ABSTRACT

EWING, SARAH JANE. C/EBPβ Represses p53 to Promote Cell Survival Downstream of DNA Damage Independent of Oncogenic Ras and p19Arf. (Under the direction of Dr. Robert C. Smart.)

CCAAT/enhancer binding proteins are members of the basic leucine zipper (bZIP) transcription factor family and function in a number of cellular processes such as proliferation, differentiation, senescence, survival, inflammatory responses, metabolism, and tumorigenesis. C/EBPβ is an important mediator of cell survival and tumorigenesis. This is most apparent in C/EBPβ-/- mice, as these mice respond to carcinogens that produce oncogenic Ras mutations in skin, with vast increases in keratinocyte apoptosis and a complete block in skin tumorigenesis. While elevated apoptosis in C/EBPβ-/- mice results from aberrant increases in p53 levels and function, it is not known whether these increases in p53 result from oncogenic Ras-induced p19Arf, oncogenic Ras-induced activation of DNA damage response pathways, or direct carcinogen-induced DNA damage. We report that p19Arf is dramatically up-regulated in C/EBPβ-/- mouse epidermis and is highly expressed in a sub-population of keratinocytes within the hair follicle. C/EBPβ also repressed a p19Arf promoter reporter in keratinocytes. To determine whether p19Arf is required for altered p53 regulation and apoptosis in C/EBPβ-/- mice, C/EBPβ-/-;p19Arf-/- mice were generated. These mice responded to carcinogen treatment similar to C/EBPβ-/- mice, indicating p19Arf is not required. Moreover, generation of K14-ER:Ras;C/EBPβ-/- mice revealed that oncogenic Ras activation, itself, does not trigger abnormal increases in p53 or apoptosis. In contrast, treatment of C/EBPβ-/- mice with DNA damaging agents such as MNNG and etoposide resulted in
deregulated p53. Similarly, treatment with ultraviolet B radiation produced aberrant increases in p53 and apoptosis in C/EBPβ⁻/⁻ mice compared to wild type. These results indicate that C/EBPβ represses p53 to promote cell survival downstream of DNA damage independent of oncogenic Ras and p19Arf. Additionally, gene expression analysis was conducted to identify putative C/EBPβ target genes in response to DMBA treatment. Our results revealed a novel group of differentially expressed genes in C/EBPβ⁻/⁻ mouse epidermis compared to wild type. The identified genes are involved in processes such as metabolism, keratinization, protein phosphorylation, apoptosis, and gene regulation.
C/EBPβ Represses p53 to Promote Cell Survival Downstream of DNA Damage

Independent of Oncogenic RAS and p19^Arf

by

SARAH JANE EWING

A dissertation submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the Degree of
Doctor of Philosophy

COMPARATIVE BIOMEDICAL SCIENCES

Raleigh, North Carolina

2007

APPROVED BY:

Ronald E. Cannon

Jun Ninomiya-Tsuji

Marjorie F. Oleksiak

Robert C. Smart
Chair Advisory Committee
DEDICATION

I dedicate this dissertation to my grandmother, Goldie Jane Hawthorne Raymond.

She was the first female chemical engineer to graduate from Grove City College in Grove City, Pennsylvania on May 20th, 1944. She was a wonderful person and an inspiration to all who knew her.
BIOGRAPHY

Sarah Jane (Fry) Ewing was born on June 20, 1979 in Greenville, Pennsylvania. She grew up in Espyville, Pennsylvania and graduated in the Class of 1997 from Linesville High School as Valedictorian and Class President. She attended Pennsylvania State University, The Behrend College in Erie, Pennsylvania where she received a Bachelor of Science degree with honors in Biology in 2001, as a University Schreyer Scholar. While pursuing her undergraduate studies, she also conducted and presented research on the genetic diversity of black cherry, Prunus serotina, using microsatellite markers at the International Plant and Animal Genome Conference in San Diego, California in 2001. This work was funded, in part, by a competitive Council of Undergraduate Research Fellowship awarded to Sarah in 2000. Sarah began graduate studies in the Comparative Biomedical Sciences program at North Carolina State University with Dr. Jon Horowitz in 2001. She received a Graduate Assistance in Areas of National Need - Biotechnology Fellowship in December 2001 and her work during this time was later published in the Journal of Biological Chemistry by Dr. K. Scott Moorefield and Dr. Jon M. Horowitz. In January 2003, Sarah began her dissertation research under the
direction of Dr. Robert C. Smart. She actively participated in the North Carolina State University, College of Veterinary Medicine – Graduate Student Association as Vice-President and President as well as the North Carolina State University Graduate Student Association as Vice President of External Affairs. Additionally, she has volunteered as a workshop presenter for the Expanding Your Horizons in Science and Mathematics program for the past six years.
ACKNOWLEDGEMENTS

I would like to extend my sincere gratitude to my advisor, Dr. Robert C. Smart for training me as a scientist. I thank Dr. Jun Ninomiya-Tsuji and Dr. Ron Cannon for serving on my advisory committee and providing valuable advice. I thank Dr. Margie Oleksiak for also serving on my advisory committee and importantly, for her encouragement and guidance. I wish to thank Dr. Rebeca Rufty for much needed encouragement and knowing what I needed to hear as well as Dr. Barbara Sherry for her support and advice. I thank my colleagues, Dr. Songyun Zhu, Dr. Minsub Shim, Dr. Kyungsil Yoon, Tina Powers, Kari Loomis, Elizabeth Thompson, Rakesh Ranjan, Dominique Williams, John House, and Jeanne Burr for their help and support. I would like to thank my parents, parents-in-law, siblings and friends for their support and love. Lastly, my utmost thanks go to my husband, Clint Ewing, for enduring these years with me, his endless support and belief in me, and the many valuable discussions on life.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF TABLES</td>
<td>viii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>ix</td>
</tr>
<tr>
<td>GENERAL INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td><strong>CHAPTER 1</strong> C/EBPβ represses p53 to promote cell survival downstream of DNA damage independent of oncogenic Ras and p19Arf</td>
<td>48</td>
</tr>
<tr>
<td>Abstract</td>
<td>49</td>
</tr>
<tr>
<td>Introduction</td>
<td>51</td>
</tr>
<tr>
<td>Results</td>
<td>55</td>
</tr>
<tr>
<td>Discussion</td>
<td>63</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>68</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>77</td>
</tr>
<tr>
<td>References</td>
<td>78</td>
</tr>
<tr>
<td><strong>CHAPTER 2</strong> Gene expression analysis of carcinogen-treated C/EBPβ−/− mouse epidermis</td>
<td>96</td>
</tr>
<tr>
<td>Introduction</td>
<td>97</td>
</tr>
<tr>
<td>Results</td>
<td>100</td>
</tr>
<tr>
<td>Discussion</td>
<td>104</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>108</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>110</td>
</tr>
<tr>
<td>References</td>
<td>111</td>
</tr>
<tr>
<td><strong>GENERAL DISCUSSION</strong></td>
<td>125</td>
</tr>
</tbody>
</table>
Decreased survival of C/EBPβ-deficient keratinocytes is due to aberrant regulation of p53 levels and function..................................................178

Diminished expression of C/EBPα in skin carcinomas is linked to oncogenic Ras and re-expression of C/EBPα in carcinoma cells inhibits proliferation ......................219
# LIST OF TABLES

## CHAPTER 2

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1</td>
<td>List of genes significantly up-regulated in C/EBPβ^-/- mouse epidermis compared to wild type.</td>
<td>122</td>
</tr>
<tr>
<td>Table 2</td>
<td>List of genes significantly down-regulated in C/EBPβ^-/- mouse epidermis compared to wild type</td>
<td>123</td>
</tr>
<tr>
<td>Table 3</td>
<td>Functional classification of genes differentially expressed in C/EBPβ^-/- mouse epidermis compared to wild type</td>
<td>124</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

CHAPTER 1

Figure 1  p19Arf is specifically deregulated in C/EBPβ−/− mouse epidermis....86

Figure 2  p19Arf is deregulated in C/EBPβ−/− primary epidermal keratinocytes and highly expressed in a sub-population of keratinocytes within C/EBPβ−/− mouse skin .................................................................88

Figure 3  p19Arf is not required for carcinogen-induced anomalous Increases in p53 and apoptosis in C/EBPβ−/− mice.........................90

Figure 4  Oncogenic Ras activation is not sufficient to trigger atypical Increases in p53 and apoptosis in C/EBPβ−/− mice.........................92

Figure 5  DNA damaging agents induce aberrant increases in p53 and apoptosis in C/EBPβ−/− mouse epidermis........................................94

CHAPTER 2

Figure 1  Data normalization and characterization .................................118

Figure 2  Hierarchical clustering .................................................................120

GENERAL DISCUSSION

Figure 1  C/EBPβ−/− mice display aberrant basal and suprabasal epidermal keratinocyte proliferation..................................................132

APPENDIX

Decreased survival of C/EBPβ-deficient keratinocytes is due to aberrant regulation of p53 levels and function

Figure 1  Apoptosis is greatly increased in the epidermis of DMBA-treated C/EBPβ−/− mice.................................................................209

Figure 2  p53 levels are deregulated in C/EBPβ−/− epidermal keratinocytes in response to DMBA treatment ..............................................211
Diminished expression of C/EBPα in skin carcinomas is linked to oncogenic Ras and re-expression of C/EBPα in carcinoma cells inhibits proliferation

Figure 1 C/EBPα protein and mRNA levels are greatly diminished in SCC cell lines ................................................................. 252
Figure 2 Re-expression of C/EBPα induces growth arrest in SCC cell lines ............................................................................. 254
Figure 3 C/EBPα expression is down-regulated in SCCs ................... 256
Figure 4 Decreased C/EBPα expression is associated with oncogenic ras ................................................................. 258
GENERAL INTRODUCTION

Cell signaling and gene regulation are enormously complex processes that are linked through multi-faceted signaling networks and ultimately control a cell’s response to its environment. Studies on the molecules involved in these processes further our understanding of cellular homeostasis and help us elucidate the cellular events that go awry when an organism is overcome by disease. Cells receive their signals from small extracellular molecules, such as growth factors, cytokines or hormones that bind to their receptors or from cell-matrix or cell-cell interactions. Additionally, cells can respond to signals initiated from exposure to environmental stimuli. These signals are transmitted into and through the cell by complex signaling networks that are interconnected to allow for feedback and signal amplification, resulting in the appropriate cellular response. Cells respond to these varying signals by regulating gene expression, altering cell metabolism, stimulating cell movement and polarity, or regulating cell proliferation, differentiation, or death. Regulation of gene expression is critical in facilitating most, if not all, of these cellular responses and is in itself a highly regulated process that can fine-tune the response depending on the signal or cell type involved. Site-specific transcription factors that can be activated or repressed in response to different stimuli are the
major targets of these pathways and are required to activate or inhibit the expression of target genes that ultimately elicit the appropriate cellular response.
CCAAT/enhancer binding proteins (C/EBPs)

CCAAT/enhancer binding proteins are a family of basic leucine zipper (bZIP) transcription factors that respond to a number of physiological and pathophysiological signals by regulating the expression of genes involved in many cellular processes such as proliferation, differentiation, survival, inflammatory responses, and metabolism. The first C/EBP, later designated C/EBPα, was identified as a heat-stable, rat liver nuclear protein capable of binding to regulatory elements, commonly containing the CCAAT box motif, located within promoters and enhancer elements of viral genes [1, 2]. Studies on the C/EBPα sequence led to the discovery of the basic leucine zipper (bZIP) class of transcription factors [3, 4], which also includes Jun/Fos, CREB/ATF, and PAR-domain proteins [5].

There are six known C/EBP family members, C/EBPα (C/EBP) [6-9], C/EBPβ (NF-IL6, IL-6DBP, LAP, CRP2, NF-M, AGP/EBP, ApC/EBP) [7, 8, 10-14], C/EBPδ (NF-IL6β, GRP3, CELF) [7, 8, 15, 16], C/EBPε (CRP-1) [8, 17], C/EBPγ (Ig/EBP-1, GPE1-BP) [18, 19], and C/EBPζ (CHOP, CHOP10, DDIT3, GADD153) [20, 21]. The bZIP domain, located at the C-terminus, is highly homologous (>90%) between C/EBP family members [22], with a notable exception of C/EBPζ, which lacks a
canonical basic region. The basic region consists of ~30 amino acids with a high degree of basicity and dictates the ability of C/EBPs to recognize and bind specific DNA sequences [23]. The optimal C/EBP DNA binding motif was identified as a dyad symmetrical repeat RTTGCGYAAY, where R is A or G, and Y is C or T, although considerable variation is allowed [24]. Protein dimerization through the leucine zipper domain is also required for C/EBP interaction with DNA, as deletion or mutation of this region completely abrogates C/EBP’s DNA binding ability [25]. This domain, consisting of ~35 amino acids, forms an amphipathic parallel coiled coil structure following homo- or heterodimerization between C/EBP family members, which is characterized by four to five heptad leucine repeats, where each heptad consists of two $\alpha$-helical turns or seven amino acids [25-27]. The electrostatic interactions between amino acids along the dimerization interface determine dimer specificity between C/EBPs as well as other bZIP transcription factors [26, 28].

The N-terminus of C/EBPs is much less conserved (<20%) and contains regions responsible for activation or repression of C/EBP activity. These regions are referred to as C/EBP trans-activation domains (TADs) or repression domains
(RDs) [22, 29]. C/EBP\(\gamma\) lacks any known functional domains outside of the bZIP region and functions as a dominant negative inhibitor of C/EBP activity by forming inactive heterodimers with C/EBP family members [30]. C/EBP\(\zeta\) also lacks trans-activation and repression domains and inhibits normal C/EBP function due to its distinct bZIP domain. However, under certain conditions of cellular stress C/EBP\(\zeta\) forms homo- and heterodimers with C/EBP family members to regulate the expression of a unique set of genes [20, 31]. An additional level of complexity arises from the ability of C/EBP\(\alpha\) and C/EBP\(\beta\) transcripts to generate different sized polypeptides, a result of internally initiated translation start sites. The shorter differentially translated products have altered trans-activation potential and/or function as dominant negative inhibitors of C/EBP activity [32-34]. C/EBP\(\alpha\) is translated into two proteins (42 kDa (p42) and 30 kDa (p30)), where p30 has lower activation potential and has been shown to function as an inhibitor of C/EBP activity [33, 34]. C/EBP\(\beta\) generates three isoforms (38 kDa (LAP*), 35 kDa (LAP), and 20 kDa (LIP)). LAP and LIP are the predominantly expressed isoforms, and LIP functions as a dominant negative inhibitor [32].
C/EBPs have simple gene structures. C/EBPα, -β, and -δ genes are intronless, while C/EBPγ, C/EBPε and C/EBPζ genes contain two, two, and four exons, respectively [6-21, 35, 36]. C/EBPγ and C/EBPζ are ubiquitously expressed [18, 20]; whereas, C/EBPε expression is restricted to granulocytic cells of the hematopoietic system [17, 35-37]. C/EBPβ is rather widely expressed; its expression is particularly high in skin, liver, intestine, lung, adipose tissue, heart, kidney, spleen, and hematopoietic tissue [7, 10-13, 38-40]. C/EBPα expression is found at high levels, particularly in terminally differentiated cells, in adipose tissue, liver, intestine, lung, placenta, and skin [7, 9, 38, 39, 41]. C/EBPδ is detected in adipose tissue, lung and intestines, and can be induced in most cell types by inflammatory and/or stress stimuli [7, 16, 42-47]. The expression and activity of C/EBP family members is carefully regulated at both the transcriptional and post-translational levels by a multitude of signal transduction pathways, modulating C/EBP activity to effectively target the expression of genes required to induce the appropriate cellular response [22, 48].
CCAAT/enhancer binding protein beta (C/EBPβ)

C/EBPβ (NF-IL6) was originally identified and cloned as a nuclear factor capable of binding to an IL-1 responsive element of the IL-6 gene from human monocytes [10]. These studies revealed that C/EBPβ expression is increased by acute-phase inducers, lipopolysaccharide (LPS), IL-1, or IL-6, and that C/EBPβ binds to regulatory regions of genes involved in the acute-phase response and inflammation [10]. Three subsequent reports, published within the same year, revealed the identification and cloning of the rat (LAP and IL6-DBP) [12, 13] and mouse (AGP-EBP) [11] homologs. These studies provided additional support that C/EBPβ is an IL-6-responsive DNA binding protein and trans-activator of acute-phase genes and cytokines [11, 13] and demonstrated that C/EBPβ is involved in the regulation of albumin gene expression [12]. Since the identification and initial characterization of C/EBPβ, many studies have sought to elucidate the function of C/EBPβ in diverse cell types using target gene promoter analysis, inhibition or overexpression of C/EBPβ in cell-culture model systems, and genetically modified mice. These studies have revealed important functions for C/EBPβ in the processes of metabolism, inflammatory responses, cellular differentiation, and
proliferation/cell survival.

- **Metabolism**

  C/EBP\(\beta^{-/-}\) mice were initially reported to appear healthy when kept in specific pathogen-free facilities. However, these studies noted that homozygous C/EBP\(\beta^{-/-}\) mice were observed at a frequency lower than the expected Mendelian ratio [49, 50]. Further studies by Croniger et al [51] described two distinct C/EBP\(\beta^{-/-}\) mouse phenotypes (A and B), characterized by their ability to mobilize glycogen shortly after birth. C/EBP\(\beta^{-/-}\) (A) mice have normal glucose homeostasis, whereas, C/EBP\(\beta^{+/-}\) (B) mice have severe hypoglycemia, fail to induce phosphoenolpyruvate carboxykinase (PEPCK) expression and die within two hours after birth. The basis for the different C/EBP\(\beta^{-/-}\) phenotypes is not known. However, it is assumed that “modifier genes” present in the genetic background of C/EBP\(\beta^{-/-}\) (A) mice, which are not inbred, are responsible [52]. The surviving, adult C/EBP\(\beta^{-/-}\) (A) mice display a critical reduction in hepatic cyclic-adenosine monophosphate (cAMP) production, even after stimulation with glucagon [53]. Additionally C/EBP\(\beta^{-/-}\) (A) mice display hypoglycemia and reduced levels of hepatic glucose production and plasma free fatty acids after fasting and demonstrate impaired adipose tissue lipolysis following
administration of epinephrine [53]. These studies suggested that the abnormalities observed in C/EBP\(\beta^{-/-}\) (B) mice may be due to the inability to maintain normal hepatic cAMP levels, providing an explanation for the lack of normal glucose homeostasis in these mice. Accordingly, further studies revealed that the phenotype of C/EPB\(\beta^{-/-}\) (B) newborn pups could be immediately reversed following administration of cAMP, and that C/EBP\(\beta^{-/-}\) (A) mice display increased levels of phosphodiesterase activity, resulting in enhanced degradation of cAMP following glucagon treatment [54]. cAMP is a potent inducer of C/EBP\(\beta\) expression [55-58].

C/EBP\(\beta\)’s ability to indirectly facilitate cAMP degradation may function as a negative feedback loop to control cAMP-mediated cell signaling. It should also be noted, that C/EBP\(\beta^{-/-}\) mice have lower than normal levels of blood urea nitrogen (BUN) and ammonia after fasting, indicating C/EBP\(\beta\) may also play an important role in amino acid metabolism [54].

- **Inflammatory Responses**

  C/EBP\(\beta\) activity and expression is regulated by a number of inflammatory agents, and a wide range of cytokines [59]. Increased C/EBP\(\beta\) expression by inflammatory agents generally coincides with the induction of C/EBP\(\delta\) [42, 45].
Together these two C/EBPs play an important role in the induction of acute-phase
genes as well as various other genes involved in inflammation [45, 59, 60]. Indeed,
as described previously, C/EBPβ was identified by its ability to respond to IL-6 and
IL-1 by binding to the regulatory elements of acute-phase genes [10-13]. C/EBP β
and –δ expression are strongly induced at the transcriptional level by inflammatory
stimuli such as LPS and cytokines such as IL-6, IL-1, and TNF-α [59]. Whereas,
increased C/EBPδ expression, alone, is capable of inducing the expression of
acute-phase target genes, it appears that post-translational modification of
increased C/EBPβ protein is important in eliciting C/EBPβ’s full potential as a
transactivator of acute-phase genes [45]. For example, IL-6-induced
phosphorylation of C/EBPβ through a Ras/mitogen-activated protein kinase (MAPK)
pathway augments C/EBPβ’s trans-activation potential [61].

C/EBP binding sites have been identified in the regulatory regions of a large
number of genes involved in the inflammatory response, including genes coding for
cytokines and their receptors such as IL-6, IL-1β, TNF-α, IL-8, and IL-12 as well as
most class I acute-phase genes such as hemopexin, haptoglobin, α1-acid
glycoprotein, serum amyloid A1, A2, and A3, complement C3, and C-reactive
protein [45]. Additionally, C/EBP binding sites are found in genes that encode for proteins important in macrophage and granulocytic functions such as the inducible nitric oxide synthase, lysozyme, myeloperoxidase, and neutrophil elastase [45]. The use of C/EBPβ−/− mice has defined a critical role for C/EBPβ in the regulation of a subset of these genes, although functional compensation by other family members as well as other regulatory transcription factors has impeded the identification of additional C/EBPβ target genes following activation by inflammatory stimuli. C/EBPβ−/− mice display reduced serum amyloid A and P expression, α1-acid glycoprotein, G-CSF and TNF-α, and the expression of complement C3 is completely abrogated [22, 62]. These studies also demonstrate that C/EBPβ is more important in maintaining the induced levels of serum amyloid A and P and α1-acid glycoprotein, rather than eliciting the initial induction of these genes during the acute-phase response [62].

Surprisingly, C/EBPβ−/− mice display high levels of IL-6, indicating C/EBPβ is dispensable for IL-6 expression [50]. Accordingly, C/EBPβ−/− mice develop a pathology similar to mice overexpressing IL-6, which is nearly identical to Multicentric Castleman’s disease in humans and is characterized by splenomegaly,
peripheral lymphadenopathy, and enhanced hematopoiesis [50]. C/EBP\(\beta\)\(^{-/-}\) mice are highly susceptible to infection with Candida albicans, Listeria monocytogenes, and Salmonella typhi and reduced survival from these pathogens is partly due to defects in macrophage activation, resulting in impaired macrophage function [49, 50]. Macrophages from C/EBP\(\beta\)\(^{-/-}\) mice also display impaired tumoricidal and tumoristic activity [49], and splenic macrophages display reduced nitric oxide production and low levels of IL-12, resulting in an impaired Th1 immune response [50].

- **Cellular Differentiation**

  C/EBPs play an essential role in the differentiation of a wide range of cell types including adipocytes, myelomonocytic cells, hepatocytes, mammary epithelial cells, ovarian luteal cells, keratinocytes, neuronal cells, and intestinal epithelial cells [40, 63-72]. C/EBP\(\beta\) is involved in the differentiation and maturation of many of these cell types. Adipocyte differentiation has been studied extensively, using the 3T3-L1 pre-adipocyte/adipocyte cell line [73]. These cells are induced to differentiate by the addition of adipogenic hormones that propel the cells into two rounds of synchronized proliferation/mitosis (referred to as mitotic clonal
expansion), followed by growth arrest and expression of adipocyte-specific
differentiation markers [73]. C/EBPβ and –δ are rapidly induced following
stimulation of adipocyte differentiation [7]; these cells then undergo mitotic clonal
expansion followed by transcriptional activation of C/EBPα [73]. Induction of

C/EBPα results in the expression of many genes involved in the differentiation and
maturation of these cells into adipocytes [70].

C/EBPβ is an important mediator of adipocyte differentiation as ectopic
expression of C/EBPβ, but not C/EBPδ, in 3T3-L1 pre-adipocytes is sufficient to
induce adipocyte differentiation, in the absence of key adipogenic hormones [74].
Additionally, mouse embryonic fibroblasts lacking both C/EBPβ and –δ are unable
to differentiate into adipocytes in response to stimulation with adipogenic hormones,
and fail to induce the expression of C/EBPα as well as other important adipocyte
regulatory proteins and differentiation specific genes [75]. C/EBPβ−/− mice have mild
defects in adipocyte differentiation, while deletion of C/EBPβ and –δ in vivo
produced a more severe differentiation defect; however, these mice induce normal
levels of C/EBPα [75]. Thus, although C/EBPβ is an important factor during the
initiation of adipocyte differentiation, the exact role(s) for C/EBPβ, especially
involving the induction of C/EBP$\alpha$, is not clearly established. Studies have shown that C/EBP$\beta$ is required for mitotic clonal expansion during adipogenesis [76], and that during this time, C/EBP$\beta$ DNA-binding ability is cell cycle-dependent as a result of interaction with hypophosphorylated Rb [77]. These studies provide a link between mitotic clonal expansion and initiation of adipocyte differentiation through the regulation of C/EBP$\beta$, but it is not known why inhibition of C/EBP$\beta$’s DNA-binding ability by Rb is required.

C/EBPs also have an important role in the differentiation of myeloid cells. C/EBP$\beta$, -$\alpha$, -$\delta$, and -$\epsilon$ are up-regulated during the differentiation of myeloid cells [40]. C/EBP$\beta$ is induced during macrophage differentiation [68] and plays an important role in activation of macrophages as described above [49]. C/EBP$\beta^{-/-}$ mice do not display defects in normal granulopoiesis [49, 50], however, C/EBP$\beta$ is essential for cytokine- and infection-induced granulopoiesis [78].

Studies using C/EBP$\beta^{-/-}$ mice have also defined a role for C/EBP$\beta$ in the differentiation of mammary epithelial cells [66, 67]. C/EBP$\beta^{-/-}$ mice lack the ability to activate the expression of milk protein genes, and have multiple defects in mammary gland development such as impaired ductal morphogenesis and severe
inhibition of lobuloalveolar development [66, 67]. Additionally, adult C/EBPβ−/− female mice are sterile due to a defect in the ability of granulosa cells to differentiate into luteal cells in response to luteinizing hormone, in the ovary [65].

C/EBPβ is abundantly expressed in mouse epidermis and its expression pattern in mouse skin is suggestive of a role for C/EBPβ in the differentiation of epidermal keratinocytes [39]. C/EBPβ expression is highly ordered and relatively exclusive to the nuclei of three-cell clusters of suprabasal keratinocytes found at regular intervals throughout the epidermis, a pattern that is consistent with the central suprabasal column of the epidermal proliferative unit (EPU) [39, 79, 80]. Within the EPU, differentiation is strictly coordinated with proliferation, specifically allowing movement of a post-mitotic basal keratinocyte to migrate into the suprabasal layer, eventually forming a stack of three post-mitotic keratinocytes at different stages of terminal differentiation. In cultured keratinocytes, terminal differentiation can be induced with high levels of extracellular calcium [81, 82]. C/EBPβ expression in keratinocytes is induced by high calcium and its expression is required for calcium-induced growth arrest [38, 39, 63]. Additionally, ectopic expression of C/EBPβ in keratinocytes induces growth arrest and differentiation-
associated morphological changes [63]. C/EBPβ−/− mice display reduced levels of keratin 1 (K1) and keratin 10 (K10) gene expression [63], two early markers of epidermal keratinocyte differentiation. Further studies demonstrated that C/EBPβ can modulate the expression of K1 and K10 [63], and identified functional C/EBP binding sites within the K10 promoter [83]. Terminal differentiation of keratinocytes can also be induced by detachment of keratinocytes from the basement membrane, and integrin expression is normally down-regulated during this time [84]. As such, C/EBPβ inhibits the expression of α2 and α5 integrin genes through C/EBP binding sites identified within their promoter regions [85]. C/EBPβ also represses the expression of peroxisome proliferator-activated receptor β/δ (PPARβ/δ), which normally functions downstream of proliferative stimuli, a process that is incompatible with keratinocyte differentiation [86]. Thus, C/EBPβ functions to promote epidermal keratinocyte differentiation through activation and repression of gene expression.

- **Proliferation/Cell Survival**

C/EBPβ has both positive and negative effects on cell proliferation. In keratinocytes, C/EBPβ is involved in growth arrest associated with differentiation [63]. Additionally, C/EBPβ−/− mice display mild epidermal hyperplasia and primary
C/EBPβ−/− epidermal keratinocytes proliferate more rapidly and grow to a higher saturation density in culture compared to wild type cells [63]. Forced expression of C/EBPβ has also been shown to induce growth arrest of HepG2 hepatocarcinoma cells [87]. C/EBPβ−/− mouse embryonic fibroblasts (MEFs) display increased proliferation in culture and fail to undergo oncogenic Ras-induced senescence [88]. C/EBPβ induces growth arrest in MEFs through a mechanism requiring Rb and E2F repressor complexes and may involve repression of E2F target genes involved in proliferation [88]. C/EBPβ also appears to inhibit cell proliferation in specific types of leukemic cells [89, 90]. For example, ectopic expression of C/EBPβ in BCR/ABL-transformed granulocytic cells inhibited proliferation and promoted differentiation [89], and C/EBPβ expression was required for all-trans retinoic acid (ATRA)-induced differentiation of acute promyelocytic leukemia cells that carry the PML-RARA gene fusion [90].

In contrast, many studies reveal a critical role for C/EBPβ in the positive regulation of proliferation. As mentioned previously, C/EBPβ expression is required for mitotic clonal expansion during adipocyte differentiation [76]. C/EBPβ is also important in the proliferation of mammary epithelial cells, as C/EBPβ−/− female mice
display impaired proliferation of these cells during ductal morphogenesis [66, 67], and C/EBPβ is critical for hormone-induced proliferation of epithelial and stromal cells of the uterus [91]. In hepatocytes, C/EBPβ functions to promote TGF-α induced proliferation and its expression is required for proliferation and liver regeneration following partial hepatectomy [92, 93]. Lastly, C/EBPβ−/− B cells display impaired expansion in long-term cultures, and decreased proliferative responses to IL-7 [94].

C/EBPβ expression and function is also involved in promoting proliferation related to tumorigenesis. Studies utilizing C/EBPβ−/− mice provided the first genetic evidence that C/EBPβ is required for tumor development, as these mice are completely refractory to skin tumorigenesis initiated by carcinogens such as 7, 12-dimethylbenz[a]anthracene (DMBA) that function through their ability to produce oncogenic Ras mutations [95]. Additionally, C/EBPβ−/− mice that carry the v-Ha-ras transgene displayed significantly reduced tumorigenesis [95], linking oncogenic Ras to C/EBPβ \textit{in vivo}. C/EBPβ also cooperates with oncogenic Ras to promote cellular transformation, and this requires cell cycle-dependent phosphorylation of C/EBPβ
on Ser^{64} and Thr^{189} [96]. Other cell types are also involved in C/EBPβ-mediated cellular transformation. For example, C/EBPβ^-/- macrophages are resistant to transformation as a result of decreased proliferation and survival, whereas overexpression of C/EBPβ in transformed macrophages results in hyper-proliferation [97]. Additionally, ectopic expression of C/EBPβ in mammary epithelial cells induces aberrant proliferation and transformation and promotes an epithelial to mesenchymal transition [98].

In humans, overexpression or aberrant activity of C/EBPβ is associated with breast, colorectal, ovarian, and gastric tumorigenesis as well as invasiveness of renal cell carcinoma and prognosis in glioma patients [98-106]. Additionally, increased expression of C/EBPβ in Wilms tumors, or nephroblastomas, is associated with primary tumor relapse [107]. Although many of these studies provide a link between C/EBPβ overexpression or increased activity and human cancer, causality has not yet been demonstrated. C/EBPβ is also a key mediator of Cyclin D1 activity in human cancer [108]. C/EBPβ interacts with Cyclin D1 and this contributes to the unique gene expression patterns observed in human cancers overexpressing Cyclin D1 [108]. Collectively, these studies provide ample evidence
indicating that C/EBPβ has normal proliferative functions as well as pro-oncogenic functions in various cell types. However, it is less clear how C/EBPβ promotes tumor development and/or tumor maintenance.

C/EBPβ has been shown to be an important mediator of cell survival, which provides one mechanism through which C/EBPβ may function to promote tumorigenesis. For example, C/EBPβ promotes mouse epidermal keratinocyte cell survival in response to topical treatment with the carcinogen, DMBA, through a mechanism that requires repression of p53 levels and function [95, 109]. It was proposed from these studies that C/EBPβ may be required for survival of DMBA-initiated tumor precursor cells containing oncogenic Ras [95]. Accordingly, C/EBPβ-deficiency would result in apoptosis of tumor precursor cells, accounting for the lack of tumor development in these mice. In support of this, a recent study using a tetracycline system to conditionally express A-CEBP, a dominant negative that inhibits DNA binding activity of C/EBP family members, showed that inhibition of C/EBP DNA binding activity in mouse epidermis inhibited tumorigenesis involving oncogenic Ras. In addition, similar inhibition in DMBA-initiated tumors led to increased p53, apoptosis, and tumor regression [110]. Although these studies
demonstrate that C/EBP activity is important for tumor development and/or tumor maintenance involving oncogenic Ras, these studies fail to determine which C/EBP family member is required for these effects or the mechanism through which C/EBPs function to promote tumor development.

Additional studies have demonstrated the importance of C/EBP\(\beta\) in cell survival and provide more evidence indicating C/EBP\(\beta\)-mediated cell survival is involved in the maintenance of tumor cells. In CCl\(\text{4}\)-treated hepatic stellate cells, phosphorylation of C/EBP\(\beta\) on Thr\(^{217}\) by ribosomal protein 6-kinase (RSK) produces a functional XEXD caspase substrate/inhibitor box that is required for survival [111]. Survival of osteocytes following bisphosphonate treatment also requires RSK-phosphorylation of C/EBP\(\beta\) at this site [112]. In Myc/Raf-transformed macrophages, C/EBP\(\beta\) promotes proliferation and survival through an auto-regulatory pathway involving IGF-1 [97]. Additionally, survival following stimulation with tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)), is in part mediated through the regulation of manganous superoxide dismutase (MnSOD) gene expression by C/EBP\(\beta\) [113]. In human cells, acute loss of C/EBP\(\beta\) in a cell line derived from a human metastatic Wilms tumor led to spontaneous apoptosis [107], and C/EBP\(\beta\) is necessary to sustain growth and
survival of anaplastic lymphoma kinase-positive (ALK+) anaplastic large cell lymphoma (ALCL) cells [114].

Alternative mechanisms through which C/EBPβ may promote tumorigenesis include C/EBPβ-mediated inhibition of oncogene-induced senescence or inhibition of differentiation in tumor precursor cells. Results from previous studies (summarized above) suggest C/EBPβ is unlikely to function through these mechanisms. Briefly, C/EBPβ functions to promote differentiation of many cell types, and C/EBPβ is required for oncogenic Ras-induced senescence in mouse embryonic fibroblasts. However, it is noteworthy to mention that evidence does exist supporting the notion that DNA-binding proteins can have opposing functions with regards to oncogene-induced senescence, depending on cell type. For example, DRIL1, a member of the ARID family of DNA binding proteins, inhibits oncogenic Ras-induced senescence in mouse fibroblasts, whereas it induces senescence in human fibroblasts [115, 116]. Additionally, JunD, a member of the Jun family and a fellow bZIP transcription factor, displays positive and negative effects on proliferation, depending on the cellular and genetic context [117]. Although further studies are required to determine the exact mechanism(s) through
which C/EBPβ functions to promote tumor development and/or tumor maintenance, current evidence suggests that C/EBPβ-mediated cell survival is likely to contribute.

C/EBPβ’s ability to promote cell survival and tumorigenesis is readily apparent in C/EBPβ−/− mice, which respond to carcinogens that produce oncogenic Ras mutations in skin with vast increases in apoptosis and a complete block in tumorigenesis. While it is known that elevated apoptosis in C/EBPβ−/− mice is a result of aberrant increases in p53 levels and function, many questions still remain regarding the role of C/EBPβ in cell survival. For example, what signals elicit C/EBPβ’s role as a pro-survival/anti-apoptotic protein? What is the mechanism through which C/EBPβ represses p53 levels and function? Are there novel C/EBPβ target genes involved in this response?

**Cell Signaling and Regulation of C/EBPβ**

C/EBPβ is regulated at multiple levels including through transcriptional, translational, and post-translational mechanisms as well as through protein-protein interactions [22, 48]. Post-translational modifications and protein-protein interactions can affect C/EBPβ’s cellular localization, DNA-binding ability, and activation potential [22, 48]. Regulation of C/EBPβ can occur downstream of
various signaling factors such as growth factors, cytokines, hormones, nutrients, and agents that cause cellular stress [22, 48].

C/EBPβ is regulated at the transcriptional level by several factors involved in activating the acute-phase response (IL-1, IL-6, LPS, IFN-γ), stimulating carbohydrate metabolism (cAMP, glucagon), or initiating other cellular responses such as differentiation and proliferation (glucocorticoids, growth hormone, nerve growth factor (NGF)) [22, 48]. Two regulatory cAMP-response element (CRE)-like sequences in the C/EBPβ promoter mediate the response to cAMP through protein kinase A (PKA)-induced phosphorylation and activation of cAMP-response element binding protein (CREB) [56]. These sites are also required for IL-6-induced C/EBPβ expression in the acute-phase response by a mechanism involving signal transducer and activator of transcription-3 (STAT-3) [118]. Lastly, the C/EBPβ promoter is subject to auto-regulation [119-121].

C/EBPβ can be regulated by post-translational modifications such as phosphorylation [22, 48], acetylation [122-125], and sumoylation [126-128]. C/EBPβ has three N-terminal transcriptional activation domains and two negative regulatory domains that form a tightly folded conformation [129, 130]. This allows
the negative regulatory domains to interact with the trans-activation domains or the bZIP domain, thereby repressing C/EBPβ transcriptional activity [129, 130]. Negative regulation of C/EBPβ can be relieved through phosphorylation of the repression domains [130]. Many studies have demonstrated that C/EBPβ’s trans-activation potential can be enhanced by phosphorylation, although the phosphorylation site(s) has not always been elucidated. Phosphorylation can augment C/EBPβ’s trans-activation potential by targeting it for nuclear localization, enhancing its DNA-binding activities, or stimulating its intrinsic trans-activation potential [22, 48].

Phosphorylation of C/EBPβ was first described in rat PC12 cells treated with forskolin [131]. Forskolin-induced phosphorylation of C/EBPβ stimulated translocation of C/EBPβ from the cytosol to the nucleus [131]. It was proposed that forskolin-induced phosphorylation of C/EBPβ occurs on Ser299 through cAMP-dependent kinase (PKA) [131]. Similar redistribution of C/EBPβ may, in part, be responsible for regulating C/EBPβ during macrophage activation; although C/EBPβ may be constitutively phosphorylated during this time [132-134]. Additionally, translocation of C/EBPβ to the nucleus occurs downstream of TNF-α in hepatocytes.
and in response to antioxidants in human colon cancer cells [135, 136]. Antioxidant-induced redistribution of C/EBPβ is also likely to occur through PKA-mediated phosphorylation of C/EBPβ at Ser^{299} [136]. PKC phosphorylates this site as well [137, 138], suggesting that PKC activation may modulate C/EBPβ's activity by altering its localization within the cell. Studies examining mouse C/EBPβ phosphorylation and activity in response to growth hormone revealed that C/EBPβ phosphorylation by mitogen-activated protein kinase (MAPK) at Thr^{188} results in the redistribution of C/EBPβ within the nucleus. Phosphorylation of this site led to a punctate pattern of C/EBPβ-localization in the nucleus, where C/EBPβ bound to C/EBP binding sequences within peri-centromeric heterochromatin [139, 140]. In contrast, TNF-α-induced phosphorylation of C/EBPβ on Ser^{239} negatively regulates its activity by inducing its nuclear export [141].

PKA and PKC phosphorylation of rat C/EBPβ at Ser^{240} (Ser^{299} in human C/EBPβ) inhibits C/EBPβ DNA binding ability in vitro, suggesting that although phosphorylation of C/EBPβ at this site stimulates translocation of C/EBPβ to the nucleus, once there, C/EBPβ may not be able to efficiently bind to its target DNA binding motif(s) [137]. Additional studies have provided conflicting data regarding
cAMP/PKA-dependent phosphorylation of C/EBPβ [142, 143], bringing in to question the role of PKA in regulating C/EBPβ activity and function. Phosphorylation of C/EBPβ by glycogen-synthase kinase 3 (GSK-3) also inhibits C/EBPβ DNA binding activity [144]. Inhibition of GSK-3 by growth hormone-induced activation of the phosphatidylinositol 3-kinase (PI3K)/AKT pathway results in dephosphorylation of C/EBPβ and stimulation of its DNA binding ability in a pre-adipocyte/adipocyte cell line [144]. Thus, growth hormone can modulate C/EBPβ DNA binding ability and transcriptional activation through both dephosphorylation, involving inhibition of GSK-3, and phosphorylation, mediated by MAPK [140, 144]. Similarly, inhibition of GSK-3 by cGMP-dependent protein kinase also facilitates dephosphorylation of C/EBPβ, stimulating its DNA binding and transcriptional activities [145].

Much evidence supports a role for phosphorylation in the regulation of C/EBPβ’s intrinsic trans-activation potential. For example, phosphorylation of mouse C/EBPβ on Ser276 by calcium/calmodulin-dependent kinase stimulates C/EBPβ’s transcriptional activity, without altering C/EBPβ’s localization or DNA binding ability [142]. Phosphorylation of C/EBPβ on Ser105 downstream of PKC
activation by phorbol esters also induces C/EBPβ trans-activation capacity without affecting its DNA binding activity [143]. Phorbol ester-induced activation of PKC has also been shown to stimulate p90 ribosomal S6 kinase (RSK)-mediated phosphorylation of C/EBPβ, which in this case results in increased C/EBPβ DNA binding and activation of C/EBP elements within the IGF-1 promoter [146]. C/EBPβ transcriptional activation is greatly induced following phosphorylation on Thr^{235} (Thr^{188} in mouse) after stimulation of the Ras/MAPK pathway by IL-6 [61] and phosphorylation of this site following activation of MAPK by several additional factors results in derepression of C/EBPβ activity [130]. This site is also phosphorylated in response to glucocorticoid treatment in lung epithelial cells; although the exact mechanism involved in glucocorticoid-induced phosphorylation of this site is unclear [147].

These studies have facilitated our understanding of C/EBPβ activation, but the physiological role of C/EBPβ downstream of these modifications is less clear. Further analysis and the use of C/EBPβ^{-/-} mice have helped to elucidate how C/EBPβ phosphorylation mediates C/EBPβ’s ability to induce specific cellular responses. Indeed, studies have shown that regulation of C/EBPβ downstream of
GSK-3β, TGF-α, RSK, and Ras/MAPK is important for the physiological and pathophysiological functions of C/EBPβ [93, 95, 96, 111, 112, 148-152]. For example, regulation of C/EBPβ through these pathways can modulate C/EBPβ's ability to function in adipocyte [148] and myelomonocytic cell differentiation [151], cortical cell fate [149, 150], survival [97, 111, 112], proliferation [93, 152], cellular transformation [95, 96], and tumorigenesis [95].

Phosphorylation of C/EBPβ by RSK is required for cell survival in CCl4-treated hepatocytes as well as in osteoblasts following bisphosphonate treatment [111, 112], and RSK is a downstream target of Ras-MAPK/ERK signaling [153, 154]. Additionally, C/EBPβ functions in macrophage cell survival downstream of oncogenic Raf, which is a key mediator of Ras-induced MAPK/ERK signaling [97, 155]. These studies imply that C/EBPβ may function to promote cell survival downstream of aberrant Ras-Raf-MAPK/ERK signaling and in some cases, involves phosphorylation by RSK. However, other signaling pathways may also contribute to C/EBPβ-mediated cell survival. For example, although the biological significance of PKC-mediated regulation of C/EBPβ trans-activation of IGF-1 expression is not
known, it could function to promote C/EBPβ-mediated cell survival, as was seen in macrophages transformed with Myc/Raf [97, 146].

Treatment with the carcinogen, DMBA, induces dramatic increases in apoptosis in C/EBPβ−/− mice as a result of aberrant p53 levels and function [95]. DMBA is a polycyclic aromatic hydrocarbon that is metabolized to produce a bay-region diol epoxide that attaches to DNA, forming a DNA bulky adduct. Replication of DNA prior to repair of the DMBA-induced DNA damage, or misrepair of the lesions, results in mutation of the DNA at these sites. Importantly, treatment of mouse skin with DMBA produces DNA-adducts within the Ras gene resulting in oncogenic mutations in Ras and its constitutive activation [156-159]. The ability of DMBA to produce oncogenic Ras mutations is critical for its role in the initiation of chemical carcinogenesis [160-162]. It is not clear whether DMBA-induced activation of oncogenic Ras or direct DMBA-induced DNA damage is responsible for increased p53 and apoptosis in C/EBPβ−/− mice.

Ras signaling

Ras is a member of a large superfamily of small guanosine triphosphatases (GTPases) that is divided into five major branches, Ras, Rho, Rab, Ran, and Arf
In the Ras sub-family, only H-Ras, N-Ras, and K-Ras are commonly mutated in human cancer and carcinogen-induced rodent tumors. In humans, mutations are observed in approximately 30% of all cancers, and occur in high frequency in cancers of the pancreas (60%), colon (32%), lung (19%), ovary (17%), and bladder (11%) [155, 164].

Ras proteins are targeted to the plasma membrane where they function as binary molecular switches through their ability to switch between guanosine diphosphate (GDP)-bound and guanosine triphosphate (GTP)-bound states. Ras-GDP is inactive. When stimulated by upstream signals, guanine nucleotide exchange factors (GEFs) stimulate release of GDP from Ras in exchange for GTP, resulting in Ras activation. This process is reversed by Ras’s intrinsic GTPase activity and other GTPase activating proteins (GAPs), which promote hydrolysis of GTP back to GDP.

Ras proteins function within large signaling networks, coupling receptor activation by diverse extracellular stimuli to a multitude of divergent cytoplasmic signaling cascades that control cellular processes such as proliferation, differentiation, senescence, and survival, often through regulation of gene
Activated Ras binds to and activates Raf kinase, initiating the Ras/Raf/MAPK signaling cascade. Raf transfers the signal by serine phosphorylation of MAP kinase-extracellular signal regulated kinase (MEK1), a dual specificity kinase that phosphorylates threonine and tyrosine residues on extracellular regulated MAP kinases (ERK1 and ERK2). ERK1/ERK2, serine/threonine kinases, in turn, phosphorylate a vast number of cellular substrates, including RSK as well as a large number of transcription factors such as C/EBPβ, ELK1, SAP-1, and ETS. Phosphorylation of these transcription factors result in the expression of genes needed to elicit the appropriate cellular response induced by the initial activation of Ras. For example, activation of this pathway is sufficient to induce cellular transformation or differentiation in many cell types [165-167]. An important gene induced by Ras signaling is Cyclin D1. Cyclin D1 is rapidly induced downstream of Ras to promote entrance into the G1 phase of the cell cycle and functions by binding to and activating the cyclin-dependent kinases Cdk4 and Cdk6. Cdk4/Cdk6 further promote cell cycle progression through phosphorylation and inhibition of the expression [163]. Major downstream effectors of Ras signaling include Raf, PI3K, Ral-GDS, and RASSF [163].
tumor suppressor gene product, pRb [168]. Additional Ras effector pathways can induce Cyclin D1 expression depending on cell type, highlighting the importance of Cyclin D1 regulation by Ras [166]. Cyclin D1 is over-expressed in numerous human cancers through Ras-dependent and –independent mechanisms [168]. Additionally, loss of Cyclin D1 in mouse skin, leads to reduced tumor formation initiated by carcinogens that function through oncogenic activation of Ras signaling, highlighting the importance of this pathway in tumor development [169]. Activation of the PI3K pathway has an important role in promoting cell survival, whereas, RASSF activation by Ras induces an apoptotic response [166, 170]. Thus, Ras regulation of cell proliferation and survival is complex and involves an intricate balance of multiple signaling networks to achieve the appropriate cellular response, and activation of oncogenic Ras can wreak havoc on this balance resulting in loss of cellular control and tumorigenesis.

Oncogenic point mutations in Ras are most commonly found in the 12th, 13th, and 61st codons, which impair Ras’s intrinsic GTPase activity and prevent other GAPs from promoting GTP hydrolysis. The result of this is that Ras remains bound to GTP and constitutively active, resulting in deregulation of downstream signaling
pathways. Aberrant signaling downstream of oncogenic Ras promotes cell proliferation and survival, facilitating tumor development through constitutive activation of its effector pathways [155]. Fortunately, cells have evolved to deal with proliferative stress through activation of tumor suppressive mechanisms such as apoptosis and senescence [171]. In this regard, excessive signaling as a result of oncogenic Ras can stimulate a response from p53 in order to induce growth arrest, apoptosis, or senescence so as to remove the threat of unbalanced oncogenic signaling [170, 172-174]. Ras provokes p53 function through a well-established mechanism involving the up-regulation of the tumor suppressor gene p19\textsuperscript{Arf}. Induced p19\textsuperscript{Arf} binds to and blocks the activity of Mdm2, a potent inhibitor of p53 stability and activity [175]. p19\textsuperscript{Arf} functions by promoting Mdm2 degradation, sequestering Mdm2 in the nucleolus, and blocking nucleocytoplasmic shuttling of Mdm2 [176-179], thereby, promoting p53 stabilization. The mechanism through which oncogenic Ras induces p19\textsuperscript{Arf} is not well-defined, but may involve stimulation of E2F1 and DMP1 transcription factor activity and regulation of the p19\textsuperscript{Arf} promoter [180-184]. Other mechanisms through which oncogenic Ras induces p53 and its downstream responses have been described. For example, oncogenic Ras has
been shown to induce p53 through activation of the p38 mitogen-activated protein kinase pathway [185], PML protein [173, 186], PEA-15 [187], and seladin-1 [188]. Additionally, increasing evidence indicates that oncogenic Ras also activates DNA damage response pathways [189-191], which are well-known inducers of p53 stabilization and function [192]. It is important to note, and not surprising, that oncogenic Ras inhibits p53 stability and activity to promote cell survival. For example, oncogenic Ras signaling promotes the transcription factor JunD’s ability to block p53 and apoptosis through a mechanism involving inhibition of p19Arf induction [117] and acetylation of p53 at Lys317 downstream of oncogenic Ras signaling promotes cell survival [193].

**DNA Damage Response Pathways**

Genomic integrity is constantly challenged by exposure to endogenous and exogenous agents that result in DNA damage through alteration of DNA structure [194]. Cells respond to DNA damage by inducing DNA-repair pathways [194], activating cell cycle checkpoints/growth arrest [195], or stimulating apoptosis [196]. Proper regulation of these pathways is vital to ensure genome stability, as improper
control can result in the accumulation of mutations, leading to genomic instability and eventually disease such as tumorigenesis.

DNA can be altered by hydrolysis of bases to produce abasic sites (AP sites), deamination, oxidative damage, bulky adduct formation, alkylation, and the formation of DNA single- and double-strand breaks and DNA strand crosslinks. Under physiological conditions DNA damage occurs through the hydrolysis of nucleotide residues or spontaneous deamination of cytosine, adenine, guanine, or 5-methylcytosine. Additionally, normal cellular metabolism can result in the formation of by-products that induce DNA damage such as reactive oxygen species and products of lipid peroxidation. Environmental agents such as ultraviolet radiation (UV) from sunlight, ionizing radiation (IR), and a plethora of genotoxic agents induce many types of DNA damage [194].

DNA damage induces DNA damage response pathways that initiate cell cycle checkpoints. Cell cycle checkpoints allow time for DNA repair mechanisms to remove the damage and restore genomic integrity. However, if the damage is too severe, the cell initiates an apoptotic response to remove the damaged cell from the tissue. DNA damage response pathways form a large signaling network that is
simplified by grouping the proteins involved into three main categories: sensors, transducers, and effectors [197]. Sensors are involved in initially recognizing aberrant DNA structures and are capable of initiating the DNA damage signaling response. A number of proteins have been identified as putative DNA damage sensors. These include Rad9, Rad1, and Hus1, which comprise what is referred to as the 9-1-1 complex, Rad17, and BRCA1, which is part of a large complex called BASC (BRCA1-associated genome surveillance complex) that contains ATM, Nbs1-MRE11-RAD50 complex, mismatch proteins (MSH2/6 and MLH2) and the Bloom’s helicase (BLM) [192, 197]. ATM and ATR, two members of the large family of PI3K-like kinases (PIKK) and central components of DNA damage response pathways, also function as DNA damage sensors. ATM is present in the BASC complex described above and is recruited to and adheres to double-stranded DNA breaks (DSBs) [198, 199]. However, DNA damage also results in activation of ATM by triggering ATM intermolecular phosphorylation that induces ATM dimer dissociation, exposing ATM’s kinase domain; activation through this mechanism appears to result from changes in chromatin structure, independent of ATM interaction with DNA [200]. ATR is recruited to sites of DNA damage in conjunction with the ATR
interacting protein (ATRIP) [201] and this plays an essential role in the activation of DNA damage response pathways. ATM and ATR are also the major signal transducers of the DNA damage signaling network [197]. ATM responds exclusively to double strand DNA breaks, whereas ATR is activated in response to various types of DNA damage and participates in the response to replication stress [192, 202]. ATM and ATR control the response to DNA damage, in part, through activation of downstream transducer kinases, Chk1 and Chk2, which subsequently phosphorylate and modulate the activity of various downstream effector proteins [192, 202].

Effector proteins include p53, Mdm2, BRCA1, Nbs1, Cdc25C, Cdc25A, and E2F1 [203]. p53 is a major effector protein in the DNA damage response [192]. p53 undergoes a multitude of post-translational modifications in response to activation of DNA damage response pathways [192, 204]. For example, p53 can be phosphorylated at multiple sites by ATM, ATR, Chk1, and Chk2 [192, 204]. These modifications augment p53 stabilization and activation, facilitating p53’s ability to regulate the expression of genes necessary for activation of the G1 cell cycle checkpoint and/or apoptosis. Additionally, proteins involved in regulation of p53
levels and function are also post-translationally modified by ATM, such as Mdm2 and E2F1, and these modifications function to promote p53 stabilization in the cell [192]. Interestingly, post-translational modification of Mdm2 by ATM is the most rapid response to ionizing radiation, and may inhibit p53 degradation [205]. Once activated, p53 regulates the expression of numerous genes that elicit a growth arrest or apoptotic response. p53 target genes that play an important role in activation of cell cycle checkpoints and growth arrest include p21, GADD45, 14-3-3σ, and Reprimo [206]. To promote apoptosis, p53 functions by inducing the expression of several pro-apoptotic genes such as Bax, Fas, Perp, Noxa, and Killer/Dr5 [206].

Recent studies demonstrated that the C/EBP family member, C/EBPα, is induced by DNA damage through a p53-dependent mechanism and functions in the G1 checkpoint in keratinocytes [207]. C/EBPβ protein levels were also shown to be induced in keratinocytes following exposure to UVB [207]. Thus, it will be important to further characterize the ability of DNA damage response pathways to modulate C/EBPβ levels and/or activity and to determine whether this regulation may alter
C/EBPβ function, especially whether or not it may contribute to DMBA-induced repression of p53 and apoptosis.

**p53 and C/EBPβ**

p53 and C/EBPβ proteins were first linked in a study by Margulies et al [208] that demonstrated that IL-6 promoter activity and C/EBPβ transcriptional activity is regulated by p53. Wild type p53 repressed IL-6 promoter activity, whereas, mutant p53 (Val-135 and Phe-132) enhanced activity of this promoter through the repression or activation of C/EBPβ transcriptional activity, respectively [208]. The authors proposed that mutation of p53 at these sites may contribute to the deregulation of cytokine expression in tumor cells through a mechanism involving C/EBPβ [208]. Similar studies demonstrated that p53 could repress C/EBPβ-mediated activation of IL-6-induced promoter activity of various acute-phase genes [209], insulin receptor promoter activity [210], and expression of the liver specific gene, albumin [211]. Collectively, these studies suggest that p53 repression of C/EBPβ may represent a common mechanism through which p53 represses the expression of acute-phase genes to maintain cellular and tissue homeostasis in response to inflammatory stimuli.
p53 also antagonizes C/EBPβ’s regulation of the decidual prolactin promoter downstream of cAMP activation in human endometrial stromal cells [212]. Further analysis demonstrated that C/EBPβ and p53 physically interact and are capable of mutual trans-repression [213]. p53 repressed C/EBPβ transcriptional activation of the dPRL promoter and could inhibit C/EBPβ DNA binding activity to the C/EBP binding sites within the promoter [213]. Surprisingly, C/EBPβ was found to repress p53’s DNA binding and transcriptional activity, suggesting that there is negative crosstalk between C/EBPβ and p53 [213]. However, the results described thus far regarding p53 and C/EBPβ were all obtained using ectopic expression of C/EBPβ and p53 and exogenous promoter reporter constructs and thus, may not represent a physiologically relevant association between p53 and C/EBPβ.

Further characterization of the DMBA-induced apoptotic response in C/EBPβ−/− mouse epidermis demonstrated that aberrant increases in p53 levels and function are required [109]. This study provided the first genetic evidence indicating that C/EBPβ is involved in the negative regulation of p53, through a transcription-independent mechanism, and that repression of p53 by C/EBPβ is required for keratinocyte cell survival in response to DMBA treatment [109]. However, the
mechanism through which C/EBPβ represses p53 following DMBA exposure is not known.

**p53 Regulation**

p53 is the most commonly inactivated tumor suppressor gene in human cancer, and loss of p53 function provides a profound proliferative and survival advantage to the cell [214-216]. Cellular stress arising from DNA damage, aberrant proliferation, hypoxia, and nutrient deprivation stabilize and activate p53, which functions to maintain cellular and tissue homeostasis and to suppress tumorigenesis by safeguarding genomic integrity and activating cellular processes such as cell cycle arrest, differentiation, senescence and apoptosis [171, 192, 217, 218]. p53 functions mainly through its ability to positively and negatively regulate genes involved in cell cycle checkpoint activation/growth arrest, senescence, and/or apoptosis, although transcription-independent mechanisms have been described [171, 192, 219, 220]. Although p53 is essential for tumor suppression, strong negative regulation of p53 is required for normal growth and development [206, 221]. Regulation of p53 is complex, involving at least 10 positive and negative feedback loops, that modulate p53 expression, stability and activity [217, 222].
Many post-translational modifications of p53 such as phosphorylation, acetylation, methylation, glycosylation, ribosylation, sumoylation, and neddylation have been identified, and p53 phosphorylation and acetylation are proposed to be the major contributing factors to p53 protein stability and activation in response to stress [204, 217]. However, in vivo mouse models have demonstrated that at least some of these modifications may play more subtle roles in regulating p53 function [223, 224], whereas, protein-protein interactions between p53 and proteins involved in regulating p53 stability and activity may have a more critical role in regulating the p53 response [223].

p53 is controlled mainly through protein stability and subcellular localization. p53 is a target for ubiquitination and proteasomal degradation, and a number of ubiquitin ligases have been shown to regulate p53 through this mechanism [217]. The most extensively studied ubiquitin ligase involved in p53 ubiquitination and degradation is Mdm2. Mdm2 is a RING-finger protein that is essential for p53 regulation during normal development [225, 226]. Mdm2 is a transcriptional target of p53 and one of the major negative feedback loops that helps to maintain low cellular p53 levels under normal conditions and facilitate the return to normal p53
levels following activation of a p53 response [222, 227, 228]. Mdm2 inhibits p53 by targeting p53 for ubiquitination and proteasomal degradation or inhibiting p53 transcriptional activity [229]. DMBA-induced increases in p53 in C/EBPβ−/− mouse epidermis occur despite elevated Mdm2 levels [109], suggesting that Mdm2 protein activity in C/EBPβ−/− mouse epidermis may be attenuated. Mdm2 function is abated by the induction and activation of p19Arf. As mentioned above, p19Arf is a key mediator of oncogene-induced activation of p53. p19Arf is a small protein generated from the CDKN2a locus, the same region that encodes the tumor suppressor gene and CDK inhibitor, p16INK4a [230]. p19Arf negatively regulates Mdm2 function by promoting Mdm2 degradation, sequestering Mdm2 in the nucleolus, and blocking nucleocytoplasmic shuttling of Mdm2 [176-179], thereby, promoting p53 stabilization. p19Arf may also promote p53 activation by inhibiting the ability of Mdm2 to block the acetyltransferase activity of p300/CBP, thereby supporting acetylation of p53 and the chromatin surrounding p53 response elements, resulting in enhanced p53 trans-activation potential [231]. Interestingly, p19Arf is not required for activation of p53 in response to DNA damage, although, there is evidence to suggest induction of p19Arf by oncogenic stress may cooperate with signaling
through the DNA damage signal transducers, ATM/ATR, to induce p53 responses [232, 233]. ATM/ATR activation of p53 downstream of DNA damage was described above. Additional signaling proteins involved in the regulation of p53 include MdmX, which is structurally related to Mdm2 and contributes to activation of p53’s transcriptional ability [217], and PTEN, which functions to activate p53 through inhibiting AKT-induced phosphorylation and activation of Mdm2 [217, 222].

Once stabilized and activated, p53 must then choose how the cell is to respond. There are many factors that can modulate p53-mediated gene expression and response [234] such as cell type, cell environment, strength and/or nature of the p53-activating stimulus, and genetic context [220, 221]. For example in mouse embryonic fibroblasts, E1A can induce p53-mediated apoptosis, while oncogenic Ras promotes p53-induced senescence [235, 236]. In both cases, p53 levels are increased through a mechanism requiring p19Arf. Additionally, in cells harboring oncogenic Ras mutations, aberrant Ras signaling can induce Mdm2 expression in a p53-dependent manner and thereby inhibit p53 activation in response to DNA damage compared to cells with normal Ras [237]. Lastly, microarray analysis revealed that activation of p53 by UV-light or IR can generate unique gene
expression patterns [234]. Thus, upstream signals can impact the downstream p53 response.

p53 is clearly required for tumor suppression. p53 activity is induced by many types of stress through different mechanisms, and it is unclear how much each contributes to p53 tumor suppressor function. DNA damage response pathways have been shown to be activated in early pre-cancerous lesions [238-240]. Induction of p53 in these pre-cancerous lesions may function to inhibit tumor growth and proliferation through activation of senescence and/or apoptosis [240-242]. However, recent studies suggest that p19ARF induction may be critical for p53-induced tumor suppression, while early activation of p53-mediated DNA damage responses may not be necessary [243-245]. Additional studies are required to further characterize the contribution of each of these pathways in p53 tumor suppression.
Research Hypothesis and Objectives

C/EBPβ−/− mice are completely refractory to skin tumorigenesis and display vast increases in keratinocyte apoptosis following DMBA treatment, which produces oncogenic Ras mutations in skin [95]. DMBA-induced aberrant apoptosis in C/EBPβ−/− mouse epidermis is a result of derepression of p53 levels and activity [109]. Furthermore, C/EBPβ activity is modulated by oncogenic Ras signaling, and regulation of C/EBPβ by oncogenic Ras promotes cellular transformation [61, 95, 96]. Early evidence also suggested that C/EBPβ−/− mice do not respond to UVB with abnormally high levels of apoptosis, intimating that DNA damage is not sufficient to induce a pro-apoptotic response in C/EBPβ−/− mice [95]. Therefore, we hypothesized that C/EBPβ functions downstream of oncogenic Ras signaling to promote cell survival through a p53-dependent mechanism. Our objectives were to determine whether increased p53 in C/EBPβ−/− mice is a result of oncogenic Ras-induced p19Arf, oncogenic Ras-induced activation of DNA damage response pathways or direct DMBA-induced DNA damage. Additionally, we began studies to identify putative C/EBPβ target genes that may contribute to DMBA-induced aberrant regulation of p53 and apoptosis in C/EBPβ−/− mice.
CHAPTER 1

C/EBPβ represses p53 to promote cell survival downstream of DNA damage independent of oncogenic Ras and p19$^\text{Arf}$

Sarah J. Ewing$^{1,2}$, Songyun Zhu$^1$, Robert C. Smart$^{1,2}$

$^1$Cell Signaling and Cancer Group, Department of Environmental and Molecular Toxicology, $^2$Comparative Biomedical Sciences Program, North Carolina State University, Raleigh, NC

Running title; C/EBPβ negatively regulates p53 downstream of DNA damage

Key words; apoptosis, p53, C/EBPβ, p19$^\text{Arf}$, DNA damage, keratinocytes

CORRESPONDING AUTHOR:

Robert C. Smart

Department of Environmental and Molecular Toxicology

North Carolina State University

Raleigh, NC 27695-7633

USA

Phone: (919) 515-7245, Fax: (919) 515-7169

Email: rcsmart@unity.ncsu.edu
Abstract

C/EBP\(\beta\) is an important mediator of cell survival and tumorigenesis. This is readily apparent in C/EBP\(\beta^{\text{--/}}\) mice, which respond to carcinogens that produce oncogenic Ras mutations, with vast increases in keratinocyte apoptosis and a block in tumorigenesis. While carcinogen-induced elevated apoptosis in C/EBP\(\beta^{\text{--/}}\) mice results from aberrant increases in p53 levels and function, it is not known whether increased p53 results from oncogenic Ras-induced p19\(^{\text{Arf}}\), oncogenic Ras-induced activation of DNA damage response pathways, or direct carcinogen-induced DNA damage. We report that p19\(^{\text{Arf}}\) is dramatically up-regulated in C/EBP\(\beta^{\text{--/}}\) mouse epidermis and that C/EBP\(\beta\) represses p19\(^{\text{Arf}}\) promoter activity. To determine whether p19\(^{\text{Arf}}\) is necessary for increased p53 and apoptosis in C/EBP\(\beta^{\text{--/}}\) mice, C/EBP\(\beta^{\text{--/}}; p19^{\text{Arf}^{-/-}}\) mice were generated. These mice responded to carcinogen treatment similar to C/EBP\(\beta^{\text{--/}}\) mice, indicating p19\(^{\text{Arf}}\) is not required. Moreover, generation of K14-ER:Ras;C/EBP\(\beta^{\text{--/}}\) mice revealed that oncogenic Ras activation, itself, does not trigger anomalous increases in p53 and apoptosis. In contrast, treatment of C/EBP\(\beta^{\text{--/}}\) mice with DNA damaging agents such as MNNG or etoposide resulted in deregulated p53. Similarly, treatment with ultraviolet B
radiation produced aberrant increases in p53 and apoptosis in C/EBPβ−/− mice compared to wild type. These results indicate that C/EBPβ represses p53 to promote cell survival downstream of DNA damaging agents independent of oncogenic Ras and p19Arf.
Introduction

CCAAT/enhancer binding protein β (C/EBPβ), a member of the basic leucine zipper transcription factor (bZIP) family, is an important mediator of cell proliferation, differentiation, and survival of several cell types and functions in inflammatory responses, metabolism, cellular transformation, oncogene-induced senescence, and tumorigenesis [1, 2]. C/EBPβ promotes mouse keratinocyte cell survival in response to carcinogen treatment [3, 4]. Additionally, C/EBPβ, functions in the survival of CCl₄-treated hepatic stellate cells [5] and is required for proliferation and growth-factor dependent survival of Myc/Raf-transformed macrophages through an auto-regulatory pathway involving IGF-1 [6]. Increased expression of C/EBPβ in Wilms tumors, or nephroblastomas, is associated with primary tumor relapse, and acute loss of C/EBPβ in a cell line derived from a human metastatic Wilms tumor led to spontaneous apoptosis [7]. Similarly, C/EBPβ is necessary to sustain growth and survival of anaplastic lymphoma kinase-positive (ALK+) anaplastic large cell lymphoma (ALCL) cells [8]. Together these studies demonstrate that C/EBPβ is an important mediator of cell survival, and that C/EBPβ’s role in cell survival may provide a mechanism through which C/EBPβ promotes tumorigenesis.
C/EBPβ is required for skin tumorigenesis initiated by carcinogens such as 7,12-dimethylbenz[a]anthracene (DMBA) [3], which functions through its ability to produce oncogenic Ras mutations [9, 10]. Additionally, C/EBPβ−/− mice carrying the v-Ha-Ras transgene display a significant reduction in tumorigenesis, directly linking oncogenic Ras signaling to C/EBPβ in vivo [3]. C/EBPβ is activated by Ras [3, 11], and C/EBPβ cooperates with oncogenic Ras to induce cellular transformation, which requires cell cycle dependent phosphorylation of C/EBPβ on Ser64 and Thr189 [3, 12]. DMBA treatment of mouse skin produces benign papillomas of which greater than 95% contain an activating A→T mutation in codon 61 of Ras [10]. Carcinogen-induced oncogenic mutation of codon 61 can occur in 0.1-5% of Ras genes by error-prone repair within 1-3 days after treatment [13, 14]. Treatment of C/EBPβ−/− mouse skin with DMBA results in aberrant increases in p53 levels and function, which is required for the induction of apoptosis in 1-2% of C/EBPβ−/− keratinocytes within one day of treatment [4]. Collectively, these studies suggest that C/EBPβ may be required for the survival of DMBA-initiated oncogenic Ras containing tumor precursor cells in mouse skin through the regulation of p53 levels and function.
p53 is stabilized and activated in response to different types of stress including oncogenic stress, DNA damage, hypoxia, or nutrient deprivation [15]. Oncogenic Ras regulates p53 levels and activity through multiple mechanisms [16]. An important, well-established mechanism involves the induction of p19\textsuperscript{Arf} [15]. Oncogenic Ras induces p19\textsuperscript{Arf} gene expression through regulation of transcription factors such as Dmp1, AP-1 (JunD, c-Jun, FRA1), and E2F1 [17-20]. Induced p19\textsuperscript{Arf} functions by inhibiting the negative feedback loop between p53 and Mdm2, resulting in p53 stabilization and eventually growth arrest or apoptosis [21]. Oncogenic Ras also triggers p53-dependent senescence both in culture and in vivo, in part, through activation of DNA damage response pathways [22-28]. The mechanism through which oncogenic Ras activates DNA damage response pathways is not clear, but may be due to aberrant DNA replication, resulting in the generation of DNA strand breaks followed by activation of DNA-damage response kinases, ATM/ATR [22, 24, 26]. ATM/ATR are the central components of the DNA damage response induced by both replication stress or DNA damaging agents that directly alter DNA structure [29]. ATM/ATR activation results in phosphorylation of p53, through direct and indirect mechanisms [29]. ATM and ATR also contribute to
p53 activation by phosphorylating Mdm2, MdmX, and/or E2F1, modulating their ability to regulate p53 [29]. These post-translational modifications result in increased p53 stability and activity, facilitating p53’s ability to activate cell cycle checkpoints, senescence, or apoptosis [29].

The fact that C/EBPβ is a target for regulation by oncogenic Ras coupled with ample evidence linking oncogenic Ras to p53 prompted us to investigate whether carcinogen-induced aberrant increases in p53 and apoptosis in C/EBPβ−/− mice is a result of oncogenic Ras-induced signaling. Although we demonstrate that p19Arf is deregulated in C/EBPβ−/− mouse epidermis, oncogenic Ras activation and deregulated p19Arf expression are not required for the atypical induction of p53 and apoptosis in C/EBPβ−/− mice. In contrast, treatment of C/EBPβ−/− mice with DNA damaging agents, which produce DNA damage in the form of alkylated DNA, double-stranded DNA breaks, and cyclobutane pyrimidine dimers, resulted in anomalous increases in p53 and apoptosis, indicating that C/EBPβ functions downstream of DNA damage to repress p53 and apoptosis.
Results

p19\textsuperscript{Arf} is specifically deregulated in C/EBP\textsuperscript{β\textasciitilde} mouse epidermis and primary epidermal keratinocytes.

To begin to determine whether C/EBP\textsuperscript{β} regulates p53 through a mechanism involving p19\textsuperscript{Arf}, we first examined p19\textsuperscript{Arf} expression in untreated C/EBP\textsuperscript{β\textasciitilde} and wild type mouse epidermis. Surprisingly, we observed a dramatic 35-fold increase in p19\textsuperscript{Arf} mRNA levels in the epidermis of C/EBP\textsuperscript{β\textasciitilde} mice compared to wild type (Figure 1a). As shown in Figure 1b, p19\textsuperscript{Arf} protein levels were also significantly increased in epidermal lysates from C/EBP\textsuperscript{β\textasciitilde} mice. The INK4a-Arf locus encodes both p19\textsuperscript{Arf} and p16\textsuperscript{INK4a} [30]. Although they have unique promoters, p19\textsuperscript{Arf} and p16\textsuperscript{INK4a} tumor suppressor genes are often coordinately regulated [31]. To determine whether p19\textsuperscript{Arf} expression is specifically up-regulated in C/EBP\textsuperscript{β\textasciitilde} mice, or whether p16\textsuperscript{INK4a} expression is similarly altered, we examined p16\textsuperscript{INK4a} mRNA levels from untreated C/EBP\textsuperscript{β\textasciitilde} and wild type mouse epidermis (Figure 1c). p16\textsuperscript{INK4a} expression was not significantly altered, indicating that p19\textsuperscript{Arf} is specifically deregulated in C/EBP\textsuperscript{β\textasciitilde} mouse epidermis. Analysis of the proximal 2.6kb of the p19\textsuperscript{Arf} promoter revealed the identification of putative C/EBP binding sites. Co-
transfection of C/EBPβ and a 2.6kb-p19Arf promoter reporter construct demonstrated that C/EBPβ significantly repressed the activity of the p19Arf promoter reporter by 40% (Figure 1d), suggesting that C/EBPβ may directly repress p19Arf expression.

Our observation that p19Arf is expressed in C/EBPβ-/- mouse epidermis was unexpected, as p19Arf is not expressed in most normal adult tissues. However, p19Arf expression can be induced by oncogenic stress in vivo or by “culture shock” when primary cells are placed in culture [32-34]. To investigate whether p19Arf expression is also deregulated in C/EBPβ-/- keratinocytes in response to “culture shock”, we examined p19Arf mRNA and protein levels in wild type and C/EBPβ-/- primary epidermal keratinocytes. C/EBPβ-/- primary keratinocytes displayed a consistent, significant increase in both p19Arf mRNA and protein levels over that observed in wild type keratinocytes (Figure 2a and 2b). These results suggest that increased p19Arf in C/EBPβ-/- keratinocytes is a keratinocyte-intrinsic defect.

To determine whether increased p19Arf is expressed within all keratinocytes of C/EBPβ-/- mouse skin, or within a sub-population of keratinocytes, we performed p19Arf immunohistochemistry. p19Arf was detected in C/EBPβ-/- mouse skin, but not
in wild type or in the negative control, p19Arf-/- mouse skin (Figure 2c and data not shown). p19Arf was highly expressed in a sub-population of C/EBPβ-/- suprabasal keratinocytes located within the upper infundibulum area of the hair follicle, adjacent to the interfollicular epidermis. p19Arf localized to nuclei and displayed distinct nucleolar staining. Collectively, these results demonstrate that p19Arf is greatly increased in C/EBPβ-/- mouse epidermis and primary keratinocytes and is most highly expressed within a sub-population of keratinocytes within the infundibulum of the hair follicle.

**p19Arf is not required for carcinogen-induced anomalous increases in p53 and apoptosis in C/EBPβ-/- mice.**

Since p19Arf levels are dramatically increased in C/EBPβ-/- mouse epidermis, we hypothesized that C/EBPβ-/- epidermal keratinocytes may be “primed” to rapidly stabilize and activate p53 following DMBA-induced oncogenic Ras activation, resulting in increased cell death compared to wild type epidermis. In order to determine whether elevated p19Arf in C/EBPβ-/- mouse epidermis is involved in the anomalous increases in p53 and apoptosis in response to DMBA treatment, C/EBPβ-/-;p19Arf-/- mice were generated. Following DMBA-treatment, the number of
p53-positive and apoptotic epidermal keratinocytes were quantified in wild type, p19\textsuperscript{Arf}{-/-}, C/EBP\textsuperscript{\beta}{-/-}, and C/EBP\textsuperscript{\beta}{-/-};p19\textsuperscript{Arf}{-/-} mouse epidermis (Figure 3a and 3b). As previously described, C/EBP\textsuperscript{\beta}{-/-} mice displayed a significant increase in p53 and apoptosis compared to wild type following carcinogen treatment [4]. C/EBP\textsuperscript{\beta}{-/-};p19\textsuperscript{Arf}{-/-} mice displayed similar, anomalous increases in p53 and apoptosis levels compared to C/EBP\textsuperscript{\beta}{-/-} mice, indicating that deregulation of p19\textsuperscript{Arf} in C/EBP\textsuperscript{\beta}{-/-} mouse epidermis is not required for the abnormal induction of p53 or apoptosis following DMBA treatment.

**Oncogenic Ras activation is not sufficient to trigger atypical increases in p53 and apoptosis in C/EBP\textsuperscript{\beta}{-/-} mice.**

Forced expression of oncogenic Ras in C/EBP\textsuperscript{\beta}{-/-} primary keratinocytes does not elicit increased apoptosis (data not shown), suggesting that an *in vivo* environment is required for oncogenic Ras to induce aberrant apoptosis in C/EBP\textsuperscript{\beta}{-/-} epidermal keratinocytes or direct DNA damage is responsible for inducing apoptosis in DMBA-treated C/EBP\textsuperscript{\beta}{-/-} mice. To elucidate whether activation of oncogenic Ras signaling, itself, can elicit aberrant increases in p53 and apoptosis in C/EBP\textsuperscript{\beta}{-/-} mice, we generated K14-ER:Ras;C/EBP\textsuperscript{\beta}{-/-} mice. K14-ER:Ras mice display
targeted expression of the ER:Ras fusion protein to mouse epidermis, where its activity is regulated through topical treatment with the synthetic ER antagonist, 4-hydroxytamoxifen (4-OHT) [35]. The toggle-on of oncogenic Ras with a single, topical application of 4-OHT induced oncogenic Ras-mediated proliferation in K14-ER:Ras and K14-ER:Ras;C/EBPβ-/- mouse epidermis, as we observed a significant increase in the number of 5-bromo-2-deoxyuridine (BrdU)-positive interfollicular basal epidermal keratinocytes (Figure 4a and 4b).

To determine whether activation of oncogenic Ras elicits altered regulation of p53 and apoptosis in C/EBPβ-/- mice, we examined p53 and apoptosis levels in skin sections from wild type, C/EBPβ-/-, K14-ER:Ras, and K14-ER:Ras;C/EBPβ-/- mice treated with 4-OHT (Figure 4c and 4d). The toggle-on of oncogenic Ras by 4-OHT-treatment did not trigger an increase in p53 or apoptosis in K14-ER:Ras;C/EBPβ-/- mice. The number of apoptotic keratinocytes following oncogenic Ras activation was very low, especially compared with the apoptotic response observed in C/EBPβ-/- mice after treatment with DMBA (Figure 4d). Moreover, multiple treatments with 4-OHT was not sufficient to induce aberrant apoptosis in K14-ER:Ras;C/EBPβ-/- mice, despite the induction of significant epidermal hyperplasia.
consisting of four nucleated cell layers and a BrdU interfollicular basal keratinocyte labeling index of approximately 35% (data not shown). These data indicate that activation of oncogenic Ras does not induce atypical increases in p53 and apoptosis in K14-ER:Ras;C/EBPβ−/− mouse epidermis.

DNA damaging agents induce aberrant increases in p53 and apoptosis in C/EBPβ−/− mouse epidermis.

To determine whether direct carcinogen-induced DNA damage elicits altered regulation of p53 and apoptosis in C/EBPβ−/− mice, we examined the effects of various types of DNA damaging agents in C/EBPβ−/− mice compared to wild type. As shown in Figure 5a, topical treatment with MNNG produced a significant increase in the number of p53-positive keratinocytes in C/EBPβ−/− mouse epidermis compared to wild type mice. Similarly, C/EBPβ−/− mice displayed an anomalous increase in apoptosis when compared to wild type (Figure 5b). In general, these increases were observed across the entire 16-hour time course. Since MNNG is known to produce oncogenic Ras mutations in mouse skin, we examined the effects of etoposide, a topoisomerase II inhibitor that produces single and double strand breaks in DNA and does not produce oncogenic Ras mutations. As shown in
Figure 5c, etoposide treatment also resulted in increased p53-positive keratinocytes in C/EBPβ⁻/⁻ mice compared to wild type mice. Etoposide treatment induced a significant increase in apoptosis, over time, in both genotypes. However, it was not sufficient to induce deregulated apoptosis in C/EBPβ⁻/⁻ mice (Figure 5d).

Next, we examined whether treatment of C/EBPβ⁻/⁻ mice with ultraviolet B radiation (UVB) could induce deregulated p53 and apoptosis. UVB produces DNA damage mostly in the form of cyclobutane pyrimidine dimers that do not involve mutation of Ras. C/EBPβ⁺/+ and C/EBPβ⁻/⁻ mice were treated with 100mJ/cm² of UVB and a time course was conducted to examine the number of p53-positive or apoptotic interfollicular basal keratinocytes. p53 was rapidly and significantly increased in C/EBPβ⁻/⁻ mice compared to wild type as early as three hours after UVB treatment (Figure 5e). Similarly, UVB exposure induced a significant increase in apoptosis in C/EBPβ⁻/⁻ mice compared to wild type. The difference in apoptosis levels between C/EBPβ⁻/⁻ mice and wild type mice continued to increase throughout the 18-hour time course (Figure 5f). Collectively, these results demonstrate that DNA damaging agents that produce different types of DNA damage such as
alkylated DNA, DNA strand breaks, and cyclobutane pyrimidine dimers induce anomalous increases in p53 and apoptosis in C/EBPβ−/− mice.
DISCUSSION

C/EBPβ is an important mediator of keratinocyte cell survival in response to topical DMBA treatment and is required for skin tumorigenesis initiated by carcinogens such as DMBA and MNNG, which induce oncogenic Ras mutations [3]. The pro-apoptotic response in C/EBPβ−/− mice following DMBA treatment is due to deregulated p53 levels and function [3, 4]. As discussed in the introduction, there is a substantial body of evidence that suggested the enhanced apoptotic response in C/EBPβ−/− epidermal keratinocytes is likely a result of carcinogen-induced oncogenic Ras activation. Using K14-ER:Ras;C/EBPβ−/− mice, we observed that conditional activation of oncogenic Ras within epidermal basal keratinocytes of C/EBPβ−/− mouse epidermis was not sufficient to induce anomalous increases in p53 and apoptosis. Our results indicate that the pro-apoptotic phenotype in carcinogen-treated C/EBPβ−/− mice is not due to oncogenic Ras, but rather is a response to DNA damage.

We observed that treatment of C/EBPβ−/− mice with DNA damaging agents such as DMBA, MNNG, etoposide, and UVB, which produce DNA damage in the form of bulky adducts, alkylated DNA, double-strand DNA breaks, and cyclobutane...
pyrimidine dimers all result in abnormal increases in p53, and with the exception of etoposide, aberrant increases in apoptosis. There are many factors that can modulate p53-mediated gene expression and cellular response [36] such as cell type, cell environment, strength and/or nature of the p53-activating stimulus, and genetic context [37, 38]. DNA damage produced by etoposide treatment may not be sufficient to reach the threshold required to trigger apoptosis by p53 in C/EBPβ−/− mouse epidermis [39]. Nevertheless, our results indicate that C/EBPβ has a role in the DNA damage response pathway and functions to promote cell survival by repressing p53 levels and function in response to DNA damage.

Our results also reveal that p19Arf is deregulated in untreated C/EBPβ−/− mouse epidermis and primary keratinocytes. The magnitude of p19Arf expression in C/EBPβ−/− mouse epidermis was unexpected because p19Arf is not detectably expressed during embryonic development and is found at very low levels and only in a small subset of normal adult tissues, such as the testes [33, 34]. p19Arf was highly detectable in the nucleoli of keratinocytes within the suprabasal layers of the infundibulum area of the hair follicle. The biological significance of de-regulated p19Arf in C/EBPβ−/− mouse skin and exactly how C/EBPβ regulates p19Arf expression
remains to be determined. \( p19^{Arf} \) expression is under strict regulation, partially due to the ability of an increasing number of transcription factors such as Pokemon, E2F3b, p53, Twist, Bmi1, Tbx2/3 and JunD, to repress \( p19^{Arf} \) promoter activity [31]. Our results suggest that C/EBP\( \beta \) may also contribute to the negative regulation of \( p19^{Arf} \) through a mechanism involving repression of the \( p19^{Arf} \) proximal promoter.

While we demonstrate that direct DNA damage is important in eliciting C/EBP\( \beta \)-mediated repression of p53 to promote keratinocyte cell survival, additional mechanisms in other cell types can contribute to C/EBP\( \beta \)-mediated cell survival [5, 6]. For example, CCl\( _4 \) treatment, which induces DNA damage through the generation of free radicals, results in phosphorylation of C/EBP\( \beta \) by ribosomal protein 6-kinase (RSK), producing a functional XEXD caspase substrate/inhibitor box that is critical for survival of hepatic stellate cells [5]. RSK is an important effector protein in the cell survival response downstream of the Ras/MAPK/ERK pathway [40]. Additionally, C/EBP\( \beta \) is required for growth-factor dependent survival of Myc/Raf-transformed macrophages through an auto-regulatory pathway involving IGF-1 [6]. Activation of several oncogenes including Myc, Ras, Raf, cyclin E, Cdc25A, and E2F1 can engage DNA damage response pathways, resulting in
activation of p53 and the induction of apoptosis or senescence [22, 23, 26, 27, 41].
The important decision of cell survival or cell death in response to different stimuli is likely to be controlled by more than one signal. Thus, it is possible that there is overlap of these pathways in their ability to contribute to C/EBPβ-mediated cell survival depending on the cellular and genetic context.

DNA damage is a potent inducer of p53 through the activation of ATM/ATR kinases, which ultimately result in phosphorylation of p53 as well as several proteins involved in p53 regulation, including Mdm2 and MdmX [29]. Phosphorylation of these proteins results in stabilization and activation of p53. Regulation of p53 is complex, involving at least 10 positive and negative feedback loops that modulate p53 expression, stability and activity [42]. It is not yet known how C/EBPβ participates within the DNA damage signaling cascade to repress p53 levels and function. DNA damage response pathways may modulate C/EBPβ levels or activity, altering C/EBPβ’s ability to regulate the expression of genes or activity of proteins involved in post-translational modification of p53. Accordingly, C/EBPβ protein levels are induced in keratinocytes following exposure to UVB [43]. Elevated levels of C/EBPβ may repress p53 by contributing to phosphorylation,
acetylation, or sumoylation of p53 following DNA damage. Alternatively, C/EBPβ has been shown to interact with p53, disrupting p53 DNA binding and transcriptional activity [44]. This mechanism could also contribute to the negative regulation of p53 by C/EBPβ.

In summary, our results indicate that C/EBPβ represses p53 to promote cell survival downstream of DNA damage. These findings provide a novel link between DNA damage response pathways, C/EBPβ, and the repression of p53 to promote cell survival. Moreover, inhibition of C/EBPβ levels or activity combined with chemotherapy treatment may represent a novel molecular intervention strategy to treat p53-proficient tumors, as this could conceivably promote apoptosis, resulting in tumor regression.
Materials and Methods

Animals. B6.129-C/EBPβ⁻/⁻ mice have been previously described [3, 4]. B6.129-Cdkn2a<sup>tm1Cjs</sup> (p19<sup>Arf⁻/⁻</sup>) mice were obtained from the Mouse Models of Human Cancers Consortium (MMHCC), generously made available by Charles Sherr (St. Jude’s Research Hospital, TN, USA) [45]. Mice were genotyped as described by the MMHCC PCR protocol for strain #01XG7. p19<sup>Arf⁻/⁻</sup> female mice were crossed with C/EBPβ⁻/⁻ male mice. Offspring (C/EBPβ⁺/⁻;p19<sup>Arf⁺/⁻</sup>) were crossed to generate all genotypes utilized in these studies. K14-ER:Ras mice were a kind gift from Dr. Paul Khavari (Stanford University, CA, USA) [35]. K14-ER:Ras female mice were crossed with C/EBPβ⁻/⁻ male mice to generate K14-ER:Ras;C/EBPβ⁺/⁻ mice. K14-ER:Ras;C/EBPβ⁺/⁻ mice were crossed with B6.129-C/EBPβ⁺/⁻ mice to generate all genotypes used in these studies. Mice were genotyped for ER:Ras by PCR using the following primers: Forward: CACCACCAGCTCCACTTCACCAT, Reverse: CGCACCAACGTGAAGCAGCCACCTC. Mice (7-11 weeks old) were clipped with electric clippers at least two days prior to use. Mice were treated as described in the text. 7,12-dimethylbenz[a]anthracene (Catalog #40818, Fisher Scientific USA - Acros Organics, Pittsburgh, PA, USA) was dissolved in acetone (400 nmol/200 µl).
4-hydroxytamoxifen (4-OHT) (Catalog #H7904), 1-Methyl-3-nitro-1-nitrosoguanidine (MNNG) (Catalog #12994-1), and etoposide (Catalog #E1383) were purchased from Sigma (St. Louis, MO, USA). 4-OHT was dissolved in 95% ethanol (1 mg/100 µl). MNNG was dissolved in acetone (2.5 µmol/200 µl of acetone). Etoposide was dissolved in dimethyl sulfoxide (DMSO) (1 mg/200 µl). The UV lamp used in this study, purchased from UVP, Inc. (Catalog #34-0039-01, Upland, CA, USA), emits wavelengths between 280 and 350 nm with a spectrum peak at 312 nm. The light intensity of the lamp was measured using the IL-1400A radiometer (International Light, Newburyport, MA, USA) equipped with the SEL240/UVB-1 sensor that detects wavelengths between 265 and 332 nm. Mice were treated with 100mJ/cm² of ultraviolet B radiation (UVB).

**Cell lines and cell culture.** Balb/MK2 mouse keratinocytes (Bernard Weissman, University of North Carolina, NC, USA) were cultured as described previously [43]. Mouse primary epidermal keratinocytes were isolated from newborn C/EBPβ+/+ or C/EBPβ−/− littermates as described previously [43]. Isolated keratinocytes were plated at 3 x 10⁶ cells/well in 6-well culture dishes in complete media (Ca²⁺-free EMEM supplemented with 10% non-Chelex-treated FBS, 10ng of hEGF per ml,
100U of penicillin/ml, 100µg of streptomycin/ml, and 250ng of amphotericin B/ml) for 4 hours. Cultures were gently washed with Mg$^{2+}$- and Ca$^{2+}$-free PBS and re-fed with keratinocyte serum free medium (Ca$^{2+}$-free K-SFM supplemented with 2.5µg of hEGF, 25mg bovine pituitary extract, 5µg of gentimycin/ml, and calcium chloride to a final concentration of 0.05 mM). Three independent pools of isolated primary keratinocytes were examined for p19Arf mRNA and protein expression at each time point. Primary keratinocytes collected at time 0 were isolated as above; 1 x 10$^6$ cells were pelleted by centrifugation and subsequently used to harvest total RNA or protein lysates as described.

Construction of pGL4.10-p19Arf2.6kb promoter reporter and luciferase assay.

pGL2-p19Arf2.6kb promoter reporter was kindly provided by Kazushi Inoue (Wake Forest University, NC, USA). The p19Arf-2.6kb promoter fragment was cloned into pGL4.10 using Kpn I and Bgl II. Balb/MK2 keratinocytes were co-transfected with pGL4.10-p19Arf2.6kb or pGL4.10 and the indicated amounts of pcDNA3.1(-)-C/EBPβ-expression plasmids using Lipofectamine (Invitrogen, Carlsbad, CA, USA) in triplicate. Cells were washed with ice cold PBS, lysed in 1X Cell Culture Lysis Buffer (Promea, Madison, WI, USA), harvested by scraping, and cleared by
centrifugation prior to analyzing for luciferase activity. Data were normalized to total protein and activity of the empty reporter construct. The average relative fold change compared to p19Arf basal promoter activity, arbitrarily set to 1, for three independent experiments are shown ± S.E. A two-factor ANOVA was conducted on the relative fold change values. To test for repression, a t-test, using the pooled ANOVA Error, was carried out for each treatment to test the null hypothesis that mean relative fold change = 1 versus the alternative that mean relative fold change < 1.

**Quantitative real-time RT-PCR.** Total RNA was collected and prepared for quantitative real-time RT-PCR as previously described [4]. Detection of p19Arf mRNA levels was conducted using a Cdkn2a (p19Arf) TaqMan® Gene Expression Assay (Catalog #Mm01257348_m1, Applied Biosystems, Foster City, CA, USA). Sequences for p16INK4a primers and dual-labeled probe were provided by Bryan Betz (National Institute of Environmental Health Sciences, NC, USA), synthesized by Sigma Genosys, and used at a final concentration of 0.5 μM (Forward Primer: CCCAACGCACCAGAACT, Reverse Primer: GTGAACGTTGCCCATCATCA, Probe: [FAM]TTTCGTCGTACCCCAGGTG[TAM]). Each independent sample
was run in triplicate. Expression levels for *in vivo* samples were normalized to 18S (Applied Biosystems), and expression levels for primary keratinocyte samples were normalized to GAPDH (Applied Biosystems). Data were analyzed using the Comparative Cₜ method and presented relative to the control, C/EBPβ⁺/⁺ or C/EBPβ⁻/⁻ at time zero. N=4-8 mice/genotype for *in vivo* analysis, and N=3 independent pooled keratinocytes/time for the primary keratinocyte experiment.

**Preparation of protein lysates.** Isolation of protein lysates from epidermal homogenates were prepared as previously described [4], with the exception that samples were homogenized on ice prior to sonication. For preparation of protein lysates from cultured primary keratinocytes, cells were washed with cold PBS, harvested by scraping, and collected by centrifugation. Cells were lysed on ice in radio-immunoprecipitation buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and 1X protease inhibitor cocktail (Roche, Bael, Switzerland) in PBS), sonicated and cleared by centrifugation at 14000 *g* for 10 minutes. Supernatants were stored at -80°C until use. Protein concentration was determined by Bio-Rad Protein Assay reagent (Bio-Rad, Hercules, CA, USA).
**Immunoblot analysis.** Equal amounts of protein (40 µg) were dissolved in SDS sample buffer, boiled and separated on 12% SDS-polyacrylamide gel electrophoresis (PAGE). Immunoblot analysis was conducted as described previously [4]. Membranes were probed with antibody for p19Arf (1:400, sc-32748) or C/EBPβ (1:2000, sc-150) from Santa Cruz Biotechnology (Santa Cruz, CA, USA) or β-actin (1:20000, A-5441) from Sigma. The membranes were washed and probed with a horseradish peroxidase-conjugated secondary antibody for rat (1:2000, sc-2006, Santa Cruz Biotechnology), rabbit (1:2500, NA934V, GE Healthcare UK Ltd, Buckinghamshire, England), or mouse (1:10000, NXA931, GE Healthcare UK Ltd), respectively.

**Immunohistochemical staining.** Untreated or treated, dorsal skin was excised and fixed for 24 hours in 10% neutral-buffered formalin. Four independent areas of skin were collected, embedded in paraffin, and cut into sections (≈ 5 µM). For p53 immunohistochemical staining, sections were deparaffinized, treated with 3% H₂O₂, and subjected to antigen retrieval with citrate buffer (pH 6.0) in a 95°C water bath for 30 minutes. Sections were blocked with normal goat serum for 30 minutes at room temperature and then incubated with rabbit polyclonal anti-p53 antibody
(Catalog #sc-6243, 1:500), purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), at 4°C for 24 hours. Sections were subsequently incubated with biotinylated goat anti-rabbit IgG at room temperature for 30 minutes. Staining was detected using the rabbit Vectastatin Elite ABC kit (Catalog #PK-6101) from Vector Laboratories (Burlingame, CA, USA) and 3, 3’-diaminobenzidine (DAB) (BioGenex, San Ramon, CA) following the manufacturers’ protocols. The sections were counterstained with hematoxylin, dehydrated, and mounted. Data are expressed as the average number of p53-positive staining interfollicular basal keratinocytes per centimeter of mouse skin ± standard error. p19Arf-immunohistochemical staining was modified from a previously described method [46] by Bryan Betz (National Institute of Environmental Health Sciences, NC, USA). Briefly, paraffin-embedded sections from mouse skin were deparaffinized and subjected to antigen retrieval in antigen decloaker citrate buffer (pH 6.0) (Biocare, Concord, CA, USA) for 45 minutes in the decloaking chamber (Biocare). After cooling, slides were treated with 3% H2O2 to quench endogenous peroxide activity. Sections were blocked with normal rabbit serum in 0.1% Triton X-100/phosphate buffered saline (PBS) for approximately 1 hr at room temperature. Rat polyclonal anti-p19Arf primary antibody
(Catalog #05-929), purchased from Upstate (Chicago, IL, USA), was diluted 1:15 in 0.1% Triton X-100/PBS and added to sections for incubation at 4°C overnight. Sections were subsequently incubated with biotinylated rabbit anti-rat IgG at room temperature for 30 minutes. Staining was detected using the rat Vectastatin Elite ABC kit (Catalog #PK-6104) and DAB following the manufacturers’ protocols. The sections were counterstained with hematoxylin, dehydrated, and mounted. In vivo BrdU labeling and immunohistochemical staining were conducted as previously described [47]. Immunohistochemical staining for BrdU was quantified and summarized as the average number of BrdU-positive interfollicular basal keratinocytes per centimeter of mouse skin ± standard error, N ≥ 3.

Detection of apoptotic keratinocytes. H&E stained mouse skin sections were used to quantify the presence of apoptotic keratinocytes as previously described [3, 4]. TUNEL-staining was conducted as previously described [4] to confirm the apoptosis results obtained using H&E stained sections. Data are presented as the average number of apoptotic keratinocytes per centimeter of mouse skin ± standard error.
**Statistical analysis.** Statistical analysis was performed as described in the text using SASv9.1.3 Service Pack 4.
Acknowledgements

We would like to thank Dr. Cavell Brownie for help with statistical analysis, Bryan Betz for his assistance with p19\textsuperscript{Arf}-immunohistochemistry and providing p16\textsuperscript{INK4a} primers and probe sequences, Sara Dixon for quantification of proliferation in the K14-ER:Ras;C/EBP\textsuperscript{β\textsuperscript{-/-}} mice and Larissa Williams for help with p53-immunohistochemical staining following etoposide treatment. This work was supported by a grant from the National Cancer Institute (CA046637) and a grant from the National Institute of Environmental Health Sciences (ES012473).
References


Figure 1 p19\textsuperscript{Arf} is specifically deregulated in C/EBPβ\textsuperscript{-/-} mouse epidermis.  
(a) Relative p19\textsuperscript{Arf} mRNA levels in C/EBPβ\textsuperscript{+/+} and C/EBPβ\textsuperscript{-/-} mouse epidermis. *, significantly different from C/EBPβ\textsuperscript{+/+} as determined by Student's t-test (N=8, p<0.05). Data represent mean ± standard error. 
(b) Representative Immunoblot analysis of p19\textsuperscript{Arf} and C/EBPβ from lysates isolated from C/EBPβ\textsuperscript{+/+} and C/EBPβ\textsuperscript{-/-} mouse epidermis. β-actin levels are shown as a loading control. 
(c) Relative p16\textsuperscript{INK4a} mRNA levels in C/EBPβ\textsuperscript{+/+} and C/EBPβ\textsuperscript{-/-} mouse epidermis (N=4). Data represent mean ± standard error. No significant difference was observed (p=0.376).  
(d) Balb/MK2 keratinocytes were co-transfected with a p19\textsuperscript{Arf}-promoter reporter construct and either empty vector or a C/EBPβ-expression vector. Data represent mean ± standard error. *, significantly different from basal activity of the p19\textsuperscript{Arf}-promoter reporter, as determined by two-factor ANOVA (N=3 independent experiments, p<0.05).
Figure 2  p19Arf is deregulated in C/EBPβ−/− primary epidermal keratinocytes and highly expressed in a sub-population of keratinocytes within C/EBPβ+/− mouse skin. (a) Relative p19Arf mRNA levels in C/EBPβ+/+ and C/EBPβ−/− primary keratinocytes, over time. Data represent mean ± standard error. Two-factor ANOVA demonstrated significant interaction between genotype and time (p<0.05). *, significantly different from C/EBPβ+/+ at indicated time points (N=3, p≤0.05). (b) Representative immunoblot analysis of p19Arf from lysates harvested from C/EBPβ+/+ and C/EBPβ−/− primary keratinocytes. A non-specific band is shown as a loading control. (c) Representative p19Arf immunohistochemical staining of wild type and C/EBPβ−/− mouse epidermis. Arrows indicate p19Arf-positive nucleolar staining. Four skin sections from each of six independent C/EBPβ+/+ and C/EBPβ−/− mice were examined for p19Arf-positive staining.
**Figure a:**

- **Graph:** The graph shows the relative mRNA levels of p19Arf over time (days in culture) for C/EBPβ⁺/⁺ and C/EBPβ⁻/⁻ mice. There are significant differences observed at 8 and 12 days.

**Figure b:**

- **Table:** The table compares p19Arf levels at different time points (0, 4, 8, 12 days) for C/EBPβ⁺/⁺ and C/EBPβ⁻/⁻ mice. There is a notable difference in p19Arf levels between the two groups at 8 and 12 days.

**Figure c:**

- **Images:** Images of C/EBPβ⁺/⁺ and C/EBPβ⁻/⁻ tissues stained with 20 μM. The images show differences in the tissue structure, indicated by black arrowheads.
**Figure 3** p19\textsuperscript{Arf} is not required for carcinogen-induced anomalous increases in p53 and apoptosis in C/EBP\textsuperscript{β-/-} mice. Skin sections from wild type, p19\textsuperscript{Arf-/-}, C/EBP\textsuperscript{β-/-}, and C/EBP\textsuperscript{β-/-}/p19\textsuperscript{Arf-/-} mice collected 20 hours after treatment with 400 nmol DMBA were used to quantitate p53-positive (a) or apoptotic (b) basal keratinocytes per centimeter of mouse skin. Data represent mean ± standard error. There were no significant differences observed in p53-positive keratinocytes / cm (p=0.769) or apoptotic keratinocytes / cm (p=0.505) between C/EBP\textsuperscript{β-/-} mice compared to C/EBP\textsuperscript{β-/-};p19\textsuperscript{Arf-/-} mice; whereas, both genotypes displayed a significant increase in p53-positive keratinocytes and apoptotic keratinocytes compared to wild type or p19\textsuperscript{Arf-/-} control mice as determined by ANOVA (N≥3, p<0.05).
Figure 4 Oncogenic Ras activation is not sufficient to trigger atypical increases in p53 and apoptosis in C/EBPβ−/− mice. (a) Representative BrdU-immunohistochemical staining of wild type and K14-ER:Ras mice collected before (0 hrs) or after treatment with 4-OHT (24 hrs). (b) Quantitation of BrdU-positive basal keratinocytes in skin sections collected untreated (0 hr) or 4-OHT-treated (24 hr) mice. Data represent mean ± standard error. Two-way ANOVA demonstrated significance for genotype, time, and the interaction between genotype and time. K14-ER:Ras and K14-ER:Ras;C/EBPβ−/− mice displayed significant increases in BrdU-positive keratinocytes compared to wild type or C/EBPβ−/− control mice 24 hrs after treatment (N≥3, p<0.05). (c) p53-positive keratinocytes were quantitated in skin sections collected 12 hrs after treatment with 4-OHT. Data represent mean ± standard error (N≥3). No significant differences were observed using two-factor ANOVA (p=0.554). (d) Keratinocyte apoptosis was quantitated in wild type, C/EBPβ−/−, K14-ER:Ras, and K14ER:Ras;C/EBPβ−/− mice treated with 4-OHT. Data represent the mean ± standard error (N≥3, for all time points). No significant differences were observed as determined by two-factor ANOVA (p=0.251). For comparison, keratinocyte apoptosis was quantitated in wild type and C/EBPβ−/− mouse skin collected 20 hr after treatment with 400nmol DMBA. C/EBPβ−/− mice displayed a significant increase in apoptosis compared to wild type as determined by Student’s t-test (N=3, p<0.05).
Figure 5 DNA damaging agents induce aberrant increases in p53 and apoptosis in C/EBPβ−/− mouse epidermis. C/EBPβ+/+ and C/EBPβ−/− mice were treated with 2.5 µmole of MNNG (a, b), 1 mg etoposide (c, d), or 100 mJ/cm² of UVB (e, f) and the treated, dorsal skin was collected at the indicated times following treatment. p53-positive (a, c, e) or apoptotic interfollicular basal keratinocytes (b, d, f) were quantitated. Data represent mean ± standard error. Two-way ANOVA demonstrated significance for the increase in p53-positive keratinocytes for genotype, time, and the interaction between genotype and time following MNNG, etoposide, and UVB treatment. Two-way ANOVA demonstrated significance for increased apoptosis for genotype, time, and the interaction between genotype and time following MNNG and UVB treatment. *, significantly different from C/EBPβ+/+ at the same time point (N≥3 for all time points, p<0.05).
CHAPTER 2

Gene expression analysis of carcinogen-treated C/EBPβ⁻/⁻ mouse epidermis

Sarah J. Ewing¹,², Robert C. Smart¹,²
¹Cell Signaling and Cancer Group, Department of Environmental and Molecular Toxicology, ²Comparative Biomedical Sciences Program, North Carolina State University, Raleigh, NC

Running title; Gene expression analysis of C/EBPβ⁻/⁻ mice

Key words; C/EBPβ, microarray, carcinogen

CORRESPONDING AUTHOR:

Robert C. Smart
Department of Environmental and Molecular Toxicology
North Carolina State University
Raleigh, NC 27695-7633
USA
Phone: (919) 515-7245, Fax: (919) 515-7169
Email: rcsmart@unity.ncsu.edu
Introduction

C/EBPβ is a member of the basic leucine zipper (bZIP) family of transcription factors and regulates the expression of genes involved in numerous physiological and pathophysiological cellular processes such as proliferation, differentiation, metabolism, inflammation, survival, and tumorigenesis [1, 2]. C/EBPβ functions in these processes through both positive and negative effects on gene expression [1-5]. For example, C/EBPβ functions in inflammatory responses through the regulation of a number of class I acute-phase genes and cytokines such as α1-acid glycoprotein, serum amyloid A and P, complement C3, G-CSF, TNF-α, and IL-6 [6]. C/EBPβ is critical for growth dependent survival of Myc/Raf-transformed macrophages through an auto-regulatory loop involving IGF-1 [7], and regulation of manganous superoxide dismutase (MnSOD) gene expression by C/EBPβ promotes survival of fibroblasts in response to TNFα [8]. C/EBPβ is a key mediator of cyclin D1 activity in human cancer through the ability of C/EBPβ to interact with cyclin D1, contributing to the unique gene expression patterns observed in human cancers overexpressing cyclin D1. Additionally, repression of TFF1 gene expression by C/EBPβ is associated with human gastric cancer [5, 9].
C/EBPβ is abundantly expressed in mouse epidermis where it functions in the early stages of squamous differentiation through the regulation of keratin 1 (K1) and keratin 10 (K10) expression [10-13]. C/EBPβ-/- mouse epidermis displays moderate hyperplasia, and C/EBPβ-/- primary keratinocytes grow more rapidly and to a higher saturation density when placed in culture [10]. C/EBPβ-/- mice are completely refractory to skin tumorigenesis initiated by carcinogens such as DMBA that function by producing oncogenic Ras mutations [14], and C/EBPβ-/- mice respond to a single, topical application of DMBA with vast increases in keratinocyte apoptosis as a direct result of aberrant increases in p53 levels and function [15]. Despite having an important role in epidermal proliferation, differentiation and carcinogen-induced keratinocyte survival and skin tumorigenesis, little is known regarding the target genes responsible for mediating C/EBPβ’s function in these processes.

Several approaches have been used to identify C/EBPβ target genes including the use of cell culture systems, C/EBPβ-/- mice, and promoter analysis of candidate genes [1]. Recent studies have successfully identified biologically relevant C/EBPβ target genes involved in brain injury, liver proliferation following
partial hepatectomy, and growth factor dependent survival of Myc/Raf-transformed macrophages using microarray analysis [7, 16, 17]. We hypothesized that C/EBPβ regulates the expression of a unique set of genes needed to promote both normal epidermal homeostasis as well as keratinocyte cell survival in response to carcinogen treatment. Therefore, we conducted microarray analysis to identify putative in vivo C/EBPβ target genes in mouse epidermis. Our results reveal the identification of a novel group of differentially expressed genes in C/EBPβ−/− mouse epidermis compared to wild type independent of carcinogen treatment. The identified genes participate in a number of important cellular processes such as keratinization, metabolism, protein phosphorylation, apoptosis and gene regulation.
Results

To identify putative C/EBPβ target genes involved in epidermal homeostasis and cell survival in response to DMBA treatment, we performed microarray analysis using total RNA collected from wild type or C/EBPβ−/− mouse epidermis 20 hours after treatment with either DMBA or vehicle control. Total RNA was pooled from two mice per genotype per treatment for hybridization to Affymetrix MOE430A GeneChips®. Four independent pooled samples were processed for each genotype per treatment, resulting in hybridization to a total of 16 arrays. Samples were processed in four “batches”, where each batch consisted of one independent sample per genotype per treatment (WT-acetone, WT-DMBA, C/EBPβ−/−-acetone, C/EBPβ−/−-DMBA). Samples were prepared and hybridized to mouse MOE430A probe arrays exactly as described by the manufacturer. The raw intensity data for perfect match probes were used to calculate a normalized expression value for each probe set using two different statistical methods, dChip or RMA, or for each probe, using mixed model normalization. Based on the data distribution plots, we chose to use the dChip normalized expression values for further analysis.
Although the distribution plot of normalized expression values calculated using dChip do not indicate extreme GeneChip® effects (Figure 1a), they are not easily modeled. For example, gene-by-gene linear models to test for differential expression among the four conditions, including genotype (wild type or C/EBP\(\beta^-\)) and treatment (acetone or DMBA), produce a histogram of p-values that indicate a violation of assumptions necessary for these models. As shown in Figure 1b, there is an apparent departure from the theoretical uniform distribution for p-values corresponding to non-differentially expressed genes. There is a slight excess near 0, but an unexplainable abundance near 1. An alternative approach to testing for differential expression is to use permutation tests. The optimal discovery procedure uses this method [18-20]. It calculates a generalization of the likelihood ratio for normally distributed expressions, but then obtains p-values strictly by permutation of the treatment groups across all arrays. The permutation-based approach to compute p-values makes this procedure robust for data that does not fit a normal distribution. Indeed, a histogram of p-values from this approach looks like a mixture of a uniform distribution and a sample of p-values close to 0 (Figure 1c).
Using the permutation-based approach, and declaring genes with p-values less than 0.00048 to be significant, leads to an estimated false discovery rate (FDR) of 0.05 using Storey’s EDGE software [21]. There are 161 genes that are declared significant using this approach, with an expected false positive count of approximately 8 genes (0.05*161). The expected number of differentially expressed genes is considerably higher, but the number that may be declared significant while controlling the false discovery rate is 161. Analysis using EDGE also revealed that the 161 genes are significant as a result of differential expression between genotypes independent of carcinogen treatment. In other words, no genes were declared significantly altered based on treatment alone or the interaction between genotype and treatment. Of the 161 identified differentially expressed genes, 136 are unique.

Hierarchical clustering, which essentially classifies the significant differentially expressed genes into groups that display similar expression patterns, illustrates the notion that the most pronounced effect contributing to differential gene expression is the main effect of genotype (Figure 2a). The identified up-regulated and down-regulated genes in C/EBPβ−/− mouse epidermis compared to wild type are
listed in Table 1 and 2, respectively. Functional classification of the differentially expressed genes demonstrated enrichment for genes with similar biological functions (Table 3). For example, several keratin genes involved in wound-healing and the response to aberrant proliferation were significantly increased in C/EBPβ−/− mouse epidermis (K6a, K6b, K16, K17). Expression of several cytochrome P450 genes were also differentially expressed in C/EBPβ−/− mouse epidermis compared to wild type epidermis, including Cyp2f2, Cyp1b1, Cyp2g1, and Cyp2b19. Several identified differentially expressed genes are also associated with apoptosis such as cyclin G1, JunD, Egln3, Camk1d, Apoe, Angptl4, Gpx1, ATF5. In general, the identified genes appear to be enriched for several cellular processes in which C/EBPβ has previously been shown to function such as metabolism, apoptosis, gene regulation, and the regulation of cytoskeletal genes.
Discussion

C/EBPβ is an important mediator of epidermal keratinocyte proliferation, differentiation and cell survival [10-15]. C/EBPβ is clearly involved in the early stages of epidermal differentiation, in part through the induction of K1 and K10 [10-13]. Additionally, C/EBPβ may contribute to epidermal differentiation through repression of α2 and α5 integrin gene expression [22]. In response to DMBA, C/EBPβ-/- mice display aberrant increases in apoptosis as a direct result of deregulated p53 levels and function [14, 15]. However, this is not due to modulation of p53 mRNA levels and the mechanism by which C/EBPβ represses p53 in response to DMBA treatment is not known [15]. Our results reveal a novel group of differentially expressed genes in C/EBPβ-/- mouse epidermis compared to wild type that may contribute to C/EBPβ function in normal epidermal homeostasis and/or keratinocyte cell survival in response to carcinogen treatment.

The Affymetrix MOE430A probe arrays have 22626 probe sets that are estimated to represent approximately 18000 transcripts from approximately 14000 genes. Analysis of our data revealed that 9120 probe sets were expressed above background levels in mouse epidermis. Due to the redundancy with respect to the
genes represented in the probe sets on the arrays, approximately 6400 probe sets represent unique genes. Using the complete data set containing all 22626 probe sets, we identified 161 genes differentially expressed in C/EBPβ−/− mouse epidermis compared to wild type. These genes were significantly altered based on genotype alone. None of the identified differentially expressed genes were significant based on treatment alone or the interaction between genotype and treatment. The lack of significantly altered genes based on an interaction between genotype and treatment may be a result of limitations in detection of these genes, as approximately only 1% of C/EBPβ−/− keratinocytes undergo apoptosis 20 hours after DMBA treatment. Thus, RNA collected from the surrounding keratinocytes could dilute the differences in gene expression occurring in the apoptotic keratinocytes, limiting our ability to measure these gene differences. Alternatively, the DMBA-induced p53-mediated apoptosis in C/EBPβ−/− mice may occur through a transcription-independent mechanism [23]. Nevertheless, these studies provide valuable information regarding novel, putative C/EBPβ regulated genes in mouse epidermis.

An interesting group of keratin genes (K6a, K6b, K16, K17) were found to be significantly up-regulated in C/EBPβ−/− mouse epidermis. These genes are not
expressed in normal mouse epidermis, but are rapidly up-regulated during wound repair and diseased skin [24]. K6 as well as Sprr2a expression are up-regulated in C/EBPβ-/- mammary epithelium [25], suggesting C/EBPβ may function in the negative regulation of these genes in epithelial tissues. However, in contrast to our results, K6b promoter activity has been shown to be positively regulated by C/EBPβ in response to TNF-α in keratinocytes [26]. Several cytochrome P450 genes were also significantly altered in C/EBPβ-/- mouse epidermis compared to wild type epidermis. Similarly, C/EBPβ and C/EBPα have been shown to regulate the expression of human cytochrome P450, CYP3A4 [27-29], and rat cytochrome P450, Cyp2d5, or its mouse homolog, Cyp2d11 [30]. Lastly, cyclin G1 and JunD gene expression were up- and down-regulated in C/EBPβ-/- mouse epidermis, respectively, and both function in the negative regulation of p53 and apoptosis [31, 32]. Cyclin G1 functions in a negative feedback loop to regulate p53. p53 induces cyclin G1 gene expression and this induction coincides with activation of p53 by various DNA damaging agents [33-37]. Additionally, cyclin G1 has been shown to augment the induction of apoptosis [33]. JunD-/- mouse embryonic fibroblasts display deregulated p19Arf expression and increased apoptosis in response to DNA
damage, similar to C/EBPβ−/− mouse epidermal keratinocytes [15, 31]. Further studies are required to determine whether these putative C/EBPβ-regulated genes are bona fide C/EBPβ targets and whether C/EBPβ-mediated regulation of these genes contributes to C/EBPβ’s function in epidermal homeostasis and regulation of p53 and cell survival in response to carcinogen treatment.
Materials and Methods

Animals. B6.129-C/EBPβ+/− mice have been previously described [14, 15]. Mice (7-11 weeks old) were clipped with electric clippers at least two days prior to use. Mice were treated with 7,12-dimethylbenz[a]anthracene (Catalog #40818, Fisher Scientific USA - Acros Organics, Pittsburgh, PA, USA) dissolved in acetone (400 nmol/200 µl), or vehicle control (200 µl) and total RNA was prepared from mouse epidermis 20 hours after treatment.

RNA isolation and processing for microarray hybridization and scanning. Total RNA from C/EBPβ+/+ or C/EBPβ−/− in vivo mouse epidermis treated with DMBA, or vehicle control, acetone, was extracted using Tri® Reagent (Sigma, St. Louis, MO, USA) following the manufacturers’ protocol. Total RNA was processed and hybridized to Affymetrix GeneChip® MOE430A probe arrays (Affymetrix, Santa Clara, CA, USA) as described below. Total RNA (2.5 µg) from two independent mice were pooled, for a total of 5 µg, for hybridization to each array. Four independent pooled replicates were collected for each experimental group: Wild Type - acetone (4), Wild Type - DMBA (4), C/EBPβ−/− - acetone (4), and C/EBPβ−/− - DMBA (4), for a total of 16 arrays. Total RNA was collected and prepared in four
separate “batches,” where each batch contained one biological replicate from each experimental group. Total RNA for each “batch” was purified using the Qiagen RNeasy® Mini Kit (Qiagen, Chatsworth, CA, USA), quantified, and examined for RNA quality using formaldehyde denaturing agarose gel electrophoresis. Following confirmation of RNA quality, total RNA was prepared for hybridization to the MOE430A probe arrays following the manufacturer’s instructions. Fragmented, biotinylated cRNA was hybridized overnight to the arrays in the Affymetrix GeneChip® Hybridization Oven 640, and were subsequently washed, stained, and scanned according to the Affymetrix GeneChip® manual. Affymetrix GeneChips® were scanned using the Affymetrix GeneChip® Scanner. Raw intensity data files (.cel) were utilized for data analysis.

**Statistical Analysis.** As described in the text, data normalization was conducted on .cel files using dChip, statistical analysis was performed using Storey’s EDGE software, and graphs were produced in JMP 6 Genomics. Functional classification of differentially expressed genes was conducted using the online annotation software, DAVID [38].
Acknowledgements

We would like to thank Dr. Margie Oleksiak, Dr. Dahlia Nielson, Chris Smith, and Dr. Jason Osborne for their assistance with the design and implementation of the statistical analysis. This work was supported by a grant from the National Cancer Institute (CA046637) and a grant from the National Institute of Environmental Health Sciences (ES012473).
References


12. Oh, H.S. and R.C. Smart, *Expression of CCAAT/enhancer binding proteins (C/EBP) is associated with squamous differentiation in epidermis and*


Figure 1 Data normalization and characterization. (a) Data distribution plot of dChip normalized probe set expression values. Each line represents an independent array. (b) Distribution of p-values after conducting a mixed model analysis to determine differentially expressed genes. (c) Distribution of p-values after using Storey’s EDGE software to perform permutation tests for the detection of differentially expressed genes.
**Figure 2** Hierarchical Clustering. Hierarchical clustering of 161 significant differentially expressed genes. Genes that are up-regulated or down-regulated in C/EBPβ−/− mice compared to wild type mice are shown in the red and green clusters, respectively.
122


### Table 2: List of genes significantly down-regulated in C/EBPζ-/- mouse epididymis compared to wild type.

<table>
<thead>
<tr>
<th>Probe Set ID</th>
<th>Gene Title</th>
<th>Gene Symbol</th>
<th>Fold Change</th>
<th>p Value</th>
<th>q Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1446565_at</td>
<td>cytochrome P450 1A1, family 2, subfamily y,</td>
<td>Cyp2b11</td>
<td>1.20</td>
<td>1.6E-07</td>
<td>1.8E-07</td>
</tr>
<tr>
<td>144665_at</td>
<td>cytochrome P450 1A1, family 2, subfamily y,</td>
<td>Cyp2b11</td>
<td>1.20</td>
<td>1.6E-07</td>
<td>1.8E-07</td>
</tr>
</tbody>
</table>

**Probes and q-values were calculated using Storey's EDGE software.**

*Note: The data includes probe IDs, gene titles, gene symbols, fold changes, p-values, and q-values.*

**Reference:**
<table>
<thead>
<tr>
<th>Functional classification</th>
<th>Gene Title</th>
<th>Gene Symbol</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytoskeleton</td>
<td>keratin complex 1, acidic, gene 14</td>
<td>Krt1-14</td>
<td>2.39</td>
</tr>
<tr>
<td></td>
<td>keratin complex 2, basic, gene 6a</td>
<td>Krt2-6a</td>
<td>4.62</td>
</tr>
<tr>
<td></td>
<td>keratin complex 1, acidic, gene 17</td>
<td>Krt1-17</td>
<td>1.76</td>
</tr>
<tr>
<td></td>
<td>cDNA sequence BC031593</td>
<td>BC031593</td>
<td>1.76</td>
</tr>
<tr>
<td></td>
<td>keratin complex 1, acidic, gene 16</td>
<td>Krt1-16</td>
<td>7.31</td>
</tr>
<tr>
<td></td>
<td>keratin complex 2, basic, gene 6b</td>
<td>Krt2-6b</td>
<td>5.57</td>
</tr>
<tr>
<td></td>
<td>small proline-rich protein 2A</td>
<td>Sprn2a</td>
<td>6.50</td>
</tr>
<tr>
<td></td>
<td>cystatin E/M</td>
<td>Cst5</td>
<td>1.66</td>
</tr>
<tr>
<td></td>
<td>repentin</td>
<td>Rptn</td>
<td>2.98</td>
</tr>
<tr>
<td></td>
<td>tropomyosin 2, beta</td>
<td>Tpm2</td>
<td>1.62</td>
</tr>
<tr>
<td></td>
<td>nuclear distribution gene E homolog 1 (A nidulans)</td>
<td>Nde1</td>
<td>1.38</td>
</tr>
<tr>
<td></td>
<td>troponin T2, cardiac</td>
<td>Tnt2</td>
<td>2.56</td>
</tr>
<tr>
<td></td>
<td>myosin IB</td>
<td>Myo1b</td>
<td>-1.55</td>
</tr>
<tr>
<td></td>
<td>utrophin</td>
<td>Utm</td>
<td>-1.53</td>
</tr>
<tr>
<td></td>
<td>lysosomal trafficking regulator</td>
<td>Lyst</td>
<td>-1.51</td>
</tr>
<tr>
<td></td>
<td>shm pulmonary</td>
<td>Shmm</td>
<td>-1.31</td>
</tr>
<tr>
<td>Metabolism</td>
<td>pyruvate kinase, muscle</td>
<td>Pkm2</td>
<td>1.66</td>
</tr>
<tr>
<td></td>
<td>fructosephosphate isomerase 1</td>
<td>Tpi1</td>
<td>1.73</td>
</tr>
<tr>
<td></td>
<td>phosphofructokinase, platelet</td>
<td>Pfkl</td>
<td>1.73</td>
</tr>
<tr>
<td></td>
<td>phosphoglycerate kinase 1</td>
<td>Pkg1</td>
<td>1.77</td>
</tr>
<tr>
<td></td>
<td>phosphoglycerate mutase 1</td>
<td>Pgam1</td>
<td>1.69</td>
</tr>
<tr>
<td></td>
<td>enolase 1, alpha non-neuron</td>
<td>Eno1</td>
<td>1.87</td>
</tr>
<tr>
<td></td>
<td>phosphomannomutase 1</td>
<td>Pmm1</td>
<td>1.44</td>
</tr>
<tr>
<td></td>
<td>dicarboxyl L-xylulose reductase</td>
<td>Dcxr</td>
<td>1.12</td>
</tr>
<tr>
<td></td>
<td>phosphofructokinase, platelet</td>
<td>Pfkl</td>
<td>1.73</td>
</tr>
<tr>
<td></td>
<td>arachidonate lipoxygenase, epidermal</td>
<td>Alox12a</td>
<td>2.44</td>
</tr>
<tr>
<td></td>
<td>stearyl-coenzyme A desaturase 3</td>
<td>Sod3</td>
<td>-2.58</td>
</tr>
<tr>
<td></td>
<td>3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1</td>
<td>Hmgcs1</td>
<td>-1.31</td>
</tr>
<tr>
<td></td>
<td>phosphomevalonate kinase</td>
<td>Pmvk</td>
<td>-1.59</td>
</tr>
<tr>
<td></td>
<td>mevalonate (diphospho) decarboxylase</td>
<td>Mvd</td>
<td>-1.52</td>
</tr>
<tr>
<td>Cytochrome P450s</td>
<td>cytochrome P450, family 1, subfamily b, poly peptide 1</td>
<td>Cyp1b1</td>
<td>2.59</td>
</tr>
<tr>
<td></td>
<td>cytochrome P450, family 2, subfamily b, poly peptide 19</td>
<td>Cyp2b19</td>
<td>-1.84</td>
</tr>
<tr>
<td></td>
<td>cytochrome P450, family 2, subfamily f, poly peptide 2</td>
<td>Cyp2f2</td>
<td>-3.28</td>
</tr>
<tr>
<td></td>
<td>cytochrome P450, family 2, subfamily g, poly peptide 1</td>
<td>Cyp2g1</td>
<td>-4.34</td>
</tr>
<tr>
<td>Phosphorylation</td>
<td>FMS-like tyrosine kinase 1</td>
<td>Fli1</td>
<td>3.62</td>
</tr>
<tr>
<td></td>
<td>CD 81 antigen</td>
<td>Cdb1</td>
<td>2.29</td>
</tr>
<tr>
<td></td>
<td>calcium/calmodulin-dependent protein kinase I</td>
<td>Camki</td>
<td>-1.92</td>
</tr>
<tr>
<td></td>
<td>calcium/calmodulin-dependent protein kinase ID</td>
<td>Camk1d</td>
<td>-1.49</td>
</tr>
<tr>
<td></td>
<td>NIMA (never in mitosis gene a)-related expressed kinase 3</td>
<td>Nek3</td>
<td>-1.20</td>
</tr>
<tr>
<td>Transcription Factors</td>
<td>basic helix-loop-helix domain containing, class B2</td>
<td>Bhlhb2</td>
<td>1.83</td>
</tr>
<tr>
<td></td>
<td>nuclear receptor subfamily 1, group D, member 2</td>
<td>Nr1d2</td>
<td>-1.23</td>
</tr>
<tr>
<td></td>
<td>homebox D1</td>
<td>Hoxd1</td>
<td>-1.37</td>
</tr>
<tr>
<td></td>
<td>activating transcription factor 5</td>
<td>Atf5</td>
<td>-1.46</td>
</tr>
<tr>
<td></td>
<td>jun proto-oncogene related gene d1</td>
<td>Jund1</td>
<td>-1.35</td>
</tr>
<tr>
<td>Apoptosis</td>
<td>Cyclin G1</td>
<td>Cyclin G1</td>
<td>1.35</td>
</tr>
<tr>
<td></td>
<td>EGL nine homolog 3 (C. elegans)</td>
<td>Egln3</td>
<td>2.78</td>
</tr>
<tr>
<td></td>
<td>apolipoprotein E</td>
<td>Apeo</td>
<td>1.77</td>
</tr>
<tr>
<td></td>
<td>angiotensin-like 4</td>
<td>Angpt4</td>
<td>1.60</td>
</tr>
<tr>
<td></td>
<td>glutathione peroxidase 1</td>
<td>Gpx1</td>
<td>2.00</td>
</tr>
<tr>
<td></td>
<td>calcium/calmodulin-dependent protein kinase ID</td>
<td>Camk1d</td>
<td>-1.49</td>
</tr>
<tr>
<td></td>
<td>activating transcription factor 5</td>
<td>Atf5</td>
<td>-1.46</td>
</tr>
<tr>
<td></td>
<td>jun proto-oncogene related gene d1</td>
<td>Jund1</td>
<td>-1.35</td>
</tr>
</tbody>
</table>
GENERAL DISCUSSION

Cell signaling depends on the activity of site-specific transcription factors to regulate the expression of genes needed to elicit the intended response. Cells are inundated with a plethora of signals at any given time and must carefully balance these signals through the activation of signaling networks, which provide overlap allowing for balance and signal amplification as needed. Site-specific transcription factors function to fine-tune the response to these many signals, helping to maintain cellular homeostasis.

C/EBPβ is clearly important in maintaining cellular and tissue homeostasis by functioning in processes such as proliferation, differentiation, metabolism, inflammatory responses, and cell survival. This is most apparent in C/EBPβ−/− mice, which display abnormalities in many of these processes in several cell types. Additionally, it is clear that C/EBPβ functions in the regulation of genes involved in these cellular processes downstream of a multitude of signals that activate various signal transduction cascades. In the work presented here, we add to this increasing body of research surrounding the role of C/EBPβ in mediating cellular responses by demonstrating for the first time that C/EBPβ functions in the DNA damage response
pathway to repress p53 levels and function. Additionally, we show that C/EBPβ is involved in the negative regulation of p19^Arf in mouse epidermis and identify a novel set of putative C/EBPβ target genes in mouse epidermis.

In Chapter 1, we reveal two important, novel findings regarding C/EBPβ-function in mouse skin. First, C/EBPβ represses p53 to promote cell survival downstream of DNA damage. Second, C/EBPβ is involved in the negative regulation of p19^Arf. Previous studies from our lab demonstrated that C/EBPα is a p53-inducible mediator of the G1-checkpoint downstream of DNA damage. Our results provide the first demonstration that C/EBPβ also has a role in the DNA damage response pathway. How C/EBPβ levels and/or activity are regulated by UVB or other DNA damaging agents is unknown. Analysis of the C/EBPβ protein sequence revealed a putative PIKK (ATM/ATR/DNA-PK) and Chk1/2 consensus phosphorylation motif ([S/T]Q). It will be important to determine how C/EBPβ levels and/or activity are regulated by DNA damage response kinases and whether this regulation facilitates C/EBPβ-mediated repression of p53 and apoptosis in response to DNA damage.
Carcinogen-induced aberrant increases in p53 and apoptosis in C/EBPβ−/− mice occur independent of changes in p53 mRNA levels [109]. C/EBPβ has been shown to interact with p53 and thereby inhibit p53 DNA binding and transcriptional activities, providing one mechanism through which C/EBPβ may repress p53 levels and function [213]. Alternatively, C/EBPβ may regulate the expression of genes or the activity of proteins that are responsible for regulating p53 through post-translational modification. Previous studies from our lab demonstrated that Mdm2 levels are increased in C/EBPβ−/− mice in response to carcinogen treatment, indicating C/EBPβ-mediated regulation of Mdm2 is not involved in the regulation of p53 by C/EBPβ [109]. However, C/EBPβ may regulate levels or activity of proteins involved in the phosphorylation, acetylation, or sumoylation of p53. Indeed, C/EBPβ has been shown to modulate the activity of p300, which is a major factor involved in p53 acetylation [246]. Further studies are needed to elucidate whether specific post-translational modifications of p53 are altered in C/EBPβ−/− mice following carcinogen treatment and if so, how C/EBPβ contributes to the regulation of p53 at these sites.
C/EBPβ is required for skin tumorigenesis involving oncogenic Ras [95]. C/EBPβ-mediated repression of p53 to promote cell survival in response to carcinogen treatment represents one mechanism through which C/EBPβ may be required for tumorigenesis. Therefore, understanding the molecular events surrounding C/EBPβ’s ability to promote cell survival may provide the opportunity to develop novel molecular intervention strategies to inhibit C/EBPβ and block p53-proficient tumor cell survival, resulting in inhibition of tumor development and/or tumor regression.

Despite significant increases in p19Arf in C/EBPβ−/− mouse epidermis, p19Arf is not required for C/EBPβ-mediated repression of p53 and apoptosis in response to DNA damage. However, p19Arf is a critical mediator of oncogene-induced senescence [247] and could contribute to the complete block in skin tumorigenesis in C/EBPβ−/− mice through this mechanism. Recent studies have begun to address whether DNA damage or oncogenic signaling is more important in inducing p53-mediated tumor suppression. These studies have demonstrated that oncogenic signaling through p19Arf may provide the key to unlocking p53’s major tumor suppressor function [243, 245]. Our results are the first to demonstrate that p19Arf
levels are deregulated in C/EBPβ−/− mice. The biological significance of increased p19Arf in C/EBPβ−/− mice and the exact mechanism through which C/EBPβ negatively regulates p19Arf expression remain to be determined. Preliminary results suggest that C/EBPβ may contribute to the inhibition of p19Arf through repression of p19Arf promoter activity.

In Chapter 2 we began a study to identify novel, putative C/EBPβ target genes in mouse epidermis, particularly genes involved in the DMBA-induced aberrant p53 and apoptotic response in C/EBPβ−/− mice. Using the complete data set containing all 22626 probe sets from the Affymetrix MOE430A probe arrays, we identified 161 genes differentially expressed in C/EBPβ−/− mouse epidermis compared to wild type. The identified genes participate in a number of important cellular processes such as keratinization, metabolism, protein phosphorylation, apoptosis and gene regulation.

Surprisingly, all of the identified differentially expressed genes were significantly altered based on genotype alone. None of the identified differentially expressed genes were significant based on treatment alone or the interaction between genotype and treatment. The lack of significantly altered genes based on
an interaction between genotype and treatment may be a result of limitations in detection of these genes, as approximately only 1% of C/EBPβ−/− keratinocytes undergo apoptosis 20 hours after DMBA treatment. Thus, RNA collected from the surrounding keratinocytes could dilute the differences in gene expression occurring in the apoptotic keratinocytes, limiting our ability to measure these gene differences. Alternatively, the DMBA-induced p53-mediated apoptosis in C/EBPβ−/− mice may occur through a transcription-independent mechanism [248]. Nevertheless, these studies provide valuable information regarding novel, putative C/EBPβ regulated genes in mouse epidermis.

Our results indicate that several groups of related genes are differentially expressed in C/EBPβ−/− mouse epidermis compared to wild type epidermis. One interesting related group of genes is a subset of keratin genes (K6, K16, K17) that are not expressed under normal conditions, but rather are up-regulated during wound repair, in response to aberrant proliferation, and in diseased skin [249]. Interestingly, data demonstrate that C/EBPβ−/− mice display an atypical increase in the number of S-phase proliferative cells in the suprabasal layers of mouse epidermis as a result of an intrinsic keratinocyte defect (Figure 1a and 1b), and
display moderate hyperplasia [63]. Further studies are required to determine whether these keratin genes are direct targets of C/EBPβ or whether their expression is altered as a result of the aberrant proliferation in C/EBPβ−/− mouse epidermis. Another differentially regulated gene of interest is JunD. JunD is a bZIP protein and part of the AP-1 family of regulatory transcription factors. JunD positively and negatively regulates cell proliferation depending on cell type and genetic context. JunD−/− MEFs display deregulated p19Arf under normal conditions and aberrant increases in p53 and apoptosis in response to UVB, similar to C/EBPβ−/− epidermal keratinocytes [117]. Additionally, C/EBPβ and JunD can cooperate to regulate gene expression [250]. Further studies will be required to determine whether altered expression of these genes contribute to C/EBPβ function in mouse epidermis.
Figure 1 C/EBPβ⁻/⁻ mice display aberrant basal and suprabasal epidermal keratinocyte proliferation. (a) Untreated C/EBPβ⁺/⁺ and C/EBPβ⁻/⁻ mice were injected with BrdU solution one hour prior to sacrificing. Shaved, dorsal skin was fixed and prepared for immunohistochemical staining with a BrdU monoclonal antibody. (b) BrdU-immunohistochemical staining was conducted as in (a) using untreated epidermal specific C/EBPβ-null mice.
### a

<table>
<thead>
<tr>
<th>Genotype</th>
<th>( n )</th>
<th>Number of BrdU Positive Suprabasal Keratinocytes/cm(^b)</th>
<th>Number of BrdU Positive Basal Keratinocytes/cm(^b)</th>
<th>Nucleated Cell Layers(^b)</th>
<th>Epidermal Thickness ((\mu m))(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C/EBP(\beta)(^{+/+})</td>
<td>5</td>
<td>0.13 ± 0.06</td>
<td>46.52 ± 6.38</td>
<td>1.48 ± 0.06</td>
<td>8.62 ± 0.71</td>
</tr>
<tr>
<td>C/EBP(\beta)(^{-/-})</td>
<td>6</td>
<td>3.96 ± 1.94(^c)</td>
<td>136.27 ± 26.69(^d)</td>
<td>2.43 ± 0.16(^i)</td>
<td>14.22 ± 1.76</td>
</tr>
</tbody>
</table>

\(^{a}\)Number of animals per group.

\(^{b}\)Data is expressed as mean ± standard error.

\(^{c}\)Statistically significant at \(p<0.01\) from C/EBP\(\beta\)\(^{+/+}\) using Wilcoxon rank sum test.

\(^{d}\)Statistically significant at \(p<0.05\) from C/EBP\(\beta\)\(^{-/-}\) using two-tailed Student's t-test.

### b

**Increased S-phase BrdU-Positive Suprabasal Keratinocytes is an intrinsic Epidermal Keratinocyte Defect**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>( n )</th>
<th>Number of BrdU Positive Suprabasal Keratinocytes/cm(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Type</td>
<td>7</td>
<td>0.25 ± 0.10</td>
</tr>
<tr>
<td>C/EBP(\beta)(^{-/-})</td>
<td>4</td>
<td>0.25 ± 0.10</td>
</tr>
<tr>
<td>K5-Cre-C/EBP(\beta)</td>
<td>4</td>
<td>0.13 ± 0.10</td>
</tr>
<tr>
<td>K5-Cre-C/EBP(\beta)(^{-/-})</td>
<td>7</td>
<td>1.21 ± 0.40(^i)</td>
</tr>
</tbody>
</table>

\(^{a}\)Number of animals per group.

\(^{b}\)Data is expressed as mean ± standard error.

\(^{i}\)Statistically significant at \(p<0.05\) from wild type using Student's t-test.
GENERAL REFERENCES


39. Oh, H.S. and R.C. Smart, *Expression of CCAAT/enhancer binding proteins (C/EBP) is associated with squamous differentiation in epidermis and*


79. Potten, C.S. and T.D. Allen, *Control of epidermal proliferative units (EPUs).*

   An hypothesis based on the arrangement of neighbouring differentiated cells.


115. Prieur, A., Peeper, D.S. *Opposite functions of DRIL1, a member of the ARID family DNA-binding proteins, in human and mouse fibroblast senescence*. in *American Association for Cancer Research*. 2007. Los Angeles, California.


125. Cesena, T.I., et al., CCAAT/enhancer-binding protein (C/EBP) beta is acetylated at multiple lysines: acetylation of C/EBPbeta at lysine 39


222. Harris, S.L. and A.J. Levine, *The p53 pathway: positive and negative


Decreased Survival of C/EBPβ-Deficient Keratinocytes is Due to Aberrant
Regulation of p53 Levels and Function

Kyungsil Yoon1,2, Songyun Zhu1, Sarah J. Ewing and Robert C. Smart
Cell Signaling and Cancer Group, Department of Environmental and Molecular
Toxicology, North Carolina State University, Raleigh, NC

Running title; C/EBPβ has a role in the negative regulation of p53

Key words; apoptosis, p53, C/EBPβ, keratinocytes

CORRESPONDING AUTHOR:

Robert C. Smart, Ph.D.
Department of Environmental and Molecular Toxicology
North Carolina State University
Raleigh, NC 27695-7633
USA
Phone: (919) 515-7245, Fax: (919) 515-7169
Email: rcsmart@unity.ncsu.edu

1These authors contributed equally to this manuscript

2Present address; Institute of Biosciences and Technology, Texas A&M University
Health Science Center, Houston, TX

Published in Oncogene 26:360-367 2007.
ABSTRACT

Recent studies have identified roles for C/EBPβ in cellular survival and tumorigenesis; however, the mechanisms through which C/EBPβ regulates these processes are not fully understood. Previously, we demonstrated that C/EBPβ−/− mice are resistant to carcinogen-induced skin tumorigenesis and in response to topical carcinogen treatment display a 17-fold increase in keratinocyte apoptosis compared to wild type mice. Here we have investigated the mechanisms through which C/EBPβ regulates apoptosis in response to carcinogenic stress. Analysis of carcinogen-treated C/EBPβ−/− mouse skin revealed a striking increase in the number of p53 immunopositive keratinocytes in the epidermis of C/EBPβ−/− mice compared to wild type mice and this increase was temporally associated with a concomitant anomalous increase in apoptosis. The increased levels of p53 were functional as Mdm2, Bcl-2, C/EBPα and p21 were differentially regulated in the epidermis of carcinogen-treated C/EBPβ−/− mice. The increase in p53 protein was not associated with an increase in p53 mRNA levels. To determine whether p53 is required for the increased apoptosis in C/EBPβ−/− mice, C/EBPβ/p53 compound knockout mice were generated. Carcinogen-treated C/EBPβ/p53 compound knockout mice did not
display increased apoptosis demonstrating p53 is required for the pro-apoptotic phenotype in C/EBPβ-/- mice. Our results demonstrate that altered keratinocyte survival in C/EBPβ-/- mice results from aberrant regulation of p53 protein and function and indicate C/EBPβ has a role in the negative regulation of p53 protein levels in response to carcinogen-induced stress.
INTRODUCTION

CCAAT/enhancer binding proteins (C/EBPs) are members of the basic leucine zipper (bZIP) class of transcription factors and are involved in fundamental processes including cell growth, differentiation, survival, inflammation, immune response and metabolism (Ramji & Foka, 2002). C/EBPβ is expressed in many tissues, including epidermis (Maytin & Habener, 1998; Oh & Smart, 1998) where it plays a role in the early stages of stratified squamous differentiation (Zhu et al., 1999). Recent studies have identified a role for C/EBPβ in tumor development (Zhu et al., 2002), senescence (Sebastian et al., 2005), cell survival (Buck et al., 2001; Li et al., 2005; Wessells et al., 2004; Zhu et al., 2002) and cellular transformation (Zhu et al., 2002). In terms of cell transformation, C/EBPβ can cooperate with Ras12V to transform NIH3T3 cells (Zhu et al., 1999) and more recently, it has been shown that cell-cycle dependent phosphorylation of C/EBPβ on Ser64 and Thr189 is required to promote Ras-induced transformation of NIH3T3 cells (Shuman et al., 2004).

C/EBPβ-deficient mice are completely refractory to skin tumorigenesis induced by carcinogens such as 7,12-dimethylbenz[a]anthracene (DMBA) that
produce skin tumors containing oncogenic Ras mutations (Zhu et al., 2002). Importantly, apoptosis is dramatically elevated in epidermal keratinocytes of DMBA-treated C/EBPβ-null mice compared to similarly treated wild type mice (Zhu et al., 2002) suggesting that C/EBPβ regulates cell survival by suppressing apoptosis in response to DNA damage/oncogenic stress. Studies utilizing epidermis-specific C/EBPβ knockout mice (keratin 5-Cre;C/EBPβfl/fl mice) demonstrated that C/EBPβ exerts a keratinocyte intrinsic role in cell survival in response to carcinogen treatment (Sterneck et al., 2005).

C/EBPβ is important for the survival of numerous cell types in response to various stressors. For example, C/EBPβ has a prosurvival function in Myc/Ras transformed macrophages through an auto-regulatory pathway involving IGF-1 (Wessells et al., 2004). C/EBPβ also has a pro-survival/anti-apoptosis function in CCl₄-treated hepatic stellate cells (Buck et al., 2001). In Wilms tumors, or nephroblastomas, C/EBPβ is expressed at high levels in relapsing and metastatic tumors and blocking C/EBPβ expression in a Wilms tumor cell line results in spontaneous apoptosis (Li et al., 2005). Increased expression of C/EBPβ is associated with human breast, colorectal and ovarian tumorigenesis (Bundy &
Sealy, 2003; Rask et al., 2000; Sundfeldt et al., 1999). In addition, cyclin D1 can interact with C/EBPβ to alter gene expression and there is evidence that this interaction is required for the unique patterns of gene expression observed in human cancers that over-express cyclin D1 (Lamb et al., 2003). Collectively these studies indicate that C/EBPβ has a significant role in the proliferation/survival of a variety of human and rodent tumor cells.

In the current study we investigated the mechanisms through which C/EBPβ regulates cell survival in response to carcinogenic stress. We observed that altered keratinocyte survival in C/EBPβ−/− mice results from aberrant regulation of p53 protein and function and our results indicate that C/EBPβ has a critical role in the negative regulation of p53 protein levels in response to carcinogen-induced stress.
RESULTS

C/EBPβ−/− mice display a 17-fold increase in apoptosis in epidermal keratinocytes at 24 hrs following a single topical treatment with DMBA compared to similarly treated wild type mice (Zhu et al., 2002). To begin to define this apoptotic response in C/EBPβ−/− mice and to determine whether the increase in apoptosis involves p53, a time course for DMBA-induced apoptosis and p53 protein expression in C/EBPβ−/− and wild type mice was conducted. Apoptotic keratinocytes were identified in hematoxylin and eosin stained skin sections and were characterized by the simultaneous presence of pyknotic nuclei, eosinophilic cytoplasm and some loss of cellular attachment. A representative hematoxylin and eosin stained skin section from DMBA-treated wild type and C/EBPβ−/− mice (24 hrs post DMBA treatment) is shown in Figure 1A and 1B. Numerous apoptotic keratinocytes were detected in DMBA-treated C/EBPβ−/− mice while apoptotic cells were scarce in similarly treated wild type mice. Similar results, both in terms of numbers and location of apoptotic keratinocytes, were obtained in wild type and C/EBPβ−/− mice utilizing terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) staining (Figure 1C and 1D). Within the interfollicular
epidermis, most (>95%) of the apoptotic keratinocytes appeared in the basal layer. Within the follicular epidermis, increased numbers of apoptotic keratinocytes were observed in the infundibulum (Figure 1E) and isthmus region of the hair follicles of DMBA-treated C/EBPβ−/− mice and while an increase was also observed in wild type mice, it occurred to a lesser degree. As shown in Figure 1F, the number of interfollicular basal apoptotic keratinocytes was quantified every 4 hours post topical DMBA treatment up to 32 hours in wild type and C/EBPβ−/− mice. The overall apoptotic response of C/EBPβ−/− mice was significantly different from that of wild type mice (Two factor ANOVA p<0.05). Apoptosis was significantly increased in DMBA-treated C/EBPβ−/− mice at 16, 20 and 24 hrs post-DMBA treatment compared to similarly treated wild type mice. The number of apoptotic keratinocytes in C/EBPβ−/− mice peaked at 24 hours post-DMBA treatment and this number corresponds to ~2% of total interfollicular basal keratinocytes and represents a 60-fold increase in apoptotic keratinocytes in DMBA-treated C/EBPβ−/− mice. In comparison, the maximum increase in the number of apoptotic keratinocytes in DMBA-treated wild type mice was only 5-fold. After 24 hrs the number of apoptotic keratinocytes in C/EBPβ−/− mice decreased sharply which is likely due to the
clearance (cutaneous/systemic absorption, metabolism and excretion) of topically applied DMBA from the epidermis. At 28 and 32 hours the levels of apoptotic keratinocytes were similar in both genotypes.

To determine whether the increased aberrant apoptotic response in the C/EBPβ⁻/⁻ mice is associated with alterations in p53 levels, immunohistochemical staining for p53 in DMBA-treated wild type and C/EBPβ⁻/⁻ mouse skin was conducted. In vehicle treated mice of both genotypes the number of p53 immunostained cells was low and they displayed weak nuclear staining indicating low levels of p53 protein (Figure 2A). However, in DMBA-treated C/EBPβ⁻/⁻ mice numerous interfollicular basal keratinocytes displayed intense nuclear p53 staining while similarly treated wild type mice displayed relatively infrequent p53 staining in basal cells of the epidermis (Figure 2A). Increased numbers of p53 positive keratinocytes were also observed in the hair follicles of DMBA-treated C/EBPβ⁻/⁻ mice compared to similarly treated wild type mice (Figure 2B). The number of p53 positive basal keratinocytes in DMBA-treated C/EBPβ⁻/⁻ and wild type mice was quantified over the 32 hour time course (Figure 2C). The response of C/EBPβ⁻/⁻ mice was significantly different from that of wild type mice (Two factor ANOVA
p<0.05). The number of p53 positive interfollicular keratinocytes was significantly increased at 4, 8, 16, 20 and 24 hrs post-DMBA treatment in C/EBPβ⁻/⁻ mice compared to wild type mice. These results indicate that p53 levels are deregulated in C/EBPβ⁻/⁻ mice in response to DMBA treatment. The time course for the DMBA-induced increase in p53-positive keratinocytes (Figure 2C) and the time course for apoptosis (Figure 1F) in C/EBPβ⁻/⁻ mice is similar suggesting a causal relationship between the increased p53 levels and apoptosis.

To determine whether the DMBA-induced increase in p53 protein levels in C/EBPβ⁻/⁻ keratinocytes is associated with increased p53 activity, we examined p53 regulated downstream gene products. Western blot analysis of epidermal lysates prepared from DMBA-treated wild type and C/EBPβ⁻/⁻ mice demonstrated that p53 levels were preferentially increased in C/EBPβ⁻/⁻ epidermis (Figure 3A) confirming our immunohistochemical observations of differential induction of p53 protein. The increase in p53 levels as detected by immunoblot analysis appears modest compared to the increased numbers of p53 positive cells in DMBA-treated C/EBPβ⁻/⁻ mouse epidermis (Figure 2C). However, it should be noted that epidermal lysates contain protein derived from suprabasal and basal cells that express low p53.
protein levels and this produces a large dilution of the p53-positive basal keratinocytes which account for approximately 7% of basal keratinocytes. Thus, a 10-fold increase in p53 in 7% of the cells would result in less than a 2-fold induction upon immunoblot analysis of total epidermal protein. p21 and C/EBPα are both known to be positively regulated by p53 in response to DNA damage. As shown in Figure 3A, both p21 and C/EBPα protein levels were induced to higher levels by DMBA treatment in C/EBPβ−/− epidermis compared to similarly treated wild type mice. Immunohistochemical staining for epidermal p21 revealed a significant increase in the number of p21-positive cells in DMBA-treated C/EBPβ−/− mice (Figure 3B) and this increase is similar to that observed for p53 immunopositive cells (Figure 2C). The large increase in p21 immunopositive cells provides additional evidence that immunoblot analysis of total epidermal protein results in a dramatic under representation of the actual changes. Bcl-2 has anti-apoptotic activity and is negatively regulated by p53. As shown in Figure 3A, Bcl-2 protein levels were decreased in the epidermis of DMBA-treated C/EBPβ−/− mice compared to wild type mice (Figure 3A). Collectively, these results demonstrate that the increased p53 levels observed in the epidermis of DMBA-treated C/EBPβ−/− mice are associated
with increased p53 function. Our immunohistochemical staining results indicate that a select minor population of keratinocytes within the epidermis is responding to carcinogen treatment with increased p53 protein and p53 target gene expression. Analysis of total epidermal protein/mRNA for p53 and p53 target gene products results in a significant under representation of the response of target cells to carcinogen-induced stress due to a poor signal to noise ratio.

To determine whether the anomalous increase in apoptosis in the C/EBPβ−/− mice is dependent upon p53, we crossed C/EBPβ−/− mice with p53 deficient mice to produce C/EBPβ/p53 compound knockout mice. C/EBPβ/p53 compound knockout mice as well as C/EBPβ−/−, p53−/− and wild type littermates were treated with a single dose of DMBA and the number of apoptotic keratinocytes was quantified. As shown in Figure 4, DMBA-treated C/EBPβ−/− mice displayed their characteristic elevated levels of apoptosis. In contrast, DMBA-treated C/EBPβ/p53 compound knockout mice displayed low levels of apoptosis similar to that observed in DMBA-treated wild type mice. Thus, the deletion of p53 in C/EBPβ−/− mice (C/EBPβ−/−;p53−/− ) rescues keratinocytes from DMBA-induced apoptosis. These results demonstrate that p53
is an absolute requirement for the anomalous increase in apoptosis in C/EBPβ−/− keratinocytes.

Our results reveal that p53 protein levels are deregulated in C/EBPβ−/− epidermis in response to DMBA and that this de-regulation is responsible for the increased aberrant apoptotic response in C/EBPβ−/− mice. However, it is not known how C/EBPβ represses p53 levels in response to carcinogen stress. Because C/EBPβ can function as a transcriptional repressor of certain genes we measured p53 mRNA levels with quantitative TaqMan® RT-PCR. Basal levels of p53 mRNA in epidermis of C/EBPβ+/− mice were ~2-fold greater than that in wild type mice suggesting C/EBPβ has a repressor function in the regulation of basal p53 mRNA expression (Figure 5A). However, treatment of wild type and C/EBPβ−/− mice with DMBA did not significantly induce p53 mRNA in either genotype (Figure 5A) indicating that the increased p53 protein levels in DMBA-treated C/EBPβ−/− epidermis (Figure 2C) are not due to the up-regulation of p53 mRNA levels. These results suggest C/EBPβ has a role in the regulation of p53 protein levels independent of regulation of p53 mRNA levels. Mdm2 is a p53 target gene and major negative regulator of p53 protein levels. If C/EBPβ is involved in the positive
regulation of Mdm2 levels, then C/EBPβ deficiency would result in decreased Mdm2 levels and increased p53 protein levels. We measured Mdm2 mRNA levels with quantitative TaqMan® RT-PCR and conducted immunohistochemical staining for mdm2 protein. As shown in Figure 5B, Mdm2 mRNA levels were greater in the DMBA-treated mice and we observed increased numbers of Mdm2 immunopositive cells with strong nuclear staining in DMBA-treated C/EBPβ−/− mice compared to similarly wild type mice (Figure 5C). Collectively, these results indicate p53 protein levels and function are increased in C/EBPβ−/− mice despite elevated levels of Mdm2.
DISCUSSION

Previously we demonstrated that C/EBPβ−/− mice display elevated levels of apoptosis in response to topical DMBA treatment and that these mice are completely refractory to skin tumorigenesis induced by carcinogens such as DMBA (Zhu et al., 2002). These studies suggested the possibility that C/EBPβ is required for the survival of DMBA-induced tumor precursor cells. Accordingly, C/EBPβ deficiency would result in apoptosis of the tumor precursor cells and account for the resistance to skin tumor development in carcinogen-treated C/EBPβ−/− mice. Thus, understanding the pro-apoptotic response in C/EBPβ−/− mice has important implications for early tumor development. In the current study we demonstrated that the abnormal increase in apoptosis in DMBA-treated C/EBPβ−/− keratinocytes requires p53 and is due to the aberrant up-regulation of p53 levels and function. Our results indicate that in wild type mice, C/EBPβ suppresses carcinogen-induced apoptosis in keratinocytes through repression of p53 protein levels/function. In the absence of C/EBPβ, carcinogen treatment results in the de-repression of p53 protein levels resulting in increased p53 protein levels, increased p53 regulated gene expression and increased C/EBPβ−/− keratinocyte apoptosis. We propose
that the pro-apoptotic phenotype in C/EBPβ−/− mice results from a defect in the ability of C/EBPβ−/− keratinocytes to suppress p53-mediated apoptosis in response to carcinogen-induced stress. DMBA produces oncogenic mutations in Ras in keratinocytes of treated mouse skin. Currently, it is not known whether the increase in DMBA-induced apoptosis in C/EBPβ−/− keratinocytes is in response to DNA damage and/or oncogenic Ras-induced oncogenic stress.

Our results indicate that the aberrant increase in p53 protein levels induced by carcinogen treatment in C/EBPβ−/− epidermis occur independent of changes in p53 mRNA levels. Regulation of p53 protein levels and p53 transcriptional activity is complex and approximately 10 regulatory feedback loops have been identified (Harris & Levine, 2005). One key regulator of p53 protein levels is Mdm2. Mdm2 negatively regulates p53 protein by mediating nuclear export and subsequent ubiquitination and proteosomal degradation and also decreases p53 transcriptional activity by direct binding to p53 (Freedman & Levine, 1998; Honda et al., 1997). We initially hypothesized that if C/EBPβ were involved in the positive regulation of Mdm2 expression, then loss of C/EBPβ in DMBA-treated C/EBPβ−/− epidermis would likely result in decreased Mdm2 levels and increased p53 protein levels. However,
our results show the opposite, that is, Mdm2 mRNA and protein are increased in carcinogen-treated C/EBPβ−/− mice compared to carcinogen-treated wild type mice. Thus, despite increased Mdm2 protein in C/EBPβ−/− epidermis there is increased levels of functional p53 as determined by increased p53-target gene expression and apoptosis. These results suggest that the activity of Mdm2 protein is attenuated in C/EBPβ−/− epidermis in response to carcinogen-induced stress. Several mechanisms are known to modulate Mdm2 activity including posttranslational modification of p53 and Mdm2 as well as interactions with cellular factors including p19Arf, MdmX and Arf-BP1 (Brooks & Gu, 2006). Acetylation of C-terminal lysines on p53 blocks Mdm2 induced ubiquitination of p53 and p53 degradation (Ito et al., 2002). ATM and/or ATR dependent phosphorylation of Mdm2 (Maya et al., 2001) as well as p53 (Shieh et al., 1999) blocks p53-Mdm2 interaction and stabilizes p53 . The tumor suppressor p19Arf, promotes Mdm2 degradation, sequesters Mdm2 in nucleolus and blocks nucleocytoplasmic shuttling of Mdm2 resulting in the accumulation of p53 (Kamijo et al., 1998; Pomerantz et al., 1998; Tao & Levine, 1999; Zhang et al., 1998). MdmX can form dimers with Mdm2 and stabilize p53 (Jackson & Berberich, 2000). It is possible that C/EBPβ may regulate genes
involved in posttranslational modifications of p53 such as those involved in p53 phosphorylation, methylation, acetylation, ubiquitination or sumoylation all of which can influence the stability and activity of the p53 protein (Appella & Anderson, 2001). Future studies in our laboratory will examine the expression of regulators of p53 and Mdm2 proteins as well as specific post-translational modifications in p53 and Mdm2 in C/EBPβ−/− and wild type epidermis in response to carcinogen-induced stress.

DNA damage and oncogenic stress, among other types of cellular stress, induce and activate p53 and based on the integration of incoming stress signals, cells will determine whether to undergo growth arrest, senescence or apoptosis (Vogelstein et al., 2000; Vousden & Lu, 2002). At the peak of apoptosis in DMBA-treated C/EBPβ−/− epidermis, we observed that approximately 1 of 4 cells with increased p53 levels underwent apoptosis compared to only 1 of 12 in similarly treated wild type mice. These results suggest that in response to carcinogen-induced stress the cellular decision in p53-positive C/EBPβ-deficient keratinocytes is heavily weighted in favor of cell death. As discussed above, this result could be due
to specific post-translational modifications and/or protein interactions of p53 that enhance its activity and are differentially regulated in the two genotypes.

In terms of basal p53 mRNA expression, we observed that basal p53 mRNA levels were significantly increased in untreated C/EBPβ−/− mouse epidermis compared to untreated wild type epidermis suggesting that C/EBPβ has a role in the negative regulation of basal p53 mRNA levels. Consistent with this notion are several recent studies that have identified a repressor function for C/EBPβ in the regulation of gene expression (Burkart et al., 2005; Corbi et al., 2000; Di-Poi et al., 2005; Sankpal et al., 2005). Moreover, we have identified putative C/EBPβ binding sites in the p53 proximal promoter. Additional studies are required to determine whether C/EBPβ is a direct repressor of basal p53 expression.

In summary, our results demonstrate that p53 protein levels are deregulated in DMBA-treated C/EBPβ−/− mice and we have provided genetic evidence that p53 is required for the pro-apoptotic phenotype of C/EBPβ−/− mice. Our results indicate that C/EBPβ has a role in the negative regulation of p53. While additional studies are required to understand how C/EBPβ represses p53, our current study reveals a novel link between C/EBPβ, p53 and apoptosis. C/EBPβ may be a potential
molecular target for cancer therapy as blocking C/EBPβ function in a p53 proficient tumor cell may result in apoptosis and tumor regression.
MATERIALS AND METHODS

Animals/carcinogen treatment

The C/EBPβ⁻/⁻ mice used in this study have been described (Sterneck et al., 1997).

The C/EBPβ⁻/⁻ and wild-type mice were generated by mating C/EBPβ⁺/⁻ females to C/EBPβ⁺/⁺ males (C57BL/6;129/sv). Male p53⁻/⁻ mice (C57BL/6) were crossed with female C/EBPβ⁺/⁻ mice; F1 p53⁺/⁻ C/EBPβ⁺/⁻ mice were crossed to generate the four genotypes used experimentally. The mice were genotyped using the following PCR primers: for p53 wild type (Forward: TGCCCTGTGCAGTTGTGGGTCA, Backward: ATTTCTTTCCACCCGGGATAGATG); p53 knockout (Forward: ATGACTGCCATGGAGGAGTCACAGTC, Backward: TTTACGGGAGCCCTGGCGCTCGATGT); C/EBPβ wild type (Forward: AGCCCTACCTGGAGGCGCGCG, Backward: GCGCAGGGCGGAGCCGCTCGCG), and C/EBPβ knockout (Forward: GCTCCAGACTGCGTGGGAAAAG, Backward: GGCCCGGCTAGACAGTTACAGC). The hair of the dorsal skin of the mice (7-11 weeks old) was clipped with electric clippers at least two days before each
experiment. Mice were treated with a single dose (400 or 200 nmol) of DMBA in 200 ul acetone or 200 ul acetone alone as vehicle control.

**Detection of Apoptotic Cells/TUNEL**

Wild-type and C/EBPβ−/− mice were treated with 400 nmol DMBA (at least three mice/group) and at each time point after treatment the treated dorsal skin was excised and fixed for 24 hours in 10% neutral buffered formalin phosphate. Four different areas of dorsal skin were taken, processed, embedded in paraffin, and 5 µm sections were cut and stained with hematoxylin and eosin. More than 4,000 basal keratinocytes were examined for each mouse. Apoptotic keratinocytes in the interfollicular stratum basale were scored based on the simultaneous presence of the following three criteria: dark pyknotic nuclei, cytoplasmic eosinophilia, and some loss of cellular attachment. Data are presented as the number of apoptotic keratinocytes/cm length of mouse skin. TUNEL (TdT-mediated dUTP Nick-End Labeling) assay on skin sections was conducted using DeadEnd™ Fluorometric TUNEL System (Promega) following manufacturer’s protocol.

**Immunohistochemistry**

Mouse skins were fixed in 10% neutral buffered formalin phosphate and embedded
in paraffin. After deparaffinization, tissue sections (5 µm) were subjected to antigen retrieval with citrate buffer in 95°C degree water bath for 20 minutes followed by treatment with 0.1% H₂O₂ to block endogenous peroxidase activity. For Mdm2 immunostaining antigen retrieval was conducted using Dako Antigen Retrieval Buffer in 95°C water bath for 30 minutes. Sections were incubated with either rabbit polyclonal anti-p53 antibody (1:1000) (Santa Cruz Biotechnology, sc-6243), anti-p21 antibody (1:1000) (Santa Cruz Biotechnology sc-471) or anti-mdm2 antibody (1:500) (Santa Cruz sc-965) at 4°C for 20-24 hours after blocking with normal goat serum. This was followed by incubation with biotinylated goat anti-rabbit IgG at room temperature for 30 minutes. Detection was made with Vectastatin Elite ABC kit (Vector Laboratories) and 3, 3’-diaminobenzidine (BioGenex Laboratories) as the chromagen following manufacturer’s protocol. The sections were counterstained with hematoxylin, dehydrated and mounted. p53, p21 and Mdm2 positive basal keratinocytes were scored in 3 sections/mouse for at least 3 mice/group and data are expressed as p53-, p21- and Mdm2-positive cells/cm length of mouse skin.
Statistical Analysis

A two-factor ANOVA with interaction was conducted on the square root transformed numbers of apoptotic cells and on log transformed numbers of p53 positive cells. The factors were genotype and time. Cell numbers were transformed to reduce heterogeneity of the error variances. When interaction was significant, single factor ANOVA was conducted to compare genotypes at each fixed time point.

Preparation of epidermal homogenates

The hair on the dorsal skin of the mice was clipped with an electric clipper at least 2 days before each experiment. Mice were treated, killed and the shaved dorsal skin was removed. The skin was spread on an index card and immediately immersed in liquid nitrogen. The epidermis was scraped from the dermis with a surgical scalpel and placed in RIPA buffer (1 % Nonidet P-40, 0.5 % sodium deoxycholate, 0.1 % SDS, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1X protease inhibitor cocktail (Roche) and 1 mM sodium orthovanadate in PBS). The samples were sonicated on ice, vortexed and centrifuged at 14,000 g for 10 min at 4°C. Supernatants were stored at –80 °C until use. Protein concentration was determined using Bio-Rad DC protein assay reagent.
Immunoblot analysis

Equal amounts of protein 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 antibody for C/EBPα (sc-61), C/EBPβ (sc-520), p53 (sc-6243), p21 (sc-397) from Santa Cruz, Bcl-2 (554279, Becton Dickinson) or β-actin (A5441, Sigma). 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.

Quantitative real time RT-PCR

Total RNA from DMBA-treated (400 nmol) wild type and C/EBPβ−/− epidermis was extracted using Tri Reagent (Sigma) 20 hours after treatment and purified with RNeasy® Mini Kit (Qiagen). cDNA was synthesized using ImProm-II™ reverse transcription (RT) system (Promega) following manufacturer’s protocol. cDNA from 50ng total RNA was used as template to perform quantitative real-time PCR using mouse p53, Mdm2 or 18S TaqMan® Gene Expression Assay (Applied Biosystems)
and TaqMan® Universal PCR Master Mix (Applied Biosystems) in the ABI Prism 7000 Sequence Detection System. Samples were run in triplicate and prepared according to manufacturer's protocol. The expression levels for all samples were normalized to the endogenous control 18S (Applied Biosystems). Data were analyzed using the Comparative $C_T$ Method and presented relative to the wild type acetone control. $N = 4$-8 mice/genotype/treatment.
ACKNOWLEDGEMENTS

We would like to thank Dr. Cavell Brownie for help with the statistical analysis. This work was supported by a grant CA46637 from the National Cancer Institute.
REFERENCES


Figure 1) Apoptosis is greatly increased in the epidermis of DMBA-treated C/EBPβ⁻/⁻ mice. A. H&E staining of wild type skin at 24 hours after DMBA (400 nmol) treatment. B. H&E staining of C/EBPβ⁻/⁻ skin at 24 hours after DMBA (400 nmol) treatment. Arrows indicate apoptotic keratinocytes and photographs were taken at a magnification of 400x. C. TUNEL staining of wild type skin at 24 hours after DMBA (400 nmol) treatment. D. TUNEL staining of C/EBPβ⁻/⁻ skin at 24 hours after DMBA (400 nmol) treatment. Arrows indicate TUNEL positive keratinocytes and photographs were taken at a magnification of 200x. E. H&E staining of wild type and C/EBPβ⁻/⁻ hair follicles at 24 hrs after DMBA (400 nmol) treatment. Arrows indicate apoptotic keratinocytes and photographs were taken at a magnification of 400x. F. Time course apoptosis study with wild type and C/EBPβ⁻/⁻ mice treated with a single dose of DMBA. At least three mice per group were treated with 400 nmol DMBA. At the indicated times after treatment, skins were collected. H&E sections were examined and the number of apoptotic interfollicular basal keratinocytes/cm length skin was scored. Data are expressed as mean ± standard error. Two-factor ANOVA demonstrated significant interaction between genotype and time (p<0.05). *, significantly different from DMBA-treated wild type at the same time point as determined by ANOVA (p<0.05). Standard error bars are present at all time points, in some cases it so small it is masked by open and closed boxes.
Figure 2) p53 levels are deregulated in C/EBPβ−/− epidermal keratinocytes in response to DMBA treatment. A. p53 immunohistochemical staining in wild type (left) and C/EBPβ−/− (right) epidermis treated with acetone vehicle or 400 nmol DMBA at 24 h after treatment. Photographs were taken at a magnification of 400x. B. p53 immunohistochemical staining in wild type (left) and C/EBPβ−/− (right) hair follicle 24 hrs after DMBA treatment. C. Time course study for p53 positive keratinocytes in wild type and C/EBPβ−/− mice treated with DMBA. At least three mice per group were treated with 400 nmol DMBA. At the indicated times after treatment, skins were collected and p53 immunohistochemical staining was conducted. The number of p53-stained interfollicular basal keratinocytes/cm length skin was scored. Data are expressed as mean ± standard error. Two-factor ANOVA demonstrated significant interaction between genotype and time (p<0.05). *, significantly different from DMBA-treated wild type at the same time point as determined by ANOVA (p<0.05). Standard error bars are present at all time points, in some cases it so small it is masked by open and closed boxes.
Figure 3) C/EBPα, Bcl-2 and p21 proteins are differentially expressed by DMBA in C/EBPβ-/- epidermis compared to wild type. A. Wild type and C/EBPβ-/- mice were treated with 400 nmol DMBA or acetone alone. At the indicated times after treatment, epidermal lysates were prepared, and equal amounts of protein were subjected to immunoblot analysis with rabbit polyclonal anti-p53, -C/EBPβ, -C/EBPα, -p21 and -Bcl-2 antibody and mouse monoclonal β-actin antibody for loading control. B. Quantitation of p21-immunopositive interfollicular basal keratinocytes/cm length mouse skin at 24 hrs after DMBA treatment. Data are expressed as mean ± standard error. *, significantly different from DMBA-treated wild type as determined by Student's t test (p<0.05).
A

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>ACET</th>
<th>DMBA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>KO</td>
<td>24</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Protein</th>
<th>WT</th>
<th>KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>C/EBPβ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p53</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C/EBPα</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bcl-2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

![Graph showing p21 positive cells/cm comparison between C/EBPβ^+/+ and C/EBPβ^-/- groups.](image)

* indicates a significant difference.
Figure 4) Increased DMBA-induced apoptosis in C/EBPβ⁻/⁻ mice is dependent upon p53. Mice (7-10 weeks of age; 3 mice/group) were treated with 200 nmol DMBA or acetone alone. After 24 hours the skins were collected, and H&E sections were examined for the number of apoptotic interfollicular basal keratinocytes/cm length skin was scored. Data are expressed as mean ± standard error. *, significantly different from DMBA-treated wild type mice as determined by Student’s t-test (p<0.05). Similar results were obtained from an independent repeat experiment. Wild type standard error is masked by bar and is 0.01. The number of apoptotic cells in acetone treated mice was between 0.008-0.016 apoptotic cells/cm for all genotypes.
Figure 5) *p53* mRNA and *Mdm2* mRNA/protein levels in epidermis in response to carcinogen-induced stress. Total RNA was isolated from wild type and C/EBP\(\beta^{+/-}\) mouse epidermis 20 hrs after treatment with DMBA or acetone alone and quantitative RT-PCR was conducted for A) *p53* mRNA levels and B) *Mdm2* mRNA levels. Data were analyzed using the Comparative \(C_T\) Method and data are presented relative to the wild type acetone control. N = 8 mice (p53) N=4 mice (Mdm2) mice/genotype/treatment. *, significantly different from WT Acetone (p<0.05), #, significantly different from WT DMBA as determined by Student’s t test (p<0.05). C. Quantitation of Mdm2-positive interfollicular basal keratinocytes/cm length mouse skin after DMBA treatment. Data are expressed as mean ± standard error. a, significantly different from control mice within each genotype as determined by Student’s t test (p<0.05), b, significantly different between the genotypes at the same time point as determined by Student’s t test (p<0.05).
Diminished Expression of C/EBPα in Skin Carcinomas is Linked to Oncogenic Ras and Re-Expression of C/EBPα in Carcinoma Cells Inhibits Proliferation

Minsub Shim, Kristina L. Powers, Sarah J. Ewing, Songyun Zhu
and Robert C. Smart

Cell Signaling and Cancer Group, Department of Environmental and Molecular Toxicology, North Carolina State University

CORRESPONDING AUTHOR:
Robert C. Smart, Ph.D.
Department of Environmental and Molecular Toxicology
North Carolina State University
Raleigh, NC 27695-7633
Phone: (919) 515-7245, Fax: (919) 515-7169, email: rcsmart@unity.ncsu.edu

Running Title; Loss of C/EBPα expression in SCC
Key Words; C/EBPα, Ras, keratinocytes, skin

Abbreviations; C/EBPα: CCAAT/enhancer binding protein-alpha, C/EBPβ: CCAAT/enhancer binding protein-beta, SCC: squamous cell carcinoma

Published in Cancer Res 65(3):861-867 2005.
ABSTRACT

The bZIP transcription factor, C/EBPα is involved in mitotic growth arrest and has been implicated as a human tumor suppressor in acute myeloid leukemia. We have previously shown that C/EBPα is abundantly expressed in mouse epidermal keratinocytes. In the current study, the expression of C/EBPα was evaluated in seven mouse skin squamous cell carcinoma (SCC) cell lines that contain oncogenic Ha-Ras. C/EBPα mRNA and protein levels were greatly diminished in all seven SCC cell lines compared to normal primary keratinocytes while C/EBPβ levels were not dramatically changed. Re-expression of C/EBPα in these SCC cell lines resulted in the inhibition in SCC cell proliferation. To determine whether the decrease in C/EBPα expression observed in the SCC cell lines also occurred in the carcinoma itself, immunohistochemical staining for C/EBPα in mouse skin SCCs was conducted. All fourteen SCCs evaluated displayed negligible C/EBPα protein expression and normal C/EBPβ levels compared to normal epidermis and all fourteen carcinomas contained mutant Ras. To determine whether oncogenic Ras is involved in the down regulation of C/EBPα, BALB/MK2 keratinocytes were infected with a retrovirus containing Ras12V, and C/EBPα protein, mRNA and DNA binding levels were determined. Keratinocytes infected with the retrovirus containing
oncogenic Ras12V displayed greatly diminished C/EBPα protein, mRNA and DNA binding levels. In addition, BALB/MK2 cells containing endogenous mutant Ras displayed diminished C/EBPα expression and the ectopic expression of a dominant negative RasN17 partially restored C/EBPα levels in these cells. These results indicate that oncogenic Ras negatively regulates C/EBPα expression and the loss of C/EBPα expression may contribute to the development of skin SCCs.
INTRODUCTION

The CCAAT/enhancer binding proteins (C/EBP) are members of the basic leucine zipper (bZIP) class of transcription factors that contain a basic DNA binding domain and a leucine zipper domain involved in homo- or heterodimerization (1, 2). There are six members of the C/EBP family [C/EBPα, C/EBPβ, C/EBPγ, C/EBPδ, C/EBPε and C/EBPζ] (3-5). C/EBPα plays an important role in metabolism as well as in the regulation of cell proliferation and differentiation in a variety of cell types (5). C/EBPα protein can interact with several cell-cycle regulatory proteins and it has been proposed that such interactions are responsible for inhibiting cell proliferation. C/EBPα can regulate p21 expression as well as to directly interact and enhance the ability of p21 to inhibit CDK2 (6, 7). In addition, C/EBPα has been shown to inhibit cell growth through its interaction with Rb family proteins (8, 9). More recent studies suggest that C/EBPα can block cell proliferation independent of its transcriptional activity by forming a complex with CDK2 and CDK4 thereby blocking cyclin-CDK interactions and cell cycle progression (10). C/EBPα can also directly repress E2F function through its physical association with E2F and this repression is necessary for growth arrest and adipocyte and granulocyte
differentiation (11, 12). Most recently the antimitotic activity of C/EBPα was shown to require a SWI/SWF complex, however events downstream of this interaction important in the regulation of cell proliferation are currently unknown (13).

The anti-proliferative effects of forced C/EBPα expression have been shown in a variety of non-transformed cells (14, 15) as well as transformed cells including SaOS2 osteosarcoma cells which lack functional Rb and p53, (16), SV-40 transformed fibroblasts (16), HepG2 hepatocarcinoma cells (17) and lung cancer cell lines (18). Thus, C/EBPα appears to have antimitotic activity in a variety of tumor cell types and Rb and p53 appear to be dispensable for its antimitotic activity (16). The loss of C/EBPα expression or function in certain cancers is emerging as an important event in the development of certain cancers. For example, C/EBPα is inactivated by mutation (19) or through its association with oncoprotein AML-1-ETO (20, 21) in human acute myeloid leukemia (AML). The inactivation of C/EBPα is thought to result in differentiation block of the granulocytic blasts and has implicated C/EBPα as a putative tumor suppressor gene in AML. C/EBPα expression is greatly reduced in human hepatocellular carcinomas (22), lung cancer cell lines and lung cancers (18), particularly adenocarcinoma and poorly differentiated lung
cancers. Collectively these studies support the notion that loss of C/EBPα expression may be permissive for tumor cell proliferation.

C/EBPα and C/EBPβ are abundantly expressed in mouse and human epidermal keratinocytes (23-25). In mouse keratinocytes, C/EBPβ is involved in the regulation of the early stages of squamous differentiation (26). Recently, C/EBPβ has been shown to play a critical role in Ras-mediated mouse skin tumorigenesis and keratinocyte survival (27). Unlike C/EBPβ, C/EBPα does not cooperate with Ras to induce transformation of NIH 3T3 cells (27). However, the forced expression of C/EBPα in keratinocytes inhibits their growth (26), and preliminary data from our laboratory on a limited number of SCCs suggested that both C/EBPα and C/EBPβ levels are reduced in mouse squamous cell carcinomas (SCCs) (23). In the current study, we have examined C/EBPα and C/EBPβ levels in seven mouse skin SCC cell lines and fourteen SCCs that contain oncogenic Ras. Our results indicate that C/EBPα but not C/EBPβ protein levels are greatly diminished in SCC cell lines and SCCs. We observed that oncogenic Ras negatively regulates C/EBPα levels and re-expression of C/EBPα in oncogenic Ras containing SCC cell lines blocks
proliferation. Our results suggest the loss of C/EBPα expression may contribute to the deregulation of cell proliferation in SCCs.
MATERIALS AND METHODS

Cell lines and cell culture

Primary keratinocytes were isolated from newborn CD-1 mice (less than 3 days old) by overnight trypsin flotation at 4 °C and keratinocytes were cultured in low-calcium medium (Ca²⁺-free EMEM supplemented with 4% Chelex-treated fetal bovine serum, 10 ng/ml of hEGF, 100 U/ml of penicillin, 100 µg/ml of streptomycin, 250 ng/ml of amphotericin B [Fungizone], with added calcium chloride to a final concentration of 0.05 mM). Mouse SCC cell lines were isolated from skin SCCs in CD-1 mice that were induced by DMBA-initiation followed by TPA and/or mirex promotion. Following trypsin treatment of the SCCs, SCC cells were plated at low density in low-calcium medium without EGF. Individual colonies were ring cloned and expanded (28). All five SCC cell lines (MT2.5, MT2.6, MT23r3, T6, and M9.6) contain oncogenic H-Ras mutations in the 61st codon. FVBN-217 and TGAC-43 mouse skin SCC cell lines were a kind gift from Dr. Ron Cannon (NIEHS, Research Triangle) and were cultured in DMEM supplemented with 10% fetal bovine serum, 100 U/ml of penicillin, 100 µg/ml streptomycin per ml, and 250 ng/ml of amphotericin B [Fungizone]. BALB/MK2 and BALB/MK2-DMBA keratinocytes (a gift from Dr. B.
Weissman, University of North Carolina, Chapel Hill) were cultured in low-calcium medium (Ca\(^{2+}\) free EMEM supplemented with 8 % Chelex-treated fetal bovine serum, with or without 4 ng of hEGF per ml, and 0.05 mM calcium chloride).

**Immunohistochemistry for C/EBP\(\alpha\) and C/EBP\(\beta\)**

Paraffin-embedded tissue sections of normal skin, SCC and keratoacanthomas derived from DMBA-initiation/TPA promotion were subjected to an antigen retrieval protocol (95°C for 30 min in 10 mM citrate buffer pH 6.0) followed by incubation with anti-C/EBP\(\alpha\) antibody (1:250) (sc-61, SantaCruz Biotechnology) or anti-C/EBP\(\beta\) antibody (1:250,) (sc-150, SantaCruz Biotechnology). A biotinylated goat anti-rabbit IgG was used as the secondary antibody. Detection was made by ABC kit (Vector Laboratories) and 3,3'-diaminobenzidine (DAB) as the chromagen (BioGenex). Sections were counterstained with hematoxylin. No C/EBP\(\alpha\) or C/EBP\(\beta\) staining was observed when the primary antibody was omitted and the control rabbit serum was applied.

**Preparation of cell lysates**

Nuclear extracts were prepared as previously described by Schreiber et al (29). For the preparation of whole cell lysates, cells were washed with cold PBS, harvested
by scraping, and collected by brief centrifugation. Cells were lysed in lysis buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1mM PMSF, 100 µg/ml aprotinin, 100 µg/ml leupeptin, 1mM sodium orthovanadate, 0.6 % NP-40) by sonication and then one-tenth volume of 5 M NaCl was added. Lysates were vortexed, incubated for 15 min on ice, and centrifuged (14,000g, 10 min, 4°C). Supernatants were stored at –80°C until use. Protein concentration was determined using the 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 acetone (-20°C). Protein samples were solubilized and boiled in SDS sample buffer for 2 min, and then separated by SDS-PAGE. The separated proteins were transferred to an Immobilon-P membrane (Millipore). Following incubation in blocking buffer (PBS with 1 % bovine serum albumin, 5 % non fat dry milk, and 0.1 % Tween-20) for 1 hr at room temperature, the membranes were probed for 2 hr at room temperature with rabbit polyclonal IgG raised against C/EBPα (1:2000) (sc-61, SantaCruz Biotechnology) or C/EBPβ (1:2000) (sc-150, SantaCruz Biotechnology). The membranes were washed and
then probed with an HRP-linked secondary antibody (1:2500) for 1 hr at room temperature. Detection was made with an enhanced chemiluminescence reagent followed by exposure of membrane to film. All membranes were stained to confirm equal loading.

**Northern blot analysis**

Total RNA was isolated using Promega’s SV total RNA isolation kit. C/EBPα cDNA was labeled with [α-32P] dCTP by using Ready-To-Go labeling beads (Amersham). RNA was electrophoresed on agarose gel containing formaldehyde and transferred to zeta-probe GT membrane (BioRad) and UV cross-linked. Blots were incubated at 65°C in hybridization buffer (0.25 M Na2HPO4 pH 7.2, 7 % SDS) and sequentially washed with washing buffer 1 (20 mM Na2HPO4 pH 7.2, 5 % SDS) and washing buffer 2 (20 mM Na2HPO4, 1% SDS) at room temperature. Films were exposed to membranes at –80°C and developed.

**Transfection of SCC cell lines**

SCC cell lines were transfected when they reached 30 to 40% confluence in 60-mm-diameter dishes with 2 µg of DNA (pcDNA3 or pcDNA3-C/EBPα) and 16 µg of Lipofectamine (Invitrogen). Transfection was performed in serum-free EMEM
(containing 0.05 mM calcium) at 37°C and 5% CO₂ for 4 h, after which the cells were re-fed with low-calcium medium. Forty-eight hours later, the cultures were split (1:3) and re-plated in the above medium containing 300 µg/ml G418 and this selection medium was changed every other day. On days 3, 5, and 7 after G418 selection, the total number of colonies in 30 random grid squares was counted and then converted to colonies per dish. The number of cells per colony was scored directly from 30 randomly chosen colonies.

Virus infection of Balb/MK2 cells

φNX cells were cultured with DMEM medium plus 10% FBS, and transfected with pBabe-puro control vector or pBabe-puro-Ras (12V) vector (20 ug/dish) by calcium precipitation method for 6 hours. The cells were then maintained in EMEM medium containing 8% chelexed FBS, 4ng/ml EGF 4 and 0.05 mM calcium chloride for overnight. Virus containing medium was collected from φNX cell cultures 3 times, 4 hours apart, filtered through a 0.45 um filter, and overlaid onto BALB/MK2 cells with 5 ug/ml polybrene. Cultures were maintained with viral containing medium overnight and 24 hrs later cells were shifted to a selection medium containing
2μg/ml puromycin for 4 days before the cells were processed for nuclear extract preparation.

*Electrophoretic mobility shift assay (EMSA)*

Nuclear protein (4 μg per sample in 10 μl sample buffer) was incubated at room temperature for 30 minutes with 10 μl of master binding mix with $^{32}$P-labeled C/EBP probe (Santa Cruz, SC-2525). Samples were loaded onto 4% polyacrylamide gel, and subjected to electrophoresis in 0.025X TBE buffer at 200V for 3-4 hours. The gel was transferred to Whatman paper, dried in a 80°C gel dryer for 1 hour, then exposed to film for appropriate time to check the DNA binding pattern.
RESULTS

C/EBPα protein and mRNA levels are greatly diminished in SCC cell lines

We have previously shown that C/EBPα and C/EBPβ are abundantly expressed in mouse keratinocytes (23). In the current study, we examined C/EBPα protein levels in seven mouse SCC cell lines. SCC cell lines (MT23r3, MT2.5, MT2.6, T6, FVBN-217 and M9.6) were isolated from various mouse skin SCCs which were generated by in vivo treatment with 7,12-dimethylbenz[a]anthracene (DMBA) and then promoted with 12-O-tetradecanoylphorbol-13-acetate (TPA) and/or mirex. All of these SCC cell lines contain oncogenic H-Ras A182->T mutations in at least one allele of the 61st codon as determined by allele specific hybridization (28). SCC cell line TgAC-43 was isolated from a skin carcinoma that developed on transgenic Tg.AC mouse skin. TgAC-43 expresses high levels of the v-H-Ras transgene. As shown in Figure 1A, the C/EBPα protein levels were greatly diminished in all seven SCC cell lines compared to normal primary mouse keratinocytes. The average decrease in C/EBPα protein levels was 90% (Figure 1A). In contrast to C/EBPα protein levels, C/EBPβ protein levels were not dramatically changed (25% decrease on average) compared to normal primary
keratinocytes (Figure 1A and B). These results demonstrate that C/EBPα protein levels are greatly diminished or nearly undetectable in all seven SCC cell lines containing oncogenic Ras compared to normal primary keratinocytes and that C/EBPα and C/EBPβ are differentially regulated in SCC cells. We also compared the levels of C/EBPα in BALB/MK2 keratinocytes, an immortalized but not tumorigenic cell line to the levels in the SCC cell lines. Compared to BALB/MK2 keratinocytes, C/EBPα protein was decreased in all seven SCC by an average of 67% (range 32-89% decrease).

C/EBPα can be regulated at the transcriptional level by numerous transcription factors, however, C/EBPα protein is also degraded via a ubiquitin-dependent proteasomal pathway (30). To determine whether the decrease in C/EBPα protein level in the SCC cells is associated with decreased C/EBPα mRNA expression, we examined C/EBPα mRNA levels in the SCC cell lines. As shown in Figure 1C, C/EBPα mRNA levels in all seven SCC cell lines were also greatly diminished compared to normal primary keratinocytes. The average decrease in C/EBPα mRNA was 90% (Figure 1D). In general the decreased level of C/EBPα
mRNA expression was proportional to the decreased level of C/EBPα protein in each SCC cell line.

**Re-Expression of C/EBPα induces growth arrest in SCC cell lines**

We have previously reported that forced expression of C/EBPα induces growth arrest and morphological changes in BALB/MK2 keratinocytes (26). In order to examine the effect of C/EBPα on the growth of SCC cell lines, we transfected MT2.5 and MT3r3 SCC cell lines with pcDNA3-C/EBPα. Transfected SCC cells were selected in the presence of G418 and the number of colonies per dish and the number of cells per colony were counted during the selection period. As shown in Figure 2A, 2B, and 2C, the number of colonies in C/EBPα transfected SCC cell lines was greatly reduced (>75%) compared to pcDNA3-transfected SCC cells. The number of cells per colony was also decreased (>90%) in the C/EBPα transfected cells compared to pcDNA3 transfected cells. Moreover, during the selection period the number of cells/colony in C/EBPα transfected SCC cells increased very slowly compared to the empty vector transfected cells. The above experiment was also conducted with MT2.6, T6, FVBN-217, M9.6 and TgAC43 and similar results to those reported above for MT2.5 and MT3r3 were obtained (data not shown). These
results indicate that C/EBPα inhibits the proliferation of SCC cells containing oncogenic Ras suggesting that antimitotic activity of C/EBPα is dominant over the proliferative effects of oncogenic Ras.

\textit{C/EBPα expression is down-regulated in SCCs}

To confirm that the decrease in C/EBPα expression observed in the SCC cell lines also occurs in the primary cancer lesion itself, immunohistochemical staining for C/EBPα in mouse skin SCCs was conducted. We examined fourteen SCCs and two keratoacanthomas that were generated by DMBA/TPA initiation-promotion protocol. All 14 SCC and both keratoacanthomas contained oncogenic H-Ras with mutations in the 61\textsuperscript{st} codon as determined by SSCP analysis of exon 2 followed by XbaI restriction analysis (31). As shown in Figure 3A, in normal untreated epidermis there is nuclear and cytoplasmic C/EBPα staining in the suprabasal layer of epidermis where post-mitotic keratinocytes undergo differentiation as well as perinuclear C/EBPα staining in basal layer. In contrast to normal epidermal keratinocytes, all SCCs (14/14) demonstrated little to no C/EBPα immunostaining as did the two keratoacanthomas (data not shown). A representative micrograph of a SCC is shown in Figure 3B with intact epidermis above the tumor for comparison.
of normal C/EBPα immunostaining intensity. A micrograph of the SCC using higher
magnification is shown in Figure 3C. These data confirm the decrease in C/EBPα
levels observed in the SCC cell lines also occurs in the primary SCC tumor itself.

As previously reported (23), C/EBPβ immunostaining in the epidermis occurs
primarily in the nuclei of the suprabasal cells (Figure 3D). Representative
micrographs of C/EBPβ immunostaining in a SCC are shown in Figure 3E and 3F.

In contrast to C/EBPα, CEBPβ was abundantly expressed in 14/14 SCCs. These
immunostaining results in SCCs are in accord with the SCC cell line Western
analysis results and clearly demonstrate C/EBPα and C/EBPβ protein levels are
differentially regulated in the SCCs.

**Decreased C/EBPα expression is associated with oncogenic ras**

Our results in the SCC cell lines as well as in SCCs suggest an association
between decreased C/EBPα expression and the presence of oncogenic Ras. This
association between oncogenic Ras containing SCC cell lines and decreased
C/EBPα is apparent whether oncogenic Ras results from the mutated endogenous
Ras or from the expression of transgenic v-Ha-Ras. Since papillomas are the
precursor tumor of SCC and since the mutation of Ras is an early initiating event in
DMBA-initiated/TPA promoted mouse skin tumorigenesis we examined by western analysis the levels of C/EBPα in DMBA-initiated/TPA-promoted papillomas. We observed that the C/EBPα levels were decreased by ~70% in 4/4 pooled papilloma samples compared to whole skin and immunohistochemical staining for C/EBPα also revealed a substantial decrease compared to uninvolved epidermis (data not shown). To determine whether oncogenic Ras is involved in the down regulation of C/EBPα, BALB/MK2 keratinocytes were infected with pBABEpuro retrovirus or pBABEpuro containing Ras12V and then C/EBPα protein, mRNA and DNA binding levels were determined. As shown in Figure 4A, C/EBPα protein levels were decreased greater than 90% in cells infected with pBABEpuro containing oncogenic Ras12V compared to cells infected with empty retrovirus (Figure 4A). Oncogenic Ras12V expressing cells displayed approximately a 50% decrease in C/EBPα mRNA as determined by RT-PCR. As shown in Figure 4B, keratinocytes infected with pBABEpuro-Ras12V displayed decreased C/EBPα DNA binding as determined by EMSA analysis. To provide additional evidence that Ras is involved with the down regulation of C/EBPα expression as well as to avoid the possible influence of supra-physiologic levels of Ras12V that may occur with the pBABEpuroRas12V
retroviral infection we examined C/EBPα levels in BALB/MK2-DMBA cells that contain endogenous H-Ras A^{182}->T mutation in the 61^{st} codon which was induced by DMBA treatment (32). C/EBPα expression was greatly diminished (Figure 4C) in the mutant Ras containing BALB/MK2-DMBA cells compared to BALB/MK cells. Moreover, the expression of a dominant negative RasN17 in BALB/MK2-DMBA cells (Figure 4D) resulted in an increase in C/EBPα protein levels. Collectively these results indicate that oncogenic Ras produces the down-regulation of C/EBPα expression.
DISCUSSION

In the current study, we have examined fourteen mouse skin SCCs and seven SCC cell lines all of which contain mutant oncogenic Ras and found that C/EBPα levels, but not C/EBPβ levels, were greatly diminished in all carcinomas and SCC cell lines. These results clearly establish that loss of CEBPα expression in Ha-ras containing SCC is a frequent event and that C/EBPα and C/EBPβ are differentially regulated in SCCs. This divergence in expression of the two highly related bZIP transcription factors in SCCs may reflect their emerging roles in tumorigenesis. For example, C/EBPβ has a critical role in Ras-mediated mouse skin tumorigenesis and keratinocyte survival (27). C/EBPβ can transform mammary epithelial cells (33) and cooperate with Ras to transform NIH 3T3 fibroblasts (27). In addition, increased expression of C/EBPβ is associated with increased invasiveness of human colorectal cancer (34) and the progression of ovarian cancer (35). More recently it has been shown that cyclin D1 can interact with C/EBPβ to alter gene expression and it appears that C/EBPβ is important in the unique patterns of altered gene expression observed in the human cancers that overexpress cyclin D1 (36). Collectively these studies suggest that the retention of
C/EBPβ expression within the tumor cells contributes to tumor cell proliferation/survival. In direct contrast, the loss of C/EBPα expression appears to be permissive for tumor growth. C/EBPα is anti-mitotic in numerous cell types and C/EBPα levels are decreased in human hepatocellular carcinomas (22), lung cancer and lung cancer cell lines (18) as well as a variety of transformed cell lines (16, 17). Moreover, C/EBPα function is abrogated by mutation or through its association with oncoprotein AML-1-ETO in human acute myeloid leukemia (AML) implicating C/EBPα as a putative human tumor suppressor (19-21). Collectively these studies support the idea that loss of C/EBPα expression may be permissive for tumor cell growth. Our findings that C/EBPα levels are greatly diminished in skin SCC cell lines and SCCs are in accord with a tumor suppressor-like function for C/EBPα.

Ras proteins function as key regulators of signaling pathways that control cell proliferation/differentiation. Ras signaling is altered in the majority of human cancers due to the mutational activation of Ras or alterations in upstream or downstream signaling pathways (37). Oncogenic Ras is known to alter the expression of genes that stimulate cell proliferation, such as cyclin D1 which
participates with CDK4/6 in propelling the cell through the G1/S transition of the cell cycle. C/EBPα is thought to negatively regulate the cell cycle by binding to CDK4 and CDK2 and preventing complex formation with cyclin D and E, respectively (10). C/EBPα can also inhibit E2F transcription activity and stimulate the activity of the CDK inhibitor p21. In the current study, we demonstrate that Ras is a potent negative regulator of C/EBPα mRNA levels, protein levels and DNA binding activity. Thus, Ras signaling appears to couple the up-regulation of genes that positively regulate proliferation (e.g. cyclin D1) with the down-regulation of genes that negatively regulate cell proliferation (e.g. C/EBPα). Importantly, we found that re-expression of C/EBPα in mutant Ras containing SCC cell lines can block cell proliferation even though these cells contain oncogenic Ras. Our results suggest the Ras-induced down regulation of C/EBPα expression is a permissive event for cell proliferation. While we have shown that C/EBPα inhibits proliferation of SCC cell lines, we do not know the mechanism by which C/EBPα induces growth arrest in SCC cell lines. As described in the introduction, C/EBPα negatively regulates cell proliferation by multiple mechanisms (6-13) and additional studies are required to determine which of mechanism(s) are operative in keratinocytes.
In skin, oncogenic Ras is a potent stimulator of epidermal keratinocyte proliferation and an inhibitor of stratified squamous differentiation (38). C/EBPα is involved in mitotic growth arrest and differentiation of numerous cell types. If C/EBPα has both these activities in keratinocytes then the down regulation of C/EBPα by Ras would not only be permissive for cell proliferation but also block squamous differentiation, perhaps contributing to clonal expansion of oncogenic Ras containing cells. C/EBPα likely contributes to squamous differentiation as it can regulate the expression of involucrin, a marker of squamous differentiation (39). We have observed that in BALB/MK2 cells that overexpress C/EBPα, involucrin levels are increased (unpublished results M.S. and RCS). While oncogenic Ras is frequently mutated in a variety of human epithelial cancers and in chemical carcinogen-induced mouse skin SCC, it is not frequently mutated in human skin tumors. However, oncogenic Ras is frequently mutated in certain human cancers including; pancreas (90%), colorectal (45%) and lung adenocarcinoma (35%), and liver (30%) (37). It is noteworthy that reports of decreased C/EBPα expression in human cancers occurred in hepatocellular carcinoma (22) and lung cancer, with lung adenocarcinomas demonstrating the most significant and frequent decreases
Thus, there may be a relationship between human tumors that exhibit frequent mutations in Ras and those that have been reported to exhibit diminished C/EBPα expression.

The regulation of C/EBPα can occur at the transcriptional and post-transcriptional level (30). Our results demonstrate that Ras produces decreased mRNA levels suggesting that Ras produces a decrease in the transcription of C/EBPα. The C/EBPα promoter has been shown to be subject to auto-regulation by C/EBPα (40) and negative regulation by c-myc (41, 42). The exact mechanism through which Ras negatively regulates C/EBPα levels in keratinocytes remains to be determined but could involve c-myc. In terms of post-translational modification, transfected C/EBPα has been shown to be phosphorylated on serine 248 in a Ras dependent manner involving PKC in 293T kidney cells (43). This modification enhanced transcription activity of C/EBPα. We have also observed that transfected Ras stimulates transcription activity of transfected C/EBPα in Balb/MK2 keratinocytes. However this regulation may not be relevant in non-transfected keratinocytes as endogenous C/EBPα expression is down regulated by Ras. Recently PKCδ and PKCη have been reported to positively regulate C/EBPα
expression through a mechanism involving p38 (39). Future studies in our lab will address the signaling mechanism(s) through which Ras down-regulates C/EBPα expression.

In summary, our results suggest that the loss of C/EBPα expression contributes to the altered growth characteristics of skin SCCs. Importantly we have observed that oncogenic Ras down-regulates the expression of C/EBPα. While additional studies are required to determine how Ras negatively regulates C/EBPα expression, our studies describe a novel link between these two important proteins. Our results contribute to the emerging evidence that C/EBPα may have a tumor suppressor-like function in numerous types of cancer and identify a novel mechanism for negative regulation by Ras.
ACKNOWLEDGMENTS

This study was funded by a grant from the National Cancer Institute (Grant CA 46637) and in part by a National Institute of Environmental Health Sciences Training Grant (ES007046). We would like to thank Dr. Sharon Meyer for the isolation of the SCC cell lines and Dr. Peter Johnson for the pBabe-C/EBPα constructs.
REFERENCES


**Figure 1** C/EBPα protein and mRNA levels are greatly diminished in SCC cell lines.

A) Nuclear extracts were prepared from normal mouse keratinocytes and SCC cell lines and equal amounts of protein (20 μg) were subjected to western blot analysis using rabbit polyclonal anti-C/EBPα or anti-C/EBPβ antibody. C/EBPα protein standard is histidine-tagged and runs slightly slower than non-tagged C/EBPα. B) Densitometric analysis of results from A normalized to C/EBPα or C/EBPβ in normal keratinocytes. C) Total cellular RNA was isolated from normal keratinocytes and SCC cell lines and northern blot analysis was conducted. The membrane was reprobed with 7S RNA cDNA to verify the equal loading of RNA. D) Densitometric analysis of results from C normalized to 7S RNA.
Figure 2 Re-expression of C/EBPα induces growth arrest in SCC cell lines.

A) MT2.5 SCC cells were transfected with pcDNA3 or pcDNA3-C/EBPα and subsequently subcultured in the presence of 300 µg/ml G418. The number of colonies per dish (left panel) and the number of cells per colony (right panel) were determined at days 3, 5, and 7 of G418 selection. Data are expressed as the mean +/- standard deviation of a representative experiment done in triplicate. B) MT23r3 SCC cells were transfected with pcDNA3 or pcDNA3-C/EBPα and subsequently subcultured in the presence of 300 µg/ml G418. The number of colonies per dish (left panel) and the number of cells per colony (right panel) were determined at days 3, 5, and 7 of G418 selection. Data are expressed as the mean +/- standard deviation of a representative experiment done in triplicate. C) MT23r3 SCC cells were transfected with pcDNA3 or pcDNA3-C/EBPα and subcultured in the presence of 300 µg/ml G418. On days 5 and 7 of G418 selection, the cells were stained with crystal violet and photographs of colonies were taken.
Figure 3 C/EBPα expression is down-regulated in SCCs. 
A) C/EBPα immunostaining in normal epidermis. B) C/EBPα immunostaining in SCC with epidermis above the tumor (lower magnification). C) C/EBPα immunostaining in SCC. D) C/EBPβ immunostaining in normal epidermis. E) C/EBPβ immunostaining in SCC with epidermis above the tumor (lower magnification). F) C/EBPβ immunostaining in SCC.
Figure 4 Decreased C/EBPα expression is associated with oncogenic ras.

A) C/EBPα protein levels in whole cell extracts prepared from BALB/MK2 cells infected pBabe-puro or pBabe-puro-RasV12
B) EMSA analysis of nuclear extracts prepared from BALB/MK2 cells infected pBabe-puro or pBabe-puro-RasV12
C) C/EBPα protein levels in BALB/MK2 keratinocytes and BALB/MK2-H-Ras keratinocytes which contain endogenous H-Ras mutation
D) C/EBPα proteins levels in cells transfected with RasN17. NSB = non-specific binding.