ABSTRACT

HOUSE, JOHN STEVEN. Simultaneous Removal of C/EBPα and C/EBPβ Reveals Their Critical Roles in Sebocyte and Keratinocyte Differentiation. (Under the direction of Dr. Robert C. Smart.)

C/EBPα and C/EBPβ are bZIP transcription factors and are highly expressed in the interfollicular epidermis and sebaceous glands of skin with known and emerging roles in proliferation, tumorigenesis, metabolism, and differentiation in a multitude of cell types/tissues. Despite their expression levels in skin, past experiments with germ line deletion in skin of C/EBPα or C/EBPβ alone had only a mild or no effect on keratinocyte and sebocyte biology. To address possible functional redundancies and reveal functional roles of C/EBPα and C/EBPβ in postnatal skin, mouse models were developed in which either family member could be acutely ablated alone or together in the epidermis and sebaceous glands of adult mice. Co-ablation of C/EBPα and C/EBPβ in postnatal epidermis resulted in disruption of stratified squamous differentiation characterized by hyperproliferation of basal and suprabasal keratinocytes and a defective basal to spinous keratinocyte transition involving an expanded basal compartment and a diminished and delayed spinous compartment. Acute co-ablation of C/EBPα and C/EBPβ in sebaceous glands resulted in severe morphological defects, and sebocyte differentiation was blocked as determined by lack of sebum production and reduced expression of stearoyl-CoA desaturase3 (SCD3) and melanocortin 5 receptor (MC5R), two markers of terminal sebocyte differentiation. In order to assess whether C/EBPα and C/EBPβ have a role in human sebocyte differentiation, human immortalized sebocytes (SEB-1) were used. To begin to determine whether C/EBPα and C/EBPβ have a role in human sebocyte differentiation and sebaceous gland lipid

production, we first examined the spatial distribution of C/EBPα and C/EBPβ within human sebaceous glands. C/EBPα and C/EBPβ were expressed in distinct spatial patterns in human and mouse sebocytes in vivo; C/EBPα was expressed in differentiating interior sebocytes but rarely present in the peripheral undifferentiated proliferative population while C/EBPβ was expressed in both peripheral undifferentiated and differentiated sebocytes. Mouse sebaceous glands displayed a similar expression pattern and when C/EBPα and C/EBPβ were concurrently ablated from adult mouse sebaceous glands in vivo, the number of S-phase sebocytes more than tripled suggesting C/EBPα and C/EBPβ influence peripheral sebocyte cell cycle withdrawal. SEB-1 cells expressed C/EBPα and C/EBPβ and when induced to differentiate, the transcription activity of C/EBPs increased as did the levels of C/EBPβ. When C/EBPα and C/EBPβ were knocked down concurrently with siRNA, sebocyte differentiation and subsequent lipogenesis were inhibited as measured by Oil Red O and Nile Red staining. The expression of enzymes critical in the synthesis of lipids in maturing sebocytes such as fatty acid synthase (FASN), peroxisome proliferator activated receptor gamma (PPARγ) and stearoyl-CoA desaturase1 (SCD1) were all significantly decreased. These studies show C/EBPα and C/EBPβ are critically involved in epidermal homeostasis and function to regulate the basal to spinous transition in early keratinocyte differentiation, to regulate sebocyte differentiation and lipogenesis and cooperate in the regulation of cell cycle withdrawal in both keratinocytes and sebocytes.

Simultaneous Removal of C/EBPα and C/EBPβ Reveals Their Critical Roles in Sebocyte and Keratinocyte Differentiation

by
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DEDICATION

I dedicate this dissertation to my father, John C. House III PhD, and my high school teacher Mona Decker. My father, like all parents, did some things wrong, and did some things right. I was a very curious child and my father always took the time to answer thoughtfully, patiently, and in ways I could understand, the myriad of questions I asked; he still does. That is a very right thing. Mona Decker was my high school world history and speech teacher. I was a very troubled teen and she never stopped believing in me. It is in large part, through the influence these two individuals have had on my life, that this body of work exists.

BIOGRAPHY

John S. House was born in Maine and immediately moved to Virginia where he lived in and around Chesapeake Bay Area until he was 9. He comes from an unbroken line of first sons named John going back at least a dozen generations. In 1980, his hippie parents decided to get “back to the land” as some were wont to do then and they moved to the Ozark Mountains, purchased 40 acres, and promptly planted a 7,000 square foot garden that grew an abundance of rocks, as well as some vegetables. This venture proved unviable, and his parents eventually had to return to reality. John attended and graduated from Ava High School, in Ava, Missouri and ventured off to the University of Missouri in Columbia with aspirations of becoming an aeronautical engineer. The ability to do that and enjoy his freshman year of college to the fullest were in conflict, so John changed his major to Liberal Arts, undecided. This was the first of many. John moved to Tennessee to attend University of Tennessee, Knoxville and graduated in 1994 with his B.S. in Biology. For reasons unbeknownst to many and himself at times, he decided go to graduate school in statistics. He graduated in 1997 with his M.Stat and a minor in toxicology from NCSU. He married his lovely wife Eva in 1998 and he spent the next 9 years in various business jobs becoming an expert in demand planning for manufacturing companies and eventually the Director of Forecasting for a billion dollar company in Atlanta, Georgia. Despite the money and the luxuries it afforded, with permission from Eva, they decided to downsize and allow John to pursue a PhD in cancer research at NCSU. This was in 2006. This tome is what occupied him for the next 5 years.

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I would like to express my sincere gratitude for many of the people who have supported, influenced and mentored me in my endeavor to become a scientist. First, to my mentor and advisor Dr. Robert C. Smart, for training me as a scientist and inspiring me by his work ethic and compassion for others; I don't think I could have found a better fit. I also would like to thank my high school science teacher John Deakins, who introduced me to science and made it incredibly interesting; thank you! I thank my colleagues, Dr. Songyun Zhu, Jeanne Burr, Dr. Sarah Ewing, Dr. Kari Loomis, Dr. Elizabeth Thompson, Dr. Rakesh Ranjan, Dr. Jonathan Hall, Hann Tam, and Zachary Messenger for their help and support. I would like thank my parents, siblings, in-laws and friends for their support and encouragement. Lastly, my deepest gratitude goes to my wife Eva, who has been my best friend, supporter, confidant, and partner before and during these last five years. It is much better with two, and much much better with her.

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GENERAL INTRODUCTION

Organism development, differentiation, growth, and homeostasis are fundamental and yet tremendously complex biological processes that function through cell signaling and gene regulation. These complex processes are finely coordinated and linked and respond to affect responses to environmental and intrinsic stimuli. It is when these processes are perturbed or fail to function as intended that disease states arise. The central dogma of cellular biology dictates that DNA encodes RNA (transcription), which is then processed and translated into proteins. It is these proteins that function to maintain cellular homeostasis and metabolism, coordinate complex biological processes, regulate the expression of additional genes, provide structural support, in addition to functioning as catalysts and as signaling molecules. Cells are able to exert fine control over these complex processes enabled through post-translational modifications (PTMs) of proteins, including but not limited to: phosphorylation, sumoylation, methylation, acetylation, hydroxylation, myristolation, amidation, glycosylation and ubiquitination. These modifications result in conformational changes to proteins that increase or decrease their ability to interact with other biological molecules, or enhance or delay their turnover rate, or in the case of enzymes, affect their catalytic activity. Additional control is exerted by alterations in protein expression through message stabilization and trafficking, targeted proteosomal degradation and through alternative start translation and post transcript spliceosomal processing.

Regulation of the expression of genes into proteins during these processes of homeostasis, development, growth and differentiation therefore becomes paramount

in the ability of a cell to respond properly to intrinsic and extrinsic stimuli and is in itself, a highly regulated process. Transcription factors are specialized proteins that recognize specific DNA motifs and function to regulate the expression of genes. This regulation can result in more or less transcription of genes. Some transcription factors function to prevent RNA polymerase transcription apparatus from binding and therefore preventing transcription; some recruit RNA polymerase and promote transcription, while others function in either capacity depending on how they are modified. Although the primary function of transcription factors involves their interactions with DNA, they are not precluded from having additional functions through protein-protein interactions.

Differentiation

Simply put, differentiation is a determination of cell fate, and is a process whereby daughter cells acquire new abilities different and more specialized than the parent cell. This is almost always an irreversible process that results in cells and their machinery becoming more specific for the lineage and fate they have been directed to through the activation of certain genes and inactivation of others. It is usually preceded by withdrawal from the cell cycle. After fertilization and through the first few cell divisions, cells are considered totipotent, meaning they can literally become any cell type in the organism including germ cells. Eventually, through subsequent divisions, cells become more committed to specific lineages, but still retain the capacity to become several different types; these are characterized as pluripotent or multipotent. For example, stem cells in the bulge region of the hair

follicle are able to become sebocytes or keratinocytes or hair follicle keratinocytes and are therefore multipotent as are granulopoetic stem cells in bone marrow that give rise to various blood cell lineages. A cell is considered a stem cell if it can divide and produce one cell that goes on to differentiate and another daughter cell that maintains this ability. Differentiation is also a process whereby cells lose their ability to replicate and become mitotically inactive. In skin, it is this balance between differentiation and proliferation that is key to having a self-renewing organ and the ongoing process of maintaining that balance is termed homeostasis.

Skin

In mammals, skin is the largest organ in the body and is responsible for numerous biological functions. First and foremost, the skin is at the surface of the body and serves as a protective barrier to the environment and prevents dehydration. The skin manufactures vitamin D with the aid of sunlight, is involved in thermo regulation, and contains sensory receptors for pain, fine touch and pressure. The skin is also the first defense against infection and must be able to repair or re-epithelialize itself after injury. To adequately sustain a largely airtight and waterproof barrier, while maintaining flexibility and providing protection from physical and ultraviolet radiation assaults, skin relies on various structures and supporting cells to constantly replenish itself. This is a process of maintaining homeostasis. Skin homeostasis is maintained by various stem cells (SCs) populations that are defined by their ability to self-renew and restore tissue and various cell types after injury. The embryonic origins of the skin come from ectodermal progenitor cells that

express Wnt proteins. These prevent the ectoderm from responding to fibroblast growth factor signaling allowing bone morphogenic proteins to be expressed instead, sealing their fate to become epidermis [3]. Skin is divided into an outermost layer termed the epidermis, a supporting layer underneath termed the dermis and a hypodermis under the dermis which is responsible for lipid storage, thermogenesis and providing additional elasticity to skin. The hypodermis is also referred to as the sub-cutaneous layer (Fig. 1).

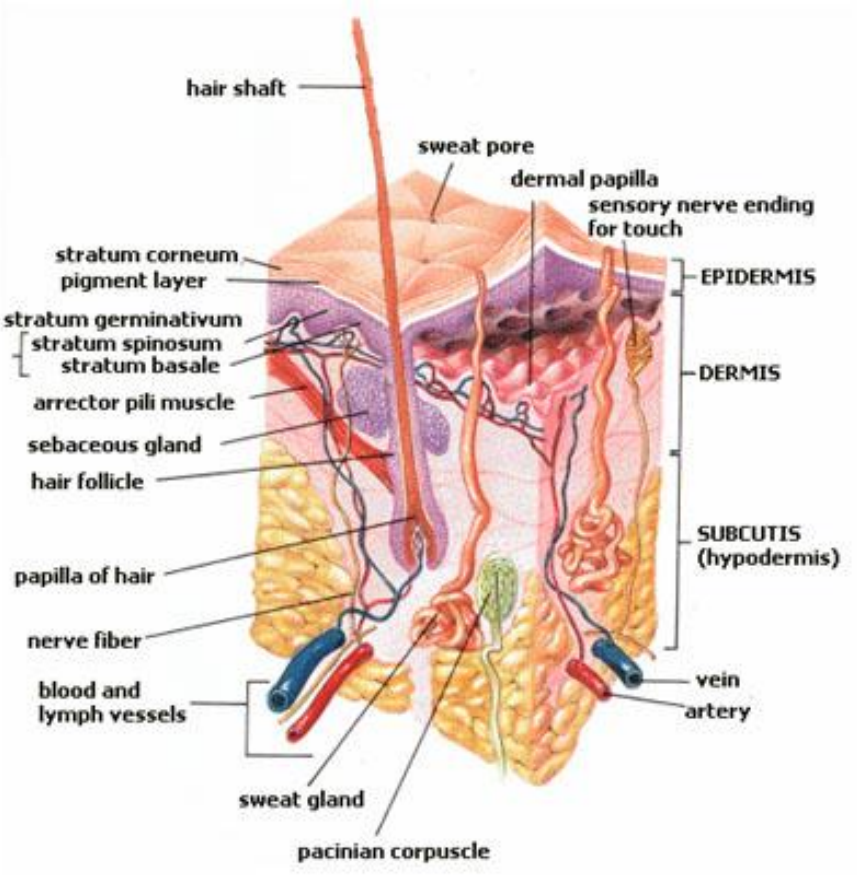

Figure 1. Diagram of human skin (common use license).

Epidermis

The epidermis is comprised largely of keratinocytes with a small amount of Merkel cells, melanocytes (in human) and Langerhans cells. The epidermis does not have a direct blood supply and is instead nourished by diffusion from blood capillaries in the upper part of the dermis. Lineage tracing experiments have demonstrated the interfollicular epidermis (IFE) has different stem cells responsible for maintaining epidermal stratified squamous differentiation that are separate from the stem cells residing the bulge of the hair follicle [4, 5]. Classical experiments with tritiated thymidine labeling have demonstrated less than five percent of the epidermal keratinocytes are slow cycling and that homeostasis in the skin is controlled by these basal label retaining cells and the epidermal proliferating unit around them [6]. These slow cycling cells occasionally divide to give rise to transient amplifying cells that are the workhorse of skin proliferation. These transient amplifying cells have a finite number of divisions, but can divide quickly to replenish cells lost to stratified squamous differentiation and injury. After exhausting their replicative capacity, transient amplifying cells undergo a change in the expression of surface integrins that allows detachment from the basement membrane, withdrawal from the cell cycle and subsequent entry into stratified squamous differentiation.

The epidermis is separated from the dermis by a basement membrane, and is subdivided into four layers. Starting with the layer of cells residing on the basement membrane and progressing to the surface of the skin, they are: *stratum basale*,

stratum spinosum, *stratum granulosum*, and finally *stratum corneum*. It is because of these layers, or strata, that the homeostasis of skin is termed *stratified* squamous differentiation. In skin, stratified squamous differentiation involves keratinocytes losing attachment to the basal lamina, becoming post-mitotic, and turning on a complex, highly coordinated program that involves the regulation of a myriad of genes. It is this attachment to the basement membrane by the expression of integrins that allows keratinocytes to maintain an active Ras-MAPK (mitogen-activated protein kinase) pathway through expression of epidermal growth factor receptor (EFGR) [7], which is needed for cellular proliferation [8]. Epidermal homeostasis involves the intricate regulation and balance between proliferation of basal cells and their commitment to terminally differentiate. Notch signaling [9-11] and AP-2α/AP-2γ [12] have essential roles in epidermal development and postnatal epidermal differentiation. In addition, C/EBPs [13-17], Oct proteins [18, 19] and Kruppel-like factor 4 (Klf4) [20] have also been shown to be critical in regulation of keratinocyte differentiation and homeostasis. A calcium gradient exists in the four layers of the epidermis with basal keratinocytes having the least concentration and progressively increased calcium concentration as keratinocytes differentiate [21].

As basal keratinocytes detach from the basement membrane and enter the stratum spinosum, the expression of keratins 5 and 14 are turned off as well as the afore mentioned integrins. This is commensurate with withdrawal from the cell cycle [22], an increase in intracellular calcium, and concomitant with expression of keratins 1 and 10. Cells in the stratum spinosum and stratum granulosum are still

transcriptionally active. The keratins 1 and 10 in the spinous layer form strong intermediate filament networks that link with desmosome junctions between cells enhancing skin integrity during physical stress [22]. As keratinocytes migrate to the stratum granulosum, they start accumulating keratohyalin and laminar granules and aren't yet compressed into flattened layers or squames. The granules contain lipids as well as proteins that organize the keratin filaments inside the cell, namely profilaggrin, loricrin and intermediate keratin filaments. Profilaggrin is processed to generate filaggrin, which then bundles keratin filaments into extremely strong cables. In addition, cells in the granular layer produce transglutaminases and glutamine and lysine rich proteins. As keratinocytes exit the granular layer and enter the stratum corneum, metabolic activity is shut down and intracellular calcium levels rise, resulting in activation of transglutaminases that cross link the glutamine and lysine rich proteins to form the cornified envelope. The lamellar granules are then extruded on this scaffold of cross linked proteins to form a lipid layer that serves as a final water and air tight barrier at the surface of the body. Thus, this stratum corneum is composed layers of dead flattened keratinocytes cross-linked with keratins and ceramides and it is this layer that is largely waterproof and airtight. Many disease states arise from mutations in genes involved in stratified squamous differentiation including but not limited to: cancer and degenerative blistering diseases such as epidermolysis bullosa simplex (EBS) caused by mutations in keratins 5 and 14 and palmoplantar keratoderma caused by mutations in keratins 9 and 16, and ichthyosis vulgaris caused by mutations in filaggrin.

Many factors have been identified in epidermal proliferation. These include, obviously, epidermal growth factor (EGF) [23], as well as keratinocyte growth factor (KGF) [24], transforming growth factor α (TGFα) [25] and multiple cytokines (i.e. IL-6, IL-1[26]) [27]. TGFα and EGF stimulate EGF receptors, members of the receptor tyrosine kinase family, which are expressed on the cell surface of basal keratinocytes attached to the basement membrane. Keratinocytes also can autoregulate their own growth by the production of TGFα (stimulatory) [28] and TGF-β_1 (inhibitory) [29].

Dermis

The dermis is of mesenchymal origin, is located between the hypodermis and epidermis and is separated from the epidermis by a shared layer of specialized extracellular matrix called the basement membrane. The basement membrane is comprised of specialized extracellular matrix proteins consisting of type IV collagen fibers, microfibrils, fibronection, laminin 5, and heparan sulfate clusters. The basement membrane is also rich in tyrosine kinase growth factors that provide mitotic stimuli to the basal keratinocytes in the epidermis. Fibroblasts in the dermis adjacent to the basement membrane respond to basal keratinocyte interleukin-1 production and c-jun activation by production and secretion of keratinocyte growth factor (KGF) and granulocyte macrophage stimulating colony factor (GM-CSF) [30] which promotes the overlying keratinocytes to proliferate and differentiate [31]. Hemidesmosome junctions containing $\alpha_6\beta_4$ integrins anchor the basal keratinocytes in the epidermis to the basement membrane.

The dermis is largely comprised of fibroblasts that secrete an extracellular matrix (sometimes referred to as ground substance) of collagen, reticular fibers and elastin. The skin's specialized cells, structures and appendages are housed in the dermis; these include sweat glands, sebaceous glands, hair follicles, nerve endings, arrector pili muscles, and blood and lymph vessels (Figure 1). These constituents allow inference about the major functions of the dermis including but not limited to innervation, nutrient delivery to the epidermis, thermoregulation and feedback, and maintenance of hydration. The hair follicle also contains the largest reservoir of stem cells in skin. This region is known as the *bulge* region and is located directly under the sebaceous gland appendage where it attaches to the hair follicle canal, and is part of the outer root sheath keratinocytes that are also contiguous with the interfollicular epidermis (IFE). The bulge region was identified under the premise that stem cells would divide infrequently, and thus would be slow cycling and once labeled with tritiated thymidine, would retain that label labeling for a long time, i.e. label retaining cells (LRCs) [32]. A subset of LRCs from the bulge region are responsible for generating the hair follicle in anagen [33]. The bulge region of keratinocytes is so multipotent that a single LRC from this region in rat whiskers, even after multiple passages in vitro, was able to generate epidermis, hair follicles and sebaceous glands and even a new bulge region stem cell niche when grafted onto the dorsal skin of *Nude* mice [34, 35].

Lastly, the hypodermis is sometimes referred to as the subcutaneous layer. The hypodermis is the innermost layer of skin, is comprised of adipocytes,

fibroblasts, macrophages and connective tissue. The hypodermis functions to fasten skin to the underlying surface, as a shock absorber to physical impact, as well as a storage depot for energy reserves and insulation from environmental temperature changes.

Sebaceous glands

In human skin, sebaceous glands are an appendage resulting from an outgrowth of the hair follicle, located below the hair follicle opening and above the arrector pili muscle and can have several acini or lobules. In mice, the progenitor cells to the sebaceous gland emerge just before birth, and the gland fully matures in the first few days after birth. cMyc [36, 37], hedgehog signaling [38] and AP-2α/AP-2γ [12] are involved in regulating sebaceous gland development/differentiation. All sebaceous glands function similarly; sebocytes in the acini, as they differentiate, produce a waxy, oily substance (sebum) through the accumulation of lipids in terminally differentiated sebocytes and eventually self-destruct spilling their contents and membranes alike into associated ducts in a process called holocrine secretion [39] (Fig. 2).

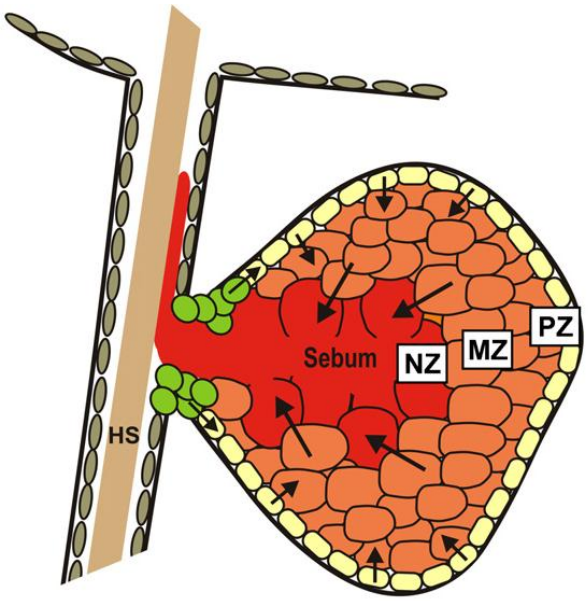

Figure 2. Diagram of Sebaceous Gland and Associated Hair Follicle. HS-Hair Shaft, PZ-Proliferating Zone, MZ-Maturation Zone, NZ-Necrotizing Zone [2].

Sebum is composed of wax esters, triglycerides, free fatty acids, squalene and the debris of dead sebocytes and the composition is species specific. Several exocrine glands exist in mammals that are comprised of specialized sebocytes such as the Harderian and Meibomian glands of the eye, the Zymbal glands of the ear, and preputial glands of some male mammals. These are sometimes called *free* sebaceous glands because they are not associated with a hair follicle, but instead secrete their lipid contents directly onto the surface of the associated organ through ductal epithelium. The activity of these glands is regulated by the binding of ligands to receptors expressed in these cells including androgens, estrogens, vitamin D, retinoids, proopiomelanocortins and PPARs [40]. As mentioned, these glands are holocrine glands, and maintaining homeostasis involves a constant flux of maturing cells that become lipid filled, undergo systematic programmed cell death and lyse their contents into associated hair follicles or ducts and out onto adjacent surface organs [39]. In the case of skin, these lipids lubricate the hair canal and skin surface and provide suppleness and barrier function as well as bactericidal benefits.

Multiple human diseases are related to improper function of sebaceous and related glands. These include acne, seborrhoeic dermatitis, blepharitis, dry eye syndrome, and sebaceous cancer. Acne vulgaris alone affects over 85% of adolescents in the United States [41], and is characterized by excessive sebum production as a result of androgen signaling and dietary habits. More severe cases result in significant scarring and psychological distress. Seborrhoeic dermatitis is a common inflammatory skin disorder characterized by white, flaky skin scales, and

excessive oily skin and is thought to be associated with an abnormal immune response to a common yeast *Malassezia*, present on skin [42]. Meibomian and Harderian glands in the eye supply lipids essential to preventing microbial infection and evaporation of tear film and blockages of their ducts can result in styes. Blepharitis is one of the most common eye disorders characterized by inflammation of the eyelids causing redness, tearing, itching and burning and is often associated with Meibomian gland dysfunction with obstructed Meibomian ducts and resulting limited secretions. Disruption of sebaceous gland lipid formation also leads to compromised barrier function in skin [43]. The organization of sebaceous gland acini includes mitotically active undifferentiated sebocytes in contact with the basement membrane, differentiating sebocytes that have exited the cell cycle and started synthesis of lipid and mature terminally differentiated sebocytes ready to lyse their contents into associated ducts or follicles (Fig. 2).

Sebaceous glands extend off of the upper hair follicle and are formed from a sebocyte progenitor (Fig. 2 green cells) that expresses Blimp 1, keratin 14 (K14) and keratin 5 (K5) [44] and these cells produce a proliferative population of sebocytes which in turn terminally differentiate to produce the lipid/sebum producing cells that lyse and spill their contents into associated hair follicles or ducts [37] (Fig. 2). Although C/EBPα and C/EBPβ are abundantly expressed in sebocytes of the sebaceous glands [45, 46], no function for these transcription factors in sebocytes has been described and no sebaceous gland phenotype has been reported in C/EBPα or C/EBPβ knockout mice. In fact, for many years, understanding of the

function of sebaceous glands was limited to their role in the barrier function. Recent work in sebocyte cell biology including the development of several in-vivo cell lines [47] has enabled considerable progress on elucidating aspects of the roles these glands play in human health and skin homeostasis. In humans, sebaceous glands create and secrete the vernix caseosa around the 20th week of gestation. The vernix caseosa is a white creamy coating on newborns and provides a thermal and dehydration barrier in utero and functions to facilitate passage through the birth canal [48]. It is comprised of water, corneocytes from the periderm, and sebum from sebaceous glands and functions to provide a barrier from dehydration as well as protection from microbes [49]. After gestation, sebaceous glands lipids are responsible for assisting in the three-dimensional organization of surface lipids of the skin lending to the integrity of the skin barrier [50, 51]. Sebum from sebaceous glands has a photoprotective effect through platelet-activating factor acetyl hydrolase II which functions to protect epidermal keratinocytes from UVB radiation and oxidative stress induced bioactive phospholipids [52]. Sebum secretion after birth slowly subsides through around 9 years of age when adrenarche occurs. Adrenarche is the production of androgens from the adrenal cortex at around 8 or 9 years of age. At this time, sebum production ramps up through 17 years of age when young adulthood is reached [53]. Peroxisome proliferators-activating receptor (PPAR) ligands, insulin-like growth factor (IGF), IGF receptors, estrogens and adrogens have all been shown to affect lipid synthesis in sebaceous glands [54].

Immune Response

Mounting evidence suggests sebaceous glands are a key player in innate immune response in skin. Innate immunity partners such as CD1d, CD14, and toll-like receptor 2 (TLR2) and TLR4 are expressed in immortalized human sebocytes [55-57]. Sebaceous glands exhibit a response to gram positive bacteria via signaling by TLR2 by producing more sapienic acid (C16:1Δ6), which is an isomer of palmitoleic acid and has been shown to have innate antimicrobial activity [58, 59]. In fact, sebaceous glands express many proinflammatory cytokines and chemokines and antimicrobial peptides including interleukin-1 alpha, interleukin-1 beta, and tumor necrosis factor-alpha [60]. Additional studies in human sebocytes have shown sebocytes play a role in the innate immune response by demonstrating *Propionibacterium acnes* and LPS induce sebocyte differentiation and beta-defensin-2 production as well as the production of other proinflammatory cytokines/chemokines [61]. Lastly, in addition to sapienic acid, sebaceous glands manufacture monounsaturated fatty acids (MUFA), primarily oleic and palmitic acid, that are bactericidal to gram positive bacteria [59]. MUFAs are synthesized in part by stearoyl coenzyme A desaturase 1 (SCD1) and which is expressed in the sebaceous gland [59].

Acne and sebaceous glands

Acne is the most common skin disease and its pathology expresses itself in the pilosebaceous unit, encompassed by the sebaceous gland and the associated follicle. The pathology of acne is associated with many factors including excess

sebum production, sebum composition, inflammatory mediator release in skin, follicular hyperkeratinization and follicular colonization by *Propionibacterium acnes* populations [62]. Increased sebum production results from the onset of puberty and accompanying increase in androgen production in both sexes. This excessive sebum production results in an increased propensity to clog the duct and an increase in a food source (sebum) for *P. acnes*. A clogged pore, or comedo forms and develops into whiteheads where the contents are not exposed to air, and blackheads where the contents are exposed to air and subsequent sebum oxidation. Another attribute of comedones is inflammation and associated hyperkeratization that lends to the clogging of the pore. *P. acnes* have a soluble factor different from lipopolysaccharide (LPS) but with similar characteristics that is recognized by CD14, a pattern recognition receptor for LPS and other lipid ligands [63]. This soluble factor induces production of IL-1β and tumor necrosis factor α (TNFα), acting through TLR-2 [64]. Studies have shown inflammatory cytokines identified in acne lesions act in a paracrine and autocrine fashion that eventually activates activator protein 1 (AP-1) transcription factor which is known to regulate metallo-matrix proteinases (MMPs) that degrade the dermal matrix [65]. Current treatment for acne consists of a two pronged approach of antibiotics that kill *P.acne* in conjunction with retinoids that have been shown to induce apoptosis of sebocytes but not keratinocytes and to induce monocytes to differentiate into CD209(+) macrophages more effective at uptake of *P. acne* [62, 66, 67]. However, 13-cis-retinoic acid, while

effective at reducing sebum production, is also teratogenic [68], lending urgency to developing additional methods for treatment.

Understanding molecular mechanisms regulating sebaceous gland (and their specialized family members: Meibomian, Harderian, zymbol) differentiation, and lipid synthesis/accumulation will provide critical knowledge in the development of treatment options for acne, seborrhea, eye styes, and other sebaceous gland related disease conditions and subsequent human health.

Sebaceous glands and the neuroendocrine system

The hypothalamic/pituitary/adrenalcortex (HPA) axis responds to environmental stress, either physical or psychological; neurons in the hypothalamus respond to stress stimuli by producing corticotrophin-releasing hormone (CRH), which immediately binds to receptors in the pituitary gland causing the production of adrenocorticotropic hormone (ACTH). ACTH travels to the adrenal glands where it stimulates release of cortisol and other adrenal hormones. It is well established that the sebaceous glands not only respond to endocrine sex hormone signaling, but also function to synthesize steroids from cholesterol via various cytochrome p450 enzymes [69]. In the last decade, research has shown that sebaceous glands are intricately involved in a neuroendocrine axis in the skin involving corticotropin-releasing hormone (CRH) and its receptors and associated melanocortin signaling peptides and receptors. CRH results in the production of the proopiomelanocortin (POMC) peptide which is then cleaved into at least five different products, one of which is the afore mentioned ACTH, each with different specificity for various

melanocortin receptors. Melanocortin receptors (MCxRs) are a family of seven transmembrane G-protein coupled receptors (GPCRs) whose canonical signaling pathway consists of G-alpha stimulation resulting in the accumulation of cAMP through the upregulation of adenylyl cyclase and ultimately, PKA activation. Classically, this network is activated by the HPA axis in response to stress and early studies gave the first clue to the importance of the HPA axis in sebogenesis. Thody et.al removed the neurointermediate lobe of the pituitary and noted decreased sebum in rats [70] and went on to show application of α-melanocyte stimulating hormone (α-MSH), one of the cleavage products from POMC peptide, and androgen restored sebaceous gland lipogenesis [71]. This system can also be activated in skin, primarily by pro-inflammatory cytokines, causing the synthesis and release of CRH from sebocytes and other dermal cells [72]. Chen et.al first characterized MC5R deficient mice with having defective epidermal barrier function and a resulting defect in thermal regulation in addition to characterizing a role for MC5R in the production of proteins secreted in specialized sebaceous glands of the eye (Harderian and lacrimal) [73]. Subsequently, Zhang et.al showed in two different studies that targeted deletion of MC5R in mice resulted in down-regulation of sebum in mice [74], and stimulation of MC5R by its endogenous ligand, α-MSH, induced differentiation and production of squalene and wax esters [75] which are lipids unique to sebaceous glands. Despite the proven role of MC5R in sebocyte differentiation and induction of sebaceous lipids, the mechanistic pathways downstream of MC5-receptor activation are not known.

CCAAT/enhancer binding proteins

CCAAT/enhancer binding proteins (C/EBPs) are a set of highly conserved transcription factors and are members of the basic leucine zipper (bZIP) transcription factor superfamily that also include AP-1, fos, jun, CREB, ATF and others. Since the discovery and subsequent cloning of C/EBPα in the late 80's by Steven McKnight's lab [76-78] as a heat-stabile factor in rat liver nuclei, an additional five family members have been characterized. Subsequent study of C/EBPα led to the discovery and characterization of the basic leucine zipper (bZIP) class of transcription factors [79].

Structure

C/EBPs share a dimerization domain and a basic region leucine zipper (bZIP) DNA binding domain where they are able to accomplish transcriptional repression and activation of target genes. Cao and colleagues proposed the current naming convention of using Greek letters in the order of C/EBP discovery [80] to avoid confusion. The six currently identified members of the CCAAT/enhancer binding protein (C/EBP) family are named : C/EBPα (*C/EBP, RcC/EBP-1*), C/EBPβ (*NF-IL6, IL-6DBP, LAP, CRP2, NF-M, AGP/EBP, ApC/EBP*), C/EBPγ (*Ig/EBP-1*), C/EBPδ (*NF-IL6b, CRP3, CELF, RcC/EBP2*), C/EBPε (*CRP-1*) and C/EBPζ (*CHOP-10, GADD153*) [81-88]. C/EBPε contains two introns and C/EBPζ four, while the remaining family members are intronless. In addition, several family members have multiple variable length polypeptides produced by regulated proteolysis, leaky ribosomal scanning, alternative translation start sites (C/EBPα, C/EBPβ) [89-92], and

by use of alternative promoters and differential splicing C/EBPε [93, 94]. C/EBPs function critically in fundamental cellular processes including proliferation, apoptosis, differentiation, inflammation, senescence and energy metabolism [95, 96]. The C-terminus contains the bZIP domain and all C/EBPs share more than 90% sequence homology in the 55-65 amino acids of this region. This domain contains a string of basic amino acids used in DNA binding and specificity followed by the 'leucine zipper' consisting of a heptad repeat of four or five leucines that form a α-helical motif with two repeats able to interdigitate to form a coiled-coil structure [97-99]. Dimerization of C/EBPs is required for DNA binding. The accepted bZIP model proposes that dimerization forms a Y shaped structure where the arms of the Y

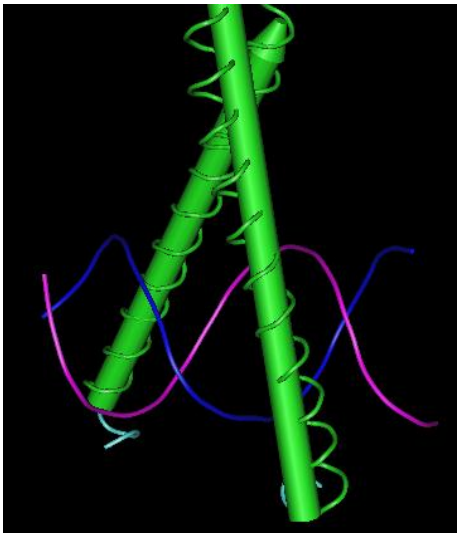

Figure 3. Crystal Structure of C/EBP Dimer Bound to High Affinity DNA Fragment. [1].

consist of the basic region that each bind one half of a palindromic sequence in the major groove of DNA (Fig. 3) [98, 99]. All C/EBPs with the exception of C/EBPζ recognize the same sequence of DNA for binding, RTTGCGYAAY, where R

represents A or G and Y represents C or T. However, substantial variance is allowed [100]. Steven McKnight's lab also showed dimerization through the bZIP domain is a requisite for C/EBPs to interact with DNA [97].

The N-terminus of C/EBPs are rather more diverse among family members (<20% homology) and it is this portion of C/EBPs that is responsible for activation and repression of C/EBP transcriptional activity [101]. There are regions within the N-terminus of C/EBPs that are conserved (with the exception of C/EBPγ) that represent specific transactivation and repression domains that function to interact with basal transcription apparatus components (TBP/TFIID) [102-106]. As noted, C/EBPγ lacks any such domains and its dimerization with other C/EBPs typically results in repression of transcription [107].

The complexity of C/EBPs and their interactions are further compounded by various isoforms produced by differential splicing and leaky ribosomal scanning of start translation sites. C/EBPα, C/EBPβ, [89-92] and C/EBPε [93, 94] each have several isoforms active in cells at any given time, each with different activation potentials, and all isoforms are capable of heterodimerizing with each other and other family members [81-88]. Furthermore, C/EBPs can also form protein-protein interactions with other bZIP [108, 109] and non-bZIP transcription factors [110, 111]. The use of cell culture models and knockout mice has demonstrated multiple roles for C/EBPs in the regulation of genes, although this process has been hindered by compensation by other family members.

Expression

C/EBPs are expressed in a multitude of tissues and cells types. C/EBPα is expressed in liver, adipocytes, keratinocytes, and sebocytes, with the highest amounts in terminally differentiated lineages [17, 45, 86, 87, 112], in addition to expression in epidermis, intestine, lung, adrenal glands, placenta and myeloid cells [17, 86, 87, 113]. C/EBPβ is expressed widely; it has been found to be constitutively expressed in skin, liver, lung, adipose tissue, heart, kidney, spleen, hematopoietic cells and chondrocytes [80-84, 87, 114-116]. C/EBPδ expression has been characterized in adipose, intestine and lung [86, 87, 117, 118]. Whereas C/EBPγ and C/EBPζ are expressed in most tissues, the expression of C/EBPε is primarily found in myeloid and lymphoid cells [119].

CCAAT/enhancer binding protein alpha (C/EBPα)

Structure

C/EBPα has multiple isoforms generated by regulated alternative start translation initiation sites. C/EBPα is an intronless gene and exists as a 42 kDa full length protein and as a 30 kDa protein (referred to as p42 and p30) when expressed. Both isoforms retain the basic region leucine zipper (BR-LZ) DNA-binding domain, with p30 missing a portion of the N-terminus transactivation domain region (see [96] for diagram of all forms of C/EBPs). p30 has less transactivation potential and has been shown to inhibit C/EBP activity [90, 91] but has also been shown to have a different regulatory profile than p42 [120]. Recent studies have indicated C/EBPα might have an additional isoform through an upstream non AUG

codon and have characterized this extended form of C/EBPα. This extended C/EBPα contains a nucleolar localization signal and is translocated to the nucleolus where stimulates synthesis of ribosomal RNA before mitosis [121], allowing for a novel new function for C/EBPα.

C/EBPα and differentiation

Subsequent to its discovery as a liver-enriched binding protein, the expression of C/EBPα was found highly expressed in epidermis, lung, liver, white adipose tissue, and bone marrow myeloid cells. Knockout mice for C/EBPα died within eight hours of birth as a result of hypoglycemia from failure to store hepatic glycogen [122]. The C/EBPα null mice had greatly reduced levels of the gluconeogenic enzymes glucose-6-phosphatase and physphoenolpyruvate carboxykinase (PEKCK). In addition, there was a lack of lipid accumulation in adipocytes and hepatocytes. These dysfunctions pointed to a role for C/EBPα in differentiation. Subsequent research showed C/EBPα induced adipogenesis through its induction of peroxisome proliferator activated receptor-γ (PPARγ) [123], as fibroblasts lacking PPARγ could not be induced to differentiate by C/EBPα. Zhang et.al [124] characterized a complete block in differentiation of neutrophils and eosinophils in C/EBPα null mice. Instead, white blood cells were arrested as myeloid blasts, due in part, from a lack of granulocyte colony-stimulating factor receptor, a gene target of C/EBPα. Given that a feature of myelogenous leukemia (AML) is a block in differentiation of myeloid precursors, the authors surmised C/EBPα null mice would be a useful tool to study the disease. Indeed, C/EBPα was

later shown to have dominant negative mutations in AML [125] and Gombart et.al [126] showed that C/EBPα was a tumor suppressor in AML and was mutated in ~10% of AML cases. Further cementing its role as a master regulator of differentiation, C/EBPα was found to be critical for proper type II alveolar differentiation in lung epithelium [127]. Lung specific C/EBPα null mice suffered respiratory failure at birth because of block in alveolar type II differentiation resulting in a lack of surfactant secreting type II cells with a substantial increase in proliferating cells with a concomitant decrease in apoptotic cells. C/EBPα plays a dual role in the differentiation associated with its ability to induce cell cycle arrest [128]. In addition coupling with co-factors to induce differentiation specific genes, C/EBPα also interacts with and suppresses E2F complex [129] and contains a SWI/SNF interactive domain needed for its anti-proliferative ability [130, 131]. This suppression of E2F complex S-phase transcription factors is required for both adipocyte and granulocyte differentiation [132]. [53]. Micro RNAs (miRNAs) have been getting more attention as of late. miRNAs are, after processing by Dicer/Drosha complexes, 22-26 nucleotide RNA molecules that can positively and negatively regulate multiple genes. Recently, miRNA-34a was identified as a target of C/EBPα in granulopoiesis and miRNA-34a was responsible for targeting E2F3, effectively blocking myeloid cell proliferation. Given C/EBPα's expression in skin and its role as a master regulator of differentiation, it was surprising that the first epidermal conditional knockout of C/EBPα resulted in no discernable changes in skin homeostasis or differentiation [133].

C/EBPα and tumorigenesis

The fact that C/EBPα can suppress the E2F complex and interferes with cdk2 and cdk4 functions [134], is mutated in AML, and induces growth arrest in most cell lines suggested that C/EBPα might be a global tumor suppressor in multiple tissues. In fact, greatly diminished C/EBPα expression had been noted in lung, liver, endometrial, skin and breast tumors, but little direct evidence for C/EBPα as a tumor suppressor in epithelial tissues was available because no mutations of C/EBPα were found. Direct genetic evidence for C/EBPα as an epithelial tumor suppressor came from a study in which C/EBPα was removed from the skin of mice with a keratin 5 driven conditional knockout. Those mice exhibited greatly increased tumor occurrence, multiplicity, and size, along with decreased latency in two-stage carcinogenesis experiments with DMBA/TPA [133]. Further evidence for C/EBPα acting as a global tumor suppressor comes from studies showing C/EBPα induced growth arrest is overcome in hepatic tumor cells by activation of PI3K/Akt and subsequent phosphorylation of C/EBPα on serine 193, preventing C/EBPα from interacting/inhibiting E2F and cyclin dependent kinases (CDKs). In addition, C/EBPα expression is downregulated in human lung cancer cell lines and lung primary tumors due to silencing of its upstream promoter region by DNA methylation and histone acetylation [135]. Lastly, Thompson et.al have revealed C/EBPα is a largely absent in human squamous cell carcinomas and functions as a tumor suppressor in UVB induced tumorigenesis in SKH1 hairless mice [136].

C/EBPα and lipogenesis

Lipogenesis is a generic term to describe certain biological processes in cell types that involve the bio-synthesis and accumulation of lipids. Adipocytes, sebocytes, and hepatocytes are the most common cell types that undergo lipogenesis. Classically, C/EBPα, C/EBPβ and C/EBPδ were among of the first transcription factors identified as important in adipocyte differentiation [137]. Adipogeneis, without going into an exhaustive review (please see [138] for review), involves *mesenchymal precursors* going through several stages of differentiation including *committed preadipocyte, growth arrested preadipocyte, mitotic clonal expansion, terminal differentiation* and finally, *mature adipocyte*. When preadipocytes are induced to differentiate, C/EBPβ and C/EBPδ expression are immediately upregulated as preadipocytes undergo two rounds of mitotic clonal expansion. C/EBPβ is phosphorylated/activated by GSK3β [139] and with C/EBPδ then induces the expression of C/EBPα and PPARγ. C/EBPα is responsible for growth arrest via afore mentioned mechanisms [132] and a genome wide analysis of PPARγ binding sites revealed an almost complete co-localization with C/EBPα on the promoters of most genes induced in adipogenesis [140]. C/EBPα and PPARγ upregulate each other and additional late differentiation specific adipocyte genes. Recently, it was demonstrated that forkhead transcription factor Foxo1 is a coactivator with C/EBPα of gluconeogenetic gene expression [111] of PEPCK and that Foxo1's ability to act as a C/EBPα coactivator was prevented by insulin linking C/EBPα to insulin signaling during liver development. When C/EBPα was ablated in

adult liver, hepatic expression of lipogenic genes including Acc1, Fas, Scd1, and Acly were decreased resulting in a reduction of fatty liver formation in genetically obese mice [141, 142]. Mouse knockin experiments with a deletion of a proline-histidine rich domain in C/EBPα dysregulates control of Acly, Me1, Got1, and Acas2, all genes involved in metabolite synthesis for lipogenesis [143]. Lastly, C/EBPα is involved in lipogenesis responses through miRNA378/378* induction during lipogenesis and overexpression of miRNA378/378* increases lipogenesis. Although overexpression of miRNA378/278* in ST2 mesenchymal precursor cells did not cause increase in message levels of C/EBPs, it did increase transcriptional activity of C/EBPα and C/EBPβ on the promoters of adipocyte genes [144].

CCAAT/enhancer binding protein beta (C/EBPβ)

Like C/EBPα, C/EBPβ is also intronless and has multiple isoforms produced from the same transcript: 38 kDa LAP*, 36 kDa LAP, and 17 kDa LIP [145]. LAP and LAP* both contain transactivation and bZIP domains, while LIP only contains the latter. Since LIP is missing its transactivation domain, it typically functions as a negative regulator of C/EBP function by forming inactive heterodimers with C/EBP family members. C/EBPβ's first name was NF-IL6 and was discovered and cloned as a nuclear factor able to bind to the IL-1 responsive element in the IL-6 gene [146]. C/EBPβ was found to be involved in the acute phase immune response, by being induced by IL-1, IL-6, and lipopolysacchride (LPS) and in turn binding to the regulatory domains of additional acute phase response and inflammation genes [146]. Multiple studies in multiple cell types using targeted gene promoter analysis,

overexpression or knockdown of C/EBPβ, and genetically modified mice soon followed, and critical roles for C/EBPβ have been revealed in the processes of differentiation, inflammation, metabolism, proliferation/cell survival and more recently, memory formation and endoplasmic reticulum stress response.

C/EBPβ and differentiation

C/EBPβ has a demonstrated role in the differentiation process in mammary epithelial cells [147, 148], adipocytes [118, 149], ovarian granulosa cells [150], ovarian luteal cells, keratinocytes [14], neuronal cells [151, 152], and macrophages [114, 153]. C/EBPβ's role in adipocyte differentiation has already been discussed, but it is important to note that although C/EBPδ cooperates in early differentiation to propel preadipocytes through two days of mitotic clonal expansion followed by inducement of C/EBPα and PPARγ, overexpression of C/EBPβ alone is able to drive this process in the absence of adipogenic hormones, but not C/EBPδ [118]. In addition, during the mitotic clonal expansion phase, C/EBPβ DNA-binding is a result of its cell cycle dependent interaction with hypophosphorylated Rb [154]. Female mice deficient in C/EBPβ are sterile, in spite of normal development [150] and no effect on the fertility of adult males exists. Sterneck et.al demonstrated C/EBPβ was required for proper granulosa cell differentiation after stimulation by luteinizing hormone and C/EBPβ was one of the first non-steroid hormone receptor transcription factors demonstrated to be required for ovarian follicle development [150]. Additional studies in C/EBPβ deficient mice revealed a defect in ductal morphogenesis in mammary gland development, with the ducts displaying reduced

branching. Furthermore, the activation of milk protein genes was inhibited in C/EBPβ null mice [147].

Like C/EBPα, C/EBPβ expression is abundant in skin and sebaceous glands of mouse and human [13, 45] and appears to be correlated in keratinocytes with stronger expression in nuclei of ordered triple stacks of cells found at regular intervals in the epidermis of mice, very similar to the proposed epidermal proliferative unit (EPU) [6]. Zhu et.al demonstrated a role for C/EBPβ in the early events of stratified squamous differentiation with a reduction in the levels of keratin 1 and keratin 10 that accompany exit from the cell cycle and migration off the basement membrane [155]. Despite altered levels of K1 and K10 and a modest increase in the number of S-phase cells, C/EBPβ null mice displayed no other changes to epidermal homeostasis in skin. Further implicating C/EBPβ's role in keratinocyte differentiation is research that showed C/EBPβ can inhibit the expression of integrin genes [156], and differentiation of keratinocytes can be induced by the release of keratinocytes from the basement membrane, when integrin expression is downregulated [157]. Compensation from other family members could explain why the epidermal phenotype in a C/EBPβ knockout mice isn't more severe.

C/EBPβ and inflammation

As described, C/EBPβ was initially named and identified by its ability to bind to promoters of acute-phase genes in response to IL-6 and IL-1 [82, 146]. LPS and other inflammatory stimuli such as IL-6, IL-1 and TNFα increase the expression of

C/EBPβ and C/EBPδ [158], and unlike C/EBPδ, post-translational modification of C/EBPβ enhances its ability to transactivate acute-phase genes [159]. Binding sites for C/EBPs have been identified in many inflammatory response genes, including but not limited to the following cytokines and receptors: TNFα, IL-8, IL-6, IL-1β, IL-12, haptoglobin, serum amyloid proteins, complement C3, hemopexin, and C-reactive protein [159]. C/EBP binding sites are also found in genes responsible for critical macrophage and granulocytic functions including lysozyme, myeloperoxidase, inducible nitric oxide synthase, and neutrophil elastase [159]. This is in concert with observations that C/EBPβ deficient mice are more susceptible to infection with various pathogens including *C. albicans, L. monocytogenes,* and *S. typhi* [160, 161]. While C/EBPβ null mice have allowed for the elucidation of critical roles of C/EBPβ regulation of target genes in inflammation, compensation by other C/EBPs that bind to the identical DNA consensus sequence and homo- and hetero-dimerize with C/EBPβ and each other have hindered full elucidation of target genes.

C/EBPβ and proliferation

Whereas conceptually the functions of C/EBPα are consistent and seem in alignment, (cell cycle exit, blocking of proliferation, promotion of differentiation, suppression of tumorigenesis), C/EBPβ does not fit into one neat paradigm. C/EBPβ, like C/EBPα, is able to suppress genes required for cell proliferation and this activity is dependent on RB family members [162]. Likewise, C/EBPβ is able to block proliferation in certain leukemic cells [163, 164]. Furthermore, C/EBPβ is able to function similarly to C/EBPα in studies where C/EBPβ was knocked into C/EBPα's

locus, thereby rescuing C/EBPα null lethality [165, 166]. However, C/EBPβ is usually associated with proliferative and survival functions. C/EBPβ functions to mediate keratinocyte survival downstream of RAS signaling [167]. Mice missing C/EBPβ in skin displayed greatly increased apoptosis in keratinocytes in response to oncogenic RAS signaling [167]. Further research demonstrated this increase in apoptosis was the result of C/EBPβ negatively regulating p53 protein levels after carcinogen induced stress [168]. Ewing et.al [169] followed up by demonstrating C/EBPβ was a repressor of p53 after DNA damage and this function was not dependent on oncogenic RAS or $p19^{arf}$. C/EBPβ phosphorylation is required for TGFα induced RSK proliferation of hepatocytes [170] and for RAS-induced transformation of NIH 3T3 cells [171]. C/EBPβ levels in hepatocytes increase after partial hepactomy as the liver regenerates itself, and hepatocytes in mice deficient in C/EBPβ display impaired regeneration [172]. Likewise, C/EBPβ has been shown function downstream of nuclear factor kappaB (NF-kappaB) signaling to inhibit tumor necrosis factor-α (TNF-α) induced caspace dependent apoptosis in hepatocytes [173]. Further supporting the notion that C/EBPβ usually functions in a pro-survival role is a study where gene expression data was mined from human tumor samples. C/EBPβ, but not C/EBPα, was found to be expressed with an overexpression cyclin D1 signature in epithelial tumors [174], but not brain tumors, suggesting an epithelial specific context for C/EBPβ and survival.

C/EBPβ and tumorigenesis

C/EBPβ's role in epidermal cell survival is apparent in tumorigenesis experiments in C/EBPβ knockout mice. Whether C/EBPβ was systemically deleted or epidermally deleted, two stage carcinogenesis protocols to induce tumors were countered by a tremendous increase in apoptosis concomitant with a complete block in tumorigenesis [167]. The survival of initiated cells downstream of RAS mutations functions through activation of C/EBPβ by ERK1/ERK2 RAS signaling by phosphorylation on threonine188 and mice containing C/EBPβ with a threonine to alanine conversion (T188A) displayed greatly reduced tumorigenesis [167]. Likewise, the acute loss of C/EBPβ in human metastatic Wilms tumor cell lines resulted in spontaneous apoptosis while increased expression of C/EBPβ is associated with tumor relapse [175]. Lastly, in multiple human cancers, including breast, colorectal, ovarian, renal, and gastric, C/EBPβ is over-expressed or functions aberrantly [176-180].

C/EBPs and compensation

C/EBPα and C/EBPβ were found to be highly expressed in mouse and human epidermis [13, 15, 181], keratinocytes [133] and sebaceous glands [45], so it was quite surprising when Loomis et.al [133] demonstrated C/EBPα in mouse epidermis was completely dispensable for stratified squamous differentiation in skin, noting no change in epidermal thickness, proliferation and a proper expression pattern of genes. Furthermore, the sebaceous glands of these mice, which also were deficient in C/EBPα (data not shown) displayed no discernable differences in

size and function. Likewise, the analysis of germline C/EBPβ knockout mouse epidermis revealed changes, albeit modest, in keratinocyte differentiation involving decreased K1 and K10 expression and increased basal keratinocytes proliferation, indicating a role for C/EBPβ in the early events of stratified differentiation [14]. Because these changes in C/EBPβ deficient epidermis were modest it led to the notion that C/EBPβ has supportive rather than a major role in epidermal keratinocyte differentiation. However, another possibility for the lack of a major skin phenotype in the C/EBPα and C/EBPβ knockout mouse is that functional redundancies between the transcription factors mask their roles in the single knockouts. In support of this notion are studies demonstrating the knockin of C/EBPβ into the C/EBPα locus can rescue C/EBPα$^{-/-}$ mice from perinatal lethality and restore granulocytic differentiation and glycogen metabolism [166]. More evidence for compensation comes from double knock-outs for C/EBPβ and C/EBPδ. Although most of the mice died as neonates, those that did survive had normal levels of PPARγ and C/EBPα in their white adipose tissue (WAT) [182].

Research Hypothesis and Rationale

C/EBPα and C/EBPβ are critical regulators of differentiation and cellular growth in a multitude of cell types and tissues [96] including but not limited to keratinocytes, hepatocytes, myeloid differentiation, neuronal cells, mammary epithelial cells, adipocytes, granulosa cells, type II alveolar cells, and macrophages. Both C/EBPα and C/EBPβ are expressed in skin and yet mice deficient in C/EBPβ revealed a modest differentiation phenotype in skin [183], and a profound lack of tumors resulting from carcinogenesis protocols [167]. Targeted epidermal deletion of C/EBPα, while revealing its role as an epithelial tumor suppressor, resulted in no discernable alterations in skin homeostasis or stratified squamous differentiation [133]. Furthermore, sebocytes, which also share an epidermal lineage, and express high levels of C/EBPα and C/EBPβ [45] (unpublished data on mouse), displayed no changes in epidermal specific knock-outs for C/EBPα and a modest effect (mild atrophy in aged mice) in C/EBPβ deficient mice was observed (data unpublished). Given recent demonstration of compensation and rescue of hematopoiesis in a C/EBPα null phenotype by a knockin of C/EBPβ into C/EBPα's locus [165], combined with the fact that C/EBPs bind to the same consensus sequence and attempts to generate epidermal specific double knockouts were unsuccessful (data unpublished), we hypothesized that removing both C/EBPα and C/EBPβ concurrently would reveal their roles in skin and sebocyte differentiation and homeostasis. Our objectives were to create a model in vivo in which we could conditionally ablate C/EBPα and C/EBPβ singly and concurrently in adults animals,

address whether C/EBPα and C/EBPβ were functionally redundant while revealing their functional roles in adult epidermis and sebaceous gland, and to determine how C/EBPα and C/EBPβ functioned human sebaceous glands.

CHAPTER 1

C/EBPα and C/EBPβ are Required for Sebocyte Differentiation and Stratified Squamous Differentiation in Adult Mouse Skin

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Abstract

C/EBPα and C/EBPβ are bZIP transcription factors that are highly expressed in the interfollicular epidermis and sebaceous glands of skin and yet germ line deletion of either family member alone has only a mild or no effect on keratinocyte biology and their role in sebocyte biology has never been examined. To address possible functional redundancies and reveal functional roles of C/EBPα and C/EBPβ in postnatal skin, mouse models were developed in which either family member could be acutely ablated alone or together in the epidermis and sebaceous glands of adult mice. Acute removal of either C/EBPα or C/EBPβ alone in adult mouse skin revealed modest to no discernable changes in epidermis or sebaceous glands. In contrast, co-ablation of C/EBPα and C/EBPβ in postnatal epidermis resulted in disruption of stratified squamous differentiation characterized by hyperproliferation of basal and suprabasal keratinocytes and a defective basal to spinous keratinocyte transition involving an expanded basal compartment and a diminished and delayed spinous compartment. Acute co-ablation of C/EBPα and C/EBPβ in sebaceous glands resulted in severe morphological defects, and sebocyte differentiation was blocked as determined by lack of sebum production and reduced expression of stearoyl-CoA desaturase (SCD3) and melanocortin 5 receptor (MC5R), two markers of terminal sebocyte differentiation. Specialized sebocytes of Meibomian glands and preputial glands were also affected. Our results indicate that in adult mouse skin, C/EBPα and C/EBPβ are critically involved in regulating sebocyte differentiation and

epidermal homeostasis involving the basal to spinous keratinocyte transition and basal cell cycle withdrawal.

Introduction

There are six members of the CCAAT/enhancer binding protein (C/EBP) family: C/EBPα, C/EBPβ, C/EBPγ, C/EBPδ, C/EBPε and C/EBPζ. C/EBPs are members of the basic leucine zipper (bZIP) class of transcription factors. C/EBPs play important roles in fundamental cellular processes including proliferation, apoptosis, differentiation, inflammation, senescence and energy metabolism [1,2]. In terms of differentiation, C/EBPα plays a role in the differentiation of myeloid cells [3,4], alveolar type II cells [5], hepatocytes [6,7] and adipocytes [8-10] while C/EBPβ has a role in the differentiation process in mammary epithelial cells [11,12], adipocytes [13], ovarian granulosa cells [14], and macrophages [15,16].

The interfollicular epidermis (IFE) of skin is a stratified squamous epithelium composed primarily of keratinocytes that undergo a highly coordinated program of sequential changes in gene expression as they migrate from the proliferating basal keratinocyte layer through morphologically distinct spinous and then granular suprabasal keratinocyte layers, ending in the production of a nonviable stratum corneum. A discrete population of IFE stem cells replenishes the basal cell compartment in the epidermis[17]. Epidermal homeostasis involves the intricate regulation and balance between proliferation of basal cells and their commitment to terminally differentiate. Notch signaling [18-20] and AP-2α/AP-2γ [21] have essential roles in epidermal development and postnatal epidermal differentiation.

C/EBPα and C/EBPβ have been shown to be highly expressed in mouse and human epidermis [22-24] suggesting a possible role for these transcription factors in

stratified squamous differentiation. However, the analysis of an epidermal specific germline C/EBPα knockout mouse revealed that C/EBPα is completely dispensable for epidermal homeostasis involving squamous differentiation and keratinocyte proliferation [25] and the analysis of germline C/EBPβ knockout mouse epidermis revealed changes, albeit modest, in keratinocyte differentiation involving decreased K1 and K10 expression and increased basal keratinocytes proliferation, indicating a role for C/EBPβ in the early events of stratified differentiation [26]. Because these changes in C/EBPβ deficient epidermis were modest it led to the notion that C/EBPβ has supportive rather than a major role in epidermal keratinocyte differentiation. However, another possibility for the lack of a major skin phenotype in the C/EBPα and C/EBPβ knockout mouse is that functional redundancies between the transcription factors mask their roles in the single knockouts. In support of this notion are studies demonstrating that the knockin of C/EBPβ into the C/EBPα locus can rescue C/EBP$\alpha^{-/-}$ mice from perinatal lethality [27] and restore granulocytic differentiation and glycogen metabolism [27]. A recent study in developing mouse skin in which C/EBPα and C/EBPβ were co-deleted during epidermal development produced mice that were only viable for a few hours after birth, and the analysis of epidermis from these mice revealed hyperplasia and decreased expression of spinous and granular markers of differentiation [28]. Thus, while C/EBPα and C/EBPβ are critical for epidermal development, their roles in stratified squamous differentiation in the adult epidermis remain poorly understood.

Sebaceous glands extend off of the upper hair follicle and are formed from a sebocyte progenitor that expresses Blimp 1, keratin 14 (K14) and keratin 5 (K5) [29] and these cells produce a proliferative population of sebocytes which in turn differentiate to produce the lipid/sebum producing cells [30]. These differentiated sebocytes lyse and release their contents of lipids/sebum into the hair follicle canal and make their way to surface of the skin where they prevent drying of the skin and hair [31]. cMyc [30,32], hedgehog signaling [33] and AP-2α/AP-2γ [21] regulate sebaceous gland development/differentiation. C/EBPα and C/EBPβ are abundantly expressed in sebocytes of the sebaceous glands [34,35], however, no function for these transcription factors in sebocytes has been described and no sebaceous gland phenotype was reported in C/EBPα or C/EBPβ knockout mice.

To address possible functional redundancies and to reveal the functional roles of C/EBPα and C/EBPβ in the adult epidermis and sebaceous gland, mouse models were developed in which either family member could be ablated alone or together in the epidermis and sebaceous glands of adult mice. Our results demonstrate C/EBPα and C/EBPβ are essential for sebocyte differentiation and epidermal squamous differentiation involving the basal to spinous transition.

Methods

Ethics Statement

All animal work described in the study involving animal husbandry, experimentation, and care/welfare have been conducted according to NIH guidelines and approved by NCSU Institutional IACUC committee.

Animals and treatments

K14-CreER^{tam} homozygous mice [36] (CD1) were obtained from Jackson Labs (#005107) and crossed with either floxed-α (B6.129)[37], floxed-β (B6.129)[38] or floxed-$\alpha\beta$ (B6.129). Floxed-α and floxed-β mice were genotyped as previously described [37,38]. Mice were genotyped for K14-CreER^{tam} using the following primers: forward, CGATGCAACGAGTGATGAGGTTC; reverse, GCACGTTCACCGGCATCAAC. Mice 6-8 weeks of age in telogen were treated topically to clipped skin with 4OHT (Sigma catalog # T176-50MG) 1.0mg/day for days 1-5 and again on days 13-17 dissolved in 95% ethanol (1.0mg/200ul) and skin collected for IHC on day 21.

Preparation of epidermal homogenates

Mice were killed by cervical dislocation and dorsal skin was removed and subjected to 15 seconds heat shock in 65^{o}C deionized water followed by 15 seconds in ice water. Water was removed from skin by blotting between paper towels and epidermis was scraped from dermis. For C/EBPα and C/EBPβ proteins, epidermal scrapes were homogenized on ice in RIPA buffer (1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1.5mM sodium orthovanadate, 1.5mM

phenylmethylsufonyl fluoride, 1.5mM dithiothreitol, and 1x protease inhibitor cocktail (Roche Diagnostics GmbH ref 11 836 001) in PBS), and centrifugated at 14,000g for 20 minutes at 4 ^{o}C. Supernatants were stored at -80^{o}C prior to use. Protein quantification was determined by Bio-Rad Protein Assay reagent (Bio-Rad, Cat# 500-0006). For keratins and cornified envelope precursors, epidermal scrapes were homogenized in 20% 2-mercaptoethanol and 5% SDS, boiled 5 minutes, allowed to cool and centrifugated at 14,000g for 10 minutes.

Immunoblot analysis

Equal amounts of protein were denatured in sample buffer, loaded onto 12% tris-glycine gels (Invitrogen, EC6005), separated by gel electrophoresis and transferred to PVDF membrane. Membranes were blocked for one hour (PBS with 0.1% tween/5.0% milk/1.0% BSA), and incubated overnight at 4^{o}C (PBS with 0.1% tween/1.0% BSA) with one of the following rabbit polyclonal antibodies: C/EBPα (Santa Cruz, SC-61, 1:2000), C/EBPβ (Santa Cruz, SC-150, 1:2000), K1 (Covance, 1:10,000), K5 (Covance, 1:10,000), K10 (Covance, 1:10,000), K14 (Covance, 1:10,000), involucrin (Covance, 1:10,000), loricrin (Covance, 1:10,000), filaggrin (Covance, 1:10,000). Membranes were then rinsed one hour in PBS-T (0.1% tween) and subjected to one hour incubation (PBS-T, 1.0% BSA) with anti-rabbit IgG, horseradish peroxidase-linked secondary (GE Healthcare, NA934V, 1:2500), incubated one minute with Western Lightening Plus-ECL (PerkinElmer NEL105001EA) and subsequently exposed to film. Each blot represents $n \geq 3$ mice per genotype. Membranes were stripped and re-probed for β-actin (Sigma A-5441,

1:25,000, mouse) for one hour followed by one hour incubation anti-mouse horseradish peroxidase-linked secondary (GE Healthcare, NXA931, 1:25,000), incubated one minute with Western Lightening Plus-ECL (PerkinElmer NEL105001EA) and subsequently exposed to film.

Cell proliferation analysis

Mice were injected intraperitoneally with bromodeoxyuridine (BrdU, Sigma B5002, 100mg/kg in PBS) and killed 1 hour later. Skin was fixed in 10% neutral buffered formalin phosphate (NBF) for 20h and moved to 70% ethanol and subsequently embedded in paraffin. Immunohistochemical (IHC) staining for BrdU was performed as previously described [23] on 5μM sections with anti-BrdU (BD Biosciences 27644, 1:25).

Immunohistochemical (IHC) staining

Tissues were fixed in 10% NBF for 20h, switched to 70% ethanol and embedded in paraffin. Tissue sections (5μM) were stained for H&E or specific IHC as described [23,26,39] with the following antibodies: C/EBPα (Santa-Cruz SC-61, 1:1000), C/EBPβ (Santa-Cruz SC-7962, 1:1000), K5 (Covance PRB-160P, 1:2000), K10 (Covance PRB-159P, 1:2000), involucrin (Covance PRB-140C, 1:2000), loricrin (Covance PRB-145P, 1:2000), K6 (Covance PRB-169P, 1:2000) and fatty acid synthase (FASN)(Santa-Cruz SC-20140, 1:1000).

Oil Red O staining

Fresh unfixed epidermis sections were frozen on liquid nitrogen in Tissue-Tek® O.C.T (Sakura Finetek, #4583) compound and stored at -80^{o}C. Frozen sections (10

microns) were washed successively in distilled water, 30% isopropanol, and 60% isopropanol. Slides were then incubated in Oil Red O for 15 minutes and subsequently washed in 60% isopropanol, 30% isopropanol, and water. Slides were counterstained with hematoxylin, rinsed in water for 10 minutes and coverslipped with glycerin jelly.

Co-immunofluorescence staining

Tissue slides were deparaffinized and hydrated in successive washes (Xylene, 95% ethanol, 70% ethanol, PBS), blocked (K5,K10: 1h with 1.5% natural goat serum (NGS), 1.5% natural horse serum (NHS), 1% bovine serum albumin (BSA) in PBS. C/EBPα,C/EBPβ: 2h with 6%NGS, 6%NHS, 1%TritonX in PBS), and incubated overnight at 4^{o}C with primary antibodies: K5 (rabbit polyclonal, Covance, 1:1000), K10 (mouse monoclonal, Covance, 1:1000), C/EBPα (rabbit polyclonal, Santa-Cruz SC-61, 1:400), and C/EBPβ (mouse monoclonal, Santa-Cruz SC-7962, 1:200). Slides were washed in PBS and incubated 45 minutes with fluorophore-linked secondary antibodies (Alexa Fluor® 568 goat anti-mouse (1:1000), Alexa Fluor® 488 goat anti-rabbit (1:1000)) in PBS, dehydrated and coverslipped with Dako Faramount Aqueous Mounting Medium.

RNA and quantitative PCR

In brief, mice were killed by cervical dislocation and skin removed. Whole skin was minced in Sigma Tri-Reagent®, T-2494, sonicated on ice 4x10s, centrifuged 14,000g/10m/4^{o}C, and RNA was extracted with chloroform. Pelleted RNA was solubilized in water and then purified with DNase on Qiagen RNeasy® columns.

cDNA was created with equal amounts of starting template with reverse transcriptase with appropriate no-RT controls. cDNA was then subjected to Real Time TaqMan® PCR (Applied Biosystems: keratin 10 (Mm03009921_m1), SCD3 (Mm00470480_m1), MC5R (Mm00442970_m1)) using GAPDH (Mm99999915_g1) as a housekeeping gene) and semi-quantitative PCR (all others) using the primers given in Figure S1. For semi-quantitative PCR, PCR product was then run on 2% agarose gels with .01% ethidium bromide. After confirming PCR amplification was in the linear range by varying the amount of starting template, optical density of bands were determined by ImageJ™. Each experiment included 3 or more animals of each genotype.

Results

C/EBPα and C/EBPβ are co-expressed in skin

The bZIP transcription factors, C/EBPα and C/EBPβ are expressed in mouse epidermis and sebaceous glands [23,24,34]. To determine whether C/EBPα and C/EBPβ are co-expressed within the same cell and not just within the same tissue or gland we performed co-immunofluorescence labeling for C/EBPα and C/EBPβ in untreated animals. As shown in Fig. 1A, C/EBPα and C/EBPβ are co-expressed in the nuclei of many interfollicular epidermal (IFE) keratinocytes. Most IFE suprabasal and basal keratinocytes that expressed C/EBPβ also expressed C/EBPα. However, some basal keratinocytes predominately expressed C/EBPα. The majority of outer root sheath (ORS) follicular keratinocytes co-expressed C/EBPα and C/EBPβ in their nuclei as did the vast majority of sebocytes of the sebaceous gland which displayed the highest levels of staining intensity for C/EBPα and C/EBPβ (Fig. 1B).

Acute ablation of either C/EBPα or C/EBPβ alone in adult mouse skin does not produce a major skin phenotype

To ablate C/EBPα and C/EBPβ in postnatal adult mouse skin we used a conditional inducible approach utilizing K14-CreER^{tam} mice [36]. The K14 promoter directs the expression CreER^{tam} to the basal layer of the epidermis, ORS follicular keratinocytes of the hair follicle as well as the progenitor and proliferative populations of sebocytes of the sebaceous gland of mouse skin. The activity of CreER^{tam} is regulated through topical treatment with the synthetic ER antagonist, 4-hydroxytamoxifen (4OHT).

While the overall goal was to simultaneously acutely ablate C/EBPα and C/EBPβ in adult mouse skin, it was first necessary to characterize the epidermis of adult mice in which C/EBPα or C/EBPβ were individually acutely ablated. While the phenotypes of the epidermal specific germline knockouts of C/EBPα and C/EBPβ have been previously characterized [25,26], it could not be assumed that acute ablation in fully-developed adult skin would mimic the germline embryonic knockouts where developmental compensatory events could have occurred in the embryo to mask a postnatal phenotype. Moreover, in order to accurately define and interpret the phenotype of the double knockout it was important to determine the phenotype of single C/EBP knockouts in the same experimental model system. K14-CreER^{tam};C/EBPα^{ff} mice and K14-CreER^{tam};C/EBPβ^{ff} mice were generated and we first analyzed K14-CreER^{tam};C/EBPβ^{ff} mice (hereafter referred to as (inducible knock out) IKOβ mice). Before treating mice with 4OHT, the levels of C/EBPβ protein in the epidermal homogenates of untreated IKOβ and untreated C/EBPβ^{ff} mice (β^{ff}) were examined. Immunoblot analysis demonstrated epidermal C/EBPβ levels were similar in the two genotypes indicating the CreER^{tam} fusion protein was not significantly active in the absence of 4OHT treatment (Fig. 1C). 4OHT treatment (1.0 mg 4OHT/day for days 1-5 and 13-17; skin was collected on day 21), had no significant effect on C/EBPα or C/EBPβ levels in floxed-β mice (Fig. 1C), however, 4OHT treatment resulted in near complete ablation of C/EBPβ in IKOβ epidermis and this was consistently accompanied by a modest increase in C/EBPα levels

suggesting possible compensatory upregulation (Fig. 1C). C/EBPβ is expressed in three forms (LAP* (39kD), LAP (36kD) and LIP (20kDa) due to the use of alternative translation initiation sites and all forms of C/EBPβ were decreased in 4OHT-treated IKOβ mice (data not shown). Immunohistochemical (IHC) staining for C/EBPβ confirmed the loss of C/EBPβ in the IFE and revealed the loss of C/EBPβ in the ORS follicular keratinocytes of the hair follicle and sebocytes of sebaceous gland (Fig. 1D). The 4OHT-treated IKOβ epidermis displayed a moderate increase in: epidermal thickness (Fig. 1E right), the number of nucleated cell layers (Fig. 1F) and the number of BrdU positive basal keratinocytes following a 1 hour pulse was increased (Fig. 1G first panel). Overall, with the exception of the epidermal thickness and the increase in nucleated cell layers, the morphology of the IFE, ORS of the hair follicle and sebaceous glands of the 4OHT-treated IKOβ epidermis were indistinguishable from 4OHT-treated β^{ff} mice (Fig. 1E). IHC staining for K5, K10, loricrin and involucrin in C/EBPβ^{ff} and IKOβ epidermis treated with 4OHT revealed the correct spatial expression patterns, however, similar to germline C/EBPβ knockout mice [26], we observed a modest decreased staining intensity for K10 (Fig. 1H).

Next, K14-CreER^{tam};C/EBPα^{ff} (IKOα) were examined. Baseline levels of C/EBPα protein in the epidermis of untreated IKOα mice and control C/EBPα^{ff} (α^{ff}) mice were similar and the topical application of 4OHT to IKOα mice resulted in greatly reduced levels of C/EBPα and a modest increase in C/EBPβ (Fig. 2A). Both

isoforms of C/EBPα ((p42 (42 kD) and p30 (30kD)) were similarly decreased after treatment with 4OHT (data not shown). IHC staining for C/EBPα demonstrated the loss of C/EBPα in the IFE, ORS cells of the hair follicle and sebocytes in 4OHT treated IKOα animals (Fig. 2B). Epidermal thickness and basal cell proliferation as measured by BrdU incorporation was moderately increased in 4OHT-treated IKOα vs. 4OHT-treated C/EBPα^{ff} (Fig. 2D). With the exception of a slight increase epidermal thickness proliferation, the morphology of the IFE, ORS follicular epidermis and sebaceous glands of the 4OHT-treated IKOα epidermis were indistinguishable from 4OHT-treated C/EBPα^{ff} mice (Fig. 2C) as was the IHC staining for K5, K10, loricrin and involucrin in epidermis (data not shown). In summary, with the exception of modest increases in epidermal proliferation in 4OHT-treated IKOα mice, the acute single ablation of C/EBPα or C/EBPβ in adult mouse epidermis results in a phenotype similar to the germline knockout phenotype [25,26], indicating developmental compensatory events do not have a major role in masking the postnatal germline knockout phenotype.

Acute co-ablation of C/EBPα and C/EBPβ results in severe morphological defects in epidermis involving hyperplasia, dysplasia and hyperkeratosis

To address potential functional redundancies between C/EBPα and C/EBPβ and unmask their function in epidermis and sebaceous glands, K14-CreER^{tam};C/EBPα^{ff};C/EBPβ^{ff} (IKOαβ) and C/EBPα^{ff};C/EBPβ^{ff} ($\alpha^{ff}\beta^{ff}$) mice were generated and characterized. Immunoblot analysis demonstrated the levels of

C/EBPα and C/EBPβ in the untreated epidermis of IKOαβ mice and $\alpha^{ff}\beta^{ff}$ mice were similar (Fig. 3A), and the topical application of 4OHT to IKOαβ and $\alpha^{ff}\beta^{ff}$ mice resulted in dramatic decreases in both C/EBPα and C/EBPβ in IKOαβ but not 4OHT-treated $\alpha^{ff}\beta^{ff}$ mice (Fig. 3A). All forms of C/EBPβ (LAP*, LAP and LIP) and C/EBPα (p42 and p30) were similarly decreased (data not shown). Loss of C/EBPα and C/EBPβ in the IFE, ORS follicular keratinocytes and sebocytes was demonstrated by co-IF staining for C/EBPα and C/EBPβ in 4OHT-treated IKOαβ and 4OHT-treated $\alpha^{ff}\beta^{ff}$ mouse skin (Fig. 3B). In normal IFE, basal keratinocytes progress upwards through morphologically distinct spinous and then granular suprabasal layers, eventually ending in the production of a nonviable stratum corneum or cornified layer. Histological analysis of dorsal skin sections from 4OHT-treated IKOαβ and 4OHT-treated $\alpha^{ff}\beta^{ff}$ mice revealed $\alpha^{ff}\beta^{ff}$ IFE was normal (Fig. 3D). In contrast, the 4OHT-treated IKOαβ IFE and infundibular ORS keratinocytes were hyperplastic (Fig. 3E&F) and the IFE displayed a significant increase in the number of nucleated epidermal cell layers (Fig. 3C). In addition, the basal and spinous layers of the 4OHT-treated IKOαβ epidermis were dysplastic as characterized by the disorganization of the layers and by highly abnormal variations in cell and nucleus size (Fig. 3G&H). There were also focal and regional areas of moderate to severe hyperplasia of the follicular infundibular epithelium and ORS keratinocytes (Fig. 3F) in the 4OHT-treated IKOαβ epidermis. In contrast to the disorganized basal and spinous layers, the granular layer of keratinocytes in 4OHT-treated IKOαβ mice

appeared less affected and keratohyalin granules, a hallmark of granular layer, were present at levels similar to 4OHT-treated control mice (Fig. 3I-J). The stratum corneum in 4OHT-treated IKO$\alpha\beta$ epidermis was significantly thickened (hyperkeratosis) (Fig. 3E-G) and displayed low levels of parakeratosis. Collectively, these morphological and histological alterations in the 4OHT-treated IKO$\alpha\beta$ epidermis indicate the acute loss of C/EBPα and C/EBPβ disrupts epidermal homeostasis, and within the viable epidermis, the basal and spinous layers appear to be most severely affected. We also examined mice at different times after the start of 4OHT treatment to determine when epidermal abnormalities appeared. We observed focal areas of hyperplasia in as early as four days from start of treatment and these areas became more extensive and severe at 8, 12, and 18 days from start of treatment (Fig 3K).

Acute co-ablation of C/EBPα and C/EBPβ results in specific molecular defects in squamous differentiation.

4OHT-treated IKO$\alpha\beta$ epidermis was analyzed for specific molecular defects in differentiation using IHC staining for molecular markers of specific stages of squamous differentiation. IHC staining in $\alpha^{ff}\beta^{ff}$ and IKO$\alpha\beta$ epidermis from mice treated with 4OHT were conducted side by side and 3,3'-diaminobenzidine (DAB) incubation time was standardized to 4OHT-treated $\alpha^{ff}\beta^{ff}$ epidermis. In 4OHT-treated IKO$\alpha\beta$ epidermis, the basal cell markers, K5 and K14, were no longer restricted to the basal layer, but were aberrantly expressed throughout all suprabasal layers of

the epidermis (Fig. 4A). However, K14 and especially K5 IHC staining were significantly less intense in 4OHT-treated IKO$\alpha\beta$ epidermis than in 4OHT-treated $\alpha^{ff}\beta^{ff}$ epidermis. Immunoblot analysis of epidermal lysates from 4OHT treated $\alpha^{ff}\beta^{ff}$ and 4OHT-treated IKO$\alpha\beta$ epidermis (Fig. 4B) revealed increased levels of K14 in IKO$\alpha\beta$ epidermis, while K5 showed similar levels to $\alpha^{ff}\beta^{ff}$ controls substantiating the IHC results showing reduced levels but a greatly expanded K5 compartment (Fig. 4A). K10, an early marker of the spinous layer and of the basal to spinous transition was significantly reduced in 4OHT-treated IKO$\alpha\beta$ epidermis (Fig. 4A). K1, another early marker of the spinous layer was delayed in its expression in 4OHT-treated IKO$\alpha\beta$ epidermis and instead of being expressed in the layer adjacent to the basal cell layer, K1 was not expressed until ~2-3 layers of cells above the basal layers (Fig. 4A). Immunoblot analysis for K1 and K10 confirmed the IHC results (Fig. 4B). In general, markers of later stages of differentiation such as involucrin and filaggrin were not decreased. Involucrin, a component of the cornified envelope and a marker of the granular layer, was expressed in the appropriate layers but at higher levels compared to similarly treated $\alpha^{ff}\beta^{ff}$ epidermis (Fig. 4A-C). Filaggrin, a marker of the granular layer was also expressed at higher levels in 4OHT-treated IKO$\alpha\beta$ epidermis than in similarly treated $\alpha^{ff}\beta^{ff}$ epidermis (Fig. 4B). In contrast, the expression of loricrin, a component of the cornified envelope, was significantly diminished in 4OHT-treated IKO$\alpha\beta$ epidermis (Fig. 4A-C). In general, mRNA levels of the various differentiation markers were in accord with immunoblot and IHC levels

(Fig. 4C). Overall the molecular changes in K5, K14, K1, and K10 in the 4OHT-treated IKOαβ epidermis are consistent with a defect in the basal to spinous transition.

Acute co-ablation of C/EBPα and C/EBPβ results in hyperproliferative basal and suprabasal keratinocytes.

Cell proliferation analysis was conducted using a 1h pulse label with BrdU, followed by IHC analysis for BrdU positive S-phase cells. 4OHT-treated IKOαβ epidermis demonstrated a significant increase in the numbers of BrdU positive S-phase cells in the IFE and ORS keratinocytes (Fig. 5 A&B) over 4OHT-treated $\alpha^{ff}\beta^{ff}$ epidermis. In 4OHT-treated IKOαβ IFE, there was a 4.7 fold increase in BrdU positive basal S-phase keratinocytes compared to similarly treated $\alpha^{ff}\beta^{ff}$ mice (Fig. 5C). In normal epidermis, suprabasal proliferating keratinocytes are infrequent as proliferating keratinocytes are restricted to the basal layer. Analysis of 4OHT-treated IKOαβ epidermis revealed suprabasal BrdU positive S-phase keratinocytes were greatly increased and were observed at a ~27 fold increase over 4OHT-treated $\alpha^{ff}\beta^{ff}$ epidermis (Fig. 5B&C). Collectively, these results demonstrate hyperproliferative basal and suprabasal keratinocytes and suggest an impairment of basal keratinocyte cell cycle withdrawal in mice missing C/EBPα and C/EBPβ.

Acute co-ablation of C/EBPα and C/EBPβ results in molecular, morphological and differentiation defects in sebocytes.

In untreated mouse skin, sebaceous glands are associated with the upper follicle and these glands contained numerous sebocytes with a large light staining, finely vacuolated, clear translucent cytoplasm and a distinct round nucleus. Small reserve cells, (proliferative undifferentiated sebocytes) were less numerous and were peripheral in the glands (Fig. 6A). In the skin of 4OHT-treated IKOαβ mice, hair follicles contained distinct sebaceous gland lobules in the typical location on the hair follicle (Fig. 6B); however, these glands contained unusual looking sebocytes that appeared undifferentiated, had reduced cytoplasmic volume and lacked clear fine vacuolation supportive of lipid accumulation. Affected glands had an increased cellular density with more closely apposed nuclei and the cells were morphologically reminiscent of the reserve undifferentiated cells**.** To determine whether other sebaceous type glands were similarly affected, we examine the Meibomian and preputial glands for C/EBPα and C/EBPβ expression and for alterations in 4OHT-treated IKOαβ mice. Meibomian glands are found at the rim of the eyelid of mammals and the Meibomian sebocytes produce and release a sebum-like substance onto the eye that functions to prevent the evaporation of the tear film on the eye [40]. The preputial gland, found in some male mammals and located in the subcutaneous tissue of the inguinal area, is involved in the production and secretion of pheromones and lipids. Co-IF staining revealed C/EBPα and C/EBPβ were co-expressed in sebocytes of both the Meibomian and preputial glands (Fig. 6C&D).

Gross examination of 4OHT-treated IKO$\alpha\beta$ mice revealed that eyes of these mice appeared dry, swollen, and partially closed. Histological analysis revealed atrophied Meibomian glands characterized by greatly reduced lobule size and diminished numbers of differentiated sebocytes with clear vacuolated cytoplasm (Fig. 6E). In addition, there was severe regional epidermal hyperplasia of the haired skin eyelid epidermis as well as hyperplasia of the Meibomian ducts and follicular infundibula, which was similar to skin epithelium (Fig. 6E right panel). In contrast, eyes of 4OHT-treated $\alpha^{ff}\beta^{ff}$ mice were normal and the mice contained normal appearing Meibomian glands and ducts (Fig. 6E). Preputial glands from 4OHT-treated IKO$\alpha\beta$ mice were also abnormal. The 4OHT-treated $\alpha^{ff}\beta^{ff}$ mice displayed preputial glandular cells with typical, abundant, clear, finely vacuolated, translucent cytoplasm and distinct round nuclei (Fig. 6F circle). In contrast, 4OHT-treated IKO$\alpha\beta$ mice had marked atrophy of preputial gland lobules and decreased numbers of clear finely vacuolated sebocytes (Fig. 6F right). Thus, the acute lost of C/EBPα and C/EBPβ severely impairs sebaceous, Meibomian, and preputial gland homeostasis and results in the loss of morphologically distinct differentiated sebocytes in all of these glands. To provide biochemical evidence for altered sebocyte differentiation, histological sections of skin of 4OHT-treated $\alpha^{ff}\beta^{ff}$ and 4OHT-treated IKO$\alpha\beta$ mice were subjected to Oil Red O staining. Oil Red O stains lipid and sebum and positive Oil Red O staining of sebaceous glands is a hallmark of sebocyte differentiation. In 4OHT-treated $\alpha^{ff}\beta^{ff}$ mice, Oil Red O stained all sebaceous glands, demonstrating the presence of

terminally differentiated sebum producing sebocytes associated with nearly every hair follicle (Fig. 6G). In contrast, the sebaceous gland lobules associated with hair follicles in 4OHT-treated IKO$\alpha\beta$ mice did not stain with Oil Red O (Fig. 6G right and bottom right panels) indicating the absence of sebum and terminally differentiated sebocytes. To further characterize the sebaceous gland defect in 4OHT-treated IKO$\alpha\beta$ mice, we examined K14, fatty acid synthase (FASN), stearoyl-CoA desaturase (SCD3) and melanocortin 5 receptor (MC5R). K14 is expressed in the basal IFE keratinocytes and is also expressed in the undifferentiated peripheral sebocytes in sebaceous glands, while SCD3 and MC5R have been characterized as specific markers of sebocyte differentiation. Within the sebaceous glands of 4OHT-treated $\alpha^{ff}\beta^{ff}$ mice, K14 is appropriately expressed in the undifferentiated sebocytes around the periphery of sebaceous gland (Fig. 6I). In contrast, the entire 4OHT-treated IKO$\alpha\beta$ sebocyte lobule uniformly expressed K14 suggesting that the lobule may be composed of a population of undifferentiated sebocytes. To further identify these cells within the lobule as sebocytes we conducted IHC staining for FASN. Sebaceous glands in 4OHT-treated $\alpha^{ff}\beta^{ff}$ mice expressed FASN as did 4OHT-treated IKO$\alpha\beta$ sebocyte lobules indicating that the lobules are comprised of sebocytes (Fig. 6J). The intense FASN staining of 4OHT-treated IKO$\alpha\beta$ sebocyte lobules compared to control glands is likely due to reduced cytoplasmic volume of IKO$\alpha\beta$ sebocytes. FASN mRNA levels were similar in skin between the two genotypes (Fig. 6H). SCD3 and MC5R are markers of terminally differentiated sebocytes and real time

quantitative PCR revealed mRNA of both were greatly reduced in 4OHT-treated IKO$\alpha\beta$. Collectively these morphological, biochemical and molecular results indicate that acute co-ablation of C/EBPα and C/EBPβ in adult mice results in a significant sebaceous gland defect in which sebocyte terminal differentiation and lipid production is impaired.

Discussion

The co-ablation of C/EBPα and C/EBPβ in adult mouse skin revealed critical biological functions for these transcription factors in keratinocytes and sebocytes that are not present in germ line single knockout mice [25,26] as well as the inducible conditional single knockout mice described in this study. For example, sebaceous glands of C/EBPα or C/EBPβ knockout mice were indistinguishable from wild type sebaceous glands. In contrast, the co-ablation of C/EBPα and C/EBPβ resulted in severe sebaceous gland morphological defects and a failure of sebocyte differentiation. Likewise, acute co-deletion of C/EBPα and C/EBPβ produces profound changes in epidermal homeostasis not seen in single KOs that involves the disruption of stratified squamous differentiation, disorganization of the epidermis and the hyperproliferation of the IFE keratinocytes as well as ORS follicular keratinocytes. Thus the co-ablation of C/EBPα and C/EBPβ has revealed previously unknown functional roles for these transcription factors in sebocyte biology and has confirmed and eclipsed earlier studies in C/EBPβ knockout mice that indicated a role for C/EBPβ in early events of keratinocyte differentiation involving K1/K10 expression and growth arrest [26]. Our results indicate that functional redundancies exist between C/EBPα and C/EBPβ and/or these two transcription factors cooperate to regulate basal keratinocyte cell cycle withdrawal and early differentiation in keratinocytes and sebocytes.

Sebaceous glands are formed from a sebocyte progenitor that expresses Blimp 1, keratin 14 (K14) and K5 [29], and these cells produce a proliferative population of sebocytes [30,32] which in turn differentiate into the lipid/sebum producing cells. Through the inducible conditional co-ablation of C/EBPα and C/EBPβ we unmasked novel roles for these transcription factors in sebaceous gland differentiation. Sebocytes lacking C/EBPα and C/EBPβ were morphologically altered and sebum production was blocked. IKOαβ mice retained distinct sebaceous gland lobules, but these cells did not produce sebum, a hallmark of sebocyte differentiation. It appears the proliferating cells located on the periphery of the gland continued dividing but their progeny were unable to initiate terminal differentiation. Our observation that the cells in the lobules of IKOαβ mice express K14 and FASN is consistent with this idea, providing compelling evidence that the cells within the lobule are sebocytes. In addition, we observed decreased levels of SCD3 and MC5R, two markers of differentiated sebocytes. Our results indicate the adipogenic transcription factors, C/EBPα and C/EBPβ, also have critical roles in sebocyte differentiation through the regulation of lipogenesis and sebum production. The critical importance of these transcription factors in sebocyte homeostasis was further strengthened by the profound effect of their co-ablation on the specialized sebocytes of the Meibomian and preputial glands. Meibomian glands of mice lacking C/EBPα and C/EBPβ were severely disorganized, dysplastic and there was evidence of bacterial infection. Posterior blepharitus is a human disease often

characterized by improper sebum production by Meibomian glands, lending an environment favorable for bacterial growth, and accompanied by inflammation [41]. Acne, seborrheic dermatitis, blepharitis, and sebaceous cancer are among several human health conditions involving altered sebocyte homeostasis. It seems plausible the transcription factors C/EBPα and C/EBPβ could have involvement in these disease conditions.

Molecular analysis of IKOαβ epidermis revealed that K5 and K14, markers of basal keratinocytes, were expressed throughout the basal and suprabasal layers, while suprabasal markers K1 and K10 were delayed or weakly expressed. A concomitant increase in hyperproliferative basal and suprabasal IFE keratinocytes was observed in IKOαβ epidermis. These results indicate an expansion of the basal compartment and reduced ability of these basal keratinocytes to initiate the early events of stratified squamous differentiation involving K1 and K10 expression and cell cycle withdrawal. Both C/EBPα and C/EBPβ can bind to and activate the K10 promoter and C/EBP binding sites [42] have been identified in both the K1 and K10 promoters [26] suggesting differences in K1 and K10 expression could be regulated directly by C/EBPs at the promoter level. Chromatin immuno-precipitation experiments will be required to resolve these issues.

In general, the granular layers of IKOαβ epidermis appeared less affected; keratohyalin granules were present and involucrin and filaggrin were expressed, albeit at increased levels. These changes in increased involucrin and filaggrin expression could be a response to the disruption of epidermal homeostasis due to

the defective basal to spinous transition or it is possible that C/EBPα and C/EBPβ contribute to the suppression of granular differentiation as has been reported for Hes1 in epidermal development [19].

Mechanisms of epidermal development and postnatal differentiation are not necessarily synonymous. For example, ablation of Notch in epidermal development produces the loss of spinous and granular layers and a hypoproliferative phenotype [18], while postnatal ablation of Notch1 produce an opposite effect involving a hyperproliferative phenotype and an increased granular layer [20]. Hes1 ablation dramatically alters epidermal development involving the spinous to granular transition, but appears to have a minimal role in adult skin [19]. Recently the co-deletion of C/EBPα and C/EBPβ in developing mouse skin produced mice that died soon after birth, and the analysis of epidermis from these mice revealed hyperplasia, decreased expression of spinous and granular markers of differentiation and a role for E2F in the hyperplastic epidermis [28]. While we observed similar changes in basal cell hyperproliferation and defects in spinous markers of differentiation, we did not observe the granular changes reported in developing skin. For example, we did not observe diminished keratohyalin granules, filaggrin, and involucrin expression in adult epidermis and we did observe an uncoupling of involucrin and loricrin expression in adult epidermis. Collectively, these results indicate that there are significant differences between the effects of co-deletion of C/EBPα and C/EBPβ in embryonic development and postnatal differentiation.

Notch signaling is important in both the early and later stages of epidermal development where it regulates the basal to spinous transition in a Hes1 independent manner and the spinous to granular transition in a Hes1 dependent manner [18-20] . AP-2α and AP-2γ also have essential roles in both the early and later stages of epidermal development and recently it was reported that both AP-2 and Notch signaling converge on the regulation of the expression of C/EBPα and C/EBPβ [21]. Results reported in our current study demonstrate C/EBPα and C/EBPβ have critical roles in the early stages of squamous differentiation and our results are consistent with a model where C/EBPs function downstream of AP-2 and Notch signaling to regulate the basal to spinous transition.

As described in the introduction, the knockin of C/EBPβ into the C/EBPα locus rescues the lethality of C/EBP$\alpha^{-/-}$ mice and supports the notion of functional redundancies between these family members [27,43]. However, not all of the phenotypes of C/EBP$\alpha^{-/-}$ mouse were reversed suggesting that there are also unique C/EBP family member functions. Likewise our current study provides evidence for functional redundancies between C/EBPα and C/EBPβ in epidermal and sebocyte homeostasis. However, in terms of skin tumorigenesis, these family members have unique functions. For example, C/EBPα is a skin tumor suppressor [25] and a regulator of the G1 checkpoint in keratinocytes in the DNA damage response [44]. In contrast, C/EBPβ is a mediator of keratinocyte survival and is required for skin tumorigenesis [45]. Understanding the redundant and unique roles these two

transcription factors have will be important in the eventual elucidation of their roles in tumorigenesis.

Acknowledgements

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Figure Legends

Figure 1. C/EBPα/β are co-expressed in skin and acute ablation C/EBPβ does not produce a major skin phenotype

Co-IF staining of untreated mouse skin in: (A) interfollicular epidermis (IFE), and (B) the hair follicle outer root sheath (HF-ORS) and sebaceous gland (SG). (C) Immunoblot analysis of epidermal lysates from untreated and 4OHT-treated C/EBPβ^{ff} and IKOβ mice (n=3 mice/genotype/treatment). (D) IHC staining for C/EBPβ in 4OHT-treated C/EBPβ^{ff} and 4OHT-treated IKOβ mouse skin. (E) H&E staining of 4OHT-treated C/EBPβ^{ff} and 4OHT-treated IKOβ mouse skin. (F) Quantification of nucleated cell layers in 4OHT-treated C/EBPβ^{ff} and IKOβ mice (n=3 mice/genotype/treatment). (G) Quantification of basal BrdU positive cells in 4OHT-treated C/EBPβ^{ff} and IKOβ mice (n=3 mice/genotype/treatment). (H) IHC staining for BrdU and markers of squamous differentiation. All scale bars represent 30 microns. *indicates significantly different from controls p<.05, Student's t test.

Figure 2. Acute ablation C/EBPα does not produce a major skin phenotype

(A) Immunoblot analysis of epidermal lysates from untreated and 4OHT-treated C/EBPα^{ff} and IKOα mice (n=3 mice/genotype/treatment). (B) IHC staining for C/EBPα in 4OHT-treated C/EBPα^{ff} and 4OHT-treated IKOα mouse skin. (C) H&E staining of 4OHT-treated C/EBPα^{ff} and 4OHT-treated IKOα mouse skin. (D) Quantification of nucleated cell layers and number of basal BrdU positive cells in 4OHT-treated C/EBPα^{ff} and IKOα mice (n=3 mice/genotype/treatment). All scale

bars represent 30 microns. *indicates significantly different from controls p<.05, Student's t test.

Figure 3. Acute co-ablation of C/EBPα/β results in epidermal morphological defects.

(A) Immunoblot analysis of epidermal lysates from untreated and 4OHT-treated $\alpha^{ff}\beta^{ff}$ and IKOαβ mice (n=3 mice/genotype/treatment). (B) Co-IF staining for C/EBPα and C/EBPβ in 4OHT-treated $\alpha^{ff}\beta^{ff}$ and 4OHT-treated IKOαβ mouse skin. (C) Quantification of epidermal nucleated cell layers in 4OHT-treated mice. (D) H&E staining of 4OHT-treated $\alpha^{ff}\beta^{ff}$ mice. (E) H&E staining of 4OHT-treated IKOαβ mice. (F) Characteristic H&E staining of lesion in 4OHT-treated IKOαβ mouse. (G) H&E staining of 4OHT-treated IKOαβ epidermis with (H) region enlarged displaying dysplasia. (I) H&E staining and TEM micrograph of keratohyalin granules in 4OHT-treated (I) $\alpha^{ff}\beta^{ff}$ and 4OHT-treated (J) IKOαβ epidermis. (K) H&E staining of lesions from 4OHT-treated animals from different days after start of 4OHT treatment. Scale bars represent 30 microns unless otherwise notated. *indicates significantly different from controls p<.05, Student's t test.

Figure 4. Co-ablation of C/EBPα/β results in defects in epidermal squamous differentiation.

(A) IHC staining for markers of stratified squamous differentiation in 4OHT-treated controls and 4OHT-treated inducible double knockouts (IKOαβ). (B) Immunoblot analysis of epidermal lysates from animals treated with 4OHT for markers of

stratified squamous differentiation (n=3 mice/genotype). (C) Taqman® real time quantitative PCR (K10) and reverse transcriptase semi-quantitative PCR (all others) on mRNA from 4OHT-treated whole skin (n=5 mice/genotype). Scale bars represent 30 microns.

Figure 5. Co-ablation of C/EBPα/β results in hyperproliferative basal and suprabasal keratinocytes.

(A) IHC staining for BrdU-positive keratinocytes in HF-ORS and IFE in 4OHT-treated $\alpha^{ff}\beta^{ff}$ and 4OHT-treated IKOαβ epidermis. (B) Suprabasal BrdU-positive S-phase keratinocytes in 4OHT-treated IKOαβ mice. (C) Quantification of BrdU-positive basal and suprabasal IFE keratinocytes in 4OHT-treated $\alpha^{ff}\beta^{ff}$ and 4OHT-treated IKOαβ mice (n=3 mice/genotype, 3 strips per mouse; *$p<0.05$). Scale bars represent 30 microns.

Figure 6. Co-ablation of C/EBPα/β results in morphological and molecular defects in sebaceous, Meibomian and preputial glands.

(A) H&E staining of sebaceous glands/lobules of 4OHT-treated $\alpha^{ff}\beta^{ff}$ and (B) 4OHT-treated IKOαβ mice. Co-IF staining in (C) Meibomian glands and (D) preputial glands in untreated controls. H&E staining of (E) Meibomian glands (circled) and (F) preputial glands (circled) of 4OHT-treated $\alpha^{ff}\beta^{ff}$ and 4OHT-treated IKOαβ mice. (G) Oil Red O staining for lipids in 4OHT-treated $\alpha^{ff}\beta^{ff}$ (arrows point to sebaceous glands) and 4OHT-treated IKOαβ mice (arrows point to sebaceous lobules). (H) FASN: Semi-quantitative reverse transcriptase PCR on whole skin mRNA from

4OHT-treated $\alpha^{ff}\beta^{ff}$ and 4OHT-treated IKO$\alpha\beta$ mice. SCD3 and MC5R: TaqMan® Real Time PCR. (n=5 mice/treatment/genotype; *$p<0.05$). IHC staining for (I) keratin 14 and (J) FASN in 4OHT-treated $\alpha^{ff}\beta^{ff}$ and 4OHT-treated IKO$\alpha\beta$ mouse epidermis (AD-Adipose Tissue). Scale bars represent 30 microns.

Figure 1.

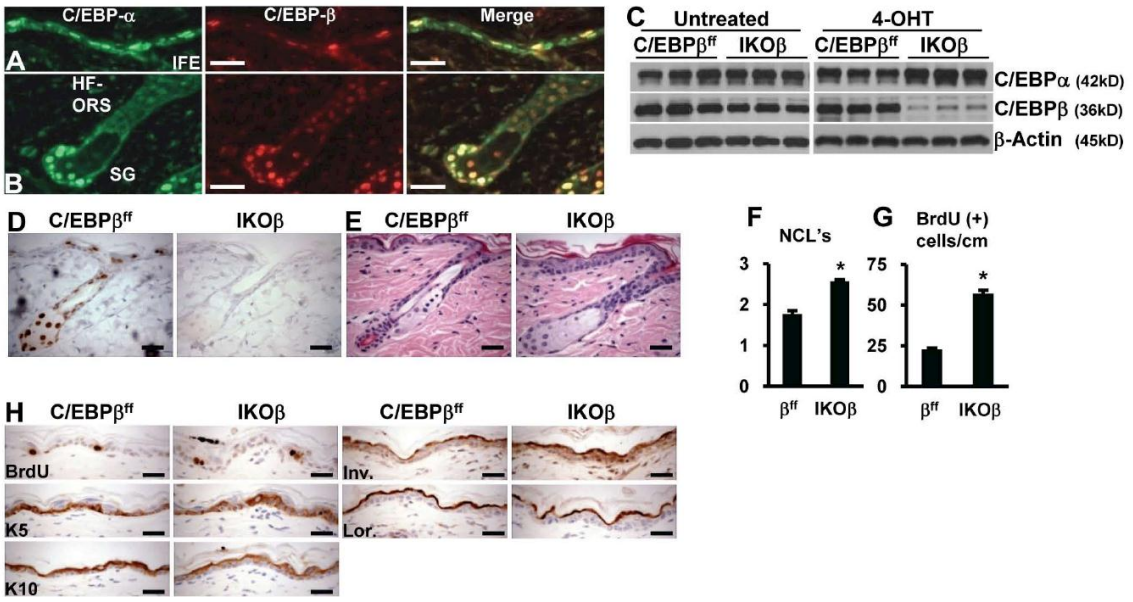

Figure 1. C/EBPα/β are co-expressed in skin and acute ablation C/EBPβ does not produce a major skin phenotype

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Figure 2

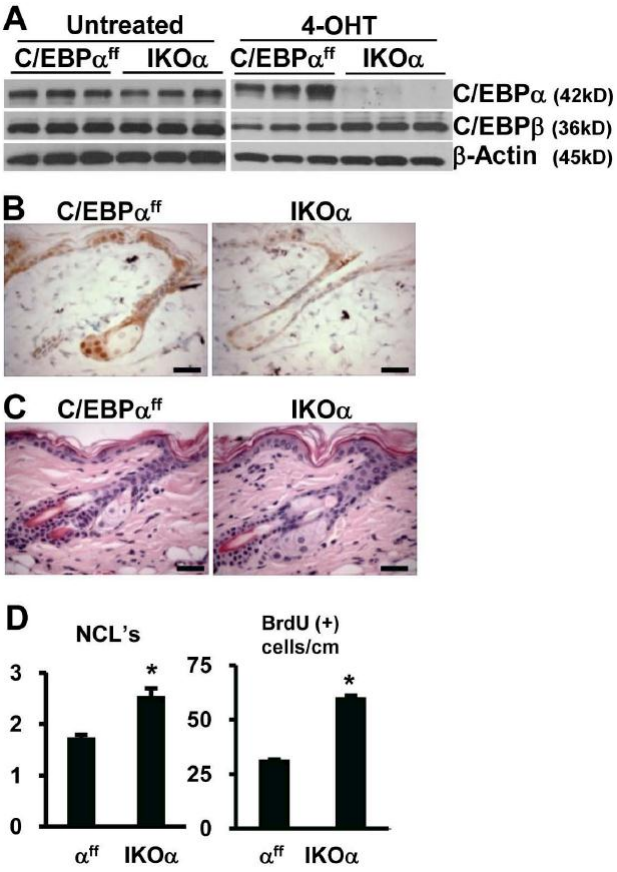

Figure 2. Acute ablation C/EBPα does not produce a major skin phenotype

(A) Immunoblot analysis of epidermal lysates from untreated and 4OHT-treated C/EBPα^{ff} and IKOα mice (n=3 mice/genotype/treatment). (B) IHC staining for C/EBPα in 4OHT-treated C/EBPα^{ff} and 4OHT-treated IKOα mouse skin. (C) H&E staining of 4OHT-treated C/EBPα^{ff} and 4OHT-treated IKOα mouse skin. (D) Quantification of nucleated cell layers and number of basal BrdU positive cells in 4OHT-treated C/EBPα^{ff} and IKOα mice (n=3 mice/genotype/treatment).

Figure 3

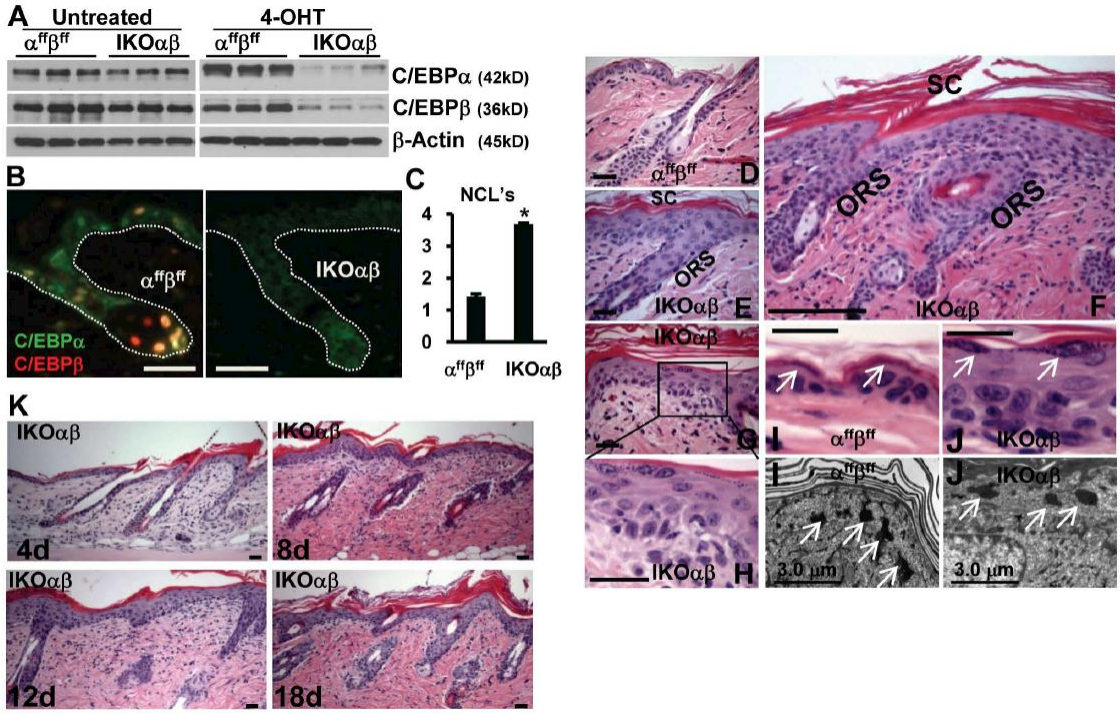

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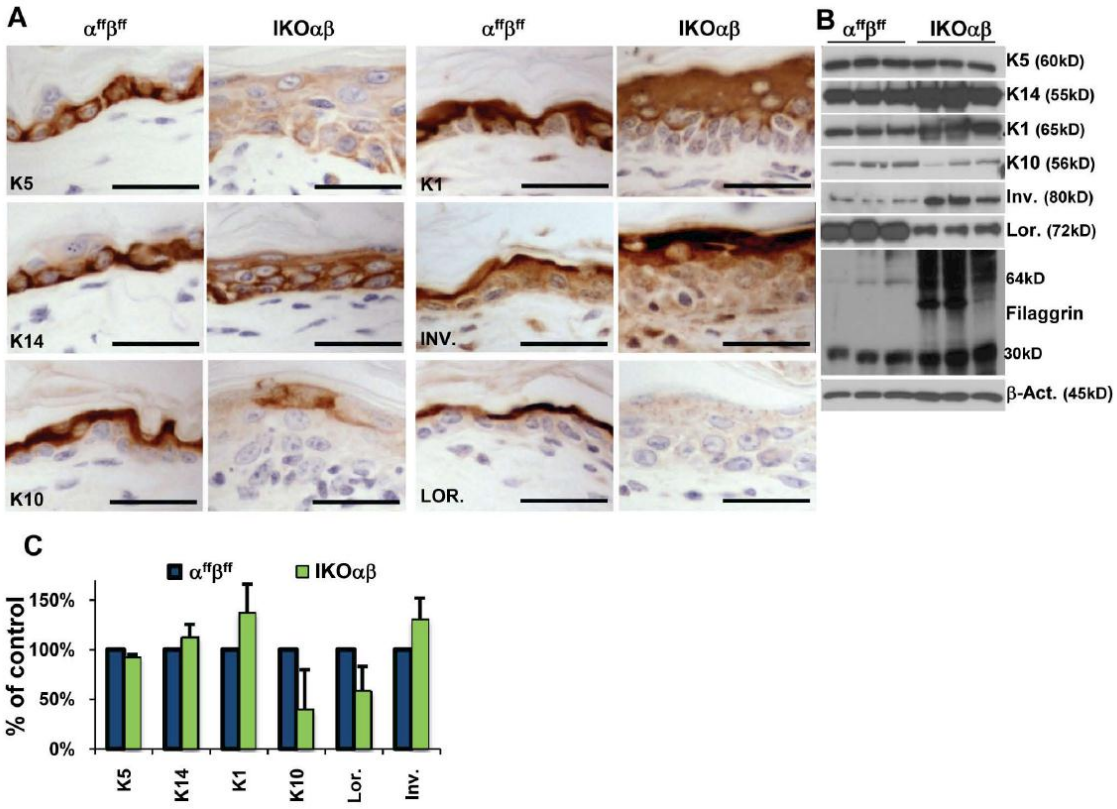
Figure 4



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(A) IHC staining for markers of stratified squamous differentiation in 4OHT-treated controls and 4OHT-treated inducible double knockouts (IKOαβ). (B) Immunoblot analysis of epidermal lysates from animals treated with 4OHT for markers of stratified squamous differentiation (n=3 mice/genotype). (C) Taqman® real time quantitative PCR (K10) and reverse transcriptase semi-quantitative PCR (all others) on mRNA from 4OHT-treated whole skin (n=5 mice/genotype). Scale bars represent 30 microns.

Figure 5

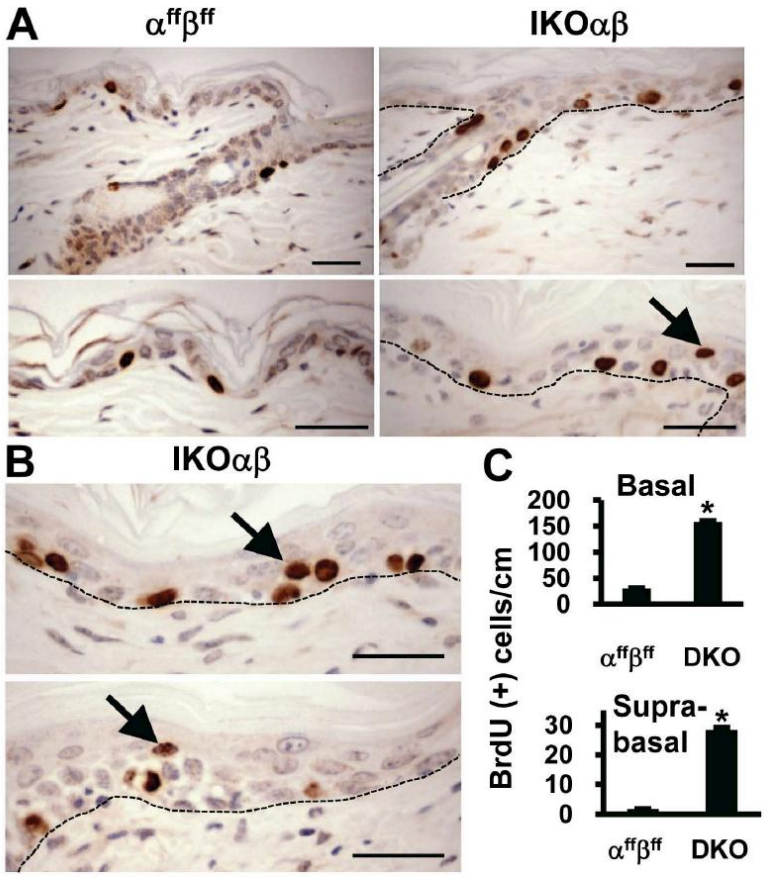

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Figure 6

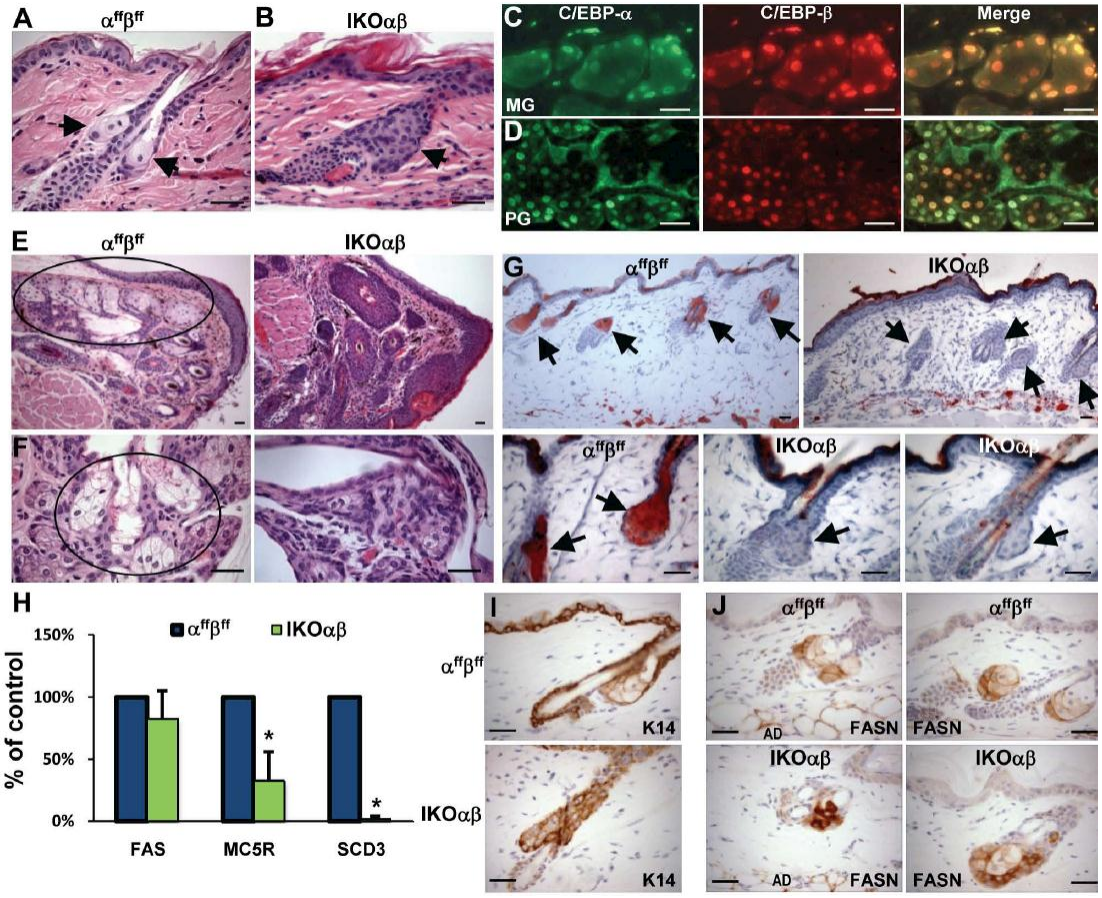

Figure 6. Co-ablation of C/EBPα/β results in morphological and molecular defects in sebaceous, Meibomian and preputial glands.
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Figure S1

Gene	5'-3' Forward Primer	5'-3' Reverse Primer
FASN	AGCGGCCATTTCCATTGCCC	CCATGCCCAGAGGGTGGTTG
Keratin 1	GACACCACAACCCGGACCCAAAACTTAGAC	ATACTGGGCCTTGACTTCCGAGATGATG
Keratin 5	AACCTCCAGAACGCCATTGC	GCCAGAAGAGACACTGTTTGTAACG
Keratin 14	TTGGTAGTGGATTTGGTGGTCG	GATGGTCTTGAAGTAGGGGCTGTAG
Loricrin	TCCTCTCAGCAGACCAGTCAG	GGTAGTCATTCAGAAACCAAGATG
Involucrin	GAGCGTGAAGGTTATCAAGGACC	GGTGTGGTTGCTTTAGTTTTGGC
GAPDH	GAAGGTCGCTGTGAACGGA	GTTAGTGGGGTCTCGCTCCT

Figure S1. Primers used for reverse transcriptase PCR

CHAPTER 2

C/EBPα and C/EBPβ Regulate Production of Neutral Lipids in Human Sebocyte Differentiation

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Abstract

C/EBPα and C/EBPβ are bZIP transcription factors with known and emerging roles in proliferation, tumorigenesis, metabolism, and differentiation in a multitude of cell types/tissues. The concurrent ablation of C/EBPα and C/EBPβ in sebaceous glands of adult mice results in the inability of sebocytes to differentiate and produce sebum. To begin to determine whether C/EBPα and C/EBPβ have a role in human sebocyte differentiation and sebaceous gland lipid production, we first examined the spatial distribution of C/EBPα and C/EBPβ within human sebaceous glands. C/EBPα and C/EBPβ are expressed in distinct spatial patterns; C/EBPα is expressed in differentiating interior sebocytes but rarely present in the peripheral undifferentiated proliferative population while C/EBPβ is expressed in both peripheral undifferentiated and differentiated sebocytes. Mouse sebaceous glands displayed a similar expression pattern and when C/EBPα and C/EBPβ were concurrently ablated from adult mouse sebaceous glands in vivo, the number of S-phase sebocytes more than tripled suggesting C/EBPα and C/EBPβ influence peripheral sebocyte cell cycle withdrawal. Immortalized human sebocytes (SEB-1 cells) expressed C/EBPα and C/EBPβ and when induced to differentiate, the transcription activity of C/EBPs increased as did the levels of C/EBPβ. When C/EBPα and C/EBPβ were knocked down concurrently with siRNA, sebocyte differentiation and subsequent lipogenesis were inhibited as measured by Oil Red O and Nile Red staining. The expression enzymes critical in the synthesis of lipids in maturing sebocytes such as fatty acid synthase (FASN), peroxisome proliferator

activated receptor gamma (PPARγ) and stearoyl-CoA desaturase1 (SCD1) were all significantly decreased. These results indicate C/EBPα and C/EBPβ participate in the regulation of cell cycle withdrawal and subsequent differentiation and lipid production in human sebocytes.

Introduction

C/EBPα and C/EBPβ are members of CCAAT/enhancer binding proteins (C/EBPs) family; a family of six highly conserved transcription factors that are members of the larger class of basic leucine zipper (bZIP) class of transcription factors [1]. C/EBP family members share a C-terminus conserved dimerization domain and a basic region leucine zipper (bZIP) DNA binding domain that recognize the same consensus DNA sequence (RTTGCGYAAY, where R represents A or G and Y represents C or T) [2]. The N-terminus region is highly variable among family members and contains transactivation and repression domains that allow for activation and inactivation of transcriptional activity (for review see [3]). In many cases the activity of family members is regulated through post translational modifications such as phosphorylation, acetylation and sumoylation as well as interaction with other proteins [3-5].

In human and mouse skin, sebaceous glands are an appendage usually located as an outgrowth of the hair follicle, located below the hair follicle opening and above the arrector pili muscle and typically have several acini or lobules. cMyc [6, 7], hedgehog signaling [8] and AP-2α/AP-2γ [9] have been shown to be involved in regulation of sebaceous gland development in mice. Sebaceous glands are formed from sebocyte progenitor cells that expresses Blimp 1, keratin 14 (K14) and keratin 5 (K5) [10] and these cells produce a proliferative population of sebocytes which in turn terminally differentiate to produce the lipid/sebum producing cells [7]. C/EBPα and C/EBPβ are highly expressed in sebocytes of human and mouse

sebaceous glands. The concurrent ablation of C/EBPα and C/EBPβ in sebaceous glands of adult mice resulted in the inability of sebocytes to differentiate and produce sebum demonstrating an essential role for these transcription factors in mouse sebocyte differentiation [11].

The organization of sebaceous gland acini includes peripherally located mitotically active undifferentiated sebocytes in contact with the basement membrane and differentiating sebocytes that have exited the cell cycle and lost adhesion to the basement membrane. As these sebocytes move into the interior of the gland the maturing sebocytes differentiate and produce and accumulate a waxy, oily substance (sebum). Eventually, the fully differentiated sebocytes self-destruct releasing their lipid contents and membranes alike into associated hair follicles or ducts in a process termed holocrine secretion (see [12] for review). These lipids lubricate the hair canal and skin surface and provide suppleness and barrier function as well as bactericidal benefits [13].

Multiple human diseases are related to improper function of sebaceous and related glands. These include acne, seborrheic dermatitis, blepharitis, dry eye syndrome, and sebaceous cancer. Acne vulgaris alone affects over 85% of adolescents in the United States [14], and is characterized by excessive sebum production as a result of androgen signaling brought on by adrenarche and dietary habits [15]. More severe cases result in significant scarring and psychological distress. Seborrheic dermatitis is a common inflammatory skin disorder characterized by white, flaky skin scales, and excessive oily skin and is thought to

be associated with an abnormal immune response to a common yeast *Malassezia*, present on skin [16].

The goal of this study was to determine whether C/EBPα and C/EBPβ have critical roles in differentiation and lipogenesis in human sebocytes. Although C/EBPα and C/EBPβ are abundantly expressed in sebocytes of the human sebaceous glands [17, 18], no function for these transcription factors in human sebocytes has been described. Collectively our results indicate that C/EBPα and C/EBPβ are essential for human sebocyte differentiation and lipogenesis.

Results

C/EBPα and C/EBPβ are expressed in a distinct spatial pattern in human and mouse sebaceous glands

Differentiation in sebaceous glands is a continuous process whereby the peripheral sebocytes which are adherent to the basement membrane withdraw from the cell cycle, detach from the basement membrane, initiate differentiation specific transcription events and synthesize neutral lipids before their eventual destruction through cell lysis. To begin to determine whether C/EBPα and C/EBPβ are functioning in human sebocyte differentiation and sebaceous gland lipid production, we first examined the spatial distribution of C/EBPα and C/EBPβ within human sebaceous glands using immunohistochemical (IHC) staining. As shown in Figure 1A and B, C/EBPα and C/EBPβ were detected in the nuclei of differentiated sebocytes within the interior of the sebaceous gland. These differentiated sebocytes were characterized by a large finely vacuolated translucent cytoplasm with a distinct round nucleus. Strikingly, peripheral sebocytes, which are a small, proliferative, undifferentiated sebocyte population that circumscribe the gland, rarely expressed C/EBPα (Fig 1A) but did express C/EBPβ (Fig 1B). Similar to human sebaceous glands, mouse sebaceous glands displayed C/EBPα and C/EBPβ expression in interiorly located differentiated sebocytes while C/EBPβ was detectable in the majority of peripheral sebocytes and C/EBPα was seldom observed (Fig 1C/D). Previously we developed mouse models utilizing K14-CreER^{tam} mice [19] to ablate C/EBPα and C/EBPβ alone or together in postnatal adult mouse skin. The K14

promoter directs the expression CreER^{tam} to the basal layer of the epidermis, ORS follicular keratinocytes of the hair follicle as well as the progenitor and proliferative populations of sebocytes of the sebaceous gland of mouse skin. The activity of CreER^{tam} is regulated through topical treatment with the synthetic ER antagonist, 4-hydroxytamoxifen (4OHT). We utilized these mice to determine whether ablation of C/EBPα and C/EBPβ alone and together in vivo in mouse sebaceous glands alters sebocytes proliferation as measured by BrdU pulse labeling. When C/EBPα and C/EBPβ were concurrently ablated from adult mouse sebaceous glands in vivo, a condition that blocks differentiation [11], the number of BrdU positive S-phase sebocytes more than tripled (Fig 1E) indicating C/EBPα and C/EBPβ influence peripheral sebocyte cell cycle withdrawal.

Differentiation of human sebocytes is accompanied by increased C/EBPβ protein levels and C/EBP transcription activity

Immortalized human sebocytes (SEB-1) have been shown to be a useful cell culture model to study human sebocyte differentiation [20]. Immunoblot analysis revealed SEB-1 cells express both C/EBPα and C/EBPβ (Fig 2A). SEB-1 cells can be induced to differentiate with the addition of high serum and insulin to their medium [21]. The production of sebum, which is a collection of neutral lipids, is a hallmark of sebocyte differentiation. As shown in Figure 2C, SEB-1 cells cultured in high serum and insulin, displayed increased Oil Red O (ORO) staining compared to control cells (Fig 2B) and this treatment resulted in a statistically significant increase in the number of ORO positive cells (Fig 2D). Because the quantification of ORO

positive cells per field of view did not take into account the number or size of ORO droplets/cell, we utilized Nile Red staining and ImageJ to quantify the increase in neutral lipid synthesis by measuring the luminosity of neutral lipid droplets stained with Nile Red, with and without MDI treatment. Nile Red fluoresces green or yellow for neutral lipids depending on the excitation/emission spectra and red for polar lipids. MDI cocktail (0.5mM isobutyl-methyl-xanthin , 0.3uM dexamethasone, 1.74uM insulin, 10% FBS) induces adipocyte differentiation [22] as well as sebocyte differentiation [21] and as shown in Fig 2E-G, SEB-1 cells treated with MDI cocktail (48 hrs) displayed a significant increase in neutral lipid synthesis (sebum) when compared to cells in control medium and this increase was sustained and further increased through six days of MDI treatment (Fig 2H). C/EBPβ protein levels were increased by MDI treatment and C/EBPα protein levels were unaffected or slightly decreased with MDI treatment while both C/EBPα and C/EBPβ protein levels increased with days in culture (Fig 2J). In sebocytes transfected with the C/EBP responsive promoter reporter construct MGF82 [23, 24], MDI treatment resulted in increased C/EBP transcription activity (Fig 2I). Collectively, these results indicate that increased levels of C/EBP transcription activity and increased levels of C/EBPβ protein are associated with sebocyte differentiation.

C/EBPα and C/EBPβ have a role in lipid synthesis and differentiation of human sebocytes

To test the hypothesis that C/EBPα and C/EBPβ are required for human sebocyte differentiation and the synthesis of neutral lipids, siRNAs for C/EBPα and

C/EBPβ were introduced into SEB-1 cells to knockdown either C/EBPα or C/EBPβ singly or both concurrently (Fig. 3A). At 24 hrs after transfection of siRNAs, sebocytes were treated with or without MDI media for 48 hours to induce differentiation. Short interfering RNA for green fluorescent protein (siGFP) was used as a control. As shown in Figure 3A, knockdown of C/EBPα and C/EBPβ was successful and densitometric scanning of immunoblots for C/EBPα and C/EBPβ protein demonstrated that knockdown was generally greater than 75% (data not shown). Next we quantified Nile Red staining in the sebocyte cultures using ImageJ in each treatment group using eight or more random fields of view (Fig. 3B,C). The concurrent knockdown of C/EBPα and C/EBPβ significantly decreased lipogenesis by greater than 50% in MDI-treated cells and by 30% in cells grown in normal media. Knockdown of either C/EBPα or C/EBPβ alone had no effect on MDI-induced lipogenesis. These results demonstrate C/EBPα and C/EBPβ have critical roles and cooperate to regulate human sebocyte lipogenesis and differentiation.

Loss of C/EBPα and C/EBPβ in SEB-1 cells results in decreased expression of lipid synthesis genes

To examine possible downstream effectors of C/EBPs in lipogenesis in human sebocytes, protein levels of fatty acid synthase (FASN) were examined. FASN is a large multi-unit protein responsible for synthesizing long chain fatty acids from acetyl-CoA and malonyl-CoA. As shown in Figure 4A and B, FASN was decreased by greater than 50% in MDI-treated sebocytes in which both C/EBPα and C/EBPβ were knocked down. Next we examined expression of peroxisome

proliferated-activated receptor gamma (PPARγ) and steroyl CoA desaturase1 (SCD1). PPARγ is a well characterized protein involved in adipocyte differentiation and works in concert with C/EBPs in adipocytes to regulate biosynthesis of lipids in adipocytes (for review [25]). SCD genes code for a family of enzymes which function in the biosynthesis of unsaturated fatty acids. We utilized Taqman RT PCR to examine PPARγ and SCD1 mRNA levels. siRNA mediated knockdown of C/EBPα and C/EBPβ resulted in decreases in PPARγ and SCD1 expression. SCD1 and PPARγ mRNA levels were decreased by approximately 50% and 25% in the MDI-treated sebocytes in which both C/EBPα and C/EBPβ were knocked down. These results indicate that decreased expression of C/EBPα and C/EBPβ results in decrease expression of genes involved in sebum production.

Discussion

Our results demonstrate the co-knockdown of C/EBPα and C/EBPβ expression impairs human sebocyte differentiation as measured by the production of neutral lipids, a hallmark of sebocyte differentiation. The co-knockdown of C/EBPα and C/EBPβ in human SEB-1 sebocytes also resulted in decreased expression of genes known to be important in lipogenesis. For example, FASN, a large multi-unit protein responsible for synthesizing long chain fatty acids from acetyl-CoA and malonyl-CoA was significantly decreased. SCD1, which is important in the synthesis of the monounsaturated fatty acids oleate and palmitoleate, precursors of cholesterol esters and triglycerides, was also significantly decreased. It is of note that SCD1 knockout mice display impaired sebocyte differentiation and sebaceous and Meibomian gland defects [26], a similar phenotype to that observed when C/EBPα and C/EBPβ were concurrently ablated in mouse sebaceous and Meibomian glands [11]. We also observed that PPARγ, a well characterized transcription factor involved in adipocyte differentiation and one that works in concert with C/EBPs in adipocytes to regulate biosynthesis of lipids in adipocytes (for review [25]) was also decreased. Collectively, these results demonstrate C/EBPα and C/EBPβ are required for human sebocyte differentiation and lipogenesis.

Sebaceous glands contain several discrete populations of cells to maintain homeostasis. Progenitor stem cells at the mouth of gland that divide slowly to replenish peripherally located sebocytes that are mitotically active and in contact with the basement membrane, maturing interior sebocytes that have detached from

the basement membrane and have started differentiation and subsequent lipid accumulation, and finally, fully mature lipid filled differentiated sebocytes near the exit of the gland ready to lyse their contents into associated ducts or hair follicles [12]. Although the expression of C/EBPs has been previously noted in human and mouse sebaceous glands [17, 18], the contrasting spatial distribution has not been reported. The spatial distribution of C/EBPα and C/EBPβ in human and mouse sebocytes provide some clues as to their roles in differentiation/lipogenesis in sebocytes. While both C/EBPα and C/EBPβ were abundantly expressed in the maturing sebocytes, the expression of these transcription factors in the mitotically active periphery sebocytes was largely limited to C/EBPβ. However, we did observe a small number of peripheral sebocytes per sebaceous gland that expressed C/EBPα, suggesting that perhaps C/EBPα's early role in sebocyte differentiation is to initiate cell cycle withdrawal. C/EBPα expression was always and without exception abundantly expressed in the nucleus every sebocyte adjacent to the peripheral sebocytes suggesting C/EBPα expression is either initiated as peripheral sebocytes become post mitotic as suggested above, or that C/EBPα is concurrently expressed with detachment from the basement membrane. The fact that we observed a 60% increase in S-phase sebocytes when C/EBPα alone is ablated in mouse sebaceous glands is consistent with a role in peripheral sebocyte cell cycle withdrawal. In support of this notion are studies showing C/EBPα is required for cell cycle arrest for both adipogenesis and granulopoiesis [27, 28]. Interestingly, we observed further increases in sebocyte S-phase cells when C/EBPα and C/EBPβ

were co-ablated suggesting the two transcription factors compensate for each other or cooperate to induce cell cycle withdrawal.

Compensation of C/EBPα for C/EBPβ or vice versa is not uncommon. For example, C/EBPα deficient mice are perinatal lethal due to defects in adipogenesis, glucose metabolism, and hematopoiesis [29, 30] and experiments where C/EBPβ was knocked into C/EBPα's locus were able to rescue liver abnormalities [31] and hematopoiesis [32] dysfunction exhibited by C/EBPα null mice. In addition, it was not until both C/EBPα and C/EBPβ were deleted from adult mouse skin that critical roles in epidermal development [33] and both keratinocyte and sebocyte differentiation and homeostasis in mouse were revealed [11]. With respect to human sebocyte differentiation, we observed that C/EBPα and C/EBPβ are co-expressed in almost every interior sebocyte (Figure 1A and B), and yet loss of C/EBPα alone or C/EBPβ alone did not affect the ability of SEB-1 cells to differentiation and synthesize neutral lipids (Figure 3). Concurrent knockdown of both transcription factors were required to impair sebocyte differentiation and lipogenesis, suggesting functional redundancy or a compensatory mechanism.

While research into control of development of sebaceous glands has implicated many transcription factors including AP2, Sox9, Blimp1, and the Wnt/BMP and hedgehog pathways [9, 10, 34, 35], the pathways regulating homeostasis of sebocytes consisting of the continual renewal of cells lost to self-destruction are less well understood. It is known that sebum production and cell proliferation are increased by androgen signaling [36] and that retinoids, particularly 13-cis-retinoic

acid, reduces sebum production and selectively causes apoptosis in sebocytes [37]. In addition, melanocortins, insulin-like growth factor-1 (IGF-1) and PPARγ have been shown to affect sebocyte activity [38-40]. The IGF-1 pathway elucidated by Smith et.al [38] activates both MAPK/ERK and PI3-K pathways resulting in activation of sterol response element-binding protein-1 (SREBP-1) and subsequent transcription of genes involved in lipogenesis. Of potential significance are studies demonstrating that C/EBPs regulate SREBP1c in adipogenesis [41]. Future work is planned to examine whether C/EBPs regulate SREBP1 and act downstream of PI3K/AKT signaling from IFG in human sebocytes.

We have shown for the first time that C/EBPα and C/EBPβ are important in the regulation of lipid production in human sebocytes and this result could have important human health relevance. Acne is the most common skin disease and in the United States, and affects up to 85% of all adolescents and young adults. Currently, the only treatment for acne is a combination therapy of antibiotics to treat *Propionibacterium acnes* bacteria that uses sebum as a food source and exacerbates inflammation, and retinoids, that have been shown to selectively cause apoptosis in sebocytes [37, 42]. Retinoids are teratogenic so finding alternative ways to reduce sebum production and subsequent acne is highly desirable. It is possible that C/EBPα and C/EBPβ could be biological targets for treatment of sebaceous gland disorders, notably acne.

Figure Legends

Figure 1. C/EBPα and C/EBPβ are expressed in in a distinct spatial pattern in mouse and human sebaceous. (A) Representative IHC staining for C/EBPα in human sebaceous glands (arrows point to negative staining in peripheral sebocytes; n=9 subjects, multiple acini per subject). (B) Representative IHC staining for C/EBPβ in human sebaceous glands. (C) Representative IHC staining for C/EBPα in mouse sebaceous glands (arrows point to negative staining in peripheral sebocytes; n=9 mice, multiple acini per mouse). (D) Representative IHC staining for C/EBPβ in mouse sebaceous glands. (E) Quantification of the number of BrdU positive cells per sebaceous gland in inducible knockout mice for C/EBPα alone, C/EBPβ alone and both together (n = 3 mice/genotype, 3 skin section strips (minimum 1cm per section) per mouse; * p<.03, # p<.001).

Figure 2. Lipogenesis in human sebocytes is accompanied by increased C/EBPβ levels and C/EBP transcription activity. (A) Immunoblot analysis of SEB-1 cell lysate for C/EBPα (42 kDa) and C/EBPβ (Lap, 41 kDa;Lap*,43 kDa)with associated standards. (B) Oil Red O (ORO) staining for neutral lipids of SEB-1 cells 48 hours after confluence in normal growth media (arrows point to ORO staining). (C) ORO staining of SEB-1 cells 48 hours after confluence treated with 12.5% FBS and 1uM insulin. (D) Quantification of the number of ORO positive cells per field of view (n=26 40x objective views per treatment) of cells in (B) and (C). (E) Nile red staining of SEB-1 cells at ~60% confluence for polar lipids (red), neutral lipids

(green) and merged field. (F) Nile red staining 48 hours later after normal growth media. (G) Nile red staining 48 hours later treated with MDI. (H) ImageJ quantification of nile red neutral (green) fluorescence of SEB-1 cells treated with or without MDI. (I) Quantification of the fold change of the C/EBP responsive promoter reporter MGF82 in SEB-1 cells 48 hours after treatment with MDI or 12.5% FBS and 1uM insulin (n=3). (J) Immunoblot analysis of time course of SEB-1 cell lysates treated with or without MDI (Beta Actin is provided as a loading control).

Figure 3. Co-knockdown of C/EBPα and C/EBPβ decreases synthesis of neutral lipids. (A) Immunoblot analysis of SEB-1 cell lysates for C/EBPα and C/EBPβ treated with siRNA for C/EBPα (100nM), C/EBPβ (100nM) or C/EBPα and C/EBPβ (50nM/50nM) with or without MDI for 48 hours. siRNA for green fluorescent protein (GFP) was used as a control for transfection; beta actin is represented as a loading control. (B) SEB-1 cells were treated at ~75% confluency with or without MDI for 48 hours and stained with nile red to visualize neutral lipids. (C) Quantification of neutral lipid fluorescence in SEB-1 cells with ImageJ (n=8 random 10x objective fields of view per treatment; * $p<.05$, # $p<.001$).

Figure 4. Loss of C/EBPα and C/EBPβ in SEB-1 cells results in decreased expression of lipid synthesis genes. (A) Immunoblot analysis of SEB-1 cell lysates for fatty acid synthase (FASN) treated with siRNA for C/EBPα (100nM), C/EBPβ (100nM) or C/EBPα and C/EBPβ (50nM/50nM) for 24 hours followed by

treatment with or without MDI for 48 hours. siRNA for green fluorescent protein (GFP) was used as a control for transfection; beta actin is represented as a loading control. (B) Denstiometric quantification of FASN levels in (A) compared to siGFP. (C) PPARγ levels in GFP control, C/EBPα knockdown, C/EBPβ knockdown, and C/EBPα/C/EBPβ knockdown. Taqman® real time quantitative PCR analysis of SEB-1 cells treated as described in (A) for expression of PPARγ represented as percent of siGFP. (D) SCD levels in GFP control, C/EBPα knockdown, C/EBPβ knockdown, and C/EBPα/C/EBPβ knockdown. Taqman® real time quantitative PCR analysis of SEB-1 cells treated as described in (A) for expression of SCD represented as percent of siGFP.

Methods

Ethics Statement

All animal work described in the study involving animal husbandry, experimentation, and care/welfare have been conducted according to NIH guidelines and approved by NCSU Institutional IACUC committee.

Human Skin Sections

Human specimens were obtained from de-identified patients seen at the University of Chicago under approval of the IRB (Institutional Review Board) and in accordance with the Declaration of Helsinki Principles. Tissues were formalin fixed and sections from paraffin-embedded blocks were stained as described below.

Animals and treatments

K14-CreER^{tam} homozygous mice [19] (CD1) were obtained from Jackson Labs (#005107) and crossed with either floxed-α (B6.129) [43], or floxed-β (B6.129). Floxed-α and floxed-β mice were genotyped as previously described [43, 44]. Mice were genotyped for K14-CreER^{tam} and treated with 4-OHT as described in [45].

Cell Culture

SEB-1 human immortalized sebocytes [20] between passage 27 and 32 were cultured in 3:1 Low Glucose DMEM (Invitrogen #11885-084) : Ham's F12 (Invitrogen # 11765-054) with 2.5% Characterized FBS (Hyclone # SH30071.03), 24ug/ML

Adenine (Sigma # A-9795), 0.0452ug/mL Hydrocortisone (Sigma # H-0888), 10ng/mL Insulin (Sigma # I-1882), 3ng/mL EGF (Invitrogen PHG0311), $1.2x10^{-10}$M Cholera Toxin (Sigma # C-8052), and 1X antibiotics (Invitrogen # 15240-062) until 100 percent confluent before splitting or experiments. All references to normal media are as described above. MDI media is normal media supplemented with 0.5mM isobutyl-methyl-xanthin (Sigma #I7108), 0.3uM dexamethasone (Sigma #D4902), 1.74uM insulin (Sigma #I1882), and 10% FBS.

Immunoblot analysis

Equal amounts of protein were denatured in sample buffer, loaded onto 12% tris-glycine gels (FASN; 5%), separated by gel electrophoresis and transferred to PVDF membrane. Membranes were blocked for one hour (TBS with 0.1% tween/5.0% milk), and incubated overnight at 4^{o}C (TBS with 0.1% tween/5.0% milk) with one of the following rabbit polyclonal antibodies: C/EBPα (Novus Biologicals, NB110-55640, 1:2500), C/EBPβ (Santa Cruz, SC-150, 1:4000). Membranes were then rinsed 30 minutes (0.1% tween, 5% milk in TBS) and subjected to one hour incubation (TBS-T, 5.0% milk) with anti-rabbit IgG, horseradish peroxidase-linked secondary (GE Healthcare, NA934V, 1:2500), incubated one minute with Western Lightening Plus-ECL (PerkinElmer NEL105001EA) and subsequently exposed to film. Membranes were stripped and re-probed for β-actin (Sigma A-5441, 1:50,000, mouse) and anti-mouse horseradish peroxidase-linked secondary (GE Healthcare, NXA931, 1:50,000), incubated one minute with Western Lightening Plus-ECL

(PerkinElmer NEL105001EA) and subsequently exposed to film. A 5% tris-glycine gel, along with twice the transfer time, was used to probe for FASN.

Cell proliferation analysis

Mice were injected intraperitoneally with bromodeoxyuridine (BrdU, Sigma B5002, 100mg/kg in PBS) and killed 1 hour later. Skin was fixed in 10% neutral buffered formalin phosphate (NBF) for 20h and moved to 70% ethanol and subsequently embedded in paraffin. Sections were then used with co-IF and IHC staining.

Immunohistochemical (IHC) staining

For mouse, tissues were fixed in 10% NBF for 20h, switched to 70% ethanol and embedded in paraffin. Human normal skin sections were paraffin embedded.In brief, tissue sections (5μM) were warmed 65^{o}C for 20 minutes, successively hydrated (Xylene, 95% ethanol, 70% ethanol, PBS), endogenous peroxidase activity blocked10 minutes with 3% H_2O_2, followed by 6% serum for 1 hour followed by citrate buffer antigen retrieval in *Retriever 2100*for two hour cycle. Sections were incubated overnight with following antibodies: C/EBPα (mouse: Santa-Cruz SC-61, 1:1000; human: Novus Biologicals, NB110-55640, 1:2500), C/EBPβ (mouse and human: Santa-Cruz SC-7962, 1:1000), BrdU (BD Biosciences 27644, 1:200). *Vectastain* (mouse – 6102, rabbit – 6101) ABC kit was used in conjunction with *Biogenex* DAB substrate kit (HK153-5K) according to manufacturer's instructions.

Sections were then counterstained with hemotoxylin, successively dehydrated from PBS to Xylene, and coverslipped with Permount (Fisher SP-15).

Oil Red O staining

Cells were washed in PBS and fixed 40 minutes in 10% neutral buffered formalin. Cells were again washed in PBS for 5 minutes followed by 5 minutes in 60% 2-propanol followed by 10 minutes in Oil Red O (3 parts .3% ORO in 2-propylnol with 2 parts water and filtered). Cells were then rinsed in tap water until clear, counterstained with hemotoxylin and again rinsed until clear and visualized.

Co-immunofluorescence staining

Tissue slides were deparaffinized and hydrated in successive washes (Xylene, 95% ethanol, 70% ethanol, PBS), blocked (C/EBPα or C/EBPβ with BrdU: 6%NGS, 6%NHS, 1%TritonX in PBS), citrate buffer antigen retrieval in *Retriever 2100*® and incubated overnight at 4^{o}C with primary antibodies: C/EBPα (rabbit polyclonal, Santa-Cruz SC-61, 1:400), and C/EBPβ (mouse monoclonal, Santa-Cruz SC-7962, 1:200). Slides were washed in PBS and incubated 45 minutes with fluorophore-linked secondary antibodies (Alexa Fluor® 568 goat anti-mouse (1:1000), Alexa Fluor® 488 goat anti-rabbit(1:1000)) in PBS, dehydrated and coverslipped with Dako Faramount Aqueous Mounting Medium.

RNA and quantitative PCR

In brief cells were collected in Tri-Reagent®, (Sigma T-2494) and RNA was extracted with chloroform. Pelleted RNA was solubilized in water and then purified with DNase on Qiagen RNeasy® columns. cDNA was created with equal amounts of starting template with reverse transcriptase with appropriate no-RT controls. cDNA was then subjected to Real Time TaqMan® PCR (Applied Biosystems: PPARγ (Hs01115513_m1), C/EBPα (Hs00269972_s1) C/EBPβ (Hs00270923_s1), SCD1 (Hs01682761_m1), and GAPDH (Hs_0392097_g1) as a housekeeping gene).

Nile Red Staining

500x Nile Red (Sigma #19123) stock was made in acetone (0.5mg/mL 500x). Cells in culture were washed one time in ice cold PBS, fixed in 10% neutral buffered formalin for 15 minutes, rinsed one time in PBS and 3mL/p60 plate of 1x Nile Red in PBS was added. After a minimum of 10 minutes, lipids were visualized at 470nm excitation, 525-550 emission (green) for non-polar lipids and 540nm excitation, 600-660 emission (red) for polar lipids. ImageJ©, from NIH, was used to quantify fluorescence with n = 8 or more random fields of view at 100x magnification.

siRNA Experiments

All knockdown experiments were done with DharmaFECT® 1 transfection reagent (Thermo Scientific # T-2001-03) according to the manufacturer's instructions. For lipogenesis endpoints, cells 75-80% confluent were transfected 24 hours with 100nM

concentrations of siGFP (5’-GCU UAC GUC CAG GAG CGC ACC) or siC/EBPα(5’-CGA CGA GUU CCU GGC CGA C) or siC/EBPβ (5’-GAA AAG AGG CGU CUG UAU AUU) for single transfections or 50nM each of siC/EBPα andsiC/EBPβ. MDI cocktail (1.74uM insulin, 0.3uM Dexamethasone, 10% FBS, 0.5mM Isobutyl-Methyl-Xanthin) was added or not for 48 additional hours before protein/RNA collection and Nile Red staining.

Plasmid Transfection and Luciferase Assay

All transfections were done on 75-90% confluent SEB-1 cells in 12 well plates using Transfast™ transfection reagent according to the manufacturer’s instructions (Promega) for 48 hours before collection with either 200 ng MGF40 or MGF82 [23, 24] promoter reporter in concert with 100 ng of : C/EBPα [46] C/EBPβ [47]. Counts were normalized to protein content.

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Competing Interests

The authors have declared that no competing interests exist.

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Figure 1

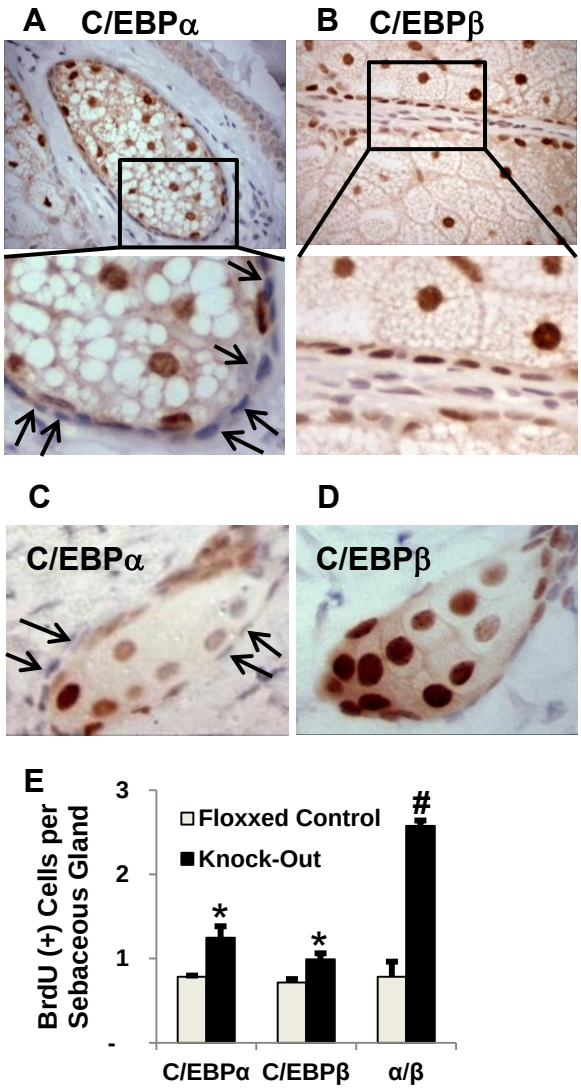

Figure 1. C/EBPα and C/EBPβ are expressed in in a distinct spatial pattern in mouse and human sebaceous. (A) Representative IHC staining for C/EBPα in human sebaceous glands (arrows point to negative staining in peripheral sebocytes; n=9 subjects, multiple acini per subject). (B) Representative IHC staining for C/EBPβ in human sebaceous glands. (C) Representative IHC staining for C/EBPα in mouse sebaceous glands (arrows point to negative staining in peripheral sebocytes; n=9 mice, multiple acini per mouse). (D) Representative IHC staining for C/EBPβ in mouse sebaceous glands. (E) Quantification of the number of BrdU positive cells per sebaceous gland in inducible knockout mice for C/EBPα alone, C/EBPβ alone and both together (n = 3 mice/genotype, 3 skin section strips (minimum 1cm per section) per mouse; * p<.03, # p<.001).

Figure 2

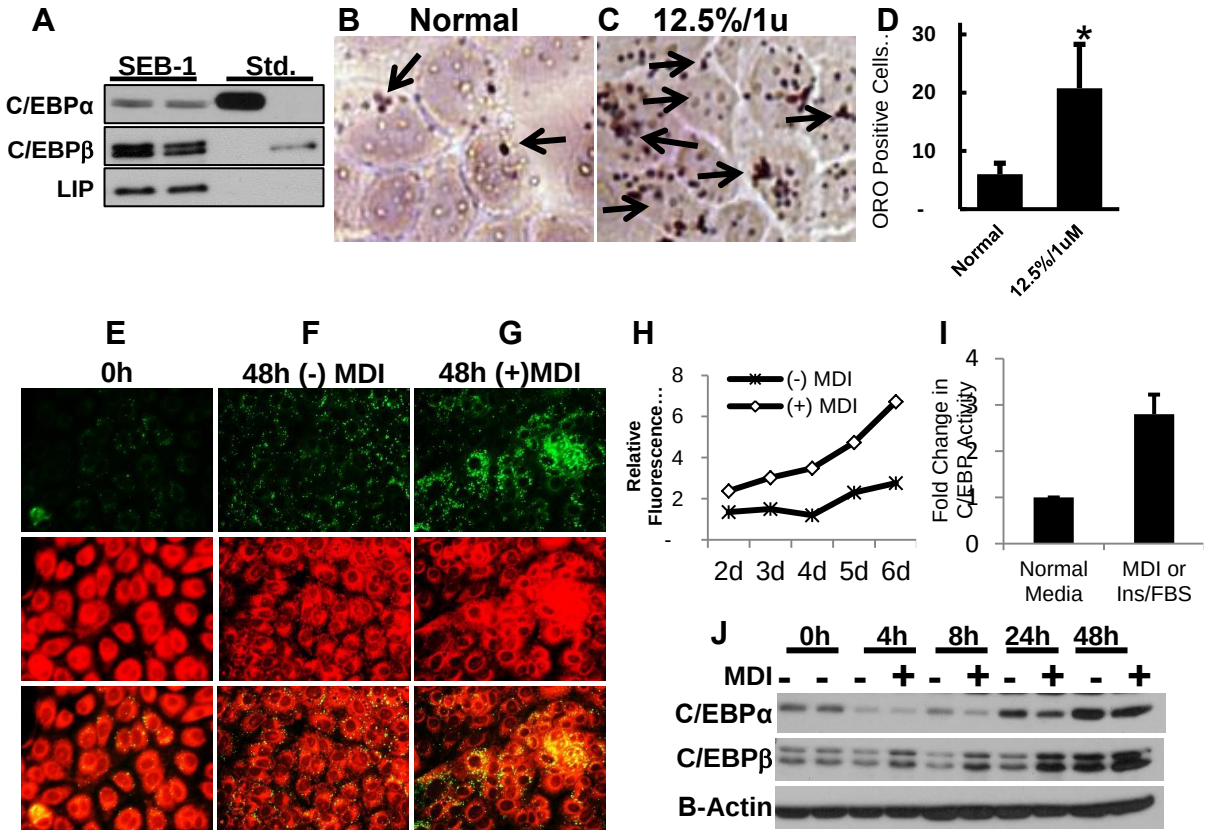

Figure 2. Lipogenesis in human sebocytes is accompanied by increased C/EBPβ levels and C/EBP transcription activity. (A) Immunoblot analysis of SEB-1 cell lysate for C/EBPα (42 kDa) and C/EBPβ (Lap, 41 kDa;Lap*,43 kDa)with associated standards. (B) Oil Red O (ORO) staining for neutral lipids of SEB-1 cells 48 hours after confluence in normal growth media (arrows point to ORO staining). (C) ORO staining of SEB-1 cells 48 hours after confluence treated with 12.5% FBS and 1uM insulin. (D) Quantification of the number of ORO positive cells per field of view (n=26 40x objective views per treatment) of cells in (B) and (C). (E) Nile red staining of SEB-1 cells at ~60% confluence for polar lipids (red), neutral lipids (green) and merged field. (F) Nile red staining 48 hours later after normal growth media. (G) Nile red staining 48 hours later treated with MDI. (H) ImageJ quantification of nile red neutral (green) fluorescence of SEB-1 cells treated with or without MDI. (I) Quantification of the fold change of the C/EBP responsive promoter reporter MGF82 in SEB-1 cells 48 hours after treatment with MDI or 12.5% FBS and 1uM insulin (n=3). (J) Immunoblot analysis of time course of SEB-1 cell lysates treated with or without MDI (Beta Actin is provided as a loading control).

Figure 3

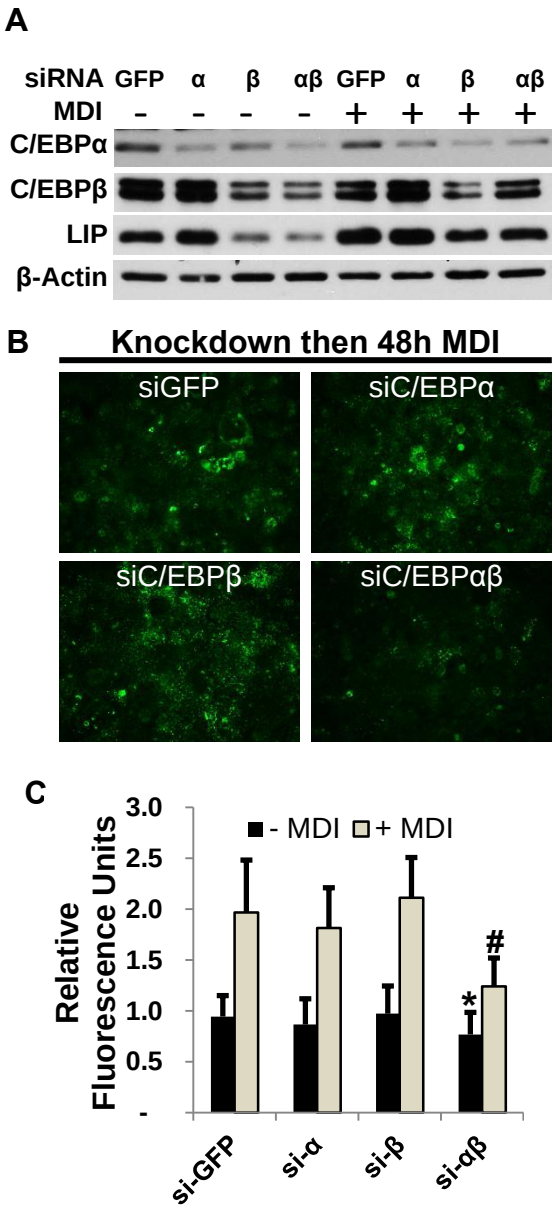

Figure 3. Co-knockdown of C/EBPα and C/EBPβ decreases synthesis of neutral lipids. (A) Immunoblot analysis of SEB-1 cell lysates for C/EBPα and C/EBPβ treated with siRNA for C/EBPα (100nM), C/EBPβ (100nM) or C/EBPα and C/EBPβ (50nM/50nM) with or without MDI for 48 hours. siRNA for green fluorescent protein (GFP) was used as a control for transfection; beta actin is represented as a loading control. (B) SEB-1 cells were treated at ~75% confluency with or without MDI for 48 hours and stained with nile red to visualize neutral lipids. (C) Quantification of neutral lipid fluorescence in SEB-1 cells with ImageJ (n=8 random 10x objective fields of view per treatment; * p<.05, # p<.001).

Figure 4

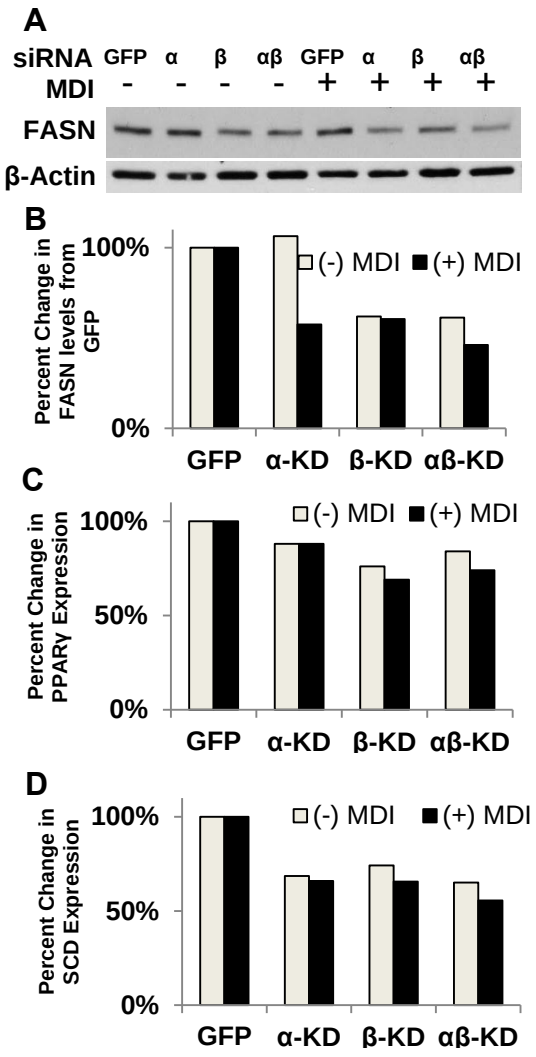

Figure 4. Loss of C/EBPα and C/EBPβ in SEB-1 cells results in decreased expression of lipid synthesis genes. (A) Immunoblot analysis of SEB-1 cell lysates for fatty acid synthase (FASN) treated with siRNA for C/EBPα (100nM), C/EBPβ (100nM) or C/EBPα and C/EBPβ (50nM/50nM) for 24 hours followed by treatment with or without MDI for 48 hours. siRNA for green fluorescent protein (GFP) was used as a control for transfection; beta actin is represented as a loading control. (B) Denstiometric quantification of FASN levels in (A) compared to siGFP. (C) PPARγ levels in GFP control, C/EBPα knockdown, C/EBPβ knockdown, and C/EBPα/C/EBPβ knockdown. Taqman® real time quantitative PCR analysis of SEB-1 cells treated as described in (A) for expression of PPARγ represented as percent of siGFP. (D) SCD levels in GFP control, C/EBPα knockdown, C/EBPβ knockdown, and C/EBPα/C/EBPβ knockdown. Taqman® real time quantitative PCR analysis of SEB-1 cells treated as described in (A) for expression of SCD represented as percent of siGFP.

GENERAL DISCUSSION

The processes of cell signaling and cellular differentiation are complex, interwoven and highly coordinated. In order to study cellular signaling, scientists often drill down to specific interactions to tease out function. Although this is necessary, cells are not binomial systems; they are incredibly complex, diverse and the signaling that goes on in a single cell consists of literally hundreds of signaling molecules, receptors, co-factors, transcription factors, some of which are redundant, and the interplay between them. Transcription factors recognize specific DNA sites and function to delineate and fine-tune the response in this milieu of signals. This body of work adds to the body of research that the transcription factors C/EBPα and C/EBPβ have in keratinocyte and sebocyte differentiation and homeostasis.

Keratinocyte Differentiation and Homeostasis

The expression of C/EBPα and C/EBPβ in skin is among the highest of any tissue examined in our laboratory (data unpublished), and epidermal specific knockouts for C/EBPα and C/EBPβ revealed contrasting roles in cell survival and tumorigenesis with C/EBPβ deficient mice being refractory to chemical carcinogenesis [167] while C/EBPα deficient mice were highly susceptible [133]. Our results presented here are the first to show co-expression of C/EBPα and C/EBPβ in not only interfollicular epidermal (IFE) and outer root sheath (ORS) keratinocytes but also in sebocytes. Interestingly, in spite of substantial expression

of both transcription factors in keratinocytes, prior work using knockouts of C/EBPα or C/EBPβ alone in the epidermis revealed only a modest effect for C/EBPβ null mice and no effect for C/EBPα null mice with regard to epidermal homeostasis and differentiation [133, 155]. Attempts were made to generate double knockouts for C/EBPα and C/EBPβ in skin from development by crossing mice carrying floxed alleles for C/EBPα and C/EBPβ to mice harboring the K5 directed expression of Cre targeting the epidermis, and no double knockout progeny were born, indicating we were on the right track. In order to circumvent the developmental affects preventing viable progeny, mice were created in which C/EBPα and C/EBPβ could be knocked out in epidermis of adult animals through the application of 4-OHT. It was not until both C/EBPα and C/EBPβ, transcription factors which have been characterized as key effectors in the differentiation of many different tissues and cell types, were able to be knocked out concurrently in skin using this model, that critical and essential functions in the homeostasis and differentiation in skin were revealed. The results from ablating only C/EBPα or only C/EBPβ were similar to germline single knockout animals (skin specific for C/EBPα and either skin or systemic knockout for C/EBPβ), with little changes in the expression of keratins, loricrin and involucrin. For C/EBPα, inducible ablation revealed an increase in S-phase cells in the interfollicular epidermis (IFE) commensurate with a slight increase in the number of nucleated cell layers that was not present in the work by Loomis et.al with developmental deletion of C/EBPα in epidermis. As this was not seen in mice with C/EBPα deleted in

epidermis during development, it hinted at some form of compensation during development for the loss of C/EBPα.

The results from ablating both C/EBPα and C/EBPβ at the same time in adult animals were striking and demonstrated C/EBPα and C/EBPβ were requisite for proper keratinocyte differentiation and homeostasis. When both C/EBPα and C/EBPβ were removed from epidermis of adult mice, lesions appeared in as little as 4 days characterized by IFE and ORS keratinocyte hyperplasia, dysplasia and hyperkeratosis and progressed dramatically through our longest time point of 25 days after start of treatment to ablate C/EBPα and C/EBPβ. Examination of markers of stratified squamous differentiation demonstrated defects in the basal to spinous transition. Normally, K5 and K14 expression are confined to keratinocytes in contact with the basement membrane, and as keratinocytes begin to differentiate, they lose adherence to the basement membrane, turn off expression of K5 and K14 and turn on expression of K1 and K10. In mice where C/EBPα and C/EBPβ were conditionally ablated in adult animals, we saw an expansion of the basal compartment as indicated by K5 and K14 staining in suprabasal cell layers commensurate with a delay in the expression of the spinous markers of differentiation, K1 and K10. This indicated C/EBPα and C/EBPβ were involved in the proper transition from basal to spinous differentiation in keratinocytes. In general, the granular layers of epidermis with C/EBPα and C/EBPβ ablated appeared less affected; keratohyalin granules were present and involucrin and filaggrin were expressed, albeit at increased levels. These changes in increased

involucrin and filaggrin expression could be a response to the disruption of epidermal homeostasis due to the defective basal to spinous transition or it is possible that C/EBPα and C/EBPβ contribute to the suppression of granular differentiation as has been reported for Hes1 in epidermal development [10].

Mechanisms of epidermal development and postnatal differentiation are not necessarily synonymous. For example, ablation of Notch in epidermal development produces the loss of spinous and granular layers and a hypoproliferative phenotype [9], while postnatal ablation of Notch1 produce an opposite effect involving a hyperproliferative phenotype and an increased granular layer [11]. Hes1 ablation dramatically alters epidermal development involving the spinous to granular transition, but appears to have a minimal role in adult skin [10]. Recently the co-deletion of C/EBPα and C/EBPβ in developing mouse skin produced mice that died soon after birth, and the analysis of epidermis from these mice revealed hyperplasia, decreased expression of spinous and granular markers of differentiation and a role for E2F in the hyperplastic epidermis [16]. While we observed similar changes in basal cell hyperproliferation and defects in spinous markers of differentiation, we did not observe the granular changes reported in developing skin. For example, we did not observe diminished keratohyalin granules, filaggrin, and involucrin expression in adult epidermis and we did observe an uncoupling of involucrin and loricrin expression in adult epidermis. Collectively, these results indicate that there are significant differences between the effects of co-deletion of C/EBPα and C/EBPβ in embryonic development and postnatal differentiation.

Another striking defect in stratified squamous differentiation when both C/EBPα and C/EBPβ were ablated from adult mice was the presence of S-phase cells in suprabasal layers of epidermis. As described in the introduction, proliferation of keratinocytes is normally restricted to cells in contact with the basement membrane through attachments via integrins. In addition to more overall proliferation, we observed an almost 30 fold increase in suprabasal S-phase cells when both C/EBPα and C/EBPβ were ablated suggesting an impairment of basal keratinocyte cell cycle withdrawal. Similar to the defects described in differentiation, single knockouts for C/EBPα and C/EBPβ showed no changes in suprabasal proliferating cells.

Notch signaling is important in both the early and later stages of epidermal development where it regulates the basal to spinous transition in a Hes1 independent manner and the spinous to granular transition in a Hes1 dependent manner [9-11] . AP-2α and AP-2γ also have essential roles in both the early and later stages of epidermal development and recently it was reported that both AP-2 and Notch signaling converge on the regulation of the expression of C/EBPα and C/EBPβ [12]. Results reported here demonstrate C/EBPα and C/EBPβ have critical roles in the early stages of squamous differentiation and our results are consistent with a model where C/EBPs function downstream of AP-2 and Notch signaling to regulate the basal to spinous transition in epidermal keratinocytes.

As described in the introduction, the knockin of C/EBPβ into the C/EBPα locus rescues the lethality of C/EBP$\alpha^{-/-}$ mice and supports the notion of functional redundancies

between these family members [165, 166]. However, not all of the phenotypes of C/EBP$\alpha^{-/-}$ mouse were reversed suggesting that there are also unique C/EBP family member functions. Likewise our current study provides evidence for functional redundancies between C/EBPα and C/EBPβ in epidermal and sebocyte homeostasis. However, in terms of skin tumorigenesis, these family members have unique functions. For example, C/EBPα is a skin tumor suppressor [133] and a regulator of the G1 checkpoint in keratinocytes in the DNA damage response [184]. In contrast, C/EBPβ is a mediator of keratinocyte survival and is required for skin tumorigenesis [167]. Understanding the redundant and unique roles these two transcription factors have will be important in the eventual elucidation of their roles in tumorigenesis.

In summary, the co-ablation of C/EBPα and C/EBPβ confirmed and eclipsed earlier studies in C/EBPβ knockout mice that indicated a role for C/EBPβ in early events of keratinocyte differentiation involving K1/K10 expression and growth arrest [14]. Our results indicate that functional redundancies exist between C/EBPα and C/EBPβ and/or these two transcription factors cooperate to regulate basal keratinocyte cell cycle withdrawal and early differentiation in keratinocytes and sebocytes.

How C/EBPα and C/EBPβ function to compensate for the loss of the other in developmental knockouts is still unknown. It will be important to determine who the native binding partners are for these transcription factors during processes of basal keratinocyte cell cycle withdrawal and early differentiation. Given that C/EBPs

require homo- or hetero-dimerization to bind DNA, experiments conducted with fluorescence resonance energy transfer (FRET) tagging other C/EBPs or additional bZIP proteins could reveal dimerization partners in wild type and knockout mice.

Cell culture systems are much different than animal models and of less relevance, but if the defects observed in the double knockouts were intrinsic keratinocyte defects, then primary cells isolated from inducible double knockouts for alpha and beta (IKOαβ) should repeat the changes in expression of genes in stratified squamous differentiation we observed in vivo. When we tried to replicate the double knockout in vivo knockout phenotype with primary cells, we were unable to do so; keratin expression in both controls and treatments did not respond properly, indicating our culture system was a poor replacement for our animal model. Another confounding factor could be compromised barrier function. Recent research has suggested that compromised barrier function and resultant trans-epithelial water loss in epidermis results in disruptions in markers of stratified squamous differentiation and proliferation similar to that observed in IKOαβ mice [185]. Further evidence of compromised barrier function contributing to the IKOαβ phenotype is unpublished data in our lab indicating expression of several genes involved in synthesis of ceramides and lipids of the stratum corneum are dysregulated in C/EBPβ deficient skin. Follow up in how C/EBPα and C/EBPβ cooperate in the control of barrier function lipid synthesis needs to be investigated.

Sebocyte Differentiation and Homeostasis

C/EBPα and C/EBPβ are also heavily expressed in sebaceous glands of mouse and human. Our results showed C/EBPα and C/EBPβ were not only expressed but co-expressed in mouse sebaceous glands and several other specialized sebaceous glands including Meibomian and preputial. The model system we used to knockout C/EBPα and C/EBPβ in the epidermis by the application of 4-OHT was K14 directed, and as mentioned in the introduction, the progenitor and peripheral cells of the sebaceous glands express K14. The ablation of C/EBPα and C/EBPβ in adult mouse skin resulted in a block in neutral lipid synthesis in sebocytes of sebaceous glands, Meibomian glands and preputial glands as measured by Oil Red O staining commensurate with ductal epithelium hyperplasia in Meibomian and preputial glands. Similar with results in keratinocytes, this phenotype was observed only after both C/EBPs were ablated and wasn't observed in single knockouts that had C/EBPα or C/EBPβ acutely ablated, lending further credence to the hypothesis that C/EBPα and C/EBPβ compensate for each other in skin. As mentioned in chapter one, SCD3 and MC5R are markers of terminal differentiation in mouse sebocytes and further examination of these two genes by TaqMan® RT-PCR revealed their expression was significantly reduced. Expression of MC5R was decreased by two thirds and SCD3 expression was nearly abolished. Further work needs to be done in mouse to determine if C/EBPα and C/EBPβ directly regulate expression of these genes or indirectly through some other

mechanism such as through sterol-regulatory element-binding protein 1 (SREBP1), a transcription factor known to regulate fatty acid synthesis genes, and one in which one isoform has been shown to be regulated by C/EBPs in adipocytes [186].

The fact that C/EBPα and C/EBPβ are also profoundly involved in sebaceous gland differentiation was an important and novel contribution. Although C/EBPs role in adipocyte differentiation is well characterized, no such observations have been made showing C/EBPα and C/EBPβ play a fundamental role in sebaceous glands as well, and again, this role wasn't revealed until both transcription factors could be ablated concurrently in mouse suggesting compensation or functional redundancy.

In chapter 2, we tested the hypothesis that C/EBPα and C/EBPβ control differentiation and subsequent lipogenesis in human sebocytes. As mentioned in the introduction, many human disease conditions are related to improper function of sebaceous glands including acne, seborrhoeic dermatitis, blepharitis, dry eye syndrome, and sebaceous cancer. To test whether C/EBPα and C/EBPβ participate in regulation of differentiation of sebocytes in human we used an immortalized human sebocyte cell line, SEB-1; a kind gift from Diane Thiboutot. Our results demonstrate the co-knockdown of C/EBPα and C/EBPβ expression impairs human sebocyte differentiation as measured by the production of neutral lipids, a hallmark of sebocyte differentiation. The co-knockdown of C/EBPα and C/EBPβ in human SEB-1 sebocytes also resulted in decreased expression of genes known to be important in lipogenesis. For example, FASN, a large multi-unit protein responsible for synthesizing long chain fatty acids from acetyl-CoA and malonyl-CoA

was significantly decreased. SCD1 is the only identified family member of SCDs in human, but is also important in the synthesis of the monounsaturated fatty acids oleate and palmitoleate, precursors of cholesterol esters and triglycerides. SCD1 was also significantly decreased in human sebocytes, similar to results of SCD3 in mouse. It is of note that SCD1 knockout mice display impaired sebocyte differentiation and sebaceous and Meibomian gland defects [187], a similar phenotype to that observed when C/EBPα and C/EBPβ were concurrently ablated in mouse sebaceous and Meibomian glands [188]. We also observed that PPARγ, a well characterized transcription factor involved in adipocyte differentiation and one that works in concert with C/EBPs in adipocytes to regulate biosynthesis of lipids in adipocytes (for review [138]) was also decreased. Collectively, these results demonstrate C/EBPα and C/EBPβ are required for human sebocyte differentiation and lipogenesis.

Sebaceous glands contain several discrete populations of cells to maintain homeostasis. Progenitor stem cells at the mouth of gland that divide slowly to replenish peripherally located sebocytes that are mitotically active and in contact with the basement membrane, maturing interior sebocytes that have detached from the basement membrane and have started differentiation and subsequent lipid accumulation, and finally, fully mature lipid filled differentiated sebocytes near the exit of the gland ready to lyse their contents into associated ducts or hair follicles [2]. Although the expression of C/EBPs has been previously noted in human and mouse sebaceous glands [45, 46], the contrasting spatial distribution has not been reported.

The spatial distribution of C/EBPα and C/EBPβ in human and mouse sebocytes provide some clues as to their roles in differentiation/lipogenesis in sebocytes. While both C/EBPα and C/EBPβ were abundantly expressed in the maturing sebocytes, the expression of these transcription factors in the mitotically active periphery sebocytes was largely limited to C/EBPβ. However, we did observe a small number of peripheral sebocytes per sebaceous gland that expressed C/EBPα, suggesting perhaps C/EBPα's early role in sebocyte differentiation is to initiate cell cycle withdrawal. C/EBPα expression was always and without exception abundantly expressed in the nucleus of every sebocyte adjacent to the peripheral sebocytes suggesting C/EBPα expression is either initiated as peripheral sebocytes become post mitotic as suggested above, or that C/EBPα is concurrently expressed with detachment from the basement membrane. We envision a role in human sebocytes whereby C/EBPα is up regulated as soon as peripheral cells exit the cell cycle and begin differentiation and subsequent lipogenesis (Figure 4). The fact that we observed a 60% increase in S-phase sebocytes when C/EBPα alone is ablated in mouse sebaceous glands is consistent with a role in peripheral sebocyte cell cycle withdrawal. In support of this notion are studies showing C/EBPα is required for cell cycle arrest for both adipogenesis and granulopoiesis [95, 132]. Interestingly, we observed further increases in sebocyte S-phase cells when C/EBPα and C/EBPβ were co-ablated suggesting the two transcription factors compensate for each other or cooperate to induce cell cycle withdrawal.

Compensation of C/EBPα for C/EBPβ or vice versa is not uncommon. For example, C/EBPα deficient mice are perinatal lethal due to defects in adipogenesis, glucose metabolism, and hematopoiesis [122, 124] and experiments where C/EBPβ was knocked into C/EBPα's locus were able to rescue liver abnormalities [189] and hematopoiesis [165] dysfunction exhibited by C/EBPα null mice. In addition, it was not until both C/EBPα and C/EBPβ were deleted from adult mouse skin that critical roles in epidermal development [190] and both keratinocyte and sebocyte differentiation and homeostasis in mouse were revealed [188]. With respect to human sebocyte differentiation, we observed that C/EBPα and C/EBPβ are co-expressed in almost every interior sebocyte and yet loss of C/EBPα alone or C/EBPβ alone did not affect the ability of SEB-1 cells to differentiation and synthesize neutral lipids. Concurrent knockdown of both transcription factors were required to impair sebocyte differentiation and lipogenesis, suggesting functional redundancy or a compensatory mechanism.

While research into control of development of sebaceous glands has implicated many transcription factors including AP2, Sox9, Blimp1, and the Wnt/BMP and hedgehog pathways [12, 44, 191, 192], the pathways regulating homeostasis of sebocytes consisting of the continual renewal of cells lost to self-destruction are less well understood. It is known that sebum production and cell proliferation are increased by androgen signaling [193] and that retinoids, particularly 13-cis-retinoic acid, reduces sebum production and selectively causes apoptosis in sebocytes [194]. In addition, melanocortins, insulin-like growth factor-1 (IGF-1) and PPARγ

have been shown to affect sebocyte activity [47, 195, 196]. The IGF-1 pathway elucidated by Smith et.al [195] activates both MAPK/ERK and PI3-K pathways resulting in activation of sterol response element-binding protein-1 (SREBP-1) and subsequent transcription of genes involved in lipogenesis. Of potential significance are studies demonstrating that C/EBPs regulate SREBP1c in adipogenesis [186]. Future work examining whether C/EBPs regulate SREBP1 and act downstream of PI3K/AKT signaling from IFG in human sebocytes is merited.

We have shown for the first time that C/EBPα and C/EBPβ are important in the regulation of lipid production in human sebocytes and this result could have important human health relevance. Acne is the most common skin disease and in the United States, and affects up to 85% of all adolescents and young adults. Currently, the only treatment for acne is a combination therapy of antibiotics to treat *P.* acne bacteria that uses sebum as a food source and exacerbates inflammation, and retinoids, that have been shown to selectively cause apoptosis in sebocytes [194, 197]. Retinoids are teratogenic so finding alternative ways to reduce sebum production and subsequent acne is highly desirable. It is possible that C/EBPα and C/EBPβ could be biological targets for treatment of sebaceous gland disorders, notably acne.

Additional experiments to determine how C/EBPα and C/EBPβ regulate differentiation and lipogenesis in humans are needed. Thin layer chromatography (TLC) or mass spectrometer (MS) analysis of lipids before and after knockdown of C/EBPα and C/EBPβ followed up by ChIP would give pertinent clues to specific

enzymes regulated directly or indirectly by C/EBPs. In addition, one could envision a role for C/EBPδ, which has been well established in adipogenesis, in sebocyte differentiation. It is possible C/EBPα or C/EBPβ dimerize with C/EBPδ in the absence of only C/EBPα or only C/EBPβ, and when both C/EBPα and C/EBPβ are removed, C/EBPδ is insufficient for keratinocyte and/or sebocyte differentiation and lipogenesis. Previously mentioned experiments with FRET could reveal native and compensatory dimerization partners after removal of C/EBPα or C/EBPβ in the process of sebocyte differentiation and lipogenesis. In addition, it seems likely that AP-2 transcription factors could be regulating C/EBPα and C/EBPβ in sebocyte differentiation and experiments to determine that would be straightforward.

In summary, we have shown C/EBPα and C/EBPβ cooperate and/or are functionally redundant for both keratinocyte and sebocyte differentiation. These findings further cement established roles for C/EBPα in cell cycle withdrawal as a precursor to differentiation and the roles that both C/EBPα and C/EBPβ have as master regulators of differentiation in many different cell types and tissues.

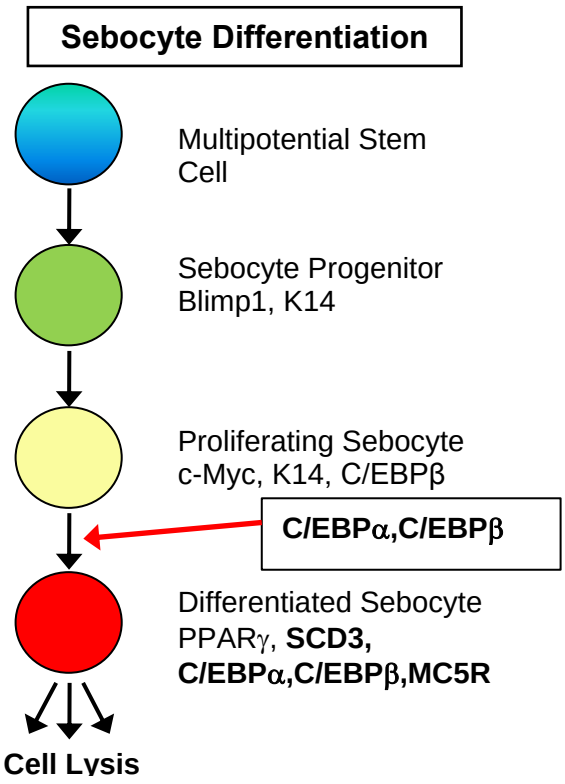

Figure 4. Proposed model for the function of C/EBPα and C/EBPβ on sebocyte differentiation in human sebocytes

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