. The XRCC4 gene product is a target for and interacts with the DNA-dependent protein kinase. J Biol Chem, *273:* 1794-1801, 1998.
164. Pellegrini, L., Yu, D. S., Lo, T., Anand, S., Lee, M., Blundell, T. L., and Venkitaraman, A. R. Insights into DNA recombination from the structure of a RAD51-BRCA2 complex. Nature, *420:* 287-293, 2002.
165. Tauchi, H., Kobayashi, J., Morishima, K., van Gent, D. C., Shiraishi, T., Verkaik, N. S., vanHeems, D., Ito, E., Nakamura, A., Sonoda, E., Takata, M., Takeda, S., Matsuura, S., and Komatsu, K. Nbs1 is essential for DNA repair by homologous recombination in higher vertebrate cells. Nature, *420:* 93-98, 2002.
166. Abraham, R. T. Cell cycle checkpoint signaling through the ATM and ATR kinases. Genes Dev, *15:* 2177-2196, 2001.
167. Rouse, J. and Jackson, S. P. Interfaces between the detection, signaling, and repair of DNA damage. Science, *297:* 547-551, 2002.
168. Cortez, D., Guntuku, S., Qin, J., and Elledge, S. J. ATR and ATRIP: partners in checkpoint signaling. Science, *294:* 1713-1716, 2001.
169. Downs, J. A., Lowndes, N. F., and Jackson, S. P. A role for Saccharomyces cerevisiae histone H2A in DNA repair. Nature, *408:* 1001-1004, 2000.

170. Weiss, R. S., Matsuoka, S., Elledge, S. J., and Leder, P. Hus1 acts upstream of chk1 in a mammalian DNA damage response pathway. Curr Biol, *12:* 73-77, 2002.
171. Smith, G. C., Cary, R. B., Lakin, N. D., Hann, B. C., Teo, S. H., Chen, D. J., and Jackson, S. P. Purification and DNA binding properties of the ataxia-telangiectasia gene product ATM. Proc Natl Acad Sci U S A, *96:* 11134-11139, 1999.
172. Bakkenist, C. J. and Kastan, M. B. DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. Nature, *421:* 499-506, 2003.
173. Lavin, M. F. and Shiloh, Y. The genetic defect in ataxia-telangiectasia. Annu Rev Immunol, *15:* 177-202, 1997.
174. Brown, E. J. and Baltimore, D. ATR disruption leads to chromosomal fragmentation and early embryonic lethality. Genes Dev, *14:* 397-402, 2000.
175. Cliby, W. A., Roberts, C. J., Cimprich, K. A., Stringer, C. M., Lamb, J. R., Schreiber, S. L., and Friend, S. H. Overexpression of a kinase-inactive ATR protein causes sensitivity to DNA-damaging agents and defects in cell cycle checkpoints. Embo J, *17:* 159-169, 1998.
176. Wright, J. A., Keegan, K. S., Herendeen, D. R., Bentley, N. J., Carr, A. M., Hoekstra, M. F., and Concannon, P. Protein kinase mutants of human ATR increase sensitivity to UV and ionizing radiation and abrogate cell cycle checkpoint control. Proc Natl Acad Sci U S A, *95:* 7445-7450, 1998.
177. Zou, L., Cortez, D., and Elledge, S. J. Regulation of ATR substrate selection by Rad17-dependent loading of Rad9 complexes onto chromatin. Genes Dev, *16:* 198-208, 2002.
178. Unsal-Kacmaz, K., Makhov, A. M., Griffith, J. D., and Sancar, A. Preferential binding of ATR protein to UV-damaged DNA. Proc Natl Acad Sci U S A, *99:* 6673-6678, 2002.
179. Lindsey-Boltz, L. A., Bermudez, V. P., Hurwitz, J., and Sancar, A. Purification and characterization of human DNA damage checkpoint Rad complexes. Proc Natl Acad Sci U S A, *98:* 11236-11241, 2001.
180. Thelen, M. P., Venclovas, C., and Fidelis, K. A sliding clamp model for the Rad1 family of cell cycle checkpoint proteins. Cell, *96:* 769-770, 1999.
181. Cortez, D., Wang, Y., Qin, J., and Elledge, S. J. Requirement of ATM-dependent phosphorylation of brca1 in the DNA damage response to double-strand breaks. Science, *286:* 1162-1166, 1999.
182. Tibbetts, R. S., Cortez, D., Brumbaugh, K. M., Scully, R., Livingston, D., Elledge, S. J., and Abraham, R. T. Functional interactions between BRCA1 and the checkpoint kinase ATR during genotoxic stress. Genes Dev, *14:* 2989-3002, 2000.
183. Yarden, R. I., Pardo-Reoyo, S., Sgagias, M., Cowan, K. H., and Brody, L. C. BRCA1 regulates the G2/M checkpoint by activating Chk1 kinase upon DNA damage. Nat Genet, *30:* 285-289, 2002.
184. Lee, J. S., Collins, K. M., Brown, A. L., Lee, C. H., and Chung, J. H. hCds1-mediated phosphorylation of BRCA1 regulates the DNA damage response. Nature, *404:* 201-204, 2000.

185. Moynahan, M. E., Chiu, J. W., Koller, B. H., and Jasin, M. Brca1 controls homology-directed DNA repair. Mol Cell, *4:* 511-518, 1999.
186. Wang, Y., Cortez, D., Yazdi, P., Neff, N., Elledge, S. J., and Qin, J. BASC, a super complex of BRCA1-associated proteins involved in the recognition and repair of aberrant DNA structures. Genes Dev, *14:* 927-939, 2000.
187. Gowen, L. C., Avrutskaya, A. V., Latour, A. M., Koller, B. H., and Leadon, S. A. BRCA1 required for transcription-coupled repair of oxidative DNA damage. Science, *281:* 1009-1012, 1998.
188. Rappold, I., Iwabuchi, K., Date, T., and Chen, J. Tumor suppressor p53 binding protein 1 (53BP1) is involved in DNA damage-signaling pathways. J Cell Biol, *153:* 613-620, 2001.
189. Joo, W. S., Jeffrey, P. D., Cantor, S. B., Finnin, M. S., Livingston, D. M., and Pavletich, N. P. Structure of the 53BP1 BRCT region bound to p53 and its comparison to the Brca1 BRCT structure. Genes Dev, *16:* 583-593, 2002.
190. Anderson, L., Henderson, C., and Adachi, Y. Phosphorylation and rapid relocalization of 53BP1 to nuclear foci upon DNA damage. Mol Cell Biol, *21:* 1719-1729, 2001.
191. Kim, S. T., Lim, D. S., Canman, C. E., and Kastan, M. B. Substrate specificities and identification of putative substrates of ATM kinase family members. J Biol Chem, *274:* 37538-37543, 1999.
192. Sanchez, Y., Wong, C., Thoma, R. S., Richman, R., Wu, Z., Piwnica-Worms, H., and Elledge, S. J. Conservation of the Chk1 checkpoint pathway in mammals: linkage of DNA damage to Cdk regulation through Cdc25. Science, *277:* 1497-1501, 1997.
193. Liu, Q., Guntuku, S., Cui, X. S., Matsuoka, S., Cortez, D., Tamai, K., Luo, G., Carattini-Rivera, S., DeMayo, F., Bradley, A., Donehower, L. A., and Elledge, S. J. Chk1 is an essential kinase that is regulated by Atr and required for the G(2)/M DNA damage checkpoint. Genes Dev, *14:* 1448-1459, 2000.
194. Zhao, H., Watkins, J. L., and Piwnica-Worms, H. Disruption of the checkpoint kinase 1/cell division cycle 25A pathway abrogates ionizing radiation-induced S and G2 checkpoints. Proc Natl Acad Sci U S A, *99:* 14795-14800, 2002.
195. Gatei, M., Sloper, K., Sorensen, C., Syljuasen, R., Falck, J., Hobson, K., Savage, K., Lukas, J., Zhou, B. B., Bartek, J., and Khanna, K. K. Ataxia-telangiectasia-mutated (ATM) and NBS1-dependent phosphorylation of Chk1 on Ser-317 in response to ionizing radiation. J Biol Chem, *278:* 14806-14811, 2003.
196. Xiao, Z., Chen, Z., Gunasekera, A. H., Sowin, T. J., Rosenberg, S. H., Fesik, S., and Zhang, H. Chk1 mediates S and G2 arrests through Cdc25A degradation in response to DNA-damaging agents. J Biol Chem, *278:* 21767-21773, 2003.
197. Zhao, H. and Piwnica-Worms, H. ATR-mediated checkpoint pathways regulate phosphorylation and activation of human Chk1. Mol Cell Biol, *21:* 4129-4139, 2001.
198. Ahn, J. Y., Li, X., Davis, H. L., and Canman, C. E. Phosphorylation of threonine 68 promotes oligomerization and autophosphorylation of the Chk2 protein kinase via the forkhead-associated domain. J Biol Chem, *277:* 19389-19395, 2002.

199. Matsuoka, S., Rotman, G., Ogawa, A., Shiloh, Y., Tamai, K., and Elledge, S. J. Ataxia telangiectasia-mutated phosphorylates Chk2 in vivo and in vitro. Proc Natl Acad Sci U S A, *97:* 10389-10394, 2000.
200. Lu, X. and Lane, D. P. Differential induction of transcriptionally active p53 following UV or ionizing radiation: defects in chromosome instability syndromes? Cell, *75:* 765-778, 1993.
201. Stewart, Z. A. and Pietenpol, J. A. p53 Signaling and cell cycle checkpoints. Chem Res Toxicol, *14:* 243-263, 2001.
202. Banin, S., Moyal, L., Shieh, S., Taya, Y., Anderson, C. W., Chessa, L., Smorodinsky, N. I., Prives, C., Reiss, Y., Shiloh, Y., and Ziv, Y. Enhanced phosphorylation of p53 by ATM in response to DNA damage. Science, *281:* 1674-1677, 1998.
203. Canman, C. E., Lim, D. S., Cimprich, K. A., Taya, Y., Tamai, K., Sakaguchi, K., Appella, E., Kastan, M. B., and Siliciano, J. D. Activation of the ATM kinase by ionizing radiation and phosphorylation of p53. Science, *281:* 1677-1679, 1998.
204. Chao, C., Hergenhahn, M., Kaeser, M. D., Wu, Z., Saito, S., Iggo, R., Hollstein, M., Appella, E., and Xu, Y. Cell type- and promoter-specific roles of Ser18 phosphorylation in regulating p53 responses. J Biol Chem, *278:* 41028-41033, 2003.
205. Dumaz, N. and Meek, D. W. Serine15 phosphorylation stimulates p53 transactivation but does not directly influence interaction with HDM2. Embo J, *18:* 7002-7010, 1999.
206. Maya, R., Balass, M., Kim, S. T., Shkedy, D., Leal, J. F., Shifman, O., Moas, M., Buschmann, T., Ronai, Z., Shiloh, Y., Kastan, M. B., Katzir, E., and Oren, M. ATM-dependent phosphorylation of Mdm2 on serine 395: role in p53 activation by DNA damage. Genes Dev, *15:* 1067-1077, 2001.
207. Ryan, K. M., Phillips, A. C., and Vousden, K. H. Regulation and function of the p53 tumor suppressor protein. Curr Opin Cell Biol, *13:* 332-337, 2001.
208. Tibbetts, R. S., Brumbaugh, K. M., Williams, J. M., Sarkaria, J. N., Cliby, W. A., Shieh, S. Y., Taya, Y., Prives, C., and Abraham, R. T. A role for ATR in the DNA damage-induced phosphorylation of p53. Genes Dev, *13:* 152-157, 1999.
209. Siliciano, J. D., Canman, C. E., Taya, Y., Sakaguchi, K., Appella, E., and Kastan, M. B. DNA damage induces phosphorylation of the amino terminus of p53. Genes Dev, *11:* 3471-3481, 1997.
210. Painter, R. B. and Young, B. R. Radiosensitivity in ataxia-telangiectasia: a new explanation. Proc Natl Acad Sci U S A, *77:* 7315-7317, 1980.
211. Falck, J., Mailand, N., Syljuasen, R. G., Bartek, J., and Lukas, J. The ATM-Chk2-Cdc25A checkpoint pathway guards against radioresistant DNA synthesis. Nature, *410:* 842-847, 2001.
212. Takisawa, H., Mimura, S., and Kubota, Y. Eukaryotic DNA replication: from pre-replication complex to initiation complex. Curr Opin Cell Biol, *12:* 690-696, 2000.
213. Bell, D. W., Varley, J. M., Szydlo, T. E., Kang, D. H., Wahrer, D. C., Shannon, K. E., Lubratovich, M., Verselis, S. J., Isselbacher, K. J., Fraumeni, J. F., Birch, J. M., Li, F. P., Garber, J. E., and Haber, D. A. Heterozygous germ line hCHK2 mutations in Li-Fraumeni syndrome. Science, *286:* 2528-2531, 1999.

214. Carney, J. P., Maser, R. S., Olivares, H., Davis, E. M., Le Beau, M., Yates, J. R., 3rd, Hays, L., Morgan, W. F., and Petrini, J. H. The hMre11/hRad50 protein complex and Nijmegen breakage syndrome: linkage of double-strand break repair to the cellular DNA damage response. Cell, *93:* 477-486, 1998.
215. Nelms, B. E., Maser, R. S., MacKay, J. F., Lagally, M. G., and Petrini, J. H. In situ visualization of DNA double-strand break repair in human fibroblasts. Science, *280:* 590-592, 1998.
216. Zhao, S., Weng, Y. C., Yuan, S. S., Lin, Y. T., Hsu, H. C., Lin, S. C., Gerbino, E., Song, M. H., Zdzienicka, M. Z., Gatti, R. A., Shay, J. W., Ziv, Y., Shiloh, Y., and Lee, E. Y. Functional link between ataxia-telangiectasia and Nijmegen breakage syndrome gene products. Nature, *405:* 473-477, 2000.
217. Gatei, M., Young, D., Cerosaletti, K. M., Desai-Mehta, A., Spring, K., Kozlov, S., Lavin, M. F., Gatti, R. A., Concannon, P., and Khanna, K. ATM-dependent phosphorylation of nibrin in response to radiation exposure. Nat Genet, *25:* 115-119, 2000.
218. Yazdi, P. T., Wang, Y., Zhao, S., Patel, N., Lee, E. Y., and Qin, J. SMC1 is a downstream effector in the ATM/NBS1 branch of the human S-phase checkpoint. Genes Dev, *16:* 571-582, 2002.
219. Kim, S. T., Xu, B., and Kastan, M. B. Involvement of the cohesin protein, Smc1, in Atm-dependent and independent responses to DNA damage. Genes Dev, *16:* 560-570, 2002.
220. Pines, J. Cyclins and cyclin-dependent kinases: a biochemical view. Biochem J, *308 (Pt 3):* 697-711, 1995.
221. Parker, L. L., Atherton-Fessler, S., and Piwnica-Worms, H. p107wee1 is a dual-specificity kinase that phosphorylates p34cdc2 on tyrosine 15. Proc Natl Acad Sci U S A, *89:* 2917-2921, 1992.
222. Booher, R. N., Holman, P. S., and Fattaey, A. Human Myt1 is a cell cycle-regulated kinase that inhibits Cdc2 but not Cdk2 activity. J Biol Chem, *272:* 22300-22306, 1997.
223. Draetta, G. and Eckstein, J. Cdc25 protein phosphatases in cell proliferation. Biochim Biophys Acta, *1332:* M53-63, 1997.
224. Peng, C. Y., Graves, P. R., Thoma, R. S., Wu, Z., Shaw, A. S., and Piwnica-Worms, H. Mitotic and G2 checkpoint control: regulation of 14-3-3 protein binding by phosphorylation of Cdc25C on serine-216. Science, *277:* 1501-1505, 1997.
225. Jin, P., Hardy, S., and Morgan, D. O. Nuclear localization of cyclin B1 controls mitotic entry after DNA damage. J Cell Biol, *141:* 875-885, 1998.
226. Matsuoka, S., Huang, M., and Elledge, S. J. Linkage of ATM to cell cycle regulation by the Chk2 protein kinase. Science, *282:* 1893-1897, 1998.
227. Scott, D., Spreadborough, A. R., and Roberts, S. A. Radiation-induced G2 delay and spontaneous chromosome aberrations in ataxia-telangiectasia homozygotes and heterozygotes. Int J Radiat Biol, *66:* S157-163, 1994.
228. Hirao, A., Kong, Y. Y., Matsuoka, S., Wakeham, A., Ruland, J., Yoshida, H., Liu, D., Elledge, S. J., and Mak, T. W. DNA damage-induced activation of p53 by the checkpoint kinase Chk2. Science, *287:* 1824-1827, 2000.

229. Bunz, F., Dutriaux, A., Lengauer, C., Waldman, T., Zhou, S., Brown, J. P., Sedivy, J. M., Kinzler, K. W., and Vogelstein, B. Requirement for p53 and p21 to sustain G2 arrest after DNA damage. Science, *282:* 1497-1501, 1998.
230. Hermeking, H., Lengauer, C., Polyak, K., He, T. C., Zhang, L., Thiagalingam, S., Kinzler, K. W., and Vogelstein, B. 14-3-3 sigma is a p53-regulated inhibitor of G2/M progression. Mol Cell, *1:* 3-11, 1997.
231. Chan, T. A., Hermeking, H., Lengauer, C., Kinzler, K. W., and Vogelstein, B. 14-3-3Sigma is required to prevent mitotic catastrophe after DNA damage. Nature, *401:* 616-620, 1999.
232. Wang, X. W., Zhan, Q., Coursen, J. D., Khan, M. A., Kontny, H. U., Yu, L., Hollander, M. C., O'Connor, P. M., Fornace, A. J., Jr., and Harris, C. C. GADD45 induction of a G2/M cell cycle checkpoint. Proc Natl Acad Sci U S A, *96:* 3706-3711, 1999.
233. Zhan, Q., Antinore, M. J., Wang, X. W., Carrier, F., Smith, M. L., Harris, C. C., and Fornace, A. J., Jr. Association with Cdc2 and inhibition of Cdc2/Cyclin B1 kinase activity by the p53-regulated protein Gadd45. Oncogene, *18:* 2892-2900, 1999.
234. Ohki, R., Nemoto, J., Murasawa, H., Oda, E., Inazawa, J., Tanaka, N., and Taniguchi, T. Reprimo, a new candidate mediator of the p53-mediated cell cycle arrest at the G2 phase. J Biol Chem, *275:* 22627-22630, 2000.
235. Taylor, W. R., DePrimo, S. E., Agarwal, A., Agarwal, M. L., Schonthal, A. H., Katula, K. S., and Stark, G. R. Mechanisms of G2 arrest in response to overexpression of p53. Mol Biol Cell, *10:* 3607-3622, 1999.
236. de Toledo, S. M., Azzam, E. I., Keng, P., Laffrenier, S., and Little, J. B. Regulation by ionizing radiation of CDC2, cyclin A, cyclin B, thymidine kinase, topoisomerase IIalpha, and RAD51 expression in normal human diploid fibroblasts is dependent on p53/p21Waf1. Cell Growth Differ, *9:* 887-896, 1998.
237. Norbury, C. J. and Hickson, I. D. Cellular responses to DNA damage. Annu Rev Pharmacol Toxicol, *41:* 367-401, 2001.
238. Ziegler, A., Jonason, A. S., Leffell, D. J., Simon, J. A., Sharma, H. W., Kimmelman, J., Remington, L., Jacks, T., and Brash, D. E. Sunburn and p53 in the onset of skin cancer. Nature, *372:* 773-776, 1994.
239. Owen-Schaub, L. B., Zhang, W., Cusack, J. C., Angelo, L. S., Santee, S. M., Fujiwara, T., Roth, J. A., Deisseroth, A. B., Zhang, W. W., Kruzel, E., and et al. Wild-type human p53 and a temperature-sensitive mutant induce Fas/APO-1 expression. Mol Cell Biol, *15:* 3032-3040, 1995.
240. Miyashita, T. and Reed, J. C. Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. Cell, *80:* 293-299, 1995.
241. Nakano, K. and Vousden, K. H. PUMA, a novel proapoptotic gene, is induced by p53. Mol Cell, *7:* 683-694, 2001.
242. Oda, E., Ohki, R., Murasawa, H., Nemoto, J., Shibue, T., Yamashita, T., Tokino, T., Taniguchi, T., and Tanaka, N. Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. Science, *288:* 1053-1058, 2000.
243. Sax, J. K., Fei, P., Murphy, M. E., Bernhard, E., Korsmeyer, S. J., and El-Deiry, W. S. BID regulation by p53 contributes to chemosensitivity. Nat Cell Biol, *4:* 842-849, 2002.

244. Miyashita, T., Krajewski, S., Krajewska, M., Wang, H. G., Lin, H. K., Liebermann, D. A., Hoffman, B., and Reed, J. C. Tumor suppressor p53 is a regulator of bcl-2 and bax gene expression in vitro and in vivo. Oncogene, *9:* 1799-1805, 1994.
245. Hengartner, M. O. The biochemistry of apoptosis. Nature, *407:* 770-776, 2000.
246. Moroni, M. C., Hickman, E. S., Denchi, E. L., Caprara, G., Colli, E., Cecconi, F., Muller, H., and Helin, K. Apaf-1 is a transcriptional target for E2F and p53. Nat Cell Biol, *3:* 552-558, 2001.
247. Wu, G. S., Burns, T. F., McDonald, E. R., 3rd, Jiang, W., Meng, R., Krantz, I. D., Kao, G., Gan, D. D., Zhou, J. Y., Muschel, R., Hamilton, S. R., Spinner, N. B., Markowitz, S., Wu, G., and el-Deiry, W. S. KILLER/DR5 is a DNA damage-inducible p53-regulated death receptor gene. Nat Genet, *17:* 141-143, 1997.
248. Maecker, H. L., Koumenis, C., and Giaccia, A. J. p53 promotes selection for Fas-mediated apoptotic resistance. Cancer Res, *60:* 4638-4644, 2000.
249. Ashkenazi, A. and Dixit, V. M. Apoptosis control by death and decoy receptors. Curr Opin Cell Biol, *11:* 255-260, 1999.
250. Muller, M., Wilder, S., Bannasch, D., Israeli, D., Lehlbach, K., Li-Weber, M., Friedman, S. L., Galle, P. R., Stremmel, W., Oren, M., and Krammer, P. H. p53 activates the CD95 (APO-1/Fas) gene in response to DNA damage by anticancer drugs. J Exp Med, *188:* 2033-2045, 1998.
251. O'Connor, L., Harris, A. W., and Strasser, A. CD95 (Fas/APO-1) and p53 signal apoptosis independently in diverse cell types. Cancer Res, *60:* 1217-1220, 2000.
252. Fuchs, E. J., McKenna, K. A., and Bedi, A. p53-dependent DNA damage-induced apoptosis requires Fas/APO-1-independent activation of CPP32beta. Cancer Res, *57:* 2550-2554, 1997.
253. Stambolic, V., MacPherson, D., Sas, D., Lin, Y., Snow, B., Jang, Y., Benchimol, S., and Mak, T. W. Regulation of PTEN transcription by p53. Mol Cell, *8:* 317-325, 2001.
254. Marchenko, N. D., Zaika, A., and Moll, U. M. Death signal-induced localization of p53 protein to mitochondria. A potential role in apoptotic signaling. J Biol Chem, *275:* 16202-16212, 2000.
255. Brash, D. E. Sunlight and the onset of skin cancer. Trends Genet, *13:* 410-414, 1997.
256. Bowden, G. T. Prevention of non-melanoma skin cancer by targeting ultraviolet-B-light signalling. Nat Rev Cancer, *4:* 23-35, 2004.
257. Kielbassa, C., Roza, L., and Epe, B. Wavelength dependence of oxidative DNA damage induced by UV and visible light. Carcinogenesis, *18:* 811-816, 1997.
258. Miller, D. L. and Weinstock, M. A. Nonmelanoma skin cancer in the United States: incidence. J Am Acad Dermatol, *30:* 774-778, 1994.
259. Marks, R. An overview of skin cancers. Incidence and causation. Cancer, *75:* 607-612, 1995.
260. Moller, R., Reymann, F., and Hou-Jensen, K. Metastases in dermatological patients with squamous cell carcinoma. Arch Dermatol, *115:* 703-705, 1979.
261. Willis, I., Menter, J. M., and Whyte, H. J. The rapid induction of cancers in the hairless mouse utilizing the principle of photoaugmentation. J Invest Dermatol, *76:* 404-408, 1981.

262. Brash, D. E., Rudolph, J. A., Simon, J. A., Lin, A., McKenna, G. J., Baden, H. P., Halperin, A. J., and Ponten, J. A role for sunlight in skin cancer: UV-induced p53 mutations in squamous cell carcinoma. Proc Natl Acad Sci U S A, *88:* 10124-10128, 1991.
263. Kanjilal, S., Pierceall, W. E., Cummings, K. K., Kripke, M. L., and Ananthaswamy, H. N. High frequency of p53 mutations in ultraviolet radiation-induced murine skin tumors: evidence for strand bias and tumor heterogeneity. Cancer Res, *53:* 2961-2964, 1993.
264. Ananthaswamy, H. N. and Pierceall, W. E. Molecular mechanisms of ultraviolet radiation carcinogenesis. Photochem Photobiol, *52:* 1119-1136, 1990.
265. Brash, D. E., Ziegler, A., Jonason, A. S., Simon, J. A., Kunala, S., and Leffell, D. J. Sunlight and sunburn in human skin cancer: p53, apoptosis, and tumor promotion. J Investig Dermatol Symp Proc, *1:* 136-142, 1996.
266. Bode, A. M. and Dong, Z. Mitogen-activated protein kinase activation in UV-induced signal transduction. Sci STKE, *2003:* RE2, 2003.
267. Kato, T., Jr., Delhase, M., Hoffmann, A., and Karin, M. CK2 Is a C-Terminal IkappaB Kinase Responsible for NF-kappaB Activation during the UV Response. Mol Cell, *12:* 829-839, 2003.
268. Adler, V., Pincus, M. R., Polotskaya, A., Montano, X., Friedman, F. K., and Ronai, Z. Activation of c-Jun-NH2-kinase by UV irradiation is dependent on p21ras. J Biol Chem, *271:* 23304-23309, 1996.
269. Herrlich, P., Ponta, H., and Rahmsdorf, H. J. DNA damage-induced gene expression: signal transduction and relation to growth factor signaling. Rev Physiol Biochem Pharmacol, *119:* 187-223, 1992.
270. Pearson, G., Robinson, F., Beers Gibson, T., Xu, B. E., Karandikar, M., Berman, K., and Cobb, M. H. Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. Endocr Rev, *22:* 153-183, 2001.
271. Chouinard, N., Valerie, K., Rouabhia, M., and Huot, J. UVB-mediated activation of p38 mitogen-activated protein kinase enhances resistance of normal human keratinocytes to apoptosis by stabilizing cytoplasmic p53. Biochem J, *365:* 133-145, 2002.
272. Nakamura, S., Takahashi, H., Kinouchi, M., Manabe, A., Ishida-Yamamoto, A., Hashimoto, Y., and Iizuka, H. Differential phosphorylation of mitogen-activated protein kinase families by epidermal growth factor and ultraviolet B irradiation in SV40-transformed human keratinocytes. J Dermatol Sci, *25:* 139-149, 2001.
273. Assefa, Z., Garmyn, M., Bouillon, R., Merlevede, W., Vandenheede, J. R., and Agostinis, P. Differential stimulation of ERK and JNK activities by ultraviolet B irradiation and epidermal growth factor in human keratinocytes. J Invest Dermatol, *108:* 886-891, 1997.
274. Cooper, S. J., MacGowan, J., Ranger-Moore, J., Young, M. R., Colburn, N. H., and Bowden, G. T. Expression of dominant negative c-jun inhibits ultraviolet B-induced squamous cell carcinoma number and size in an SKH-1 hairless mouse model. Mol Cancer Res, *1:* 848-854, 2003.
275. Young, M. R., Li, J. J., Rincon, M., Flavell, R. A., Sathyanarayana, B. K., Hunziker, R., and Colburn, N. Transgenic mice demonstrate AP-1 (activator

protein-1) transactivation is required for tumor promotion. Proc Natl Acad Sci U S A, *96:* 9827-9832, 1999.
276. Chen, W., Borchers, A. H., Dong, Z., Powell, M. B., and Bowden, G. T. UVB irradiation-induced activator protein-1 activation correlates with increased c-fos gene expression in a human keratinocyte cell line. J Biol Chem, *273:* 32176-32181, 1998.
277. Chen, W. and Bowden, G. T. Activation of p38 MAP kinase and ERK are required for ultraviolet-B induced c-fos gene expression in human keratinocytes. Oncogene, *18:* 7469-7476, 1999.
278. Chen, W. and Bowden, G. T. Role of p38 mitogen-activated protein kinases in ultraviolet-B irradiation-induced activator protein 1 activation in human keratinocytes. Mol Carcinog, *28:* 196-202, 2000.
279. Robertson, L. M., Kerppola, T. K., Vendrell, M., Luk, D., Smeyne, R. J., Bocchiaro, C., Morgan, J. I., and Curran, T. Regulation of c-fos expression in transgenic mice requires multiple interdependent transcription control elements. Neuron, *14:* 241-252, 1995.
280. Stein, B., Angel, P., van Dam, H., Ponta, H., Herrlich, P., van der Eb, A., and Rahmsdorf, H. J. Ultraviolet-radiation induced c-jun gene transcription: two AP-1 like binding sites mediate the response. Photochem Photobiol, *55:* 409-415, 1992.
281. van Dam, H., Duyndam, M., Rottier, R., Bosch, A., de Vries-Smits, L., Herrlich, P., Zantema, A., Angel, P., and van der Eb, A. J. Heterodimer formation of cJun and ATF-2 is responsible for induction of c-jun by the 243 amino acid adenovirus E1A protein. Embo J, *12:* 479-487, 1993.
282. Pentland, A. P., Schoggins, J. W., Scott, G. A., Khan, K. N., and Han, R. Reduction of UV-induced skin tumors in hairless mice by selective COX-2 inhibition. Carcinogenesis, *20:* 1939-1944, 1999.
283. Buckman, S. Y., Gresham, A., Hale, P., Hruza, G., Anast, J., Masferrer, J., and Pentland, A. P. COX-2 expression is induced by UVB exposure in human skin: implications for the development of skin cancer. Carcinogenesis, *19:* 723-729, 1998.
284. Higashi, Y., Kanekura, T., and Kanzaki, T. Enhanced expression of cyclooxygenase (COX)-2 in human skin epidermal cancer cells: evidence for growth suppression by inhibiting COX-2 expression. Int J Cancer, *86:* 667-671, 2000.
285. Chen, W., Tang, Q., Gonzales, M. S., and Bowden, G. T. Role of p38 MAP kinases and ERK in mediating ultraviolet-B induced cyclooxygenase-2 gene expression in human keratinocytes. Oncogene, *20:* 3921-3926, 2001.
286. Tang, Q., Chen, W., Gonzales, M. S., Finch, J., Inoue, H., and Bowden, G. T. Role of cyclic AMP responsive element in the UVB induction of cyclooxygenase-2 transcription in human keratinocytes. Oncogene, *20:* 5164-5172, 2001.
287. Thomson, S., Clayton, A. L., Hazzalin, C. A., Rose, S., Barratt, M. J., and Mahadevan, L. C. The nucleosomal response associated with immediate-early gene induction is mediated via alternative MAP kinase cascades: MSK1 as a potential histone H3/HMG-14 kinase. Embo J, *18:* 4779-4793, 1999.

288. Zhong, S. P., Ma, W. Y., and Dong, Z. ERKs and p38 kinases mediate ultraviolet B-induced phosphorylation of histone H3 at serine 10. J Biol Chem, *275:* 20980-20984, 2000.
289. Zhong, S., Jansen, C., She, Q. B., Goto, H., Inagaki, M., Bode, A. M., Ma, W. Y., and Dong, Z. Ultraviolet B-induced phosphorylation of histone H3 at serine 28 is mediated by MSK1. J Biol Chem, *276:* 33213-33219, 2001.
290. She, Q. B., Ma, W. Y., and Dong, Z. Role of MAP kinases in UVB-induced phosphorylation of p53 at serine 20. Oncogene, *21:* 1580-1589, 2002.
291. Tournier, C., Hess, P., Yang, D. D., Xu, J., Turner, T. K., Nimnual, A., Bar-Sagi, D., Jones, S. N., Flavell, R. A., and Davis, R. J. Requirement of JNK for stress-induced activation of the cytochrome c-mediated death pathway. Science, *288:* 870-874, 2000.
292. She, Q. B., Ma, W. Y., Zhong, S., and Dong, Z. Activation of JNK1, RSK2, and MSK1 is involved in serine 112 phosphorylation of Bad by ultraviolet B radiation. J Biol Chem, *277:* 24039-24048, 2002.
293. Zha, J., Harada, H., Yang, E., Jockel, J., and Korsmeyer, S. J. Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X(L). Cell, *87:* 619-628, 1996.
294. Zhang, Q. S., Maddock, D. A., Chen, J. P., Heo, S., Chiu, C., Lai, D., Souza, K., Mehta, S., and Wan, Y. S. Cytokine-induced p38 activation feedback regulates the prolonged activation of AKT cell survival pathway initiated by reactive oxygen species in response to UV irradiation in human keratinocytes. Int J Oncol, *19:* 1057-1061, 2001.
295. Roymans, D. and Slegers, H. Phosphatidylinositol 3-kinases in tumor progression. Eur J Biochem, *268:* 487-498, 2001.
296. Wan, Y. S., Wang, Z. Q., Shao, Y., Voorhees, J. J., and Fisher, G. J. Ultraviolet irradiation activates PI 3-kinase/AKT survival pathway via EGF receptors in human skin in vivo. Int J Oncol, *18:* 461-466, 2001.
297. Kulik, G., Klippel, A., and Weber, M. J. Antiapoptotic signalling by the insulin-like growth factor I receptor, phosphatidylinositol 3-kinase, and Akt. Mol Cell Biol, *17:* 1595-1606, 1997.
298. Gonzales, M. and Bowden, G. T. The role of PI 3-kinase in the UVB-induced expression of c-fos. Oncogene, *21:* 2721-2728, 2002.
299. Tang, Q., Gonzales, M., Inoue, H., and Bowden, G. T. Roles of Akt and glycogen synthase kinase 3beta in the ultraviolet B induction of cyclooxygenase-2 transcription in human keratinocytes. Cancer Res, *61:* 4329-4332, 2001.
300. Cohen, P. and Frame, S. The renaissance of GSK3. Nat Rev Mol Cell Biol, *2:* 769-776, 2001.
301. Haertel-Wiesmann, M., Liang, Y., Fantl, W. J., and Williams, L. T. Regulation of cyclooxygenase-2 and periostin by Wnt-3 in mouse mammary epithelial cells. J Biol Chem, *275:* 32046-32051, 2000.
302. Grotewold, L. and Ruther, U. The Wnt antagonist Dickkopf-1 is regulated by Bmp signaling and c-Jun and modulates programmed cell death. Embo J, *21:* 966-975, 2002.
303. Liu, J., Stevens, J., Rote, C. A., Yost, H. J., Hu, Y., Neufeld, K. L., White, R. L., and Matsunami, N. Siah-1 mediates a novel beta-catenin degradation pathway

linking p53 to the adenomatous polyposis coli protein. Mol Cell, *7:* 927-936, 2001.
304. Matsuzawa, S. I. and Reed, J. C. Siah-1, SIP, and Ebi collaborate in a novel pathway for beta-catenin degradation linked to p53 responses. Mol Cell, *7:* 915-926, 2001.
305. Watcharasit, P., Bijur, G. N., Zmijewski, J. W., Song, L., Zmijewska, A., Chen, X., Johnson, G. V., and Jope, R. S. Direct, activating interaction between glycogen synthase kinase-3beta and p53 after DNA damage. Proc Natl Acad Sci U S A, *99:* 7951-7955, 2002.
306. Ross, S. E., Hemati, N., Longo, K. A., Bennett, C. N., Lucas, P. C., Erickson, R. L., and MacDougald, O. A. Inhibition of adipogenesis by Wnt signaling. Science, *289:* 950-953, 2000.
307. Hutchins, J. R., Hughes, M., and Clarke, P. R. Substrate specificity determinants of the checkpoint protein kinase Chk1. FEBS Lett, *466:* 91-95, 2000.
308. Jacks, T. and Weinberg, R. A. The expanding role of cell cycle regulators. Science, *280:* 1035-1036, 1998.
309. Xiong, Y., Hannon, G. J., Zhang, H., Casso, D., Kobayashi, R., and Beach, D. p21 is a universal inhibitor of cyclin kinases. Nature, *366:* 701-704, 1993.
310. Kagawa, S., Fujiwara, T., Kadowaki, Y., Fukazawa, T., Sok-Joo, R., Roth, J. A., and Tanaka, N. Overexpression of the p21 sdi1 gene induces senescence-like state in human cancer cells: implication for senescence-directed molecular therapy for cancer. Cell Death Differ, *6:* 765-772, 1999.
311. Missero, C., Cunto, F. D., Kiyokawa, H., Koff, A., and Dotto., G. P. The absence of p21Cip1/WAF1 alters keratinocyte growth and differentiation and promotes *ras*-tumor progression. Genes Dev., *10:* 3065-3075, 1996.
312. Deng, C., Zhang, P., Harper, J. W., Elledge, S. J., and Leder, P. Mice lacking p21CIP1/WAF1 undergo normal development, but are defective in G1 checkpoint control. Cell, *82:* 675-684, 1995.
313. Brugarolas, J., Chandrasekaran, C., Gordon, J. I., Beach, D., Jacks, T., and Hannon, G. J. Radiation-induced cell cycle arrest compromised by p21 deficiency. Nature, *377:* 552-557, 1995.
314. Medrano, E. E., Im, S., Yang, F., and Abdel-Malek, Z. A. Ultraviolet B light induces G1 arrest in human melanocytes by prolonged inhibition of retinoblastoma protein phosphorylation associated with long-term expression of the p21Waf-1/SDI-1/Cip-1 protein. Cancer Res, *55:* 4047-4052, 1995.
315. Lee, J. H., An, H. T., Chung, J. H., Kim, K. H., Eun, H. C., and Cho, K. H. Acute effects of UVB radiation on the proliferation and differentiation of keratinocytes. Photodermatol Photoimmunol Photomed, *18:* 253-261, 2002.
316. Lu, Y. P., Lou, Y. R., Yen, P., Mitchell, D., Huang, M. T., and Conney, A. H. Time course for early adaptive responses to ultraviolet B light in the epidermis of SKH-1 mice. Cancer Res, *59:* 4591-4602, 1999.

APPENDIX

CCAAT/Enhancer Binding Protein-β (C/EBPβ) is a Mediator of Keratinocyte Survival and Skin Tumorigenesis Involving Oncogenic Ras Signaling

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ABSRACT

The basic leucine zipper (bZIP) transcription factor CCAAT/enhancer binding protein-β (C/EBPβ is expressed in many cell types, including keratinocytes. C/EBPβ activity can be increased by phosphorylation through pathways stimulated by oncogenic Ras, although the biological implications of Ras-C/EBPβ signaling are not understood. We report here that C/EBPβ-nullizygous mice are completely refractory to skin tumor development induced by a variety carcinogens and carcinogenesis protocols, including DMBA-initiation/TPA promotion, that produce tumors containing oncogenic Ras mutations. No significant differences in TPA-induced epidermal keratinocyte proliferation were observed in C/EBPβ-null versus wild type mice. However, apoptosis was significantly elevated (17 fold) in the epidermal keratinocytes of DMBA-treated C/EBPβ-null mice compared to wild type mice. In v-Ha-*ras* transgenic mice C/EBPβ deficiency also led to greatly reduced skin tumor multiplicity and size, providing additional evidence for a tumorigenesis pathway linking Ras and C/EBPβ. Oncogenic Ras potently stimulated C/EBPβ to activate a C/EBP-responsive promoter-reporter in keratinocytes and mutating an ERK1/2 phosphorylation site (T188) in C/EBPβ completely abolished this Ras effect. Finally, we observed that C/EBPβ participates in oncogenic Ras-induced transformation of NIH-3T3 cells. These findings indicate that C/EBPβ has a critical role in Ras-mediated tumorigenesis and cell survival and implicate C/EBPβ as a novel target for tumor inhibition.

INTRODUCTION

The Ras family of GTP binding proteins function as intracellular mediators of extracellular signals to regulate cell proliferation, apoptosis, survival, senescence and differentiation (1-5). *Ras* proto-oncogenes are frequently mutated in tumors and approximately 25% of human cancers contain transforming mutations in *ras*. Therefore, understanding oncogenic Ras signaling pathways is critical for elucidating the mechanisms that underlie cellular transformation and for designing effective therapeutic strategies to prevent the development or block the growth of many classes of tumors. Ras has numerous effectors and its pathways are multifaceted (3, 6, 7). Ras activation by growth factors or oncogenic mutations elicits activation of several transcription factors, which in turn regulate the expression of genes that control the cellular responses to Ras signaling, including oncogenesis. The transcription factors Ets, c-jun, c-myc and NF-κB are known to have roles in oncogenic ras-induced cellular transformation (8-11).

The basic leucine zipper (bZIP) transcription factor C/EBPβ (also known as NF-IL6, IL-6DBP, LAP, CRP2, and NF-M) is expressed in a variety of cell types (12, 13) including keratinocytes (14, 15), where it plays a role in squamous differentiation (16). C/EBPβ is also involved in regulating differentiation of specific mesenchymal, epithelial and hematopoietic cell types (17-21). C/EBPβ activity can be activated/derepressed by phosphorylation through pathways stimulated by oncogenic Ras in fibroblasts, erythoblasts and P19 embryonal carcinoma cells (22, 23), suggesting a role for C/EBPβ as nuclear effector of Ras signaling. However, the physiological functions of C/EBPβ as downstream target of Ras are unclear.

The fact that C/EBPβ is present in numerous epithelial and hematopoietic cells and some of these cell types give rise to human and rodent tumors containing mutant ras (24, 25), prompted us to investigate whether C/EBPβ has a role in oncogenic Ras mediated tumorigenesis and transformation. To address this question we have examined C/EBPβ-nullizygous mice in the mouse skin model of multistage carcinogenesis. The mouse skin model is one of the best defined *in vivo* paradigms of experimental epithelial carcinogenesis (26, 27) and there is ample evidence that the mutational activation of Ras plays a central role in skin tumor development induced by a variety of carcinogens (25, 27-29). For example, initiation with a single dose of the carcinogen, 7,12-dimethylbenz[a]anthracene (DMBA), followed by repetitive treatment with the tumor promoter, TPA, results in the appearance of squamous papillomas, 95-100% of which contain an A->T^{182} mutation in Ha-*ras* (25, 28, 29). Furthermore, transgenic mice which express an oncogenic Ras transgene in their epidermis develop skin tumors, demonstrating a causal role for activated Ras in squamous papilloma development (30, 31).

Here we report that C/EBPβ-null mice are completely refractory to carcinogen-induced skin-tumors involving mutant Ras and that v-Ha-*ras* transgenic mice that carry the C/EBPβ-null mutation also show a significant reduction in tumorigenesis. Apoptosis was significantly elevated in C/EBPβ-null mice in response to DMBA but not to UVB treatment. These findings reveal a novel role for C/EBPβ in tumorigenesis and cell survival.

METHODS

Tumor experiments in wild type and C/EBPβ mutant mice

The C/EBPβ deficient mice used in our studies were described previously (32). The mutant and wild type mice were generated by mating heterozygous 129/Sv females to heterozygous males from the 6^{th}-8^{th} generation backcross into the C57BL/6 strain. No significant sex difference in tumor response was observed.

Reporter Assays

DNA fragments encoding mouse C/EBPβ (p35 and LIP isoforms) were inserted into pCDNA3.1. The T188A mutation was generated using the QuickChange system (Stratagene). BALB/MK2 keratinocytes (a gift from B.E, Weissman, UNC Chapel Hill NC) at ~25-30% confluence were transfected using Lipofectin reagent (GIBCO BRL Gaithersburg, MD) with pcDNA3-C/EBPβ (0.5 μg) and/or pcDNA3-Ha-ras(12V) (0.5 μg) (gift from C.J. Der, UNC Chapel Hill, NC) and 1.0 μg of the specified C/EBP dependent promoter/reporter as described in text. The total amount of DNA among all groups was kept constant using empty vector. After 4 hours, cultures were washed and grown in low calcium EMEM containing 4 ng/ml EGF, 0.05 mM calcium chloride and 8% chelexed FBS. Forty eight hours later cells were harvested and luciferase activity was determined (16). Primary keratinocytes were isolated from C/EBPβ-/- or C/EBPβ +/+ newborn littermates (2-3 days old) and cultured as previously described (14, 16). At ~100% confluence cells were transfected using the PerFect Lipid, Pfx-3 (Invitrogen San Diego, CA). Cells were processed for reporter assays as described above.

Detection of Apoptotic Cells

Wild type and C/EBPβ-null mice were treated with a single dose of 400 nmol DMBA and 24 hrs later the treated dorsal skin was excised and fixed for 24 hours in a 10% neutral buffered formalin, processed and embedded in paraffin. Five μm hematoxylin and eosin stained sections were examined. Apoptotic keratinocytes in the stratum basale were scored using the following criteria; dark pyknotic nuclei, cytoplasmic eosinophilia and absence of cellular contacts.

NIH-3T3 Cell Focus Assay

NIH-3T3 cells (gift from C.J. Der, UNC, Chapel Hill, NC) were plated at $5x10^5$ cells/60 mm dish in DMEM medium containing 10% calf serum. One day later the cells were transfected using a calcium phosphate precipitation method (33). Transformed foci were identified 14 days after transfection as defined by Clark et al (33).

Western Analysis

BALB/MK2 keratinocytes were transfected with pcDNA3-C/EBPβ (0.5 μg) and/or pcDNA3-Ha-ras(12V) (0.5 μg) as described above. Forty eight hours later lysates were prepared, equal amounts of each protein sample were loaded on 10% polyacrylamide Tris-Glycine gels (Novex) and separated by electrophoresis and western analysis conducted with a rabbit polyclonal IgG raised against C/EBPβ or Ras (1:2,000) (Santa Cruz Biotechnologies, Santa Cruz CA).

Tg.AC x C/EBPβ$^{-/-}$ crosses

Female Tg.AC mice (R. Cannon, NIEHS) were crossed with C/EBPβ$^{-/-}$ mice and F1 mice were backcrossed to C/EBPβ$^{-/+}$ mice. Mice were genotyped by southern analysis for v-

Ha-ras and C/EBPβ. All v-Ha-ras positive mice were also genotyped to verify they were of the responder genotype (34).

RESULTS

Effect of C/EBPβ deficiency on skin tumor development.

Initiation with a single dose of DMBA followed by TPA treatment produces squamous papillomas, 95-100% of which contain an A->T^{182} mutation in Ha-*ras* (25, 28, 29). Therefore, C/EBPβ nullizygous and wild-type littermates were initiated with 200 nmol DMBA and one week later these mice were promoted thrice weekly with 5 nmol TPA for 25 weeks. Wild-type mice developed an average of 15 squamous papillomas/mouse and exhibited a 100% incidence of papillomas (Fig. 1A and B). In contrast, C/EBPβ nullizygous mice were completely refractory to papilloma development and no papillomas appeared after 25 weeks of promotion. In some groups of mutant mice TPA promotion was continued for 35 weeks, but no tumors developed within this time (data not shown). C/EBPβ heterozygous mice express a level of C/EBPβ protein in keratinocytes that is intermediate between that of wild-type and C/EBPβ-deficient animals (14). C/EBPβ heterozygous mice were partially resistant to DMBA/TPA-induced carcinogenesis (Fig. 1A and B), indicating that the tumor modifying effect of C/EBPβ is gene dosage dependent.

Since C/EBPβ can be phosphorylated via a PKC pathway (35) and in hepatocytes this event is required for TPA-induced mitogenesis (36), we examined whether TPA-induced keratinocyte proliferation was altered in epidermis of C/EBPβ-null mice. No significant differences were observed between wild-type and C/EBPβ nullizygous mice after single or multiple treatments with TPA (Table 1). C/EBPβ has been implicated in the regulation of COX2 (37) and TNFα (38) expression and both TNFα null (39) and COX2 null mice (40) are partially resistant to DMBA/TPA-induced carcinogenesis.

However, TNFα mRNA and COX2 protein expression was not different in untreated or TPA-treated C/EBPβ deficient mice compared to similarly treated wild type mice (data not shown). These results indicate that TNFα and COX2 expression as well as TPA-induced proliferative responses in the epidermis of C/EBPβ-null mice are normal and thus, are not responsible for the resistance of C/EBPβ-null mice to DMBA/TPA-induced tumorigenesis.

To test the possibility that C/EBPβ nullizygous mice are refractory to DMBA initiation due to their inability to convert DMBA to the carcinogenic species, we subjected the mice to the direct-acting carcinogen N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) followed by TPA promotion. The MNNG/TPA carcinogenesis protocol produces papillomas, 85-90 % of which contain oncogenic mutations in the 12^{th} codon of either Ha-*ras* (25, 41) or Ki-*ras* (41). Wild-type mice displayed a 100% incidence of papillomas with approximately 3 papillomas/mouse, while C/EBPβ null littermates did not develop any tumors (Fig. 1C and D). Since both MNNG- and DMBA-initiated mice were treated with TPA, it was possible that the inability of C/EBPβ-null mice to develop tumors resulted from a defective response to TPA or to an initiation/promotion protocol. Therefore, we used a complete carcinogenesis protocol in which wild-type and C/EBPβ-null mice were treated weekly with DMBA. All of the wild-type mice developed papillomas with an average of 12 papillomas/mouse while C/EBPβ mutant mice were again completely resistant to carcinogenesis (Fig. 1E and F). We also observed that C/EBPβ null mice were refractory to DMBA-initiation followed by promotion with the non-phorbol ester tumor promoter, mirex (data not shown). Thus, C/EBPβ nullizygous mice are fully resistant to tumorigenesis induced by a variety of carcinogens, tumor

promoters and carcinogenesis protocols that, in normal mice, cause tumors that contain mutant oncogenic Ha-ras or Ki-ras (25, 27-29, 41). These results suggest that C/EBPβ is an essential downstream mediator of oncogenic Ras tumorigenesis.

The lack of tumor development in carcinogen treated C/EPBβ-null mice could be due to apoptosis of C/EBPβ-deficient keratinocytes that have acquired oncogenic Ha-ras lesions. To examine this possibility we treated mice with DMBA and scored the number of apoptotic keratinocytes in C/EBPβ-null and wild type epidermis using the cytological parameters described in the Methods. Compared to wild type mice, C/EBPβ-null mice exhibited a 17-fold increase in the number of basal apoptotic keratinocytes (Table 2), indicating that C/EBPβ functions as a survival factor in DMBA/Ras-induced oncogenesis. Similar fold increases in apoptotic cells were observed using TUNEL staining (data not shown). To determine if C/EBPβ-null mice also display increased apoptosis in response to UVB irradiation, a potent inducer of apoptosis and DNA damage, wild type and C/EBPβ-null mice were irradiated with UVB doses of 50, 100 and 200 mJ/cm^2. While all UVB doses increased the number of apoptotic epidermal keratinocytes, there was no difference between wild type and mutant mice (data not shown). These results show that the enhanced apoptosis in DMBA-treated C/EBPβ-null mice is stimulus specific and that DNA damage alone is not sufficient to elicit increases in apoptosis in epidermal keratinocytes of C/EBPβ-null mice.

Impaired skin tumorigenesis in C/EBPβ-/- v-Ha-ras transgenic mice

To provide additional evidence for a relationship between Ras and C/EBPβ in skin tumorigenesis, we crossed C/EBPβ-null mice with Tg.AC transgenic mice. Tg.AC mice contain a v-Ha-ras transgene under the control of a partial ζ-globin promoter and

are susceptible to skin tumor development in the absence of carcinogen exposure (31). Tumorigenesis in Tg.AC mice does require a promoting stimulus such as wounding or treatment with a tumor promoter. As shown in Figure 2A, TPA-treated C/EBPβ deficient mice carrying the v-Ha-ras transgene developed ~65% fewer skin tumors than C/EBPβ +/+ transgene positive mice and the tumor size (Fig 2B) was significantly reduced by 60% in the C/EBPβ null mice (4.1 ± 2.4 mm C/EBPβ +/+ vs 1.7 ±1.0 mm C/EBPβ -/- $p<0.01$ student t-test). While there was not a complete ablation of tumor development in the C/EBPβ null mice carrying the v-Ha-ras transgene, it is clear that C/EBPβ significantly affects the development and growth of Ras-induced papillomas. These results support a direct role for C/EBPβ as a nuclear effector of Ras-mediated tumorigenesis.

C/EBPβ activation by Ha-Ras signaling in keratinocytes

To ascertain if oncogenic Ha-ras signaling can stimulate C/EBPβ activity in epidermal keratinocytes, we transfected BALB/MK2 keratinocytes with C/EBPβ and/or Ha-ras(12V) and a luciferase reporter gene fused to different lengths of the C/EBP-dependent myelomonocytic growth factor (MGF) promoter(16, 42). pMGF-40 contains a 40 bp portion of the MGF promoter that lacks C/EBP sites while pMGF-82 contains an additional 42 bp region of the promoter harboring two C/EBP binding sites. Co-transfection of Ha-ras(12V) and C/EBPβ resulted in 30- and 80-fold increases, respectively, in pMGF-82 reporter activity over that observed with C/EBPβ or Ha-ras(12V) alone (Fig. 3A). In contrast, cotransfection of Ha-ras(12V) and C/EBPβ caused only a 5-fold increase in transcription from the MGF-40 promoter, demonstrating that C/EBP binding sites are required for the synergistic response. Similar results were

obtained using a minimal albumin promoter with four tandem C/EBP sites {$(DE1)_4$-Alb-luc} (43) (Fig. 3B). Western blot analysis of cell lysates from BALB/MK2 cells co-transfected with C/EBPβ and Ha-ras(12V) demonstrated that the observed synergistic effect on C/EBP-responsive promoter-reporter activity is not due to increased Ha-ras(12V) or C/EBPβ expression (Fig 3C).

Co-transfection of Ha-ras(12V) with a truncated form of C/EBPβ (LIP; liver inhibitory protein) that lacks the N-terminal activation domain but retains the bZIP DNA-binding and leucine zipper domain (44) did not increase the activity of the pMGF-82 reporter (Fig. 4A). In fact, LIP inhibited the activation of wild-type C/EBPβ by Ha-ras(12V) by ~50%, which is consistent with its known role as a dominant negative inhibitor of C/EBPβ (44) (Fig 4A). Previous studies have identified an ERK1/2 phosphorylation site (T 188) in C/EBPβ and substituting T188 with alanine diminished Ras activation of C/EBPβ (22). Therefore, we tested the Ras-responsiveness of a C/EBPβ mutant containing the T188A substitution. Oncogenic Ha-ras-induced stimulation of C/EBPβ activity was abolished in this mutant. (Fig. 4B) Thus, an oncogenic Ha-ras pathway can activate C/EBPβ in keratinocytes and this activation is dependent upon threonine 188 of C/EBPβ.

To determine if endogenous C/EBPβ can mediate Ras signaling, we transfected primary keratinocytes isolated from C/EBPβ-nullizygous and wild-type mice with oncogenic Ha-ras and the C/EBP promoter-reporter constructs. Transfection of Ha-ras(12V) into wild-type keratinocytes resulted in a 30-fold increase in pMGF-82 reporter activity while in C/EBPβ-nullizygous keratinocytes Ha-ras(12V) caused less than a 4-fold increase (Fig. 5A and B). The Ras-induced increase in promoter activity required

C/EBP binding sites (Fig. 5A). Similar results were obtained with the $(DE1)_4$-Alb-luc reporter (data not shown). Ectopic expression of C/EBPβ in C/EBPβ-null keratinocytes restored responsiveness to oncogenic Ras (Fig 5B). Thus, endogenous C/EBPβ is a downstream mediator of oncogenic Ha-ras signaling in primary keratinocytes.

C/EBPβ augments Ras-induced transformation of NIH-3T3 cells

The NIH-3T3 focus assay has been widely used to identify pathways and genes that cooperate with Ras to induce transformation (33). To examine the role of C/EBPβ in NIH-3T3 transformation, we first confirmed that oncogenic Ras could stimulate C/EBPβ to activate a C/EBP-responsive promoter-reporter in NIH-3T3 cells (22) (data not shown). Next we examined whether C/EBPβ has the capacity to transform cells and/or cooperate with oncogenic Ha-ras to increase its transforming potential in the NIH-3T3 focus formation assay. Transfection of C/EBPβ alone did not induce NIH 3T3 transformation, (Table 3) showing that this transcription factor does not possess intrinsic transforming activity. Co-transfection of 5 or 10 ng C/EBPβ enhanced the transformation potential of oncogenic Ha-ras(12V), producing a ~1.3 and 1.7 fold increase, respectively, in the number of transformed foci compared to Ha-ras(12V) alone (Table 3). Paradoxically, co-transfection of 50 ng of C/EBPβ with Ha-ras(12V) inhibited transformation by 20% suggesting the enhancing effect of C/EBPβ is not only saturable, but that high levels of C/EBPβ can inhibit ras transformation, perhaps due to a nonspecific effect of transfecting too high levels of C/EBPβ. Importantly, we observed that co-transfection of 10 ng LIP or 10 ng C/EBPβ T188A inhibited Ha-ras (12V)-induced transformation, indicating an important role for endogenous C/EBPβ in ras-induced transformation of NIH-3T3 cells. C/EBPβ also enhanced the transforming

potential of oncogenic Raf, lending further support for a Ras-Raf-ERK-C/EBPβ pathway. In contrast to C/EBPβ, neither C/EBPα nor C/EBPδ enhanced the transforming activity of oncogenic Ha-ras (12V) (Table 3). Thus not all C/EBP family members are capable of augmenting Ras transformation.

DISCUSSION

We have identified C/EBPβ as a critical gene in two established *in vivo* models of Ras-mediated epithelial tumorigenesis as well as in Ras-induced transformation of NIH-3T3 fibroblasts. C/EBPβ was found to be essential for skin tumorigenesis induced by a variety of carcinogens that are known to cause the mutational activation of Ha-ras and K-ras. Moreover, C/EBPβ deficiency in oncogenic v-Ha-ras transgenic mice inhibited v-Ha-*ras*-induced tumorigenesis by 60%. Substitution of C/EBPβ T188 with alanine, which disrupts an ERK1/2 phosphorylation site, blocked the ability of Ras to stimulate C/EBPβ transactivation function and also inhibited Ras-induced NIH 3T3 transformation. Although our studies cannot rule out the existence of a Ras-independent pathway responsible for the observed responses in the C/EBPβ-deficient mice, our collective results do implicate C/EBPβ as a critical component of a Ras-dependent tumorigenesis/transformation pathway. Future studies using of C/EBPβ -/- MEFs may provide further insight into the exact role of C/EBPβ in Ras-induced transformation and apoptosis.

Depending upon the cellular context, strength of signal and pathways engaged, oncogenic Ras can regulate cell proliferation, differentiation, senescence, apoptosis or survival (3, 45, 46). Ras-induced apoptosis is suppressed by its activation of pro-survival pathways involving NF-κB (2) or Rac GTPase (31); if these pathways are blocked, apoptosis results. Moreover, in PC-12 cells the MEK/ERK pathway promotes cell survival (47). We have observed that C/EBPβ-null mice display a 17-fold increase in the number of apoptotic keratinocytes in DMBA-treated epidermis compared to similarly treated wild

type epidermis. Studies in mouse epidermis have indicated that 0.1-5% of Ha-*ras* genes sustain a codon 61 mutation within 1-3 days of topical carcinogen application (48). The observed increase in apoptotic cells in DMBA-treated C/EBPβ deficient epidermis is consistent with a survival/anti-apoptotic role for C/EBPβ in oncogenic Ras expressing cells. Previously we demonstrated that C/EBPβ positively regulates the program of squamous differentiation in the epidermis and in isolated keratinocytes (16). Therefore, we suggest that in normal keratinocytes, C/EBPβ regulates differentiation as well as survival which is required to complete the differentiation program. However, in the presence of oncogenic Ha-ras the C/EBPβ pro-survival response may predominate over the differentiation pathway and clonal expansion occurs, ultimately resulting in tumor formation. When the C/EBPβ pro-survival signaling pathway is deleted as in the C/EBPβ-deficient mice, cells containing oncogenic Ras undergo apoptosis and tumorigenesis is blocked.

In addition to *ras*, there is evidence for additional DMBA target genes that cooperate with oncogenic ras to induce skin tumorigenesis and it is conceivable that C/EBPβ is also a component of non-ras oncogenic circuitry (49). Such a notion is consistent with our observation that C/EBPβ deficiency in the v-Ha-ras transgenic mice did not result in the complete inhibition of tumorigenesis. Alternatively, it is possible that high levels of expression of v-Ha-ras transgene in Tg.AC epidermal keratinocytes and/or the expression of the transgene within a different subpopulation of keratinocytes could account for the differences between the degree of inhibition of tumorigenesis in v-Ha-*ras* and carcinogen-treated C/EBPβ deficient mice. C/EBPβ-null keratinocytes could also have a defective DNA repair mechanism which contributes to the elevated levels of DMBA-

induced apoptosis. However, such a mechanism would not involve all forms DNA damage as we did not observe differences in UVB-induced apoptosis in wild type versus C/EBPβ-null keratinocytes. Moreover, since both UVB-induced pyrimidine dimers and DMBA bulky adducts are repaired by the same nucleotide excision repair mechanism it is unlikely that defects in nucleotide excision repair account for the elevated level of DMBA-induced apoptosis in C/EBPβ null mice. MNNG-induced DNA damage is repaired by a base excision repair mechanism suggesting that activation of ras and not altered DNA repair is the common link between the two carcinogens. Collectively, our results tend to support a role for C/EBPβ in the survival of oncogenic Ha-ras cells.

The complete inhibition of tumorigenesis in carcinogen-treated C/EBPβ deficient mice is striking and is, to our knowledge, the most profound inhibitory effect of a single gene deletion on skin tumorigenesis reported to date. The potent effect of C/EBPβ in an experimental mouse model raises the question of whether C/EBPβ plays a similar role in human cancers. Interestingly, C/EBPβ levels are strongly increased in human colorectal tumors (50), and are also associated with human ovarian epithelial tumor progression (51). Thus, the induction of C/EBPβ may be an important event in the development epithelial tumors, perhaps by providing an essential anti-apoptotic signal.

Recent studies have implicated another C/EBP family member, C/EBPα, in myeloid leukemogenesis. However, in contrast to C/EBPβ, C/EBPα functions as a tumor suppressor by promoting granulocytic differentiation and growth arrest. Thus, C/EBPα inactivation by mutation (52) or by its association with the oncoprotein AML-1-ETO (53, 54) contributes to myeloid leukemogenesis by maintaining cells in an extended

proliferative state. It is notable that, despite their structural relatedness, C/EBPβ and C/EBPα apparently play very different roles in transformed cells and have opposite effects on tumorigenesis.

While further studies are required to discern the downstream pathways and genes through which C/EBPβ regulates tumor development, our study reveals a heretofore unknown function for C/EBPβ as a critical component of the tumorigenesis pathway initiated by activated Ras. C/EBPβ may therefore represent an attractive target for antineoplastic pharmacological agents.

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1. Serrano, M., Lin, A. W., McCurrach, M. E., Beach, D. & Lowe, S. W. (1997) *Cell* **88,** 593-602.

2. Mayo, M. W., Wang, C. Y., Cogswell, P. C., Rodgers-Graham, K. S., Lowe, S. W., Der, C. J. & Baldwin, A. S. (1997) *Science* **278,** 1812-1815.

3. Cambell, S. L., Khosravi-Far, R., Rossman, K. L., Clark, G. J. & Der, C. J. (1998) *Oncogene* **17,** 1395-1413.

4. Gille, H. & Downward, J. (1999) *J. Biol. Chem.* **274,** 22033-22040.

5. Bonni, A., Brunet, A., West, A. E., Datta, S. R., Takasu, M. A. & Greenberg, M. E. (1999) *Science* **286,** 1358-1362.

6. Katz, M. E. & McCormick, F. (1997) *Curr. Opin. Genet. Dev.* **7,** 75-79.

7. Marshall, C. J. (1996) *Curr. Opin. Cell Biol.* **8,** 197-204.

8. Sklar, M. D., Tompson, E., Welsh, M. J., Liebert, M., Harney, J., Grossman, H. B., Smith, M. & Prochownik, E. V. (1991) *Mol. Cell. Biol.* **11,** 3699-3710.

9. Langer, S. L., Bortner, D. M., Roussel, M. F., Sherr, C. J. & Ostrowski, M. C. (1992) *Mol. Cell Biol.* **12,** 5355-5362.

10. Johnson, R., Spiegelman, B., Hanahan, D. & Wisdom, R. (1994) *Mol. Cell. Biol.* **16,** 4501-4511.

11. Finco, T. S., Westwick, J. K., Norris, J. L., Beg, A. A., Der, C. J. & Baldwin, A. S. (1997) *J. Biol. Cem.* **272,** 24113-24116.

12. Williams, S. C., Cantwell, C. A. & Johnson., P. F. (1991) *Genes Dev* **5,** 1553-1567.

13. Lekstrom-Himes, J. & Xanthopoulos, K. G. (1998) *J. Biol. Chem.*. **273,** 28545-28548.

14. Oh, H.-S. & Smart, R. C. (1998) *J. Invest. Dermatoll.* **110,** 939-945.

15. Maytin, E. V. & Habener, J. F. (1998) *J. Invest. Dermatoll.* **110,** 238-246.

16. Zhu, S., Oh, H. S., Shim, M., Sterneck, E., Johnson, P. F. & Smart, R. C. (1999) *Mol. Cell. Biol.l* **19,** 7181-7190.

17. Cao, Z., Umek, R. M. & McKnight, S. L. (1991) *Genes Dev.* **5,** 1538-1552.

18. Natsuka, S., Akira, S., Nishio, Y., Hashimoto, S., Sugita, T., Isshiki, H. & Kishimoto, T. (1992) *Blood* **79,** 460-466.

19. Scott, L. M., Civin, C. I., Roth, P. & Friedman, A. D. (1992) *Blood* **80,** 1725-1735.

20. Seagroves, T. N., Krnacik, S., Raught, B., Gay, J., Burgess-Beusse, B., Darlington, G. J. & Rosen, J. M. (1998) *Genes Dev.* **12,** 1917-1928.

21. Robinson, G. W., Johnson, P. F., Hennighausen, L. & Sterneck, E. (1998) *Genes Dev*. **12,** 1907-1916.

22. Nakajima, T., Kinoshita, S., Sasagawa, T., Sasaki, K., Naruto, M., Kishimoto, T. & Akira, S. (1993) *Proc. Natl. Acad. Sci.. USA* **90,** 2207-2211.

23. Kowenz-Leutz, E., Twamley, G., Ansieau, S. & Leutz, A. (1994) *Genes Dev.* **8,** 2781-2791.

24. Bos, J. L. (1989) *Cancer Res.* **49,** 4682-4689.

25. Balmain, A. & Brown, K. (1988) *Ad. Cancer Res.* **51,** 147-181.

26. Frame, S., Crombie, R., Liddell, J., Stuart, D., Linardopoulos, S., Nagase, H., Portella, G., Brown, K., Street, A., Akhurst, R. & Balmain, A. (1998) *Philos Trans R Soc Lond B Biol Sci* **353,** 839-845.

27. Yuspa, S. H. (1994) *Cancer Res* **54,** 1178-89.

28. Quintanilla, M., Brown, K., Ramsden, M. & Balmain, A. (1986) *Nature* **322,** 78-80.

29. Moser, G. J., Robinette, C. L. & Smart, R. C. (1993) *Carcinogenesis* **14,** 1153-1160.

30. Brown, K., Strathsee, D., Bryson, S., Lambie, W. & Balmain, A. (1998) *Curr. Biol.* **8,** 516-524.

31. Leder, A., Kuo, A., Cardiff, R. D., Sinn, E. & Leder, P. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 9178-9182.

32. Sterneck, E., Tessarollo, L. & Johnson., P. F. (1997) *Genes Dev* **11,** 2153-2162.

33. Clark, G. J., Cox, A. D., Graham, S. M. & Der, C. J. (1995) *Meth. Enzmoll.* **255,** 395-412.

34. Kantz, D. C., Lacks, G. D. & Cannon, R. E. (1999) *Biotechniques* **27,** 278-280.

35. Trautwein, C., Geer, P. v. d., Karin, M., Hunter, T. & Chojkier, M. (1994) *J. Clin. Invest.* **93,** 2554-2561.

36. Buck, M., Poli, V., Geer, P. v. d., Chojkier, M. & Hunter, T. (1999) *Mol. Cell* **4,** 1087-1092.

37. Reddy, S. T., Wadleigh, D. J. & Herchman, H. R. (2000) *J. Biol. Chem.* **275,** 3107-3113.

38. Drouet, C., Shakhov, A. N. & Jongeneel, C. V. (1991) *J. Immunoll.* **147,** 1694-1700.

39. Moore, R. J., Owens, D. M., Stamp, G., Arnott, C., Burke, F., East, N., Holdsworth, H., Turner, L., Rollins, M. B., Pasparakis, Kollia, G. & Balkwill, F. (1999) *Nat Med* **5,** 828-31.

40. Langenbach, R., Loftin, C., Lee, C. & Tiano, H. (1999) *Biochem. Pharmacol.* **58,** 1237-1246.

41. Rehman, I., Lowry, D. T., Adams, C., Abdel-Fattah, R., Holly, A., Yuspa, S. H. & Hennings, H. (2000) *Mol. Carcinogenesis* **27,** 298-307.

42. Sterneck, E., Muller, C., Katz, S. & Leuz, A. (1992) *EMBO. J* **11,** 115-126.

43. Williams, S. C., Baer, M., Dillner, A. J. & Johnson, P. F. (1995) *EMBO J.***14,** 3170-3183.

44. Descombes, P. & Schibler, U. (1991) *Cell* **67,** 569-579.

45. Shields, J. M., Pruit, K., McFall, A., Shaub, A. & Der, C. J. (2000) *Trends Cell Biol.* **10,** 147-154.

46. Joneson, T. & Bar-Sagi, D. (1999) *Mol. Cell. Biol.* **19,** 5892-5901.

47. Xia, Z., Dickens, M., Raingeaud, J., Davis, R. J. & Greenberg, M. E. (1995) *Science* **270,** 1326-1331.

48. Chakravarti, D., Mailander, P., Franzen, J., Higginbotham, S., Cavalieri, E. L. & Rogan, E. G. (1998) *Oncogene* **16,** 3201-3210.

49. Owens, D. M., Spalding, J. W., Tennant, R. W. & Smart, R. C. (1995) *Cancer Res.* **55,** 3171-3178.

50. Rask, K., Thorn, M., Poten, F., Kraaz, W., Sundfeldt, K., Hedin, L. & Enerback, S. (2000) *Inter. J. Cancer* **86,** 337-343.

51. Sundfeldt, K., Ivarsson, K., Carlsson, M., Enerback, S., Janson, P. O., Brannstrom, M. & Hedin, L. (1999) *British J. Cancer* **79,** 1240-1248.

52. Pabst, T., Mueller, B. U., Zhang, P., Radomska, H. S., Narravula, S., Schnittger, S., Behre, G., Hiddemann, W. & Tenen, D. G. (2001) *Nature Genetics* **27,** 263-270.

53. Westendorf, J. J., Yamamoto, C. M., Lenny, N., Downing, J. R., Selsted, M. E. & Hiebert, S. W. (1998) *Mol. Cell. Biol.* **18,** 322-333.

54. Pabst, T., Muller, B. U., Harakawa, N., Schoch, C., Haferlach, T., Behre, G., Hiddemann, W., Zhang, D. E. & Tenen, D. G. (2001) *Nature Med.* **7,** 444-451.

Table 1 Effect of TPA treatment on epidermal cell proliferation in wild-type and C/EBPβ-null mice.

	Nucleated Cell Layers	BrdU Positive Cells (%)
Single Treatment		
Acetone		
Wild-type	1.3 ± 0.1	4.6 ± 1.3
C/EBPβ -/-	1.5 ± 0.1	7.4 ± 3.7
TPA		
Wild-type	1.8 ± 0.3	39.7 ± 8.5
C/EBPβ -/-	2.0 ± 0.9	43.9 ± 3.7
Multiple Treatment		
Acetone		
Wild-Type	1.3 ± 0.1	6.0 ± 1.2
C/EBPβ -/-	1.7 ± 0.2	10.9 ± 5.6
TPA		
Wild-type	3.8 ± 1.6	32.2 ± 7.7
C/EBPβ -/-	3.7 ± 0.6	30.6 ± 8.7

Mice were treated with a single application or thrice weekly for 1 month with 5 nmol TPA/200 μl acetone or with acetone alone. BrdU labeling was conducted by a single dose i.p. injection of BrdU 18 hours after the last TPA treatment, one hour later the animals were euthanized and immunochemical staining of BrdU positive cells was performed (14, 16). Data are expressed as the mean ± SD from at least 3 mice. Each value for wild type mice and similarly treated C/EBPβ null mice within each category was not significantly different ($p>0.05$) as determined by the student's t-test.

Table 2 Apoptosis is significantly elevated in epidermal keratinocytes of DMBA-treated C/EBPβ-null mice.

	Apoptotic Keratinocytes (%)	
	Wild type	C/EBPβ-/-
Acetone treated	0.02 ± 0.02	0.04 ± 0.01
DMBA treated	0.10 ± 0.02[a]	1.73 ± 0.14[ab]

Mice (3/group) were treated with a single application of 400 nmol DMBA/200 µl acetone of acetone alone. More than 4000 basal keratinocytes were counted for each individual mouse.[a] significantly different from acetone-treated group ($p<0.01$) as determined by Student t-test. [b] significantly different from wild type DMBA-treated group ($p<0.01$) as determined by Student's t-test.

Table 3 C/EBPβ enhances oncogenic Ha-ras-induced transformation of NIH-3T3 cells

	Transformed Foci/Dish
10 ng pcDNA3	0.0 ± 0.0
10 ng C/EBPβ	0.0 ± 0.0
10 ng Ha-ras(12V)	35.3 ± 3.5
+ 5 ng C/EBPβ	46.0 ± 7.0*
+ 10 ng C/EBPβ	57.7 ± 1.5*
+ 50 ng C/EBPβ	28.0 ± 5.6
10 ng Ha-ras(12V)	34.7 ± 3.5
+ 10 ng C/EBPβ	58.9 ± 3.8*
+ 10 ng LIP	20.7 ± 2.4*
+ 10 ng C/EBPβ(T188A)	14.0 ± 2.0*
10 ng Ha-ras	25.0 ± 1.0
+ 10 ng C/EBPβ	25.0 ± 4.4
+ 10 ng C/EBPβ	22.3 ± 2.3
100 ng Raf(22W)	29.3 ± 4.5
+ 10 ng C/EBPβ	47.0 ± 1.7*

Data are expressed as transformed foci/plate and each value represents the mean ± SD of triplicate dishes per treatment. All experiments were repeated at least two times and similar results were obtained in each experiment. C/EBPβ, C/EBPβ(T188A) or LIP did not produce any transformed foci at all doses examined (1ng-1000ng/plate). * significantly different from the value of cells transfected with Ha-ras(12V) or Raf (22W) alone as determined by the Student's t-test, $p<0.01$.

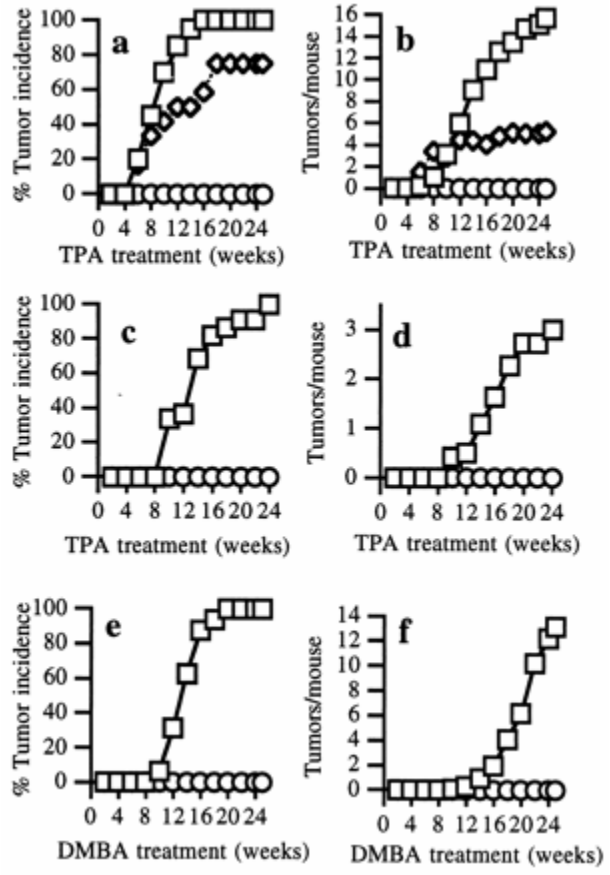

Figure 1 C/EBPβ-null mice are completely refractory to carcinogen-induced skin tumorigenesis.

C/EBPβ null ❍, wild type □ or heterozygous ◊ mice littermates (7-8 weeks old) were treated with: (Panel A/B) a single application of 200 nmol DMBA followed one week later with thrice weekly treatment with 5 nmol TPA (n = 20 C/EBPβ+/+, 21 C/EBPβ -/-, 12 C/EBPβ +/-); (Panel C/D) a single application of 2.5 μmol MNNG followed one week later with thrice weekly application of 5 nmol (n = 22 C/EBPβ +/+, 19 C/EBPβ -/-); (Panel E/F) 100 nmol DMBA once a week for 25 weeks (n = 16 C/EBPβ +/+, 18 C/EBPβ -/-). All agents were applied in 200 μl acetone and all experiments were repeated twice and similar results were obtained.

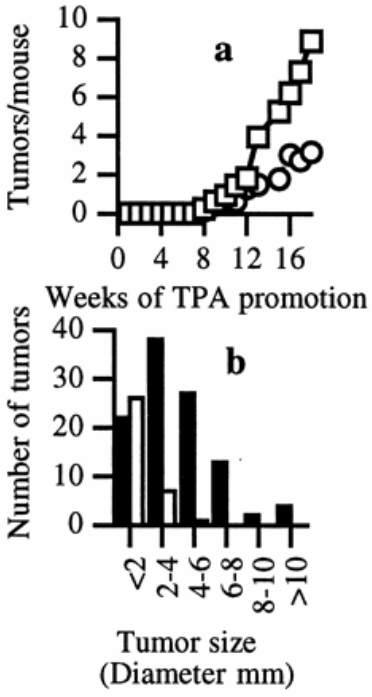

Figure 2 C/EBPβ deficient v-Ha-ras transgenic mice display decreased tumor multiplicity and tumor size

v-Ha-ras +/- C/EBPβ+/+ mice (n=16) and v-Ha-ras+/- C/EBPβ-/- mice (n=14) were treated twice weekly with 5 nmol TPA in 200 ul of acetone. A) Tumor multiplicity in v-Ha-ras+/- C/EBPβ-/- mice ❍ is decreased compared v-Ha-ras +/- C/EBPβ +/+ mice □ ($p<0.05$, F-test) B) Tumor size distribution in v-Ha-ras +/- C/EBPβ+/+ mice ■ and v-Ha-ras+/- C/EBPβ-/- mice □ ($p<0.05$, Fisher's Exact Test).

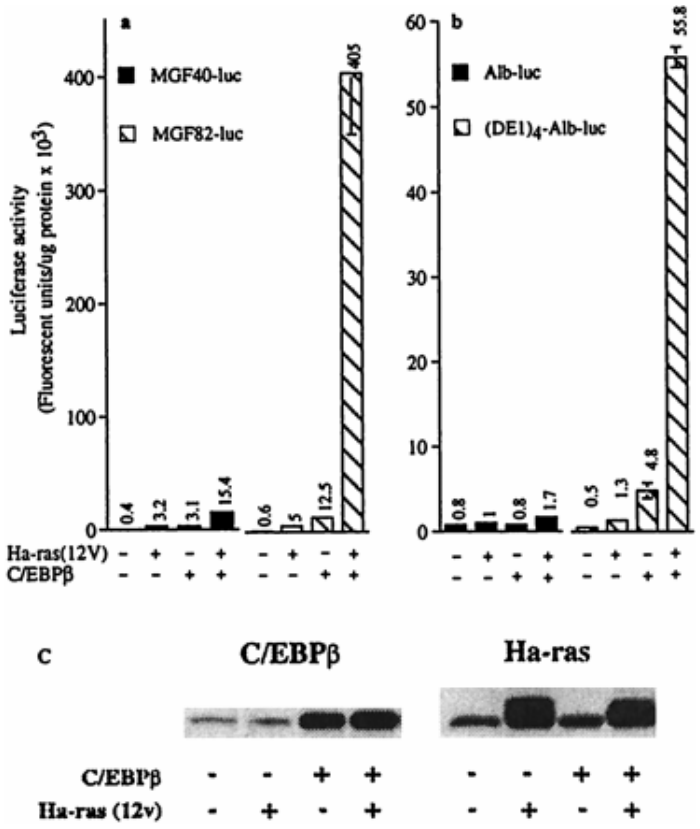

Figure 3 Oncogenic Ha-ras stimulates C/EBPβ transactivation function.

Panel A/B - BALB/MK2 keratinocytes were transfected with pcDNA3-C/EBPβ (0.5 μg) and/or pcDNA3-Ha-ras(12V) (0.5 μg) and 1.0 μg of the specified C/EBP dependent promoter/reporter as described in text. Luciferase activity is expressed as fluorescent units/μg protein and each value represents the mean ± SD of triplicate dishes per treatment. Similar results were obtained from 2 repeat experiments. Inclusion of pSV-β-galactosidase and subsequent normalization of luciferase to β-galactosidase activity produced similar results to those normalized to protein levels. Panel C – BALB/MK2 keratinocytes were transfected with Ha-ras(12V) and/or C/EBPβ and 48 hour later lysates were prepared and western analysis conducted.

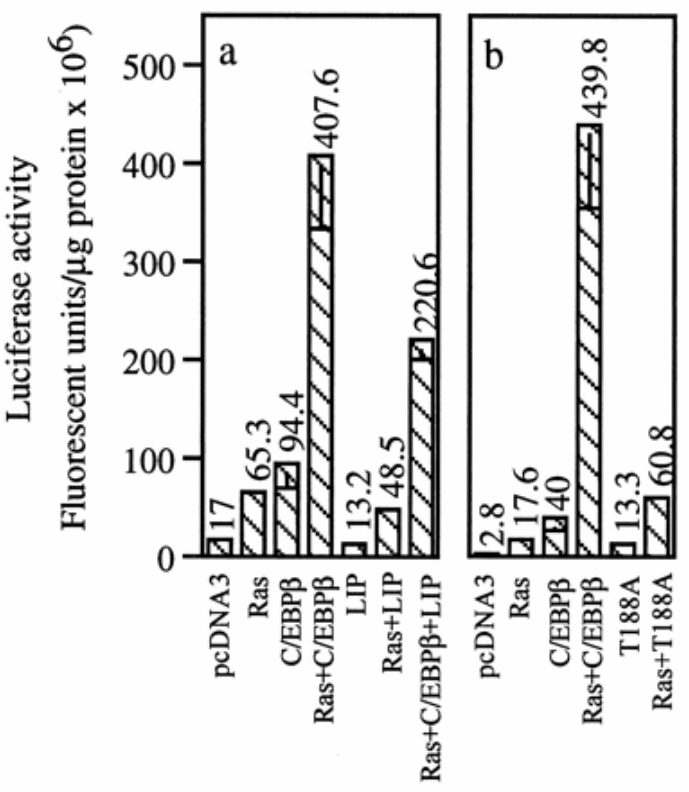

Figure 4 Activation of C/EBPβ by oncogenic Ha-ras involves a threonine 188 and requires the presence of the C/EBPβ transactivation domain.

BALB/MK2 keratinocytes were transfected with 1.0 μg of the promoter/reporter MGF82-luc and 0.5 μg of one or more of the specified vectors. The experimental procedures were carried out as described in Figure 3. Each value represents the mean ± SD of triplicate dishes per treatment. Similar results were obtained from 2 repeat experiments. Inclusion of pSV-β-galactosidase and subsequent normalization of luciferase to β-galactosidase activity produced similar results to those normalized to protein levels.

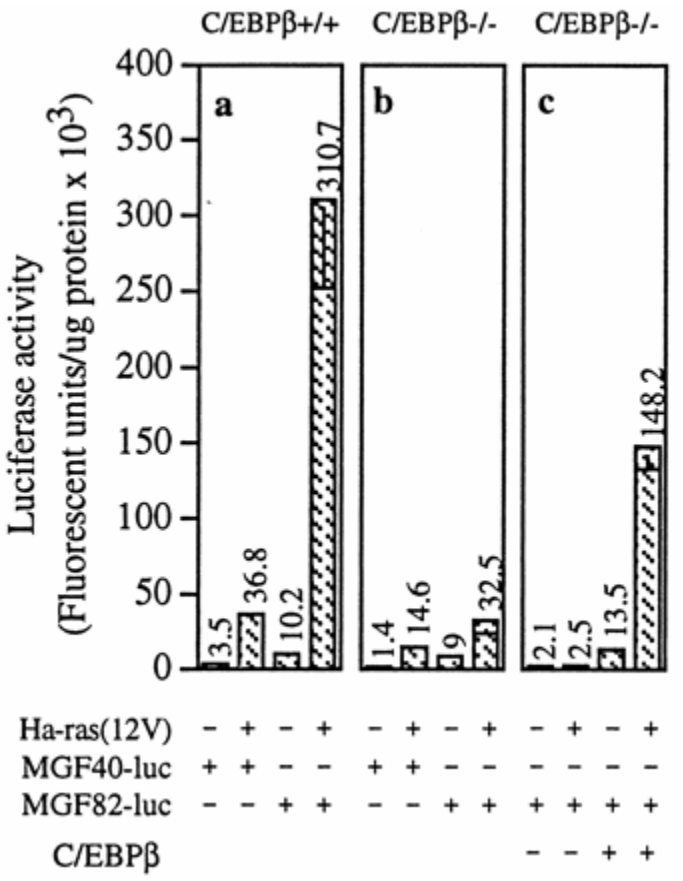

Figure 5 Endogenous C/EBPβ is a downstream mediator of oncogenic Ha-ras signaling in keratinocytes.

Primary keratinocytes were isolated from C/EBPβ-/- or C/EBPβ +/+ newborn littermates (2-3 days old) and cultured as previously described (14, 16). Primary keratinocytes were transfected with the specified vector (0.5 μg each) and 1.0 μg of the C/EBP dependent promoter/reporter vector and processed as described in Figure 3. Each value represents the mean ± SD of triplicate dishes per treatment. Similar results were obtained from 2 repeat experiments.