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The Tumor Suppressor PKC δ Is Critical for UV-Induced G2/M Checkpoint Activation and Apoptosis

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LOYOLA UNIVERSITY CHICAGO

THE TUMOR SUPPRESSOR PKC δ IS CRITICAL FOR UV-INDUCED G2/M
CHECKPOINT ACTIVATION AND APOPTOSIS

A DISSERTATION SUBMITTED TO THE FACULTY OF THE
GRADUATE SCHOOL IN CANDIDACY FOR THE DEGREE OF
DOCTOR OF MOLECULAR AND CELLULAR BIOCHEMISTRY
PROGRAM IN MOLECULAR AND CELLULAR BIOCHEMISTRY

BY

EDWARD LEWIS LAGORY

CHICAGO, ILLINOIS

JUNE 2010

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List of Abbreviations

APC/C: Anaphase promoting complex/cyclosome

A-T: Ataxia Telangiectasia

ATM: Ataxia telangiectasia mutated kinase

ATR: Ataxia telangiectasia and Rad3 related kinase

Cdk: Cyclin-dependent kinase

G2/M: Boundary between G2 and mitosis

HR: Homologous recombination

KC: Primary human keratinocytes

NER: Nucleotide excision repair

NHEJ: Non-homologous end joining

NMSC: Non-melanoma skin cancer

PKC δ : Protein Kinase C δ

PKC δ -cat: Protein Kinase C δ catalytic fragment

PKC δ -cat-ER: Protein Kinase C δ catalytic fragment/estrogen receptor ligand binding domain fusion protein.

P~Cdk1(Y15): Cdk1 phosphorylation on tyrosine 15

P~H3(S10): Histone H3 phosphorylation on serine 10

P~p53(S15): p53 phosphorylation on serine 15

UV: ultraviolet radiation

XP: Xeroderma pigmentosum

Abstract

Protein kinase C delta (PKC δ) is an essential component of the intrinsic apoptotic program. Following DNA damage, such as exposure to UV radiation, PKC δ is cleaved in a caspase-dependent manner, generating a constitutively active catalytic fragment (PKC δ -cat) which is necessary and sufficient for keratinocyte (KC) apoptosis. We found that in addition to inducing apoptosis, expression of PKC δ -cat caused a pronounced G2/M cell cycle arrest in both primary human KCs and immortalized HaCaT cells. Consistent with a G2/M arrest, PKC δ -cat induced phosphorylation of Cdk1 (Tyr15), a critical event in the G2/M checkpoint. Treatment with the ATM/ATR inhibitor caffeine was unable to prevent PKC δ -cat induced G2/M arrest, suggesting that PKC δ -cat is functioning downstream of ATM/ATR in the G2/M checkpoint. To better understand the role of PKC δ and PKC δ -cat in the cell cycle response to DNA damage, we exposed wild type and PKC δ null MEFs to UV radiation. Wild type MEFs underwent a pronounced G2/M arrest, Cdk1 phosphorylation, and induction of apoptosis following UV exposure, while PKC δ null MEFs were resistant to these effects. Expression of PKC δ -GFP, but not caspase-resistant or kinase inactive PKC δ , was able to restore G2/M checkpoint integrity in PKC δ null MEFs. The function of PKC δ in the DNA damage-induced G2/M cell cycle checkpoint may be a critical component of its tumor suppressor function.

In light of recent reports suggesting the importance of nuclear localized PKC δ in the apoptotic response, we examined changes in PKC δ sub-cellular localization following exposure to UV radiation. Using a PKC δ -GFP fusion protein we determined that nuclear PKC δ was present after UV exposure, and that it formed foci coinciding with regions of intense DAPI staining, suggesting localization to condensed chromatin. We have previously shown that expression of the PKC δ catalytic fragment induced phosphorylation of Histone H3 (Ser10), an important event for mitotic chromatin condensation. Interestingly, PKC δ -cat induction of P~H3(S10) was not prevented by inhibition of the mitotic H3 kinase Aurora B, and was induced throughout all phases of the cell cycle, supporting the idea that this event is distinct from the P~H3(S10) induction during mitosis. *In vitro* phosphorylation studies using recombinant proteins demonstrated that PKC δ -cat is capable of directly phosphorylating H3 on Ser10 raising the possibility that PKC δ may function as a Histone H3 kinase in the cell. Using confocal microscopy we found that UV-induced PKC δ -GFP foci co-localized with regions of positive P~H3(S10) staining. To avoid any artifacts associated with over-expression of the PKC δ -GFP fusion protein, we confirmed the formation of endogenous PKC δ foci after UV radiation in normal keratinocytes. This novel chromatin modifying activity of PKC δ may be an important component of its tumor suppressor function for UV-induced skin cancers in the human epidermis.

Chapter One

I. Introduction

1.1 Epidermal Biology and Cancer

1.1.1 Epidermal Structure and Function

The epidermis is the outermost layer of the skin and the largest organ by weight in the human body. The skin performs many critical functions including providing a protective barrier against infection and the environment as well as maintaining a waterproof layer that prevents loss of water from the human body. The skin is composed of two major layers, the dermis and the epidermis. The dermis is a largely acellular region, consisting primarily of extra-cellular matrix (ECM) proteins, dermal fibroblasts, blood vessels, hair follicles, and sebaceous glands. Dermal fibroblasts secrete the ECM components and other factors that nourish and support the epidermis. The epidermis represents the outermost layer of the skin, and largely consists of the structural cell of the skin, the keratinocyte. Also residing within the epidermis are pigment producing melanocytes and antigen-presenting Langerhaan cells.

Separating the epidermis and dermis and providing a critical anchoring site for basal keratinocytes is the basement membrane. This membrane is composed of ECM proteins and cell membrane receptors including $\alpha 6\beta 4$ integrins and laminin-5 and prevents invasion of keratinocytes and melanocytes into the dermis (Niessen et al., 1994). Importantly, during carcinogenesis, matrix-metalloproteinases secreted by neoplastic

keratinocytes or melanocytes can degrade the basement membrane, allowing invasion of the transformed cells into the dermis and eventually the bloodstream.

The epidermis is a self-renewing tissue, ensuring efficient wound healing post-injury and constant replenishment of the tissue during normal homeostasis. Residing in the basal layer of the epidermis, proliferating keratinocytes detach from the basement membrane, undergo irreversible growth arrest, and begin a sequential differentiation program that ultimately results in cell death and cornification. The epidermis consists of several distinct layers of keratinocytes at various stages of differentiation. These layers include the basal layer, which contains self-renewing undifferentiated keratinocytes, the intermediate spinous and granular layers, and finally the outermost cornified layer which contains dead keratinocytes and the barrier proteins of the skin, including loricrin, involucrin, and filaggrin (Dale and Holbrook, 1987; Presland and Dale, 2000). In direct relation to the increasing level of differentiation during progression from basal to apical regions of the epidermis is a calcium gradient (Menon et al., 1985). The increasing levels of calcium in the suprabasal layer are critical for epidermal barrier function and keratinocyte differentiation through the activation of the classical protein kinase C isoform, PKC α (Elias et al., 1998; Jaken and Yuspa, 1988; Jerome-Morais et al., 2009). Evidence for the critical role of calcium in keratinocyte differentiation is further bolstered by the common use of elevated Ca²⁺ to induce the differentiation of cultured keratinocytes (Hennings et al., 1980).

During the keratinocyte differentiation process there is a progressive downregulation of cell cycle and proliferation genes such as cyclin D1 and upregulation of differentiation genes such as keratin 1 and 10, desmoglein 1 and ultimately, barrier proteins such as loricrin and profilaggrin. It is believed that the coordinated array of gene activation and repression during differentiation is controlled by epigenetic regulators such as DNMT1, DNMT3a, and DNMT3b as well as the histone demethylase, JMJD3 (Sen et al., 2010; Sen et al., 2008). In addition, the classical protein kinase C isoform, PKC α , is critical for activating the initial growth arrest following detachment of the differentiating keratinocyte from the basement membrane (Jerome-Morais et al., 2009). PKC δ and η have also been implicated in the keratinocyte differentiation program (Adhikary et al., 2010; Kamioka et al., 2010; Szegedi et al., 2009; Cabodi et al., 2000). PKC δ , for example, is critical for the proper epidermal differentiation associated upregulation of involucrin in human keratinocytes (Deucher et al., 2002).

1.1.2 Epidermal Tumorigenesis and Cancer

According to the National Cancer Institute (NCI), epidermal tumors are by far the most commonly diagnosed malignancies in the United States of America with over one million new cases diagnosed each year. There are several types of skin cancer that vary in clinical prognosis depending on the type of cell giving rise to the tumor, as well as the stage of cancer progression at the time of diagnosis. It has been understood for some time that there was a relationship between sun exposure and skin cancers in humans, early work proved the direct link between ultraviolet radiation and skin carcinogenesis in mice in the

1960's (Epstein and Epstein, 1963; Fisher and Kripke, 2002; Cleaver and Crowley, 2002).

1.1.3 Ultraviolet Radiation and Carcinogenesis

The sun emits a wide range of radiation on the electromagnetic spectrum. This includes high-energy wavelengths such as ionizing radiation as well as lower energy wavelengths including the visual spectrum and infrared radiation. Included in the spectrum of light emitted by the sun is ultraviolet (UV) radiation. UV radiation encompasses 100-400 nm wavelengths and is further divided into UV A, B, and C wavelengths. UVC is the highest energy, lowest wavelength form of UV radiation and is commonly used in germicidal lights for sterilization. UVC is emitted from the sun, but does not reach the surface of the Earth because it is absorbed by the outer atmosphere. In contrast, UVA and B are also emitted from the sun, but are less efficiently absorbed by the atmosphere and reach the Earth's surface at appreciable levels. Notably, the thinning of the ozone layer correlates with increased levels of UVA and UVB radiation reaching the surface of the Earth. This has led to a significant increase in skin cancer incidence in areas such as Australia where the thinning of the ozone layer is more pronounced than other regions (de Gruijl, 1999).

UVB, and possibly to a lesser extent UVA radiation, is highly carcinogenic in humans (de Gruijl et al., 1993). Exposure of DNA to UVB radiation generates bulky DNA adducts, including thymine dimers and 6-4 photoproducts, as well as oxidative damage to the DNA represented by formation of 8-oxo-2'-deoxyguanosine (Hemminki, 1993). The presence of these adducts on a DNA molecule distorts the double helix and can cause

errors in DNA replication and thus result in potentially carcinogenic mutations being incorporated into the genome of damaged keratinocytes or melanocytes (Sinha and Hader, 2002). Because the damage caused by UV radiation is sequence specific, specific UV signature mutations have been characterized, the most common being CC to TT and C to T mutations, with GC to AT mutations also commonly detected (Miller, 1985; Brash et al., 1987). Mutations that follow these patterns have been detected throughout critical cancer related genes such p53, indicating why p53 function is altered in many cases of NMSC (Brash et al., 1991).

UVA has long been considered to be less harmful than the higher energy forms of UV radiation, however recent evidence has revealed that UVA can also have a carcinogenic effect on the skin, although the mechanism is less understood. The direct mutagenic effect of UV radiation exposure on DNA molecules underscores the vital role of cell cycle checkpoints in recognizing damage, preserving genomic integrity. These checkpoints will be discussed in depth later in this document.

1.1.4 The Epidermal Keratinocyte

Keratinocytes are the structural cell of the epidermis and are the most common cell type in the skin. In the basal layer of the epidermis, self-renewing keratinocytes divide asymmetrically, allowing for maintenance of the proliferative compartment while giving rise to post-mitotic keratinocytes that make up the supra-basal layers (Lechler and Fuchs, 2005). Supra-basal keratinocytes undergo a sustained growth arrest and differentiation

program that ultimately results in cell death and cornification. This differentiation process results in expression of key proteins such as loricrin, filaggrin, and involucrin, which provide the skin with its barrier function.

Disruption of this growth arrest and differentiation process can result in keratinocyte neoplasias and non-melanoma skin cancers (NMSC). NMSCs include both basal and squamous cell carcinomas, which together account for over 1 million new cases diagnosed within the United States each year. This makes non-melanoma skin cancer the most common form of cancer by far. Generally, these cancers are associated with a relatively low rate of lethality with approximately 1000 deaths each year being attributed to metastatic growth of NMSCs. Despite this, preventing their formation using prophylactic treatments with sunscreens or lotions would decrease the significant morbidity associated with the treatment and removal of these tumors.

1.1.5 The Epidermal Melanocyte

Melanocytes originate from the neural crest during development. In the epidermis, melanocytes are responsible for producing skin pigmentation through generation of melanins. Once produced, these pigmented compounds are passed to keratinocytes where they absorb UV radiation, thus protecting epidermal cells from the carcinogenic effects of UV radiation exposure.

The most deadly skin cancer, malignant melanoma, arises from epidermal melanocytes. According to the NCI, approximately 70,000 new cases of cutaneous melanoma are diagnosed within the United States each year. While melanoma patients diagnosed with pre-metastatic disease have a favorable 5 year survival rate, those diagnosed with advanced metastatic disease have a 5 year survival rate of only 15.8% (Altekruse SF et al., 2009). This is because melanomas often display significant resistance to standard treatments such as chemotherapy and radiation. They also present with a high rate of metastasis to vital organs such as the brain and liver, making this form of skin cancer highly lethal.

1.2 Cell Cycle and Proliferation

1.2.1 Cell Cycle Overview

Multi-cellular eukaryotes, such as humans and mice, undergo a tightly controlled cell cycle in which the entire genome and cellular contents are replicated and equal halves are divided into two daughter cells (Figure 1). While it has been understood for decades that cells undergo a mitotic cell division process, the idea of a cell cycle in which the cells commit to DNA replication and division is more recent (Smith and Martin, 1973). Proper control of the cell cycle is critical in preventing aberrant proliferation, as well as ensuring that genomic mutations are not propagated to daughter cells. The majority of differentiated cells in the body are in a state of senescence or quiescence, depending on the tissue and cell type. These cells have for the large part, permanently exited the cell cycle and undergone terminal differentiation, allowing them to support the organ and tissue architecture in which they exist.

Despite the predominance of post-mitotic cells in the human body, proliferating cell populations make up a significant component of self-renewing tissues. For example, proliferating basal layer keratinocytes are critical for the replenishment of the suprabasal layers of the epidermis and are absolutely required for epidermal integrity (Regnier et al., 1986). Similarly, crypt cells in the small intestine migrate away from the basal region of the crypt and undergo terminal differentiation to become one of a number of cell types critical for barrier function and nutrient absorption in the colon (Humphries and Wright,

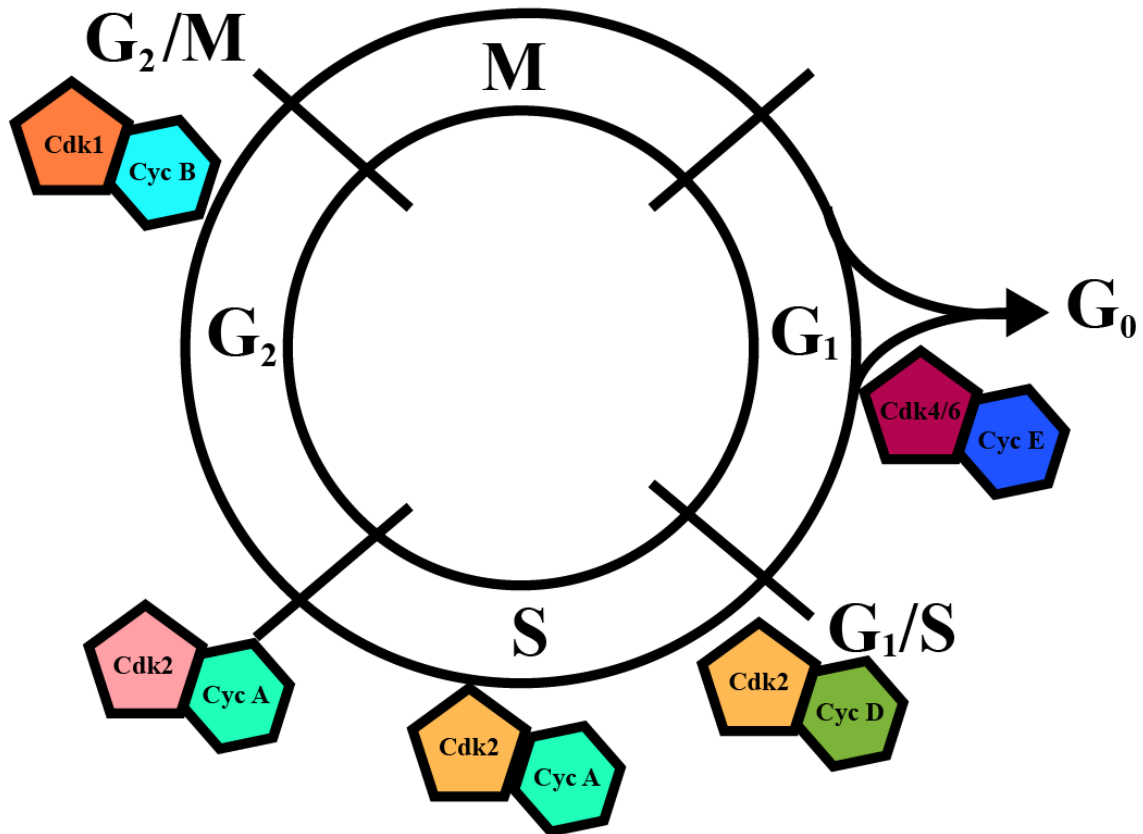


Figure 1 – Overview of the Eukaryotic Cell Cycle. The eukaryotic cell cycle is tightly controlled. Progression through several “checkpoints” at key transitions in the cell cycle requires the coordinated activation/inhibition of various cyclin-dependent kinase activity. These kinases are tightly controlled, and can only be activated upon binding of cyclin proteins, whose expression is strictly limited to specific cell cycle phases, ensuring that Cdk activity is only activated in the proper cell cycle compartment.

2008). The signal responsible for driving this growth arrest and differentiation program is likely unique to each individual tissue and cell type.

The eukaryotic cell cycle can be broken down into several stages. These stages are known as G1, S, G2, and finally mitosis (M). There is an additional cell cycle stage G0, in which the cell has permanently (senescence), or reversibly (quiescence) exited the cell cycle. The G1, or gap 1 phase of the cell cycle is tightly regulated by factors such as nutrient availability, cell-cell contact, and other extrinsic and intrinsic criterion that must be satisfied before the cell commits to undergoing a cell cycle. Once a cell has proceeded through the restriction checkpoint in early G1 phase, it has committed to division and now contains only intrinsic barriers to checkpoint progression. Importantly, the presence of mitogenic factors is critical for passage through the restriction point and the commitment to cell division. When a cell has determined that there are sufficient bio-materials available, the cell passes through the G1/S boundary and begins S phase.

The cell cycle stage known as S phase encompasses the period of genomic replication that occurs before a cell can undergo division. During S phase, the cell precisely replicates each and every one of its chromosomes. In this way the cell exits S phase having completely replicated the genome only once and is prepared to pass through the G2 phase into mitosis.

During G2 phase, a gap phase significantly shorter than G1, the cell prepares to enter mitosis. Once all required mitotic proteins are synthesized, the cell passes through G2 into mitosis where it undergoes chromatin condensation, metaphase alignment and

segregation of the chromatin into two equal halves. Once proper chromosome segregation has taken place, the cell completes the division process separating the two daughter cells which are now in a G0/G1 cell cycle stage. At this stage, one full cell cycle is completed.

1.2.2 Cell Cycle Checkpoints: Critical Guardians of the Genome

As previously mentioned, cell cycle progression is a tightly regulated process. Throughout the cell cycle, specific Cyclin-dependent kinases (Cdks) are required for cell cycle progression (Figure 1). These Cdks are dependent upon binding of cyclin proteins for their kinase activity and are tightly regulated by phosphorylation and cytoplasmic sequestration by 14-3-3 chaperone proteins. Thus, while the Cdk proteins are expressed fairly evenly across all phases of the cell cycle, their activity is constrained to specific stages by the regulation of cyclin expression and binding. The first cyclin was identified as a protein which was rapidly degraded upon the onset of cell division (Evans et al., 1983). The importance of cyclins for cell cycle progression was realized when it was described that their injection into *Xenopus* oocytes induced meiosis or mitosis (Swenson et al., 1986).

The critical importance of proper Cdk/cyclin complex regulation is revealed in the fact that many cancers overexpress one or multiple Cdks and cyclins. For example, cyclin D and Cdk4 are two commonly overexpressed oncogenes in many cancer types (Kim and Diehl, 2009; An et al., 1999). The balance between cell cycle arrest and progression is struck by the degree to which Cdk/cyclin complexes are activated or inhibited and this

balance is governed by cell cycle checkpoints. Cell cycle checkpoints are coordinated signal transduction cascades that respond to a wide variety of stimuli and exert a negative force on cell cycle progression. Checkpoint activation halts the cell cycle, allowing time for the cell to resolve the checkpoint stimuli before checkpoint activation is alleviated, and cell cycle progression can resume.

The misregulation of cellular proliferation can give rise to hyperproliferative disorders and ultimately, malignant cancers. It is therefore critical that mechanisms are in place to prevent cell cycle misregulation from occurring. As a result, higher eukaryotes have developed multiple cell cycle checkpoints that are able to halt cell cycle progression in response to a wide range of stimuli, including but not limited to nutrient deprivation, DNA damage, or unattached mitotic spindles. Loss of checkpoint function is known to be a causative effect in many types of cancer. For example, over 50% of all tumors have mutated p53 function (Gasco et al., 2002). Similarly, many cancers have mutations in some component of the Rb signaling pathway resulting in premature G1/S phase transition (Nevins, 2001). Finally, many familial cancer syndromes involve mutations to critical cell cycle and DNA repair genes, including Li-Fraumeni Syndrome (p53), Fanconi's anemia (FANC genes), ataxia telangiectasia (ATM), xeroderma pigmentosa (XP genes), Nijmegen Breakage Syndrome (Nbs1), Cockayne (ERCC) syndrome and many others (Li and Fraumeni, Jr., 1969; Strathdee et al., 1992; Savitsky et al., 1995b; Savitsky et al., 1995a; Cleaver et al., 2009; Weemaes et al., 1981; Tauchi et al., 1999; Weeda et al., 1990). Many of the genes involved in these disorders will be discussed more extensively in later sections.

1.2.3 Intrinsic Versus Extrinsic Checkpoints

Cell cycle checkpoints can be sorted into two encompassing categories, namely intrinsic and extrinsic checkpoints. Extrinsic checkpoints can only be satisfied by external stimuli. For example, mitogenic growth factors must be present and bound to their corresponding receptors to initiate a signal transduction cascade that drives a cell through said checkpoint. An example of an extrinsic checkpoint in eukaryotic cells is the restriction point in G1, in which growth factor stimulation drives a cell to commit to the cell cycle (Pardee, 1974). In contrast, intrinsic cell cycle checkpoints are satisfied by meeting requirements that arise within the cell itself. These checkpoints include the G1/S and G2/M phase cell cycle checkpoints.

1.2.4 The G1/S Phase Checkpoint

The G1/S checkpoint is a critical guardian of the genome and prevents premature initiation of genomic replication. The main effectors of the G1/S phase checkpoint include such vital tumor suppressors as p53 and the retinoblastoma pocket protein family. The critical effector step in the G1/S checkpoint is the binding of Rb family proteins to the transcription factor E2F1. This binding prevents the transcriptional activation of E2F1 target genes that are critical for initiating S phase and DNA replication. The mechanism by which Rb inhibits E2F1 dependent transcription likely involves the recruitment of co-repressor complexes containing among other things histone methyltransferases and deacetylases (Nielsen et al., 2001; Petrie et al., 2008; Wang et al., 2002).

The Rb protein was first identified as the gene mutated in familial retinoblastoma, and the genetic studies of these cancers led to the two-hit paradigm of carcinogenesis (Knudson, Jr., 1971). The Rb protein exists in several phosphorylation states within a cell depending on the cell cycle status (Buchkovich et al., 1989; Chen et al., 1989). Interestingly, senescent and quiescent G0 cells contain a completely unphosphorylated Rb, suggesting complete inactivation of Cdk4/6 and Cdk2 activity (Futreal and Barrett, 1991). Cells in early- to mid-G1 contain primarily hypo-phosphorylated Rb. This hypo-phosphorylation of Rb is catalyzed by Cdk4/cyclin D although whether this enhances or inhibits Rb activity remains controversial. As cells progress through late G1 phase, gradual activation of Cdk2/cyclin E complexes leads to the hyperphosphorylation of Rb. This hyperphosphorylation of Rb inhibits Rb activity, and leads to dissociation of the RB/E2F1 complex (Harbour et al., 1999). The release of E2F1 from Rb results in the E2F1 dependent transcription of critical S phase genes like DNA polymerase and PCNA (DeGregori et al., 1995).

In addition to regulation of cell cycle progression by inhibiting transcription of E2F target genes, the Cdk/cyclin complexes are themselves subjected to tight regulation by the binding of Cdk inhibitor proteins (CKI). These proteins include p21, p16INK4A, and p27. CKIs are inhibitors of Cdk/cyclin complexes and suppress kinase activity to these complexes upon binding. Following DNA damage, p21 is transcribed in a largely p53-dependent manner and induces G1/S phase arrest by inhibiting the activity of both Cdk4 and Cdk2 (El Deiry et al., 1993; Harper et al., 1993; He et al., 2005a). Similarly to p21,

the CKI p16 was first identified as a 16 kDa protein that bound to and inhibited Cdk4 in response to SV40 or oncogenic Ras transformation in fibroblasts (Serrano et al., 1993; Serrano et al., 1997). Interestingly, the p16INK4a gene locus overlaps with another tumor suppressor, p19ARF, which stabilizes p53 by limiting the ability of MDM2 to induce p53 degradation (Quelle et al., 1997; Serrano et al., 1996; Kamijo et al., 1997; Pomerantz et al., 1998). p27kip1 is an additional Cdk inhibitor that appears to have specificity for the inhibition of Cdk2/cyclin E complexes (Polyak et al., 1994).

Adding a further layer of complexity to the regulation of G1/S cell cycle progression is the presence of Cdk activating kinases (CAKs) which phosphorylate the T-loop of Cdk1 and 2 (Kamijo et al., 1997; Fesquet et al., 1993). The precise identity of the CAK acting at various cell cycle stages remains controversial, however it is believed that Cdk7/cyclin H may function as a CAK during both G1/S and G2/M cell cycle progression (Jeffrey et al., 1995; Matsuoka et al., 1994; Fisher and Morgan, 1994).

1.2.5 Intra-S-Phase Checkpoints and the Regulation of DNA Replication

It is well known that the treatment of cells actively replicating DNA in S phase with hydroxyurea or DNA damage causes a decrease in the rate of nascent DNA synthesis (Harper and Elledge, 1998). This suggests that a system is in place to monitor the genome for damage, even following commitment to the cell cycle and DNA replication. Indeed, much work has been done to characterize the so-called intra-S phase checkpoints. This work has revealed the critical importance of Mec1 and Rad53 in decreasing late-origin

firing during DNA replication after damage is detected (Grallert and Boye, 2008; Tercero and Diffley, 2001). This results in an overall decreased rate of DNA synthesis, allowing time for the repair of DNA damage.

1.2.6 G2/M Cell Cycle Regulation

Similar to the series of checkpoints in place at the G1/S phase boundary, progression into mitosis from the G2 phase is a tightly regulated process. The G2/M checkpoint has evolved to prevent cells from prematurely entering mitosis, or from entering mitosis in the presence of DNA damage. Prior to mitotic entry cells must first satisfy a series of requirements before entry into mitosis is permitted, the ultimate requirement being the activation of the cyclin dependent kinase, Cdk1.

1.2.7 Cdk1 Activity and Inhibition during G2 and Mitosis

Cdk1 was first identified as the protein cdc2, which was identified as a critical factor for mitotic entry in *Schizosaccharomyces pombe* (Nurse and Thuriaux, 1980). Cdc2 was subsequently identified in higher eukaryotes as a component of the mitosis promoting factor in *Xenopus laevis* (Dunphy et al., 1988). Additional studies identified cdc2 as a cyclin dependent kinase, capable of binding both cyclin A and B (Dunphy et al., 1988; Draetta et al., 1989). Several conditions must be met for Cdk1 activation to occur. The first is the synthesis and binding of cyclin B to Cdk1. As cyclin B protein levels are regulated by cell cycle stage, this ensures that Cdk1 activation can only occur in late S

and G2 phases of the cell cycle. In addition to cyclin B binding, Cdk1 is also phosphorylated on its T-loop by Cdk7, an event that is important for cyclin binding and subsequent Cdk1 activation (Draetta and Beach, 1989).

1.2.8 The G2/M DNA Damage Checkpoint

Opposing the activation of Cdk1/cyclin B and entry into mitosis is a signal transduction cascade that prevents the premature or inappropriate entry into mitosis (Figure 2). This pathway culminates with the inhibitory phosphorylation of Cdk1 on Thr 14 and Tyr 15 and is known as the G2/M checkpoint. The G2/M checkpoint ensures that cells do not enter mitosis in the presence of DNA damage, and thus preserves genomic integrity. Conversely, the G2/M DNA damage checkpoint is a target of cancer therapy, as the inhibition of this checkpoint in the presence of DNA damage is known to induce cancer cell death.

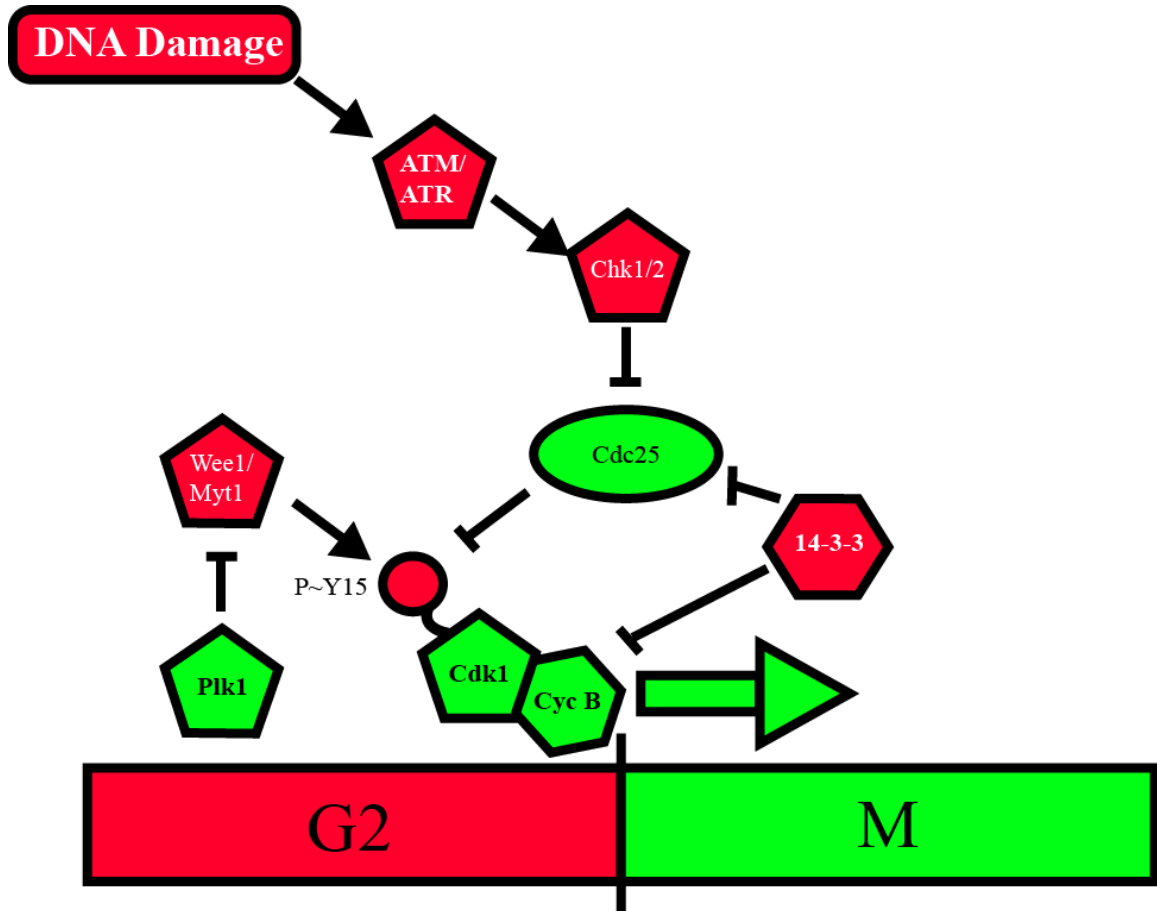


Figure 2–The G2/M DNA Damage Checkpoint. DNA damage in G2 activates a signal transduction cascade that culminates in the inhibition of Cdk1/cyclin B complex, which is required for cells to enter into the prophase of mitosis. Some of the key components of the G2/M checkpoint are displayed above. Proteins and events that promote G2/M checkpoint activation are displayed in red. Proteins that promote mitotic entry are displayed in green.

1.2.9 The Cdk1 Kinases, Wee1 and Myt1

During progression through the G2-phase, and during conditions in which the G2/M checkpoint is active, Cdk1 is phosphorylated on Tyr15 and Thr14. These phosphorylation events are catalyzed by the kinases Wee1 and Myt1 (Parker et al., 1995; Parker and Piwnica-Worms, 1992; Piwnica-Worms et al., 1991; Mueller et al., 1995; Booher et al., 1997). This phosphorylation event is critical for the inhibition of Cdk1 activity that prevents cells from inappropriately entering mitosis (Rhind et al., 1997). The expression and activity of Wee1 and Myt1 increase as cells pass through S and into the G2 phase until entry into mitosis, when Wee1 is degraded as a result of Plk1 mediated hyperphosphorylation (Watanabe et al., 1995; van Vugt et al., 2004). As the inhibitory phosphorylation of Cdk1 is the ultimate outcome of G2/M checkpoint signaling, it is not surprising that the checkpoint kinase, Chk1, can phosphorylate and activate Wee1 directly (Raleigh and O'Connell, 2000).

1.2.10 Cdc25 Family Phosphatases

During the entry into prophase, Cdk1 becomes sequentially dephosphorylated on Thr 14 and Tyr 15 (Borgne and Meijer, 1996). Cdc25 phosphatases are absolutely required for this dephosphorylation event to take place. The Cdc25 phosphatase family contains three isoforms, A, B, and C that differ in pattern of expression across the cell cycle, as well as their mode of regulation. These phosphatases are responsible for removing inhibitory phosphorylation marks on Cdk kinases, and thus are positive regulators of the cell cycle

(Gautier et al., 1991; Lee et al., 1992; Dunphy and Kumagai, 1991). Due to their positive regulation of Cdk activity, Cdc25 family phosphatases can be classified as putative oncogenes (Galaktionov et al., 1995). Indeed, Cdc25 A and B are commonly overexpressed and associated with poor prognosis in many cancer types, including colorectal, gastric, head and neck, and non-small cell lung cancers (Gasparotto et al., 1997; Kudo et al., 1997; Wu et al., 1998; Takemasa et al., 2000; Kristjansdottir and Rudolph, 2004).

As described in section 1.2.13, Cdc25 phosphatases are negatively regulated by Chk1/2 phosphorylation. Chk1/2 phosphorylation of Cdc25 leads to cytoplasmic sequestration and inhibition of phosphatase activity via binding to 14-3-3 chaperone proteins (Conklin et al., 1995; Peng et al., 1997; Dalal et al., 1999). Cdc25A is rapidly degraded during checkpoint activation in response to phosphorylation by Chk1 (Mailand et al., 2000; Molinari, 2000). It is now understood that the ubiquitin ligase, SCF binds to phosphorylated Cdc25A following DNA damage, and promotes ubiquitination degradation of Cdc25A (Donzelli et al., 2002). In contrast, Cdc25C is primarily regulated by hyperphosphorylation, and is not detectably degraded following checkpoint activation.

Complex genetic studies in mice have demonstrated that the three Cdc25 isoforms are at least somewhat redundant in their function. The loss of Cdc25 B or C individually, or Cdc25B and C together demonstrated no detectable phenotype, suggesting that Cdc25A is able to compensate for loss of B and C (Chen et al., 2001). In contrast, the Cdc25A^{-/-} mutation was embryonic lethal. Furthermore, conditional knockout of Cdc25A in adult

mice lacking Cdc25B and C resulted in severe intestinal hypoplasia, and rapid death due to loss of intestinal crypts (Lee et al., 2009). This indicates that Cdc25A can complement loss of Cdc25B or C, but that Cdc25A has important and necessary roles during early embryogenesis and normal tissue homeostasis. It is therefore likely that the aberrant expression of even one Cdc25 isoform would be sufficient to override cell cycle checkpoint activation in response to DNA damage.

1.2.11 The ATM and ATR Checkpoint Kinases

Several kinases are activated downstream of the recognition of DNA damage. Ataxia telangiectasia mutated kinase (ATM) was discovered based on its mutation in the congenital ataxia telangiectasia (A-T) disease (Savitsky et al., 1995b; Savitsky et al., 1995a). A-T is a rare, congenital disorder that is associated with severe hypersensitivity to ionizing radiation. This sensitivity occurs due to defects in the ATM protein which result in impaired checkpoint signaling following ionizing radiation exposure. This defective signaling results in genomic instability and increased cancer incidence. Ataxia telangiectasia and Rad3 related kinase (ATR) is another DNA damage-activated kinase that is critical for proper checkpoint signaling following UV-radiation exposure (Hall-Jackson et al., 1999; Cimprich et al., 1996). ATR binds to sites of UV-damaged DNA via interaction with ATR-interacting protein ATRIP (Cortez et al., 2001). ATR binding to ATRIP results in ATR activation and propagation of a signal transduction cascade that promotes cell cycle arrest, and DNA repair.

1.2.12 Differential Activation of ATM and ATR

Depending on the type of DNA present in the cell, the response towards that damage can vary greatly. This includes the activation of different repair mechanisms, as well as the coordination of different cell cycle regulators. This can be seen by the differential activation of ATM and the related kinase, ATR (Helt et al., 2005). Following double stranded break formation, such as occurs during ionizing radiation exposure, ATM is activated, and exerts a negative force on the cell cycle by phosphorylating Chk2 on Thr 68 and p53 on Ser 15 (Matsuoka et al., 2000; Canman et al., 1998). In contrast, ATR responds to UV-induced DNA adducts and replication fork collapse by phosphorylating and activating Chk1 on Ser 345 and p53 on Ser 15 (Zhao and Piwnicka-Worms, 2001; Tibbetts et al., 1999). While activation of both Chk 1 and 2 have the effect of halting the cell cycle, the differential activation of ATM and ATR allows the cell to detect a wide array of genotoxic events, and respond accordingly. Interestingly, caffeine, a radiosensitizing agent with known anti-cancer properties is an inhibitor of both ATM and ATR kinase activity (Sarkaria et al., 1999) For example, treatment with caffeine sensitizes keratinocytes to apoptosis following UV exposure by inhibiting the ATR-Chk1 signaling axis (Heffernan et al., 2009; Lu et al., 2008; Lu et al., 2004; Lu et al., 2002; Conney et al., 2008; Conney et al., 2007).

1.2.13 The Checkpoint Kinases, Chk1 and Chk2

Immediately downstream of ATM and ATR kinases in the DNA damage cell cycle checkpoints are the checkpoint kinases Chk1 and Chk2. Upon activation, both kinases phosphorylate and inactivate the Cdc25 family of phosphatases and thus indirectly inhibit Cdk/cyclin complex activity. Which Chk kinase is activated depends on the upstream checkpoint kinases activated, as well as the cell cycle stage in which the damage was detected. Chk2 is primarily activated by ATM phosphorylation on Thr68, and is primarily activated during G1 and S phase checkpoint responses. Once activated by ATM, Chk2 inhibits Cdc25C by direct phosphorylation on Ser 216, resulting in cell cycle arrest (Matsuoka et al., 1998). In addition to inhibiting Cdc25 activity, Chk2 also phosphorylates and activates p53 during the DNA damage response (Hirao et al., 2000).

Chk1 is activated primarily by phosphorylation on Ser345, and is critically important for the G2/M cell cycle checkpoint. Chk1 is a critical downstream mediator of ATM checkpoint signaling, as the ectopic expression of Chk1 can restore G2/M checkpoint integrity in ataxia-telangiectasia cells, which are known to have impaired ATM function (Chen et al., 1999b). The critical importance of these genes is revealed by the finding that Chk1 null mice die at E2.5 from p53-independent apoptosis. Furthermore, it was demonstrated that Chk1 was essential for G2/M checkpoint activation following ionizing radiation in murine ES cell lines. Chk1 is a bona fide tumor suppressor, as Chk1 heterozygous null mice have an increased incidence of mammary tumor formation in the presence of WNT-1 ligand expression (Liu et al., 2000).

1.2.14 Mitosis and Regulation of Mitotic Progression

It is now well established that Cdk1/cyclin B activity is critical for mitotic entry. The cell cycle phase known as mitosis consists of at least 6 distinct stages, prophase, prometaphase, metaphase, anaphase, telophase, and finally cytokinesis. In addition to the extensive regulation of mitotic entry, there are highly complex regulatory pathways within mitosis that ensure the equal distribution of chromosomes to each newly forming daughter cell.

The first stage of mitosis is the prophase. During prophase, Aurora B kinase becomes active and phosphorylates histone H3 on Ser10 and Ser28 (Giet and Glover, 2001; Goto et al., 2002). This phosphorylation is thought to promote mitotic chromatin condensation through the recruitment of condensin proteins (Schmiesing et al., 2000). Notably, however, yeast possessing an H3(S10A) mutation still efficiently condense chromatin during prophase, and are able to complete mitosis comparably to their wild type counterparts (Hsu et al., 2000; Prigent and Dimitrov, 2003). While this may call into question the direct requirement for H3(S10) phosphorylation during mitotic chromatin condensation, this discrepancy may reflect differences in mitotic regulation between yeast and higher eukaryotes. Simultaneous to the condensation of chromatin during prophase, is the reversible breakdown of the nuclear envelope which is associated with depolymerization of the nuclear lamina (Gerace and Blobel, 1980). Dissociation of the nuclear lamina occurs following phosphorylation of lamin B envelope proteins by

Cdk1/cyclin B (Peter et al., 1990; Courvalin et al., 1992). Notably, this reversible depolymerization differs from the irreversible dissociation that occurs during apoptosis.

The process of chromatin condensation and nuclear envelope breakdown occurs throughout prophase and prometaphase. Another key process that begins during prometaphase is the generation of bipolar mitotic spindles from the centrosomes. Centrosomes contain γ -tubulin and provide the seeding site for α - and β -tubulin polymerization in the formation of the mitotic spindle apparatus. As spindle formation occurs, the leading edge of the spindle connects to the kinetochore of one of a pair of sister chromatids. It is critically important that during spindle attachment, each pair of sister chromatids are attached to a spindle associated with opposing centrosomes. This ensures equal chromosomal segregation during the later stages of mitosis. The correct bipolar attachment of spindles to each kinetochore is a key factor monitored by the spindle-attachment checkpoint during the next stage of mitosis, metaphase. The importance of spindle assembly and attachment to the successful completion of mitosis is evident in the success of microtubule poisons such as paclitaxel and docetaxel as anti-cancer agents. These compounds disrupt microtubule dynamics by preventing $\alpha\beta$ -tubulin depolymerization and thus prevent proper kinetochore attachment and chromosomal segregation (Schiff and Horwitz, 1980).

1.2.15 Metaphase and the Spindle Assembly Checkpoint

As nuclear envelope breakdown and chromatin condensation completes, the kinetochores of each chromosome associate in a bipolar manner with the mitotic spindle apparatus. As this association occurs, the chromatin migrates to the metaphase plate, which is perpendicular to the axis between the two centrosomes. Cells maintain a metaphase arrest until each kinetochore is attached to a mitotic spindle in what is known as the spindle assembly checkpoint (Wells and Murray, 1996; Wells, 1996). Amazingly, the presence of even one unattached kinetochore is sufficient to maintain activation of the spindle assembly checkpoint and prevent the cell from progressing into anaphase. The signal emanated from unattached kinetochores is thought to arise from binding to Mad2 protein (Chen et al., 1996). This binding efficiently prevents activation of the Anaphase Promoting Complex, whose activity is critical for anaphase progression.

In order for the cell to overcome a metaphase arrest, and proceed into anaphase, the activity of Cdk1/cyclin B must first be inhibited (Lorca et al., 1992). Once conditions for satisfying the spindle assembly checkpoint have been met, Cdc20 accumulates, binds to and activates the Anaphase Promoting Complex/Cyclosome (APC) (Fang et al., 1998b; Fang et al., 1998a). The APC/C is a ubiquitin ligase, that targets many mitotic regulators, including cyclin B for proteasomal degradation (King et al., 1995).

Another key substrate of APC/C is securin protein. While unattached kinetochores are present in the mitotic cell, securin binds to and inactivates separase proteins

(Waizenegger et al., 2002). This inactivation prevents separase from cleaving the cohesin protein complexes that maintain sister chromatid attachment. Thus APC/C promotes anaphase entry through promoting degradation of multiple proteins, including cyclin B and securin (Leismann and Lehner, 2003).

1.2.16 Chromatin Segregation and Cytokinesis

Once all kinetochores are properly attached to the bipolar mitotic spindle, the spindle assembly checkpoint is satisfied, and the cell enters anaphase. The onset of anaphase occurs following degradation of the cohesin complexes that connect sister chromosomes and prevent their separation (Michaelis et al., 1997). Cohesin removal allows the centrosomes to pull the sister chromosomes in opposite directions, ensuring equal copies of each chromosome will be present in both daughter cells. As the chromatin is drawn towards the centrosomes, the cell progresses through anaphase and telophase into the final phase of mitosis, cytokinesis, during which the process of cell division is completed.

Cytokinesis is the physical process of cell division, and can occur only upon completion of chromosomal segregation. Once segregation is completed, a contractile ring containing actin and myosin filaments forms between the two newly forming daughter cells and begins to form what is known as the cleavage furrow (Sanger, 1975). This contractile ring then pinches off the membrane between the two daughter cells, resulting in the completion of mitosis and formation of two independent daughter cells. A complex signal transduction pathway dubbed the mitotic exit network tightly regulates the cytoskeletal

reorganization necessary for cytokinesis to occur (Bardin and Amon, 2001). While the mitotic exit network has been well described in *S. cerevisiae* and *S. pombe* (where it is known as the septation initiation network), the regulatory pathway in higher eukaryotes remain unclear. Some components that have been identified in higher eukaryotes include the large tumor suppressor 1 (LATS1), Mob1, and the Rab family of GTPases (Bothos et al., 2005; Kouranti et al., 2006; Chow et al., 2010).

Factors that can interfere with successful completion of cytokinesis include chromosome bridging between the newly forming daughter cells. This bridging prevents the completion of cytokinesis, and if it cannot be resolved, will lead to cleavage furrow regression and cytokinesis failure. The result of cytokinesis failure is the generation of a binucleated tetraploid cell. It is thought that following cytokinesis failure, the resulting tetraploid cell then exits the cell cycle due to a p53-dependent checkpoint in early-G1 phase.

1.2.17 Do Mammalian Cells Possess a Tetraploidy Checkpoint?

There remains a great deal of controversy over whether there is a checkpoint in place within the G0/G1 phase of the cell cycle to protect against further cycling of tetraploid cells (Uetake and Sluder, 2004). This checkpoint, located in early-G1, is thought to prevent further risk for genomic instability caused by altered chromosomal numbers in the tetraploid cell. p53 deficient cells exhibit increased levels of genomic instability and polyploidy following mitotic defects, leading some to postulate that the tetraploidy

checkpoint is p53 dependent (Sphyris and Harrison, 2005; Vitale et al., 2007). Non-transformed cells were demonstrated to arrest in G1 following induction of tetraploidy dependent on their p53 status, suggesting such a checkpoint does indeed exist (Andreassen et al., 2001). However, other groups question whether such a checkpoint exists at all in mammalian cells (Wong and Stearns, 2005).

1.2 Programmed Cell Death

1.3.1 Programmed Cell Death Overview

Multi-cellular organisms require additional safeguards compared to unicellular organisms since the health and fitness of the organism as a whole must take precedence over the survival of an individual cell. As a result, higher eukaryotic organisms have evolved complex programmed cell death pathways that ensure the death of cells in order to preserve the organism as a whole. Programmed cell death pathways are involved in processes as diverse as development to the response to DNA damage from ultraviolet radiation. The earliest form of cell death to be discovered, necrosis, was described to occur following tissue injury. For many years, however, it was recognized that a non-necrotic cell death pathway must exist to account for homeostasis in tissues containing proliferating cell populations. The earliest forms of this type of cell death were described as shrinking necrosis and later became known by the current term of apoptosis (Kerr et al., 1972).

1.3.2 Apoptotic Cell Death

Apoptosis is characterized morphologically by decreased cell size, nuclear envelope breakdown, chromatin condensation and fragmentation, and membrane blebbing (Earnshaw, 1995). A primary attribute of apoptosis is that it allows for cell death with minimal effects on the surrounding tissue. This is because the highly coordinated process that induces cell death during apoptosis packages intra-cellular contents in such a way

that they are not released into the extracellular space. This is in contrast to necrosis, a process in which the cell membrane ruptures, spilling intra-cellular contents in the extracellular space. The release of intracellular contents induces a massive inflammatory response and can damage the surrounding tissue.

A critical step in the apoptotic process is permeabilization of the outer mitochondrial membrane. This permeabilization releases mitochondrial contents into the cytosol including cytochrome C (Adachi et al., 1997). Cytochrome C, which participates in the electron transport chain during oxidative metabolism, binds to APAF-1 to form the apoptosome complex responsible for promoting processing and activation of procaspase 9 (Zou et al., 1997; Cain et al., 2000). Caspase 9 then activates the major effector caspase, caspase 3, which cleaves a number of proteins to set in place a series of events that will result in nuclear envelope breakdown, chromatin condensation and degradation and ultimately, cell death (Li et al., 1997a). For some time, it has been understood that a network of caspase activation is critical to the apoptotic process (Yuan et al., 1993; Miura et al., 1993).

1.3.3 Caspases: The Harbingers of Programmed Cell Death

Ced-3, a homolog of Interleukin 1 converting enzyme (ICE), now known as caspase 1, was the first protease identified as a player in the apoptotic process (Yuan et al., 1993). Since this discovery, 11 additional caspases have been identified in mammals. These

caspases play important roles in processes as diverse as apoptosis and cellular differentiation.

The caspase cleavage network is a complex and highly controlled pathway that involves a number of distinct caspases that are activated at different stages in the cell death pathway (Danial and Korsmeyer, 2004). In addition, the specific caspases that are activated depends on the cell death stimulus. The apoptotic caspases can be loosely grouped into the initiator caspases (those which cleave/activate of other caspases), and the effector caspases (those which cleave other proteins to promote apoptosis). The initiator caspases are caspase 2, 8, 9, and 10. The effector caspases are caspases 3, 6, and 7. While caspase 1 was the first identified pro-apoptotic caspase, it is now thought to be primarily involved in promoting inflammation (Miller et al., 1995). Caspase 14 is not involved in apoptosis, but instead promotes keratinocyte differentiation (Eckhart et al., 2000). The functions of caspases 4 and 5 have not been completely described at this point.

Many substrates of the effector caspases have been described in the literature. For example, it is well known that caspase 3 binds and cleaves PKC δ to generate a constitutively active catalytic fragment that will be discussed in depth in section 1.5.9. Other caspase substrates important for the execution of apoptosis include DNase enzymes, PARP, the nuclear lamins and many others (Enari et al., 1998; Lazebnik et al., 1994; Orth et al., 1996; Zhivotovsky et al., 1997).

Regulation of apoptosis also occurs at the level of the caspases. The IAP proteins were identified as anti-apoptotic proteins that function by directly or indirectly inhibiting the activity of caspases (Clem et al., 1991; Deveraux et al., 1997). For example, XIAP binds directly to, and inhibits, caspase 9 (Srinivasula et al., 2001). Adding further complexity to caspase regulation, is the fact that the IAP proteins themselves are regulated by binding of the SMAC and DIABLO proteins. Binding of SMAC or DIABLO to XIAP prevents XIAP from inhibiting caspase activity, and thus promotes apoptosis (Du et al., 2000; Verhagen et al., 2000). In addition to cytochrome C, caspase-independent inducers of apoptosis are also released from the mitochondria. These include endonuclease G and AIF, both of which travel to the nucleus and induce chromatin condensation and degradation (Li et al., 2001; Susin et al., 1999).

1.3.4 Caspases and Cell Cycle Checkpoint Activation

Interestingly, caspases may be important for cellular processes outside of apoptosis. For example, there is evidence that caspase 2 has an important function in regulating the G2/M transition following exposure to γ -irradiation. In this study, it was demonstrated that the loss of caspase 2 activity compromised G2/M checkpoint activation (Shi et al., 2009). Furthermore, it was demonstrated that after IR exposure, caspase 2 is incorporated into a complex with DNA-PK and PIDD (which has been previously shown to activate caspase 2). This complex, dubbed the DNA-PK-PIDDosome, promotes caspase 2 autoprocessing and activation. Interestingly, no substrates for caspase 2 have yet been identified to explain how it would have influence over cell cycle progression. Caspase 2

may also be unique amongst caspases in that it is the only caspase with at least partial constitutive nuclear localization, making it primed to participate in a nuclear DNA damage response (Zhivotovsky et al., 1999). Supporting a role for caspase 2 in cell cycle control, it has been demonstrated that the loss of caspase 2 causes an increase in proliferation and tumorigenicity in Ras/E1A transformed MEFs (Ho et al., 2009).

1.3.5 BCL-2 Family Members

The Bcl-2 family of proteins includes a number of proteins that play both pro- and anti-apoptotic roles in the cell. The first family member, Bcl-2, was identified as an oncogene located at a chromosomal breakpoint in B cell lymphoma (Pegoraro et al., 1984). Although this putative oncogene had no discernible effect on proliferation, it was soon discovered that the expression of Bcl-2 conferred resistance to various apoptotic stimuli (Vaux et al., 1988). Since the discovery of Bcl-2, other pro-survival family members, including but not limited to Bcl-XL and Mcl-1 have been identified (Boise et al., 1993; Kozopas et al., 1993). The primary antiapoptotic activity of anti-apoptotic Bcl-2 family members comes from their ability to bind to Bax and Bak, and prevent the oligomerization of these pro-apoptotic proteins. By preventing Bax/Bak oligomerization, the anti-apoptotic Bcl-2 proteins prevent inappropriate permeabilization of the outer mitochondrial membrane, and thus execution of the apoptotic cascade.

In addition to the pro-survival Bcl-2 family members, a number of pro-apoptotic proteins, including Bax and Bak have been described (Oltvai et al., 1993). These proteins contain

oligomerization domains that upon activation can form pores on the outer mitochondrial membrane, allowing the release of cytochrome C and activation of the apoptosome. The oligomerization of Bax and Bak is prevented by binding of the pro-survival Bcl-2 proteins, with this interaction representing a key component of their anti-apoptotic function. The pro-survival family members contain domains BH1-4 which form a pocket capable of binding the BH3 domains of Bax and Bak, thus preventing their oligomerization (Muchmore et al., 1996).

1.3.6 Pro-Apoptotic BH3 Only Bcl-2 Family Members

Another class of proteins in Bcl-2 family are the pro-apoptotic BH3-only proteins. The first BH3 only family member, Bid, was identified as a pro-apoptotic protein that upon expression could bind to Bax and promote mitochondrial membrane permeabilization (Wang et al., 1996). Two other important BH3-only proteins are the p53-dependent Noxa and Puma (Oda et al., 2000; Nakano and Vousden, 2001). Upon activation, p53 binds to the promoters of these genes and activates their transcription. Other BH3-only proteins include both BIM and BAD. BH3-only proteins promote apoptosis by disrupting the interaction between Bcl-2, Bcl-XL, and Mcl-1 with Bax and Bak.

1.3.7 Intrinsic versus Extrinsic Apoptosis

Apoptosis can be classified into at least two general classes depending on the initiating death stimulus. Extrinsic apoptosis occurs in response to autocrine or paracrine signaling

via death receptor activation. Death receptors are transmembrane proteins and include Fas and FADD. Death receptors are activated upon binding of death ligands such as Fas Ligand, and transmit an intracellular apoptotic signal by stimulating the processing of pro-caspase 8 zymogen into the cleaved and activated caspase 8 (Kischkel et al., 1995).

In contrast, intrinsic apoptosis occurs in response to intracellular stimuli such as high levels of DNA damage or oxidative stress. The intrinsic apoptotic pathway involves the permeabilization of the mitochondria and activation of the apoptosome that was described in sections 1.2.3 and 1.2.4.

The boundaries between intrinsic and extrinsic apoptosis are not completely clear. For example, while death receptor induction of caspase 8 is sufficient to induce caspase 3 and 7 activation independent of mitochondrial permeabilization in some cell types, others require cleavage of Bid to an active truncated form (t-Bid). Cleavage of Bid allows the t-Bid protein to travel to the mitochondria where it promotes permeabilization by inducing Bax/Bak oligomerization (Li et al., 1998).

1.3.8 Chromatin Condensation During Apoptosis

During the late stages of apoptosis, the nuclear envelope is broken down via the phosphorylation and cleavage of lamin B and the chromatin becomes condensed and is cut into oligo-nucleosomal fragments (Neamati et al., 1995; Enari et al., 1998). The molecular events behind this condensation are not clearly understood, but warrant further

investigation. Whether there is a role for histone H3 phosphorylation in apoptotic chromatin condensation in an analogous manner to that which occurs during prophase of mitosis is unclear.

The protein Acinus is cleaved by caspases during apoptosis and promotes condensation of chromatin in the absence of DNA fragmentation, suggesting that chromatin condensation and degradation are independent during apoptosis (Sahara et al., 1999). Furthermore, AKT protects against chromatin condensation and apoptosis by phosphorylating acinus on Ser 422 and 573, thereby preventing caspase cleavage of acinus during apoptosis (Hu et al., 2005). One group has reported that PKC δ -mediated phosphorylation of histone H2B on Thr 14 is a critical downstream mediator following acinus cleavage and activation (Hu et al., 2007). However, the molecular mechanism behind the importance of H2B phosphorylation and chromatin condensation remains elusive. Whether other histone phosphorylation events (such as H3 S10 and S28) are important for chromatin condensation during apoptosis remains unclear.

1.3.9 Mitotic Catastrophe

Mitotic Catastrophe was first characterized in *S. pombe* as gross abnormalities in chromosomal segregation that occurred in Cdc2/wee1 mutant strains. This study demonstrated that mutations in positive regulators of the G2/M checkpoint, such as cdc25, could suppress this phenotype, implicating inappropriate mitotic entry as a causative factor (Molz et al., 1989). The hallmark of mitotic catastrophe is the formation

of polyploid multi-micronucleated cells that arise after several cycles of mitotic failure and endo-reduplication (Castedo et al., 2002; Castedo et al., 2004a; Erenpreisa et al., 2005). Whether multi-micronucleated cells are the intended consequence of mitotic catastrophe, or are a byproduct of an escape from mitotic cell death remains the subject of much controversy (Castedo et al., 2004b). Cancer cells are often sensitized to cell death by mitotic catastrophe since they often lack intact cell cycle checkpoints that are necessary to protect against inappropriate mitotic entry in response to DNA damage (Park et al., 2005; Eom et al., 2005; Chan et al., 1999; Castedo et al., 2004c). As a consequence of this sensitivity, the induction of mitotic catastrophe in tumors is an attractive therapeutic target.

Chapter 1.4 DNA Damage Responses and Repair

1.4.1 Sources of DNA Damage

Throughout the lifetime of an organism, frequent exposure to DNA damage in various forms is inevitable. DNA damage can be caused by many different factors including endogenous factors arising from within the cell and exogenous sources of DNA damage in the environment. The importance of DNA damage repair is evident in the findings that many components of DNA repair pathways are mutated in disorders that cause cancer predisposition. For example, the ATM mutation results in the disorder ataxia telangiectasia which, among other symptoms causes a severe hypersensitivity to ionizing radiation exposure (Savitsky et al., 1995a). Furthermore, mutations to the base excision repair machinery results in a disorder known as xeroderma pigmentosum which is associated with UV hypersensitivity and increased incidence of skin malignancy (Cleaver et al., 2009).

All types of DNA damage, however, are not equal in terms of the response elicited by the cell. In fact, the response and detection of DNA damage within a cell varies depending on the type and source of DNA damage. For example, exposure to γ -radiation induces double stranded breaks in the DNA backbone which are recognized differently than the bulky DNA adducts that occur following exposure to UVB radiation.

1.4.2 The 9-1-1 Complex is Critical for DNA Damage Detection and Repair

The DNA repair proteins Rad9, Rad1, and Hus1 make up the so called 9-1-1 complex that is essential for both DNA repair and cell cycle checkpoint integrity (St Onge et al., 1999; Hopkins et al., 2004). The complex of Rad9, Rad1, and Hus1 forms a heterotrimeric ring-like complex which acts similarly to PCNA as a clamp. An additional protein, Rad17, acts as the clamp loading complex for 9-1-1, and is essential for 9-1-1 function (Venclovas and Thelen, 2000). The 9-1-1 complex is multifunctional, in that it is critical both in activating cell cycle checkpoints by recruiting ATM/ATR to sites of damage as well as participating directly in DNA repair. Rad9 is also thought to have a direct role in DNA repair, as it is known to interact with components of the nucleotide excision repair pathway, such as FLAP endonuclease, and DNA polymerase (Friedrich-Heineken et al., 2005; Toueille et al., 2004). Interestingly, the activation of Rad9 following treatment with 5-azacytidine required activation of PKC δ and ATM (Yoshida et al., 2003).

1.4.3 Nucleotide Excision Repair

The nucleotide excision repair (NER) pathway is critical in the repair of UVB-induced DNA damage. UVB radiation exposure induces the formation of bulky DNA adducts within the nucleus. These adducts include pyrimidine dimers and 6,4-photoproducts. If these adducts are not adequately repaired, they can cause stalling of DNA polymerase during S-phase resulting in erroneous DNA replication. This can result in hotspot mutations in tumor suppressor genes like p53 at sites of adjacent pyrimidine nucleotides (de Gruijl and Rebel, 2008). Mutations in components of the base excision repair

machinery cause increased skin cancer incidence as a direct result of the inability to remove UV-induced DNA adducts (Berg et al., 2000).

NER can be divided into two processes, global NER, and transcription-coupled NER. During global NER, the XPC-Rad23 complex recognizes and binds to distortions in the DNA double helix caused by bulky DNA adducts (Masutani et al., 1994). Once bound, XPC recruits XPB and D, components of the TFIIH transcription factor, which unwind the double helix surrounding the lesion (Drapkin et al., 1994). The endonucleases XPF and XPG then make incisions in the backbone on either side of the lesion, allowing for removal of the damaged DNA (Bessho et al., 1997). DNA polymerase then fills in the excised region using the other strand as a template.

Transcription coupled NER differs in the mechanism by which the damage is recognized. Rather than relying on the recognition of lesions by XPC, transcription coupled repair likely begins with stalling of RNA polymerase at the site of the DNA adduct (Mu and Sancar, 1997). The subsequent steps after recognition are conserved between the two types of NER. Notably, patients suffering from Cockayne Syndrome have defects in transcription coupled NER (Schmickel et al., 1977; Venema et al., 1990).

1.4.4 Repair of DNA Double Stranded Breaks: HR Versus NHEJ Pathways

While DNA adducts pose a significant mutagenic risk to a cell, DNA double stranded breaks are potentially much more deleterious. The presence of DNA double stranded

breaks can induce genomic instability by leading to chromosomal translocations. Indeed many cancers, including leukemias, are associated with distinct patterns of chromosomal translocations. These translocations, in which two chromosomes are fused together at the site of the double stranded break, can be highly mutagenic depending on their locations within the chromosomes. For example, in chronic myelogenous leukemia, the reciprocal translocation of chromosomes 9 and 22 results in the expression of the fusion protein BCR-ABL, the expression of which is potently oncogenic (Rowley, 1973). Due to the risk posed to a cell by double stranded breaks, cells have evolved multiple mechanisms for their detection and repair.

Two repair pathways are critical for the repair of double stranded breaks. These pathways are non-homologous end joining (NHEJ) and homologous recombination (HR). HR utilizes homologous DNA sequences to properly align the DNA sequences prior to ligation. Since this process requires the presence of sister chromosomes, it is thought to occur only in cells which are in the S, G2, and M phases of the cell cycle. In contrast to HR, NHEJ is slightly more error prone, but does not require homologous chromosomes and can therefore function prior to DNA replication.

1.4.5 DNA Dependent Protein Kinase (DNA-PK) is Critical for NHEJ

The recognition of DNA DSBs involves the assembly of the Ku70/80 proteins into a heterodimeric complex at the sites of the break. The Ku proteins have a high affinity for exposed DNA ends, and following assembly at the site of damage, act as a scaffold for

the recruitment of the NHEJ repair machinery (Walker et al., 2001). The presence of Ku heterodimers at break sites is sufficient to recruit DNA-PK, a kinase related to the ATM and ATR checkpoint kinases, which is critical for completion of NHEJ (Spagnolo et al., 2006). Recruitment and activation of DNA-PK is critical for assembly and activation of enzymes such as Artemis, XRCC4, and DNA ligase IV, all of which are critical for end processing and ligation (Ahnesorg et al., 2006; Hentges et al., 2006; Goodarzi et al., 2006; Meek et al., 2007).

1.4.6 BRCA1/2, the MRN Complex and Homologous Recombination

The second mode of DSB repair is homologous recombination. This process utilizes homologous sister chromosomes to accurately identify and ligate the appropriate DNA ends, and involves distinct protein complexes from those involved in NHEJ. The MRN complex consists of Mre11, Rad50, and Nbs1 (Xiao and Weaver, 1997). The importance of this complex to DNA repair is evident in patients suffering from Nijmegen Break Syndrome, a genetic disease associated with Nbs1 mutations that results in massive genetic instability. Following DNA DSB formation the MRN complex is recruited to the break site via direct interaction of Mre11 with the exposed DNA end (de Jager et al., 2001b; de Jager et al., 2001a). Following assembly at the break site, The MRN complex then process the DNA ends to expose RPA coated stretches of single stranded DNA for use in subsequent steps of HR-mediated repair (Trujillo and Sung, 2001).

Also critical to the HR repair process are the tumor suppressor Brca proteins. The Brca1 and 2 genes have been identified in hereditary breast cancers, in which individuals carrying Brca mutations have an elevated risk for developing breast cancer (Castilla et al., 1994; Thorlacius et al., 1998). Brca1 and 2 are clearly important for homologous recombination, as cells with impaired Brca1 or Brca2 function have depleted levels of HR following DNA damage (Moynahan et al., 2001b; Moynahan et al., 2001a; Chen et al., 1999a).

Chapter 1.5 Protein Kinase C Signaling and Biology

1.5.1 Protein Kinase C Family of Serine/Threonine Kinases

Protein kinase C was first discovered in bovine brain extracts as a proteolytically activatable kinase (Inoue et al., 1977). The protein kinase C (PKC) family consists of at least 9 isoforms which differ in pattern of tissue expression and overall signaling biology. The 9 isoforms can be further separated into the classical isoforms, novel isoforms, and atypical PKC isoforms. PKC isoforms play diverse roles in processes including cellular differentiation, proliferation, apoptosis, and migration. The human epidermis expresses 5 distinct PKC isoforms, α , δ , ϵ , η , and ζ (Denning, 2004).

1.5.2 The Classical PKC Isoforms

The classical PKC isoforms were the first PKC isoforms to be identified, and include PKC α , PKC β , and PKC γ . Classical PKC isoforms are activated by intracellular Ca²⁺ release from the endoplasmic reticulum in response to binding of inositol triphosphate to the inositol triphosphate receptor. In addition to activation by Ca²⁺, classical PKC isoforms are activated by binding to diacylglycerol. Inositol triphosphate and diacylglycerol are generated when phospholipase C cleaves phosphatidyl inositol biphosphate following phosphorylation of phosphatidyl inositol by phosphatidyl inositol-3 kinase (PI3K) (Berridge and Irvine, 1984).

Interestingly, the lone classical PKC isoform expressed in the human epidermis is PKC α . As there is a positive Ca²⁺ gradient in the epidermis, it is interesting to note that PKC α has been demonstrated to play a critical role in the induction of differentiation induced cell cycle withdrawal (Jerome-Morais et al., 2009).

1.5.3 The Novel PKC Isoforms

The novel PKC isoforms include PKC δ , PKC ϵ , PKC η , and PKC θ (Ono et al., 1987). Unlike the cPKC isoforms, nPKCs do not respond to Ca²⁺ stimulus as they contain a C2-like domain that varies significantly from the Ca²⁺ responsive C2 domain of the classical PKC isoforms (Pappa et al., 1998). nPKC isoforms do, however, retain responsiveness to diacylglycerol stimulation. The precise function of the C2-like domain of PKC δ remains unclear, but it has been purported to bind to and enable actin redistribution in neutrophils (Lopez-Lluch et al., 2001). In addition, the C2-like domain of PKC δ has been demonstrated to bind to phospho-tyrosine residues, enabling phosphorylation specific interactions with other proteins (Benes et al., 2005). In contrast to the anti-proliferative effects of PKC δ , PKC ϵ promotes proliferation (Cacace et al., 1996). The positive role for PKC ϵ in proliferation is bolstered by the finding that PKC ϵ transgenic mice are more susceptible to formation of squamous cell carcinoma when treated with the DMBA/TPA carcinogenesis regimen (Reddig et al., 2000; Jansen et al., 2001). While PKC ϵ is a potential oncogene, another novel PKC isoform, η , localizes to the granular layer of the epidermis and is important for normal epidermal differentiation (Koizumi et al., 1993; Osada et al., 1993; Cabodi et al., 2000).

1.5.4 The Atypical PKC Isoforms

The third class of PKC isoforms, the atypical PKCs include PKC ζ and PKC ι/λ (Ono et al., 1987; Ono et al., 1989; Selbie et al., 1993). These isoforms are perhaps the least well understood of the three classes and lack responsiveness to either Ca²⁺ or diacylglycerol. In the epidermis, PKC ζ has been implicated in establishing cell polarity and in the formation of intercellular tight junctions (Helfrich et al., 2007; Izumi et al., 1998).

1.5.5 Structural Domains of Protein Kinase C

PKCs are multi-domain proteins, and the domain makeup of each PKC class differs (Figure 3). Generally, the structure of PKC proteins can be broken down into the regulatory and catalytic domains, each of which constitutes roughly half of the protein. The PKC catalytic domain contains the C3 and C4 domains (Newton, 1997). The C4 domain of PKC provides the protein with its catalytic activity and substrate binding domain. The C3 domain of PKC binds to ATP, bringing it in proximity to the catalytic site in the C4 domain allowing for transfer of the γ -phosphate of ATP to PKC substrates.

There is a great deal of variation between PKC isoforms in the makeup of the regulatory domain of the protein. Classical PKC isoforms contain a calcium binding C2 domain in the regulatory domain of the protein. The C2-like domain of the novel PKC isoforms lacks Ca²⁺ responsiveness, but likely confers unique substrate binding properties for these isoforms. Both classical and novel, but not atypical PKC isoforms contain phorbol ester

binding C1b domains (Hunn and Quest, 1997). These allow the protein to localize to the membrane upon activation where it can interact with and phosphorylate a variety of downstream effectors.

Another key structural component of protein kinase C is the pseudosubstrate domain. Thus far, every PKC isoform, with the possible exception of PKC μ contains a pseudosubstrate domain in within the regulatory domain of the protein (House and Kemp, 1987). Under conditions in which PKC is inactive, the pseudosubstrate binds to the substrate binding catalytic site of PKC, and inhibits PKC activity. Mutation to the pseudosubstrate domain, or disruption of pseudosubstrate domain binding using blockin antibodies is sufficient to induce PKC activity, indicating the importance of this intramolecular interaction in preventing inappropriate PKC activation (Pears et al., 1990; Makowske and Rosen, 1989). Upon activation of PKC, the interaction of the pseudosubstrate domain and the catalytic domain is disrupted, thus freeing the catalytic site to phosphorylate PKC targets.

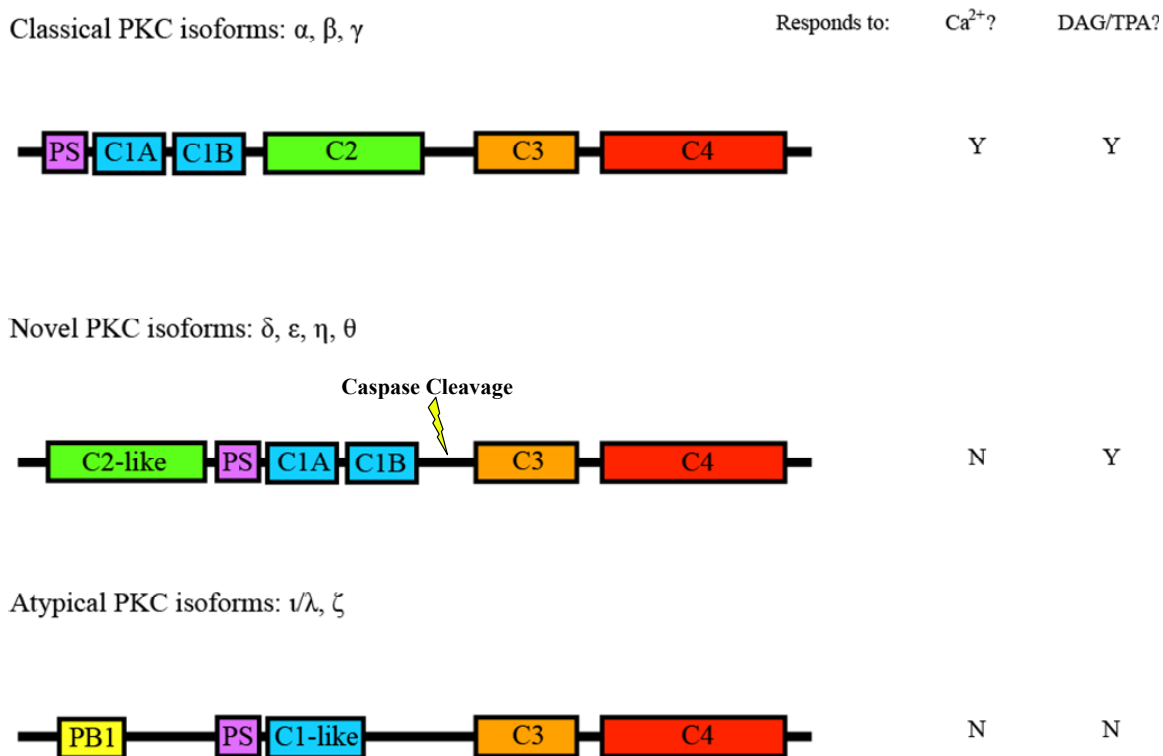


Figure 3 – Structural Domains of the Protein Kinase C Family. Shown above is the basic domain makeup of the classical, novel, and atypical PKCs. The C1A and B domains provide binding to diacylglycerol and phorbol esters and are present on novel and classical isoforms. The C2 domain binds Ca^{2+} and is incomplete in the novel isoforms. The PB1 domain of the atypical PKCs is thought to mediate protein-protein interactions. Also labeled is the caspase cleavage site that is unique to PKC δ among all PKC isoforms.

1.5.6 The Novel PKC isoform, δ

Protein Kinase C δ was first cloned from a rat brain cDNA library using probes designed to recognize classical PKC isoforms (Olivier and Parker, 1991). Further characterization led to the classification of PKC δ as being unresponsive to Ca²⁺ stimulation and predominantly expressed in the murine epidermis and other epithelial tissues, as well as the brain (Leibersperger et al., 1991). The characterization of human PKC δ followed shortly after the discovery of this isoform in rats and mice (Aris et al., 1993). PKC δ protein has a molecular weight of approximately 78 kDa. PKC δ tends to phosphorylate substrates on the following consensus sequence:

S/TXXR/K (X represents any amino acid residue)

PKC δ is unique among PKC isoforms in several ways. First, PKC δ contains a conserved nuclear localization sequence (NLS) within the C4 domain (DeVries et al., 2002). Several groups have reported the importance of nuclear translocation of PKC δ during apoptosis (Scheel-Toellner et al., 1999; Yoshida et al., 2003; Eitel et al., 2003). In addition, it has been demonstrated that mutation of this nuclear localization sequence abrogates the ability of PKC δ to induce apoptosis (DeVries et al., 2002; DeVries-Seimon et al., 2007). The importance of the NLS for PKC δ pro-apoptotic activity suggests that there are key substrates for PKC δ within the nucleus. Interestingly, the V5 region of PKC δ has also been reported to regulate PKC δ sub-cellular localization, suggesting that other factors than the putative NLS may control nuclear translocation of PKC δ (Wang et al., 2004).

Another unique aspect of PKC δ is that it is capable of undergoing activation independently of membrane localization (Steinberg, 2004). This may be, at least in part, due to activation of PKC δ by caspase cleavage.

PKC δ contains a conserved caspase cleavage site within the hinge region that separates the regulatory and catalytic domains of the protein. This cleavage event generates a constitutively activated catalytic fragment of approximately 40 kDa in molecular weight (Emoto et al., 1995). The fate of the regulatory domain of PKC δ following caspase cleavage is not well understood. This caspase cleavage site and the associated biological functions of PKC δ cleavage will be discussed below.

PKC δ protein function varies significantly depending on the tissue and cellular context. For example, PKC δ expression is lost in many squamous cell carcinomas and PKC δ has a well characterized role in promoting apoptosis (Reyland et al., 1999; Reyland et al., 2000; Matassa et al., 2001; Carpenter et al., 2002; Denning et al., 2002; Lasfer et al., 2006). In contrast, PKC δ expression has been associated with poor prognosis in breast cancers (McKiernan et al., 2008). Additionally, PKC δ activity was demonstrated to be a pro-survival protein in some breast cancer cell lines (McCracken et al., 2003).

1.5.7 Post-Translational Mechanisms of PKC δ Regulation

Full length PKC δ activity is extensively modulated by a number of phosphorylation events that occur on serine, threonine, and tyrosine residues located throughout the protein. PDK1 phosphorylates PKC δ on Thr505 of the activation loop to prime the protein for activation (Le Good et al., 1998; Balendran et al., 2000; Flynn et al., 2000). This PDK1-mediated phosphorylation promotes the autophosphorylation of Ser 643 and 662, generating a catalytically competent “mature” PKC δ protein (Li et al., 1997b; Stempka et al., 1999). It is important to note that while these phosphorylation events are critical for the maturation of PKC δ to a catalytically competent protein, they do not activate the full length protein in the absence of other stimuli/phosphorylation events.

In addition to serine and threonine phosphorylation, PKC δ can also be extensively phosphorylated on key tyrosine residues located throughout the protein (Denning et al., 1993; Li et al., 1994). Tyrosine phosphorylation of PKC δ can be catalyzed by the Src family of tyrosine kinases (Gschwendt et al., 1994; Zang et al., 1997). In addition, tyrosine phosphorylation is stimulated by activation of the epidermal growth factor receptor (EGFR) (Denning et al., 1996; Denning et al., 2000; Bossi et al., 2008a). Tyrosine phosphorylation sites on PKC δ that have been identified include Tyr 187, 311, 332 (Szallasi et al., 1995; Li et al., 1996; Blake et al., 1999; Lu et al., 2007b; Lu et al., 2007a). Whether the tyrosine phosphorylation of PKC δ activates or inhibits the kinase activity of PKC δ remains the subject of controversy (Denning et al., 1993; Fukunaga et al., 2001; Blass et al., 2002). In keratinocytes, the expression of oncogenic ras induces

tyrosine phosphorylation and inhibition of PKC δ activity (Denning et al., 1993). Interestingly, phosphorylation at Tyr311 and 332 may modulate caspase cleavage due to the proximity to the caspase cleavage site in the hinge region.

1.5.8 Alternative Splicing of PKC δ

In addition to being regulated at the transcriptional and post-translational levels, PKC δ can also be regulated by alternative splicing. To date, eight different PKC δ splice variants have been identified in different species. Although the function of the various isoforms has yet to be clearly described, at least some of the variants may display resistance to caspase cleavage. For example, the PKC δ II and VIII isoform cannot be cleaved by caspase 3, and appear to be anti-apoptotic in function (Sakurai et al., 2001; Jiang et al., 2008). PKC δ VIII expression, for example, is capable of rescuing NT2 cells from etoposide-induced apoptosis.

1.5.9 Caspase Cleavage of PKC δ

PKC δ is unique among PKC isoforms in that it is proteolytically activated by caspase cleavage during apoptosis in many cell types (Emoto et al., 1995; Ghayur et al., 1996; Denning et al., 1998). Occurring on highly conserved aspartic acid residues (see below), this cleavage generates a constitutively active catalytic fragment, PKC δ -cat, by separating the regulatory and catalytic domains of PKC δ . This catalytic fragment has been convincingly demonstrated to be both necessary and sufficient for UV-induced

apoptosis. For example, the ectopic expression of the PKC δ -cat is sufficient to induce apoptosis in human keratinocytes (Denning et al., 2002). Conversely, expression of a cleavage resistant PKC δ mutant protects cells against UV-induced apoptosis, suggesting that the cleavage of PKC δ is a critically important step in the apoptotic cascade (D'Costa and Denning, 2005).

PKC δ Conserved Caspase Cleavage Sequence **DMQD₃₃₀N**

The primary caspase responsible for cleaving PKC δ has been reported to be caspase 3, although it has also been reported that caspase 2 can also cleave PKC δ at this region (Panaretakis et al., 2005).

It is likely that in addition to conferring constitutive catalytic activity to the PKC δ catalytic domain, caspase cleavage also allows access to a host of new substrates as PKC δ activity no longer requires phorbol ester binding following cleavage. This cleavage therefore generates a soluble pool of activated PKC δ -cat that may phosphorylate proteins that are not phosphorylated by the membrane bound full length PKC δ (Denning et al., 1998).

1.5.10 PKC δ is a Critical Mediator of Apoptosis

Protein Kinase C δ is critically important for a diverse range of functions within the human body. In the epidermis, and many other tissues, PKC δ is necessary and sufficient

for the induction of apoptosis in response to a wide variety of stimuli and therefore has important tumor suppressive function. This work, largely using cell culture, has been extended into PKC δ null mice, where it was demonstrated that the loss of PKC δ confers a resistance to etoposide or γ -irradiation induced apoptosis (Humphries et al., 2006).

A great deal of effort has been exerted toward understanding the downstream substrates of PKC δ that make this protein so important for the apoptotic cascade. This work has yielded insight into many interesting targets of PKC δ signaling that may explain why this protein is so potently pro-apoptotic. For example, the PKC δ has been shown to phosphorylate and activate p53 on Ser 15 and 46 (Lee et al., 2006; Yoshida et al., 2006a). In addition to regulating p53 activation at the post-translational level, PKC δ has also been implicated in the transcriptional activation of p53 via induction of the apoptotic protein Btf (Liu et al., 2007). PKC δ has also been implicated in the regulation of the p53 family member, p73 during apoptosis (Ren et al., 2002). In addition, PKC δ also phosphorylates Mcl-1, to promote Mcl-1 degradation and Bax activation during UV-induced apoptosis (Sitailo et al., 2004; Sitailo et al., 2006). PKC δ activity is also important for nuclear envelope breakdown during apoptosis, since PKC δ functions as an apoptotic lamin kinase (Cross et al., 2000). The pro-apoptotic role of PKC δ also likely reflects PKC δ -mediated induction of p38 signaling (Tanaka et al., 2003).

It has also been postulated that PKC δ activity stimulates an pro-apoptotic autocrine signaling loop via the induction of TRAIL and TNF α signaling (Gonzalez-Guerrico and

Kazanietz, 2005). Interestingly, at least in LNCaP prostate cancer cells, this pro-apoptotic function appears to be independent of PKC δ cleavage (Fujii et al., 2000).

1.5.11 PKC δ and Regulation of the Cell Cycle

In addition to regulating apoptosis, PKC δ also has a role in regulating cell cycle progression following DNA damage. Over-expression studies have demonstrated that PKC δ can induce cell cycle arrest at multiple stages in the cell cycle. For example, PKC δ overexpressing Chinese hamster ovary cells underwent a cell division arrest in mitosis following phorbol ester stimulation (Watanabe et al., 1992). Subsequent work demonstrated that the inhibition of cytokinesis by PKC δ likely involves the inhibition of Lats1, an integral component of the mitotic exit network (Takahashi et al., 2006). In addition, the treatment of metastatic melanoma cells with TPA results in inhibition of Cdk1 and a G2/M arrest, although the specific PKC isoforms involved in this arrest were not identified (Arita et al., 1998).

Besides regulating progression through the G2 and M phases of the cell cycle, PKC δ has also been implicated in regulating the G1/S phase of the cell cycle. PKC δ was shown to induce p27 and delay cyclin D expression following serum stimulation of quiescent rat microvascular endothelial cells (Ashton et al., 1999). Treatment of anaplastic human thyroid cancer cells with TPA induced a G1 arrest associated with upregulation of p21 and p27, and this effect could be blocked by siRNA mediated depletion of PKC δ (Afrasiabi et al., 2008). Similar work in lung adenocarcinoma cells demonstrated TPA

induced G1 arrest was dependent on PKC δ but not PKC α , and was associated with upregulation of p21 (Nakagawa et al., 2005). Conversely, in Wistar rat thyroid cells, PKC δ has been demonstrated to induce apoptosis by first inducing G1 progression followed by S phase arrest (Santiago-Walker et al., 2005).

1.5.12 PKC δ and the DNA Damage Response

PKC δ has recently been implicated in the DNA damage response where it was phosphorylated and activated by ATM following treatment with 5-azacytidine and subsequently phosphorylated and activated Rad9 to promote DNA repair (Yoshida et al., 2003). Interestingly, PKC δ was also discovered downstream of ATM in the activation of NF- κ B transcription following UV-irradiation, making PKC δ a bona-fide target of ATM following DNA damage (Ravi et al., 2007). Specific ATM phosphorylation sites in PKC δ remain to be identified.

In contrast to the positive role in DNA repair, PKC δ may also act as a signal to shut down DNA repair pathways. It was reported that PKC δ -cat phosphorylates and inactivates DNA-PK during apoptosis (Bharti et al., 1998). Since shutdown of repair mechanisms is known to occur during apoptosis, DNA-PK may be an additional substrate important for PKC δ apoptotic activity.

1.5.13 PKC δ as a Tumor Suppressor

Given the importance of PKC δ to apoptosis, cell cycle regulation, and DNA repair, it is not entirely surprising that PKC δ has been identified as a putative tumor suppressor. In human squamous cell carcinomas, the expression of PKC δ is lost, and the re-expression of PKC δ in SCC cell lines suppresses their tumorigenicity (D'Costa et al., 2006). Furthermore, PKC δ transgenic mice are resistant to both skin papilloma and squamous cell carcinoma formation in response to treatment with the DMBA-TPA carcinogenesis regimen (Reddig et al., 1999). Interestingly, while PKC δ transgenic mice were resistant to the chemical carcinogenesis regimen, they showed no resistance to a UV carcinogenesis regimen (Aziz et al., 2006). This surprising finding could illustrate a true discrepancy between the requirement for PKC δ as a tumor suppressor in chemical versus UV carcinogenesis, or it may be an indication that the endogenous levels of PKC δ are sufficient for UV tumor suppression. If the latter is true, then increasing PKC δ levels would not be expected to have an additional protective effect over that provided by the endogenous protein. In addition, PKC δ levels were reduced in the SCCs generated by UV radiation, suggesting that even in the transgenic mice, PKC δ expression was lost during tumorigenesis. In order to address this question further, the use of PKC δ knockout mice should be utilized with the UV carcinogenesis regimen to determine whether the loss of PKC δ function confers susceptibility to SCC formation.

The loss of PKC δ in squamous cell carcinomas likely occurs at the transcriptional level (Yadav et al., 2010). PKC δ expression is also lost or decreased in a number of other

cancer types, including endometrial, and colon cancer (Reno et al., 2008; Kahl-Rainer et al., 1994; Craven and DeRubertis, 1994). In contrast, PKC δ is overexpressed in mammary tumors and is associated with metastatic progression (Kiley et al., 1999a; Kiley et al., 1999b). How PKC δ can be both oncogenic and tumor suppressive remains to be determined.

1.6 Significance

While PKC δ clearly functions as a tumor suppressor in the skin, the mechanism of tumor suppression by PKC δ is not completely understood. Here, we seek to investigate and characterize additional tumor suppressor mechanisms of PKC δ in relation to G2/M cell cycle regulation and apoptosis. By gaining a better understanding of PKC δ function in the epidermis, we may be able to identify new therapies in the treatment of squamous cell carcinomas of the skin. Further characterization of the PKC δ signaling network will aid in the development of treatments that could restore PKC δ expression, or activation of important downstream effectors whose regulation may be altered in the absence of PKC δ signaling.

Chapter 2

Materials and Methods

2.1 Retroviral Constructs, Packaging, and Infection

PKC δ -GFP, PKC δ (D327A)-GFP, and PKC δ (K376R)-GFP constructs were graciously provided by Dr. Mary E. Reyland (University of Colorado Health Sciences Center) and were previously described (DeVries et al., 2002). These constructs were sub-cloned in XhoI/NotI sites of the LZRS-Linker retroviral expression vector for infection of cultured KCs and mouse embryonic fibroblasts (MEFs). LZRS-PKC δ -cat-FLAG, and the 4-hydroxytamoxifen-activatable LZRS-PKC δ -cat-ER were previously described (Sitailo et al., 2004). A diagram of the LZRS-Linker retroviral plasmid is displayed in Figure 4. Please note that the multiple cloning site used for subcloning of PKC δ constructs into the LZRS-Linker plasmid is located between the 5' and 3' long terminal repeat sequences (LTR). Upon infection with the LZRS retrovirus the LTR sequences, along with any intervening sequences, are incorporated randomly into the genome of the infected cell. The 5' LTR then drives transcription of the target gene of interest.

Retroviral supernatant was generated by calcium phosphate-mediated transfection of Phoenix-Ampho packaging cells as described previously (Sitailo et al., 2002). Following transfection, Phoenix-Ampho cells were positively selected for plasmid incorporation by treatment with 10 ng/mL puromycin until the culture dishes reached approximately 75% confluency. Phoenix-Ampho cells were then refed with DMEM minus puromycin and

incubated at 32 °C overnight to generate retrovirus. Retroviral infection of target cells using Phoenix-Ampho retroviral supernatant was done for 1 hr at 1200 rpm and 32°C. Schematic diagrams of the PKC δ full length and catalytic fragment constructs used in this study are displayed in Figure 5.

2.2 Cell Culture and UV Treatment

Primary human KCs were isolated from neonatal foreskins with Loyola Institutional Review Board approval as described previously (Mitra and Nickoloff, 1994; Tibudan et al., 2002). KCs and HaCaT cells were cultured in Medium 154CF with 0.07 mM calcium and HKGS (Cascade Biologics). Spontaneously immortalized MEFs from wild type or PKC δ null mice were cultured in DMEM (Invitrogen) containing 10% fetal bovine serum, and were kindly provided by Dr. Anning Lin (University of Chicago). PKC δ -cat-ER was activated by treatment with 10 μ M 4-hydroxytamoxifen (Alexis Biochemicals) (Sitailo et al., 2004). In all experiments using PKC δ -cat-ER, control (Linker) transduced cells were also treated with 4-hydroxytamoxifen. ATM/ATR inhibition was achieved by the addition of 2 mM caffeine in water to the culture medium (Sarkaria et al., 1999). The inhibition of Aurora kinases was achieved by the addition of 10 μ M Aurora Kinase Inhibitor II (189404, Calbiochem) in the culture medium. UV irradiation was done using a UV Panelite Unit with approximately 65% emission in the UVB spectrum as previously described (D'Costa et al., 2006).

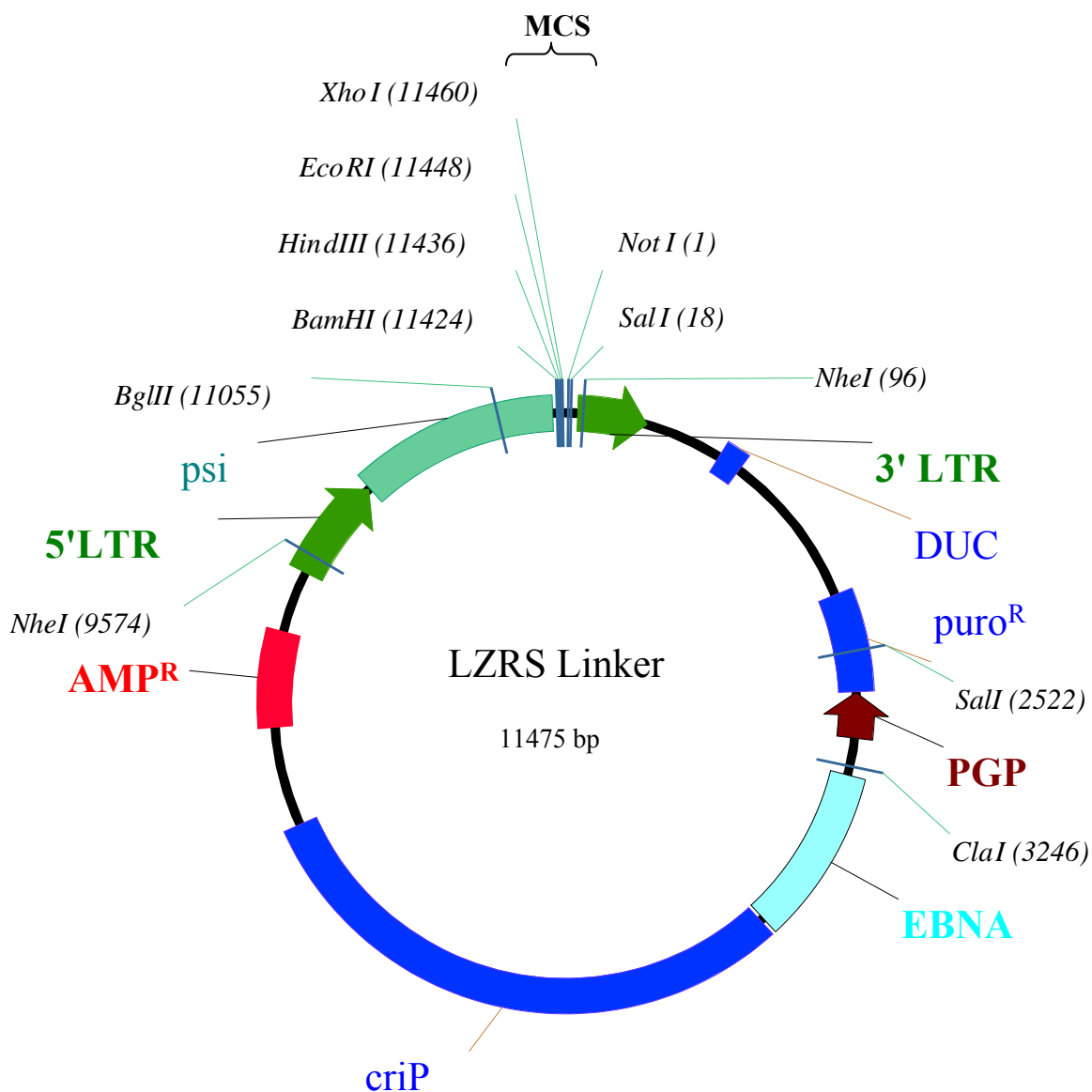


Figure 4 – LZRS-Linker Plasmid Map. Shown above is a schematic of the LZRS-Linker plasmid used in the subcloning and retrovirus generation of all PKC δ constructs used within this document. Please note the multiple cloning site (MCS) is located between the 5' and 3' LTR sequences, resulting in the incorporation of the target sequence upon retroviral recombination into the genome of infected cells. The puro^R construct allows for selection of transfected Phoenix-Ampho cells using puromycin.

PKC δ Constructs





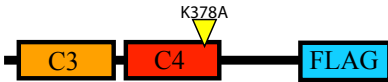

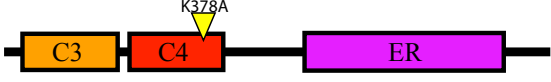
	Kinase Activity	Caspase Cleavage
PKC δ -GFP 	Yes/Reversible	Yes
PKC δ KD-GFP 	No	Yes
PKC δ CR-GFP 	Yes/Reversible	No
PKC δ -cat-FLAG 	Yes/Constitutive	NA
PKC δ KD-cat-FLAG 	No	NA
PKC δ -cat-ER 	Yes/Tamoxifen Inducible	NA
PKC δ -cat-KD-ER 	No	NA

Figure 5–Retroviral PKC δ Constructs. Schematic diagrams of the PKC δ constructs used throughout this document. PKC δ -cat indicates the PKC δ catalytic fragment. ER is the estrogen receptor ligand binding domain. GFP is green fluorescent protein.

2.3 Cell Cycle Synchronization

For mitotic synchronization experiments, HaCaT cells were treated with 10 ng/mL nocodazole for 18 hours to induce metaphase arrest (Chou and Omary, 1994). Cells were released from nocodazole arrest by removal of media, washing in PBS, and refeeding with nocodazole-free 154CF containing 10 μ M 4-hydroxytamoxifen. Exit from mitosis was monitored using propidium iodide staining and FACS analysis as described in the flow cytometry section of this Chapter. An increased percentage of cells with 2N DNA content was interpreted to mean that successful completion of mitosis had occurred.

For G1/S synchronization, cells were treated for 18 hrs with the DNA polymerase inhibitor aphidicolin at a concentration of 10 μ M (Berger et al., 1979; Pedrali-Noy et al., 1980). Following incubation, cells were released by removing the media, washing with PBS, and refeeding with aphidicolin-free 154CF plus 10 μ M 4-hydroxytamoxifen. Progression through S phase was measured by propidium iodide staining and FACS analysis.

2.4 siRNA Transfection of HaCaT Cells

HaCaT cells were transfected with control (SC-37007, Santa Cruz) or PKC δ siRNA (SC-44229, Santa Cruz) using the Lipofectamine 2000 transfection reagent (11668, Invitrogen) in antibiotics-free 154CF. HaCaT cells were plated at a density of 100,000 cells per well in a 6-well plate the day prior to transfection. The next day, 100 pmol of

siRNA per well was combined with 250 μ L of Opti-MEM serum free medium and mixed gently. Meanwhile, 5 μ L per well of Lipofectamine 2000 was diluted in 250 μ L Opti-MEM and incubated at room temperature for 5 min. After incubation, the diluted Lipofectamine 2000 solution was mixed with the diluted siRNA solution, and incubated at room temperature for 20 min. 500 μ L of the Lipofectamine/siRNA solution was added to each well containing 1.5 mL of Opti-MEM and the HaCaT cells. After overnight incubation, cells were washed and refed with normal 154CF media. 48 hrs after transfection, cells were collected for flow cytometry to measure apoptosis, and Western blot analysis to verify successful knockdown of PKC δ .

2.5 Antibodies and Western Blotting

Cell lysates were collected by scraping cells in RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1% Sodium Deoxycholate). Lysates were briefly sonicated and centrifuged at 14,000 rpm for 5 min to remove cellular debris. Protein concentrations were determined using standard Bradford Reagent methodology.

Antibodies used for Western blotting include: Actin (69100, MP Biomedical), Cdc25A (SC-97, Santa Cruz) Cdk1 (SC-747, Santa Cruz), Phosphorylated Cdk1(Y15) (SC-7989R, Santa Cruz), γ H2A.X (UBI 05-636, Upstate), Histone H3 (ab1791, Abcam), Phosphorylated histone H3(S10) (UBI 06-570, Upstate), P~p53(S15) (9284S, Cell Signaling), PKC δ (SC-937, Santa Cruz) (610397, BD), Rad9 (SC-74464, Santa Cruz), α -tubulin (UBI 05-829, Upstate), and Vinculin (V4505, Sigma). Secondary antibodies used for Western blotting were goat anti-rabbit IgG-Alexa Fluor 680 (A21076, Invitrogen),

donkey anti-rabbit IgG-IRDye 800 (611-732-127, Rockland) and goat anti-mouse IgG conjugated to AlexaFluor-680 (A21057, Molecular Probes) and donkey anti-mouse IgG IRDye 800 (610-732-124, Rockland) for detection using the LI-COR infrared scanning system (LI-COR Biosciences).

2.6 Recombinant PKC δ -cat Kinase Reaction

To generate recombinant PKC δ -cat, 400 ng of active caspase 3 (201-038-C005; Alexis Biochemicals) was incubated with 200 ng of recombinant full length PKC δ (Panvera Corp) in caspase assay buffer (25 mM HEPES pH 7.4, 0.25 M sucrose, 2 mM EDTA, 2.5 M DTT) for 30 min at 37 °C. 25 ng of full length or cleaved PKC δ was then incubated with 1 μ g of recombinant histone H3 (New England Bio) in PKC kinase buffer (50 mM Tris-HCl, pH 7.4, 250 mg/mL BSA, 1 mM EDTA, 1.5 mM MgCl₂, 25 mM ATP, 1 mg/mL 4:1 phosphatidyl choline:phosphatidyl serine, 10 nM 12-O-tetradecanoylphorbol-13-acetate) then incubated at 30 °C for 30 min. To measure histone H3 Ser 10 phosphorylation, reactions were subjected to SDS-PAGE Western blot analysis using anti-P~H3(S10) primary antibody.

2.7 Flow Cytometry

Cells were collected by trypsinization, pelleted and washed in FACS buffer (phosphate buffered saline, 5% fetal bovine serum). Cells were then pelleted and resuspended in 100 μ L of FBS. Cells were fixed by addition of ice-cold 100% ethanol for at least 30 minutes.

RNA was digested by treatment with 10 $\mu\text{g}/\text{mL}$ RNase for 15 minutes at 37°C. Propidium iodide was added to a final concentration of 50 $\mu\text{g}/\text{mL}$ and samples were incubated on ice for at least 1 hr. Cell cycle profiles were analyzed using a Beckman Coulter EPICS XL-MCL flow cytometer. Histogram overlays were generated using FlowJo software.

2.8 Phospho-H3(S10)/DNA PI Staining and Mitotic Index

For mitotic index measurements in MEFs, the cells were treated with or without UV followed by 10 ng/mL nocodazole to trap any cells that had overcome UV-induced G2/M arrest and entered mitosis. Mitotic index measurements in HaCaT cells were performed 3 days after PKC δ -cat-ER transduction, and thus were not treated with nocodazole. Cells were collected and washed in FACS buffer before being fixed in 3.7% formaldehyde for 10 minutes. After fixation, cells were permeabilized in 70% EtOH at 4 °C for 30 min. After washing in FACS buffer, cells were incubated for 2 hrs in 100 μL of P-H3(S10) antibody. Cells were washed and incubated on ice in 100 μL of diluted Alexa Fluor-488 anti-rabbit secondary for 30 min. Following secondary antibody incubation, cells were washed twice and incubated in 500 μL of solution containing 10 $\mu\text{g}/\text{mL}$ RNase and 50 $\mu\text{g}/\text{mL}$ PI for 30 minutes prior to FACS analysis.

2.9 Immunofluorescent Staining and Confocal Microscopy

Cells were cultured on glass coverslips, fixed in 3.7% formaldehyde, and permeabilized with 0.1% Triton X-100. Alternatively, for staining with certain antibodies, cells grown on coverslips were fixed and permeabilized using ice-cold acetone/methanol (1:1). Following fixation, coverslips were washed in FA buffer then incubated with primary antibody diluted in goat serum to block non-specific binding for 1 hr. Following primary antibody incubation, coverslips were washed in FA buffer and incubated in secondary-fluorophore conjugated secondary antibody diluted 1:400 in goat serum for 1 hr. After staining with primary and secondary antibodies, coverslips were washed in FA then incubated in 100 ng/mL DAPI for 5 min to stain chromatin. Coverslips were mounted onto slides using Gelvatol. Images were generated using a Carl Zeiss LSM-510 confocal microscope with 1 μ m optical slice at 40x magnification or an Olympus AX80 epifluorescent microscope, equipped with a QImaging Retiga 4000R digital camera. Secondary antibodies used for immunofluorescent microscopy were goat anti-rabbit IgG-Alexa Fluor 488 (A11008, Molecular Probes) and 568 (A11036, Molecular Probes) and goat anti-mouse IgG-Alexa Fluor 488 (A11001, Molecular Probes) and 568 (A21144, Molecular Probes).

2.10 Chromosomal Spreads

Primary keratinocytes were plated into 6-well dishes for retroviral infection with LZRS-Linker or LZRS-PKC δ -cat-ER retrovirus. Cells were infected the day after plating, and

treated with 10 μ M 4-hydroxytamoxifen the day following infection. 3 days post-infection, cells were collected by trypsinization, pelleted, and resuspended in 5 mL hypotonic buffer (75 mM KCl) to induce cell swelling. Cells were incubated in hypotonic buffer at room temperature for 30 minutes, and then pelleted. Cells were resuspended and fixed in 3.7% formaldehyde for 10 minutes. Following fixation, cells were pelleted and permeabilized in 1% Triton X detergent for 10 minutes. Cells were pelleted and resuspended in 300 μ L PBS and pelleted onto microscope slides using a cytopsin. After allowing slides to dry, cells were stained with α -P~H3(S10) antibody for 1 hr at room temp, followed by staining of the chromatin with 100 ng/mL DAPI.

2.11 Nuclear/Cytoplasmic Fractionation

Cells were collected in PBS and incubated in hypotonic buffer (10 mM KCl, 50 mM HEPES pH 7.9, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, Complete Protease Inhibitor (Roche) and 40 mM glycerophosphate, 2 mM sodium fluoride, and 1 mM sodium orthovanadate) for 15 min on ice. Triton-X 100 was added to a final concentration of 0.5% to complete lysis. Samples were centrifuged and the resulting supernatant contained enriched cytoplasmic proteins. The pellet (containing nuclei) was washed in hypotonic buffer plus 1 M sucrose and lysed in high-salt buffer (400 mM KCl, 50 mM HEPES pH 7.0, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1 mM DTT, Complete Protease Inhibitor and phosphatase inhibitors). Samples were centrifuged, and the resulting supernatant contained enriched nuclear proteins.

2.12 Thymine Dimer Southwestern Blot

WT and PKC δ null MEFs were plated into p100 tissue culture dishes. Cells were then exposed to 75 mJ/cm² UV radiation. At the indicated times, DNA was harvested from cells by scraping the cells into 500 μ L of DNA lysis buffer (10 mM Tris-HCl pH = 7.8, 5 mM EDTA, 0.3 M NaOAC, 1% SDS, 20 μ g/mL proteinase K). Protein digestion was performed by incubating lysates overnight at 55 $^{\circ}$ C. Following overnight incubation, 500 μ L of phenol/chloroform/isoamyl alcohol (25:24:1) was added to each tube with shaking for 2 minutes. Tubes were spun in eppendorf centrifuge at 13,000 rpm for 10 minutes. The aqueous layer (containing DNA) was transferred to a fresh tube, and 1 mL of ice-cold 100% EtOH was added to precipitate DNA. Tubes were spun at 13,000 rpm for 2 min to pellet DNA, and EtOH was carefully decanted following centrifugation. DNA pellets were then resuspended in 300 μ L of TE buffer.

After resuspension, DNA concentrations were measured using a UV spectrophotometer. In preparation for sample loading, 200 ng of DNA were added to a final volume of 500 μ L of TE. DNA was denatured by incubation at 100 $^{\circ}$ C for 10 min. Using a slot blot apparatus, samples were applied to Hybond-N+ membrane (Amersham) using vacuum pressure to pull TE through the membrane. After loading, membrane was allowed to dry. DNA was then crosslinked to the membrane using 70,000 J of UVC in a UV stratalinker. After crosslinking, the membrane was blocked overnight using Odyssey blocking buffer (LI-COR Biosciences). Membranes were then incubated in α -thymine dimer primary antibody overnight, washed for 15 min in TBS, followed by goat anti-rabbit IgG-Alexa

Fluor 680 (A21076, Invitrogen) for 1hr. Following secondary antibody incubation, the membrane was washed in TBST for 1hr then scanned using Licor infrared scanner.

Densitometry was done using Licor software, and intensities were corrected by removing the background membrane intensity.

2.13 Crystal Violet Staining

For focus forming assays, WT and PKC δ null MEFs were plated in p100 tissue culture dishes at low density. Cells were cultured to confluent conditions, and maintained at confluency for 7 days. To visualize confluency-induced colony formation, cells were fixed in 3.7% formaldehyde for 5 minutes at room temperature, and then stained with 0.05% crystal violet stain for 5 minutes. Plates were then washed extensively in water to decrease background staining. After allowing plates to airdry, images were generated using a flatbed scanner.

2.14 Soft Agar Colony Formation Assay

PKC δ null MEFs were plated into 6-well dishes for infection with LZRS-PKC δ -GFP retrovirus. Three days post-infection, media/soft agar mixture was prepared by combining 40 mL of 2x DMEM with antibiotics, 10 mL FBS, and 40 mL of melted 44 °C, 1.25% Difco agar for a final concentration of 0.5% agar medium. 1 mL of prepared DMEM-agar medium was poured into each well of a 6-well plate and allowed to solidify at room temperature for 30 min. Meanwhile, PKC δ null control MEFs, and PKC δ null MEFs

expressing PKC δ -GFP were resuspended to a final concentration of 100,000 cells per mL of DMEM-agar medium, and 1 mL of this suspension was added on top of each well. Cells were then grown in soft agar over 10 days then stained using a 1 mg/mL solution of p-iodonitrotetrazolium violet (INT) staining to label metabolically active colonies. 500 μ L of INT solution was added on top of each well, and plates were incubated at 37 °C overnight. Labelled colonies were counted using a bright field microscope.

Chapter Three

The PKC δ catalytic fragment induces multiple cell cycle checkpoints

3.1 Ectopic Expression of PKC δ -cat Induces G2/M Cell Cycle Arrest

Many tumor suppressors have critical roles in both the regulation of apoptosis and cell cycle regulation (ie. p53, ATM and ATR). This tight link between cell cycle regulation is not entirely surprising considering that there are common stimuli between the two responses. For example, DNA damage can potently induce both cell cycle checkpoint activation and apoptosis. Furthermore, the onset of apoptosis is often preceded by a period of cell cycle arrest while the cell attempts to repair the damaged DNA. Since the two processes are so tightly intertwined, there should be proteins which share regulation over both pathways. These proteins are expected to play important roles in tumor suppression, as the misregulation of either cell cycle checkpoints or apoptosis promote genomic instability and tumor development.

As discussed in Chapter 1.5.13, PKC δ is a known tumor suppressor in many cancer types, including squamous cell carcinoma. The importance of PKC δ for squamous cell carcinoma tumor suppression likely reflects, at least in part, the key role for PKC δ in the apoptotic cascade of keratinocytes. However, like p53 and many other tumor suppressors, PKC δ has also been reported to regulate cell cycle progression in many cell types, but whether the ability of PKC δ to regulate cell cycle progression is important for

PKC δ -mediated tumor suppression is unclear (Watanabe et al., 1992; Takahashi et al., 2006; Arita et al., 1998).

While the role of the PKC δ in apoptosis has been extensively studied little is understood about the role of PKC δ in regulating cell cycle progression, particularly with regards to G2/M checkpoint activation (Watanabe et al., 1992; Takahashi et al., 2006; Arita et al., 1998). To determine the effect, if any, of the PKC δ catalytic fragment (PKC δ -cat) on the cell cycle, we retrovirally expressed PKC δ -cat or an inducible PKC δ -cat/estrogen receptor fusion protein (PKC δ -cat-ER) in cultured primary human KCs, HaCaT cells (Figure 4), and MEFs (Figure 7A). Propidium iodide staining revealed that PKC δ -cat expression increased G2/M KCs approximately 2-fold ($p < 0.005$) compared to the control Linker virus population (Figure 5A and B). PKC δ -cat expression had a similar effect on the immortalized HaCaT cell line, which harbors mutant p53, although the effect was slightly diminished (Lehman et al., 1993). A similar but diminished effect was also detected in WT and PKC δ null MEFs, suggesting that the effect of PKC δ -cat on the cell cycle is present in multiple species (Figure 7B). Surprisingly, in these experiments, we did not detect a significant induction of apoptosis (ie. cells with sub-G1 DNA content) following PKC δ -cat induction.

To distinguish between arrest in G2 or M, mitotic index measurements were performed on PKC δ -cat-ER transduced HaCaT cells by measuring P~H3(S10) levels and DNA content. Using this procedure, cells in mitosis should stain positively for P~H3(S10) and have 4N DNA content. In this experiment, a decrease in mitotic index would indicate that

PKC δ -cat is inducing G2/M arrest outside of mitosis, while an increase in mitotic index would indicate a mitotic arrest. Figure 6A and 6B show that PKC δ -cat-ER causes a significant ($p < 0.001$) reduction in mitotic cells, indicating that PKC δ -cat-ER induces a G2/M rather than mitotic arrest.

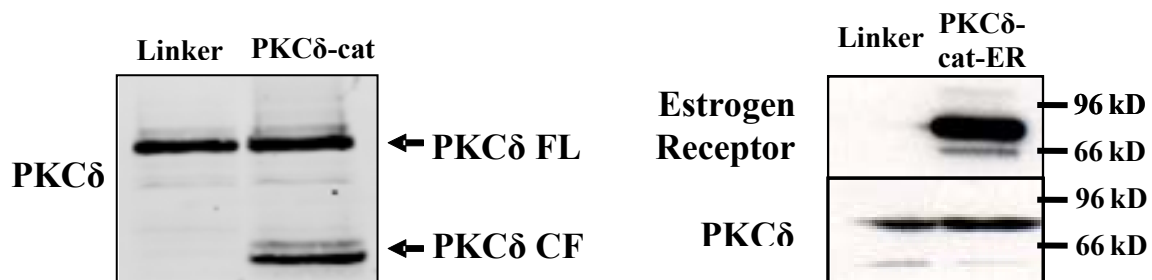


Figure 4—Ectopic Expression of PKC δ -cat-FLAG and PKC δ -cat-ER Proteins.

Western blots displaying relative levels of full length (PKC δ -FL) and catalytic fragment (PKC δ -cat) of PKC δ protein are shown in control Linker, PKC δ -cat-FLAG, and PKC δ -cat-ER transduced KCs. PKC δ -cat-ER transduced cells display a strong estrogen receptor, ER, positive band at the same position as the PKC δ protein, indicating successful expression of the ER fusion protein.

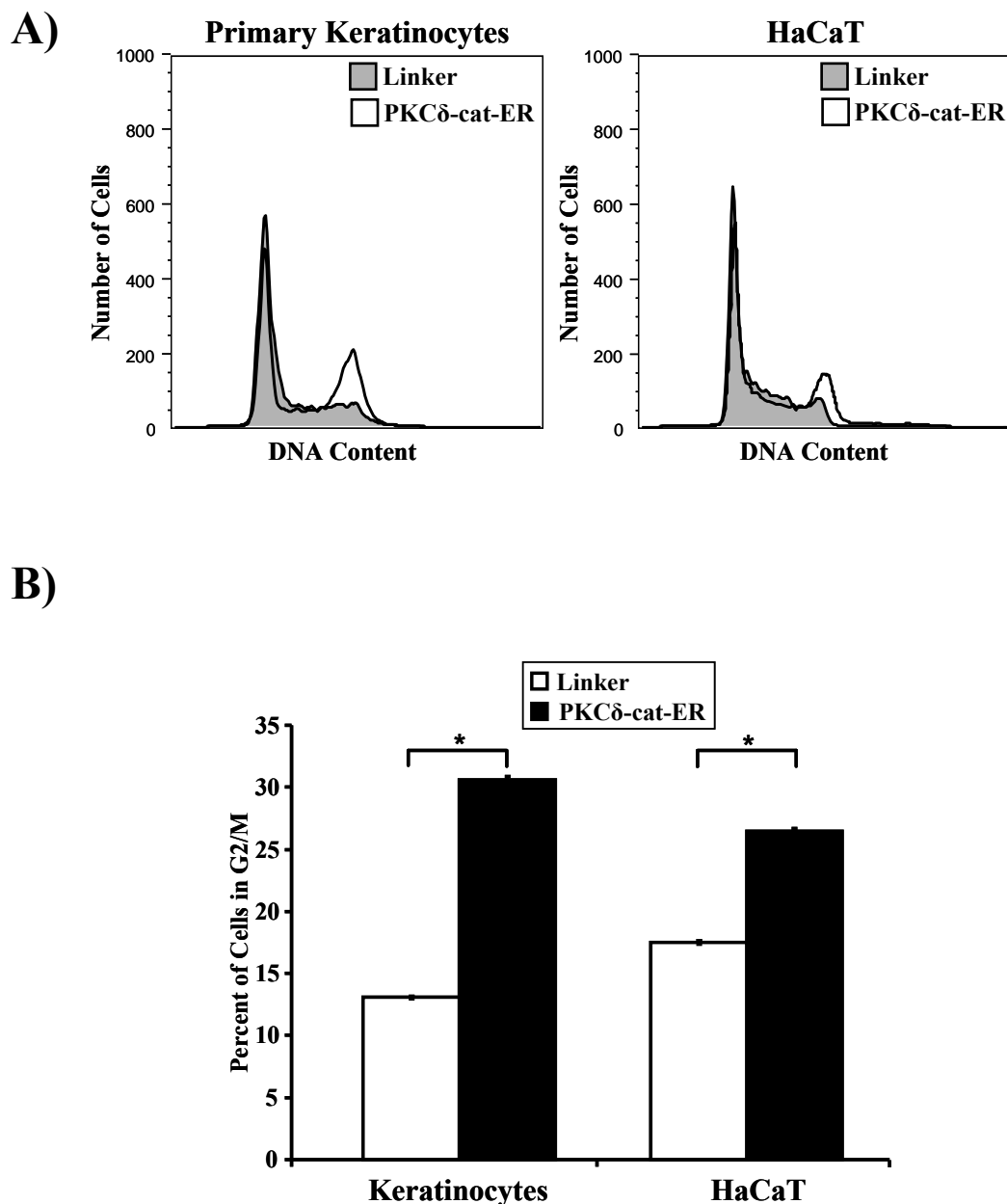
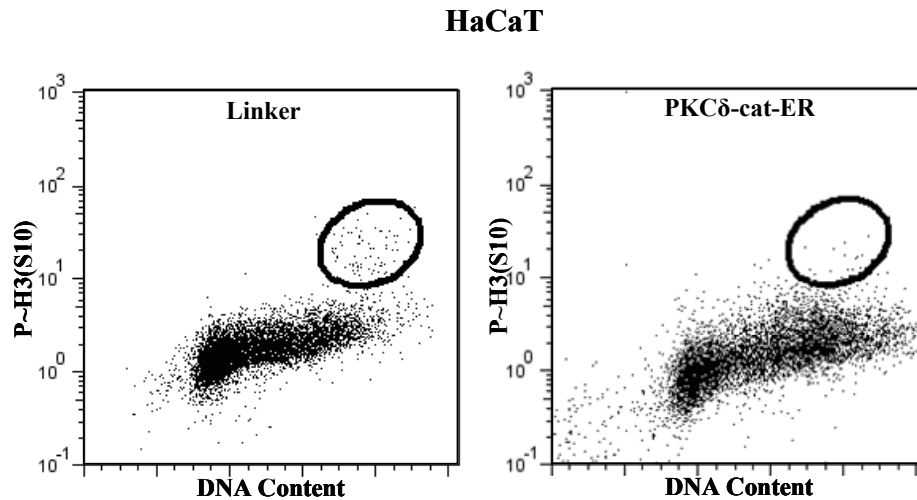


Figure 7—PKC δ -cat Induces G2/M Cell Cycle Arrest in Primary Keratinocytes and HaCaT Cells. A) Representative DNA content histograms of primary KCs and HaCaT cells transduced with either Linker (gray) or PKC δ -cat-ER (white) retrovirus are shown. Propidium iodide staining was performed 2-3 days following infection. Similar results were obtained in at least 3 independent experiments. B) Quantitation of the percentage of cells containing G2/M DNA content in KCs and HaCaT cells 2-3 days after infection with either Linker control or PKC δ -cat-ER retrovirus. Graphs represent experiments done in triplicate. * Student's t-test value of $p < 0.005$.

A)



B)

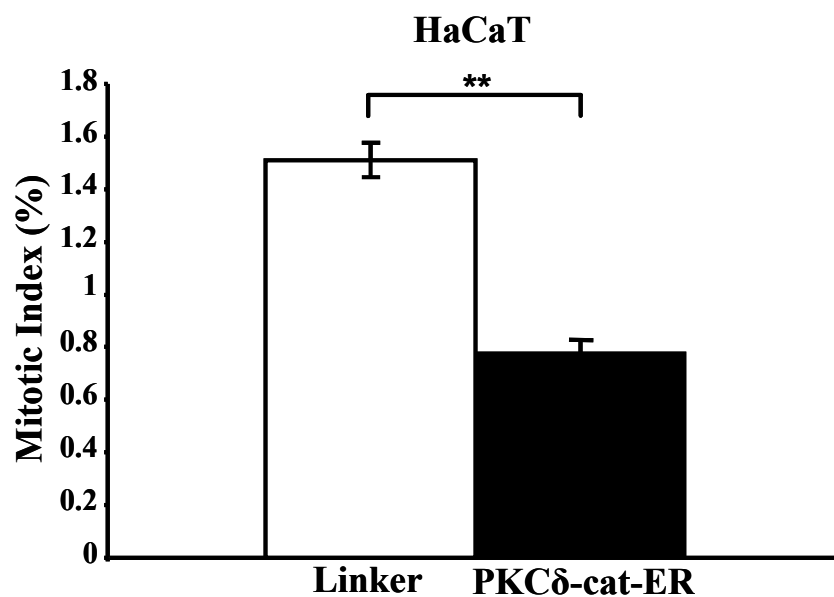
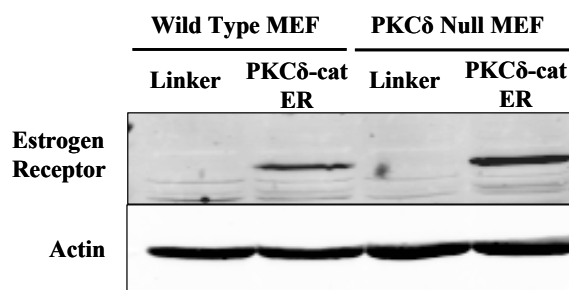


Figure 8—PKC δ -cat Induces a Decrease in Mitotic Index in HaCaT Cells

A) HaCaT cells transduced with either Linker or PKC δ -cat-ER retrovirus were harvested and stained for P~H3(S10) and propidium iodide after 3 days, and analyzed by flow cytometry. B) Quantitation of mitotic indices from HaCaT cells treated as in A. ** Student's t-test value of $p < 0.001$. Error bars denote the standard deviation.

A)



B)

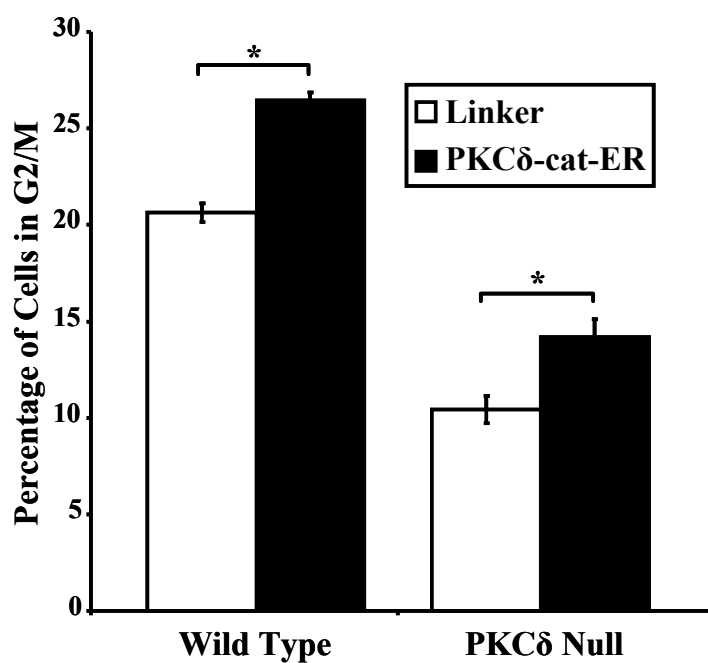


Figure 9 –PKC δ -cat-ER induces G2/M Cell Cycle Arrest in WT and PKC δ Null MEFs. A) Western Blot showing retrovirally transduced expression of PKC δ -cat-ER in wild type and PKC δ null MEFs. B) Wild type and PKC δ null MEFs were transduced with either Linker or PKC δ -cat-ER retrovirus, and after 3 days stained with PI and DNA content was measured using flow cytometry. The percentage of cells in the G2/M phase of the cell cycle is shown. * Student's t-test value of $p < 0.01$

3.2 PKC δ -cat Expression Induces G2/M Checkpoint Signaling

Since many of the components that positively and negatively regulate G2/M cell cycle progression are localized to the nucleus, it is likely that PKC δ -cat would need to have at least some nuclear localization to participate in the G2/M checkpoint. In addition, it has been reported by several groups that upon exposure to various apoptotic stimuli, PKC δ translocates to the nucleus, and this nuclear translocation is critical for the apoptotic function of PKC δ (Scheel-Toellner et al., 1999; Yoshida et al., 2003; Eitel et al., 2003).

To examine PKC δ sub-cellular localization, we performed nuclear/cytoplasmic fractionation on HaCaT cells before and 18 hours after exposure to 30 mJ/cm² UV radiation. Fractionation results revealed that both the full length PKC δ and PKC δ -cat were present in the nucleus, with PKC δ -cat detected only after UV exposure (Figure 8A). The decrease in levels of the full length PKC δ after UV exposure are likely a consequence of UV-induced cleavage of PKC δ . UV radiation caused a significant fraction of histone H3 to be detected in the cytoplasmic extract, suggesting apoptotic degradation of the nuclear membrane occurred in the cells exposed to UV radiation. Confocal microscopy using a carboxyl-terminal GFP fusion of PKC δ (PKC δ -GFP) and DAPI staining was used to confirm the sub-cellular fractionation data. PKC δ -GFP was localized to both the cytoplasm and nucleus before and after UV irradiation (Figure 8B). This localization provides a means by which PKC δ may interact with important components of the G2/M cell cycle checkpoint pathway.

We next tested whether PKC δ -cat induced molecular changes consistent with G2/M checkpoint activation. One key aspect of this checkpoint involves the phosphorylation of Cdk1 on tyrosine 15 by Wee1 and Myt1 kinases, an event which has been shown to inhibit Cdk1 activity and prevent entry into mitosis (Parker et al., 1995; Parker and Piwnica-Worms, 1992; Piwnica-Worms et al., 1991; Mueller et al., 1995; Booher et al., 1997). We measured relative levels of P~Cdk1(Y15) in PKC δ -cat expressing KCs and found that PKC δ -cat expression induced elevated levels of P~Cdk1(Y15) compared to the control Linker-transduced cells. The PKC δ -cat kinase dead (K378A) mutant did not induce P~Cdk1(Y15) (Figure 9). As a positive control, UV radiation also induced P~Cdk1(Y15).

PKC δ signaling is known to promote the production of reactive oxygen species (ROS), and elevated intracellular levels of ROS can induce oxidative DNA damage (Bossi et al., 2008b). The induction ROS following PKC δ -cat expression might then induce indirect checkpoint activation by causing oxidative damage to DNA. If this were true, and PKC δ -cat-induced G2/M checkpoint activation occurred by stimulating DNA damage, then inhibition of ATM and ATR, two genes critical for recognizing DNA damage and activating cell cycle checkpoints, should block PKC δ -cat induced cell cycle arrest. To examine this, we treated primary human KCs and HaCaT cells ectopically expressing the PKC δ -cat-ER with 2 mM caffeine, a known inhibitor of ATM and ATR (Figure 10) (Sarkaria et al., 1999). Interestingly, we found that inhibition of ATM/ATR activity by caffeine treatment did not block PKC δ -cat induced G2/M arrest in primary KCs or

HaCaT cells. This finding provides substantial evidence that PKC δ -cat induced G2/M checkpoint activation occurs independently of ATM/ATR signaling.

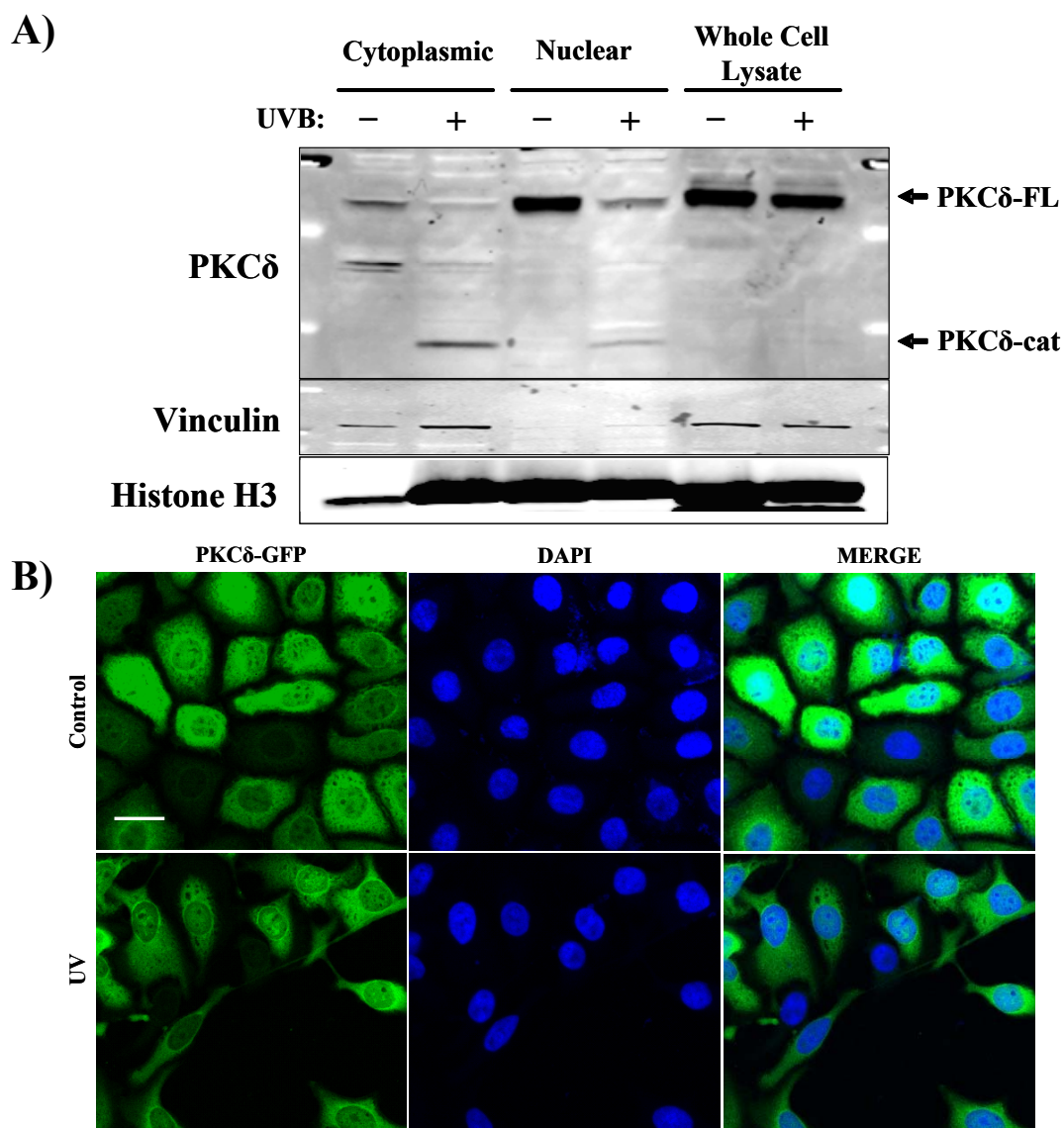


Figure 10–PKC δ Protein Levels are Present in both Cytoplasm and Nucleus Before and After UV irradiation. A) Western blot showing levels of PKC δ protein in cytoplasmic and nuclear extracts of untreated or 30 mJ/cm² UV exposed KCs after 18 hrs. The full length (FL) and catalytic fragment (CF) of PKC δ are indicated by arrows. Vinculin and histone H3 are presented as cytoplasmic and nuclear markers, respectively. B) Confocal microscope images were taken showing cytoplasmic and nuclear staining of PKC δ -GFP fusion protein both before and 18 hrs after exposure to 30 mJ/cm² UV radiation. DAPI staining is displayed to demonstrate nuclear localization. Images were taken using 40x objective and 1 μ m optical slice. Scale bar denotes 25 μ m.

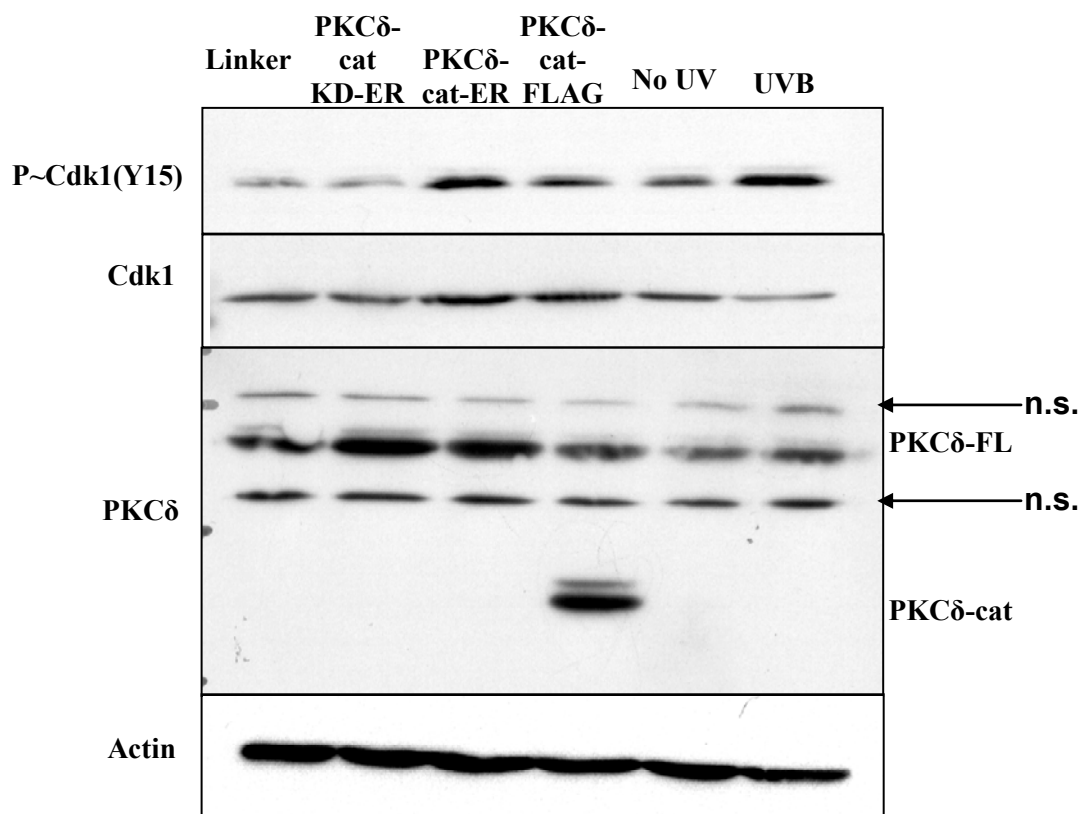


Figure 11—UV and PKCδ-cat Expression Cdk1 Induce Tyrosine Phosphorylation. Western blot showing levels of P~Cdk1(Y15), total Cdk1, and PKCδ after retroviral transduction of either PKCδ-cat-ER, PKCδ-cat, or the kinase dead PKCδ(K378A)-cat-ER. The PKCδ-cat-ER fusion proteins run to the same position in the gel as the full length PKCδ. KC lysate harvested 18 hrs after exposure to 30 mJ/cm² UV radiation is shown as a positive control for P~Cdk1(Y15) induction. Actin protein levels are displayed as a loading control. n.s. indicates the detection of a non-specific band on the Western Blot.

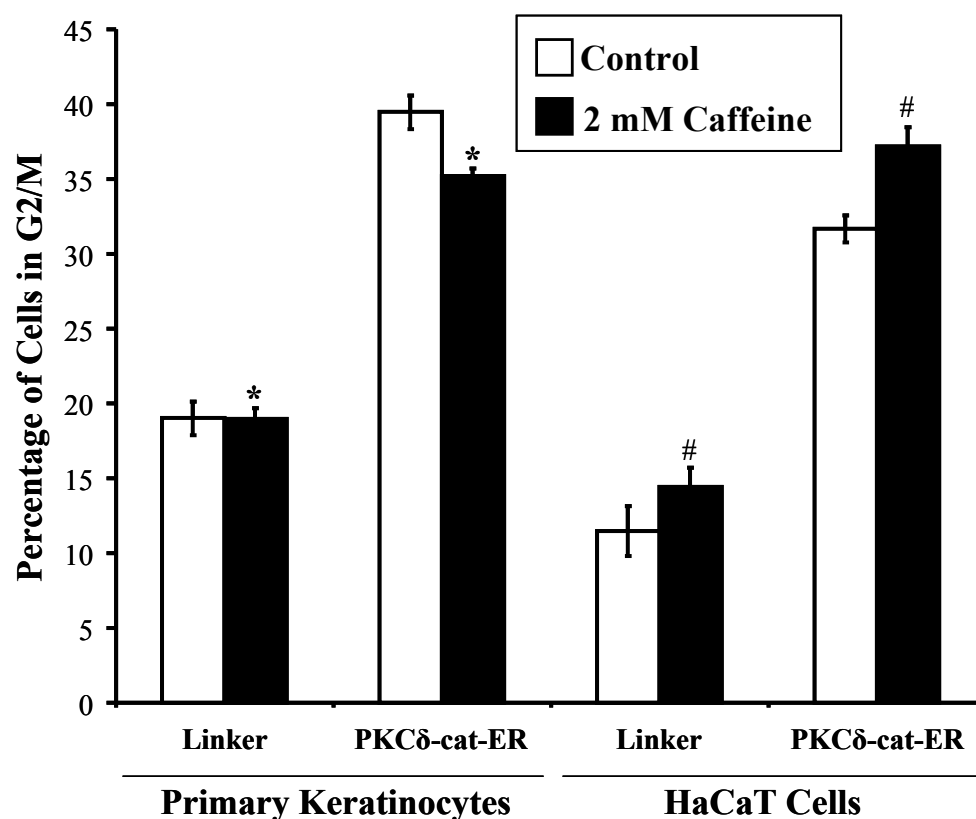


Figure 12—PKC δ -cat Induced G2/M Arrest Occurs Independently of ATM/ATR.PI cell cycle analysis of LZRS-Linker or LZRS-PKC δ -cat-ER transduced primary KCs and HaCaT cells incubated with 2 mM caffeine and 10 μ M 4 OH-Tamoxifen for 48 hours. The percentage of cells in G2/M phase of the cell cycle is displayed from experiments performed in triplicate. * Student's t-test value of $p < 10^{-5}$; # Student's t-test value of $p < 10^{-4}$.

3.3 PKC δ Controls Multiple Cell Cycle Checkpoints

To further investigate the role for PKC δ in controlling overall cell cycle progression, we utilized the cell cycle synchronizing agents nocodazole, and aphidicolin. The combination of these agents with our PKC δ -cat-ER tamoxifen-inducible constructs allowed us to specifically activate PKC δ -cat-ER activity at various cell cycle stages, and determine whether PKC δ -cat could influence cell cycle progression in cell cycle stages other than G2/M.

Nocodazole, a microtubule depolymerizing agent causes cells to arrest in mitosis by activating the spindle assembly checkpoint (Chou and Omary, 1994). The subsequent removal of nocodazole from the culture medium allows cells to form mitotic spindles and complete chromosomal segregation and mitosis. It has been reported by several groups that PKC δ is a negative regulator of cytokinesis (Watanabe et al., 1992; Takahashi et al., 2006). To investigate whether PKC δ -cat expression can induce late-stage mitotic arrest, we treated Linker and PKC δ -cat-ER expressing HaCaT cells with nocodazole overnight to arrest cells at metaphase. The next day, between 70-85% of cells had arrested within mitosis based on DNA content and observed cell morphology. Next, we simultaneously released the cells from metaphase arrest and treated with 4-hydroxytamoxifen to activate the PKC δ -cat-ER fusion protein. Cells were harvested at various time points post-release to measure the return of both cell populations to 2N DNA content, indicative of the successful completion of mitosis.

Strikingly, while Linker infected control HaCaT cells were able to largely recover from nocodazole arrest, complete mitosis, and return to G1 phase, PKC δ -cat-ER activation significantly prevented HaCaT cells from completing mitosis (Figure 11). This was evidenced by the maintenance of 4N DNA content in the PKC δ -cat-ER experimental group. This suggests that in addition to regulating the G2/M checkpoint, and in agreement with previously published work, PKC δ also plays a role in regulating progression through the late stages of mitosis.

In addition to nocodazole-mediated mitotic synchronization, we utilized the DNA polymerase inhibitor aphidicolin to arrest cells in the G1/S phase of the cell cycle (Berger et al., 1979; Pedrali-Noy et al., 1980). Upon overnight incubation of both Linker and PKC δ -cat-ER infected HaCaT cells with 10 μ M aphidicolin, approximately 70-75% of cells were arrested in G1 or early S phase (Figure 12). The removal of aphidicolin from the culture medium allowed cells to resume DNA replication, progress through S phase and enter into G2 and mitosis. As in our nocodazole release experiments, we simultaneously removed aphidicolin and treated with 4-hydroxytamoxifen to activate PKC δ -cat-ER. We then measured the progression of cells through S phase and into G2/M by collecting at various timepoints, and staining with propidium iodide.

Strikingly, while Linker cells successfully completed S phase, entered mitosis, and began to return to G1 over the time, PKC δ -cat-ER cells entered S phase, but never progressed through S phase into G2 (Figure 12). This conclusion can be observed by the diminished increase in the percentage of PKC δ -cat-ER transduced cells with G2/M DNA

content after aphidicolin release (Linker: 15% after arrest increased to 40% 9 hours after release; PKC δ -cat-ER: 10% after arrest increased to less than 20% after release). Similarly, following the initial entry into S-phase following aphidicolin release, PKC δ -cat-ER cells appear unable to accumulate, as only approximately 2-3% exit S-phase and enter G2/M phase based on DNA content analysis. These results indicate that PKC δ -cat can impede progression through S-phase, and are consistent with the recent finding that PKC δ could stimulate premature G1 phase cell cycle progression followed by S phase arrest and apoptosis (Santiago-Walker et al., 2005).

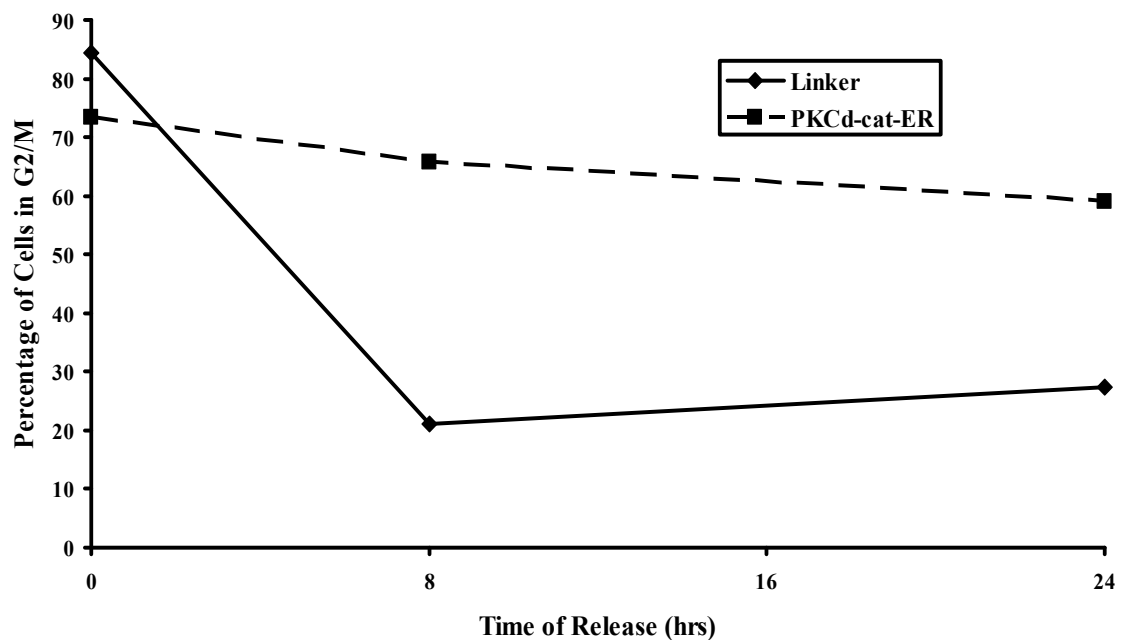


Figure 13—PKC δ -cat-ER Activation Delays Mitotic Exit After Release from Nocodazole Induced Metaphase Arrest. Linker (solid) and PKC δ -cat-ER (dashed) transduced HaCaT cells were incubated with 10 ng/mL nocodazole overnight to induce metaphase arrest. Cells were then washed and re-fed with nocodazole-free media + 10 μ M 4-hydroxytamoxifen to activate PKC δ -cat-ER. Cells were collected at indicated timepoints, fixed and stained with propidium iodide to measure DNA content.

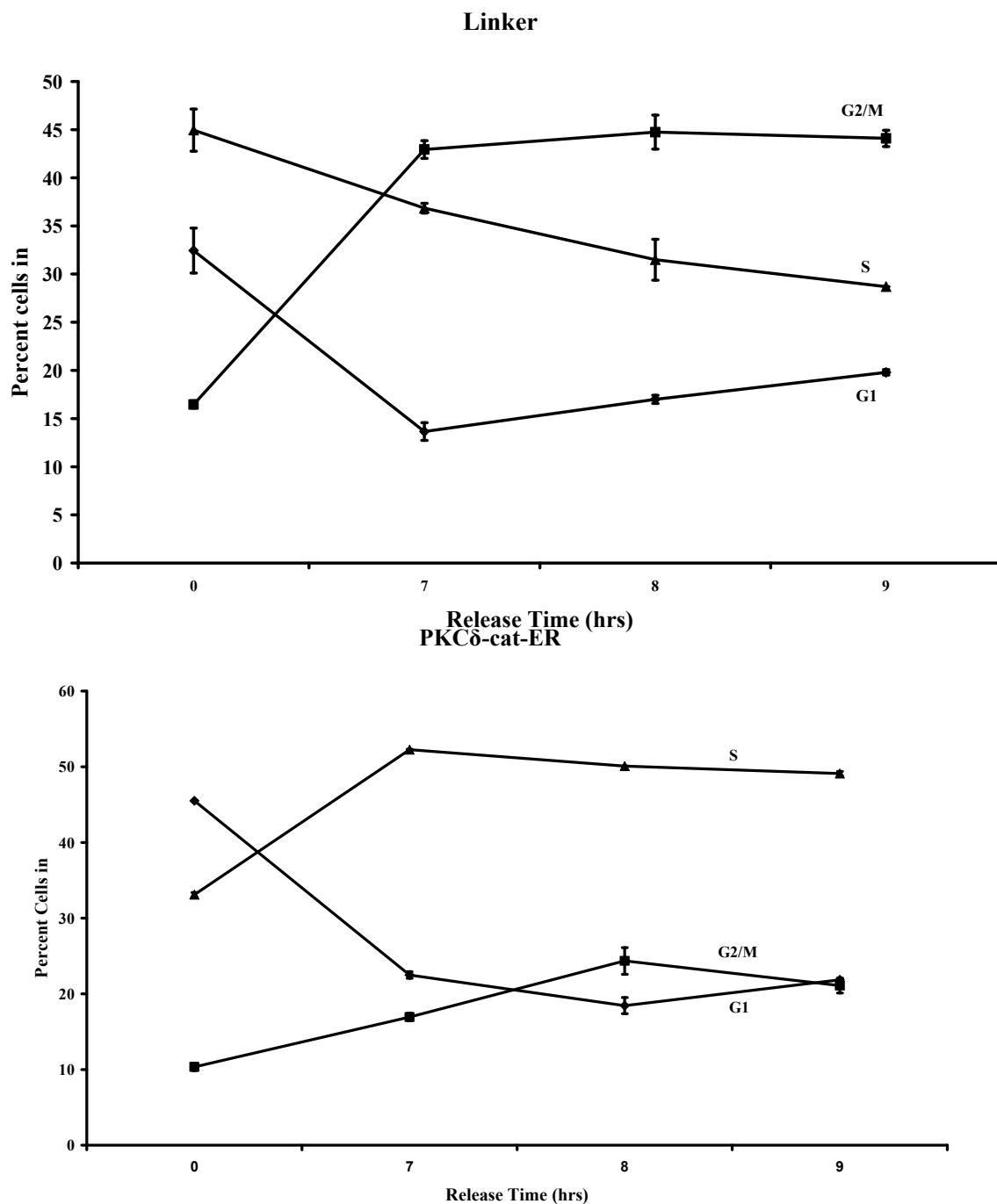


Figure 14—PKC δ -cat-ER Activation Delays Progression Through S-phase After Release from Aphidicolin Arrest. Linker and PKC δ -cat-ER transduced HaCaT cells were incubated with 10 μ M aphidicolin overnight to induce G1/S phase arrest. Cells were then washed and refed with aphidicolin-free media + 10 μ M 4-hydroxytamoxifen to activate PKC δ -cat-ER. Cells were then collected at indicated timepoints, fixed and stained with propidium iodide to measure DNA content. Error bars represent the standard deviation from the mean of the experiment done in triplicate.

Chapter 4

PKC δ is Required for UV-Induced G2/M Checkpoint Activation

4.1 UV-Induced G2/M Cell Cycle Arrest Requires PKC δ

Since expression of PKC δ -cat was capable of inducing a pronounced G2/M arrest and checkpoint activation in KCs and HaCaT cells, we next determined the requirement for PKC δ in DNA damage-induced cell cycle arrest. To address this issue, wild type and PKC δ null MEFs were exposed to UV radiation (Figure 13 and 14). We found that exposure of the wild type MEFs to 30 mJ/cm² UV radiation induced a pronounced G2/M arrest which persisted up to 24 hours after exposure (Figure Figure 14 and 15). Strikingly, UV exposure failed to induce G2/M arrest in PKC δ null MEFs although there was a slight increase in the percentage of cells in S phase. The slight increase in the percentage of cells in the G2/M phase of the cell cycle 12 hours following UV-irradiation may be a reflection of an initial PKC δ -independent G2/M arrest (Figure 15 and 17). Importantly, PKC δ null MEFs did undergo a G2/M arrest when transduced with PKC δ -cat-ER virus, indicating that they were competent to arrest in G2/M (Figure 7).

Mitotic indices were measured by staining for P~H3(S10) levels (a known marker of mitosis) and DNA content. Measurement of mitotic indices revealed that UV caused a significant ($p < 0.05$) decrease in mitotic wild type MEFs, but not PKC δ null MEFs, at 6 hours, indicating that the UV-induced arrest was in G2 (Figure 16). In contrast, when mitotic indices were gathered 1 hr after UV exposure, both WT and PKC δ null MEFs

displayed a comparable decrease in mitotic index (Figure 17). This suggests that PKC δ is not required for early UV-induced G2/M arrest, but that maintenance of the G2/M arrest over time requires PKC δ . G2/M checkpoint activation compared to maintenance in the response to DNA damage has been described for other proteins, including p53, and will be elaborated upon in the discussion.

Interestingly, G2/M checkpoint override by the ATM/ATR inhibitor caffeine drove UV-irradiated wild type MEFs into apoptosis (as measured by sub-G1 DNA content), but did not induce apoptosis in PKC δ null MEFs (Figure 18). The lack of apoptosis after caffeine-mediated inhibition of checkpoint activation in PKC δ null MEFs is likely a reflection of the crucial pro-apoptotic functions of PKC δ . Interestingly, PKC δ null cells treated with UV plus caffeine accumulated in S-phase ($p < 10^{-4}$), indicating a possible S-phase checkpoint independent of PKC δ and ATM/ATR function.

We were also interested in the requirement of PKC δ in the cell cycle response to other forms of DNA damage. Ionizing radiation exposure induces both double and single stranded DNA breaks, and is well known to be a potent inducer of G2/M cell cycle arrest. We decided to use ionizing radiation as a source of DNA damage in our MEF system to determine the effect of PKC δ loss on G2/M checkpoint response. As occurred following exposure to UV radiation, WT MEFs underwent a pronounced G2/M cell cycle arrest following exposure to low (4 Gy) and high (20 Gy) doses of γ -radiation (Figure 19). Similar to the response to UV radiation, PKC δ null MEFs exposed to 4 Gy of γ -radiation had a diminished G2/M cell cycle arrest compared to WT MEFs. Strikingly, even after

exposure to high doses of γ -radiation (20 Gy), PKC δ null MEFs demonstrated a diminished ability to undergo G2/M arrest. This finding suggests that PKC δ is important for the G2/M checkpoint response to both UV and ionizing radiation. It will be interesting to determine the universality of PKC δ in the G2/M checkpoint response to other types of DNA damage (ie. ROS, inter-strand crosslinks, etc...).

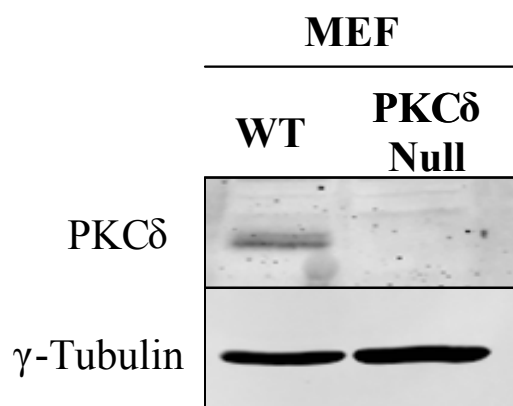


Figure 15–PKC δ Protein Levels in WT and PKC δ Null MEFs. Western blot displaying PKC δ protein levels in wild type (WT) and PKC δ null MEF whole cell lysates. α -Tubulin levels are shown as a loading control.

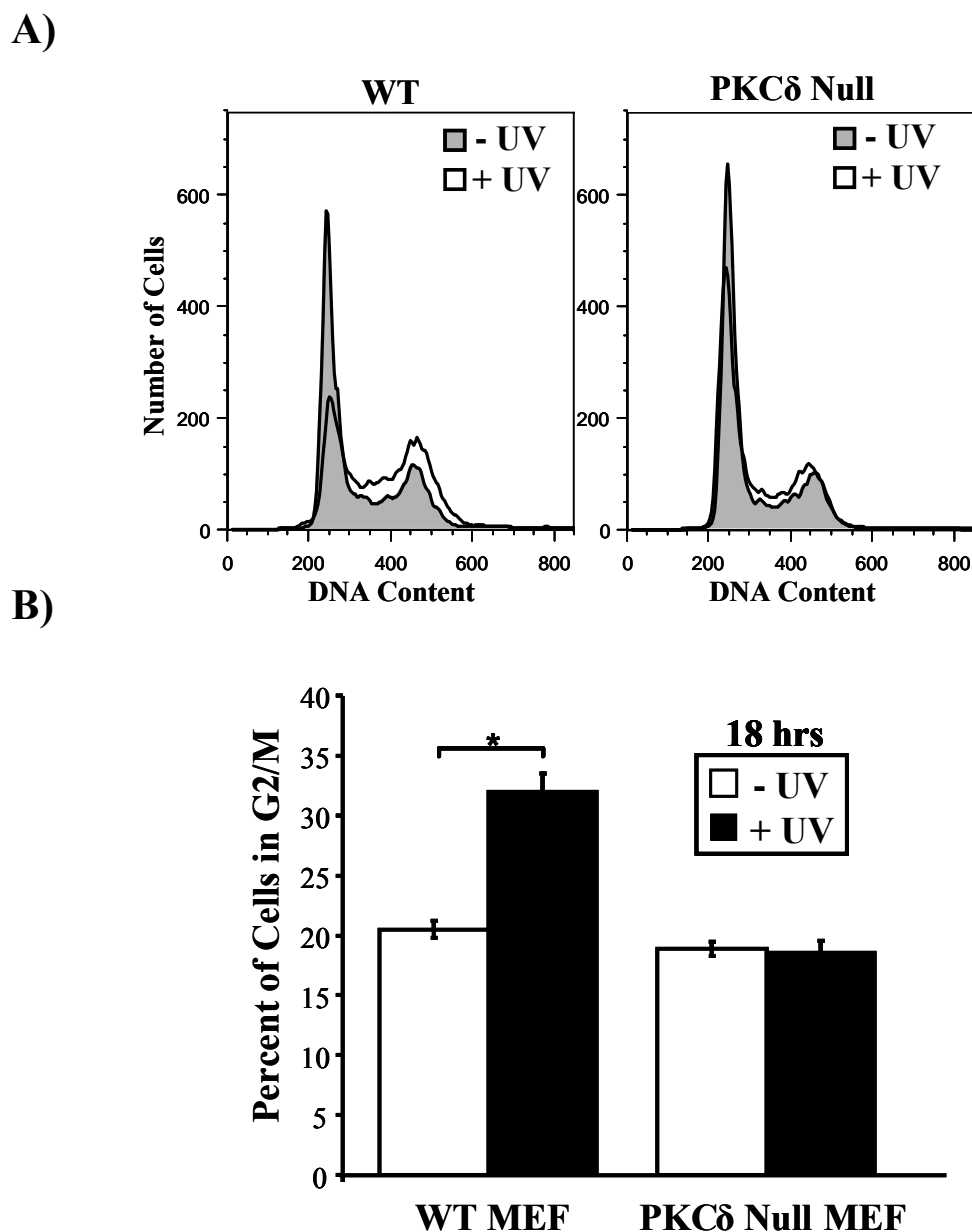


Figure 16—WT, but not PKC δ Null MEFs Undergo a G2/M Cell Cycle Arrest Following Exposure to UV Radiation. A) Representative DNA content histograms of WT and PKC δ null MEFs before and 18 hrs after exposure to 30 mJ/cm². B) The percentage of WT and PKC δ null MEFs in the G2/M phase of the cell cycle before and after exposure to 30 mJ/cm² UV radiation is displayed. Error bars denote the standard deviation of the mean from experiments performed in triplicate. * Student's t-test value of $p < 0.005$.

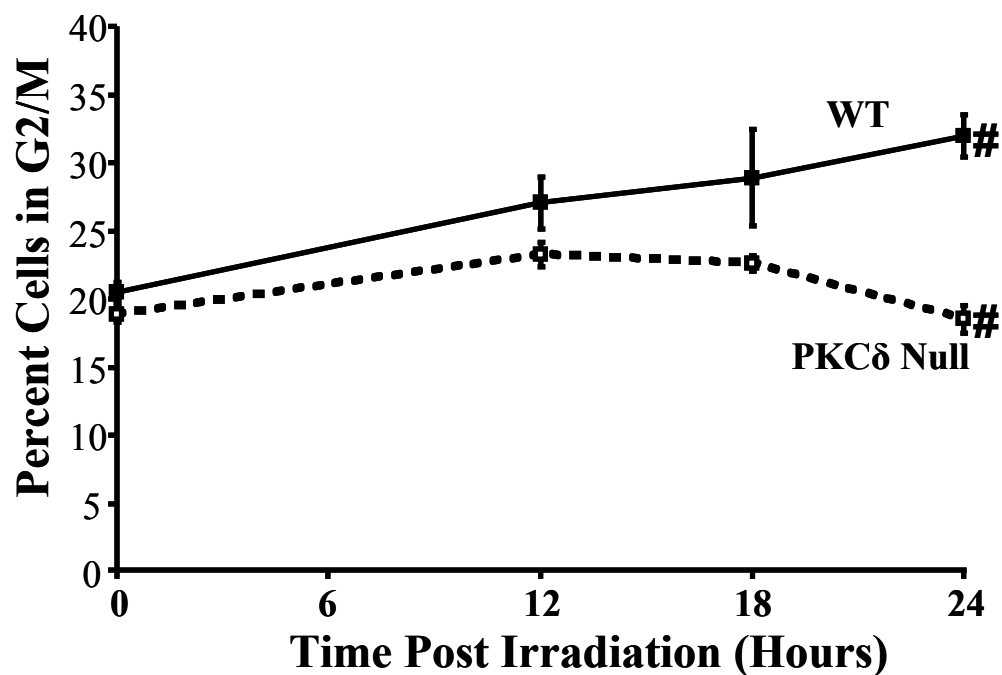


Figure 17—PKC δ Null MEFs Fail to Arrest in G2/M Over 24 Hours Following UV-Radiation Exposure. The percentage of WT and PKC δ null MEFs with G2/M DNA content at various times following exposure to 30 mJ/cm² UV radiation is displayed. Error bars denote the standard deviation of the mean from experiments performed in triplicate. # Student's t-test value of p<0.005.

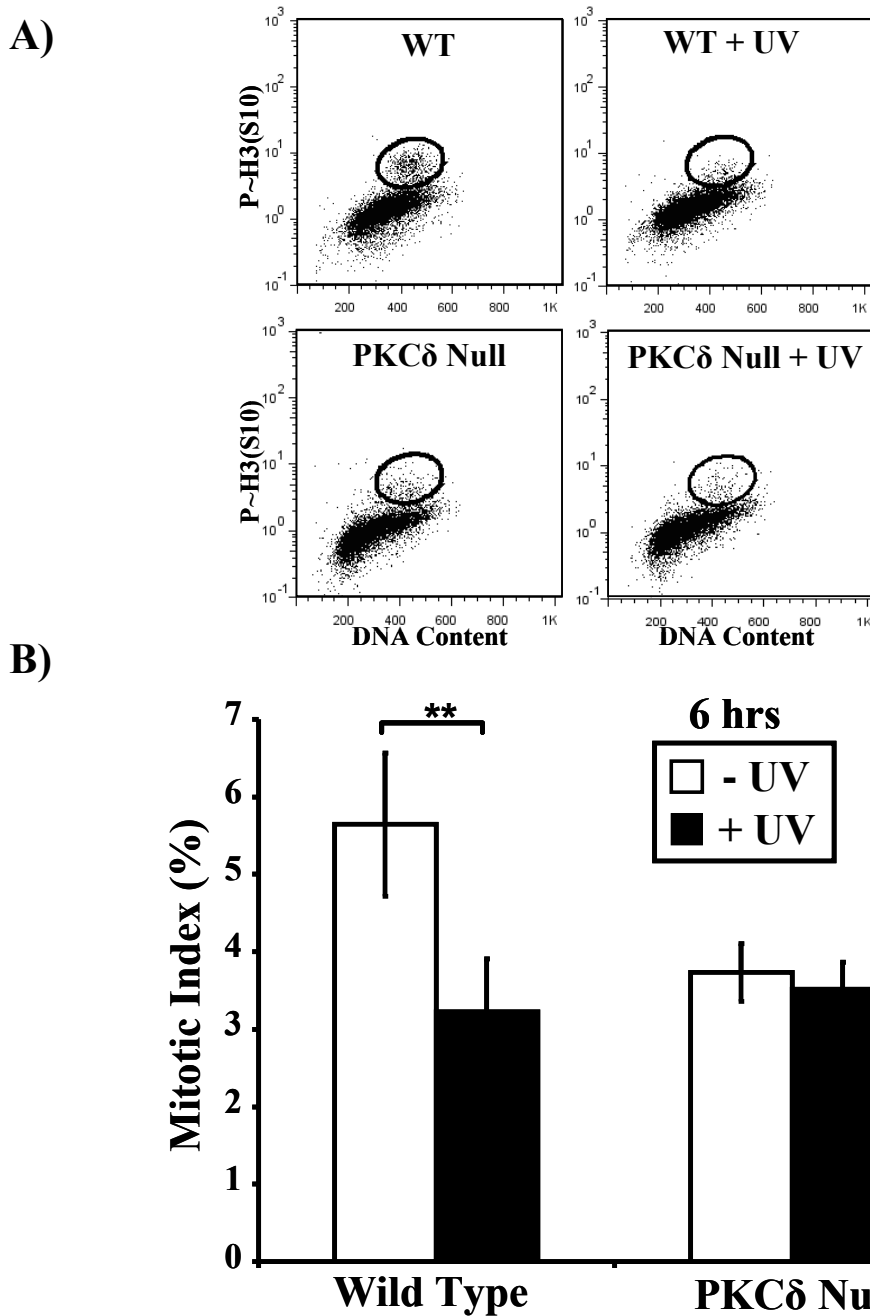


Figure 18—UV Induces a Decrease in Mitotic Index in WT but not PKC δ Null MEFs A) WT and PKC δ null MEFs were exposed to 10 mJ/cm² UV and treated with 100 ng/mL nocodazole and stained for P~H3(S10) and propidium iodide 6 hrs after UV exposure. Flow cytometry analysis is shown, with the P~H3(S10) positive, G2/M DNA content cells circled as the mitotic cells. B) Quantitation of mitotic indices from HaCaT cells treated as in A. ** Student's t-test value of p<0.05.

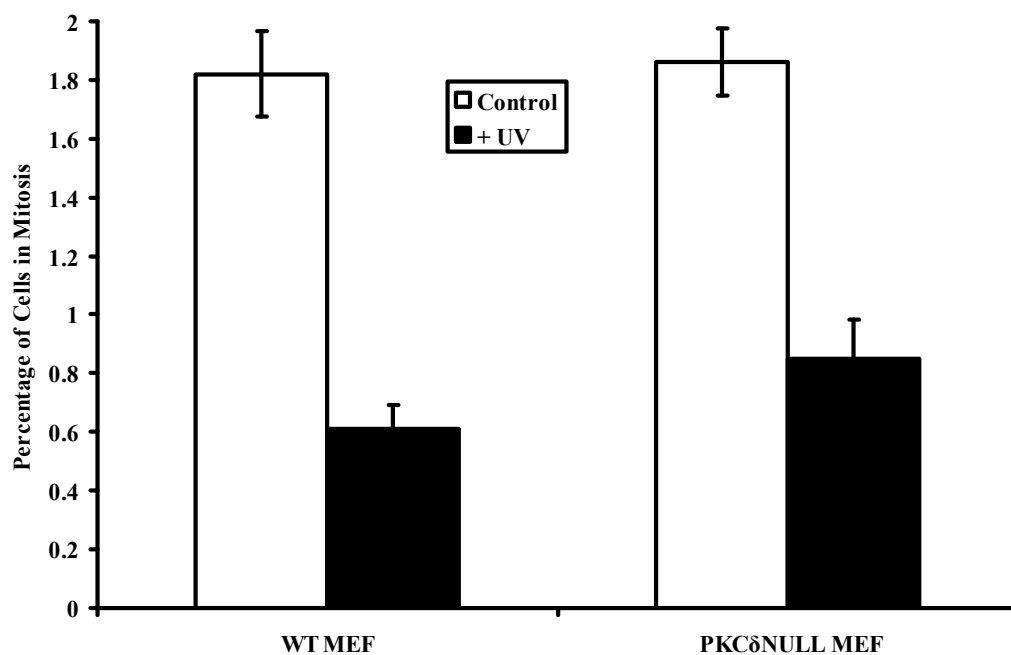


Figure 19—UV Induced Decrease in Mitotic Index 1 Hour After UV-Irradiation Occurs in Both Wild Type and PKC δ Null MEFs. 1 hr after exposure to 30 mJ/cm² UV radiation, cells were collected, and mitotic index was determined by flow cytometry. Note the apparent decrease in mitotic index occurred in both WT and PKC δ Null MEFs, in contrast to what is observed at later timepoints.

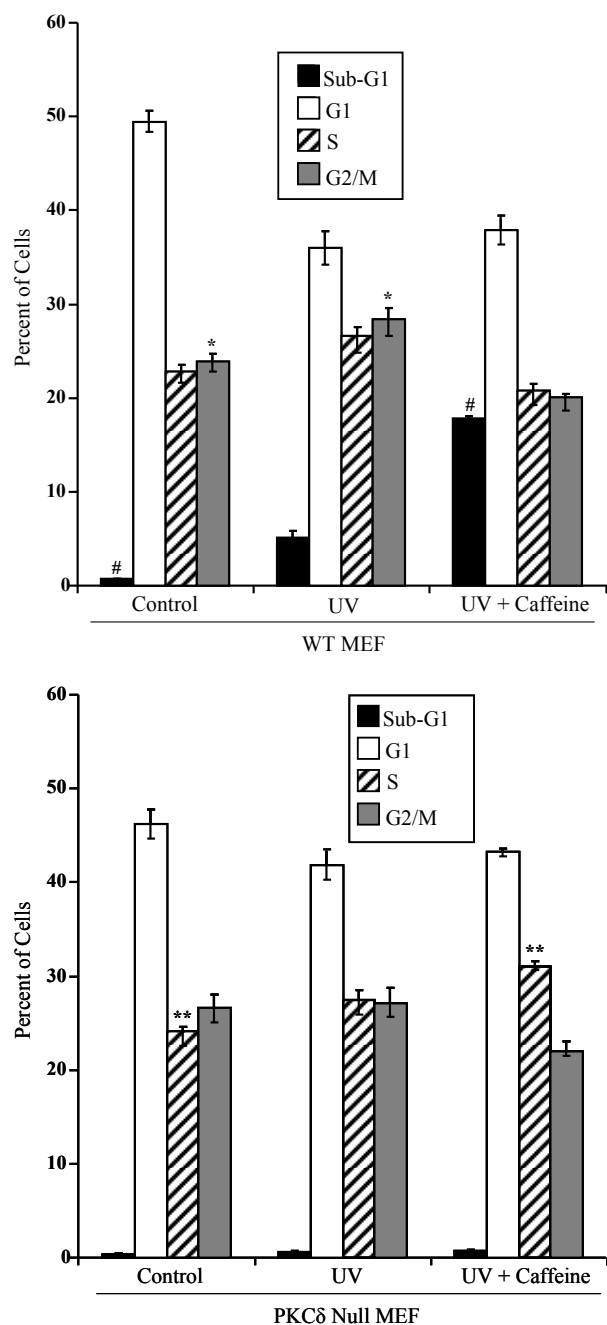


Figure 20—Caffeine sensitizes WT but not PKC δ null MEFs to UV-induced apoptosis. WT and PKC δ null MEFs were exposed to 30 mJ/cm² UV radiation in the presence or absence of 2 mM caffeine. The Percentage of cells in each phase of the cell cycle is displayed. Error bars denote the standard deviation of the mean from experiments performed in triplicate. * Student's t-test value of $p < 0.05$; # Student's t-test value of $p < 0.05$; ** Student's t-test value of $p < 10^{-4}$.

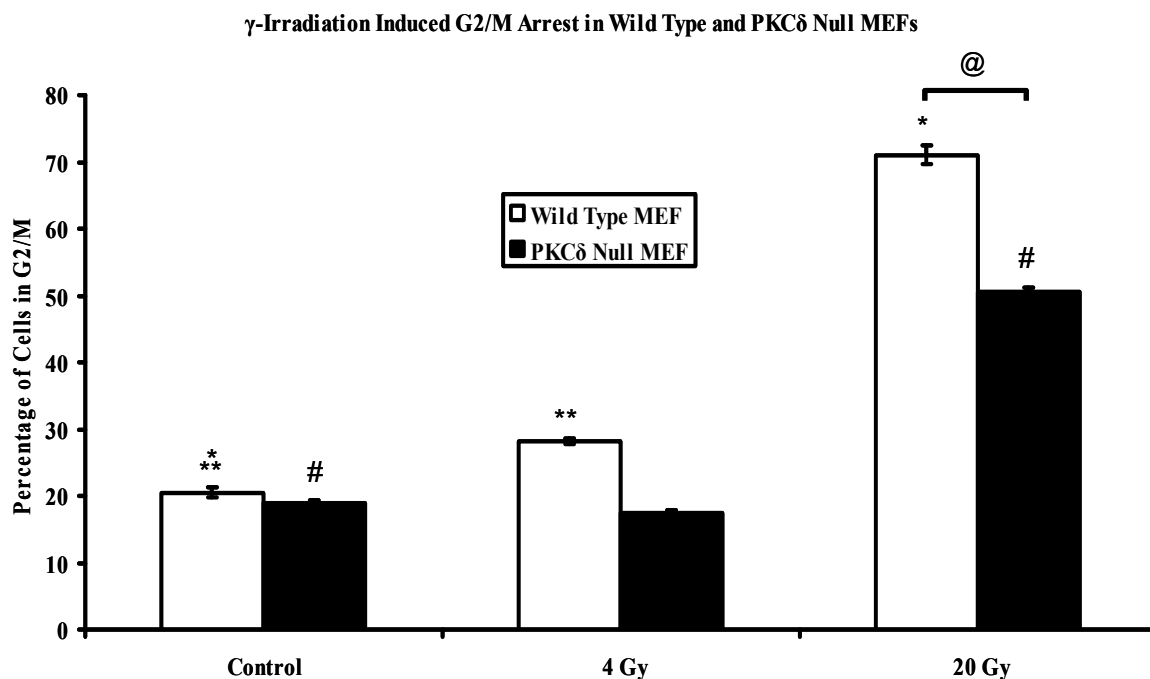


Figure 21— γ -Radiation Induces a G2/M Arrest in WT and PKC δ Null MEFs

WT and PKC δ null MEFs were exposed to 4 or 20 Gy γ -radiation. Cells were collected 18 hrs after irradiation, and DNA content was analyzed. The percentage of cells in the G2/M phase is displayed. Error bars denote the standard deviation of the mean from experiments performed in triplicate. * Student's t-test value of $p < 10^{-4}$; ** Student's t-test value of $p < 0.0005$, # Student's t-test value of $p < 0.001$; @ Student's t-

4.2 UV-Induced G2/M Arrest Requires PKC δ Cleavage and Kinase Activity

We next attempted to rescue G2/M checkpoint integrity in PKC δ null MEFs by retrovirally transducing them with the catalytically competent full-length PKC δ -GFP (Figure 20). To address whether PKC δ kinase activity or caspase cleavage are required to restore the G2/M checkpoint response, we also transduced a kinase dead mutant, PKC δ (K376R)-GFP, or a kinase competent mutant that possesses a mutated caspase cleavage site, PKC δ (D327A)-GFP. All PKC δ -GFP constructs were expressed at similar levels (Figure 20). Expression of PKC δ -GFP in PKC δ null MEFs had little effect on the cell cycle distribution of non-damaged cells (Figure 21). As expected, expression of PKC δ -GFP in PKC δ null MEFs restored the G2/M checkpoint response to UV radiation suggesting the altered cell cycle response of PKC δ null MEFs was a direct consequence of PKC δ loss of function (Figure 21). In contrast, the kinase dead PKC δ (K376R)-GFP mutant was not able to restore the G2/M checkpoint in the PKC δ null MEF background, indicating that kinase activity is necessary for this PKC δ checkpoint function. Strikingly, expression of the cleavage site mutant PKC δ was also unable to restore UV-induced G2/M arrest in the PKC δ null MEFs (Figure 21). This suggests that PKC δ cleavage is a critically important event in the enforcement of the G2/M checkpoint after exposure to UV radiation. The dose of UV radiation (30 mJ/cm²) used in these experiments was sufficient to induce caspase 2 and 3 activity, both of which are capable of cleaving PKC δ (unpublished results from Dr. Leonid Sitailo).

4.3 UV-induced G2/M checkpoint activation requires PKC δ

We next examined components of the G2/M checkpoint pathway before and after UV exposure in wild type and PKC δ null MEFs. In agreement with the cell cycle analysis in Figures 14-16, we found that UV exposure induced G2/M checkpoint activation as assessed by increased levels of P~Cdk1(Y15) in wild type but not PKC δ null MEFs (Figure 22). In agreement with the cell cycle data presented in Figure 21, re-expression of PKC δ -GFP, but not either the PKC δ kinase dead or caspase cleavage mutant PKC δ , restored phosphorylation of P~Cdk1(Y15) after UV exposure (Figure 22). This supports the idea that both kinase activity and caspases cleavage of PKC δ are important events in the activation of the G2/M checkpoint after DNA damage.

Despite lacking an intact G2/M checkpoint response, PKC δ null MEFs exhibited UV-induced γ H2A.X and P~p53(S15), both of which are direct substrates of ATM and ATR kinases (Figures 22 and 23). This indicates that ATM/ATR are activated successfully in PKC δ null MEFs, but that there is a disruption in downstream signaling when PKC δ is lost. Successful activation of ATM/ATR in PKC δ null MEFs reinforces the findings in Figure 10, that PKC δ -cat affects the G2/M checkpoint downstream, or independently of ATM and ATR. Furthermore, the treatment of MEFs with caffeine partially repressed the UV-induced phosphorylation of p53(S15), supporting the direct role of ATM and ATR in this phosphorylation event (Figure 23). Please note that the slightly repressed levels of γ H2AX induction after UV in the PKC δ null MEFs were not reproducible between multiple experiments.

In an attempt to identify a known cell cycle regulator that was misregulated in PKC δ null MEFs, we performed immunofluorescent staining for Cdc25A in WT and PKC δ null MEFs (Figure 24). Misregulation of Cdc25A after UV exposure could be one explanation for the inability of PKC δ null MEFs to phosphorylate Cdk1(Y15) in response to DNA damage. However, despite the fact that PKC δ null MEFs lack an intact G2/M DNA damage checkpoint they appeared to regulate Cdc25A in the same way as WT MEFs. In both WT and PKC δ null MEFs, Cdc25A was sequestered in the cytoplasm after UV radiation exposure (Figure 24). The possibility remains that PKC δ may be responsible for regulating a different Cdc25 isoform, as the misregulation of one isoform would be sufficient to block elevated levels of P~Cdk1(Y15) (Varmeh and Manfredi, 2009).

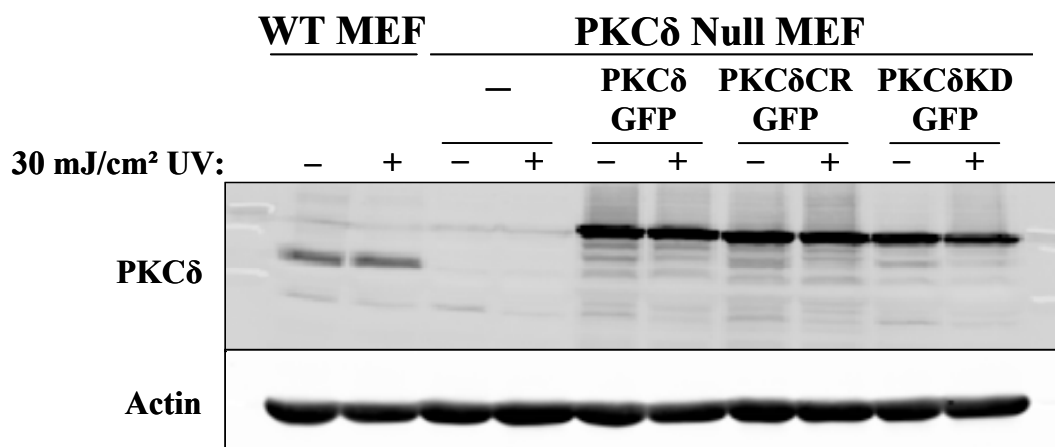


Figure 22—Expression of PKC δ -GFP Fusion Proteins in PKC δ Null MEFs. WT and PKC δ null MEFs were untransduced, or transduced with either WT PKC δ -GFP, the cleavage resistant PKC δ (D327A)-GFP, or the kinase inactive PKC δ (K376R)-GFP and exposed to 30 mJ/cm² UV. Protein lysates were analyzed for expression of PKC δ and Actin.

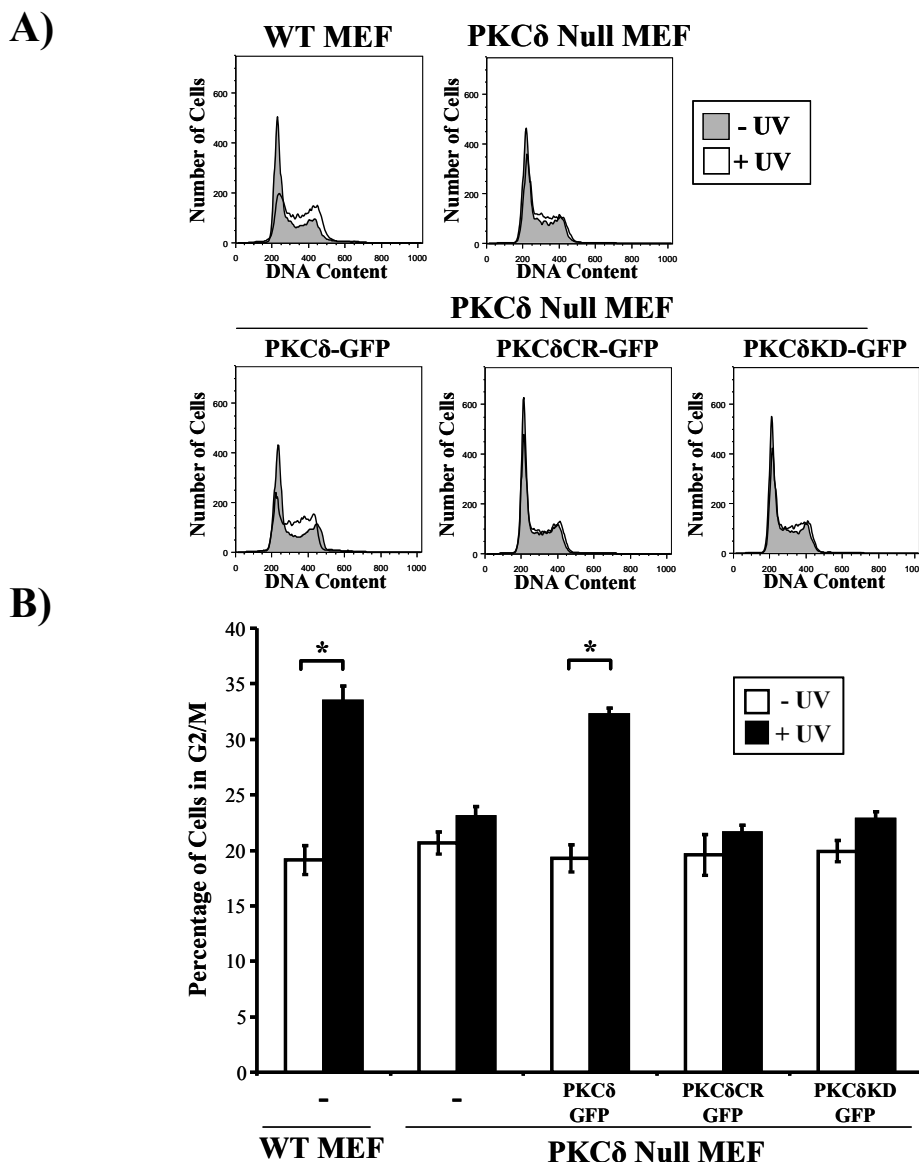


Figure 23—WT PKC δ , but not Caspase Resistant or Kinase Dead PKC δ Rescues G2/M Checkpoint Function in PKC δ Null MEFs. A) Representative DNA content histograms of WT and PKC δ null MEFs before and 18 hrs after exposure to 30 mJ/cm² UV radiation. B) The percentage of WT and PKC δ null MEFs in G2/M after transduction with the indicated PKC δ -GFP fusion protein before and after exposure to 30 mJ/cm² UV radiation is displayed. Error bars denote the standard deviation of the mean from experiments performed in triplicate. * Student's t-test value of p<0.005.

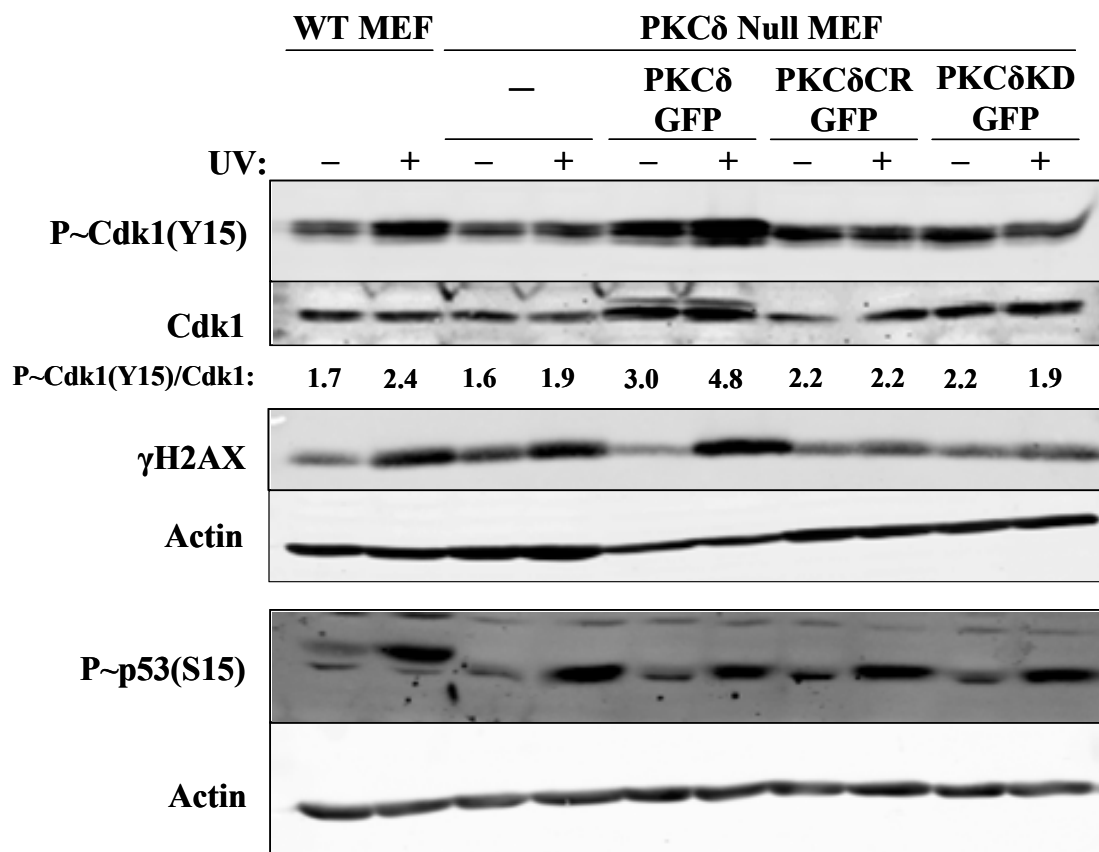


Figure 24—WT PKC δ but not Caspase Resistant or Kinase Dead PKC δ Mutants restores Cdk1(Y15) Phosphorylation After UV. WT and PKC δ null MEFs expressing PKC δ -GFP, PKC δ (D327A)-GFP, or PKC δ (K376R)-GFP were exposed to 10 mJ/cm² UV radiation. Protein lysates were collected 18 hrs after irradiation and analyzed for levels of P~Cdk1(Y15), total Cdk1, γ H2A.X, and Actin. P~Cdk1(Y15)/Cdk1 densitometry ratios are displayed, and were similar in multiple experiments.

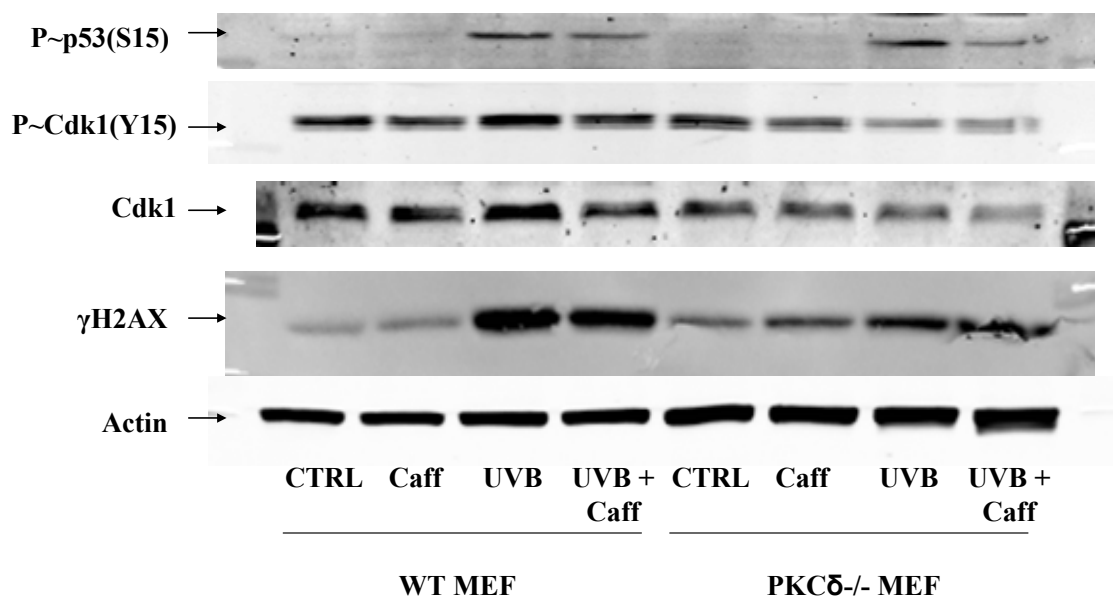
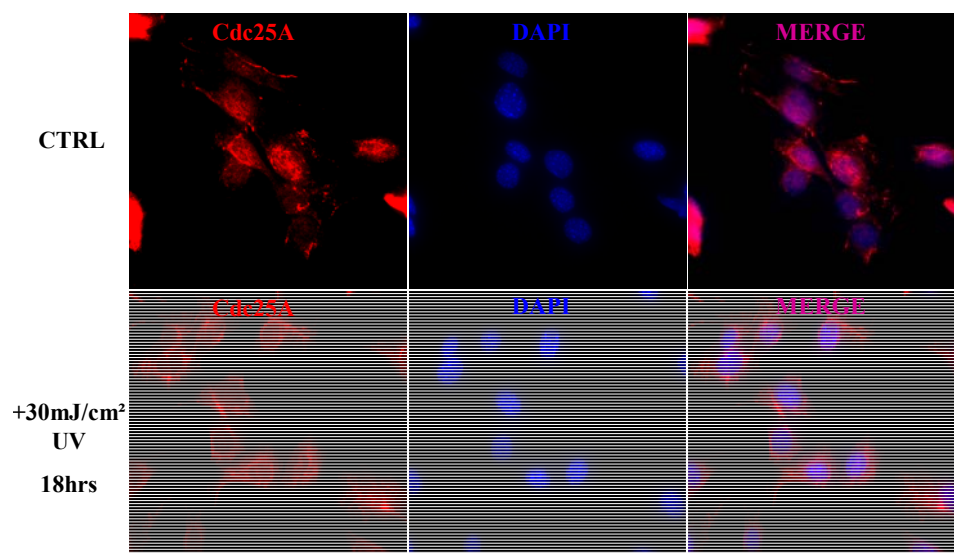


Figure 25—Phosphorylation of Checkpoint Proteins 6hrs after UV Irradiation in WT vs. PKCδ^{-/-} MEFs. WT and PKCδ null MEFs were exposed to 10 mJ/cm² UV radiation and protein lysates were collected after 6 hours for detection of the phosphorylation status of p53, γH2AX, and Cdk1. Note the suppression of P~p53(S15) and γH2AX levels by caffeine, indicating successful inhibition of ATM/ATR activity.

A) Effect of UV Exposure on Cdc25A Levels in Wild Type MEFs



B) Effect of UV Exposure on Cdc25A Levels in PKC δ ^{-/-} MEFs

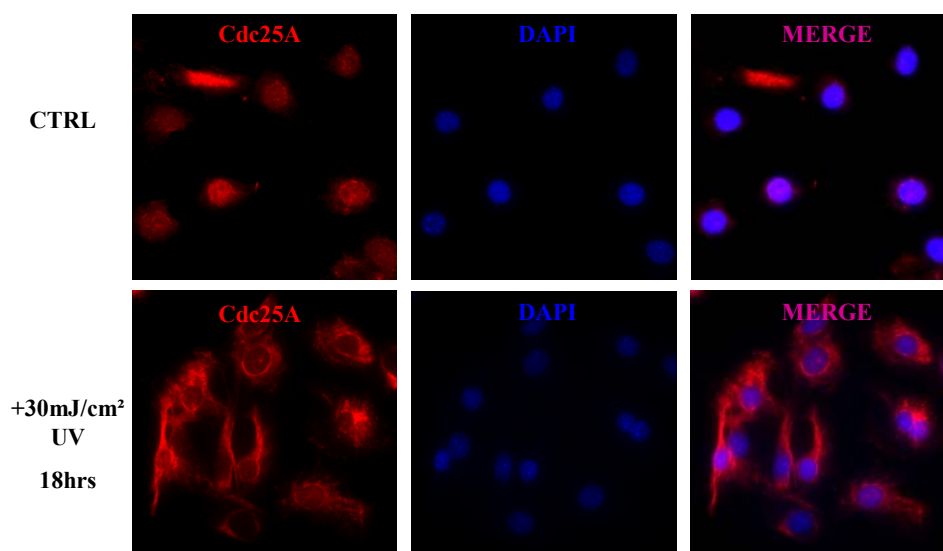


Figure 26 Both Wild Type and PKC δ Null MEFs. A) WT and B) PKC δ null MEFs were exposed to 30 mJ/cm² UV radiation. Cells were fixed and stained for Cdc25A protein 18 hours post UV exposure. Images are taken at 40x magnification using an epifluorescent microscope.

Chapter 5

The PKC δ Catalytic Fragment Induces Phosphorylation of Histone H3(S10)

5.1 PKC δ -cat Induces Histone H3(S10) Phosphorylation in Interphase Cells

While investigating PKC δ -cat-induced G2/M arrest within G2 and mitosis we discovered that while PKC δ -cat expression blocks mitotic entry by stimulating activation of the G2/M checkpoint, it also results in the induction of histone H3(S10) phosphorylation (Figure 25). Massive phosphorylation of Histone H3 Ser 10 and 28 occurs during prophase and is tightly linked with mitotic chromatin condensation, so an increase in P~H3(S10) levels would seem to indicate a mitotic rather than G2 arrest. While this result at first seemed contradictory to our work showing PKC δ -cat induced G2 arrest, it became clear upon further investigation that PKC δ -cat-induced phosphorylation occurred in cells that lacked the characteristic morphological features of mitotic cells (Figure 26). That is, they did not present with mitotic chromatin, they lacked any semblance of a bipolar mitotic spindle apparatus, and they maintained intact nuclear lamina (Figures 26 and 27).

To confirm that PKC δ -cat induced histone H3 phosphorylation outside of mitosis, we utilized a pharmacologic approach to inhibit Aurora B, the kinase responsible for phosphorylating histone H3(S10) during the prophase of mitosis (Giet and Glover, 2001; Goto et al., 2002). Strikingly, while inhibition of Aurora B almost completely abrogated P~H3(S10) levels in the Linker control cells, it caused no significant change in the PKC δ -cat induced P~H3(S10) (Figure 28). To directly determine the cell cycle status of PKC δ -cat induced P~H3(S10) positive cells, we simultaneously measured P~H3(S10) levels and DNA content using propidium iodide. While P~H3(S10) positive cells were almost completely restricted to the G2/M phase in Linker cells, we discovered that PKC δ -cat expression induced a significant percentage of P~H3(S10) positive cells with both interphase and mitotic DNA content (ie. 2N, 4N and intermittent content) (Figure 29). Together, these findings demonstrate that PKC δ -cat induces P~H3(S10) outside of mitosis, and independently of the mitotic H3 kinase, Aurora B.

In addition to mitotic H3 phosphorylation by Aurora B, several other kinases, including IKK α , MSK3 and 6, and Fyn have been implicated as Histone H3 kinases in interphase cells (Duncan et al., 2006; Yamamoto et al., 2003). To investigate whether PKC δ -cat may be directly phosphorylating Histone H3(S10) we decided to test the ability of recombinant PKC δ -cat to phosphorylate histone H3 on Ser10 *in vitro*. We utilized recombinant PKC δ and recombinant active caspase 3 to generate constitutively activated, recombinant PKC δ -cat. We next incubated this reaction with recombinant histone H3 and non-radiolabelled ATP and subsequently analyzed histone H3(S10) phosphorylation by Western blot. Strikingly, PKC δ -cat, and to a lesser extent, full length PKC δ were able to

phosphorylate histone H3 on Ser 10 *in vitro*, raising the possibility that PKC δ may be a histone H3 kinase in the cell (Figure 30). It remains possible that the PKC δ phosphorylation *in vitro* may be an artifact of the reaction conditions and further experiments are needed to verify that PKC δ is a true H3 kinase.

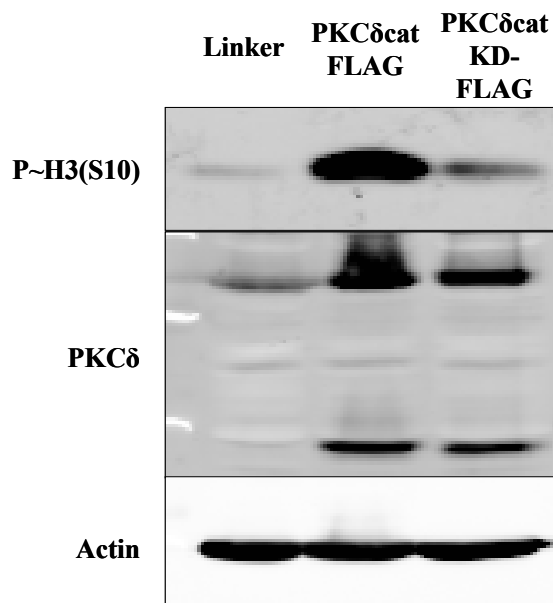


Figure 27– Expression of PKC δ -cat-ER induces phosphorylation of histone H3 on Ser10. Primary keratinocytes were retrovirally transduced with either Linker, PKC δ -cat-FLAG or the kinase dead PKC δ -catKD-FLAG. 1 day post-infection, all cells were treated with 10 μ M 4-hydroxytamoxifen to induce activation of ER fusion proteins. Protein lysates were collected 3 days post-infection and subjected to Western blot analysis of protein levels with the indicated antibodies.

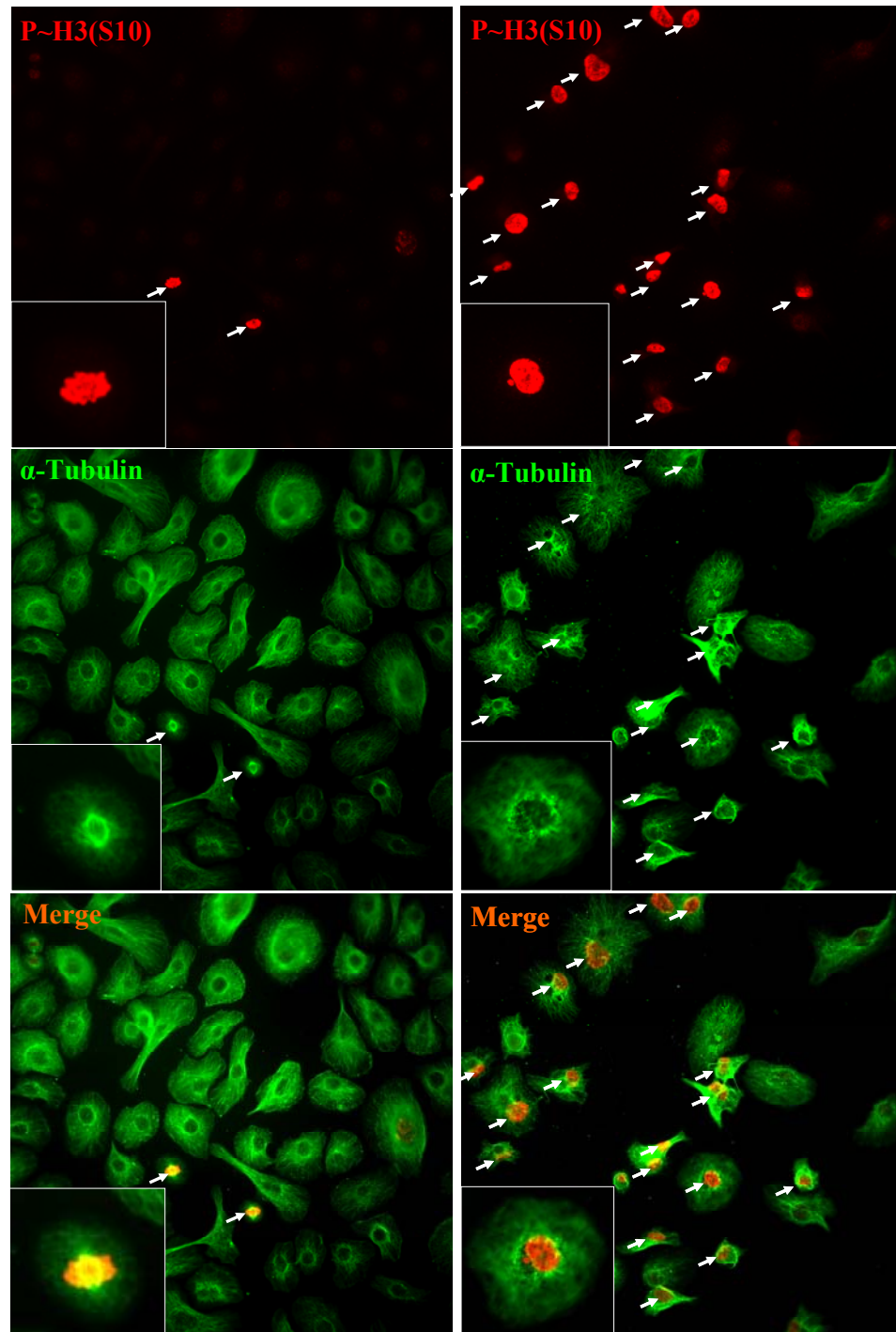


Figure 28– PKC δ -cat-Induced P~H3(S10) Positive Cells Lack Mitotic Morphology. Primary keratinocytes were retrovirally transduced with either Linker, or PKC δ -cat-ER. 1 day post-infection, all cells were treated with 10 μ M 4-OH tamoxifen to induce activation of ER fusion proteins. 3 days post-infection, cells were fixed and stained with the indicated antibodies. Arrows indicate P~H3(S10) positive cells. Pictures taken at 40x magnification. Inlayed images are displayed at 160x.

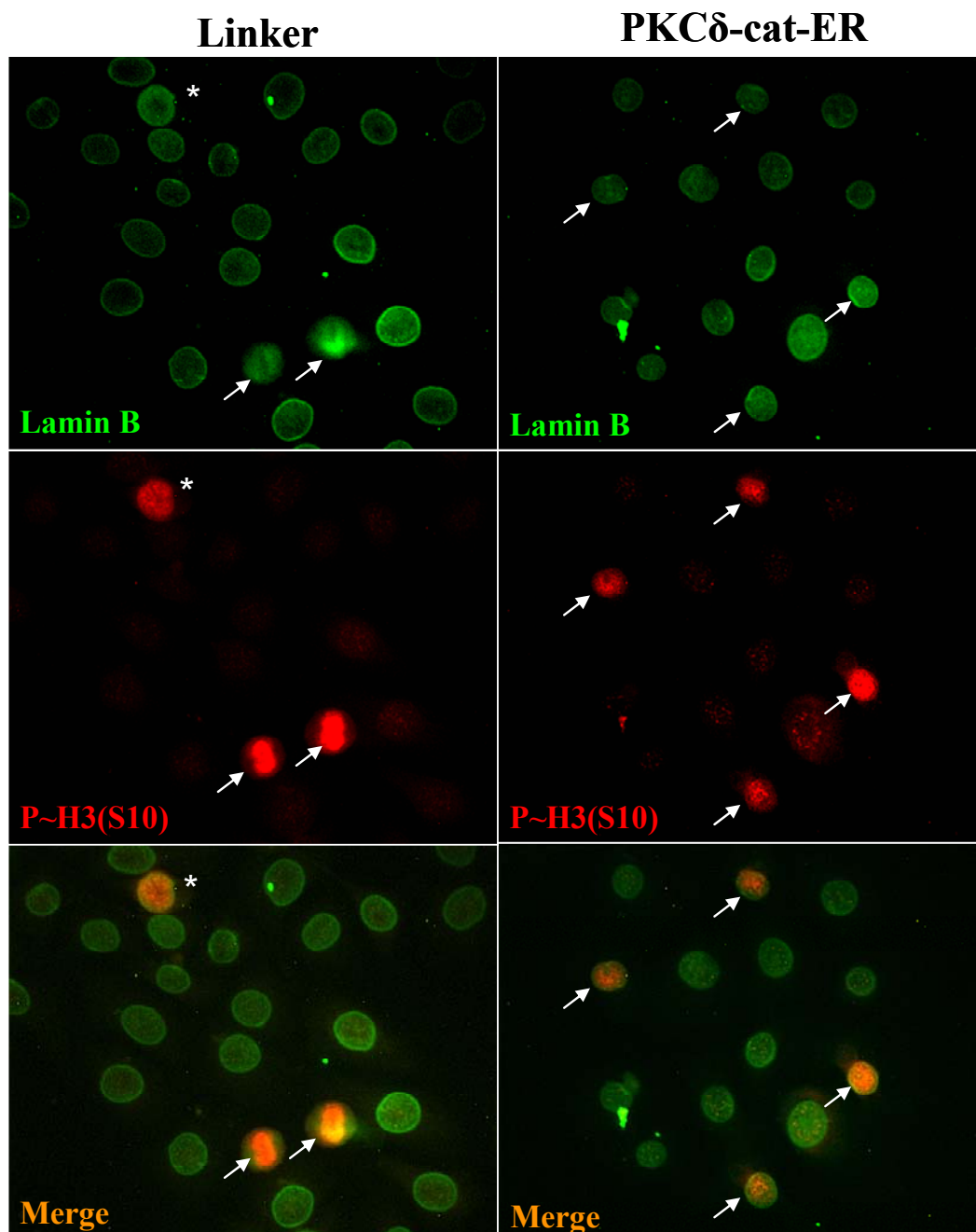


Figure 29– PKC δ -cat Induced P~H3(S10) Positive Cells Have Intact Nuclear Envelopes. Linker and PKC δ -cat-ER infected keratinocytes were treated with 10 μ M 4-hydroxytamoxifen. Cells were fixed 3 days after infection and stained with anti-P~H3(S10) and anti-Lamin B2. Arrows indicate P~H3(S10) positive nuclei. Note the intact nuclear envelope in PKC δ -cat-ER induced P~H3(S10) positive cells. * represents a P~H3(S10) positive Linker infected cell with an intact nuclear envelope that is likely in prophase, prior to nuclear envelope breakdown. Images were taken at 40x magnification.

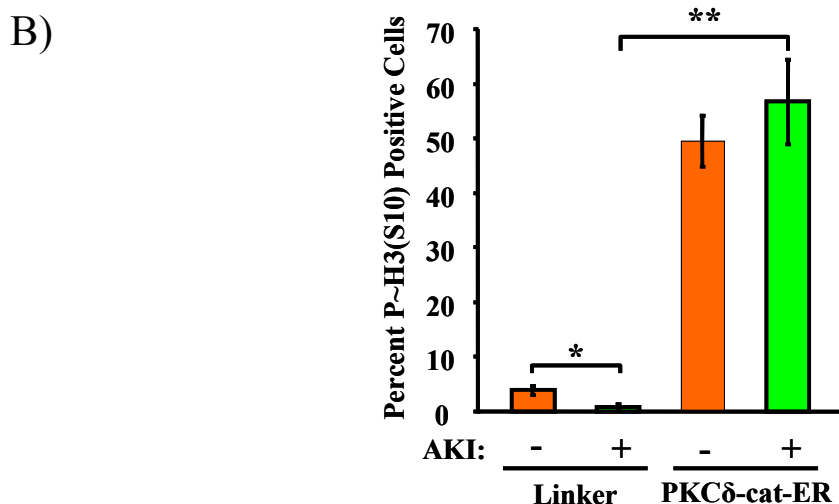
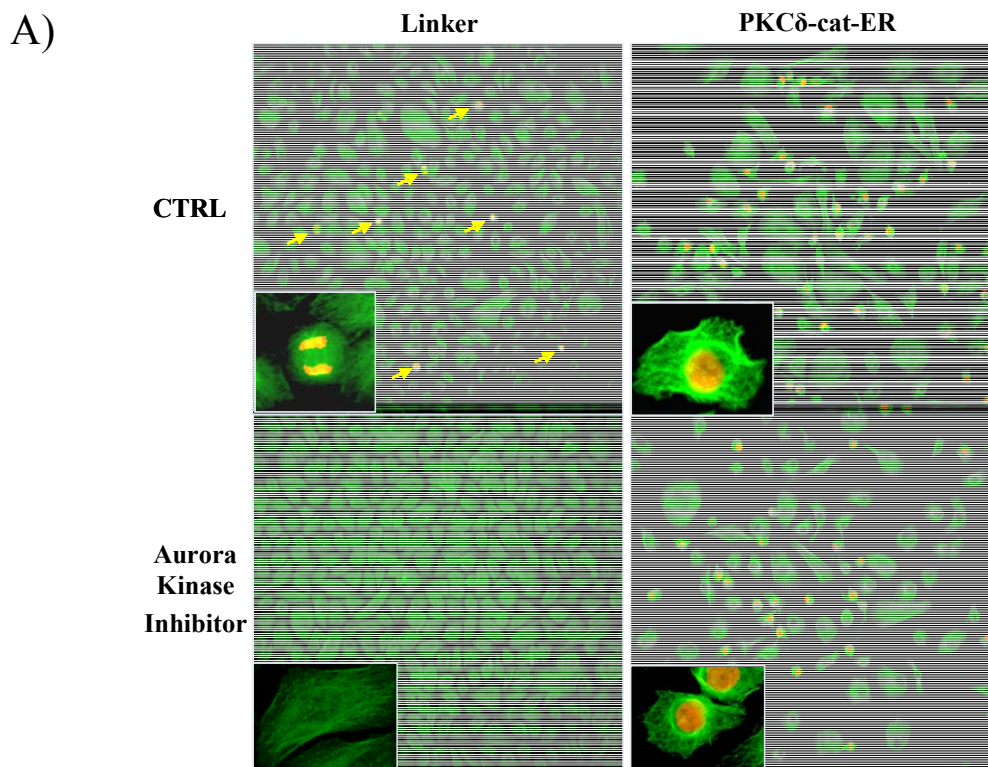
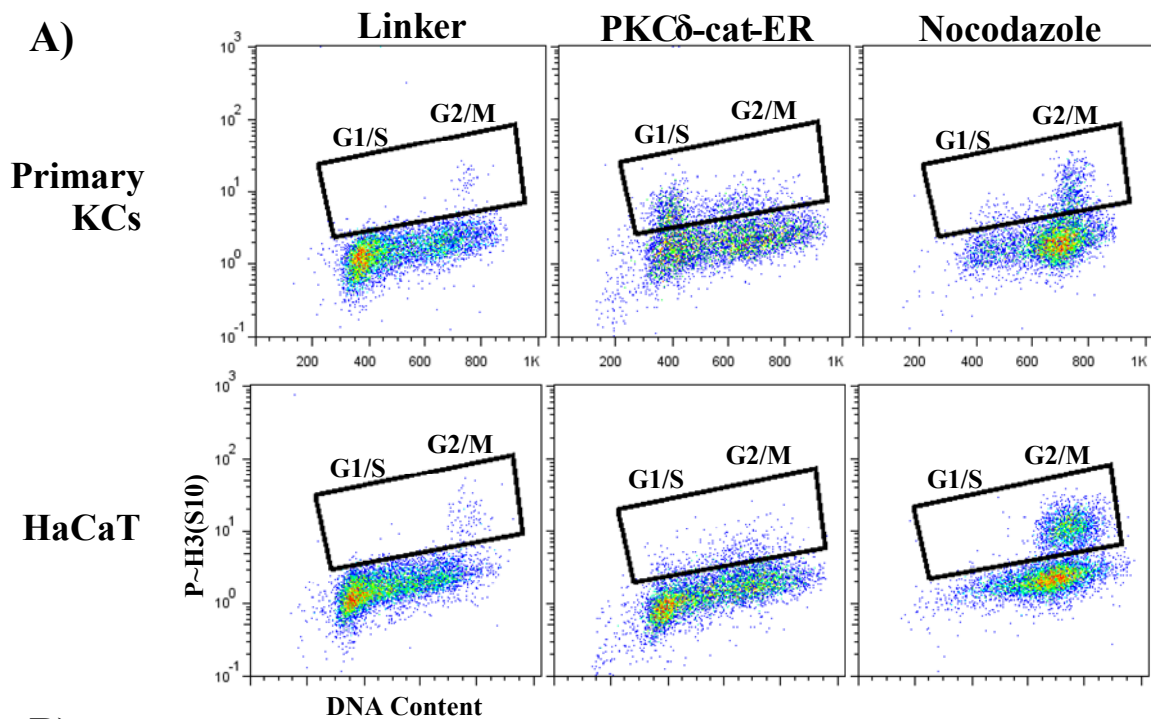


Figure 30– Inhibition of Aurora Kinase does not Block PKC δ -cat Induction of P~H3(S10). Linker and PKC δ -cat-ER infected keratinocytes were treated with 10 μ M 4-hydroxytamoxifen with or without 10 μ M Aurora Kinase inhibitor. Cells were fixed 3 days after infection and stained with anti-P~H3(S10) and anti- α -tubulin. Pictures displayed at 20x magnification. A) Representative images showing the failure of AKI treatment to prevent PKC δ -cat induced P~H3(S10). B). Percentage of P~H3(S10) positive cells per 20x field. 3 fields per experimental group were counted in blinded manner. * Student's t-test value of $p < 0.05$. ** Student's t-test value of $p < 0.01$



B)

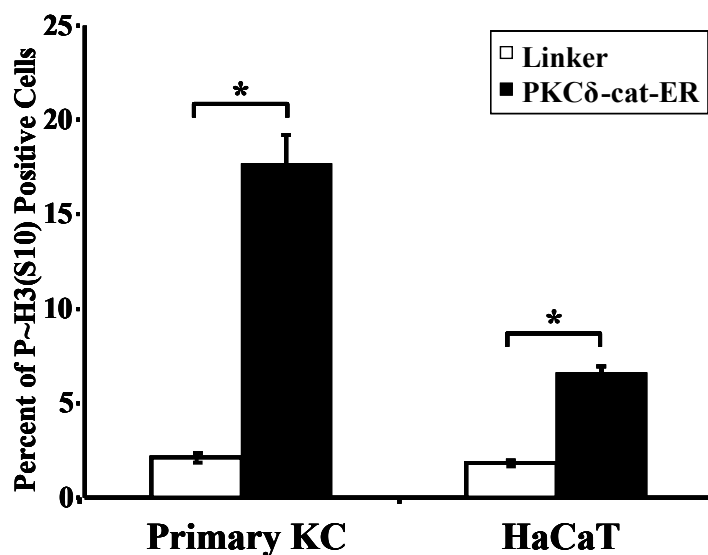


Figure 31– PKC δ -cat-Induced P~H3(S10) Positive Cells Have Variable DNA Content. Primary keratinocytes were retrovirally transduced with either Linker, or PKC δ -cat-ER. Cells were treated with 10 μ M 4-hydroxytamoxifen on day 1 and collected for staining on day 3. Cells were stained with anti-P~H3(S10) and propidium iodide for flow cytometry analysis. A) representative histograms from 3 separate experiments. B) Error bars denote the standard deviation of the mean from experiments performed in triplicate. * Student's t-test value of $p < 0.005$.

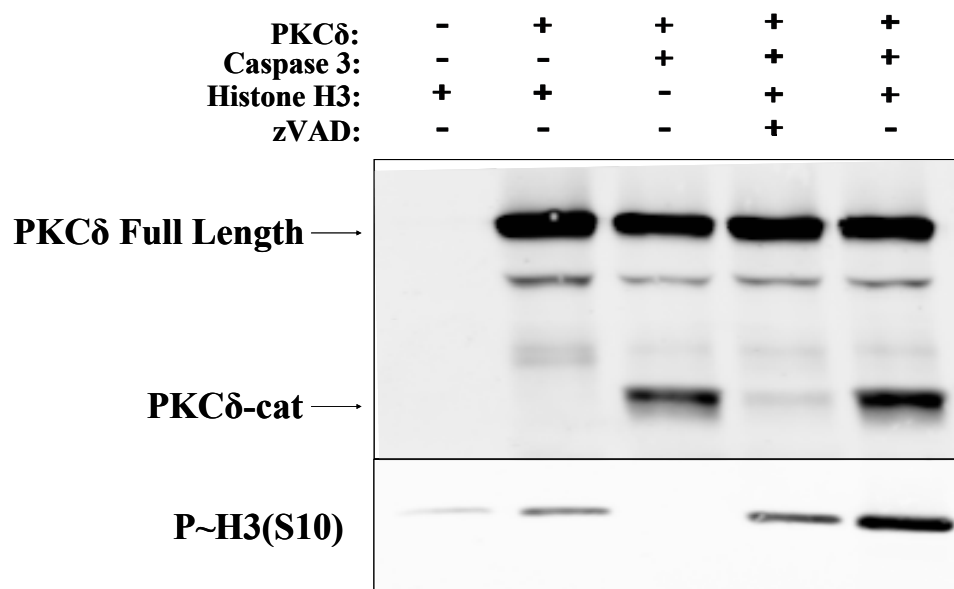


Figure 32– PKC δ -cat Phosphorylates Histone H3 (S10) *in vitro*. Recombinant PKC δ -cat was generated by incubation with recombinant activated caspase 3. Reactions were then incubated with recombinant histone H3 and ATP. Proteins from the reactions were subjected to Western blotting to measure the cleavage of PKC δ and phosphorylation of Histone H3 on Ser10 using anti-P~H3(S10) monoclonal antibody.

5.2 PKC δ is Required for UV Radiation-Induced Phosphorylation of Histone H3

Since we had determined that PKC δ -cat induced P~H3(S10) in interphase cells, and that PKC δ could directly phosphorylate histone H3 *in vitro*, we next addressed the question of whether PKC δ was required for the phosphorylation of histone H3. Since it has been previously reported that UV radiation can induce the phosphorylation of histone H3, and UV radiation is known to activate PKC δ , we decided on a model of UV-induced histone H3 phosphorylation to test the requirement for PKC δ (He et al., 2005b). As in our cell cycle studies described in Chapter 4, we utilized WT and PKC δ null MEFs for our PKC δ loss of function studies with regards to histone H3 phosphorylation.

As expected, UV-irradiation of WT MEFs resulted in elevated levels of P~H3(S10) as measured both by Western blot, and P~H3(S10) FACS analysis (Figure 31 and 32). Furthermore, the UV induced phosphorylation occurred in cells across the cell cycle. Strikingly, PKC δ null MEFs did not demonstrate an increase in phosphorylated histone H3 after exposure to UV radiation, suggesting that PKC δ is required for this event (Figure 31 and 32).

Similarly, treatment of HaCaT cells with PKC δ siRNA not only protected the cells from UV-induced apoptosis, but also resulted in decreased levels of P~H3(S10) before and after UV radiation, suggesting that the findings in the MEF system can be applied to human cells (Figure 33). Please note that in this experiment, the cells were not subjected to growth factor withdrawal, resulting in an elevated level of baseline P~H3(S10) prior to

UV due to the high percentage of cells within mitosis at the time of the assay. This results in an apparent decrease in P~H3(S10) levels after UV radiation exposure, and this decrease can likely be explained by a UV-induced decrease in mitotic index.

5.3 UV Radiation Exposure Induces Nuclear Foci Containing PKC δ /P~H3(S10)

The discovery that, at least *in vitro*, PKC δ -cat was capable of directly phosphorylating histone H3(S10) led us to investigate whether any co-localization between PKC δ and phosphorylated H3 could be detected after exposure of HaCaT cells to UV radiation. To visualize PKC δ protein, we retrovirally transduced PKC δ -GFP into HaCaT cells. In control HaCaT cells, PKC δ -GFP was detected in both the cytoplasm and the nucleus, however, the localization pattern was diffuse in both compartments (Figure 34).

Following exposure to UV, nuclear PKC δ -GFP localization shifted to a much more punctate pattern. Furthermore, the PKC δ -GFP foci that formed following UV exposure appeared to co-localize with regions of intense DAPI staining, suggesting that PKC δ -GFP was recruited to condensed chromatin following UV-irradiation.

We also investigated P~H3(S10) staining patterns before and after cells were exposed to 30 mJ/cm² UV radiation. In control HaCaT cells, significant histone H3 phosphorylation was only detected in mitotic cell populations, and no noticeable co-localization between P~H3(S10) and PKC δ -GFP was observed (Figure 35). In contrast, HaCaT cells exposed to UV radiation had an overall higher level of P~H3(S10). Furthermore, the P~H3(S10) staining indicated that the phosphorylation of histone H3 was occurring on distinct sub-nuclear foci, and these foci co-localized with regions that also had elevated levels of

PKC δ -GFP (Figure 35B). This finding demonstrates that following UV radiation exposure, PKC δ is in the proper location to phosphorylate histone H3(S10).

We considered the possibility that following UV-irradiation, PKC δ might co-localize with proteins that have more clearly defined roles in the DNA damage response. By establishing additional patterns of co-localization, we may be able to develop a better understanding for the role of histone H3 phosphorylation in the response to UV radiation. With this in mind, we decided to investigate two well characterized markers of the DNA repair, Rad9 and γ H2AX. Strikingly, both were induced by UV-radiation exposure, however, in contrast to the P~H3(S10) staining after UV, there appeared to be no obvious co-localization with PKC δ nuclear foci (Figures 36 and 37).

5.4 Are There Structural Changes to P~H3(S10) Associated Chromatin?

Histone H3 phosphorylation on Ser 10 and 28 is tightly linked to chromatin condensation during mitosis. It is also well known that many nuclear changes, including chromatin condensation, occur during mitosis, but the mechanism of apoptotic chromatin condensation is not well understood. Given the role of histone H3 phosphorylation during mitosis, we questioned whether there may be an analogous role for this phosphorylation event during apoptotic chromatin condensation. To study this hypothesis, we generated chromosome spreads from Linker and PKC δ -cat-ER expressing primary keratinocytes. We stained these spreads with DAPI and anti-P~H3(S10) to visualize the chromatin architecture of PKC δ -cat induced P~H3(S10) positive cells. However, despite the fact

that PKC δ -cat induced P~H3(S10) positive cells lacked mitotic chromosomes, they could not be distinguished from normal interphase nuclei, suggesting that there were no detectable gross changes to chromatin structure in these cells (Figure 38). This finding does not rule out the possibility PKC δ induced P~H3(S10) causes structural changes to the chromatin, and may simply be a reflection of the limitations of this assay to detect changes to chromatin architecture.

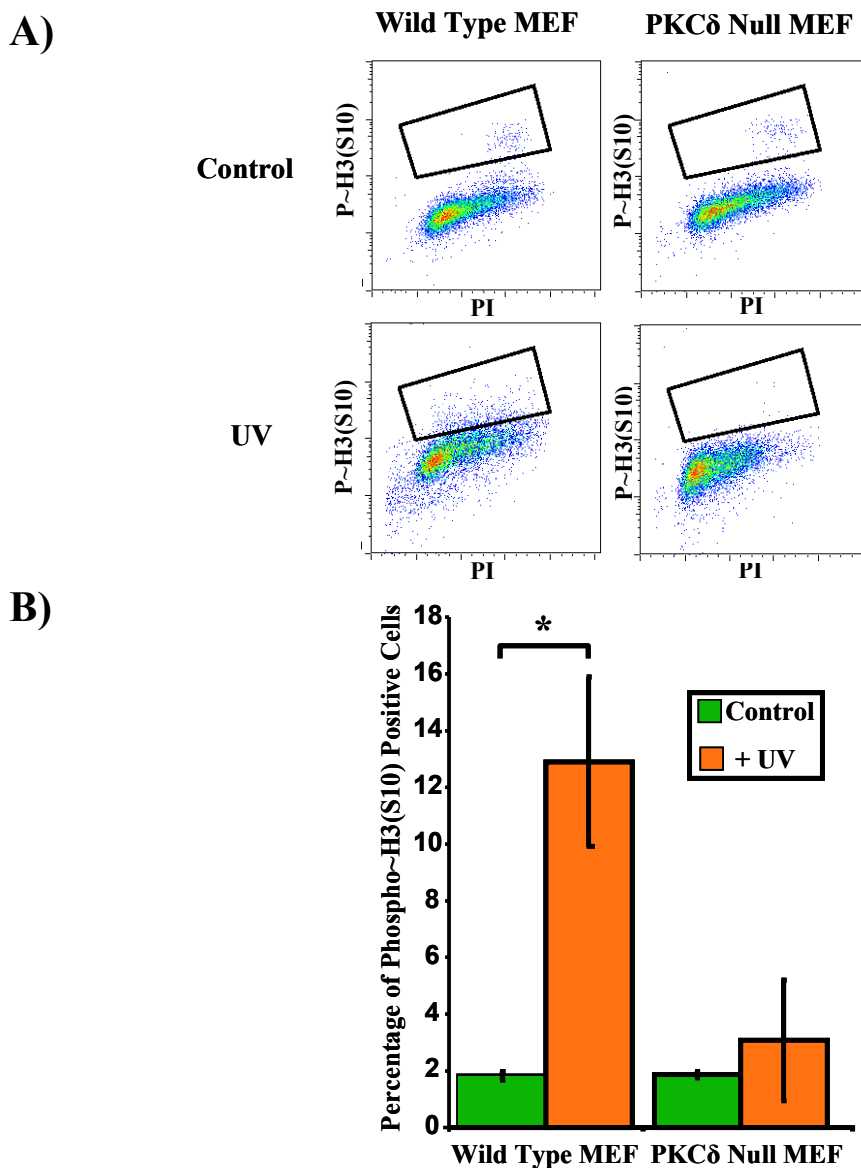


Figure 33– PKC δ is Required for the UV-Induced Phosphorylation of Histone H3(S10). WT and PKC δ Null MEFs were treated with or without 30mJ/cm² UV radiation. Cells were collected 18hrs post-irradiation, fixed, and stained with anti-P~H3(S10) and propidium iodide. A) Representative histograms showing UV-induced P~H3(S10) in WT but not PKC δ null MEFs. B) Error bars denote the standard deviation of the mean from experiments performed in triplicate. * Student's t-test value of $p < 0.005$.

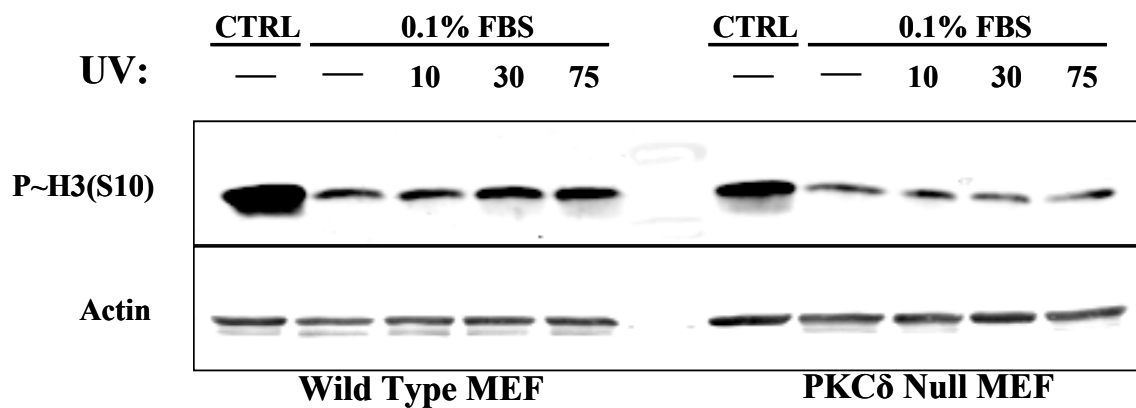
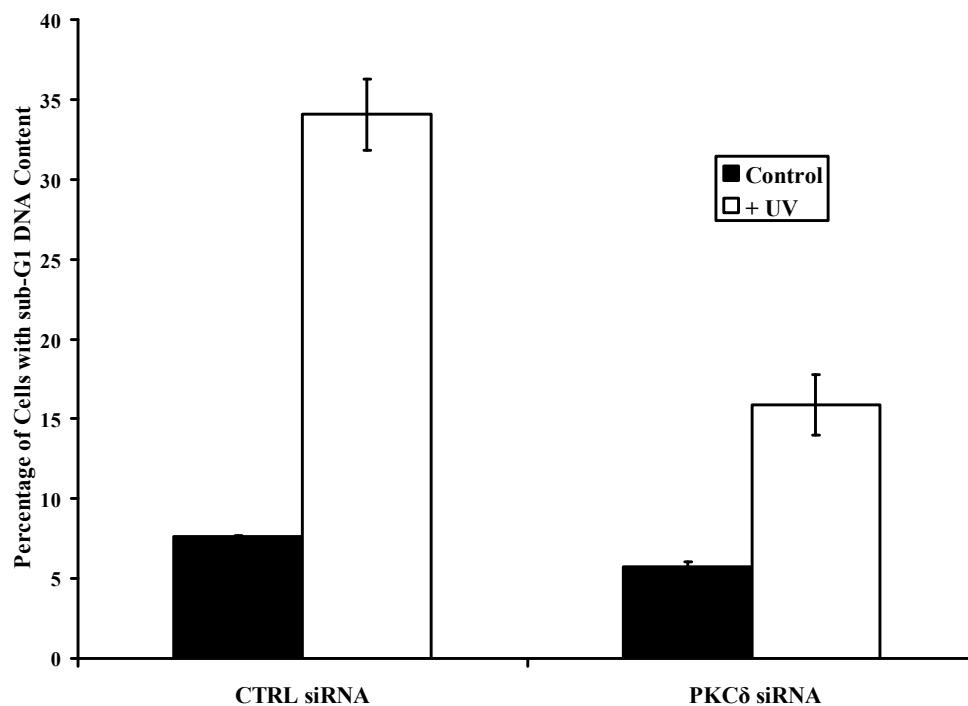


Figure 34– PKC δ is Required for the UV-Induced Phosphorylation of Histone H3(S10). WT and PKC δ Null MEFs were grown in DMEM plus 0.1% FBS to induce G1 growth arrest for 48hrs. Cells were then treated with or without 30mJ/cm² UV radiation. Protein lysates were collected 18hrs post-irradiation and subjected to Western blot analysis to measure levels of P~H3(S10)

A)



B)

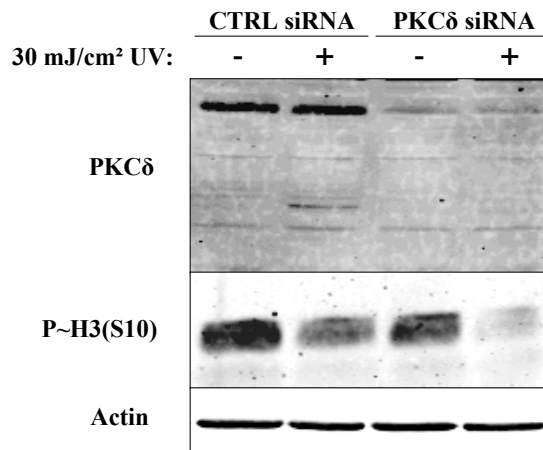


Figure 35—Knockdown of PKCδ Suppresses Apoptosis and UV-Induced Phosphorylation of Histone H3(S10) in HaCaT Cells. HaCaT cells were transfected with CTRL or PKCδ siRNA. 48 hrs post-transfection, cells were treated with or without UV. 18 hrs post-UV-irradiation A) cells were collected for FACS analysis of apoptosis, and B) protein lysates were gathered and subjected to Western blot analysis of PKCδ and P~H3(S10) levels.

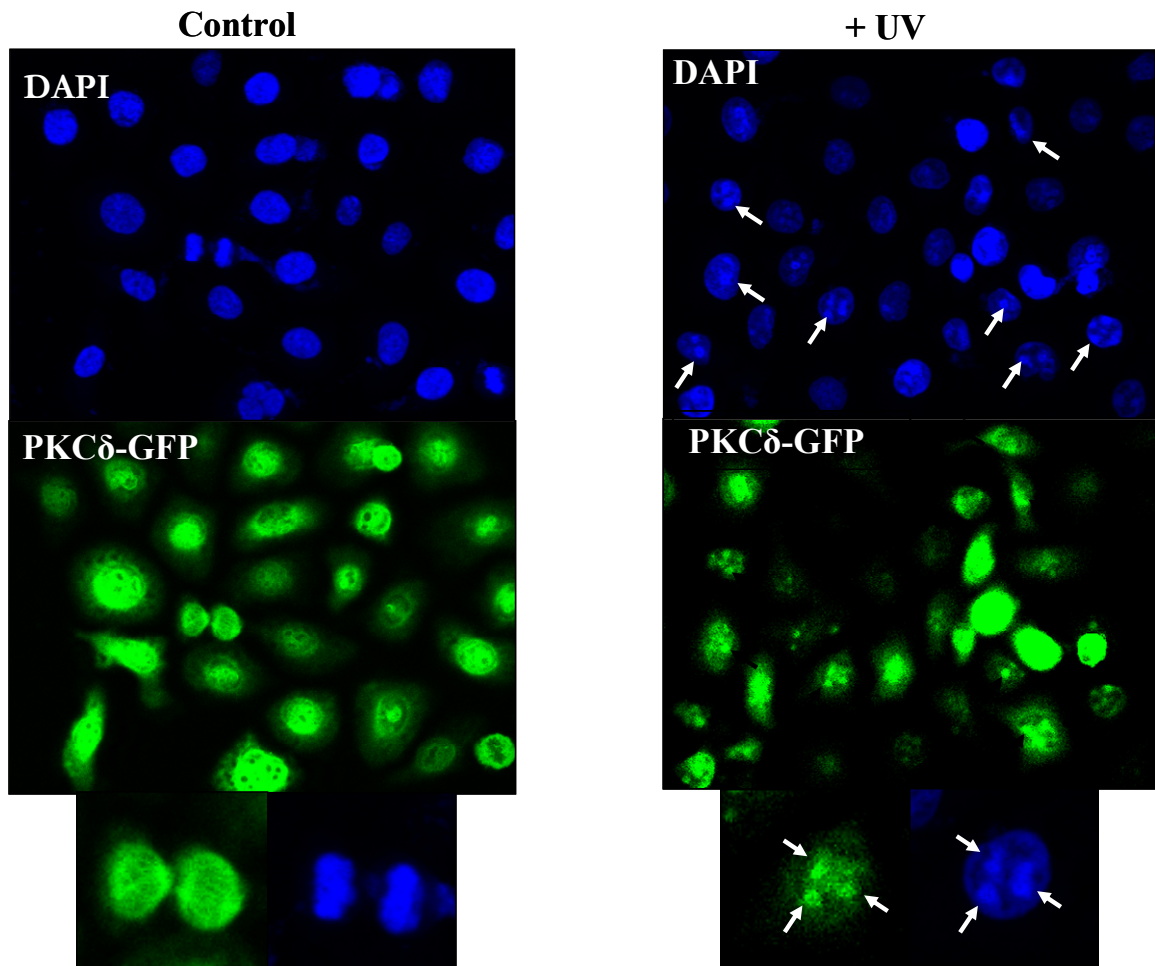
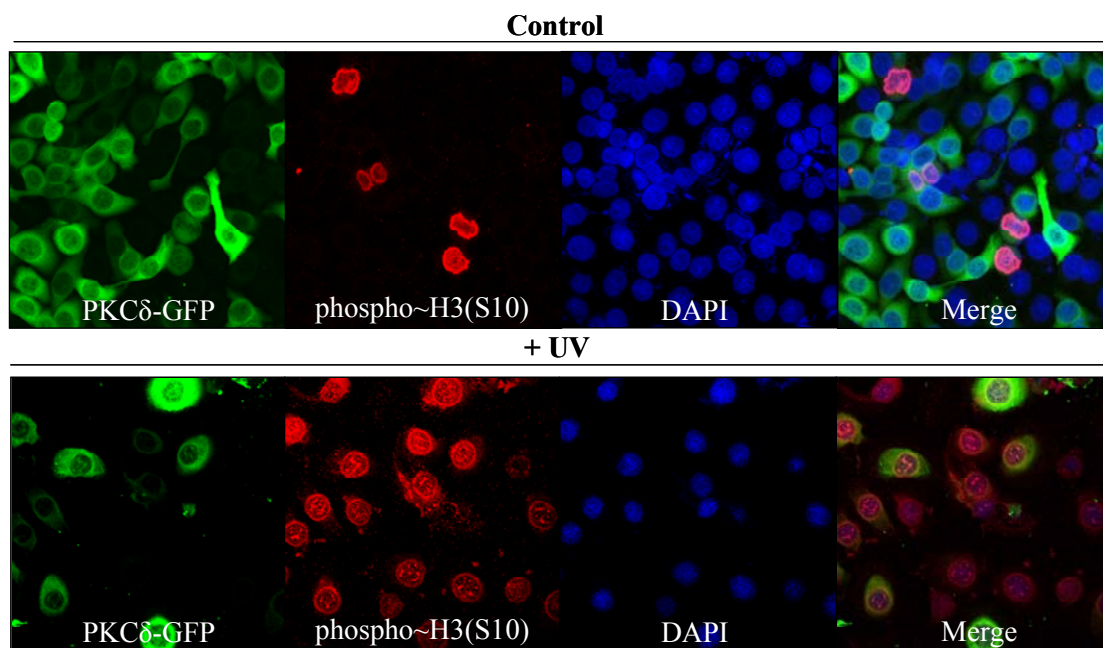


Figure 36—UV Induces PKC δ Recruitment to Nuclear Foci. HaCaT cells were retrovirally transduced to express PKC δ -GFP. Following infection cells were treated with or without 30 mJ/cm² UV radiation. Cells were fixed 18hrs post irradiation, stained with DAPI to visualize chromatin, and photographed using a confocal microscope. Images were taken at 40x magnification.

A)



B)

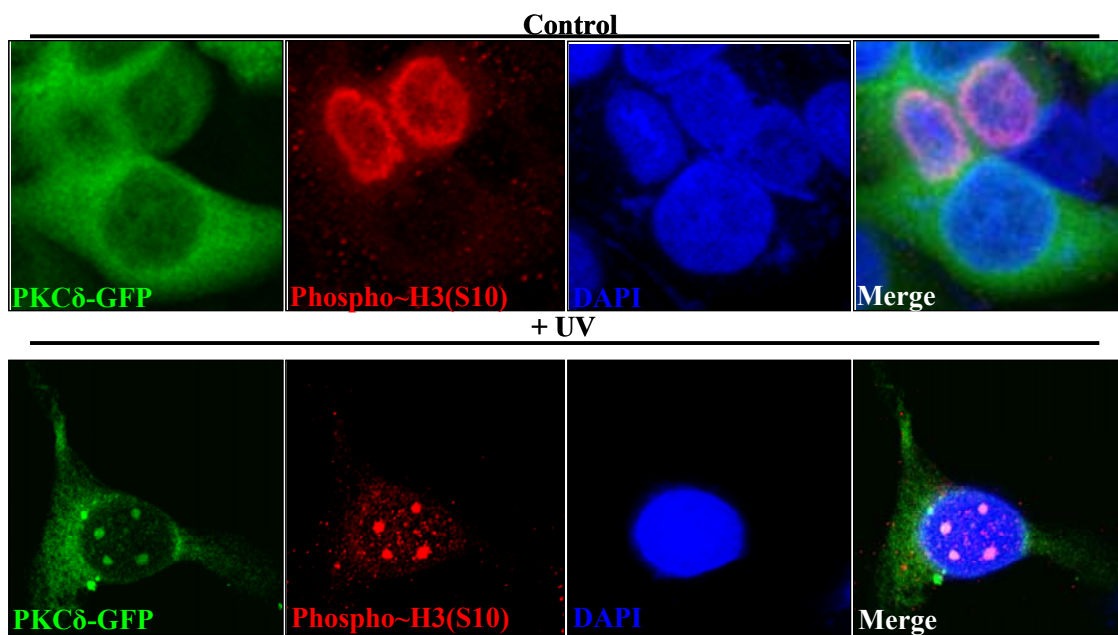


Figure 37—UV-Induced Co-localization of PKC δ with P-H3(S10). HaCaT cells were retrovirally transduced to express PKC δ -GFP. Following infection cells were treated with or without 30 mJ/cm² UV radiation. Cells were fixed 18hrs post irradiation, stained with anti-P-H3(S10) and DAPI and photographed using a confocal microscope. A) 40x magnification. B) 160x magnification.

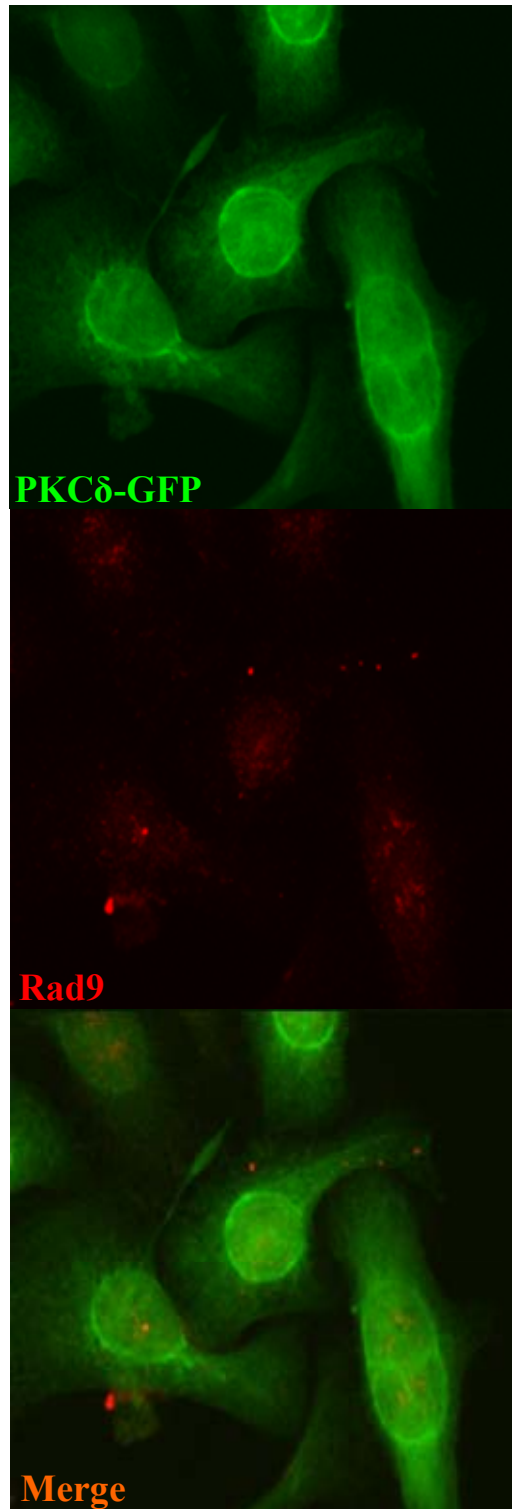


Figure 38A—Control HaCaT-PKCδ-GFP cells display diffuse PKCδ-GFP nuclear localization, and weak Rad9 staining. HaCaT cells expressing PKCδ-GFP were plated on coverslips, fixed and stained for Rad9 and DAPI. Images are displayed at 80x magnification.

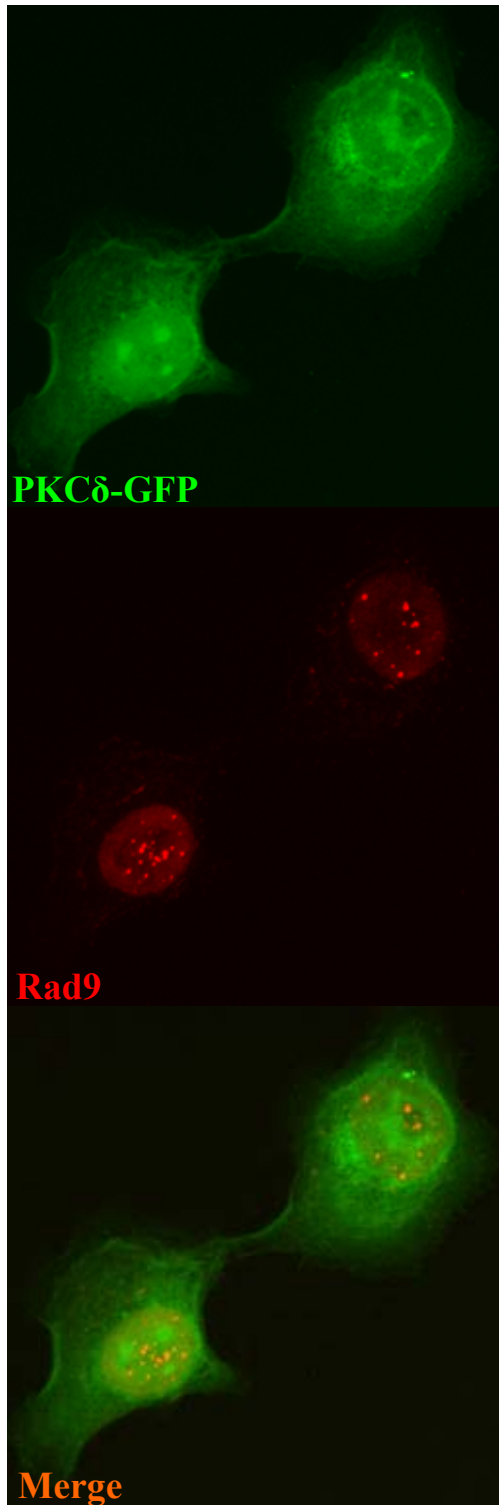


Figure 38B—UV-Induced Rad9 foci do not colocalize with PKCδ-GFP HaCaT cells expressing PKCδ-GFP were plated on coverslips, exposed to 30 mJ/cm² UV radiation, fixed 18 hrs post UV, and stained for Rad9 and DAPI. Images are displayed at 80x magnification.

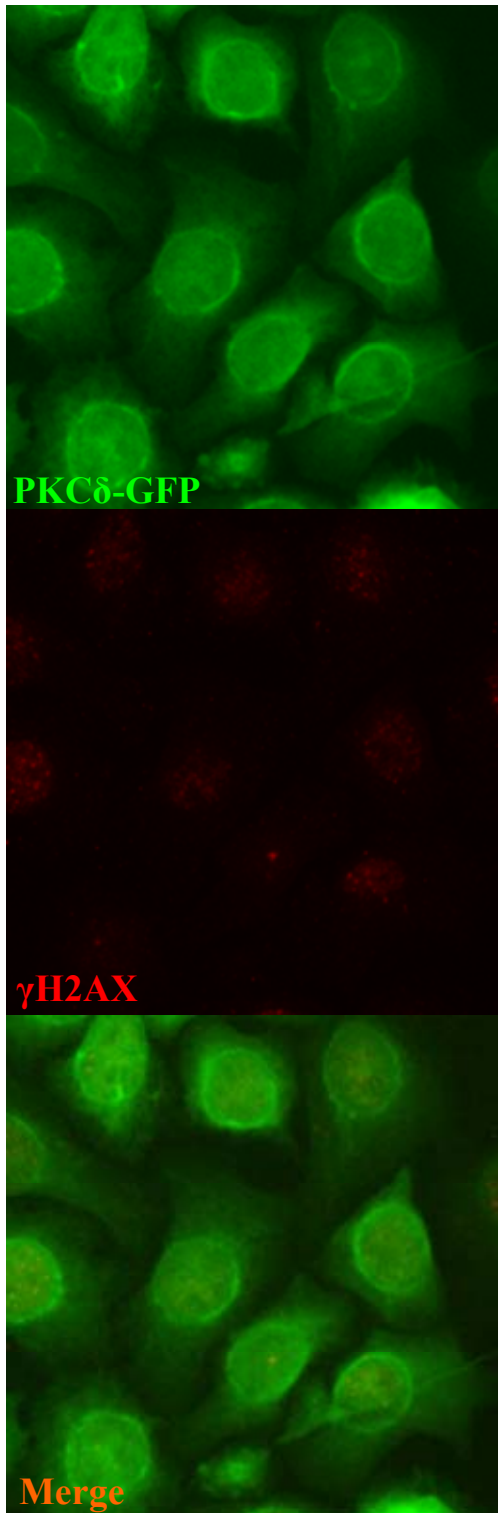


Figure 39A—Control HaCaT-PKCδ-GFP cells display diffuse PKCδ-GFP nuclear localization, and weak γH2AX staining. HaCaT cells expressing PKCδ-GFP were plated on coverslips, fixed and stained for γH2AX and DAPI. Images are displayed at 80x magnification.

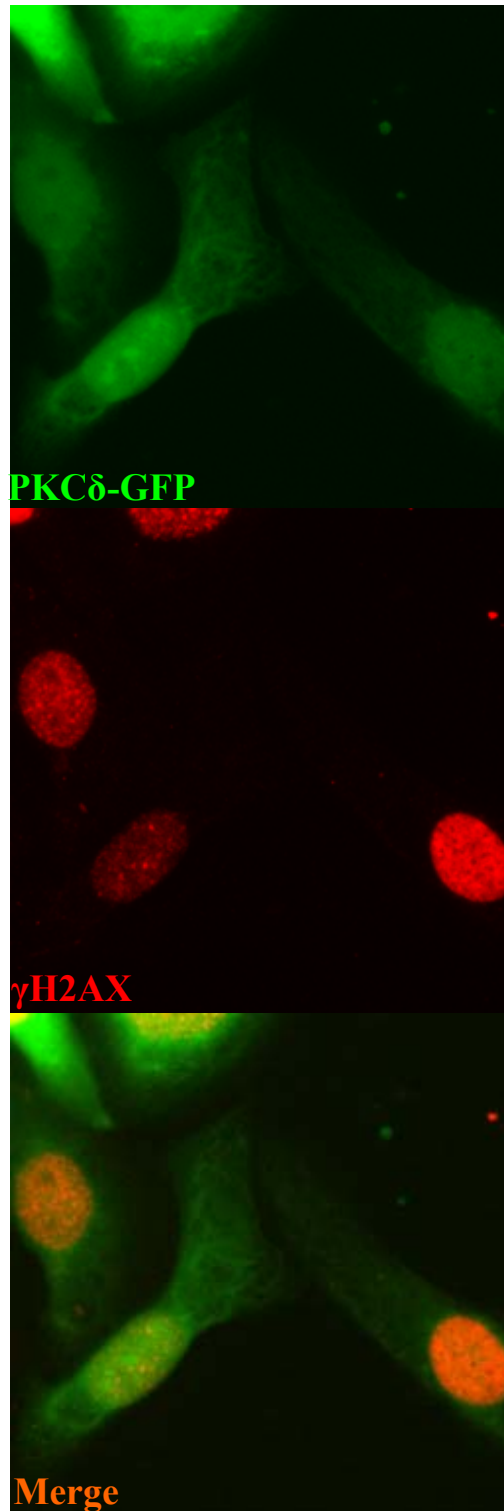


Figure 39B—UV-induced γ H2AX foci do not colocalize with PKC δ -GFP.
HaCaT cells expressing PKC δ -GFP were plated on coverslips, exposed to 30 mJ/cm² UV radiation, fixed 18 hrs post UV, and stained for γ H2AX and DAPI. Images are displayed at 80x magnification.

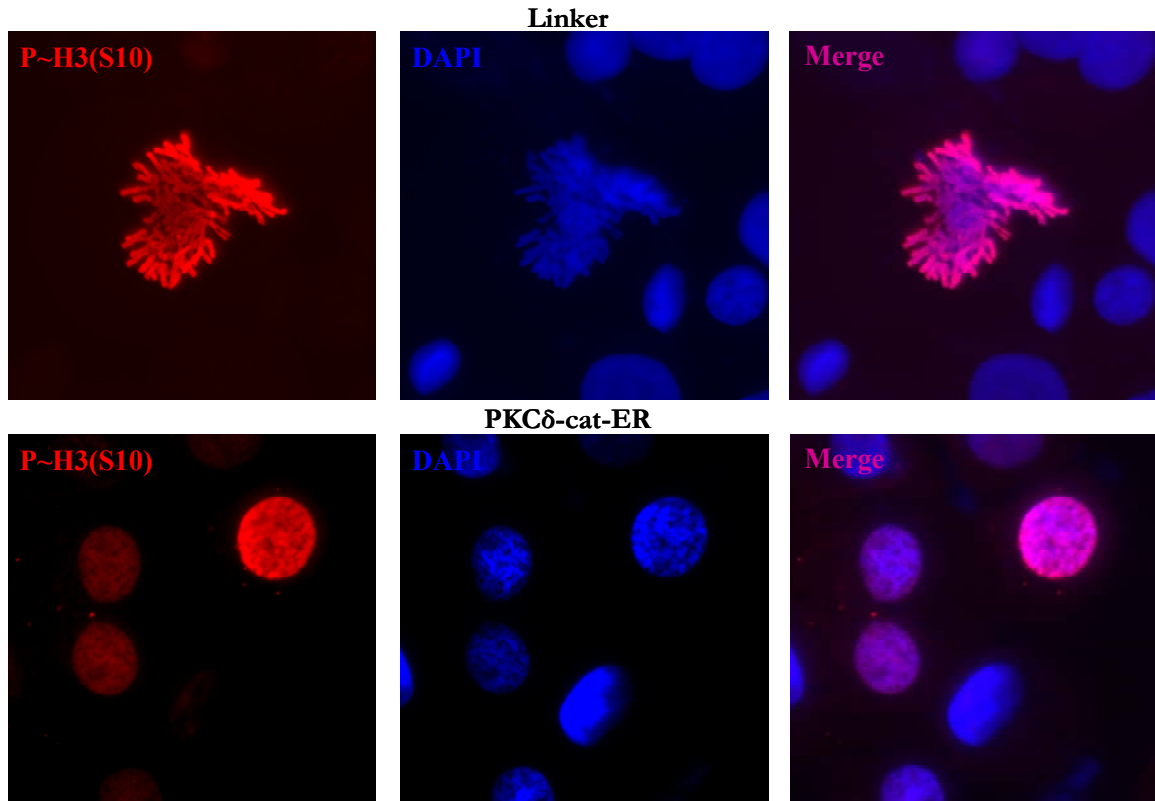


Figure 40—PKCδ-cat-ER Induced P~H3(S10) Positive Nuclei do not show Obvious Alterations to Chromatin Morphology. Primary Keratinocytes expressing either Linker or PKCδ-cat-ER were treated with 4-hydroxytamoxifen, fixed and chromosomal morphology was analyzed using chromosome spreading technique.

Chapter 6

Other Findings of Note

6.1 PKC δ is Required for Efficient Repair of UV-Induced Thymine Dimers

It has been previously reported that PKC δ is important for the DNA damage-induced activation of the DNA repair/cell cycle checkpoint protein Rad9 (Yoshida et al., 2003). We postulated that if PKC δ is important for the activation of critical components of the DNA repair machinery, then PKC δ loss may impair the cells ability to repair damaged DNA. This would be particularly deleterious in the case of the epidermis, since the exposure to UV radiation from the sun is known to be highly mutagenic. To investigate the role, if any, of PKC δ in the repair of UV damaged DNA, we exposed WT and PKC δ null MEFs to 75 mJ/cm² UV radiation. We then used a Southwestern blotting technique to directly measure thymine dimer levels over time, since thymine dimer formation is a key step in the mutagenic effects of UV. The successful repair of UV damaged DNA should correlate with decreased thymine dimer levels. We found that compared to the WT MEFs, PKC δ null MEFs were impaired in their ability to remove thymine dimers following UV exposure (Figure 39). This suggests that PKC δ has an important function in promoting the repair of UV damaged DNA, although whether this role is connected to the ability of PKC δ to promote cell cycle arrest remains to be determined.

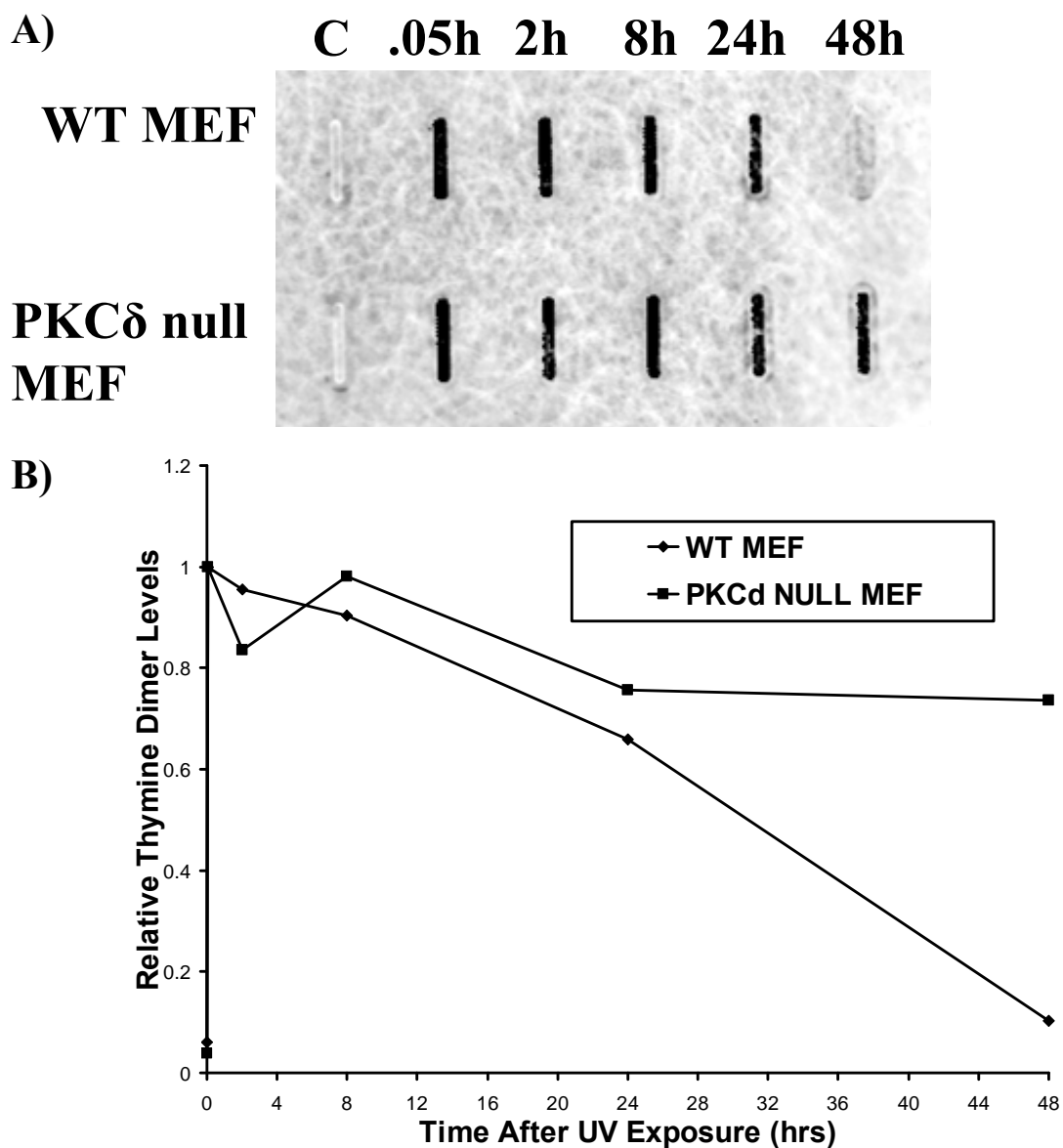


Figure 41—Delayed Removal of Thymine Dimers in PKC δ Null MEFs. WT and PKC δ null MEFs were exposed to 75 mJ/cm² UV radiation and DNA was collected at the indicated timepoints and subjected to Southwestern blot analysis of thymine dimer levels. A) Thymine dimer southwestern blot showing thymine dimer levels at indicated time points post-UV irradiation. B) Densitometric analysis of bands in A shows delayed removal of thymine dimers in PKC δ null MEFs.

6.2 PKC δ Suppresses Colony Formation and Anchorage Independent Growth

A common *in vitro* measure of tumorigenicity is the ability of cells to evade contact inhibition and form foci after growth to confluency, and the ability of cells to grow in an anchorage-independent manner (ie. in soft agar or methylcellulose). Since PKC δ can suppress proliferation by inducing cell cycle arrest, and apoptosis, we sought to determine whether the loss of PKC δ would enhance these characteristics in PKC δ null MEFs compared to their WT counterparts. Strikingly, while WT MEFs underwent a sustained confluency induced growth arrest, PKC δ null MEFs formed numerous foci that were clearly visible after crystal violet staining (Figure 40). This suggests that the loss of PKC δ allows MEFs to escape from contact inhibition, allowing them to continue to proliferate, even when grown under confluent conditions.

When grown in soft agar, WT MEFs formed no detectable colonies. In contrast, PKC δ null MEFs formed numerous colonies detectable by MTT staining, and the ability to grow in soft agar could be re-expressed by ectopically expressing PKC δ -GFP (Figure 41). The ability of PKC δ -GFP expression to suppress growth suggests that the ability to grow in soft agar was a direct consequence of PKC δ loss, and not an artifact of the MEF immortalization process.

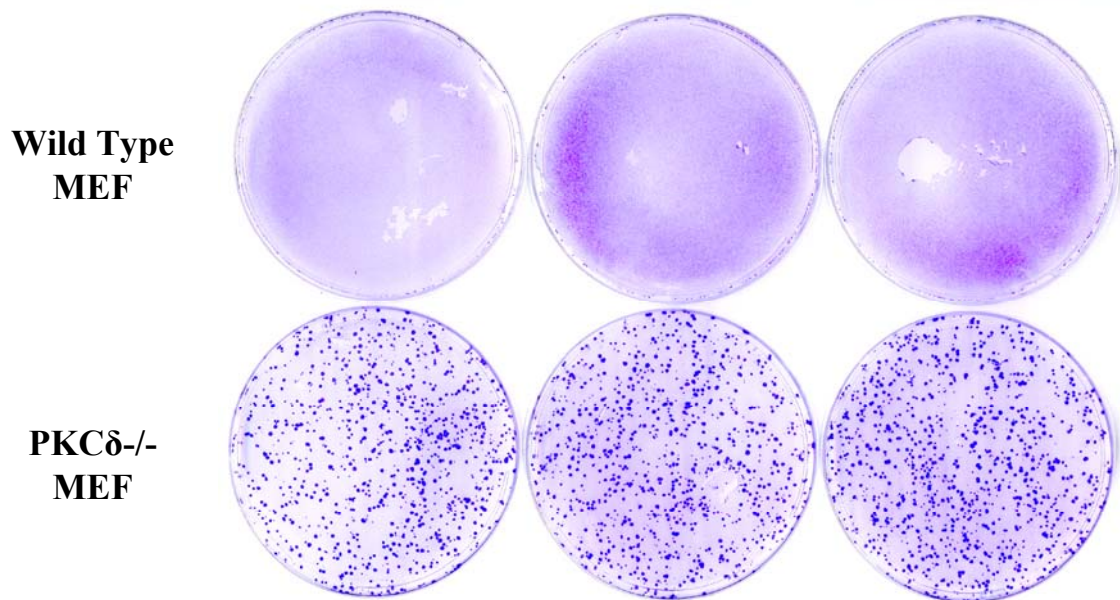


Figure 42—PKC δ loss allows escape from contact inhibition. WT and PKC δ null MEFs were maintained under confluent culture conditions for 7 days, fixed, and stained with crystal violet to visualize confluency induced foci. Note that while WT MEFs appear to have arrested upon reaching confluency, PKC δ null MEFs have formed numerous colonies across the plates.

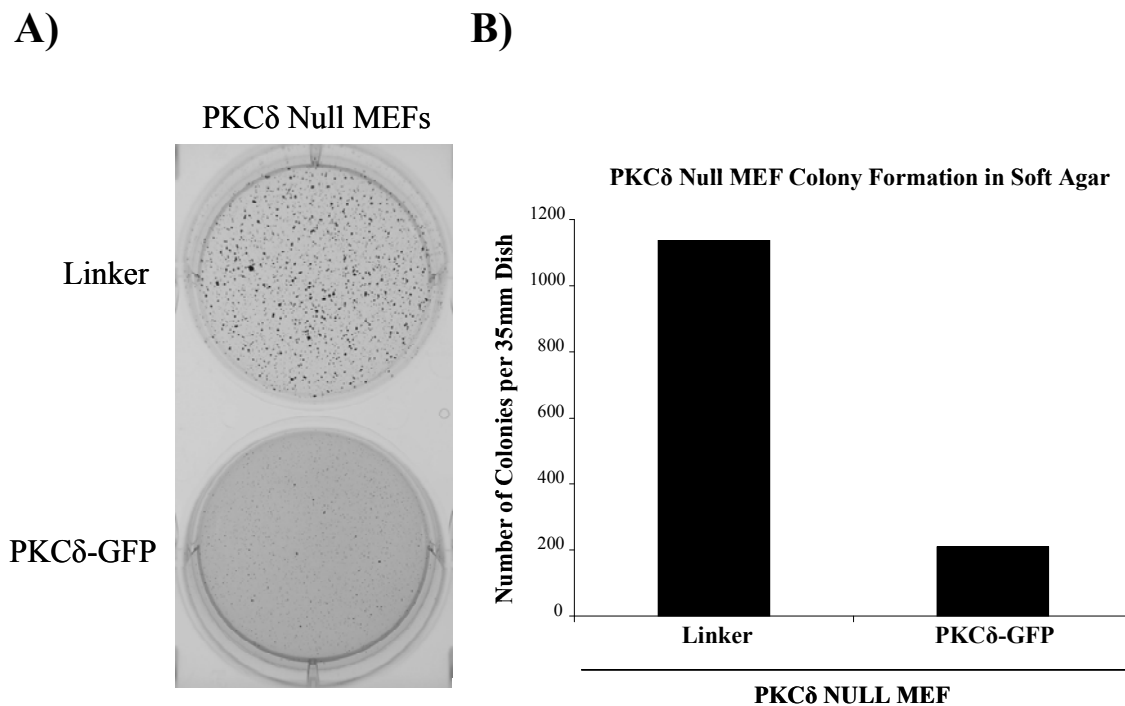


Figure 43—PKC δ -GFP Suppresses the Growth of PKC δ Null MEFs in Soft Agar. Control and PKC δ -GFP transduced PKC δ null MEFs were suspended in soft agar and cultured for 7 days prior to addition of DTT to label metabolically active cells. A) Representative image of DTT labeled colonies in Control and PKC δ -GFP transduced PKC δ null MEFs. B) Colonies in Control and PKC δ -GFP transduced PKC δ null MEFs per 35mm culture dish were counted.

Chapter 7

Discussion

7.1 The PKC δ Catalytic Fragment is a Critical Regulator of the G2/M Checkpoint.

The majority of studies on PKC δ function have focused on the pro-apoptotic role that the kinase plays both in vivo and in vitro. We have now demonstrated that in addition to these pro-apoptotic effects, PKC δ also regulates cell cycle progression by participating in the G2/M DNA damage checkpoint. We have shown that retroviral expression of PKC δ -cat in primary KCs, immortalized KCs with mutant p53, and MEFs is sufficient to induce G2/M checkpoint activation and cell cycle arrest. PKC δ -cat induced G2/M checkpoint activation requires kinase competent PKC δ since a kinase dead PKC δ mutant failed to activate the G2/M checkpoint (Figure 21 and 22).

Based on our studies, PKC δ appears to also play an important role in the response to γ -radiation (Figure 19). Although PKC δ null MEFs underwent a significant G2/M arrest following high dose γ -radiation (20 Gy), the arrest was significantly diminished when compared to the arrest which occurred in the WT MEFs. Furthermore, no detectable G2/M arrest occurred in the PKC δ null MEFs following exposure to low dose γ -radiation (4 Gy).

It is well known that the type of DNA damage caused by the ultraviolet and ionizing radiation are very different, and as a result, different DNA repair pathways are activated

in response to each. Ionizing radiation induces both single and double stranded breaks in the DNA, and activates NHEJ or HR DNA repair responses (See Chapter 1.4.4-1.4.6). In contrast, UV radiation causes the formation of bulky DNA adducts (ie. pyrimidine dimers), and the nucleotide excision repair pathway is required for the proper removal of these adducts (1.4.3). Similarly, UV and ionizing radiation activate different upstream components of cell cycle checkpoint pathways, although many of the mediators of these pathways are shared. Ionizing radiation activates cell cycle checkpoints through ATM, and a lesser extent ATR activation. In contrast, UV radiation induces a checkpoint response primarily through ATR. The use of multiple upstream kinases to respond to different types of DNA damage likely allows the cell to respond to a wide variety of genotoxic stresses with the same outcome, that is cell cycle arrest, DNA repair, and if the damage is of a high enough severity, apoptosis.

The relative requirement for PKC δ to elicit proper checkpoint signaling in response to UV as compared to γ -radiation, indicates that PKC δ activation likely occurs downstream of both ATM and ATR mediated checkpoint responses. Interestingly, ATM has been reported to phosphorylate and activate PKC δ in response to 5-azacytidine treatment (Yoshida et al., 2003). Whether ATR can similarly activate PKC δ in response to UV exposure remains to be determined. Regardless of the activation of PKC δ downstream of ATM or ATR, the diminished G2/M cell cycle arrest after both UV and γ -radiation in PKC δ null MEFs suggests that PKC δ may be required for the response to a wide variety of DNA damaging agents, and may even be universally required for maintenance of the G2/M checkpoint over time. The role for PKC δ in checkpoint maintenance may be

critical for providing the cell with enough time to properly repair all DNA damage before resuming cell cycle progression, indicating that PKC δ is critical for preserving genomic integrity.

7.2 Caspase Cleavage is Critical for PKC δ Induced Checkpoint Function

In addition to kinase activity, the cleavage of PKC δ appears to be critical to checkpoint integrity since the expression of a caspase-resistant PKC δ (D327A)-GFP mutant was unable to restore the G2/M checkpoint in PKC δ null MEFs (Figures 21 and 22). PKC δ is cleavable by numerous proteases including caspases 2 and 3, but the protease responsible for cleaving PKC δ in this circumstance remains unclear (Denning et al., 1998; Emoto et al., 1995; Panaretakis et al., 2005). It is possible that PKC δ may be a substrate for the caspase 2 containing DNA-PKcs-PIDDosome complex which was recently demonstrated to be integral to proper G2/M checkpoint maintenance after ionizing radiation exposure (Shi et al., 2009). Indeed, previous work in our lab has demonstrated that both caspase 2 and 3 can cleave PKC δ *in vitro* (unpublished results from Dr. L. Sitailo). Caspase 2 appears to be unique among the caspases in that it is the only caspase with constitutive nuclear localization, and appears to play an important role not only in the apoptotic cascade, but also in regulating cell cycle progression (Shi et al., 2009; Zhivotovsky et al., 1999; Ho et al., 2009). Further studies will need to be done to verify whether caspase 2 mediates the cleavage of PKC δ *in vivo*, during UV-induced activation of the G2/M checkpoint.

Interestingly, PKC δ appears to be required for G2/M checkpoint maintenance rather than induction, since a decrease in mitotic index was observed in both wild type and PKC δ null MEFs 1 hour after UV exposure (Figure 17), but not after 6 hours (Figure 16). Proteins such as p53, p21 and even the DNA-PKcs-PIDDosome complex have been similarly implicated in G2/M checkpoint maintenance rather than initial checkpoint activation (Shi et al., 2009; Bunz et al., 1998). This discrepancy in early compared to late time points of checkpoint activation likely correlates with the appearance of the PKC δ catalytic fragment, which will only appear once caspases have become activated. The role for PKC δ in maintenance may reflect a bimodal activation of the G2/M checkpoint following UV radiation exposure. Initially, checkpoint activation likely occurs through PKC δ -independent activation of an ATR/Chk1 signaling axis which results in the phosphorylation and inhibition of Cdk1. This early PKC δ -independent arrest appears to be transient, as the loss of PKC δ allows cells to escape UV-induced G2/M arrest at later timepoints. Because caspase cleavage of PKC δ is important for G2/M checkpoint function, it is likely that at these later timepoints, PKC δ is cleaved and can participate in checkpoint maintenance.

7.3 Potential Interactions of PKC δ with DNA Repair and Cell Cycle Machinery

Several PKC δ substrates involved in the DNA damage response have been identified. These include both p53 and the p53 family member, p73 β (Yoshida et al., 2006a; Liu et al., 2007; Ren et al., 2002). PKC δ has been demonstrated to phosphorylate and activate the DNA repair protein Rad9 after exposure to the DNA damaging agent 5-azacytidine in

an ATM-dependent manner (Yoshida et al., 2003). It is possible ATR, a major kinase activated by UV radiation, is also capable of activating PKC δ in a similar fashion. Other components of the DNA damage response that have been identified as PKC δ substrates include DNA-PKcs and topoisomerase II α (Bharti et al., 1998; Yoshida et al., 2006b). Together, these studies reveal the importance of PKC δ in the DNA damage repair pathway and indicate it may be functioning at several levels. Nuclear localization of PKC δ is important for DNA damage-induced apoptosis, and may be required during multiple processes following DNA damage, including checkpoint activation (DeVries et al., 2002; DeVries-Seimon et al., 2007).

The novel cell cycle regulatory role for PKC δ described in Chapters 3 and 4 has important implications for the role of this protein as a tumor suppressor. Based on these results, the loss of PKC δ during tumor development would allow for the cell to progress through the cell cycle, even in the presence of DNA damage, while at the same time evading apoptosis. This is illustrated by wild type MEFs being driven into apoptosis by caffeine-induced G2/M checkpoint override after UV irradiation, while PKC δ null MEFs do not arrest, nor undergo apoptosis even after treatment with caffeine (Figure 18). Similar to wild type MEFs, KCs are highly sensitive to caffeine treatment after UV irradiation (Lu et al., 2008; Heffernan et al., 2009). Thus, KCs lacking PKC δ , such as in squamous papillomas and carcinomas, are also likely to have this dual defect in the response to DNA damage, resulting in both reduced cell cycle arrest and reduced apoptosis (D'Costa and Denning, 2005; Reddig et al., 1999; Aziz et al., 2006).

Furthermore, concomitant treatment of LZRS-PKC δ -cat-ER transduced cells with the ATM/ATR inhibitor caffeine was unable to prevent PKC δ -cat-ER induced G2/M arrest. This suggests that PKC δ -cat is inducing G2/M checkpoint activation independent of the ATM/ATR kinases, and may actually function internally to the G2/M checkpoint signal cascade (Figure 41). Consistent with this, UV induced γ H2A.X and P~p53(S15) in PKC δ null MEFs but did not induce P~Cdk1(Y15) (Figure 22).

It will be important in the future to identify substrates for PKC δ within the G2/M checkpoint signaling hierarchy. We demonstrated that the misregulation of UV-induced G2/M checkpoint activation that occurs in the absence of PKC δ cannot be explained by misregulation of Cdc25A (Figure 24). Possible alternatives to Cdc25A include, but are not limited to, the other Cdc25 phosphatases, the checkpoint kinases Chk1 and Chk2, and the Cdk1 kinase Wee1 (Russell and Nurse, 1987; Sancar et al., 2004). The importance of cell cycle checkpoint function to cancer cells has prompted intensive study of small molecule inhibitors targeted towards components of the G2/M checkpoint such as ATM/ATR, Chk1 and 2, as well as the Aurora family for potential use as cancer therapeutics (Schmidt and Bastians, 2007; Bucher and Britten, 2008; Hose et al., 2009). Better understanding of how PKC δ participates in this cell cycle checkpoint is important for developing better cancer treatments in the future.

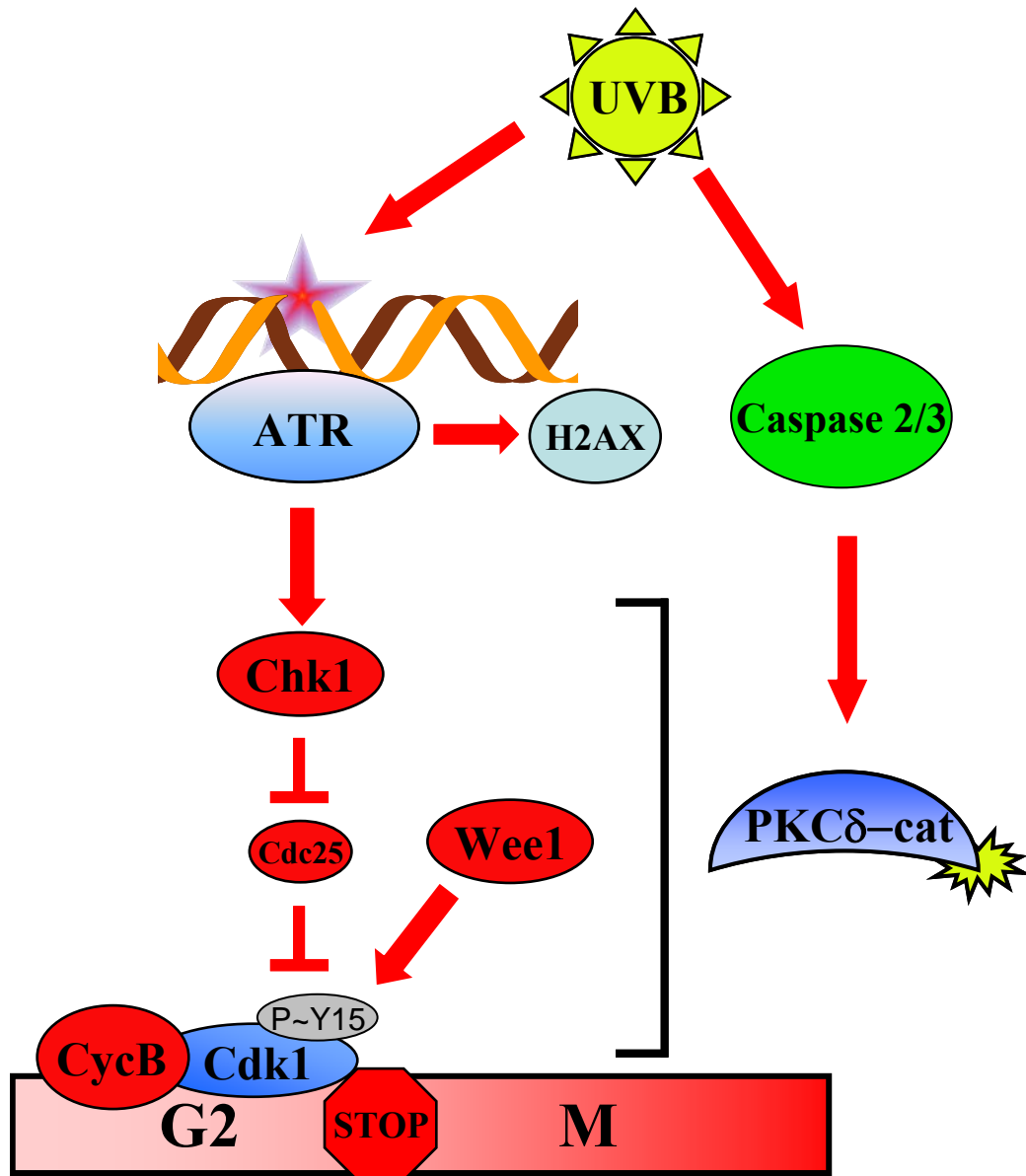


Figure 44—PKC δ -cat is Important for G2/M Checkpoint Signaling

Downstream or Independent of ATR Signaling. UV-induced activation of the G2/M DNA damage checkpoint requires PKC δ cleavage. The substrates of PKC δ within the checkpoint pathway remain unclear, but it is likely that they function downstream of ATR signaling since caffeine could not block PKC δ -cat induced G2/M arrest.

7.4 PKC δ -cat Induces Histone H3(S10) Phosphorylation Outside of Mitosis

The discovery that PKC δ -cat expression induced the phosphorylation of histone H3 on Ser10 outside of mitosis, and independently of Aurora B activity was very surprising (Figures 25-29). Our findings suggest that this phosphorylation event occurred on a global scale in an analogous manner to that which occurs during mitosis; however, we did not detect any obvious changes to the chromatin architecture after PKC δ -cat-ER induced H3 phosphorylation. In addition to its role in mitotic chromatin condensation, there is increasing evidence for histone H3 phosphorylation during interphase, where it is important for the transcription of certain genes (Paulson and Taylor, 1982).

Early evidence of histone H3 phosphorylation outside of mitosis came from the discovery that phosphorylation of the N-terminal tail of histone H3 rapidly occurred in response to a number of stimuli, including growth factors, phorbol esters, okadaic acid, and protein synthesis inhibitors (Mahadevan et al., 1991). Histone H3 phosphorylation was then identified at transcriptionally active heat shock-induced puffs on polytene chromosomes of *D. melanogaster* (Nowak and Corces, 2000). Now, specific H3 kinases have been identified within various pathways of transcriptional activation. For example, TNF α stimulation of NF- κ B target genes involved the phosphorylation of Ser 10 by IKK α (Yamamoto et al., 2003). Furthermore, EGFR induced gene transcription of c-jun and c-fos was accompanied by MSK1 and 2 dependent phosphorylation of histone H3(S10) at the promoters of these genes (Duncan et al., 2006; Cheung et al., 2000). Histone H3 phosphorylation has now been implicated in a host of additional transcriptional activation

pathways and at specific gene promoters, including some signaling pathways that are dependent upon PKC signaling (Lefebvre et al., 2002; DeManno et al., 1999; Huang et al., 2004; Karrasch et al., 2006).

It remains unclear whether H3 phosphorylation is directly important for transcriptional activation, or whether it functions as a precursor to other post-translational modifications. For example, there is a correlation between histone H3 phosphorylation and acetylation status on the promoters of some genes (Clayton et al., 2000). Indeed the phosphorylation of histone H3 has been implicated in the recruitment of the histone acetyltransferase GCN5 (Lo et al., 2000; Espinos et al., 1999; Clements et al., 2003). Other findings, however, implicate H3(S10) phosphorylation as preventing H3(K9) acetylation (Edmondson et al., 2002). It should be noted that this finding may be an artifact of the S10 phosphate shielding the lysine 9 acetylation mark from detection using the lysine 9 acetylated antibody.

How histone H3 phosphorylation can lead to opposing chromatin states, that is open during trans-activation, and condensed during mitosis, remains a matter of debate. It is possible that the mark itself does not confer a certain status upon chromatin, but recruits other proteins and co-factors that mediate the outcome. These mediators may be regulated in a cell cycle dependent manner, explaining why condensation is the outcome of H3 phosphorylation during mitosis. It is also possible that the difference arises from the pattern of phosphorylation. During mitosis, histone H3 is phosphorylated across

entire chromosomes. In contrast, during gene activation, H3 phosphorylation is present on specific gene promoters.

UV exposure is known to induce the phosphorylation of histone H3 on Ser10, but the biological consequence of this phosphorylation event in the context of the response to UV is not well understood (Allison and Milner, 2003; Zhong et al., 2000). Of note, one group has described the phosphorylation of histone H3 on Ser 10 during gleotoxin induced apoptosis, and a correlation between P~H3(S10) and enhanced sensitivity to nuclease digestion of the chromatin was reported (Waring et al., 1997). Indeed, we found that UV induced the phosphorylation in WT but not PKC δ null MEFs, suggesting a requirement for PKC δ in this event (Figures 31-32). Furthermore, PKC δ co-localizes with P~H3(S10) in the nuclei of UV-irradiated HaCaT cells, raising the possibility that PKC δ may be the kinase responsible for H3 phosphorylation in response to UV radiation (Figure 35).

Other histone phosphorylation events have also been implicated in apoptosis. Apoptotic stimuli induce the phosphorylation of histone H2B on T14, and this phosphorylation has been implicated in apoptotic chromatin condensation and fragmentation (Ajiro, 2000; Ura et al., 2007). Since the phosphorylation of H2B and H3 occur during biologically similar circumstances, it is appealing to speculate that histone H3(S10) phosphorylation may also be involved in apoptotic chromatin condensation. The kinases implicated in H2B(T14) phosphorylation during mitosis include Mst1 and particularly interesting in light of these studies, PKC δ (Cheung et al., 2003; Hu et al., 2007). Interestingly, phosphorylated

histone H2B co-localizes with γ H2AX and components of the DNA damage machinery during death receptor induced apoptosis, putting it in the proper context to interact with components of the cell cycle and DNA repair apparatus (Solier and Pommier, 2009; Solier et al., 2009). PKC δ has also been implicated in the phosphorylation of histone H3 on Thr45 during differentiation induced apoptosis in neutrophils, although it was not formally proven that this phosphorylation event played a causative role in the cell death (Hurd et al., 2009).

Additional studies are clearly needed to better understand the biological context of UV-induced phosphorylation of histone H3. That is, when histone H3 becomes phosphorylated in the response to UV radiation, what is the status of the cell? We are currently undertaking studies using inhibitors of DNA repair (caffeine), and late stage apoptosis (z-VAD.fmk) to attempt to block the UV-induced phosphorylation of histone H3 in WT MEFs. In doing so, we hope to gain a better understanding of when histone H3 becomes phosphorylated in the UV response, as well as what role it may be playing in said response. Unfortunately, the abundant nature of histone proteins in the cell does not lend itself to genetic manipulation, and so we are limited to correlations at this point, barring the extension of these studies into budding yeast, which only have one histone H3 genetic locus.

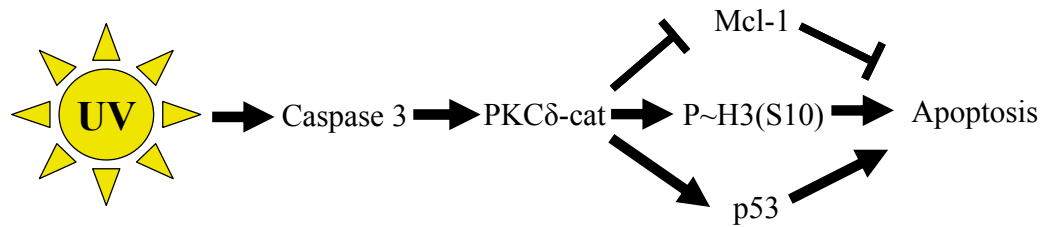


Figure 45–UV-Induced Apoptosis Requires PKC δ Cleavage. Apoptotic doses of UV radiation initiate a cascade of events that converge upon the cleavage of PKC δ by caspases. Cleaved PKC δ then phosphorylates a number of targets, which we now believe includes histone H3 to promote apoptosis. The requirement and consequence of histone H3 phosphorylation remains unclear.

7.5 Tumor Suppressor Mechanisms of PKC δ

Given the well characterized role of PKC δ in promoting apoptosis, it is not entirely surprising that most attention on PKC δ as a tumor suppressor has focused on its role in the apoptotic cascade (Reyland et al., 1999; Reyland et al., 2000; Matassa et al., 2001; Carpenter et al., 2002; Denning et al., 2002; Lasfer et al., 2006). While the ability to induce apoptosis is a common characteristic of tumor suppressors, many tumor suppressors also function by limiting cell cycle progression. PKC δ has been previously implicated in regulating the progression through multiple stages of the cell cycle, including G1/S, and mitosis (Lagory et al., 2009; Watanabe et al., 1992; Takahashi et al., 2006; Ashton et al., 1999; Afrasiabi et al., 2008; Nakagawa et al., 2005).

We believe that in addition to promoting apoptosis, the expanding role of PKC δ in promoting cell cycle arrest, particularly through the G2/M phase of the cell cycle, may represent an additional mechanism of PKC δ mediated tumor suppression. The dual function of PKC δ in both the promotion of apoptosis and cell cycle arrest implies that the loss of PKC δ expression during tumorigenesis would be especially deleterious. Many, if not all cancers demonstrate elevated levels of genomic instability. Despite the increased risk of cancer progression associated with genomic instability, it is inherently toxic to the cell and can promote both apoptosis and cell cycle arrest. A cell that has lost PKC δ expression, however, would be naturally resistant to both apoptosis and cell cycle arrest, allowing the cell to continue to proliferate, accumulating more mutations, and ultimately allowing the cancer to become more aggressive. The loss of PKC δ would also have

important implications early during tumorigenesis, as the inability to execute cell cycle arrest or apoptosis may increase the likelihood that initiating mutations to genes like p53 would be allowed to escape repair.

It will be critical to determine how PKC δ -cat influences G2/M checkpoint activation in the future, as a better understanding of this checkpoint may uncover novel targets for cancer therapy. By better understanding the downstream targets of PKC δ -cat, it may be possible to devise therapeutic strategies to activate important downstream components even in the absence of PKC δ , a possibility that may be particularly effective in those tumors which have lost PKC δ expression.

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