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Role of Pkc Delta in Uv Radiation Dna Damage Repair

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ROLE OF PKC DELTA IN UV RADIATION DNA DAMAGE REPAIR

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVATIONS

UV: Ultraviolet radiation

NMSC: Non-melanoma skin cancers

BCC: Basal cell carcinoma

SCC: Squamous cell carcinoma

CDKN2A: Cyclin-Dependent Kinase Inhibitor 2A

MLL3: Mixed-lineage Leukemia protein 3

RB: Retinoblastoma

PREX2: Phosphatidylinositol-3, 4, 5-Trisphosphate-Dependent Rac Exchange Factor 2

HPV: Human papilloma virus

HIV: Human immunodeficiency virus

CPDs: Cyclobutane pyrimidine dimers

6-4 PP: 6-(1, 2)-dihydro-2-oxo-4-pyrimidyl)-5-methyl-2, 4-(1H, 3H) photoproducts

NER: Nucleotide excision repair

XP: Xeroderma Pigmentosum

ATR: Ataxia telangiectasia and Rad3-related protein

GG-NER: Global genomic nucleotide excision repair

TC-NER: Transcription coupled nucleotide excision repair

DDB2: DNA-damage binding factor 2

XPC: Xeroderma Pigmentosum complementation group C

XPC-RAD23B-CENT2: Xeroderma Pigmentosum complementation group C, UV excision repair protein RAD23 homolog B, Centrin 2

CSA: Cockayne syndrome proteins A

CSB: Cockayne syndrome proteins B

XPA: Xeroderma Pigmentosum- A

TFIIH: Transcription initiation factor II H

RPA: Replication protein A

PCNA: Proliferating cell nuclear antigen

USP7: Ubiquitin specific protease 7

PKCδ: Protein kinase C delta

DAG: Diacylglycerol

PI (4, 5) P2: Phosphatidylinositol 4, 5-bisphosphate

PLCγ: Phospholipase C γ

PLCβ: Phospholipase C β

Ara-c: 1-[beta-D-arabinofuranosyl] cytosine

CF: Catalytic fragment

Tyr: Tyrosine

Mcl-1: Myeloid leukemia cell differentiation protein 1

c-Abl: Abelson murine leukemia viral oncogene homolog 1

MAPK: Mitogen activated protein kinases

Btf: Bcl-2-associated transcription factor

CDKN1B: Cyclin-dependent kinase inhibitor 1 B

ATM: Ataxia telangiectasia mutated

TAD: Transcription activation domain/transactivation domain

DBD: DNA binding domain

TET: Tetramerization domain

CTD: C-terminal domain

Mdm2: Mouse double minute 2 homolog

Lys: Lysine

YY1: Yin Yang 1

EHMT1: Euchromatic histone-lysine N-methyltransferase 1

H3K9: Histone 3 Lys 9

Mdm4/MdmX: Mouse double minute 4 homolog/Mouse double minute X homolog

DNA-PK: DNA-dependent protein kinase

Chk2: Checkpoint kinase 2

Chk1: Checkpoint kinase 1

Tip-60: Tat-interactive protein 60

hMOF: Males absent on the first

PCAF: P300/CBP-associated factor

HDAC: Histone deacetylase

SIR2: Silent information regulator 2

SET8: SET-domain containing protein 8

Smyd2: SET and MYND domain containing 2

PRMT: Protein arginine methyltransferase

FBXO11: F-Box protein 11

CDK: Cyclin-dependent Kinase

PTPRV: protein tyrosine phosphatase receptor type V

Gadd45a: Growth arrest and DNA-damage-inducible alpha

MSH2: MutS protein homolog 2

AP: Apurinic/apyrimidinic

DSB: Double strand breaks

HR: Homologous recombination

BLM: Bloom syndrome associated helicase

PIDD: p53-induced death domain

DEC1: Differentiated embryo-chondrocyte expressed gene 1

PML: Promyelocytic leukemia protein

PTEN: Phosphatase and tensin homologue

Map4: Microtubule-associated protein 4

MEFs: Murine embryonic fibroblasts

NHEK: Normal human epidermal keratinocytes

WT: Wild type

RefSeq: Reference Sequences

6-TG: 6-Thioguanine

Hprt: Hypoxanthine phosphoribosyltransferase

rpS3: Ribosomal protein S3

Pol η: Polymerase eta

BPDE: Benzo[a]pyrene diol epoxide

TPA: 12-O-tetradecanoylphorbol-13-acetate

KLF4: Kruppel-like transcription factor

EGF: Epidermal growth factor

ROS: Reactive oxygen species

PTCH1: Patched 1

PDGFRB: Platelet-derived growth factor receptor beta

TRAIL: TNF-related apoptosis-inducing ligand

PRL-3: Phosphatase of regenerating liver 3

ABL1: Abelson murine leukemia viral oncogene homolog

CHAPTER ONE

INTRODUCTION

1.1 Skin Cancer and Ultraviolet Radiation DNA Damage

Skin is one of the vital organs of our body and act as a protective barrier from the external world. Because of its primary barrier function, the skin is exposed to environmental carcinogens, such as ultraviolet radiation (UV), which can lead to the malignant transformation of several skin cell types [1]. UV is divided into three wavelengths, UVA (315-400 nm) is the longest wavelength, which is penetrates deep into the dermis of the skin and is primary responsible for photoaging and actinic elastosis [2, 3]. UVB (280-315 nm) wavelength is shorter than UVA and is absorbed by the epidermis of the skin causing reactive oxygen species (ROS), immunosuppression as well as DNA damage. UVB is most carcinogenic and principal cause of skin cancer [2, 4, 5]. The shortest wavelength UVC (100 -280 nm) is a high energy UV and can cause substantial DNA damage [2, 6]. The majority of the UVC and some of the UVB is absorbed by ozone layer, thus the solar UV which reaches the earth's surface is about 95% UVA and 5% UVB [6-9].

In part, due to its environmental exposure, skin cancer is the most common type of cancer in United States [10]. Skin cancer can be divided into malignant melanomas and

non-melanoma skin cancers (NMSC). With an estimated 3.5 million cases each year, NMSC put a large burden on our healthcare system [11]. NMSC are further subdivided into basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) [12, 13]. Basal cell carcinoma is believed to arise from the hair follicle stem cells. Homozygous loss of function of tumor suppressor gene *PATCHED 1* (PTCH1) and activation of hedgehog signaling, or overexpression of transcription factors Gli1/Gli2 in the hair follicle stem cells induces BCC [14-17]. About quarter of a million new cases of SCC are diagnosed every year [18]. SCC originates from the interfollicular epidermis to form squamous differentiating cancers of keratinocytes [19]. In cutaneous SCC, tumor cells are believed to arise in the keratinocyte stem cells localized to the basal layer of the interfollicular epidermis [20]. Aggressive forms of SCC involve multiple genetic alterations such as mutated *HRAS, TP53,* Cyclin-dependent kinase inhibitor 2A (*CDKN2A*), *NOTCH1* and Mixed-lineage leukemia protein 3 (*MLL3*) [21].

Melanoma is the third form of skin cancer and it is derived from the pigment producing melanocytes which are present in the basal layer of epidermis. With poor prognosis and high incidences of metastasis, melanoma is the most deadly of the three skin cancer subtypes [22]. Skin cancers, including melanoma have a higher incidence of mutations than most other cancers and the majority of these mutation are caused by UV radiation. The most prominent driver mutations in melanoma are in *BRAF,* Neuroblastoma Ras Viral (*V-Ras*) Oncogene Homolog (*NRAS*), Retinoblastoma (*RB*), Phosphatidylinositol-3, 4, 5-Trisphosphate-Dependent Rac Exchange Factor 2 (*PREX2*) and *TP53* [23-25]. UV radiation from the sun is the prevalent environmental cause of skin

cancer [26-28]. Apart from sunlight, tanning beds and other forms of UV lamps are common sources of exposure to artificial UV [29, 30].

In addition to UV, therapeutic ionizing radiation or occupational exposure to radiation can cause NMSC. Skin cancers can be also caused by exposure to hazardous chemicals such as arsenic or polycyclic hydrocarbons [31, 32]. Organ transplant recipients who are on immunosuppressive medications have high risk of developing skin cancers, including fatal metastatic melanomas [33]. Infection with Human papilloma virus (HPV) [34], Human immunodeficiency virus (HIV) [35] or Merkel cell polyomavirus [36] in immunosuppressed patients is also linked to skin cancer development.

Exposure to UV radiation is dangerous because UV radiation causes DNA damage by creating DNA adducts such as dimers between adjacent pyrimidine residues of DNA. These adducts are called as cyclobutane pyrimidine dimers (CPDs) and 6-(1, 2)-dihydro-2-oxo-4-pyrimidyl)-5-methyl-2, 4-(1H, 3H) photoproducts (6-4 PP). ROS are generated in the cells exposed to UV radiation as well as a byproduct of cell metabolism, and this creates oxidative stress-induced cyclopurines [37-44]. All of these DNA adducts, if not repaired, can give rise to mutation by the activity of trans-lesion DNA polymerases. The Y family trans-lesion polymerases seldom utilize high fidelity while synthesizing new strands and can polymerize through the damaged bases, introducing mutation in the process [45]. This event is very dangerous because mutations in tumor suppressor genes like TP53 can promote skin cancer development [46, 47]. All types of skin cancers exhibit such UV specific mutations, for example, more than 58% of invasive squamous cell carcinoma show *TP53* mutations [24, 48-50]. It has been observed that CPDs are more

mutagenic than 6-4 PP after UV-induced DNA damage, thus CPD are the most carcinogenic UV-induced DNA adducts [51].

1.2 Nucleotide Excision Repair

UV-induced DNA adducts are repaired by a DNA damage repair pathway called nucleotide excision repair (NER). It is a multi-step pathway employing a large number of proteins [43]. When this repair pathway is impaired, the DNA damage is not repaired completely, leading to development of large number of disorders, including cancers, neurodevelopment and photo-sensitivity disorders [52]. For example, Xeroderma Pigmentosum (XP) patients have a defect in XP complementary group components leading to extreme UV sensitivity and skin cancers [53]. Additionally, expression of NER components was reduced in head and neck SCC samples [54]. Multiple melanoma cell lines also displayed defects in Ataxia telangiectasia and Rad3-related protein (ATR) mediated DNA damage repair signaling [55].

NER repairs the damage at two levels, the whole genome i.e. global genomic NER (GG-NER) and actively transcribing region i.e. transcription coupled NER (TC-NER) [56]. After UV inflicts damage in DNA, the damage is recognized and verified. In GG-NER, Xeroderma Pigmentosum complementation group C (XPC) and ultraviolet radiation DNA-damage binding factor 2 (DDB2) are the early DNA damage recognition proteins [57]. The damage is recognized by XPC-RAD23B-CENT2 (Xeroderma Pigmentosum complementation group C, UV excision repair protein RAD23 homolog B, Centrin 2) complex in co-ordination with DDB2 [57-60]. Whereas in TC-NER, RNA polymerase gets blocked by the distorted DNA adduct to trigger damage recognition. Cockayne

syndrome proteins A and B (CSA & CSB) form a complex, and are summoned when RNA polymerase II is stalled in the transcribing region. They engage DNA damage repair proteins such as Transcription factor II H (TFIIH) to the site of damage [61].

Henceforth, the GG-NER and TC-NER have similar steps of damage verification, excision, and the gap filling using a undamaged strand as a template. Ligation is done in the end to seal the nick [56]. DNA repair protein complementing Xeroderma Pigmentosum- A (XPA) is one of the important proteins in NER. XPA coordinates multiple proteins at damage verification and excision steps. That is why XPA is also termed as a 'rate limiting factor' of NER [62, 63]. XPA, TFIIH, Xeroderma pigmentosum-D (XPD) and Replication protein A (RPA) work together to verify the damaged and undamaged strand [64, 65]. Xeroderma pigmentosum-G (XPG) and Xeroderma pigmentosum-F (XPF) endonucleases are recruited to excise the damage by XPA or RPA [66, 67]. DNA polymerases, with the help from Proliferating cell nuclear antigen (PCNA), fill the gap with correct bases using a template strand. DNA ligases seal the nick [43, 56, 68, 69].

These NER DNA damage response proteins can be regulated by different factors and at different levels (i.e. transcriptional, post-translational). For example, early DNA damage recognition protein XPC is regulated by ubiquitination by DDB-ubiquitin ligase complex and de-ubiquitination by Ubiquitin specific protease 7 (USP7) [70, 71]. XPA protein is also heavily controlled by multiple factors. ATR phosphorylates XPA on serine 196 and physically interacts with XPA at lysine 188 to facilitate nuclear translocation of XPA. In the nucleus, ubiquitin ligase HERC2 ubiquitinates and degrades XPA in the

nucleus [72-75].The XPA- RPA activity is enhanced by deacetylation of XPA by Sirtuin-1 (SIRT1) [76].

Additionally, the tumor suppressor protein p53 is also involved in regulating NER proteins [53]. p53 is involved in the recruitment of proteins at the DNA damage site, such as XPC and TFIIH after DNA damage [77]. p53 is also involved in the DNA damage-induced gene expression of NER factors DDB2 and XPC [78-80]. Furthermore, cells harboring homozygous *TP53* mutation are defective in global genomic repair of CPD and 6-4 PP [81].

1.3 Protein Kinase C Delta (PKCδ)

Structure of PKCδ

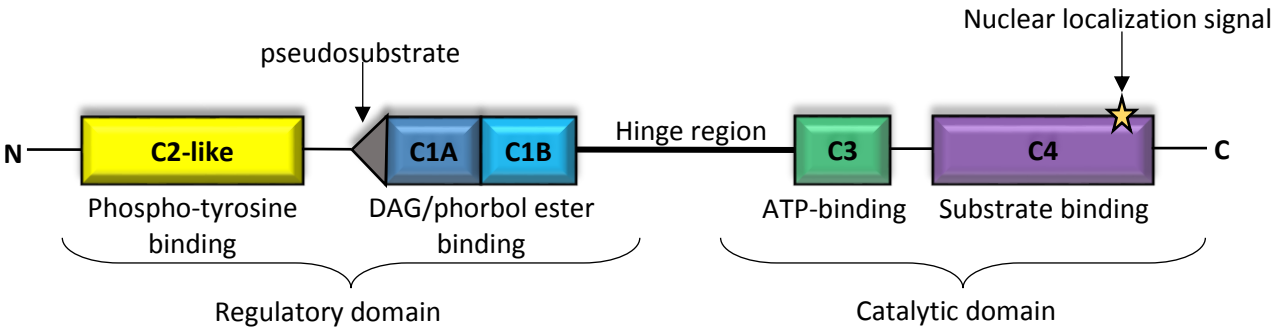


Figure 1. Domain Structure of PKCδ

PKCδ is a 78 kD serine/threonine calcium-independent protein kinase made up of a regulatory domain and a catalytic domain connected by a hinge region. The regulatory domain at the amino terminal contains two C1 regions, C1A and C1B one after the other, and a non-calcium binding-C2-like region. The C1 domains are involved in diacylglycerol (DAG) and phorbol esters binding. The C2-like region is a phosphotyrosine binding domain and implicated in protein-protein interactions. The catalytic domain at the carboxyl terminal contains a C3 region and a C4 region. The C3

region contains an ATP- binding site and the C4 region contains the PKCδ substrate binding site and a nuclear localization signal. The regulatory domain of PKCδ also contains a pseudo-substrate sequence which binds to the substrate binding part at the catalytic domain at C4 region [82-86]. This induces the catalytic domain to fold over the regulatory domain and this folded conformation is the inactive state of PKCδ.

1.3.1 Regulation of PKCδ

PKCδ can be activated by membrane bound diacylglycerol (DAG) binding to the C1 region. DAG is produced by hydrolysis of phosphatidylinositol 4, 5-bisphosphate [PI(4, 5)P2] by receptor tyrosine kinase activated Phospholipase C γ (PLCγ) or by G protein-coupled receptors activated Phospholipase C β (PLCβ). Furthermore, PKCδ can be activated by treatment with pharmacological analog of DAG such as phorbol esters. PKCδ is also activated by external stimuli such as UV, ionizing radiation and genotoxic agents like 1-[beta-D-arabinofuranosyl] cytosine (ara-c) [82, 86-94]. Upon activating stimuli such as UV, caspase 3 proteolytically cleaves full length PKCδ at the hinge region and produces a PKCδ active catalytic fragment (CF) of 40 kD [89, 95-98]. This PKCδ catalytic fragment is constitutively active and has been localized to both the mitochondria and the nucleus. A proteolytic cleavage resistant isoform of PKCδ has also been reported. The caspase 3 cleavage site structure at the hinge region is abrogated by insertion of new amino acid sequence in this splice variant. This change has decreases caspase 3 proteolytic sensitivity, resulting in a cleavage-resistant isoform of PKCδ variant [99].

Tyrosine phosphorylation is also a mechanism for PKCδ regulation which is independent of DAG-related activation. For example, H_2O_2 treatment induces PKCδ

phosphorylation at Tyrosine-311 (Tyr- 311), Tyr- 332 and Tyr-512 resulting in increased enzymatic activity [100]. PKCδ also auto phosphorylates itself at serine 643[101] and there is a correlation between tyrosine phosphorylation of PKCδ by Platelet-derived growth factor receptor beta (PDGFRB) [102] and increased PKCδ enzymatic activity. In contrast, tyrosine phosphorylation can be an inhibiting mechanism for PKCδ, such as phosphorylation by Src family kinases such as c-Src and c-Fyn [103-105]. Without physically associating with PKCδ, these membrane bound Src kinases inactivate PKCδ by tyrosine phosphorylation [104].

1.3.2 Functions of PKCδ

Apoptosis is one of the most effective tumor suppressive mechanisms to eliminate cells with mutations and to maintain genetic integrity of a tissue, when repair of DNA damage is no longer possible [46]. PKCδ has a major role in inducing apoptosis [106]. Generally, upon apoptotic stimuli, caspase 3 is activated and it cleaves full length PKCδ at the hinge region to create a 40 kD constitutively active catalytic fragment (PKCδ-cat) [91]. Also, PKCδ has been found working upstream of caspases. Our lab found that this PKCδ-cat can cause apoptosis by localizing to mitochondria, phosphorylating anti-apoptic protein Myeloid leukemia cell differentiation protein (Mcl-1) and thus decreasing Mcl-1 protein half-life [107]. The reduction of Mcl-1 promotes activation of the pro-apoptotic protein Bax and disruption of the outer mitochondrial membrane releasing cytochrome c and stimulating further activation of caspases [93, 108]. Tyrosine phosphorylation of PKCδ is required for etoposide-mediated activating cleavage of caspase 3 indicating a positive feedback loop [106, 109]. The PKCδ-cat is also sufficient for

apoptosis. Apoptosis can be induced if PKCδ-cat is expressed in the human keratinocytes [93]. Our lab has also shown that re-expression of full-length PKCδ in Ras-transformed HaCaT keratinocytes which have lost expression of PKCδ, induces apoptosis and suppresses tumorgenicity in nude mice [110]. Additionally, PKCδ induces apoptosis in keratinocytes through p38delta-ERK1/2 complex [111]. It has been observed that upon ionizing radiation-induced DNA damage, a p53-dependent apoptosis cascade is activated by Abelson murine leukemia viral oncogene homolog 1 (c-Abl) where PKCδ is found to be activated [112]. PKCδ also induces apoptosis in an endometrial cancer cell line and inhibits their transformation [113]. In some cases of PKCδ-dependent apoptosis is induced upon etoposide treatment [114], Fas-ligation and cytokine deprivation in T cells [115, 116]. PKCδ is also involved in activation of Mitogen activated protein kinases (MAPK) in 3T3-F442A cells as well as in salivary epithelial cells where PKCδ induces apoptosis via MAPK activation [117, 118]. PKCδ also induces apoptosis by activating topoisomerase IIα [119] and death promoting transcription factor Bcl-2-associated transcription factor (Btf) [120]. Additionally, apoptosis is also started by phosphorylation of Lamin B and p53 by PKCδ[121, 122]. Furthermore, PKCδ interacts with Abelson murine leukemia viral oncogene homolog 1 (ABL1) [123], TNF-related apoptosis-inducing ligand (TRAIL)/CDKN1A [124] and topoisomerase II α [119] to induce apoptosis. Thus, PKCδ is activated by a wide range of apoptotic stimuli and promotes apoptosis through multiple mechanisms.

PKCδ is also found to be involved in promoting cell cycle checkpoints. Upon DNA damage or acute stress-induced by chemical agents, radiation, or internal metabolic sources, the cell cycle is halted at various checkpoints. These cell cycle checkpoints are

vital to allow time for DNA damage repair and for mutation free survival of the damaged cell. Overexpression of PKCδ induces cell cycle arrest in late G1 phase in BALB/MK-2 mouse keratinocytes [125]. PKCδ overexpression in capillary endothelial cells arrested the cells in S-phase. This arrest was mediated through Cyclin-dependent kinase inhibitor 1 B (CDKN1B) [126]. Furthermore, our lab has shown that PKCδ catalytic fragment is involved in the maintenance of G2/M cell cycle checkpoint after UV-induced DNA damage [127]. The Nishizuka lab found similar results in the Chinese hamster ovary cells (CHO) cells where PKCδ overexpressing CHO cells arrested at the G2/M after treatment with phorbol ester [127, 128]. Additionally we found that inhibiting Ataxia telangiectasia mutated (ATM)/ATR signaling in primary keratinocytes or HaCaT cells with the ATM/ATR inhibitor caffeine had no effect on PKCδ-cat-induced G2/M checkpoint, indicating that PKCδ is working downstream of ATM/ATR in the G2/M checkpoint[127].

While analyzing human SCC samples, previous members of our lab found that the PKCδ is lost at the mRNA and protein levels in about 30% of human SCCs, but the PKCδ gene was not deleted [110]. Additionally, PKCδ expression is also found to be decreased in endometrial tumor samples, and the reduction was associated with high tumor grade [129]. Furthermore, rat fibroblast cells displayed increased 12-O-Tetradecanoylphorbol-13-acetate (TPA)-induced transformation when PKCδ was depleted [130] and reduction in tumorigenicity was observed when PKCδ was transduced in the human colon cancer cells [131]. PKCδ is found to be decreased in colon carcinoma cells and tumors developed in nude mice when xenografted with PKCδ knocked down

colon carcinoma cells [132, 133]. Taken together, these observations support a tumor suppressive function for PKCδ and call for further investigation of this kinase

1.4 p53

1.4.1 Structure of p53

p53 is a 53 kD tumor suppressor protein encoded by the *TP53* gene located on the short arm of chromosome 17 [134]. p53 acts as a transcription factor for a large number of genes spanning diverse functions including cell cycle arrest, apoptosis, senescence, autophagy and DNA damage repair [135, 136]. p53 is a 393 residues protein made up of transcription activation domain/transactivation domain (TAD) (residues 1-61) and a proline rich domain (residues 64-92) at the N-terminus. The DNA binding domain (DBD) (residues 94-293) is situated in the center followed by a nuclear localization signal domain (residues 312-323), tetramerization domain (TET) (residues 326-355) and a regulatory C-terminal domain (CTD) (residues 363-393) at the C-terminus. The TAD domain is further subdivided into TAD1 and TAD2 [135, 137]. The TAD is involved in transcription activation as well as repression through binding to transcription factors and co-activators, such as TATA-binding protein [138-140]. The proline rich domain contains five PXXP (P-proline, X-any amino acid) motifs [141] and is involved in apoptosis and oxidative stress response [142]. The p53 DNA binding domain (DBD) is made up of immunoglobulin-like-β sandwich which binds to major and minor grooves of particular DNA response elements [143]. The nuclear localization signal next to the DBD, enables p53 to translocate to nucleus and interact with DNA or transcription machinery proteins [144]. The TET domain is involved in building of p53 tetramer which is essential for its activation and function, and this tetramer formation is independent of presence or absence of DNA [137, 145]. These tetramers, two at a time, can bind to one DNA response

elements at the same time [146]. Usually the CTD is modified by post-translational modifications depending on internal or external stimuli [147]. The CTD regulates the function of p53 by controlling binding of DBD to specific DNA response elements [148]. The CTD does that by creating steric hindrance with the conformation of p53 protein or binding to long segments of non-specific DNA, which prevents binding of DBD to specific sequences of DNA [149, 150].

1.4.2 Regulation of p53

p53 regulation is thoroughly supervised because in the absence of an inhibitory regulator, p53 is embryonic lethal [151]. p53 activity is supervised on two levels; regulating p53 protein level and regulating the p53-mediated transcription of target genes. First the E3 ubiquitin ligase Mouse double minute 2 homolog (Mdm2) is the major negative regulator of p53 protein levels. Under normal DNA damage and stress free cellular conditions, p53 levels are kept low by Mdm2 [152]. Mdm2 binds and poly-ubiquitinates the TAD and CTD of p53 protein and transports p53 from nucleus to the cytoplasm where p53 is degraded through the ubiquitin-mediated proteosomal degradation pathway. Thus, lack of Mdm2 causes accumulation of the p53 protein [153-156]. In the CTD, more than 6 lysine (Lys) residues of p53 are targets for ubiquitination and degradation by Mdm2. Ubiquitination requires multiple Lys residues for successful ubiquitination of p53 [157, 158]. This regulation is controlled by a negative feedback mechanism where activated p53 activates transcription of Mdm2 by binding to its promoter region. Increased Mdm2 levels in turn inhibit p53 [159]. This negative regulation is disrupted by inhibitory binding of p19Arf (ARF) to Mdm2 resulting in activation of p53

[160]. Opposing this, Yin Yang 1 (YY1) creates a complex with Mdm2 to promote p53 degradation [161]. Additionally, Mdm2 in complex with nuclear matrix associated protein Scaffold/matrix-associated region-binding protein 1 (SMAR1) recruits Histone deacetylase 1 (HDAC1) to deacetylate and thus converts p53 to a less active form [162]. Furthermore, Mdm2 via NEDD8 E3 ligase promotes NEDDylation of p53 on Lys 370, Lys 372 and Lys 373 and this modification impede p53 activity [163].

On the other hand, after DNA damage, p53 needs to be activated and stabilized. Phosphorylation of Mdm2 by ATM on serine 395 [164] or by c-Abl on tyrosine 394 [165] inhibits Mdm2 and promotes p53 accumulation. In response to the ribosomal stress, p53 activation is induced by inhibition of Mdmd2 by ribosomal proteins L5, L11 and L23 [166-168]. Furthermore, Mdm2 also self-ubiquitinates itself to control its levels in the cells [169].

p53 governs the transcription of large number of target genes [135]. Mdm2 also inhibit transcription of p53 target genes by binding to the N-terminus of p53 and restricting its role as a transcription factor [170]. For example, Mdm2 competes with transcriptional coactivators such as p300 for binding sites located in the p53 TAD. Transcription of p53 target genes is thus abrogated because their coactivators cannot bind to p53 anymore [171]. Additionally, Mdm2 creates a complex with Euchromatic Histone-Lysine N-Methyltransferase 1 (EHMT1) to inhibit p53 target gene transcription by methylating histone 3 Lys 9 (H3K9) on promoters of p53 target genes as well as mono-methylating p53 at Lys 373 [172]. Mdm2 independent ubiquitin ligases, such as COP1 and Pirh2, supervise p53 regulation by degrading p53 through the ubiquitin-

mediated proteosomal degradation pathway [173, 174]. Mdm2 is assisted by the Mdm2 splice variant Mouse double minute 4 homolog (Mdm4/MdmX). MdmX lacks the E3-ubiquitin ligase activity and thus cannot degrade p53, but it can bind to p53 and help Mdm2 to bind to p53. MdmX is termed as 'negative regulator of p53' and lack of MdmX induces aberrant apoptosis in embryonic neuro-epithelium [175, 176].

Upon acute stress or DNA damage, p53 needs to be activated rapidly in order to start transcription of its downstream genes, followed by repression when its role is fulfilled. For this quick response, p53 is regulated by numerous post-translational modifications, such as phosphorylation, acetylation, ubiquitination, neddylation, methylation and sumoylation [155]. Numerous p53 phosphorylation sites have been discovered spanning the TAD, DBD and CTD. Upon DNA damage, p53 is phosphorylated by DNA-dependent protein kinase (DNA-PK) [177] or by ATR [178] on serine 15 and serine 37 or phosphorylated by ATM [179] on serine 15 in the TAD. These are p53 stabilizing phosphorylations. By creating Mdm2-binding-resistant conformation changes in the p53 structure, these phosphorylations protect p53 from Mdm2-mediated degradation [177][178][179]. Additionally, DNA damaging agents such as UV also induce phosphorylation of p53 by Checkpoint kinase 2 (Chk2) on serine 20; Casein 1-like kinase (CK1) on threonine 18 [180] or on Serine 6 and 9 [181]; PKCδ on serine 46 [122], Jun NH-2 terminal kinase (JNK) on threonine 81 [182], and p38 kinase on serine 33 and serine 46 [183]. Furthermore, after DNA damage, Checkpoint kinase 1 (Chk1) and Chk2 phosphorylate the CTD of p53 on serine 366, serine 378 and threonine 387, and these phosphorylations are important for promoting acetylation (Lys 382) of p53 on CTD [184].

Apart from phosphorylation, p53 is acetylated on multiple sites. Mdm2 cannot bind to acetylated p53, thus acetylation and deacetylation are used as a switch to turn on or turn off p53 activity quickly [185]. Furthermore, acetylation is a crucial step for p53-mediated target gene transcription [186] and induction of apoptosis [187]. DNA damage induces Lys 120 acetylation in the DBD of p53 by ATM downstream effectors Tat-interactive protein 60 (Tip-60) or by Males absent on the first (hMOF) and promotes p53-mediated apoptosis [188, 189]. Coactivator p300 acetylates p53 on 6 different lysines [292, 305 [190], 370, 372, 373, 381 [191], and 382 [192]] spanning the DBD, TET and CTD. Additionally, p300 in complex with CBP acetylates p53 at Lys 164 which prevents Mdm2 binding and p53 degradation [187]. Lys 320 in the nuclear localization signal of p53 is acetylated by P300/CBP-associated factor (PCAF) resulting in increased DNA binding [192]. Conversely, Histone deacetylase (HDAC) deacetylates p53 to make it more vulnerable for Mdm2 binding and subsequent degradation and reduces p53 target gene transcription [193, 194]. Similarly, Silent information regulator 2 (SIR2) protein family deacetylases Sir2α and SIRT1 also deacetylates p53 residues and decrease p53 functions such as DNA damage induced apoptosis and target gene activation [195, 196].

Apart from acetylation, p53 lysines are also targeted for methylation and neddylation. Methylation at different sites of p53 yields different outcomes. p53-mediated gene activation is inhibited by mono-methylation of p53 on either Lys 382 by SET-domain containing protein 8 (SET8) [197] or at Lys 370 by methyltransferase SET and MYND Domain Containing 2 (Smyd2) [198]. The methyltransferase enzyme Set9 mono-methylates p53 on Lys 372, where this methylation protects p53 from repressing

methylation by Smyd2 (Lys 370) as well as confines p53 to the nucleus, where p53 activates target gene transcription [199]. Protein arginine methyltransferase (PRMT) 5 methylates p53 on arginine (Arg) 333, 335 and 337 in p53-mediated DNA damage responses [200]. F-Box protein 11 (FBXO11) represses p53-mediated target gene activation by NEDDylating p53 on Lys 320 and Lys 321 [201].

1.4.3 Functions of p53

p53 protects cells from oncogenic transformation by implementing various anti-proliferative programs such as inducing cell cycle checkpoints, promoting DNA damage repair and promoting apoptosis or senescence as a final resort, to protect the integrity of the genome. Upon DNA damage, p53 triggers reversible arrest of cell cycle at various checkpoints so that the damage can be repaired. Upon DNA damage, p53 transcriptionally activates p21 which inhibits CDK to execute G1 checkpoint arrest [202]. Additionally, p53-dependent activation of Phosphatase of regenerating liver-3 (Prl-3) [203] and protein tyrosine Phosphatase receptor type V (PTPRV) [204] induce G1 cell cycle arrest. p53 also imposes G2/M cell cycle checkpoint after UV-induced DNA damage via activation of Growth arrest and DNA-damage-inducible alpha (Gadd45a) as well as upregulation of 14-3-3σ [205, 206].

As p53 arrests damaged cells at various checkpoints, it also assist in repair of the damage by participating in NER, mismatch repair, base excision repair non-homologous end joining and homologous recombination. As mentioned previously, p53 regulates NER DNA damage repair proteins such as XPC, TFIIH [77] and DDB2 [79, 80, 207]. In mismatch repair, p53 preferentially binds to insertion/deletion mismatch DNA structures [208]and

interacts with DNA mismatch repair protein MutS protein homolog 2 (MSH2) [209]. Additionally, in complex with c-Jun, p53 initiates transcription of MSH2 upon UV-induced DNA damage [210]. p53 is also involved in base excision repair of DNA damage, where lack of p53 decreases base excision repair of methyl methanesulfonate-induced damage [211]. This decrease in repair may be because p53 is found to be interacting with apurinic/apyrimidinic (AP) endonuclease as well as enhancing interaction between DNA polymerase β and AP DNA [212]. Furthermore, p53 can facilitate non-homologous end joining of double strand breaks (DSB) [213]. This activity was attributed to the affinity of p53 CTD towards non-specific DNA strands [214]. p53 actively contributes to homologous recombination (HR) where it interacts with many HR proteins such as, RAD51, RAD54 [215] and Bloom syndrome associated helicase (BLM) [216]. Furthermore, p53 inspects HR fidelity and halts HR in case of mismatches [217, 218].

When DNA damage cannot be repaired completely, for a mutation-free genome, p53 induces apoptosis [219]. p53-mediated apoptosis is carried out by the ability of p53 to act as a transcription factor i.e. transcription-dependent, or transcription-independent activities. In the transcription-dependent apoptosis pathway, upon external apoptotic stimuli, cellular stress, unrepairable DNA damage, genotoxin treatment or hypoxia [220-222], p53 is activated and translocate to nucleus, where it activates transcription of apoptotic proteins such as Bax [223], Bid [224], p53 upregulated modulator of apoptosis (Puma) [225], Noxa [226] and p53-induced death domain (PIDD)[227]. p53 also induces transcription of microRNA-34a (mir-34a) which is responsible for apoptosis [228]. Additionally, p53 can also activate transcription of death

receptors such as KILLER/DR5 [229] or Fas [230, 231]. Furthermore, apoptosis is also induced via p53-mediated activation of caspase 6 [232] and caspase 9 [233]. In the transcription-independent apoptosis pathway, activated p53 translocates to mitochondria and interacts with Bcl_{XL} to release cytochrome c and compromises the integrity of the mitochondrial outer membrane [234]. p53 also activate caspase 8 which in turn, activates pro-caspase 3 to induce apoptosis [235, 236].

Senescence is another p53-mediated anti-cancer activity where the senescence is induced upon replicative stress, oncogene activation or genotoxic exposure such as chemotherapy agents [237]. Cellular senescence induced by telomere shortening is also mediated through p53 where ATM/ATR kinases induce constant cell cycle arrest [238]. Activation of oncogenes such as *Ras* activates p53 to induce either ARF-dependent senescence [239], or activates transcription of Differentiated embryo-chondrocyte expressed gene 1 (DEC1) which activates senescence [240]. Additionally, Ras activates Promyelocytic leukemia protein (PML) which induces acetylation of p53 and formation of a p53-CBP-PML complex which generates senescence in the cells [241]. Tumor suppressor inactivation can also induce senescence, for example inactivation of Phosphatase and tensin homologue (PTEN) generates p53-dependent senescence [242].

1.4.4 Mutations in *TP53*

Unfortunately, the *TP53* anti-cancer gene is susceptible to enormous numbers of mutations, where more than 10,000 somatic mutations have been reported [243]. The majority of the *TP53* mutations have been reported in its DNA binding domain (mentioned in review hotspot mutations at R175, R248, R249, R273, R282 and G245),

where the presence of a mutation abrogates the ability of p53 to bind DNA and promote the transcription of p53 target genes. These mutations are divided into two types, “contact mutants” which have mutations in the residues which directly bind to the DNA, and “structural mutants” where mutation changes the structure of p53 in a way that it can no longer bind to the DNA [244-248].

Mutation in *TP53* can change its structure in a way that partner proteins cannot bind to it, or the affinity of p53 for a different set of protein increases, and this changes the transcriptional outcome. Mutant p53 can repress or activate genes abnormally. For example, the p53 repressed gene Microtubule-Associated Protein 4 (*MAP4*) is activated when p53 corepressor mSin3a removes p53 from promoter of *MAP4*. Mutations modify p53 so that the corepressor cannot bind to it and p53 remains on the promoter region of *MAP4* constantly repressing its transcription [249]. Similarly, mutant p53 binds to p63 and p73 and prevents transcription of its target genes [250]. Furthermore, the p53 TAD also associates with various transcription factors and promotes expression of genes which might advance oncogenic transformation such as PML which helps the proliferation of mutant *TP53* carrier cancer cells [251]. Additionally, gain-of-function mutant *TP53* can acquire certain characteristics which differ from its normal function, and thus is responsible for catastrophic biological outcomes such as inducing tumors [252], genomic instability by polyploidy [253], gene amplification [254] and non-reciprocal chromosome translocations [255]. p53 exists in tetrameric form. If mutation arises in one mutant allele, then 50% of the tetramer will be composed of mutant p53. This semi-mutant tetramer decreases the affinity of p53 for DNA binding especially [256-258] to

genes involved in apoptosis thus cells carrying mutant *TP53* are resistant to genotoxin-induced mediated apoptosis [259, 260].

1.4.5 UV Mutations of *TP53*

UV causes dipyrimidine site C--T substitution or CC--TT double base change or G:C-A:T transition mutations [261, 262]. UV-induced mutations have a particular sequence specificity and can be distinguished from other mutations. They are termed as UV signature mutations. Mutated *TP53* in skin cancer display this UV signature in mutations [263]. *TP53* is found to be mutated in 53% of actinic keratosis [264], 58% of cutaneous SCC [265] and 50% of BCC [266]. Additionally normal appearing epidermis was found to be harboring patches of *TP53* mutated cells where each clone measured about 60 to 3000 cells [267] with frequency of 40 cells per cm^2 [268]. Furthermore, with continuing UV exposure, the *TP53* mutant clones can invade nearby stem cell compartments [269]. It has been postulated that SCC originates in stem cells harboring early mutations, such as *TP53* mutations induced by UV. Lack of *TP53* reduces destruction of mutation carrier cells and these stem cells expand to produce pre-neoplastic clones. These cells, usually at the actinic keratosis level, proliferate and give rise to SCC [270-272].

Moreover, mice lacking *Tp53* spontaneously developed tumors [273]. Taken together, p53 fulfills its role as the 'guardian of the genome' by suppressing oncogenic transformation via multitude of mechanisms such as triggering cell cycle arrest after damage, aiding in DNA damage repair and inducing apoptosis or senescence if the repair fails.

SUMMARY

RATIONALE AND HYPOTHESIS

PKCδ is found to be lost or mutated in many cancers, including cutaneous SCC. PKCδ is activated by wide array of DNA damaging agents including UV. Upon UV radiation induced DNA damage, PKCδ is involved in the maintenance of G2/M cell cycle checkpoint arrest, while the damage repair machinery, such as NER, repair the damage. Cells lacking PKCδ are defective in maintaining the G2/M cell cycle checkpoint. Cell cycle checkpoints are linked to DNA damage repair. Therefore we hypothesize that cells lacking PKCδ will have a defect in repair of UV-induced DNA damage. We further wanted to investigate the mechanism behind the defective DNA damage repair. Being a major regulator of DNA damage repair as well as cycle checkpoints, p53 was a prime suspect for the investigation. Additionally, some published reports describe regulation of p53 by PKCδ [122, 274-276].

CHAPTER TWO

MATERIALS AND METHODS

2.1 Cell Culture

a. Cell Lines- In this project, we have used murine embryonic fibroblasts (MEFs), HaCaT cells and normal human epidermal keratinocytes (NHEK). We have wild type (WT) MEFs and PKCδ knockout MEFs (PKCδ null MEFs), which helped us to investigate DNA damage repair in the absence of PKCδ. These MEFs were obtained from Dr. Anning Lin, University of Chicago [277]. HaCaTs are spontaneously immortalized human keratinocytes with biallelic *TP53* mutations [278, 279]. MEFs and HaCaTs were grown in DMEM (Gibco) with 4.5g/L D-glucose, L-glutamine and without sodium pyruvate + 10% FBS +1% Penicillin/Streptomycin (Gibco). NHEKs from foreskins were obtained from the Loyola University Medical Center nursery and used below passage five. NHEKs were fed with Medium 154 CF (Gibco Invitrogen M-154CF-500) with 0.07 mM calcium and Human keratinocyte growth supplement kit (HKGS Kit S-001-K).

b. UV Exposure- Cells were irradiated with either UVB or UVC. Before irradiation, the cells were washed with PBS+ (PBS with calcium and magnesium. UV irradiation was carried out in the presence of PBS+ (volume used was similar to amount of media used). After UV irradiation, the cells were fed with warm media and incubated at 37 °C for the indicated time period. UVB was generated from a UV light box (Ultralite Enterprises Inc. Lawrenceville, GA, USA) (34% UVA, 65% UVB, 1% UVC) with broadband UVB bulbs (Light

Emission Tech FS36T12/UVB/ VHO). The UVB dose was measured using radiometer/photometer model IL 1400A (International Light Inc. Newburyport, MA, USA) with a UVB detector attachment. UVC was generated with Bio-Rad GS GENE LINKER (Five G8T5 bulbs installed. 8W each. >2 mW/cm^2 output).

2.2 Western Blotting

a. Protein was harvested after cells were lysed in lysis buffer (20 mM Tris-HCl pH 7.5, 1% Triton-X 100, 5 mM EDTA) with 22 µM sodium fluoride, 1 mM sodium orthovanadate, Complete Protease Inhibitor (Santa Cruz Biotechnology SC-29130 1X) and phosphatase inhibitor cocktail (Pierce phosphatase inhibitor mini tablets 88667SPCL, 1 tablet in 200 µL for 50X stock). Protein concentration was determined using Bradford protein assay. 40-50 µg of protein was loaded in each well and proteins were separated using the SDS-PAGE technique. Proteins were transferred to nitrocellulose membrane and protein transfer was confirmed by Ponceau-S stain. Membranes were blocked with Odyssey blocking buffer for 1 hour. Primary antibody was diluted in PBS+ 1:1 with Odyssey blocking buffer according to the recommendations of the manufacturer, and the membrane was stained overnight at 4 °C. Secondary antibody was diluted 1:10,000 in PBS+ 1:1 with Odyssey blocking buffer, and the membrane was stained for 1 hour. Membrane was washed with Tris buffered saline (TBS) (Bio-Rad Cat#170-6435) and TBS –T (TBS+ 0.05% Tween 20). The intensity of protein bands was detected using Odyssey LI-COR scanner.

b. Antibody Details-

Primary Antibody	Cat# /Company	Dilution	Species
p53	FL393 SC-6243 Santa Cruz	1:1000	Rabbit
p-p53 serine 15	9284 Cell Signaling	1:1000	Rabbit
Mcl-1	SC-56152 RC13 Santa Cruz	1:250	Mouse
Mcl-1	D35A5 5453 Cell Signaling	1:1000	Rabbit
Actin	691002 MP Biomedicals	1:5000	Mouse
p-MARCKS	2741 Cell Signaling	1:500	Rabbit
PKCδ	SC-937 Santa Cruz	1:1000	Rabbit
PKCδ	610397 BD Biosciences	1:1000	Mouse
XPC	SC-30156 Santa Cruz	1:100	Rabbit
XPA	12F5, MC-340 clone	1:500	Mouse
Secondary antibody	**Cat# /Company**	**Dilution**	**Species**
IgG, anti-Mouse 680	A21057 Molecular Probes Alexa	1: 10,000	Mouse
IgG, anti-Mouse 800	610-131-121 IR Dye	1: 10,000	Mouse
IgG, anti-Rabbit 680	A21076 Molecular Probes Alexa	1: 10,000	Rabbit
IgG, anti-Rabbit 800	611-131-122 IR Dye	1: 10,000	Rabbit

2.3 Immunofluorescence

a. Cells were plated on flame sterilized glass coverslips (Fisherbrand 12-541-B 22x22-1.5) and used for immunofluorescence.

Micropore Filters- 5 µm TMTP micropore filters (Millipore, ISOPORE Membrane Filters Catalog number TMTP02500) were used to induce DNA damage at selective parts of a cell. Cultured cells on coverslips were washed with PBS+ and a drop of PBS+ was left on the coverslip. Micropore filter was gently placed on the coverslip with help of flame

sterilized forceps. After irradiation with UVC, the micropore filter is gently removed without scrapping on the cells on the coverslip.

Cells were fixed with either Acetone-methanol (1:1) (20 minutes at 20 °C) or 3.7% formaldehyde in PBS+ (10 minutes at room temperature). Cells were permeabilized using 0.1% Triton-X 100 in PBS+ (10 minutes at room temperature). For CPD staining, cells were treated with antigen retrieval chemicals (see below). Normal goat serum in PBS+ 1:20 was used to block the non-specific binding of antibody. Blocking was done for one hour at room temperature in a humidor. Primary antibody was diluted according to manufacturer's recommendations in 1:20 normal goat serum in PBS+. The staining was done for 1-2 hours in a humidor. Secondary antibody was diluted in 1:20 normal goat serum and PBS+ at dilution of 1:400. Staining was done for 1 hour followed by counterstaining the DNA for 10 minutes with 300 nM DAPI stain. Coverslips were washed with FA buffer (Difco FA Buffer BD Biosciences) between antibodies for 30 times, changing solution after every 10^{th} wash. After washing, the coverslips were mounted on slides in gelvatol. Staining was observed in microscope (Olympus AX80 Fluorescent Microscope) and photographs were taken using a Retiga-4000R monochrome digital camera (QImaging).

b. CPD Antigen Retrieval- CPD staining requires denaturing of the chromatin, so the anti-thymine dimer antibody can reach the CPD adduct and recognize the epitope. For this purpose, we used 2 M HCl (for 30 minutes) or NaOH (70mM in 70% ethanol in PBS+; followed by 0.1% Triton-X 100 in FA buffer). After that coverslips were washed multiple times with PBS+ to remove residual chemicals prior to blocking and antibody staining.

c. Antibody Details-

Primary Antibody	**Cat# /Company**	**Dilution**	**Species**
Thymine Dimer	H3, NB600-1141 NovusBio	1:500	Mouse
Secondary antibody	**Cat# /Company**	**Dilution**	**Species**
IgG, anti-Mouse 488	A11001 Molecular Probes Alexa	1: 400	Mouse
IgG, anti-Mouse 594	A31623 Molecular Probes Alexa	1: 400	Mouse

d. Processing of Images- Pictures were taken using QIMAGING RETIGA 4000R FAST 1394 cooled Mono 12-bit camera. The camera is interfaced to a Precision T3400 computer workstation loaded with Olympus CellSens image capture software. Images were processed with Adobe Photoshop software. ImageJ software was used to quantify intensity of fluorescence. Fluorescence signal of each cell was quantified manually. A circle was drawn around nucleus and the area was kept constant for all the cells in the sample. ImageJ measures the fluorescence in the selected circle. Blank reading was used to subtract the background fluorescence.

2.4 Flow Cytometry

a. Cells were trypsinized and resuspended in PBS- (PBS without calcium and magnesium). Cells were fixed with ice-cold 100% ethanol on ice for 30 minutes. For antigen-retrieval, cells were incubated with 2 M HCl in 0.5% Triton-X 100, for 30 minutes. The acid was neutralized using 0.1 M Borax pH 9. Washing with 2 mL PBS-T (0.5% Tween-20 in PBS-) was done after every antibody staining. Primary antibody was diluted in 1% BSA in PBS-T at 1:200. Staining with primary antibody was done for 1 hour at room temperature. Secondary antibody was diluted in 1% BSA in PBS-T (0.5% tween-

20 in PBS-) at 1:400. Staining with secondary antibody was done for 1 hour at room temperature.

b. Antibody Details-

Primary Antibody	**Cat# /Company**	**Dilution**	**Species**
Thymine Dimer	H3, MC-062 Kamiya Bio	1:200	Mouse
Secondary antibody	**Cat# /Company**	**Dilution**	**Species**
IgG, anti-Mouse 488	A11001 Molecular Probes Alexa	1: 400	Mouse

c. Data Analysis Using FlowJo- Data files were opened in FlowJo and the cell population of interest was selected. Geometric mean of fluorescence from each sample (about 10,000- 30,000 cells) was calculated. Those values were used as measurement of CPD levels present in each sample. The amount of fluorescence was selected on X- axis versus number of cells on Y-axis to create histograms.

2.5 Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

a. Primer Details- All primers were ordered from Integrated DNA Technologies. GAPDH, *Gadd45a*, *XPC*, *DDB2*, *Tp53* and *PKCδ* primers were mouse species because they were used in MEFs samples. NCBI Reference Sequences (RefSeq) was used as reference to create specific primers. 'http://genome.ucsc.edu/' website's In-Silico PCR tool was used to test the selected primers before ordering. Two sets of primers (batch 1 and batch 2) were used to avoid off target effects.

Primer name	Sequence	Tm °C	GC content
Gadd45a BATCH 1 FORWARD	5’-TGC TGC TAC TGG AGA ACGAC-3’	56.9	55%
Gadd45a BATCH 1 REVERSE	5’TCC ATG TAG CGA CTT TCC CG-3’	57	55%
Gadd45a BATCH 2 FORWARD	5’-CTG CTG CTA CTG GAG AAC GA-3’	56.7	55%
Gadd45a BATCH 2 REVERSE	5’-ACC CAC TGA TCC ATG TAG CG-3’	56.9	55%
GAPDH BATCH 1 FORWARD	5’-GCG ACT TCA ACA GCA ACT CC-3’	56.7	55%
GAPDH BATCH 1 REVERSE	5’-CCC TGT TGC TGT AGC CGT AT-3’	57.1	55%
GAPDH BATCH 2 FORWARD	5’-ATG TGT CCG TCG TGG ATC TG-3’	57	55%
GAPDH BATCH 2 REVERSE	5’-GTG TAG CCC AAG ATG CCC TT-3’	57.5	55%
PKCδ BATCH 1 FORWARD	5’-AGG AAA CAT CAG GAT TCA CCC C-3’	56.9	50%
PKCδ BATCH 1 REVERSE	5’-GTT GCT GTA GTC TGA AGG GGA-3’	56.5	52.4%
PKCδ BATCH 2 FORWARD	5’-CAG GAA ACA TCA GGA TTC ACC C-3’	55.7	50%
PKCδ BATCH 2 REVERSE	5’-AGT TGC TGT AGT CTG AAG GGG-3’	56.5	52.4%
p21 BATCH 1 FORWARD	5’-GCT GTC TTG CAC TCT GGT GT-3’	57.7	55%
p21 BATCH 1 REVERSE	5’-TGG GCA CTT CAG GGT TTT CT-3’	56.9	50%
p21 BATCH 2 FORWARD	5’-TTG CAC TCT GGT GTC TGA GC-3’	57.5	55%
p21 BATCH 2 REVERSE	5’-AGA CCA ATC TGC GCT TGG AG-3’	57.6	55%
p53 BATCH 1 FORWARD	5’-ACT TGA TGG AGA GTA TTT CAC CCT-3’	55.5	41.7%
p53 BATCH 1 REVERSE	5’-TCT GTA GCA TGG GCA TCC TTT-3’	56.5	47.6%
p53 BATCH 2 FORWARD	5’-CCT CTC CCC CGC AAA AGA AA-3’	57.7	55%
p53 BATCH 2 REVERSE	5’-GGG CAT CCT TTA ACT CTA AGG C-3’	55.5	50%

b. mRNA was isolated using TRIZOL RNA extraction technique. RNA concentration and quality was determined using a spectrophotometer (Thermo Scientific ND 1000 NANODROP). cDNA was prepared using 1 µg of RNA, cDNA synthesis kit (iScript cDNA synthesis kit 1708891) and thermal cycler (Applied Biosystem 2720). Bio-Rad iTaq Universal SYBR Green Supermix was used with above created cDNA. QuantStudio 6 qRT-PCR instrument with Fast 96 well PCR plates (AXYGEN PCR MICROPLATE PCR-96-LP-AB-C) were used for PCR reaction. Comparative ΔΔCT was calculated by QuantStudio 6 flex system. *GAPDH* was used as a housekeeping control and all the values of mRNA were normalized to *GAPDH*.

2.6 CPD ELISA

a. The CPD ELISA was carried out using the protocol recommendations from Cosmo Bio Co LTD. A 96 well microplate (Costar 3590) was coated with 70 µl 0.003% protamine sulfate solution in distilled water and incubated at 37 °C overnight. Each well was washed 3 times with 100 µL of distilled water. DNA was harvested using QIAGEN DNeasy Blood and Tissue kit 69504. DNA concentration and quality were determined using a spectrophotometer (Thermo Scientific ND 1000 NANODROP). DNA was heat denatured at 100 °C for 10 minutes and chilled rapidly on ice for 15 minutes. Two hundred ng DNA diluted in 50 µl was loaded on the 96 well plate coated with protamine sulfate incubated at 37 °C overnight. Blocking was performed using 2% FBS in PBS- (150 µl) for 1 hour. Hundred µl primary antibody was diluted in PBS- and incubation was done for 2 hours at 37 °C. Biotinylated horse anti-Mouse IgG secondary antibody (Vectastain Elite PK- 6102 ABC kit, Vector Laboratories Inc.) was diluted in 5% BSA in PBS+ 1:200 and

incubated for 2 hours at 37 °C. Between antibodies, wells were washed 5 times with 150 µL of PBS-T (0.05% Tween 20 in PBS-). Peroxidase-Streptavidin reaction step was performed using ABC reagents from the Vectastain kit. Five µL Elite Reagent A was diluted in 250 µL of 0.1% Tween in PBS+. Five µL of Elite Reagent B was added to the diluted Reagent A. Solution was incubated for 30 minutes at room temperature. Hundred µL of ABC reagent was added to each well and incubated for 30 minutes at room temperature. Wells were washed 3 times with 150 µL of PBS-T. Seventy five µL of TMB substrate (BioLegend) was added to the wells and development of reaction was observed by development of color. The reaction was stopped by adding 50 µL of 1 M H_3PO_4. The plates were read at 450 nm (minus 570 nm for wavelength correction) and values were plotted in graph in Microsoft Excel.

b. Antibody Details-

Primary Antibody	**Cat# /Company**	**Dilution**	**Species**
Anti-Cyclobutane Pyrimidine Dimer	Clone TDM2 Cosmo Bio Co LTD	1:1000	Mouse
Secondary antibody	**Cat# /Company**	**Dilution**	**Species**
Biotinylated horse anti-Mouse IgG,	Vectastain Elite PK- 6102 ABC Kit, Vector Laboratories Inc.	1:200	Mouse

2.7 Mutagenesis Assay

a. To determine the optimum concentration of 6-Thioguanine (6-TG) for the endogenous resistance, WT and PKCδ null MEFs were plated 1 X 10^4/well in a 6-well plate. Increasing concentration of 6-TG (10 µM to 60 µM) was used to feed the MEFs through media. After 14 days, MEFs were fixed with 3.7% formaldehyde in PBS+ and stained with 0.05% crystal violet stain.

b. WT and PKCδ null MEFs were plated at an average of 5 X 10^5 cell/100 mm tissue culture plate and irradiated with UVB 5 mJ/cm^2 or UVC 1 mJ/cm^2. After they have recovered from the irradiation (24-48 hours), cells were replated for 6-TG treatment (preferably 3 X 10^5 cells/100 mm plate) as well as for colony forming efficiency (plating efficiency) (50 to 200 cells/ 60 mm plate). After 24 hours, the mutagenesis plates were continuously treated with 30 µM of 6-Thioguanine (6-TG) (Abcam Biochemicals ab142729). Colony forming efficiency plates were not treated with 6-TG. After 14 days, the cells were fixed with 3.7% formaldehyde in PBS+ and stained with 0.05% crystal violet stain. Visible colonies were counted and mutation rate was calculated from the number of 6-TG resistant colonies divided by plating efficiency.

2.8 Retroviral Transduction

a. Virus Details- Retrovirus was produced in Phoenix-Ampho retroviral packaging cell line by transfection of plasmids by calcium phosphate transfection method [108]. PKCδ was expressed as MYC-tagged full length PKCδ (LZRS-myc-PKCδ) [108, 280]. PKCδ was also produced in LZRS based retroviral vector as a pEGFP fusion protein (LZRS-pEGFP- PKCδ WT) based on constructs provided by Dr. Mary Reyland (University of Colorado Health

Sciences Center). As a negative control, LZRS-pEGFP was transduced. Transduction efficiency was determined by observing GFP fluorescence in cells.

pSUPER.retro.puro.PKCδ.toker shRNA [107, 281, 282] was used to knockdown PKCδ in cells. pSUPER.retro.puro.control (empty) was used as a negative control.

(Virus details- pSUPER.retro.puro.control 8/22/2013+ pSUPER.retro.puro.control 11/29/2014; pSUPER.retro.puro.PKCδ toker 8/22/2013+ pSUPER.retro.puro.PKCδ toker 11/29/2014; pSUPER.retro.puro.control 8.22.13; pSUPER.retro.puro.PKCδ toker 8.21.13 DS3p.91-92).

b. Cells were plated at a concentration of 10^5 cells/well in a 6 well plate the day before viral transduction. A Jouan centrifuge (CR412) was pre-warmed with plate holders by spinning at 3000 rpm for 15 minutes. The virus was thawed rapidly at 37 °C and 4 µg/mL polybrene (Hexadimethrine Bromide, Sigma H-9268) was added to the virus. Media was removed from cells, and cells were washed with PBS+ followed by addition of 0.5 mL of virus to each well. The plate was sealed with parafilm and spun at 1300 rpm for 1 hour at 32 °C. After 1 hour, virus was removed and fresh pre-warmed media was fed to the cells.

c. Puromycin-resistance gene was encoded with the pSUPER.retro.puro virus and transduced cells were selected with 1 µg/mL of puromycin 48 hours after the infection. Transduction was confirmed by detecting the target protein using western blot analysis.

2.9 UVC Dose Response Curve with MTT Cell Proliferation Assay

a. NHEK NN1185 passage 2 were irradiated with increasing amounts of UVC (0, 1, 2, 3, 4 or 5 mJ/cm^2) and incubated for 72 hours. Cell viability was measured using MTT assay-

Cell Proliferation Kit I (MTT) [Roche Diagnostics GmbH Cat. No. 11465 007001] according to instructions by the manufacturer.

CHAPTER THREE

RESULTS

3.1 PKCδ is Required For Repair of UV-Induced DNA Damage

We assessed the role of PKCδ in repair ofUV-induced DNA damage by using cells lacking expression of PKCδ. If PKCδ is required for repair of the DNA damage, then cells lacking PKCδ will have a defect in the repair of the DNA damage. We have used immunofluorescence microscopy to detect UV-induced CPDs. Cultured WT and PKCδ null MEFs were irradiated with a non-apoptotic dose of UVB (5 mJ/cm^2) to avoid any confounding effects of cell death on CPD detection. The maximum initial damage was detected after 1-2 hours of incubation and repair was observed at 48 hours post-UV (*Figure 2A*). As seen in the immunofluorescence images, WT and PKCδ null MEFs accumulated large amounts of DNA damage after 1 hour post-UV. After 48 hours, WT MEFs repaired the vast majority of the CPD damage as seen by the greatly reduced CPD staining. In contrast, PKCδ null MEFs still harbored large amounts of DNA damage after 48 hours.

We were able to quantify the fluorescence intensity using ImageJ software as shown in *Figure 2B*. The WT and PKCδ null MEFs display similar levels of CPD fluorescence at 1 hour post-UV. After 48 hours, the WT MEFs repaired the damage ($p<0.001$) but the PKCδ null MEFs have a significantly high amount of DNA damage still

present. We confirmed the reduced repair of UV-induced CPDs in PKCδ null MEFs using flow cytometry (data not shown). This confirms that PKCδ promotes repair of UV-induced DNA damage.

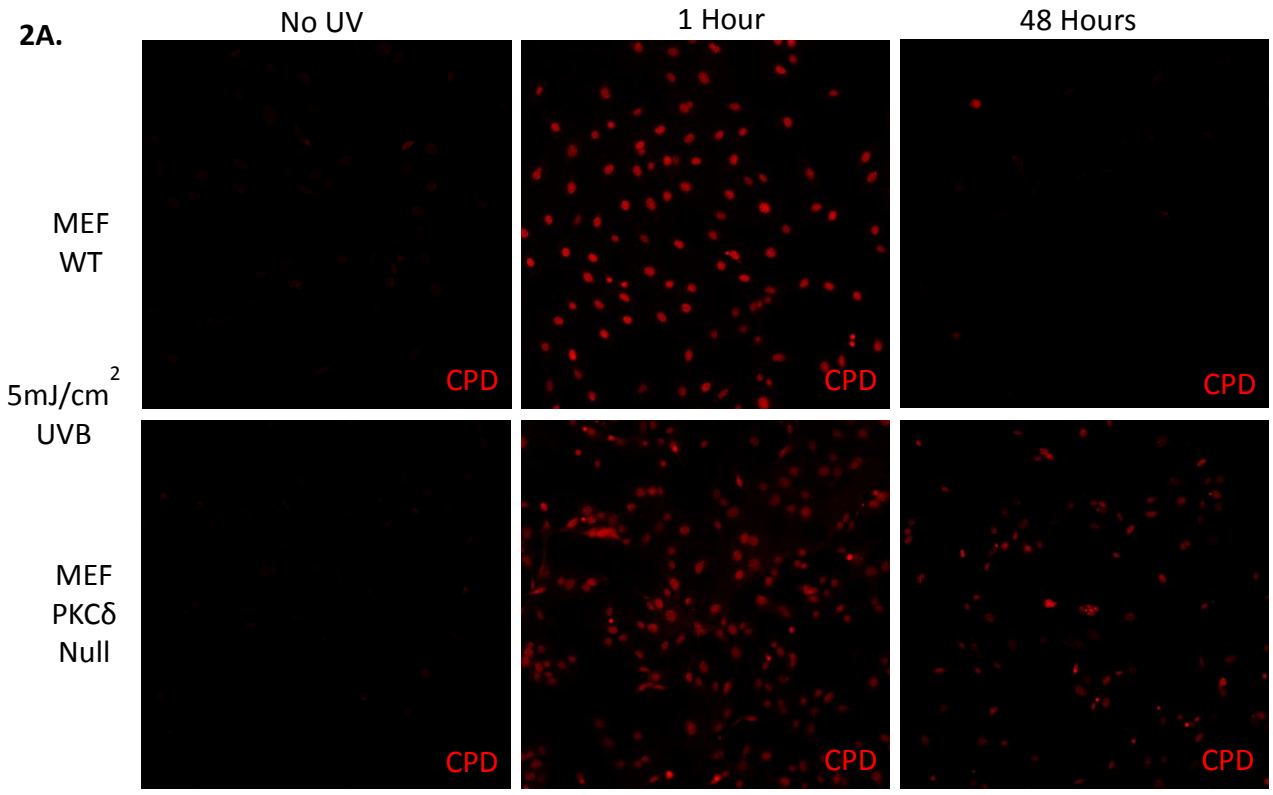


Figure 2. PKCδ is Required For the Repair of UV Induced DNA Damage

Immunofluorescence staining of CPD in MEFs. **A**) WT and PKCδ null MEFs were irradiated with 5 mJ/cm^2 UVB and stained with an anti-CPD antibody at indicated times.

2B.

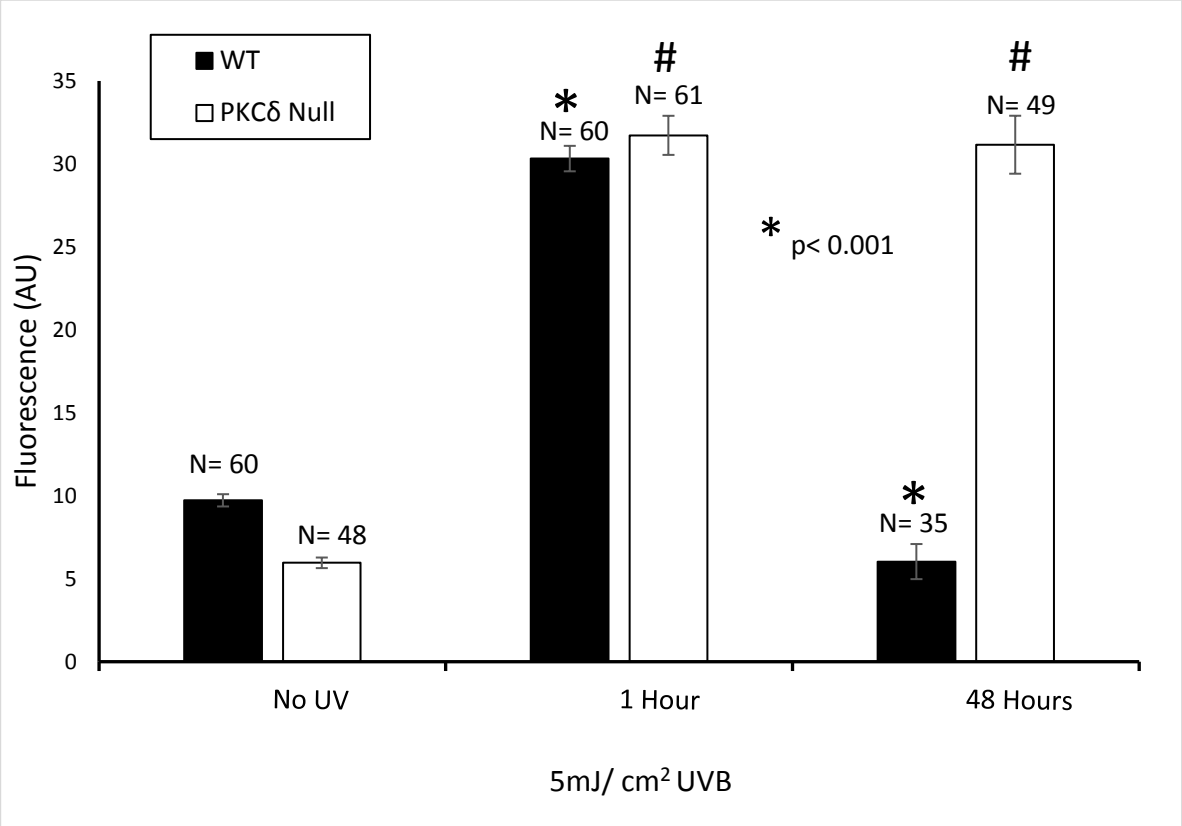


Figure 2. PKCδ is Required For the Repair of UV Induced DNA Damage

Immunofluorescence staining of CPD in MEFs. **A.** WT and PKCδ null MEFs were irradiated with 5 mJ/cm^2 UVB and stained with an anti-CPD antibody at indicated times. **B.** Nuclear CPD fluorescence was quantified using ImageJ. Only WT MEFs significantly repaired the damage while PKCδ null MEFs failed to repair the damage. 'N' indicate number of nuclei quantified. ***** p value<0.001 (T-Test); **#** N.S.=Not Significant.

3.2 PKCδ Reduces UV Mutagenesis Frequency

Cells lacking PKCδ are defective in repairing the UV-induced DNA adducts thus we hypothesized that these cells will harbor more UV-induced mutations. To test our hypothesis, we have used the Hypoxanthine phosphoribosyltransferase (*Hprt*) mutagenesis assay.

When mutations arise in the *Hprt* gene, and are not repaired, it can lead to loss of the only expressed copy of *Hprt* on X-chromosome. Cells that failed to repair the DNA damage will have more mutations on the *Hprt* locus and thus cells will be incompetent to utilize the 6-TG and will survive. The resultant colonies were counted [283-288]. The mutation frequency was determined by seeding known number of cells in media containing 6-TG to detect the mutant cells, and in media without 6-TG to determine the cloning efficiency.

3.

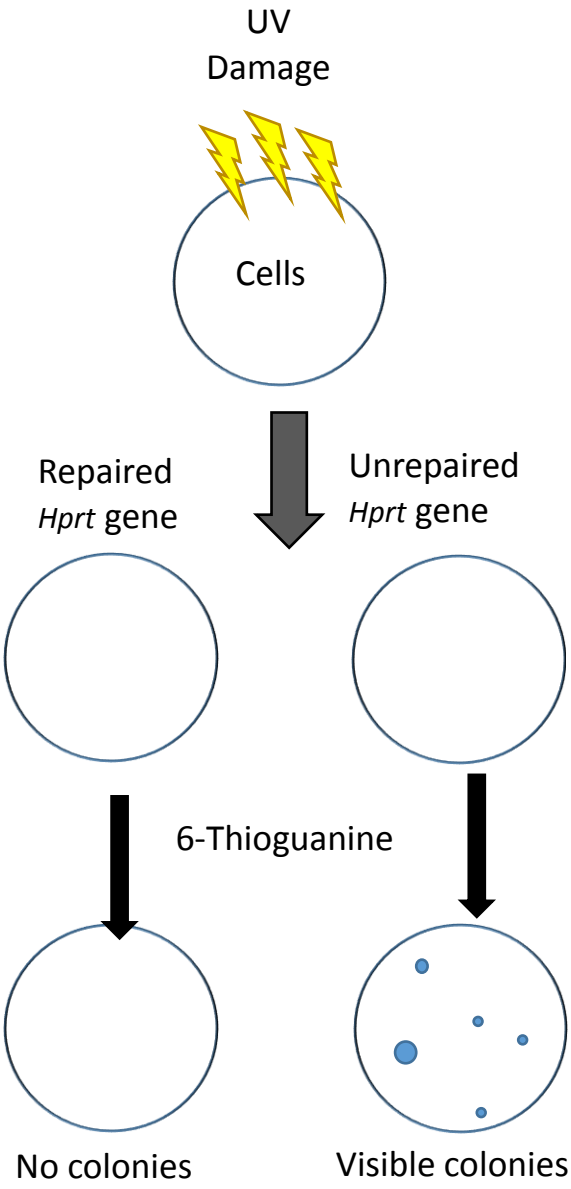


Figure 3. Hypoxanthine Guanine Phosphoribosyltransferase Mutagenesis Scheme:

Hprt is a gene located on the X-chromosome, and thus cells have only one functional copy of the gene. *Hprt* encodes the Hypoxanthine guanine phosphoribosyltransferase (HGPRT) enzyme which recycles the purines through purine salvage pathway. If a toxic purine analogue [6-Thioguanine (6-TG)] is fed to a normal cell carrying *Hprt* gene, it incorporates the toxic purine into its DNA, which results in death of the cell. A mutated *Hprt* gene cannot produce the HGPRT enzyme and thus cells cannot utilize the toxic purine analogue and survive.

To determine the optimum dose of 6-TG and to determine the endogenous 6-TG resistance frequency, MEFs were continuously treated with various concentrations of 6-TG (10 µM to 60 µM) for 14 days. No visible colonies were observed in both WT and PKCδ null MEFs at any 6-TG doses suggesting low (Less than 2 X 10^{-5}) endogenous resistance in MEFs, as seen in *Figure 4.*

In the mutagenesis experiment, the colony forming efficiency (CFE) of non-irradiated WT MEFs was 1.3 and PKCδ null MEFs was 1.2. This is surprising because the values of the CFE are over the maximum of the theoretical impossible. However, the values were similar to each other indicating that PKCδ does not influence the CFE of non-irradiated MEFs. The CFE of UV irradiated WT MEFs was 0.045, significantly lower ($p<0.05$) than 0.218 for PKCδ null MEFs, indicating that the PKCδ null MEFs were more resistant to UVB than WT MEFs. UV-induced mutations in both WT and PKCδ null MEFs, but the PKCδ null MEFs have significantly ($p<0.05$) higher frequency of mutations at the *Hprt* locus (*Figure 5*), about 5 time higher compared to WT MEFs (*Figure 5*). About 5 cells per 10^4 cells of PKCδ null MEFs had UV-induced mutations compared to less than 1 cell per 10^4 cells in WT MEFs. The experiment was repeated for 4 independent times and displayed similar trend.

A mutagenesis assay was also carried out in WT and PKCδ null MEFs with the mutations induced by UVC. High cell death was observed initially with 2 or 3 mJ/cm^2 of UVC stopping the experiment. We chose 1 mJ/cm^2 UVC as the irradiation dose because it is the lowest dose we can deliver and induces minimal cell death.

UVC also induced mutations at the *Hprt* locus but again PKCδ null MEFs had elevated mutation frequency than WT MEFs (*Figure 6*). Thus PKCδ reduces mutation frequency after UV.

We found that the WT and PKCδ null MEFs had very low (less than 2 X 10^{-5}) endogenous *Hprt* mutation frequency. UVB-irradiation induced mutations at the *Hprt* locus in both WT and PKCδ null MEFs, however PKCδ null MEFs showed a significantly increased mutation frequency, Also, PKCδ null MEFs showed higher colony forming efficiency than WT MEFs after UVB irradiation, indicating that they are more resistant to UV than the WT MEFs.

The UV dose used here (5 mJ/cm^2 UVB or 1 mJ/cm^2 UVC) was high enough to induce DNA damage but was low enough to not start the apoptosis pathway. Thus the cells will survive and will need to repair the damage or produce mutations. In the PKCδ null cells, the mutations may have developed due to defective NER machinery leading to incomplete DNA damage repair. These damages then would accumulate as well as would be passed down to the daughter cells. This is the first study done to investigate the role of PKCδ in UV-induced mutagenesis and to demonstrate that the reduced NER in PKCδ null cells leads to enhanced mutagenesis, thereby contributing to carcinogenesis.

4.

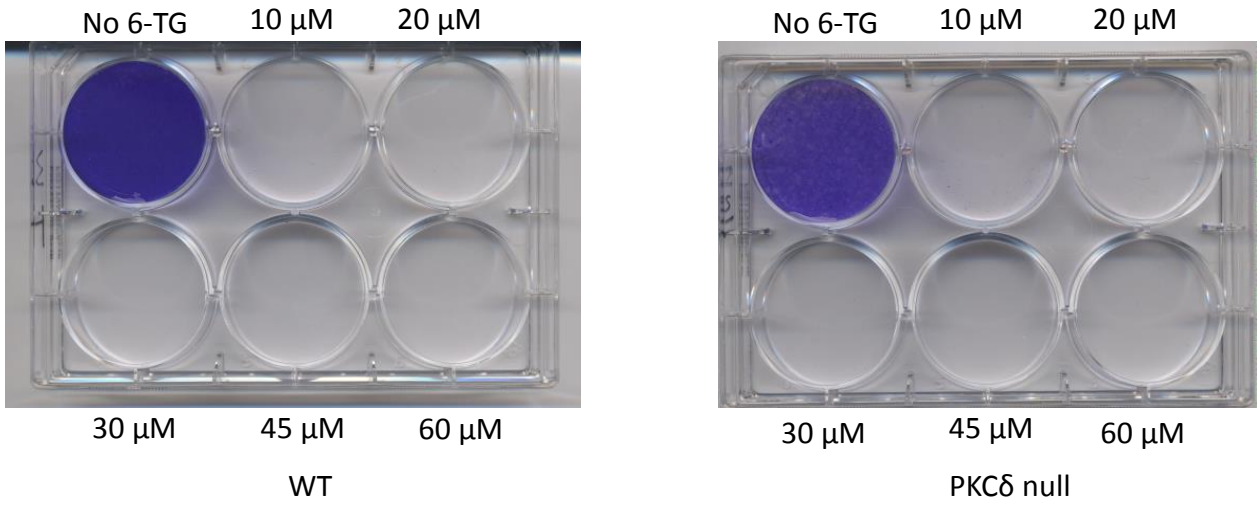


Figure 4. MEFs Have Low Endogenous 6-Thioguanine Resistance

Dose response of 6-TG for toxicity was determined in MEFs. WT and PKCδ null MEFs were continuously treated with indicated concentrations of 6-TG to investigate endogenous resistance in MEFs. After 14 days, MEFs were fixed with formaldehyde and stained with crystal violet to see colonies. The absence of colonies indicated very low endogenous 6-TG resistance in both WT and PKCδ null MEFs.

5.

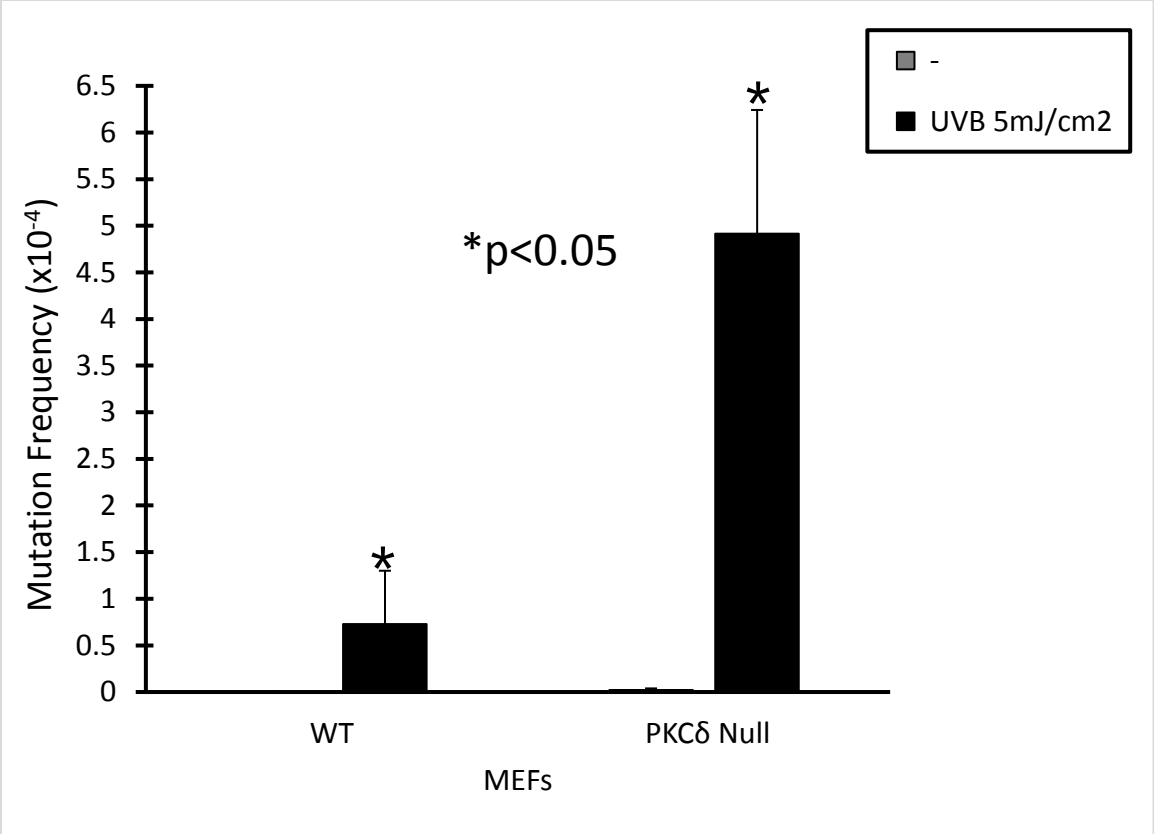


Figure 5. PKCδ Reduces UVB-induced Mutagenesis

Hprt mutagenesis assay was used in WT and PKCδ null MEFs to investigate mutation frequency after UVB irradiation. This graph is an average of four experiments with $p<0.05$ (T-Test). PKCδ null MEFs harbor significantly higher mutations at *Hprt* locus than WT MEFs. The mutagenesis rate was calculated by the number of 6-TG resistant colonies divided by plating efficiency.

6.

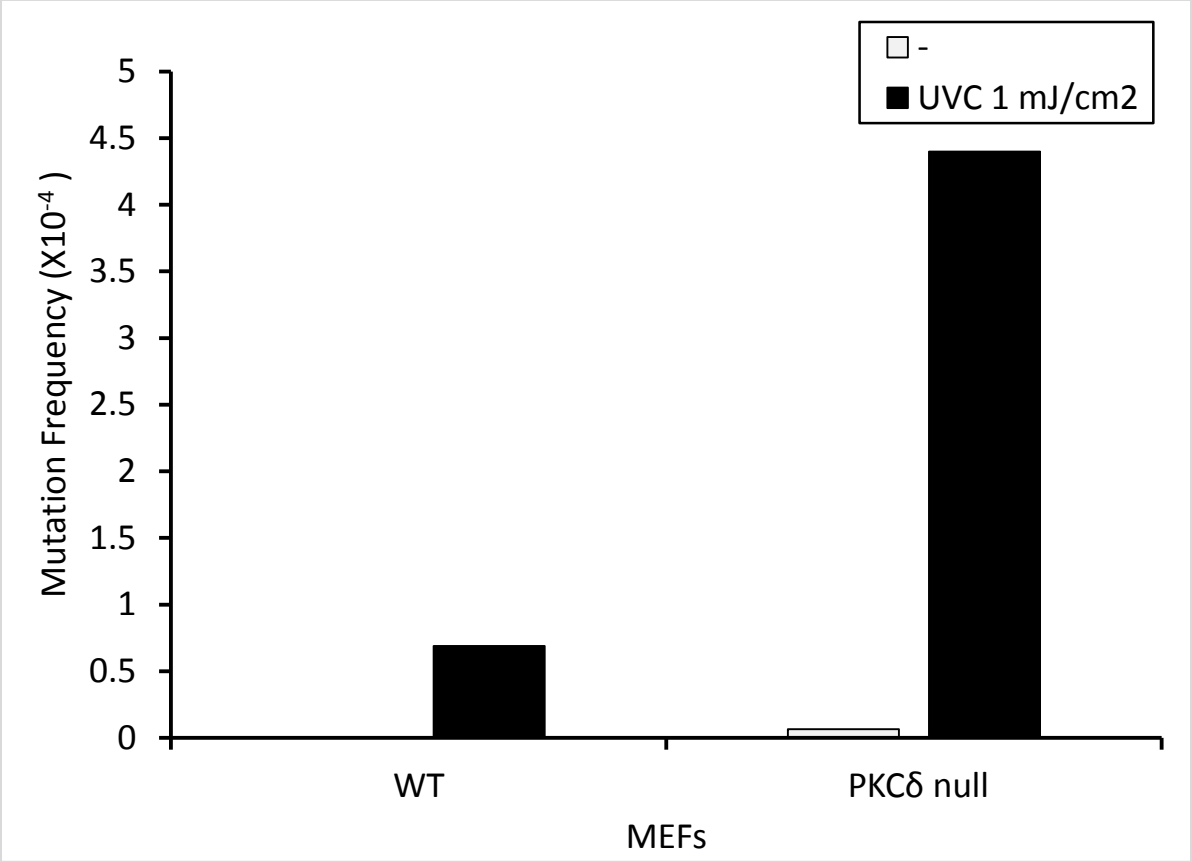


Figure 6. PKCδ Reduces UVC-induced Mutagenesis

Hprt mutagenesis assay was used in WT and PKCδ null MEFs to investigate mutation frequency after UVC irradiation. PKCδ null MEFs displayed higher amounts of mutations at *Hprt* locus compared to WT MEFs. Note, mutation frequency was normalized to plating efficiency.

3.3 Mechanistic Role of PKCδ in DNA Damage Repair

Upon UV-induced DNA damage, NER proteins are recruited to the sites of damage. If PKCδ promotes recruitment of NER proteins at sites of DNA damage then lack of PKCδ will reduce recruitment of these repair proteins. To find out more about the mechanistic role of PKCδ in UV DNA damage repair, we used micropore filter immunofluorescence. UV radiation can pass only through the small holes in the TMTP micropore filter and induces DNA damage at specific parts of the nucleus whereas the rest of the cell remains undamaged and acts as an internal negative control. The small damaged part can be then stained with an anti-CPD antibody (immunofluorescence) co-stained with an antibody against NER proteins. This way we can observe and compare the recruitment of proteins at the damage sites in the WT or cells lacking PKCδ. To test the micropore filters, we used CPD immunofluorescence in HaCaT keratinocytes (*Figure 7*) irradiated with 50 mJ/cm^2 UVB or 10/100 mJ/cm^2 UVC. As a negative control we had non-irradiated cells. Without the micropore filter, both UVB and UVC-induced large amounts of CPD damage which was absent in the non-irradiated cells. Surprisingly, the micropore filter efficiently blocks only UVC and not UVB. In UVC-irradiated cells with the micropore filter, damage was observed only at small regions of the cells whereas micropore filter could not obstruct the UVB and thus CPD staining was observed in almost the entire nucleus.

7.

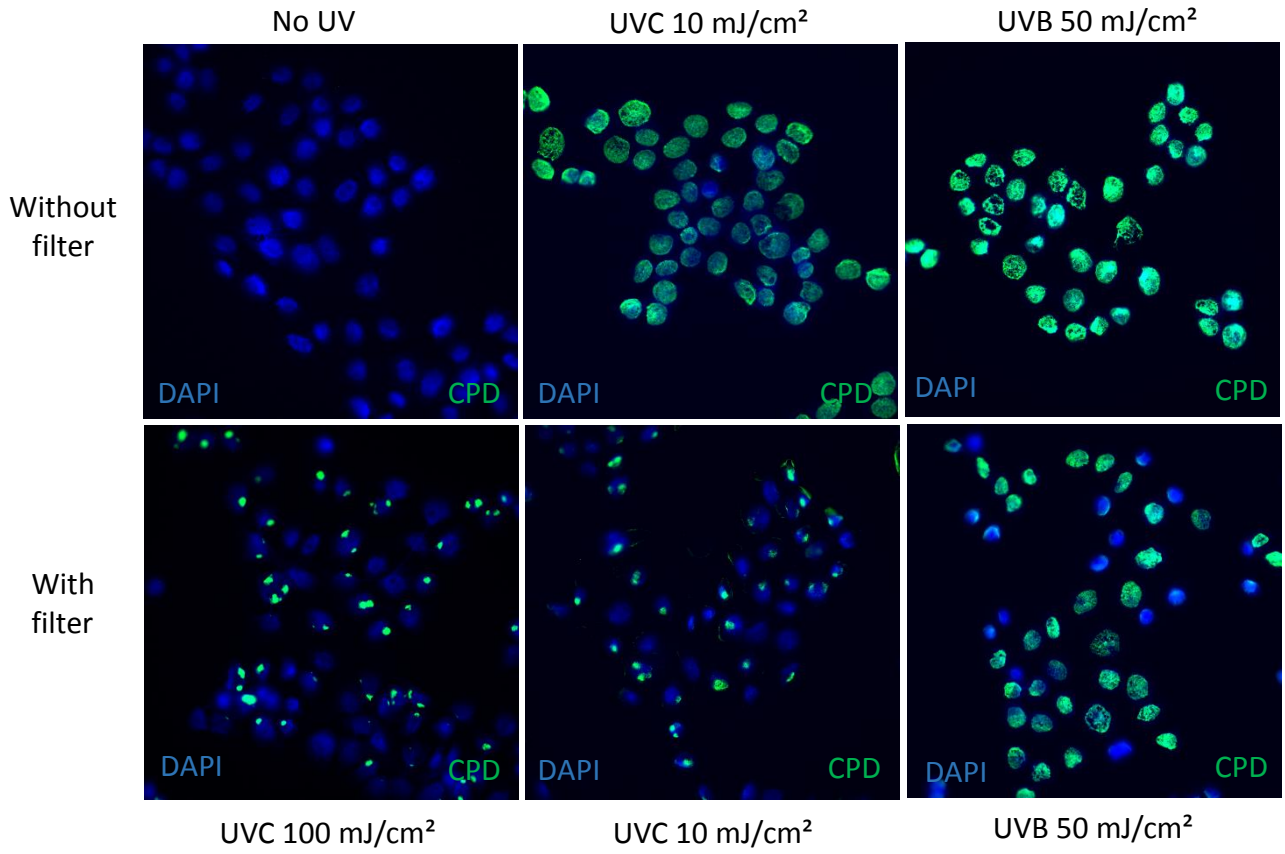


Figure 7. Micropore Filters Block UVC Effectively But Not UVB

HaCaT cells were exposed to UVC and UVB in the presence or absence of TMTP micropore filter as indicated. Immunofluorescence staining with an anti-CPD antibody was carried out at 2 hours post-UV. Micropore filter was successful in blocking the UVC however it only partially blocked the UVB.

We had chosen to examine the XPC and XPA proteins for their recruitment to the damage sites because XPC an important early DNA damage recognition protein, and XPA, being at the center of the NER process, interacts with multiple NER factors. Additionally, a former student of our lab, Chris Negro, found a defect on nuclear translocation of XPC and XPA in PKCδ null MEFs using immunofluorescence microscopy [289]. Initially, however the chromatin denaturing agent (70% NaOH in 70% EtOH) used for CPD staining destroyed the proteins staining making it impossible to co-localize CPDs with the repair proteins. To optimize this co-staining technique, we tested combinations of chemicals (Table 1) to fix and denature the chromatin to identify conditions that would not destroy the protein staining (*Figure 8*). NHEK were irradiated with 10 mJ/cm^2 UVC, fixed and permeabilized using combination of chemicals, followed by staining with anti-CPD and anti- PKCδ antibodies (Santa Cruz SC-937).

Table 1: Methods For Optimizing Immunofluorescence Co-staining of CPD and Protein

	Fixative agent	Antigen Retrevial/Permeabilizing agent
1	Acetone/Methanol at -20 °C for 10 mins	-
2	Acetone/Methanol at -20 °C for 10 mins	70mM NaOH in 70% EtOH for 2 mins +0.1% Triton-x 100 in FA buffer
3	Acetone/Methanol at -20 °C for 10 mins	70mM NaOH in 70% EtOH for 1 min +0.1% Triton-x 100 in FA buffer
4	Acetone/Methanol at -20 °C for 10 mins	2 N HCl for 20 mins
5	3.7% Formaldehyde +PBS for 10 mins	2 N HCl for 20 mins

Only acetone/methanol or formaldehyde+ triton-X 100 treatment with 2N HCl produced no CPD staining. Although acetone/methanol followed by 70 mM NaOH gave the strongest CPD staining, the protein staining (PKCδ) was very weak. Acetone/methanol at -20 °C for 10 minutes followed by 2N HCl for 20 minutes gave weaker CPD staining but was found to be effective for co-staining of proteins with CPD. However, because MEFs are a mouse cell line and most of the XPC or XPA antibodies were not compatible with mouse antigens, the XPC and XPA staining produced non-specific staining or no staining in MEFs. Similar weak staining results were observed when XPC or XPA protein levels were detected using western blot analysis in MEFs (data not shown).

8.

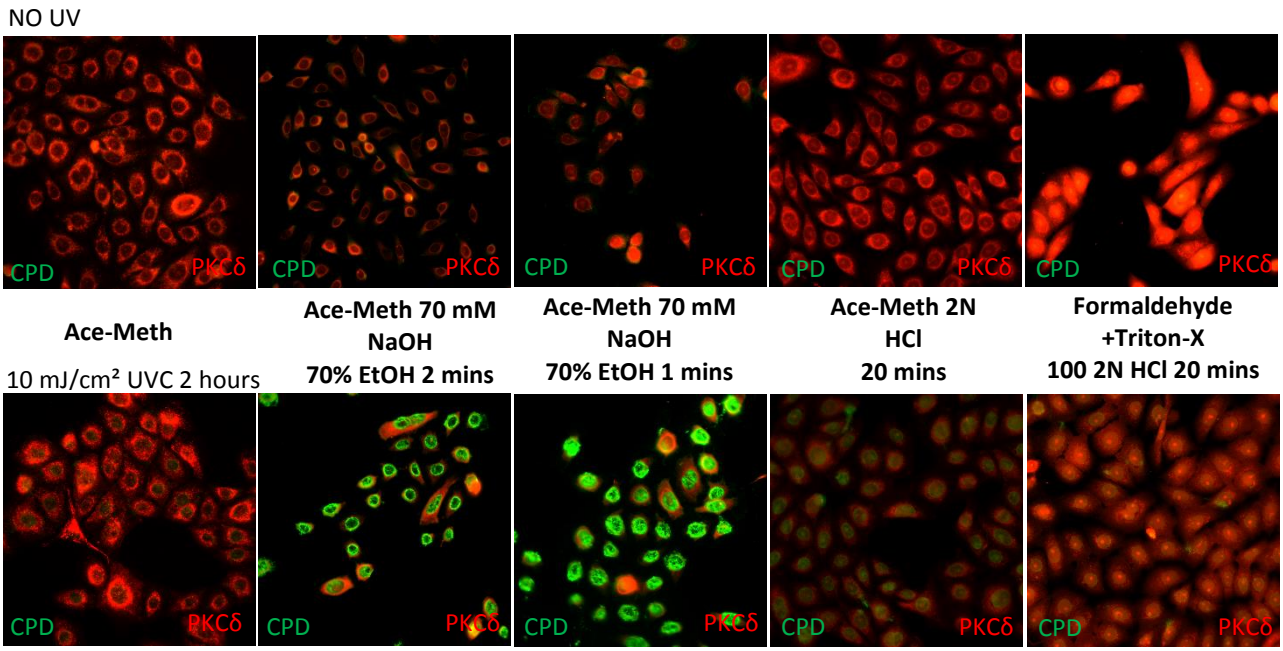


Figure 8. Combinations For Optimizing Immunofluorescence Co-staining of CPD and Protein

NHEK were irradiated with 10 mJ/cm^2 UVC and incubated for two hours. A variety of combinations of fixative and antigen retrieval chemicals were used to find the best method to co-stain CPD and proteins. Acetone-Methanol with 2 N HCl was found to be effective in preserving the red PKCδ staining and green CPD staining

Since the micropore filter immunofluorescence technique was not helpful, we next decided to observe protein levels of XPC and XPA in MEFs by western blot. If PKCδ influences protein levels of NER proteins in response to UV-induced damage, then lack of PKCδ will alter protein levels of XPC or XPA. As the antibodies against XPA/XPA were not compatible with mouse cells, we used NHEK for our investigation. We transduced NHEK with control or PKCδ shRNA to knock down PKCδ (*Figure 9*) and the protein levels were observed at 1, 3 and 6 hours post 5 mJ/cm^2 UVB.

As can be observed in *Figure 9*, the shRNA knocked down the PKCδ effectively (64% knockdown). XPA protein levels were found to increase 3 and 6 hours post-UV in WT MEFs. However, no difference was observed in the XPA protein induction between control and PKCδ knockdown samples indicating that there was no effect of PKCδ knockdown on basal levels or UV-mediated induction of XPA.

Without UV irradiation, PKCδ knockdown sample had slightly higher basal levels of XPC proteins than control sample. UV-induced increase in XPC protein levels in both control and PKCδ shRNA samples. But the increase was similar in both control and PKCδ knockdown samples. Taken together, PKCδ knockdown did not affect basal or UV induction of XPC.

In these NHEK, we came across an observation that phosphorylated p53 on serine 15 was not induced by UV as much in the samples with knockdown of PKCδ (*Figure 9*). With UV, total p53 levels increased 1-6 hours in both control and PKCδ shRNA samples, but p-p53 S15 was induced only in the control group and not in the PKCδ shRNA samples.

9.

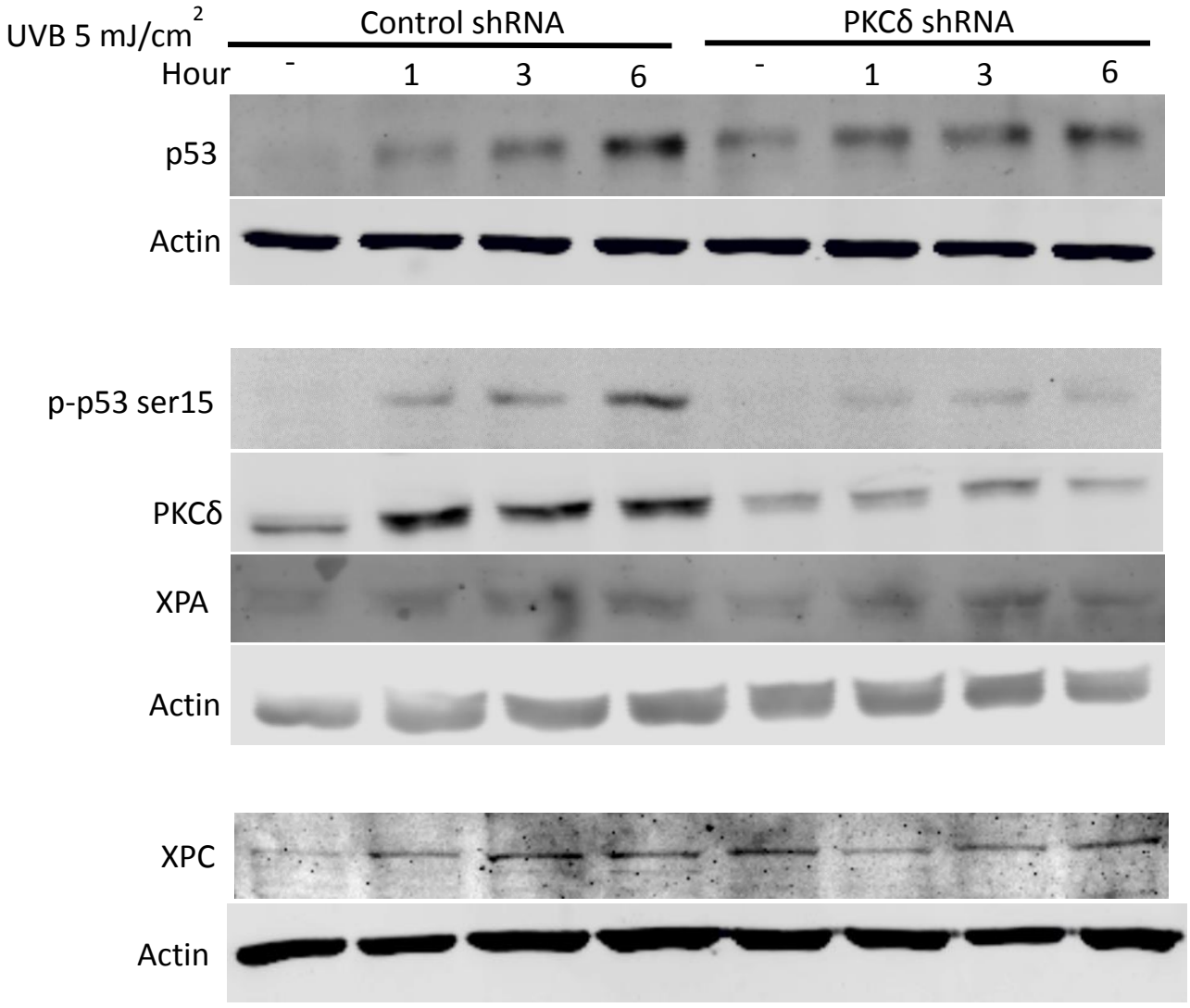


Figure 9. XPC and XPA Protein Levels Were Not Changed After UV and PKCδ Knockdown Decreases Phosphorylated p53 at Serine 15 in NHEKs

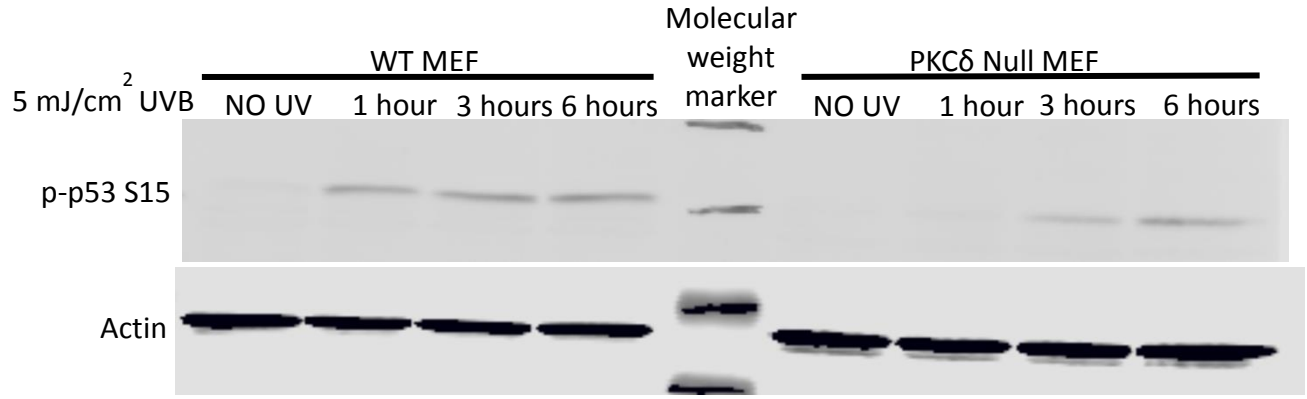
Normal human epidermal keratinocytes were transduced with control or PKCδ shRNA. NHEK were irradiated with 5 mJ/cm^2 UVB and lysates were analyzed using western blotting. PKCδ shRNA reduced the PKCδ levels in the knockdown samples. UV was able to induce p53 protein in both control and PKCδ shRNA samples. However, p53 S15 levels were lower in the PKCδ knockdown samples compared to control. XPA and XPC protein levels were induced by UV irradiation (3 and 6 hours) however no significant difference in XPA and XPC protein levels between the control and PKCδ knockout samples was observed.

3.4 PKCδ Null MEFs Have Lower Levels of p-p53 S15 and Total p53 Protein

We also explored p53 levels and S15 phosphorylation in MEFs. PKCδ null MEFs also had delayed UV-induced serine 15 phosphorylation of p53 (p53 S15) (*Figure 10A*). This S15 phosphorylation of p53 is important for activation and stabilization of the p53 protein. UV-induced p53 phosphorylation was delayed in the PKCδ null MEFs compared to WT MEFs. Additionally, the total levels of basal and UV-induced p53 were also greatly reduced in the PKCδ null MEFs compared to WT (*Figure 10B*).

To investigate whether this reduction in the p53 total levels in MEFs was at the transcription level, *Tp53* mRNA levels were analyzed by qRT- PCR (*Figure 11*). PKCδ null MEFs had more *Tp53* mRNA than WT MEFs and thus the reduced p53 protein in PKCδ null MEFs is not due to reduced *Tp53* mRNA. *GAPDH* levels were not significantly different between non-irradiated and UV irradiated MEFs.

10A.



10B.

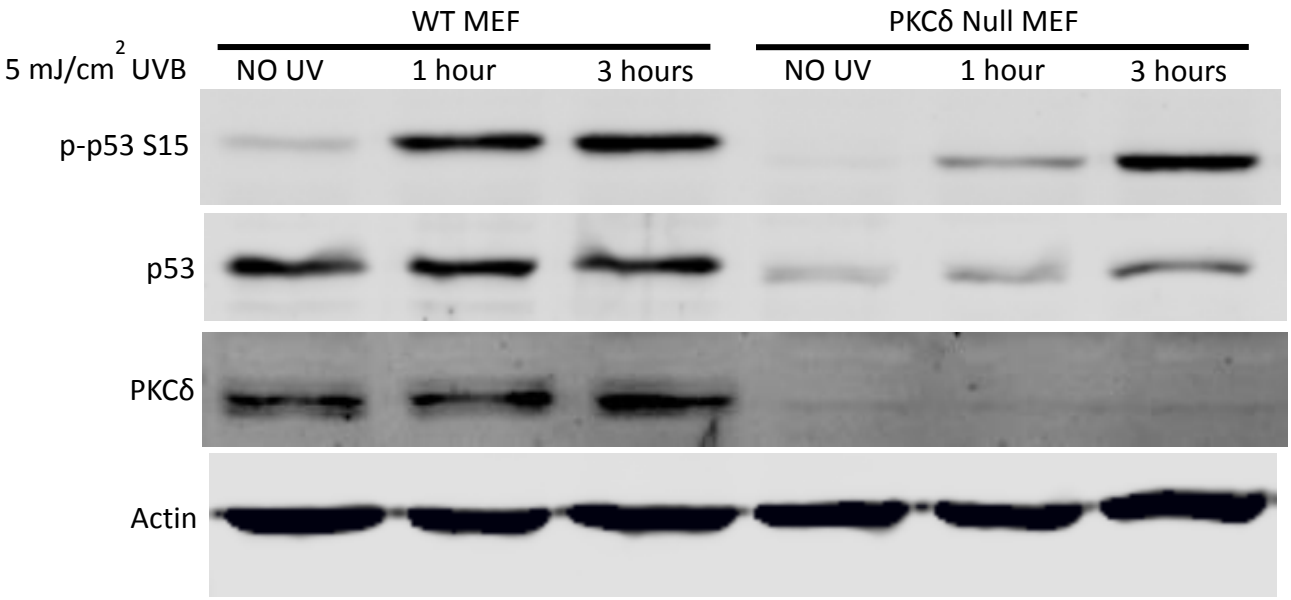


Figure 10. PKCδ Null MEFs Have Delayed Phosphorylation of p53 At S15 and Reduced Total p53

p53 levels in MEFs. WT and PKCδ Null MEFs were irradiated with 5 mJ/cm^2 UVB and incubated for indicated times. Protein lysates were analyzed by western blotting.
A. PKCδ Null MEFs had lower levels of phosphorylated p53 serine 15 (p-p53 S15) compared to WT.
B. PKCδ Null MEFs had lower levels of p53 protein as well as phosphorylated p53 serine 15.

11.

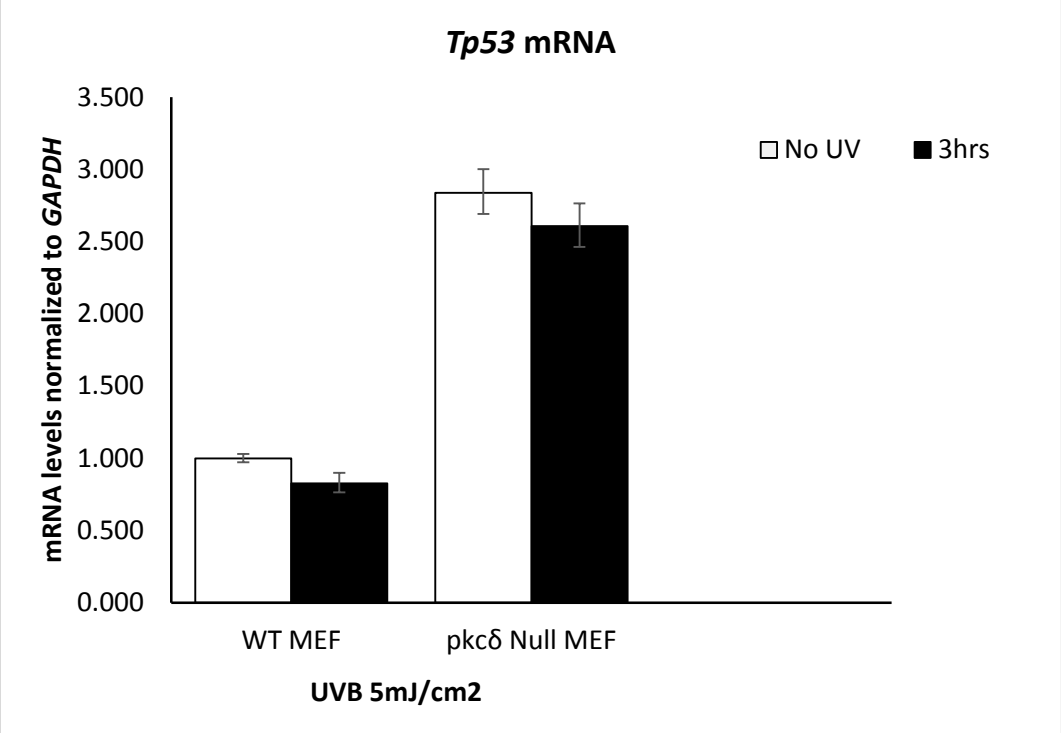


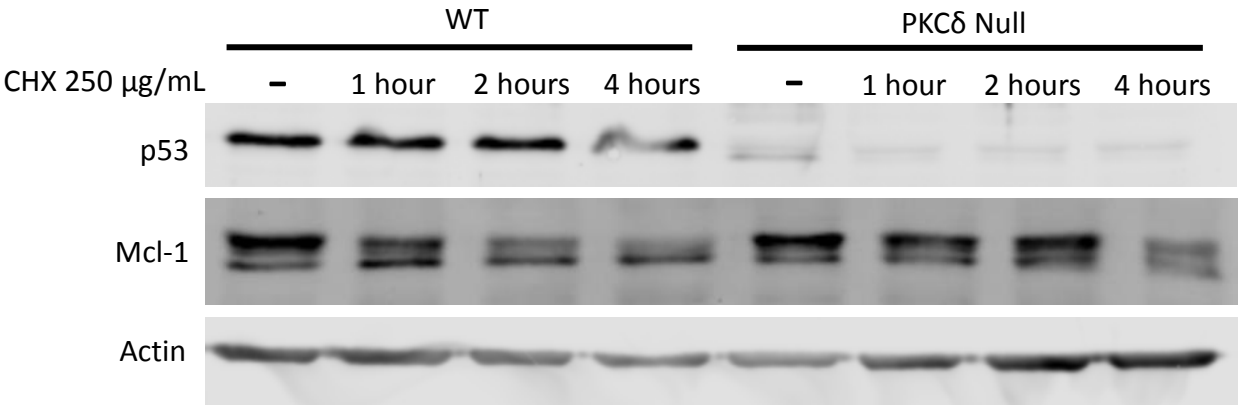
Figure 11. *Tp53* mRNA Levels in WT and PKCδ null MEFs

WT and PKCδ null MEFs were irradiated with/without 5 mJ/cm^2 UVB and incubated for 3 hours. RNA was extracted, cDNA was prepared and levels of *Tp53* mRNA were determined using qRT-PCR. mRNA levels are normalized to *GAPDH* control.

3.5 p53 in WT MEFs Has a Longer Half-Life Than in PKCδ Null MEFs

To investigate the p53 protein stability in the MEFs, the protein translation inhibitor cyclohexamide (CHX) was used. WT and PKCδ null MEFs were treated with 250 μg/mL CHX for 1, 2 or 4 hours and p53 protein levels were analyzed using western blotting (Figure 12A). Mcl-1 protein which was used as a positive control, which started to degrade at 2 hours in WT MEFs whereas the degradation started at 4 hours in PKCδ null MEFs. The half-life of the p53 protein was determined by plotting the levels of p53 after CHX treatment average from 2 experiments on Y-axis and time of incubation with CHX on X-axis in both WT and PKCδ null MEFs (Figure 12B). The half-life of p53 was found to be longer in WT MEFs (4.6 hours) compared to PKCδ null MEFs (2.6 hours). The p53 protein was less stable in PKCδ null MEFs compared to WT, and this may explain the lower levels of p53 in PKCδ null MEFs.

12A.



12B.

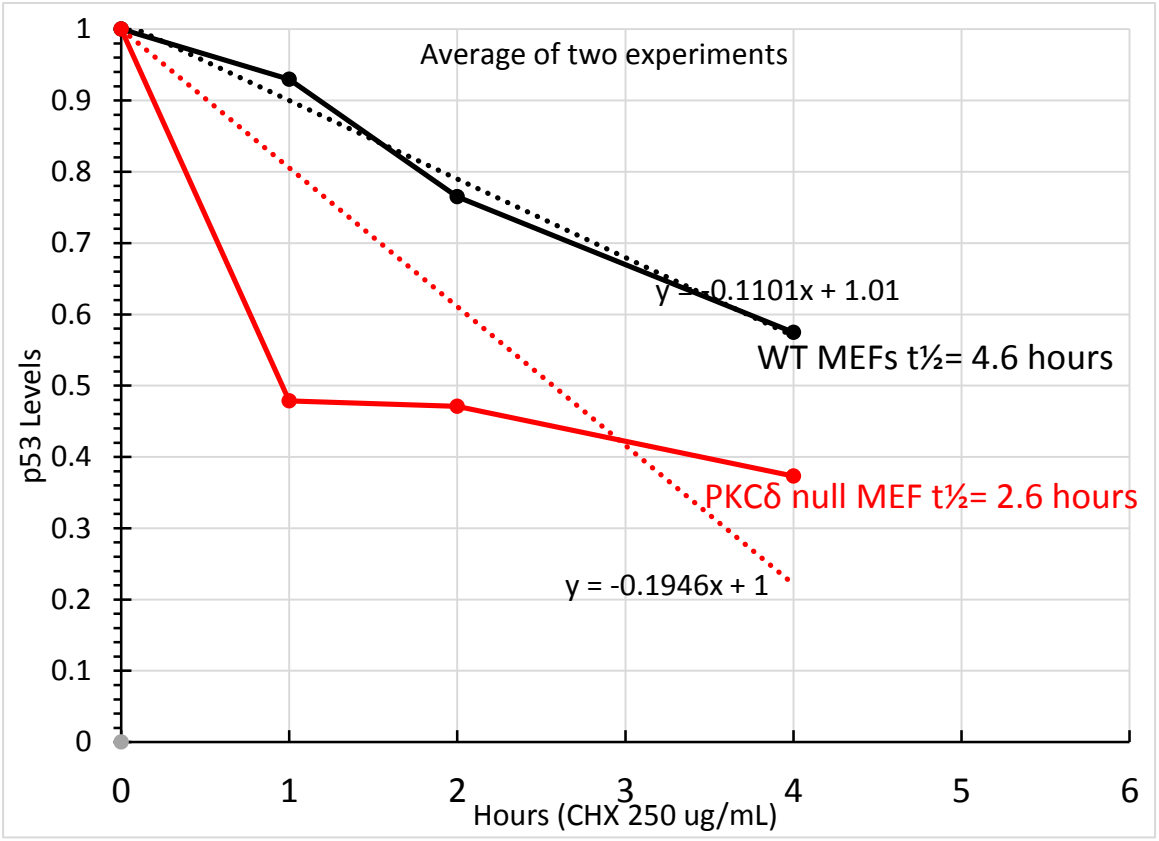


Figure 12. p53 in WT MEFs Has a Longer Half-Life than in PKCδ Null MEFs

A. MEFs were treated with/without CHX for indicated times and protein lysates were analyzed using western blotting. Mcl-1 was used as a CHX positive control.
B. Half-life of p53 plotted in graph from two independent experiments. Linear regression was used to calculate the half-life of p53. Trendline was plotted by computer.

3.6 Re-expression of PKCδ in PKCδ Null MEFs Did Not Rescue the Lower Levels of p53

To determine if ectopic PKCδ expression could rescue the decreased p53 levels in PKCδ null MEFs. Myc-tagged PKCδ was re-expressed in the PKCδ null MEFs and the p53 protein expressions were investigated using western blotting (*Figure 13*). Transduction of PKCδ into PKCδ null cells was successful indicated by elevated PKCδ in the western blot, however the total p53 levels were similar to that of un-transduced PKCδ null MEFs. Additionally, delayed phosphorylation of p53 at S15 was also not rescued by PKCδ re-expression in the null MEFs. Thus, PKCδ does not appear to be a direct positive regulator of p53 levels or S15 phosphorylation in MEFs, although the PKCδ transduction efficiency is not known, and may be low. Furthermore, we do not know if the PKCδ re-expression here restored UV-induced CPD repair in PKCδ null MEFs.

13.

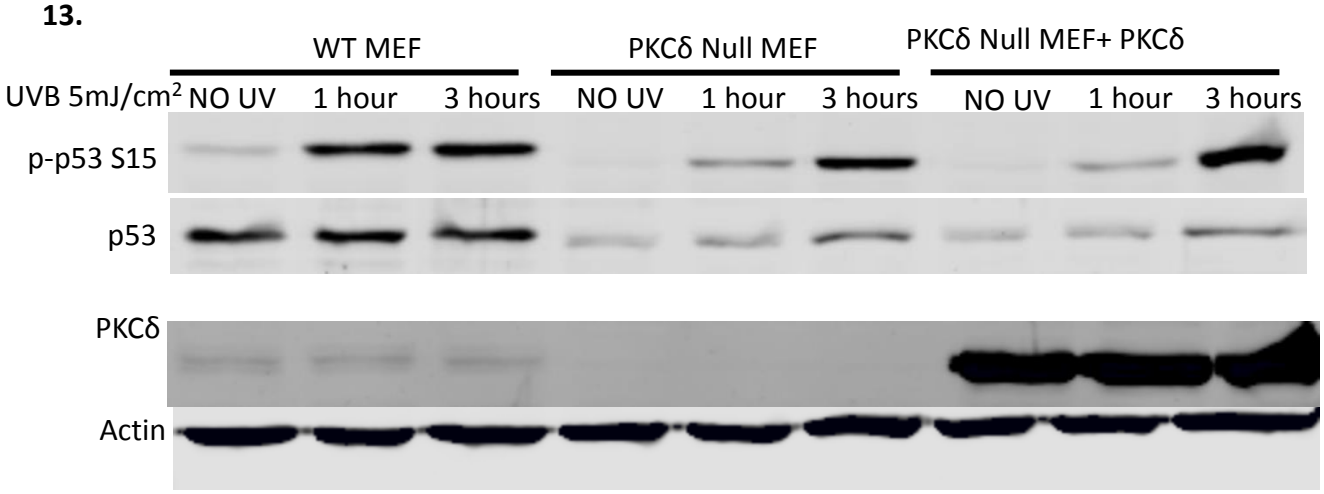


Figure 13. Re-expression of PKCδ in PKCδ Null MEFs Did Not Rescue the Lower Levels of p53

Myc tagged PKCδ was transduced into PKCδ null MEFs and irradiated with 5 mJ/cm^2 UVB for the indicated times. Protein lysates were analyzed by western blotting.

3.7 p53 Was Not Directly Phosphorylated By PKCδ On Serine 15 *In Vitro*

Since serine 15 phosphorylation is important for the activation and stabilization of p53 and was delayed in PKCδ null MEFs following UV exposure, the possibility of PKCδ directly phosphorylating p53 was investigated using an *in vitro* kinase assay (*Figure 14*). One μg of recombinant GST-p53 was incubated with 200 ng recombinant PKCδ in the presence of complete assay buffer containing phosphatidylcholine/phosphatidylserine (PC/PS) and ATP, with or without of 100 nM TPA. The GST-p53 was tested for being able to be phosphorylated on S15 by incubating it with whole cell lysates (WCL) from NHEK with/without 5 mJ/cm^2 UVB exposure. Phosphorylation was detected using anti-p-p53 S15 or p-MARCKS (Myristoylated alanine-rich C-kinase substrate). We found that PKCδ was not able to phosphorylate p53 on S15 *in vitro* as indicated by the absence of p-p53 S15 bands on the blot. PKCδ successfully phosphorylated MARCKS *in vitro* although TPA did not increase MARCKS phosphorylation further. Unknown kinases in the WCL were able to phosphorylate GST-p53 (75 kD) even in the absence of UV exposure, and endogenous p-p53 S15 (53 kD) phosphorylation in NHEK was induced with UV irradiation, indicating that the GST-p53 was competent for S15 phosphorylation.

14.

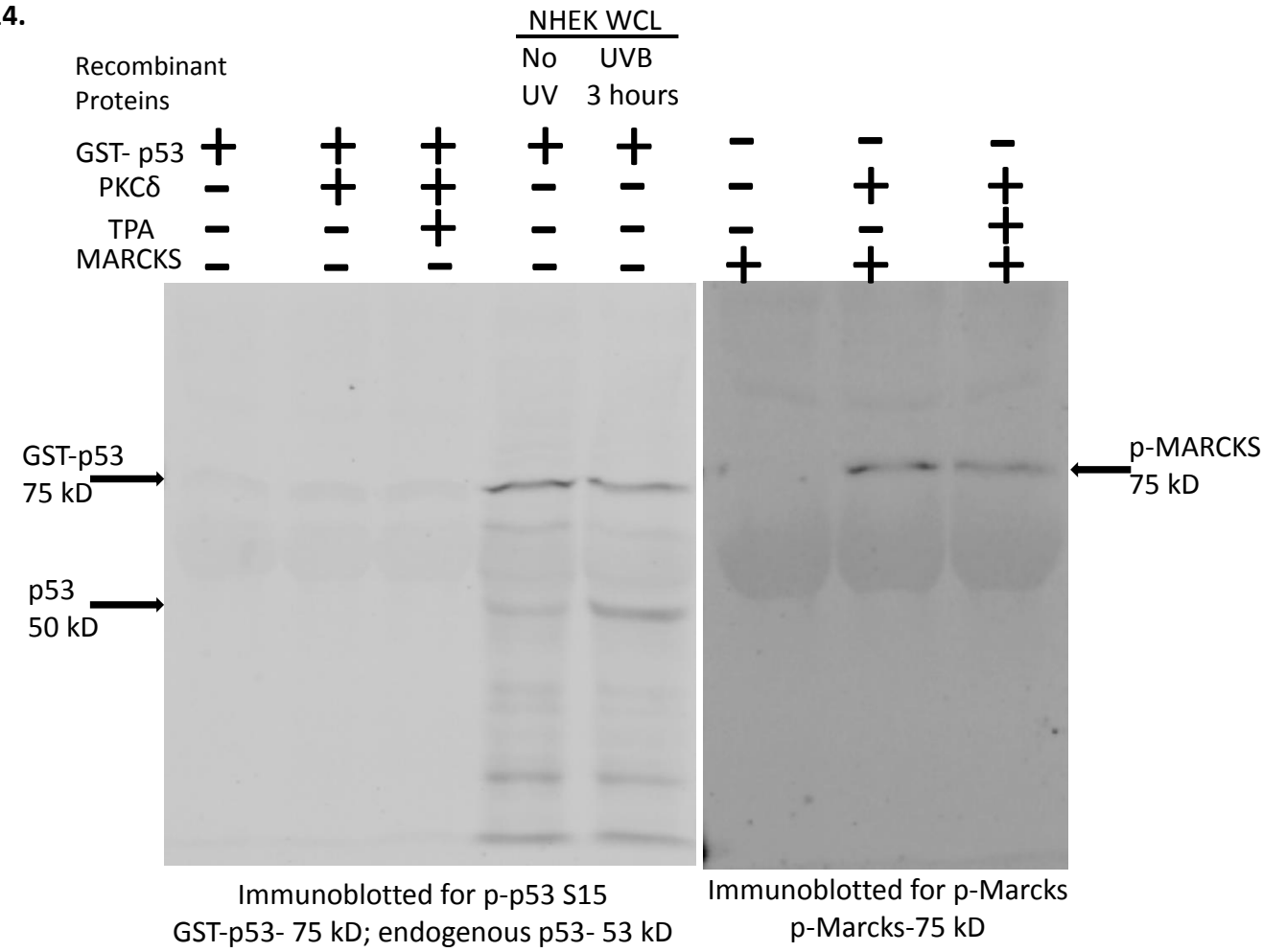


Figure 14. p53 Was Not Phosphorylated By PKCδ On Serine 15 *In vitro*

Recombinant GST-p53 was incubated with recombinant PKCδ, in complete assay buffer containing PC/PS and ATP, with or without TPA as indicated. PKCδ did not phosphorylate p53 on S15 as deduced by absence of p-p53 S15 band. The GST-p53 was inspected for being able to phosphorylated by incubating with whole cell lysates (WCL) of NHEK with/without 5mJ/cm^2 UVB. PKCδ activity was observed with PKCδ phosphorylation target protein MARCKS. Phosphorylation was detected using anti-p-p53 S15 or p-MARCKS.

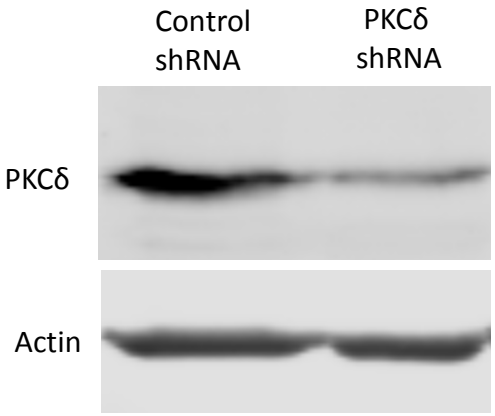
3.8 PKCδ Knockdown Did Not Affect Repair of UV-Induced DNA Damage in HaCaTs with Mutant *TP53*

UV induces mutations in the *TP53* gene and it was found that normal appearing epidermis can be harboring patches of *TP53* mutated cells with each clone measured about 60 to 3000 cells [267][268]. Since HaCaTs have UV signature mutations in the *TP53* gene, they can be used as a model to investigate DNA damage repair role of PKCδ in cells with mutant *TP53*.

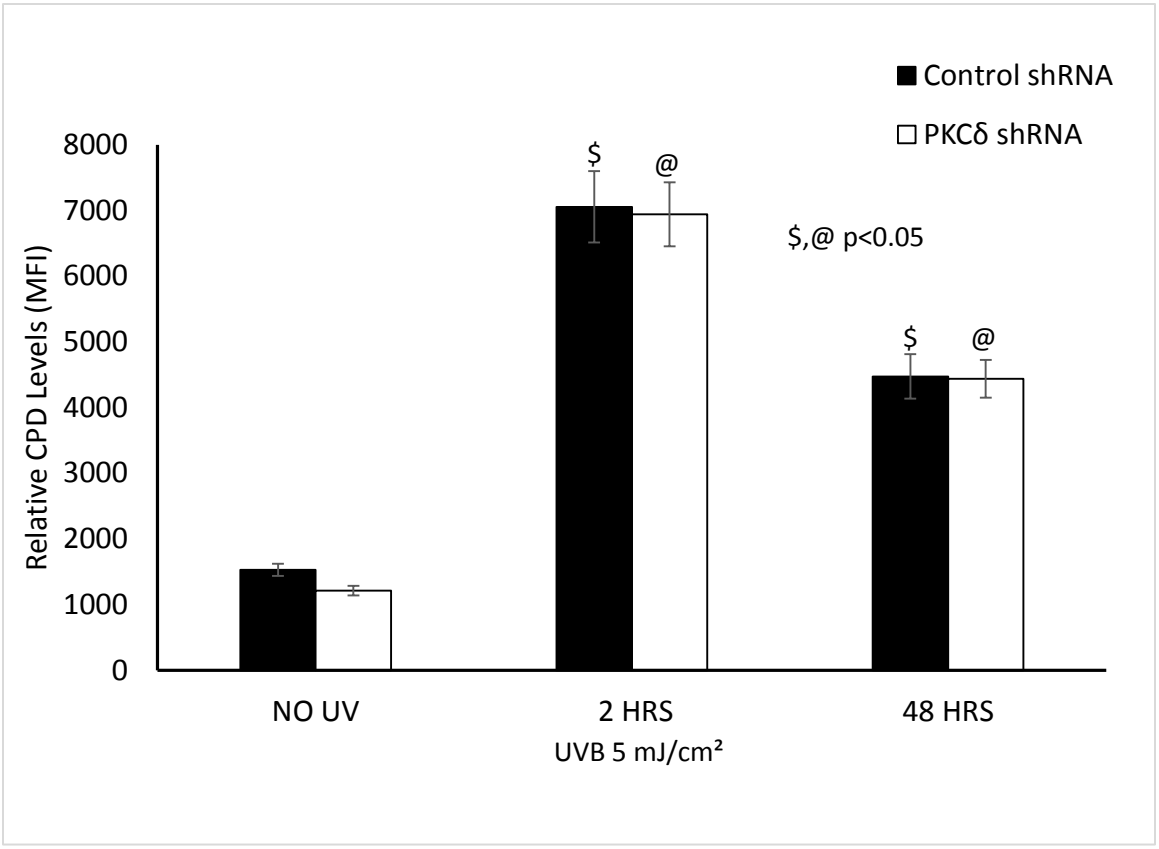
To investigate the role of PKCδ in repair of UV-induced CPDs in HaCaTs, PKCδ shRNA was used to knockdown PKCδ (*Figure 15A*). shRNA reduced the PKCδ protein levels 83% (analyzed by ImageJ) as seen in the blots. Flow cytometry technique was used to confirm the CPD repair the HaCaTs (*Figure 15B*). UV-induced concomitant CPD damage in both control and PKCδ shRNA samples at 2 hours post-UV. The damage was significantly and equally repaired at 48 hours in control as well as in PKCδ shRNA samples. This confirms that knockdown of PKCδ did not affect repair of UV-induced DNA damage in cells with *TP53* mutation. Immunofluorescence technique was also used to look at the CPD DNA damage repair (*Figure 15C*). HaCaTs were irradiated with 5 mJ/cm^2 UVB and immunofluorescence microscopy was used to detect UV-induced CPDs. UV-induced DNA damage in both control and PKCδ shRNA samples as seen at 1 hour post-UV. Reduction of CPD fluorescence at 48 hours indicated repair of CPD, however the repair was similar in both control and PKCδ shRNA samples. Almost all the CPDs were repaired by 72 hours in both control and PKCδ shRNA samples indicating PKCδ

knockdown did not affect UV-induced CPD repair in HaCaTs. The fluorescence from the immunofluorescence experiment was calculated using the ImageJ software (*Figure 15D*).

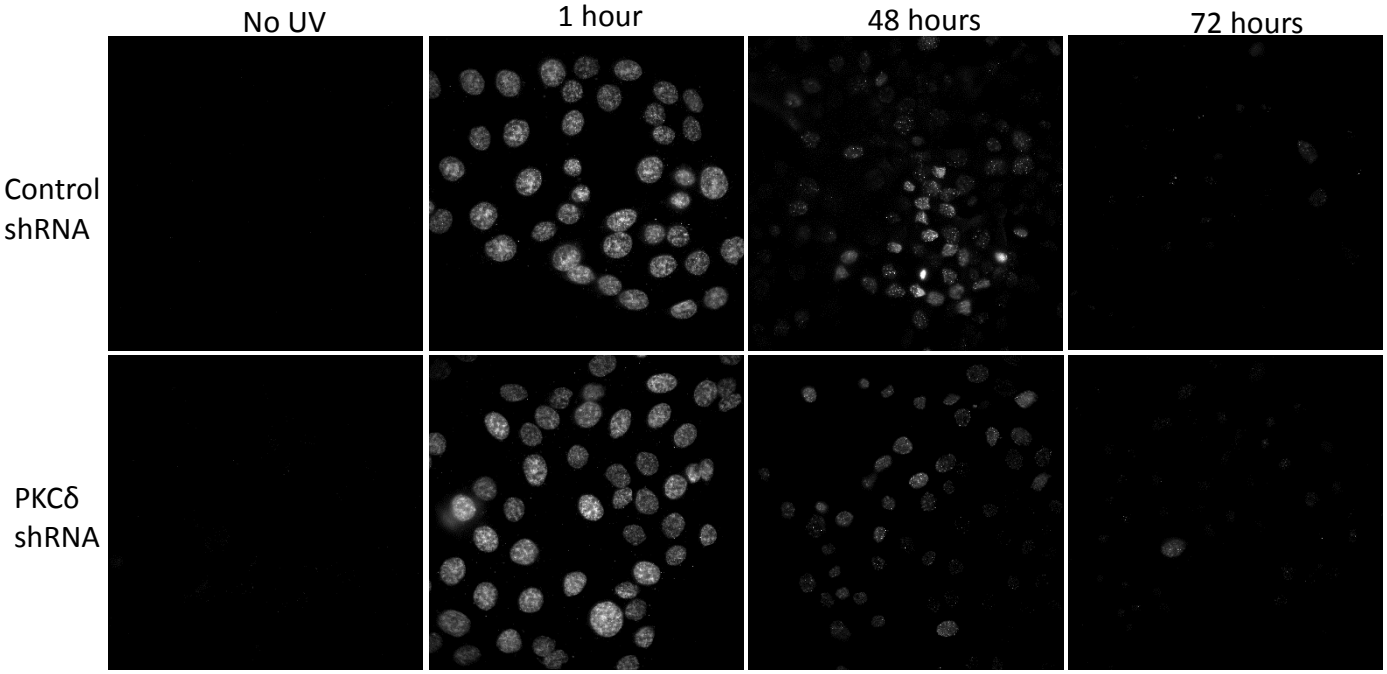
15A.



15B. Flow Cytometry



15C.



15D. Quantification of fluorescence

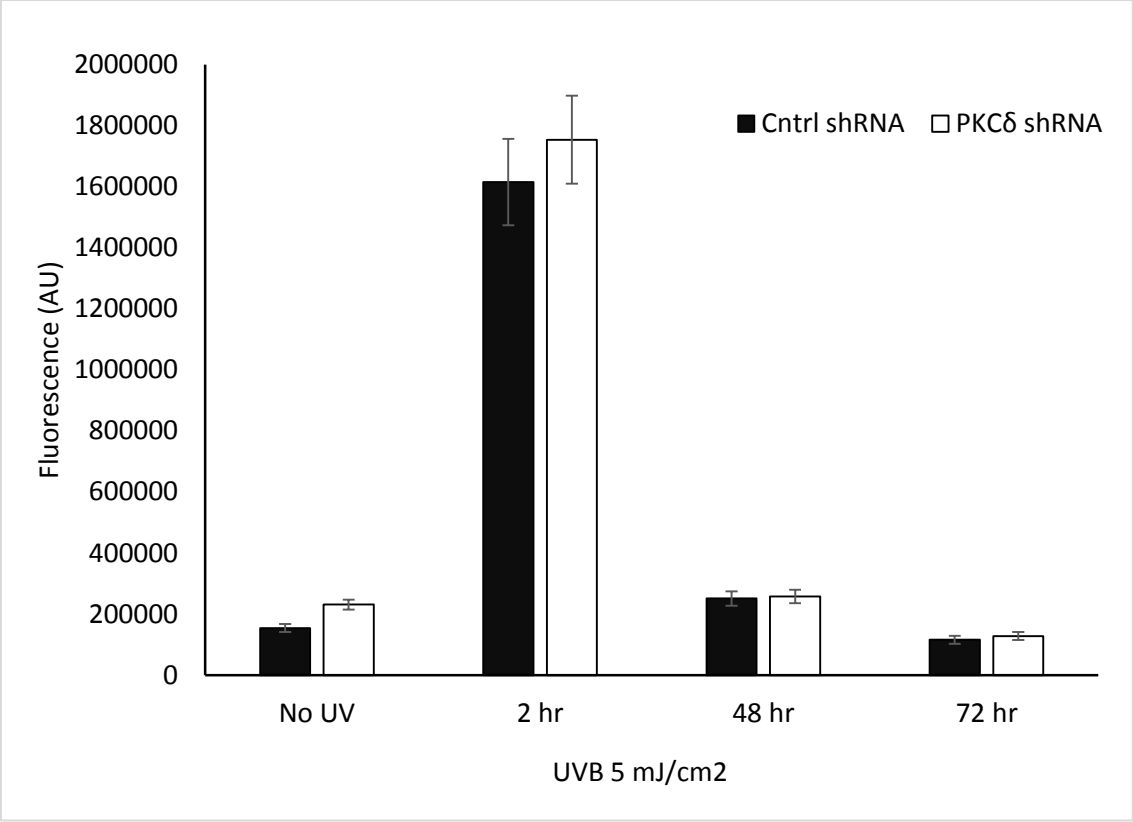


Figure 15. PKCδ Knockdown Did Not Affect Repair of UV Induced DNA Damage in HaCaTs with Mutant *TP53*

A. Western blot analysis of PKCδ knockdown. PKCδ was knocked down in HaCaTs using shRNA and protein lysates were harvested. PKCδ protein levels were analyzed using western blotting. PKCδ levels were decreased in samples treated with PKCδ shRNA (83% knockdown analyzed by ImageJ).
B. Flow cytometry analysis of CPD in HaCaTs. Cells were irradiated with 5 mJ/cm^2 UVB and incubated as indicated. Flow cytometry was used to detect CPD fluorescence intensity in cells. CPD levels were determined by calculating geometric mean of fluorescence values of 30,000 cells in each sample. $,@ p value< 0.05.
C. Immunofluorescence microscopy of CPD in HaCaTs. HaCaTs were irradiated with 5 mJ/cm^2 UVB and incubated as indicated. Cells were fixed, permeabilized and stained with an anti-CPD antibody, followed by incubation with secondary antibody.
D. Quantification of fluorescence from immunofluorescence using ImageJ software.

3.9 Investigation of Transcript Levels of p53 Target Genes

Because p53 protein levels were reduced in the PKCδ null MEFs, we investigated whether p53-target genes had reduced induction following UVB exposure. mRNA of p53-target genes involved in cell cycle arrest (*p21* and *Gadd45a*) [202][205] as well as in NER (*XPC* and *DDB2)* [79, 80, 290] were investigated in WT and PKCδ null MEFs. MEFs were exposed with 5 mJ/cm^2 UVB and incubated for 24 hours. mRNA levels were investigated using qRT-PCR.

50-fold UV induction of *p21* mRNA was observed in WT MEFs, however PKCδ null MEFs had only 14-fold induction. Thus, WT MEFs had 3.5-fold higher UV induction of *p21* mRNA than PKCδ null MEFs 24 hours after UV exposure (*Figure 16*). However there was no statistical significant difference between the UV groups.

UV induction was observed in *Gadd45a* mRNA as well. WT MEFs had 7-fold UV induction while PKCδ null MEFs had only 4-fold induction of *Gadd45a* mRNA (*Figure 17*). WT MEFs had significantly ($p<0.05$) higher UV induction of *Gadd45a* transcript than PKCδ null MEFs.

16.

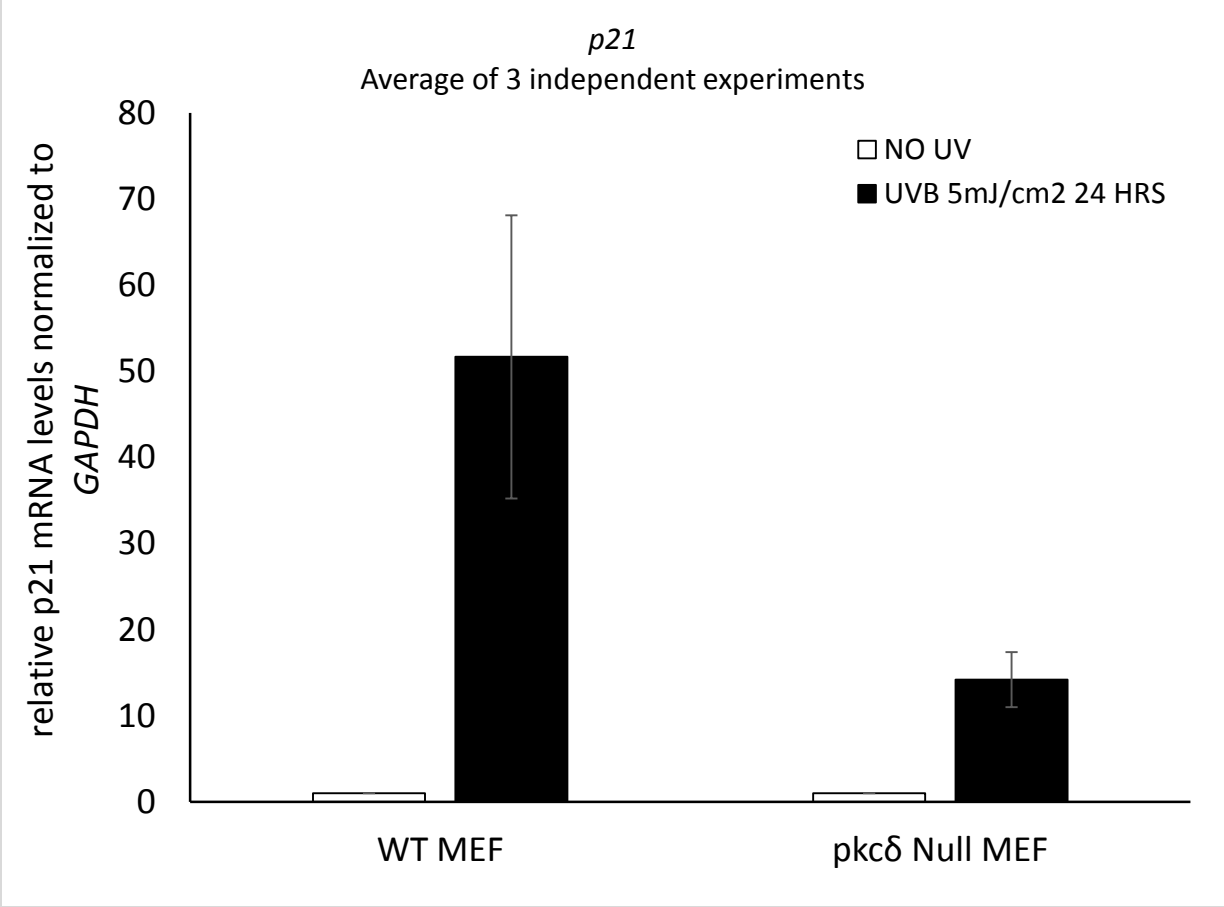


Figure 16. PKCδ Null MEFs Have Reduced UV Induction of *p21* mRNA

WT and PKCδ null MEFs were exposed to 5 mJ/cm^2 UVB as indicated and incubated for 24 hours. *p21* mRNA levels were analyzed using qRT-PCR and normalized to *GAPDH* control. Average of 3 independent experiments is shown in this graph. WT MEFs had 50-fold UV induction of *p21* mRNA while PKCδ null MEFs had only 14-fold UV induction. However, there was no statistically significant difference between the UV groups.

17.

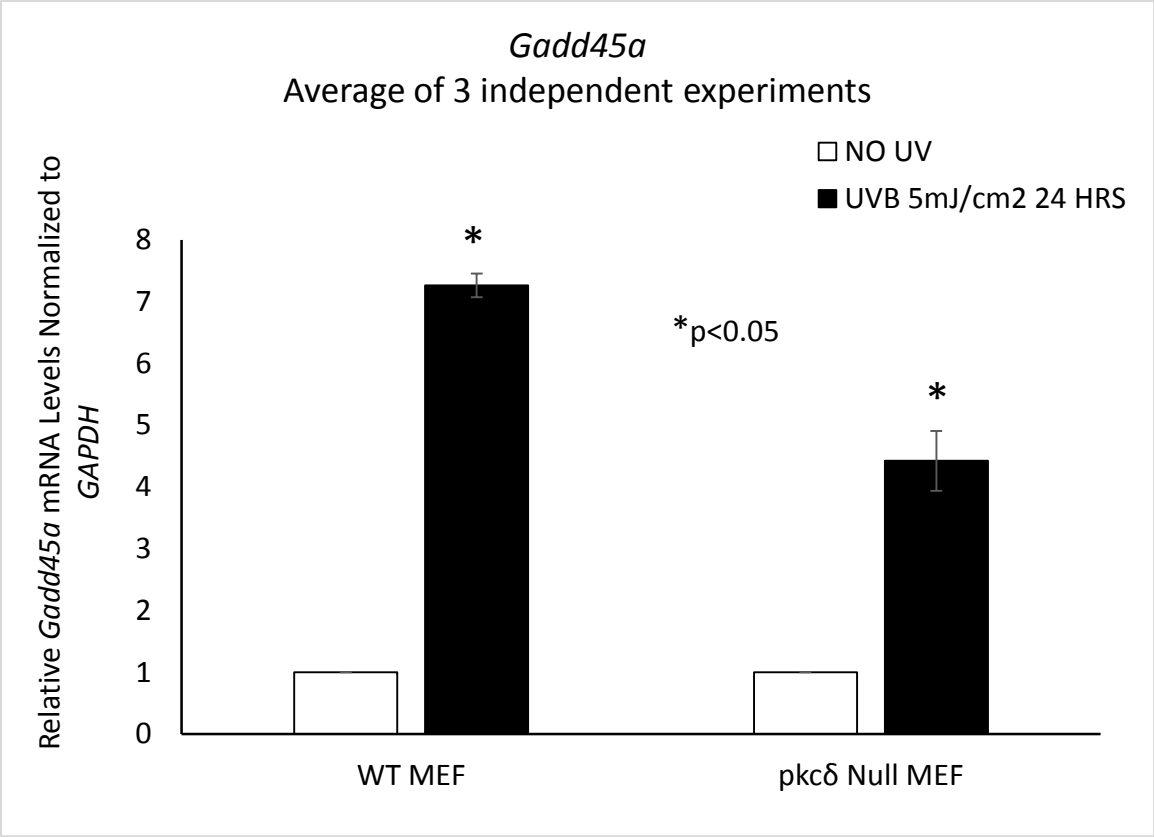


Figure 17. PKCδ Null MEFs Have Reduced UV Induction of *Gadd45a* mRNA

WT and PKCδ null MEFs were exposed to 5 mJ/cm^2 UVB as indicated and incubated for 24 hours. *Gadd45a* mRNA levels were investigated using qRT-PCR and were normalized to *GAPDH* control. This graph is showing average of 3 independent experiments. 7-fold UV induction of *Gadd45a* mRNA was observed in the WT MEFs whereas only 4-fold UV induction was observed in the PKCδ null MEFs. WT MEFs had significantly higher ($p<0.05$) UV induction of *Gadd45a* than PKCδ null MEFs.

PKCδ Null MEFs Have elevated UV Induction of *XPC* and *DDB2* mRNA

Additionally, UV induction of mRNA of NER proteins *XPC* and *DDB2* was investigated. PKCδ null MEFs had 4-fold UV-induction of *XPC* mRNA whereas only 1.5-fold increase was observed in the WT MEFs (*Figure 18*). There was no statistically significant difference in the UV induction of *XPC* mRNA between WT and PKCδ null MEFs. PKCδ null MEFs had 3-fold UV induction of *DDB2* mRNA, however UV failed to induce increase in mRNA levels of *DDB2* in WT MEFs (*Figure 19*) 24 hours after UV exposure. There was no statistically significant difference between the groups.

The basal expression of *XPC* mRNA were 1.4-fold higher in the PKCδ null MEFS than WT MEFs but the basal *DDB2* mRNA levels were almost similar in the WT and PKCδ null MEFs (1.1-fold difference). There was no statistically significant difference in the basal levels of the *XPC* and *DDB2* mRNA between WT and PKCδ null MEFs.

18.

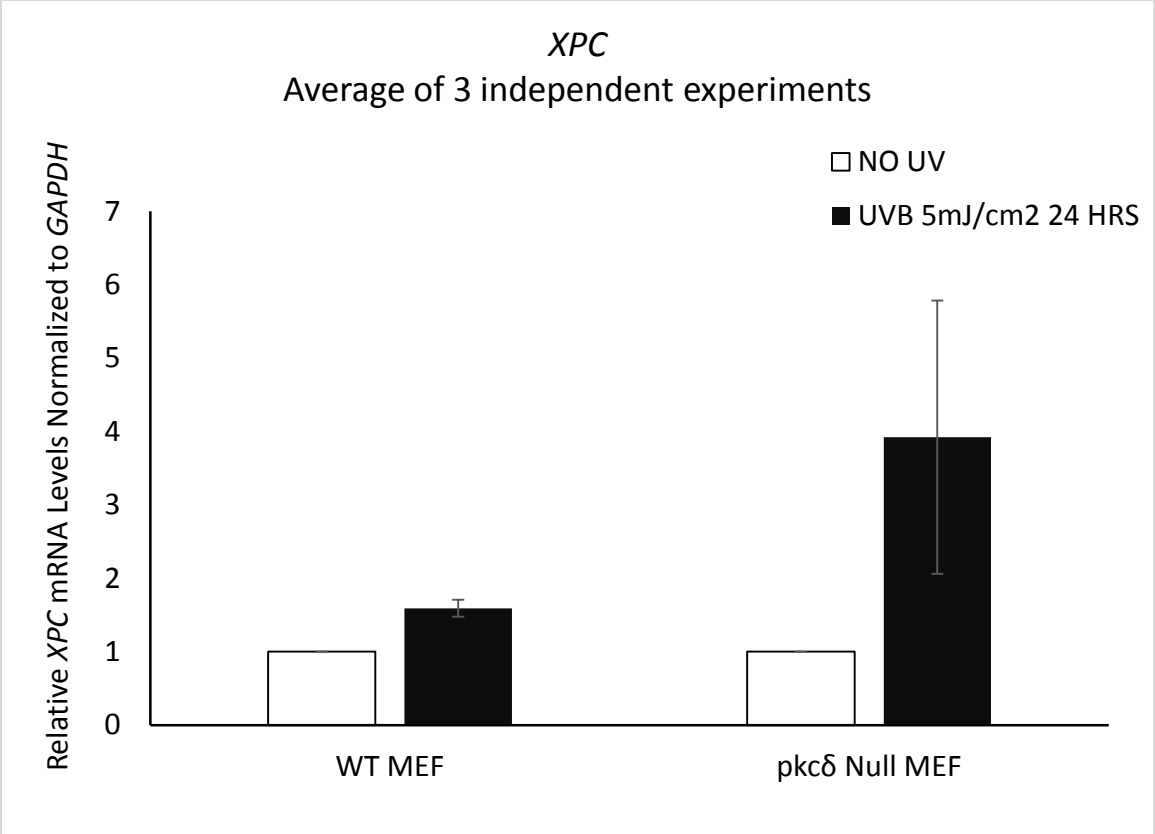


Figure 18. PKCδ Null MEFs Have Increased UV Induction of *XPC* mRNA

WT and PKCδ null MEFs were exposed to 5 mJ/cm^2 UVB as indicates and incubated for 24 hours. *XPC* mRNA levels were investigated using qRT-PCR and normalized to *GAPDH* control. This graph is indicating average of 3 independent experiments. No statistical difference was observed between the UV-induction of *XPC* mRNA in WT and PKCδ null MEFs.

19.

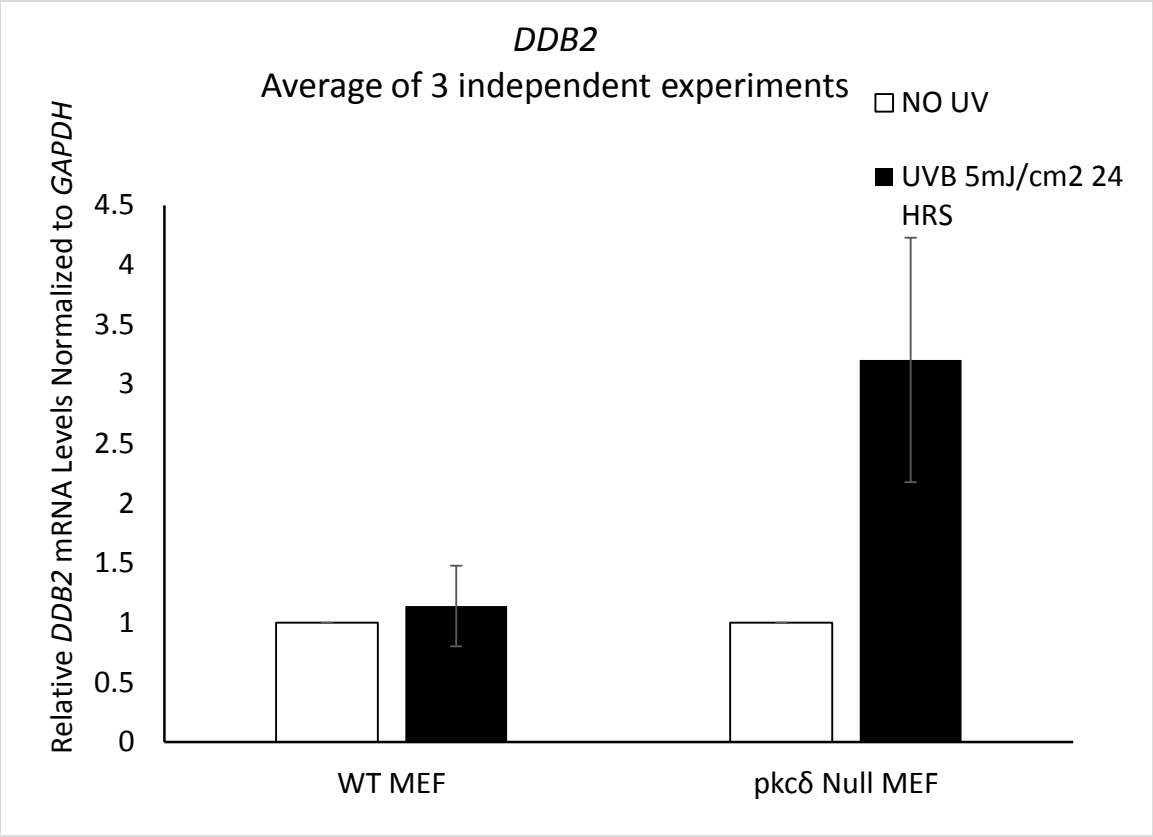


Figure 19. PKCδ Null MEFs Have Increased UV Induction of *DDB2* mRNA

WT and PKCδ null MEFs were exposed to 5 mJ/cm^2 UVB as indicated and incubated for 24 hours. *DDB2* mRNA levels were investigated using qRT-PCR and normalized to *GAPDH* control. Average of 3 independent experiments exhibited here.

CHAPTER FOUR

DISCUSSION

PKCδ may repress tumorigenicity in cells by activating cell cycle arrest at various checkpoints after DNA damage or inducing apoptosis, when the repair is not possible. However, very little is known about the involvement of PKCδ in DNA damage repair and the DNA damage repair interacting partners of PKCδ. In this project, we investigated the role of PKCδ in UV-induced DNA damage repair. PKCδ maintains genetic integrity and suppresses oncogenic transformation in cells after DNA damage using various mechanisms. PKCδ overexpression was found to be inducing G1 [125] or p27 (Kip1) dependent S-phase cell cycle arrest [291]. Additionally, PKCδ is also responsible for the maintenance of G2/M checkpoints after DNA damage [127].

For its pro-apoptotic function, PKCδ is cleaved and transformed into a constitutively, pro-apoptotic active catalytic fragment by caspase 3 [98]. Additionally, upon DNA damage, PKCδ promotes apoptosis by activating the MAPK pathway [117, 118]. PKCδ also interacts with pro-apoptotic proteins such as Bax [93], BAK [292] and Btf [120] to induce apoptosis. PKCδ also promotes apoptosis by phosphorylating the anti-apoptotic protein Mcl-1 and inducing proteosomal degradation [107]. Upon ionizing radiation induced DNA damage, PKCδ translocates to the nucleus and induces p53-dependent apoptosis in association with c-Abl with PKCδ nuclear localization sequence [293].

TP53 mutations are a common phenomenon in skin cancer and in fact normal appearing skin harbors patches of *TP53* mutated cells [294]. These *TP53* mutant clones are particularly dangerous because they can invade neighboring stem compartments called epidermal proliferative units after repeated UV exposure [295]. PKCδ re-expression induces apoptosis in HaCaT keratinocytes exhibiting *TP53* mutations and suppresses their tumorigenicity in nude mice [280]. PKCδ plays very important role in eliminating these *TP53* mutated cells by inducing apoptosis to maintain genetic integrity of the tissue.

PKCδ is implicated in interacting with proteins involved in DNA damage repair. PKCδ is found to be physically interacting with Ribosomal protein S3 (rpS3), which is a part of 40S ribosomal subunit. rpS3 usually functions in the translation machinery but can also act as an endonuclease to cleave a DNA adduct [296]. Upon genotoxin-induced of DNA damage, PKCδ-mediated phosphorylation of rpS3 enhances its endonuclease activity and nuclear localization [297]. The G2 checkpoint protein Rad9 forms a complex with Rad1 and Hus1 and formation of this Rad9-Rad1-Hus1 (9-1-1) complex is important for the G2 checkpoint control [298]. Upon genotoxin ara-C treatment, PKCδ phosphorylates Rad9 and promotes formation of 9-1-1 complex. PKCδ is also required for the induction of Rad9-mediated apoptosis after ara-C treatment [299]. Similarly, PKCδ knockdown reduced activating phosphorylation of Checkpoint kinase 2 (chk2) on threonine 68 [300, 301] upon genotoxin treatment. Thus PKCδ can participate in multiple DNA damage repair pathways, but no published studies have explored its involvement in NER.

PKCδ Is Involved In Repair of UV-Induced DNA Damage

To investigate the involvement of PKCδ in UV-induced DNA damage repair, immunofluorescence microscopy and flow cytometry techniques were used in WT and PKCδ null MEFs. Both techniques showed similar trend of reduced CPD repair in MEFs lacking PKCδ indicating that PKCδ is involved in the repair of UV-induced DNA damage (*Figure 2A and B*). UV-induced CPDs are repaired by NER, where the repair of the DNA damage can be either via the TC or GG [43] components of NER. We can speculate whether UV DNA damage repair in WT MEFs was TC or GG-NER. If the DNA damage repair defect observed in the PKCδ null MEFs was only TC, then a very small amount of damage would have been unrepaired. However, we saw widespread repair defect in the PKCδ null MEFs. Thus we can be fairly confident to conclude that the UV DNA damage repair requiring PKCδ was GG NER. We still cannot exclude a role for PKCδ in TC NER.

PKCδ is required for the maintenance of G2/M cell cycle checkpoint following UV exposure [127]. Arresting DNA damaged cells at G2/M checkpoint provides time to repair the DNA damage. PKCδ null MEFs are not able to sustain the G2/M cell cycle checkpoint compared to WT MEFs, suggesting in these MEFs, the NER machinery does not get enough time provided by the cell cycle arrest required for the DNA damage repair, and thus the NER machinery is functionally compromised resulting in defect in the DNA damage repair. Furthermore, PKCδ null MEFs are resistant to the DNA damage due to reduced apoptosis and thus have a survival advantage over WT MEFs.

NER is a promiscuous DNA damage repair pathway and thus cells lacking PKCδ may be resistant to other forms of DNA damaging agents such as chemotherapy drugs.

NER repairs the DNA adducts formed by the platinum based chemotherapy drugs such as cisplatin and thus reduces the effectivity of the drug [302-304]. It is unknown whether PKCδ participates in the repair of the DNA adducts formed by the chemotherapy drugs. However, it has been found that PKCδ is activated by cisplatin treatment and induces apoptosis in renal cells [305, 306]. 5-fluorouracil (FU) is used in treatment of skin cancer and many skin cancers have mutated *TP53*. PKCδ induces apoptosis in colorectal cancer cells upon treatment with FU [307]. Additionally, PKCδ and c-Abl are involved in FU-mediated apoptosis in Hep3B cells in the absence of p53, p73 and Fas receptor [308]. On the contrary, PKCδ is also found to be responsible for chemo-resistance in cisplatin treated thyroid [309, 310] and breast cancer cells [305] where pharmacological inhibition of PKCδ increased sensitivity to chemotherapy. Thus, the role of PKCδ in chemotherapy-induced apoptosis pathways might be organ dependent.

PKCδ Reduces UV-Induced Mutagenesis

Previously, UV *Hprt* mutagenesis assay has been used in diverse contexts in multiple cell lines to investigate the mutagenic potential of a chemical agents and the requirement of a protein in DNA damage repair and to compare genotoxic agents [311-315]. PKCδ null MEFs have a defect in maintaining the G2/M cell cycle checkpoint as well as a defect in repairing the UV-induced DNA damage (*Figure 2*). Because we found a defect in the DNA damage repair in the PKCδ null MEFs, we investigated the influence of PKCδ on the frequency of UV-induced mutations at the *Hprt* locus (*Figure 4*) in WT and PKCδ null MEFs. If the DNA damage were not repaired completely, DNA damage would be accumulated, thus leading to mutations. We found that the WT and PKCδ null MEFs

had very low (less than 2 X 10^{-5}) endogenous *Hprt* mutation frequency. UVB-irradiation induced mutations at the *Hprt* locus in both WT and PKCδ null MEFs however, PKCδ null MEFs showed a significantly increased mutation frequency, 5 time higher compared to WT MEFs (*Figure 5*). Also, PKCδ null MEFs showed higher colony forming efficiency than WT MEFs after UVB irradiation, indicating that they are more resistant to UV than the WT MEFs.

The UV dose used here (5 mJ/cm^2 UVB or 1 mJ/cm^2 UVC) was high enough to induce DNA damage but was low enough to not start the apoptosis pathway. Thus the cells will survive and will need to repair the damage or produce mutations. In the PKCδ null cells, the mutations may have developed due to defective NER machinery leading to incomplete DNA damage repair. These damages then would accumulate as well as would be passed down to the daughter cells. This is the first study done to investigate the role of PKCδ in UV-induced mutagenesis and to demonstrate that the reduced NER in PKCδ null cells leads to enhanced mutagenesis, thereby contributing to carcinogenesis.

Other investigators found a role of polymerase ζ subunits hREV3, hRev7 and REV1 in UV-induced mutagenesis using the *Hprt* mutagenesis assay [311, 312, 316]. The involvement of trans-lesion polymerase eta (Pol η) in UV-induced mutagenesis is well established [317]. Likewise involvement of Pol η in benzo[a]pyrene diol epoxide (BPDE)-induced mutagenesis was investigated using *Hprt* mutagenesis assay, where Pol η was found to be promoting mutagenesis by bypassing UV DNA damages [313].

DNA Damage Repair Interacting Partners of PKCδ

While investigating the mechanistic role of PKCδ in UV DNA damage repair, we found that MEFs lacking PKCδ had reduced total p53 and phosphorylated p53 S15 protein levels (*Figure 10A and B*). This is very significant given the central role of p53 in DNA damage responses, including NER. Notably, *Tp53* mRNA levels were not reduced in the PKCδ null MEFs (*Figure 11*) indicating that the cause for reduction in p53 protein levels was not reduced *Tp53* mRNA. However some studies have shown that PKCδ positively regulates *TP53* basal transcription upon 12-O-tetradecanoylphorbol-13-acetate (TPA) or genotoxin doxorubicin treatment, and inhibition of PKCδ reduces *TP53* transcript levels. Additionally, PKCδ promotes *TP53* transcription by interacting with the transcription factor Btf upon genotoxin exposure. The researchers used a reporter assay and chromatin immune-precipitation (ChIP) assay to look at the *TP53* transcriptional activity and found that PKCδ upregulates *TP53* transcription by co-occupying the *TP53* core promoter element with Btf upon genotoxin treatment [318][120]. Above mechanisms indicate that PKCδ can be involved in transcriptional upregulation of p53 upon DNA damage, but we did not find lower levels of *TP53* mRNA in PKCδ null MEFs

PKCδ null MEFs have impaired DNA damage repair, loss of G2/M cell cycle checkpoint and reduced apoptosis. Since p53 is involved in apoptosis, cell cycle regulation, and involved in transcription of various DNA damage response factors, decreased p53 levels in the PKCδ null MEFs may enhance the susceptibility to mutations due to compromised NER, reduce cell cycle arrests and diminished apoptosis.

Furthermore, due to decreased p53 in PKCδ null MEFs, the cells may have great risk of oncogenic transformation.

Surprisingly, the p53 levels in the WT MEFs were not induced after UV exposure (*Figure 10B*). In the absence of UV, the p53 basal levels were also higher in WT MEFs and appeared to be stabilized. The mutation status of p53 is unknown in the WT or PKCδ null MEFs. It is highly unlikely that the p53 in the WT MEFs in mutated because the WT MEFs are capable of repair the UV DNA damage and mutated p53 would hinder the repair. Determining the mutation status of the p53 in WT and PKCδ null MEFs is a future direction in this investigation.

p53 in WT MEFs Has a Longer Half-Life Than in PKCδ Null MEFs

The stability of p53 protein was investigated using the protein translation inhibitor cyclohexamide in MEFs (*Figure 12A and B*), which revealed that the p53 in WT MEFs had longer half-life (4.6 hours) than in the PKCδ null MEFs (2.6 hours). It is unknown why p53 in WT MEFs is more stable than p53 in PKCδ null MEFs. p53 half-life is predominantly regulated by E3-ubiquitin ligase Mdm2 [152]. Mdm2 binds and poly-ubiquitinates/degrades p53 protein through the ubiquitin-mediated proteosomal degradation pathway [153-156]. Upon stress, p53 needs to be stabilized rapidly to perform its functions, thus upon genotoxic stress, p53 is stabilized by Herpesvirus-associated ubiquitin-specific protease (HAUSP/USP7). It forms a direct complex with p53 and deubiquitinates it, thus rescuing p53 from proteosomal degradation. HAUSP keeps deubiquitinating p53 even in the presence of excess Mdm2 protein [319, 320]. Similarly, Ubiquitin-specific protease 42 (USP42) and Ubiquitin-specific protease 29 (USP29)

physically bind to p53 and deubiquitinate it [321, 322]. Furthermore, Ubiquitin-specific protease 10 (USP10) also deubiquitinates p53 and stabilize its levels [323]. Apart from deubiquitination, p53 protein is also stabilized by phosphorylation by DNA-PK, ATM and ATR on serine 15 or serine 37. These phosphorylations induce conformation change in the p53 structure in a way that Mdm2 cannot bind it anymore [177, 324, 325].

Various direct and indirect interactions between PKCδ and p53 have been previously reported. Apart from transcription regulation, PKCδ indirectly positively regulates p53 through IKKα [274] and p53DINP1 [326]. PKCδ induces p53 accumulation and translocation to the mitochondria to initiate apoptosis upon H_2O_2 treatment induced damage [276, 327]. Thus PKCδ indirectly stabilize the p53 levels but a direct mechanism is not known yet.

The half-life of p53 is highly variable in different treatments and cells lines. Determination of p53 half-life using CHX had been carried out in the past in colon carcinoma cell line RKO cells. One group reported p53 half-life in UV-irradiated (UV 50 J/cm^2) RKO cells was found to be 1-2 hours [328] whereas another study determined the p53 half-life to be greater than 3.5 hours in UV-irradiated (UV 20 J/cm^2) RKO cells [329]. Gamma radiation-induced (10 Gy) p53 stabilization in the RKO cells was found to result in a half-life longer than 3.5 hours [329]. Similarly, p53 half-life was found to be 33.42 minutes in untreated mouse melanoma cell line 8B20 [330] and 20 minutes in un-transformed NIH-3T3 cells [331, 332]. Furthermore, p53 half-life was 72.5 minutes in etoposide treated nuclear lysates of MCF-7 cells [333] and 22.5 minutes control transfected MCF7 cells [334].

PKCδ Did Not Phosphorylate p53 on Serine 15 *In Vitro*

PKCδ null MEFs had reduced phosphorylated p53 S15 protein levels compared to WT MEFs (*Figure 10A and B*). We speculated that the decreased p53 S15 levels in PKCδ null MEFs were because PKCδ may phosphorylate p53 on S15. *In vitro* kinase assays were performed to test this. In our experiments, PKCδ did not directly phosphorylate p53 on serine 15 *in vitro*; whereas, PKCδ phosphorylation target MARCKS was found to be phosphorylated by PKCδ (*Figure 14*). Thus, PKCδ is not responsible for p53 phosphorylation on Ser 15. Additionally, the S15 site is not close to a consensus PKCδ phosphorylation site [335].

The S15 phosphorylation is usually induced upon genotoxin exposure or stress, such as UV. In the p53 half-life experiment (*Figure 12A and B*), the p53 half-life was determined without UV and p53 S15 phosphorylation was not different between unexposed WT and PKCδ null MEFs. Thus, phosphorylation on serine 15 is not the mechanism for the PKCδ-mediated stabilization of p53 in MEFs and cannot explain the difference in the half-life between WT and PKCδ null MEFs.

It has been observed that PKCδ phosphorylates p53 on C-terminal domain in other system [335]. PKCδ was reported to be involved in phosphorylation of p53 on serine 15 in sodium nitroprusside-treated dopaminergic neuron cells SN4741, however this was not demonstrated to be direct [336]. Similarly, it has been previously reported that PKCδ can phosphorylate p53 on serine 46 *in vitro* [122]. To initiate apoptosis after DNA damage p53 is phosphorylated *in vitro* on serine 46 by ATM kinase [337].

PKCδ Knockdown Did Not Affect Repair of UV-Induced DNA Damage in HaCaTs with Mutant *TP53*

UV-induced DNA damage repair was examined in the human keratinocyte cell line HaCaT with or without PKCδ knockdown using immunofluorescence and flow cytometry techniques (*Figure 16A, B and C*). Coequal DNA damage repair was observed in control and PKCδ shRNA HaCaTs at 48 and 72 hours post-UV. This observation is in agreement with PKCδ being involved in DNA damage repair via p53. If PKCδ-mediated DNA damage repair involves p53, then knockdown of PKCδ should not affect the UV-induced DNA damage repair in HaCaTs because they harbor mutant *TP53* [278, 279]. Note, PKCδ knockdown inhibits UV-DNA damage repair in normal human keratinocytes, which contain wild type p53 (Jack O'Sullivan unpublished observation).

Investigation of Transcript Levels of p53 Target Genes

Since p53 is a transcription factor, reduced p53 protein levels in PKCδ null MEFs raised questions about the transcription of its target genes. We investigated the expression of p53 target genes in WT and PKCδ null MEFs upon UVB irradiation. UV irradiation-induced 3.5-fold higher mRNA levels of the cell cycle checkpoint protein *p21* in WT MEFs than PKCδ null MEFs (*Figure 17A*). Indirect regulation of *p21* by PKCδ had been previously reported in colon cancer cell [338]. In keratinocytes, PKCδ regulates *p21* transcription by controlling *p21* transcription factor Krüppel-like transcription factor (KLF4) [339]. However, the basal expression levels of *p21* mRNA were on average higher (31-fold) in PKCδ null MEFs than in WT, but this was highly variable and was not statistically significant. One possible explanation for the higher basal levels of the *p21* in

PKCδ null MEFs could be that the MEFs lacking PKCδ have impaired DNA damage repair as well as tendency for increased mutagenesis. This induces constant genomic stress on PKCδ null MEFs and that might be the reason for their slower growth than WT MEFs, and may induce elevated basal transcription of cell cycle inhibitor *p21*.

Gadd45a is activated by stress or DNA damage and is involved in cell cycle arrest and apoptosis [340]. WT MEFs had significantly ($p<0.05$) higher levels of UV-induced *Gadd45a* mRNA compared to PKCδ null MEFs (*Figure 18*). WT and PKCδ null MEFs had approximately the same levels of basal expression of the *Gadd45a* mRNA. Thus, p53 or PKCδ in WT MEFs may be responsible for higher levels of *Gadd45a* mRNA. *Gadd45a* is responsible for dissociation of cdc2/cyclin B1 complex and thus inhibiting the cell cycle at G2/M [341]. While PKCδ-mediated UV *Gadd45a* regulation has not been reported before, PKCδ was found to be involved in the stabilization of *Gadd45a* protein under Epidermal growth factor (EGF) treatment [342].

UV induction of NER factors *XPC* and *DDB2* transcript levels were also analyzed in WT and PKCδ null MEFs (*Figure 19 and 20*). PKCδ null MEFs had elevated UV induction of *XPC* and *DDB2* mRNA compared to WT MEFs was not induced by UV. This is surprising because the WT MEFs were more proficient in repair of UV DNA damage than the PKCδ null MEFs, and the mRNA levels of NER factors should have reflected that. However, NER proteins get recruited to the sites of DNA damage and the PKCδ null MEFs had higher levels of CPD DNA damage; thus, their mRNA levels may be induced higher in the PKCδ null MEFs to compensate for the persistent DNA damage. On the other hand, the

WT MEFs had repaired the CPD damage and the NEF factors are no longer required and thus their mRNA levels decrease.

Thus, despite PKCδ being required for repair of UV-induced DNA damage, it appears to not be required for the UV induction of p53 target NER genes *XPC* and *DDB2*.

Concluding Remarks

Here we investigated the involvement of PKCδ in repair of UV-induced DNA damage. We found that PKCδ is involved in the repair of UV-induced CPDs and protects from UV-induced mutagenesis. This is the first study to show that PKCδ reduces UV mutation frequency. We also found that p53 may be involved in the PKCδ-mediated DNA damage repair. PKCδ was found to be required for the cell cycle checkpoint genes and not for NER genes. Thus it can be speculated that the cell cycle defect is more important for PKCδ DNA damage response. It is still unknown how p53 is directly regulated by PKCδ in UV DNA damage repair but future investigations may clarify the UV DNA damage-induced interplay between p53 and PKCδ.

The reason behind the decreased half-life of p53 in PKCδ null MEFs compared to the p53 in WT MEFs, may be the decreased stability of p53 protein. Further role of PKCδ in mediating p53 protein turnover in the MEFs needs to be investigated. The p53 stability is dependent on it's negative regulators, major regulators which are directly involved in the p53 protein destabilization such as Mdm2 and USPs (7, 42, 29 10). It might be possible that the PKCδ is destabilizing Mdm2 or USPs by either phosphorylating or physically binding to them. This binding might cause structural changes in a way that Mdm2 or USPs cannot binds to p53 and ubiquitinates it. Thus, in

the absence of PKCδ, the Mdm2 or USPs can actively ubiquitinate and degrade p53. On contrary, p19/ARF binds to MDM2 and inhibits Mdm2 activity against p53 thus stabilizing the levels of p53 [160]. Activated PKCδ can translocate to nucleus and might act as a coactivator of p19/ARF. Additionally, PKCδ might be involved in stabilization of the p19/ARF by phosphorylating it. Thus, Mdm2, USPs and p19/ARF are prominent suspects for fiture investigations.

Taken together, PKCδ is involved in multiple tumor suppressing mechanisms. It is well established that PKCδ induces apoptosis after DNA damage to eliminate cells with catastrophic DNA damage. PKCδ is also involved in arresting cell cycle at G1 or S-phase as well as maintenance of G2/M checkpoint. Additionally, lack of PKCδ has been observed in many cancers and decrease in tumorigenicity was observed when PKCδ was re-introduced into tumor cells. Finally, PKCδ participates in UV-induced DNA damage repair, reduces UV mutagenesis and is required for cell cycle checkpoint genes. Thus, DNA damage repair-coupled cell cycle checkpoints may be an important tumor suppressor mechanism for PKCδ.

20. Working Model

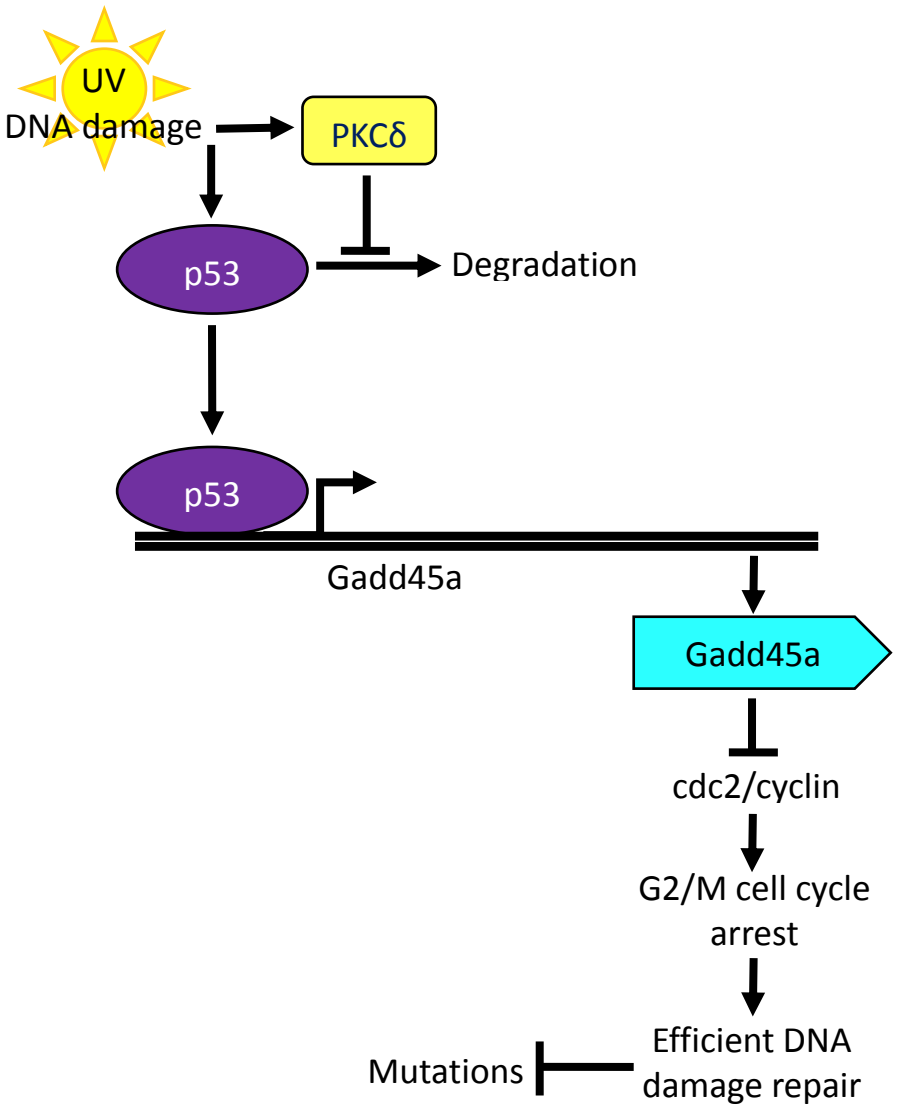


Figure 20. PKCδ Mediated DNA Damage Repair

PKCδ increase stability of p53 protein by inhibiting its degradation. This increases levels of the p53 and p53 starts transcription of *Gadd45a*. *Gadd45a* is known to disrupt and inhibit cdc2/cyclin B1 complex. This arrests the cell cycle at G2/M checkpoint and the DNA damage repair machinery gets ample time to repair the DNA damage. Additionally, because the cell cycle is arrested, the DNA damage is not passed down to the daughter cells and that protects the genetic integrity of the cell. In the absence of this cell cycle checkpoint, the DNA damage repair machinery would not have enough time to repair

the damage and the damage will be passed down to the daughter cells leading to mutations.

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