ABSTRACT

LOOMIS, KARI DANIELLE. C/EBPα is an Epithelial Tumor Suppressor Gene, and Mitogenic Stimulation Reciprocally Regulates C/EBPα and C/EBPβ. (Under the direction of Dr. Robert C. Smart.)

CCAAT/enhancer binding protein α (C/EBPα) and C/EBPβ are basic leucine zipper transcription factors that function in the inhibition and stimulation of cell cycle progression, and regulate differentiation in various cell types. C/EBPα is inactivated by mutation in acute myeloid leukemia (AML) and is considered a human tumor suppressor in AML. While C/EBPα mutations have not been observed in malignancies other than AML, greatly diminished expression of C/EBPα occurs in numerous human epithelial cancers including those of lung, liver, endometrium, skin and breast suggesting a possible tumor suppressor function. However, direct evidence for C/EBPα as an epithelial tumor suppressor is lacking due to the absence of C/EBPα mutations in epithelial tumors and the lethal effect of C/EBPα deletion in mouse model systems. To examine the function of C/EBPα in epithelial tumor development, an epidermal-specific C/EBPα knockout mouse was generated. The epidermal-specific C/EBPα knockout mice survived and displayed no detectable abnormalities in epidermal keratinocyte proliferation, differentiation or apoptosis; demonstrating that C/EBPα is dispensable for normal epidermal homeostasis. In spite of this, the epidermal-specific C/EBPα knockout mice were highly susceptible to skin tumor development involving oncogenic Ras. These mice displayed decreased tumor latency and striking increases in tumor

incidence, multiplicity, growth rate and the rate of malignant progression. Mice hemizygous for C/EBPα displayed an intermediate enhanced tumor phenotype. Our results suggest decreased expression of C/EBPα contributes to deregulation of tumor cell proliferation. C/EBPα had been proposed to block cell cycle progression through inhibition of E2F activity. We observed that C/EBPα blocked Ras-induced and epidermal growth factor–induced E2F activity in keratinocytes and also blocked Ras-induced cell transformation and cell cycle progression.

Next the effects of mitogenic stimulation on C/EBPα and C/EBPβ expression were studied. Stimulation of cells in culture with EGF resulted in decreased C/EBPα expression, as well as a reciprocal increase in C/EBPβ expression. Further, loss of C/EBPβ results in an inhibited transition from the G1 to S phase of the cell cycle after treatment with EGF, and this reduction was associated with decreased expression of E2F target genes. Both C/EBPα and C/EBPβ have been proposed to affect cell cycle progression through interactions with E2F. We observed that C/EBPα blocked while C/EBPβ stimulated Ras- and EGF-induced E2F activity in keratinocytes. My results demonstrate that C/EBPα is dispensable for epidermal homeostasis, that C/EBPα and C/EBPβ are reciprocally regulated by mitogenic stimulation, and provide genetic evidence that C/EBPα is a haploinsufficent suppressor gene in epithelial tumorigenesis.

C/EBPα is an Epithelial Tumor Suppressor Gene, and Mitogenic Stimulation Reciprocally Regulates C/EBPα and C/EBPβ

by
Kari Danielle Loomis

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

Functional Genomics

Raleigh, North Carolina

2008

APPROVED BY:

David M. Bird	Barbara Sherry
Marcelo Rodriguez-Puebla	Robert C. Smart Chair of Advisory Committee

DEDICATION

I dedicate this thesis to my grandfather, Orval Lucier. Orval was a true lifelong learner who took classes at the University of Illinois, Urbana/Champagne every semester until he passed away in 1996 at age 81. I will always strive to maintain that kind of enthusiasm for life and learning.

BIOGRAPHY

Kari Danielle Loomis was born on February 5, 1976 in Massachusetts where she graduated from Duxbury High School in 1994. She then attended Clemson University in South Carolina where she earned a BS in Biology Education in 1998. Kari then taught middle school science and math in Brewster, NY, followed by three years of teaching high school biology at Bethel High School in Connecticut. Within this time, Kari worked to update the curriculum, designed a new course in the evolution of animal behavior, and coordinated internships with Boehringer Ingelheim Pharmaceuticals, a local pharmaceutical company. Kari sought out summer internships with this company, which became the launching pad for her research career. In 2003, Kari began her doctoral studies at North Carolina State University under the direction of Dr. Robert Smart. Kari was awarded the Graduate Assistance for Areas of National Need (GAANN) biotechnology fellowship, and taught a biotechnology PCR course. Kari was an active participant in the Functional Genomics graduate student association where she served as both vice president and president. Kari presented her research at a number of local and national meetings, and she was awarded first and second place for poster presentations at the North Carolina Society of Toxicology and North Carolina State University Graduate Student research symposia, respectively. Kari has accepted a tenure track assistant professor position in Cellular and Molecular Biology at Mars Hill College, in Mars Hill, NC.

ACKNOWLEDGEMENTS

I would first and foremost like to thank my advisor, Dr. Robert C. Smart for the rigorous and thorough training I have received in his lab. I would also like to thank my committee members, Dr. David Bird, Dr. Marcelo Rodriguez-Puebla, and Dr. Barbara Sherry for their time and advice throughout my doctoral studies. I would especially like to thank Dr. Sherry as she has been a great support and inspiration to me from the moment I came for my admission interview to my doctoral defense. I thank my colleagues in the Smart lab for their help and support, including Dr. Songyun Zhu, Dr. Kyungsil Yoon, Dr. Sarah Ewing, Kristina Powers, Jeanne Burr, Elizabeth Thompson, Rakesh Ranjan, and John House. I would like to thank my family and friends for supporting me in my quest, as well as my dogs Jewels and Oscar who have had to sacrifice many nice hikes in the woods for my work. Lastly, I would like to thank my fiancé, Matthew T. Dziedzic. Matt's support and belief in me have given me the strength I needed to complete this work.

TABLE OF CONTENTS

page

LIST OF FIGURES

page

GENERAL INTRODUCTION

Extracellular stimuli induce a multitude of cellular responses through the specific activation of transcriptional apparatus to induce changes in gene expression: a cascade of modifications to signal-transducing molecules direct site-specific transcription factors to activate or repress gene transcription and elicit an appropriate physiological response. Cellular responses include those involving metabolism, proliferation, apoptosis/survival, differentiation, cell adhesion, inflammatory responses, and locomotion, to name a few. This complex system of gene regulation is tightly controlled and often varies significantly between cell types and organisms. Deregulation of important signal transduction pathways can result in numerous disorders and diseases, including cancer. Here I will focus on the function and regulation of the site-specific transcription factors, CCAAT/enhancer binding proteins α and β (C/EBPα and C/EBPβ), in normal epidermal homeostasis, as well as in epidermal neoplasia formation.

Skin

Skin is composed of three distinct layers: epidermis, dermis, and hypodermis. The hypodermis, or subcutaneous fat layer, is the innermost layer of the skin. The hypodermis contains adipocytes as well as connective tissue serving to house larger blood vessels and nerves. The hypodermis serves as an energy reserve and participates in shock absorption and thermoregulation. The dermis lies between the hypodermis and the epidermis, and is a strong but

flexible connective tissue layer which helps bind the entire body together. The dermis contains fibroblasts and macrophages organized in a semi-fluid matrix of collagen, elastin, and reticular fibers. This layer houses most of the skin's specialized cells and structures including blood and lymph vessels, sweat glands, hair follicles, sebaceous glands, and nerve endings. The dermis functions to regulate temperature, maintain hydration, and supply the epidermis with nutrient-rich blood. An extracellular matrix component known as the basement membrane forms a barrier which separates the epidermis and its appendages from the dermis. The basement membrane is also rich in receptor tyrosine kinase growth factors, which provide proliferative stimuli to the innermost basal layer of the epidermis[1].

Small finger-like projections known as dermal papillae indent the overlying epidermis carrying pain and touch receptors as well as capillary loops. Follicular dermal papillae are composed of specialized fibroblasts that specifically populate the base of the hair follicle bulb[2]. The dermal papillae contain cells involved in important epithelial-mesenchymal interactions which direct various processes including the initiation of epidermal stem cell differentiation into hair follicles or other epidermal appendages[1].

Epidermis

The epidermis is a thin layer of keratinized stratified squamous epithelium and is the outermost protective shield of the body. Keratinocytes are the primary epidermal cell type, but melanocytes, Merkel cells, and Langerhans'

cells are also present. In mammals, the epidermis comprises the interfollicular epidermis as well as the adnexal structures including the hair follicle and sebaceous gland.

As a self-renewing tissue, the epidermis must balance proliferation and differentiation[3, 4]. Proliferative keratinocytes of the innermost interfollicular epidermal layer become post-mitotic, move up to the suprabasal layers, and undergo terminal differentiation where they become flattened and eventually lead to the production of the dead, cornified epidermal layers that provide the protective covering of the skin. In addition to its role in protection from the environment, the skin and its appendages also function in sensation, communication, respiration, locomotion, immune responses, and thermoregulation[5]. Epidermal keratinocytes are organized into four sub-layers: the basal layer, spinous layer, granular layer, and stratum corneum.

The basal layer of the epidermis consists of a single layer of proliferating keratinocytes that give rise to terminally differentiating post-mitotic stratified layers. Keratinocytes of the basal layer are firmly attached to the extracellular matrix-rich basement membrane through focal contacts and hemidesmosomes, and are attached to one another through adherens junctions and desmosomes[6]. The basal layer follows a path along the surface of the interfollicular epidermis, as well as along the outer root sheath of the hair follicle. Keratinocytes in the basal layer of the interfollicular epidermis express genes encoding integrins necessary for basement membrane attachment, growth factor receptors

(particularly epidermal growth factor receptor, or EGFR), as well as the structural keratins 5 and 14 (K5 and K14)[6, 7]. The basal layer contains both proliferative and post mitotic keratinocytes which move upward and leave the basal cell layer. Once cells detach from the basement membrane, they are characterized as suprabasal cells, and all of the cells become post-mitotic.

The suprabasal layer consists of the spinous layer, granular layer, and stratum corneum. In these layers, cells repress basally expressed genes and switch to expressing a set of differentiation-associated proteins, including keratins 1 and 10 (K1 and K10) in early stages of differentiation and loricrin and involucrin in the later stages of differentiation. As keratinocytes continue their trek upward to the stratum corneum, they further adjust their transcriptional program, all metabolic activity ceases, and cytoplasmic organelles are lost, culminating in the production of dead, flattened squames which form the stratum corneum. The resulting cornified envelope is composed of highly insoluble, cross-linked proteins with covalently attached lipids essential for the mechanical integrity and water impermeability of the skin[8]. Cells of the stratum corneum are eventually sloughed from the skin surface as new cells moving outward replace them[4].

Epidermal Homeostasis

The epidermis is a continuously renewing tissue derived from multipotent stem cells that self-renew and also generate progeny that undergo terminal differentiation[9]. The best characterized stem cells reside in a region of the hair

follicle known as the bulge region[10], although stem cells also reside in the interfollicular epidermis and the potentially the sebaceous gland[11]. The bulge region is found in a portion of the hair follicle outer root sheath just below the sebaceous gland, and the stem cells residing there are capable of producing keratinocytes, hair follicles, and sebaceous glands[11]. Although any epidermal stem cell can generate all of the epidermal lineages, it can be argued that the choice of lineage is determined by the specific location or microenvironment of the cell[3, 12, 13]. Mesenchymal stimulation from dermal papillae cells, specifically, has been shown to induce interfollicular epidermal cells to differentiate into hair and sebocyte lineages[14, 15].

Although it has been proposed that stem cells from the bulge region were largely responsible for basal keratinocyte replenishment[6], recent studies show that distinct stem cell populations regenerate the hair follicle and interfollicular epidermis[16,]. The bulge region stem cells do however participate in interfollicular regeneration in wound repair[16, 17]. Keratinocytes exiting the bulge region populate the outer root sheath, and are thought to fuel the highly proliferative matrix cells that are adjacent to the mesenchymal dermal papillae and are involved in hair follicle development. β1-integrin extracellular matrix receptors are important in epidermal stem cell proliferation and maintenance [1], contribute to the ability of stem cells to bind to the extracellular matrix of the basement membrane, and are potential markers of interfollicular epidermal stem cells[18]. In addition to high surface expression of β1 integrins, high α6

integrin, low CD71 expression, high expression of Delta1, low expression of desmoglein 3, and low expression of the EGF receptor 1 are also markers for interfollicular epidermal stem cells[19].

In the widely accepted mechanism for interfollicular epidermal homeostasis, a small number of slow cycling stem cells produce a larger number of highly proliferative transit-amplifying cells[3]. Transit-amplifying cells have a limited replicative potential before undergoing terminal differentiation[18]. The stem cells are surrounded by transit-amplifying cells to form proliferative units, or EPUs, composed of hexagonally packed cells[1]. In vivo pulse-chase experiments identified label retaining cells of the hair follicle as cells that cycle less frequently than those of nearby basal keratinocytes. The Cairns hypothesis argues that stem cells preserve their genetic message by using selective strand segregation to maintain a constant template strand and prevent mitotic crossing over[20]. Due to this strand retention, mutations in the DNA of stem cells pose a greater risk for cancer development. While a large percentage of hair follicle bulge cells are label retaining cells, only a small percentage of interfollicular cells are label retaining cells. The low rate and even dispersal of label retaining cells in the interfollicular epidermis was taken as circumstantial evidence in support of the EPU mechanism described above. It is unclear however whether basal epidermal label retaining cells represent stem cells[21]. Comparisons between pulse-chase label retention experiments and cellular profiling using various epidermal stem cell markers such as β1

integrin, showed far greater β1 high expressing cells than label retaining cells in human skin[1].

Recent studies suggesting another possible mechanism for maintenance of epidermal homeostasis show that as basal epidermal cells divide, they can polarize and localize key regulatory proteins in the cell to create an asymmetrical partition of cellular components, thus specifying the cell fate of each daughter cell as stem cell or differentiating cell[22, 23]. With this second mechanism, many if not all basal cells may have the capacity for self-renewal and epidermal stratification and differentiation[1]. Appropriate stem cell markers and a proper definition of what constitutes a stem cell are central issues in determining the true mechanism for epidermal homeostasis.

C/EBP Family of Transcription Factors

CCAAT/enhancer binding proteins, or C/EBPs, are a family of basic leucine zipper transcription factors that act as master regulators of many cellular responses. There are currently 6 C/EBP family members represented by greek letters assigned chronologically according to each gene's date of identification (α, β, γ, δ, ε, and ζ). In the late 1980's, the McKnight lab first identified and cloned the founding member C/EBPα (know at the time simply as C/EBP)[24, 25]. The protein was named according to its role in binding the CCAAT cis-regulatory promoter sequence as well as in binding an enhancer core element common to SV40, murine sarcoma virus, and polyomavirus[26, 27]. Further characterization of the C/EBP genes revealed their role not only in

binding to a variety of cis-regulatory DNA sequences, but also in binding to and controlling the activity of key regulatory proteins. The promiscuous and unexpected nature of C/EBP protein and DNA binding has led McKnight to describe the name *C/EBP* as "A silly acronym he wears around his neck like a rotting albatross[27]". The next section will give an overview of the structure, expression, and function of all C/EBP family members; with a focus on the roles of C/EBPα and C/EBPβ in the epidermis.

Structure

C/EBPα, -β, -δ, and –γ are intronless genes, whereas C/EBPε and –ζ contain two and four exons, respectively[28]. Multiple isoforms of C/EBPε protein are produced through alternative use of promoters and differential splicing of the RNA transcript[29, 30]. Despite the fact that C/EBPα and C/EBPβ are intronless genes, they have multiple isoforms due to regulation of translation initiation [29, 31, 32]. C/EBP family members share greater than 90% sequence identity in their C-terminal bZIP domain, consisting of a basic amino acid rich DNA binding region followed by a dimerization motif termed the leucine zipper[28]. Family members form homo- and heterodimers with one another through their leucine zipper domains; C/EBP dimerization is required for DNA binding. C/EBP dimerization interactions can contribute to the differential regulation of C/EBP activity[31-35]. The largely conserved C/EBP DNA binding domain, with the exception of C/EBPζ, results in a common but flexible C/EBP-DNA binding recognition motif of (A/G)TTGCG(C/T)AA(C/T)[36, 37].

The exact mechanism by which C/EBPs achieve specificity of function while maintaining similar binding recognition domains is not yet fully elucidated, but has been shown to involve numerous processes including: 1. protein conformational changes resulting from post-translational modification, as when phosphorylation of C/EBPα at Ser21 alters C/EBPα conformation to favor monocyte differentiation and inhibit granulopoiesis through a transcriptional mechanism[38], 2. protein-protein interactions with the basal transcription machinery, other transcription factors, and chromatin remodeling complexes, as when C/EBPβ binds to specific components of the RNA-polymerase-II holoenzyme to activate or repress gene transcription[39], and 3. Differential regulation of C/EBP expression, as shown with the decreased expression of C/EBPα after partial hepatectomy to promote C/EBPβ rather than C/EBPα transcription[40], and in C/EBPβ knock-in mice where the temporal expression of C/EBPβ from the *C/ebpα* gene locus was able to substitute for C/EBPα expression and rescue a lethality phenotype[41]. All of these mechanisms contribute to the ability of individual family members to achieve specificity of DNA binding and transcriptional regulation.

C/EBP proteins are primarily regulated through the N-terminal transactivation domain where C/EBP family members share less than 20% sequence identity. Within the N-terminus are three semi-conserved activation domains and regulatory domains, whose presence varies between family members. These conserved domains are involved in interactions between

C/EBPs and the basal transcription apparatus (TBP/TFIID), transcriptional coactivators (CBP/p300), and in sumoylation. Exceptions include C/EBPγ which lacks any functional transactivation domain outside of the basic region, and C/EBPζ which has a shortened N-terminal and does not contain any of the conserved activation or repressive domains[33, 42]. Also, the smaller isoforms of C/EBPα and C/EBPβ lack the major N-terminal functional domains.

Differences in the N-terminal functional domains of specific C/EBP isoforms can result in highly varied transactivation potential between family members. Heterodimerization between a relatively more and less active C/EBP can result in the ability of the less active C/EBP to dominantly inhibit the activating C/EBP. The N-terminal domain is generally considered to be the activation domain, and proteins lacking the domain are largely considered to have little to no capacity for intrinsic activity. However, this paradigm seems to be shifting somewhat as dimerization interactions between the leucine zipper domains of specific family members have been shown to activate C/EBPs lacking the N-terminal region. For instance, it has been shown that C/EBPβ and C/EBPγ heterodimerization results in the specific activation of C/EBPγ stimulatory activity in a cell type and gene promoter specific manner[35]. The ability of dimerization interactions in the leucine zipper to activate C/EBPγ's transcriptional potential also supports the idea that the leucine zipper domain plays a larger role than simply enabling dimerization[35]. Further, protein-protein interactions take place in both the C- and N-terminal of the protein. Gene

regulation, heterodimerization, protein-protein interactions, and post-translational modifications, all contribute to the functional versatility of individual C/EBP family members to specifically respond to their cellular environments with unique and overlapping roles.

Expression

C/EBP family members are differentially expressed in numerous cells, tissues, and organisms, and in response to a variety of stimuli. The precise expression profile for each C/EBP family member is not yet fully elucidated and there are some conflicting studies in regards to these C/EBP expression patterns. The general consensus will be presented here. C/EBPα is expressed at high levels in skin, adipose tissue, liver, intestine, lung, mammary gland, adrenal gland, peripheral-blood mononuclear cells, limbal and corneal epithelial cells, ovary, and placenta[28, 43-47]. In both liver and adipose tissue, C/EBPα message is highest in the most differentiated post mitotic cells[48, 49]. C/EBPβ, also known as NF-IL6 or LAP, is expressed in most tissues, but its steady state expression levels are greatest in the skin, liver, intestine, lung, adipose tissue, spleen, kidney, and myelomonocytic cells[43, 47, 48, 50-56]. C/EBPγ, or Ig/EBP-1, is characterized as ubiquitously expressed in various tissues, and has been thought to be somewhat unresponsive to extracellular stimuli, in part due to the absence of any functional N-terminal domain[35, 57]. More recent studies have shown a high inter-individual variation in C/EBPγ transcript abundance, and a correlation between C/EBPγ and antioxidant and DNA repair genes in normal

individuals, but not in individuals with bronchogenic carcinoma[58]. Although C/EBPδ, also known as NF-IL6β, is expressed at low to undetectable levels in most cell types and tissues under physiological conditions [47, 48, 59, 60], it is highly inducible by a variety of extracellular stimuli including growth hormone, insulin, INFγ, IL-1, IL-6, LPS, TNFα, dexamethasone, noradrenalin, glutamate, and luteinizing hormone[60, 61]. C/EBPδ induction is best characterized in adipose tissue, lung, intestine, and mammary epithelium[28, 62]. C/EBPε has a more limited range of expression specific to myeloid and lymphoid cells[28]. Finally, C/EBPζ, better known as CHOP-10/GADD153 (C/EBP Homologous Protein-10/Growth Arrest- and DNA Damage-inducible gene 153), is ubiquitously expressed at very low levels, but is highly inducible by a number of extracellular growth arrest and/or DNA damaging factors including calcium ionophores, glucose deprivation, oxidative stress, reductive stress, and endoplasmic reticulum stress/activation of the acute phase response[63]. Although C/EBPγ, and possibly C/EBPζ and C/EBPδ are expressed in the skin, C/EBPα and C/EBPβ are the most prevalent C/EBP family members in epidermal keratinocytes (unpublished data, and[43, 44, 64, 65]).

Function

C/EBP family members function as transcriptional activators or repressors of many key genes involved in cellular processes such as energy metabolism, inflammation, and liver regeneration. C/EBPs are also well known regulators of differentiation and proliferation in various cell types including

adipocytes, granulocytes, hepatocytes, and keratinocytes. Although many of the functions of C/EBPs are in line with traditional roles for transcription factors, many C/EBP functions are fully independent of DNA binding and transcriptional activation or repression[28].

There is considerable crosstalk between family members, as C/EBPs can either homo- or hetero-dimerize, and heterodimerization can result in modification of function. A certain level of redundancy in C/EBP protein function is also suggested by the fact that the post-natal lethality of C/EBPα knockout mice can be rescued by expression of C/EBPβ from the *C/ebpα* gene locus[41]. C/EBPs have been shown to transcriptionally self-regulate their promoters and/or regulate the promoters of other C/EBP family members, further adding to the complex modulation of C/EBP function. As a result, studying the function of individual C/EBP family members in isolation can be somewhat difficult. A general overview of all C/EBP family members' functions will be given here, and a more detailed look specifically at the functions of C/EBPα and C/EBPβ in the epidermis and in cancer will follow.

C/EBPα plays a role in inducing genes characteristic of the terminally differentiated state in several cell types including adipocytes, lung, liver, mammary gland, skin, neurons, and within the hematopoietic system[66-68]. Another primary function of C/EBPα is its ability to seemingly slow or arrest mitotic growth. Much attention has been given to the ability of C/EBPα to couple these two main features of terminal differentiation[66]. C/EBPα also plays

a critical role in energy metabolism, as evidenced by the fact that mice with a germline mutation of the C/EBPα gene die before or shortly after birth due to severe hypoglycemia resulting from reduced expression of glycogen synthase[69]. The role for C/EBPα as a tumor suppressor will be discussed in a later section.

C/EBPβ functions in differentiation, specifically of mammary epithelial cells, adipocytes, ovarian granulosa cells, neurons, keratinocytes, in the lymphoid cell compartment and in the hematopoietic system[28, 68]. C/EBPβ is essential for ovarian follicle development in vivo, and female C/EBPβ$^{-/-}$ mice are sterile[70]. C/EBPβ is also required for the functional differentiation of mammary epithelial cells and for normal mammary gland development[71]. The role of C/EBPβ in proliferation arrest is less clear however, as C/EBPβ has been shown to both induce proliferation and mitotic growth arrest, as well as to induce senescence and oncogenic transformation[72]. Metabolism defects are also evident in one of two strains of C/EBPβ knockout mice, where mice die soon after birth owing to hypoglycemia due to an inability to mobilize their hepatic glycogen and express PEPCK[73]. C/EBPβ plays an important role in the innate and acquired immune response[28]. C/EBPβ has also been implicated in cellular transformation, cell survival, anchorage independence, and other invasive properties. Specific roles for C/EBPβ in cancer will be discussed in a later section.

C/EBPγ has been characterized primarily as a dominant negative inhibitor of other activating C/EBP family members through heterodimerization[33].

More recent studies have shown that C/EBPγ modulates other C/EBPs in a cell- and isoform-specific manner[74], and have begun to elucidate more activating functions for C/EBPγ including activation of the IL-6 and IL-8 promoters[35, 75]. C/EBPγ has been shown to have a role in protecting lungs from oxidant damage, as C/EBPγ knockout mice begin to die shortly after birth due to emphysematous lung conditions[76]. Further results supporting a specific activating role for C/EBPγ include its correlation with antioxidant and DNA repair genes in bronchial epithelia[58].

C/EBPδ, like C/EBPα and C/EBPβ, is also associated with differentiation, specifically in adipocytes, lung epithelial cells, and myelomonocytic cells[48, 77, 78]. C/EBPδ also functions in mammary gland biology. However, instead of promoting mammary gland proliferation and differentiation as C/EBPβ does, C/EBPδ is associated with mammary epithelial cell G_0 growth arrest and programmed cell death[79]. C/EBPδ has also been shown to regulate mitotic quiescence of limbal keratinocytes of the cornea by forcing cells into the G_0/G_1 phase of the cell cycle and promoting the self-renewal of human limbal stem cells[80]. C/EBPδ has also been shown to be involved in late stages of differentiation in human epidermis, although the status of C/EBPδ expression in the epidermis is questionable (unpublished data and [80, 81]). C/EBPδ knockout mice, however do not exhibit an altered gross phenotype under normal physiological conditions, or after carcinogen-induced skin tumorigenesis[82]. Other functions of C/EBPδ involve the regulation of the acute phase

response[83, 84], as well as in learning and long-term memory in the central nervous system[85, 86]. Loss of C/EBPδ has been shown to promote chromosomal instability[60], and it has been identified as a tumor suppressor gene in acute myeloid leukemia[87].

C/EBPε has a much more limited range of expression than the other C/EBP family members, and is expressed solely in the myeloid lineage[28, 88]. In myelopoiesis, C/EBPε is required for normal granulocytic differentiation. This was evidenced in C/EBPε knockout mice, where mice undergo early lethality due to impaired granulopoiesis and myelodysplasia[89]. C/EBPε has been shown to be a critical transcription factor in acute promyelocytic leukemia cells for the TNFα-induced up-regulation of PHGPx, a selenium dependent antioxidant enzyme[90]. Also, the N-terminal region of C/EBPε has been shown to be critical for cell cycle arrest, apoptosis, and functional maturation during myeloid differentiation[91].

C/EBPζ was first characterized as a dominant negative inhibitor of other activating C/EBPs through heterodimerization[92]. CHOP has however been shown to activate other, non-C/EBP, transcription factors such as AP-1, while inhibiting C/EBP DNA binding and transcriptional activation[93]. CHOP has been implicated in cell cycle arrest and apoptosis, where microinjection of GADD153 induces 3T3 cells to arrest at the G_1/S boundary[94], while ectopic expression of GADD153 causes induction of apoptosis in a p53 dependent manner in myeloblastic leukemia cells[95]. The role for CHOP in apoptosis was further

elucidated by the fact that CHOP knockout mice exhibit reduced apoptosis in response to ER stress[63, 96, 97].

As outlined above, the C/EBP transcription factors are involved in numerous cellular functions, some of which are shared while some are specific to an individual C/EBP family member. C/EBPs are differentially regulated through a complex network of signal transduction pathways in order to induce various cellular responses such as differentiation, mitosis, proliferation arrest, and apoptosis. The differential regulation of these processes by C/EBPs promotes tissue homeostasis and allows cells to respond to cellular stressors. The following section will review some of the specific signal transduction networks which interact with C/EBPs in the epidermis.

Signal Transduction Networks

Cells have developed elaborate signal transduction pathways for communication to help individual cells respond to extracellular stimuli. There are multiple components to this communication system including extracellular signal molecules which are produced by cells to signal to other cells, cell surface receptors which bind to the signal molecule, plus a variety of intracellular signaling proteins that distribute the signal to multiple parts of the cell. Target proteins that lie at the end of signal transduction pathways are modified in some way in order to induce a physiological response such as differential gene regulation, ion channel opening/closing, metabolic responses, or cytoskeletal changes to induce altered cell shape or movement. The

following section will focus on specific signal transduction pathways that C/EBPα and C/EBPβ are involved with in the epidermis and in epithelial tumorigenesis.

ErbB Family of Receptor Tyrosine Kinases

Receptors of the tyrosine kinase superfamily of proteins play pivotal roles in relaying extracellular signals to intracellular signaling networks. Members of the RTK family specifically bind peptide ligands and properly integrate extracellular messages with the appropriate intracellular signal transduction pathways to invoke a fitting cellular response[98]. The ErbB proteins belong to subclass I of the superfamily of RTKs. There are four members of the ErbB family, ErbB1, or epidermal growth factor receptor (EGFR), ErbB2, ErbB3, and ErbB4 (also known as HER1-4 in humans). Three of these family members are expressed in the skin: EGFR, ErbB2, and ErbB3, but not ErbB4[99-102].

ErbB ligands are referred to as EGF-related peptide growth factors, and each contain an EGF-like domain that is sufficient to confer binding specificity[103]. EGF family members expressed by epidermal keratinocytes include transforming growth factor-α (TGFα)[104] and amphiregulin (AR)[105], which both specifically bind EGFR[103]; and heparin-binding EGF-like growth factor (HB-EGF)[106] and epiregulin (EPR)[107], which specifically bind EGFR and ErbB4[103]. EGF is also a potent mitogen in epidermal keratinocytes and specifically binds to EGFR[108]. Induction of one EGF family member generally results in the upregulation of other family members, creating an auto- and cross-induction

mechanism. These ligands are however, differentially expressed under various cellular conditions such as proliferation and differentiation to convey distinct, non-redundant biological functions[107]. Although all five ligands activate EGFR, each ligand evokes a unique cellular response. ErbB ligands achieve specificity of interactions and diversity of signaling through structural features that direct the interactions of ErbB receptors with one another, such as through the promotion of specific dimerization partners[103].

ErbB receptors have a common extracellular ligand-binding domain, a single membrane spanning region, and a cytoplasmic protein tyrosine kinase domain[103]. Ligand binding stimulates specific ErbB family members to form homo- and hetero-dimers, thereby activating the cytoplasmic tyrosine kinase domain. The homo-and heterodimerization of each family member is specific to the family member and ligand present. EGFR can form homodimers, and can heterodimerize with ErbB2. ErbB2 lacks any known ligands and therefore does not homodimerize, but only heterodimerizes with the other three family members and has no known ligands[103]. ErbB3 receptors lack a functional tyrosine kinase domain, and therefore only form inactive homodimers. ErbB2/ErbB3 heterodimers do however form a functional unit[103].

EGFR knockout mice display defective proliferation, differentiation, and maintenance of cutaneous, gustatory and gut epithelia as well as altered patterning of cutaneous innervation[109]. EGFR is an important regulator of interfollicular but not follicular proliferation, and of hair follicle but not

interfollicular epithelial differentiation[110]. In keratinocytes, ErbB1 activation promotes proliferation/survival while ErbB2 activation promotes differentiation[111]. ErbB2 and ErbB3 have been shown to form heterodimers in human keratinocytes in response to the ErbB3 specific ligand, heregulin, creating a potential autocrine–paracrine system involved in epidermal homeostasis and repair, as well as in hyperproliferative pathologies[112].

Activating mutations can lead to the ability of EGFR to signal to downstream effectors in the absence of an activating ligand. Cell proliferation in the absence of growth factor stimulation is a hallmark of cancer, and inappropriate EGFR or ErbB2 expression or activation is implicated in a wide range of cancers including breast cancer[103, 113]. Targeted disruption of EGFR in epidermal keratinocytes alters phenotypic responses to the v-Ha-ras oncogene in vitro and in vivo but does not block v-Ha-ras-induced tumor development[114]

Receptor Tyrosine Kinase Signaling

Receptor tyrosine kinase activation initiates many cellular signaling pathways involved in development, proliferation, and differentiation in numerous tissues of epithelial, mesenchymal, and neuronal origin. Three major signaling pathways activated by RTKs include the PI3K/AKT pathway, the Ras/MAP kinase pathway, and the JAK/STAT pathway.

Ras: From activation to cell cycle progression

Activation of the Ras/MAP kinase signaling cascade can transmit signals from activated EGFR to the cell nucleus, thereby inducing cell proliferation.

EGFR couples to the MAP kinase pathway through adaptor proteins. Adaptor proteins contain Src homology 2 (SH2) or phosphotyrosine binding (PTB) domains, and dock to phosphorylated regions of the tyrosine kinase domain[103]. Examples of proteins known to bind these domains include phospholipase Cγ (PLCγ), PI3K, SRC, GTPase activating protein (GAP p120), and the adaptor proteins Shc and Grb2. Adaptor proteins function to recruit additional proteins to the activated RTK. GRB2 recruits a guanine nucleotide exchange factor (GEF) known as Son of Sevenless (SOS) to the inner leaflet of the plasma membrane.

Ras is tethered to the plasma membrane, and GRB2 recruitment of SOS to the activated EGFR, brings the GEF into close proximity to Ras. Ras acts as a molecular switch, bound to GDP in its inactive state and bound to GTP in its active state. The exchange of GDP for GTP is facilitated by the guanine exchange factor SOS, tying EGFR activation and GRB2 recruitment of SOS, to Ras activation. Ras exhibits intrinsic GTPase activity which is largely stimulated by the GTPase activating protein GAPp120. This mechanism of activation and subsequent inactivation of Ras allows for a tightly controlled system of signal transduction. The active GTP-bound form of Ras undergoes a conformational change which allows it to interact with downstream effectors such as RAF, PI3K, RALGDS, and RAC.

Ras-GTP binds to and activates the serine threonine kinase RAF-1, which is free to move about the cytoplasm and carry on the signal. RAF-1 then

phosphorylates MAP kinase-extracellular signal regulated kinase, or MEK1, which consequently activates its intrinsic threonine and tyrosine kinase abilities. MEK furthers the signal by then phosphorylating and activating ERK1 and ERK2 (extracellular regulated MAP kinase), which have numerous cellular substrates and activate a number of transcription factors. Within a few minutes of mitogenic stimulation, activated ERK isoforms translocate to the nucleus thereby transmitting mitogenic signals to the cell nucleus[115]. One important target for MAP kinase signaling is cyclin D1.

Cyclin D1 is an important cell cycle regulator whose induction leads to the de-repression of E2F transcription through activation of Cdk4 and Cdk6 to phosphorylate pRb and release E2F. E2F transcription leads to the upregulation of genes required for progression from G_1, through the restriction point, into S-phase of the cell cycle. Once cells have passed through the restriction point of the cell cycle, further cell cycle progression is growth factor independent.

Ras/MAP kinase signaling can thereby transmit proliferative signals to the nucleus and translate growth factor stimulation into cellular proliferation. It is apparent, then how deregulation or aberrant activation of proteins at any point along the MAP kinase pathway could result in uncontrolled growth. Characteristic activating Ras mutations in the 12^{th}, 13^{th}, or 61^{st} codon impair Ras's intrinsic and GAPp120-induced GTPase activity, thereby allowing Ras to remain in an active, GTP-bound state. This sort of growth factor independent

proliferation is a hallmark of cancer[113], and Ras is mutated in approximately 25% of all human tumors. This is of course only one of many possible signaling pathways activated by EGFR, and there is considerable cross talk between pathways further adding to the complexity of growth factor induced cell signaling networks.

Cell Cycle

The numerous processes that must take place as cells undergo mitotic expansion characterize the three stages of cell cycle progression: G_1, S, and G_2/M. Another stage known as G_0, is said to be a stage where non-proliferating cells perform basic cellular functions, and that cells are only considered to be in G_1 upon induction of proliferation. The G_1 or gap phase 1 of the cell cycle is the longest phase of the cell cycle and is the period in which the cell increases in size and begins to prepare for cell division. The S- phase, or synthesis phase of the cell cycle is the period of time when cells replicate their DNA, and the G_2 (gap-2) phase allows for final preparations before entering mitosis. There are numerous stopping points or checkpoints in the cell cycle

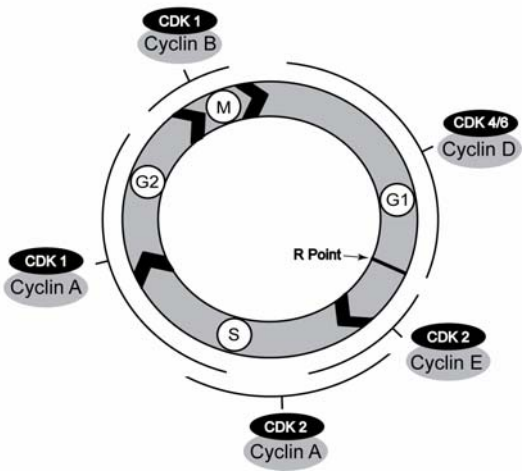


Figure 1. Cyclin/CDK complexes [116]

which allow the cell to halt proliferation in response to cytogenic stressors such as UVB irradiation.

As described above, entry into the cell cycle is tightly regulated by upstream signaling pathways that culminate in the increased expression of numerous genes including cyclin D1. Cyclins bind to and activate cyclin dependent kinases (Cdks) to promote cell cycle progression. Cdk expression is constant, however its kinase activity is not stimulated until it interacts with the

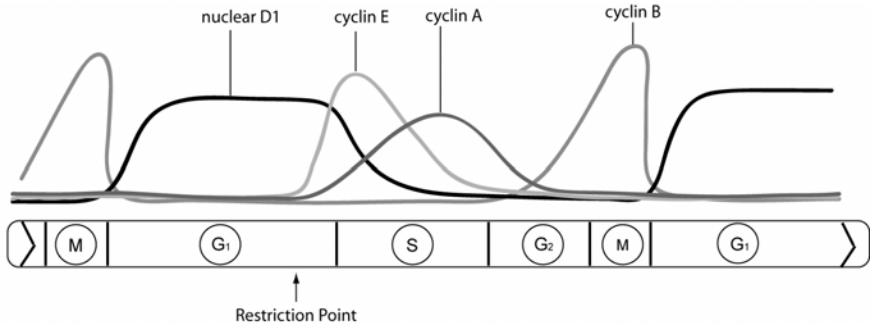


Figure 2. Cyclin expression throughout the cell cycle[116]

appropriate cyclin. The increased expression of specific cyclins at different phases of the cell cycle is associated with activation of Cdks involved various phases of the cell cycle. As shown in figures 1 and 2, cyclin D1 levels are induced as cells enter G_1, and cyclin D1 binds to and activates Cdk4 and Cdk6. The activation of Cdk4 and Cdk6 by cyclin D1 leads to phosphorylation of the tumor suppressor Rb.

The Rb/E2F cell cycle regulatory mechanism generally inhibits G_1 to S-phase transition through Rb's repression of E2F-mediated transcription, as depicted in figure 3. The repressive effects of Rb on E2F transcription are released upon sequential hyperphosphorylation of Rb by Cdk4 and Cdk6, followed by Cdk2. E2F transcription induces genes required for entry into S phase, including cyclin E, thereby further phosphorylating Rb and derepressing E2F's transcriptional activities. As shown in figures 1-3, the point beyond which cell cycle progression continues without growth factor stimulation is known as

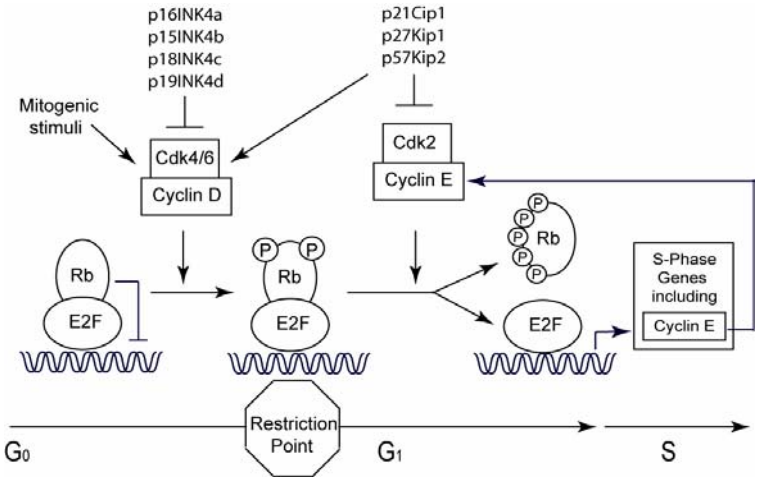


Figure 3. Gene transcription control by E2F and Rb[116]

the restriction, or R point, and it falls between mid and late G_1. Further oversight of cellular proliferation is enforced through specific inhibitors of the cell cycle such as $p16^{INK4A}$ and $p21^{Cip1}$. Replicative senescence and

differentiation of keratinocytes are associated with increased levels of $p16^{INK4A}$ and involucrin, which indicate irreversible exit from the cell cycle and onset of terminal differentiation, respectively[117, 118].

Cancer

Cancer is not a single disease, but is rather a group of diseases characterized by cells that have acquired a number of unifying characteristics. Hanahan and Weinberg propose that there are six hallmarks of cancer which alter cell physiology to promote malignant growth: 1. Self sufficiency in growth signals, 2. Insensitivity to growth-inhibitory signals, 3. Tissue invasion and metastasis, 4. Limitless replicative potential, 5. Sustained angiogenesis, and 6. Evading apoptosis[113]. They propose that cancer cells must acquire all 6 characteristic hallmarks of cancer, and that the acquisition of these traits occurs through somatic mutations of DNA. These traits could be acquired from as few as 2 to 3 mutations, or as many as 8, depending on the specific gene mutations and the interactions between that gene and other mutated genes. All of these characteristics can be regulated by signal transduction pathways, and the perturbation of these signaling pathways can result in cancer development. For instance, mutation of p53 could promote both angiogenesis and resistance to apoptosis, allowing the cell to acquire 2 characteristic hallmarks of cancer through a single mutation. Upon acquisition of each new acquired trait, the cell becomes more out of control, feeding an environment of instability and promoting further gene mutations, otherwise known as genomic instability.

Specific genes involved in cancer have been deemed oncogenes and tumor suppressor genes. Oncogenes are genes which promote tumorigenesis, while tumor suppressor genes inhibit carcinogenesis. The interplay between oncogenes, tumor suppressor genes, and genes that promote genomic stability such as DNA repair genes drive the cancer process and result in the acquisition of the characteristic hallmarks of cancer. As shown in figure 4, Oncogenic activation can lead to altered expression of proteins to promote increased survival/proliferation, tumor suppressor inactivation results in the loss of

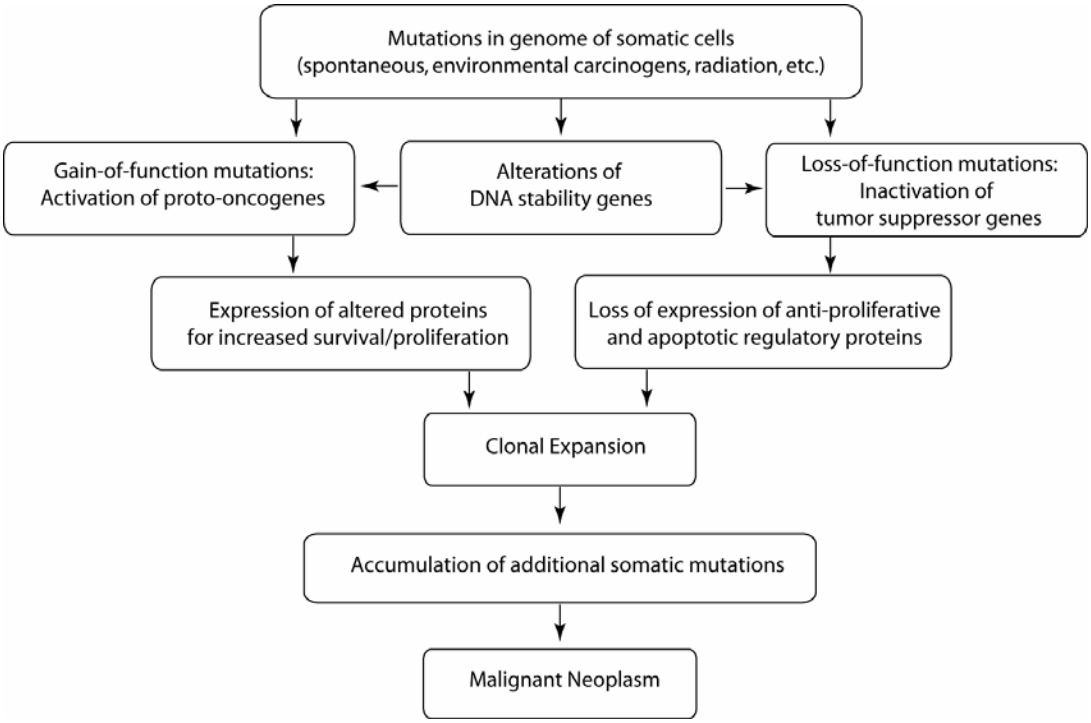


Figure 4. Overview of cancer process[116]

expression of anti-proliferative and apoptotic regulatory proteins, and faulty DNA repair genes can contribute to these changes. As stated above, once the cell has acquired a hallmark of cancer the cell starts to lose control, resulting in an increased rate of acquisition of further mutations leading towards malignant neoplasia formation.

There are a number of mechanisms by which oncogenes can be activated, as shown in figure 5. A point mutation in an oncogene can lead to its activation, as with the characteristic mutations in the Ras oncogene mentioned above. Chromosome rearrangements can result in the overproduction of normal protein by altered regulation of gene expression due to translocation of a foreign sequence to the promoter of an oncogene. Translocation of a portion of a chromosome can also lead to the production of a fusion protein, where the

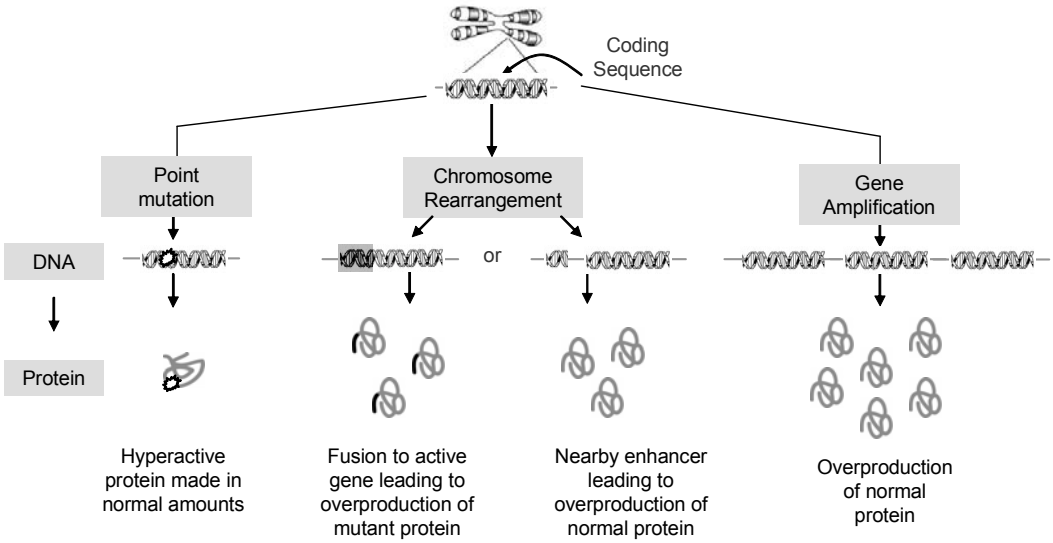


Figure 5. Oncogene activation[116]

mutant protein has acquired additional oncogenic characteristics. Another mechanism of oncogene activation by mutation is through gene amplification, where multiple copies of the gene become inserted into and expressed from the genome. All of these mechanisms lead to the activation/overexpression of oncoproteins to promote proliferation and survival, and further neoplastic progression.

This idea of a single cell acquiring cumulative mutations as it progresses towards malignancy is known as the somatic mutation theory of clonal expansion. Figure 6 depicts how a single cell with cumulative acquired somatic mutations can clonally expand to produce a primarily clonal neoplasm. For instance, the first mutation may have inactivated a tumor suppressor gene,

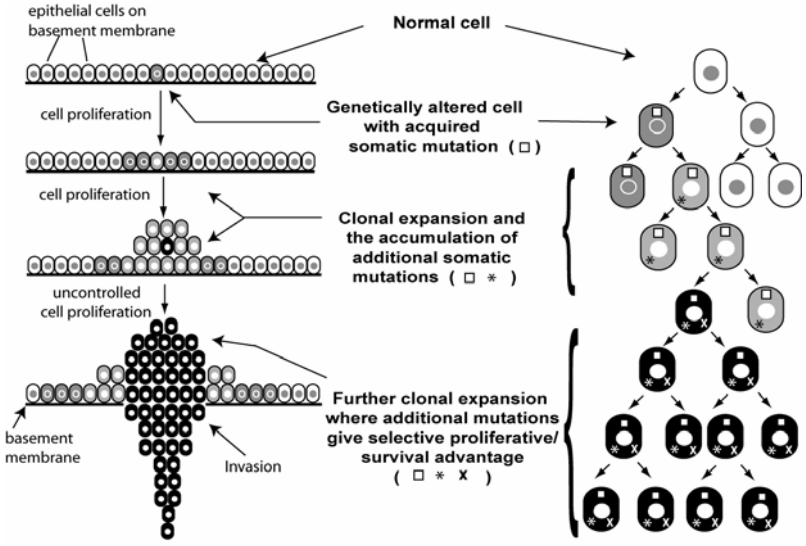


Figure 6. Monoclonal Nature of Cancer. A tumor develops through successive rounds of proliferation and mutation. As a cell acquires a proliferative/survival advantage through mutation, it becomes more susceptable to further mutation. Eventually, an individual cell with successive mutations gives rise to a population of identical cells that have the proliferative/survival capacity to form a tumor[116].

resulting in unchecked growth. The cell is now replicating its DNA without any checks on proliferation making it much more susceptible to further mutation. A further mutation involving the activation of an oncogene would thereby promoting excessive proliferation. The drive for proliferation is now fully unchecked by the mutated tumor suppressor genes, thereby resulting in further mutations, including mutations of DNA repair genes. The uncontrolled proliferation of cells renders cells more prone to errors in DNA replication, but if the DNA repair genes are inactive they are unable to repair the damage. The mutated cells pass on the acquired somatic mutations, eventually leading to possible further mutation and neoplasm formation. One requirement for cancer development is that at some point cells must replicate and pass on their acquired mutations. This taken with other evidence has led to the theory that epithelial cancers are derived from highly proliferative stem cells that have a limitless replicative potential[119]. Further, oncogenes such as c-Myc are capable of immortalizing cells. Limitless replicative potential is in fact one of the hallmarks of cancer, thereby giving stem cells one of the hallmark characteristics of cancer even without mutation.

C/EBPs and Cancer

C/EBPα and C/EBPβ have been implicated in the cancer process through a number of studies in numerous tissues and cell types. C/EBPα has been characterized as a tumor suppressor which is inactivated by mutation in ~10% of all acute myeloid leukemia (AML)[66]. C/EBPα expression is also diminished in

numerous tumors of various cell types[66]. C/EBPα was first identified as an inhibitor of mitotic growth in fibroblasts by the McKnight lab[120], and many further studies have confirmed this function in multiple cell types[66]. The mechanism by which C/EBPα inhibits growth arrest is not yet fully elucidated, although a number of possible mechanisms has been proposed: 1. stabilization of the Cdk inhibitor p21, 2. promotion of a shift in Rb complex constitution, from proliferative E2F-p107 complexes to resting E2F-p130 complexes, 3. direct binding to E2F and repression of E2F transcription, 4. direct binding to Cdk2 and Cdk4 and repression of their kinase activity, and 5. a requirement for interactions with the SWI/SNF chromatin remodeling complex[66]. These mechanisms suggest a primarily non-transcriptional role for C/EBPα in growth arrest. As stated previously, it is not yet determined which if any of these proposed mechanisms is responsible for the growth inhibitory role of C/EBPα.

The role for C/EBPβ in cancer is not quite as clear as that for C/EBPα. C/EBPβ$^{-/-}$ primary keratinocytes express partial resistance to calcium induced growth arrest associated with induction of differentiation and forced expression of C/EBPα or C/EBPβ inhibits the proliferation of keratinocytes in culture[64]. A tumor suppressor function for C/EBPβ has been suggested by its important role in Ras^{v12}-induced senescence involving Rb/E2F complexes. Opposing roles for C/EBPα and C/EBPβ are seen in the suppression and stimulation, respectively, of ras-induced cellular transformation[121, 122]. Ras signaling downregulates C/EBPα but not C/EBPβ expression, and co-expression of Ras and C/EBPβ

results in the synergistic activation of a C/EBP-responsive reporter reporter[121, 123]. C/EBPβ is required for transition from G1 to S phase of the cell cycle in hepatocytes induced to proliferate after partial hepatectomy, and in adipocytes clonal expansion[72]. An important piece of evidence which truly highlights the importance of C/EBPβ in cancer is that loss of C/EBPβ renders mice completely refractory to carcinogen-induced skin tumorigenesis[121]. Similar oncogenic functions of C/EBPβ can be observed in macrophages, mammary epithelial cells, uterine epithelium and stroma, B lymphocytes, and human tumors with elevated cyclin D1[72]. The oncogenic and tumor suppressive functions of C/EBPβ may allow C/EBPβ to serve as a key regulator involved in the balance between proliferation and growth inhibition.

C/EBPα and C/EBPβ may play key roles in epidermal homeostasis through the regulation of differentiation and cellular proliferation, and these functions equip C/EBPα and C/EBPβ with the ability to have both oncogenic (C/EBPβ) and tumor suppressive (C/EBPα and C/EBPβ) roles in oncogenesis.

Research Hypothesis and Objectives

C/EBPα and C/EBPβ are key regulators of cellular growth and differentiation in numerous tissues and cell types[28]. The anti-proliferative function of C/EBPα is widely established, but the implications for its growth inhibitory role in normal epidermal homeostasis are not yet known[66]. Diminished expression of C/EBPα is also associated with epithelial tumor development[124]. Causal or genetic evidence that C/EBPα can function as an epithelial tumor suppressor is lacking as C/EBPα mutations have not been detected in epithelial tumors and C/EBPα deficient mice die before or shortly after birth[69]. I therefore hypothesized that C/EBPα's anti-proliferative function is associated with normal epidermal homeostasis and that C/EBPα acts as a tumor suppressor in epithelial tissue. C/EBPβ is involved with both the inhibition and stimulation of proliferation, and C/EBPβ$^{-/-}$ mice are completely refractory to carcinogen-induced skin tumorigenesis[72, 121]. I also hypothesized then, that C/EBPα and C/EBPβ are differentially regulated and may have opposing roles in epidermal homeostasis and tumorigenesis. My objectives were to generate an epidermal specific C/EBPα$^{-/-}$ mouse to determine the effects of C/EBPα loss on epidermal homoestasis and tumorigenesis. I also sought to explore the regulation of C/EBPα and C/EBPβ in the keratinocytes by mitogenic stimulation.

CHAPTER 1

Genetic Ablation of C/EBPα in Epidermis Reveals its Role in Suppression of Epithelial Tumorigenesis

Kari D. Loomis[1,2], Songyun Zhu[1], Kyungsil Yoon[1], Peter F. Johnson[3], and Robert C. Smart[1,2]*

[1] Cell Signaling and Cancer Group, Department of Environmental & Molecular Toxicology, [2]Functional Genomics Program, North Carolina State University, Raleigh, NC 27695-7633; [3]Laboratory of Protein Dynamics and Signaling, National Cancer Institute, Frederick, MD 21702-1201

Running Title: C/EBPα is a suppressor of epithelial tumorigenesis

Key Words: Tumor Suppressor, C/EBP, Keratinocytes

Financial Support: NCI Grant # CA46637, NIEHS Grant # ES12473, GAANN Fellowship

CORRESPONDING AUTHOR:

Robert C. Smart

North Carolina State University Campus Box 7633

Raleigh, NC 27695

rcsmart@unity.ncsu.edu

PUBLISHED IN:

Cancer Res 2007; 67: (14). July 15, 2007

Abstract

C/EBPα is a b-zip transcription factor that inhibits cell cycle progression and regulates differentiation in various cell types. C/EBPα is inactivated by mutation in acute myeloid leukemia (AML) and is considered a human tumor suppressor in AML. While C/EBPα mutations have not been observed in malignancies other than AML, greatly diminished expression of C/EBPα occurs in numerous human epithelial cancers including lung, liver, endometrial, skin and breast suggesting a possible tumor suppressor function. However, direct evidence for C/EBPα as an epithelial tumor suppressor is lacking due to the absence of C/EBPα mutations in epithelial tumors and the lethal effect of C/EBPα deletion in mouse model systems. To examine the function of C/EBPα in epithelial tumor development, an epidermal-specific C/EBPα knockout mouse was generated. The epidermal-specific C/EBPα knockout mice survived and displayed no detectable abnormalities in epidermal keratinocyte proliferation, differentiation or apoptosis; demonstrating that C/EBPα is dispensable for normal epidermal homeostasis. In spite of this, the epidermal-specific C/EBPα knockout mice were highly susceptible to skin tumor development involving oncogenic Ras. These mice displayed decreased tumor latency and striking increases in tumor incidence, multiplicity, growth rate and the rate of malignant progression. Mice hemizygous for C/EBPα displayed an intermediate enhanced tumor phenotype. Our results suggest decreased expression of C/EBPα contributes to deregulation of tumor cell proliferation. C/EBPα had been proposed to block

cell cycle progression through inhibition of E2F activity. We observed that C/EBPα blocked Ras- and EGF-induced E2F activity in keratinocytes and also blocked Ras-induced cell transformation and cell cycle progression. Our study demonstrates that C/EBPα is dispensable for epidermal homeostasis and provides genetic evidence that C/EBPα is a suppressor gene in epithelial tumorigenesis.

INTRODUCTION

C/EBPα is a basic leucine zipper transcription factor and has a role in energy metabolism, differentiation and mitotic growth arrest (1). Forced expression of C/EBPα results in the inhibition of cell cycle progression in most cell types including those with activated oncogenes and inactivated tumor suppressor genes (2, 3). C/EBPα has been reported to inhibit cell proliferation through mechanisms involving; i) regulation, stabilization and activation of the CDK inhibitor p21 (4, 5), ii) direct inhibition of CDK4 and CDK2 activity (6), iii) interaction with Rb family members (7, 8), iv) interaction with and repression of E2F-mediated transcription activity (9, 10) and v) interaction with a SWI/SNF complex (11). Whether all of these possible mechanisms are operative in all cells or whether certain cells utilize a specific subset of C/EBPα inhibitory mechanisms is not known (12).

C/EBPα is inactivated through specific somatic mutations in approximately 10% of acute myeloid leukemia (AML) patients (13, 14) and these studies along with work demonstrating that C/EBPα is required for granulopoiesis in C/EBPα mutant mice (15) provide compelling evidence that C/EBPα is a tumor suppressor in AML. While C/EBPα mutations have not been observed in malignancies other than AML, loss or greatly decreased expression occurs in numerous epithelial cancers, including lung (16), skin (17, 18), liver (19), endometrial (20) and breast cancer (21). Re-expression of C/EBPα in hepatoma cell lines (22), lung cancer lines (16) or skin squamous cell

carcinomas (SCC) cell lines (18) blocks cell cycle progression. Thus, it appears that diminished expression of C/EBPα is associated with epithelial tumor development (23). However, causal or genetic evidence that C/EBPα can function as an epithelial tumor suppressor is lacking as C/EBPα mutations have not been detected in epithelial tumors and C/EBPα deficient mice die before or shortly after birth, presumably from altered hepatic glucose and glycogen metabolism (24). Conditional or tissue specific knockout of C/EBPα in a tissue/organ in which tumors derived from this tissue are known to display decreased C/EBPα expression would be an ideal model system for testing whether C/EBPα has tumor suppressor function. However, this approach has also been problematic as the lung specific loss of C/EBPα in mice results in respiratory failure at birth (25).

C/EBPα is expressed in epidermal keratinocytes of human and mouse skin (17, 26, 27). Forced expression of C/EBPα in keratinocytes inhibits cell cycle progression (28). In terms of stress responses, C/EBPα is induced in keratinocytes by a variety of DNA damaging agents and has a role in the G1/S checkpoint in response to UVB-induced DNA damage (29). C/EBPα is expressed primarily in the suprabasal layers of the epidermis where post-mitotic keratinocytes undergo differentiation, and to a lesser extent, in a subpopulation of basal keratinocytes (17, 29). The location of C/EBPα expression within the epidermis suggests that it may be involved in cell cycle exit associated with stratified squamous differentiation and/or the regulation of differentiation

specific genes. However, a role for C/EBPα in squamous differentiation remains unidentified.

The mouse skin model of multistage **chemical-induced** carcinogenesis is a well defined in vivo model of epithelial neoplasia where oncogenic Ras mutations precede p53 and INK4A/ARF mutations during tumor development and progression (30, 31). Carcinogen-induced oncogenic Ras mutation is the initial critical event responsible for the development of the squamous papilloma (32). To examine the function of C/EBPα in epithelial tumor development as well as in epidermal homeostasis, we generated an epidermal specific C/EBPα knockout mouse using the Cre-*loxP* recombination system. We observed that C/EBPα is dispensable for normal epidermal homeostasis, however, in spite of this the epidermal specific C/EBPα knockout mice are highly susceptible to Ras-induced skin tumorigenesis. Either reduced or ablated expression conferred increased susceptibility to tumorigenesis. Thus, C/EBPα functions as a haploinsufficient tumor suppressor gene in epithelial tumorigenesis and our results suggest that C/EBPα suppresses Ras-mediated tumorigenesis through repression of E2F activity.

RESULTS

C/EBPα expression is ablated in epidermis, hair follicles and sebaceous glands of K5-Cre;C/EBPα$^{fl/fl}$ mice

To achieve epidermal-specific ablation of C/EBPα, C/EBPα$^{fl/fl}$ mice (33) were crossed with K5-Cre transgenic mice in which Cre recombinase expression is directed to the epidermis and other stratified epithelia by the keratin 5 promoter (34). Immunoblot analysis of epidermal lysates prepared from wild type, C/EBPα$^{fl/fl}$, K5Cre and K5Cre;C/EBPα$^{fl/fl}$ mice was conducted (Fig 1A) . C/EBPα protein was not detectable in the epidermal lysates prepared from K5Cre;C/EBPα$^{f/fl}$ mice, while it was expressed at normal levels in the three other genotypes. To document the specificity of the ablation of C/EBPα in epidermis, we examined C/EBPα protein levels in liver, lung and fat; three tissues known to express relatively high levels of C/EBPα (Fig 1A). Immunoblot analysis revealed that C/EBPα was expressed at normal levels in all three tissues of all four genotypes. To examine the efficiency and location of Cre-induced recombination within the epidermis, we conducted immunohistochemical staining. In C/EBPα$^{fl/fl}$ mouse skin, C/EBPα staining was observed in the nuclei of interfollicular epidermal basal and suprabasal keratinocytes (Fig 1B) as well as in hair follicles and sebaceous gland cells (Fig 1C). In contrast, C/EBPα was not detected in any of the above structures in K5Cre;C/EBPα$^{fl/fl}$ mice (Fig 1B and 1C), reflecting the fact that epithelial cells of the epidermis and its appendages are all derived from a common pluripotent K5

expressing stem cell (43). Epidermal stem cells are considered to be the target precursors cells for skin tumor development (44) and our results indicate that C/EBPα is ablated in these cells.

Ablation of C/EBPα in the epidermis has no effect on epidermal homeostasis

Mice with an epidermal specific ablation of C/EBPα were born at normal Mendelian frequency. These mice did not display a visible phenotype and were grossly indistinguishable from control mice. To determine whether the loss of C/EBPα in the epidermis has any effect on epidermal homeostasis we examined epidermal keratinocyte proliferation, apoptosis and squamous differentiation. Surprisingly, K5Cre;C/EBPα$^{fl/fl}$ mice did not display any detectable alterations in epidermal keratinocyte proliferation as determined by epidermal thickness, number of nucleated cell layers (data not shown) and the number of BrdU positive S-phase cells (Fig 2A) when compared to control mice. Similarly there were no differences in the number of apoptotic keratinocytes between control and K5Cre;C/EBPα$^{fl/fl}$mice (Fig 2A). To determine whether the loss of C/EBPα expression results in alterations in epidermal stratified squamous differentiation we examined the expression of keratin 5 (K5), keratin 10 (K10), keratin 1 (K1), involucrin and loricrin. K5 is expressed in the basal layer keratinocytes while K10 and K1 are first expressed in the transition from the basal to spinous layer and involucrin and loricrin are expressed later in the differentiation program. Immunoblot analysis revealed that all of these markers

were expressed at normal levels in the absence of epidermal C/EBPα (Fig 2B). Immunohistochemical staining of the epidermis demonstrated that the spatial expression of these markers was also normal in the K5Cre;C/EBPα$^{fl/fl}$ mice (Fig 2C). Collectively, these results indicate that the ablation of C/EBPα in the epidermis does not alter epidermal keratinocyte proliferation, squamous differentiation or apoptosis, demonstrating that C/EBPα is dispensable for normal epidermal homeostasis.

C/EBPβ and p21 are up-regulated in C/EBPα deficient epidermis.

The lack of effect of C/EBPα deficiency on epidermal proliferation and differentiation was unexpected and could be due to the compensatory up-regulation of genes with similar functions. C/EBPβ is expressed in the epidermis and is involved in squamous differentiation (42, 45). Therefore, we examined C/EBPβ protein levels in K5Cre;C/EBPα$^{fl/fl}$ epidermis. As shown in Figure 2D, C/EBPβ was up-regulated ~2 fold in C/EBPα deficient epidermis compared to control epidermis. The cyclin dependent kinase inhibitor, p21, a regulator of the G1 to S phase transition in the cell cycle, was also up regulated in the K5Cre;C/EBPα$^{fl/fl}$ epidermis. Increased expression of C/EBPβ and p21 may compensate for the loss of C/EBPα and potentially mask the role of C/EBPα in keratinocyte differentiation and proliferation.

Loss of C/EBPα in the epidermis results in increased susceptibility to Ras-induced skin tumorigenesis.

The loss of C/EBPα in the epidermis and presumably in the epidermal stem cell compartment is not sufficient in itself for skin tumor development, as untreated K5Cre;C/EBPα$^{fl/fl}$ mice held for one year did not develop any skin tumors. These results indicate that additional events are required for skin tumor development. The mouse skin model of multistage carcinogenesis involves treatment of mouse skin with DMBA followed by weekly TPA treatments and results in the production of squamous papillomas, the majority of which (>95%) contain an A->T^{182} transversion in Ha-ras (32). To determine whether K5Cre;C/EBPα$^{fl/fl}$ mice have an altered susceptibility to tumorigenesis involving oncogenic Ras, we subjected mice to a DMBA/TPA two-stage carcinogenesis protocol. As shown in Fig 3A, wild type mice developed their first tumor at week 7 and at week 19 developed their maximum tumor incidence of 90% with a tumor multiplicity of approximately 10 tumors/mouse. C/EBPα$^{fl/fl}$ and K5-Cre mice displayed similar tumor latency, incidence and multiplicity as wild type mice (data not shown). In contrast, K5Cre;C/EBPα$^{fl/fl}$ mice developed their first tumor at week 4, which is nearly 50% earlier than that of wild type mice. All of K5Cre;C/EBPα$^{fl/fl}$ mice developed papillomas by week 8 and these mice developed ~40 tumors/mouse. Dramatic differences in both tumor number and size were evident in the K5Cre;C/EBPα$^{fl/fl}$ mice (Fig 3B). Mice that were hemizygous for epidermal C/EBPα (K5Cre;C/EBPα$^{fl/+}$) had reduced levels of C/EBPα in their epidermis (Fig 3C) and displayed an intermediate tumor phenotype between wild type mice and mice completely deficient in epidermal

C/EBPα (Fig 3A and B). Collectively, these results demonstrate that ablation or reduced expression of epidermal C/EBPα has a multifaceted effect on tumor development involving decreased tumor latency, increased tumor incidence and increased tumor multiplicity in DMBA/TPA-treated mice.

DMBA-induced mutation of Ha-Ras in epidermal stem cells is considered the critical event for skin tumor development (32, 44) and earlier studies demonstrated that forced expression of C/EBPα can override the proliferative effects of oncogenic Ras in skin SCC cell lines (18). Therefore, tumors of the epidermal-specific C/EBPα knockout and control mice were examined by mutation specific PCR to verify the presence of the DMBA-induced oncogenic Ras precursor tumor cell lesions (46). An A->T transversion in the 61st codon of H-Ras was present in all tumors isolated from K5Cre;C/EBPα$^{fl/fl}$ mice (Fig 3D). Collectively, these results indicate that reduced or ablated expression of epidermal C/EBPα results in increased susceptibility to Ras-induced tumorigenesis.

Premalignant tumors of K5Cre;C/EBPα$^{fl/fl}$ mice display increased growth rate and an increased rate of malignant progression

During the course of the tumor experiments, it was evident that there were striking differences in the tumor growth rate as indicated by tumor size. Grossly these tumors were identified as papillomas. Measurement of tumor diameters showed that at 14 weeks only 22% of the wild type mouse tumors were greater than 2 mm in diameter; in contrast, 71% of the K5Cre;C/EBPα$^{fl/fl}$ tumors were

greater than 2 mm in diameter (Fig 4A). The average tumor volume of K5Cre;C/EBPα$^{fl/fl}$ tumors was 5-fold greater than control tumor volume (data not shown). Similar to tumor multiplicity results, we observed that mice hemizygous for epidermal C/EBPα (K5Cre;C/EBPα$^{fl/+}$) also displayed an intermediate tumor size phenotype between wild type mice and mice deficient in epidermal C/EBPα (Fig 4A) . Tumors of K5Cre;C/EBPα$^{fl/fl}$ continued to increase in size and the tumor experiment described in Figure 3A was terminated at 25 weeks due to the large size of some papillomas/keratoacanthomas (>15 mm), as well as the presence of squamous cell carcinomas (>18 mm) in the epidermal specific C/EBPα knockout mice. In the mouse skin model, papillomas and keratoacanthomas are considered premalignant tumors which can progress to malignant squamous cell carcinomas (35). Histological analysis revealed that the tumors were a similar combination of papillomas and keratoacanthomas in both wild type and K5Cre;C/EBPα$^{fl/fl}$ mice. BrdU pulse labeling studies were conducted in vivo to examine tumor cell proliferation. Histological sections of the papillomas/keratoacanthomas revealed increased numbers of BrdU S-phase positive suprabasal cell layers in K5Cre;C/EBPα$^{fl/fl}$ tumors compared to wild type tumors (Figure 4B and C). While the increase in the number of suprabasal BrdU positive cell layers contributes to the increased growth rate of the tumors, it is also known to be associated with papillomas that have a higher probability of progression to malignancy (47). No major differences in the

number of apoptotic tumor cells between the genotypes was detected using TUNEL staining (data not shown).

Progression of papillomas/keratoacanthomas to malignant squamous carcinomas is a rare and late event (>30 weeks) and C57BL6 mice are considered to be a resistant strain. Histological examination of tumors revealed that none of the wild type, K5-Cre or C/EBPα$^{fl/fl}$ mice developed SCCs or carcinoma in situ (total of 39 control mice;13 mice/group) (Fig 4D). In contrast, 2/13 K5Cre;C/EBPα$^{fl/fl}$ mice developed SCC and both of these mice displayed two SCCs each. In addition, 3/13 of these mice displayed carcinoma in situ. One of thirteen hemizygous mice for epidermal C/EBPα (K5Cre;C/EBPα$^{fl/+}$) developed a SCC and one developed a carcinoma in situ (Fig 4D). All SCCs were highly dysplastic and displayed malignant invasion into the panniculus muscle. In another smaller tumor study containing only two genotypes (K5Cre;C/EBPα$^{fl/fl}$ and C/EBPα$^{fl/fl}$ mice N=6/group) that was carried out for 30 weeks we observed no SCCs or carcinoma in situ in C/EBPα$^{fl/fl}$ mice, while 5/6 K5Cre;C/EBPα$^{fl/fl}$ mice displayed at least one SCC or carcinoma in situ (2/6 mice developed SCC). Collectively, these findings demonstrate that reduced expression of epidermal C/EBPα results in squamous papillomas with an increased tumor growth rate and increased rate of malignant progression.

C/EBPα blocks Ras-induced transformation, E2F activity and cell cycle progression

C/EBPα has been reported to inhibit cell proliferation in some cell types through the direct repression of E2F-mediated transcription (9, 10). To determine whether C/EBPα can inhibit E2F1-mediated transcription in keratinocytes, we conducted transient transfection studies in BALB/MK2 keratinocytes using E2F1 and an E2F1 promoter/reporter (36). E2F1 is an important mediator of the G1 to S-phase transition and is autoregulated at the transcriptional level during the G1 to S transition (36). Transfection of keratinocytes with E2F1/DP1 stimulated the E2F1 promoter (Fig 5A). As shown in Figure 5A, C/EBPα inhibited the ability of E2F1/DP1 to stimulate the E2F1 promoter in a dose-dependent manner. In contrast, C/EBPβ a related member of the C/EBP family, did not inhibit the ability of E2F1/DP1 to stimulate the E2F1 promoter indicating that the inhibitory effect of C/EBPα is isoform specific. Mutational inactivation of the E2F sites in the E2F1 promoter abolished the ability of E2F1/DP1 to stimulate the promoter/reporter as well as the inhibitory activity of C/EBPα (Fig 5A). In contrast to its inhibitory action on the E2F1 promoter reporter, C/EBPα potently stimulated MGF82, a well-characterized C/EBP promoter reporter (Fig 5A). We conducted studies to determine whether C/EBPα could inhibit oncogenic Ras-induced E2F1 promoter reporter activity. As shown in Figure 5B, oncogenic Ras potently stimulated the E2F1 promoter and co-transfection of C/EBPα with Ras blocked the ability of Ras to stimulate the E2F1 promoter. Control

experiments with the E2F1 mutant construct showed diminished Ras-induced E2F activity. EGF is a potent epithelial cell mitogen, and stimulates endogenous Ras through a well characterized EGFR dependent pathway, a pathway deregulated in many epithelial tumors. As shown in Figure 5B, C/EBPα can also inhibit EGF-induced E2F1 activity. Collectively, these results indicate that C/EBPα can inhibit E2F1/DP1 as well as Ras and EGF-induced E2F activity in keratinocytes.

Next, we examined the effect of C/EBPα on Ras-induced transformation. As shown in Fig 5C, C/EBPα blocked Ras-induced transformation of NIH-3T3 cells. Similar to the Ras mutation detected in the DMBA-induced skin papillomas, BALB/MK2-Ras keratinocytes also contain endogenous oncogenic Ras with an A->T transversion in codon 61. As shown in Figure 5C, forced expression of C/EBPα blocked cell cycle progression of BALB/MK2-Ras keratinocytes as determined by a colony formation assay. Collectively these above results suggest that C/EBPα suppresses Ras-mediated tumorigenesis through repression of E2F activity.

DISCUSSION

The discovery of loss-of-function mutations in C/EBPα in human AML (13, 14) as well as seminal observations in genetically modified C/EBPα mutant mice involving hematopoiesis (10, 15) have implicated C/EBPα as a tumor suppressor in AML. So far, C/EBPα mutations have not been detected in epithelial tumors; however, decreased expression of C/EBPα has been reported in numerous human and mouse epithelial tumors (23). While decreased expression of C/EBPα is consistent with a tumor suppressor function it has not been possible to distinguish whether decreased C/EBPα expression is a cause or consequence of epithelial tumor development. Our study provides the first genetic evidence that C/EBPα has tumor suppressor activity in an epithelial tissue. Our results demonstrate that either reduced or abrogated expression of C/EBPα is permissive for Ras-induced epithelial tumorigenesis in the mouse skin tumorigenesis model. Deletion of C/EBPα in the epidermis produced a profound and multifaceted effect on carcinogen-induced tumor development, as tumor incidence, tumor multiplicity, tumor growth rate and the rate of malignant progression were all substantially increased. These results lend credence to the functional importance of the observed decreased C/EBPα expression in skin carcinomas (17, 18) and could have important implications for other epithelial cancers including liver, lung, breast and endometrial where C/EBPα expression is absent or greatly diminished (16, 18-21).

C/EBPα and epidermal homeostasis

Our results indicate that C/EBPα expression is abrogated in the epidermal stem cells of K5Cre;C/EBPα$^{fl/fl}$ mice as C/EBPα is no longer expressed in the epidermis and epidermal appendages which are all derived from the pluripotent epidermal stem cells (43). The ablation of C/EBPα in epidermis had no effect on normal epidermal homeostasis, as epidermal keratinocyte proliferation, differentiation and apoptosis were not altered. This is particularly surprising in light of the relatively high level of C/EBPα in epidermal keratinocytes (17) as well as the potent anti-mitotic effect of forced C/EBPα expression in isolated keratinocytes (28). C/EBPβ, another member of the C/EBP family, is co-expressed with C/EBPα within keratinocytes of the epidermis (17, 26). C/EBPβ has a role in the early stages of squamous differentiation and forced expression of C/EBPβ in keratinocytes blocks cell cycle progression (28). Our finding that C/EBPβ is up-regulated in the epidermis of C/EBPα deficient mice suggests that C/EBPβ may compensate for the lack of C/EBPα and thereby mask a phenotype and function of C/EBPα in epidermal homeostasis. In support of this notion are studies demonstrating that C/EBPβ can partially compensate for the loss of C/EBPα when C/EBPβ is knocked-into the C/EBPα locus (48). Future studies in our laboratory involving the generation and utilization of compound knockout of C/EBPα and C/EBPβ in the epidermis will address this important issue. Other compensatory responses in C/EBPα deficient epidermis could involve the observed up-regulation of p21 levels. p21, a cyclin dependent

kinase inhibitor and member of the Cip/Kip family, is a multifunction protein in epidermis where it has a role in the regulation of cellular proliferation and differentiation (49). Both C/EBPα and p21 inhibit cell cycle progression by inhibiting the G1 to S-phase transition. In the absence of C/EBPα, an increase in p21 may prevent a hyperproliferative epidermal phenotype and thus contribute to the apparent epidermal homeostasis in the C/EBPα mutant epidermis.

C/EBPα and Ras-induced tumor development

We observed that the loss of C/EBPα in the epidermis is not sufficient in itself for skin tumor development indicating that additional events are required for skin tumor development. The mouse skin tumorigenesis model is a well-characterized model of epithelial tumorigenesis in which DMBA-induced mutations of Ras in epidermal stem cells is a stochastic event and is considered to be the critical oncogenic lesion in the development of the premalignant squamous papilloma (30, 31, 50). Our results demonstrate that C/EBPα is a tumor suppressor and is haploinsufficent in this in vivo epithelial tumorigenesis model. All tumors examined from mice deficient in epidermal C/EBPα displayed oncogenic Ras mutations, emphasizing the underlying relevance of oncogenic Ras in the development of C/EBPα deficient tumors. The increase in tumor multiplicity (~4-fold) in mice lacking epidermal C/EBPα suggests the possibility that greater numbers of Ras tumor precursor cells were capable of clonally expanding to produce premalignant tumors. The notion that the loss of C/EBPα

augments Ras-induced clonal expansion is supported by the observed increase in tumor growth rate in C/EBPα-deficient tumors and by our results showing that C/EBPα can inhibit Ras-induced transformation of NIH3T3 cells and block Ras-induced E2F activity. Additional support comes from previous studies demonstrating that forced expression of C/EBPα inhibits cell cycle progression in cells containing activated Ras (3, 9, 18).

C/EBPα is highly induced by a variety of DNA damaging agents in keratinocytes and has a role in the G1 checkpoint in response to UVB-induced DNA damage (29). It is possible that increased tumor multiplicity in the C/EBPα epidermal specific knockout mice is due to a diminished G1 checkpoint in response to DMBA-induced DNA damage. A diminished G1 checkpoint could increase the numbers of initiated oncogenic Ras containing tumor precursor cells available for clonal expansion. Thus, C/EBPα ablation may have dual effects on the early stages of tumor development by increasing the number of initiated oncogenic Ras cells and augmenting Ras-induced clonal expansion.

C/EBPα's role in tumor growth and malignant progression

Most human cancer involves alterations in the cyclin D-Cdk4,6/IN4A/Rb/E2F pathway. Perturbation of the "Rb" pathway results in uncontrolled cell proliferation and often involves the functional inactivation of Rb by phosphorylation due to either the activation of Ras, overexpression of D cyclins or CDKs, or inactivation of Ink4a (51). Significantly, C/EBPα has been proposed to inhibit cell cycle progression through its interaction with several

proteins in this critical pathway including p21(4), Cdk4 (6), members of the Rb family (7) and E2F proteins (9). The repression of E2F activity by C/EBPα has been shown to be important in the inhibition of cell proliferation in isolated cells (9) as well as in vivo as mice expressing mutant forms of C/EBPα defective in the repression of E2F display abnormalities in cell proliferation and differentiation (10). Our finding that C/EBPα can inhibit oncogenic Ras-induced E2F activity in keratinocytes is consistent with the E2F repression model, although it does not rule out other possibilities. E2F has been shown to cooperate with Ras to induce transformation of MEFs (52) and various E2Fs cooperate with Ras in epithelial tumorigenesis (53, 54). Moreover, cyclin D1 or CDK4 deficiency results in decreased Ras-induced tumorigenesis (55, 56) while increased CDK4 activity increases tumor susceptibility (57). K5-CDK4 transgenic mice display a similar tumor phenotype to C/EBPα epidermal specific knock out mice as these mice are susceptible to carcinogen-induced skin tumorigenesis involving Ras and display increased tumor size, increased numbers of BrdU positive tumors cells as well as increased malignant progression (57). Our results suggest that the loss of C/EBPα cooperates with oncogenic Ras to contribute to the dysregulation of the “Rb” pathway via derepression of E2F, resulting in an increased tumor growth rate in C/EBPα-deficient tumors. In the mouse skin model, papillomas are considered premalignant lesions which progress toward SCC formation at different rates (35). The increased proliferative rate in C/EBPα deficient premalignant lesions

coupled with a diminished C/EBPα-regulated G1 checkpoint response would likely contribute to the acquisition of additional mutations and enhance malignant progression.

It is informative to compare the tumor phenotypes of epidermal-specific C/EBPα knockout mice to C/EBPβ knockout mice (42). While C/EBPα and C/EBPβ are 90% similar in their bZIP domain and are considered to bind the same DNA consensus sequence (1), they have opposite effects on skin tumor development. C/EBPβ knockout mice are completely refractory to skin tumorigenesis involving Ras and our previous studies indicate that C/EBPβ can cooperate with Ras to induce transformation (42, 58). Thus, it is possible that increased expression of C/EBPβ in C/EBPα deficient epidermis contributes to the enhanced tumor phenotype observed in C/EBPα deficient mice. Similarly, C/EBPβ deficiency results in C/EBPα being the predominant form of C/EBP and this may contribute to the observed resistance to skin tumorigenesis in C/EBPβ knockout mice. Thus, it appears that these two family members have a yin-yang relationship in tumorigenesis such that removing one member disrupts the balance and has profound effects on the activity of the other.

We observed that reduced or abrogated expression of C/EBPα in epidermis has a profound effect on many aspects of tumor development but has no effect on normal epidermal differentiation and proliferation. These results are in contrast to AML where loss of C/EBPα function results in a block in the differentiation of granulocytic blasts and this is considered a critical event in

expansion of the myeloid precursor population (59). Our findings suggest that the loss of C/EBPα contributes to epidermal tumorigenesis through a mechanism that results in the deregulation of tumor cell proliferation independent of an effect on cellular differentiation. In summary, our results provide genetic evidence that C/EBPα is a tumor suppressor gene in epithelial tumorigenesis and suggest that C/EBPα suppresses Ras-mediated tumorigenesis through repression of E2F activity. Reduced C/EBPα expression in some epithelial tumors appears to be the result of promoter hypermethylation (60) and negative regulation via Ras signaling (18). These epigenetic mechanisms may provide novel therapeutic opportunities for suppression of tumor progression through drug-targeted reconstitution of C/EBPα expression in cancers depleted of C/EBPα.

Materials and Methods

Cell culture

BALB/MK2 and BALB/MK2-Ras keratinocytes were cultured as described (18). For luciferase experiments involving Ras and the addition or omission of EGF, cells were placed in medium deprived of growth factors (0.1% FBS, no EGF, and 0.05mM $CaCl_2$).

Mice

To achieve the epidermal-specific ablation, C/EBPα$^{fl/fl}$ mice (C57BL/6;129/SV) (33) were crossed with K5Cre transgenic mice (C57BL/6;DBA), in which Cre recombinase expression is directed to the epidermis by the keratin 5 promoter (34). F1 K5Cre;C/EBPα$^{fl/+}$ mice were crossed with C/EBPα$^{fl/+}$ littermates to produce the 5 genotypes used in all experiments. C/EBPα$^{fl/fl}$ and K5Cre mice were genotyped by PCR as described (33, 34).

Immunoblot Analysis

Immunoblot analysis was conduced as described (18) using the following antibodies; C/EBPα sc-61 (1:2000), C/EBPβ sc-150 (1:2500), or p21 sc-471 (1:600) rabbit polyclonal antibodies (sc-# antibodies from SantaCruz) followed by HRP-linked donkey anti-rabbit immunoglobulin (1:2500) from Amersham (NA934V). Immunoblot analysis for detection of differentiation markers was performed by incubation with involucrin (Covance PRB-140C), loricrin (Covance PRB-145P), keratin 5 (Covance PRB-160P), or keratin 10 (Covance PRB-159P)

rabbit polyclonal antibodies at a 1:2000 dilution followed by anti-rabbit secondary antibody at 1:2500.

Cell Proliferation and Apoptosis

Mice were injected with BrdU (100mg/kg body weight) then killed 1 hour later and immunohistochemical staining was performed as described (17). Apoptotic keratinocytes in the interfollicular basal epidermis were scored in hematoxylin and eosin stained sections and scored positive if all three of the following criteria were present: dark pyknotic nuclei, cytoplasmic eosinophilia, and absence of cellular contacts.

Tumor Experiments

Wild type, C/EBPα$^{fl/fl}$, K5Cre, K5Cre;C/EBPα$^{fl/+}$, and K5Cre;C/EBPα$^{fl/fl}$ mouse littermates (6–9 weeks old; 13 mice/group) were treated with a single application of 200 nmol DMBA (Acros) followed 1 week later with thrice weekly treatment of 5 nmol of tetradecanoyl-phorbol-13-acetate (TPA)(LC Laboratories). All agents were applied in 200ul of acetone. Mice were killed 25 weeks after start of TPA promotion and tumors were harvested for histological analysis and/or DNA isolation. Two additional tumor experiments were conducted utilizing only C/EBPα$^{fl/fl}$ and K5Cre;C/EBPα$^{fl/fl}$ genotypes.

Immunohistochemical Staining

Mouse skins and/or tumors were fixed in 10% neutral buffered formalin phosphate for 24 hours and embedded in paraffin. Tissue sections (5um) were

subjected to hematoxylin and eosin staining or specific immunohistochemistry as described (17, 18, 28)

Tumor Pathology

Squamous carcinomas were identified histologically as described (35) and confirmed by veterinary pathologists. Squamous carcinomas were identified based on the following criteria: severely dysplastic to anaplastic growth, marked atypia in all cell layers, lack of differentiation patterns, and most importantly invasion through the muscle layer. Tumors that exhibited these characteristics but that did not penetrate through the muscle layer were classified as carcinomas *in situ*.

Reporter Assays

BALB/MK2 keratinocytes at 25–40% confluence were transfected in 12 well plates using TransFast Transfection Reagent (Promega). Cells were transfected in serum free medium with 200ng E2F1 promoter reporter construct or E2F mutant promoter reporter (Masa-Aki Ikeda) (36) with or without the following constructs: E2F1 in pcDNA1 (37), DP1 in pCMV (38), rat C/EBPα (39) or C/EBPβ (28) in pcDNA3.1, or Ha-Ras (12V) in pcDNA3 (40). The total amount of DNA among all groups was kept constant by using empty pcDNA3.1 (promega). For the C/EBP-responsive promoter reporter assays, cells were transfected similarly as above with 200ng MGF82 promoter-reporter construct (41) with or without 100ng of C/EBPα or C/EBPβ.

Colony Formation Assay and NIH 3T3 Focus Assay

BALB/MK2-Ras cells were transfected and 48 hours later the cells were trypsinized and replated at $5x10^5$ cells per p60 dish in selection medium containing 300ug/ml G418. NIH 3T3 focus assay was conducted as described (42).

ACKNOWLEDGEMENTS

The authors would like to thank Drs. David Malarkey and Mac Law for their advice and histological analysis of the tumors. This work was supported by grants from the NCI (CA46637) and from the NIEHS (ES12473). Support was also granted in part by the GAANN Fellowship.

References

1. Ramji, D. P. and Foka, P. CCAAT/enhancer-binding proteins: structure, function and regulation. Biochem J 2002;365:561-575.
2. Umek, R. M., Friedman, A. D., and McKnight, S. L. CCAAT-enhancer binding protein: A component of a differentiation switch. Science 1991;251:288-292.
3. Hendricks-Taylor, L. R. and Darlington, G. J. Inhibition of cell proliferation by C/EBP alpha occurs in many cell types, does not require the presence of p53 or Rb, and is not affected by large T-antigen. Nucleic Acids Res 1995;23:4726-4733.
4. Timchenko, N. A., Wilde, M., Nakanishi, M., Smith, J. R., and Darlington, G. J. CCAAT/enhancer-binding protein alpha (C/EBPalpha) inhibits cell proliferation through the p21 (WAF-1/CIP-1/SDI-1) protein. Genes & Dev 1996;10:804-815.
5. Harris, T. E., Albrecht, J. H., Nakanishi, M., and Darlington, G. J. CCAAT/enhancer-binding protein-alpha cooperates with p21 to inhibit cyclin-dependent kinase-2 activity and induces growth arrest independent of DNA binding. J Biol Chem 2001;276:29200-29209.
6. Wang, H., Iakova, P., Wilde, M., Welm, A., Goode, T., Roesler, W. J., and Timchenko, N. A. C/EBPalpha arrests cell proliferation through direct inhibition of Cdk2 and Cdk4. Mol Cell 2001;8:817-828.

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

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

9. Slomiany, B. A., D'Arigo, K. L., Kelly, M. M., and Kurtz, D. T. C/EBPalpha inhibits cell growth via direct repression of E2F-DP-mediated transcription. Mol Cell Biol 2000;20:5986-5997.

10. Porse, B. T., Pedersen, T. A., Xu, X., Lindberg, B., Wewer, U. M., Friis-Hansen, L., and Nerlov, C. E2F repression by C/EBPalpha is required for adipogenesis and granulopoiesis in vivo. Cell 2001;107:247-258.

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

12. Johnson, P. F. Molecular stop signs: regulation of cell-cycle arrest by C/EBP transcription factors. J Cell Sci 2005;118:2545-2555.

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

14. Gombart, A. F., Hofmann, W. K., Kawano, S., Takeuchi, S., Krug, U., Kwok, S. H., Larsen, R. J., Asou, H., Miller, C. W., Hoelzer, D., and Koeffler, H. P. Mutations in the gene encoding the transcription factor CCAAT/enhancer binding protein alpha in myelodysplastic syndromes and acute myeloid leukemias. Blood 2002;99:1332-1340.

15. Zhang, D. E., Zhang, P., Wang, N. D., Hetherington, C. J., Darlington, G. J., and Tenen, D. G. Absence of granulocyte colony-stimulating factor signaling and neutrophil development in CCAAT enhancer binding protein alpha-deficient mice. Proc Natl Acad Sci U S A 1997;94:569-574.

16. Halmos, B., Huettner, C. S., Kocher, O., Ferenczi, K., Karp, D. D., and Tenen, D. G. Down-regulation and antiproliferative role of C/EBPalpha in lung cancer. Cancer Res 2002;62:528-534.

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

18. Shim, M., Powers, K. L., Ewing, S. J., Zhu, S., and Smart, R. C. Diminished expression of C/EBPalpha in skin carcinomas is linked to oncogenic Ras and reexpression of C/EBPalpha in carcinoma cells inhibits proliferation. Cancer Res 2005;65:861-867.

19. Xu, L., Hui, L., Wang, S., Gong, J., Jin, Y., Wang, Y., Ji, Y., Wu, X., Han, Z., and Hu, G. Expression profiling suggested a regulatory role of liver-

enriched transcription factors in human hepatocellular carcinoma. Cancer Res 2001;61:3176-3181.

20. Takai, N., Kawamata, N., Walsh, C. S., Gery, S., Desmond, J. C., Whittaker, S., Said, J. W., Popoviciu, L. M., Jones, P. A., Miyakawa, I., and Koeffler, H. P. Discovery of epigenetically masked tumor suppressor genes in endometrial cancer. Mol Cancer Res 2005;3:261-269.

21. Gery, S., Tanosaki, S., Bose, S., Bose, N., Vadgama, J., and Koeffler, H. P. Down-regulation and growth inhibitory role of C/EBPalpha in breast cancer. Clin Cancer Res 2005;11:3184-3190.

22. Watkins, P. J., Condreay, J. P., Huber, B. E., Jacobs, S. J., and Adams, D. J. Impaired proliferation and tumorigenicity induced by CCAAT/enhancer-binding protein. Cancer Res 1996;56:1063-1067.

23. Schuster, M. B. and Porse, B. T. C/EBPalpha: A tumour suppressor in multiple tissues? Biochim Biophys Acta 2006.

24. Wang, N. D., Finegold, M. J., Bradley, A., Ou, C. N., Abdelsayed, S. V., Wilde, M. D., Taylor, L. R., Wilson, D. R., and Darlington, G. J. Impaired energy homeostasis in C/EBP alpha knockout mice. Science 1995;269:1108-1112.

25. Basseres, D. S., Levantini, E., Ji, H., Monti, S., Elf, S., Dayaram, T., Fenyus, M., Kocher, O., Golub, T., Wong, K. K., Halmos, B., and Tenen, D. G. Respiratory failure due to differentiation arrest and expansion of

alveolar cells following lung-specific loss of the transcription factor C/EBPalpha in mice. Mol Cell Biol 2006;26:1109-1123.

26. Maytin, E. V. and Habener, J. F. Transcription factors C/EBPa, C/EBPb, and CHOP (Gadd153) expressed during the differentiation program of keratinocytes in vivo and in vitro. J. Invest. Dermatol. 1998;110:238-246.

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

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

29. Yoon, K. and Smart, R. C. C/EBPalpha is a DNA damage-inducible p53-regulated mediator of the G1 checkpoint in keratinocytes. Mol Cell Biol 2004;24:10650-10660.

30. DiGiovanni, J. Multistage carcinogenesis in mouse skin. Pharmacol Ther 1992;54:63-128.

31. Balmain, A. and Harris, C. C. Carcinogenesis in mouse and human cells: parallels and paradoxes. Carcinogenesis 2000;21:371-377.

32. Quintanilla, M., Brown, K., Ramsden, M., and Balmain, A. Carcinogen specific mutation and amplification of Ha-ras during mouse skin carcinogenesis. Nature 1986;322:78-80.

33. Lee, Y. H., Sauer, B., Johnson, P. F., and Gonzalez, F. J. Disruption of the c/ebp alpha gene in adult mouse liver. Mol Cell Biol 1997;17:6014-6022.

34. Ramirez, A., Page, A., Gandarillas, A., Zanet, J., Pibre, S., Vidal, M., Tusell, L., Genesca, A., Whitaker, D. A., Melton, D. W., and Jorcano, J. L. A keratin K5Cre transgenic line appropriate for tissue-specific or generalized Cre-mediated recombination. Genesis 2004;39:52-57.

35. Aldaz, C. M., Conti, C. J., Klein-Szanto, A. J., and Slaga, T. J. Progressive dysplasia and aneuploidy are hallmarks of mouse skin papillomas: relevance to malignancy. Proc Natl Acad Sci U S A 1987;84:2029-2032.

36. Johnson, D. G., Ohtani, K., and Nevins, J. R. Autoregulatory control of E2F1 expression in response to positive and negative regulators of cell cycle progression. Genes Dev 1994;8:1514-1525.

37. Cress, W. D., Johnson, D. G., and Nevins, J. R. A genetic analysis of the E2F1 gene distinguishes regulation by Rb, p107, and adenovirus E4. Mol Cell Biol 1993;13:6314-6325.

38. Helin, K., Wu, C. L., Fattaey, A. R., Lees, J. A., Dynlacht, B. D., Ngwu, C., and Harlow, E. Heterodimerization of the transcription factors E2F-1 and DP-1 leads to cooperative trans-activation. Genes Dev 1993;7:1850-1861.

39. Williams, S. C., Cantwell, C. A., and Johnson, P. F. A family of C/EBP-related proteins capable of forming covalently linked leucine zipper dimers in vitro. Genes Dev 1991;5:1553-1567.

40. Der, C. J., Finkel, T., and Cooper, G. M. Biological and biochemical properties of human rasH genes mutated at codon 61. Cell 1986;44:167-176.

41. Sterneck, E., Muller, C., Katz, S., and Leuz, A. Autocrine growth induced by kinase type oncogenes in myeloid cells requires AP-1 and NF-M, a myeloid specific, C/EBP-like factor. EMBO. J 1992;11:115-126.

42. Zhu, S., Yoon, K., Sterneck, E., Johnson, P. F., and Smart, R. C. CCAAT/enhancer binding protein-beta is a mediator of keratinocyte survival and skin tumorigenesis involving oncogenic Ras signaling. Proc Natl Acad Sci U S A 2002;99:207-212.

43. Taylor, G., Lehrer, M. S., Jensen, P. J., Sun, T. T., and Lavker, R. M. Involvement of follicular stem cells in forming not only the follicle but also the epidermis. Cell 2000;102:451-461.

44. Owens, D. M. and Watt, F. M. Contribution of stem cells and differentiated cells to epidermal tumours. Nat Rev Cancer 2003;3:444-451.

45. Maytin, E. V., Lin, J. C., Krishnamurthy, R., Batchvarova, N., Ron, D., Mitchell, P. J., and Habener, J. F. Keratin 10 gene expression during

differentiation of mouse epidermis requires transcription factors C/EBP and AP-2. Dev Biol 1999;216:164-181.

46. Nelson, M. A., Futscher, B. W., Kinsella, T., Wymer, J., and Bowden, G. T. Detection of mutant Ha-ras genes in chemically initiated mouse skin epidermis before the development of benign tumors. Proc Natl Acad Sci U S A 1992;89:6398-6402.

47. Tennenbaun, T., Weiner, A. K., Belanger, A. J., Glick, A. B., Hennings, H., and Yuspa, S. H. The suprabasal expression of alpha6beta4 integrin is associated with a high risk for malignant progression in mouse skin carcinogenesis. Cancer Res 1993;53:4803-4810.

48. Chen, S. S., Chen, J. F., Johnson, P. F., Muppala, V., and Lee, Y. H. C/EBPbeta, when expressed from the C/ebpalpha gene locus, can functionally replace C/EBPalpha in liver but not in adipose tissue. Mol Cell Biol 2000;20:7292-7299.

49. DiCunto, F., Topley, G., Calautti, E., Hsiao, J., Ong, L., Seth, P. K., and Dotto, G. P. Inhibitory function of p21Cip1/WAF1in differentiation of primary mouse keratinocytes independent of cell cycle control. Science 1998;280:1069-1072.

50. Yuspa, S. H. The pathogenesis of squamous cell cancer: lessons learned from studies of skin carcinogenesis--thirty-third G. H. A. Clowes Memorial Award Lecture. Cancer Res 1994;54:1178-1189.

51. Malumbres, M. and Barbacid, M. To cycle or not to cycle: a critical decision in cancer. Nat Rev Cancer 2001;1:222-231.

52. Johnson, D. G., Cress, W. D., Jakoi, L., and Nevins, J. R. Oncogenic capacity of the E2F1 gene. Proc Natl Acad Sci U S A 1994;91:12823-12827.

53. Pierce, A. M., Fisher, S. M., Conti, C. J., and Johnson, D. G. Deregulated expression of E2F1 induces hyperplasia and cooperates with ras in skin tumor development. Oncogene 1998;16:1267-1276.

54. Paulson, Q. X., McArthur, M. J., and Johnson, D. G. E2F3a stimulates proliferation, p53-independent apoptosis and carcinogenesis in a transgenic mouse model. Cell Cycle 2006;5:184-190.

55. Yu, Q., Geng, Y., and Sicinski, P. Specific protection against breast cancers by cyclin D1 ablation. Nature 2001;411:1017-1021.

56. Rodriguez-Puebla, M. L., Miliani de Marval, P. L., LaCava, M., Moons, D. S., Kiyokawa, H., and Conti, C. J. Cdk4 deficiency inhibits skin tumor development but does not affect normal keratinocyte proliferation. Am J Pathol 2002;161:405-411.

57. Miliani de Marval, P. L., Macias, E., Conti, C. J., and Rodriguez-Puebla, M. L. Enhanced malignant tumorigenesis in Cdk4 transgenic mice. Oncogene 2004;23:1863-1873.

58. Sterneck, E., Zhu, S., Ramirez, A., Jorcano, J. L., and Smart, R. C. Conditional ablation of C/EBPbeta demonstrates its keratinocyte-specific

requirement for cell survival and mouse skin tumorigenesis. Oncogene 2005.

59. Nerlov, C. C/EBPalpha mutations in acute myeloid leukaemias. Nat Rev Cancer 2004;4:394-400.

60. Tada, Y., Brena, R. M., Hackanson, B., Morrison, C., Otterson, G. A., and Plass, C. Epigenetic modulation of tumor suppressor CCAAT/enhancer binding protein alpha activity in lung cancer. J Natl Cancer Inst 2006;98:396-406.

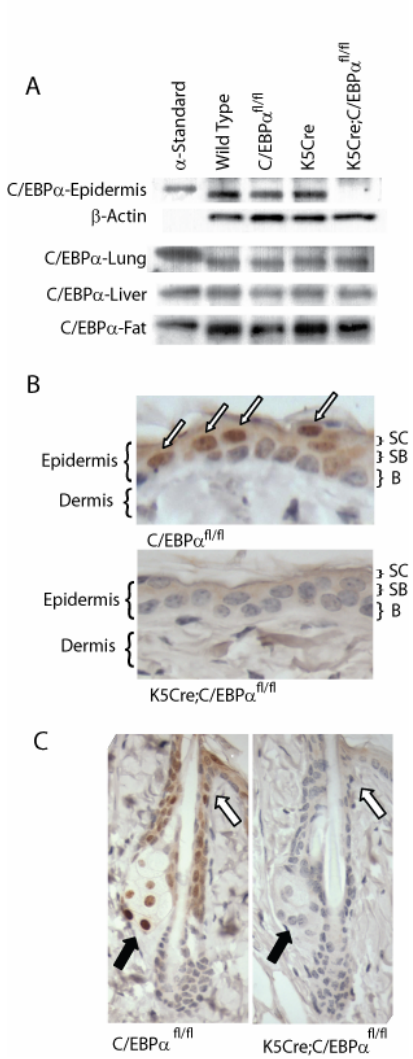


Figure 1. C/EBPα is not expressed in the epidermis, hair follicle, and sebaceous gland of K5Cre/C/EBPα$^{fl/fl}$ mice. *A*, immunoblot analysis of C/EBPα. *B*, immunohistochemical staining for C/EBPα in epidermis. *SC*, stratum corneum; *SB*, suprabasal layer; *B*, basal layer. *Arrows*, nuclear C/EBPα staining. *C*, immunohistochemical staining for C/EBPα. *Black arrows*, sebaceous glands; *White arrows*, infundibulum area of the hair follicle.

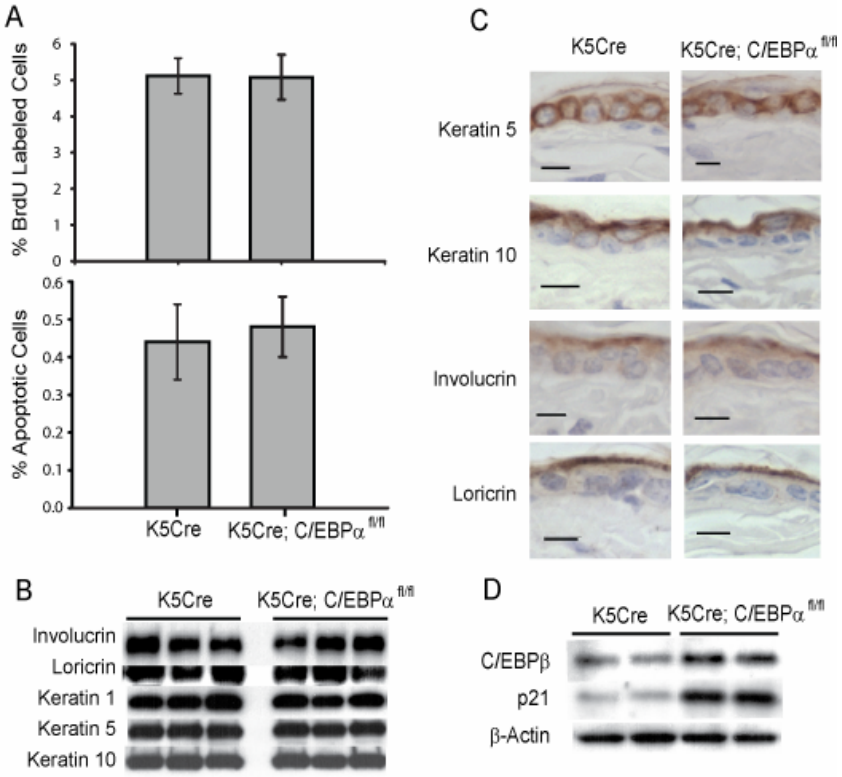


Figure 2. C/EBPα is dispensable for normal epidermal homeostasis. *A,* percentage BrdUrd-positive S-phase keratinocytes (*top*) and percentage apoptotic keratinocytes in the interfollicular basal epidermis (*bottom*). *Columns*, mean (n = 5 mice/group); *bars,* SE. *B,* immunoblot analysis of various markers of differentiation. *C,* immunostaining for various markers of squamous differentiation. *Bar*, 10 μm. *D*, immunoblot analysis of epidermal C/EBPβ and p21.

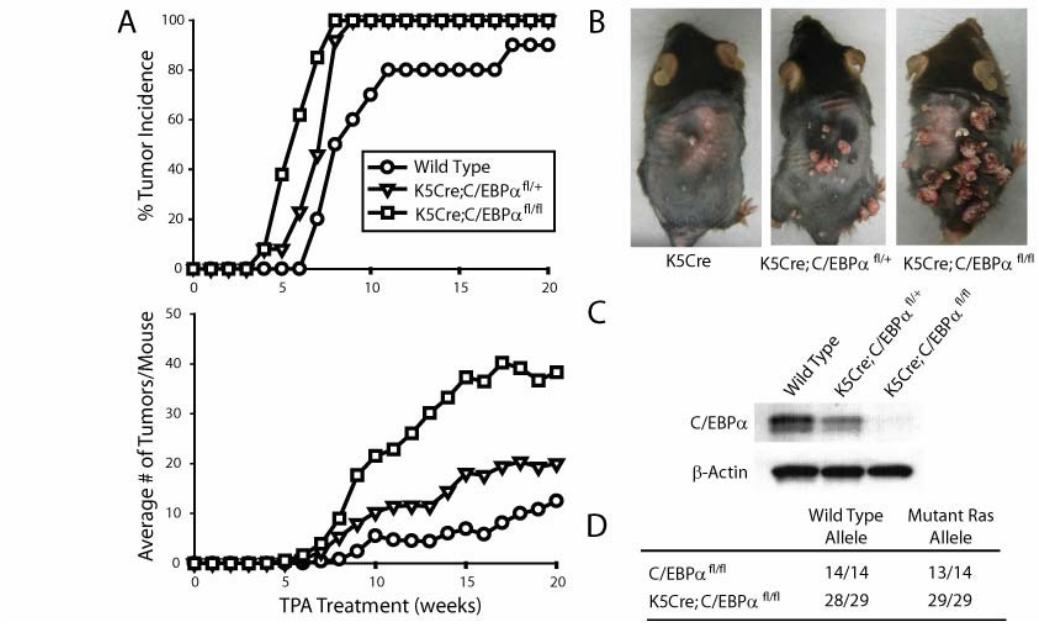
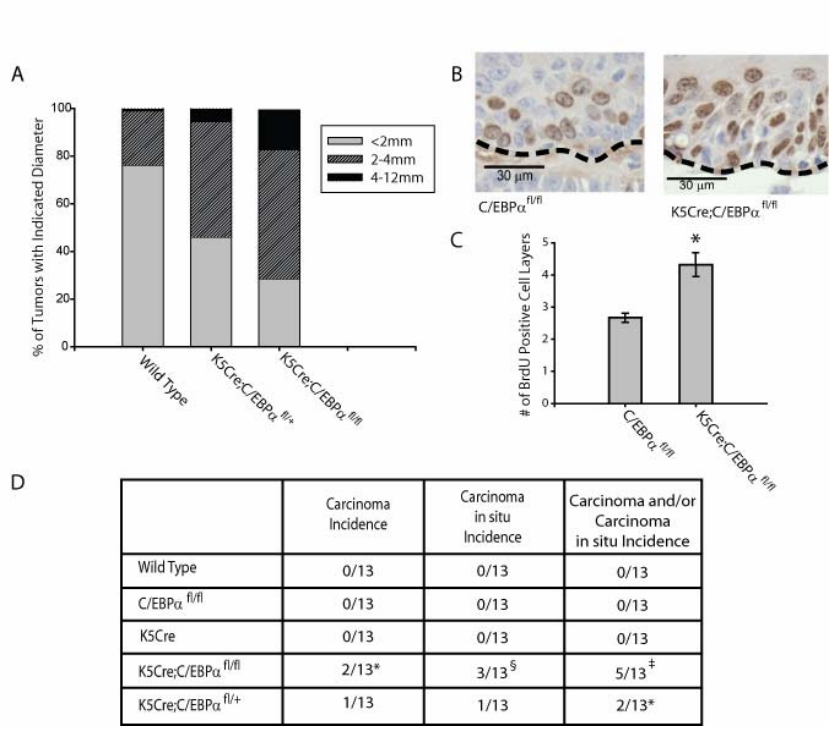


Figure 3. K5Cre;C/EBPα$^{fl/fl}$ mice are more susceptible to carcinogen-induced skin tumor development involving oncogenic Ras. *A,* tumor incidence and multiplicity (*n* = 13 mice/group). *B,* representative appearance of mice at 14 wks. *C,* immunoblot analysis of epidermal C/EBPα. *D,* activating Ras mutations were identified in codon 61 (CAA→CTA).



D

	Carcinoma Incidence	Carcinoma in situ Incidence	Carcinoma and/or Carcinoma in situ Incidence
Wild Type	0/13	0/13	0/13
C/EBPα $^{fl/fl}$	0/13	0/13	0/13
K5Cre	0/13	0/13	0/13
K5Cre;C/EBPα $^{fl/fl}$	2/13*	3/13 §	5/13 ‡
K5Cre;C/EBPα $^{fl/+}$	1/13	1/13	2/13*

Figure 4. K5Cre;C/EBPα$^{fl/fl}$ mice display a significant increase in tumor growth rate and the rate of malignant progression. *A,* tumor diameter at 14 wks of TPA promotion. *B,* immunohistochemical staining for BrdUrd in tumors harvested 25 wks after start of TPA promotion. The BrdUrd-positive cells are represented by the dark staining nuclei. *C,* number of BrdUrd-positive cell layers in tumors. Tumors were matched in size between genotypes and harvested 25 wks after start of TPA promotion. Forty fields of view per tumor (12 tumors per genotype) were analyzed. *Bars*, SE. *, P<0.01, Student's *t* test. *D*, chart representing the carcinoma and carcinoma *in situ* incidence at 25 wks after TPA promotion. *, P=0.059 for K5Cre;C/EBPα$^{fl/fl}$ mice vs control mice; §, P=0.013 for K5Cre;C/EBPα$^{fl/fl}$ mice versus wild-type mice; ‡, P=0.003 for K5Cre;C/EBPα$^{fl/fl}$ mice versus wild-type mice (Fisher's exact test).

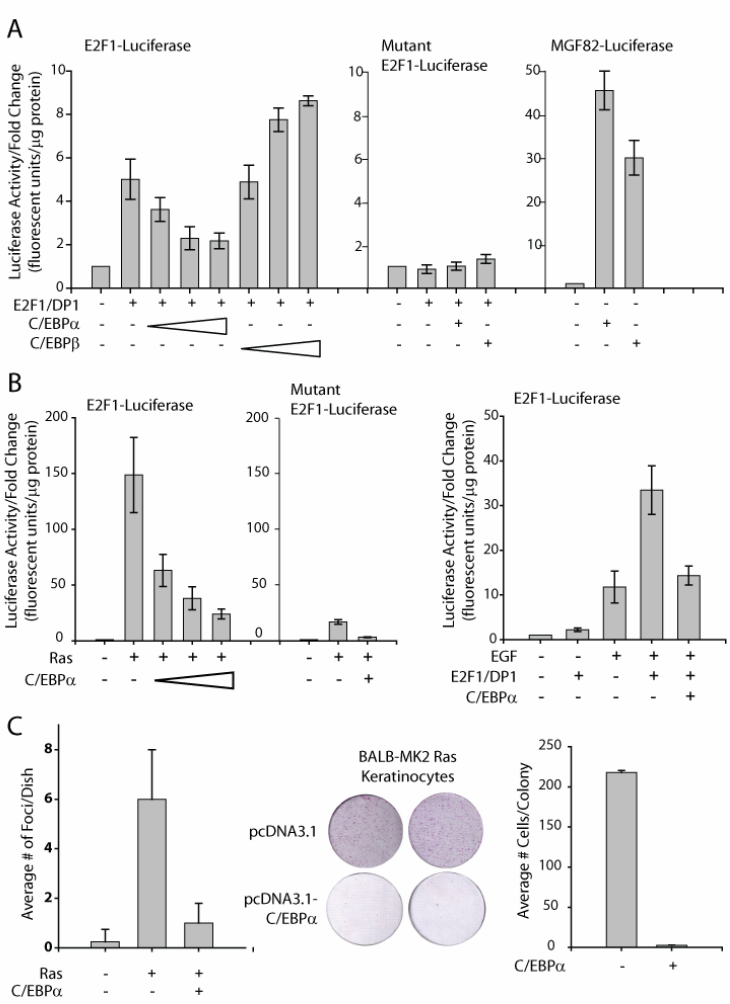


Figure 5. C/EBPα inhibits Ras-induced E2F transcription activity, transformation, and cell cycle progression. *A*, reporter assay using the E2F1 promoter/reporter (left), E2F1 promoter reporter with mutant E2F sites (middle), or MGF82, a C/EBP-responsive promoter reporter (right). BALB/MK2 cells were transfected with 5 ng E2F1 and 5 ng DP1. Increasing amounts of C/EBPα or C/EBPβ were transfected (10, 30, and 100 ng) or 100 ng when one amount was used. *B*, reporter assay using E2F1 promoter reporter and cotransfection with 5 ng Ras (left) or with EGF treatment (right). Following transfection, cells were maintained in growth factor–depleted medium for 24 h. For EGF studies, 4 ng/mL EGF was added to cells in growth factor/serum-deprived medium following the 24 h in growth-factor depleted medium and cells were harvested 16 h after the addition of EGF. All luciferase reporter assays done in triplicate. Data are representative of at least three independent experiments. *Bars*, SD. *C*, left, C/EBPα inhibits Ras-induced transformation of NIH3T3 cells. NIH-3T3 cells were transfected with 10 µg Ras and 5 µg C/EBPα as indicated. *Columns,* average of four dishes per group; *bars*, SD. Middle and right, C/EBPα inhibits proliferation of BALB/MK2-Ras keratinocytes. Keratinocytes were transfected with 2 µg empty pcDNA3.1 or C/EBPα. Cells were fixed and stained with crystal violet at 7 d after start of G418 selection.

CHAPTER 2

Stimulation with EGF induces reciprocal regulation of C/EBPα and C/EBPβ to specifically activate C/EBPβ and enhance E2F-driven cell cycle progression

Kari D. Loomis[1,2], Songyun Zhu[1], Kristina L. Powers[1],
and Robert C. Smart[1,2]*

[1] Cell Signaling and Cancer Group, Department of Environmental & Molecular Toxicology, [2]Functional Genomics Program, North Carolina State University, Raleigh, NC 27695-7633

*Corresponding author - rcsmart@ncsu.edu
Phone- (919)515-7245
Fax- (919)515-7169

Running Title: Reciprocal Regulation of C/EBPα and C/EBPβ

Abstract

C/EBPα and C/EBPβ are important regulators of epidermal homeostasis and skin tumorigenesis. We sought to determine the regulation and function of C/EBPα and C/EBPβ in response to EGF treatment and EGF-induced keratinocyte proliferation. C/EBPα and C/EBPβ are the major C/EBP isoforms expressed in keratinocytes and stimulation of keratinocytes with EGF resulted in the activation of endogenous C/EBPs as determined by an increase in a C/EBP responsive promoter reporter. EGF treatment of cells either overexpressing C/EBPα or C/EBPβ resulted in the activation of both isoforms, indicating a lack of specificity of EGF on C/EBP activation. However, EGF preferentially activates endogenous C/EBPβ in part through the up-regulation of C/EBPβ mRNA and protein and the down regulation of C/EBPα mRNA and protein, and also through the MEK/ERK dependent phosphorylation of C/EBPβ at Thr^{188}. EGF induced reciprocal regulation of C/EBPα and C/EBPβ coincided with S-phase entry, and C/EBPβ$^{-/-}$ keratinocytes exhibit a diminished rate of progression from G_1 to S-phase of the cell cycle. C/EBPβ stimulates E2F1 transcriptional activation through an EGFR and Ras dependent pathway, and loss of C/EBPβ results in diminished expression of numerous E2F target proteins. These data demonstrate that EGFR signaling diminishes the anti-proliferative presence of C/EBPα while preferentially upregulating and activating C/EBPβ to promote E2F-induced cell cycle entry.

Keywords: C/EBP, Cell Cycle, EGFR, Keratinocytes, E2F

Introduction

C/EBPα and C/EBPβ are key regulators of cellular growth and differentiation in numerous tissues and cell types[1]. The anti-proliferative function of C/EBPα is widely established, but its exact mechanism of action is not yet fully elucidated although numerous mechanisms have been proposed[2]. C/EBPβ is involved with both the inhibition and stimulation of proliferation[3]. In the epidermis, C/EBPα and C/EBPβ are expressed at high levels in the postmitotic suprabasal layer, and C/EBPβ is specifically expressed in virtually all basal cells whereas C/EBPα is expressed in select basal cell populations[4, 5]. These expression patterns suggest a role for C/EBPα and C/EBPβ in the development and maintenance of an anti-proliferative/differentiated state, as well as a potential role for C/EBPβ in the proliferative basal layer. Genetic ablation of C/EBPα from mouse epidermis has no apparent effect on epidermal proliferation or differentiation in vivo[6], while ablation of C/EBPβ results in epidermal hyperplasia, decreased expression of keratin 1 and keratin 10, and an unkempt hair phenotype[7]. Further, C/EBPβ$^{-/-}$ primary keratinocytes are partially resistant to calcium induced growth arrest associated with induction of differentiation[7]. UVB irradiation induces both C/EBPα and C/EBPβ expression, as well as C/EBPα induced cell cycle arrest[8]. These data are consistent with a similar role for C/EBPα and C/EBPβ in epidermal growth suppression, but also suggest a potential role for C/EBPβ in the proliferation of basal keratinocytes.

Divergent phenotypic responses to loss of C/EBPα or C/EBPβ in Ras-induced epithelial tumorigenesis allow further insight to the roles of these transcription factors in the epidermis. Loss of C/EBPα results in increased carcinogen-induced tumor multiplicity, decreased tumor latency, increased tumor growth rate, and accelerated tumor progression to malignancy[6]. Loss of C/EBPβ, on the other hand, renders mice completely refractory to carcinogen-induced skin tumorigenesis[9]. These tumor studies both involved oncogenic Ras, and C/EBPα and C/EBPβ have opposing roles in the suppression and stimulation, respectively, of Ras-induced NIH-3T3 cellular transformation[6, 9]. Ras signaling also downregulates C/EBPα but not C/EBPβ expression, and co-expression of Ras and C/EBPβ results in the synergistic activation of a C/EBP-responsive reporter[9, 10].

Epidermal keratinocytes are directed to proliferate through a number of signaling mechanisms, including activation of the ErbB family of receptor tyrosine kinases. ErbB1, or epidermal growth factor receptor (EGFR), is expressed in the epidermal keratinocytes, as are ErbB2 and ErbB3. Epidermal EGFR dimerization is stimulated by members of the EGF family of peptide growth factors including epidermal growth factor (EGF), transforming growth factor-α (TGFα), and amphiregulin (AR). EGFR homodimerization or EGFR/ErbB2 heterodimerization results in activation of each receptor's intrinsic tyrosine kinase activity. EGFR and ErbB2 play critical roles in proliferation, differentiation, and survival through activation of key regulatory pathways

including the Ras/MAP kinase, JAK/STAT, and PI3K/AKT pathways[11]. EGFR and ErbB2 are also important in the development of many epithelial cancers, including SCCs of the skin[12].

In the current study, we sought to determine the regulation and function of C/EBPα and C/EBPβ in response to EGF treatment and EGF-induced keratinocyte proliferation. We found that EGF treatment preferentially upregulated and activated C/EBPβ mRNA, protein levels, DNA binding and transcriptional activity, while reciprocally diminishing C/EBPα expression. Further, the induction of C/EBPβ expression, phosphorylation, and transcriptional activation was associated with cell cycle entry, and loss of C/EBPβ resulted in diminished expression of numerous E2F target genes and diminished cell cycle progression.

Results

EGF treatment induces endogenous keratinocyte C/EBP transcriptional activity, as well as that of the forced expression of C/EBPα and C/EBPβ. C/EBPα and C/EBPβ are each highly expressed in mouse epidermal keratinocytes. To examine the effects of EGF treatment on endogenous C/EBP transcriptional activity, BALB/MK2 keratinocytes were transfected with the C/EBP-responsive promoter reporter construct (MGF-82), serum deprived for 24 hours, and stimulated with EGF. As shown in figure 1A, C/EBP activity was increased 10 fold by EGF treatment. C/EBP specificity was confirmed through use of a promoter-reporter construct which lacks C/EBP binding sites (MGF-40). Since C/EBPα and C/EBPβ are both highly expressed in epidermal keratinocytes, it was not clear which C/EBP isoform(s) was responsible for the increase in EGF-induced C/EBP activity. In order to assess the ability of EGF to activate C/EBPα or C/EBPβ, the promoter reporter assay was conducted with forced expression of each isoform. As shown in figure 1B, EGF treatment significantly activated both C/EBPα and C/EBPβ, demonstrating that EGF treatment can stimulate both endogenous C/EBP activity as well as transfected C/EBPα and C/EBPβ in keratinocytes.

C/EBPβ is the predominant endogenous C/EBP isoform in the presence of EGF. Although EGF signaling is able to similarly stimulate C/EBPα and C/EBPβ following their forced expression, this does not necessarily reflect a physiologically relevant relationship. For a better understanding of the

individual contributions of C/EBPα and C/EBPβ to overall endogenous C/EBP activity, we performed a gel shift assay using a C/EBP consensus sequence probe. BALB-MK2 keratinocytes were serum/growth factor deprived and then stimulated with EGF. As shown in figure 2A, C/EBP-DNA binding was greatly increased by EGF treatment, and the vast majority of both untreated and EGF treated C/EBP-DNA binding can be supershifted with a C/EBPβ-specific antibody. It was surprising to find that C/EBPα represented a small portion of the overall C/EBP-DNA binding complexes in the presence of EGF, since EGF was able to activate C/EBPα to an extent similar to C/EBPβ.

Because C/EBPα appeared to be under-represented in EMSA analysis, particularly after EGF treatment, we examined the effects of EGF treatment on C/EBPα and C/EBPβ, message and protein levels. BALB-MK2 keratinocytes were serum deprived for 24 hours, then treated with EGF and cells were collected at various time points. As shown in figure 2B, EGF treatment decreased levels of C/EBPα protein, and reciprocally increased levels of C/EBPβ protein expression. The increases in C/EBPβ protein were observed as early as 2 hours after treatment with EGF and remained elevated throughout the timecourse. In contrast, C/EBPα protein levels were decreased by EGF treatment throughout the entire timecourse. Interestingly, C/EBPα protein levels were significantly increased in the absence of EGF.

C/EBPα and C/EBPβ are intronless genes, but each express multiple isoforms as a result of alternate translational start sites[13]. C/EBPα expresses a

42 and 30kd isoform, while C/EBPβ has 37, 35, and 20kd isoforms known as Lap*, Lap, and Lip, respectively. Differential expression of either p30 or Lip has been observed in various tissues, and both p30 and Lip have been shown to act as dominant negative inhibitors of their full length counterparts[13, 14]. As shown in figure 2C, EGF does not appear to induce differential regulation of alternate C/EBP isoforms from their parent isoforms.

In order to determine if the reciprocal regulation of C/EBPα and C/EBPβ also occurs at the mRNA level, quantitative real time PCR (qRT-PCR) was performed on EGF treated samples. Reciprocal regulation of C/EBPα and C/EBPβ was observed at the mRNA level. EGF produces a 300% increase in C/EBPβ mRNA and a 400% decrease in C/EBPα mRNA 2 hours after treatment (figure 2D). This up- and down-regulation of C/EBPβ and C/EBPα, respectively, remained significantly altered through 16 hours of treatment (figure 2D); and these changes were completely blocked by Actinomycin D, indicating that EGF treatment regulates C/EBPs at the transcriptional level (data not shown). Taken together, these data demonstrate that while EGF treatment is able to efficiently activate both C/EBPα and C/EBPβ, the EGF-induced reciprocal regulation of C/EBPα and C/EBPβ levels results in the preferential expression and activation of C/EBPβ.

EGF regulates C/EBPβ through an EGFR, Ras, and MEK dependent pathway. The transactivation potential of C/EBPβ has been shown to be enhanced by post-translational modifications, including its phosphorylation by

ERK, GSK3β, and CDK 1 and CDK2[15-17]. Numerous C/EBPβ phosphorylation sites have been identified including Thr[188], which has been shown to be essential for Ras-dependent MAP kinase activation of the protein[18]. ERK phosphorylation of C/EBPβ at Thr[188] has been well characterized, and is implicated in the control of many key C/EBPβ functions. Further, the increases in C/EBPβ-DNA binding after treatment with EGF exceed the increases observed in C/EBPβ protein expression, suggesting that EGF-induced post-translational modification may increase the ability of C/EBPβ to bind DNA. We therefore sought to determine whether ERK signals to C/EBPβ downstream of EGFR. We observed a multifaceted effect of MEK inhibition as the EGF-induced increase in C/EBPβ mRNA and protein as well as DNA binding and protein phosphorylation, were all significantly decreased (figures 3A and B). Surprisingly, MEK inhibition had little effect on EGF's downregulation of C/EBPα message or protein levels, suggesting that EGF regulates C/EBPα and C/EBPβ expression through distinct signaling pathways.

To determine whether U0126's inhibition of protein expression, phosphorylation, and DNA binding translate into altered EGF-induced C/EBPβ activity, BALB-MK2 keratinocytes were transfected with C/EBPβ and the C/EBP-responsive MGF-82 promoter reporter. As shown in figure 3C, U0126's inhibition of MEK-induced ERK activation results in the statistically significant inhibition of EGF-induced C/EBPβ activity. As mentioned, EGF is an EGFR ligand and activation of EGFR leads to many events including stimulation of the

Ras-MAP kinase signaling pathway to phosphorylate ERK and induce proliferation. To confirm the role of EGFR and Ras in the EGF-induced increase in C/EBPβ activity we utilized an EGFR inhibitor and a dominant negative Ras inhibitor. As shown in figures 3D and E, EGF's induction of C/EBPβ activity was significantly blocked with by the EGFR inhibitor AG1478, and by expression of the Ras dominant negative RasN17. Taken together, these data indicate that EGF regulates C/EBPβ protein and message levels, phosphorylation, DNA binding, and transcriptional activities through an EGFR/Ras/MEK/ERK signaling pathway.

EGF signaling upregulates and activates C/EBPβ to promote cell cycle entry. EGF and MAP kinase signaling are well known to induce cell proliferation. To begin to determine what if any role C/EBPβ plays in EGF-induced keratinocyte cell cycle progression, BALB-MK2 keratinocytes were synchronized in the G_0/G_1 phase of the cell cycle by serum and growth factor deprivation for 24 hours, followed by stimulation with EGF. In figure 4A, EGF treatment was sufficient to cause cells to enter S-phase as determined by pulse labeling with tritiated thymidine. Nuclear extracts were prepared and analyzed for C/EBP-DNA binding, C/EBPβ phosphorylation, and C/EBPα and C/EBPβ protein levels at various time points after treatment with EGF. As shown in Figure 4B, there was a large (~10 fold) induction of C/EBPβ-DNA binding 6 hours after stimulation with EGF, which corresponded to an increase of C/EBPβ protein levels and phosphorylation at Thr188. These changes in C/EBPβ in

response to EGF treatment occurred prior to S-phase entry (figure 6A), and then diminished as cells progressed through S-phase. In contrast, C/EBPα protein levels were reciprocally downregulated 6 hours after treatment with EGF, but were gradually increased as the cells progressed through S-phase (figure 4B). A highly reproducible but subtle increase in C/EBPα-DNA binding can also be observed at later time points.

To determine whether EGF-induced increases in C/EBPβ protein, DNA binding, and phosphorylation reflect an important role for C/EBPβ in cell cycle entry, we compared the ability of EGF to induce S-phase entry in serum deprived C/EBPβ$^{-/-}$ and wild type primary keratinocytes. Wild type keratinocytes entered S-phase at 10 hours post EGF treatment and continued to progress at 12 and 16 hours. In contrast, C/EBPβ$^{-/-}$ keratinocytes entered S-phase later and demonstrated significantly lower levels of ^{3}H thymidine incorporation. Taken together, these data support the hypothesis that EGF stimulation upregulates and activates C/EBPβ in epidermal keratinocytes to promote the progression from the G1 to S phase of the cell cycle.

C/EBPβ deficiency results in the impaired induction of E2F target genes by EGF stimulation. The significant reduction in C/EBPβ$^{-/-}$ keratinocytes entering S-phase, concomitant with the increased DNA binding and activity of C/EBPβ in proliferating keratinocytes after stimulation with EGF, suggest a possible role for C/EBPβ in the transcription of genes involved in entry into S phase. A cell cycle specific qRT-PCR array was used to identify which, if any

genes are differentially regulated by EGF in cells lacking C/EBPβ. Primary keratinocytes were serum deprived and then stimulated with EGF and harvested at 10 hours. A number of genes were altered in the C/EBPβ$^{-/-}$ samples, and their fold down- or up-regulation are listed in figures 1A and B of the appendix, respectively. The downregulated genes are involved in various functions including regulation of the cell cycle and cell cycle machinery, DNA damage response, mitotic growth arrest, and survival. Interestingly, 10 out of 12 of the most down-regulated genes in the C/EBPβ$^{-/-}$ samples are also E2F target genes (indicated by asterisks in figures 1A and B of the appendix). These data are somewhat preliminary, but suggest a possible relationship between C/EBPβ and increased E2F-induced gene expression.

To determine whether C/EBPβ can alter the expression of E2F-regulated genes, we used an E2F1 promoter-reporter construct. E2F1 is a well characterized auto-regulated gene involved in many processes including the transition from G1 to S Phase of the cell cycle. EGF signaling is known to induce E2F target gene expression, and is able to activate an E2F1 promoter reporter construct as shown in figure 5A. Transfection with E2F1/DP1 in the presence of EGF further stimulated this activity. Co-expression of C/EBPβ with E2F1/DP1 further increased the activation of an E2F1 promoter reporter construct (Figure 5A). Interestingly, C/EBPβ expression was capable of activating the E2F1 promoter independently of exogenous E2F1 expression, suggesting that increased C/EBPβ expression may cooperate with endogenous

E2Fs to activate transcription (data not shown). Ras signaling is also known to activate E2F1 transcription, and Ras was able to induce a 200 fold increase in E2F1 activity as measured by the E2F1 promoter-reporter, and co-expression of C/EBPβ with Ras produced a further increase in E2F1 promoter activity as shown in figure 5B. In contrast to C/EBPβ, C/EBPα inhibited both EGF- and Ras-induced E2F1 activity (Figures 5A and B). These data demonstrate that C/EBPβ cooperates with EGF and Ras signaling to activate E2F target gene transcription.

Discussion

The results of this study establish C/EBPβ as an important effector of EGF-induced keratinocyte proliferation. We described the ability of EGF signaling to reciprocally regulate C/EBPα and C/EBPβ in keratinocytes, promoting C/EBPβ expression. EGF induced phosphorylation and activation of C/EBPβ through an EGFR/Ras/MEK/ERK pathway to promote progression from G_1 to S-phase of the cell cycle in part though the increased transactivation of E2F target genes.

The effects of mitogenic stimulation on C/EBPβ vary greatly, and are often dose dependent[3]. We found EGF capable of activating both C/EBPα and C/EBPβ after their forced expression in keratinocytes. Further analysis revealed, however, that mitogenic stimulation preferentially upregulated and activated endogenous C/EBPβ, while C/EBPα levels were diminished. Similar reciprocal regulation of C/EBPα and C/EBPβ has been observed in hepatocytes induced to proliferate after partial hepatectomy[19-21]. Upon partial hepatectomy, there is a shift in the ratio of C/EBPα to C/EBPβ, from an α-predominant state in normal liver to a β-predominant state during liver regeneration. Further studies identified that C/EBPβ is required for normal hepatocyte proliferation in mice after partial hepatectomy[22]. C/EBPβ is also required for mitotic clonal expansion of adipocytes[23] and is associated with induction of proliferation in the endometrium, mammary epithelial cells, macrophages, and lymphocytes[3, 24, 25].

C/EBPβ is known to participate with a number of key regulators of the cell cycle. The work of Lamb et al. revealed a relationship between C/EBPβ and Cyclin D1. They found that C/EBPβ represses Cyclin D1-dependent changes in gene expression, and that cyclin D1 acts by antagonizing this repressor function[26]. An association has also been established between C/EBPβ and the Rb-E2F cell cycle machinery. Rb interacts with and regulates the transcriptional activity of C/EBPβ in a similar manner to its regulation of E2F, where hypo-but not hyper-phosphorylated forms of Rb were able to bind to and repress C/EBPβ-DNA binding [27]. C/EBPβ has been shown to associate with E2F target genes in both the liver and in MEFs. In the liver, C/EBPβ activates E2F-regulated genes by recruitment on the coactivator CREB-binding protein/p300[28]. The studies in MEFs were focused on the elucidation of Ras^{v12}-induced cellular senescence, and found that with overexpression of Ras^{v12}, C/EBPβ binds to and represses E2F target gene expression to promote RB:E2F-dependent senescence [29].

The link between C/EBPβ and E2F transcription and function may explain some of the seemingly contradictory roles of C/EBPβ in cellular differentiation, proliferation, and survival, as E2F proteins not only mediate the tumor suppressor roles of Rb, but also promote the progression of cells from G_1 to S phase by activating genes required for this transition[30]. E2F dependent gene expression has been shown to determine the balance between proliferation and cell death[31]. The data presented here suggest at least two

viable hypotheses: 1. that loss of C/EBPβ results in diminished E2F expression and consequently to decreased expression of E2F target genes, or that 2. C/EBPβ is part of the transcriptional apparatus that interacts with E2F and Rb to enhance the expression of E2F target genes including E2F. The functional duality of C/EBPβ suggested by its distinct roles in E2F-driven cell cycle progression, taken with the data presented here, suggest that C/EBPβ may be an important component of the RB:E2F cell cycle regulatory network which enables cells to respond appropriately to varying stimuli.

Although the association of and cooperation between C/EBPβ and Cyclin D1 in human tumors suggests a possible link between C/EBPβ, cell cycle regulation, and cancer; the connection between C/EBPβ's role in proliferation and its known oncogenic functions remains to be determined[26]. Ras-induced epidermal tumorigenesis is completely abrogated in the absence of C/EBPβ, and there is an increased incidence of p53-induced apoptosis in these mice in response to carcinogen treatment[32]. This increase in apoptosis has not yet been linked directly to the inhibition of tumorigenesis in C/EBPβ$^{-/-}$ mice, although it remains a viable mechanism. The enhanced epithelial tumorigenic response in C/EBPα$^{-/-}$ mice also suggests an important role for C/EBPβ in tumorigenesis, as it could be argued that the observed tumorigenic phenotypes have as much to do with the loss of C/EBPα as with the unchecked effects of C/EBPβ (and vica versa in C/EBPβ$^{-/-}$ mice). Specifically, there is an increased rate of proliferation in suprabasal cells of C/EBPα$^{-/-}$ tumors, which may be

related to the unchecked proliferative function of C/EBPβ. Presumably, the antiproliferative capabilities of C/EBPα could balance the proliferative capabilities of C/EBPβ through inhibition of E2F target gene expression. The observation that the increases in C/EBPα mRNA and protein levels in the absence of EGF did not correlate with an increase in C/EBPα-DNA binding in the gel shift analysis, are consistent with a possible non-DNA binding function of C/EBPα in response to serum and growth factor deprivation. Clearly further work is required to determine the mechanistic roles of C/EBPα and C/EBPβ in epithelial tumorigenesis, however this study poses that the balance between the pro-and anti-proliferative functions of C/EBPα and C/EBPβ may be a contributing factor in this mechanism. C/EBPβ may function in epidermal tumorigenesis to both stimulate proliferation and promote survival, as is the case in macrophage tumor cells[3].

In conclusion, this study demonstrates that EGF treatment activates and upregulates C/EBPβ, while reciprocally downregulating C/EBPα, to promote the induction of E2F target gene expression and cell cycle progression. Further elucidation of the pro-and anti-proliferative roles C/EBPα and C/EBPβ in the tumorigenic response will aid in the identification and development of novel cancer therapies.

Materials and Methods

Cell culture

Balb/MK2 keratinocytes were maintained in low Ca^{2+} EMEM (BioWhittaker) containing 8% chelexed FBS, 4ng/ml EGF (Gibco) and 0.05mM $CaCl_2$. Mouse primary keratinocytes were isolated from the epidermis of 1-2 day old newborn mice by overnight flotation in trypsin at 4°C[33, 34]. Isolated keratinocytes were placed in Ca^{2+} -free EMEM supplemented with 10% non-Chelex-treated FBS and 10ng of hEGF/ml. Four hours later, cultures were re-fed with Keratinocyte-SFM (GIBCO) containing BPE (10ug/ml), EGF (5ng/ml), Ca^{2+} 0.05M and gentamycin (5µg/ml). For luciferase experiments involving Ras and the addition or omission of EGF, cells were placed in medium deprived of growth factors (0.1% FBS, no EGF, and 0.05mM $CaCl_2$).

Transfection

BALB-MK2 cell transfection:

One day prior to transfection cells were plated in 12 well plates at a density of $1.3x10^5$ cells per well. Cells were transiently transfected with indicated vectors using Tfx-10 or Transfast transfection reagent (Promega). Transfections were performed according to manufacturers' protocol, initially using serum free medium containing no EGF; after 1 hour 2% FBS medium was added. After 24 hours medium was removed then replaced with serum free medium +/- EGF. Cells were incubated for 16 hours the assayed for luciferase activity.

Primary keratinocyte transfection:

Reporter Assays

Cells were transfected in serum free medium with 200ng MGF82 promoter-reporter construct or mutant [35] or 200ng E2F1 promoter reporter construct or mutant (Masa-Aki Ikeda) [36] with or without the following constructs: E2F1 in pcDNA1 [37], DP1 in pCMV [38], rat C/EBPα [39] or C/EBPβ [40] in pcDNA3.1, or Ha-Ras (12V) in pcDNA3 [41]. The amounts of each contruct used are specified in the figure legends, and the total amount of DNA among all groups was kept constant by using empty pcDNA3.1 (Promega).

Electrophoretic mobility shift assay (EMSA) and supershift.

C/EBP consensus oligonucleotide (Santa Cruz) was labeled with [γ-^{32}P]ATP by kinase reaction with T4 polynucleotide kinase. Radiolabeled oligonucleotide was incubated with 4μg of nuclear extract at room temperature in binding buffer containing poly(dI-dC). DNA-protein complexes were loaded onto a 6% polyacrylamide gel and run in 0.25x Tris-borate-EDTA buffer. The gel was transferred to Whatman 3mm chromatography paper, dried, and exposed to film. Supershifts were performed with the inclusion of a 30 minute pre-incubation of the nuclear extract with 3μl of the selected antibody or PBS prior to incubation of the sample with the radiolabeled probe. Antibodies used for supershift analysis are the same as those listed in the Immunoblot section of Material and methods.

Immunoblot Analysis

Immunoblot analysis was conduced as described[10] using the C/EBPα sc-61 (1:2000), C/EBPβ sc-150 (1:2500), or PO_4-T188 specific C/EBPβ sc-16993-R (1:1000) rabbit polyclonal antibodies (sc-# antibodies from SantaCruz) followed by HRP-linked donkey anti-rabbit immunoglobulin (1:2500) from Amersham (NA934V). β-Actin was detected with a 1:20,000 dilution of a mouse monoclonal antibody (Sigma, A2228), followed by HRP-linked donkey anti-mouse immunoglobulin from Amersham (1:10,000).

Quantitative real-time RT–PCR

Total RNA from was extracted using Tri Reagent (Sigma), treated with DNase (Qiagen) and purified with RNeasy Mini Kit (Qiagen). cDNA was synthesized using ImProm-II reverse transcription (RT) system (Promega) following the manufacturer's protocol. cDNA from 50 ng total RNA was used as template to perform quantitative realtime PCR using mouse C/EBPα, C/EBPβ, 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 the manufacturer's protocol. The expression levels for all samples were normalized to the endogenous control 18S (Applied Biosystems). Data were analysed using the Comparative CT Method.

Thymidine incorporation assay

Cells were pulse-labeled with 3μCi of [^{3}H-methyl] thymidine per ml (20Ci/mmol) for 1 hour before collection (Perkin Elmer). Cells were collected by

trypsinization, resuspended in 1mM EDTA buffer, and sonicated. Aliquots were collected onto APFC glass fiber filters (Millipore) and washed with 4% perchloracetic acid followed by increasing concentration of EtOH. The washed filters were placed in liquid scintillation cocktail and subjected to scintillation counting. For DNA quantitation, an aliquot of each sample and 5µl of 0.1 mg of Hoechst 33258 solution/ml were mixed in 1ml of 0.01M tris (pH7.0)-0.1MNaCl-0.01M EDTA buffer. The fluorescence units were determined with a fluorometer (excitation at 365 nm and emission at 450 nm.

Mice

Primary cells were isolated from newborn pups of a C/EBPβ$^{+/-}$ x C/EBPβ$^{+/-}$ mating resulting in a 50% C57BL/6 and 50%129/SV strain background[42]. C/EBPα$^{-/-}$ primary cells were derived from the skin of epidermal specific C/EBPα$^{-/-}$ mice[6].

Acknowledgements

This work was supported by grants from the NCI (CA46637) and from the NIEHS (ES12473). Support was also granted in part by the GAANN Fellowship.

References

1. Ramji, D. P. & Foka, P. CCAAT/enhancer-binding proteins: structure, function and regulation. Biochem J 365, 561-75 (2002).
2. Schuster, M. B. & Porse, B. T. C/EBPalpha: a tumour suppressor in multiple tissues? Biochim Biophys Acta 1766, 88-103 (2006).
3. Sebastian, T. & Johnson, P. F. Stop and go: anti-proliferative and mitogenic functions of the transcription factor C/EBPbeta. Cell Cycle 5, 953-7 (2006).
4. Oh, H. S. & Smart, R. C. Expression of CCAAT/enhancer binding proteins (C/EBP) is associated with squamous differentiation in epidermis and isolated primary keratinocytes and is altered in skin neoplasms. J Invest Dermatol 110, 939-45 (1998).
5. Maytin, E. V. & Habener, J. F. Transcription factors C/EBP alpha, C/EBP beta, and CHOP (Gadd153) expressed during the differentiation program of keratinocytes in vitro and in vivo. J Invest Dermatol 110, 238-46 (1998).
6. Loomis, K. D., Zhu, S., Yoon, K., Johnson, P. F. & Smart, R. C. Genetic ablation of CCAAT/enhancer binding protein alpha in epidermis reveals its role in suppression of epithelial tumorigenesis. Cancer Res 67, 6768-76 (2007).

7. Zhu, S. et al. C/EBPbeta modulates the early events of keratinocyte differentiation involving growth arrest and keratin 1 and keratin 10 expression. Mol Cell Biol 19, 7181-90 (1999).
8. Yoon, K. & Smart, R. C. C/EBPalpha is a DNA damage-inducible p53-regulated mediator of the G1 checkpoint in keratinocytes. Mol Cell Biol 24, 10650-60 (2004).
9. Zhu, S., Yoon, K., Sterneck, E., Johnson, P. F. & Smart, R. C. CCAAT/enhancer binding protein-beta is a mediator of keratinocyte survival and skin tumorigenesis involving oncogenic Ras signaling. Proc Natl Acad Sci U S A 99, 207-12 (2002).
10. Shim, M., Powers, K. L., Ewing, S. J., Zhu, S. & Smart, R. C. Diminished expression of C/EBPalpha in skin carcinomas is linked to oncogenic Ras and reexpression of C/EBPalpha in carcinoma cells inhibits proliferation. Cancer Res 65, 861-7 (2005).
11. Olayioye, M. A., Neve, R. M., Lane, H. A. & Hynes, N. E. The ErbB signaling network: receptor heterodimerization in development and cancer. Embo J 19, 3159-67 (2000).
12. Rittie, L. et al. Differential ErbB1 Signaling in Squamous Cell versus Basal Cell Carcinoma of the Skin. Am J Pathol 170, 2089-2099 (2007).
13. Descombes, P. & Schibler, U. A liver-enriched transcriptional activator protein, LAP, and a transcriptional inhibitory protein, LIP, are translated from the same mRNA. Cell 67, 569-79 (1991).

14. Cleaves, R., Wang, Q.-f. & Friedman, A. D. C//EBP[alpha]p30, a myeloid leukemia oncoprotein, limits G-CSF receptor expression but not terminal granulopoiesis via site-selective inhibition of C//EBP DNA binding. Oncogene 23, 716-725.

15. Trautwein, C. et al. Transactivation by NF-IL6/LAP is enhanced by phosphorylation of its activation domain. Nature 364, 544-547 (1993).

16. Nerlov, C. C/EBPs: recipients of extracellular signals through proteome modulation. Curr Opin Cell Biol 20, 180-5 (2008).

17. Shuman, J. D. et al. Cell cycle-dependent phosphorylation of C/EBPbeta mediates oncogenic cooperativity between C/EBPbeta and H-RasV12. Mol Cell Biol 24, 7380-91 (2004).

18. Nakajima, T. et al. Phosphorylation at Threonine-235 by a ras-Dependent Mitogen-Activated Protein Kinase Cascade is Essential for Transcription Factor NF-IL6. Proceedings of the National Academy of Sciences 90, 2207-2211 (1993).

19. Rana, B., Mischoulon, D., Xie, Y., Bucher, N. L. & Farmer, S. R. Cell-extracellular matrix interactions can regulate the switch between growth and differentiation in rat hepatocytes: reciprocal expression of C/EBP alpha and immediate-early growth response transcription factors. Mol. Cell. Biol. 14, 5858-5869 (1994).

20. Greenbaum, L. E., Cressman, D. E., Haber, B. A. & Taub, R. Coexistence of C/EBP alpha, beta, growth-induced proteins and DNA

synthesis in hepatocytes during liver regeneration. Implications for maintenance of the differentiated state during liver growth. J Clin Invest 96, 1351-65 (1995).

21. Mischoulon, D., Rana, B., Bucher, N. L. & Farmer, S. R. Growth-dependent inhibition of CCAAT enhancer-binding protein (C/EBP alpha) gene expression during hepatocyte proliferation in the regenerating liver and in culture. Mol. Cell. Biol. 12, 2553-2560 (1992).
22. Greenbaum, L. E. et al. CCAAT enhancer- binding protein beta is required for normal hepatocyte proliferation in mice after partial hepatectomy. J Clin Invest 102, 996-1007 (1998).
23. Tang, Q.-Q., Otto, T. C. & Lane, M. D. CCAAT/enhancer-binding protein beta is required for mitotic clonal expansion during adipogenesis. Proceedings of the National Academy of Sciences 100, 850-855 (2003).
24. Arnett, B., Soisson, P., Ducatman, B. & Zhang, P. Expression of CAAT Enhancer Binding Protein Beta (C/EBP beta) in Cervix and Endometrium. Molecular Cancer 2, 21 (2003).
25. Bundy, L., Wells, S. & Sealy, L. C/EBPbeta-2 confers EGF-independent growth and disrupts the normal acinar architecture of human mammary epithelial cells. Mol Cancer 4, 43 (2005).
26. Lamb, J. et al. A mechanism of cyclin D1 action encoded in the patterns of gene expression in human cancer. Cell 114, 323-34 (2003).

27. Cole, K. A., Harmon, A. W., Harp, J. B. & Patel, Y. M. Rb regulates C/EBPbeta-DNA-binding activity during 3T3-L1 adipogenesis. Am J Physiol Cell Physiol 286, C349-354 (2004).

28. Wang, H. et al. C/EBPbeta Activates E2F-regulated Genes in Vivo via Recruitment of the Coactivator CREB-binding Protein/P300. J. Biol. Chem. 282, 24679-24688 (2007).

29. Sebastian, T., Malik, R., Thomas, S., Sage, J. & Johnson, P. F. C/EBPbeta cooperates with RB:E2F to implement Ras(V12)-induced cellular senescence. Embo J 24, 3301-12 (2005).

30. Johnson, D. G. The paradox of E2F1: oncogene and tumor suppressor gene. Mol Carcinog 27, 151-7 (2000).

31. Hallstrom, T. C., Mori, S. & Nevins, J. R. An E2F1-dependent gene expression program that determines the balance between proliferation and cell death. Cancer Cell 13, 11-22 (2008).

32. Yoon, K., Zhu, S., Ewing, S. J. & Smart, R. C. Decreased survival of C/EBP beta-deficient keratinocytes is due to aberrant regulation of p53 levels and function. Oncogene 26, 360-7 (2007).

33. Dlugosz, A. A., Glick, A. B., Tennenbaum, T., Weinberg, W. C. & Yuspa, S. H. Isolation and utilization of epidermal keratinocytes for oncogene research. Methods Enzymol 254, 3-20 (1995).

34. Hennings, H. et al. Calcium regulation of growth and differentiation of mouse epidermal cells in culture. Cell 19, 245-54 (1980).

35. Sterneck, E., Muller, C., Katz, S. & Leuz, A. Autocrine growth induced by kinase type oncogenes in myeloid cells requires AP-1 and NF-M, a myeloid specific, C/EBP-like factor. EMBO. J 11, 115-126 (1992).

36. Johnson, D. G., Ohtani, K. & Nevins, J. R. Autoregulatory control of E2F1 expression in response to positive and negative regulators of cell cycle progression. Genes Dev 8, 1514-25 (1994).

37. Cress, W. D., Johnson, D. G. & Nevins, J. R. A genetic analysis of the E2F1 gene distinguishes regulation by Rb, p107, and adenovirus E4. Mol Cell Biol 13, 6314-25 (1993).

38. Helin, K. et al. Heterodimerization of the transcription factors E2F-1 and DP-1 leads to cooperative trans-activation. Genes Dev 7, 1850-61 (1993).

39. Williams, S. C., Cantwell, C. A. & Johnson, P. F. A family of C/EBP-related proteins capable of forming covalently linked leucine zipper dimers in vitro. Genes Dev 5, 1553-67 (1991).

40. Zhu, S. et al. C/EBPb modulates the early events of keratinocyte differentiation involving growth arrest and keratin 1 and keratin 10 expression. Mol Cell Biol 19, 7181-7190 (1999).

41. Der, C. J., Finkel, T. & Cooper, G. M. Biological and biochemical properties of human rasH genes mutated at codon 61. Cell 44, 167-176 (1986).

42. Sterneck, E., Tessarollo, L. & Johnson, P. F. An essential role for C/EBPbeta in female reproduction. Genes Dev 11, 2153 - 2162 (1997).

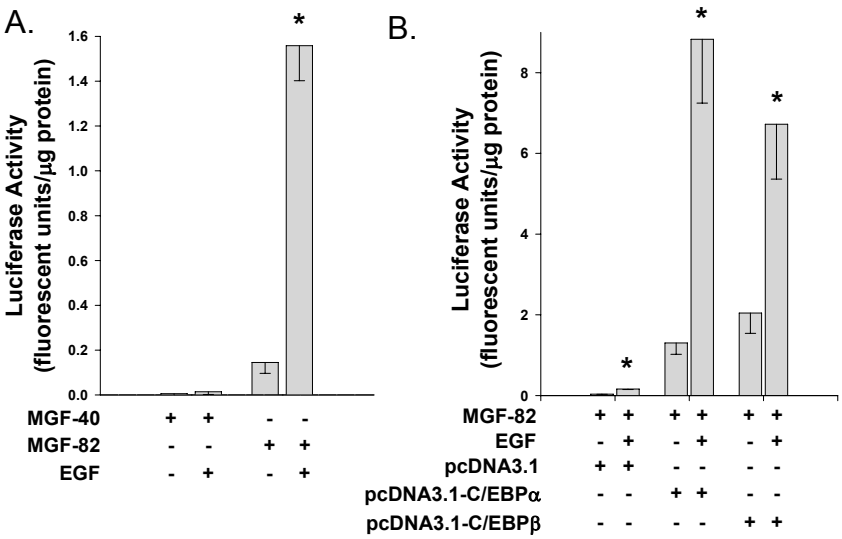


Figure 1: C/EBP activity is stimulated by EGF in keratinocytes
A. EGF stimulates the transcription activity of endogenous C/EBPs in keratinocytes. Balb/MK2 keratinocytes were transfected with 250ng of pMGF-82 a C/EBP dependent promoter-reporter or pMGF-40 promoter-reporter that lacks the C/EBP binding sites. Twenty four hours after transfection, medium was replaced with serum free medium +/- 4ng/ml EGF. After 16 hours, cells were lysed and luciferase activity was determined. Each value represents the mean $\pm$ SD of triplicate treatment groups. *Statistically significant increase in C/EBP activity upon treatment with EGF, Student's t-test ($p<0.0001$). B. Balb/MK2 keratinocytes were transfected with 250ng of pMGF-82 and 50ng of empty vector (pcDNA3), pcDNA3-C/EBPα or pcDNA3-C/EBPβ. Twenty four hours after transfection, medium was replaced with serum free medium +/- 4ng/ml EGF. After 16 hours, cells were lysed and luciferase activity was determined. Each value represents the mean $\pm$ SD of triplicate treatment groups. *Statistically significant increase in C/EBP activity upon treatment with EGF, Student's t-test ($p<0.005$). All experiments were confirmed in at least three independent studies.

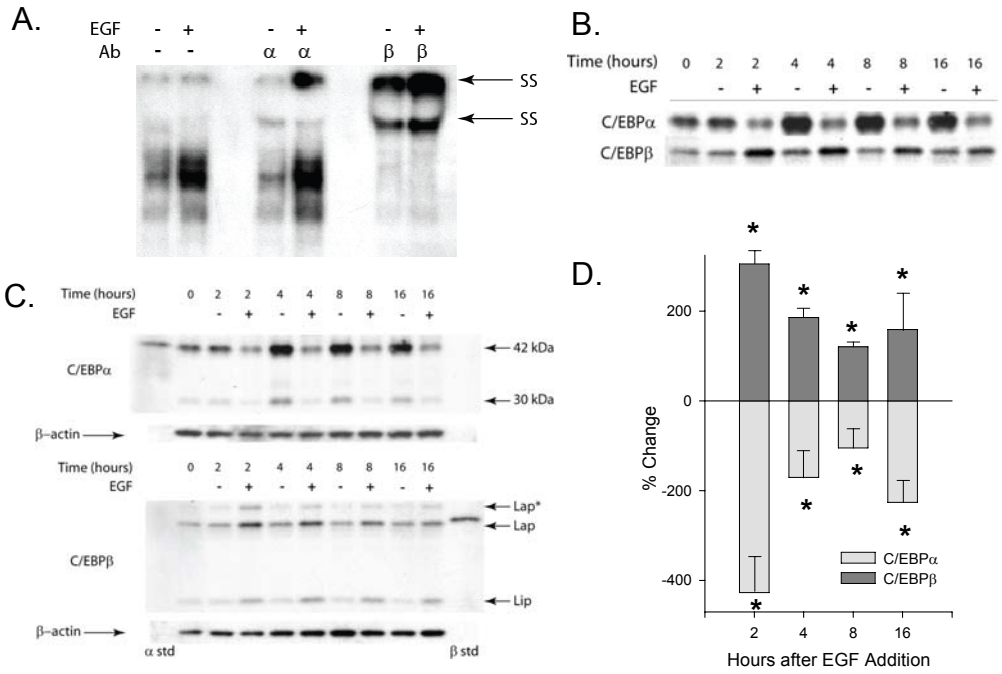
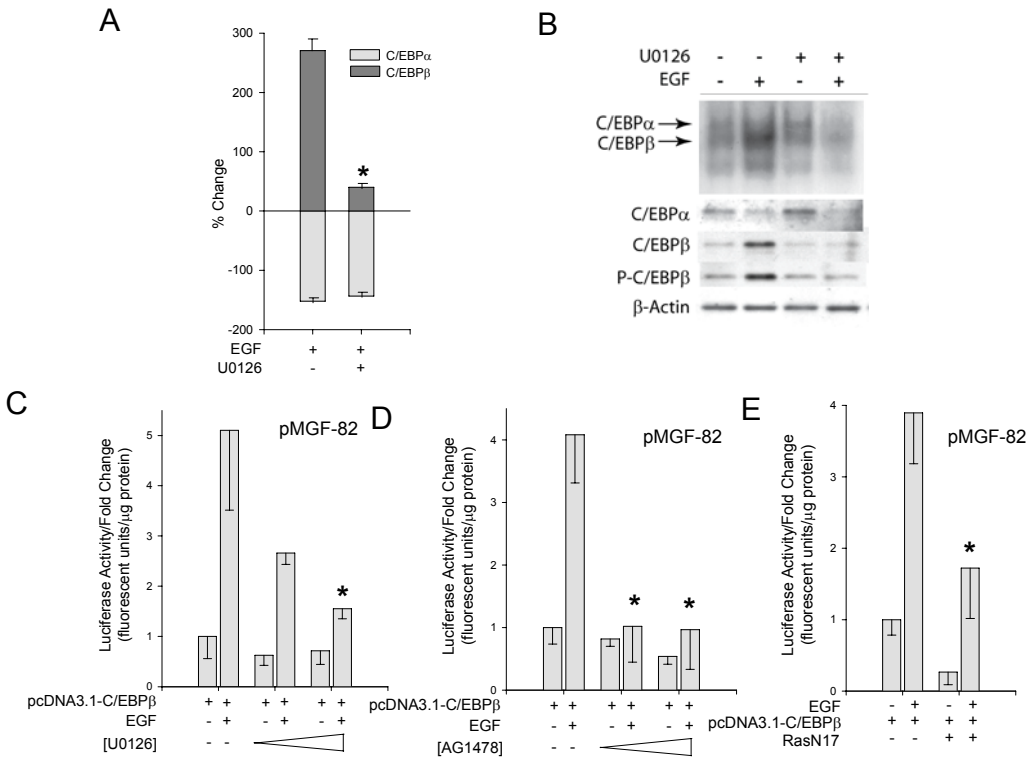


Figure 2: C/EBPα and C/EBPβ mRNA levels, protein levels, and DNA binding are reciprocally regulated by EGF

A. C/EBPβ-DNA binding is increased in response to EGF treatment. Balb/MK2 keratinocytes were serum deprived in 1% FBS medium for 24 hours. Medium was then replaced with serum free EMEM with either +/- 4ng/ml EGF. Nuclear extracts were prepared 2 hours after EGF treatment. Supershifted complexes (SS) formed from the addition of the indicated antibody (Ab where α is C/EBPα, SC-61, and β is C/EBPβ, sc-150) are indicated by arrows. B. EGF treatment decreases C/EBPα (p42) protein levels and increases C/EBPβ (LAP) protein levels. Figure 2B is a cropped version of the Immunoblot found in Figure 2C. Balb/MK2 keratinocytes were serum deprived in 1% FBS medium for 24 hours. After 24 hours (time zero), medium was replaced with serum free medium +/- 4ng/ml EGF. Cells were harvested at time points indicated and western analysis was performed with 10μg nuclear protein. β-actin is included as protein loading control. C. EGF treatment decreases C/EBPα p42 and p30 and increases LAP*, LAP, and LIP. Figure C is a full gel image of the cropped image in figure B. D. EGF induces the reciprocal regulation of C/EBPα and C/EBPβ message levels. BALB-MK2 keratinocytes were treated as described above for figure 2B, total RNA was isolated, and quantitative RT–PCR was conducted for C/EBPα and C/EBPβ. Data were analyzed using the Comparative CT Method and data are presented as percent change from time zero. Each value represents the mean ± SD of triplicate treatment groups. *Significantly different from time zero, Student's t-test ($p<0.05$). All experiments were confirmed in at least three independent studies.

Figure 3: C/EBPβ expression levels and transcriptional activity are upregulated through an EGFR/Ras/MEK/ERK pathway

A. EGF treatment upregulates C/EBPβ message levels through a MEK-dependent pathway and downregulates C/EBPα through a MEK-independent pathway. qRT-PCR was performed as described above for figure 2D. BALB-MK2 keratinocytes were serum deprived and released as described in figure 2B, however cells were treated with 5μM of the MEK inhibitor U0126 15 minutes prior to stimulation with EGF and cells were collected 2 hours post EGF treatment. Each value represents the mean ± SD of triplicate treatment groups. *Significantly different from control, Student's t-test ($p<0.0001$). B. C/EBPβ is phosphorylated at Thr^{188}, after treatment with EGF, and EGF-induced C/EBPβ phosphorylation, protein induction, and DNA binding are blocked by the MEK inhibitor U0126. Cells were treated as described above for figure 3A. Nuclear extracts were harvested 2 hours after treatment with EGF, and used for immunoblot and EMSA analysis. β-actin expression was included as a protein loading control. C. EGF's activation of C/EBPβ is blocked by the MEK inhibitor U0126. Balb/MK2 keratinocytes were co-transfected with 150ng of pMGF-82 and 50ng of C/EBPβ. Twenty four hours after transfection, medium was replaced with 500μl serum free medium containing 1 or 5μM of U0126 or DMSO (control). One hour later an additional 500μl serum free medium +/- 4ng/ml EGF was added. After 16 hours, cells were lysed and luciferase activity was determined. Each value represents the mean ± SD of triplicate treatment groups. *Significantly different from the control sample (+ EGF, No U0126), Student's t-test ($p<0.0001$). D. EGF's activation of C/EBPβ is dependent on EGFR. MGF-82 promoter reporter analysis was performed as described in figure 3C, except 1 and 5μM of the EGFR inhibitor AG1478 were used in lieu of the U0126. Each value represents the mean ± SD of triplicate treatment groups. *Significantly different from the control sample (+ EGF, No AG1478), Student's t-test ($p<0.006$). E. EGF-induced C/EBPβ activity is blocked by expression of the Ras dominant negative, RasN17. Balb/MK2 keratinocytes were transfected in 1% FBS medium with 250ng of pMGF-82 and 250ng of C/EBPβ, RasN17, empty vector (pcDNA3) or combination (total 500ng transfected into each sample). Twenty four hours after transfection, medium was replaced with serum free medium +/- 4ng/ml EGF. After 16 hours, cells were lysed and luciferase activity was determined. Each value represents the mean ± SD of triplicate treatment groups. *Significantly different from the control sample (+ EGF, No RasN17), Student's t-test ($p=0.020$). All experiments were confirmed in at least three independent studies.


A
B
C
D
E
C/EBPα
C/EBPβ
% Change
EGF
U0126
P-C/EBPβ
β-Actin
pMGF-82
Luciferase Activity/Fold Change
(fluorescent units/μg protein)
pcDNA3.1-C/EBPβ
[U0126]
[AG1478]
RasN17

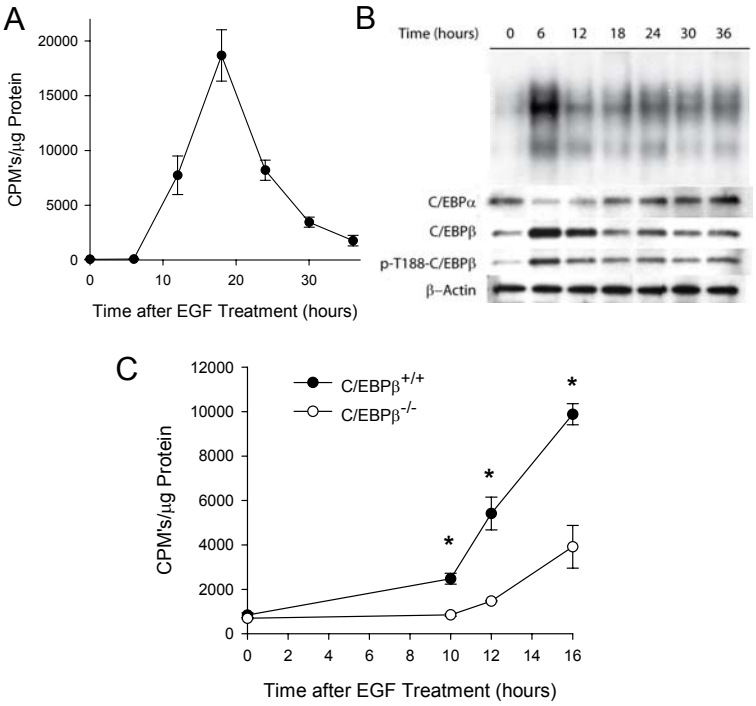


Figure 4: EGF signaling upregulates and activates C/EBPβ to promote cell cycle entry.

A and B. EGF is sufficient to induce cell cycle entry. Balb/MK2 cells were serum deprived in 1% FBS for 24 hours to synchronize the cells. After 24 hours (time zero), the medium was replaced with EMEM with 4ng/ml EGF. Matched samples were pulse labeled for 1 hour with ^{3}H-Thymidine and harvested simultaneously at the indicated times for thymidine incorporation (A) and EMSA and immunoblot analysis (B). Each value in figure 4A represents the mean $\pm$ SD of triplicate treatment groups. C. C/EBPβ$^{-/-}$ primary keratinocytes have a diminished entry into S-phase of the cell cycle. WT and C/EBPβ$^{-/-}$ keratinocytes were synchronized through incubation in serum free EMEM supplemented with 1mg/ml BSA and HEPES pH 7.4 for 40 hours. At time zero, the medium was replaced with the EMEM with BSA and HEPES, as well as with 4ng/ml EGF. Cells were pulse labeled for 1 hour with ^{3}H-Thymidine, collected at time points indicated and subjected to scintillation counting. Each value represents the mean $\pm$ SD of triplicate treatment groups. *Significant differences between C/EBPβ$^{+/+}$ and C/EBPβ$^{-/-}$ readings at each time point were determined by the Student's t-test ($p<0.001$). All experiments were confirmed in at least three independent studies.

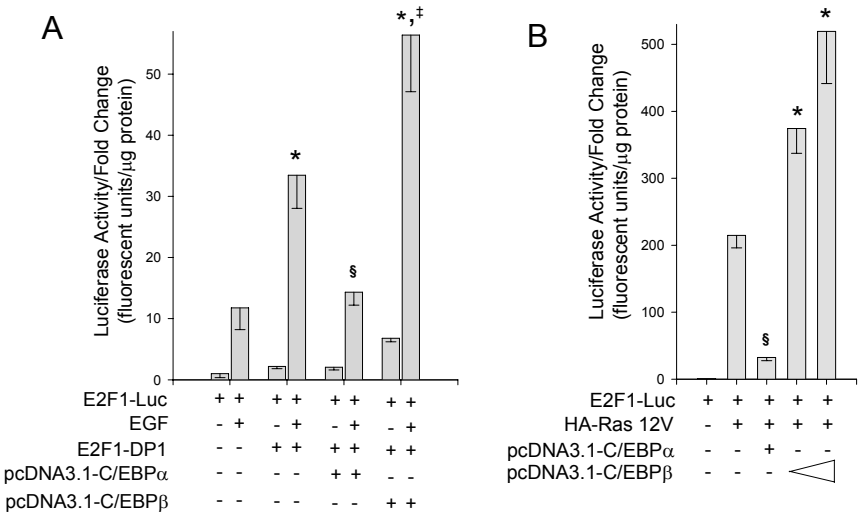


Figure 5: C/EBPβ can enhance EGF and Ras-induced E2F transcription activity. A. C/EBPβ enhances EGF-induced E2F activity. The E2F1 promoter reporter was cotransfected with 5ng E2F1 and 5ng DP1, and 100ng C/EBPα or C/EBPβ as indicated. Following transfection, 4ng/ml EGF was added to cells in growth factor/serum deprived medium as indicated. Each value represents the mean ± SD of triplicate treatment groups. * Significant differences between indicated sample and sample with EGF alone. § Significant inhibition compared to the sample with EGF, E2F1, and DP1. ‡ Significant increase compared to the sample with EGF, E2F1 and DP1, Student's t-test ($p<0.05$). B. C/EBPβ enhances Ras-induced E2F activity. The E2F1 promoter reporter was transfected as described above, however 5ng Ras was transfected in lieu of E2F and DP1, and cells were not exposed to EGF. Each value represents the mean ± SD of triplicate treatment groups. * Significant increase compared to Ras transfected sample. § Significant inhibition compared to the Ras transfected sample, Student's t-test ($p<0.003$).

GENERAL DISCUSSION

Tissue homeostasis is maintained through an intricate balance between cell proliferation, differentiation, and survival. Cancer develops through the distortion of these normal processes, resulting in cells that no longer respond appropriately to extracellular signals. C/EBPα and C/EBPβ are key regulators of these processes in the epidermis, and have been shown to exhibit oncogenic and tumor suppressor functions in epithelial tumorigenesis[66, 72].

C/EBPα's antiproliferative and differentiation-associated functions have been widely established in numerous cell types and tissues. However, the function of C/EBPα in the epidermis is not yet fully elucidated. The localization of C/EBPα expression in primarily suprabasal cells of the epidermis suggests a role in terminal differentiation and/or maintenance of the post-mitotic state[43, 44]. C/EBPα is not, however, essential for normal epidermal differentiation or homeostasis as we have shown that mice with an epidermal-specific ablation of C/EBPα exhibit no apparent abnormalities in tissue morphology, or alterations in the levels or localization of differentiation markers. Further, proliferation and apoptosis levels in interfollicular basal epidermal keratinocytes remained unchanged in the absence of C/EBPα[122]. These findings argue that C/EBPα is either uninvolved in epidermal homeostasis, or, more likely, that C/EBPα contributes in a redundant/non-essential manner.

The presumably compensatory upregulation of C/EBPβ and p21 in C/EBPα$^{-/-}$ epidermis potentially masks the effect of C/EBPα loss, but also

suggests aspects of C/EBPα function[122]. Specifically, both C/EBPβ and p21 are implicated in the inhibition of proliferation and induction of differentiation in keratinocytes[64, 126]. An important function for C/EBPα in the epidermis was revealed when UVB-induced DNA damage resulted in C/EBPα-dependent inhibition of cell cycle progression[127]. Serum deprivation also results in the induction of high levels of C/EBPα, although this expression has not been directly linked to inhibition of proliferation. These studies point to an antiproliferative role for C/EBPα in response to various cellular stressors, and suggest a potential for tumor suppressor function.

The multifaceted increase in carcinogen-induced skin tumorigenesis found in epidermal specific C/EBPα knockout mice demonstrated here is the first genetic evidence for C/EBPα as tumor suppressor in epithelial tissues. Analysis of C/EBPα$^{-/-}$ tumors revealed an increased rate of growth, an expanded proliferative compartment, and an increased conversion to malignancy compared to control tumors. C/EBPα was also able to inhibit ras-induced proliferation and both Ras and EGF-induced E2F1 transcription[122]. Previous work revealed that decreased expression of C/EBPα was associated with tumors containing oncogenic Ras, and that re-expression of C/EBPα in SCC cell lines results in inhibited proliferation[123]. These data support the hypothesis that C/EBPα's growth inhibitory abilities are involved in its epithelial tumor suppressor functions.

C/EBPβ has been attributed with pro-oncogenic functions such as proliferation and survival, as well as with tumor-suppressive properties such as growth arrest, differentiation, and senescence[128]. In the epidermis specifically, the role for C/EBPβ is similarly confounding. C/EBPβ is required for the normal expression of the differentiation markers keratins 1 and 10, and C/EBPβ deficiency results in modest epidermal hyperproliferation[64]. Forced expression studies also indicate an antiproliferative function for C/EBPβ, although their physiological relevance is questionable. These seemingly tumor suppressive properties of C/EBPβ however do not translate into suppression of epidermal tumorigenesis in response to carcinogen treatment. Rather, C/EBPβ$^{-/-}$ mice are completely refractory to carcinogen-induced tumorigenesis involving oncogenic Ras[121].

The mechanism by which C/EBPβ$^{-/-}$ mice are able to resist tumorigenesis is not yet determined. An important clue towards elucidating the mechanism of tumor resistance is the increased p53-dependent apoptosis observed in C/EBPβ$^{-/-}$ epidermis in response to DMBA treatment[121, 129]. Theoretically, C/EBPβ could promote survival of initiated cells after carcinogen treatment, thereby promoting the onset of tumorigenesis. The number/percentage of initiated cells harboring the requisite Ras mutations is very low even in WT animals, limiting the ability to test this hypothesis. Further, as tumors do not develop in C/EBPβ$^{-/-}$ epidermis, there are no tumors with which to study.

In the absence of C/EBPα, C/EBPβ function is potentially unmasked as C/EBPs do not function in isolation, but rather form homo-and hetero-dimers with other C/EBP family members[28]. Observations from tumor studies in epidermal-specific C/EBPα$^{-/-}$ mice are therefore relevant in the study of C/EBPβ function. C/EBPα$^{-/-}$ mouse carcinogen-induced tumorigenesis studies revealed an increase in tumor number, decreased tumor latency, increased tumor growth rate, and an increased rate of malignant progression. Promotion of survival is alone not sufficient to induce these multifaceted changes. It seems perhaps then, that multiple C/EBPβ functions could be contributing to its cancer phenotype.

C/EBPα and C/EBPβ may contribute to tumorigenesis through the inhibition and enhancement of E2F-induced proliferation, respectively. C/EBPα and C/EBPβ have both been implicated in the regulation of proliferation through interactions with E2F[66, 72]. C/EBPα has been implicated in inhibition of proliferation through association with an E2F-DNA binding complex, while C/EBPβ has also been shown to interact with E2F but in a growth promoting manner. These data are supported by the E2F1 promoter assays presented here which show similar results. The balance between the repressive role of C/EBPα and the stimulatory role of C/EBPβ on E2F transcription is theoretically of key importance in regulating normal cellular proliferation.

Mitogenic stimulation results in the reciprocal regulation of C/EBPα and C/EBPβ mRNA, protein, and DNA binding. Reciprocal regulation of C/EBPα

and C/EBPβ is a prerequisite for liver regeneration after partial hepatectomy, and a decreased ratio of C/EBPα to C/EBPβ in these cells was associated with increased cell cycle progression[130]. C/EBPα levels are diminished in numerous epithelial tumors associated with activating mutations in Ras, and Ras signaling specifically downregulates C/EBPα levels while not affecting C/EBPβ expression[123]. The reciprocal regulation of C/EBPα and C/EBPβ by an EGFR/Ras/MEK/ERK pathway as described here identifies a specific mechanism by which these mitogenic signals can preferentially activate C/EBPβ and promote cell cycle progression. EGFR signaling is capable of activating both C/EBP isoforms, but the downregulation of C/EBPα negates the relevance of its activation. The downregulation of C/EBPα and the upregulation of C/EBPβ occur through independent EGFR/Ras signaling pathways, as a

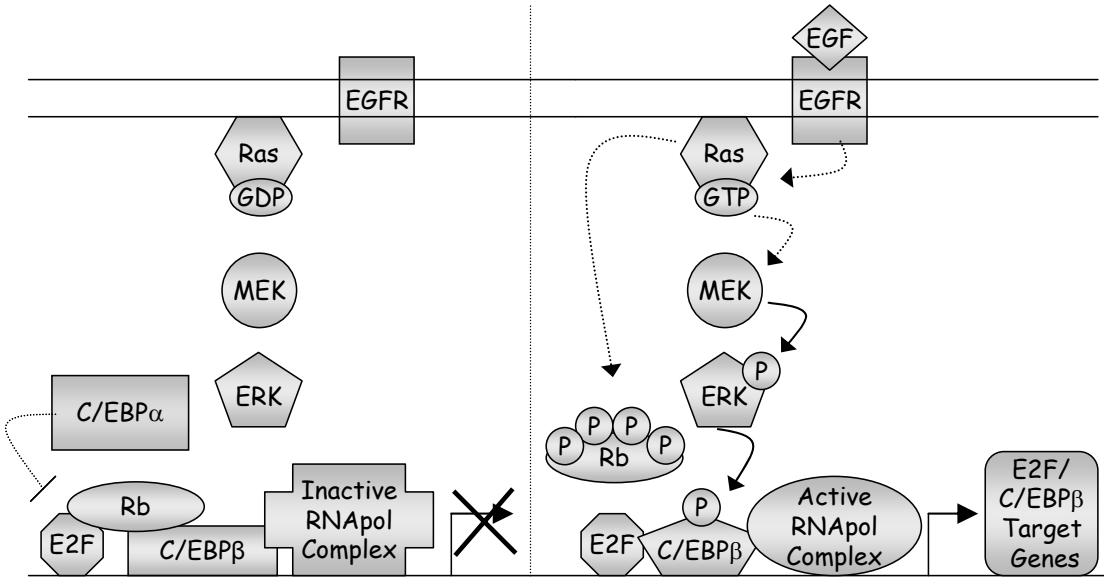


Figure 1. Proposed mechanistic role for C/EBPα and C/EBPβ in the regulation of E2F transcription and cell cycle progression

MEK inhibitor was able to block EGF's induction of C/EBPβ expression, but not of EGF's inhibition of C/EBPα expression. A proposed model for C/EBPα and C/EBPβ in the EGF induced up-regulation of E2F1 trancription and cell cycle progression is shown in figure 1.

These data demonstrate that C/EBPα is an epithelial tumor suppressor gene, and that C/EBPα expression is diminished while C/EBPβ expression is enhanced by EGFR and Ras signaling. The EGF- and Ras-induced reciprocal regulation of E2F activity by C/EBPα and C/EBPβ poses a potential mechanism by which C/EBPα and C/EBPβ may function in the regulation of proliferation. EGFR and Ras are key proteins implicated in epithelial cancer development, and their reciprocal regulation of C/EBPα and C/EBPβ suggests that the balance between the growth suppressive properties of C/EBPα and the proliferative properties of C/EBPβ are important in their induction of cell cycle progression and possibly in tumorigenesis.

GENERAL REFERENCES

1. Fuchs, E. & Horsley, V. More than one way to skin. Genes Dev. 22, 976-985 (2008).
2. Hedrich, H. J. & Bullock, G. R. The laboratory mouse (Elsevier Academic Press, Amsterdam ; Boston, 2004).
3. Niemann, C. & Watt, F. M. Designer skin: lineage commitment in postnatal epidermis. Trends Cell Biol 12, 185-92 (2002).
4. Dai, X. & Segre, J. A. Transcriptional control of epidermal specification and differentiation. Curr Opin Genet Dev 14, 485-91 (2004).
5. Chuong, C. M. et al. What is the 'true' function of skin? Exp Dermatol 11, 159-87 (2002).
6. Fuchs, E. & Raghavan, S. Getting under the skin of epidermal morphogenesis. Nat Rev Genet 3, 199-209 (2002).
7. Atit, R., Conlon, R. A. & Niswander, L. EGF signaling patterns the feather array by promoting the interbud fate. Dev Cell 4, 231-40 (2003).
8. Koster, M. I. & Roop, D. R. Mechanisms Regulating Epithelial Stratification. Annual Review of Cell and Developmental Biology 23, 93-113 (2007).
9. Watt, F. M. Epidermal stem cells: markers, patterning and the control of stem cell fate. Philos Trans R Soc Lond B Biol Sci 353, 831-7 (1998).
10. Taylor, G., Lehrer, M. S., Jensen, P. J., Sun, T. T. & Lavker, R. M. Involvement of follicular stem cells in forming not only the follicle but also the epidermis. Cell 102, 451-61 (2000).

11. Watt, F. M., Celso, C. L. & Silva-Vargas, V. Epidermal stem cells: an update. Current Opinion in Genetics & Development 16, 518-524 (2006).

12. Ghazizadeh, S. & Taichman, L. B. Multiple classes of stem cells in cutaneous epithelium: a lineage analysis of adult mouse skin. Embo J 20, 1215-22 (2001).

13. Claudinot, S., Nicolas, M., Oshima, H., Rochat, A. & Barrandon, Y. From The Cover: Long-term renewal of hair follicles from clonogenic multipotent stem cells. Proceedings of the National Academy of Sciences 102, 14677-14682 (2005).

14. Reynolds, A. J. & Jahoda, C. A. Cultured dermal papilla cells induce follicle formation and hair growth by transdifferentiation of an adult epidermis. Development 115, 587-93 (1992).

15. Ferraris, C., Bernard, B. A. & Dhouailly, D. Adult epidermal keratinocytes are endowed with pilosebaceous forming abilities. Int J Dev Biol 41, 491-8 (1997).

16. Levy, V., Lindon, C., Harfe, B. D. & Morgan, B. A. Distinct stem cell populations regenerate the follicle and interfollicular epidermis. Dev Cell 9, 855-61 (2005).

17. Ito, M. et al. Stem cells in the hair follicle bulge contribute to wound repair but not to homeostasis of the epidermis. Nat Med 11, 1351-1354 (2005).

18. Jones, P. H., Harper, S. & Watt, F. M. Stem cell patterning and fate in human epidermis. Cell 80, 83-93 (1995).

19. Jensen, K. B. & Watt, F. M. Single-cell expression profiling of human epidermal stem and transit-amplifying cells: Lrig1 is a regulator of stem cell quiescence. Proceedings of the National Academy of Sciences 103, 11958-11963 (2006).

20. Cairns, J. Cancer and the Immortal Strand Hypothesis. Genetics 174, 1069-1072 (2006).

21. Potten, C. S. Keratinocyte Stem Cells, Label-Retaining Cells and Possible Genome Protection Mechanisms. J Investig Dermatol Symp Proc 9, 183-195 (2004).

22. Lechler, T. & Fuchs, E. Asymmetric cell divisions promote stratification and differentiation of mammalian skin. Nature 437, 275-80 (2005).

23. Clayton, E. et al. A single type of progenitor cell maintains normal epidermis. Nature 446, 185-9 (2007).

24. Graves, B. J., Johnson, P. F. & McKnight, S. L. Homologous recognition of a promoter domain common to the MSV LTR and the HSV tk gene. Cell 44, 565-76 (1986).

25. Johnson, P. F., Landschulz, W. H., Graves, B. J. & McKnight, S. L. Identification of a rat liver nuclear protein that binds to the enhancer core element of three animal viruses. Genes Dev 1, 133-46 (1987).

26. Landschulz, W. H., Johnson, P. F., Adashi, E. Y., Graves, B. J. & McKnight, S. L. Isolation of a recombinant copy of the gene encoding C/EBP. Genes Dev 2, 786-800 (1988).

27. McKnight, S. L. McBindall--a better name for CCAAT/enhancer binding proteins? Cell 107, 259-61 (2001).

28. Ramji, D. P. & Foka, P. CCAAT/enhancer-binding proteins: structure, function and regulation. Biochem J 365, 561-75 (2002).

29. Yamanaka, R. et al. CCAAT/enhancer binding protein epsilon is preferentially up-regulated during granulocytic differentiation and its functional versatility is determined by alternative use of promoters and differential splicing. Proc Natl Acad Sci U S A 94, 6462-7 (1997).

30. Lekstrom-Himes, J. A. The role of C/EBP(epsilon) in the terminal stages of granulocyte differentiation. Stem Cells 19, 125-33 (2001).

31. Descombes, P. & Schibler, U. A liver-enriched transcriptional activator protein, LAP, and a transcriptional inhibitory protein, LIP, are translated from the same mRNA. Cell 67, 569-79 (1991).

32. Ossipow, V., Descombes, P. & Schibler, U. CCAAT/enhancer-binding protein mRNA is translated into multiple proteins with different transcription activation potentials. Proc Natl Acad Sci U S A 90, 8219-23 (1993).

33. Cooper, C., Henderson, A., Artandi, S., Avitahl, N. & Calame, K. Ig/EBP (C/EBP gamma) is a transdominant negative inhibitor of C/EBP family transcriptional activators. Nucleic Acids Res 23, 4371-7 (1995).

34. Agre, P., Johnson, P. F. & McKnight, S. L. Cognate DNA binding specificity retained after leucine zipper exchange between GCN4 and C/EBP. Science 246, 922-6 (1989).

35. Gao, H., Parkin, S., Johnson, P. F. & Schwartz, R. C. C/EBPgamma Has a Stimulatory Role on the IL-6 and IL-8 Promoters. J. Biol. Chem. 277, 38827-38837 (2002).

36. Osada, S., Yamamoto, H., Nishihara, T. & Imagawa, M. DNA binding specificity of the CCAAT/enhancer-binding protein transcription factor family. J Biol Chem 271, 3891-6 (1996).

37. Johnson, P. F. Identification of C/EBP basic region residues involved in DNA sequence recognition and half-site spacing preference. Mol Cell Biol 13, 6919-30 (1993).

38. Ross, S. E. et al. Phosphorylation of C/EBP{alpha} Inhibits Granulopoiesis. Mol. Cell. Biol. 24, 675-686 (2004).

39. Mo, X., Kowenz-Leutz, E., Xu, H. & Leutz, A. Ras induces mediator complex exchange on C/EBP beta. Mol Cell 13, 241-50 (2004).

40. Skrtic, S. et al. Changes in expression of CCAAT/enhancer binding protein alpha (C/EBP alpha) and C/EBP beta in rat liver after partial hepatectomy but not after treatment with cyproterone acetate. J Hepatol 27, 903-11 (1997).

41. Jones, L. C. et al. Expression of C/EBPbeta from the C/ebpalpha gene locus is sufficient for normal hematopoiesis in vivo. Blood 99, 2032-6 (2002).

42. Ubeda, M. et al. Stress-induced binding of the transcriptional factor CHOP to a novel DNA control element. Mol Cell Biol 16, 1479-89 (1996).

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

primary keratinocytes and is altered in skin neoplasms. J Invest Dermatol 110, 939-45 (1998).

44. Maytin, E. V. & Habener, J. F. Transcription factors C/EBP alpha, C/EBP beta, and CHOP (Gadd153) expressed during the differentiation program of keratinocytes in vitro and in vivo. J Invest Dermatol 110, 238-46 (1998).

45. Bull, J. J. et al. Contrasting Expression Patterns of CCAAT//Enhancer-Binding Protein Transcription Factors in the Hair Follicle and at Different Stages of the Hair Growth Cycle. 118, 17-24 (2002).

46. Swart, G. W., van Groningen, J. J., van Ruissen, F., Bergers, M. & Schalkwijk, J. Transcription factor C/EBPalpha: novel sites of expression and cloning of the human gene. Biol Chem 378, 373-9 (1997).

47. Williams, S. C., Cantwell, C. A. & Johnson, P. F. A family of C/EBP-related proteins capable of forming covalently linked leucine zipper dimers in vitro. Genes Dev 5, 1553-67 (1991).

48. Cao, Z., Umek, R. M. & McKnight, S. L. Regulated expression of three C/EBP isoforms during adipose conversion of 3T3-L1 cells. Genes Dev 5, 1538-52 (1991).

49. Antonson, P. & Xanthopoulos, K. G. Molecular cloning, sequence, and expression patterns of the human gene encoding CCAAT/enhancer binding protein alpha (C/EBP alpha). Biochem Biophys Res Commun 215, 106-13 (1995).

50. Akira, S. et al. A nuclear factor for IL-6 expression (NF-IL6) is a member of a C/EBP family. Embo J 9, 1897-906 (1990).

51. Poli, V., Mancini, F. P. & Cortese, R. IL-6DBP, a nuclear protein involved in interleukin-6 signal transduction, defines a new family of leucine zipper proteins related to C/EBP. Cell 63, 643-53 (1990).

52. Descombes, P., Chojkier, M., Lichtsteiner, S., Falvey, E. & Schibler, U. LAP, a novel member of the C/EBP gene family, encodes a liver-enriched transcriptional activator protein. Genes Dev 4, 1541-51 (1990).

53. Chang, C. J., Chen, T. T., Lei, H. Y., Chen, D. S. & Lee, S. C. Molecular cloning of a transcription factor, AGP/EBP, that belongs to members of the C/EBP family. Mol Cell Biol 10, 6642-53 (1990).

54. Katz, S. et al. The NF-M transcription factor is related to C/EBP beta and plays a role in signal transduction, differentiation and leukemogenesis of avian myelomonocytic cells. Embo J 12, 1321-32 (1993).

55. Kousteni, S., Kockar, F. T., Sweeney, G. E. & Ramji, D. P. Characterisation and developmental regulation of the Xenopus laevis CCAAT-enhancer binding protein beta gene. Mech Dev 77, 143-8 (1998).

56. Wang, H. et al. C/EBPbeta is a negative regulator of human papillomavirus type 11 in keratinocytes. J Virol 70, 4839-44 (1996).

57. Roman, C., Platero, J. S., Shuman, J. & Calame, K. Ig/EBP-1: a ubiquitously expressed immunoglobulin enhancer binding protein that is similar to C/EBP and heterodimerizes with C/EBP. Genes Dev. 4, 1404-1415 (1990).

58. Mullins, D. N. et al. CEBPG transcription factor correlates with antioxidant and DNA repair genes in normal bronchial epithelial cells but not in individuals with bronchogenic carcinoma. BMC Cancer 5, 141 (2005).

59. Kinoshita, S., Akira, S. & Kishimoto, T. A member of the C/EBP family, NF-IL6 beta, forms a heterodimer and transcriptionally synergizes with NF-IL6. Proc Natl Acad Sci U S A 89, 1473-6 (1992).

60. Huang, A. M. et al. Loss of CCAAT//enhancer binding protein [delta] promotes chromosomal instability. Oncogene 23, 1549-1557 (2004).

61. Huang, A. M. et al. The Cebpd (C/EBPÎ´) Gene Is Induced by Luteinizing Hormones in Ovarian Theca and Interstitial Cells But Is Not Essential for Mouse Ovary Function. PLoS ONE 2, e1334 (2007).

62. O'Rourke, J., Yuan, R. & DeWille, J. CCAAT/Enhancer-binding Protein-delta (C/EBP-delta) Is Induced in Growth-arrested Mouse Mammary Epithelial Cells. J. Biol. Chem. 272, 6291-6296 (1997).

63. Oyadomari, S. & Mori, M. Roles of CHOP//GADD153 in endoplasmic reticulum stress. Cell Death Differ 11, 381-389 (2003).

64. Zhu, S. et al. C/EBPbeta modulates the early events of keratinocyte differentiation involving growth arrest and keratin 1 and keratin 10 expression. Mol Cell Biol 19, 7181-90 (1999).

65. Bull, J. J. et al. Contrasting expression patterns of CCAAT/enhancer-binding protein transcription factors in the hair follicle and at different stages of the hair growth cycle. J Invest Dermatol 118, 17-24 (2002).

66. Schuster, M. B. & Porse, B. T. C/EBPalpha: a tumour suppressor in multiple tissues? Biochim Biophys Acta 1766, 88-103 (2006).

67. Di-Poi, N., Desvergne, B., Michalik, L. & Wahli, W. Transcriptional Repression of Peroxisome Proliferator-activated Receptor {beta}/{delta} in Murine Keratinocytes by CCAAT/Enhancer-binding Proteins. J. Biol. Chem. 280, 38700-38710 (2005).

68. Calella, A. M. et al. Neurotrophin/Trk receptor signaling mediates C/EBPalpha, -beta and NeuroD recruitment to immediate-early gene promoters in neuronal cells and requires C/EBPs to induce immediate-early gene transcription. Neural Develop 2, 4 (2007).

69. Wang, N. D. et al. Impaired energy homeostasis in C/EBP alpha knockout mice. Science 269, 1108-12 (1995).

70. Sterneck, E., Tessarollo, L. & Johnson, P. F. An essential role for C/EBPbeta in female reproduction. Genes Dev. 11, 2153-2162 (1997).

71. Seagroves, T. N. et al. C/EBPbeta , but not C/EBPalpha , is essential for ductal morphogenesis, lobuloalveolar proliferation, and functional differentiation in the mouse mammary gland. Genes Dev. 12, 1917-1928 (1998).

72. Sebastian, T. & Johnson, P. F. Stop and go: anti-proliferative and mitogenic functions of the transcription factor C/EBPbeta. Cell Cycle 5, 953-7 (2006).

73. Liu, S. et al. Hypoglycemia and impaired hepatic glucose production in mice with a deletion of the C/EBPbeta gene. J Clin Invest 103, 207-13 (1999).

74. Parkin, S. E., Baer, M., Copeland, T. D., Schwartz, R. C. & Johnson, P. F. Regulation of CCAAT/Enhancer-binding Protein (C/EBP) Activator Proteins by Heterodimerization with C/EBPgamma (Ig/EBP). J. Biol. Chem. 277, 23563-23572 (2002).

75. Crawford, E. L. et al. CEBPG regulates ERCC5/XPG expression in human bronchial epithelial cells and this regulation is modified by E2F1/YY1 interactions. Carcinogenesis 28, 2552-9 (2007).

76. Kaisho, T. et al. Impairment of natural killer cytotoxic activity and interferon gamma production in CCAAT/enhancer binding protein gamma-deficient mice. J Exp Med 190, 1573-82 (1999).

77. Breed, D. R., Margraf, L. R., Alcorn, J. L. & Mendelson, C. R. Transcription Factor C/EBP{delta} in Fetal Lung: Developmental Regulation and Effects of Cyclic Adenosine 3',5'-Monophosphate and Glucocorticoids. Endocrinology 138, 5527-5534 (1997).

78. Scott, L. M., Civin, C. I., Rorth, P. & Friedman, A. D. A novel temporal expression pattern of three C/EBP family members in differentiating myelomonocytic cells. Blood 80, 1725-1735 (1992).

79. O'Rourke, J. P., Newbound, G. C., Hutt, J. A. & DeWille, J. CCAAT/Enhancer-binding Protein delta Regulates Mammary Epithelial Cell G0 Growth Arrest and Apoptosis. J. Biol. Chem. 274, 16582-16589 (1999).

80. Barbaro, V. et al. C/EBP{delta} regulates cell cycle and self-renewal of human limbal stem cells. J. Cell Biol. 177, 1037-1049 (2007).

81. Smith, C. et al. Regulation of desmocollin gene expression in the epidermis: CCAAT/enhancer-binding proteins modulate early and late events in keratinocyte differentiation. Biochem J 380, 757-65 (2004).

82. Sterneck, E., Zhu, S., Ramirez, A., Jorcano, J. L. & Smart, R. C. Conditional ablation of C/EBP beta demonstrates its keratinocyte-specific requirement for cell survival and mouse skin tumorigenesis. Oncogene 25, 1272-6 (2006).

83. Cantwell, C. A., Sterneck, E. & Johnson, P. F. Interleukin-6-specific activation of the C/EBPdelta gene in hepatocytes is mediated by Stat3 and Sp1. Mol Cell Biol 18, 2108-17 (1998).

84. Yamada, T., Tobita, K., Osada, S., Nishihara, T. & Imagawa, M. CCAAT/Enhancer-Binding Protein {delta} Gene Expression Is Mediated by APRF/STAT3. J Biochem 121, 731-738 (1997).

85. Sterneck, E. et al. Selectively enhanced contextual fear conditioning in mice lacking the transcriptional regulator CCAAT/enhancer binding protein delta. Proc Natl Acad Sci U S A 95, 10908-13 (1998).

86. Taubenfeld, S. M. et al. Fornix-dependent induction of hippocampal CCAAT enhancer-binding protein [beta] and [delta] Co-localizes with phosphorylated cAMP response element-binding protein and accompanies long-term memory consolidation. J Neurosci 21, 84-91 (2001).

87. Agrawal, S. et al. The C/EBP{delta} tumor suppressor is silenced by hypermethylation in acute myeloid leukemia. Blood 109, 3895-3905 (2007).

88. Chumakov, A. M. et al. Cloning of the novel human myeloid-cell-specific C/EBP-epsilon transcription factor. Mol. Cell. Biol. 17, 1375-1386 (1997).

89. Yamanaka, R. et al. Impaired granulopoiesis, myelodysplasia, and early lethality in CCAAT/enhancer binding protein varepsilon -deficient mice. Proceedings of the National Academy of Sciences 94, 13187-13192 (1997).

90. Hattori, H. et al. Identification of a responsible promoter region and a key transcription factor, CCAAT/enhancer-binding protein Îµ, for up-regulation of PHGPx in HL60 cells stimulated with TNFÎ±. Biochem J 408, 277-286 (2007).

91. Nakajima, H. et al. N-terminal Region of CCAAT/Enhancer-binding Protein {epsilon} Is Critical for Cell Cycle Arrest, Apoptosis, and Functional Maturation during Myeloid Differentiation. J. Biol. Chem. 281, 14494-14502 (2006).

92. Ron, D. & Habener, J. F. CHOP, a novel developmentally regulated nuclear protein that dimerizes with transcription factors C/EBP and LAP and functions as a dominant-negative inhibitor of gene transcription. Genes Dev. 6, 439-453 (1992).

93. Ubeda, M., Vallejo, M. & Habener, J. F. CHOP enhancement of gene transcription by interactions with Jun/Fos AP-1 complex proteins. Mol Cell Biol 19, 7589-99 (1999).

94. Barone, M. V., Crozat, A., Tabaee, A., Philipson, L. & Ron, D. CHOP (GADD153) and its oncogenic variant, TLS-CHOP, have opposing effects on the induction of G1/S arrest. Genes Dev 8, 453-64 (1994).

95. Matsumoto, M., Minami, M., Takeda, K., Sakao, Y. & Akira, S. Ectopic expression of CHOP (GADD153) induces apoptosis in M1 myeloblastic leukemia cells. FEBS Lett 395, 143-7 (1996).

96. Oyadomari, S. et al. Targeted disruption of the Chop gene delays endoplasmic reticulum stress-mediated diabetes. J Clin Invest 109, 525-32 (2002).

97. Oyadomari, S. et al. Nitric oxide-induced apoptosis in pancreatic beta cells is mediated by the endoplasmic reticulum stress pathway. Proceedings of the National Academy of Sciences 98, 10845-10850 (2001).

98. Marques, M. M., Martinez, N., Rodriguez-Garcia, I. & Alonso, A. EGFR family-mediated signal transduction in the human keratinocyte cell line HaCaT. Exp Cell Res 252, 432-8 (1999).

99. Hackel, P. O., Zwick, E., Prenzel, N. & Ullrich, A. Epidermal growth factor receptors: critical mediators of multiple receptor pathways. Curr Opin Cell Biol 11, 184-9 (1999).

100. Press, M. F., Cordon-Cardo, C. & Slamon, D. J. Expression of the HER-2/neu proto-oncogene in normal human adult and fetal tissues. Oncogene 5, 953-62 (1990).

101. Prigent, S. A. et al. Expression of the c-erbB-3 protein in normal human adult and fetal tissues. Oncogene 7, 1273-8 (1992).

102. Rittie, L. et al. Differential ErbB1 Signaling in Squamous Cell versus Basal Cell Carcinoma of the Skin. Am J Pathol 170, 2089-2099 (2007).

103. Olayioye, M. A., Neve, R. M., Lane, H. A. & Hynes, N. E. The ErbB signaling network: receptor heterodimerization in development and cancer. Embo J 19, 3159-67 (2000).

104. Coffey, R. J., Jr. et al. Production and auto-induction of transforming growth factor-alpha in human keratinocytes. Nature 328, 817-20 (1987).

105. Shoyab, M., Plowman, G. D., McDonald, V. L., Bradley, J. G. & Todaro, G. J. Structure and function of human amphiregulin: a member of the epidermal growth factor family. Science 243, 1074-6 (1989).

106. Higashiyama, S., Abraham, J. A., Miller, J., Fiddes, J. C. & Klagsbrun, M. A heparin-binding growth factor secreted by macrophage-like cells that is related to EGF. Science 251, 936-9 (1991).

107. Shirakata, Y. et al. Epiregulin, a novel member of the epidermal growth factor family, is an autocrine growth factor in normal human keratinocytes. J Biol Chem 275, 5748-53 (2000).

108. Barnard, J. A. et al. Auto- and cross-induction within the mammalian epidermal growth factor- related peptide family. J. Biol. Chem. 269, 22817-22822 (1994).

109. Miettinen, P. J. et al. Epithelial immaturity and multiorgan failure in mice lacking epidermal growth factor receptor. Nature 376, 337-341 (1995).

110. Hansen, L. A. et al. The Epidermal Growth Factor Receptor Is Required to Maintain the Proliferative Population in the Basal Compartment of Epidermal Tumors. Cancer Res 60, 3328-3332 (2000).

111. Stoll, S. W. et al. Differential utilization and localization of ErbB receptor tyrosine kinases in skin compared to normal and malignant keratinocytes. Neoplasia 3, 339-50 (2001).

112. De Potter, I. Y., Poumay, Y., Squillace, K. A. & Pittelkow, M. R. Human EGF receptor (HER) family and heregulin members are differentially expressed in epidermal keratinocytes and modulate differentiation. Exp Cell Res 271, 315-28 (2001).

113. Hanahan, D. & Weinberg, R. A. The hallmarks of cancer. Cell 100, 57-70 (2000).

114. Woodworth, C. D., Gaiotti, D., Michael, E., Hansen, L. & Nees, M. Targeted Disruption of the Epidermal Growth Factor Receptor Inhibits Development of Papillomas and Carcinomas from Human Papillomavirus-immortalized Keratinocytes. Cancer Res 60, 4397-4402 (2000).

115. L'Allemain, G., Sturgill, T. W. & Weber, M. J. Defective regulation of mitogen-activated protein kinase activity in a 3T3 cell variant mitogenically nonresponsive to tetradecanoyl phorbol acetate. Mol. Cell. Biol. 11, 1002-1008 (1991).

116. Smart, R. C., Ewing, S.J., and Loomis, K.D. . Carcinogenesis (ed. Smart, R. C., and Hodgson, E) (Wiley Interscience, New York, 2008).

117. Harvat, B. L., Wang, A., Seth, P. & Jetten, A. M. Up-regulation of p27Kip1, p21WAF1/Cip1 and p16Ink4a is associated with, but not sufficient for, induction of squamous differentiation. J Cell Sci 111 (Pt 9), 1185-96 (1998).

118. Dellambra, E. et al. Downregulation of 14-3-3{sigma} Prevents Clonal Evolution and Leads to Immortalization of Primary Human Keratinocytes. J. Cell Biol. 149, 1117-1130 (2000).

119. Adams, J. M. & Strasser, A. Is Tumor Growth Sustained by Rare Cancer Stem Cells or Dominant Clones? Cancer Res 68, 4018-4021 (2008).

120. Umek, R. M., Friedman, A. D. & McKnight, S. L. CCAAT-Enhancer Binding Protein: A Component of a Differentiation Switch. Science 251, 288-292 (1991).

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

122. Loomis, K. D., Zhu, S., Yoon, K., Johnson, P. F. & Smart, R. C. Genetic ablation of CCAAT/enhancer binding protein alpha in epidermis reveals its role in suppression of epithelial tumorigenesis. Cancer Res 67, 6768-76 (2007).

123. Shim, M., Powers, K. L., Ewing, S. J., Zhu, S. & Smart, R. C. Diminished expression of C/EBPalpha in skin carcinomas is linked to oncogenic Ras and reexpression of C/EBPalpha in carcinoma cells inhibits proliferation. Cancer Res 65, 861-7 (2005).

124. Schuster, M. B. & Porse, B. T. C/EBPalpha: A tumour suppressor in multiple tissues? Biochim Biophys Acta (2006).

125. Lamb, J. et al. A mechanism of cyclin D1 action encoded in the patterns of gene expression in human cancer. Cell 114, 323-34 (2003).

126. Di Cunto, F. et al. Inhibitory Function of p21Cip1/WAF1 in Differentiation of Primary Mouse Keratinocytes Independent of Cell Cycle Control. Science 280, 1069-1072 (1998).

127. Yoon, K. & Smart, R. C. C/EBPalpha is a DNA damage-inducible p53-regulated mediator of the G1 checkpoint in keratinocytes. Mol Cell Biol 24, 10650-60 (2004).

128. Johnson, P. F. Molecular stop signs: regulation of cell-cycle arrest by C/EBP transcription factors. J Cell Sci 118, 2545-55 (2005).

129. Yoon, K., Zhu, S., Ewing, S. J. & Smart, R. C. Decreased survival of C/EBP beta-deficient keratinocytes is due to aberrant regulation of p53 levels and function. Oncogene 26, 360-7 (2007).

130. Takiguchi, M. The C/EBP family of transcription factors in the liver and other organs. International Journal of Experimental Pathology 79, 369-391 (1998).

APPENDIX

C/EBPβ deficiency results in the impaired induction of E2F target genes by EGF stimulation. The significant reduction in C/EBPβ$^{-/-}$ keratinocytes entering S-phase, concomitant with the increased DNA binding and activity of C/EBPβ in proliferating keratinocytes after stimulation with EGF, suggest a possible role for C/EBPβ in the transcription of genes involved in entry into S phase (chapter 2). A cell cycle specific qRT-PCR array was used to identify which, if any genes are differentially regulated by EGF in cells lacking C/EBPβ. Primary keratinocytes were serum deprived and then stimulated with EGF and harvested at 10 hours. A number of genes were altered in the C/EBPβ$^{-/-}$ samples, and their fold down- or up-regulation are listed in figures 1A and B, respectively. The downregulated genes are involved in various functions including regulation of the cell cycle and cell cycle machinery, DNA damage response, mitotic growth arrest, and survival. Interestingly, 10 out of 12 of the most down-regulated genes in the C/EBPβ$^{-/-}$ samples are also E2F target genes (indicated by asterisks in figures 1A and B). These data are somewhat preliminary as reflected in the absence of p-values, but suggest a possible role for C/EBPβ in the stimulation of E2F-induced transcription. Taken with the decreased EGF-induced cell cycle progression of C/EBPβ$^{-/-}$ keratinocytes and C/EBPβ's ability to stimulate E2F-induced transcription, the results shown here are consistent with the hypothesis that C/EBPβ and E2F cooperate to induce genes required for cell cycle entry. Further studies are required to confirm this data, and future experiments are planned to determine the exact relationship

between the EGF-induced upregulation and activation of C/EBPβ, and E2F induced gene transcription including: 1. Chromatin immunoprecipitation (ChIP) using a C/EBPβ-specific antibody and amplification of E2F target gene promoter regions to determine whether C/EBPβ is binding to E2F target gene promoters in living cells, 2. EMSA supershift analysis and co-immunoprecipitation (Co-IP) of C/EBPβ and E2F and/or Rb to determine whether C/EBPβ is in a binding complex with either protein, and 3. Assess the effects of EGF treatment on these potential binding complexes.

MATERIALS AND METHODS

Cell Cycle Specific qRT-PCR array

Differential expression of EGF-induced genes in C/EBPβ$^{-/-}$ vs C/EBPβ$^{+/+}$ primary keratinocytes was assessed by using the cell cycle specific qRT-PCR array from Superarray (PAMM-020A). RNA was isolated, treated with DNase, and cleaned up as described for qRT-PCR above. Analysis was performed according to the manufacturer's protocol, using 1µg of total RNA for the RT reaction. Data analysis was performed using the Superarray web-based data analysis tool, and is based on the 2^^CT method.

A

Gene Symbol	Fold Down Regulation WT/ KO	P-value
Mad2l1*	2.06	
Sesn2	2.03	
E2F2*	2.01	
Chek1*	1.77	
Mki67*	1.77	
Atm*	1.70	
Mre11a	1.59	
Ccna2*	1.57	
Nek2*	1.57	
E2f1*	1.54	

B

Gene Symbol	Fold Up-Regulation WT/ KO	P-value
Cdkn2a*	7.65	
Apbb1	3.26	
Gadd45a	3.06	
Ccnd1*	2.74	
Ak1	2.17	
Pmp22	1.82	
Mdm2	1.77	
Inha	1.6	
CamK2a	>8	
Ccna1	>8	

Figure 1. C/EBPβ$^{-/-}$ keratinocytes have diminished expression of E2F target genes

A and B. Numerous genes are differentially regulated in C/EBPβ$^{-/-}$ primary keratinocytes after stimulation with EGF. Results represent fold down (A) or up (B) regulation of cell cycle related genes as determined by a cell cycle specific qRT-PCR array. WT and C/EBPβ$^{-/-}$ keratinocytes were synchronized through incubation in serum free EMEM supplemented with 1mg/ml BSA and HEPES pH 7.4 for 40 hours. At time zero, the medium was replaced with the EMEM with BSA and HEPES, as well as with 4ng/ml EGF. Total RNA was isolated 10 hours after EGF treatment as matched samples with the cell cycle entry experiment shown in figure 4C of chapter 2.