ABSTRACT

RANJAN, RAKESH. Role and Regulation of C/EBPα in Response to DNA Damage. (Under the direction of Dr. Robert C. Smart.)

C/EBPα and C/EBPβ are members of basic leucine zipper (bZIP) class of transcription factors. They are abundantly expressed in epidermis. C/EBPα expression is diminished in mouse and human squamous cell as well as basal cell carcinomas. Recently, C/EBPα has been shown to be an epithelial tumor suppressor gene in genetically engineered mouse model. siRNA experiment has suggested a role for C/EBPα in DNA damage G_1 checkpoint response. But definitive genetic evidence is lacking. Here, we report that C/EBPα is highly inducible in primary dermal fibroblasts by DNA damaging agents that induce strand breaks, alkylate and crosslink DNA as well as those that produce bulky DNA lesions. Fibroblasts deficient in C/EBPα (C/EBPα$^{-/-}$) display an impaired G_1 checkpoint as evidenced by inappropriate entry into S-phase in response to DNA damage and these cells also display an enhanced G_1 to S transition in response to mitogens. The induction of C/EBPα by DNA damage in fibroblasts does not require p53. EMSA analysis of nuclear extracts prepared from UVB- and MNNG-treated fibroblasts revealed increased binding of C/EBPβ to a C/EBP consensus sequence and ChIP analysis revealed increased C/EBPβ binding to the C/EBPα promoter. To determine whether C/EBPβ has a role in the regulation of C/EBPα we treated C/EBPβ$^{-/-}$ fibroblasts with UVB or MNNG. We observed C/EBPα induction was impaired in both UVB- and MNNG- treated C/EBPβ$^{-/-}$ fibroblasts. Our study reveals a novel role for C/EBPβ in the regulation of C/EBPα in response to DNA damage and provides definitive genetic evidence that C/EBPα has a critical role in the DNA damage G_1 checkpoint.

Since, the evidence for C/EBPα as a tumor suppressor gene in human skin is mounting; we decided to further study the signaling pathway of C/EBPα induction in response to DNA damage in keratinocytes. C/EBPα has been shown to be induced by DNA damage in human and mouse skin, and in primary and immortalized keratinocyte cell lines. In keratinocytes, the induction of C/EBPα requires p53; p53 directly binds to C/EBPα promoter and is responsible for increases in C/EBPα mRNA expression in response to DNA damage. We show that GSK3β inhibitors block C/EBPα protein and message induction in response to DNA damage without altering p53 protein levels. Further, we found that GSK3β interaction with p53 increased in response to DNA damage. In addition, UVB treatment of keratinocytes resulted in post-translation modification of C/EBPα protein. Our results suggest that GSK3β regulates C/EBPα expression, interacts with p53, and that C/EBPα protein undergoes post-translational modification in response to DNA damage.

Hence, from these two studies we have provided evidence that

i) C/EBPα has a role in DNA damage G_1 checkpoint response and in mitogen induced G_1/S transition.

ii) C/EBPα is induced in response to various DNA damage in different cell types and regulation of C/EBPα is cell type specific.

iii) GSK3β might have a role in C/EBPα induction in response to DNA damage in keratinocytes.

Role and Regulation of C/EBPα in Response to DNA Damage

by
Rakesh Ranjan

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APPROVED BY:

_____	_____
Robert C. Smart Chair of Advisory Committee	Marcelo L. Rodriguez-Puebla
_____	_____
Yoshiaki Tsuji	Jun Ninomiya-Tsuji

DEDICATION

I dedicate this dissertation to

My parents Sri Laldeo Singh and Smt. Kanti Devi, for their unconditional love, encouragement, support and sacrifice;

My wife Pratibha Verma, for her patience, understanding and bottomless love;

All my gurus who instilled their knowledge, to help me to complete the journey to PhD.

Thanks all for your valuable time, support and encouragement.

BIOGRAPHY

Rakesh Ranjan was born on 2nd March 1978 in a small city, Bokaro Steel City, in Jharkhand state of India. He completed his primary and high school education at Bokaro Steel City. He matriculated in June 1995 from high school. He was awarded Junior Research Fellowship by ICAR for his under-graduate degree in Veterinary Sciences. He received a Bachelor of Veterinary Science & Animal Husbandry degree in November 2002 from the A.N.G.R. Agricultural University, Hyderabad, AP, India. His interest in Pharmacology and Toxicology during his Veterinary Science degree became launching pad for his research career. In 2003, he was awarded Graduate teaching assistantship in College of Veterinary Science, at Oklahoma State University. After one year of study at Oklahoma State University, he moved to NC State University, Raleigh, where he was awarded Graduate Research Assistantship in the Department of Environmental and Molecular Toxicology to pursue Doctoral degree. He started his dissertation work in Molecular Toxicology under the direction of Dr. Robert C. Smart. His current research includes, understanding of the roles and regulation of two transcriptional factors CCAAT/ Enhancer binding proteins alpha and beta (C/EBPα and C/EBPβ) in response to the DNA damage. He has published one manuscript in peer reviewed journal, Oncogene and has presented his work at several national and local scientific meetings and conferences. He actively participated in Toxicology Graduate Student Association as secretary. Rakesh has accepted a scientist II position in the regulatory toxicology group at Bayer CropScience in RTP, NC.

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GENERAL INTRODUCTION

Determining how cells respond to external environmental and endogenous stimuli is a subject of great interest as well as challenging one. Although the scientific community has made remarkable progress in understanding the signaling pathways through which cells respond to environmental stress, growth factors or other stimuli, still there are a myriad of questions unanswered. Signaling pathways that determine cellular responses to external or internal stimuli are very complex and proper responses to stimuli are necessary for cell survival and growth. Perturbation in these cellular responses due to endogenous or exogenous stimuli can lead to cell death, abnormal proliferation, proliferation arrest, apoptosis and senescence. These altered pathways may lead to alterations in cellular homeostasis which may give rise to different disease processes. Better understanding of these cellular signaling pathways can provide us with innovative ideas to treat various diseases. Cells respond to various stimuli by regulating gene expression, and gene expression, at least partly, is regulated by numerous site specific transcription factors. Transcription factors play pivotal roles in numerous cell signaling pathways, and regulation of these transcription factors must be tightly controlled for maintenance of cellular homeostasis. Transcription factors are proteins that bind to specific DNA sequences in the genome and either induce or suppress the expression of particular genes. Regulation of gene expression by transcription factors can occur at many levels and varies depending on the type of stimuli and cells. Therefore, understanding these processes involved in gene regulation in response to various stimuli in different cell types is of great importance.

DNA damage is one of the most common forms of environmental stress to the cell, and here I will discuss regulation and role of two transcription factors: CCAAT/Enhancer Binding Proteins α and β (C/EBPα and C/EBPβ) in response to DNA damage.

1. DNA Damage Response and Cell Cycle Regulation

DNA is the basic unit of the genome. Hence maintaining the integrity of the genome depends on the maintenance of the DNA sequence in an organism. Maintenance of genomic integrity is a very complex process and cells are constantly challenged by endogenous and exogenous agents. Normal cellular proliferation involves numerous regulatory processes, both positive and negative, which require tight coordination to preserve genomic integrity [1]. Proteins involved in various processes such as DNA replication, DNA repair, and cell cycle progression are components of complex pathways that work synergistically to maintain cellular homeostasis [2]. Alteration in proliferative pathways and deregulation of genes involved in these pathways may lead to abnormal cellular proliferation and cancer. One of the primary reasons for the alteration in these pathways is DNA damage induced by intrinsic and extrinsic genotoxic insults [3, 4]. The ability of cells to respond to intrinsic and extrinsic DNA damage is essential to ensure the integrity of the genome [5, 6].

Unchecked, DNA damage may lead to heritable mutations, genomic instability and ultimately cancer [5, 7]. Depending upon the type and amount of DNA damage as well as the cells involved, a cell can respond by inducing cell cycle checkpoints, DNA repair, apoptosis and senescence [8, 9]. Activation of cell cycle checkpoints in G_1, S or G_2 phases of the cell cycle prevents the replication of damaged DNA and allows time for DNA repair [10]. Defects in DNA damage checkpoints and DNA repair pathways may lead to genomic

instability and cancer [2]. Depending on the nature of the cellular insult DNA damage can be initiated either through endogenous or exogenous sources. Byproducts of the normal cellular processes such as endogenous oxidants, lipid peroxidation products, alkylating agents, glycoxidation products, reactive nitrogen species, and chlorinating agents can lead to endogenous DNA damage [11, 12]. Apurinic/apyrimidinic (AP) sites are among the most common DNA lesions [13, 14]. AP sites are formed by spontaneous hydrolysis, by enzymatic removal of altered bases by specific glycosylases, or by the action of chemical or physical agents [15].

Exogenous sources of DNA damage include alkylating agents such as N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), methylmethane sulfonate (MMS), nitrosamines such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and nitrosourea, a DNA methylating agent. Polycyclic aromatic hydrocarbons such as DMBA, benzo[a]pyrene and UVB light induce bulky adducts to DNA. Nitrogen mustards, cisplatin, ultraviolet (UV) radiation, infrared radiation (IR), and X-ray induce various kinds of DNA damage such as DNA-crosslinks, single and double stranded DNA breaks and formation of bulky adducts [16-19].

The survival of the organisms depends on well regulated and accurate transmission of genetic information from one generation to the next and requires cells to overcome spontaneous and environmentally induced DNA damage. In response to DNA damage, cells initiate a cascade of signaling processes involving several groups of proteins. These proteins have been categorized in four specific groups; sensors, mediators, transducers and effectors as shown in figure 1 [20].

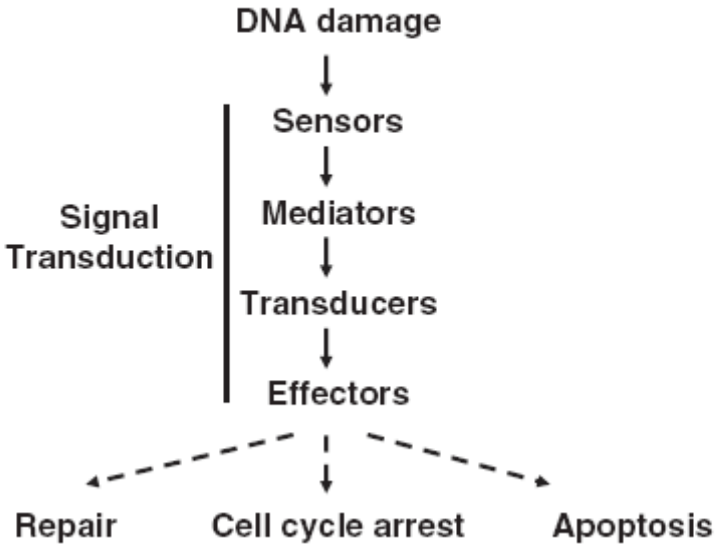

Figure 1. Components of DNA damage signaling pathway[20]

i) DNA Damage Sensors

Recognition of DNA damage by cells is the first step in response to DNA-damage induced cell cycle checkpoints. Proteins involved in the recognition of DNA damage are called sensors. Sensor proteins may directly attach to damaged DNA or may indirectly associate with other sensors already attached to damaged DNA [21]. Two groups of proteins are involved in sensing DNA damage; 1) Two members of PI3K (phosphatidyl-inositol-3-OH kinase)-like kinases (PIKKs); ATM and ATR [22] and 2) the RFC/PCNA (clamploader/polymerase clamp)-related Rad17-RFC and 9-1-1 complex [23]. In addition to ATM and ATR, mTOR and DNA-PK are also members of PI3K-like protein kinase (PIKK) family. PIKKs are on the top of the pathway and sense DNA damage. The sensor protein ATM, is exclusively activated in response to DSBs, while ATR is activated by a much

Table I. Classification of genes involved in DNA damage checkpoints	
Function	**Gene**
Sensors	**ATM**
	ATR?
	RAD17
	RFC2-5
	9-1-1 Complex
Mediators	**scRAD9,**
	Mrc1
	53BP1
	TopBP1
	H2AX, BRC1, M/R/N Complex, SMC1
Transducers	**Chk1**
	Chk2
	p53BP1
	BRCA1
Effectors	**CDC25A**
	CDC25B
	CDC25C
	p53
	p21

broader range of stimuli. [24]. In unstimulated stage ATM exist as a homodimer [25]. Upon activation by IR and double stranded DNA breaks, ATM subunits autophosphorylate and dissociate as active monomer and are ready to phosphorylate downstream substrates [25, 26]. ATM autophosphorylation occurs within minutes of IR radiation. Chromatin modification also leads to autophosphorylation of ATM [25]. Two important target of ATM are Chk2 and p53 [25]. ATM phosphorylates Chk2 only when Chk2 is in close proximity to damaged DNA [27].

At present it is not clear whether ATR acts as a DNA damage sensor protein or not. ATR binding affinity to DNA is increased in response to UV treatment [28]. Damaged DNA increases kinase activity of ATR compared to normal DNA suggesting role of ATR as a DNA damage sensor [28]. In response to DNA damage ATR and ATR-interacting protein (ATRIP) are recruited to the site of DNA damage [29]. ATRIP is one of the immediate substrate of ATR and helps in binding of ATR to damaged DNA site [29]. Rad1–Rad9–Hus1 complex recruited by Rad17 in response to DNA damage enables ATR to phosphorylate its substrates on chromatin such as Chk1 [30]. ATR can bind to damaged DNA in absence of other sensory proteins such as Rad17 suggesting potential role of ATR as a sensor protein [30].

ii) Mediators

DNA damage response mediators are the proteins which provide specificity to signal transduction from sensor to signal transducer by forming a complex with them [6]. For example, scRad9 protein functions along the signal transduction pathway from scMEC1 (ATR) to scRad53 (Chk1). scRad9 is considered a prototype mediator [31, 32]. Mrc1 is

another example of mediator protein. Examples of mediators in human are p53 binding protein: 53BP1, the topoisomerase binding protein (TopBP1), and the mediator of DNA damage checkpoint 1 (MDC1). Other proteins such as H2AX, BRC1, the M/R/N complex, and SMC1 (structural maintenance of chromatin1) also fall into category of mediators as they play essential roles in the activation of checkpoint kinases and function downstream of sensors [6].

iii) Transducers

Transducers include the protein kinases that are activated by mediators in the presence of DNA damage and initiate a signal transduction cascade that propagates and amplifies the damage signal to the effectors to induce cell cycle arrest [21]. Chk1 and Chk2 kinases are two classic transducers in cell cycle regulation and checkpoint response [23, 33, 34]. When cells experience double stranded breaks, it is sensed by ATM and then transduced by Chk2 [35, 36]. On the other hand ATR senses DNA damage induced by UVB and Chk1acts as a transducer downstream of ATR [24, 37]. In addition to Chk1 and Chk2, there are other ATM and ATR substrates such as BRCA1 and 53BP that contain a consensus motif for ATM and ATR phosphorylation [38] and can act as a transducer protein.

iv) Effectors

Effectors are the targets of transducer kinases and these proteins are directly involved in cell cycle transition and execute the signal started by DNA damage sensors. Cdc25A, Cdc25B and Cdc25C are three main effectors proteins in human which are phosphorylated by checkpoint kinases [39]. In response to UV- and hydroxyurea, Chk1 is phosphorylated by ATR and becomes activated. Chk1 phosphorylates Cdc25C which leads to translocation of

Cdc25C into cytoplasm from the nucleus [37]. Chk2 is the major kinase downstream of DNA damage which is activated by ATM in response to IR [36]. Activated Chk2 phosphorylates a number of downstream effectors such as Cdc25A, Cdc25C and p53 [39, 40]. Once these effectors are activated they induce G_1, S and G_2/M cell cycle checkpoints based on the nature of the DNA damage and the signaling pathways involved.

2. Restriction Point and Cell Cycle Checkpoints

The cell cycle can be defined as the sequence of events that occurs inside a cell between cell divisions. The cell cycle of most eukaryotic cells is divided into four phases: G_1 phase, S phase, G_2 phase and mitotic or M phase. One additional phase of cell cycle is G_0, which is exit from the active cell cycle where cells either reach a quiescent state or senescence. Interphase is a collective term that describes the G_1, S and G_2 phases of the cell cycle. G_1, S and G_2 phases are preparatory phases where cells prepare themselves for mitosis by growing in size and replicating their DNA. The M phase consists of two parts; mitosis where cellular chromosome is divided into two identical halves and cytokinesis where cellular contents of the cell are split into two daughter cells [41, 42]. The length of different phases of the cell cycle varies depending on cell type and growth conditions, but in general, a typical eukaryotic cell G_1 phase is 12 h long, S phase is 6 to 8 h long, G_2 phase is 3 to 6 h and M phase is 30 min long [43]. Two important groups of proteins, cyclins and cyclin dependent kinases(CdKs), regulate molecular events in cell cycle progression [43].

i) Restriction point (R)

The G_1 phase of cell cycle is very important since major regulatory events related to cellular proliferation occur during G_1. Cellular proliferation in cell culture is regulated by a

combination of cell density, growth factors and attachment to substrate [44]. Serum/growth factors/mitogen stimuli are essential for progression of cells through G_1 phase leading to S-phase. The term restriction point (R) was first introduced by Pardee, who defined the restriction point as the point in G_1 phase of cell cycle after which cells can proliferate independent of the mitogenic stimuli [45]. In 1985, position of the restriction point in G_1 phase and its relation to entry into S phase was determined by Zetterberg and Larsson [46, 47]. The retinoblastoma protein (Rb) regulates cellular progression through G_1 phase, and Rb protein is considered to guard the R point [48]. Cell progression through the R point depends on phosphorylation of Rb. Cells can progress until the R point and stop there if Rb is hypophosphorylated. Cells cross the R point and enter into S phase of the cell cycle only if Rb is hyperphosphorylated [48]. Normal cells require mitogen stimulus to pass the R point, but tumor cells do not require serum to progress through the R point and can enter the S phase of cell cycle independent of any mitogen stimuli [49].

ii) **Cell Cycle Checkpoints**

A DNA damage checkpoint can be defined as a temporary pause induced by biochemical pathways that delay or arrest cell cycle progression in response to DNA damage [21]. There are three important checkpoints that cells engage in response to DNA damage, G_1 checkpoint, S-phase checkpoint and G_2 checkpoint.

- **G_1 Checkpoint**

The G_1 checkpoint prevents cells with DNA damage from entering into S-phase and provides time for DNA damage repair. G_1 checkpoint in eukaryotes has two phases [50, 51]. First is an immediate response that occurs directly after DNA damage. This phase is p53

independent and initiates the G_1 checkpoint. The initiation phase includes Chk2 activation through ATM or Chk1 activation by ATR in response to DNA damage. Activated Chk1/Chk2 phosphorylates Cdc25A and leads to ubiquitination and proteosomal degradation of Cdc25A [51]. Degradation of Cdc25A leads to maintenance of inhibitory T15 phosphorylation on Cdk2 and inhibiting its association with cyclin E. Cdc25A degradation also leads to maintenance of inhibitory T17 phosphorylation on Cdk4. As a result progression of cells into S phase of cell cycle is inhibited [52, 53]. The second phase of G_1 checkpoint is the maintenance phase which was initiated by phosphorylation of Cdc25A and this phase involves p53. The maintenance phase of the G_1 checkpoint involves ATM/ATR and Chk1/Chk2 but takes longer to initiate as it is regulated at transcriptional level [50]. Activated ATM/ATR and Chk1/Chk2 pathway phosphorylates p53 at S15 and S20 respectively and stabilizes p53 [54]. Stabilization of p53 leads to p53 accumulation and subsequent increased transcription of target genes such as CDK inhibitor (p21) and leads to inhibition of S-phase promoting Cdk2-Cyclin E complex and maintains G_1/S arrest [55, 56]. p21 also prevents Rb phosphorylation by inhibiting Cdk4-CyclinD complex [57]. The G_1 checkpoint maintenance phase provides sufficient time for the repair of the damaged DNA.

- **S-phase checkpoint**

S-Phase checkpoint is initiated when cells experience any DNA damage during S-phase or by damage that cells were not able to repair during G_1 phase of the cell cycle [58]. S-phase checkpoint is initiated by DNA damaging agents by inhibition of new replication initiation and slowing down DNA replication. When cells from cancer-prone individuals affected with ataxia telangiectasia (AT) or Nijmegen breakage syndrome (NBS) were treated with IR, they

fail to initiate S-phase checkpoint following IR exposure [59]. The inability of cells to inhibit DNA synthesis in response to ionizing radiation is known as radio-resistant DNA synthesis (RDS). This finding provided the evidence for the role of ATM and NBS1 in S-phase checkpoint. There are at least two ATM mediated pathways through which IR induces S-phase checkpoint in the cells [60-62]. The first well characterized pathway is the ATM/ATR-Chk1/Chk2-Cdc25A-cyclin E(A)/Cdk2 pathway [51, 52, 60, 63]. Chk2 is activated by ATM in response to DNA damage. Activated Chk2 phosphorylates Cdc25A phosphatase on S123 and targets it for proteosomal degradation. As a result Cdc25A is not able to remove inhibitory phosphorylations (T14 and Y15) from Cdk2, which results in inactive Cdk2/Cyclin E and Cdk2/Cyclin A complexes preventing completion of DNA synthesis. The other pathway leading to S-phage checkpoint in response to DNA damage is ATM-mediated phosphorylation of Nbs1 and SMC1 protein. Nbs1 is phosphorylated by ATM on several sites including S343 [64]. IR induced DNA damage results in Nbs1 recruitment to MRN complex to the sites of DNA damage [27, 65, 66]. The ATM substrate, structure maintenance of chromosome 1 (SMC1) protein, is also involved in the ATM-Nbs1 dependent S-phase checkpoint. ATM phosphorylates SMC1 on S957 and S966 in response to IR treatment dependent on Nbs1 and required for S-phase checkpoint [61, 62]. Any alteration in phosphorylation of either Nbs1 or SMC1 leads to altered S-phase checkpoint [67]. Treatment of NRK cells and mouse embryonic fibroblasts (MEFs) with DNA damaging agents (MMS and cisplatin) induces S-phase cell cycle arrest by inactivation of Cdk2 by increasing its binding to p21. S-phase arrest was short in MMS treated $p21^{-/-}$ MEFs when compared to wild type MEFs suggesting role of p21 in S-phase checkpoint [68]. p21 is

a critical player in Bid induced S-phase checkpoint in response to DNA damage in hepatocellular carcinoma cells (HCC)[69]. The complete S-phase checkpoint also involves suppression of late-firing of origins and slowing down of replication fork progression. MEC1 mediated signaling is necessary for suppression of late-firing of origin as well as for recovery of stalled replication forks in budding yeast [70-72]. Studies have suggested role for FANCD2 [73], BRCA1 [74] and 53BP1 [75] in S-phase checkpoint as cells devoid of these genes exhibit defective S-phase checkpoint. Recently, two other targets for ATM/ATR and Chk1, Cdc7/Dbf4 [76] and T1K1 [77, 78] S-phase kinases have been reported but the exact molecular mechanism still remains elusive.

- **G_2 Checkpoint**

The G_2 checkpoint is initiated if cells experience DNA damage in G_2 phase of the cell cycle or if cells enter into G_2 phase with unrepaired DNA damage. G_2 checkpoint prevents cells from entering into M phase of cell cycle with damaged DNA. Ionization radiation and other genotoxic agents, induce G_2 checkpoint and inhibit CyclinB/Cdk1 kinase activity by phosphorylating Cdk1 on T14 and T15 [79-82]. Cdk1 is dephosphorylated by Cdc25C phosphatase and activates Cdk1 in G_2 phase, which leads to entry of cells into M phase of the cell cycle [21, 24]. In response to DNA damage the ATM/ATR- and Chk1/Chk2 mediated pathway inhibits Cdc25C by phosphorylating Cdc25C on S216 [83, 84]. Phosphorylated Cdc25C binds to 14-3-3 proteins which results in transcriptionaly a less active form and can be sequestered into cytoplasm [85, 86].

Upstream kinases which regulate Cdc25C activity such as the Polo-like kinases, Plk3 and Plk1, are also involved in G_2 checkpoint. PlK3 is activated by ATM and phosphorylates

Cdc25C at S216 and inhibits its activity [87, 88]. Other Polo-like kinase such as Plk1 activates Cdc25C by phosphorylation and thus promotes entry of cells into M phase. In response to DNA damage, ATM/ATR phosphorylate Plk1 and inhibits its ability to phosphorylate and activate Cdc25C [87, 89]. In response to DNA damage Cdc25A is completely degraded in G_2 phase of the cell cycle and has been shown to be involved in G_2 arrest through alteration in protein stability by phosphorylation [39, 90, 91]. Cdc25B is phosphorylated by p38 in response to UV. As a result Cdc25B's binding to 14-3-3 is increased and results in limited access to the substrate cyclins/cdks [92, 93]. There are studies suggesting the role of other checkpoint mediators such as 53BP1 and BRCA1 in maintaining G_2 checkpoint response [74, 75, 94, 95].

Delayed or sustained G_2 arrest is mediated through p53 regulated Cdk inhibitor p21. Other p53 regulated genes including GADD45 and 14-3-3σ, have been shown to be required for G_2 arrest [96]. Cdk1 activity is inhibited by p21 in response to IR treatment and induces G_2 arrest [97]. p21 may also inhibit Cdk1 activity by inhibiting T161 phosphorylation of Cdk1 by Cdk-activating kinase (CAK) [87, 98]. GADD45 is required for G_2 arrest in response to UV and methyl methanesulfonate (MMS) treatment in lymphocytes and GADD45 deficient lymphocytes had a diminished G_2/M checkpoint [99]. GADD45 interacts with CdkI and inhibits binding of Cdk1 with CyclinB and inhibits Cdk1 kinase activity [100].

3. C/EBP Family of Transcription Factors

CCAAT/Enhancer Binding Proteins (C/EBPs) are the members of basic leucine zipper (bZIP) class of transcription factors. The first family member was identified in the laboratory of Steve McKnight as a heat-stable factor in rat liver nuclei [101, 102]. They

found the protein was capable of interacting with the CAAT box motif present in several gene promoters as well as with core homology sequence found in certain viral enhancers [101, 102]. In 1988, the C/EBPα gene was cloned and studies on the C/EBPα sequence led to the discovery of the basic leucine zipper (bZIP) class of DNA binding and dimerization domain [103-106] as well as basic leucine zipper class of transcription factor. Other members of bZIP family of transcription factors include Jun/Fos, CREB/ATF and PAR-domain proteins [107]. CCAAT/enhancer binding proteins consists of a family of six proteins, and have been named C/EBP followed by Greek letter based on their chronological order of discovery [108], namely: C/EBPα (C/EBP) [103, 108-110], C/EBPβ (NF-IL6,IL-6DBP, LAP, CRP2, NF-M, AGP/EBP) [108, 109, 111-113], C/EBPγ (Ig/EBP-1) [113],C/EBPδ (NF-IL6β, CRP3) [108, 109], C/EBPε (CRP-1) [109], and C/EBPζ (CHOP10,GADD153) [114, 115].

i) Structure

C/EBPα [108], C/EBPβ [112], C/EBPδ [108]and C/EBPγ [115] genes are intronless. C/EBPε [116] and C/EBPζ [117] contain two and four exons, respectively. Cloning of C/EBPα revealed a basic leucine zipper (bZIP) domain involved in DNA binding and dimerization [103-106]. As shown in figure 2 bZIP domain is conserved among all C/EBP family members, and is located at the C-terminus [108, 109, 111-113, 115, 118, 119]. At the C-terminus all C/EBP isoforms with the exception of C/EBPζ, which lacks a canonical basic region, [115] share greater than 90% sequence homology [103-106, 108, 109, 111-113, 115, 118, 119]. The C-terminal domain is composed of a basic amino acid rich DNA binding region followed by a dimerization motif referred to as the ‘leucine zipper’ [103-106, 108,

109, 111-113, 115, 118, 119]. The basic region of the transcription factor preferably binds to a dyad symmetrical repeat RTTGCGYAAY, where R is A or G, and Y is C or T [120]. C/EBPs can form homodimers or heterodimers with other members of C/EBP family [109, 113]. Dimerization of C/EBPs is essential for binding to DNA, which is mediated through basic region [105, 107]. Deletion or mutation in bZIP region leads to abrogation of C/EBPs binding to DNA [104]. Specificity to DNA binding depends on amino acid sequence within the basic region [121]. The specificity of dimerization is determined by the electrostatic interaction between the amino acids along the dimerization interface [105, 122]. C/EBPζ can readily dimerize with other members of the C/EBP family through its intact leucine zipper, but dimers containing C/EBPζ cannot bind to the putative C/EBP sequence in target genes. However, C/EBPζ can bind to a different DNA sequence in the promoter of other genes due to the variant basic region of C/EBPζ [115, 123]. Thus, C/EBPζ can act as an inhibitor of C/EBPs transcriptional activity and as an activator of other genes.

In contrast to the C-terminus which is highly conserved, the N-terminus of C/EBPs is not very well conserved and shares only 20% sequence homology (Figure. 2). The N-terminus contains C/EBP trans-activation domains (TADs) and repression domains (RDs) which are responsible for activation or repression of C/EBP activity. The trans-activation domain interacts with basal transcription apparatus [124] as well as transcription co-activators [125] and stimulates transcription. C/EBPγ lacks trans-activation domain and functions as a dominant negative inhibitor of C/EBP activity by forming inactive heterodimers with C/EBP family members [126]. Another family member C/EBPζ lacks

transactivation and repression domain but can form heterodimers with other members, and because of intact bZIP region, also inhibits other C/EBPs activity [115, 123].

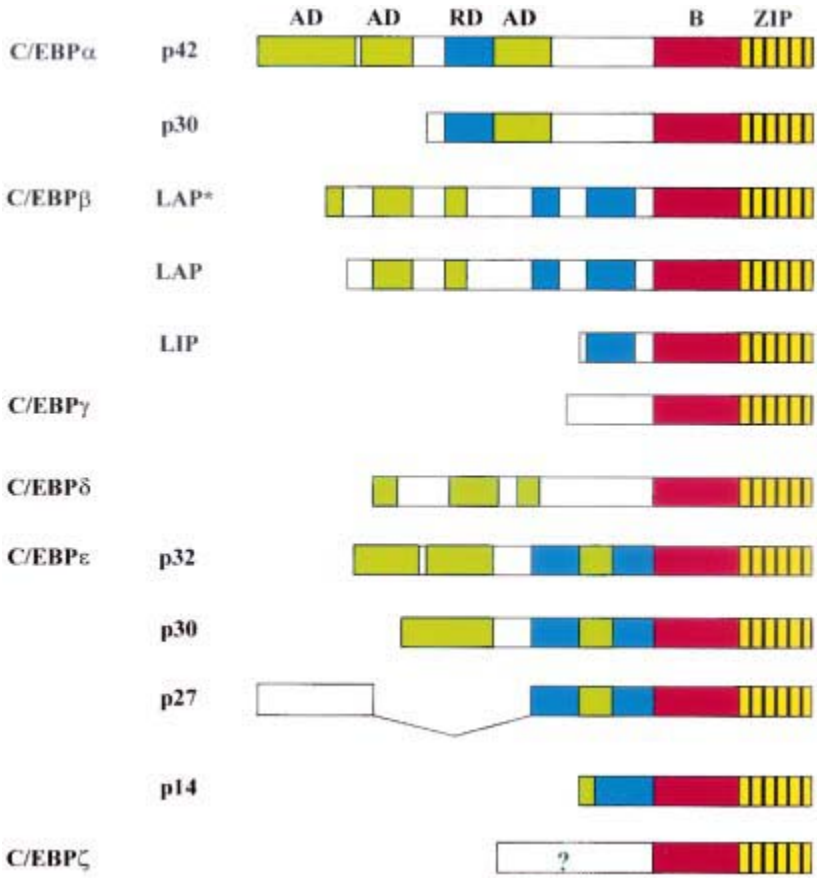

Figure 2. Schematic representation of the C/EBP family members[127]

Some of the six family members of C/EBPs also have isoforms that are expressed in the cells. This is because of alternate translation initiation codons, regulated proteolysis of the proteins, alternate use of promoters and differential splicing. Alternative use of translation intiatiation codons in the same mRNA molecule due to a leaky ribosome scanning mechanism, or regulated proteolysis, various size polypeptides of C/EBPα [128, 129] C/EBPβ [130, 131] and C/EBPε [132, 133] are produced. For C/EBPα there are two

isoforms; a 42 kDa isoform and a 30 kDa isoform [128, 129]. The larger isoform (42 kDa) of C/EBPα is considered to be the active form and the smaller isoform (30 kDa) is less active form as 30 kDa isoform lacks two transactivation domains. p30 isoform of C/EBPα forms a heterodimer with p42 isoform and the heterodimer (p30-p42) in comparison to p42 homodimer has less transactivation and DNA binding activity [128, 130, 134-136]. Interestingly ratio of the two isoforms is very important during adipocyte differentiation and hepatocyte development [128, 129]. There are three isoforms of C/EBPβ produced from the same mRNA; Liver Activating Protein* (LAP*) a 38 kDa protein, Liver Activating Protein (LAP) a 35 kDa protein, and Liver Inhibitory Protein (LIP) a 20 kDa protein [130]. In the case of C/EBPβ, the 35 kDa LAP and 20 kDa LIP isoforms are predominantly expressed and again the shorter LIP isoform lacks a trans-activation domain and functions as a dominant negative inhibitor of C/EBP function by forming non-functional heterodimers with other members [128, 129, 134]. By alternate use of promoter and differential splicing four isoforms of C/EBPε (32 kDa, 30 kDa, 27 kDa and 14 kDa) can be produced [132, 133].

ii) Expression

Some C/EBP family members are ubiquitously expressed and others have a very distinctive pattern of expression based on the stage of differentiation, cell type, tissues, and organisms. C/EBPs expression can be either constitutive or inducible in response to various stimuli. C/EBPγ and C/EBPζ are expressed ubiquitously [113, 115], whereas, C/EBPε mRNA and protein are mainly expressed in myeloid and lymphoid cells [116]. C/EBPα is highly expressed in epidermis, esophagus, lung, liver, fat, myeloid, intestine, ovary, adrenal gland and skeletal muscle [110, 127, 137-141]. In terminally differentiated cells of skin, liver and

adipose tissue, C/EBPα mRNA is expressed at high level [108, 109, 137, 142]. C/EBPβ is expressed in many cells types including liver, intestine, lung, adipose tissue, spleen, kidney, epidermis and myelomonocytic cells [108, 109, 111, 112, 137]. C/EBPδ is primarily an inducible protein and is induced in many cell types by inflammatory and stress stimuli.

4. C/EBPα

C/EBPα is the founder member of the C/EBP family of transcription factors. C/EBPα gene is present on human chromosome 19q13.1. As mentioned above, C/EBPα is an intronless gene and its two isoforms (p30 kDa and p42 kDa) are formed by an alternative translation start site using two inframe AUG codons on single mRNA. C/EBPα is expressed in various cell types including skin [137, 138], lung [110], ovary [139], intestine [110], myeloid cells [140, 141] and adrenal gland [143]. C/EBPα is involved in mitotic growth arrest and terminal differentiation in hepatocytes [144], preadipocytes [145, 146] and myeloid cells [140, 141]. C/EBPα plays a critical role in energy homeostasis and germline deletion of C/EBPα is lethal (C/EBPα mice die just before or just after birth) due to altered hepatic glucose and glycogen metabolism [147]. Neonatal mice with germline deletion of C/EBPα also displayed defects in granulopoiesis [148], white adipose tissue differentiation [147], hepatic cell proliferation [149], and lung development [150]. Gene expression analysis of C/EBPα null mice revealed reduced expression of glycogen synthase which results in reduced level of glycogen in the liver. In addition, mice deficient in C/EBPα displayed decreased expression of three gluconeogenic enzymes involved in *de-novo* synthesis of glucose, glucose -6-phosphatase, phosphoenolpyruavate carboxykinase (PEPCK) and tyrosine aminotransferase [147]. Loss of C/EBPα also leads to hyperammonemia (increased blood concentration of ammonia) as

compared to wild type littermate. Hyperammonemia in C/EBPα null results from impaired expression of ornithine-cycle enzymes [151]. Examination of adipose tissue in C/EBPα null revealed reduced lipid droplets in the adipose tissue [147]. C/EBPα conditional knock out in liver also results in reduced expression of genes involved in glycogen synthesis, gluconeogenesis, and bilirubin detoxification leading to abnormal cellular metabolism [152]. The evidence provided above strongly support the idea that C/EBPα is a central regulator of energy metabolism as speculated by McKnight and co-workers [106].

i) Role in proliferation and differentiation

C/EBPα is predominantly expressed in post-mitotic terminally differentiated cells. C/EBPα has emerged as an antiproliferative gene and activates genes involved in cellular differentiation. Over-expression studies have provided evidence for C/EBPα as a strong inhibitor of cellular proliferation [142, 153]. Abnormal cell proliferation is observed in liver and lung of C/EBPα deficient mice [149]. Christy et. al. (1989) showed that C/EBPα expression is induced during adipogenesis and activates genes expressed in differentiated fat cells [154]. Forced expression of C/EBPα in 3T3-L1 preadipoblasts leads to their differentiation into adipocytes and induces cell cycle arrest [155]. Hendric-Taylors and Darlington extended C/EBPα's antiproliferative activity to hepatocytes (Hep3B2) and Saos2 osteosarcoma cells [153]. These studies provided evidence that C/EBPα can induce proliferation arrest and also differentiation specific genes. C/EBPα regulates expression of gene products associated with differentiation of lung (surfactant A, surfactant B and uteroglobin), and C/EBPα null mice exhibit hyper proliferation of type II pneumocytes suggesting the involvement of C/EBPα in blocking cellular proliferation and inducing

differentiation in lung [149, 156-158]. Deletion of C/EBPα from respiratory epithelial cell of mouse fetal lung inhibited differentiation of the fetal lung, causing death from respiratory failure at birth. Mice lacking C/EBPα show block in type pneumocytes differentiation and increased cellular proliferation and decreased cellular apoptosis [150, 159]. C/EBP transcription factors also play important roles in differentiation of myeloid cells [132, 140, 160-162]. Many studies have suggested a role for C/EBPα in the early and late stages of myeloid differentiation. A large number of myeloid genes contain C/EBP binding site in their promoter suggesting C/EBPs role in granulopoiesis [163-165]. Forced expression studies suggested C/EBPα induces granulopoiesis in various bipotential and multipotential cell lines [140, 141]. Since C/EBPα null mice are perinatal lethal [147], it hampers the study of hematopoietic system in C/EBPα null mice. A study with fetal liver hematopoietic cells and peripheral blood of C/EBPα null mice and inducible knock out strain of mice was used to determine the role of C/EBPα in hematopoiesis. FACS analysis demonstrated that C/EBPα deficiency blocks transition from the common myeloid progenitor (CMP) to the granulocyte monocyte precursor (GMP) in the adult as well as in embryonic hematopoiesis leading to loss of granulocytes [148, 166, 167]. Thus C/EBPα mediates cell cycle arrest associated with terminal differentiation in adipocytes [145, 168], hepatocytes [144] and myeloid cells [140, 141] and regulates the expression of genes associated with the differentiated phenotype in these cell types.

ii) Role in cancer and cell cycle arrest

C/EBPα has been implicated as tumor suppressor gene in various cell types. C/EBPα has been demonstrated as tumor suppressor gene in acute myeloid leukemia (AML) where C/EBPα level is greatly diminished. In AML C/EBPα level is diminished or the gene is inactivated through somatic mutation (~10% cases), or promoter hypermethylation or transcriptional down regulation by translocation protein products such as AML1-ETO [169-171]. While somatic mutations in C/EBPα have not been observed in epithelial tumors, C/EBPα expression is lost or greatly diminished in a number of epithelial cancers including, lung [172], skin [137, 173], liver [174], head and neck [175], endometrium [176] and breast [176, 177] cancer suggesting a tumor suppressor function. Immunohistochemistry studies for C/EBPα protein expression in primary tissue derived from lung cancer patients showed approximately 40% of cancers had greatly diminished expression of C/EBPα [172], suggesting a correlation between diminished expression of C/EBPα and the malignant progression. This idea was substantiated by a study showing induction of C/EBPα in lung cancer cell line H358 results in cell cycle arrest and apoptosis [172]. In liver cancer, tissue samples display diminished C/EBPα expression when compared to normal cells suggesting the role of C/EBPα in liver cancer [174, 178]. In primary breast cancer samples, C/EBPα mRNA expression is downregulated in 83% of cases and protein expression is diminished in 30% of the samples. Overexpression of C/EBPα in mammary epithelial cells results in cell cycle arrest again suggesting the role of C/EBPα in breast cancer [177]. In terms of skin cancer, C/EBPα levels are reduced in squamous skin carcinomas (SCC) and forced expression of C/EBPα induces proliferation arrest in SCC cell lines [137, 173]. Recent

studies in our lab have provided evidence for diminished or ablated expression of C/EBPα in human squamous cell and basal cell carcinoma (unpublished data). Microarray expression profiling on head and neck squamous cell carcinoma (HNSCC) samples provided evidence of down-regulation of C/EBPα in HNSCC and down regulation of C/EBPα was correlated with poor prognosis [175]. Takai et. al. showed that the expression levels of C/EBPα were low in endometrial cancer cell lines and clinical samples but high in normal endometrial tissues suggesting anti-cancerous activity of C/EBPα in endometrial cancer [176]. Above studies suggested a role for C/EBPα in various epithelial cancers but were unable to conclude whether decreased expression of C/EBPα is the cause or outcome of cancer. Genetic evidence for C/EBPα as a suppressor of epithelial tumorigenesis has recently come from a genetically engineered mouse model in which C/EBPα was ablated specifically in the epidermis [179]. The epidermal-specific C/EBPα knockout mice are viable, but highly susceptible to skin tumor development involving oncogenic Ras, and display decreased tumor latency and a higher rate of malignant progression in two stage chemical carcinogenesis experiments [179].

C/EBPα induces cell cycle arrest in variety of cell types. McKnight and co-workers used chimeric C/EBPα-ER protein and were the first to suggest C/EBPα as an inhibitor of mitotic growth arrest in 3T3-L1 preadipocytes in G_0/G_1 [145]. As mentioned previously, numerous studies have reported the forced over-expression of C/EBPα results in the inhibition of cell cycle progression in a variety of cell types including cancer cells. Several overexpression studies have suggested a role for C/EBPα in G_1/S transition. To date different mechanisms have been proposed about C/EBPα involvement in cell cycle arrest.

Proposed mechanisms for C/EBPα-induced cell cycle arrest involve interactions with various cell cycle proteins. Timchenko et. al. first provided the evidence that C/EBPα stabilizes p21. Overexpression of C/EBPα results in 20 fold increase in p21 level. Increases in p21 levels were observed at the transcriptional and at post-transcriptional levels by association with C/EBPα which resulting in stabilization of p21 protein [180]. In C/EBPα$^{-/-}$ mice and hepatocytes from regenerating liver, p21 levels are diminished as C/EBPα expression is reduced in these cases [181, 182]. This has suggested role for C/EBPα in regulation of the p21 gene which is a cyclin dependent kinase inhibitor and participates in cell cycle arrest. The other proposed mechanism through which C/EBPα induces cell cycle arrest is by inhibition of cyclin dependent kinase (CDK) activity. Several studies have suggested C/EBPα induces cell cycle arrest by inhibiting kinase activity of CDK2 and CDK4 through various mechanisms [183, 184]. It has also been proposed that C/EBPα inhibition of CDKs is independent of C/EBPα's DNA binding activity and its mostly protein-protein interaction [184, 185]. *In vitro* studies have shown C/EBPα binds to CDK2 and CDK4 and inhibits their ability to phosphorylate proteins involved in cell cycle regulation such as histone H1. Wang et. al. (2001) proposed that C/EBPα inhibits CDKs activity by disrupting the association of CDKs and cyclins [184]. C/EBPα has also been proposed to inhibit CDKs activity by stabilizing the CDK2-p21 inhibitory complex [183]. The RB family of proteins regulates cell cycle progression and tumorigenesis through their interaction with E2F transcription factor, by forming complexes that repress genes expressed in S-phase of the cell cycle. Darlington and co-workers suggested C/EBPα disrupts the E2F-p107 complex that is present during S-phase and are associated with proliferating cells [186]. They also found ectopic expression

of C/EBPα in 3T3-L1 preadipocytes induces complex formation of proliferation inhibitory form of E2F-p130 [187]. Thus C/EBPα alters the E2F-RB complex formation in such a way that it promotes cell cycle arrest. The other proposed method of C/EBPα induced cell cycle arrest is through inhibition of E2F mediated transcription. Transcriptional activity of E2F family members is essential for the G_1-S phase progression [188, 189]. C/EBPα is proposed to make a complex with E2F and inhibit E2F mediated transcription of S-phase genes [190, 191]. This theory was further supported by a study using C/EBPα basic region mutant BRM2 and BRM5. C/EBPα mutant BRM2 and BRM5 are not present in the complex which binds to E2F site in E2F responsive genes and also have reduced antiproliferative activity in cell culture [192]. DNA binding deficient mutant of C/EBPα is able to repress cell cycle progression in myeloid differentiation assay suggesting the DNA binding activity of C/EBPα to induce cell cycle arrest is not required. The last proposed mechanism of antiproliferative activity of C/EBPα requires the SWI/SNF chromatin remodeling complex. C/EBPα directly interacts with the SWI/SNF complex and is important for C/EBPα-mediated adipocyte differentiation *in vitro* [192]. Overexpression of C/EBPα, in cell lines lacking Brm (a core ATPase of the SWI/SNF chromosome remodeling complex) was unable to induce cell cycle arrest and reexpression of the Brm subunit reinstated C/EBPα mediated cell cycle exit [193]. Recently, siRNA experiment in keratinocyte cell lines has provided evidence that C/EBPα is involved in DNA damage induced G_1 checkpoint [194]. The exact mechanism of C/EBPα involvement in G_1 checkpoint is still not clear. C/EBPα may involve more than one mechanism to induce cell cycle arrest which may be cell type specific. From literature, it is not clear whether C/EBPα involves all mechanism of cell cycle arrest in all cell type or

certain mechanism is cell type specific. Above mentioned studies strongly suggest C/EBPα's involvement in cell cycle arrest through protein-protein interaction as DNA binding deficient form of C/EBPα was able to induce cell cycle arrest.

5. Regulation of C/EBPα and C/EBPβ

C/EBPs are regulated at multiple levels. Studies have provided evidence that C/EBPs are regulated at transcriptional, translational and post-translational level including protein-protein interactions [127].

i) Transcription

C/EBPα

The proximal promoter of mouse C/EBPα was first characterized by Christy et. al. (1991) and then by Legraverend et. al. (1993). They found the mouse C/EBPα proximal promoter contain potential binding sites for C/EBP, Sp1, nuclear factor (NF)-1, NF-Y, upstream stimulating factor (USF), basic transcription element-binding protein(BTEB) and NF-κB [195, 196]. Mouse C/EBPα promoter can be auto-activated by plasmid expressing C/EBPα and C/EBPβ in transfected cells [195, 196]. Similar to mouse C/EBPα proximal promoter, rat C/EBPα proximal promoter can also be autoregulated and it shares complete sequence homology with mouse C/EBPα [197]. Human C/EBPα proximal promoter share only 53% sequence homology with mouse C/EBPα proximal promoter and lacks a C/EBP recognition sequence [198]. Human C/EBPα can still be autoregulated through USF, which interacts with a site in human C/EBPα promoter [198]. c-Myc has been shown to regulate C/EBPα transcription through interaction with the core transcription machinery [199]. During adipocytes differentiation C/EBPβ binds to C/EBPα promoter and increases C/EBPα mRNA

expression [195, 200-202]. Recently, it has shown that p53 binds directly to C/EBPα promoter and regulates its expression in response to DNA damage [194].

C/EBPβ

C/EBPβ promoter is autoregulated in many species [119, 203-205]. Transcriptional regulation of C/EBPβ is mediated through several factors such as acute phase response (IL-1,IL-6) or stimulating carbohydrate metabolism (cAMP, glucagon). In addition C/EBPβ is regulated at transcriptional level by other cellular responses such as differentiation and proliferation (glucocorticoids, growth hormone, and nerve growth factor). C/EBPβ promoter has two CREB binding sites through which CREB regulates C/EBPβ expression. These two sites are important for IL-6 mediated induction of C/EBPβ during acute phase response which involves STAT-3.

ii) Translation

As mentioned earlier, by alternative use of translation initiation codons in the same mRNA molecule due to a leaky ribosome scanning mechanism various size polypeptides of C/EBPα [57, 128] and C/EBPβ [130, 131] are produced. Both C/EBPα and C/EBPβ contain conserved short upstream open reading frame (uORF) [206] and has been shown to be essential for the leaky ribosome scanning mechanism that produces different sized polypeptides in case of C/EBPα and C/EBPβ [207].

iii) Post-translation modification

Post-translational modifications and protein-protein interaction of C/EBPs can affect their cellular localization, DNA binding ability and activation potential.

C/EBPα

C/EBPα contains several putative phosphorylation sites suggesting regulation of C/EBPα by post-translational modifications. In AML patients, activated Fms-like tyrosine kinase 3 (Flt3) inhibits C/EBPα function by extracellular signal receptor kinase (ERK)1/2 mediated inhibitory phosphorylation of C/EBPα at serine 21 and affects C/EBPα's activity to induce granulocytic differentiation [208]. S-21 of C/EBPα is also target of phosphorylation by p38 mitogen activated protein kinase and phosphorylated C/EBPα mediates induction of hepatic phosphoenolpyruvate carboxykinase [209]. C/EBPα is phosphorylated on T222, T226 and S230 by Glycogen synthase kinase-3 (GSK3) [210]. Recently, it has been shown that phosphorylation of C/EBPα at T222/T226 is essential for metallothionein (MT) gene transactivation [211]. C/EBPα is phosphorylated at S193 by Cyclin D3-cdk4/cdk6 *in vitro* and in liver; phosphorylated C/EBPα binds to cdk2 and to Brm and inhibits cellular proliferation [212]. In liver tumors, the PI3K/AKT pathway dephosphorylates C/EBPα at S193 and blocks its antiproliferative activity by accumulation of protein phosphatase 2A (PP2A) in the nuclei [213]. Phosphorylation of C/EBPα on S21 is mediated by Erk1/2, which results in inhibition of in vivo granulopoiesis [167]. The Ras pathway phosphorylates C/EBPα on S248 which stimulates *in vitro* granulopoiesis and this increased activity of C/EBPα is blocked by PKC inhibitors [214]. Phosphorylation of C/EBPα on S248, S277 and S299 by PKC leads to decreased DNA binding activity of C/EBPα *in vitro* [215].

In addition to phosphorylation C/EBPα also undergoes sumoylation and ubiquitination. C/EBPα is sumoylated at the lysine residue near the transcriptional attenuator domain [216, 217]. Sumoylated C/EBPα cannot interact with BRG1(core subunit of

SWI/SNF remodeling complex) as a result cannot induce cell cycle arrest [217]. Shim et.al. (2003) showed that C/EBPα is ubiquitinated in BALB/MK2 keratinocytes and ubiquitination of C/EBPα leads to its proteasomal degradation [218].

C/EBPβ

Seven (CR1-CR7) regulatory regions have been identified in C/EBPβ protein, and two of them CR5 and CR7 are shown to interact with the activation domain of C/EBPβ to inhibit its transcriptional activity. Regulatory domains RD1 and RD2 also inhibit transactivation potential and binding to DNA respectively by inducing a closed conformation [219, 220]. Post-translational modifications such as phosphorylation and deletion of these inhibitory domains activate C/EBPβ and increase its transcriptional activity [219, 220]. C/EBPβ undergoes different kinds of post-translational modification such as phosphorylation, acetylation, methylation and sumoylation. Ras dependent mitogen activated protein kinase (MAPK) pathway phosphorylates C/EBPβ on T235 and activates C/EBPβ mediated transcription [221]. Protein Kinase C (PKC) phosphorylates C/EBPβ on S105 in activation domain and phosphorylation of C/EBPβ on S276 in leucine zipper region by Ca^{2+}/calmodulin dependent protein kinase increases its transcriptional activity [222, 223]. PKC induced p90 ribosomal S kinase (RSK) increases C/EBPβ transcriptional activity by phosphorylation [224]. Oltipraz, a chemopreventive agent, causes the phosphorylation of C/EBPβ at T217 (mouse), S105 (rat) and T266 (human) through RSK1 and increases C/EBPβ mediated transactivation of GATA2 gene downstream of PI3 kinase [225]. TGFα activated RSK phosphorylates C/EBPβ at S105(rat) and at T217(mouse) and induces proliferation in differentiated hepatocytes [226]. Shuman et. al. (2004) showed that Cdk2

and Cdk1 phosphorylate C/EBPβ at S64 and Thr189 in cell cycle dependent manner and promote Ras-induced transformation of NIH 3T3 cells [227]. During differentiation of 3T3-L1 preadipocyte MAPK and cdk2 sequentially phosphorylate C/EBPβ at T188 which primes it for phosphorylation by GSK3β at S184 and T179 [228]. These phosphorylation events result in a conformational change in C/EBPβ and also in dimerization [229].

There are several studies which suggest that phosphorylation not only activates C/EBPβ but can also lead to decrease in C/EBPβ activity. Phosphorylation of C/EBPβ by PKA and PKC on S240 leads to decreased C/EBPβ binding to DNA [230]. Growth hormone mediated activation of PI3K/AKT and inhibition of GSK3β kinase activity results in dephosphorylation of C/EBPβ and increased binding to c-fos promoter [231]. In osteoblasts, cGMP dependent protein kinase (PKG) inhibits GSK3β mediated phosphorylation of C/EBPβ and increases C/EBPβ DNA binding activity [232]. Insulin disrupts C/EBPβ interaction with p300 probably by C/EBPβ phosphorylation by PKB and decreases C/EBPβ activity [233].

In addition to phosphorylation, C/EBPβ is modified by acetylation, methylation and sumoylation. Acetylation of C/EBPβ at K39 by growth hormone increases C/EBPβ mediated transcription of c-fos. In response to glucocorticoid treatment during preadipocyte differentiation, GCN5 acetylates C/EBPβ and stimulates C/EBPα expression by C/EBPβ [234]. Acetylation of C/EBPβ is important for adipogenesis as deacetylation of K39 by HDAC1 decreases the C/EBPβ-mediated transcription of genes involved in an adipogenesis. A mutant C/EBPβ lacking ability to be acetylated on K-39 has an impaired ability to transactivate a C/EBPα promoter reporter construct [235, 236]. Post-translational

modifications involving methylation at K39 results in repression of C/EBPβ transcriptional activity [237]. Sumoylation of LAP1 by SUMO-2/3 alters ability of LAP1 to regulate the cyclin D1 but does not affect nuclear localization of LAP1. Sumoylation of C/EBPβ in T lymphocytes regulates C/EBPβ-mediated expression of Myc and also results in redistribution of nuclear C/EBPβ [238-240]. In addition to post-translational modifications, changes in mediator complex also result in C/EBPβ activation [241]

6. GSK3

Glycogen synthase kinase 3 (GSK3) is a serine threonine kinase present in two isoforms, GSK3α and GSK3β having molecular weight 51 and 47 kDa respectively and are encoded by two independent genes [242]. They share 84% overall homology and 98% in their catalytic domain. GSK3α and GSK3β exhibit very similar biochemical activities [243]. In human, GSK3α is present at 19q13.2 and GSK3β at 3q13.3 loci of chromosome [244, 245]. Other variant GSK3β2 has been identified recently which contains a 13 amino-acids insertion in the catalytic domain [246]. GSK3 is ubiquitously expressed in all tissues and is present in all eukaryotes [242]. GSK3β null mice are embryonic-lethal underscoring the importance of this protein for survival [247].

GSK3s were first identified as kinases involved in regulation of glycogen synthase and were implicated in muscle energy storage and metabolism [248, 249]. After more than a decade of research, GSK3s have been implicated in different cellular processes such as Wnt and Hedgehog signaling, axial orientation during development, neuronal function and circadian rhythm [246, 250-252]. GSK3 is also involved in cell cycle regulation, differentiation, DNA damage response pathway, and cell death and cell survival. Several

studies have shown that GSK3 regulates proteins involved in Alzheimer disease, cancer and neurological disorder [246, 252, 253].

GSK3 can be regulated by four different mechanisms.

i) Phosphorylation:

Phosphorylation of GSK3β can either increase or decrease its catalytic activity depending on the site of phosphorylation. S 9 (S 21 in GSK3α) phosphorylation of GSK3β leads to inactivation whereas phosphorylation at T 216 (T 279 in GSK3α) leads to activation of GSK3β. GSK3β can be phosphorylated by various kinases. PKA, Akt, ILK, RSK2 phosphorylates GSK3β at S 9 and deactivates it. T 216 is phosphorylated by mitogen-activated protein kinasekinase 1 (MEK1), src-like FYN kinase and the Ca^{2+} sensitive proline-rich tyrosine kinase 2 (PYK2) and makes GSK3β more active [246].

ii) Protein- protein interaction:

GSK3β interaction with other proteins helps in docking of substrates for priming. Substrate priming is a pre-requisite for GSK3β mediated phosphorylation. Axin interacts with GSK3β and helps in substrate priming by priming kinase known as Casine Kinase 1 (CK1). Interaction with specific proteins may play a role in differential regulation of GSK3β in different cells or under different conditions. GSK3β binding protein (GBP) binds to GSK3 and inhibits its activity. As a result, GSK3 mediated degradation of β-catenin is reduced, which leads to abnormal embryonic development [254, 255].

iii) Substrate Priming:

Substrate priming is critical for optimal kinase activity of GSK3β. Priming on serine residue four amino-acid C-terminal to the GSK3β putative site is required by priming kinase such as CK1 for β-catenin and by CDK5 for Tau protein. GSK3 recognises consensus sequence S/TXXXS(P)/T(P) for phosphorylation and S(P)/T(P) is the site for priming [256]. Priming of substrate is a common phenomenon in GSK3 mediated phosphorylation and is a prerequisite for some substrates like glycogen synthase. However, there are examples where substrate priming is not necessary as in case of phosphorylation of β-catenin [257].

iv) Subcellular localization

GSK3β is primarily a cytoplasmic protein but is also present in nucleus and mitochondria. GSK3β kinase activity is regulated by its intracellular distribution and in response to various stimuli the amount of cytoplasmic or nucler GSK3β changes. For example, DNA damage increases nuclear translocation of GSK3β and increases its catalytic activity. In response to apoptotic signal nuclear GSK3β level increases and interacts with nuclear substrates [258]. GSK3β activity is very high in mitochondria and nucleus [259].

A recent study has added an additional mechanism of regulation of GSK3. This study documented that N-terminal proteolysis is a novel way of regulation of GSK3. In *in vitro* system, N-terminal cleavage of recombinant GSK3α/β by calpain resulted in increased catalytic activity of the truncated enzyme [260].

7. GSK3 and Wnt signaling

The canonical GSK3 signaling pathway is its involvement in Wnt signaling [261]. The Wnt pathway plays important role in embryonic development as well as in carcinogenesis. Alterations in Wnt signaling lead to various human cancers such as hepatomas, colon cancer, uterine cancer and melanomas [262]. GSK3 plays a pivotal role in Wnt signaling and in the regulation of the amount of proto-oncogene β-catenin. In unstimulated cell, GSK3 is always in active state. In absence of Wnt signal, GSK3, axin, APC and β-catenin form a complex, and GSK3 phosphorylates β-catenin and targets it for ubiquitin-mediated degradation [263-266]. In the presence of Wnt signal, above mentioned complex is degraded, and as a result GSK3 activity is decreased and β-catenin is no longer degraded. Hence, as cellular level of β-catenin increases, it translocates into nucleus and in association with TCF/LEF proteins increases transcription of target genes such as c-Myc and cyclin D1 which are involved in carcinogenesis [263-266].

The group of GSK3β regulated substrates includes proteins involved in metabolism and signaling such as glycogen synthase, insulin receptor substrate-1(IRS-1), cyclic AMP-dependent protein kinase, cyclinD1 and APC. Structural proteins such as microtubule associated proteins MAP1B and MAP2, and various transcription factors involved in gene expression such as AP-1, CREB, Myc, NFκB, β-catenin and C/EBPs are also substrate for GSK3β [252].

8. DNA damage, GSK3 and p53

p53 is a DNA damage responsive gene induced by various kinds of cellular stress such as UV radiation, γ-radiation, DNA crosslink and oxidative stress, and regulates genes involved

in DNA damage response pathway [267-269]. In response to DNA damage, p53 is post-translationally modified, stabilized and its transcriptional activity is increased. p53 can induce cell cycle arrest, cellular senescence or apoptosis in response to DNA damage [269]. Several recent studies suggest a role for GSK3β in the DNA damage response and other stress pathways [270-272]. These studies also suggest a link between GSK3β and p53 in response to DNA damage [270-273]. DNA damage induces nuclear localization of GSK3β and increases its catalytic activity. Nuclear GSK3 forms a complex with p53 in response to DNA damage, activates p53, which subsequently induces expression of p21 and induction of apoptosis [271]. They also suggested this induction in p53 activity is independent of GSK3β induced phosphorylation of p53 [271]. Another study from the same group showed that DNA damage induces GSK3β and p53 interaction not only in the nucleus but also in mitochondria and induces apoptosis [272]. In 2001, Turenne and Price suggested that GSK3β phosphorylates p53 at S33 and increases its transcriptional activity in response to DNA damage [273]. GSK3β interacts with p53 in the nucleus during senescence [274]. Recently, studies have shown that GSK3β interacts with p53 and induces its acetylation at L373 and L382, which in turn decreases interaction between p53 and GSK3β [275].

The other indirect pathway through which GSK3β regulates p53 level is through phosphorylation of MDM2. MDM2 phosphorylation by GSK3β induces p53 degradation by MDM2 and suggests GSK3β as a negative regulator of p53 stability. Sometimes, DNA damage inhibits GSK3 kinase activity. IR inhibits GSK3 activity and MDM2 is hypophosphorypated and as a result p53 is stabilized [276]. UVB treatment activates PI3K

pathway and inhibits GSK3β kinase activity by phosphorylating S9 of GSK3β in human keratinocytes [277].

Other signaling pathway that correlates GSK3β and p53 involves Wnt signaling pathway. In response to DNA damage such as UVB and other genotoxic stresses, p53 dependent repression of Wnt signaling results in GSK3β derepression and downregulation of LEF/TCF transcriptional activity [278-280].

In response to endoplasmic stress GSK3β phosphorylates p53 at S315 and S376 and induces cytoplasmic localization and inhibits apoptosis [270]. Thus in literature, the role of GSK3β in response to DNA damage in terms of its kinase activity and GSK3β regulation of p53 are controversial, probably because of multiple signaling pathway regulating p53 and GSK3 and also cell specific regulation.

Research Hypothesis and Objectives

C/EBPα expression is lost or greatly diminished in a number of epithelial cancers including lung [172], skin [137, 173], liver [174], head and neck [175], endometrium [176] and breast [176, 177] cancer suggesting a tumor suppressor function. Recently, the first genetic evidence for C/EBPα as an epithelial tumor suppressor gene was provided by Loomis et. al. [179]. C/EBPα is highly induced in keratinocytes by different types of DNA damaging agents including UVB, MNNG, bleomycin and etoposide [194]. This induction of C/EBPα requires p53 and p53 binds directly to the C/EBPα promoter to induce C/EBPα expression. UVB is a potent inducer of C/EBPα in primary and immortalized mouse and human keratinocytes, but not in other cell types examined including NIH 3T3, HepG2 and NRK [194]. These results suggest the induction of C/EBPα by UVB may be a cell type specific response. C/EBPα knockdown by siRNA in immortalized keratinocytes results in a diminished G_1 checkpoint after UVB-induced DNA damage [194]. However, the genetic evidence to provide a definitive answer is lacking. siRNA is a great tool to study gene function but it has some limitations and cannot replace *in-vivo* gene knock out technology. siRNA may target non specific genes and the phenotype that we observe may be due to knock down of a non target gene. In order to provide a definitive answer whether C/EBPα has a role in G_1 checkpoint or not, we wanted to provide genetic evidence using C/EBPα$^{-/-}$ primary fibroblasts to perform cell cycle regulation experiments. In light of C/EBPα's emerging roles in tumorigenesis and DNA damage response, it is important to understand the role and regulation of C/EBPα in the stress response pathway. In this study our objectives

were: i) to provide genetic evidence that C/EBPα has a critical role in the mitogen-induced G_1/S transition and in the DNA damage G_1 checkpoint response, and ii) to determine whether C/EBPα induction and the pathway involved in response to DNA damage is cell type specific. Additionally, we also wanted to increase our understanding of C/EBPα regulation in keratinocytes in response to DNA damage utilizing GSK3β specific pharmacological inhibitors.

CHAPTER 1

C/EBPα Expression is Partially Regulated by C/EBPβ in Response to DNA Damage and C/EBPα Deficient Fibroblasts Display an Impaired G_1 Checkpoint

Rakesh Ranjan[1], Elizabeth A. Thompson[1], Kyungsil Yoon[2], and Robert C. Smart[1]

Cell Signaling and Cancer Group, Department of Environmental and Molecular Toxicology, North Carolina State University, Raleigh, North Carolina 27695-7633[1] and National Cancer Center, Division of Common Cancers, Lung Cancer Branch, South Korea [2]

Address Correspondence to: Robert C. Smart, Ph.D., Cell Signaling and Cancer Group, Department of Environmental and Molecular Toxicology, North Carolina State University, Raleigh, NC 27695-7633, USA.

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

Email: rcsmart@ncsu.edu

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Abstract

We observed that C/EBPα is highly inducible in primary fibroblasts by DNA damaging agents that induce strand breaks, alkylate and crosslink DNA as well as those that produce bulky DNA lesions. Fibroblasts deficient in C/EBPα (C/EBPα$^{-/-}$) display an impaired G_1 checkpoint as evidenced by inappropriate entry into S-phase in response to DNA damage and these cells also display an enhanced G_1 to S transition in response to mitogens. The induction of C/EBPα by DNA damage in fibroblasts does not require p53. EMSA analysis of nuclear extracts prepared from UVB- and MNNG-treated fibroblasts revealed increased binding of C/EBPβ to a C/EBP consensus sequence and ChIP analysis revealed increased C/EBPβ binding to the C/EBPα promoter. To determine whether C/EBPβ has a role in the regulation of C/EBPα we treated C/EBPβ$^{-/-}$ fibroblasts with UVB or MNNG. We observed C/EBPα induction was impaired in both UVB- and MNNG- treated C/EBPβ$^{-/-}$ fibroblasts. Our study reveals a novel role for C/EBPβ in the regulation of C/EBPα in response to DNA damage and provides definitive genetic evidence that C/EBPα has a critical role in the DNA damage G_1 checkpoint.

Introduction

The CCAAT/enhancer binding proteins (C/EBPs) are members of the basic leucine zipper (bZIP) class of transcription factors that contain a C-terminal basic DNA binding domain and a leucine zipper domain involved in homo- or hetero-dimerization (Ramji & Foka, 2002). The N-terminal region contains transcription activation and regulatory domains that interact with basal transcription apparatus and transcription co-activators. There are six members of the C/EBP family and C/EBPs play important roles in fundamental cellular processes including proliferation, apoptosis, differentiation, inflammation, senescence and energy metabolism (Ramji & Foka, 2002; Johnson, 2005).

C/EBPα mediates cell cycle arrest associated with terminal differentiation in adipocytes (Umek et al., 1991; Lin & Lane, 1994), hepatocytes (Diehl et al., 1996) and myeloid cells (Radomska et al., 1998; Wang et al., 1999) and regulates the expression of genes associated with the differentiated phenotype in these cell types. Consistent with its role in the regulation of differentiation, C/EBPα has been shown to be a tumor suppressor gene in acute myeloid leukemia where it is inactivated in ~9% of AML cases through specific somatic mutations (Pabst et al., 2001; Gombart et al., 2002). The inactivating mutations in C/EBPα result in a block in granulocytic differentiation and contribute to the uncontrolled proliferation of undifferentiated immature granulocytic blasts. Ectopic or forced expression of C/EBPα inhibits cell cycle progression in nearly all cell types examined (Hendricks-Taylor & Darlington, 1995; Watkins et al., 1996; Halmos et al., 2002; Johnson, 2005; Shim et al., 2005); however, the intrinsic cellular signals and pathways that regulate

C/EBPα expression and its growth arrest properties are not fully understood. In some cases these pathways are linked to cellular differentiation.

C/EBPα expression is ablated or greatly diminished in a number of epithelial cancers including lung (Halmos et al., 2002), skin (Oh & Smart, 1998; Shim et al., 2005), liver (Xu et al., 2001), head and neck (Bennett et al., 2007), endometrial (Takai et al., 2005) and breast (Gery et al., 2005) suggesting a tumor suppressor function. In many cases, the C/EBPα gene is silenced through promoter hypermethylation (Gery et al., 2005; Tada et al., 2006; Bennett et al., 2007). Causal or genetic evidence for a suppressor role of C/EBPα in epithelial tumorigenesis is lacking due to the absence of C/EBPα mutations in epithelial tumors. Moreover, genetically engineered mouse models to document the suppressor function of C/EBPα in tumorigenesis have been problematic as germline or lung specific deletion of C/EBPα is perinatally lethal (Wang et al., 1995; Basseres et al., 2006). Recently, a genetically engineered mouse model in which C/EBPα was ablated in the epidermis was successfully developed (Loomis et al., 2007). These mice survived and were highly susceptible to carcinogen-induced skin tumorigenesis, thus providing the first genetic evidence for C/EBPα as a suppressor of epithelial tumorigenesis. Surprisingly, the epidermal specific C/EBPα knockout mice did not show alterations in stratified squamous differentiation or proliferation of epidermal keratinocytes, suggesting their enhanced susceptibility to tumorigenesis is not related to alterations in keratinocyte differentiation (Loomis et al., 2007).

To prevent or reduce stress-induced injury and cellular damage cells have evolved intricate pathways that permit them to respond to both intrinsic and extrinsic stressors. In

terms of DNA damage, cells respond by engaging cell cycle checkpoints and repairing damaged DNA in order to maintain genome integrity and to prevent heritable mutations which can lead to genomic instability, aging and cancer (Kastan & Bartek, 2004; Sancar et al., 2004; Ishikawa et al., 2006). In UVB-treated skin keratinocytes, C/EBPα expression is highly induced through a p53-dependent pathway and the partial siRNA knockdown of C/EBPα in the BALB/MK2 keratinocyte cell line resulted in a diminished G_1 checkpoint after UVB-induced DNA damage (Yoon & Smart, 2004). While C/EBPα was highly induced by UVB in keratinocytes, UVB treatment of HepG2, NRK and NIH3T3 cells failed to induce C/EBPα. Thus, C/EBPα, at least in keratinocytes, is regulated by stress involving DNA damage through a p53 pathway and C/EBPα appears to have a role in the maintenance of genomic stability via its role in the G_1 checkpoint. However, genetic evidence supporting a role for C/EBPα in the G_1 checkpoint is lacking and as mentioned above no other cell types other than keratinocytes have been reported to respond to DNA damage with the induction of C/EBPα.

In light of C/EBPα's role in tumorigenesis and DNA damage response, it is important to understand the stress pathways or mechanisms through which C/EBPα is regulated in response to DNA damage and the functional importance of C/EBPα's induction. In the current study, we show that fibroblasts respond to multiple types of DNA damage with the induction of C/EBPα and we present genetic evidence utilizing C/EBPα$^{-/-}$ cells to demonstrate a role for C/EBPα in the G_1 checkpoint. Importantly, we have identified a novel stress pathway in which C/EBPβ has a role in the induction of C/EBPα in response to DNA damage

Results

UVB Induces C/EBPα In Fibroblasts and C/EBPα$^{-/-}$ Fibroblasts Display Alterations in the G_1/S Transition and G_1 Checkpoint Initially, we attempted cell cycle studies in wild type and C/EBPα$^{-/-}$ primary keratinocytes; however, these studies were not informative due to the inherent complexity of using primary keratinocytes for cell cycle regulation studies owing to the presence of mixed populations of differentiating and proliferating primary keratinocytes. To provide direct genetic evidence for C/EBPα in the G_1 checkpoint and to extend the findings on UVB-induced expression of C/EBPα in keratinocytes to another cell type, mouse dermal wild type and C/EBPα$^{-/-}$ primary fibroblasts were utilized. Wild type primary and C/EBPα$^{-/-}$ fibroblasts were exposed to a single dose of UVB radiation (5 mJ/cm^2). UVB produced a significant increase in C/EBPα protein levels in wild type fibroblasts (Fig 1A) and C/EBPα was not expressed in C/EBPα$^{-/-}$ fibroblasts (Fig 1B). In wild type fibroblasts, C/EBPα was maximally induced at 6 h post-UVB treatment and returned to control levels by 24 h post-UVB treatment. Elevated levels of C/EBPα were detected as early as 1 h post-UVB treatment (data not shown). UVB also induced a modest transient increase in C/EBPβ at 6 h post-UVB (Fig. 1A).

To examine the effect of the genetic ablation of C/EBPα on cell cycle regulation, wild type and C/EBPα$^{-/-}$ and primary fibroblasts were synchronized by serum deprivation and then released into the cell cycle by the addition of serum containing media. Fibroblasts were either left untreated or treated with a single dose of UVB (5 mJ/cm^2) 4 h after release. Cells were pulsed with BrdU 1 h before each collection time point (4, 15, 18, 21 and 24 h post release) and FACS analysis was conducted to monitor entry into S-phase. Serum released

C/EBPα$^{-/-}$ and wild type fibroblasts not treated with UVB displayed a synchronized entry into S-phase. However, C/EBPα$^{-/-}$ fibroblasts consistently displayed 10-20% more cells in S-phase than wild type fibroblasts indicating that C/EBPα$^{-/-}$ cells have an enhanced mitogen-induced entry into S-phase (Fig. 1C, E). Serum released wild type and C/EBPα$^{-/-}$ fibroblasts treated with UVB exhibited a significant decrease in the number of S-phase cells at 15 and 18 h compared to untreated control cells and this decrease was followed by a recovery at 21 and 24 h, indicating that cells from both genotypes engaged a G_1 checkpoint response. However, UVB-treated C/EBPα$^{-/-}$ fibroblasts exhibited a significantly attenuated G_1 checkpoint as there were ~ 70% more C/EBPα$^{-/-}$ cells in S-phase at 15 and 18 h than similarly treated wild type cells indicating inappropriate entry into S-phase (Fig. 1D, F). Collectively, these results demonstrate that C/EBPα is highly induced by UVB in fibroblasts and the genetic ablation of C/EBPα results into an enhanced mitogen induced G_1/S transition in untreated cells and a significantly diminished G_1 checkpoint in response to DNA damage.

DNA damage and the regulation of C/EBPα

As shown in Fig 2A, UVB treatment of fibroblasts with 5, 10 and 20 mJ/cm^2 resulted in the induction of C/EBPα with the higher doses displaying a more prolonged induction of C/EBPα. To determine whether C/EBPα can be induced by DNA damaging agents other than UVB, we treated fibroblasts with MNNG, a direct acting mutagen that methylates DNA; cisplatin, a cancer therapeutic that cross links DNA; camptothecin, an alkaloid with anti-tumor activity that induces single stranded DNA breaks by inhibiting topoisomerase I enzyme; and bleomycin, an antineoplastic drug that induces both single and double stranded DNA breaks. As shown in Fig. 2B-E, MNNG, cisplatin, camptothecin and bleomycin were

inducers of C/EBPα. To determine whether the increases in UVB-induced C/EBPα protein levels involve altered stability of C/EBPα protein, untreated and UVB-treated fibroblasts were incubated with cycloheximide, an inhibitor of protein synthesis, and the stability of the C/EBPα protein was examined over time. The degradation of C/EBPα protein was similar in both untreated (Fig. 2F, left panel) and UVB-treated fibroblasts (Fig. 2F, right panel). Similar results were observed for C/EBPβ (Fig. 2F). To determine whether C/EBPα mRNA levels are increased by UVB treatment, we utilized quantitative TaqMan reverse transcription-polymerase chain reaction (qRT-PCR). UVB treatment (5 mJ/cm^2) of primary fibroblast resulted in significant increases in C/EBPα mRNA at 6 and 12 h post UVB (Fig. 2G) and this increase was blocked by actinomycin D, an inhibitor of transcription (data not shown). These results indicate that the increased levels of C/EBPα in UVB-treated fibroblasts are due to increased transcription of C/EBPα.

In keratinocytes, the transcription factor p53 is essential for the UVB induction of C/EBPα (Yoon & Smart, 2004). As shown in Figure 3A, UVB treatment of wild type primary keratinocytes resulted in significant increases in the protein levels of C/EBPα and p53, as well as the p53 target gene p21, while UVB treatment of $p53^{-/-}$ primary keratinocytes failed to significantly induce the expression of both C/EBPα and p21 protein (Fig. 3A). These results verify that in keratinocytes, p53 is required for C/EBPα induction by UVB. In contrast to keratinocytes, p53 was dispensable for the UVB-induction of C/EBPα in fibroblasts. As shown in Figure 3B, UVB treatment of $p53^{-/-}$ fibroblasts efficiently induced C/EBPα and as anticipated failed to induce the p53 target, p21, thus verifying the ablation of p53 activity (Fig. 3B). These results demonstrate p53 is dispensable for the UVB induction

of C/EBPα in fibroblasts indicating a p53 independent pathway contributes to the induction of C/EBPα in fibroblasts.

UVB Increases C/EBPβ Binding To C/EBP Consensus Sequence And Is Bound To The C/EBPα Promoter

During the process of L1 preadipocyte differentiation, both C/EBPβ protein levels and DNA binding activity are increased and C/EBPβ has been shown to regulate C/EBPα levels during process (Christy et al., 1991; Darlington et al., 1998; Tang et al., 1999; Tang et al., 2004). Since we observed that UVB produces an increase in C/EBPβ protein levels, we initiated studies to examine whether C/EBPβ has role in the regulation of C/EBPα expression in response to DNA damage. First, we used EMSA analysis to examine whether nuclear extracts isolated from UVB and MNNG treated fibroblasts display increases in the binding of C/EBPβ to a canonical C/EBP consensus sequence (TGCAGATTGCGCAATCTGCA) (Osada et al., 1996). As shown in Fig. 4A, nuclear extracts from cells treated with UVB and MNNG displayed increased C/EBP binding to the C/EBP consensus sequence and this increase appeared somewhat greater than the increase in C/EBPα and C/EBPβ protein levels (Fig. 4 A, lower panel). As shown in Figure 4B, no binding was detected with these nuclear extracts when a mutant C/EBP consensus sequence (TGCAGAGACTAGTCTCTGCA) was utilized and in competition studies, only the cold wild type C/EBP consensus sequence could compete for binding. Supershift assays with antibodies to C/EBPα and C/EBPβ, but not IgG control antibodies revealed that the increase in C/EBP DNA binding post-UVB was due to both C/EBPα and C/EBPβ binding with C/EBPβ being present in all complexes (Fig. 4C). Due to alternative translation start sites, C/EBPβ protein can be present in three isoforms,

termed LAP*(liver activating protein*), LAP *aka* C/EBPβ and LIP (liver inhibitory protein) which functions as a dominant negative inhibitor of LAP* and LAP. To begin to understand which C/EBPβ isoforms are responsible for the increase binding in the EMSA analysis, we first conducted immunoblot analysis of protein extracts from fibroblasts to examine the levels of the three C/EBPβ isoforms and then overexpressed LAP and LIP and examined their DNA binding location using EMSA. As shown in Fig 4D, LAP is the predominate C/EBPβ isoform in fibroblasts and is also the predominate C/EBPβ binding isoform in UVB-treated fibroblasts (Fig 4E). As shown in Fig. 4F, we also conducted EMSA analysis using the C/EBP binding sequence ((-188) GCGTTGCGCCACGATCTCTC (-169) that was previously identified as a bona-fide C/EBP site in the C/EBPα promoter (Tang et al., 1999; Tang et al., 2004). We observed increases in C/EBP binding after UVB or MNNG to the C/EBPα promoter consensus oligomer (Fig 4F) similar to those observed using the canonical C/EBP consensus sequence (Fig 4A). This binding could be competed away with cold consensus sequence (data not shown). To determine whether the C/EBPβ binds to this C/EBP site in the C/EBPα promoter in vivo, we conducted ChIP analysis utilizing a C/EBPβ antibody to immunoprecipitate C/EBPβ-DNA complexes. PCR was conducted on the input DNA and C/EBPβ immunoprecipitated DNA with primers that flank the C/EBP site in C/EBPα promoter. We observed that C/EBPβ was bound to the C/EBPα promoter in the basal untreated state and that UVB treatment consistently produced a modest increase in C/EBPβ binding (N=3) at the early time point (Fig. 4G). To further confirm the specificity of immunoprecipitation of C/EBPβ, we performed ChIP analysis on the C/EBPα promoter in C/EBPβ$^{-/-}$ fibroblasts. We observed an absence of a PCR product in the C/EBPβ

immunoprecipitated samples further supporting our results that C/EBPβ directly binds to the C/EBPα promoter in wild type fibroblasts. We also conducted ChIP analysis on the C/EBPα promoter with a C/EBPα antibody to determine whether C/EBPα is bound to its own promoter in vivo. We observed that C/EBPα is bound to its own promoter at basal state and UVB treatment resulted in increased C/EBPα binding (Fig. 4H). Taken together, these results suggest C/EBPα expression is regulated by C/EBPβ in response to DNA damage and that C/EBPα has an autoregulatory role.

C/EBPβ has a Role in the UVB- and MNNG-Induction of C/EBPα

To determine whether C/EBPβ has a functional role in the regulation of C/EBPα in response to DNA damage, we isolated dermal fibroblasts from wild type and C/EBPβ$^{-/-}$ mice and treated these cells with UVB (5 mJ/cm^2 or 10 mJ/cm^2) or MNNG (35 μM). As shown in Fig. 5A, UVB (10 mJ/cm^2) induction of C/EBPα protein in C/EBPβ$^{-/-}$ fibroblasts was impaired as both the level of protein induction was reduced and the time course for its induction was altered. While the induction of C/EBPα was impaired the increase in p53 protein was comparable in wild type and C/EBPβ$^{-/-}$ fibroblasts (Fig. 5A). The induction of C/EBPα was also impaired in C/EBPβ$^{-/-}$ fibroblasts treated with a lower dose of UVB (5 mJ/cm^2) (Fig 5B) or with the alkylating mutagen MNNG (Fig 5C). To determine whether C/EBPβ deficiency has an effect on UVB-induced C/EBPα mRNA levels, we isolated RNA from wild type and C/EBPβ$^{-/-}$ fibroblasts before and after UVB treatment and examined C/EBPα mRNA levels using quantitative TaqMan reverse transcription-polymerase chain reaction (qRT-PCR), (Fig. 5D). The UVB induction of C/EBPα mRNA was significantly decreased in C/EBPβ$^{-/-}$ fibroblasts compared to wild type fibroblasts (Fig 5D). Collectively, these results indicate

that in fibroblasts C/EBPβ functions downstream of DNA damage to partially regulate C/EBPα mRNA and protein expression.

Discussion

Previously we have shown that C/EBPα is a UVB/DNA damage-inducible gene in mouse and human primary keratinocytes, however, UVB treatment did not induce C/EBPα in three other cell lines examined (HepG2, NRK, or NIH3T3 cells) (Yoon & Smart, 2004) suggesting the induction of C/EBPα by UVB may be a keratinocytes-specific effect. In keratinocytes, UVB-induction of C/EBPα is solely dependent upon p53 and this is mediated through p53 binding to a p53 response element in the distal promoter of C/EBPα (Yoon & Smart, 2004). The results presented in this study demonstrate that C/EBPα is a DNA damage responsive gene in mouse primary fibroblasts and that p53 is dispensable for the UVB-induction of C/EBPα in fibroblasts. Instead, we observed C/EBPα is regulated by C/EBPβ in response to DNA damage and C/EBPα likely has an autoregulatory role in response to DNA damage. C/EBPα induction by DNA damage was impaired in C/EBPβ$^{-/-}$ fibroblasts, both at the protein and mRNA level and the time course for their induction was altered, however, there was not a complete ablation of C/EBPα induction indicating that other pathways are also involved. While C/EBPβ has been shown to regulate C/EBPα expression during pre-adipocyte differentiation (Christy et al., 1991; Darlington et al., 1998; Tang et al., 1999; Tang et al., 2004), our study is the first to demonstrate that C/EBPβ functions downstream of DNA damage to regulate the induction of C/EBPα. Therefore, C/EBPβ is a protein that can be activated by numerous cues including those involving differentiation (Yeh et al., 1995; Sterneck et al., 1997; Oh & Smart, 1998; Zhu et al., 1999) and DNA damage (Ewing et al., 2008) as well as oncogenes (Sundfeldt et al., 1999; Rask et al., 2000; Zhu et al., 2002; Shuman et al., 2004) and inflammatory cytokines (Akira et al., 1990; Mukaida et al., 1990;

Drouet et al., 1991). Recently, Ewing et al (Ewing et al., 2008) reported C/EBPβ represses p53 levels and function to promote cell survival downstream of DNA damage. Therefore, emerging evidence indicating that both C/EBPα and C/EBPβ participate in DNA damage response pathways.

UVB treatment produced a modest increase in C/EBPβ levels and ChIP analyses also revealed modest increases in C/EBPβ binding to the C/EBPα promoter and yet C/EBPβ$^{-/-}$ fibroblasts displayed a significantly impaired induction of C/EBPα mRNA and protein in response to UVB or MNNG treatment. Our ChIP results also revealed that C/EBPβ is constitutively bound to the C/EBPα promoter in untreated fibroblasts. Taken together these results suggest that post-translational modifications of C/EBPβ may contribute to the regulation of C/EBPα in response to UVB. It is generally accepted that C/EBPβ exists in a repressed state and post-translational modifications de-repress C/EBPβ and increase its transcriptional activity. For example, phosphorylation or deletion of the repressor domain unfolds and induces conformational change in C/EBPβ, which results in de-repression and increased transactivation activity of C/EBPβ (Kowenz-Leutz et al., 1994; Williams et al., 1995). In addition to phosphorylation, C/EBPβ is modified by acetylation and a mutant C/EBPβ that can no longer be acetylated on Lys-39 has an impaired ability to transactivate a C/EBPα promoter reporter construct (Cesena et al., 2007; Cesena et al., 2008). Post-translational modifications involving methylation (Pless et al., 2008) and sumoylation (Kim et al., 2002; Eaton & Sealy, 2003; Berberich-Siebelt et al., 2006) also have regulatory roles and changes in mediator complex also result in C/EBPβ activation (Mo et al., 2004). Recently, it was reported that C/EBPβ is involved in the opening of chromatin allowing other

transcription factors to bind to the gene promoter and increase gene expression (Plachetka et al., 2008). Further studies are required to understand how C/EBPβ is activated in response to DNA damage and how these events contribute to the regulation of C/EBPα.

Previous studies in keratinocytes utilizing siRNA knockdown of C/EBPα indicated that C/EBPα has a role in the G_1 checkpoint in response to DNA damage (Yoon & Smart, 2004). One goal of the current study was to use a genetic approach to confirm and verify the role of C/EBPα in the G_1 checkpoint as siRNA knockdown studies can be complicated by the unintentional interaction of the siRNA with other unidentified targets. We observed a diminished G_1 checkpoint response in C/EBPα$^{-/-}$ fibroblasts compared to wild type fibroblasts in response to UVB treatment, and our results provides important genetic evidence that C/EBPα is involved in DNA damage induced G_1 checkpoint. Moreover, we observed that serum deprived C/EBPα$^{-/-}$ fibroblasts display an enhanced mitogen-induced entry into S-phase compared to wild type fibroblasts suggesting that C/EBPα has a direct role in the regulation of the G_1 to S transition in response to mitogens. To our knowledge there are no previous studies that have utilized synchronous cultures of primary cells genetically deficient in C/EBPα to define a functional role for C/EBPα in the regulation of G_1/S transition in response to mitogens. Proposed mechanisms for C/EBPα-induced cell cycle arrest involve interactions with cell cycle proteins including; Rb family members (Chen et al., 1996; Timchenko et al., 1999), CDK4 and CDK2 (Wang et al., 2001), E2F (Slomiany et al., 2000; Porse et al., 2001), p21 (Timchenko et al., 1996) and the SWI/SNF chromatin remodeling complex (Muller et al., 2004). Further studies are required to understand the molecular mechanisms of C/EBPα's involvement in the G_1/S transition and how this

impinges on the DNA-damage induced G_1 checkpoint and neoplasia.

Materials and Methods

Cell lines and cell culture- Mouse primary keratinocytes were isolated from epidermis of newborn mice by overnight floatation of skin in trypsin at 4°C (Hennings et al., 1980; Dlugosz et al., 1995) and dermal fibroblasts were isolated from dermis after the removal of epidermis from skin. Primary kerotinocytes were cultured as described by Yoon et al (Yoon & Smart, 2004). For fibroblasts isolation, dermis was subjected to digestion in collagenase (0.35%) for 25 mins, followed by DNAase (250 units/skin) treatment for 5 mins at 37°C while shaking. Cells were filtered, and collected after centrifugation, and isolated fibroblasts were plated in Eagle's minimal essential medium (EMEM; BioWhittaker) (2 mM $CaCl_2$) supplemented with 10% Fetal Bovine Serum (FBS)(Sigma), 100 U/ml of penicillin, 100 µg/ml of streptomycin and 0.25 µg/ml of amphotericine B (GIBCO) in 100 mm tissue culture dish. Upon confluence, fibroblasts were passaged and plated in 60 mm tissue culture dish. When cells reached 70% confluence they were treated with UVB or other DNA damaging agents.

Animals- Germline C/EBP$\alpha^{-/-}$ mice do not survive; pups die prenatally or survive only few hours after birth (Wang et al., 1995). A keratin 5, K5Cre transgenic line mouse can be used for generalized Cre-mediated recombination or tissue specific gene ablation. When floxed females carrying transgene K5Cre are mated to floxed male animals, recombination occurs in all the tissues in all mice produced from the above mating pair (Ramirez et al., 2004). Germline C/EBP$\alpha^{-/-}$ pups were produced by mating epidermal conditional C/EBP$\alpha^{-/-}$ female (K5Cre;C/EBP$\alpha^{fl/fl}$) (C57BL/6;DBA;129SV) (Loomis et al., 2007) and C/EBPα floxed (C/EBP$\alpha^{fl/fl}$) male mice (C57BL/6;DBA;129SV) (Lee et al., 1997). Primers and PCR

conditions used to genotype mice were published previously (Lee et al., 1997; Ramirez et al., 2004). p53 $^{+/-}$ male mice were mated with p53$^{+/-}$ female mice to generate p53$^{-/-}$ as well as wild type newborn pups (C57BL/6). Primers and PCR conditions were published previously (Hulla et al., 2001). The C/EBPβ$^{-/-}$ mice used in this study have been described previously (Sterneck et al., 1997). The C/EBPβ$^{-/-}$ and wild-type new born pups were generated by mating C/EBPβ$^{+/-}$ females to

C/EBPβ $^{+/-}$ males (C57BL/6;129/SV). For all other studies not utilizing genetically modified mice, fibroblasts were isolated from the dermis of wild type 129SV mice.

Treatment of cells- *The* UV lamp (model EB 280C; Spectronics) used for treating cells emits wavelengths between 280 and 320 nm with a spectrum peak at 312 nm. The intensity of light emitted was measured by NIST Traceable Radiometer Photometer (Model IL1400A, International Light). Cells were treated with UVB as described by Yoon et al (Yoon & Smart, 2004). *N*-Methyl-*N*'-nitro-*N*-nitrosoguanidine (MNNG), camptothecin and cychloheximide were dissolved in dimethyl sulfoxide (DMSO). Cisplatin {cis-Diammineplatinum (II), and bleomycin were dissolved in water. Cells were either treated with MNNG, cycloheximide, camptothecin, bleomycin, dimethyl sulfoxide or water. Cells were treated with cisplatin for 2 h, and then media was replaced with fresh media without cisplatin. Actinomycin D was dissolved in ethanol and cells were treated with Actinomycin D or ethanol.

Preparation of cell lysates- Nuclear extracts were prepared as previously described by Schreiber et al (Schreiber et al., 1989). For preparation of whole cell lysates, cells were washed with cold PBS and harvested by scraping. Cells were collected by centrifugation and

protein was isolated in radio-immunoprecipitation assay buffer as previously described (Ewing et al., 2008).

Western blot analysis- Protein from cell lysates were loaded onto a 12% polyacrylamide Tris-glycine gel (Invitrogen), then separated by electrophoresis and transferred to an Immobilon-P membrane (Millipore). Following incubation in blocking buffer (Oh & Smart, 1998), the membranes were probed with rabbit polyclonal immunoglobulin G (IgG) raised against C/EBPα (sc-61), p53 (sc-6243), C/EBPβ (sc-150), p21 (sc-757), or mouse monoclonal raised against α tubulin (sc-8035) (1:2000) (Santa Cruz Biotechnology) and then probed with a horseradish peroxidase-linked secondary antibody (Amersham). Detection was made with an enhanced chemilluminescence reagent (Perkin Elmer life Science) followed by exposure of membrane to the film.

Quantitative real time PCR- Total RNA was isolated from either control or UVB treated fibroblasts using TRI reagent (Sigma) and then purified by RNeasy Mini Kit (Qiagen). cDNA was prepared from 50 ng RNA by ImProm-II Reverse Transcription System (Promega) following the manufacturer's protocol. cDNA was used to perform Quantitative PCR using mouse C/EBPα TaqMan Gene Expression Assays, 18S TaqMan Gene Expression Assays (Applied Biosystems) and TaqMan Universat PCR mix (Applied Biosystems). Data were analyzed using comparative C_T method.

Chromatin immunoprecipitation (ChIP) assay- Primary fibroblasts were plated in 100 mm culture plates and were left untreated or treated with UVB dose 5 mJ/cm^2. After 1 and 6 h of UVB treatment cells were treated with 1% formaldehyde, thereafter ChiP assay was performed as per manufacturer's instruction (Upstate Biotechnology). The

formaldehyde treated cells were lysed with SDS lysis buffer and sonicated to produce 200-500 bp long DNA fragments. Samples were pre-cleared with salmon sperm DNA/ protein A, then immunoprecipitated with polyclonal antibody against C/EBPβ, C/EBPα or rabbit IgG at 4°C overnight. Immunoprecipitated DNA was decrosslinked with 5 M NaCl and extracted by ethanol/ chloroform precipitation and amplified by PCR. Primer set for PCR was designed to flank C/EBP regulatory sequence in C/EBPα promoter (-188) GCGTTGCGCCACGATCTCTC (-169) (Tang et al., 1999; Tang et al., 2004). Primer set flanking the corresponding site were 5'-(-324) GGCTGGAAGTGGGTGACTTA (-305)-3' and 5'-(-115) CGCCTTCTCCTGTGACTTTC (-134)-3' to produce a 210 bp PCR product.

Electrophoretic mobility shift assay (EMSA) and supershift- Nuclear extracts, 2 μg in 10 μl buffer C, were incubated with 10 μl of master binding mix buffer with ^{32}P-labeled C/EBP probe (Santa Cruz) or ^{32}P labeled probe corresponding to C/EBP responsive element in C/EBPα promoter for 30 minutes at room temperature. For the supershift assays, samples were treated as above but incubated with either C/EBPα (sc-61), C/EBPβ (sc-150) or IgG (sc-2027) (Santa Cruz Biotechnology) antibody. For competition assays, samples were incubated for 20 minutes with cold wild type and cold mutant C/EBP consensus oligonucleotide probe (50 fold in excess) in 10 μl master mix binding buffer and then with wild type ^{32}P-labeled C/EBP probe for 20 minutes at room temperature. Samples were loaded onto 6% polyacrylamide gel, and subjected to electrophoresis in 0.025X TBE buffer at 200V at 4-8° C for 5-7 h.

5-Bromo-2'-deoxyuridine (BrdU) labeling and fluorescence-activated cell sorting (FACS) Analysis - When fibroblasts reached 30-40% confluence, cells were synchronized by

serum deprivation in 0.5% FBS for 28 h. Fibroblasts were released by adding 10% FBS containing fibroblast medium. After 4 h of release, fibroblasts were either left untreated or treated with UVB (5 mJ/cm^2). One hour before the cells were harvested at each time point, the cells were incubated with 10 μM BrdU. Cells were then fixed in 70% alcohol, treated with 2 N HCl–Triton X-100 to denature DNA, followed by neutralization with $Na_2B_4O_7$. Cells were pelleted, resuspended in 0.5% Tween 20–1% bovine serum albumin–PBS with anti-BrdU-fluorescein isothiocyanate antibody (1:50; Becton Dickinson) and 0.5 mg of RNase/ml, and incubated at 4°C overnight. Cells were pelleted and resuspended in PBS containing 5 μg/ml propidium iodide (PI) and subjected to FACS analysis.

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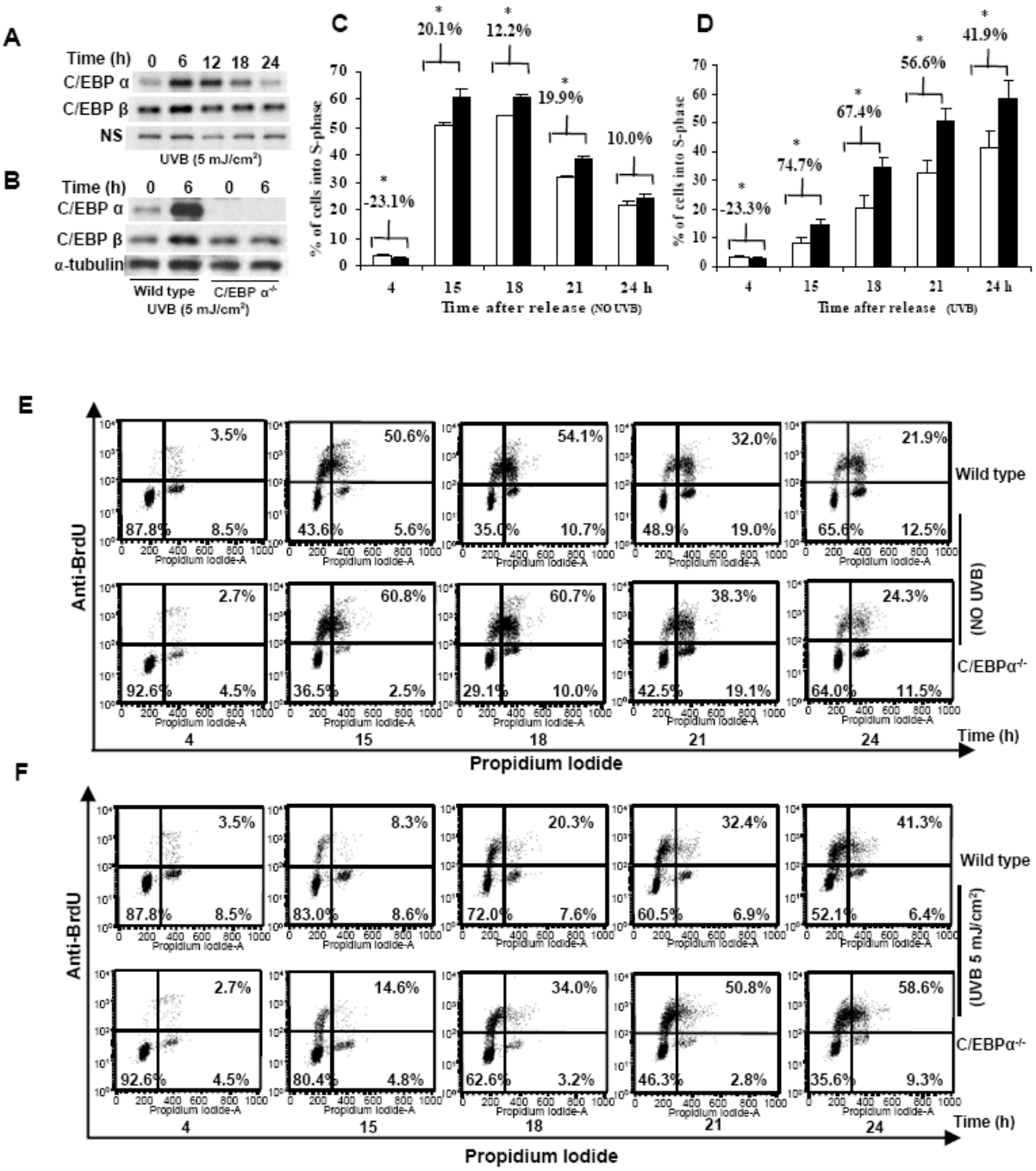
Figure. 1. UVB induces C/EBPα in primary fibroblasts and C/EBPα is involved in the G_1/S transition as well as in UVB-induced G_1 checkpoint. (A) Primary fibroblasts were exposed to UVB (5 mJ/cm^2) and cell lysates were prepared at various time points and immunoblot analysis conducted. Non-specific (NS) band is shown to confirm equal loading. (B) Primary fibroblasts from newborn wild type or C/EBPα$^{-/-}$ were treated with UVB (5 mJ/cm^2) and cell lysates were prepared at various time points and immunoblot analysis conducted. (C,D) Wild type (open column) and C/EBPα$^{-/-}$ (black column) fibroblasts were synchronized by serum deprivation for 28 h in 0.5% serum and then released into the cell cycle by the addition of serum containing medium. Fibroblasts were either not treated (C) or treated with UVB (5 mJ/cm^2) (D) at 4 h after the addition of serum containing medium. Cells were pulse labeled with 5-bromo-2'-deoxyuridine (BrdU) 1 h before collection and then the cells were fixed, incubated with anti-BrdU antibody, stained with PI, and subjected to FACS analysis. The number above each column pair represents the percent increase in S-phase cells in C/EBPα$^{-/-}$ fibroblasts compared to wild type. Data represents mean ± S.D, N=3/time point/genotype. Two-factor ANOVA demonstrated significant interaction between genotype and time for both untreated and treated cells ($P<0.05$). *Significantly different from wild type fibroblasts ($p< 0.05$) at the indicated time point as determined by Student's t-test. (E) Representative scatter plots for untreated fibroblasts (for C) after release, showing the mean percentage of cells (N=3/time point/genotype) in G_1 (lower left), S (top), and G_2M (lower right) phase of the cell cycle. (F) Representative scatter plots for UVB-treated fibroblasts (for D) showing the mean percentage of cells (N=3/time point/genotype) in G1, S and G2/M. The data presented represents one of four independent experiments all showing similar results.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C/EBP β
NS
UVB (5 mJ/cm²)
B
Time (h) 0 6 0 6
C/EBP α
C/EBP β
α-tubulin
Wild type
C/EBP α-/-
UVB (5 mJ/cm²)
C
-23.1%
20.1%
12.2%
19.9%
10.0%
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4 15 18 21 24 h
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67.4%
56.6%
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4 15 18 21 24 h
Time after release (UVB)
E
Anti-BrdU
Propidium Iodide
Wild type
C/EBPα-/-
(NO UVB)
Time (h)
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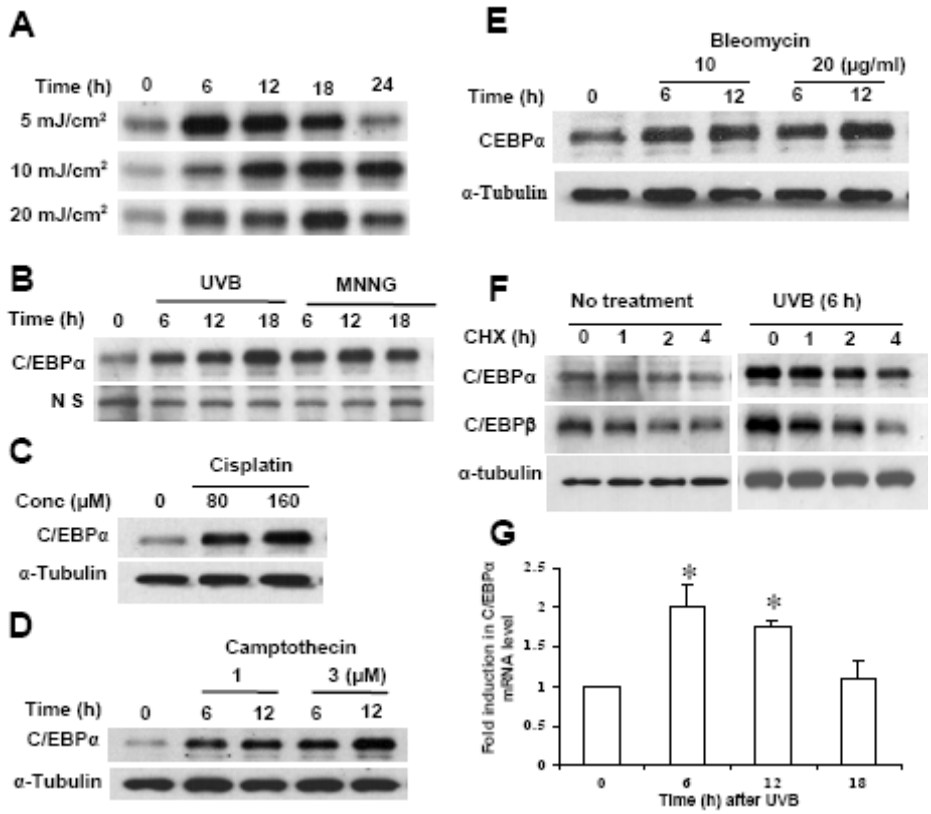
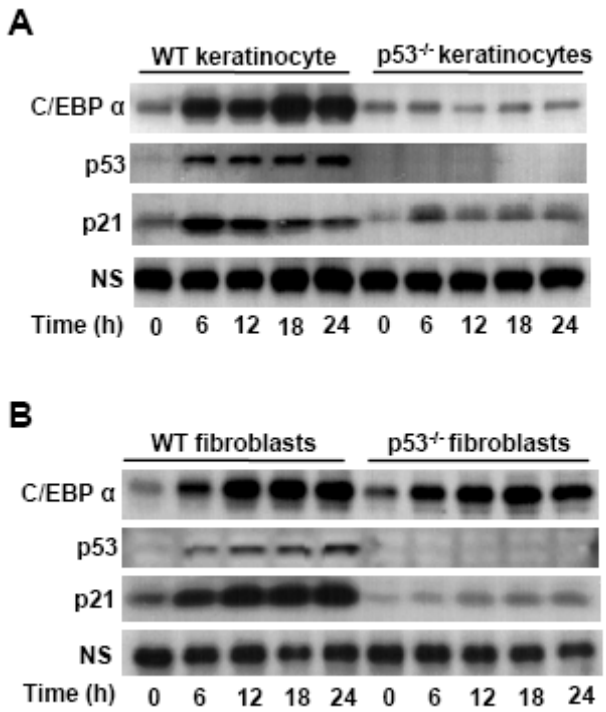
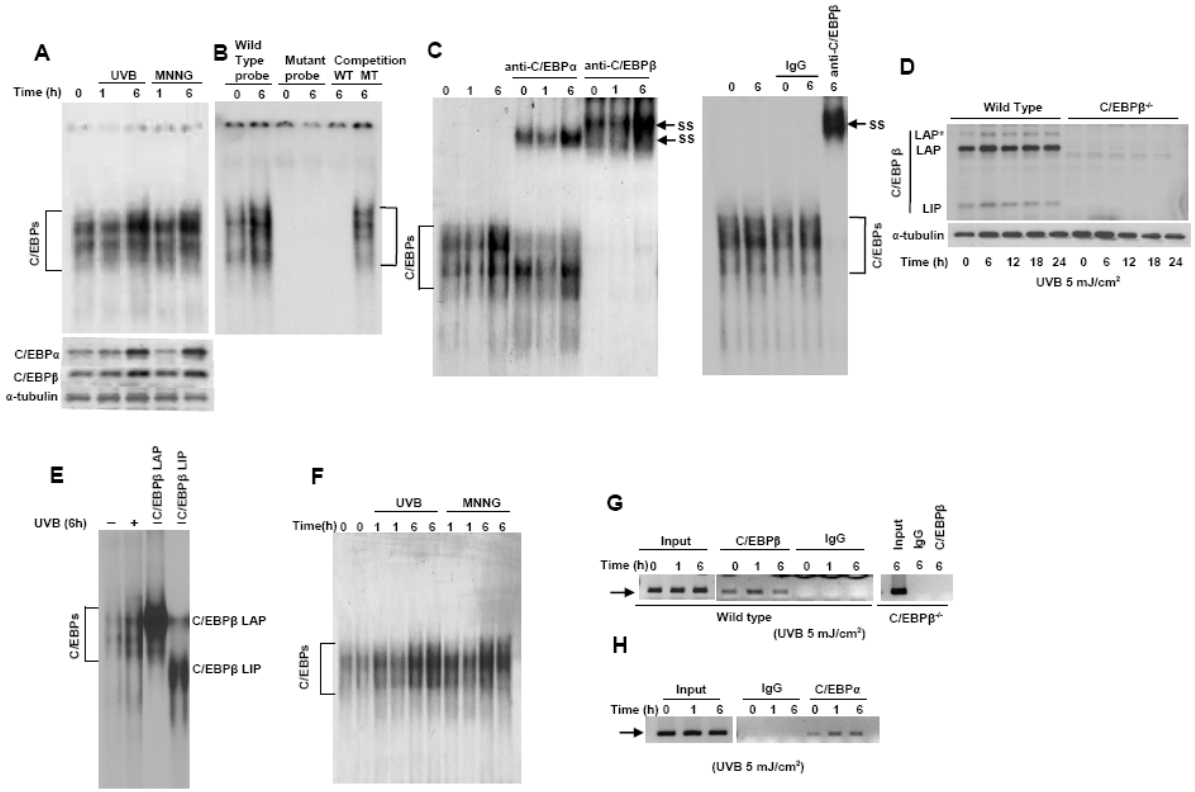


Figure. 2. DNA damaging agents induce C/EBPα in primary fibroblasts. (A) Primary fibroblasts were exposed to different doses of UVB and cell lysates were prepared at indicated time points and immunoblot analysis conducted. (B-E) Primary fibroblasts were treated with UVB (10 mJ/cm^2), MNNG (35 μM), cisplatin (80 and 160 μM), camptothecin (1 and 3 μM), bleomycin (10 and 20 μg/ml), DMSO or water alone, and immunoblot analysis conducted. Non-specific (NS) and α-tubulin band is shown to confirm equal loading. (F) Fibroblasts were either not treated (left panel) or treated (right panel) with UVB and 6 h later were incubated with cycloheximide (50 μg/ml). Cells were harvested at indicated time points after the start of cycloheximide treatment and immunoblot analysis was conducted. (G) Total RNA was isolated from fibroblasts at different time points after UVB (5 mJ/cm^2) treatment. Quantitative RT–PCR was conducted for C/EBPα and 18 S mRNA levels. Data was normalized to 18 S and analyzed using the comparative C_T method. Data is expressed as mean ± standard error (N=3) and each experiment was run in triplicate. *Significantly different from time zero ($p < 0.05$) as determined by Student's t-test



Fgure. 3. UVB induction of C/EBPα does not require p53 in fibroblasts. (A) Primary keratinocytes were isolated from wild type and $p53^{-/-}$ newborn mice and were treated with UVB (10 mJ/cm^2). Keratinocytes were harvested at the indicated time points and immunoblot analysis conducted. (B) Primary fibroblasts isolated from wild type and $p53^{-/-}$ newborn mice were treated with UVB (10 mJ/cm^2). Fiboblasts were harvested at indicated time points and immunoblot analysis conducted.

Fgure. 4. UVB and MNNG increase binding of C/EBPα and C/EBPβ to C/EBP consensus sequence and to the C/EBPα promoter in vivo. (A) Wild type fibroblasts were treated with UVB (5 mJ/cm^2) or MNNG (35 μM) and nuclear extracts were prepared. EMSA was conducted with 2 μg of nuclear extract and a labeled wild type C/EBP consensus oligonucleotide probe. (Lower panel) Nuclear extract from above experiment was used to conduct immunoblot analysis. (B) Wild type fibroblasts were treated with UVB (5 mJ/cm^2) and nuclear extracts were prepared. EMSA was conducted with 2 μg of nuclear extract and a labeled wild type or mutant C/EBP consensus oligonucleotide probe. Competition assays were performed with cold wild type (WT) or cold mutant (MT) C/EBP probe (50 fold in excess). (C) Wild type fibroblasts were treated with UVB (5 mJ/cm^2) and nuclear extracts were prepared. EMSA was conducted with 2 μg of nuclear extract and a labeled wild type C/EBP consensus oligonucleotide probe. Supershift assays were performed with anti-C/EBPα, anti-C/EBPβ antibody or IgG (SS-Supershift). (D) Primary fibroblasts from wild type and C/EBPβ$^{-/-}$ mice were treated with UVB (5 mJ/cm^2). Cells were harvested at the indicated time points and immunoblat analysis conducted. (E) Nuclear extracts were prepared from non-treated and UVB (5 mJ/cm^2) treated fibroblasts (Lane 1 and 2) and from HEK 293 cells transfeced with pcDNA3.1 C/EBPβ-LAP or pcDNA3.1-C/EBPβ LIP (Lane 3 and 4). EMSA was conducted with 2 μg of nuclear extract and a labeled C/EBP consensus oligonucleotide probe. (F) Wild type fibroblasts were treated with UVB (5 mJ/cm^2) or MNNG (35 μM) and nuclear extracts were prepared at indicated time points. EMSA was conducted with 2 μg of nuclear extract and labeled C/EBP consensus sequence corresponding to the C/EBP responsive element in C/EBPα promoter. (G) Wild type or C/EBPβ$^{-/-}$ fibroblasts were treated with UVB (5 mJ/cm^2) and ChIP assay using a C/EBPβ antibody was conducted as described in the methods section. Input control represents 5% DNA as compared to IgG or C/EBPβ samples. (H) Wild type fibroblasts were treated with UVB (5 mJ/cm^2) and ChIP assay using a C/EBPα antibody was conducted as described in the methods section. Input control represents 5% DNA as compared to IgG or C/EBPα samples.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Time (h)
UVB
MNNG
C/EBPs
C/EBPα
C/EBPβ
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Wild Type probe
Mutant probe
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C/EBPs
C
anti-C/EBPα
anti-C/EBPβ
IgG
anti-C/EBPβ
SS
SS
SS
C/EBPs
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C/EBP β
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Time (h) 0 6 12 18 24 0 6 12 18 24
UVB 5 mJ/cm²
E
UVB (6h)
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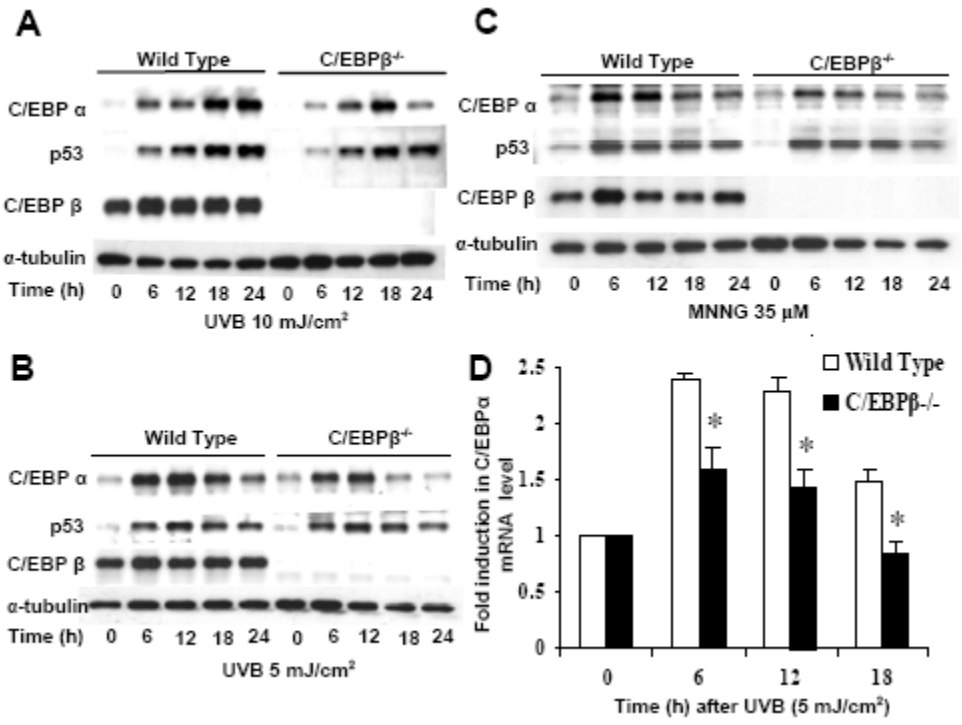

Figure. 5. C/EBPα is Regulated by C/EBPβ in Response to DNA Damage.
(A-C) Primary fibroblasts from newborn wild type or C/EBPβ$^{-/-}$ mice were treated with UVB (10 mJ/cm^2), UVB (5 mJ/cm^2) or MNNG (35 uM). Cells were harvested at the indicated time points and immunoblot analysis conducted. (D) Primary fibroblasts from newborn wild type or C/EBPβ$^{-/-}$ mice were treated with UVB (5 mJ/cm^2) and RNA isolated at the indicated time points. Quantitative RT–PCR was conducted for C/EBPα and 18 S mRNA levels. Data was normalized using 18 S and was analyzed using comparative C_T method. Data is expressed as mean ± standard error (N = 4) and each experiment was run in triplicate. Two-factor ANOVA demonstrated significant interaction between genotype and time ($P<0.05$). *Significantly different from wild type fibroblasts ($p< 0.05$) at the indicated time point as determined by Student's t-test.

CHAPTER 2

GSK3β Inhibitors Block DNA Damage Induced C/EBPα Expression

Rakesh Ranjan[1], Kyungshil Yoon[2], and Robert C. Smart[1]*

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

National Cancer Center, Division of Common Cancers, Lung Cancer Branch, Goyang-si, Gyeonggi-do, South Korea[2]

Address Correspondence to: Robert C. Smart, Ph.D., Cell Signaling and Cancer Group, Department of Environmental and Molecular Toxicology, North Carolina State University, Raleigh, NC 27695-7633, USA.

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

Email: rcsmart@ncsu.edu

Abstract

The basic leucine zipper transcription factor, CCAAT/enhancer binding protein alpha (C/EBPα) is highly expressed in skin. C/EBPα has been shown to be induced by DNA damage in human and mouse skin, and in primary and immortalized keratinocyte cell lines. Cells deficient in C/EBPα (mouse keratinocyte cell line and mouse primary fibroblasts) display a diminished DNA damage induced G_1 checkpoint underscoring the importance of C/EBPα in DNA damage response. In keratinocytes, the induction of C/EBPα requires p53; p53 directly binds to C/EBPα promoter and is responsible for increases in C/EBPα mRNA expression in response to DNA damage. We report here that treatment of keratinocytes with GSK3β inhibitors results in inhibition of C/EBPα protein and mRNA induction in response to UVB induced DNA damage. Induction of p53 protein (C/EBPα regulating protein) by UVB was unaltered after treatment with GSK3β inhibitors. However, UVB treatment of keratinocytes increased GSK3β/p53 protein-protein interaction. UVB treatment resulted in the post-translational modification of C/EBPα protein as determined by a slow migrating band using PAGE electrophoresis. Our results suggest that GSK3β regulates C/EBPα expression, interacts with p53, and that C/EBPα protein undergoes post-translational modification in response to DNA damage.

Introduction

Cells are continually exposed to intrinsic and extrinsic genotoxic insults, which cause DNA damage. The ability of cells to respond to DNA damage is essential to ensure the integrity of the genome. Unchecked DNA damage may lead to heritable mutations, genomic instability and cancer. Depending upon the type and amount of DNA damage as well as the cells involved, a cell can respond by inducing apoptosis, cell cycle checkpoints, DNA repair and senescence [1, 2]. Activation of cell cycle checkpoints in G_1, S or G_2 phases of the cell cycle prevents the replication of damaged DNA and allows time for DNA repair or in case of unrepairable damage apoptosis or senescence. Understanding the regulation of the genes involved in DNA damage and cell cycle checkpoints is important in the understanding of mutagenesis, aging and cancer. UVB radiation is a human carcinogen, and is a model agent for studying cellular responses to DNA damage. It produces cyclobutane pyrimidine dimers, 6-4 photoproducts, cytosine photohydrates, DNA strand breaks, and DNA cross links [3, 4]. In the United States solar radiation causes 1,000,000 non-melanoma skin cancers (squamous cell and basal cell carcinoma) per year and is a leading skin carcinogen [5].

The CCAAT/enhancer binding proteins (C/EBPs) are members of the basic leucine zipper (bZIP) class of transcription factors. The family includes six members: C/EBPα, C/EBPβ, C/EBPγ, C/EBPδ, C/EBPε and C/EBPζ [6]. C/EBPα is expressed in various cell types including skin [7, 8], lung [9], ovary [10], intestine [9], myeloid cells [11, 12] and adrenal gland [13]. It has been shown that C/EBPα is involved in mitotic growth arrest and terminal differentiation in hepatocytes [14], preadipocytes [15, 16] and myeloid cells [11,

12]. C/EBPα has been reported to inhibit cell cycle progression through numerous mechanisms [17-24], most of which involve interactions with cell cycle proteins. Consistent with its role in cell cycle progression and differentiation, C/EBPα has been shown to be a tumor suppressor gene in acute myeloid leukemia where it is inactivated through specific somatic mutations [25, 26]. While somatic mutations in C/EBPα have not been observed in epithelial tumors, C/EBPα expression is lost or greatly diminished in a number of epithelial cancers including, lung [27], skin [8, 28], liver [24], head and neck [29], endometrial [30] and breast [31] cancer suggesting a suppressor function. There is mounting evidence suggesting C/EBPα functions as a tumor suppressor gene in the skin [8, 28, 33]. Germline deletion of C/EBPα is perinatal lethal [32], however, genetic evidence that C/EBPα is a suppressor of epithelial tumorigenesis has recently come from studies in our laboratory using a genetically engineered mouse model in which C/EBPα was specifically ablated in the epidermis [33].

C/EBPα is highly induced in keratinocytes by numerous DNA damaging agents including UVB, MNNG, bleomycin and etoposide [34]. This induction of C/EBPα requires p53 and p53 directly binds to the C/EBPα promoter to induce C/EBPα expression. C/EBPα knockdown by siRNA in immortalized keratinocytes results in a diminished G_1 checkpoint after UVB-induced DNA damage [34]. Recently genetic evidence demonstrating the involvement of C/EBPα in the G_1 checkpoint has been provided in primary fibroblasts [35]. C/EBPα expression is diminished or is absent in mouse and human squamous cell carcinoma and basal cell carcinoma (unpublished data) and absence of C/EBPα from mouse skin results in increase tumor incidence and higher rate of malignant conversion [33].

GSK3β is a serine threonine kinase which is involved in different cellular processes such as Wnt signaling, insulin function, axial orientation during development, neuronal function and circadian rhythm [36-39]. GSK3β is also involved in cell cycle regulation, differentiation, cell death and cell survival, and in cancer [39-42]. In one study a role for GSK3β in maintaining chromosomal stability has been suggested [43]. Recent studies have indicated a role of GSK3β in the DNA damage response pathway [44, 45]. DNA damage increases GSK3β catalytic activity and GSK3β interacts with p53 and increases p53 transcriptional activity in response to DNA damage [44, 45]. Since p53 regulates C/EBPα expression in response to DNA damage, it is possible that GSK3β regulates C/EBPα expression through p53 in keratinocytes.

In light of C/EBPα's emerging role in tumorigenesis and DNA damage response, it is important to further elucidate how C/EBPα is regulated in response to DNA damage in keratinocytes. We hypothesized that GSK3β is involved in the DNA damage induced increase in C/EBPα expression. We demonstrate that GSK3β pharmacological inhibitors block C/EBPα mRNA and protein expression in response to DNA damage. Our results also suggest that GSK3β forms a complex with p53 in response to DNA damage and might regulate C/EBPα expression at message and protein level.

Results

GSK3β inhibitors block C/EBPα protein induction and are specific to DNA damage response

To determine whether GSK3β has a role in C/EBPα induction in response to DNA damage, we treated BALB/MK2 keratinocytes with the GSK3β specific pharmacological inhibitors, lithium chloride (LiCl) and SB 415286 for 1 h. After 1 h of treatment with inhibitors, cells were exposed to UVB (10 mJ/cm^2). As previously shown by Yoon and Smart in 2004, C/EBPα is highly induced by UVB treatment [34]. Interestingly C/EBPα induction was greatly diminished in the presence of GSK3β inhibitors, LiCl and SB 415286, in a dose dependent manner (Fig. 1A). We found that GSK3β inhibitors also blocked C/EBPα induction by a lower dose (5 mJ/cm^2) of UVB (Fig. 1C) as well as by MNNG, a direct acting mutagen that methylates DNA (Fig. 1B). GSK3β inhibitors had no effect on C/EBPα protein levels in the absence of DNA damage (Fig. 1D). These data suggest GSK3β may be a part of DNA damage network that regulates C/EBPα. Since p53 regulates C/EBPα induction in response to DNA damage [34], we examined p53 levels. We did not find any decrease in p53 protein level in response to DNA damage in presence of GSK3β inhibitors (Fig. 1A,B,C). This result suggests that GSK3β inhibitors are regulating C/EBPα protein level without altering p53 protein level in response to DNA damage.

UVB induces post-translational modification to the C/EBPα protein but does not affect C/EBPα protein stability

C/EBPα protein can undergo post-translational modifications such as phosphorylation, sumoylation and ubiquitination under various stimuli [46-50]. Post-translational modification of proteins may lead to increased stability of protein or altered function.

GSK3β kinase phosphorylates C/EBPα at T222 and T226 [48, 51] and NetPhos 2.0 Server analysis suggests putative sites for ATR and Chk1 phosphorylation. We wanted to examine if UVB induces post-translational modification to C/EBPα protein. We treated BALB/MK2 keratinocytes with UVB (10 mJ/cm^2) and examined the C/EBPα electrophoretic protein mobility in response to UVB treatment by PAGE electrophoresis. We found 2 h after UVB treatment half of the protein was modified (Fig. 2A) as indicated by slower migrating band, and after 12 h of UVB treatment the entire amount of protein is shifted to a slower migrating band (Fig. 2B). At this point it is unknown whether this shift is due to phosphorylation or some other DNA damage induced post-translational modification to C/EBPα protein.

Post-translational modification of proteins may lead to increased stability of protein and GSK3β inhibitors may alter the stability of C/EBPα protein post-UVB treatment. To answer this question, we first wanted to determine whether UVB stabilizes C/EBPα protein in response to UVB treatment. Untreated and UVB-treated keratinocytes were incubated with cycloheximide, an inhibitor of protein synthesis, and the stability of the C/EBPα protein was examined over time by western blot analysis (Fig. 2C). The degradation of C/EBPα protein was similar in both untreated and UVB treated samples indicating no effect of UVB treatment on C/EBPα protein stability.

GSK3β inhibitors regulate C/EBPα expression at message level in response to UVB treatment

To determine whether GSK3β inhibitors also inhibit C/EBPα mRNA after UVB treatment, we utilized quantitative TaqMan reverse transcription-polymerase chain reaction (qRT-PCR). BALB/MK2 keratinocytes were either treated or not with UVB and GSK3β inhibitors and

total RNA was isolated 12 h after UVB treatment and subjected to qRT-PCR. UVB treatment of BALB/MK2 keratinocytes resulted in ~28 fold increase in C/EBPα mRNA at 12 h post UVB. GSK3β inhibitors LiCl and SB 415286 blocked increase in C/EBPα mRNA by 60% and 40% respectively suggesting GSK3β inhibitors regulate C/EBPα expression at message level (Fig. 3A,B). Caffeine, a pharmacological inhibitor of ATM/ATR, inhibits C/EBPα protein expression in response to DNA damage [34]. We show that caffeine inhibits C/EBPα mRNA expression in response to DNA damage; possibly through inhibition of ATM/ATR mediated p53 activation.

UVB treatment induces GSK3β/p53complex formation

C/EBPα induction in response to DNA damage is solely dependent on p53 [34]. Previous study has shown that in response to DNA damage, GSK3β and p53 form a complex which results in increased p53 transcriptional activity [44, 45, 52]. Therefore, we examined whether UVB induces GSK3β/p53 complex formation in BALB/MK2 keratinocytes. BALB/MK2 keratinocytes were treated with UVB (10 mJ/cm^2), cells were harvested, cell lysates were prepared in RIPA buffer and p53 was immunoprecipitated. Levels of immunoprecipitated p53 and co-immunoprecipitated GSK3β were measured. We found GSK3β/p53 interaction increased at 12 h after UVB (10 mJ/cm^2) treatment (Fig. 4A). We also found similar results after 4 and 8 h of UVB (10 mJ/cm^2) treatment (Fig. 4B). These results suggest GSK3β interacts with p53 and might regulate p53 activity to increase C/EBPα expression in response to UVB treatment.

GSK3β knock down has no effect on C/EBPα induction in response to DNA damage

We wanted to provide molecular evidence for the involvement of GSK3β in C/EBPα induction by DNA damage. We utilized siRNA strategy to knockdown GSK3β. BALB/MK2 cells were transfected with siRNA targeting GSK3β or GFP (negative control), and 72 h after transfection cells were exposed to UVB (10 mJ/cm^2). We were able to knockdown GSK3β efficiently (~70%) in BALB/MK2 keratinocytes (Fig. 5). Surprisingly, siRNA mediated suppression of GSK3β had no effect on C/EBPα induction by UVB (Fig. 5A). This result suggested LiCl and SB 415286 compound might be inhibiting non-specific target other than GSK3β and ultimately regulating C/EBPα expression. To confirm that GSK3β activity is decreased in GSK3β knock down cells, we examined β-catenin (a GSK3β target protein) protein levels in GFP siRNA and GSK3β siRNA transfected cells. We did not find any increase in β-catenin protein levels in GSK3β knockdown group compared to siGFP group rather we saw decrease in β-catenin level (Fig. 5B). This might suggest that we did not have sufficient GSK3β knockdown in our system to completely abrogate GSK3β kinase activity.

Discussion

Previously, our laboratory has shown that C/EBPα is a UVB/DNA damage-inducible gene in mouse and human keratinocytes as well as in mouse primary dermal fibroblasts [34, 35]. In keratinocytes, UVB-induction of C/EBPα is solely dependent upon p53 and this is mediated through p53 binding to a p53 response element in the distal promoter of C/EBPα [34]. ATM/ATR inhibition by caffeine also inhibits C/EBPα induction by inhibiting p53 induction by UVB. UVB treatment did not induce C/EBPα in other cell types such as HepG2, NRK, or NIH3T3 cells [34]. Recently, we have shown that C/EBPα is inducible in mouse dermal fibroblasts in response to various types of DNA damage [35]. We have also provided genetic evidence that C/EBPα has a critical role in DNA damage G_1 checkpoint [35]. In fibroblasts C/EBPα induction does not require p53 and involves a novel pathway involving C/EBPβ [35]. These studies suggest that C/EBPα is regulated by different DNA damage pathways in different cell types. Based on these studies and the importance of C/EBPα in DNA damage response, G_1 checkpoint and skin cancer, we wanted to further our knowledge about C/EBPα induction in keratinocytes. In this study we report that GSK3β inhibitors block C/EBPα induction in response to DNA damage. DNA damage induces GSK3β interaction with p53. We also provide evidence that UVB induces post-translational modifications to C/EBPα protein but does not alter C/EBPα protein stability.

GSK3β is also involved in cell cycle regulation, differentiation, DNA damage response pathway, cell death and cell survival as well in carcinogenesis [39-41, 44]. In the presence of GSK3β inhibitors we observed decreased induction of C/EBPα protein and message without altering p53 protein level in response to DNA damage. Since p53 regulates

C/EBPα expression, we hypothesized that GSK3β catalytic activity is necessary for p53 activity. Surprisingly, we did not observe any decrease in p53 regulated genes, p21 and MDM2 by GSK3β inhibitors (data not shown). GSK3β regulates p21 protein level by phosphorylating p21 and causing p21 proteasomal degradation [53]. The observation that GSK3β inhibitors do not block p53 regulated gene expression such as p21 and MDM2 argues against our hypothesis. However, another possibility is that p53 requires an interaction with GSK3β and GSK3β catalytic activity in response to DNA damage to bind to C/EBPα promoter and initiate C/EBPα transcription but does not require GSK3β for other p53 regulated genes.

In response to DNA damage, p53 is post-translationaly modified, stabilized, and its transcriptional activity is increased [54]. p53 can induce cell cycle arrest, cellular senescence or apoptosis in response to DNA damage [54]. Recently, several studies have suggested a role for GSK3β in DNA damage response pathway and suggested a link between GSK3β and p53 in response to DNA damage [44, 45]. For example DNA damage induces nuclear localization of GSK3β and increases its catalytic activity [44]. Nuclear GSK3β forms a complex with p53 in response to DNA damage, activates p53 and induces expression of p53 regulated gene p21 and GSK3β also induces apoptosis [44, 45]. These authors also suggested that this induction in p53 activity is independent of GSK3β induced phosphorylation of p53 [44]. This DNA damage can induce GSK3β and p53 interaction not only in nucleus but also in mitochondria and this complex induces apoptosis [45]. Turenne and Price (2007) suggested GSK3β can phosphorylate p53 at S33 and this event increases the transcriptional activity of p53 [52]. Also, during senescence p53 interacts with GSK3β in

nucleus [55]. Recently it was also shown that GSK3β interacts with p53 and induces its acetylation at L373 and L382 [56]. It is possible that one of the above mechanisms is involved in regulating C/EBPα through p53/GSK3β complex formation in response to UVB treatment.

Although we have provided pharmacological evidence for the involvement of GSK3β in C/EBPα induction, we were not successful in providing the molecular evidence using siRNA targeting GSK3β. We were able to successfully knock down about 70% of the GSK3β protein but we did not observe any decrease in GSK3β kinase activity. It is possible that the remaining GSK3β protein is enough to maintain the kinase activity of GSK3β. The other possibility of not seeing any effect of GSK3β knock down on C/EBPα induction is that the other isoform of GSK3, GSK3α is involved in this pathway. LiCl and SB 415286 can inhibit kinase activity of both GSK3α and GSK3β isoforms and the phenotype that we see may involve GSK3α. It will be interesting to knock down GSK3α in BALB/MK2 cell line and look at C/EBPα induction in response to DNA damage. Another possibility is that GSK3α and GSK3β are compensating for each other. In this case knocking down both GSK3α and GSK3β will be more revealing.

We provide evidence that UVB induces post-translational modification to C/EBPα protein. The entire protein is modified and shifted to a slower migrating band as detected by PAGE electrophoresis. Known post-translational modifications of C/EBPα protein include phosphorylation [48, 57, 58], sumoylation [49, 50] and ubiquitination [59]. We are not sure if C/EBPα post-translational modification is mediated by GSK3β, ATM/ATR or Chk1. C/EBPα is phosphorylated on T222, T226 and S230 by Glycogen synthase kinase-3 (GSK3)

[48]. Recently, it has been shown that phosphorylation of C/EBPα at T222/T226 is essential for metallothionein (MT) gene transactivation [57]. NetPhos 2.0 Server analysis of C/EBPα amino acid sequence suggests consensus site for ATM, Chk1 and p38 kinase. As these kinases are involved in DNA damage response pathway, they may phosphorylate C/EBPα and can alter its activity or binding ability with other cell cycle proteins. C/EBPα has been shown to interact with cell cycle proteins such as p21, CDK2/CDK4, E2F, Rb, SWI/SNF complex and induce cell cycle arrest in many cell types [17-24]. It will be interesting to examine C/EBPα binding ability to cell cycle proteins in response to DNA damage. Understanding more about C/EBPα regulation and C/EBPα target proteins involved in G_1 checkpoint response will provide us with novel candidates that can be targeted to reduce genomic instability and ultimately cancer incidence.

Material and Methods

Cell lines and cell culture - BALB/MK2 keratinocytes (a gift from Dr. Weissman, University of North Carolina Chapel Hill, NC) were plated in 60 mm tissue culture dish. Cells were cultured in Ca^2-free Eagle's minimal essential medium (EMEM; BioWhittaker) supplemented with 8% Chelex-treated fetal bovine serum (FBS; Invitrogen), 4 ng of human epidermal growth factor (hEGF)/ml (Invitrogen), and 0.05 mM calcium. Cells were treated with UVB or other DNA damaging agents upon reaching 70% confluence.

UVB and chemical treatment - The UV lamp (model EB 280C; Spectronics) used for treating cells emits wavelengths between 280 and 320 nm with a spectrum peak at 312 nm. The intensity of light emitted was measured by NIST Traceable Radiometer Photometer (Model IL1400A, International Light). Cells were treated with UVB as described by Yoon and Smart [34]. Another DNA damaging agent used to treat cells was N-Methyl-N'-nitro-N-nitrosoguanidine (MNNG; Sigma Aldrich) which was dissolved in DMSO. GSK3β inhibitors, lithium chloride (LiCl; Sigma Aldrich) was dissolved in water and SB 415286 (Torcis) compound was dissolved in DMSO. Cells were pretreated with GSK3β inhibitors for 1 h. Subsequently media was removed, cells were treated with UVB or MNNG, incubated again with the inhibitors, and cells were harvested at different time points.

Preparation of cell lysates - Cells were washed with cold PBS (Phosphate Buffer Saline) and harvested by scraping subsequently cells were collected by centrifugation and resuspended in radio-immunoprecipitation assay (RIPA) buffer [48]. Cells were lysed by sonication after incubating on ice for 20 minutes. Lysates were centrifuged at 12,000 g for

10 min at 4°C. Supernatants were stored at -80°C until use. Protein concentration was determined using the Bio-Rad protein assay reagent.

Western blot analysis - Equal amounts (10 μg) of protein from cell lysates were loaded onto a 12% polyacrylamide Tris-glycine gels (Invitrogen) or 7.5% gels and separated by electrophoresis. The separated proteins were transferred to an Immobilon-P membrane (Millipore). Following incubation in blocking buffer, the membranes were probed with rabbit polyclonal immunoglobulin G (IgG) raised against C/EBPα (1:2000, sc-61), p53 (1:2000, sc-6243), C/EBPβ (1:2500, sc-150), p21 (1:2000, sc-757), GSK3β (1:2000, sc-9166) (Santa Cruz Biotechnology), or mouse monoclonal IgG raised against α tubulin (1:1000, sc-8035), GSK3α/β (1:2000, sc-7291) (Santa Cruz Biotechnology) or β-catenin (1:2000,BD Transduction Laboratories, 610153) and then probed with a horseradish peroxidase-linked secondary antibody (1:2500, Amersham). Bound antibody was detected with an enhanced chemiluminescence reagent (Perkin Elmer life Science) followed by exposure of membrane to the film.

Protein stability - BALB/MK2 cells were treated with UVB (10 mJ/cm^2). After 12 h of UVB treatment, cells were incubated with 50 μg/ml cycloheximide (Sigma Aldrich). Cells were harvested at various time points, and cell lysates were subjected to Western blot analysis.

Quantitative real time PCR - Total RNA was isolated from control, UVB treated and UVB + GSK3β inhibitor treated BALB/MK2 cells using TRI reagent (Sigma) and then purified by RNeasy Mini Kit (Qiagen). cDNA was prepared from 50 ng RNA by ImProm-II Reverse Transcription System (Promega) following the manufacturer's protocol. cDNA was

used to perform Quantitative RT-PCR using mouse C/EBPα TaqMan Gene Expression Assays, 18S TaqMan Gene Expression Assays (Applied Biosystems) and TaqMan Universat PCR mix (Applied Biosystems). All reactions were performed in ABI Prism 7000 Sequence Detection System. Expression levels for all the genes were normalized to the endogenous control 18S. Data were analyzed using comparative CT method.

Co-immunoprecipitation - 100 μg of whole cell extracts were brought to a volume of 500 ml in ELB buffer (.25M NaCl, .1% NP-40, 50mM HEPES pH7.0, 1mM PMSF, 5mM EDTA, .5mM DTT). Extracts were then precleared by rotating with 0.125 μg normal rabbit serum and 20 μl Protein A/G beads (SC-2003, Santa Cruz) at 4^{o}C for 30 minutes. Precleared supernatants were then incubated for 2 h with 2 μg p53 antibody (SC-6243, Santa Cruz) or 2 μg normal rabbit serum at 4^{o}C. 40 μl Protein A/G beads were added to the protein-antibody mixture, and were incubated overnight at 4^{o}C. Supernatants were discarded, and beads were washed 3 times with ELB buffer. Beads were then re-suspended in 20 μl SDS sample buffer and boiled for 5 minutes. Samples were then loaded onto 10% tris glycine gels for protein separation by gel electrophoresis. Proteins were transferred to an Immobilon-P membrane (Millipore), incubated in blocking buffer, and probed for GSK3α/β (SC-7291, Santa Cruz) or p53 (SC-6243, Santa Cruz) antibody. Membranes were washed, and probed with a horseradish peroxidase-linked secondary antibody (1:2500, Amersham). Bound antibody was detected using chemiluminescence reagent (Perkin Elmer life Scienve) followed by exposure of membrane to a film.

siRNA treatment - siRNA targeting GSK3β (MSS226317, GGAAGUCAGUUAUACAG ACACGAAA) + (Cat # MSS226318,

GGUAGCAUGAAAGUUAGCAGAGAUA) or GFP (negative control, GGUGCGCUCCUGGACGUAGCCTT) were purchased from Invitrogen. When BALB-MK2 cells were 25-30% confluent in 6-well plate, they were transfected with DharmaFECT 1 transfection reagent (Cat # T-2001-0, Dharmacon, Inc.) according to manufacturer's instructions in presence of 100 nM siRNAs. Cells were incubated with siRNAs for 72 h. Afterwards cells were treated with UVB, harvested at indicated time points and western blot analysis was conducted to determine the efficiency of knock down.

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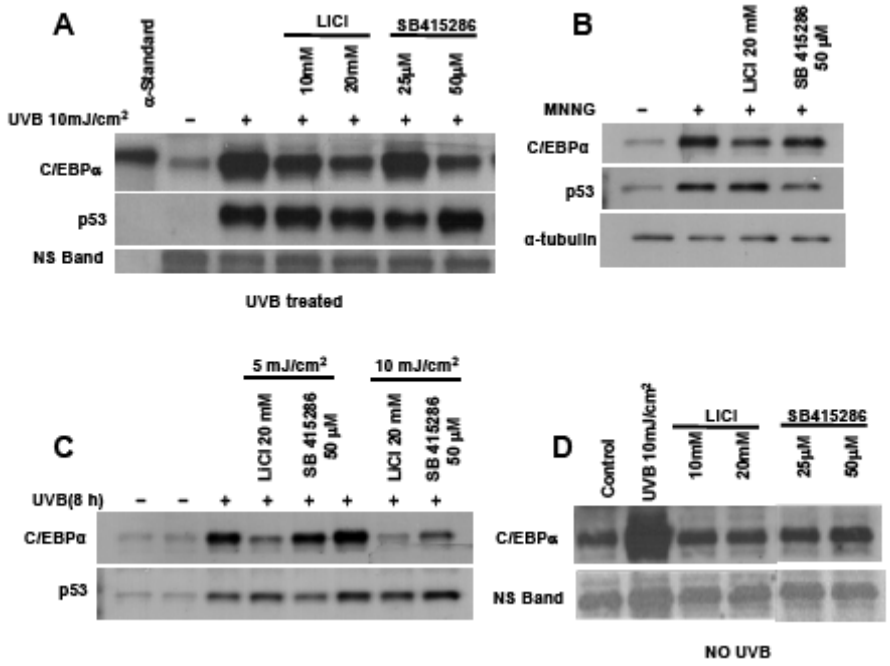

Figure. 1. GSK3β inhibitors block C/EBPα induction in response to DNA damage. A) BALB/MK2 keratinocytes were pretreated with LiCl 10 or 20 mM and SB 415286 25 or 50 μM for 1 h, then media was removed and cells were exposed to UVB (10 mJ/cm^2) and again incubated with GSK3β inhibitors for next 12 h, cell lysates were prepared and immunoblot analysis was conducted. Non-specific (NS) band is shown to confirm equal loading. B) BALB/MK2 keratinocytes were pretreated with LiCl 20 mM and SB 415286 50 μM for 1 h, and then either DMSO or MNNG was added to the media of respective plates, after 12 h cell lysates were prepared and immunoblot analysis was conducted. C) BALB/MK2 keratinocytes were pretreated with LiCl 20 mM and SB 415286 50 μM for 1 h, then media was removed and cells were exposed to UVB (5 or 10 mJ/cm^2) and again incubated with GSK3β inhibitors for next 8 h, cell lysates were prepared and immunoblot was analysis conducted. D) BALB/MK2 keratinocytes were treated with LiCl 10 or 20 mM and SB 415286 25 or 50 μM for 12 h, cell lysates were prepared and immunoblot analysis was conducted.

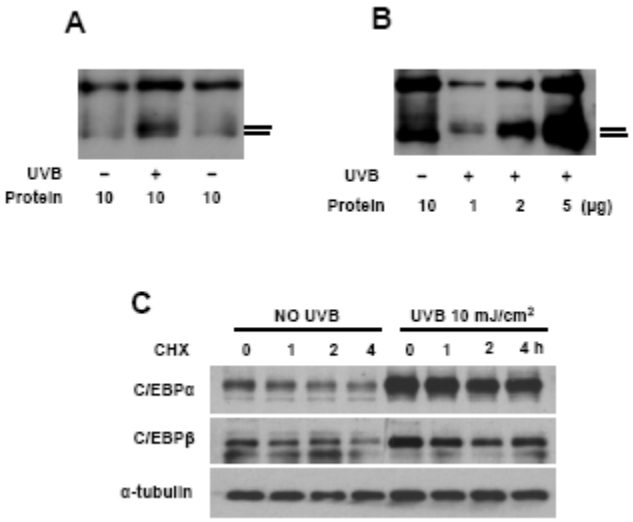

Figure. 2. UVB induces post-translational modification to C/EBPα protein but does not alter protein stability. A) BALB/MK2 keratinocytes were exposed to UVB (10 mJ/cm^2) and 2 h after exposure cell lysates were prepared and immunoblot analysis conducted. Two lines indicate C/EBPα protein bands with or without mobility shifts. B) BALB/MK2 keratinocytes were exposed to UVB (10 mJ/cm^2) and 12 h after exposure cell lysates were prepared. For immunoblot analysis 10 µg protein lysate from non-treated samples and 1, 2 or 5 µg of protein lysate from UVB treated sample were used. Two lines indicate C/EBPα protein bands with or without mobility shifts. C) BALB/MK2 keratinocytes were either not treated (left panel) or treated (right panel) with UVB (10 mJ/cm^2) and 8 h later were incubated with cycloheximide (50 µg/ml). Cells were harvested at indicated time points after the start of cycloheximide treatment and immunoblot analysis was conducted.

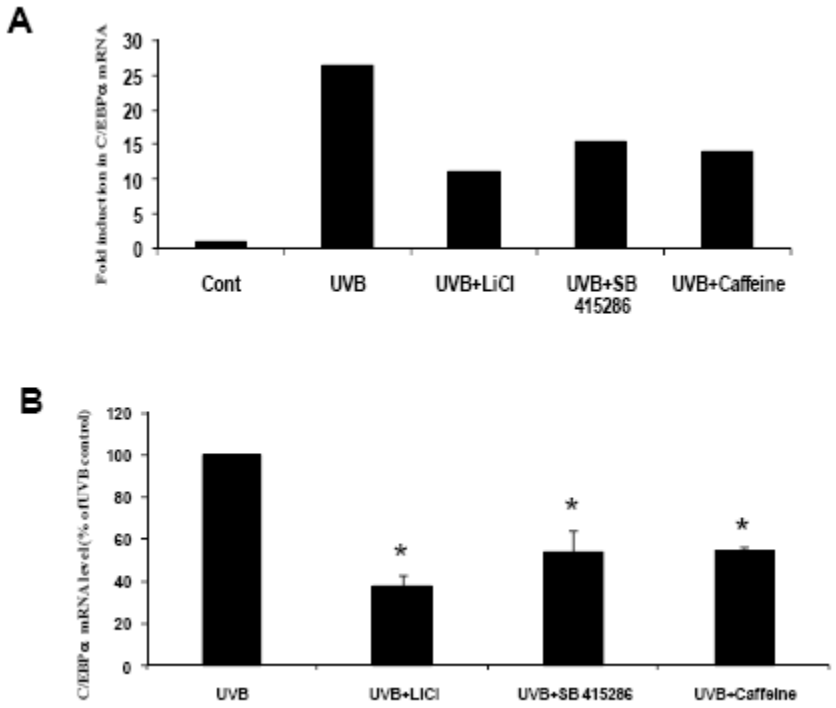

Figure. 3. GSK3β inhibitors block UVB induced increase in C/EBPα mRNA. A) BALB/MK2 keratinocytes were pretreated with LiCl 20 mM and SB 415286 50 μM for 1 h, then media was removed and cells were exposed to UVB (10 mJ/cm^2) and again incubated with GSK3β inhibitors for next 12 h. Then total RNA was isolated, cDNA was made and quantitative RT–PCR was conducted for C/EBPα and 18 S mRNA levels. Data was normalized to 18 S and analyzed using the comparative CT method. A) Representative bar graph from one of the three independent experiments and each experiment was run in triplicate. B) Average induction by UVB from three independent experiments is presented as 100% and average for each group is presented as % of UVB induction. Data is expressed as mean ± standard error (N=3) and each experiment was run in triplicate. *Significantly different from time zero ($p < 0.05$) as determined by Student's t-test.

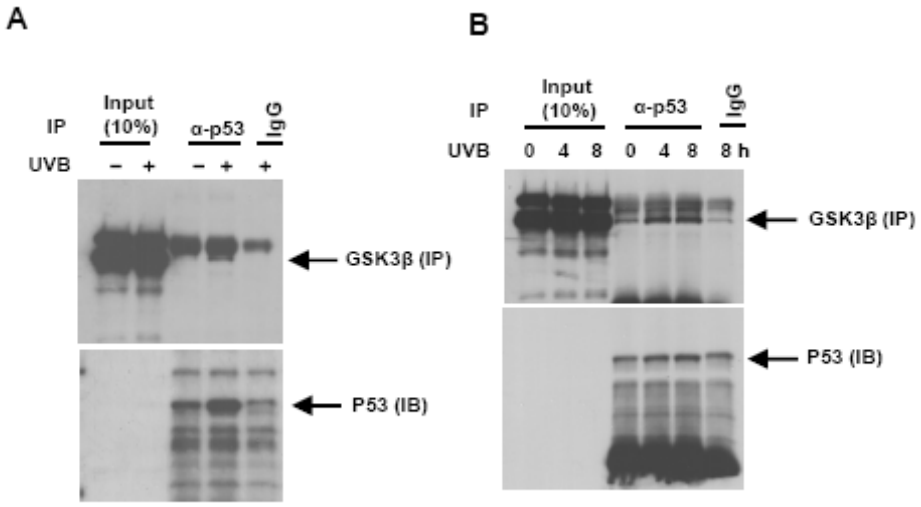

Figure. 4. UVB induces GSK3β and p53 complex formation. A) BALB/MK2 cells were treated with single dose of UVB (10 mJ/cm^2) and cells were harvested after 12 h (A) or 4 and 8 h (B) of treatment. Cell lysates were immunoprecipitated with p53 antibody or IgG by overnight incubation in the presence of protein A/G beads. Beads were washed with ELB buffer, and were resuspended in SDS buffer; boiled for 5 min and loaded on 10% gel. Western blot was conducted with GSK3α/β monoclonal antibody or p53 antibody.

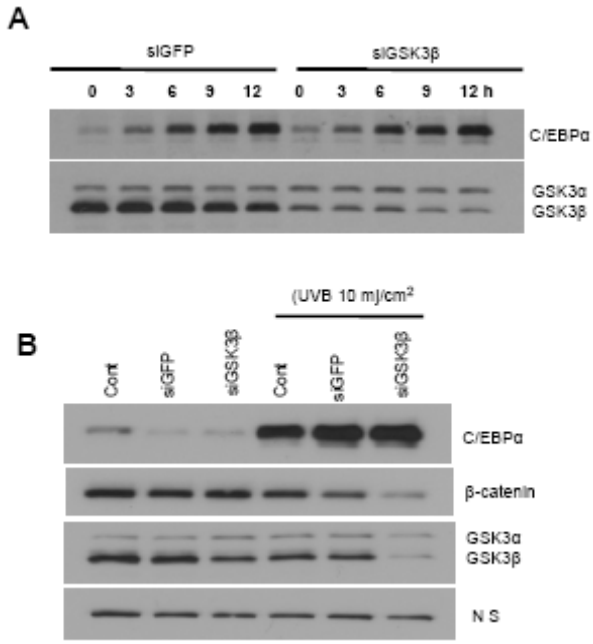

Figure. 5. GSK3β knock down by siRNA has no effect on C/EBPα induction in response to DNA damage. A) BALB/MK2 keratinocytes were transfected with GSK3β siRNA or GFP siRNA 100 nM for 72 h. Then cells were exposed to UVB (10 mJ/cm^2) and were harvested at indicated time points. Cell lysates were prepared and immunoblot analysis was conducted. B) BALB/MK2 keratinocytes were either transfected or not with GSK3β siRNA or GFP siRNA 100 nM for 72 h. Then cells were exposed to UVB (10 mJ/cm^2) and harvested after 12 h. Cell lysates were prepared and immunoblot analysis was conducted. Non-specific (NS) band is shown to confirm equal loading.

GENERAL DISCUSSION

Cell signaling is a cascade of complex pathways involving different groups of proteins that regulate numerous cellular functions such as cellular metabolism, cellular proliferation and cell cycle regulation. Transcription factors play a central role in maintaining cellular homeostasis. Regulation of transcription factors such as C/EBPs, which regulate genes involved in various cellular functions such as metabolism, cellular differentiation, DNA damage response, and tumorigenesis are very important for proper cellular homeostasis [127].

Systemic C/EBPα deficient mice die before or shortly after birth due to abnormalites in energy metabolism [147]. Recently, our lab developed genetically engineered mice lacking C/EBPα specifically from the epidermis [179]. These mice did not show any abnormality in skin development or differentiation suggesting C/EBPα is dispensable for skin differentiation. This may be because of compensation by C/EBPβ for the loss of C/EBPα in the skin. Interestingly, when epidermis specific C/EBPα deficient mice were subjected to two stage chemical carcinogenesis experiment, the epidermis specific C/EBPα deficient mice show dramatic increases in tumor incidence and size compared to wild type mice. The most interesting part of this experiment was higher rate of malignant progression of tumors in C/EBPα knockout mice [179]. These results suggested tumors in C/EBPα knockout mice were acquiring additional mutations at a faster rate, indicating genomic instability in C/EBPα knockout mice tumor. Alterations in G_1 checkpoint response that allow cells with damaged DNA to enter cell cycle can lead to heritable mutations, genomic

instability and ultimately cancer [5, 7]. Yoon and Smart (2004) suggested a role for C/EBPα in the DNA damage G_1 checkpoint response using a siRNA approach [194]. However, the genetic evidence to confirm the result was still lacking. We are the first to provide genetic evidence that C/EBPα is involved in the DNA damage G_1 checkpoint. As mentioned in the introduction, C/EBPα expression is greatly diminished in many squamous cell carcinomas as well as in basal cell carcinoma. Diminished expression of C/EBPα may lead to impaired G_1 checkpoint in the cell, as a result cells with damaged DNA will enter cell cycle and mutations will accumulate at higher rate in the cell. Due to the increased rate of mutation, cells will acquire a growth advantage resulting in accelerated tumor progression. Thus loss, diminished expression, or mutation of C/EBPα may be a kind of mutator phenotype that destabilizes the genome leading to uncontrolled cell growth and ultimately cancer. Hence, C/EBPα may work as a tumor suppressor gene through maintaining genomic integrity. Any activity/treatment that can reduce the rate of genetic changes or mutation will help in preventing cancer incidence [281]. Hence, better understanding of the pathways impinging upon induction of C/EBPα in response to DNA damage may be a potential therapeutic target to be explored to control tumorigenesis.

Studies in literature suggest involvement of C/EBPα in cell cycle arrest associated with differentiation of adipocytes [145, 146], myeloid cells [140, 141] and type II pneumocytes [159]. C/EBPα has been shown to induce cell cycle arrest in many cell types using forced expression studies [141, 145, 153, 282]. Overexpression studies can be misleading as the protein expression in these studies are not at physiological relevant level

and makes the interpretation of results complicated. To our knowledge no study, before us, has used C/EBPα deficient cells to characterize role of C/EBPα in G_1/S transition in response to mitogen stimuli. G_1/S transition plays important role in differentiation pathway as cell cycle arrest precedes differentiation. Role of C/EBPα in cell cycle arrest and G_1/S transition may be one of the pathway through which C/EBPα regulates cellular differentiation in different cell types.

As mentioned in the introduction, there are many proposed mechanisms through which C/EBPα induces cell cycle arrest involving cell cycle proteins including Rb family members [187, 283], CDK4 and CDK2 [184], E2F [190, 192], p21 [180], and the SWI/SNF chromatin remodeling complex [193], but the definitive answer is still lacking. The model system that we have developed to study C/EBPα role in G_1/S transition and DNA damage G_1 checkpoint using C/EBPα$^{-/-}$ fibroblasts can be a useful tool to elucidate the exact molecular mechanism involved.

In chapter 1, we documented that C/EBPα induction in response to DNA damage is not limited to just one cell type but can also be induced in fibroblasts and human melanocytes (unpublished data) and have extended our knowledge about C/EBPα's response to various types of DNA damage. In keratinocytes, C/EBPα induction is dependent on p53, however, in fibroblasts, C/EBPα induction does not require p53. We have also documented a novel pathway of C/EBPα regulation by C/EBPβ in response to DNA damage in fibroblasts. Thus C/EBPα induction is not only cell type specific, but also involves different signaling pathways in different cell types which may or may not require p53.

Skin cancer is one of the most common form of cancer present worldwide [284]. In mouse skin C/EBPα acts as a tumor suppressor gene [179] whereas C/EBPβ is required for tumorigenesis as mice lacking C/EBPβ are refractory to tumorigenesis in two stage chemical carcinogenesis experiments [285]. Hence C/EBPα and C/EBPβ appear to have opposing roles with regard to skin carcinogenesis. But, this is not always the case as C/EBPβ regulates C/EBPα expression during adipogenesis [195, 200, 202]. We have shown in Chapter 1 that C/EBPβ is a positive regulator of C/EBPα in response to DNA damage and is bound to C/EBPα promoter. Expression of C/EBPβ gene from C/EBPα locus is sufficient to reverse the phenotype in C/EBPα knockout mice [286]. Recently Ewing et. al. (2008) showed C/EBPβ functions in the DNA damage response pathway, suppresses p53 to promote cell survival in mouse skin [287]. Here we show that C/EBPβ is activated by DNA damage response pathway and increases C/EBPα expression, but the exact mechanism through which DNA damage increases C/EBPβ activity is not clear. It is generally accepted that C/EBPβ is present in a repressed state and different stimuli derepress C/EBPβ [219, 220]. It is possible that one of the kinases involved in DNA damage response pathway phosphorylates C/EBPβ and increases its activity. We have preliminary data suggesting C/EBPβ is a substrate for Chk1 in response to DNA damage (unpublished data). Still, biological significance of DNA damage induced C/EBPβ activation is not clear and more research is required. In C/EBPβ null fibroblasts C/EBPα induction was not completely ablated suggesting some other pathway is also involved in C/EBPα induction. One possibility is that p53 is compensating for the lack of C/EBPβ and regulating C/EBPα expression in C/EBPβ knockout fibroblasts.

Studies involving p53 and C/EBPβ double knock out fibroblasts will be helpful in answering this question.

In chapter 2, we tried to better understand the signaling pathway that leads to C/EBPα induction in response to DNA damage in keratinocytes. We found GSK3β inhibitors block C/EBPα induction in response to DNA damage suggesting GSK3β's involvement in C/EBPα's induction. In addition, we found UVB induces post-translation modifications to C/EBPα protein. In our opinion GSK3β has dual effect on C/EBPα, one at the message level and another at the protein level. GSK3β likely regulates C/EBPα mRNA expression in response to DNA damage by forming a complex with p53. GSK3β/p53 complex activates p53 and increase C/EBPα expression at message level. One possibility is that at protein level GSK3β phosphorylates C/EBPα and increases C/EBPα ability to interact with cell cycle proteins and induce cell cycle arrest and that is how C/EBPα works as a tumor suppressor gene. In support of this, recently GSK3β has been reported to be involved in the DNA damage response [271, 272] and also in skin tumorigenesis [288, 289]. The role of GSK3β in DNA damage response and in regulation of p53 has provided a new platform to study this kinase. GSK3β was originally thought to be involved in phosphorylation and regulation of glycogen synthase enzyme [248, 249]. Recently, GSK3β has been implicated in a tumor suppressive role in skin tumorigenesis [288-290]. In two-stage chemical carcinogenesis experiment and also in human skin cancer tissues, inactivation of GSK3β has been reported [288, 289]. GSK3β expression is decreased in human cutaneous squamous cell carcinoma and basal cell carcinoma [290]. An *In vitro* study has shown that re-expression of GSK3β

suppresses transformation of mouse epidermal JB6 P+ cells (promotion sensitive cells) in response to epidermal growth factor (EGF) and 12-O-tetradecanoylphorbol-13-acetate (TPA) [290]. Treatment of cells with GSK3β inhibitors promote cellular transformation [290]. Recently, one study with GSK3β inhibitors and siRNA against GSK3β has provided evidence for involvement of GSK3β in maintaining genomic stability [291]. Treatment of HeLa cells with GSK3β inhibitors and siRNA against GSK3β, induce genomic instability by chromosomal missegregation during mitosis [291]. In addition to skin, GSK3β acts as a tumor suppressor gene in mammary tumors [292]. Lastly, GSK3β's role has been suggested not only in tumorigenesis but also in cancer cell metastasis [293, 294].

Based on above mentioned studies GSK3β activity appears to be decreased in mouse and human skin cancer [288, 289]. If GSK3β activity is decreased it will not be able to increase C/EBPα expression through p53 and could explain why we observe less expression of C/EBPα in skin tumor samples. Although C/EBPα has been shown to be substrate for GSK3β, no functional significance of C/EBPα phosphorylation has been reported except one study suggesting phosphorylation of C/EBPα by GSK3β increases C/EBPα ability to bind DNA [211]. More research needs to be done to elucidate the functional significance of C/EBPα phosphorylation by GSK3β.

In summary, future research work should focus on understanding the mechanism through which C/EBPα mediates the G_1/S transition and the DNA damage G_1 checkpoint. From studies in literature it appears, a promising avenue is determining the ability of C/EBPα to bind to cell cycle proteins in response to mitogen stimulus as well as DNA

damage. In addition, understanding the fate of cells that enter the cell cycle with damaged DNA in C/EBPα$^{-/-}$ fibroblasts is also important. It will be interesting to determine the viability of cells in response to DNA damage in wild type and C/EBPα$^{-/-}$ fibroblasts. Lastly, it needs to be determined in case of C/EBPα$^{-/-}$ fibroblasts, whether cells move faster into S-phase of the cell cycle or if there are just more cells entering into S-phase.

Using siRNA targeting GSK3β, we did not observe any effect on C/EBPα protein expression in response to UVB treatment in keratinocytes. This observation suggests two possibilities; i) GSK3β knock down is not efficient enough to show the phenotype, or ii) GSK3β inhibitors are blocking activity of non-target proteins. Hence, very important and basic questions remain to be answered in order to make firm conclusions about the involvement of GSK3 in C/EBPα regulation in response to DNA damage. In my opinion knocking down GSK3α alone or in a combination with GSK3β will be more revealing.

GSK3β phosphorylation of C/EBPα may be important in regulating the G_1/S transition as well as the DNA damage G_1 checkpoint. Growth factors activate the PI3K/AKT cell survival pathway and this pathway is frequently altered in cancer [295, 296]. Activation of P13K/AKT pathway will lead to inhibition of GSK3β activity [252, 290, 297] and as a result, C/EBPα will not be phosphorylated and will not be able to bind cell cycle proteins and induce cell cycle arrest. Hence, it is important to determine if GSK3β phosphorylation to C/EBPα protein is relevant in cell cycle regulation and tumorigenesis.

Lastly, an important direction of research will be investigating if C/EBPα is important in maintain genomic integrity and whether loss of C/EBPα leads to genomic

instability and ultimately tumorigenesis. Since C/EBPα is involved in DNA damage G_1 checkpoint, cells may enter cell cycle with damaged DNA and may result in accumulation of more mutations, which might lead to genomic instability and ultimately cancer. Determining if loss of C/EBPα results in increased rate of mutation and chromosomal/genomic instability is a potential area of research.

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