ABSTRACT

TAM, HANN WEN. Overcoming the Dominant Negative Effect of Mutant p53$^{+/R172H}$ Mice. (Under the direction of Dr. Robert C. Smart.)

Transcription factor p53 is activated by various cellular stresses, DNA damage, and oncogenic stimuli to regulate cell cycle arrest, senescence, and apoptosis to prevent cancer. p53 is the most frequent mutated tumor suppressor gene in human cancer. The p53 mutations often results in loss of its tumor suppressor function, and may gain functions that promote tumor progression. Most of the p53 mutations are missense mutation with a single base substitution at the DNA binding domain lacks transcriptional activity. However, these mutant p53s retain their ability to form tetramer with other mutant or wild type p53 molecules. The mutant p53s in cells with single mutant p53 allele (p53$^{+/M}$) usually exert dominant negative effect over the remaining wild type allele and suppress the wild type p53 activity. Overcoming the dominant negative effect of mutant p53 and restoring wild type p53 activities in those p53$^{+/M}$ cells are important in develop cancer therapeutic strategies. The basic leucine zipper transcription factor, CCAAT/enhancer binding protein β (C/EBPβ), has an important role in cell survival and tumorigenesis. Early studies demonstrate C/EBPβ deficient mice are completely refractory to chemical carcinogen induced, oncogenic Ras-driving skin cancer. Upon treatment of chemical carcinogen or DNA damage agents, the C/EBPβ deficient keratinocytes display an aberrant increase in both apoptosis and p53 protein levels. In this research we utilized UVB, an inducer of DNA damage to investigate the role of C/EBPβ in p53-mediated apoptosis in epidermis and to test whether the deletion

of C/EBPβ could enhance wild type p53 apoptotic function and overcome the dominant-negative effects of mutant p53 in p53$^{+/R172H}$ mice. The R172H mutation in mice is equivalent to the R175H mutation in humans which is a hot spot p53 mutation in human cancer. In keratinocytes of UVB-treated p53$^{+/+}$ SKH1 mice the deletion of C/EBPβ increased the stability of wt p53 protein and enhanced p53's pro-apoptotic function. Deleting C/EBPβ in the epidermis of UVB-treated p53$^{+/R172H}$ mice completely overcame the dominant negative effect of the mutant p53 and enhanced apoptosis to a level that was 30% greater than that observed in UVB-treated mice with two wild type alleles of p53. In UVB-treated p53$^{+/R172H}$ mice the deletion of C/EBPβ resulted in increased levels of wt p53 protein and decreased levels of the mutant p53 protein reducing the mutant:wt protein ratio 7 fold. During skin cancer development the UVB-induced mutation of p53 is an early event where the mutant p53 protein functions as a dominant negative inhibitor to allow for the evasion of UVB-induced apoptosis thus contributing to tumor initiation/progression. Skin specific C/EBPβ knockout mice chronically treated with UVB were resistant to UVB-induced skin cancer and displayed increased tumor latency and striking decreases in tumor multiplicity and a reduced frequency of mutant p53 in UVB-induced carcinomas. Collectively these results suggest that targeting C/EBPβ could have cancer therapeutic benefits.

Overcoming the Dominant Negative Effect of Mutant p53$^{+/R172H}$ Mice

by
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BIOGRAPHY

Hann wen Tam was born on September 12, 1985 in New Brunswick, New Jersey. She grows up in Taipei, Taiwan. She moved to Pittsburgh, Pennsylvania in 2002 and graduated in the Class of 2004 from Schenley High School. She attended University of Pittsburgh, where she received a Bachelor of Science degree with in Biological Sciences in 2008. While pursuing her undergraduate studies, she involved in undergraduate research on the plants-pollinators interaction with Dr. Tia-Lynn Ashman in 2006. In 2009, Hann worked in a molecular biology lab as lab assistant in Academia Sinica, Taipei, Taiwan. Hann began graduate studies in Toxicology program in North Carolina State University in 2009. In January 2010, Hann began her dissertation research under the direction of Dr. Robert C. Smart to investigate the role of CCAAT/enhancer binding protein beta in epidermal keratinocytes in response to DNA damage.

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GENERAL INTRODUCTION

CCAAT/enhancer binding proteins (C/EBPs)

CCAAT/enhancer binding proteins (C/EBPs) are a family of basic leucine zipper (bZIP) transcription factors that respond to various pathophysiological and physiological signals. C/EBPs regulate the expression of an array of genes which participate in various cellular processes such as survival, proliferation, differentiation, metabolism, and inflammatory responses [1, 2]. The six identified members in the C/EBP family are: C/EBPα (C/EBP) [3-6], C/EBPβ (NF-IL6, IL-6DBP, LAP, CRP2, NF-M, AGP/EBP, ApC/EBP) [4, 5, 7-11], C/EBPδ (NF-IL6 β, GRP3, CELF) [4, 5, 12, 13], C/EBPε (CRP-1) [5, 14], C/EBPγ (Ig/EBP-1, GPE1-BP) [15, 16], and C/EBPζ (CHOP, CHOP10, DDIT3, GADD153)[17, 18]. The C/EBP family members are present in many tissues and expressed at various levels. The expression and activity of C/EBPs are regulated at both the transcriptional and posttranslational levels by a multitude of signal transduction pathways. C/EBP activities are modulated to alter the expression of their target genes in order to induce the appropriate cellular responses [19, 20].

The C/EBP family members share basic molecular structures. The leucine zipper and the basic region (bZIP) domains are located at the C-terminal of the proteins. With the exception of C/EBP ζ, which lacks a canonical basic region, other family members have a highly conserved (>90% homology) bZIP domain [19]. The leucine zipper domain is characterized by four to five heptad leucine repeats. Each heptad has two α-helical turns of seven amino acids. The C/EBP family members form homo- or heterodimers via these leucine zipper domains [21-23]. Dimerization is required for C/EBPs binding to DNA with

their basic regions [21, 24]. Mutation or deletion of the leucine zipper domain abrogates the C/EBP's DNA binding ability [23]. The basic region is the region used by C/EBPs to recognize and bind to specific target DNA sequences [25]. The C/EBP DNA binding motif has been identified as a dyad of symmetrical repeat with a sequence of RTTGCGYAAY, where R is A or G, and Y is C or T, with considerable variations [26].

Comparatively, the N-terminus of C/EBPs is less conserved and contains the trans-activation domains (TADs) and repression domains (RDs) to positively or negatively regulate the C/EBP's transcriptional activity [19, 27]. C/EBPγ and C/EBPζ lack transactivation and repression domains, but they can regulate cellular responses by forming heterodimers with other C/EBP family members. C/EBPγ functions as a dominant negative inhibitor by forming inactive heterodimers [28]. The activity of the C/EBP dimers is determined by the N-terminal trans-activation domains.

Some C/EBP family members have isoforms lacking the TADs and act as dominant negative inhibitors. Both C/EBPα and C/EBPβ have isoforms derived from alternative translational initiation sites. In the absence of the TADs, these shortened isoforms have altered transactivation potential [29-31]. For instance, C/EBPα has two isoforms of p42 (42 kDa) and p30 (30 kDa), where p30 has lower activation potential than p42 [29, 30]. C/EBPβ has three isoforms of LAP* (38 kDa), LAP (35 kDa), and LIP (20 kDa). LIP lacks the trans-activation domain and serves as a dominant negative inhibitor [31].

C/EBPβ

C/EBPβ is also known as NF-IL6, as it was first discovered as a nuclear factor binding to the IL-6 gene from human monocytes [7]. C/EBPβ is induced by IL-6, IL-1, and lipopolysacchride (LPS), and responds to acute-phase immune response by regulating acute phase response and inflammatory genes [7]. C/EBPβ is expressed abundantly in the epidermis [32]. It is also expressed in many tissues, including liver, lung, adipose tissue, heart, kidney, spleen, hematopoietic cells and chondrocytes [32, 33]. Many studies have been conducted to investigate the functional roles of C/EBPβ in diverse tissues and cell types. C/EBPβ participates in important cellular processes such as metabolism, inflammatory responses, cellular differentiation, proliferation, and survival [7, 34-45].

C/EBPβ is expressed in various tissues and functions in the differentiation process in mammary epithelial cells, adipocytes, ovarian granulosa cells, macrophages, sebocytes, intestinal epithelial cells and epidermal keratinocytes [34, 36-38, 46-50]. C/EBPβ plays a role in early stages of epidermal keratinocytes stratified squamous differentiation [34]. The expression of C/EBPβ in epidermis is highly ordered and shows a specific pattern in which the expression of C/EBPβ is exclusive to the nuclei of three-cell clusters of the suprabasal keratinocytes that is repeated at regular interval in the epidermis [32]. Forced expression of C/EBPβ in keratinocytes resulted in growth arrest and express of two epidermal keratinocyte differentiation early markers keratin 1 (K1) and keratin 10 (K10) [34]. The role of C/EBPβ in adipocyte differentiation is well studied. Ectopic expression of C/EBPβ can induce adipocyte differentiation in cultured 3T3-L1 pre-adipocytes without adipogenic

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hormones [39, 51]. In the absence of C/EBPβ and C/EBPδ, mouse embryonic fibroblasts are unable to differentiate into adipocytes even upon adipogenic hormone stimulation [52]. These cells also cannot express adipocyte regulatory proteins [52]. C/EBPβ deficient female mice are sterile due to the inability of ovarian granulosa cells to differentiate into luteal cells in response to luteinizing hormone [47].

C/EBPβ can either block or support cell proliferation, depending on cell types. C/EBPβ suppresses cell proliferation in certain leukemic cells [53, 54]. C/EBPβ is involved in growth arrest and is associated with differentiation in keratinocytes [34]. In HepG2 hepatocarcinoma cells, forced expression of C/EBPβ induces growth arrest [55]. C/EBPβ deficient mice display mild epidermal hyperplasia [34]. Other studies show C/EBPβ positively regulates cell proliferation [36, 37, 56-58]. For example, during adipocyte differentiation, C/EBPβ is required for mitotic clonal expansion [59]. C/EBPβ deficient female mice show impaired proliferation of mammary epithelial cells during ductal morphogenesis [36, 37].

C/EBPβ is an important mediator of cell survival and promotes tumorigenesis [41-43, 60]. C/EBPβ is highly expressed in skin, ovary, prostate, colon/rectum, kidney, breast, and lymphomatous tumors [60-66]. The increased expression of C/EBPβ in cancer is often associated with a poor prognosis and invasiveness [60]. Increased expression of C/EBPβ in human metastatic Wilms tumor cell lines associates with tumor relapse, while acute ablation of C/EBPβ in human metastatic Wilms tumor cell lines results in acute apoptosis [41]. Phosphorylation of C/EBPβ on Thr^{217} is required for the survival of CCl$_4$-treated hepatic

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stellate cells [42] and bisphosphonate treated osteocytes [43]. C/EBPβ deficient mice are refractory to chemical carcinogen-induced and oncogenic Ras-driven skin tumorigenesis [67]. C/EBPβ deficient mice display elevated in p53 levels and apoptotic cells in their epidermis in response to DNA damage agents. [67-69]. Collectively, these studies suggest that C/EBPβ regulates cell survival by suppressing p53 activity.

Skin and Skin Cancer

Skin is the largest organ in the human body. This airtight and waterproof protective barrier serves as the first line of defense against infection and dehydration. Skin helps regulate the homeostasis of body temperature and it contains sensors for pain, touch, and pressure for mammals to avoid environmental dangers. The differentiated daughter cells provide supporting cells and various structures to replenish and sustain skin structures. The mammalian skin has a waterproofing outer layer of epidermis resting on a collagen-rich dermis layer for structural support. The hypodermis is situated under the dermis layer to provide additional elasticity and lipid storage. Capillaries are located in dermis, but white blood cells can travel to epidermis to immune defense.

Epidermis is made up mainly of keratinocytes with small number of Merkel cells, Langerhans cells, and melanocytes (in human). The epidermis is subdivided into four horizontal layers: *stratum basale, stratum spinosum, stratum granulosum* and *stratum corneum*. The bottom most layer of the keratinocytes, referred to as the *stratum basale*, are attached to the basement membrane, which separates the epidermis from dermis. The

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basal layer contains undifferentiated keratinocytes that possess proliferation ability [70].

There are skin epithelial stem cells located in the interfollicular regions of the basal layer [71]

and in the bulge region of the hair follicle [71, 72]. These stem cells compose less than five

percent of the basal keratinocytes [73, 74]. The epidermal stem cells are slow cycling and

occasionally divide to give rise to transient amplifying cells which have the ability to

proliferate at a higher rate [71, 72]. The transient amplifying cells are responsible for

replenishing the basal keratinocytes that are lost to injury or stratified squamous

differentiation [71, 72]. When transient amplifying basal cells become post mitotic, they

move upward to the *stratum spinosum* layer, and begin the early stage of epidermal

stratified squamous differentiation [71]. The stratified squamous differentiation is a highly

ordered and complex process that involves numerous genes, proteins, and pathways [71].

In each stage, the differentiated keratinocytes express particular sets of genes and proteins

[71]. Keratinocytes in the *stratum granulosum* accumulate keratohyalin and laminar

granules [71]. When the keratinocytes move up to the *stratum corneum* layer, they

terminally differentiate into a cornified envelope and excrete laminar to provide an airtight

waterproofing dead cell layer for the epidermis [71, 75]. Finally, the cells shed from the skin

as the terminal event of differentiation [75]. Compares to rodents, human epidermis have

higher proportion of interfollicular epidermis relative to hair follicle epithelium [76]. The

epidermis of human skin is thicker with more cell layers and form deep epidermal ridges

[76]. These rete ridges extend downward and help anchor the epidermis to the dermis [77].

SKH1 hairless mice are extensively utilized to study UVB-induced biologic changes in skin and are an experimental model relevant to UVB-induced human squamous cell carcinoma (SCC) skin cancer [78]. Hairless gene (*Hr*) is located at mouse chromosome 14 [79]. The *Hr* gene is expressed in epithelial cells of tongue, mouth, tooth bud, nose, bladder, urethra, stomach, and submandibular salivary gland, in cartilage, and in nervous tissue in mouse embryo [80]. By the time of birth, Hr is highly expressed in hair follicle and the interforllicular epidermis [79]. SKH1 hairless mice (Crl:SKH1-*hr*) by Charles River Laboratories carry mutant allele hairless (Hr^{hr}), which is an autosomal recessive mutation. The mutation has aberrant splicing of over 95% of Hr transcripts [79]. Hairless mice develop normal hair coat in the first hair growth cycle [81]. Beginning two weeks after birth, rapid hair loss beginning at the eyelids and proceeding caudally [78]. About three weeks after birth, the mice are complete hairless except for vibrissae [78]. These hairless mice have significant hair follicle abnormality and display two characteristic structures: the dermal cyst and utriculus [78]. Dermal cyst, which is located in deep dermis, is lined by keratinized epithelium and may contain sebocytes in the wall [78]. Utriculus is arisen from infundibulum of the hair shaft. It is an ampuliform structure lined by hyperkeratotic epithelium [78]. SKH1 mouse skin is rugose and nails are long and twisted [78]. SKH1 mice are susceptible to spontaneous skin abscesses caused by β-hemolytic Group G *Streptococcus* [82].

Skin cancer is the most common cancer in the United States, and there are more new skin cancer cases each year than of breast, prostate, colon rectal, and lung cancers

combined [83]. Skin cancer can be categorized by melanoma and non-melanoma skin cancers (NMSC). NMSC is further sub-divided into basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) [84]. Basal cell carcinoma accounts for 80% of NMSC, while squamous cell carcinoma accounts for the remainder. SCC is less frequent than BCC, however, it can be invasive and metastatic [84]. More than 3.5 million cases of NMSC are diagnosed each year, and occur in about 2.2 million Americans, and the number of new NMSC diagnosed are still rising each year [85]. The major risk factor of skin cancer is the solar radiation from sunlight [86]. People with fair skin and/or those with prolonged sun exposure are highly susceptible to skin cancer. UV radiation is a complete carcinogen in that it is able to induce skin cancer on its own, acts as both initiator and promoter [87]. The multistage carcinogenesis model consists of three stages: initiation, promotion, and progression [88]. In the initiation stage, UV radiation causes irreversible DNA damage at critical genes. During promotion stage, chronic exposure of UV radiation promotes the selective clonal expansion of the initiated cells resulting in benign tumor development. In the progression stage, the benign tumor transforms into malignant skin cancer [87].

Ultraviolet B Radiation and DNA Damage Response

Skin is the first line of defense against both physical and chemical DNA damaging agents. These DNA damaging agents include exogenous ultraviolet (UV) and ionizing radiation, endogenous reactive oxygen species from metabolic byproducts, and genotoxic

chemicals such as polycyclic aromatic hydrocarbons. UV radiation from sunlight is the major

risk factor for skin cancer [89].

UV radiation that penetrate the ozone layer carry high energy. These include UVA

(400-320 nm) and UVB (320-290 nm) radiation [90]. UVC (290-200 nM), is completely

blocked by the ozone layer [91]. UVA has longer wave length and carries less energy than

UVB, but it can penetrate skin to the dermis and destroy the collagen matrix. UVB only

penetrates the epidermal layer and causes DNA damage [92]. Both UVA and UVB radiation

can cause skin cancer. Compared to UVA, UVB causes earlier onset of skin cancer [92].

When the skin cells are not able to repair UVB-induced DNA damage, it may result in

mutations in the genome, and if these mutation occur in proto-onocogene and tumor

suppressor genes then they contribute to the development of skin cancer [92].

Cells exposed to UVB radiation results in DNA damage in the form of DNA strand

breaks, DNA cross-links, and the formation of dimeric photoproducts between adjacent

pyrimidine bases on the same DNA strand [86, 93].   Cyclobutane pyrimidine dimers (CPD)

and pyrimidine (6-4) pyrimidinone (6-4 photoproduct) are two well characterized

pyrimidine dimers induced by UVB radiation [94]. UV radiation signature mutation is

defined as cytosine-thymine substitution at dipyrimidine sites [95, 96]. This UV signature

mutation is often found in the p53 tumor suppressor gene in NMSC [92, 96]. Cellular repair

of the CPDs is less efficient than 6-4 photoproducts [97]. Therefore, mutations resulting

from CPD lesions are often found in UV induced cancer cells [98]. It is important for the

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cells to repair the CPDs and 6-4 photoproducts to prevent the development of skin cancer [92].

Nucleotide excision repair (NER) is utilized by the cell to repair both CPDs and 6-4 photoproducts. NER is an ATP dependent system that removes 23-30 base pairs of nucleotides containing a bulky DNA lesion [99-101]. NER can be subdivided into global genome repair (GGR) and transcription coupled repair (TCR) pathways. The difference between the two sub-pathways is the way they recognize DNA damage. The GGR pathway removes DNA lesions from both transcribed and untranscribed DNA strands, while the TCR pathway only recognizes DNA lesions inactively transcribed genes [102]. The DNA lesion recognition step is followed by excision of the lesion and surrounding nucleotides, DNA synthesis to fill in the deleted patch of nucleotides, and then ligation [103]. Individuals deficient in NER are predisposed to cancer, such as the patients with xeroderma pigmentosum (XP) [100]. These individuals are unable to repair DNA damage, in particular the UV-induced DNA lesions [100]. Therefore, XP patients are highly susceptible to skin cancer.

Depending on the degree of DNA damage, cells can undergo cell cycle arrest, apoptosis, or senescence [104]. For cells with less severe DNA damage, they undergo cell cycle arrest and repair the damaged DNA [104]. When the DNA damage in the cell is extensive, the cells undergo apoptosis or senescence to protect genome integrity and stop the passage of damaged DNA to the next generation [104]. It is poorly understood how cells make the decision to live and repair their DNA or undergo apoptosis.

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p53

p53 Functions

p53, the guardian of genome, is a highly studied tumor suppressor gene. p53 is a tetrameric, DNA sequence specific transcription factor and is activated and stabilized by cellular stressors. These cellular stressors include DNA damage, hypoxia, ionizing radiation, aberrant proliferation, oxidative stress, oncogene activation, and UV radiation [105]. Once activated, p53 directly or indirectly represses or induces genes expression to activate cascades of different cellular signaling pathways that are involved in cell-cycle arrest, apoptosis, or senescence [105-107]. p53 plays a key role in preventing cancer development by regulating cell fate and cell cycle arrest in response to cellular stress.

The TP53 gene resides on the human chromosome 17. The Trp53 gene is its murine equivalent. p53 functions as a homotetramer, which binds to p53 response elements of target genes [108]. The p53 response element is composed of two identical motifs that are separated by 0-21 base pairs. The motif has a sequence of: 5'-RRRCWWGYYY-3', where R is purine, C is cytosine, W is adenine or thymine, G is guanine, and Y is pyrimidine [109-111]. The spectra of genes regulated by p53 to carry out its tumor-suppressive functions involving apoptosis include PUMA, BAX, NOXA, BCL-2, cell-cycle regulation include CDKN1A, MIR34A, STAT3, and senescence include CDKN1A, PAI1 [105, 106, 110, 112, 113].

p53 mutations are found in more than 50% of human cancer [114]. Many diseases are associated with p53 dysregulation, such as Alzheimer, Parkinson, cardiovascular and Li

Frameni syndrome (LFS) [115-120]. LFS is a rare autosomal-dominant disorder caused by germline mutations in TP53 [121, 122]. LFS patients are predisposed to sarcomas, breast cancer, leukemia, and other neoplasms [115-117]. Mouse models with systemic p53 knockout are cancer prone [123, 124]. These mice develop spontaneous tumors, the majority of which are lymphoma and sarcoma by 6 months of age [123, 124]. Mice with mutant p53 display a different tumor spectrum comparing to the mice with p53 deletion [125, 126]. For example, mice with a heterozygous R172H mutation, which is equivalent to human R175 hot spot mutation, show a different tumor spectrum compared to the p53$^{+/-}$ mice [127]. Tumor spectrum of the heterozygous mutant mice shows increased number of carcinoma and a decrease in lymphomas [127]. These p53$^{+/R172H}$ mutant mice show an increase in osteosarcomas and carcinomas metastasis [127].

p53 Structure

The p53 tumor suppressor is a 53 kDa protein of 393 residues [128]. The acidic N-terminus of the p53 protein contains the transactivation domain (TAD) at residues 1-63. Proximal to the TAD is a proline-rich region (64-92), which has a regulatory role enhancing p53 transcription activity [129, 130]. The DNA binding domain (93-292) sits at the center core domain of the protein [109]. The region penultimate to the C-terminus is basic and contains the tetramerization domain (307-355) [131]. The negative regulatory domain is located at the C-terminus of p53 (363-393), and it carries the acetylation and phosphorylation sites for posttranslational modification and has non-specific DNA binding ability [132, 133].

The N-terminus of p53 has two transactivation domains. The TAD interacts with the RNA polymerase machinery for transcriptional regulation of target genes. TAD can also interact with regulatory proteins, such as Mdm2 and p300/CBP. Mdm2, an E3 ubiquitin ligase, binds to p53 and mediates its proteasomal degradation. The DNA binding domain binds to specific DNA containing the p53 response elements (RE) [109].

The C-terminus of p53 regulates the subcellular localization and oligomerization of the p53 protein. Within the C-terminus, three nuclear localization signals (NLSs) (residue 302-322) reside after the core domain but before the tetramerization domain [134]. NLS mediates the nuclear transport of the p53 protein. The tetramerization domain allows the p53 protein to form a homotetramer, or dimer-dimer, which are the active and functional forms, to carry out its transcription factor function in the nucleus. A nuclear export signal (NES) (residues 340-351) is located within the tetramerization domain [135]. A study from Stommel et al. suggests that tetramerization of p53 blocks the NES and thus inhibits the export of the p53 into cytoplasm, where it undergoes proteasomal degradation.

p53 Regulation

p53 protein levels in cells are tightly regulated [128]. In unstressed cells, p53 is maintained at a low level due to its fast protein turnover rate [128]. Upon DNA damage or cellular stresses, the p53 protein is activated by posttranslational modifications (PTMs) and the half-life of the p53 protein is greatly increased [128]. A classical model for p53 activation is carried out in 3 steps: the p53 is stabilized by PTMs; it binds to target genes in a sequence-specific manor; and it interacts with the transcriptional machinery for target gene

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transcription [128]. The major mechanism for controlling p53 protein level is increasing its

protein stability through PTMs [136]. Cellular p53 protein levels can also be regulated by

increased transcription of p53[137]. Furthermore, there are cellular proteins and miRNAs

bind to p53 mRNA and affect the p53 translation rate. The p53 protein activity is regulated

by posttranslational modifications, and interactions with other cellular molecules.

Mdm2-mediated Degradation

Mouse double minute protein 2 (Mdm2), an endogenous E3 ubiquitin ligase, is a major

player in regulating p53 stabilization [128]. Mdm2 is a p53 target gene and functions as a

cellular antagonist of p53 [128]. In an unstressed cell, p53 actively transcripts Mdm2 and

then Mdm2 in turn down regulates p53 protein levels [128]. The N-terminus of Mdm2 binds

to the N-terminus transactivation domain of p53 and targets p53 for ubiquitination [128].

The level of Mdm2 in nucleus determines the ubiquitin status of p53 [138-140]. p53 is poly-

ubiquitinated by Mdm2 when Mdm2 activity level is high [138-140]. When Mdm2's activity

of level is low, it mono-ubiqitinats p53 for trafficking into cytoplasm [138-140]. The p53

protein is then poly-ubiquitinated in the cytoplasm by E4-ubiquin ligase and undergoes

proteasomal degradation [141]. When a cell is under stress, such as having DNA damage,

the Ser15 and Ser20 residues at the N-terminus of p53 can be phosphorylated and inhibit

the interaction between p53 and MDM2 [142-144]. This results in an increase in p53

stability and allows p53 to carry out cellular responses such as cell-cycle arrest, senescence,

or apoptosis.

Besides regulating p53 stability, Mdm2 also regulates p53 activity. The binding of Mdm2 on the p53 TAD region inhibits the interaction between p53 and other transcription factors, resulting in the loss of p53 transcriptional activity [128]. Mdm2 is crucial for p53 negative regulation. Mdm2 knockout mice are embryonic lethal, and this lethality is rescued by the knock out p53 [145, 146].

Mdmx, also known as Mdm4, is another member of Mdm2 family [128]. Mdmx and Mdm2 share similar molecular structures [147]. Mdmx can bind to the p53 N-terminus TAD domain and inhibit its transactivation [147]. The C-terminus RING finger domains of Mdm2 and Mdmx are conserved [148]. Mdmx forms heterodimer with Mdm2 through their C-terminus RING finger domains [148]. However, Mdmx doesn't have the intrinsic E3 ubiquitin ligase function in its RING finger domains [149]. Therefore, on its own, Mdmx can only regulate the p53 transcriptional activity, not its stability [149]. Interestingly, Mdmx forms heterodimer with Mdm2, and enhance Mdm2's ability of ubiquitinating p53 [128, 148]. This Mdm2-Mdmx heterodimer complexes with p53 on promoter regions of p53 specific target genes and repress the gene expressions [128].

p53 Posttranslational Modification Regulation

The p53 protein can undergo posttranslational modifications (PTMs) such as acetylation, phosphorylation, methylation, ubiquitination and sumoylation [140, 150]. Reportedly, approximately 50 residues on p53 can be subjected to posttranslational modifications [136]. PTMs allow for a complex regulation of p53 activity and stability [151].

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Generally, acetylation and phosphorylation activate p53 for target gene expression. Acetylation takes places at ten lysine residues. Six of them (Lys370, Lys372, Lys373, Lys381, Lys382 and Lys386) are located at the C-terminus, and two of these (Lys305 and Lys320) can be found in the tetramerization domain. The remaining two lysine residues (Lys120 and Lys164) are at the DNA-binding domain, which can be acetylated upon receiving signals after DNA damage. Acetylation of Lys320 increases DNA-binding and transcription activity while modification of C-terminus lysine residues enhances DNA-binding and stabilizes the p53 proteins [128, 152, 153]. Missense mutation on all six C-terminus lysine residues results in an impaired p53 that fails to enhance the transcription of DNA damage-response genes [153].

Phosphorylation of p53 also promotes DNA-binding, transcription activity, and protein stability [128]. There are nine serine and three threonine residues at that N-terminus, and three serine residues at the C-terminus of p53 that can be phosphorylated. Upon DNA damage, the ATM/ATR and Chk1/Chk2 kinase are activated and phosphorylate the N-terminus serine residues. Phosphorylation of Ser15 and Ser20 stabilize p53 by inhibiting the p53-Mdm2 interaction, thus preventing p53 from proteasomal degradation [142-144].

Methylation is more complex and can exert either positive or negative regulation on the p53 function depending on the site of methylation [128]. There are three lysine methylation sites at the C-terminal of the protein. Methylation of Lys370 and Lys382 suppress p53 target gene transcription, while methylation of Lys372 activates p53 medieated transcription of the DNA damage response gene, p21. In addition, methylation

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of Lys372 can block the modification at Lys370 and further enhance gene transcription [128, 152, 154].

As mentioned above, the p53 protein level is regulated by ubiquitin-mediated proteasomal degradation [155]. There are seven ubiquitin sites on the C-terminus and and four in the tetramerization domains of p53 [140, 152]. Ubiquitination can regulate both p53 stability and localization [140, 152]. Ubiquitin is a protein that can covalently attach on lysine sidechains of target proteins [140]. Mono- and poly-ubiquitination are referring to the addition of one or multiple ubiquitins onto a lysine sidechain. The six lysines on the C-terminus of p53 can be either mono- or poly-ubiquitinated by Mdm2 [140]. Mono-ubiquitinlated p53 is subject to nuclear export [128]. The cytoplasmic mono-ubiquitinated p53 is further poly-ubiquitinlated by E4 ubiquitin ligase and targeted for proteasomal degradation [140, 152, 156].

Posttranslational modifications of p53 have many layers of regulation. For example, different PTMs may compete for the same residue on p53. Under different cellular conditions, different PTMs take place. For example, Lys372 and Lys373 of p53 can be acetylated, ubiquitinated, or methylated. These sites are acetylated by p300/CBP acetyltransferases when cells encounter cellular stress. Acetylations of these lysine residues enhance the binding to DNA, and thus increases target gene transcription. The same sites can be ubiquitinated by Mdm2 for proteasomal degradation. Methylation at K372 promotes nuclear localization and drives target genes expression [152].

p53 Regulation at the RNA Level

Protein regulation in cells is a balance between synthesis and degradation. Under cellular stress, the p53 protein level increase primarily due to an increase in half-life of p53 due to PTMs and inhibition of mdm2-p53 interaction, however p53's also by mRNA translation initiation is also increased. Reports have shown that cells treated with cycloheximide, a protein synthesis inhibitor, have hindered p53 protein induction after ionizing radiation (IR) or etoposide treatment, however, the level of newly synthesized p53 labeled with [^{35}S]methionone is induced [157-159]. After DNA damage, the p53 mRNA associates with larger polysomes [159, 160], implying an increase in translation initiation for p53 protein synthesis.

Proteins can bind onto the untranslated regions of target mRNA to regulate its translation. p53 protein performs self-regulation by binding to its own mRNA [161]. p53 protein is found in cytoplasm during the G1 phase of the cell-cycle and is capable of binding to its mRNA at the 5'- untranslated region (UTR) to suppress the translation of the p53 protein [161]. Besides p53, nucleolin and ribosomal protein L26 (RPL26) are capable of binding to the 5'UTR of the p53 mRNA [158]. Nucleolin down regulates p53 translation [158]. Overexpression of nucleolin reduces p53 translation in both normal and stressed cells [158]. In contrast, p53 translation is up-regulated in cells by the over expression of RPL26 [158]. The amount of p53 mRNA associated with larger polysomes is enhanced in cells transfected with RPL26 [158]. In IR treated cells with RPL26 overexpression, the p53 protein levels increased while the p53 mRNA levels and protein half-life stay unchanged [158]. This observation implies an increase in translation initiation and p53 protein

synthesis. Conversely, the knock down of RPL26 in MCF7 cells with siRNA results in decreased p53 translation upon IR treatment [158]. These suggest that p53 translation is posttranscriptionaly regulated, both positively and negatively, by proteins binding to its 5'-UTR region.

The 3'-UTR region of p53 mRNA has many microRNA (miRNA) binding sites [162]. MicroRNAs are small, non-coding regulatory RNA molecules of 20-25 nucleotides in length. miRNAs bind mRNAs with partial complementary sequences at the 3'-UTR region. miRNAs regulate their target mRNAs by inhibiting translation or reducing the stability of the mRNAs. Hence, miRNA negatively regulates their target mRNA at the posttranscriptional level [162-165]. Many miRNAs, such as miR-125a, miR-125b, miR-504, and miR-122, can bind to the p53 mRNA and inhibit translation [164]. miR-125b is capable of binding to p53 mRNA resulting in a decrease in p53 mRNA and protein levels. Overexpressing miR-125b leads to repression of p53 at the protein level and reduces p53-mediated apoptosis. Conversely, knockdown of miR-125b results in elevated p53 protein levels [166]. Similarly, miR-504 binds to the two binding sites on the 3'-UTR of human p53 and decreases the p53 mRNA translation by 70%. Overexpression of miR-504 decreases the levels of p53 and its downstream proteins in human cell lines. Under cellular stress, p53 mediated apoptosis and G1 cell-cycle arrest are inhibited by overexpressing miR-504. All of these observations suggest that the miR-504 suppresses the translation and reduces the function of p53 [167].

Interaction with Other Cellular Molecules

Interaction between p53 and other cellular molecules regulates the p53 activity. p53 interacts with many cellular molecules, such as miRNA, ribosomal proteins, and mdm2, as described above. There are at least 80 known p53-binding proteins [168]. The interacting proteins can be p53 PTMs modulators, transcriptional coactivators or corepressors. p53 interacts with Mdm2 through its transactivation domain, which not only blocks p53 transactivation, but also directs p53 for proteasomal degradation in the cytoplasm [106, 155, 169, 170]. p300/CBP regulates p53 C-terminus residues' acetylation while E2F family members enhance p53 mediated apoptosis [171, 172].

E2F family members are transcription factors that are critical for cell cycle progression [168]. E2F1 binds to p53 at residue 347-370, which is overlapping with the nuclear export sequence (NES) at the C-terminus of p53, thus retaining Ser315 phosphorylated p53 in the nuclear [173]. p53 competes with cyclin A to bind the conserved N-terminus cyclin A binding domain of E2F1-3 [174]. Since cyclin A levels are tightly regulated by cell cycle progression, this suggests that E2F regulates p53 activity through a cell-cycle dependent manner. Studies suggest the interaction between p53 and E2F1 results in an increase in apoptosis. Increase of cyclin A levels inhibits binding between p53 and E2F and p53-dependent apoptosis [173, 174].

p53 Mutations

p53 protein is the most frequent mutation target in human cancer. p53 mutation occur in more than 50% of human cancer [107, 137, 175]. Unlike most tumor suppressor genes

that are usually inactivated by gene silencing or deletion, p53 mutations are generally

missense mutation [176]. Missense p53 mutations usually occur in the DNA binding domain

of the p53 protein, resulting in a mutated protein that lacks transcriptional activity [176].

The mutated p53 has an intact tetramerization domain, and can form heterotetramers with

the wild type p53 proteins [177, 178]. In addition, some mutation results in a gain of

function that makes the tumor more prone to metastasis [176, 179-181]. While 80% of p53

mutations in human cancer are p53 missense mutations, only 10% are p53

deletions/insertions [182]. Interestingly, p53 mutations in cancer display a different cancer

profile than tumors with a p53 deletion [176] . Tumors with mutant p53 usually have higher

rate of malignancy. Many experiments have been conducted to explore the possibility of

controlling cancerous growth via p53 regulation.

There are 6 hot-spots for p53 mutation (Arg175, Gly245, Arg248, Arg249, Arg273, and

Arg282) in human cancer, and all these hot-spot mutations are located at the DNA binding

domain [176]. These p53 mutants can be categorized into structural and contact mutants.

R248Q and R273H are "contact" mutations since these two residues are in contact with

DNA in the DNA binding domain [183]. Structural mutants, such as R175H, G245S, R249S,

and R282W, carry amino acid substitutions that change the p53 protein structure and are

referred to as "structural" mutations [183]. These mutations change the ability of p53 to

bind DNA, interact with other proteins and destabilize the core domain [183]. These

mutations alter the folding of the p53 protein [183]. For example, the R175H mutation

disrupts the zinc ion binding site resulting in a loss of DNA binding activity [183].

Often times, the mutant p53 has a dominant negative effect over the remaining wild type allele in cells with one mutant and one wild type p53 allele (p53$^{+/Mut}$) [176, 181, 184]. That is, the mutant p53 proteins oligomerize with wild type p53 proteins and blocks the wild type p53 activity. Over time, the tumors may exhibit loss of heterozygosity (LOH) at the remaining wild type allele. More than 50% of colorectal carcinomas show LOH, most of the cases are deletion of the remaining wild type allele [107, 185]. In addition, some p53 mutants allow cells to gain new abilities, which usually results in enhanced tumorigenesis progression [186]. p53 mutations can change the spectrum of the p53 regulated genes as well as the tumor spectrum [125, 126]. For example, mice with a R172H mutation, which is equivalent to human R175 hot spot mutation, show a different tumor spectrum compared to the p53$^{+/-}$ mice. Tumor spectrum of the heterozygous mutant mice shows increased number of carcinoma and a decrease in lymphomas [127].

p53 mutations are common in various types of human cancer [105, 107, 137, 187]. Mutant p53 can lose the wild type tumor suppressive activity and sometimes gains functions that promote tumor progression [176, 178]. Restoring wild type p53 activity in tumors has cancer therapeutic benefits. Potential strategies include converting the mutant p53 protein into a protein that has wild type p53 activity, decreasing the stability of the mutant p53 protein, blocking the dominant negative effect and inhibiting the interactions of the mutant p53 with other proteins it has hijacked to enhance cancer progression [105, 128, 186, 188]. Restoring wild type p53 function in tumors of genetically engineered p53 null mice results in rapid tumor regression [189-192].   However, in mice with tumors containing

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mutant p53^{R172H}, restoration of wild type p53 results in tumor stasis not regression [193].

These findings suggest the dominant negative mutant p53 attenuates the activity of the

restored wild type p53. Understanding and suppressing the mutant p53 oncogenic functions

is important for developing cancer therapeutic strategies.

Research Hypothesis and Rationale

p53 is the most frequently mutated tumor suppressor gene in human cancers [107, 137, 187]. Most of the mutant p53 proteins function as dominant negative inhibitors that block the tumor suppressing functions of the remaining wild type p53 proteins [181, 184, 194, 195]. Restoring wild type p53 function in the cancer cells may have a therapeutic benefit. Study showed that restoring wild type p53 function in in tumors of genetically engineered mice that are null for p53 results in tumor rapid regression [189, 190]. However, restoring wild type p53 function in tumors with mutant p53^{R172H} results in tumor stasis [193], indicating the dominant negative mutant p53 attenuates the activity of the restored wild type p53.

Our lab has previous shown that C/EBPβ$^{-/-}$ mice treated with DNA damaging agents display increased levels of p53 and apoptosis in their epidermis compared to similarly treated wild type mice [67-69]. These studies indicate that C/EBPβ enhances p53 activity in response to DNA damage. In my research we utilized UVB, an inducer of DNA damage to investigate the role of C/EBPβ in p53-mediated apoptosis in epidermis and to test whether the deletion of C/EBPβ could enhance wild-type p53 apoptotic function and overcome the dominant-negative effects of mutant p53 in p53$^{+/R172H}$ mice.

We hypothesized that deletion of C/EBPβ in cells with p53$^{+/R172H}$ mutation would enhance wild type p53 pro-apoptotic function and overcome the dominant-negative effects of mutant p53 in p53$^{+/R172H}$ mice. Essentially the removal of C/EBPβ would rescue the wild type p53 apoptotic function that is suppressed by the mutant p53.

To test this hypothesis we generated numerous mouse strains including

K5Cre$^{+/tg}$;LSLp53$^{+/R172H}$ SKH1 mice where both p53 wild type and mutant alleles are

expressed under normal physiological control in the epidermis [194]. We demonstrate that

deletion of C/EBPβ enhances wild type p53 apoptotic function to completely overcome the

dominant negative effects of mutant p53 in response to DNA damage in the epidermis of

p53$^{+/R172H}$ mice and the deletion of C/EBPβ in epidermis protects mice from UVB-induced

skin cancer.

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CHAPTER 1

Overcoming the Dominant Negative Effects of Mutant p53 in p53$^{+/R172H}$ Mice

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Abstract

p53 is activated by DNA damage, oncogenic stimuli and hypoxia to regulate senescence, apoptosis and cell cycle arrest which are essential to prevent cancer. p53 is frequently inactivated in cancer by mutations leading to mutant p53 proteins that lack transcriptional activity but retain an intact oligomerization domain. In p53$^{+/mutant}$ cells, the mutant p53 protein oligomerizes with the wild-type protein to block p53 activity. Overcoming this dominant-negative effect of mutant p53 could have therapeutic value. Earlier studies indicated the deletion of C/EBPβ enhances p53 activity in response to DNA damage. Here, we utilized UVB, an inducer of DNA damage, to investigate the role of C/EBPβ in p53-mediated apoptosis in epidermis and to test whether the deletion of C/EBPβ could enhance wild-type p53 apoptotic function and overcome the dominant-negative effects of mutant p53 in p53$^{+/R172H}$ mice. Deletion of C/EBPβ in UVB-treated p53$^{+/+}$ epidermis enhanced wild-type p53 activity and produced >3-fold increase in p53-dependent apoptosis. Deletion of C/EBPβ in UVB-treated p53$^{+/R172H}$ epidermis not only enhanced wild-type p53 activity to overcome the dominant-negative effect of mutant p53 but increased apoptosis 30% over UVB-treated p53$^{+/+}$ mice. Deletion of C/EBPβ in UVB-treated p53$^{+/R172H}$ epidermis differentially altered levels of wild-type and mutant p53 protein reducing the mutant:wild-type p53 ratio 7-fold. We tested whether deleting C/EBPβ protects mice from UVB-induced skin cancer as UVB-induced mutation of p53 is an early initiating event functioning as a dominant-negative inhibitor of wild-type p53. C/EBPβ$^{-/-}$ mice were resistant to UVB-induced

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skin cancer. Targeting C/EBPβ could have cancer therapeutic and cancer preventative

benefits.

Introduction

The p53 tumor suppressor gene is the most frequently inactivated gene in human cancers [1-4]. However, unlike other tumor suppressor genes which are generally inactivated by deletion or gene silencing, p53 is frequently inactivated by missense mutations [5]. Missense mutations in p53 prevent p53 from carrying out its anti-tumor function involving apoptosis, senescence and cell cycle arrest [5, 6]. These single missense point mutations usually occur in the DNA binding domain of p53, which leads to expression of mutant p53 proteins that lack transcriptional activity but retain intact oligomerization and transactivation domains [6, 7]. The mutant proteins can function as dominant negative inhibitors in cells with one mutant and one wild type p53 allele (p53$^{+/M}$). In this case, the mutant p53 protein oligomerizes with wild type p53 protein and blocks wild type p53 activity [8-11]. Thus, the dominant negative property of mutant p53 is a critical contributor to tumor development/progression in p53$^{+/M}$ cells [12].

Restoring wild type p53 activity in tumors has promise as a cancer therapy. For example, restoring wild type p53 function in tumors of genetically engineered p53 null mice results in rapid tumor regression [13-16]. However, the tumor regression effect of restoring wild type p53 function in mice with tumors containing mutant p53^{R172H} is blunted, in this case, restoration results in tumor stasis not regression [17]. These findings suggest the dominant negative mutant p53 attenuates the activity of the restored wild type p53. Due to secondary events in tumors, mutant p53 protein becomes stabilized and accumulates to high levels [18-20]. Similarly, in cells heterozygous for p53 missense mutations, DNA

damage preferentially stabilizes mutant p53 which functions as a dominant negative

inhibitor to inactivate wild type p53 activity [21-23]. Overall, the accumulation of dominant

negative mutant p53 is considered to be a critical event in tumor development/progression

and is necessary for the acquisition of the gain-of-function properties of certain p53

mutants [24, 25]. Squelching the dominant negative effect of mutant p53 in cells

heterozygous for mutant p53 could permit wild type p53 to carry out its anti-tumor

functions.

CCAAT/enhancer binding protein-β (C/EBPβ), a basic leucine zipper transcription

factor, has important roles in fundamental cellular processes including differentiation [26-

31], inflammation [32, 33], survival [34-36] and energy metabolism [37, 38]. C/EBPβ

regulates cell survival (blocks apoptosis) in response to DNA damage, toxicants or oncogenic

stress, and knockdown of C/EBPβ in certain cancer cells results in cell apoptosis [39-49].

C/EBPβ germline knockout mice treated with DNA damaging agents display increased levels

of p53 and apoptosis in their epidermis compared to similarly treated wild type mice [41].

These results suggest C/EBPβ normally functions to suppress p53 activity and its deletion

enhances p53 activity.

UVB radiation is a potent inducer of DNA damage, p53, apoptosis and skin cancer

[50]. Here, we utilize UVB radiation to examine the role of C/EBPβ in regulating p53-

mediated apoptosis and to test whether the deletion of C/EBPβ can enhance wild type p53

apoptotic function and overcome the dominant negative effects of mutant p53 in cells

containing a mutant p53 allele in vivo. To accomplish this we generated numerous mouse

strains including K5Cre$^{+/tg}$;LSLp53$^{+/R172H}$ SKH1 mice where both p53 wild type and mutant

alleles are expressed under normal physiological control in the epidermis [9]. We

demonstrate that deletion of C/EBPβ enhances wild type p53 apoptotic function to

completely overcome the dominant negative effects of mutant p53 in response to DNA

damage in the epidermis of p53$^{+/R172H}$ mice and the deletion of C/EBPβ in epidermis

protects mice from UVB-induced skin cancer.

Materials and Methods

Cells and mice

BALB/MK2 keratinocytes were a gift from B. E. Weissman (UNC) and are cultured in Ca^{2+}-free EMEM (06–174 G, Lonza), 8% Chelax-treated FBS (F2442, Sigma Aldrich), 4 ng/ml hEGF (PHG60311, Life Technologies), and 0.05 mM $CaCl_2$ [51]. A detailed listing of the epidermal specific mouse models can be found in Table 1. K5Cre mice were a gift from Angel Ramirez and Jose Jorcano [52]. p53LSL-R172H mice were purchased from The Jackson Laboratory [9]. All mouse genotypes have been backcrossed to SKH1 hairless female mice (Charles River Labs) at least 5 times [53, 54]. All aspects of animal care and experimentation described in this study were conducted according to the NIH guidelines and were approved by the NC State University Institutional Animal Care and Use Committee.

UVB Treatment and skin tumorigenesis experiment

BALB/MK2 keratinocytes were treated with 10 mJ/cm^2 UVB using UVB lamp model EB 280C (Spectronics) as described in [55]. Mice (7-10 weeks old) were treated with UVB radiation using a UVB lamp (34-0039-001 UVP, Upland, CA) as described [54]. Mice were treated with a single dose of 100 mJ/cm^2. For UVB-tumorigenesis studies, mice were treated with 50 mJ/cm^2 five times weekly for 27 weeks.

Tumor counting and measurement

Tumors with diameter equal or greater than 1mm were counted every week. Final tumor sizes were measured for all counted tumors at the end of study.

Tumor histological analysis

Mice were euthanized by cervical dislocation at two weeks after the last UVB treatment.
Dorsal skin samples were fixed with PAXgene Tissue Fix solution (Cat 765312) for 18 h
followed by storage in PAXgene Stabilizing solution (Cat 765512). The dorsal skin samples
were divided into upper part and lower part, and each cut into 2.5 cm x 0.5 cm x 4 strips
and embedded into paraffin blocks. Using H&E stained 5 μm sections, skin lesion were
identified and scored by veterinary pathologist and scored as; : (1) Papilloma: A discrete
mass greater than 1 mm in depth that displayed hyperplasia with mild dysplasia. (2)
Carcinoma *in situ*: A discrete raised hyperplasia that is greater than 1 mm in depth with
expanded dermis. The dysplasia includes the loss of transepidermal differentiation,
increased in mitotic index, and increased in nuclear to cytoplasm ratio. (3) Microinvasive
squamous cell carcinoma: In addition to the criteria in carcinoma in situ, the lesion has
increase depth in dermis with expansion through basement membrane. (4) Squamous cell
carcinoma: squamous cell carcinoma that touches or penetrates the muscle layer.
Categories (3) and (4) are considered as malignant skin tumor.

Preparation of protein lysates and immunoblot analysis

Mice were euthanized by cervical dislocation and dorsal skin was removed and subjected to
6 sec heat shock in 60°C dH$_2$0 followed by 15 sec in ice water. Epidermal and BALB/MK2
protein lysates were prepared using RIPA buffer as previously described [56]. Equal
amounts of protein were separated by SDS-PAGE and immunblot analysis was conducted
[56] using the following antibodies against C/EBPβ (sc-150, Santa Cruz Biotechnology), p53

(2524s, Cell Signaling Technology) and β-actin (A-5441, Sigma-Aldrich). Protein levels were quantified by densitometry using ImageJ and normalized to β-actin.

Tissue staining and apoptotic cells scoring

Immunohistochemistry (IHC) staining was conducted as described in [26, 56]. Briefly, mouse paraffinized dorsal skin sections (5 μm) were stained for p53 (Cell signaling, 2524s). Hematoxylin and eosin (H&E)-stained mouse skin sections were used to quantify the presence of apoptotic keratinocytes. Apoptotic keratinocytes in the interfollicular basal epidermis were scored positive if all three of the following criteria were present: dark pyknotic nuclei, cytoplasmic eosinophilia and absence of cellular contacts [41]. Data are presented as the average number of apoptotic interfollicular basal epidermal keratinocytes/cm skin. TUNEL staining [39] confirmed the apoptosis results obtained using H&E-stained sections. Data are presented as the average number of apoptotic keratinocytes per centimeter of mouse skin±S.D.

RNA and qRT-PCR

Total RNA was isolated from mouse epidermis and BALB/MK2 cells using QiaZOL lysis (Qiagen), followed by DNase I digestion. cDNA was prepared from RNA by ImProm-II Reverse Transcription System (Promega), and gene expression was determined using the indicated TaqMAN Gene Expression Assays in combination with FastStart Universal Probe Master Mix (Roche). Data were analyzed using the comparative $\Delta\Delta C_T$ method normalized to β-actin.

Small Interfering RNA

siRNA targeting mouse C/EBPβ (5'-GAAAAGAGGCGUAUGUAUAUUdTdT-3') and the negative control (GFP, 5'-GGCUACGUCCAGGAGCGCACCdTdT-3') were synthesized by Sigma-Aldrich and transfected at the final concentration of 75 nM. All transfections were performed using DharmaFECT Reagent1 according to manufacturer's recommendations. Cells were exposed to UVB 48 h post siRNA transfection.

Annexin-V staining

Harvested cells were washed twice with ice-cold PBS and then resuspended in Annexin V Binding Buffer (BioLegend) at a concentration of 1×10^6 cells per ml as previously described [53]. Cells were analyzed by flow cytometry at the NCSU Flow Cytometry and Cell Sorting Laboratory. Data were collected and presented on a scatter plot with Annexin V Pacific Blue intensity on the x-axis and PI intensity on the y-axis.

p53 mutation analysis

DNA was extracted from paraffin embedded incised tumor tissue using PAXgene Tissue DNA Kit (Cat #767134). p53 exons 3-4, 5-6, 7, and 8-9 were individually amplified by PCR as described in [57] followed by Sanger sequencing analysis.

Statistics

Differences between groups were evaluated by two-sided t-tests for paired data with the significance level set to $P<0.05$.

Results

Conditional deletion of C/EBPβ in the epidermis results in increased p53 protein levels and apoptosis in UVB-treated mouse skin.

Previously our laboratory showed that C/EBPβ germline knockout mice treated with DNA damaging agents, including UVB, displayed increased levels of p53 and apoptosis in their epidermis compared to similarly treated wild type mice [39, 41]. To develop a mouse model to further study the role/mechanism of C/EBPβ in regulating p53-mediated apoptosis and to determine if the increase in p53 and apoptosis in response to UVB in systemic C/EBPβ knockout mice is a consequence of a keratinocyte autonomous event(s), we generated conditional knockout K5Cre$^{+/tg}$;C/EBPβ$^{flox/flox}$ (C/EBPβ$^{-/-}$) SKH1 hairless mice. In this model, the keratin 5 (K5) promoter directs expression of Cre recombinase to the basal keratinocytes of the epidermis [52] We used SKH1 hairless mice because these mice are extensively utilized to study UVB-induced biologic changes in skin and are an experimental model relevant to UVB-induced human squamous cell carcinoma (SCC) skin cancer [58]. As shown in Fig 1A, C/EBPβ$^{-/-}$ mice no longer express C/EBPβ in their epidermis and UVB-treated C/EBPβ$^{-/-}$ mice displayed increased p53 protein levels in their epidermis when compared to UVB-treated C/EBPβ$^{+/+}$ mice (Fig 1A). This increase in p53 protein was not associated with an increase in p53 mRNA (data not shown). Next we conducted time course studies to examine the numbers of p53 positive keratinocytes and apoptotic keratinocytes in UVB-treated skin. UVB-treated C/EBPβ$^{-/-}$ mice displayed increased numbers of p53

positive keratinocytes (Fig 1B) and increased numbers of apoptotic keratinocytes (Fig 1C) compared to UVB-treated C/EBPβ$^{+/+}$ mice. Collectively, these data using skin specific C/EBPβ$^{-/-}$ mice demonstrate that the increased apoptosis and p53 protein levels in UVB-treated C/EBPβ$^{-/-}$ mice are specifically due to keratinocyte autonomous events.

To determine the degree to which p53 is responsible for UVB-induced apoptosis we developed conditional knockout K5Cre$^{+/tg}$;p53$^{flox/flox}$ (p53$^{-/-}$) SKH1 mice and double conditional knockout K5Cre$^{+/tg}$;p53$^{flox/flox}$;C/EBPβ$^{flox/flox}$ (p53$^{-/-}$;C/EBPβ$^{-/-}$) SKH1 mice. Comparing UVB-treated C/EBPβ$^{+/+}$ mice to p53$^{-/-}$ mice we observed that ~30% of the UVB-induced apoptosis in the epidermis of C/EBPβ$^{+/+}$ mice was p53-dependent (Fig 1D). Comparing UVB treated C/EBPβ$^{-/-}$ and p53$^{-/-}$;C/EBPβ$^{-/-}$, we found that the entire increase in UVB-induced apoptosis in mice lacking C/EBPβ in their epidermis was p53-dependent (Fig 1D). C/EBPβ$^{-/-}$ mice displayed greater than a 3-fold increase in p53-dependent apoptosis compared to UVB treated C/EBPβ$^{+/+}$ (Fig 1D). Collectively, these results demonstrate that deleting C/EBPβ in epidermis increases p53 protein levels and enhances the pro-apoptotic function of wild type p53 in vivo in mouse epidermis in response to UVB radiation.

Depletion of C/EBPβ increases the stability of the p53 protein in response to UVB.

To begin to investigate how the loss of C/EBPβ enhances UVB-induced p53 levels we utilized BALB/MK2 keratinocytes in culture. Similar to the in vivo data described above, UVB treatment of BALB/MK2 keratinocytes depleted of C/EBPβ resulted in increased p53 protein levels but not p53 mRNA levels compared to UVB treated siRNA control

keratinocytes (Fig 2 A/B). To determine whether the loss of C/EBPβ increases p53 protein

stability we inhibited protein synthesis with cycloheximide and measured the stability of the

p53 protein in keratinocytes depleted of C/EBPβ or not, both before and after DNA damage.

In untreated siRNA-control keratinocytes and untreated C/EBPβ depleted keratinocytes p53

protein stability was similar. In contrast, p53 protein stability was increased in UVB-treated

keratinocytes depleted of C/EBPβ when compared to UVB-treated siRNA-control

keratinocytes (Fig 2C). Similar to the in vivo results in epidermis, BALB/MK2 keratinocytes

depleted of C/EBPβ displayed increased UVB-induced apoptosis (Fig 2D). UVB-treated

keratinocytes depleted of C/EBPβ displayed increased expression of pro-apoptotic genes,

bax, noxa, and mcl-1 and decreased expression of anti-apoptotic gene, bcl2 (Fig 2E). These

results indicate that depletion of C/EBPβ increases both p53 protein stability and the pro-

apoptotic function of p53 in keratinocytes in response to UVB treatment and this increase in

apoptosis is associated with altered expression of pro- and anti-apoptotic genes.

Deletion of C/EBPβ in epidermis enhances wild type p53 apoptotic function to overcome

the dominant negative effect of mutant p53 in UVB-treated p53$^{+/R172H}$ mice.

Over 80% of the p53 mutations found in human cancers are missense mutations that

lead to the expression of mutant p53 proteins that often lack transcriptional activity and

can function as dominant negative inhibitors to block wild type p53 function [59, 60]. Thus,

in cells containing a single p53 allele with a missense mutation, the mutant p53 protein

oligomerizes with and inactivates wild type p53 protein to block its anti-tumor activities,

thereby contributing to cancer progression [61, 62]. To test whether the deletion of C/EBPβ can enhance wild type p53 apoptotic function even in the presence of a mutant p53 allele we developed K5Cre$^{+/tg}$;p53$^{+/LSL-R172H}$ (p53$^{+/M}$) and K5Cre$^{+/tg}$;p53$^{+/LSL-R172H}$;C/EBPβ$^{flox/flox}$ (p53$^{+/M}$;C/EBPβ$^{-/-}$) SKH1 mice. The R172H mutation in mice is equivalent to the R175H mutation in humans which is a "hot spot" p53 mutation in human cancer [9, 21]. In this model, both the wild type and mutant R172H p53 alleles are expressed under the control of their endogenous promoter and are under normal physiological control in the epidermis [9]. We treated p53$^{+/M}$ mice with UVB to test whether the mutant p53 protein functioned as a dominant negative inhibitor of p53-mediated apoptosis in our model system. As shown in Fig 3A, p53$^{+/M}$ mice displayed levels of UVB-induced apoptosis similar to UVB-treated p53$^{-/-}$ mice. Thus, the R172H mutation is a potent dominant-negative inhibitor in vivo in epidermis as it completely blocked wild type p53-mediated apoptosis in response to DNA damage. Next, we treated p53$^{+/M}$;C/EBPβ$^{-/-}$ mice with UVB and observed that the deletion of C/EBPβ in p53$^{+/M}$ mouse epidermis (p53$^{+/M}$;C/EBPβ$^{-/-}$) completely overcame the dominant negative effect of the mutant p53 (Fig3A/B) and enhanced apoptosis to a level that was 30% greater than that observed in UVB-treated mice with two wild type alleles of p53 (p53$^{+/+}$) (Fig 3A). The effect was observed at 6 and 9 h post UVB (Fig 3B). To verify this increase in apoptosis in p53$^{+/M}$;C/EBPβ$^{-/-}$ mice was due to the deletion of C/EBPβ enhancing the function of the wild type p53 and not an effect of converting mutant p53 into a protein that has wild type p53 activity, we generated K5Cre$^{+/tg}$;p53$^{flox/LSL-R172H}$;C/EBPβ$^{flox/flox}$ (p53$^{-/M}$;C/EBPβ$^{-/-}$). As

shown in Fig 3A, deletion of C/EBPβ no longer enhanced UVB-induced apoptosis in p53$^{-/M}$;C/EBPβ$^{-/-}$ mice. Collectively, these data demonstrate the deletion of C/EBPβ enhances wild type p53 apoptotic function to overcome the dominant negative effect of the mutant p53 protein in p53$^{+/M}$ mice in response to DNA damage.

Deletion of C/EBPβ has opposite effects on levels of wild type versus mutant p53 protein in UVB-treated skin.

As shown in Fig 3C, UVB-treated C/EBPβ$^{-/-}$ mice displayed an increase in p53 protein levels in their epidermis when compared to UVB-treated C/EBPβ$^{+/+}$ mice (Fig 3C).   In p53$^{+/M}$ mice, p53 protein levels were significantly increased in both the untreated and UVB-treated mice when compared to similarly treated p53 wild type mice. These results are consistent with reports showing that the mutant p53 protein is more stable, especially in response to DNA damage [18-20]. Next, we treated p53$^{+/M}$;C/EBPβ$^{-/-}$ mice with UVB. Surprisingly, the deletion of C/EBPβ in the UVB-treated p53$^{+/M}$ mice resulted in decreased levels of p53 protein compared to the UVB-treated p53$^{+/M}$ (Fig 3C) suggesting the possibility the deletion of C/EBPβ destabilizes the mutant p53 protein. To test this, we treated p53$^{-/M}$ and p53$^{-/M}$;C/EBPβ$^{-/-}$ mice with UVB. The deletion of C/EBPβ in p53$^{-/M}$ resulted in greatly diminished levels of mutant p53 protein (Fig 3D) demonstrating that the deletion of C/EBPβ destabilizes mutant p53 protein in response to UVB-induced DNA damage. Next, we compared the levels of p53 protein in p53$^{-/+}$ and p53$^{-/M}$ with and without UVB treatment in the presence and absence of C/EBPβ (Fig 3E/F) and then conducted densitometric analysis of the

immunoblots to estimate the ratio of the levels of mutant:wild type p53 protein. As shown

in Fig 3G, in the presence of C/EBPβ the ratio of mutant:wild type p53 protein was 4

mutant:1 wild type and in the absence of C/EBPβ this ratio decreased ~ 7 fold to a

mutant:wild type ratio of only 0.6. We also examined the number of p53 positive

keratinocytes in the epidermis of UVB-treated C/EBPβ$^{+/+}$ and C/EBPβ$^{-/-}$ mice with wild type

p53 or p53$^{-/M}$. UVB-treated C/EBPβ$^{-/-}$ mice with wild type p53 displayed increased numbers

of p53 positive keratinocytes compared to similarly treated C/EBPβ$^{+/+}$ mice (Fig 3H). In

contrast, UVB treated C/EBPβ$^{-/-}$ mice with mutant p53$^{-/M}$ displayed a two-fold decrease in

the numbers of p53 positive keratinocytes compared to similarly treated C/EBPβ$^{+/+}$ mice (Fig

3H). Collectively, our results demonstrate the deletion of C/EBPβ stabilizes the wild type

p53 protein and destabilizes the mutant p53 protein in response to UVB-induced DNA

damage.

C/EBPβ deletion protects mice from UVB-induced skin cancer.

In skin squamous cell carcinoma (SCC) development, UVB-induced mutation of p53 is

an early initiating event where the mutant p53 protein functions as a dominant negative

inhibitor to block subsequent UVB-induced apoptosis leading to clonal expansion and tumor

progression [63]. Accordingly, we tested whether the loss of C/EBPβ could prevent UVB-

induced skin cancer; we hypothesized that the deletion of C/EBPβ would overcome the

dominant negative effect of mutant p53 and result in apoptosis of p53$^{+/M}$ initiated cells and

decreased UVB-induced skin cancer. As shown in Fig 4, C/EBPβ$^{-/-}$ mice were resistant to

UVB-induced skin cancer and C/EBPβ$^{-/-}$ mice displayed increased tumor latency (Fig 4A), striking decreases in tumor multiplicity (Fig 4B) and tumor size (Fig 4C). While tumor multiplicity was significantly reduced in the C/EBPβ$^{-/-}$ mice, the distribution of the various tumor types ranging from papilloma to carcinoma were similar (Fig 4D). This result coupled with a reduced frequency of mutant p53 in UVB-induced carcinomas of C/EBPβ$^{-/-}$ mice (Fig 4E) suggests the deletion of C/EBPβ inhibited the initiation stage of tumorigenesis. Collectively these results suggest that targeting C/EBPβ could have cancer therapeutic and cancer preventative benefits.

Discussion

Mutant p53 proteins accumulate in tumors, function as dominant negative inhibitors to block wild type p53 activity and some mutant p53 proteins display gain-of-function properties which enhance cancer progression [1-3, 5, 6]. Thus, there is significant interest in therapeutic strategies aimed at the mutant p53 protein. Potential strategies include converting the mutant p53 protein into a protein that has wild type p53 activity, decreasing the stability of the mutant p53 protein, blocking the dominant negative effect and inhibiting the interactions of the mutant p53 with other proteins it has hijacked to enhance cancer progression [4, 19, 20, 64]. Our functional in vivo studies demonstrate that the depletion of C/EBPβ restores and enhances wild type p53 pro-apoptotic function even in the presence of a mutant p53 with a "hot spot" mutation to overcome the dominant negative effect of mutant p53 in UVB-treated epidermis in vivo. These results could have potential therapeutic benefit in p53$^{+/mut}$ cancers because; 1) overcoming the dominant negative effect of mutant p53 to restore wild type p53 function in p53$^{+/mut}$ could result in apoptosis and tumor regression and 2) the dominant negative function of mutant p53 is required for the manifestation of mutant p53 protein's neomorphic gain of function activity in tumor development [24, 25].

In most tissues, mutant p53 does not accumulate, however in tumors and in response to DNA damage, mutant p53 protein is preferentially stabilized and accumulates to high levels [19, 65, 66]. Our studies demonstrate the deletion of C/EBPβ in UVB-treated p53$^{+/R172H}$ epidermis not only restored wild type p53 activity to overcome the dominant

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effect of mutant p53 but enhanced apoptosis to a level that was 30% greater than that in

UVB-treated p53$^{+/+}$ mice. Remarkably, the deletion of C/EBPβ stabilized wild type p53

protein and destabilized mutant p53 in response to UVB-induced DNA damage resulting in a

7 fold decrease the mutant:wild type p53 protein in the epidermis of UVB-treated p53$^{+/R172H}$

mice. We speculate that this decreased ratio contributes to; 1) increases in wild type p53

homotetramers with a concomitant decrease in mutant and wild type p53 mixed-tetramers

and 2) restoration of p53's pro-apoptotic activity to overcome the dominant negative effect

of mutant p53.

The differential effect of the depletion of C/EBPβ on the stability of wild type versus

mutant p53 is significant. It is known that the mutant p53 protein is stabilized by its

interaction with Hsp70, Hsp90 and HDAC6 and disruption of the HDAC6 leads to dissociation

HSP70/90 from mutant p53 resulting in the preferential degradation of the mutant p53

protein by CHIP, an E3 ubiquitin ligase and mdm2 [67]. Further studies are required to

understand how the depletion of C/EBPβ regulates the stability of the wild type and mutant

protein and enhances the activity of the wildtype protein.

Early in skin cancer development, UVB radiation produces a mutation in a single p53

allele and patches/clones of mutant p53 keratinocytes (considered to be the precursor

lesion to skin cancer) can be detected in sun-exposed areas of human skin and in UVB-

treated mouse skin long before tumors form [57, 68, 69]. Most of these clones have a single

mutant allele of p53 (97% missense and 3% nonsense) [57, 63]. Thus, we hypothesized that

the deletion of C/EBPβ would overcome the dominant negative effect of mutant p53 and

cause apoptosis of p53$^{+/M}$ initiated cells resulting in decreased UVB-induced skin cancer. We observed that skin specific C/EBPβ$^{-/-}$ SKH1 mice were resistant to UVB-induced skin cancer and displayed a reduced frequency of mutant p53 in UVB-induced carcinomas. These results are consistent with the above hypothesis and suggest that targeting C/EBPβ could have cancer therapeutic and cancer preventative benefits.

Our future studies will be aimed determining whether deleting C/EBPβ can restore the anti-tumor activity of wild type p53 and result in the regression of p53$^{+/R172H}$ tumors. Additionally not all p53 mutations are equivalent and it will be important to determine if depletion of C/EBPβ can overcome the dominant negative activity of other p53 mutations.

Acknowledgements

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Figure Legends

Figure 1.

Deletion of C/EBPβ in UVB-treated mouse epidermal keratinocytes results in elevated p53 protein and p53-dependent apoptosis in response to UVB.

(A) Immunoblot analysis of epidermal protein lysates from untreated and UVB treated mice collected 6 h post 100 mJ/cm^2 UVB. (B) Quantification of p53 IHC positive keratinocytes in untreated and UVB-treated mouse epidermis post 100 mJ/cm^2 UVB. * denotes significantly different from UVB-treated C/EBPβ$^{+/+}$ mice as determined by Student's t-test (P<0.05). (C) Quantification of apoptotic cells in untreated and UVB-treated mouse epidermis post 100 mJ/cm^2 UVB. * denotes significantly different from UVB-treated C/EBPβ$^{+/+}$ mice as determined by Student's t-test (P<0.05). (D) Quantification of apoptotic cells in untreated and UVB treated mouse epidermis collected 6 h post 100 mJ/cm^2 UVB. * denotes significantly different from UVB-treated p53$^{+/+}$; C/EBPβ$^{+/+}$ at each time point mice as determined by Student's t-test (P<0.05). For (B), (C) and (D) N=3 mice/genotype/treatment. Data are expressed as the mean ± S.D..

Figure 2.

Depletion of C/EBPβ in UVB treated BALB/MK2 keratinocytes increases p53 protein stability and apoptotic function.

(A) Immunoblot analysis of BALB/MK2 cells treated with control or C/EBPβ siRNA, exposed to 10 mJ/cm^2 UVB. (B) qRT-PCR analysis of p53 mRNA levels in BALB/MK2 cells treated with

control or C/EBPβ siRNA, exposed to 10 mJ/cm^2 UVB (C) Quantification of p53 stability in untreated and UVB treated control and C/EBPβ siRNA knockdown BALB/MK2 cells. Cells were treated with 25 μg/ml cyclohexamide (CHX). Densitometric analysis of p53 immunoblots was conducted and normalized to β-actin. (D) FACS detection of Annexin-V and PI stained BALB/MK2 cells treated with control or C/EBPβ siRNA and exposed to 10 mJ/cm^2 UVB (E) qRT-PCR analysis of indicated p53 target genes in BALB/MK2 cells treated with control or C/EBPβ siRNA, exposed to 10 mJ/cm^2 UVB and collected 16 h post UVB treatment. For (B) and (E) N=3 and * denotes significantly different from UVB treated control as determined by Student's t-test (P<0.05). Data are expressed as the mean ± S.D.. The data shown in (C) and (D) are representative of at least two independent experiments.

Figure 3.

Genetic deletion of C/EBPβ enhances wild type p53 apoptotic function to overcome the dominant negative effects of mutant p53 following UVB exposure.

(A) Quantification of apoptotic cells in the epidermis of mice treated 100 mJ/cm^2 UVB. * denotes significantly different from UVB-treated p53$^{+/+}$;C/EBPβ$^{+/+}$ mice as determined by Student's t-test (P<0.05). (B) Quantification of apoptotic cells in the epidermis of mice treated 100 mJ/cm^2 UVB 0, 6, 9, and 12 h post UVB treatment. * denotes significantly different from 6 h UVB treated p53$^{+/M}$;C/EBPβ$^{+/+}$ as determined by Student's t-test (P<0.05). (C-F) Immunoblot analysis of epidermal protein lysates from mice treated with 100 mJ/cm^2 UVB. (G) Quantification of mutant to wild type p53 ratio in epidermal protein lysates of
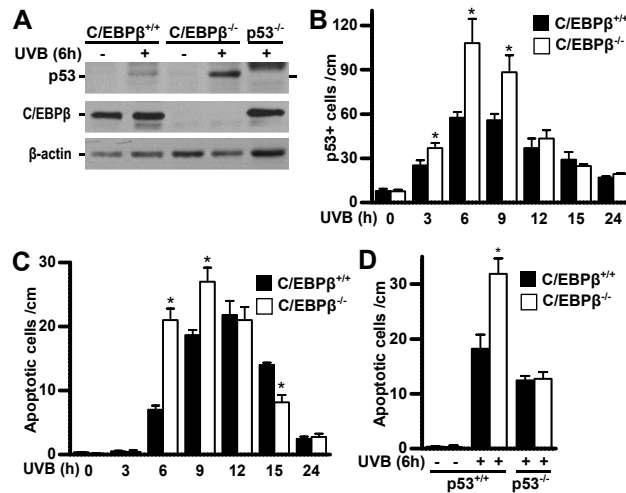
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UVB treated mice by densitometric analysis of immunoblots in 3E and 3F. (H) Quantification

of p53 IHC positive keratinocytes in the epidermis of mice treated 100 mJ/cm^2 UVB and

collected 6 h post-UVB . * denotes significantly different from UVB treated C/EBPβ$^{+/+}$ mice

as determined by Student's t-test (P<0.05). For (A), (B), and (H) N=3 mice/genotype/time

point. Data are expressed as mean ± S. D. The data in (C-F) are representative of at least 3

mice/genotype/treatment.

Figure 4.

Deletion of C/EBPβ protects mice from UVB-induced skin tumorigenesis.

C/EBPβ$^{+/+}$ and C/EBPβ$^{-/-}$ SKH1 mice were treated with 50 mJ/cm^2 UVB five days/week for 27

weeks. (N=18 C/EBPβ$^{+/+}$ and N=15 C/EBPβ$^{-/-}$). (A) Quantification of skin tumor incidence. (B)

Quantification of skin tumor multiplicity. (C) Quantification of final tumor size. Data points

represent average tumor size of each mouse. (D) Classification of tumor types into four

categories: I (papilloma), II (in situ carcinoma), III (microinvasive carcinoma), and IV

(squamous cell carcinoma). (E) Tumors in categories III and IV were sequenced for p53

mutations in exons 3-9.
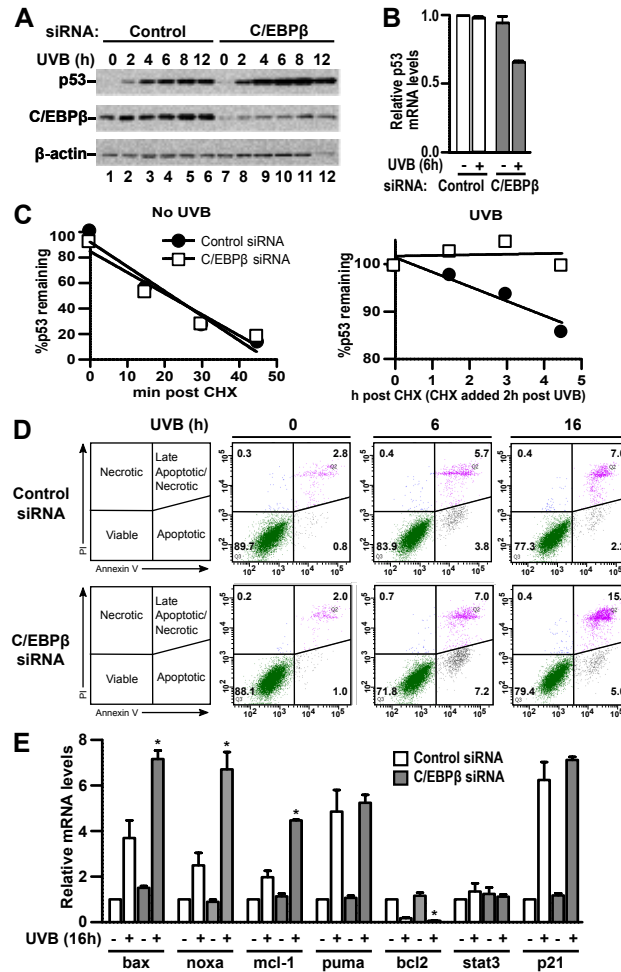
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Deletion of C/EBPβ in UVB-treated mouse epidermal keratinocytes results in elevated p53 protein and p53-dependent apoptosis in response to UVB.

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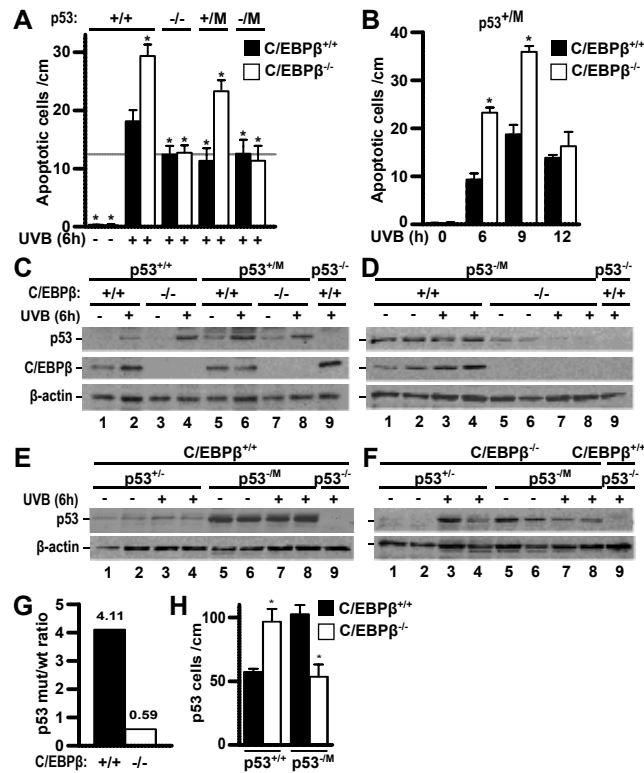
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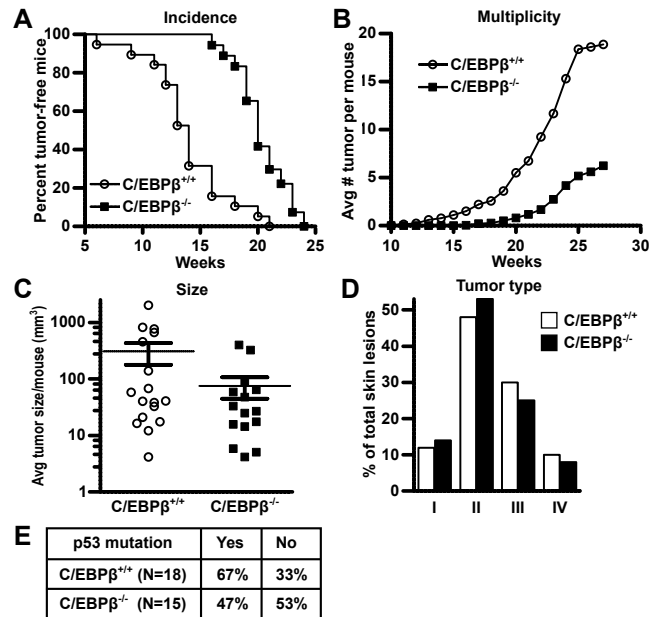
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Genetic deletion of C/EBPβ enhances wild type p53 apoptotic function to overcome the dominant negative effects of mutant p53 following UVB exposure.

(A) Quantification of apoptotic cells in the epidermis of mice treated 100 mJ/cm^2 UVB. * denotes significantly different from UVB-treated p53$^{+/+}$;C/EBPβ$^{+/+}$ mice as determined by Student's t-test (P<0.05). (B) Quantification of apoptotic cells in the epidermis of mice treated 100 mJ/cm^2 UVB 0, 6, 9, and 12 h post UVB treatment. * denotes significantly different from 6 h UVB treated p53$^{+/M}$;C/EBPβ$^{+/+}$ as determined by Student's t-test (P<0.05). (C-F) Immunoblot analysis of epidermal protein lysates from mice treated with 100 mJ/cm2 UVB. (G) Quantification of mutant to wild type p53 ratio in epidermal protein lysates of UVB treated mice by densitometric analysis of immunoblots in 3E and 3F. (H) Quantification of p53 IHC positive keratinocytes in the epidermis of mice treated 100 mJ/cm^2 UVB and collected 6 h post-UVB . * denotes significantly different from UVB treated C/EBPβ$^{+/+}$ mice as determined by Student's t-test (P<0.05). For (A), (B), and (H) N=3 mice/genotype/time point. Data are expressed as mean ± S. D. The data in (C-F) are representative of at least 3 mice/genotype/treatment.

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Deletion of C/EBPβ protects mice from UVB-induced skin tumorigenesis.
C/EBPβ^{+/+} and C/EBPβ^{-/-} SKH1 mice were treated with 50 mJ/cm^2 UVB five days/week for 27 weeks. (N=18 C/EBPβ^{+/+} and N=15 C/EBPβ^{-/-}). (A) Quantification of skin tumor incidence. (B) Quantification of skin tumor multiplicity. (C) Quantification of final tumor size. (D) Classification of tumor types into four categories: I (papilloma), II (in situ carcinoma), III (microinvasive carcinoma), and IV (squamous cell carcinoma). (E) Tumors in categories III and IV were sequenced for p53 mutations in exons 3-9.

Table 1. Mouse genotypes used

SKH1 mouse genotype	nomenclature	ref
K5Cre	C/EBPβ$^{+/+}$ or p53$^{+/+}$	[52]
K5Cre$^{+/tg}$;C/EBPβ$^{flox/flox}$	C/EBPβ$^{-/-}$	[70]
K5Cre$^{+/tg}$;p53$^{flox/flox}$	p53$^{-/-}$	[53, 71]
K5Cre$^{+/tg}$;p53$^{flox/flox}$;C/EBPβ$^{flox/flox}$	p53$^{-/-}$; C/EBPβ$^{-/-}$	
K5Cre$^{+/tg}$;p53$^{+/flox}$	p53$^{+/-}$	
K5Cre$^{+/tg}$;p53$^{+/flox}$;C/EBPβ$^{flox/flox}$	p53$^{+/-}$; C/EBPβ$^{-/-}$	
K5Cre$^{+/tg}$;p53$^{+/LSL-R172H}$	p53$^{+/M}$	[9, 53]
K5Cre$^{+/tg}$;p53$^{+/LSL-R172H}$;C/EBPβ$^{flox/flox}$	p53$^{+/M}$; C/EBPβ$^{-/-}$	
K5Cre$^{+/tg}$;p53$^{flox/LSL-R172H}$	p53$^{-/M}$	
K5Cre$^{+/tg}$;p53$^{flox/LSL-R172H}$;C/EBPβ$^{flox/flox}$	p53$^{-/M}$; C/EBPβ$^{-/-}$	

GENERAL DISCUSSION

C/EBPβ is important in maintaining cell and tissue homeostasis by functioning in processes such as metabolism, inflammatory responses, cellular differentiation, proliferation, and survival (1-13). In the work presented here, we demonstrate for the first time that the deletion of C/EBPβ in heterozygous p53$^{+/R172H}$ mutant cells can restore and enhance the wild type p53 pro-apoptotic functions. Additionally, we show that in the absence of C/EBPβ, the wild type p53 proteins are stabilized while the mutant p53 proteins are destabilized in keratinocytes treated with UVB radiation.

Previous studies in C/EBPβ deficient mice showed that these mice display increased apoptosis and p53 protein levels in response to DNA damage (14, 15). Here we show that upon UVB-induced DNA damage, the elevated apoptosis in C/EBPβ deficient keratinocytes is p53 dependent and that this is due to keratinocyte autonomous events. How C/EBPβ regulates p53 apoptotic functions and protein stability is unknown. In future studies, it will be important to determine how C/EBPβ regulates p53 at the molecular level in response to DNA damage. It has been shown that C/EBPβ physically interacts with p53 and inhibits p53 DNA binding and transcriptional activity (16). This binding of C/EBPβ to p53 may modulate the expression of the p53 regulating genes array. This suggest C/EBPβ fine tuning the expression of p53 regulating genes, and may explain the upregulation of p53-regulated pro-apoptotic genes in the C/EBPβ knockdown cells in our study. The binding between C/EBPβ and p53 may also alter the interaction between p53 and other cellular molecules. The interaction between C/EBPβ and p53 are at the C-terminus of both transcription factors (16).

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Mdm2 target p53 for proteasomal degradation by ubiquitinating p53's C-terminus. C/EBPβ

may involve in the p53–Mdm2 interaction and thus control the p53 stability. Mutant p53

proteins have altered structure and this may change their ability to interact with C/EBPβ.

The alter of C/EBPβ interaction with wild type or mutant p53 may be the mechanism of the

differential regulation on p53 stability in the C/EBPβ deficient keratinocytes.

The p53 PTM is a complex regulation on both p53 transcriptional activities and

protein stability (17). C/EBPβ may indirectly regulate p53 PTM through integration with

molecules/protein that modify p53 PTMs. For example, C/EBPB can binds to and activate

p300, which is involves in p53 acetylation (18). The CBP/p300 can acetylates the six lysine

residues at C-terminus of p53 and activates and enhance the p53 transcriptional activity

(19). The Mdm2-mediated ubiquitination is inhibited by the CBP/p300 mediated acetylation

(20). Acetylation and ubiquitination are mutually exclusive, and there's a competition of

acetylation or ubiquitination on the lysine residues (17). Therefore, the enhancement of

p300 activity inhibits the Mdm2-mediated p53 proteasomal degradation. Acetylation and

ubiquitination are only a small part in the p53 PTMs regulation. Future studies are needed

to investigate how C/EBPβ regulates p53 activities and stabilities through regulating p53

PTMs.

C/EBPβ is required for skin tumorigenesis involving oncogenic Ras (21). In the work

presented here, we have shown a delay in tumor incidence, decreases in tumor volume and

tumor multiplicity in skin specific C/EBPβ deficient mice suggesting C/EBPβ plays a key role

in the survival of pre-neoplastic lesions and that this role is an epidermal keratinocyte

autonomous event. One possible mechanism of how C/EBPβ regulates tumor cell survival could be that C/EBPβ represses p53-mediated apoptosis in the initiated cancer cells with a single copy of mutant p53. In the early stage of cancer development, UVB radiation produces a mutation in a single p53 allele (22-24). Patches/clones of mutant p53 containing keratinocytes can be detected in sun-exposed areas of human skin and in UVB-treated mouse skin long before tumors form (22-24). Most of these clones have a single mutant allele of p53 (97% missense and 3% nonsense) (24, 25). We hypothesized that the deletion of C/EBPβ would overcome the dominant negative effect of mutant p53 and cause apoptosis in p53$^{+/mutant}$ initiated cells. Understanding the molecular events of how C/EBPβ promotes cell survival in tumor may provide opportunities to develop cancer therapies. It is also important to investigate whether C/EBPβ plays a role in tumor progression and maintenance. We could utilize inducible C/EBPβ knockout mice to ablate C/EBPβ following the tumorigenesis to investigate the growth of tumor mass in the presence or absence of C/EBPβ.

p53 is highly mutated in human cancers (26-29) and results in loss of wild type p53 tumor suppressing functions of inducing apoptosis, senescence, and cell cycle arrest (28, 30). Most mutant p53 proteins function as dominant negative inhibitors that block the wild type p53 protein functions (31-34). Mutant p53 proteins frequently show gain of functions that enhance cancer progression (35, 36). Thus, there is significant interest in therapeutic strategies aimed at the mutant p53 protein. Restoring or reactivating wild type p53 function in the p53$^{+/mutant}$ cells could have significant therapeutic benefit. Potential strategies are to

destabilize the mutant p53 proteins, inactivate mutant p53 function, or reactivate wild type

p53 protein function (17, 37-39). Here we demonstrate the deletion of C/EBPβ restoring

the wild type p53 pro-apoptotic function and overcomes the dominant negative effect in

p53$^{+/mutant}$ keratinocytes with the hot spot mutation R172H. Remarkably, the deletion of

C/EBPβ stabilized wild type p53 protein and destabilized mutant p53 in response to UVB-

induced DNA damage resulting in a 7 fold decrease in the mutant:wild type p53 protein in

the epidermis of UVB-treated p53$^{+/R172H}$ mice. These results could set the stage for further

research which could uncover therapeutic benefits forp53$^{+/mutant}$ cancers. However, not all

p53 mutants are the same. Future studies using different p53 mutants are needed. This

would reveal whether the depletion of C/EBPβ is able to overcome the dominant negative

activity of other p53 mutations.

The differential regulation of C/EBPβ on wild type and mutant p53 stability is

significant. Mutant p53 does not accumulate in most tissues under normal physiological

conditions (17, 40, 41). However, in tumors and in response to DNA damage, mutant p53

protein is preferentially stabilized and accumulates to high levels (17, 40, 41). It is known

that the mutant p53 protein is stabilized by its interaction with Hsp70, Hsp90 and HDAC6

and disruption of the HDAC6 leads to dissociation HSP70/90 from mutant p53 resulting in

the preferential degradation of the mutant p53 protein by CHIP, an E3 ubiquitin ligase and

mdm2 (42). Future studies are needed to investigate the possible molecular mechanisms of

how C/EBPβ regulates p53 stability. It is possible that C/EBPβ is involved in Mdm2-mediated

p53 proteasomal degradation, or that C/EBPβ interacts with other heat shock proteins that

associate with p53 stability.

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