

# CURRICULUM VITAE

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## ABSTRACT

**Title of Dissertation:** Role of p38 $\delta$  and MEP50 in Epidermal Keratinocyte Homeostasis

**Kamalika Saha, Doctor of Philosophy, 2015**

**Dissertation Directed by:** Richard L. Eckert, Ph.D., John F.B. Weaver Distinguished

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Keratinocytes are the major cell type in the human epidermis, the outer layer of skin. These cells undergo a tightly regulated terminal differentiation program which results in the formation of the cornified envelope. This envelope is essentially the barrier which protects us from mechanical stress, extreme climate conditions and infectious agents. A fully functional epidermal barrier dictates a perfect balance between proliferation and differentiation. PKC $\delta$ , a novel PKC isoform and a key controller of epidermal differentiation regulates proliferation by increasing p21<sup>Cip1</sup> expression. However, little is known about the mediators involved in this regulation. We hypothesize that p38 $\delta$  MAPK, a downstream kinase is a mediator in this process. We observe that p38 $\delta$  regulates p21<sup>Cip1</sup> mRNA/protein levels in a p53 dependent manner. Additionally, PKC $\delta$  selectively activates p38 $\delta$  and treatment with p38 $\delta$ -siRNA or dominant negative p38 results in attenuation of the PKC $\delta$  induced p21<sup>Cip1</sup> response. Furthermore, p53 is identified as a novel target of p38 $\delta$  and is a key mediator in the p38 $\delta$ -p21<sup>Cip1</sup> signaling cascade. Moreover, the physiological relevance of this pathway is confirmed in the PKC $\delta$  and p38 $\delta$  knockdown organotypic cultures which are thicker, have additional layers and reduced cornified envelope formation as compared to the controls. We have also

identified the Protein Arginine Methyltransferase (PRMT5) and Methylosome Protein 50 (MEP50) as novel targets of PKC $\delta$  and p38 $\delta$ . MEP50 enhances keratinocyte proliferation and opposes differentiation by mechanisms involving silencing p21<sup>Cip1</sup> and involucrin. We found that symmetric demethylation of arginines in histones H3 and H4 plays a key role in this regulation. Additionally, the pro-proliferation role of MEP50 is physiologically relevant as MEP50 knockdown rafts are significantly thinner and have fewer number of Ki67 positive cells than the control rafts. These studies highlight p38 $\delta$  as a common kinase regulating the dual processes of proliferation and differentiation and characterize the role of MEP50/PRMT5 as novel players in controlling epidermal homeostasis.

Role of p38 $\delta$  and MEP50 in Epidermal Keratinocyte Homeostasis

by  
Kamalika Saha

Dissertation submitted to the faculty of the Graduate School of the  
University of Maryland, Baltimore in partial fulfillment  
of the requirements for the degree of  
Doctor of Philosophy  
2015

## **DEDICATION**

*To Ma and Baba*

*For your endless love and encouragement*

*And to Pooja and Jolyn for always being there!*

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## LIST OF ABBREVIATIONS

ADMA: Asymmetric dimethylarginine

AP-1: Activation Protein 1

AR: Androgen receptor

ASK: Apoptosis signal-regulating kinase

ATF-2: Activating transcription factor 2

ATP: Adenosine triphosphate

C/EBP: CCAAT/enhancer binding protein

CDC42: Cell division control protein 42 homolog

CDK6: cyclin dependent kinase 6

CDT1: Chromatin licensing and DNA replication factor 1

DLK: Dual leucine zipper bearing kinase

DLP: dorsolateral prostate

DNA: Deoxyribonucleic acid

DNp38 $\alpha$ : Dominant negative p38 $\alpha$

DRR: Distal regulatory region

EBS: Epidermal bullosa simplex

EGFR: Epidermal growth factor receptor

EMT: Epidermal-mesenchymal transition

ERK: Extracellular-signal related protein kinase

FEN1: Flap structure specific endonuclease 1

GAR: Glycine Arginine-rich

GTP: Guanosine triphosphate

HNSCC: Head and neck squamous cell carcinoma

JNK: c-Jun N terminal kinase

KIF: Keratin intermediate filament

KLF4: Kruppel-like factor 4

KSFM: Keratinocyte serum free medium

LI: Lamellar ichthyosis

MAPK: Mitogen activated protein kinase

MEK: MAP/ERK kinase

MEKK1: MAP/ERK kinase kinase 1

MEP50: Methylosome protein 50

MLK: Mixed-lineage kinase

MMA: Monomethyl arginine

NuRD: Nucleosome remodeling and deacetylase

PAK: p21 activated kinase

PDCD4: Programmed cell death protein 4

PDK: Pyruvate dehydrogenase kinase

PKC: Protein Kinase C

PRMT: Protein arginine methyl transferase

PRR: Proximal regulatory region

PSEK: Progressive symmetric erythrokeratoderma

RAC1: Ras-related C3 botulinum toxin substrate 1

RNA: Ribonucleic acid

SCC: Squamous cell carcinoma

SDMA: Symmetric dimethyl arginine

snRNP: Small nuclear ribonucleic proteins

SP1: Specificity protein 1

SPR: Small proline-rich protein

STAT3: Signal transducer and activator of transcription 3

TAK1: Transforming growth factor  $\beta$  activated kinase 1

TG: Transglutaminase

TNBC: Triple negative breast cancer

TNF: Tumor necrosis factor

TPA: 12-*O*-Tetradecanoylphorbol-13-acetate

UV: Ultraviolet

VP: Ventral prostate

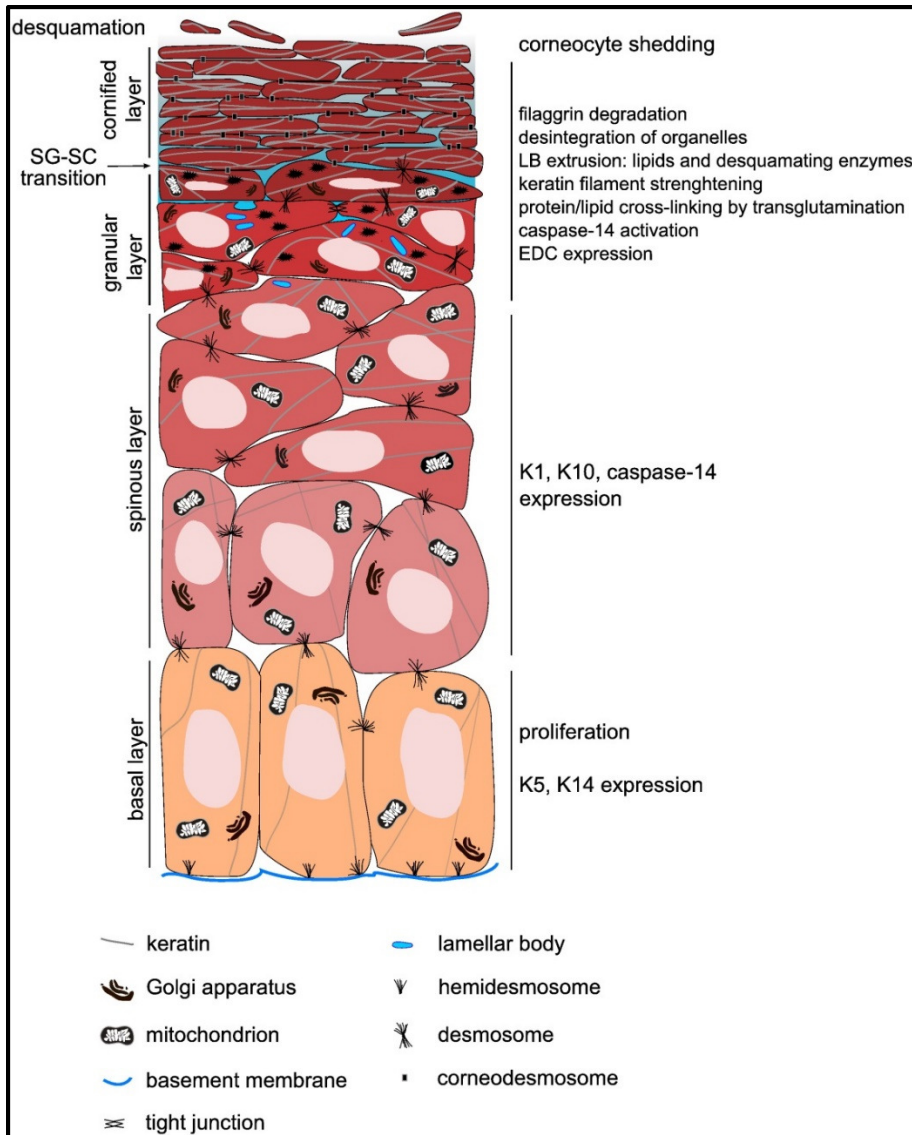
WDR77: WD repeat domain 77

## CHAPTER 1: INTRODUCTION

### *A. THE EPIDERMIS:*

Epidermis, the outermost layer of skin is the body's first line of defense against environmental hazards. This stratified squamous epithelium protects the body from pathogenic agents and prevents dehydration in extreme environments. The epidermis is separated from the underlying dermis by a basal lamina. The keratinocytes constitute the major cell type in the epidermis and they undergo an elegantly orchestrated program of terminal differentiation to form the cornified envelope (Eckert 1989, Green, Fuchs et al. 1982). The epidermis consists of four layers namely the basal, spinous, granular and the cornified layer. **Fig. 1-1** shows the different layers of the epidermis. The innermost layer, the basal layer consists of the undifferentiated stem cell population which provides a continuous supply of cells to form the upper differentiated layers (Lavker, Miller et al. 1993, Pincelli, Marconi 2010, Yang, Lavker et al. 1993). The spinous layer or the stratum spinosum immediately follows the basal layer and is characterized by the presence of desmosomal networks between cells. Involucrin and transglutaminase which are early markers of differentiation are expressed in this layer (Holbrook, Wolff 1987). The next layer is the stratum granulosum or the granular layer. As the name suggests, it is recognized by the presence of granules which contain products necessary to assemble the upper layers such as filaggrin, loricrin and the lipids necessary for formation of the cornified envelope (Steven, Bisher et al. 1990). The cells in the intermediate layer do not have any proliferative potential but they are viable and metabolically active. The final

layer is the cornified envelope which consists of dead, flattened polyhedral cells known as corneocytes.



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 Eckhart L et al, Declerq W, Biochimica et Biophysica Acta, 1833 (12), 3471-80, (December 2013)

**Figure 1-1: Morphological progression in keratinocyte differentiation.**

The human epidermis is divided into four layers namely the basal, spinous, granular and the cornified layer. The highly proliferative cells are located in the basal layer and function to replenish cells in the upper differentiated layers. This phenomenon is characterized by specific morphological changes in a strictly regulated process known as terminal differentiation. The spinous layer is characterized by the presence of desmosomal connections between the cells. The granular layer contains membrane-bound granules. The keratinocytes in the cornified layer are non-viable, devoid of the nucleus and are covalently crosslinked to form a barrier.

## ***B. CORNIFICATION – AN ORCHESTRATED EVENT:***

The efficient formation of the epidermis requires a perfect balance between the rate of proliferation in the basal layer and desquamation in the cornified layer. Any imbalance in this process leads to diverse diseases (Eckert, Crish et al. 1997). Cornification is defined as the process of keratinocyte terminal differentiation resulting in the formation of a protective barrier. The cornified envelope is an insoluble protein structure which replaces the plasma membrane of corneocytes resulting in the formation of a protective outer layer. It consists of a protein network covalently cross-linked by transglutaminase via N-( $\gamma$ -glutamyl)-lysine bonds (Eckert, Rorke 1989). The process of cornification constitutes a carefully regulated sequence of events: a) the degradation of the cellular organelles is accompanied by their subsequent replacement with a proteinaceous cytoskeleton b) peripheral proteins are enzymatically cross-linked to form the cornified envelope within the cells c) the corneocytes are linked to form a functionally relevant but non-viable structure.

The major structural proteins in the basal keratinocytes are keratin 5 (K5) and keratin 14 (K14). The basal keratinocytes commit to the differentiation module once they detach from the basal lamina and migrate to the next layer. As the cells reach the spinous layer, this keratinocyte intermediate filament network is replaced by K1 and K10. These spinous keratinocytes are connected to each other via desmosomes (Fuchs, Green 1980, Woodcock-Mitchell, Eichner et al. 1982). The spinous layer marks the initiation phase of cornified envelope formation. It involves the synthesis of structural proteins like loricrin, keratins, filaggrin, involucrin, small proline rich proteins (SPRs) and lipids which are extruded in the cell interior. The next phase which is the reinforcement phase of

differentiation occurs in the granular layer. The granular keratinocytes acquire the keratohyalin granules containing the precursor of filaggrin, profilaggrin. Filaggrin is formed by dephosphorylation and proteolysis from pro-filaggrin. It results in aggregation of the keratin intermediate filaments (KIF) into tight bundles resulting in the flattened phenotype of corneocytes (Dale, Presland et al. 1997). The reinforcement phase is also characterized by covalent attachment of lipids to the structural proteins and cross-linking of the SPRs with loricrin by transglutaminases TG1 and TG3. The lipid envelope formation occurs in the upper granular layers and involves further crosslinking of the lipids on the cross-linked proteins of the previous stages. The final product of the terminal differentiation process is the cornified envelope which consists of corneocytes enveloped in a lipid matrix and cross-linked by corneodesmosomes. The lipids serve as effective water repellants and the corneodesmosomes add to the physical strength of the envelope.

### ***C. EPIDERMAL DISEASES DUE TO IMPAIRED CORNIFICATION:***

Structural proteins, crosslinking enzymes and lipids are important components of the cornified envelope and their absence or mutation results in epidermal diseases. Interestingly, no diseased condition has been attributed to the absence of a specific structural protein. This could be due to embryonic lethality or alternative compensatory mechanisms by the other proteins. Filaggrin; a key component of the keratohyalin granules is essential for alignment of KIFs, regulation of cell morphology during cornification and maintenance of epidermal flexibility. Kawasaki et al demonstrated that filaggrin null mice displayed altered keratin filament network in the upper granular and stratum corneum layers. Additionally, the epidermis from the filaggrin null mice were

more susceptible to mechanical stress accompanied with increased cornified layer fragility and increased desquamation (Kawasaki, Nagao et al. 2012). Ichthyosis vulgaris, an autosomal dominant keratinization disorder was reported to be caused by loss-of-function in the filaggrin gene (Smith, Irvine et al. 2006). The loss-of function filaggrin mutants are major predisposing factors for atopic dermatitis (Palmer, Irvine et al. 2006). Involucrin; a 68 kDa structural protein is expressed in the spinous and granular layers (Rice, Green 1979). It is an important transglutaminase 1 target and serves as a scaffold for incorporation of other precursors of the cornified envelope (Rice, Green 1979, Eckert, Yaffe et al. 1993). Overexpression of a transgene expressing involucrin in mice lead to delayed hair growth and diffuse alopecia (Crish, Howard et al. 1993a). Interestingly, the involucrin knockout mice did not display any phenotype as compared to the wild-type mice suggesting the existence of compensatory mechanisms (Djian, Easley et al. 2000). Loricrin, a 38 kDa protein; constitutes 85-90% of the total protein mass in the cornified envelope and is expressed in the granular layer (Kalinin, Marekov et al. 2001, Steinert, Marekov 1995). Frameshift mutations in the loricrin gene leads to Vohwinkel syndrome and progressive symmetric erythrokeratoderma (PSEK) (Ishida-Yamamoto, McGrath et al. 1997). These arise from deletions in the latter half of the coding sequence resulting in a frameshift mutation. This arginine rich loricrin mutant has reduced number of glutamine and lysine crosslinking sites. Patients with Vohwinkel syndrome suffer from hyperkeratosis of the extremities, epidermal constrictions on the digits of hands and feet. PSEK patients have impaired barrier function, plaques and hyperkeratosis (Ishida-Yamamoto, McGrath et al. 1997).

Keratins are another important class of structural proteins in the epidermis. Keratins K5, K14 of the basal layer and K1 and K10 in the suprabasal layers are structural proteins which function in intermediate filament assembly (Fuchs, Green 1980, Sun, Eichner et al. 1983). Mutations in the highly conserved NH<sub>2</sub> and COOH terminal of the rod domain of these proteins results in skin blistering diseases (Vassar, Coulombe et al. 1991, Fuchs, Esteves et al. 1992). Epidermal bullosa simplex (EBS), an autosomal dominant disease occurs due to point mutations in the basal K5/K14 proteins resulting in skin blisters upon subjection to mechanical stress (Coulombe, Hutton et al. 1991, Eckert 1989). Epidermal hyperkeratosis is a similar blistering disease caused by mutations in the suprabasal K1/K10 keratins (Cheng, Syder et al. 1992, Rothnagel, Dominey et al. 1992).

Lamellar ichthyosis (LI) is a congenital disease of keratinization which results due to mutation of the transglutaminase gene (Phillips, Baden 1993). It is characterized by the presence of a collodion membrane which sheds during the early months of life. This increases the risk of sepsis and dehydration. Patients with LI express low amounts of transglutaminase which leads to a large pool of non-cross-linked involucrin and loricrin in the suprabasal layers (Hohl, Huber et al. 1993).

Impaired lipid synthesis also results in diseases affecting the cornified envelope. X-linked ichthyosis (RXLI) is caused by a mutation in the steroid sulphatase enzyme resulting in accumulation of cholesterol sulphate enzyme in the extracellular space of the cornified layer (Shapiro, Buxman et al. 1978). This impacts desquamation, involucrin crosslinking to precursor proteins and TG1 activity. Gaucher disease is caused due to a mutation in the enzyme  $\beta$ -glucocerebrosidase involved in hydrolysis of glucosylceramides to ceramides (Holleran, Ginns et al. 1994). Ceramides are a

constituent of the intracellular lamellae. Patients suffering from Gaucher disease type-2 display scaly skin which has been attributed to reduced intercellular lamellar bilayers in the cornified layer.

#### ***D. MAPK CASCADES IN KERATINOCYTE DIFFERENTIATION:***

The MAPK pathway is a pivotal regulator of keratinocyte differentiation. They are a family of evolutionary conserved serine/threonine family of kinases which regulate a host of cellular events. The three major families of MAPK which have been identified are the extracellular-signal related protein kinase (ERK), c-Jun NH<sub>2</sub>-terminal kinases (JNKs) and p38 (Raman, Chen et al. 2007). The activity of the MAPKs is regulated by the upstream cascade involving the MAPK kinase kinase (MEKK, MAP3K) and MAPK kinase (MEK, MKK, MAP2K). On activation, the MAPKs translocate to the nucleus and phosphorylate transcription factors and proteins. Involucrin, the early keratinocyte differentiation marker has been used as an effective tool to elucidate the different pathways which regulate keratinocyte differentiation. Studies by Welter et al revealed the presence of two regulatory elements which were critical for involucrin expression: the distal regulatory region (DRR) located at nucleotides (-2473/-1953) and the proximal regulatory region (PRR) located at nucleotides (-241/-7). Simultaneous deletion of both these regions resulted in the loss of 80% of promoter activity (Welter, Crish et al. 1995a). Extensive DNA-protein studies confirmed the presence of an activator protein 1 site (AP1-5) and specificity protein 1 (Sp1) site at the DRR as well as an AP1-1 and a CCAAT/enhancer binding protein (C/EBP) at the PRR. The AP1 transcription factors found to bind at these regions are JunB, JunD and Fra-1 (Welter, Crish et al. 1995a). Interestingly, mutation of the AP1-5 site leads to complete loss of promoter activity while

Sp1 site mutation leads to partial loss of activity (Banks, Crish et al. 1998). Additionally, the DRR is physiologically relevant as its deletion leads to complete loss of human involucrin expression *in vivo* (Crish, Zaim et al. 1998, Crish, Bone et al. 2002). Studies using kinase inhibitors, dominant negative and constitutively active kinases reveal that involucrin gene expression is mediated by a cascade involving protein kinase C, MEKK1, MEK isoforms (MEK3, MEK6, MEK7) and p38 MAPK (Efimova, LaCelle et al. 1998, Dashti, Efimova et al. 2001a, Dashti, Efimova et al. 2001b, Efimova, Eckert 2000).

Protein kinase C (PKC) isoforms play a key role as regulators of cell differentiation (Newton 1997). PKCs include three families. The novel PKCs (nPKC  $\delta$ ,  $\epsilon$ ,  $\eta$ , and  $\theta$ ) are activated by diacylglycerol and phospholipids, but they do not respond directly to calcium; classical PKCs ( $\alpha$ ,  $\beta$  and  $\gamma$ ) are calcium-, phospholipid-, and diacylglycerol-dependent; and atypical PKCs ( $\zeta$  and  $\lambda$ ) are calcium and diacylglycerol-independent and undergo allosteric activation (Nishizuka 1992, Rosse, Linch et al. 2010). PKC $\alpha$ ,  $\beta$ II,  $\delta$ ,  $\epsilon$ ,  $\eta$  and  $\zeta$  are expressed in human epidermal keratinocytes (Gherzi, Sparatore et al. 1992, Matsui, Chew et al. 1992, Hara, Saito et al. 2005). Several studies have emphasized the important role of PKCs in skin biology and pathogenesis of skin conditions (Denning, Dlugosz et al. 1995, Denning, Wang et al. 2002, Dlugosz, Yuspa 1994, Dlugosz, Yuspa 1991). Luciferase assays revealed that the novel PKCs were solely associated with activation of involucrin promoter activity. Additionally, overexpression of novel PKC isoforms also resulted in increased involucrin levels. Interestingly, induction of gene expression is not affected by a conventional PKC inhibitor Go-6976 while it dramatically reduces on treatment with rottlerin, a selective PKC $\delta$  inhibitor (Efimova, Eckert 2000). Subsequent studies identified that overexpression of novel PKCs

resulted in increased p38 activity and simultaneous reduction in ERK1/2 activity. No changes were observed in total MAPK levels. The p38 MAPK isoforms predominantly present in the keratinocytes are p38 $\alpha$ , p38 $\beta$  and p38 $\delta$  (Dashti, Efimova et al. 2001b). An important finding was that the novel PKCs selectively activated p38 $\delta$  resulting in increased C/EBP  $\alpha$  levels and binding to the involucrin promoter resulting in increased involucrin gene expression (Efimova, Deucher et al. 2002). The novel PKCs, PKC $\delta$  and  $\eta$ , MEKK1, MEK3, MEK6 and p38 $\delta$  serve as critical mediators of the response to differentiation agents in human epidermal keratinocytes (Adhikary, Chew et al. 2010). Another significant study reported the existence of the p38 $\delta$ -ERK1/2 complex which serves as a signaling nexus or control point. Differentiation signals result in activation of p38 $\delta$  as opposed to ERK1/2 resulting in increased expression of C/EBP and AP1 levels (Efimova, Broome et al. 2003, Balasubramanian, Efimova et al. 2002). Recent studies by Kanade et al have identified the protein arginine methyl transferase 5 (PRMT5) as a novel part of the p38 $\delta$ -ERK1/2 complex. PRMT5 expression resulted in inhibition of p38 $\delta$  activity and also blocked differentiation agent dependent activation of involucrin promoter activity emphasizing its role as an anti-differentiation factor. Interestingly, PKC $\delta$  resulted in decreased PRMT5 levels and enzymatic activity (Kanade, Eckert 2012). These studies highlight the dynamic nature of the p38 $\delta$ -ERK1/2 complex in regulating keratinocyte cell fate. Identification of additional members of this complex is currently under investigation. MAPK cascades have also been implicated in the regulation of other differentiation markers like small proline rich protein 1B (SPRR1B) and cystatin A (Takahashi, Honma et al. 2001, Vuong, Patterson et al. 2000).

Increased differentiation and cessation of proliferation are parallel events and few reports have suggested that a common pathway regulates these processes. The novel PKC  $\eta$ , a known regulator of keratinocyte differentiation has also been shown to suppress proliferation by inhibiting cdk2 activity by forming a complex with cyclin E/cdk2/p21 (Kashiwagi, Ohba et al. 2000). Additionally, PKC $\eta$  has been shown to form a complex with Fyn, a Src kinase which is known to regulate keratinocyte differentiation (Cabodi, Calautti et al. 2000). PKC $\delta$  is another novel PKC isoform which has been shown to regulate both keratinocyte differentiation and proliferation. A recent study by Chew et al showed that PKC $\delta$  regulates keratinocyte proliferation by suppression of p21<sup>Cip1</sup> in a KLF4 dependent mechanism (Chew, Adhikary et al. 2011). KLF4, a member of the Kruppel like factor family of transcription factor is expressed in the suprabasal cells and essential for epidermal barrier formation (Foster, Ren et al. 1999). PKC $\delta$  was shown to increase KLF4 mRNA and protein levels resulting in increased binding on the p21<sup>Cip1</sup> promoter (Chew, Adhikary et al. 2011). Thus keratinocyte cell fate depends on the strength and balance of input from the pro-differentiation or pro-survival cascades.

### ***E. p38 $\delta$***

p38 $\delta$  or (Stress activated protein kinase 4) SAPK4 is the fourth isoform belonging to the family of the p38 family of the mitogen activated protein kinases. The MAP3Ks which activate the p38 MAPKs are MEKK1-4, MLKs (mixed lineage kinases), ASK1 (apoptosis signal-regulating kinase), TAK1 (transforming growth factor  $\beta$  activated kinase 1) and DLK1 (dual leucine zipper bearing kinase) (Cheung, Campbell et al. 2003, Gallo, Johnson 2002, Cuevas, Abell et al. 2007). Further upstream, the GTP binding proteins Rac1 and cdc42 as well as p21 activated kinases (PAKs) regulate p38 MAPK

signaling. The primary MEKs phosphorylating the threonine-180 and tyrosine 182 residues of the p38 MAPKs are MEK3 and MEK6 (Ono, Han 2000). p38 $\delta$  is strongly activated by inflammatory cytokines like TNF $\alpha$  and IL1 $\beta$  and by environmental stressors such as oxidative stress, arsenate, UV radiation and osmotic shock (Jiang, Gram et al. 1997, Wang, Diener et al. 1997a, Goedert, Cuenda et al. 1997). The net effect of p38 signaling is stimulus and cell type dependent potentially due to differential expression patterns of the upstream activators. The p38 MAPKs are distinguished by the presence of a threonine-glycine-tyrosine (TGY) motif in the activation loop (Ono, Han 2000). Based on its amino acid sequence, p38 $\delta$  is 61%, 59% and 65% identical to p38 $\alpha$ , p38 $\beta$  and p38 $\gamma$  respectively (Goedert, Cuenda et al. 1997, Jiang, Gram et al. 1997, Wang, Diener et al. 1997b). The principal difference lies in the sequence of the ATP binding pocket which has implications in substrate specificity and inhibitor sensitivity. p38 $\alpha$  and p38 $\beta$  MAPKs are sensitive to inhibition by pyridinyl-imidazole compounds like SB203580 and SB202190. Threonine 106 in p38 $\alpha$  and p38 $\beta$  is the governing factor which helps orient the drug in the ATP binding pocket leading to inhibition of these isoforms. In p38 $\delta$ , this residue is substituted with a methionine residue which results in sensitivity to the inhibitors (Coulthard, White et al. 2009). Conversely, the amino acid sequence in the kinase domains is highly conserved across all the isoforms (Coulthard, White et al. 2009). p38 $\delta$  is highly abundant in salivary, prostate, adrenal and pituitary glands, skin, pancreas, stomach, colon, trachea and the lung (Wang, Diener et al. 1997a). Interestingly, p38 $\delta$  knockout mice have a normal phenotype, are viable and fertile (Sabio, Arthur et al. 2005). This could be attributed to compensatory mechanisms by the other p38 isoforms namely p38 $\alpha$ ,  $\beta$  and  $\gamma$ . Conversely, p38 $\alpha$  mice are embryonic lethal emphasizing its

contribution to early development (Beardmore, Hinton et al. 2005, Sabio, Arthur et al. 2005).

Few substrates of p38 $\delta$  have been reported. p38 $\delta$  phosphorylates proteins which regulate microtubule assembly. Stathmin, a regulator of microtubule dynamics, is a p38 $\delta$  substrate *in vivo* and *in vitro*. Stathmin is involved in rapid microtubule remodeling (Jourdain, Curmi et al. 1997, Curmi, Andersen et al. 1997). Stathmin interacts with two molecules of  $\alpha$ ,  $\beta$  tubulin forming a ternary complex which leads to depolymerisation of microtubules. It is postulated that stathmin phosphorylation by p38 $\delta$  leads to inhibition of this depolymerisation resulting in enhanced cell survival under stress (Parker, Hunt et al. 1998). Another important substrate of p38 $\delta$  is the microtubule associated protein Tau (Goedert, Hasegawa et al. 1997). Phosphorylation of Tau at threonine-50 was observed in neuroblastoma cells upon osmotic shock. This phosphorylation enhances its ability to assemble microtubules. Subsequent hyperphosphorylation of Tau at additional sites leads to its dissociation from the cytoskeleton resulting in self-assembly and formation of neurofibrillary tangles. The neurofibrillary tangles are an important characteristic of tauopathies, including Alzheimers.

Aberrant p38 $\delta$  signaling has been implicated in several pathogenic conditions. Psoriasis is a hyperproliferative skin condition characterized by increased secretion of inflammatory cytokines. Increased p38 $\delta$  activity as well as p38 $\alpha$  and p38 $\beta$  activity is observed in psoriatic skin lesions as compared to the normal skin counterparts (Haider, Peters et al. 2006). p38 $\delta$  has also been linked to the pathogenesis of diabetes mellitus. In these studies, p38 $\delta$  deficiency resulted in increased protection against high fat induced insulin resistance and oxidative stress stimulated pancreatic  $\beta$  cell death (Sumara,

Formentini et al. 2009). Increased p38 $\delta$  MAPK activity has been observed in type 1 and type 2 diabetes and is associated with complications of hyperglycemia (Price, Agthong et al. 2004, Komers, Lindsley et al. 2007). Protein Kinase D1 (PKD1) is a pivotal regulator of insulin exocytosis and is negatively regulated by p38 $\delta$ . Since p38 $\delta$  integrates insulin secretory pathway and survival of pancreatic  $\beta$  cells, it could serve as an attractive target for diabetes treatment.

p38 $\delta$  protein expression and activity is increased in a variety of cancers like cholangiocarcinoma, liver cancer, head and neck squamous carcinoma (HNSCC) and primary cutaneous squamous carcinoma (Haider, Peters et al. 2006, Junttila, Ala-Aho et al. 2007, Tan, Ooi et al. 2010). p38 $\delta$  has been shown to be an essential regulator of HNSCC proliferation, invasion and motility (Junttila, Ala-Aho et al. 2007). In the multistage chemical carcinogenesis model, p38 $\delta$  MAPK deficient mice showed delayed tumor development with a reduction in tumor number and size as compared to the wild type counterparts (Schindler, Hinds et al. 2009). This was attributed to reduced signaling via the proliferative ERK  $\frac{1}{2}$  pathway and reduction in STAT3 levels. Additionally, p38 $\delta$  knockout mice have shown to be less susceptible to K-Ras driven lung tumorigenesis (Schindler, Hinds et al. 2009). Similar effects were observed in cholangiocarcinoma cells upon silencing of p38 $\delta$  (Tan, Ooi et al. 2010). Conversely, p38 $\delta$  has also been shown to be a tumor suppressor in certain cell types. p38 $\delta$  levels have been reported to be downregulated in brain metastases of triple negative breast cancer (TNBC). Knockdown of p38 $\delta$  leads to reduced cell growth in TNBC (Choi, Woo et al. 2013). Studies by O'Callaghan et al showed that introduction of p38 $\delta$  into cells lacking endogenous p38 $\delta$  resulted in substantial reduction in proliferation, migration and invasion. Pronounced

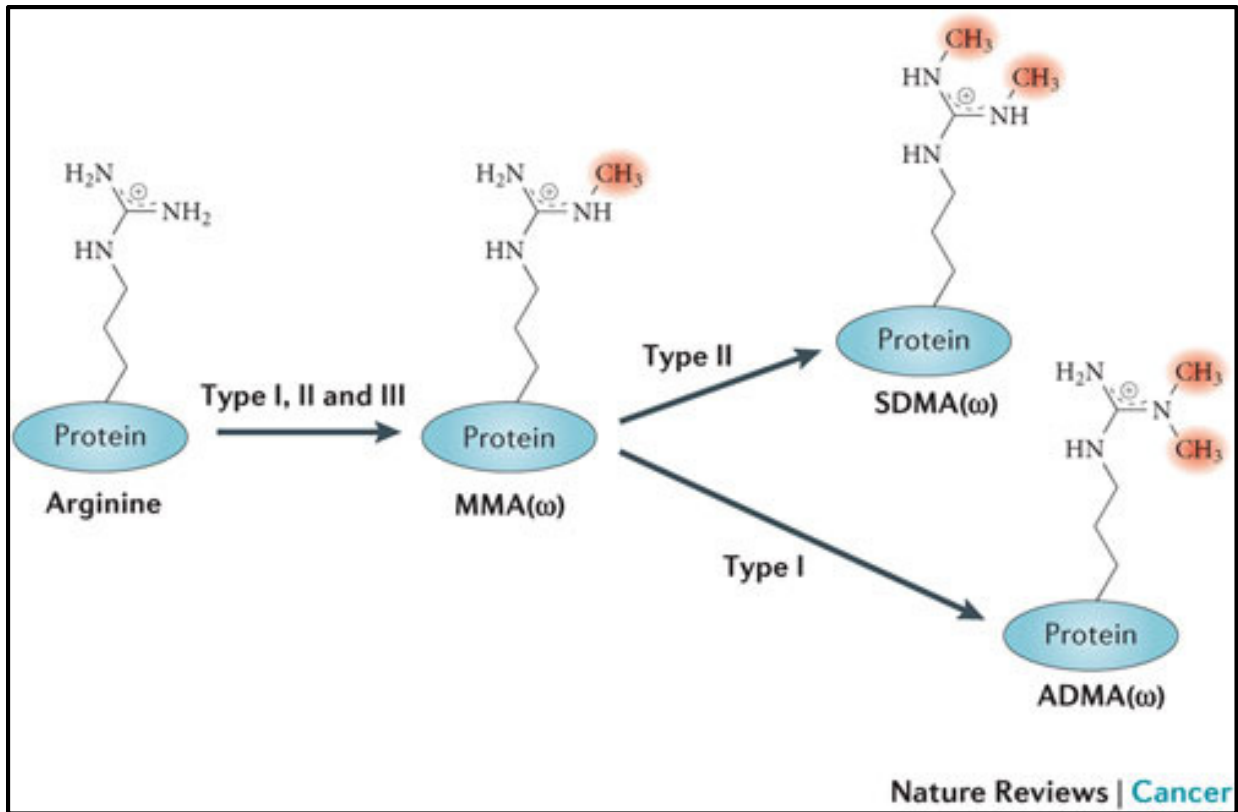
effects were observed on expression of constitutively active p38 $\delta$ . An important mechanism by which cancer cells silence the expression of tumor suppressor genes is via hypermethylation of their promoters (Merlo, Herman et al. 1995). Subsequently, hypermethylation of the MAPK13 gene promoter has been reported in cutaneous melanoma and pleural mesothelioma and correlates with decreased p38 $\delta$  mRNA and protein (Gao, Smit et al. 2013, Goto, Shinjo et al. 2009).

#### ***F. PROTEIN ARGININE METHYLATION:***

The amino acid arginine possesses a guanidino group containing five potential hydrogen bond donors positioned uniquely to mediate hydrogen bonding, aromatic interactions with DNA, RNA and proteins (Bedford, Clarke 2009). Arginine methylation is a post-translational event during which a methyl group is added to the arginine side chain. It is the most quantitatively extensive protein methylation reaction in mammalian cells (Gary, Clarke 1998). Addition of a methyl group modifies the amino acid conformation increasing its hydrophobicity. Simultaneously, it also results in loss of a potential hydrogen bond donor. Thus arginine modification impacts the protein's binding interactions which in turn affects the physiological functions. Three types of arginine methylation have been identified in eukaryotes:  $\omega$ -N<sup>G</sup>, N<sup>G</sup>-asymmetric dimethyl arginine (ADMA),  $\omega$ -N<sup>G</sup>, N<sup>G</sup>-symmetric dimethyl arginine (SDMA) and  $\omega$ -N<sup>G</sup>-monomethyl arginine (MMA) with the ADMA mark being the most prevalent (Najbauer, Johnson et al. 1993, Paik, Kim 1980). The class of proteins which catalyze the arginine methylation reactions are known as protein arginine methyltransferases (PRMTs). The PRMTs are classified as follows: Type I enzymes are PRMT1, PRMT2, PRMT3, PRMT4 (CARM1), PRMT6 and PRMT8 and they catalyze the formation of the ADMA mark. PRMT5 is the

type II enzyme which catalyzes the formation of the SDMA mark (Gary, Clarke 1998). The type III enzyme, PRMT7 catalyzes the formation of the MMA mark. All three types catalyze the MMA mark which serve as precursors for the subsequent ADMA and SDMA mark. **Fig. 1-2** distinguishes the methylation reactions catalyzed by the different PRMTs.

All PRMTs display the motifs of the seven-beta strand methyltransferases, double E (two glutamate residues) and the THW (threonine- histidine-tryptophan) sequence motifs (Bedford, Clarke 2009). The substrates of PRMTs are diverse and include RNA binding proteins, histones and signal transduction proteins such as interferons, cytokines as well as proteins essential for organelle biosynthesis (Bedford, Clarke 2009). Aberrant expression of several PRMTs results in cancer, diabetes and cardiovascular diseases. Thus, PRMTs have the potential of being important therapeutic targets.



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 Yang Y, Bedford MT, Nature Reviews Cancer, 13 (1), 37-50, (2012)

**Figure 1-2: Types of methylation on arginine residues.**

Types I, II and III protein arginine methyltransferases (PRMTs) generate monomethylarginine (MMA) on one of the terminal ( $\omega$ ) guanidino nitrogen atoms. These two nitrogen atoms are equivalent. The subsequent generation of asymmetric dimethylarginine (ADMA) is catalysed by type I enzymes (PRMT1, PRMT2, PRMT3, co-activator-associated arginine methyltransferase 1 (CARM1), PRMT6 and PRMT8), and the production of symmetric dimethylarginine (SDMA) is catalysed by type II enzymes (PRMT5 and PRMT7). On certain substrates, PRMT7 functions as a type III enzyme, which only generates MMA products. PRMT9 activity has not yet been characterized. To date, no enzyme has been found that forms both ADMA and SDMA modifications. These arginine methylation marks are regarded as very stable, and an enzyme (or enzymes) with clear demethylase activity has not yet been identified.

### ***G. PRMT5:***

Protein arginine methyltransferase 5 (PRMT5), a major type II methyltransferase; was initially identified as a part of the yeast two-hybrid assay to identify Janus Kinase 2 (JAK2) binding protein (Pollack, Kotenko et al. 1999). PRMT5 plays a pivotal role in embryonic development as PRMT5 null mice are embryonic lethal between E 3.5 and 6.5. This is attributed to the fact that PRMT5 knockdown resulted in downregulation of pluripotent genes and upregulation of differentiation-associated genes (Tee, Pardo et al. 2010). It was first identified as a transcriptional repressor as it catalyzed the formation of key repressive histone marks H4R3me2s and H3R8me2s (Fabbrizio, Messaoudi et al. 2002, Pal, Baiocchi et al. 2007a). Its role as a transcriptional regulator can be methyltransferase dependent or independent. PRMT5 interacts with chromatin remodeling complexes like SWI/SNF, NuRD and regulates transcription in a methyltransferase dependent manner to repress the transcription of suppressor of growth inhibitory genes like tumorigenicity (ST7), NM23-H1, p16<sup>INK4A</sup> and cyclin E1 (Wang, Pal et al. 2008a, Pal, Baiocchi et al. 2007b, Pal, Vishwanath et al. 2004). Conversely, PRMT5 regulates androgen receptor mediated transcription independent of its methyltransferase activity. Mutation of the key arginine residue R368 resulted in loss of its methyltransferase activity but no change in PRMT5 mediated AR promoter activity (Hosohata, Li et al. 2003).

PRMT5 plays a crucial role in cell transformation. PRMT5 overexpression or increased activity has been observed in several transformed cell lines such as melanoma, lymphoma, leukemia, breast, prostate, colorectal, gastric and lung cancer (Pal, Sif 2007, Pal, Baiocchi et al. 2007b, Powers, Fay et al. 2011, Wang, Pal et al. 2008b, Gu, Li et al.

2012, Wei, Juan et al. 2012a, Kim, Sohn et al. 2005, Zhongping, Shen et al. 2012). PRMT5 knockdown results in cessation of cell proliferation and its overexpression leads to cellular hyperproliferation (Wang, Pal et al. 2008b, Pal, Baiocchi et al. 2007b). PRMT5 has been shown to play a role in regulating epithelial to mesenchymal transition (EMT); a process which enables the cancer cells to migrate and invade effectively. Loss of E-cadherin is an important feature of EMT. SNAIL is an important transcription factor which regulates EMT by transcriptionally repressing E-cadherin. PRMT5 interacts with SNAIL via an adaptor molecule AJUBA resulting in suppression of E-cadherin. Conversely, increased E-cadherin expression was observed on PRMT5 knockdown (Hou, Peng et al. 2008). p53 is another important PRMT5 substrate. Upon DNA damage, tumor suppressor p53 is methylated by PRMT5 at R333, R335 and R337. Interestingly, this p53 triple mutant retains its apoptotic function but is unable to induce G1 cell cycle arrest (Jansson, Durant et al. 2008). Thus PRMT5 abrogation in combination with chemotherapy might be a potential approach to promote apoptosis of cancer cells. Programmed cell death protein 4 (PDCD4) is a tumor suppressor which is a prognostic indicator in several cell types (Lankat-Buttgereit, Göke 2009). Interestingly, PDCD4 can be oncogenic under certain circumstances and studies by Powers et al showed that PRMT5 might be involved in this alteration. Their studies revealed that PRMT5 binds and methylates PDCD4 at its N-terminus GAR motif. Additionally, overexpression of PRMT5 and PDCD4 enhanced tumor growth in an orthotropic model of breast cancer and this was contingent on the presence of PRMT5 as well as its target residue (Powers, Fay et al. 2011). The other proteins which impact cell transformation and are methylated by PRMT5 include E2F, RAD9, FEN1, p65 subunit of NF $\kappa$ B and EGFR (Guo, Zheng et

al. 2010, Cho, Zheng et al. 2012, Hsu, Chen et al. 2011, Wei, Wang et al. 2013, He, Ma et al. 2011).

PRMT5 and MEP50 exist in the form of tetramer of heterodimers containing four copies each of MEP50 and PRMT5 (Antonyamy, Bonday et al. 2012, Ho, Wilczek et al. 2013). MEP50 binds and positions the arginine containing substrate to the PRMT5 catalytic site (Ho, Wilczek et al. 2013). MEP50, a WD-40 protein is an important PRMT5 partner and essential for its enzymatic activity (Ho, Wilczek et al. 2013, Krause, Yang et al. 2007a). Studies by Liu et al revealed that phosphorylation of PRMT5 by mutant JAK kinases leads to disruption of the MEP50-PRMT5 interaction resulting in impaired methyltransferase activity and tumorigenesis. Conversely, phosphorylation of MEP50 by cyclin D1-CDK4 was found to increase the activity of PRMT5 leading to repression of culins CUL4A and CUL4B. These encode the scaffolding proteins for E3 ubiquitin ligase to direct the degradation of the replication licensing protein CDT1. Stabilization of CDT1 leads to increased neoplastic growth (Aggarwal, Vaiteas et al. 2010).

The versatile nature of PRMT5 function is evident by the fact that it participates in Golgi apparatus assembly and ribosome biogenesis. Studies by Zhou et al showed that PRMT5 localizes in the Golgi and methylates GM130 at R6, R18 and R23. GM130 is a matrix protein which controls the Golgi architecture by regulating stacking and ribbon assembly. Substitution of the arginine residues with lysines affected the Golgi architecture. An interesting finding was that knockdown of PRMT5 lead to fragmented Golgi apparatus further ratifying the contribution of PRMT5 (Zhou, Sun et al. 2010). PRMT5 has been shown to regulate ribosome biogenesis by methylating ribosomal

protein S10 (RPS10); a component of the ribosome 40S subunit which is essential for ribosome assembly and function (Ren, Wang et al. 2010).

The MAPK p38 $\delta$  exists in a complex with ERK and the balance between ERK and p38 $\delta$  governs keratinocyte survival status. ERK activation leads to keratinocyte survival and proliferation while p38 $\delta$  activation leads to keratinocyte differentiation or apoptosis (Efimova, Broome et al. 2004a). A recent study by Kanade et al showed that PRMT5 exists in a complex with p38 $\delta$  and regulates its activity. These studies also revealed that PRMT5 negatively impacts involucrin expression which is an early marker of keratinocyte terminal differentiation (Kanade, Eckert 2012). This was the first study which showed the function of PRMT5 in primary human epidermal keratinocytes.

#### ***H. MEP50:***

Methylosome protein 50 (MEP50), also known as WDR77 or as the androgen receptor cofactor p44. It is a WD40 protein with 7 WD domains and highly expressed in the heart, skeletal muscle, spleen, thymus, prostate, testis and the uterus (Hosohata, Li et al. 2003). WD proteins form a  $\beta$ -propeller structure which facilitates protein-protein and protein-DNA interactions (Li, Roberts 2001). MEP50 plays crucial roles in cellular processes ranging from receptor co-activator, transcriptional regulation, cell proliferation and mRNA splicing (Hosohata, Li et al. 2003, Gu, Zhang et al. 2013, Friesen, Wyce et al. 2002). It is the primary binding partner of PRMT5 and contributes to its increased stability and methyltransferase activity (Antonysamy, Bonday et al. 2012). The MEP50-PRMT5 complex has also been reported in *Xenopus laevis* and *Drosophila* (Anne, Mechler 2005, Wilczek, Chitta et al. 2011). In humans, the MEP50-PRMT5 complex exists as a hetero-octamer with PRMT5 molecules forming a tetramer at the center and

the four MEP50 molecules interacting with the TIM barrel domains of PRMT5 (Antonyamy, Bonday et al. 2012).

MEP50 is a 342 amino acid protein that was originally identified as a non-catalytic component of the protein methyltransferase complex known as the methylosome (Friesen, Wyce et al. 2002). The methylosome regulates snRNP assembly by post-translationally modifying the Sm proteins to contain SDMA. This facilitates SMN binding to the symmetrically methylated RG domains to be incorporated in the spliceosomal snRNP. MEP50 interacts with the other components of the methylosome namely PRMT5 and pICln and modulates methylosome activity (Friesen, Wyce et al. 2002) MEP50 facilitates Sm protein methylation by positioning them effectively for methylation by PRMT5.

The role of MEP50 in primary cells has been limited to the prostate and lung epithelial cells. Nuclear expression of MEP50 in the prostate epithelial cells regulates prostate function and differentiation via expression of the AR dependent genes (Zhou, Wu et al. 2006). Targeted deletion of MEP50 in the mouse prostate lead to impaired prostate development reflected by reduced number of terminal branches and duct tips in the anterior prostate (AP), ventral prostate (VP) and dorsolateral (DLP) lobes (Gao, Wu et al. 2010). Homozygous MEP50 deletion also resulted in prostatic hyperplasia (Gao, Wu et al. 2010). Studies in lung epithelial cells revealed that MEP50 expression correlated with proliferation in these cells. Additionally, MEP50 deletion dramatically reduced the growth of murine lung epithelial cells (Gu, Zhang et al. 2013). There is increasing evidence of the role of MEP50 in cancer formation. It was initially identified as an androgen receptor (AR) cofactor and an estrogen receptor (ER) cofactor in prostate

and breast cancer respectively (Peng, Chen et al. 2008, Peng, Li et al. 2010). MEP50 leads to transcriptional activation of AR regulated genes in an androgen dependent manner (Hosohata, Li et al. 2003). Another interesting facet of MEP50 function in sex hormone responsive tissues is its subcellular localization. MEP50 is found in the cytoplasm of prostate cancer (Peng, Chen et al. 2008) and testicular tumor (Liang, Wang et al. 2007) while it exhibits a nuclear localization pattern in breast (Peng, Li et al. 2010) and ovarian cancer (Ligr, Patwa et al. 2011). Forced expression of cytosolic targeted MEP50 leads to significant increase in prostate cancer cell numbers while the reverse trend is observed with forced expression of the nucleus localized mutant form of MEP50. The nuclear localized MEP50 inhibited cell proliferation by targeting p21 and p27 while the cytosolic MEP50 promoted cell proliferation by regulating cyclin D2 and cyclin dependent kinase 6 (CDK6) (Peng, Chen et al. 2008). There is evidence of the role of MEP50 in AR and ER independent cell lines. In lung adenocarcinoma cell lines, MEP50 is distributed in the nucleus as well as the cytoplasm. It is overexpressed in lung cancers and is essential for proliferation of lung cancer cells (Gu, Zhang et al. 2013, Wei, Hsia et al. 2014). MEP50 leads to G1-S cell cycle progression by negatively regulating the p21-retinoblastoma pathway (Gu, Zhang et al. 2013). Additionally, MEP50 was identified as a biomarker for colon cancer (Yan, Li et al. 2012).

## ***I. SUMMARY AND GOALS OF THE STUDY:***

The skin is a stratified squamous epithelium and is tightly regulated by the process of terminal differentiation. The efficient formation and function of the epidermis entails a perfect balance between differentiation and proliferation. Any imbalance in this homeostasis leads to diseases of cornification or hyperproliferation. The PKC $\delta$ -MEKK1-MEK3-p38 $\delta$  pathway is a crucial signaling cascade which regulates keratinocyte differentiation by regulating involucrin expression (Efimova, LaCelle et al. 1998, Efimova, Eckert 2000). This cascade directs specific transcription factors to regulatory regions on the involucrin promoter. Since triggering the differentiation pathway is associated with cessation of proliferation, it is imperative that a single cascade regulates both the processes. Very few reports have reported the involvement of novel PKCs in regulation of keratinocyte proliferation. Previous studies have indicated that Sp1 factors and KLF4 play a role in PKC $\delta$  mediated regulation of p21<sup>Cip1</sup> (Chew, Adhikary et al. 2011). Since p21<sup>Cip1</sup> is a major regulator of keratinocyte proliferation, multiple pathways might mediate its expression. The focus of our first study is to identify additional mediators of the PKC $\delta$  pathway. We present evidence of the involvement of MEK3, p38 $\delta$  and p53 as additional downstream factors in the PKC $\delta$ -p21<sup>Cip1</sup> pathway.

The MAPK p38 $\delta$ /ERK1/2 complex serves as a key signaling control point in keratinocytes (Efimova, Broome et al. 2003). Elevated p38 $\delta$  activity inversely correlates with ERK1/2 activity resulting in increased AP1 factor levels and interaction with the involucrin promoter. This pathway is significant in keratinocytes as several differentiation markers such as loricrin and filaggrin are also regulated by this mechanism. The importance of this complex in keratinocyte biology suggests that several

additional proteins might exist with p38 $\delta$  and ERK1/2 in a multiprotein complex. An important goal is to identify and characterize the function of new players both in the context of epidermal homeostasis and potential therapeutic targets in disease. Previous studies reported that PRMT5, a type II protein arginine methyltransferase is a component of this complex, antagonizes p38 $\delta$  activity and negatively regulates involucrin levels (Kanade, Eckert 2012). This was an important finding as this was the first study linking PRMT5 to regulation of epidermal homeostasis. MEP50, a WD40 protein is a crucial binding factor of PRMT5 and is essential for its enzymatic activity (Krause, Yang et al. 2007b). The goal of our second and third study is to determine the localization and function of MEP50 in regulating keratinocyte proliferation and differentiation. Also, we aim to identify the pathways regulating MEP50 expression in our model system.

## CHAPTER 2: p38 $\delta$ DEPENDENT REGULATION OF p21<sup>Cip1</sup> IN HUMAN EPIDERMAL KERATINOCYTES

### A. ABSTRACT

PKC $\delta$  suppresses keratinocyte proliferation via a mechanism that involves increased expression of p21<sup>Cip1</sup>. However, the signaling mechanism that mediates this regulation is not well understood. Our present studies suggest that PKC $\delta$  activates p38 $\delta$  leading to increased p21<sup>Cip1</sup> promoter activity and p21<sup>Cip1</sup> mRNA/protein expression. We further show that exogenously expressed p38 $\delta$  increases p21<sup>Cip1</sup> mRNA and protein, and that p38 $\delta$  knockdown or expression of dominant-negative p38 attenuates this increase. Moreover, p53 is an intermediary in this regulation, as p38 $\delta$  expression increases p53 mRNA, protein and promoter activity and p53 knockdown attenuates the activation. We demonstrate a direct interaction of p38 $\delta$  with PKC $\delta$  and MEK3, and show that exogenous agents which suppress keratinocyte proliferation activate this pathway. We confirm the importance of this regulation using a stratified epidermal equivalent model, that mimics *in vivo*-like keratinocyte differentiation. In this model, PKC $\delta$  or p38 $\delta$  knockdown results in reduced p53 and p21<sup>Cip1</sup> level and enhanced cell proliferation. We propose that PKC $\delta$  activates a MEKK1/MEK3/p38 $\delta$  MAPK cascade to increase p53 level and that p53 interacts with the p21<sup>Cip1</sup> gene to drive expression.

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<sup>2</sup> CITATION: Saha K, Adhikary G, Kanade S. R, Rorke EA, Eckert R. L. JBC 2014 Apr 18;289(16):11443-53.

## ***B. INTRODUCTION***

Protein kinase C (PKC) isoforms play a key role as regulators of cell differentiation (Newton 1997). PKCs include three families. The novel PKCs (nPKC  $\delta$ ,  $\epsilon$ ,  $\eta$ , and  $\theta$ ) are activated by diacylglycerol and phospholipids, but they do not respond directly to calcium; classical PKCs ( $\alpha$ ,  $\beta$  and  $\gamma$ ) are calcium-, phospholipid-, and diacylglycerol-dependent; and atypical PKCs ( $\zeta$  and  $\lambda$ ) are calcium and diacylglycerol-independent and undergo allosteric activation (Nishizuka 1992, Rosse, Linch et al. 2010). PKC $\alpha$ ,  $\beta$ II,  $\delta$ ,  $\epsilon$ ,  $\eta$  and  $\zeta$  are expressed in human epidermal keratinocytes (Gherzi, Sparatore et al. 1992, Matsui, Chew et al. 1992, Hara, Saito et al. 2005). PKC $\delta$  stimulates keratinocyte differentiation (Denning, Dlugosz et al. 2000, Denning 2004, Adhikary, Chew et al. 2010, Sonkoly, Wei et al. 2009, Papp, Czifra et al. 2004, Balasubramanian, Efimova et al. 2002, Kamioka, Akahane et al. 2010) by activating MAPK signaling to increase nuclear levels of key transcription factors which act to increase target gene transcription (Efimova, Eckert 2000, Eckert, Crish et al. 2003a, Efimova, Deucher et al. 2002). PKC isoforms also regulate keratinocyte proliferation by altering cell cycle control protein expression (Papp, Czifra et al. 2004, Bollag, Dodd et al. 2004, Balasubramanian, Efimova et al. 2002, Praskova, Kalenderova et al. 2002, Wheeler, Reddig et al. 2002, Bollag 2009). Since increased keratinocyte differentiation is associated with cessation of proliferation and it makes mechanistic sense that a common signaling cascade may control these processes. p21<sup>Cip1</sup> is an important suppressor of cell cycle progression and cell proliferation (Gorospe, Wang et al. 1999) and is a key PKC $\delta$  target in keratinocyte (Chew, Adhikary et al. 2011). Moreover, increased p21<sup>Cip1</sup> expression suppresses keratinocyte proliferation (Wong, Pickard et al. 2010, Cheng, McLaughlin et al. 2009,

Aliouat-Denis, Dendouga et al. 2005, Devgan, Mammucari et al. 2005, Okuyama, LeFort et al. 2004, Hauser, Ma et al. 2004).

However, in spite of this progress, we have a limited understanding of the mechanisms whereby PKC $\delta$  increases p21<sup>Cip1</sup> level. Our previous study indicates that Kruppel-like factor 4 is a downstream mediator of PKC $\delta$  action which increases p21<sup>Cip1</sup> expression, and that this involves KLF4 interaction at DNA sites located in the proximal promoter of the p21<sup>Cip1</sup> gene (Chew, Adhikary et al. 2011). Our present studies identify a second pathway that mediates PKC $\delta$  action. This involves PKC $\delta$ -dependent activation of the p38 $\delta$  kinases to activate p53 expression which interacts via the p53 sites in the distal p21<sup>Cip1</sup> promoter to drive transcription. Moreover, we confirm that this regulation is physiologically meaningful using a stratifying epidermal equivalent culture model that mimics *in vivo* epidermal differentiation. Knockdown of p38 $\delta$  in this model results in reduced p21<sup>Cip1</sup> expression, enhanced cell proliferation and reduced differentiation.

## ***C. MATERIALS AND METHODS***

### **1. Chemicals, Reagents and Antibodies**

Rabbit polyclonal antibodies for MEK3 (sc-961), PKC $\delta$  (sc-937), p53 (sc-6243), goat anti-p38 $\delta$  (sc-7587), and mouse monoclonal antibodies for p38 $\delta$  (sc-271292), p38 $\alpha$  (sc-7972) and anti-MEK3-*P* (sc-8407) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-MEKK1 (ab69533) and anti-MEKK1-*P* (T1381) (ab138662) were purchased from Abcam (Cambridge, MA). Rabbit polyclonal antibodies against p21<sup>Cip1</sup> (2947) and PKC $\delta$ -*P*(Tyr311) were obtained from Cell Signaling Technology (Danvers, MA) and mouse monoclonal antibody against  $\beta$ -actin (A-5441) and anti-FLAG M2 (F3165) were purchased from Sigma Aldrich. Peroxidase-conjugated anti-mouse IgG (NXA931) and peroxidase conjugated anti-rabbit IgG (NA934V) were obtained from GE Healthcare. Phorbol ester, 12-O-tetradecanoylphorbol-13-acetate (TPA) (524400) and rottlerin (557370) were obtained from Calbiochem (Billerica, MA).

We report results using control (sc-37007), p38 $\delta$  (sc-36456), PKC $\delta$  (sc-36253), p53 (sc-44218) and MEK3 (sc-35907) siRNA reagents obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Key findings were confirmed using additional siRNA that targets p38 $\delta$  (D-003591-01-0005 and D-003591-03-0005), PKC $\delta$  (D-003524-03-0005 and D-003524-05-0005), MEK3 (D-003509-02-0005 and D-003509-06-0005) and p53 (D-003329-05-0005 and D-003329-07-0005) which were purchased from Dharmacon, Inc (Lafayette, CO).

## **2. Cell Culture, Viruses and Plasmids**

Keratinocyte serum-free medium (KSFM), trypsin and Hank's balanced salt solution were purchased from Invitrogen (Frederick, MD). Primary cultures of human epidermal keratinocytes were obtained by separation of epidermis from the dermis with dispase followed by cell dispersion in trypsin. Cells were cultured in KSFM supplemented with epidermal growth factor and bovine pituitary extract (Efimova, Broome et al. 2003, Efimova, Broome et al. 2004b). Adenoviruses encoding MEK3, HA-p38 $\delta$ , PKC $\delta$  and empty control virus (Ad5-MEK3, tAd5-HA-p38 $\delta$ , Ad5-PKC $\delta$  and Ad5-EV) were prepared by propagation in HEK293 cells followed by cesium chloride gradient centrifugation. For experiments, keratinocytes were incubated with 5 to 15 MOI of adenovirus in KSFM containing 6  $\mu$ g/ml polybrene. Tetracycline-inducible viruses (tAd-EV, tAd5-PKC $\delta$ , tAd5-HA-38 $\delta$ ) were co-infected with Ad5-TA encoding virus and treated with doxycycline to induce to induce PKC $\delta$  and p38 $\delta$  expression (Adhikary, Chew et al. 2010).

The human p21<sup>Cip1</sup> promoter luciferase plasmid, p21-2326 was a gift from Dr. Bert Vogelstein (El-Deiry, Tokino et al. 1993). p21-124, p21-101 and p21-60 were obtained from Dr. Toshiyuki (Nakano, Mizuno et al. 1997). The other p21<sup>Cip1</sup> truncation plasmids (p21-251, p21-501, p21-1001 and p21-2001) and the p53 site mutants, p21-2326 p53( $\Delta$ 1), p21-2326 p53( $\Delta$ 2), and p21-2326 p53( $\Delta$ 1-2) were constructed in pBluescript II KS(+) (Chew, Adhikary et al. 2012). The p38 $\delta$  expression vector was pcDNA3.1-HA-p38 $\delta$ . p38 $\alpha$ , MEK3, PKC $\delta$ , and FLAG-DNp38 $\alpha$  expression plasmids were previously described (Balasubramanian, Zhu et al. 2006, Efimova, Broome et al. 2003). PG13-Luc was obtained from Dr. Nancy Colburn (Li, Cao et al. 2000).

### **3. Statistical methods**

All experiments were performed a minimum of three times and significance difference was determined using the Student's t-test.

### **4. p21<sup>Cip1</sup> promoter activity assay**

p21<sup>Cip1</sup> promoter reporter plasmid (1 µg) was mixed with 2 µl of FuGENE-6 (Roche Applied Science) diluted with 98 µl of KSFM. The mixture was incubated for 25° C for 20 min and added to a 50% confluent culture of human epidermal keratinocytes maintained in KSFM in a 9.6 cm<sup>2</sup> dish. For co-transfection experiments, 1 µg of p21<sup>Cip1</sup> promoter reporter plasmid and 1 µg of pcDNA3.1 or pcDNA3.1-HA-p38δ were mixed with FuGENE 6 and added to the cells. After 24 h, the cells were harvested for luciferase activity assay, and data was normalized based on protein content.

### **5. siRNA mediated knockdown**

Keratinocytes were electroporated with control- or p38δ-siRNA using an Amaxa electroporator and the VPD-1002 nucleofection kit (Amaxa, Cologne, Germany). Keratinocytes were harvested with trypsin and replated 1 day prior to electroporation. After 24 h, the keratinocytes were harvested with trypsin and 1 x 10<sup>6</sup> cells were centrifuged at 2000 rpm, washed in 1 ml sterile 1 x phosphate buffered saline (pH = 7.5) and suspended in 100 µl of keratinocyte nucleofection solution. Control- or p38δ-siRNA (3 µg) was added to the cell suspension, mixed by gentle pipetting and electroporated using the T-018 setting. KSFM (500 µl) was added and the suspension was transferred to a 60 mm dish containing 2 ml KSFM medium. The cells were maintained for various times before extracts were prepared for preparation of RNA or protein or activity assay.

This electroporation method delivers nucleic acid reagents to cells with greater than 90% efficiency (Adhikary, Chew et al. 2010).

## **6. Immunoblot analysis**

Equal amounts of protein were electrophoresed on a 12% denaturing polyacrylamide gel and transferred to nitrocellulose. The membranes were blocked with 5% skimmed milk in Tris-buffered saline (pH 7.5) containing 0.1% Tween 20 for 1 h. Following this, the blots were incubated with primary antibody, washed and incubated with the appropriate horseradish peroxidase conjugated secondary antibody for 2 h. Chemiluminescent detection (Amersham Biosciences) was used to detect antibody binding.  $\beta$ -actin was used as the loading control in all immunoblot experiments.

## **7. Quantitative RT-PCR**

Total RNA was isolated using Illustra RNAspin Mini Kit (GE Healthcare) and 1  $\mu$ g RNA was used for cDNA synthesis. Gene expression was measured by real time PCR using Light Cycler 480 SYBR Green I Master Mix (04-707 516 001) from Roche Diagnostics (Indianapolis, Indiana). The signals were normalized using cyclophilin A control primers. The gene specific primers used for detection of mRNA levels were as follows: p38 $\delta$  (forward, 5'-TGT GCA GAA GCT GAA CGA CAA AGC; reverse, 5'-AGG GTT CAA AGA AGG GAT GGG TGA), p21<sup>Cip1</sup> (forward, 5'-AAG ACC ATG TGG ACC TGT CAC TGT; reverse, 5'-AGG GCT TCC TCT TGG AGA AGA TCA), PKC $\delta$  (forward, 5'-GGC CAC ATC AAG ATT GCC GAC TTT; reverse, 5'-ACT GGC CAA TGA GCA TCT CGT ACA), MEK3 (forward, 5'-AGC TCA TGG ACA CAT CCT TGG ACA; reverse, 5'-ACA CAT CTT CAC ATG GCC CTC CTT) and cyclophilin A

(forward, 5'-CAT CTG CAC TGC CAA GAC TGA; reverse, 5'-TTC ATG CCT TCT TTC ACT TTG C).

## **8. p38 $\delta$ activity assay**

Kinase assays were used to determine the activity of the endogenous p38 $\delta$  isoform. Keratinocyte cell lysates were prepared under nondenaturing conditions. Equal amounts of total protein (200  $\mu$ g) were used per each kinase assay. p38 $\delta$ -specific antibodies (sc-7587 or sc-271292) were used to selectively immunoprecipitate this enzyme. The precipitated kinase was then assayed for ability to phosphorylate ATF-2 in an *in vitro* kinase reaction performed in the presence of ATP. Phosphorylation of the substrate proteins was analyzed by immunoblot using phosphorylated ATF-2-specific antibody (Efimova, Broome et al. 2003, Efimova, LaCelle et al. 1998) .

## **9. Epidermal equivalent cultures**

Keratinocytes, freshly isolated from foreskins, were harvested with trypsin and  $1.5 \times 10^6$  cells were electroporated with 3  $\mu$ g of control- or p38 $\delta$ -siRNA and replated. After an additional 72 h, the cells were harvested and  $2 \times 10^6$  cells from each group were re-electroporated with 3  $\mu$ g of control-siRNA or p38 $\delta$ -siRNA. They were then allowed to settle overnight onto the membrane in Millicell-PCF chambers (diameter 12 mm, 0.4  $\mu$ m pore size) in KSFM (Millipore, Billerica, MA). The next day, the cells were shifted to Epilife medium containing 1.4 mM calcium chloride and 5  $\mu$ g/ml of vitamin C and cultured at the air-liquid interface with addition of fresh Epilife medium every two days. After 4 d, the epidermal equivalents were harvested for preparation of RNA, protein and histological sections (Chew, Adhikary et al. 2013). Total RNA was isolated for qRT-PCR

using the Illustra RNAspin Mini kit (GE Healthcare). For protein lysates, the inserts were washed twice with PBS and the cells were harvested in 0.0625 M Tris-HCl pH 7.5 containing 10% glycerol, 5% SDS, 5% and  $\beta$ -mercaptoethanol. They were sonicated and centrifuged at 10,000 rpm for 5 min, and the supernatant was collected for immunoblot.

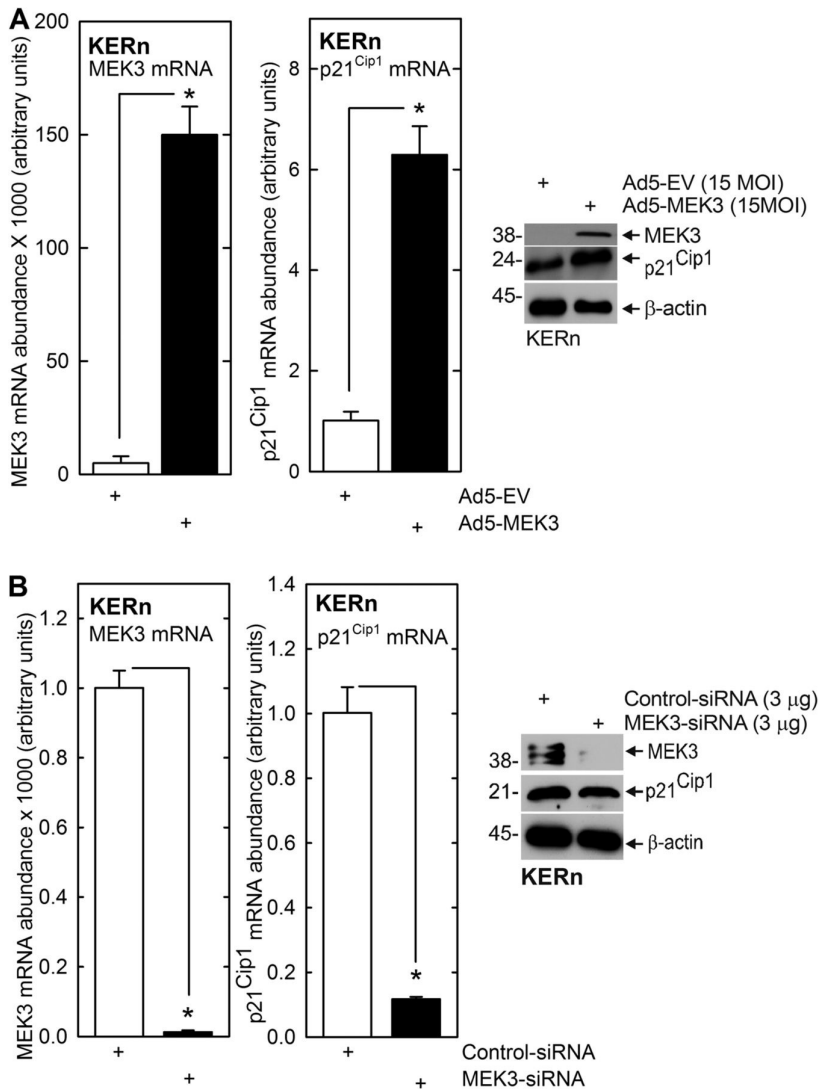
## ***D. RESULTS***

### **1. PKC $\delta$ , MEK3 and p38 $\delta$ regulate p21<sup>Cip1</sup> level**

PKC $\delta$  regulates keratinocyte proliferation by increasing p21<sup>Cip1</sup> expression via mechanisms that are not well understood (Chew, Adhikary et al. 2011). We propose that MEK3 and p38 $\delta$  may be the intervening signaling kinases that mediate this regulation. To test this, we expressed MEK3 and p38 $\delta$  and monitored the impact on p21<sup>Cip1</sup> expression. **Fig. 2-1A** and **B** show that p21<sup>Cip1</sup> expression is directly correlated with MEK3 level. MEK3 expression increases and MEK3 knockdown reduces p21<sup>Cip1</sup> mRNA and protein level. The impact on p21<sup>Cip1</sup> mRNA level is particularly dramatic. We next examined the role of p38 $\delta$ . Three p38 MAPK isoforms, p38 $\alpha$ ,  $\beta$  and  $\delta$ , are expressed in keratinocytes (Dashti, Efimova et al. 2001a, Dashti, Efimova et al. 2001b), and MEK3 activates p38 $\delta$  to stimulate differentiation-associated gene expression (Efimova, Deucher et al. 2002, Efimova, LaCelle et al. 1998). We therefore assessed whether p38 $\delta$  plays a role in regulating p21<sup>Cip1</sup> level. **Fig. 2-1 A** shows that siRNA dependent knockdown of p38 $\delta$  results in a marked reduction in p21<sup>Cip1</sup> encoding mRNA and p21<sup>Cip1</sup> protein. If p38 $\delta$  is a mediator in this pathway, we would expect that PKC $\delta$  and MEK3 should increase p38 $\delta$  activity. This was studied by measuring the ability of p38 $\delta$  to phosphorylate ATF2 transcription factor on threonine 71 (Efimova, Deucher et al. 2002, Efimova, Broome et al. 2003, Efimova, LaCelle et al. 1998). Cells were infected with empty-, PKC $\delta$ -, or MEK3-encoding adenovirus and after 24 h endogenous p38 $\delta$  was immunoprecipitated, and ability of precipitated p38 $\delta$  to phosphorylate ATF2 was measured. We used two antibodies to pull-down p38 $\delta$  - one prepared in goats and a second in mice. This analysis

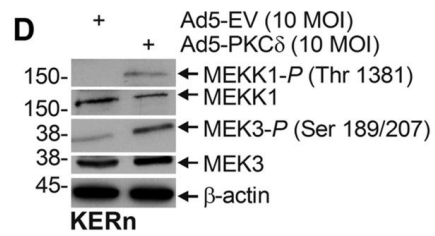
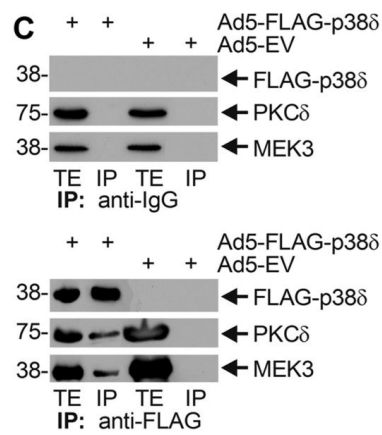
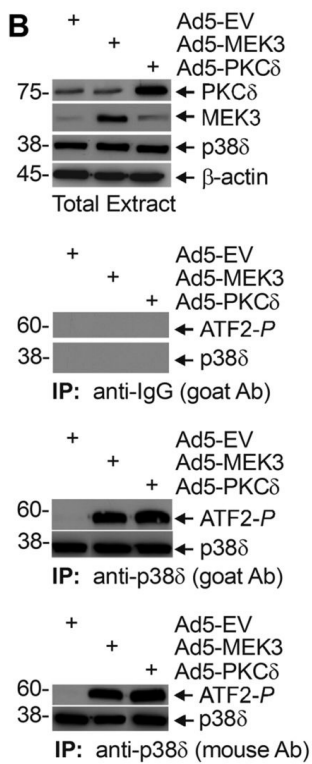
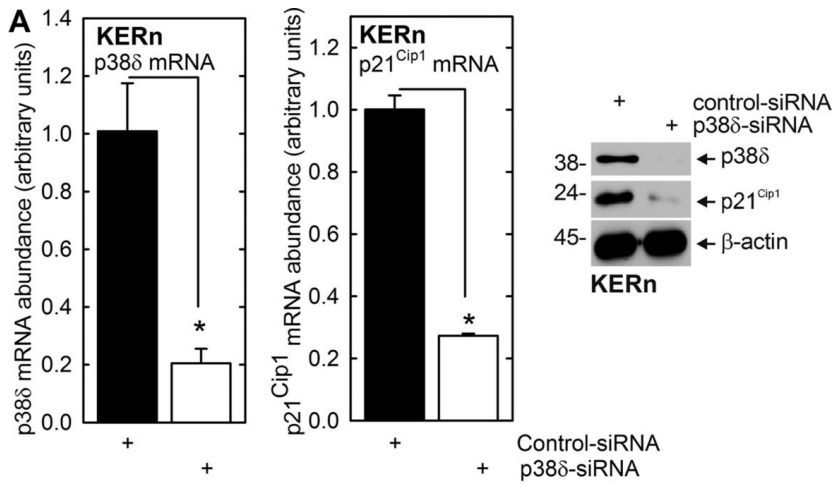
revealed that expression of PKC $\delta$  or MEK3 enhances p38 $\delta$  activity (**Fig. 2-1B**). No precipitation was observed when anti-IgG was used in the pull-down as a negative control.

The ability of these kinases to produce a change in p21<sup>Cip1</sup> expression predicts a physical interaction. To assess this, we infected cells with empty or FLAG-p38 $\delta$  encoding vector and prepared extracts for immunoprecipitation with anti-FLAG. **Fig. 2-1 C** shows that PKC $\delta$  and MEK3 co-precipitate with FLAG-p38 $\delta$ , suggesting an interaction of p38 $\delta$  with PKC $\delta$  and MEK3. No precipitation was observed when anti-IgG was substituted for anti-FLAG (**Fig. 2-1 C**). We also monitored the activity status of MEKK1 and MEK3. In the presence of increased levels of PKC $\delta$ , there is a substantial increase in MEKK1 and MEK3 activity as evidence by enhanced phosphorylation (**Fig. 2-1 D**).



**FIGURE 2-1: MEK3 regulates p21<sup>Cip1</sup> mRNA level**

**A** KERN were infected with 15 MOI of Ad5-EV or Ad5-MEK3 and after 24 h RNA was isolated and MEK3 and p21<sup>Cip1</sup> mRNA level was assessed by qRT-PCR. The values are mean  $\pm$  SEM, n = 3. In parallel identically treated cultures, protein extracts were prepared for assay of p21<sup>Cip1</sup> and MEK3 protein level by immunoblot. MEK3 is also present in Ad5-EV infected cells but is only visible at higher film exposures (not shown). **B** KERN were electroporated with 3  $\mu$ g of control-siRNA or MEK3-siRNA. After 24 h RNA was prepared and MEK3 and p21<sup>Cip1</sup> mRNA level of was measured by qRT-PCR. The values are mean  $\pm$  SEM, n = 3. The asterisks indicate significant differences, p < 0.005. In parallel identically treated cultures, protein extracts were prepared for assay of p21<sup>Cip1</sup> and MEK3 protein level by immunoblot. Similar results were observed with other MEK3-siRNA indicating that these responses are not due to off-site effects.



**FIGURE 2-2: p38 $\delta$  regulates p21<sup>Cip1</sup> protein, mRNA level and promoter activity.**

**A** KERn were electroporated with 3  $\mu$ g of control- or p38 $\delta$ -siRNA and after 24 h extracts were prepared for detection of p38 $\delta$  and p21<sup>Cip1</sup> mRNA and protein. The values are mean  $\pm$  SEM, n = 3. The asterisks indicate significant differences, p < 0.005. Similar results were observed with other p38 $\delta$ -siRNA indicating that these responses are not due to off-site actions (not shown). **B** KERn were infected with 10 MOI EV, Ad5-EV, Ad5-MEK3 or Ad5-PKC $\delta$ . After 48 h, 200  $\mu$ g of protein extract was used to immunoprecipitate p38 $\delta$  for use in an *in vitro* p38 kinase activity assay using ATF2 as substrate (Dashti, Efimova et al. 2001a). Immunoprecipitation was achieved using anti-p38 $\delta$  antibody produced in goat or mouse, or anti-IgG, as a negative-control. The level of precipitated p38 $\delta$  and ATF2 phosphorylation was monitored by immunoblot. Similar results were observed in each of three experiments. **C** KERn were infected with 10 MOI of tAd5-EV or tAd5-FLAG-p38 $\delta$  and 2.5 MOI of Ad5-TA encoding virus, and after 24 h total extract was prepared for electrophoresis or precipitation with anti-IgG or anti-FLAG. Immunoprecipitate (IP) and total extract (TE) were electrophoresed and for immunoblot with anti-FLAG, anti-PKC $\delta$  and anti-p38 $\delta$ . Similar results were observed in each of three experiments. **D** PKC $\delta$  activate MEKK1 and MEK3. KERn were infected with empty or PKC $\delta$  encoding adenovirus and after 24 h extracts were prepared for immunoblot to detect the indicated epitopes. Similar results were observed in each of three experiments. Figure 2-2 C credit: Dr. Santosh Kanade.

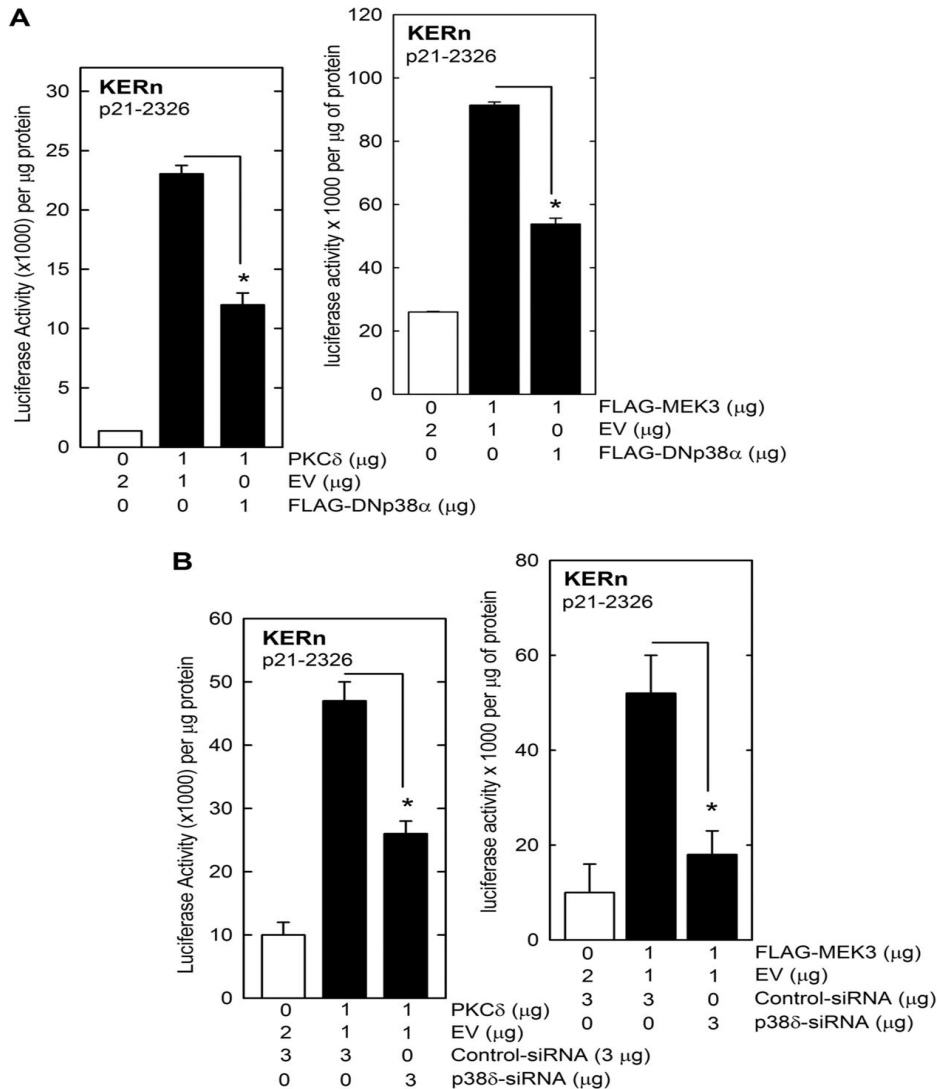
To further confirm a role for this cascade, we expressed PKC $\delta$  or MEK3 in the presence of DNp38 $\alpha$ . DNp38 $\alpha$  inhibits function of all p38 MAPK isoforms (Efimova, LaCelle et al. 1998). **Fig. 2-3 A** shows that DNp38 $\alpha$  inhibits PKC $\delta$ - and MEK3-dependent p21<sup>Cip1</sup> promoter activity, confirming that p38 $\delta$  activity is required for activation of p21<sup>Cip1</sup>. We also examined the impact of p38 $\delta$  knockdown and found that this also reduces the ability of PKC $\delta$  and MEK3 to activate p21<sup>Cip2</sup> promoter activity (**Fig. 2-3 B**). These findings suggest that a PKC $\delta$ /MEK3/p38 $\delta$  pathway regulates p21<sup>Cip1</sup> expression.

## **2. p38 $\delta$ response elements in p21<sup>Cip1</sup> promoter**

We hypothesized that p38 $\delta$  may regulate p21<sup>Cip1</sup> gene promoter activity to increase p21<sup>Cip1</sup> expression. To examine this, we transfected keratinocytes with p21-2326 and monitored the impact of p38 $\delta$  on promoter activity. p21-2326 encodes the full-length p21<sup>Cip1</sup> promoter and upstream regulatory region (Chew, Adhikary et al. 2011, Chew, Adhikary et al. 2012) (**Fig. 2-4 A**). **Fig. 2-4 B/C** shows that p38 $\delta$  overexpression increases p21-2326 activity and confirms that this is associated with an increase in p21<sup>Cip1</sup> protein. Moreover, we confirm that this activity is enhancer element-mediated, as p21-60, which encodes only the minimal promoter, is not regulated by p38 $\delta$ . These findings implicate p38 $\delta$  as regulating p21<sup>Cip1</sup> gene expression.

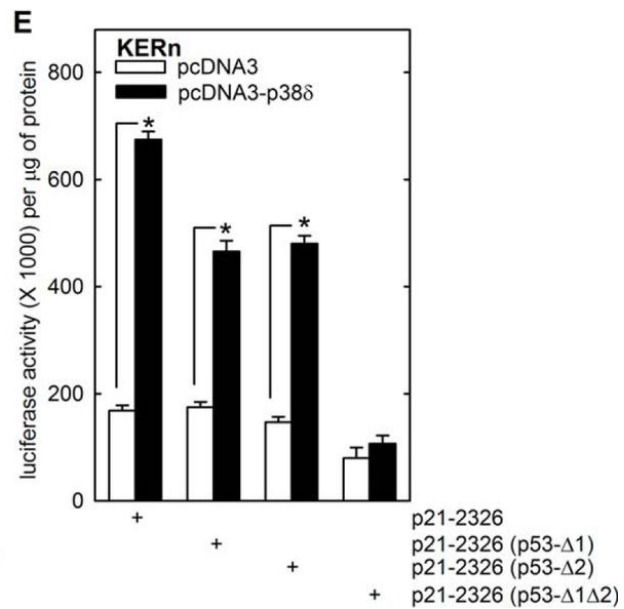
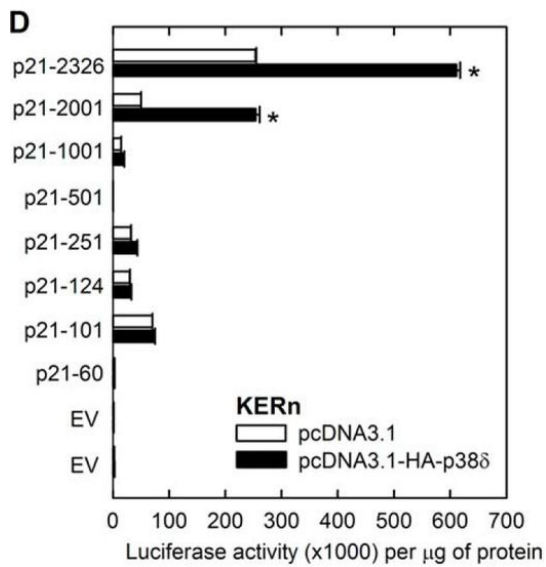
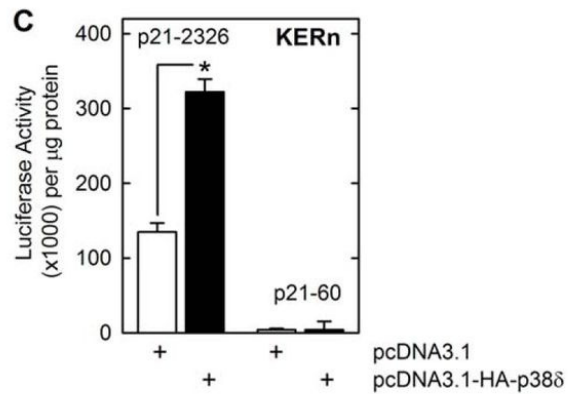
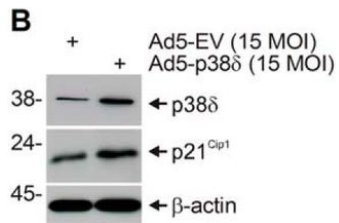
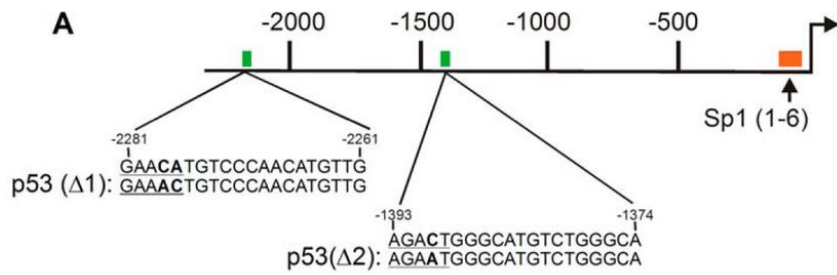
To locate p21<sup>Cip1</sup> promoter elements that mediate this regulation, we measured the impact of p38 $\delta$  on activity of a series of promoter deletion constructs. **Fig. 2-4 D** shows that activity of the p21-2326 and p21-2001 constructs are increased in response to p38 $\delta$ , but that the shorter constructs are not. This suggests that DNA elements, located between

nucleotides -2326/-1001, mediate this regulation. This region encodes two p53 response elements (**Fig. 2-4 A**). To determine whether these elements are required for this regulation, we transfected keratinocytes with empty- or p38 $\delta$ -encoding expression plasmid, and monitored the impact on activity of p21-2326 constructs encoding wild-type and mutant p53 binding sites. **Fig. 2-4 E** shows that mutation of the p53 transcription factor binding sites produces a substantial reduction in promoter response to p38 $\delta$ .



**FIGURE 2-3: p38δ is required for PKCδ and MEK3-dependent activation of p21<sup>Cip1</sup> promoter transcription**

**A** KERn were transfected with 1 µg of p21-2326 luciferase reporter plasmid in the presence of 1 µg of empty vector or vector encoding PKCδ, MEK3 or DNp38α. Levels were adjusted to a total of 3 µg per transfection by addition of empty vector (EV). At 48 h post-transfection cell extracts were prepared and assayed for promoter activity. **B** KERn (1 million cells per group) were electroporated with 3 µg of control- or p38δ-siRNA. At 48 h post electroporation the cells were harvested and counted and 0.5 million cells from each group were re-electroporated with 1 µg of endotoxin-free p21-2326 luciferase reporter plasmid in the presence of 2 µg of PKCδ or MEK3 encoding endotoxin-free plasmid or the empty vector. After an additional 24 h cell extracts were prepared for luciferase assay. The values are mean ± SEM, n = 3. The asterisks indicate significant differences, p<0.005.



**FIGURE 2-4: p38 $\delta$  acts via p53 response elements on the p21<sup>Cip1</sup> promoter.**

**A** p21<sup>Cip1</sup> promoter schematic showing Sp1 and p53 DNA response elements. The numbers indicate distance in nucleotides relative to the transcription start site. **B** p38 $\delta$  increases p21<sup>Cip1</sup> level. KERN were infected with 15 MOI of Ad5-EV or Ad5-p38 $\delta$ . After 24 h, extracts were prepared for detection of p38 $\delta$  and p21<sup>Cip1</sup> protein.  $\beta$ -actin is used as the loading control. **C** KERN were transfected with 1  $\mu$ g each p21-2326 or p21-60 p21<sup>Cip1</sup> promoter reporter plasmids and 1  $\mu$ g of pcDNA3.1 or pcDNA3.1-HA-p38 $\delta$  and after 24 h extracts were prepared for assay of luciferase activity. Values are mean  $\pm$  SEM, n = 3. The asterisk indicates a significant difference, p < 0.005. **D** KERN were transfected with 1  $\mu$ g of the indicated p21<sup>Cip1</sup> luciferase reporter plasmid and 1  $\mu$ g of pcDNA3.1 or pcDNA3.1-HA-p38 $\delta$ , and after 24 h extracts were prepared for assay of luciferase activity. The values are mean  $\pm$  SEM, n = 3 and the asterisks indicate significant differences in activity, p < 0.005. **E** p53 response elements are required for p38 $\delta$  activation of p21<sup>Cip1</sup> expression. KERN were transfected with 1  $\mu$ g of the indicated p21<sup>Cip1</sup> promoter plasmid along with 1  $\mu$ g of pcDNA3.1 or pcDNA3.1-HA-p38 $\delta$  and after 24 h extracts were prepared for luciferase activity assay. The values are mean  $\pm$  SEM, n = 3. The asterisks indicate significant difference, p < 0.005.

### **3. PKC $\delta$ , MEK3 and p38 $\delta$ suppress keratinocyte proliferation**

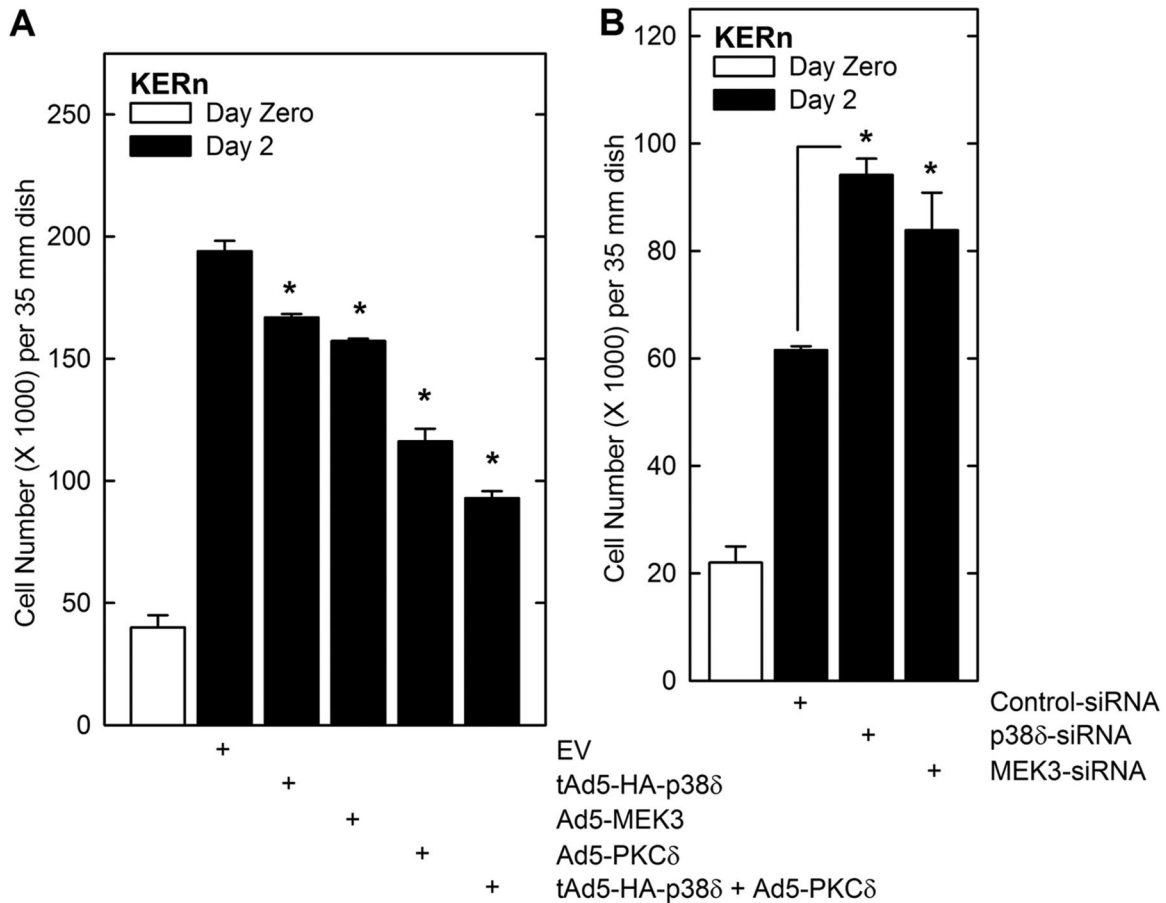
Our findings suggest that PKC $\delta$ , MEK3 and p38 $\delta$  activate p21<sup>Cip1</sup> expression and we predict that this should be associated with reduced cell proliferation. To test this, KERn were plated at low density and on day zero infected with empty (EV) adenovirus or virus encoding PKC $\delta$ , MEK3 or p38 $\delta$ . After an additional 2 d, the cells were harvested and counted. **Fig. 2-5 A** shows that PKC $\delta$  and MEK3 suppress proliferation and a more dramatic reduction is observed when both PKC $\delta$  and p38 $\delta$  are present. These findings are consistent with a role for these kinases as proliferation suppressors. We also tested the inverse experiment and determined whether p38 $\delta$ - or MEK3 knockdown enhances proliferation. Indeed, as shown in **Fig. 2-5 B**, loss of either kinase resulted in a 15 to 20% increase in cell number.

### **4. p53 level is regulated by p38 $\delta$**

The fact that the p53 transcription factor binding sites appear important for p38 $\delta$  regulation of p21<sup>Cip1</sup> gene expression suggests that p53 level may be regulated by p38 $\delta$ . **Fig. 2-6 A** shows that p38 $\delta$ -expressing keratinocytes produce increased p53 mRNA and protein and increased p21<sup>Cip1</sup> mRNA. **Fig. 2-6 B** shows that the increase in p53 mRNA is associated with increased p53 (PG13-Luc) promoter activity. Moreover, the increase is specific for the p38 $\delta$  isoform, as the other major p38 isoform present in keratinocytes, p38 $\alpha$ , does not cause a significant increase. To further confirm that the regulation is selectively dependent upon p38 $\delta$ , and not p38 $\alpha$  which is abundant in keratinocytes (Dashti, Efimova et al. 2001a, Dashti, Efimova et al. 2001b), we examined the impact of PKC $\delta$  expression on p38 $\alpha$  and p38 $\delta$  phosphorylation (activation). Cells were infected

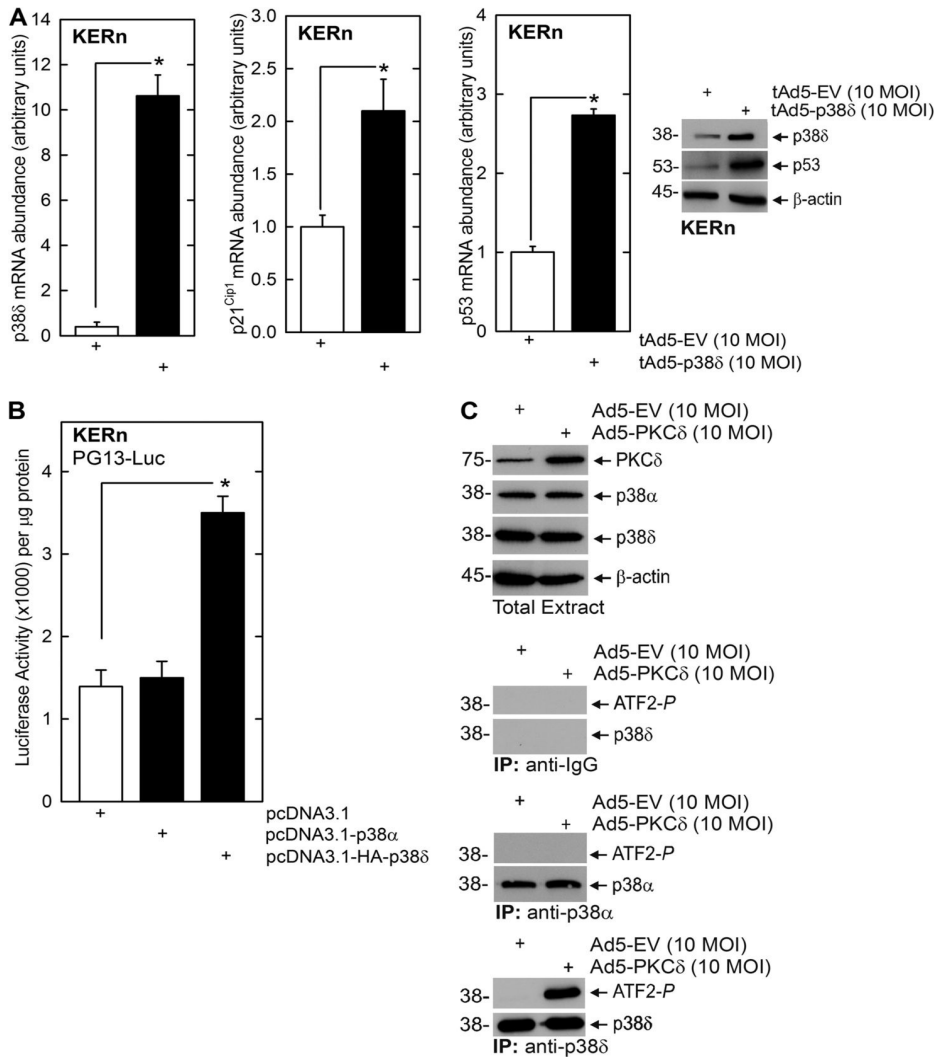
with control or PKC $\delta$ -encoding adenovirus and after 24 h p38 $\alpha$  or p38 $\delta$  were precipitated and ability of the precipitated kinases to phosphorylate ATF2 was monitored. A control precipitation was performed with anti-IgG. These results show that PKC $\delta$  does not alter p38 $\delta$  or p38 $\alpha$  level, and that only p38 $\delta$  is activated (phosphorylated) (**Fig. 2-6 C**).

To further assess the role of p53, we electroporated cells with p53-siRNA, then challenged with p38 $\delta$  encoding or empty virus and monitored the impact on p21<sup>Cip1</sup> mRNA level. As shown in **Fig. 2-7 A**, expression of p38 $\delta$  substantially increases p21<sup>Cip1</sup> mRNA level and this increase is completely inhibited in the presence of p53-siRNA, confirming that increased p53 is required for the response. **Fig. 2-7 B** shows that treatment with p53-siRNA reduces p53 encoding mRNA. These findings suggest that increased p53 level is required for PKC $\delta$ , MEK3 and p38 $\delta$  activation of the p21<sup>Cip1</sup> promoter.



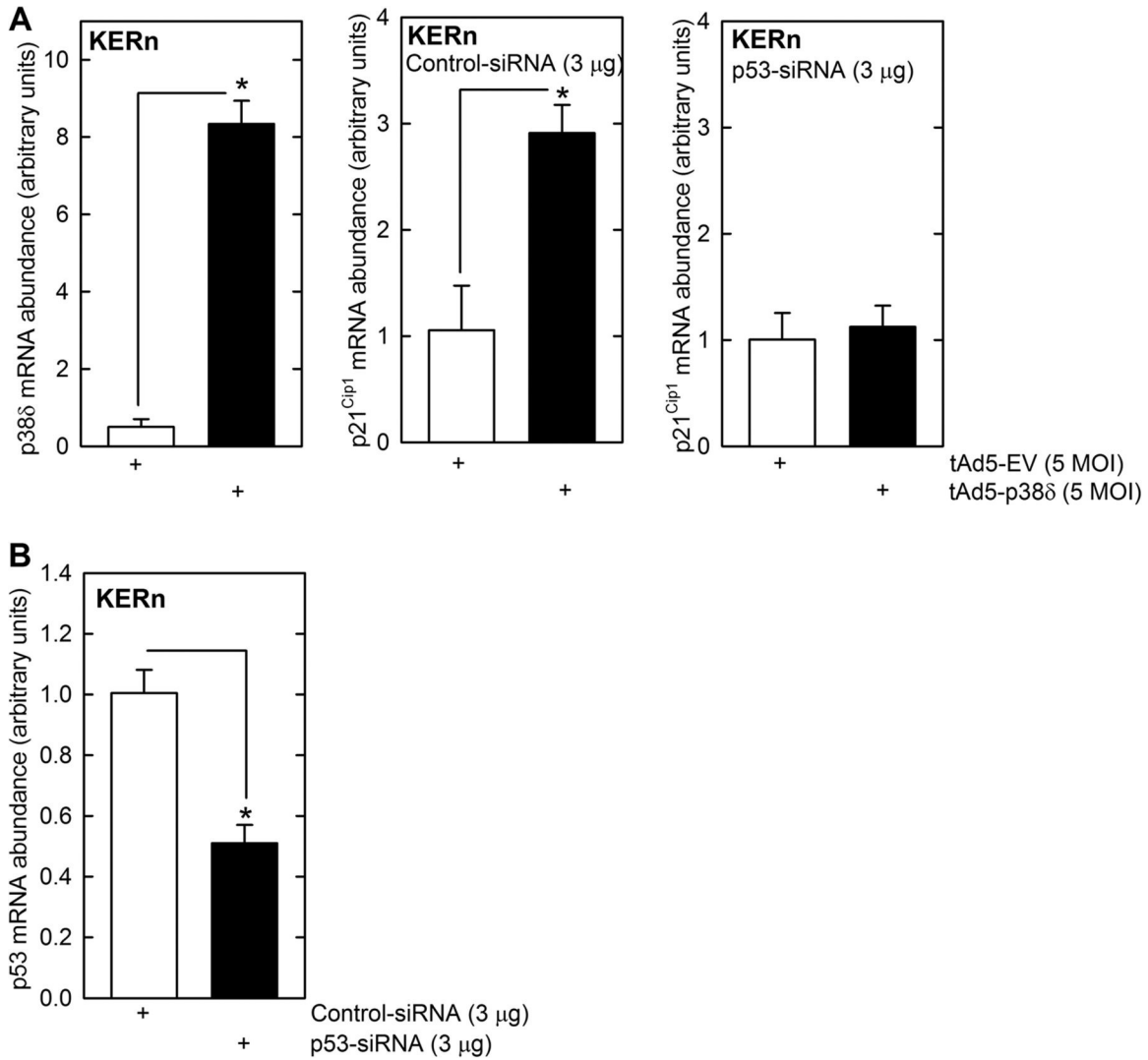
**Figure 2-5: PKC $\delta$ , MEK3 and p38 $\delta$  alter keratinocyte proliferation.**

**A** KERn were seeded at 15,000 cells per well in triplicate 35 mm dishes and permitted to attach. At time zero, the cells were infected with 10 MOI of the indicated adenovirus. After an additional 48 h, the cells were harvested and counted. The open bar indicates the cell count at time zero and the closed bars indicate the 48 h counts. Ad5-TA encoding virus (2.5 MOI) was included in each treatment (Chew, Adhikary et al. 2011). The values are mean  $\pm$  SEM, n = 3. The asterisks indicate a significant difference, p < 0.05. **B** KERn (1 million) were twice electroporated with 3  $\mu$ g of the indicated siRNA. After the second electroporation, 15,000 cells were seeded into six well cluster wells and cell number was assessed at time zero and 48 h later. The values are mean  $\pm$  SEM, n = 3. The asterisks indicate a significant difference, p < 0.05.



**Figure 2-6: p53 is a downstream target of p38δ.**

**A** p38δ increases p53 mRNA level. KERN were infected with 10 MOI of empty or p38δ-encoding adenovirus and 2.5 MOI of Ad5-TA and after 48 h extracts were prepared for qRT-PCR assay of mRNA level or immunoblot to detect the indicated targets. The values are the mean  $\pm$  SEM,  $n = 3$ . Significant differences are indicated by asterisks,  $p < 0.005$ . **B** p38δ increases p53 promoter activity. KERN were transfected with 1  $\mu$ g PG13-Luc in the presence of 1  $\mu$ g of empty plasmid plasmid or plasmid encoding p38δ or p38α, and after 24 h promoter activity was monitored. The values are mean  $\pm$  SEM,  $n = 4$  and the asterisks indicate a significant difference,  $p < 0.005$ . **C** PKCδ activates p38δ but not p38α. KERN were infected with 10 MOI of empty or PKCδ encoding adenovirus. After 48 h cell extracts were prepared, and 200  $\mu$ g of protein was immunoprecipitated with anti-p38α or anti-p38δ and ability to phosphorylate ATF2 was monitored. Similar results were observed in each of three experiments.

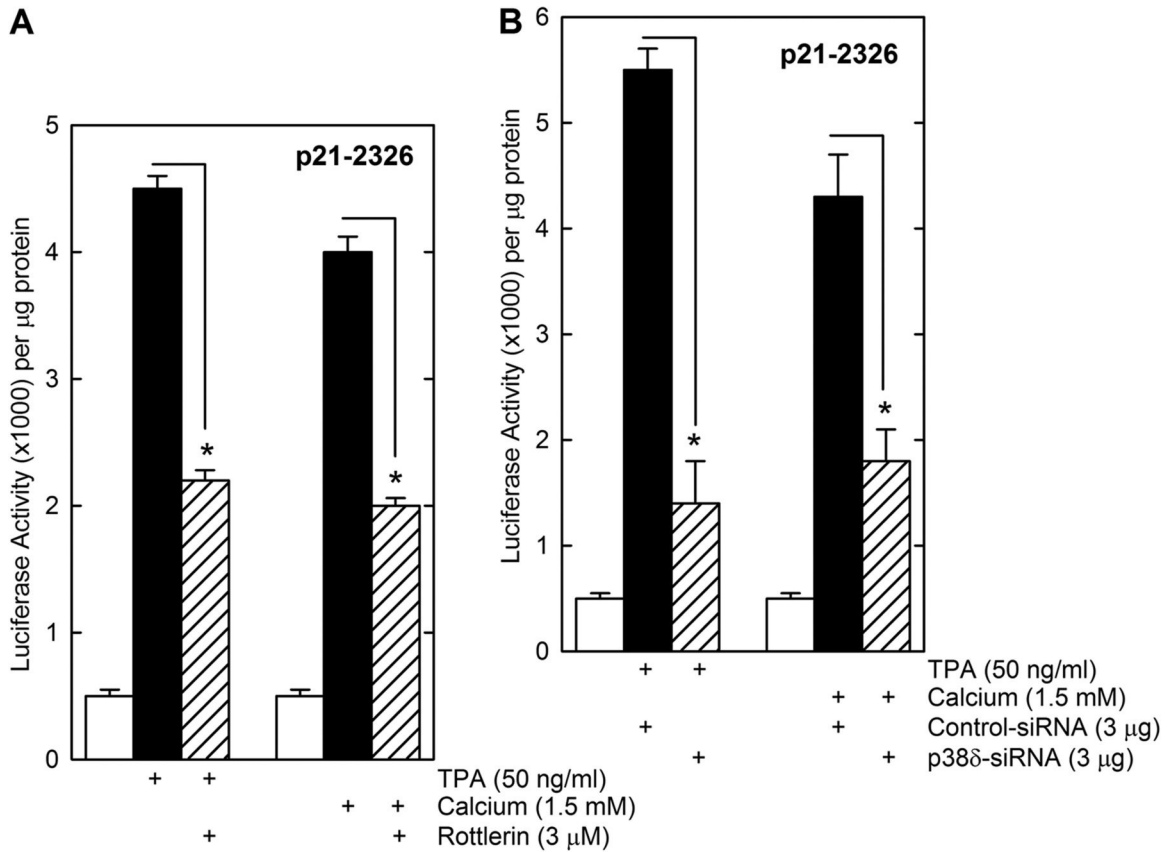


**Figure 2-7: p53 is required for the p38 $\delta$ -dependent increase in p21<sup>Cip1</sup> expression.**

**A** KERn were electroporated with 3  $\mu$ g of control (scrambled) or p53-siRNA and after 24 h infected with 5 MOI of tAd5-EV or tAd5-p38 $\delta$  with 2.5 MOI of Ad5-TA. After an additional 48 h cells were harvested to monitor mRNA levels by qRT-PCR. Values are mean  $\pm$  SEM, n = 4. The asterisks indicate a significant difference, p < 0.005. Similar results were observed with other control- and p53-siRNA indicating that these responses are not due to off-site actions (not shown). **B** p53-siRNA reduces p53 mRNA level. This plot confirms that the p53-specific siRNA, delivered as outlined above, reduces p53 expression. Values are mean  $\pm$  SEM, n = 4. The asterisks indicate a significant difference, p < 0.005.

## **5. p38 $\delta$ regulation of p53 and p21<sup>Cip1</sup> expression during differentiation**

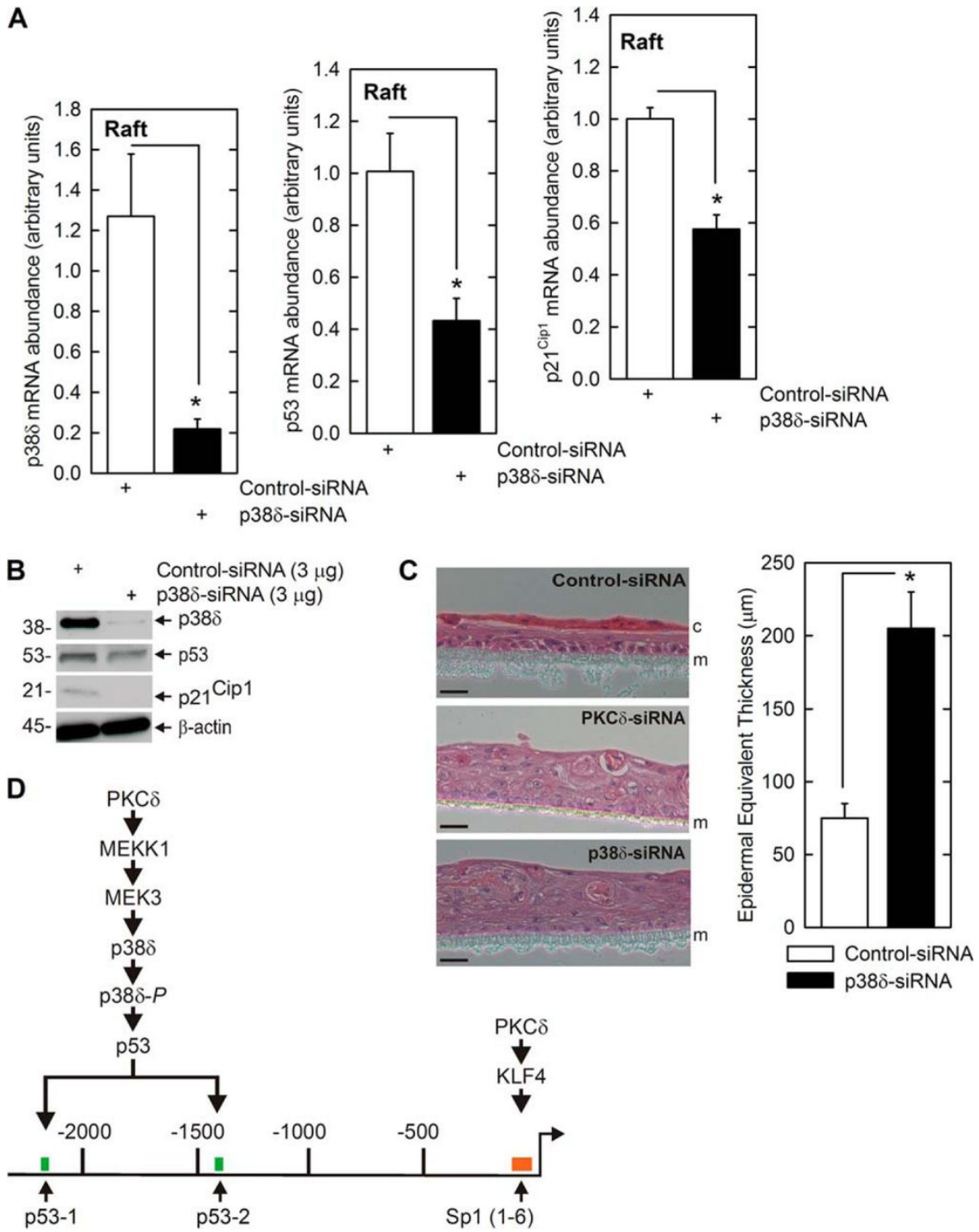
The above studies implicate PKC $\delta$  and p38 $\delta$  in a cascade that activates p21<sup>Cip1</sup> expression. We wanted to determine whether TPA and calcium, which activate keratinocyte differentiation and suppress keratinocyte proliferation (Eckert, Efimova et al. 2002a), operate via this cascade. KERN were treated with TPA or calcium in the presence of rottlerin, a PKC $\delta$  inhibitor, or p38 $\delta$  knockdown using p38 $\delta$ -siRNA, and the impact on p21-2326 activity was monitored. **Fig. 2.8 A/B** show that TPA and calcium induce p21-2326 promoter activity and that this can be reduced by treatment with rottlerin or p38 $\delta$ -siRNA.



**Figure 2-8: Activation of p21<sup>Cip1</sup> expression by keratinocyte differentiating agents.**

**A** KERN (1 million) were transfected with 0.5  $\mu\text{g}$  of p21-2326 and at 12 h after plating treated for 18 h with the indicated agent prior to preparation of extracts for luciferase assay. **B** KERN (1 million each group) were electroporated with 3  $\mu\text{g}$  of control- or p38 $\delta$ -siRNA. At 48 h post-electroporation the cells were harvested and counted and 0.5 million cells from each group were re-electroporated with 1  $\mu\text{g}$  of endotoxin-free p21-2326 luciferase reporter plasmid. At 6 h post electroporation, the cells were treated with the indicated concentration of TPA or calcium. After an additional 24 h, cell extracts were prepared for luciferase assay. The values are mean  $\pm$  SEM, n = 3. The asterisks indicate significant differences, p<0.005.

To further assess the biological relevance of this regulation, we examined the impact of altering p38 $\delta$  level using a keratinocyte epidermal equivalent system. In this model, keratinocytes are grown at the air-liquid interface to produce a stratified, multilayered and differentiated epidermal equivalent that closely mimics *in vivo* epidermis. This system can be used to assess biological response under *in vivo*-like conditions (Poumay, Dupont et al. 2004). Primary foreskin keratinocytes were electroporated with p38 $\delta$ - or control-siRNA and then transferred to Millicell chambers for growth as epidermal equivalent cultures. **Fig. 2.9 A** shows that the p38 $\delta$ -siRNA treatment reduces p38 $\delta$  mRNA, and that this is associated with reduced mRNA encoding p53 and p21<sup>Cip1</sup>. **Fig. 2.9 B** shows that forced reduction in p38 $\delta$  protein level also reduces p53 and p21<sup>Cip1</sup> protein level. To assess the biological impact on differentiation and proliferation, we monitored morphology of the epidermal equivalent cultures. **Fig. 2.9 C** shows that cultures expressing normal endogenous levels of p38 $\delta$  undergo appropriate differentiation and produce a multilayered tissue and stable cornified layer. In contrast, p38 $\delta$ -knockdown cells produce a thicker structure comprised of additional layers that is characterized by the absence of a cornified layer which is indicative of enhanced proliferation/reduced differentiation. The plot quantifies the increase in thickness that is observed in p38 $\delta$  or PKC $\delta$  knockdown cultures. These findings, confirm, using an *in vivo*-like model, that p38 $\delta$  is required for p21<sup>Cip1</sup> expression and differentiation-associated suppression of proliferation.



**Figure 2-9: p38 $\delta$  regulates p21<sup>Cip1</sup> expression and controls proliferation in epidermal equivalent model.**

**A** p38 $\delta$  is required for expression of p53 and p21<sup>Cip1</sup>. KERn were twice electroporated with control- or p38 $\delta$ -siRNA and then plated in Millicell wells to form stratified and differentiated epidermal equivalent cultures. After 4 d at the air-liquid interface, the cultures were harvested and extracts prepared for assay of p38 $\delta$ , p53 and p21<sup>Cip1</sup> mRNA. The values are mean  $\pm$  SEM, n = 4 and the asterisks indicate a significant difference, p < 0.005. Similar results were observed with other p38 $\delta$ -siRNA indicating that these responses are not due to off-site actions (not shown). **B** p38 $\delta$  is required for expression of p53 and p21<sup>Cip1</sup> proteins. Extracts were prepared from epidermal equivalents, maintained for 4 d at the air-liquid interface as in panel A, and level of the indicated proteins was monitored by immunoblot. Similar results were observed in each of three experiments. **C** p38 $\delta$  is required for appropriate cell proliferation and differentiation. KERn were twice electroporated with control- or p38 $\delta$ -siRNA and seeded for epidermal equivalent culture. After 4 d at the air-liquid interface the cultures were harvested and stained with H&E. The membrane (m) and cornified layer (c) are indicated. Similar results were observed in each of three experiments. The graph shows that loss of p38 $\delta$  expression results in reduced cornification and production of a thicker epidermal equivalent (increased proliferation). The values are mean + SEM, n = 6. The asterisk indicates a significant difference, p < 0.005. **D** A PKC $\delta$ /p53 regulatory pathway controls proliferation. Our studies suggest that PKC $\delta$  activates a MEKK1/MEK3/p38 $\delta$  mitogen-activated protein kinase module to increase p53 levels and that p53 then interacts with p53 response elements in the p21<sup>Cip1</sup> promoter to increase p21<sup>Cip1</sup> expression and reduce proliferation. Also indicated is PKC $\delta$  activation of KLF4 expression which acts via the Sp1 response elements in the p21<sup>Cip1</sup> promoter to drive transcription. This parallel PKC $\delta$ -activated pathway was previously described (Chew, Adhikary et al. 2011).

## ***E. DISCUSSION***

Protein kinase C and p38 MAPK control of cell proliferation has been studied in several systems, and p53 and p21<sup>Cip1</sup> have been implicated in some of these studies. In most cases, this regulation involves PKC-dependent covalent modification and stabilization of p53. For example, reovirus infection of target cells increases PKC $\delta$ , RAS and p38 MAPK signaling leading to increased p53 Ser-1133 phosphorylation and stabilization (Lin, Lee et al. 2009) and treatment of dopaminergic neurons with nitric oxide increases p53 Ser-15 phosphorylation to stabilize p53 against proteasome degradation (Lee, Kim et al. 2006). In vascular smooth muscle cells PKC $\delta$  increases p53 Ser-46 phosphorylation to increase p53 level, and peroxide treatment of aortic endothelial cells results in PKC $\delta$ -dependent accumulation of p53 (Niwa, Inanami et al. 2002). Some studies also implicate p38 MAPK as being important in PKC $\delta$  regulation of p53 gene expression. In smooth muscle cells PKC $\delta$  regulation of p53 expression requires p38 MAPK (Ryer, Sakakibara et al. 2005). In human endometrial cancer cells, PKC $\delta$  activation leads to increased p53 and p21<sup>Cip1</sup> expression in a process that is inhibited by GF109203X (Wu, Schally et al. 2010). Very few studies have described PKC $\delta$  activation of p53 gene transcription (Ryer, Sakakibara et al. 2005). Moreover, in those cases where alteration of p53 function involves p38 MAPK, the p38 isoform involved was not been identified (Niwa, Inanami et al. 2002).

Novel PKC isoforms are important regulators of keratinocyte function that increase MAPK signaling and the nuclear level of key transcription factors to activate expression of differentiation-associated gene (Newton 1997, Denning, Dlugosz et al. 2000, Denning 2004, Adhikary, Chew et al. 2010, Sonkoly, Wei et al. 2009, Papp, Czifra et al. 2004,

Szegedi, Páyer et al. 2009, Balasubramanian, Efimova et al. 2002, Kamioka, Akahane et al. 2010, Efimova, Eckert 2000, Eckert, Crish et al. 2003b, Efimova, Deucher et al. 2002). PKC isoforms also regulate keratinocyte proliferation (Balasubramanian, Efimova et al. 2002, Papp, Czifra et al. 2004, Bollag, Dodd et al. 2004, Bollag 2009, Praskova, Kalenderova et al. 2002, Wheeler, Reddig et al. 2002) and p21<sup>Cip1</sup> is a key target (Chew, Adhikary et al. 2011). However, in spite of this progress, we have a limited understanding of the mechanisms whereby PKC $\delta$  regulates p21<sup>Cip1</sup> level. Previous studies, from our group, indicate that Kruppel-like factor 4 and Sp1 transcription factors have a role (Chew, Adhikary et al. 2011). However, as p21<sup>Cip1</sup> is a central controller of cell proliferation, it is expected that the regulation will be complex and that multiple/reinforcing mechanisms may exist.

Our present studies identify a novel PKC $\delta$ /MEK3/p38 $\delta$ /p53/p21<sup>Cip1</sup> pathway that regulates PKC $\delta$  activation of p21<sup>Cip1</sup> expression in keratinocytes. This pathway involves PKC $\delta$ -dependent activation of MEK3 and p38 $\delta$  kinase which increases p53 expression. p53, in turn, interacts with the p21<sup>Cip1</sup> promoter via canonical p53 response elements to activate transcription which ultimately leads to a reduction in cell proliferation (**Fig. 2.9 D**). Also shown is a parallel signaling pathway wherein PKC $\delta$  acts via KFL4 to drive transcription via the proximal cluster of Sp1 binding sites (Chew, Adhikary et al. 2011) (**Fig. 2.9 D**). It is interesting that although keratinocytes express several PKC isoforms (Efimova, Eckert 2000, Efimova, Deucher et al. 2002, Efimova, Broome et al. 2004b); the form implicated as controlling proliferation in the present study is PKC $\delta$ . This is particularly intriguing, as PKC $\delta$  has been implicated as the isoform that drives keratinocyte differentiation (Efimova, Broome et al. 2003, Kraft, Efimova et al. 2007).

This finding suggests that enhanced differentiation and reduced proliferation, which are key simultaneously occurring events during keratinocyte maturation, are controlled by a common pathway that involves PKC $\delta$ . It makes sense, in terms of regulatory efficiency, that a common pathway would control both processes.

It is also interesting that p38 $\delta$  is involved as a downstream mediator of PKC $\delta$  action to increase p53 and p21<sup>Cip1</sup>. Keratinocytes express three p38 MAPK isoforms, p38 $\alpha$ ,  $\beta$  and  $\delta$ . The major forms are p38 $\alpha$  and  $\delta$  (Efimova, Broome et al. 2003, Kraft, Efimova et al. 2007). In the present study, we show that p38 $\delta$  activation, as evidenced by increased phosphorylation, is associated with increased p53 and p21<sup>Cip1</sup> expression. In contrast, p38 $\alpha$  is not important as a regulator of p53 or p21<sup>Cip1</sup>, as it produces minimal changes in expression of these genes, and is not activated (phosphorylated) in response to PKC $\delta$ . In addition, a known downstream mediator of PKC $\delta$ -dependent keratinocyte differentiation (Efimova, LaCelle et al. 1998, Dashti, Efimova et al. 2001b), MEK3, is required for activation of p53 and p21<sup>Cip1</sup> expression.

As mentioned above, previous reports in other systems indicate that PKC $\delta$  can increase p53 level. In most cases, this is associated with direct PKC $\delta$ -dependent phosphorylation of p53 (Lee, Kim et al. 2006, Lin, Lee et al. 2009, Ryer, Sakakibara et al. 2005), but in other cases this involves activation of p53 gene transcription (Ryer, Sakakibara et al. 2005). However, the mechanism whereby PKC $\delta$  increases p53 gene expression is not well understood. In the present study we show that PKC $\delta$ , MEK3 and p38 $\delta$  form a cascade that increase p53 expression as measured by increased p53 mRNA/protein level and promoter activity. We further show that knockdown of p38 $\delta$

eliminates the ability of PKC $\delta$  to increase p21<sup>Cip1</sup> mRNA, indicating that p38 $\delta$  is an essential upstream regulator of p53 level.

We also studied the link between increased p53 level and p21<sup>Cip1</sup> promoter activation. These studies show that a key response element in the p21<sup>Cip1</sup> promoter is located within nucleotides -2626/-1001 relative to the transcription start site which is located at nucleotide -1. This region encodes two p53 protein binding elements located at nucleotides -2281/-2261 and -1393/1374. Mutation of these sites results in loss of promoter activity, suggesting that p53 interaction at these sites, in response to PKC $\delta$ /MEK3/p38 $\delta$  activation, drives the increase in transcription.

To assess the physiological relevance of this regulation, we confirmed that this pathway is activated following treatment with agents (TPA and calcium) that suppress keratinocyte proliferation. We also used a stratifying epidermal equivalent model system where cells are grown at the air-liquid interface (Chew, Adhikary et al. 2013, Poumay, Dupont et al. 2004). This is a particularly useful model, since these cells closely mimic the *in vivo* epidermal differentiation process. Our studies show that knockdown of p38 $\delta$  in this system results in reduced p53 and p21<sup>Cip1</sup> expression. In addition, loss of p38 $\delta$  resulted in formation of a thicker epidermal equivalent that did not include a cornified layer. These studies strongly suggest that the PKC $\delta$ /MEK3/ p38 $\delta$ /p53/p21<sup>Cip1</sup> cascade is likely to be functional in *in vivo* epidermis.

**CHAPTER 3: METHYLOSOME PROTEIN (MEP50) AND  
PKC $\delta$ /P38 $\delta$  SIGNALING CONTROL KERATINOCYTE  
PROLIFERATION VIA OPPOSITE EFFECTS ON p21<sup>Cip1</sup> GENE  
EXPRESSION.**

**A. ABSTRACT:**

Protein arginine methyltransferase 5 (PRMT5) is a key epigenetic regulator which symmetrically dimethylates arginine residues on histone H3 and H4 to silence gene expression. PRMT5 is frequently observed in a complex with the cofactor, methylosome protein 50 (MEP50), which is required for PRMT5 activity. PKC $\delta$ /p38 $\delta$  signaling, a key controller of keratinocyte proliferation and differentiation, increases p21<sup>Cip1</sup> expression to suppress keratinocyte proliferation. We now show that MEP50 enhances keratinocyte proliferation and survival via mechanisms that include silencing of p21<sup>Cip1</sup> expression. This is associated with enhanced PRMT5/MEP50 interaction at the p21<sup>Cip1</sup> promoter and enhanced arginine dimethylation of promoter-associated histones H3 and H4. It is also associated with a MEP50-dependent reduction in the level of p53, a key controller of p21<sup>Cip1</sup> gene expression. We confirm an important biological role for MEP50 and PRMT5 in regulating keratinocyte proliferation using a stratified epidermal equivalent model that mimics *in vivo* epidermal keratinocyte differentiation. In this model, PRMT5 or MEP50 knockdown results in reduced keratinocyte proliferation. We further show that PKC $\delta$ /p38 $\delta$  signaling suppresses MEP50 expression leading to reduced H3/H4 arginine dimethylation at the p21<sup>Cip1</sup> promoter, and that this is associated with enhanced p21<sup>Cip1</sup> expression and reduced cell proliferation. These findings describe an opposing action

between PKC $\delta$ /p38 $\delta$  MAPK signaling and PRMT5/MEP50 epigenetic silencing mechanisms in regulating cell proliferation.

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<sup>3</sup> CITATION: Saha K, Eckert R. L. JBC 2015. (Submitted).

## ***B. INTRODUCTION:***

PKC $\delta$  and p38 $\delta$  are key components of a cascade that controls keratinocyte differentiation and proliferation (Chew, Adhikary et al. 2011, Chew, Adhikary et al. 2013, Efimova, LaCelle et al. 1998, Efimova, Deucher et al. 2002, Efimova, Broome et al. 2003, Efimova, Broome et al. 2004b, Adhikary, Chew et al. 2010). PKC $\delta$  activates a MEK1/MEK3/p38 $\delta$  signaling cascade which triggers events that enhance keratinocyte differentiation and suppress proliferation (Efimova, LaCelle et al. 1998, Efimova, Broome et al. 2003, Eckert, Efimova et al. 2003a, Eckert, Crish et al. 2004). Downstream events include activation of AP1, Sp1 and Kruppel-like transcription factors leading to activation of differentiation and suppression of proliferation (Adhikary, Chew et al. 2010, Chew, Adhikary et al. 2013, Welter, Eckert 1995, Welter, Crish et al. 1995b, Han, Rorke et al. 2012, Banks, Crish et al. 1999, Banks, Crish et al. 1998, Crish, Zaim et al. 1998, Crish, Bone et al. 2002, Crish, Gopalakrishnan et al. 2006, Crish, Eckert 2008). This cascade has an important role in controlling differentiation-associated gene expression (Crish, Zaim et al. 1998, Crish, Bone et al. 2002, Crish, Gopalakrishnan et al. 2006) which has been confirmed by transgenic mouse experiments demonstrating a key role for specific transcription factors and gene response elements in this regulation (Crish, Zaim et al. 1998, Crish, Bone et al. 2002, Crish, Gopalakrishnan et al. 2006, Rorke, Adhikary et al. 2010).

A recent study indicates that PRMT5, an arginine methyltransferase that symmetrically dimethylates arginine residues on target proteins, acts to antagonize PKC $\delta$ /MEK3/p38 $\delta$  signaling (Kanade, Eckert 2012). In this context, PRMT5 acts to enhance keratinocyte survival (Kanade, Eckert 2012). PRMT5 associates with a co-

activator called MEP50 (Ho, Wilczek et al. 2013, Karkhanis, Hu et al. 2011), an interaction that is required for PRMT5 activation (Antonysamy, Bonday et al. 2012, Ho, Wilczek et al. 2013). This complex then functions to dimethylate specific arginines on target proteins leading to altered protein function. Loss of either PRMT5 or MEP50 reduces this activity (Ho, Wilczek et al. 2013, Karkhanis, Hu et al. 2011). Histones are important PRMT5 targets. PRMT5 produces changes in histone dimethylation that are part of the epigenetic code that controls gene expression (Yang, Bedford 2013, Bedford, Richard 2005). Histones 3 and 4 are important PRMT5 targets. The PRMT5/MEP50 complex symmetrically dimethylates arginine 3 of histone H4 (H4R3me2s), and arginine 8 of histone H3 (H3R8me2s) and these modifications are associated with silencing of gene expression (Fabrizio, Messaoudi et al. 2002, Tae, Karkhanis et al. 2011, Bedford, Clarke 2009).

Keratinocyte proliferation and differentiation are important processes in keratinocyte lifecycle that must be balanced to produce a stratified tissue that functions as an appropriate barrier (Eckert, Crish et al. 1997) Delayed cessation of keratinocyte proliferation, for example, can lead to epidermal disease (Rorke, Adhikary et al. 2014, Rorke, Adhikary et al. 2010). In the present study, we examine the role of MEP50 as a controller of keratinocyte proliferation. We show that MEP50 enhances keratinocyte proliferation. To understand the mechanism, we study the impact of MEP50 expression on p21<sup>Cip1</sup> gene expression, a key controller of keratinocyte proliferation (Chew, Adhikary et al. 2012, Chew, Adhikary et al. 2011). We show that MEP50 has a role in stimulating dimethylation of histones associated with key regulator elements in the p21<sup>Cip1</sup> gene promoter and that this is associated with silencing of p21<sup>Cip1</sup> expression. We

further show that PKC $\delta$ /p38 $\delta$  anti-proliferation signaling reduces PRMT5 and MEP50 expression and PRMT5/MEP5 association with the p21<sup>Cip1</sup> gene. This leads to reduced H3R8me2s and H4R3me2s formation at the p21<sup>Cip1</sup> promoter leading to derepression. We propose that PRMT5/MEP50 silencing of anti-proliferation genes is an important mechanism of survival.

## ***C. MATERIALS AND METHODS:***

### **1. Antibodies and Reagents:**

Rabbit polyclonal antibodies for MEP50 (2823), PRMT5 (2252) and p21<sup>Cip1</sup> (2947) were obtained from Cell Signaling Technology (Danvers, MA), while the mouse monoclonal anti- $\beta$ -actin (A-5441), anti-FLAG antibody (8592) and anti-FLAG M2-FITC (F4049) were purchased from Sigma-Aldrich. Normal rabbit IgG was obtained from Cell Signaling (2729). Rabbit polyclonal antibodies for histone H4-symmetrically dimethylated arginine 3 (H4R3me2s) (ab5823) and Histone H3-symmetrically dimethylated arginine 8 (H3R8me2s) (PA5-27039) were obtained from Abcam (Cambridge, MA) and Thermo Fisher Scientific (Rockford, IL). Anti-MEP50 (57722) was purchased from Abcam (ab5772). Rabbit anti-PKC $\delta$  (sc-937), goat anti-PRMT5 (sc-22132) and mouse monoclonal anti-p38 $\delta$  (sc-271292) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The secondary antibodies include peroxidase-conjugated sheep anti-mouse IgG (NXA931, GE Healthcare) and peroxidase-conjugated donkey anti-rabbit IgG (NA934V, GE Healthcare). Peroxidase-conjugated donkey anti-goat IgG (sc-2033) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Phorbol ester, 12-O-tetradecanoylphorbol-13-acetate (TPA), was obtained from Calbiochem (Billerica, MA, 524400). We report results using control (D-001206-13-05), MEP50 (M-006895-01-0005) and PRMT5 (M-015817-02-0005) siRNA reagent from Dharmacon Inc (Lafayette, CO). Key findings were confirmed using additional siRNA that target MEP50 (D-006895-01-0002 and D-006895-02-0002) and PRMT5 (D-015817-01-0002 and D-015817-04-0002).

## **2. Cell culture, plasmids and viruses:**

Keratinocyte (KERn) serum-free medium (KSFM), 0.25% trypsin and Hank's balanced salt solution (HBSS) were purchased from Invitrogen. Foreskin epidermis, obtained from newborn infants, was separated from dermis by overnight dispase treatment and KERn were obtained after dispersal with trypsin and maintained in KSFM supplemented with epidermal growth factor and pituitary extract (Efimova, Broome et al. 2003, Efimova, Broome et al. 2004b). p21-2326, encoding the human p21<sup>Cip1</sup> promoter linked to luciferase, was obtained from Dr. Bert Vogelstein (El-Deiry, Tokino et al. 1993). The wild-type p21<sup>Cip1</sup> promoter and promoter mutants encoding mutant p53 transcription factor binding sites were constructed in pBluescript II KS (+) (Chew, Adhikary et al. 2011). PG13-Luc was obtained from Dr. Nancy Colburn (Li, Cao et al. 2000). The human MEP50-encoding plasmid was constructed by primer amplification using plasmid p-OTB7-FLAG-MEP50 (pOTB7-WDR77, MHS1011-202830316) from Open Biosystems (Huntsville, AL) as template. FLAG-MEP50 was amplified as a BamHI / NotI fragment using forward: 5'-GATC GGA TCC **ATG GAC TAC AAG GAC GAC GAC GAC AAG** ATG CGG AAG GAA ACC CCA and reverse: 5'-GATC GCG GCC GCC TAC TCA GTA ACA CTT GCA GG primers. The ATG start codon is bold and the FLAG epitope is underlined. The product was then cloned into pcDNA3 to produce pcDNA3-FLAG-MEP50. Adenoviruses encoding HA-p38 $\delta$ , PKC $\delta$ , and empty control virus (tAd5-HA-p38 $\delta$ , Ad5-PKC $\delta$ , Ad5-FLAG-p38 $\delta$ , and Ad5-EV) were prepared by propagation in HEK293 cells followed by cesium chloride gradient centrifugation. For experiments involving adenoviral infection, KERn were treated with 15 MOI of adenovirus in KSFM containing 6  $\mu$ g/ml polybrene. Tetracycline-inducible

viruses (tAd-EV, tAd5-PKC $\delta$ , tAd5-HA-38 $\delta$ ) were co-infected with Ad5-TA encoding virus to induce PKC $\delta$  and p38 $\delta$  expression (Adhikary, Chew et al. 2010).

### **3. Promoter luciferase assay:**

p21<sup>Cip1</sup> promoter reporter plasmid (0.5  $\mu$ g) and 1  $\mu$ g of pcDNA3 or pcDNA3-FLAG-MEP50 was mixed with 4.5  $\mu$ l FUGENE 6 (Promega) diluted in 95.5  $\mu$ l KSFM. After a 20 min incubation, this mixture was added to 2 ml of KSFM in dishes containing fifty percent confluent KERn cultures. After 24 h, cell lysates were collected and processed for luciferase activity assay (Adhikary, Chew et al. 2010).

### **4. Keratinocyte electroporation and cell proliferation assay:**

The AMAXA electroporator and VPD-1002 nucleofection kit (Cologne, Germany) was used for keratinocyte electroporation. KERn were harvested with trypsin and replated one day prior to electroporation. The cells were then re-harvested with trypsin and 1 million cells were used per electroporation. The cells were suspended in 100  $\mu$ l of keratinocyte nucleofection solution containing 3  $\mu$ g of control-, MEP50- or PRMT5-siRNA. The mixture was mixed by gentle pipetting, transferred to the electroporation cuvette, and electroporated using the T-018 setting. KSFM (500  $\mu$ l) was added and the mixture was transferred to a 55 cm<sup>2</sup> dishes containing 10 ml of KSFM. The cells were maintained for various time points before the extracts were prepared for mRNA or protein analysis. This method achieves electroporation efficiencies of > 90% efficiency (Adhikary, Chew et al. 2010).

Cells used for proliferation experiments were double-electroporated. This involved an initial electroporation with 3  $\mu$ g of appropriate siRNA, recovery in culture for 72 h, a

repeat electroporation with 3 µg of siRNA, and a 24 h recovery in culture. The cells were then harvested and seeded at low density (15,000 cells per well) in 35 mm dishes and cell number was counted at 48, 72 and 96 h.

### **5. Immunoblot:**

Cell extracts were prepared in cell lysis buffer (Cell Signaling, 9803, Danvers, MA) containing protease inhibitors (Calbiochem, 539134). Equivalent amounts of protein were electrophoresed on 12% denaturing polyacrylamide gels and transferred to nitrocellulose membrane. The membranes were blocked with 5% skimmed milk in Tris-buffered saline containing 0.1% Tween 20 and incubated overnight with primary antibody and horseradish peroxidase-conjugated secondary antibody for 2 hours. β-actin served as a gel loading control.

### **6. Quantitative RT-PCR:**

Total RNA was isolated using the RNAspin system (GE Healthcare) and reverse transcribed using the Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA). RNA (1 µg) was used for cDNA preparation. The Light Cycler 480 SYBR Green I Master mix (Roche Diagnostics) was used to measure mRNA level. The signals were normalized to the level of cyclophilin A mRNA. The gene specific primers used for detection of mRNA levels were as follows: p21<sup>Cip1</sup> (forward, 5'-AAG ACC ATG TGG ACC TGT CAC TGT; reverse, 5'-AGG GCT TCC TCT TGG AGA AGA TCA), MEP50 (forward: 5'-TTG CTC AGC AGG TGG TAC TGA GTT, reverse: 5'-AAT CTG TGA TGC TGG CTT GGG ACA), p53 (forward: 5'- TAA CAG TTC CTG CAT GGG CGG C, reverse: 5'- AGG ACA GGC ACA AAC ACG CAC C), and cyclophilin A (forward:

5'-CAT CTG CAC TGC CAA GAC TGA, reverse: 5'-TTC ATG CCT TCT TTC ACT TTGC).

## **7. Immunostaining:**

Cells, maintained on coverslips, were fixed for 20 min in phosphate buffered saline containing 4% paraformaldehyde and permeabilized with -20 C chilled methanol. The coverslips were subsequently incubated with the appropriate primary and secondary antibodies for 1 h each. Cells were then incubated with Hoechst 33258 (1:2000) for 5 minutes, washed and mounted on glass slides using Fluoromount (Sigma, F4680). An Olympus OX81 spinning-disc confocal microscope was used to collect fluorescent images. Paraffin-embedded and formalin fixed foreskin sections were immunostained as previously described using fluorescence or peroxidase methods. MEP50 antibody (ab5772) staining was detected using an appropriate fluorophore-conjugated or biotinylated secondary antibody. Biotinylated secondary antibody was obtained as part of the mouse IgG Vectastain ABC kit (Vector Laboratories, Burlingame, CA, PK-6102).

## **8. Chromatin Immunoprecipitation Assay (ChIP):**

ChIP assays were performed using the Diagenode Low Cell ChIP assay kit (C01010073: kch-maglow-G48). Cells ( $0.5 \times 10^6$ ) cells were infected with 15 MOI of empty adenovirus or adenoviruses encoding HA-p38 $\delta$  or PKC $\delta$  and after 48 h one million  $1 \times 10^6$  cells were used for shearing and  $0.1 \times 10^5$  cells were used for immunoprecipitation per antibody. Enrichment of MEP50, PRMT5, H4R3Me2s, H3R8me2s associated DNA sequences in immunoprecipitated samples was detected by qRT-PCR using sequence-specific primers and the LightCycler 480 SYBR Green I master mix. Primers were designed to detect the human p21<sup>Cip1</sup> promoter p53 binding site

located at nucleotides -1426/-1310 (forward: 5'- CCG AGG TCA GCT GCG TTA GAG G, reverse: 5'-AGA ACC CAG GCT TGG AGC AGC).

### **9. Epidermal Equivalent Cultures:**

Freshly isolated foreskin keratinocytes ( $1.5 \times 10^6$ ) were electroporated with 3  $\mu$ g of control-, MEP50- or PRMT5-siRNA. After a 72 h recovery, the cells were harvested, and  $2 \times 10^6$  cells from each group were re-electroporated with 3  $\mu$ g of the same siRNA. The cells were then seeded onto Millicell-PCF chambers (0.4  $\mu$ m, 12 mm, PIHP01250) in KSFM. After 24 h, the cells were shifted to Epilife medium containing 1.4 mM calcium chloride and 5  $\mu$ g/ml vitamin C and cultured at the air-liquid interface (Chew, Adhikary et al. 2013, Saha, Adhikary et al. 2014). Fresh Epilife medium was added every 2 days. After 5 days, the epidermal equivalents were harvested for preparation of RNA, protein, and histology. Total RNA was isolated for qRT-PCR using the Illustra RNAspin Mini kit (GE Healthcare). For protein lysates, the inserts were washed twice with phosphate-buffered saline and the cells were harvested in 0.0625 M Tris-HCl, pH 7.5, containing 10% glycerol, 5% SDS, and 5%  $\beta$ -mercaptoethanol. Next, the cells were sonicated, centrifuged at 10,000 rpm for 5 min, and the supernatant was collected for immunoblot (Saha, Adhikary et al. 2014).

## ***D. RESULTS:***

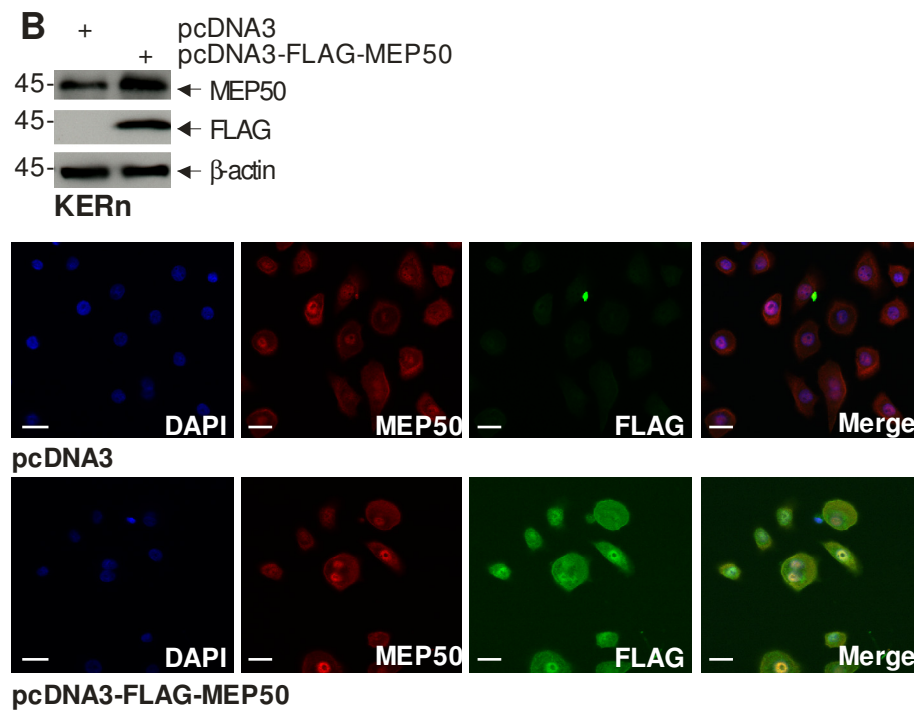
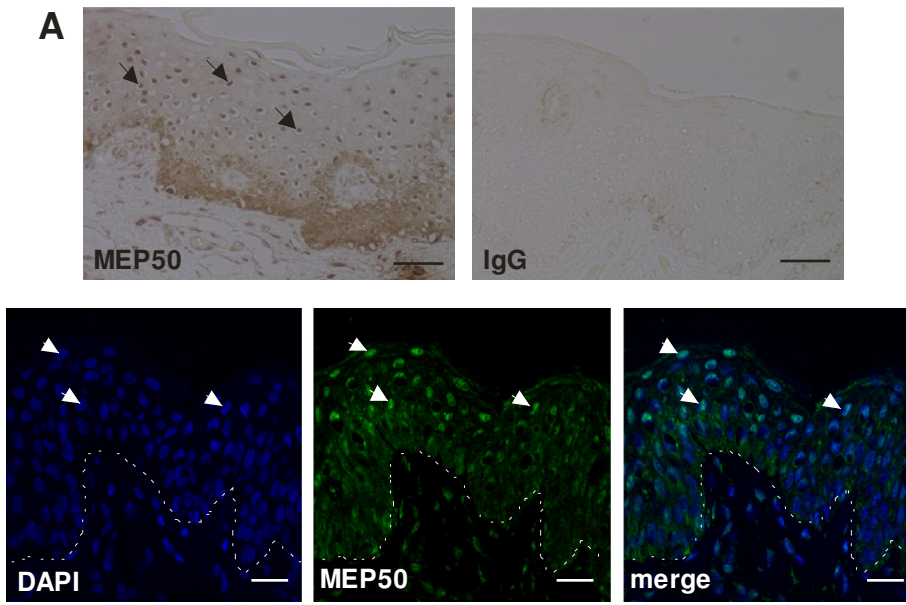
### **1. MEP50 distribution in epidermis:**

MEP50 localizes in the nucleus or cytoplasm in a tissue and cell-type dependent manner that also depends upon the environment (Peng, Chen et al. 2008, Peng, Li et al. 2010, Ligr, Patwa et al. 2011). To assess MEP50 localization in keratinocytes, we stained paraffin embedded sections of foreskin epidermis with anti-MEP50. **Fig. 3-1A** identifies MEP50 as present in all epidermal layers. Cytoplasmic localization predominates in the basal and spinous layers, while nuclear distribution is prominent in the granular layers. We next compared the distribution of endogenous and FLAG-tagged expressed MEP50 in cultured normal epidermal foreskin keratinocytes. **Fig. 3-1B** shows a mixed cytoplasmic and nuclear distribution in these cultures, and that expressed FLAG-MEP50 assumes a similar distribution to endogenous MEP50. This is consistent with a previous report showing that MEP50 localizes in the nucleus and cytoplasm in lung cancer cell lines (Wei, Hsia et al. 2014). This data also shows that vector-expressed FLAG-MEP50 co-localizes with endogenous MEP50.

### **2. MEP50 regulation of cell proliferation:**

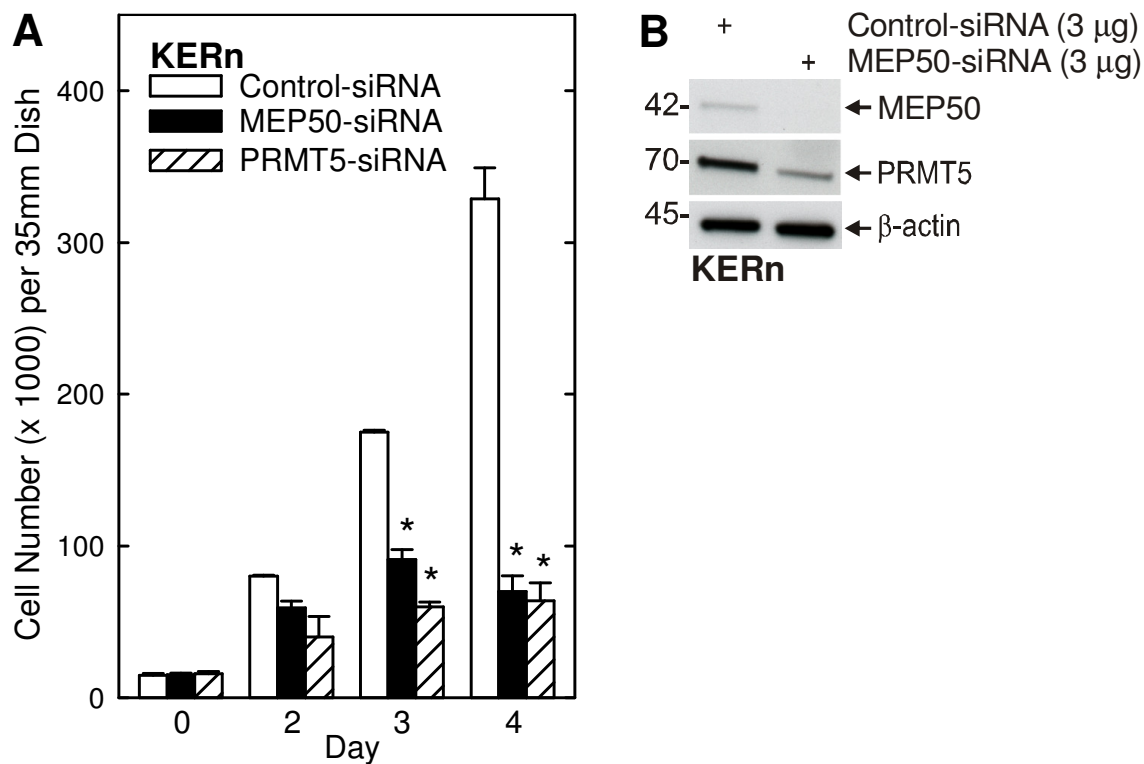
A previous study showed that PRMT5 acts to enhance keratinocyte survival and proliferation (Kanade, Eckert 2012). As MEP50 is frequently engaged as a PRMT5 cofactor that is required for PRMT5 activation, we examined whether altering MEP50 expression regulates keratinocyte proliferation. Cells were treated with control-, MEP50- or PRMT5-siRNA. **Fig. 3-2A** shows that MEP50 or PRMT5 knockdown reduces cell proliferation. **Fig. 3-2B** confirms a reduction in MEP50 expression in MEP50-siRNA treated cells. It also shows that PRMT5 levels are reduced. The reduction in PRMT5 is

not surprising that these proteins are part of a co-regulated complex (Chew, Adhikary et al. 2011, Antonysamy, Bonday et al. 2012, Ho, Wilczek et al. 2013).



**Figure 3-1: MEP50 is expressed in human epidermis and in foreskin KERn.**

**A** Foreskin tissue sections were stained with anti-MEP50 and binding was visualized peroxidase-conjugated secondary antibody (upper panels). The control is IgG. The arrows indicate MEP50 nuclear localization in suprabasal keratinocytes. Foreskin tissue sections (lower panels) were fixed and stained with anti-MEP50 and antibody binding was visualized using a FITC-conjugated secondary antibody. The arrows indicate nuclear MEP50 accumulation. Bar = 10  $\mu$ m. **B** Co-localization of endogenous and expressed MEP50. KERn were electroporated with 3  $\mu$ g of pcDNA3 or pcDNA3-FLAG-MEP50. After 48 h protein lysates were tested by immunoblot using anti-FLAG and anti-MEP50.  $\beta$ -actin was used as the loading control. After 48 h, the cells were fixed and co-stained with anti-FLAG (green) and anti-MEP50 (red). Similar results were observed in each of three experiments. The staining indicates MEP50 distribution in the nucleus and cytoplasm.



**Figure 3-2:MEP50 and PRMT5 are required for keratinocyte proliferation.**

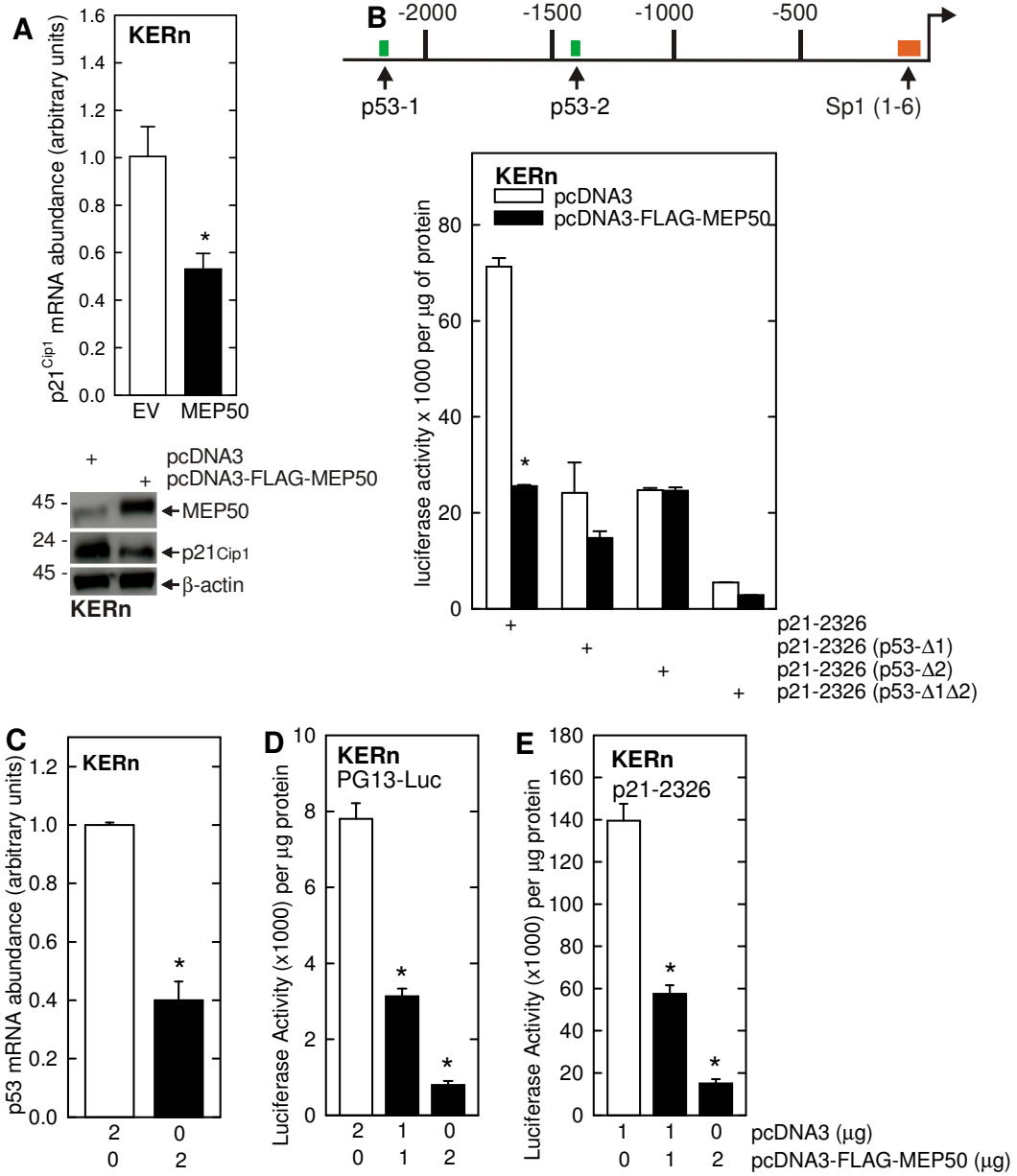
**A** KERN were twice-electroporated with control-, MEP50- or PRMT5-siRNA and 15,000 cells were plated per well. After overnight attachment, cell number was determined (day zero) and at the indicated times thereafter. The values are mean  $\pm$  SEM (n = 3). The asterisks indicate a significant difference ( $p < 0.005$ ). **B** Immunoblot detection of MEP50 and PRMT5. The immunoblot confirms a reduction in MEP50 in MEP50-siRNA treated cultures. PRMT5 is also reduced.

### **3. MEP50 control of p21<sup>Cip1</sup> expression:**

To gain insight regarding the mechanism of MEP50 regulation of proliferation, we examined the effect of MEP50 expression on p21<sup>Cip1</sup> expression. p21<sup>Cip1</sup> is an important cell cycle control regulator in keratinocytes (Chew, Adhikary et al. 2011, Chew, Adhikary et al. 2012). We have previously shown that p21<sup>Cip1</sup> is increased when keratinocyte proliferation is inhibited (Chew, Adhikary et al. 2011) via a mechanism that involves p53 interaction at the p21<sup>Cip1</sup> gene promoter (Chew, Adhikary et al. 2012). **Fig. 3-3A** shows that increased MEP50 expression is associated with reduced p21<sup>Cip1</sup> mRNA and protein expression. To examine the impact of MEP50 on p21<sup>Cip1</sup> gene expression, KERn cells were transfected with constructs encoding various p21<sup>Cip1</sup> promoter segments fused to the luciferase reporter gene (Chew, Adhikary et al. 2011, Chew, Adhikary et al. 2012), in the presence or absence of FLAG-MEP50 expression vector. **Fig. 3-3A** shows the structure of the p21<sup>Cip1</sup> promoter which includes a cluster of six Sp1 binding sites in the proximal promoter and two p53 response elements (p53-1, p53-2) in the distal promoter (Chew, Adhikary et al. 2011, Chew, Adhikary et al. 2012). **Fig. 3-3B** shows that MEP50 expression reduces activity of the full-length p21<sup>Cip1</sup> promoter by 70%. As a control, we show that mutation of one or both p53 transcription factor binding sites attenuates the MEP50 impact by reducing overall promoter activity. These findings indicate that MEP50 and p53 produce opposite effects on p21<sup>Cip1</sup> gene expression and that MEP50 expression can antagonize the action of p53.

We next examined whether MEP50 expression reduces p53 as part of the mechanism that leads to reduced p21<sup>Cip1</sup> promoter activity. **Fig. 3-3C** shows that MEP50 expression reduces the level of p53 encoding mRNA and **Fig. 3-3D** shows that this is associated with

a reduction in p53 (PG13-Luc) promoter activity. **Fig. 3-3E** confirms that this is associated with loss of p21<sup>Cip1</sup> promoter activity. These findings suggest that MEP50 suppresses p53 gene expression as part of the mechanism whereby MEP50 increases p21<sup>Cip1</sup> expression.

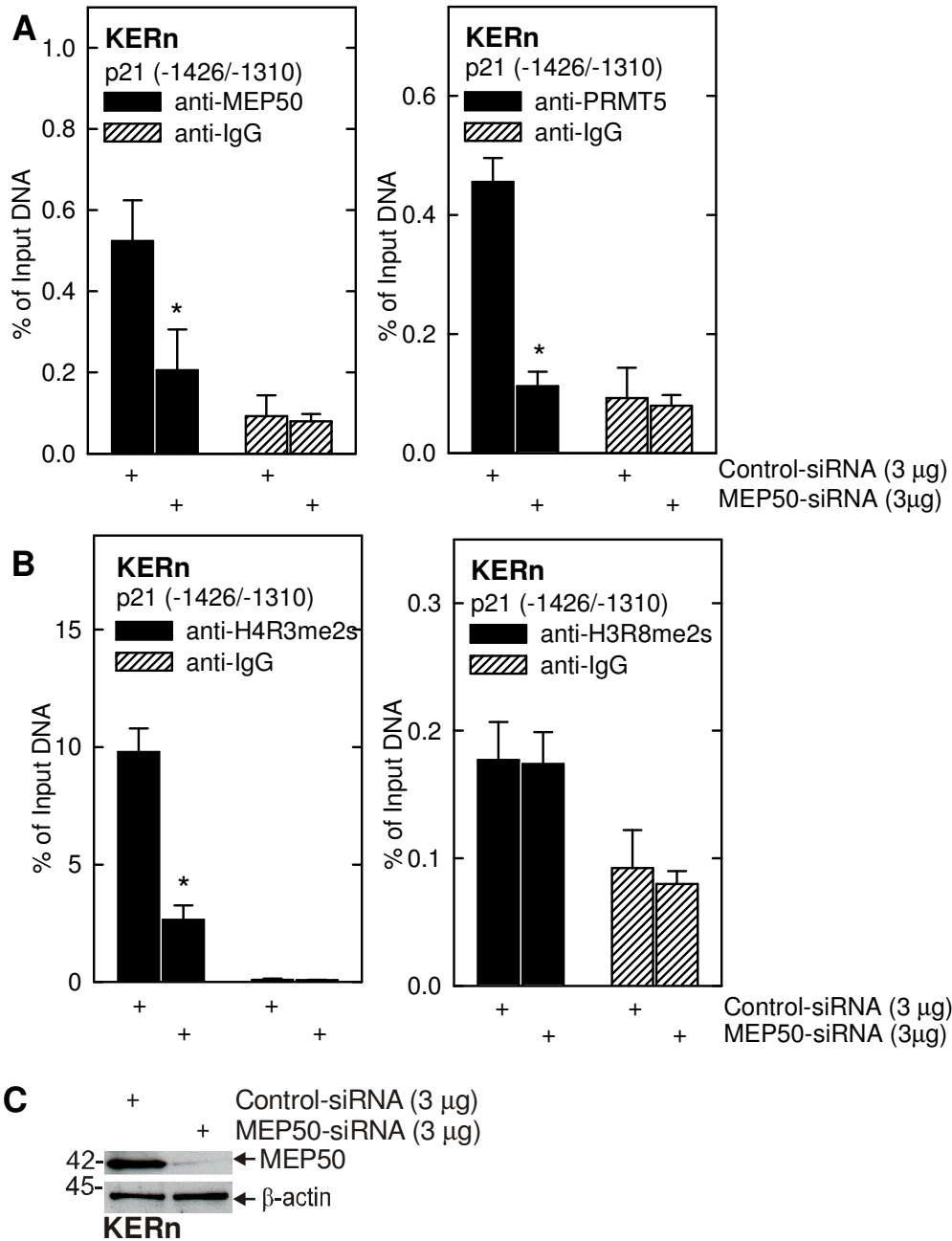


**Figure 3-3:MEP50 regulation of p21<sup>Cip1</sup> promoter activity - a role for p53.**

**A** MEP50 suppresses p21<sup>Cip1</sup> expression. KERn were electroporated with the indicated plasmid and after 24 h extracts were prepared for qRT-PCR detection of p21<sup>Cip1</sup> mRNA and immunoblot detection of p21<sup>Cip1</sup> and MEP50. **B** Opposing action of MEP50 and p53. Schematic of the human p21<sup>Cip1</sup> promoter shows the Sp1 and p53 transcription factor response elements. The numbers indicate distance in nucleotides relative to the transcription start site. KERn were transfected with 0.5 µg of p21-2316, which encodes the full-length wild-type p21<sup>Cip1</sup> promoter, or the promoter harboring mutations at the p53-1 or p53-2 sites, linked to luciferase. After 24 h extracts were prepared for luciferase activity assay. The values are mean ± SEM, n = 3. The asterisks indicate a significant change (p < 0.005). **C/D/E** KERn were transfected with 0.5 µg of the PG13-Luc (p53 gene promoter linked to luciferase) or p21-2326 and the indicated number of µg of pcDNA3 or pcDNA3-FLAG-MEP50. At 48 h post-transfection cell extracts were prepared and assayed for promoter activity. The values are mean ± SEM, n = 3. The asterisks indicate significant differences, (p < 0.005).

#### **4. MEP50 and PRMT interact at the p21<sup>Cip1</sup> promoter:**

A major role of PRMT5/MEP50 complex is catalyzing symmetric dimethylation of arginine 3 of Histone 4 (H4R3me2s) and arginine 8 of Histone 3 (H3R8me2s) (Molina-Serrano, Schiza et al. 2013). These modifications occur at histones associated with genes that are undergoing silencing (Karkhanis, Hu et al. 2011, Molina-Serrano, Schiza et al. 2013). We therefore examined the role of MEP50 in modifying H3 and H4 associated with the p21<sup>Cip1</sup> promoter. We selected the biologically important p21<sup>Cip1</sup> promoter p53-2 binding response element as a target (Chew, Adhikary et al. 2011, Chew, Adhikary et al. 2012, El-Deiry, Tokino et al. 1993). This region is known to bind p53 as a requirement for increased p21<sup>Cip1</sup> expression (El-Deiry, Tokino et al. 1993, Macleod, Sherry et al. 1995). **Fig. 3-4A** uses ChIP analysis to show that MEP50 knockdown reduces MEP50 and PRMT5 association with the p21<sup>Cip1</sup> promoter. Further ChIP analysis in **Fig. 3-4B** shows that loss of MEP50 is associated with reduced p21<sup>Cip1</sup> promoter-associated H4R3me2s formation, but no reduction in H3R8me2s formation. These findings suggest that the increase in p21<sup>Cip1</sup> expression observed in MEP50 knockdown cells is, in part, due to loss of PRMT5 and MEP50 and reduced formation of H4R3me2s in chromatin surrounding the p53-2 DNA response element in the p21<sup>Cip1</sup> promoter. It is not clear why H3R8me2s levels are not reduced.



**Figure 3-4: MEP50 knockdown reduces MEP50 and PRMT5 association at the p21Cip1 promoter.**

A/B KERn were electroporated with 3  $\mu$ g of control-siRNA or MEP50-siRNA. After 48 h, extracts were prepared from ChIP analysis. DNA from one million cells was sheared and 50,000 cell equivalents of DNA were used for immunoprecipitation. The primers span the p21<sup>Cip1</sup> promoter region that includes the p53-2 site. The values are mean  $\pm$  SEM, n = 3. The asterisks indicate significant difference (p < 0.005). C Extracts were prepared from the electroporated cells after 48 h to confirm MEP50 knockdown.

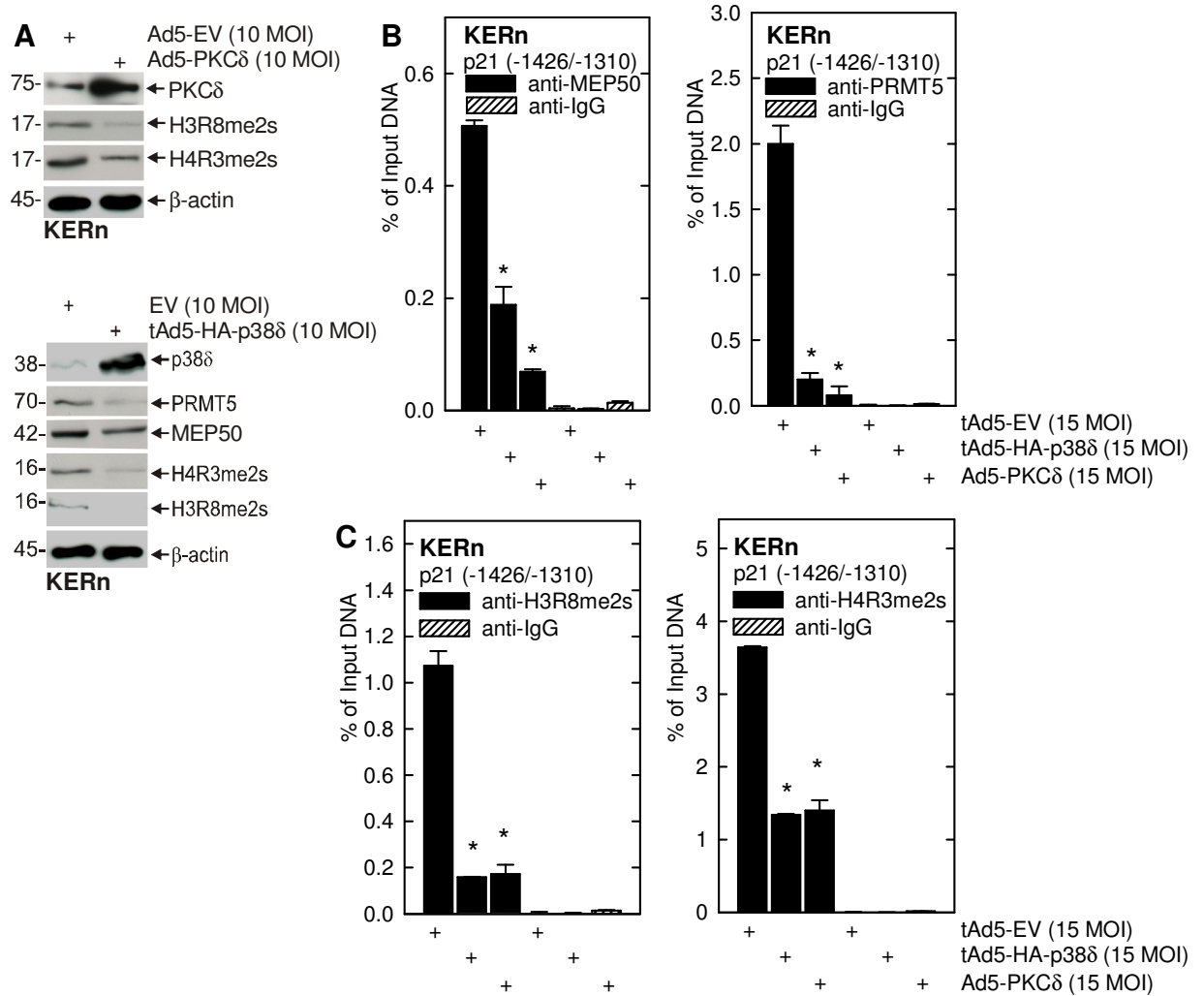
## **5. PKC $\delta$ and p38 $\delta$ regulation of MEP50 level:**

The PKC $\delta$ , MEKK1, MEK3, p38 $\delta$  signaling cascade is a central controller of keratinocyte proliferation. Activation of this cascade by overexpression of PKC $\delta$  or p38 $\delta$  suppresses proliferation and enhances differentiation (Eckert, Crish et al. 2003b) which is associated with a specific increase in p21<sup>Cip1</sup> gene expression (Adhikary, Chew et al. 2010, Chew, Adhikary et al. 2011, Chew, Adhikary et al. 2012). We wanted to assess whether activation of this cascade results in reduced expression of MEP50 or PRMT5. Keratinocytes were grown in the presence of empty, PKC $\delta$  or p38 $\delta$  encoding adenovirus, and after 48 h extracts were prepared to assess impact on total MEP50, PRMT5, H4R3me2s and H3R8me2s level. **Fig. 3-5A** shows that PKC $\delta$  or p38 $\delta$  expression reduces total MEP50 and PRMT5 level, and total cellular level of H3R8me2s and H4R3me2s. We next assessed by ChIP analysis whether PKC $\delta$  or p38 $\delta$  expression is associated with reduced MEP50 or PRMT5 interaction at the p21<sup>Cip1</sup> gene promoter p53-2 response element. **Fig. 3-5B/C** shows that PKC $\delta$  or p38 $\delta$  expression results in reduced MEP50 and PRMT5 interaction and reduced H3R8me2s and H4R3me2s histone modification at the p21<sup>Cip1</sup> promoter p53-2 site.

## **6. Impact of TPA treatment on promoter activity:**

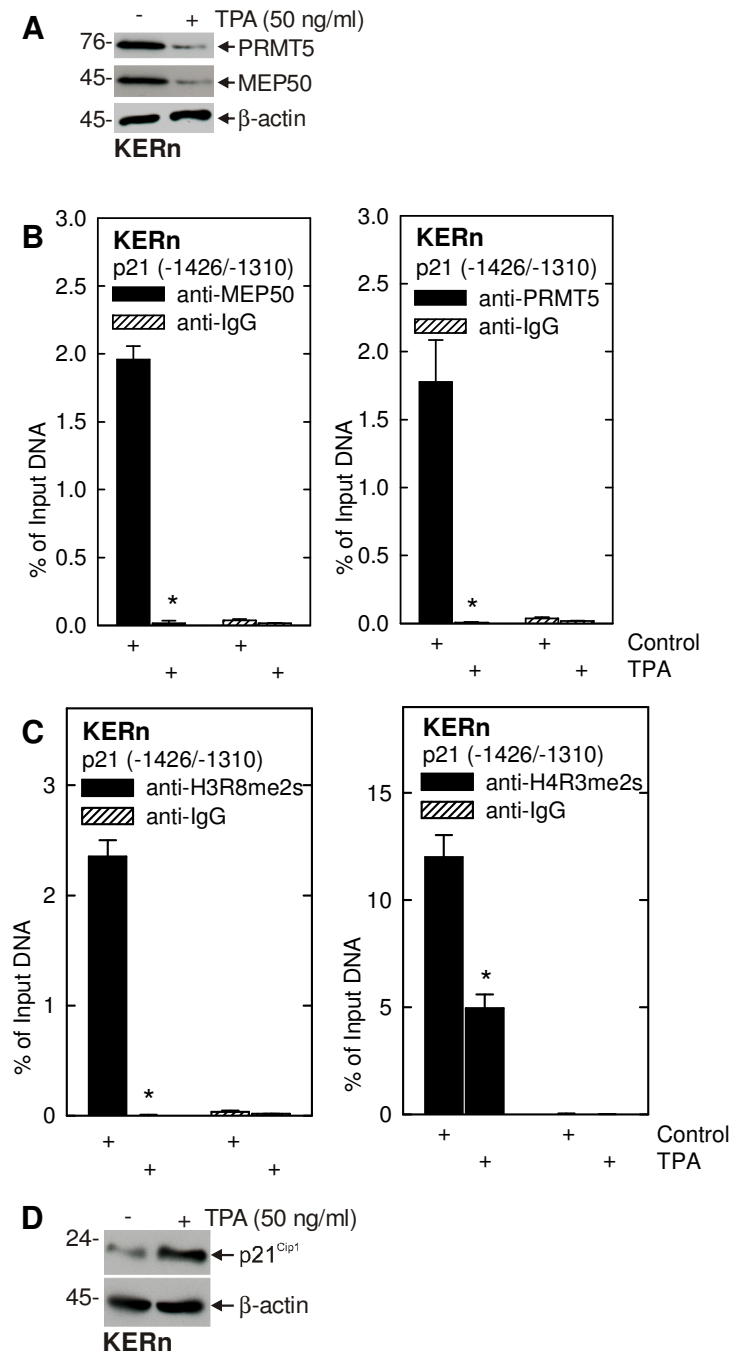
We also examined whether a known activator of the PKC $\delta$ /p38 $\delta$  cascade (Efimova, LaCelle et al. 1998) alters MEP50 and PRMT5 expression. **Fig. 3-6A** shows that TPA treatment reduces total cellular levels of PRMT5 and MEP50. Moreover, the ChIP analysis in **Fig. 3-6B/C** shows that this treatment reduces MEP50 and PRMT5 levels at the p21<sup>Cip1</sup> promoter p53-2 response element, and confirms that this is associated with reduced element-associated H4R3me2s and H3R8m2s. **Fig. 3-6D** confirms reduced

modification of methylation of histones at the p21<sup>Cip1</sup> promoter is associated with increased p21<sup>Cip1</sup> protein level.



**Figure 3-5: PKCδ and p38δ regulate MEP50 and PRMT5 level and activity.**

A KERN were infected with 10 MOI of tAd5-EV, Ad5-PKCδ or tAd5-HA-p38δ and at 48 h extracts were prepared for detection of PKCδ, MEP50, H3R8me2S and H4R3me2s. Similar results were obtained in three different experiments. B/C KERN were infected as above and at 48 h extracts were prepared for ChIP using primers spanning the p21<sup>Cip1</sup> promoter region that includes the p53-2 site. The values are mean ± SEM, n = 3. The asterisks indicate significant difference (p < 0.005).



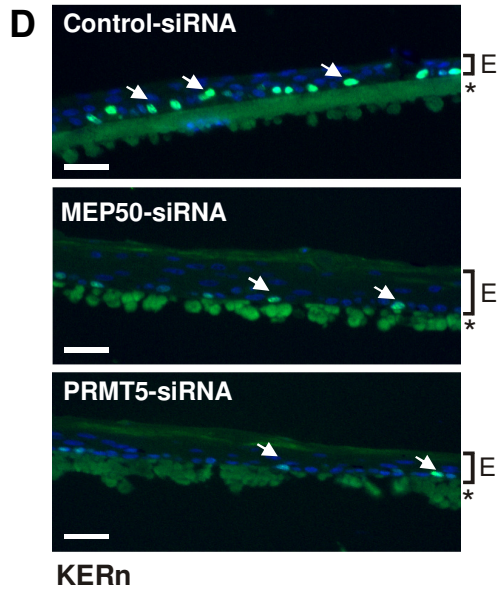
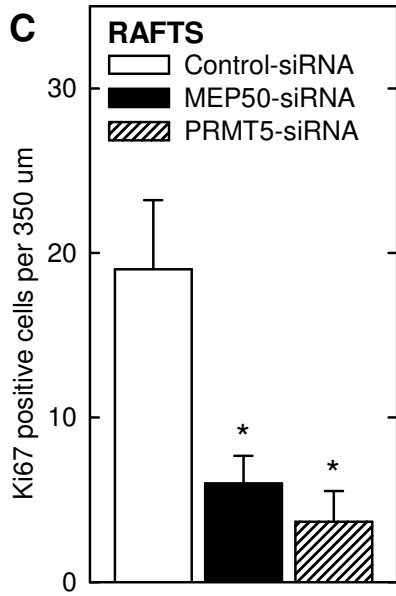
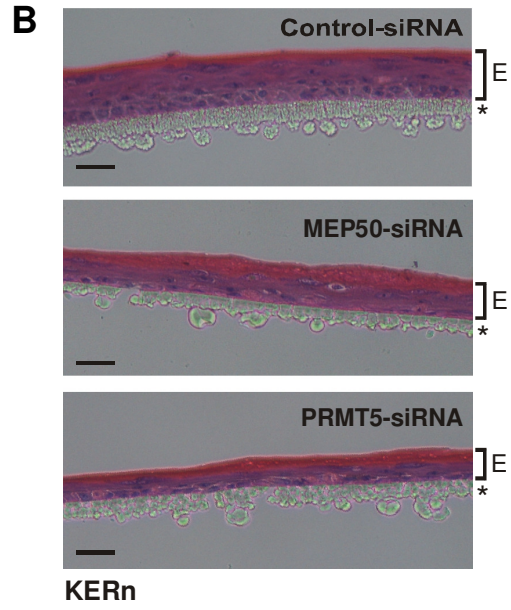
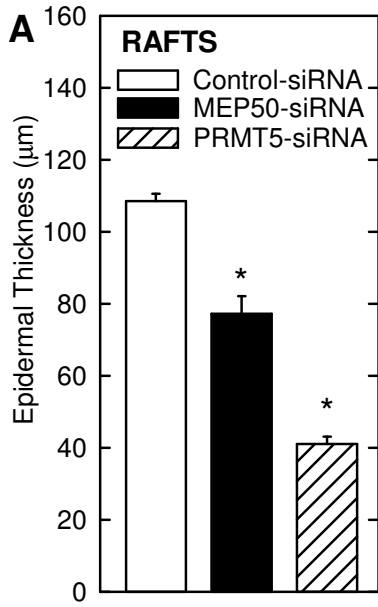
**Figure 3-6: TPA regulates MEP50 and PRMT5 levels and activity.**

**A** KERN were treated with 50 ng/ml TPA for 48 h and extracts were prepared for detection of MEP50, PRMT5, H3R8me2s and H4R3me2s. Similar results were obtained in three different experiments. **B/C** KERN were treated with 50 ng TPA/ml and after 48 h mRNA extracts were isolated for ChIP analysis and detection of MEP50 and PRMT5 interaction and H3R8-me2s and H4R3me2s formation at the p21<sup>Cip1</sup> promoter. Similar results were obtained in three different experiments. **D** Cells were treated for 48 h with TPA and p21<sup>Cip1</sup> levels are monitored.

## **7. Biological relevance of PRMT5 and MEP50 regulation:**

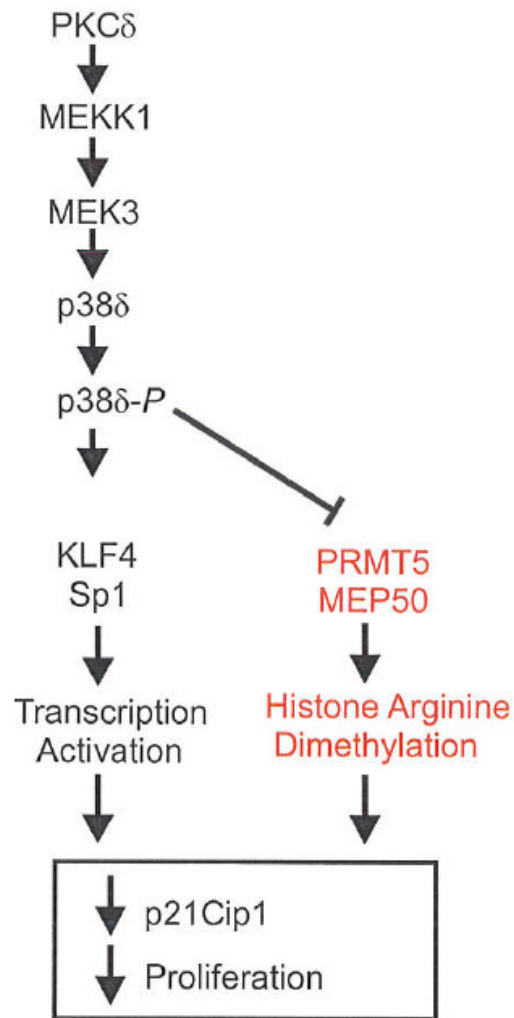
To further assess the biological relevance of this regulation, we examined the impact of altering MEP50 and PRMT5 level on proliferation using a keratinocyte epidermal equivalent model. In this model, keratinocytes are grown at the air-liquid interface to produce a stratified, multilayered and differentiated epidermal equivalent (Poumay, Dupont et al. 2004). This system is a faithful mimic of *in vivo*-like stratification (Poumay, Dupont et al. 2004). Primary foreskin keratinocytes were electroporated with control-, MEP50- or PRMT5-siRNA and then transferred to Millicell chambers for growth as epidermal equivalent cultures.

To assess the biological impact of MEP50 or PRMT5 knockdown on differentiation and proliferation, we monitored the effect on morphology of the epidermal equivalent cultures. **Fig. 3-7A/B** shows that cultures expressing normal endogenous levels of MEP50 and PRMT5 undergo appropriate proliferation and differentiation to produce a normal multilayered tissue and stable cornified layer. In contrast, knockdown of either MEP50 or PRMT5 produces a significantly thinner epidermal equivalent. In particular, the viable middle layers are reduced relative to the cornified (top) dead layers, suggestive of reduced proliferation in the MEP50 and PRMT5-deficient cultures. To test this, we generated epidermal equivalents from cells electroporated with control-, MEP50- and PRMT5-siRNA and then stained with anti-Ki67 as a marker of cell proliferation (Gerdes, Lemke et al. 1984). **Fig. 3-7C/D** shows that MEP50 or PRMT5 knockdown reduces the number of Ki67 positive (proliferating) cells (green stain, arrows).



**Figure 3-7: MEP50 and PRMT5 regulate differentiation and proliferation in an epidermal equivalent model.**

KERn were twice electroporated with control or MEP50 or PRMT5 siRNA and seeded for epidermal equivalent culture. After 4 days of exposure at the air-liquid interface, the equivalents were harvested and sectioned. **A/B** MEP50 and PRMT5 are required for appropriate skin equivalent formation. KERn were twice electroporated with control-, MEP50- or PRMT5-siRNA and seeded for epidermal equivalent culture. After 4 d of exposure at the air-liquid interface, the equivalents were harvested and stained with hemotoxylin and eosin. The nylon support membrane is indicated by an asterisk and the extent of the epidermis is indicated (E). Similar results were observed in three separate experiments. The graph compares epidermal equivalent thickness among control-, MEP50- and PRMT5-siRNA cultures. The values are mean  $\pm$  SEM, n = 3. Asterisks indicate significant differences from control, p < 0.005. **C/D** MEP50 and PRMT5 are required for cell proliferation. KERn were twice electroporated with control-, MEP50- or PRMT5-siRNA and seeded for epidermal equivalent culture. After 4 d, the equivalents were stained with Ki67 antibody and Hoescht. The membrane (\*) and extent of epidermis (E) are shown. The graph quantitates the number of Ki67 positive cells in the control, MEP50 and PRMT5-siRNA rafts. Similar results were observed in three separate experiments. Significant differences were determined using the Student t-test (p < 0.005, asterisks). Note that the blue-green spots are Ki67 staining, as compared to the larger stained circular structures which are non-specific staining to the nylon membrane.



**Figure 3-8: Proposed regulatory model.**

A balance between transcriptional and epigenetic regulation. Arrows indicate a stimulus and flat-headed bars inhibition. Details are explained in the text.

## ***E. DISCUSSION:***

### **1. Regulation of keratinocyte differentiation and proliferation:**

Keratinocytes constitute the major cell type of the epidermis (Eckert, Crish et al. 1997). These cells begin as proliferative cells in the epidermal basal layer and undergo a highly orchestrated differentiation program leading to cornified envelope formation. The result is formation of a multilayered epidermis in which differentiated cells are released from the surface (Eckert, Crish et al. 1997). Decoding the mechanisms that control epidermal homeostasis is an important goal as is understanding how deregulation of this process leads to disease. Novel PKC isoforms play pivotal roles in regulation of epidermal homeostasis (Efimova, Eckert 2000, Efimova, Deucher et al. 2002, Efimova, Broome et al. 2004b). They activate MAPK signaling to direct specific transcription factors to increase expression of differentiation associated genes (Efimova, LaCelle et al. 1998). Simultaneously, they control keratinocyte proliferation by increasing expression of growth suppressing genes, including p21<sup>Cip1</sup>. Previous studies from our group have shown that PKC $\delta$  activates a MEKK1/MEK3/p38 $\delta$  cascade that stimulates KLF4, Sp1 and p53 transcription factor association with the p21<sup>Cip1</sup> promoter leading to increased p21<sup>Cip1</sup> expression and reduced cell proliferation (Chew, Adhikary et al. 2011, Saha, Adhikary et al. 2014). However, less is known about mechanisms that antagonize this action to maintain keratinocyte proliferative potential.

### **2. A new role for MEP50 in regulating keratinocyte differentiation:**

Our recent mass spectrometry analysis identified PRMT5, a type II symmetric arginine methyltransferase, as a novel component of the PKC $\delta$ /p38 $\delta$  regulatory complex (Kanade, Eckert 2012). We further showed that PRMT5 acts to enhance cell survival by

catalyzing symmetric dimethyl arginine (SDMA) modification of target proteins in this complex leading to inhibition of p38 $\delta$  activity (Kanade, Eckert 2012). This represents a mechanism whereby PRMT5 acts to suppress activity in a growth-suppression signaling cascade.

However, PRMT5 is also known to act at the level of chromatin to increase histone arginine dimethylation as a mechanism to silence gene expression (Pal, Baiocchi et al. 2007b). Silencing is mediated by PRMT5/MEP50-dependent histone arginine dimethylation of H4R3 and H3R8 to form H4R3me<sub>2</sub>s and H3R8me<sub>2</sub>s (Ho, Wilczek et al. 2013, Pal, Baiocchi et al. 2007b, Krause, Yang et al. 2007b). In the present study, we examine the impact of PRMT5 on histone arginine dimethylation of the p21<sup>Cip1</sup> promoter as a mechanism of keratinocyte growth control. We focus on the role of MEP50, a cofactor of PRMT5 that is required for PRMT5 catalytic activity. We find that MEP50 is abundantly expressed in foreskin epidermis and monolayer keratinocyte cultures where it is present in both the nucleus and cytoplasm. This localization is similar to that observed in other cell types (Wei, Hsia et al. 2014). We show that MEP50 knockdown leads to a substantial reduction in cell proliferation. Moreover, the reduction is associated with increased expression of p21<sup>Cip1</sup>, a known inhibitor of keratinocyte proliferation (Chew, Adhikary et al. 2011, Chew, Adhikary et al. 2012). Knockdown studies show that MEP50 level is inversely correlated with p21<sup>Cip1</sup> mRNA level and promoter activity.

Previous studies show that the p53 transcription factor is a key positive activator of p21<sup>Cip1</sup> expression (Macleod, Sherry et al. 1995), and that it acts by binding to two p53 response elements, p53-1 and p53-2, in the distal region of the p21<sup>Cip1</sup> promoter. In an effort to dissect the mechanism of MEP50 action, we manipulated MEP50 expression

using siRNA and expression vectors and monitored the impact on p21<sup>Cip1</sup> gene expression, promoter activity and modification of histone surrounding the p53-2 response element in the p21<sup>Cip1</sup> promoter. These studies showed an inverse relationship between MEP50 level, and p21<sup>Cip1</sup> expression and promoter activity. We further show that MEP50 interaction at the p53-2 response element is associated with acquisition of histone silencing marks, H4R3me2s and H3R8m2s. This is a particularly important finding, as the p53-2 response element (nucleotides -1393/-1374) in the p21<sup>Cip1</sup> promoter, is a key positive regulatory element (Chew, Adhikary et al. 2012). This suggests that MEP50, acting with PRMT5, inhibits p21<sup>Cip1</sup> by producing silencing histone marks in this region of chromatin. We have not assayed the impact of these manipulations on arginine dimethylation of histones H4 and H3 at the p53-1 site (nucleotides -2281/-2261), but we assume the results would be similar.

Few studies have examined the interplay between cell cycle regulators, such as p21<sup>Cip1</sup>, and PRMT5/MEP50. However, one study suggests that MEP50 is phosphorylated by CDK4 and that this leads to increased PRMT5/MEP50 activity which enhances events leading to cyclin D1-dependent cell proliferation (Aggarwal, Vaiteas et al. 2010). A second study shows that PRMT5 accelerates cell cycle progression through G1 by upregulation of CDK4, CDK6, cyclins D1, D2 and E1, and that this was associated with activation of phosphoinositide 3-kinase (PI3K)/AKT activity (Wei, Juan et al. 2012b). Thus, our findings are consistent with cell cycle regulation being a target of PRMT5/MEP50 activity, and with PRMT5/MEP50 acting to enhance cell proliferation.

### **3. Impact of MEP50 on PKC $\delta$ /p38 $\delta$ signaling:**

A novel aspect of this work is identifying an opposing/antagonistic relationship between PKC $\delta$ /p38 $\delta$  signaling and MEP50/PRMT5 action. We show that activation of PKC $\delta$ /p38 $\delta$  signaling, by expression of either of these kinases, or by treatment with an activator of PKC $\delta$  (TPA), results in increased p21<sup>Cip1</sup> expression via direct transcriptional activation of p21<sup>Cip1</sup> gene expression (Chew, Adhikary et al. 2011, Chew, Adhikary et al. 2012). The present studies show that PRMT5/MEP50 opposes this regulation and that this is associated with SDMA modification of histones on the p21<sup>Cip1</sup> promoter. Thus, this study establishes a new biochemical link that may help explain the opposing actions of PKC $\delta$ /p38 $\delta$  signaling and MEP50/PRMT5 as regulators of the p21<sup>Cip1</sup> locus. This would be consistent with our previous report showing that PRMT5 dimethylates proteins in a p38 $\delta$  complex leading to reduced p38 $\delta$  phosphorylation and activity in this cascade (Kanade, Eckert 2012). Together, these findings suggest that PRMT5/MEP50 inhibits PKC $\delta$ /p38 $\delta$  signaling in keratinocytes via multiple mechanisms.

An additional significant finding is that PKC $\delta$ /p38 $\delta$ -dependent signaling reduces MEP50 and PRMT5 protein level and histone arginine dimethylation. Specifically, H3R8me2s and H4R3me2s levels are reduced at key transcriptional elements in the p21<sup>Cip1</sup> promoter. This suggests that activity in this pro-differentiation/anti-proliferation cascade actively suppresses function of the MEP50/PRMT5 pro-proliferation/pro-survival regulation. This, in theory, suggests that this combination of events leads to a more efficient induction of gene (p21<sup>Cip1</sup>) expression. These findings are also interesting from the perspective that PRMT5 and MEP50 have been shown to be pro-survival proteins in a variety of cancer models (Peng, Chen et al. 2008, Ligr, Patwa et al. 2011,

Peng, Li et al. 2010, Wei, Hsia et al. 2014, Aggarwal, Vaites et al. 2010, Gu, Zhang et al. 2013, Zhongping, Shen et al. 2012), which support the idea that these proteins are elevated under conditions where cells need to retain proliferative potential.

#### **4. MEP50 in epidermal equivalent models:**

In order to better assess the biological relevance of MEP50, we used an epidermal equivalent model which efficiently mimics *in vivo* keratinocyte differentiation (Poumay, Dupont et al. 2004). Our studies show that knockdown of MEP50 or PRMT5 results in a significant reduction in thickness of the epidermis. This is in agreement with the observation that MEP50 and PRMT5 knockdown decrease in keratinocyte cell number in studies of monolayer cultures. Additionally, staining of sections from the control, MEP50 and PRMT5 knockdown epidermal equivalents show fewer Ki67-positive cells in the basal layer of MEP50 or PRMT5 knockdown cells as compared to the control equivalents. This data strongly suggests that MEP50 and PRMT5 are likely to have a physiologically meaningful role in controlling keratinocyte proliferation *in vivo*.

In summary, we have shown that a PKC $\delta$ /p38 $\delta$  cascade activates p53 interaction at the p21<sup>Cip1</sup> promoter p53 response elements to increase transcription of the p21<sup>Cip1</sup> gene (**Fig. 3-8A**). p21<sup>Cip1</sup> then inhibits cyclin-dependent kinase activity to suppress cell cycle progression and reduce cell proliferation (Chew, Adhikary et al. 2011, Chew, Adhikary et al. 2012). PRMT5 and MEP50 act to antagonize this action by arginine dimethylating proteins that are part of a p38 $\delta$  regulatory complex to reduce p38 $\delta$  activity, and also act at the p21<sup>Cip1</sup> gene level to arginine dimethylate histones H3 and H4 to produce closed chromatin and p21<sup>Cip1</sup> gene silencing. We further show that activation of the PKC $\delta$ /p38 $\delta$  cascade, by PKC $\delta$ /p38 $\delta$  overexpression or TPA treatment, stimulates

p21<sup>Cip1</sup> expression by three mechanisms (**Fig. 3-8B**). First, it acts by stimulating p53 binding to the p53 response elements in the p21<sup>Cip1</sup> promoter to increase transcription. Second, it suppresses production of PRMT5 and MEP50 mRNA and protein, leading to reduced arginine dimethylation of histones H3 and H4 and derepression of p21<sup>Cip1</sup> gene expression (**Fig. 3-8B**). Third, loss of PRMT5/MEP50 reduces inhibition of p38 $\delta$  kinase activity, strengthening the stimulus to increase p21<sup>Cip1</sup> levels (not shown).

**CHAPTER 4: MEP50 AND PRMT5 SUPPRESS INVOLUCRIN  
EXPRESSION BY ARGININE METHYLATION OF PROMOTER-  
ASSOCIATED HISTONES AND THIS IS REVERSED BY PKC $\delta$ /p38 $\delta$   
PRO-DIFFERENTIATION SIGNALING**

**A. ABSTRACT:**

PKC $\delta$  and p38 $\delta$  are key proteins in a cascade that stimulates keratinocyte differentiation. This cascade activates involucrin gene transcription of the involucrin (hINV) gene and other genes associated with differentiation. Protein arginine methyltransferase 5 (PRMT5) is an arginine methyltransferase that symmetrically dimethylates arginine residues on target proteins. This protein interacts with a cofactor, MEP50, and symmetrically dimethylates arginine eight of histone 3 (H3R8me2s) and arginine three of histone 4 (H4R3me2s) to silence gene expression. We use the involucrin gene as a tool to understand the relationship between PKC $\delta$ /p38 $\delta$  signaling and PRMT5/MEP50 gene silencing. We show that MEP50 suppresses hINV mRNA level and promoter activity. This is associated with increased arginine dimethylation of hINV gene promoter-associated H3 and H4. We further show that the PKC $\delta$ /p38 $\delta$  keratinocyte differentiation-stimulatory cascade reduces PRMT5 and MEP50 expression, association with the hINV gene promoter, and H3R8me2s and H4R2me2s formation. We propose that PRMT5/MEP50 silencing of involucrin expression is an epigenetic mechanism that assists in silencing of hINV expression, and that PKC $\delta$  signaling activates gene expression by directly activating transcription and by suppressing PRMT5/MEP50 dependent arginine dimethylation of promoter associated histones. This is a novel

example of crosstalk between PKC $\delta$ /p38 $\delta$  signaling and PRMT5/MEP50 epigenetic silencing.

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<sup>4</sup> CITATION: Saha K, Adhikary G, Eckert R. L, 2015. (Submitted).

## ***B. INTRODUCTION:***

PKC $\delta$  and p38 $\delta$  are key proteins that control keratinocyte differentiation and proliferation (Chew, Adhikary et al. 2011, Adhikary, Chew et al. 2010, Chew, Adhikary et al. 2013, Efimova, LaCelle et al. 1998, Efimova, Deucher et al. 2002, Efimova, Broome et al. 2003, Efimova, Broome et al. 2004b). PKC $\delta$  activates a MEK3/p38 $\delta$  cascade which triggers events that enhance keratinocyte differentiation (Eckert, Efimova et al. 2003a, Eckert, Crish et al. 2003a, Efimova, LaCelle et al. 1998, Efimova, Broome et al. 2003). This increases AP1, Sp1 and Kruppel-like transcription factor activity leading to activation of differentiation associated gene expression (Crish, Bone et al. 2002, Crish, Gopalakrishnan et al. 2006, Crish, Zaim et al. 1998). In particular, involucrin gene expression is increased (Adhikary, Chew et al. 2010, Banks, Crish et al. 1998, Chew, Adhikary et al. 2013, Crish, Zaim et al. 1998, Crish, Bone et al. 2002, Crish, Gopalakrishnan et al. 2006, Crish, Eckert 2008, Han, Rorke et al. 2012, Welter, Eckert 1995, Welter, Crish et al. 1995b, Banks, Crish et al. 1999). This regulation is mediated via specific elements in the involucrin promoter distal regulatory region (DRR) that function both in cultured cells and *in vivo* (Crish, Zaim et al. 1998, Crish, Bone et al. 2002, Crish, Gopalakrishnan et al. 2006, Rorke, Adhikary et al. 2010).

We have recently been interested in epigenetic mechanisms that antagonize this pro-differentiation signaling mechanism. Modification of arginine side chain guanidine groups is quantitatively one of the most frequent modifications in cells (Bedford, Clarke 2009). Three distinct types of methylated arginine residues occur in cells. The most prevalent is omega-N<sup>G</sup>,N<sup>G</sup>-dimethylarginine (Paik, Paik et al. 2007) where two methyl groups are placed on one of the terminal nitrogen atoms of the guanidine group to form

asymmetric dimethylarginine. Other forms include symmetric dimethylated arginine (SDMA), where one methyl group is placed on each of the terminal guanidino nitrogens (omega-N<sup>G</sup>,N<sup>G</sup>-dimethylarginine) and the monomethylated derivative with a single methyl group on the terminal nitrogen atom (omega-N<sup>G</sup>-monomethylarginine) (Paik, Paik et al. 2007).

Protein arginine methyltransferase 5 (PRMT5) is an arginine methyltransferase that symmetrically dimethylates arginine residues on target proteins in both the cytoplasm and nucleus (Bedford, Clarke 2009). Histones H3 and H4 are important targets. PRMT5 symmetrically dimethylates H3 and H4 as part of an epigenetic mechanism (Molina-Serrano, Schiza et al. 2013). Histone H4 is symmetrically dimethylated on arginine 3 (H4R3me2s), and histone H3 is symmetrically dimethylated on arginine 8 (H3R8me2s), and this is associated with silencing of gene expression (Bedford, Clarke 2009, Tae, Karkhanis et al. 2011, Fabbrizio, Messaoudi et al. 2002).

Our recent study shows that PRMT5 antagonizes differentiation-associated signaling and reduces involucrin gene expression (Kanade, Eckert 2012). PRMT5 acts with MEP50 which forms a complex with and activates PRMT5 catalytic activity (Ho, Wilczek et al. 2013, Hosohata, Li et al. 2003). In the present study, we examine the role of MEP50 in mediating this response. We show that MEP50 suppresses involucrin (hINV) gene expression as reflected by reduced hINV mRNA level and promoter activity. This is associated with increased arginine dimethylation of the hINV gene promoter. We further show that the PKC $\delta$ /p38 $\delta$  keratinocyte differentiation cascade reduces PRMT5 and MEP50 expression and PRMT5/MEP50 association with the hINV gene promoter leading to increased promoter activity. We propose that PRMT5/MEP50 silencing of

involucrin expression is an important epigenetic mechanism that suppresses expression of differentiation-associated genes in the undifferentiated epidermal basal layers. We further propose that loss of PRMT5/MEP50 activity during differentiation is permissive for expression of differentiation-associated genes.

## ***C. MATERIALS AND METHODS:***

### **1. Antibodies and Reagents:**

Hanks' balanced salt solution and keratinocyte serum-free medium were purchased from Invitrogen. Mouse monoclonal antibodies specific for MEP50 (ab57722), p38 $\delta$  (sc-271292) and  $\beta$ -actin (A5441) were purchased from Abcam (Cambridge, MA), Santa Cruz Biotechnology (Santa Cruz, CA) and Sigma-Aldrich, respectively, while the rabbit polyclonal antibodies against MEP50 (2823) and PRMT5 (2252) were purchased from Cell Signaling Technology (Danvers, MA). Rabbit polyclonal antibodies against symmetrically dimethylated arginine<sup>3</sup> of Histone H4 (H4R3me<sub>2</sub>s) and arginine 8 of Histone H3 (H3R8me<sub>2</sub>s) were obtained from Abcam (Cambridge, MA) and Thermo Fisher Scientific (Rockford, IL). Rabbit anti- PKC $\delta$  (sc-937) was obtained from Santa Cruz Biotechnology. Normal rabbit IgG (2729) was obtained from Cell Signaling. The secondary antibodies used were peroxidase-conjugated donkey anti-goat IgG (sc-2033) from Santa Cruz Biotechnology, and peroxidase-conjugated sheep anti-mouse IgG (NXA931) and peroxidase-conjugated donkey anti-rabbit IgG (NA934V, GE Healthcare). Involucrin promoter luciferase reporter constructs were previously described (Efimova, LaCelle et al. 1998, Welter, Crish et al. 1995b). Phorbol ester, 12-O-tetradecanoylphorbol-13-acetate (TPA), was obtained from Calbiochem (Billerica, MA, 524400). Our studies use control (D-001206-13-05), MEP50 (M-006895-01-0005) and PRMT5 (M-015817-02-0005) siRNA reagent from Dharmacon Inc (Lafayette, CO). Important findings were confirmed using additional siRNAs that target MEP50 (D-006895-01-0002 and D-006895-02-0002) and PRMT5 (D-015817-01-0002 and D-015817-04-0002).

## **2. Cell culture, plasmids and viruses:**

The human epidermal keratinocytes used in our studies were obtained from foreskin epidermis. Foreskin epidermis, obtained from newborn infants, was separated from dermis by overnight dispase treatment. KERn were obtained by trypsin treatment and maintained in Keratinocyte Serum-Free Medium (KSFM) supplemented with epidermal growth factor and pituitary extract (Efimova, Broome et al. 2003, Efimova, Broome et al. 2004b). The human involucrin (hINV) promoter constructs were previously described (Banks, Crish et al. 1998, Welter, Eckert 1995). The human MEP50-encoding plasmid was constructed by primer amplification using plasmid p-OTB7-FLAG-MEP50 (pOTB7-WDR77, MHS1011-202830316) from Open Biosystems (Huntsville, AL) as template. The primers used for amplification of FLAG-MEP50 (BamHI/NotI fragment) were 5'-GATC GGA TCC **ATG GAC TAC AAG GAC GAC GAC GAC AAG** ATG CGG AAG GAA ACC CCA and 5'-GATC GCG GCC GCC TAC TCA GTA ACA CTT GCA GG. The ATG start codon is bold and the FLAG epitope is underlined. The product was then cloned into pcDNA3 to get pcDNA3-FLAG-MEP50. Adenoviruses encoding HA-p38 $\delta$ , PKC $\delta$ , and empty control virus (tAd5-HA-p38 $\delta$ , Ad5-PKC $\delta$ , Ad5-FLAG-p38 $\delta$ , and Ad5-EV) were prepared by propagation in HEK293 cells followed by cesium chloride gradient centrifugation as previously described (Chew, Adhikary et al. 2011). KERn were infected with 15 MOI of adenovirus in KSFM containing 6  $\mu$ g/ml polybrene.

## **3. Promoter luciferase assay:**

For luciferase assay, 4.5  $\mu$ l of FUGENE 6 was diluted in 95.5  $\mu$ l KSFM and the mixture was incubated at room temperature for 15 minutes. Next, the human involucrin promoter reporter plasmid (0.5  $\mu$ g) and 1  $\mu$ g of pcDNA3 or pcDNA3-FLAG-MEP50

were added to the mixture followed by a 20 min incubation. This mixture was then added to 2 ml of KSFM in dishes containing fifty percent confluent KERn cultures. After 24 h, cell lysates were collected and processed for luciferase activity assay (Adhikary, Chew et al. 2010). The readings were normalized to protein concentrations.

#### **4. Keratinocyte electroporation:**

Our studies used the AMAXA electroporator and VPD-1002 nucleofection kit (Cologne, Germany) for keratinocyte electroporation. KERn were harvested with trypsin and replated one day prior to electroporation. After an additional 24 h, the cells were harvested with trypsin and 1 million cells were used per electroporation. The cells were washed with 1 ml of PBS and suspended in 100  $\mu$ l of keratinocyte nucleofection solution containing 3  $\mu$ g of control-, MEP50- or PRMT5-siRNA. The mixture was mixed by gentle pipetting and transferred to the electroporation cuvette. The T-018 setting was used for electroporation. This was followed with addition of warm KSFM (500  $\mu$ l) and the mixture was then transferred to a 55 cm<sup>2</sup> dishes containing 10 ml of KSFM. The cells were maintained for various time points before the extracts were prepared for mRNA or protein analysis. This method achieves electroporation efficiencies of > 90% (Adhikary, Chew et al. 2010).

#### **5. Immunoprecipitation and Immunoblot:**

Cell extracts were prepared in cell lysis buffer (Cell Signaling, 9803, Danvers, MA) containing protease inhibitors (Calbiochem, 539134). For immunoprecipitation, 300  $\mu$ g of protein was used per sample. The lysate was first subjected to pre-clearing with 20  $\mu$ l of Protein A/G agarose beads for 1 hour followed by IP with the control IgG and the MEP50

antibody. The pre-clearing step is essential to reduce non-specific interactions. After an additional 24 h, the bead slurry (20  $\mu$ l) is added to the lysates followed by gentle rocking for 3 hours at 4 C. This is followed by 4 washes with lysis buffer. After the last wash, the beads were boiled with sample buffer, centrifuged and supernatants were electrophoresed on denaturing polyacrylamide gels. The proteins were then transferred on the nitrocellulose membrane and blocked with 5% skimmed milk in Tris-buffered saline containing 0.1% Tween 20. Next, the membranes were incubated overnight with primary antibody, followed by horseradish peroxidase-conjugated secondary antibody for 2 h.  $\beta$ -actin served as a gel loading control.

## **6. Quantitative RT-PCR:**

Total RNA was isolated using the RNAspin Mini Kit (GE Healthcare) and reverse transcribed using the Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA). RNA (1  $\mu$ g) was used for cDNA preparation. The Light Cycler 480 SYBR Green I Master mix (Roche Diagnostics) was used to measure mRNA level. The signals were normalized to the level of cyclophilin A mRNA. The following gene specific primers were used for detection of mRNA levels: MEP50 (forward: 5'-TTG CTC AGC AGG TGG TAC TGA GTT, reverse: 5'-AAT CTG TGA TGC TGG CTT GGG ACA), involucrin (forward: 5'-CCT CAG CCT TAC TGT GAG, reverse: 5'-GGG AGG CAG TGG AGT TGG), filaggrin (forward: 5'-ACT CAC AGG TGG GAC AGG AAC AAT, reverse: 5'-ATG GTT TCT GGA AGC AGA CCC AGA) and cyclophilin A (forward: 5'-CAT CTG CAC TGC CAA GAC TGA, reverse: 5'-TTC ATG CCT TCT TTC ACT TTGC).

## **7. Chromatin Immunoprecipitation Assay (ChIP):**

ChIP assays were performed using the Diagenode Low Cell ChIP assay kit (C01010073: kch-maglow-G48). Keratinocytes ( $0.5 \times 10^6$ ) cells were infected with 15 MOI of empty adenovirus or adenoviruses encoding HA-p38 $\delta$  or PKC $\delta$ . After 48 h, the cells were harvested and  $1 \times 10^6$  cells per group were used for sonication. Extract equivalent to 100,000 cells was used for immunoprecipitation per sample. MEP50, PRMT5, H4R3Me2s and H3R8me2s antibodies were used for ChIP analysis and binding to the distal regulatory region of the hINV promoter was detected by qRT-PCR using sequence-specific primers and the LightCycler 480 SYBR Green I master mix. The primers used to detect the distal regulatory region of the involucrin promoter (nucleotides -2218/-2055), encoding the AP1-5/Sp1 binding sites, were 5'-TCA GCT GTA TCC ACT GCC CTC TTT (forward) and 5'-TCA CAC CGG TCT TAT GGG TTA GCA (reverse).

## ***D. RESULTS:***

### **1. MEP50 and PRMT5 form a complex:**

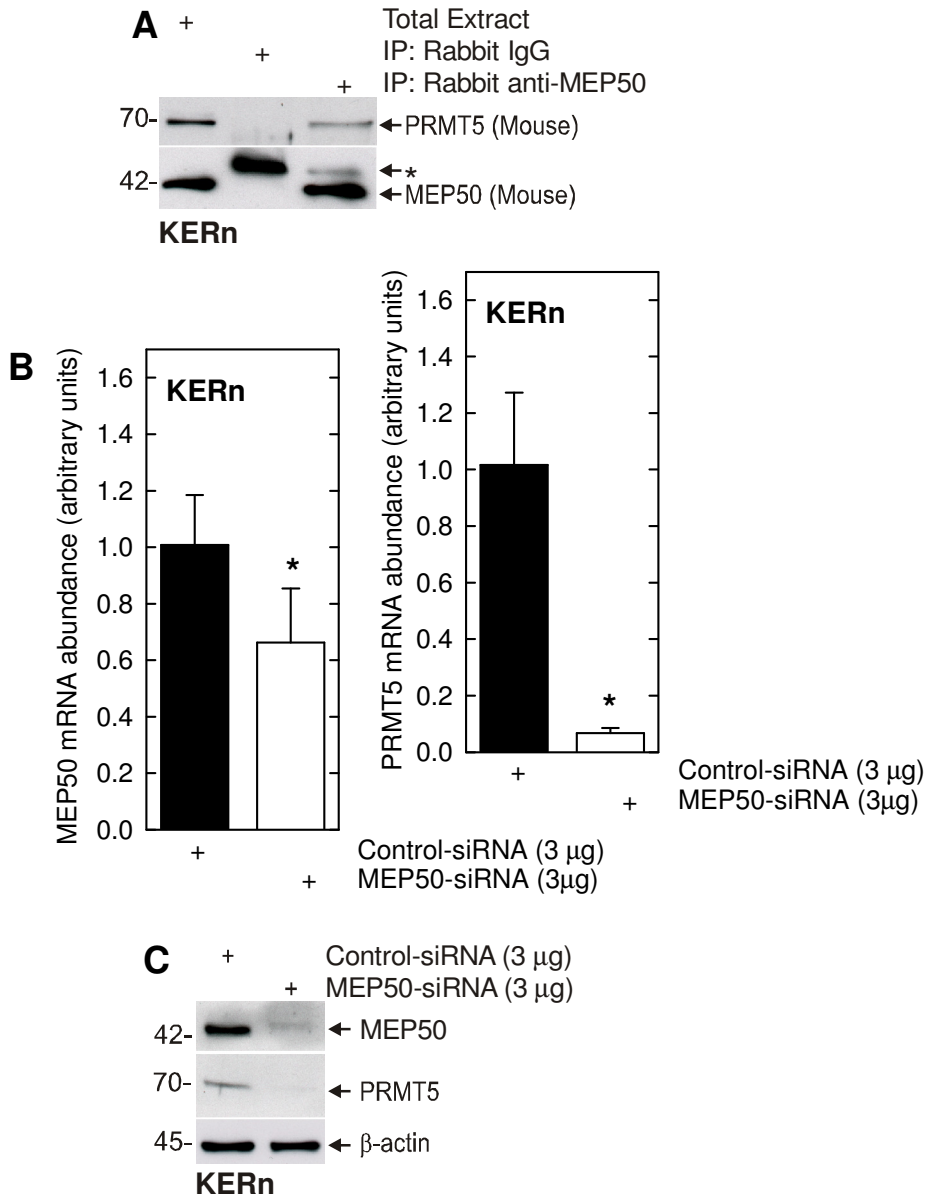
Our previous study showed that PRMT5 has an important regulatory role in keratinocytes (Kanade, Eckert 2012). In the present study, we examine the role of MEP50, a putative PRMT5 cofactor (Bedford, Clarke 2009, Karkhanis, Hu et al. 2011, Yang, Bedford 2013), as a regulator of keratinocyte differentiation. Recent studies show that MEP50 interacts with and is necessary for PRMT5 activity (Antonysamy, Bonday et al. 2012). We first examined whether a PRMT5/MEP50 complex exists in keratinocytes. Keratinocytes extracts were prepared and immunoprecipitated with anti-MEP50. **Fig. 4-1A** shows that PRMT5 co-precipitates with MEP50. Moreover, as shown in **Fig. 4-1B**, treatment with MEP50-siRNA partially reduces MEP50 mRNA level and this is associated with reduced PRMT5 mRNA expression, and a reduction in MEP50 and PRMT5 protein level (**Fig. 4-1C**). These findings suggest that the MEP50 and PRMT5 interact and are coordinately regulated in keratinocytes.

### **2. MEP50 suppresses hINV expression:**

To assess MEP50 function, we examined the impact of manipulating MEP50 expression on hINV mRNA level and promoter activity. Keratinocytes were transfected with empty vector or MEP50 expression vector and after 24 h the level of mRNA encoding involucrin (hINV) and filaggrin was monitored. These genes were selected because both are markers of terminal keratinocyte differentiation (Eckert, Crish et al. 1997, Presland, Kimball et al. 1997). We show that expression of MEP50 reduces hINV and filaggrin mRNA level (**Fig. 4-2A/B**). This suggests that MEP50 acts to suppress differentiation-associated gene expression. We have previously identified a -2471/-1

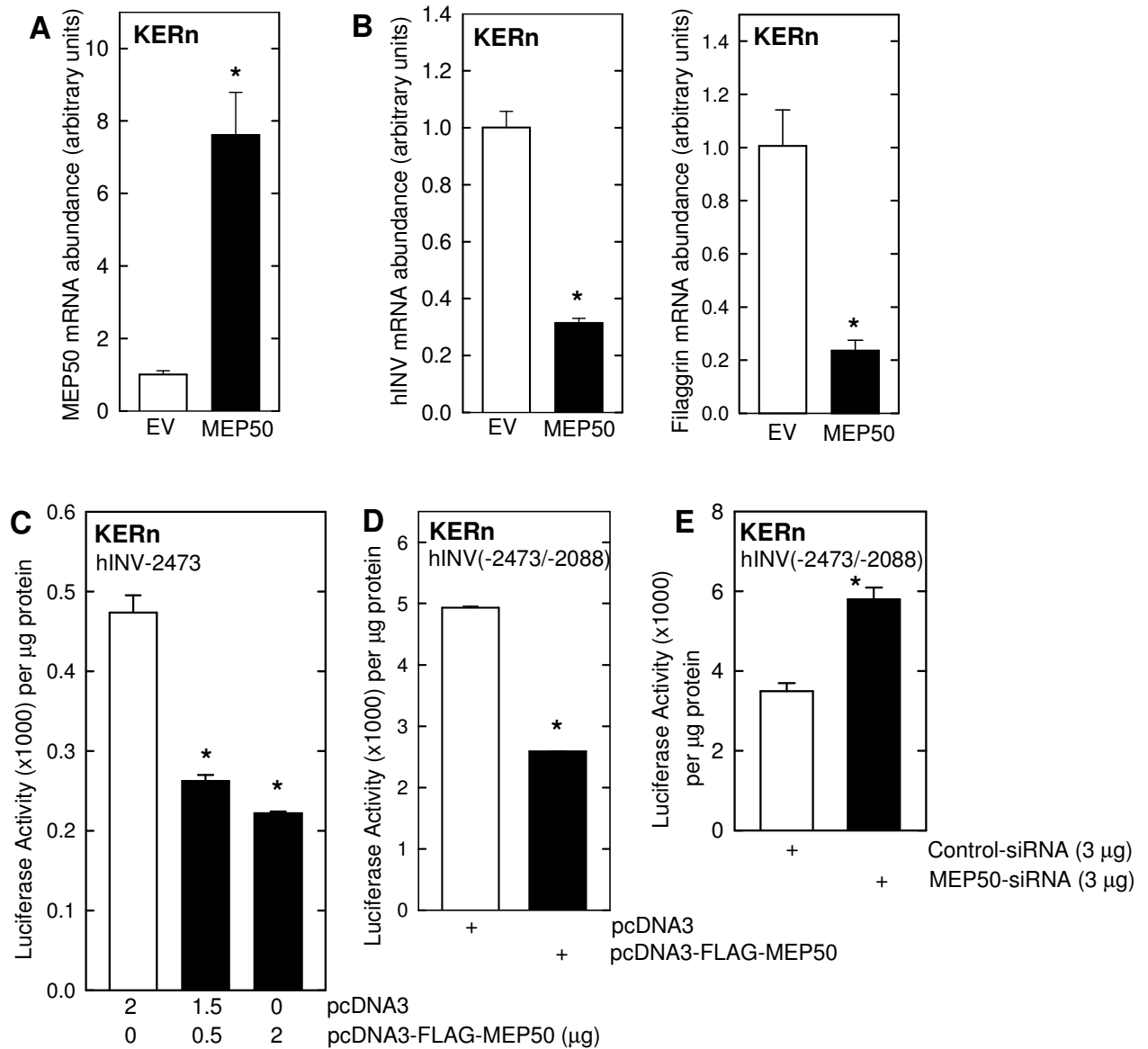
segment of the hINV promoter that mediates differentiation-appropriate hINV expression in cell culture and *in vivo* (Adhikary, Chew et al. 2010, Banks, Crish et al. 1999, Banks, Crish et al. 1998, Chew, Adhikary et al. 2013, Crish, Zaim et al. 1998, Crish, Bone et al. 2002, Crish, Gopalakrishnan et al. 2006, Crish, Eckert 2008, Han, Rorke et al. 2012, Welter, Eckert 1995, Welter, Crish et al. 1995b), and also a region of the promoter, called the distal regulatory region (DRR, nucleotides -2473/-2088), which encodes transcription factor binding elements that is essential for appropriate differentiation-associated gene expression (Banks, Crish et al. 1998, Crish, Zaim et al. 1998, Crish, Gopalakrishnan et al. 2006). Transfection of hINV-2473 (full-length promoter) in the presence of MEP50 suppresses hINV promoter activity (**Fig. 4-2C**). Moreover, MEP50 expression suppresses and MEP50-siRNA increases activity of a reporter construct (-2473/-2088) encoding the hINV DRR region (**Fig. 4-2D/E**).

The hINV promoter encodes a key activator protein 1 (AP1) transcription factor binding site, AP1-5, located within the hINV gene DRR, that is absolutely required for correct differentiation-associated expression in cultured keratinocytes and *in vivo* (Crish, Howard et al. 1993a, Crish, Bone et al. 2002, Crish, Gopalakrishnan et al. 2006). **Fig. 4-3A** shows promoter maps indicating the full length (-2473), and -241 and -41 truncation constructs, the DRR (-2473/-2088) and the AP1-5 site. **Fig. 4-3B** shows that mutation of the AP1-5 site results in a loss of promoter activity and loss of MEP50-associated suppression of promoter activity, suggesting that AP1 transcription factor signaling and the MEP50 epigenetic silencing produces opposing responses on the hINV promoter.



**Figure 4-1: MEP50 and PRMT5 form a complex in keratinocytes.**

**A** Freshly isolated foreskin keratinocyte lysates (300 μg) were used for immunoprecipitation with Rabbit IgG or rabbit anti-MEP50, and 10 μg of total extract was electrophoresed. The antibodies for immunoblot are mouse anti-MEP50 and goat anti-PRMT5. Similar results were obtained in three separate experiments. The upper band (\*) in the blot probed with MEP50 is non-specific. **B** Keratinocytes were electroporated with 3 μg of control-siRNA or MEP50-siRNA. After 48h, RNA was isolated and MEP50 and PRMT5 mRNA levels were assessed by qRT-PCR. The values are mean ± SEM, n = 3. The asterisks indicate significant differences as determined by the students t-test (\*, p<0.005). **C** MEP50 regulates PRMT5 expression. KERn were electroporated with 3 μg of control- or MEP50-siRNA. After 48 h protein lysates were prepared for immunoblot with anti-MEP50 and anti-PRMT5. β-actin was used as the loading control. Similar results were observed in each of three experiments.



**Figure 4-2: MEP50 suppresses involucrin expression.**

**A/ B** KERN were electroporated with the indicated plasmids. After 24 h RNA was isolated and MEP50, involucrin and filaggrin mRNA levels were assessed by qRT-PCR. The values are mean  $\pm$  SEM (n = 3). The asterisks indicate a significant difference ( $p < 0.005$ ). **C/D** KERN were transfected with 0.5  $\mu$ g of the indicated involucrin promoter plasmids in the presence of 1  $\mu$ g of pcDNA3 or pcDNA3-FLAG-MEP50. At 24 h post-transfection, extracts were prepared and assayed for promoter activity. **E** KERN were electroporated with 3  $\mu$ g of control-siRNA or MEP50-siRNA. After 48 h, the cells were re-electroporated with 3  $\mu$ g of endo-free involucrin promoter. After an additional 24 h, extracts were prepared and promoter activity (luciferase) assay.

### **3. MEP50 controls histone arginine methylation at the hINV promoter:**

As expected of a regulatory protein involved in arginine methylation of histones, MEP50 appears to act at various sites along the hINV promoter (**Fig. 4-3C**). However, because it is a key regulatory region (Crish, Zaim et al. 1998, Crish, Gopalakrishnan et al. 2006), we studied effect of MEP50 on arginine methylation of histones surrounding the hINV promoter DRR region. Keratinocytes were electroporated with 3  $\mu$ g of control- or MEP50-siRNA and after 48 h extracts were prepared for ChIP with anti-IgG, anti-PRMT5 or anti-MEP50. **Fig. 4-4A** shows that reducing PRMT5 or MEP50 level is associated with reduced binding of PRMT5 and MEP50 to the hINV promoter DRR region that encodes the AP1-5 and GC-rich (Sp1) binding sites (**Fig. 4-4A**). Moreover, the reduction in MEP50 level is associated with reduced H3R8me2s and H4R3me2s formation (**Fig. 4-4B**). These findings are consistent with MEP50 control of arginine methylation status at the hINV promoter.

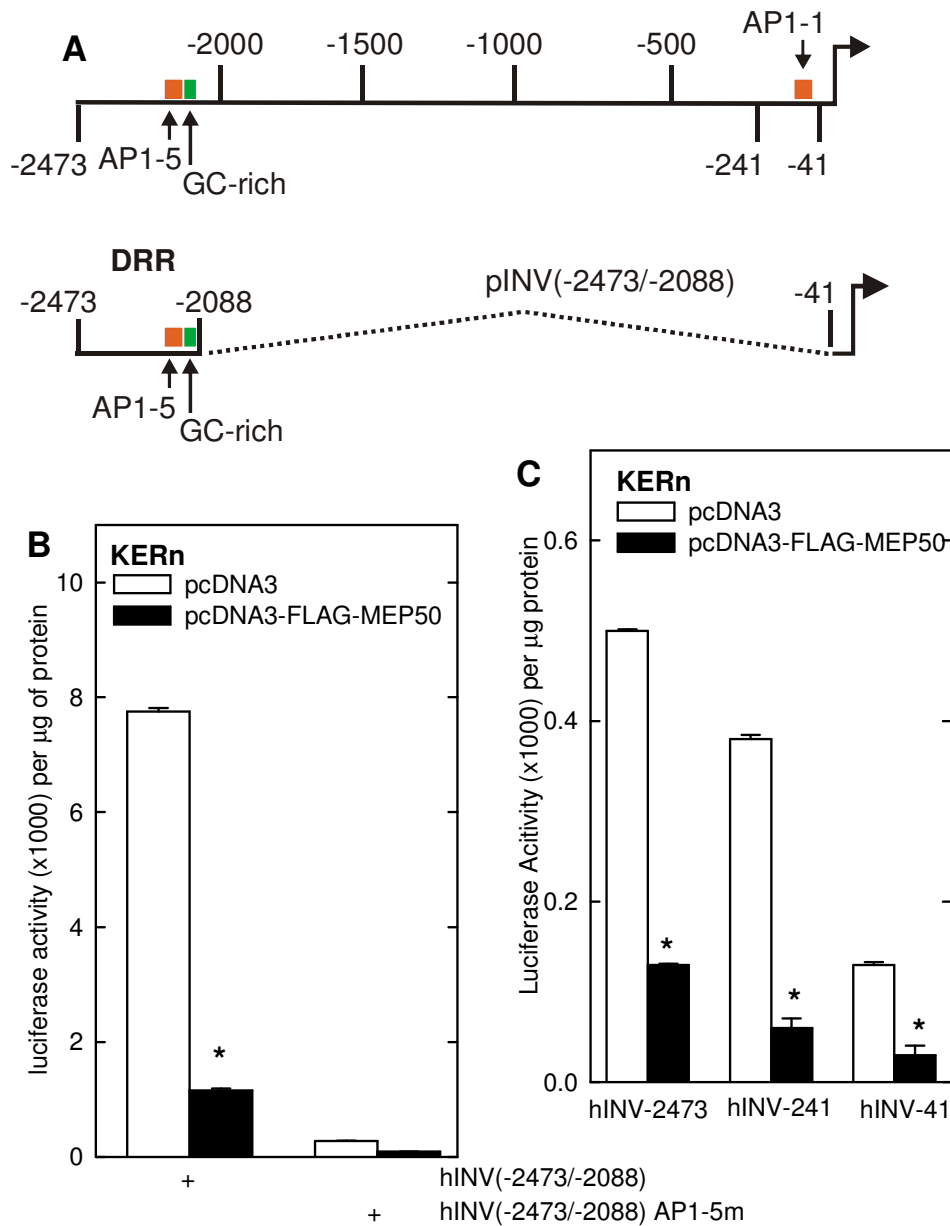
### **4. Impact of PKC $\delta$ /p38 $\delta$ signaling on MEP50 and PRMT5 interaction and activity at the hINV promoter:**

We previously described a PKC $\delta$ /p38 $\delta$  MAPK signaling pathway which increases hINV expression and promoter activity, and expression of other differentiation-associated genes in keratinocytes (Efimova, LaCelle et al. 1998, Efimova, Broome et al. 2003, Efimova, Broome et al. 2004b, Efimova, Eckert 2000). We asked whether activation of this cascade suppresses MEP50 level. Keratinocytes were infected with empty, PKC $\delta$ -encoding or p38 $\delta$ -encoding adenovirus and after 48 h extracts were prepared to monitor MEP50, PRMT5, H3R8me2s and H4R3me2s. **Fig. 4-5A** shows that PKC $\delta$  and p38 $\delta$  reduce intracellular MEP50 and PRMT5 level and PRMT5/MEP50-dependent histone

modification. **Fig. 4-5B/C** show that this is associated with loss of MEP50 and PRMT5 interaction and reduced MEP50/PRMT5-dependent histone modification at the hINV promoter DRR.

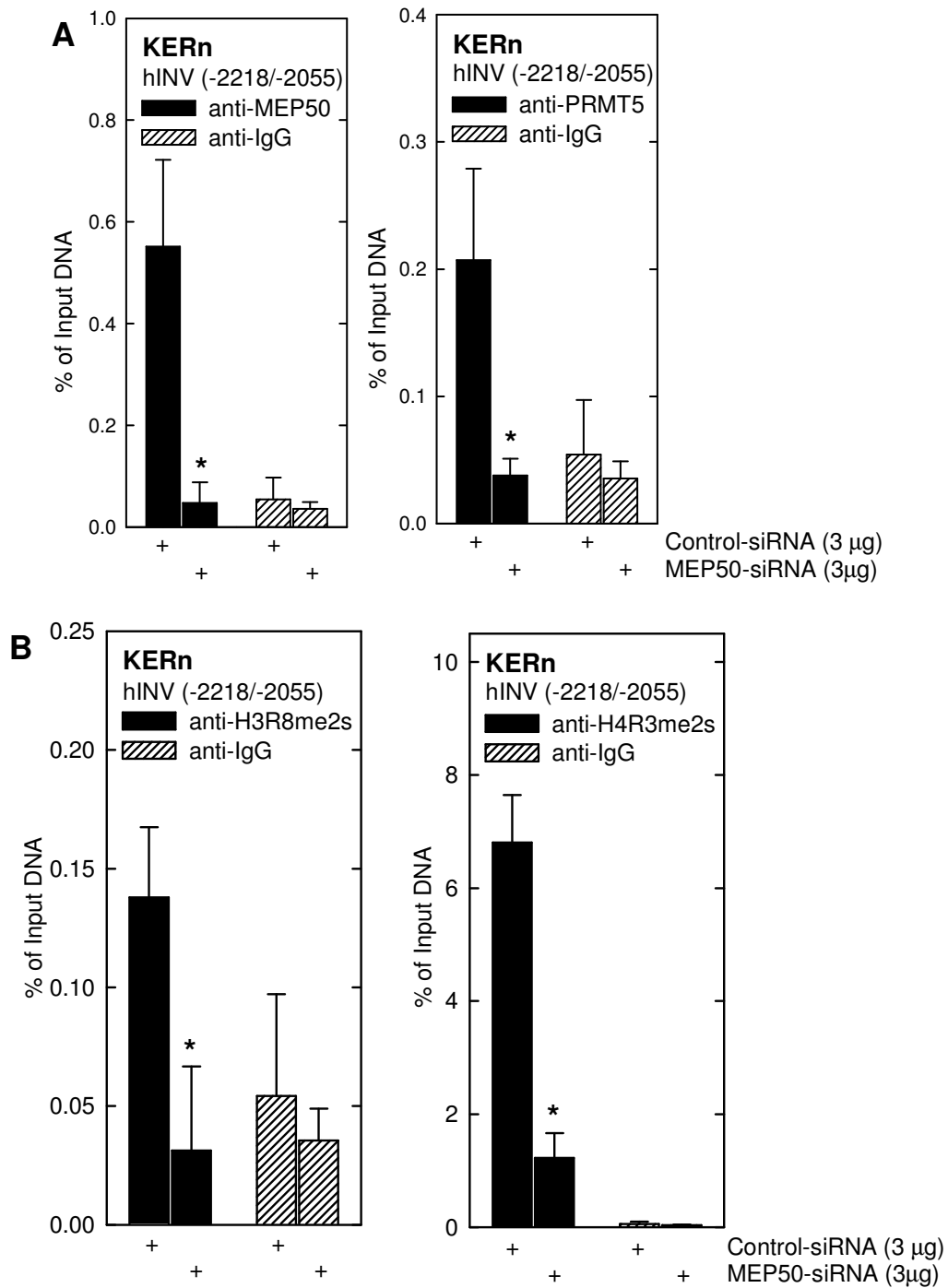
**5. Impact of phorbol ester on MEP50 and PRMT5 level and interaction and activity at the hINV promoter:**

12-O-tetradecanoylphorbol-13-acetate (TPA) is a known inducer of keratinocyte differentiation and involucrin gene expression. We wanted to assess whether an inducer of differentiation would replicate the regulation of MEP50 and PRMT5 observed following expression of PKC $\delta$  and p38 $\delta$ . Treatment with 50 ng TPA/ml for 48 h suppresses MEP50 and PRMT5 mRNA level (**Fig. 4-6A**), and this is associated with a reduced PRMT5 and MEP50 level (**Fig. 4-6B**) and increased hINV mRNA level (**Fig. 4-6C**). To examine the impact of TPA treatment on PRMT5 and MEP50 interaction with the hINV promoter DRR region, we prepared extracts for ChIP analysis. **Fig. 4-6D** shows a reduction in PRMT5 and MEP50 association with the hINV promoter that is associated with reduced promoter-associated arginine-dimethylated histone (**Fig. 4-6E**).



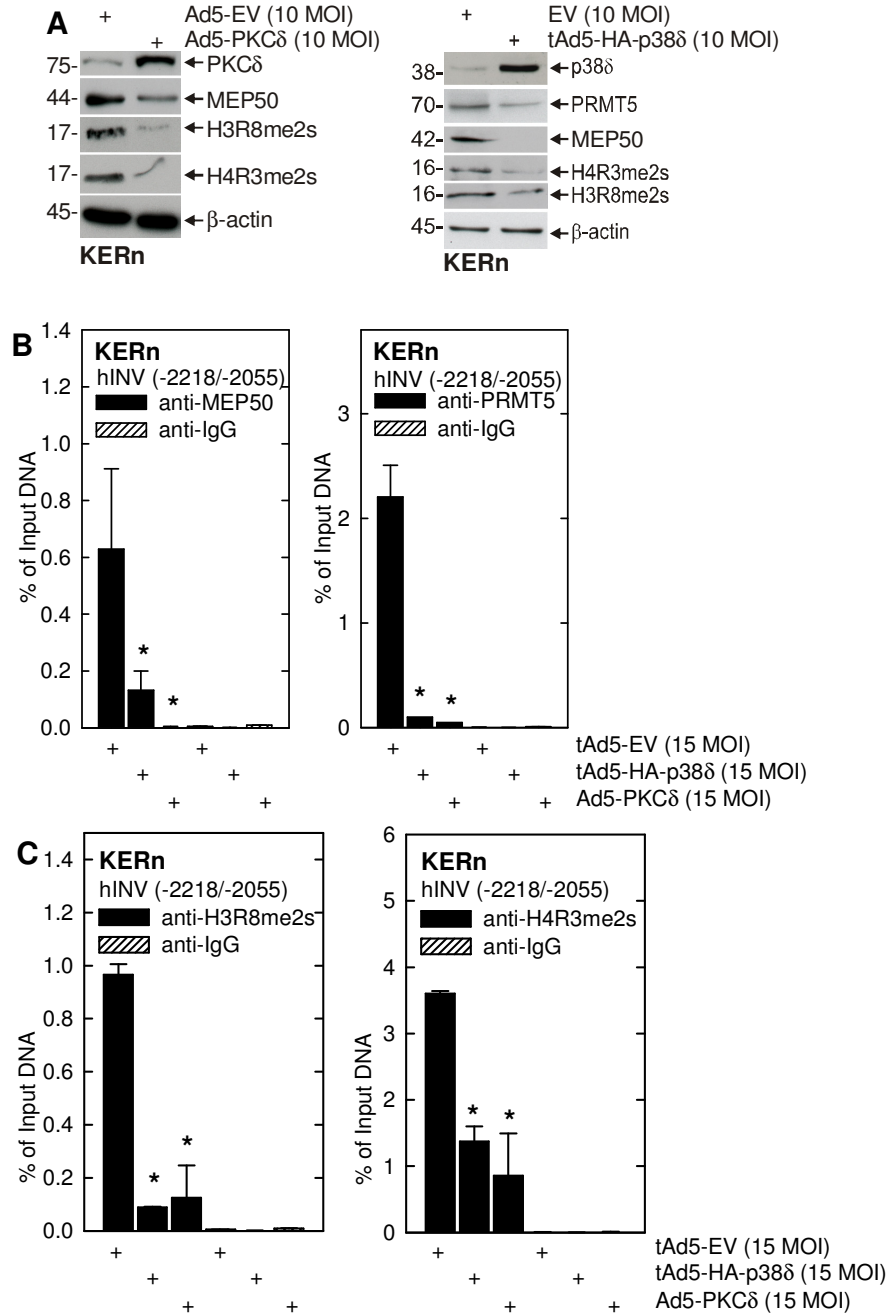
**Figure 4-3: MEP50 suppression of hINV promoter activity.**

**A** Schematic showing key regulatory elements in the hINV promoter. hINV-2473 is the full-length promoter and hINV(-2473/-2088) is a construct in which the DRR (nucleotides -2473/-2088) is linked to the hINV minimal promoter. hINV-41 encodes the minimal promoter (nucleotides -41/-1). The *dashed line* indicates the fusion. The functionally important AP1 (AP1-1 and AP1-5) sites and GC-rich (Sp1) element are indicated. The distances are in nucleotides relative to the transcription start site. **B/C** KERN were transfected with 0.5  $\mu$ g of hINV-2473, which encodes the full-length wild-type human involucrin promoter, or the promoter harboring a mutant AP1-5 site (AP1-5m), or truncated promoters (hINV-241, hINV-41) and 1  $\mu$ g of pcDNA3 or pcDNA3-FLAG-MEP50. At 24 h post-transfection cell extracts were prepared and assayed for promoter activity. The values are mean  $\pm$  SEM, n = 3. The asterisks indicate a significant change, p < 0.005.



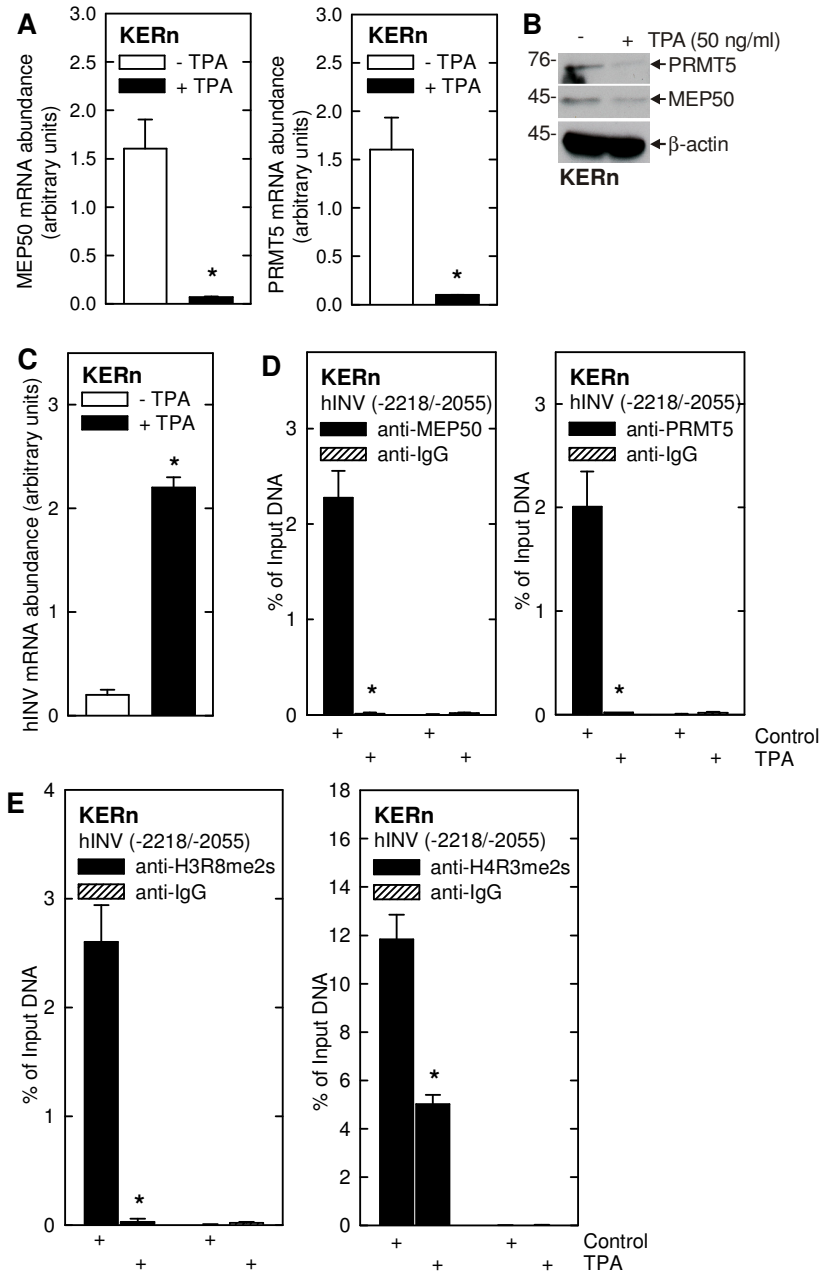
**Figure 4-4:MEP50 controls histone arginine methylation at the hINV promoter.**

A/B KERn were electroporated with 3 μg of control-siRNA or MEP50-siRNA. After 48 h, extracts were prepared for ChIP analysis. DNA from 1 million cells was sheared, collected and 100,000 cell equivalents of DNA was immunoprecipitated. The primers include nucleotides -2218/-2055 of the DRR region of the hINV promoter region that includes the AP1-5 site. The values are mean  $\pm$  SEM, n = 3 and the asterisks indicate significant difference, p < 0.005.



**Figure 4-5: PKCδ/p38δ signaling reduces MEP50 and PRMT5 level and hINV promoter activity.**

**A** KERN were infected with 10 MOI of tAd5-EV or Ad5-PKCδ and at 48 h extracts were prepared for detection of PKCδ, MEP50, H3R8me2S and H4R3me2s. β-actin was used as a loading control. Similar results were obtained in three different experiments. **B** KERN were infected as above and after 48 h ChIP was performed using the Diagenode Low Cell ChIP Kit and primers spanning nucleotides -2218/-2055 if the hINV promoter region which includes the AP1-5 site. The values are mean ± SEM, n = 3. The asterisks indicate significant difference (p < 0.005).



**Figure 4-6: TPA suppresses MEP50 and PRMT5 level and activity.**

**A/B** KERN were treated with 50 ng/ml TPA for 48 h, RNA was isolated and MEP50 and PRMT5 mRNA levels were assessed by qRT-PCR. The values are mean  $\pm$  SEM, n = 3, asterisk indicate a significant difference, p < 0.005. Simultaneously, protein extracts were prepared from identically treated cultures to detect MEP50, PRMT5, H3R8me2s and H4R3me2s. Similar results were obtained in three different experiments. **C/D/E** KERN were treated with 50 ng TPA/ml and after 48 h mRNA extracts were isolated for ChIP analysis and detection of MEP50 and PRMT5 interaction and H3R8-me2s and H4R3me2s formation at the hINV promoter. Similar results were obtained in three different experiments. The values are mean  $\pm$  SEM, n = 3. The asterisks indicate significant difference (p < 0.005).

### ***E. DISCUSSION:***

Involucrin has been extensively studied as a model to understand the mechanisms that drive gene expression during keratinocyte differentiation (Bikle, Tu et al. 2003, Bikle, Ng et al. 2001, Dlugosz, Yuspa 1994, Denning, Dlugosz et al. 1995, Eckert, Crish et al. 2004). PKC $\delta$ /p38 $\delta$  MAPK signaling is a key pro-differentiation/anti-proliferation pathway in keratinocytes (Eckert, Efimova et al. 2002b, Eckert, Efimova et al. 2003b). Genetic and inhibitor studies indicate that PKC $\delta$  and p38 $\delta$  activity stimulate hINV gene transcription and increase hINV mRNA and protein level (Efimova, LaCelle et al. 1998, Dashti, Efimova et al. 2001a, Efimova, Deucher et al. 2002, Efimova, Broome et al. 2003, Efimova, Broome et al. 2004b, Efimova, Eckert 2000, Kraft, Efimova et al. 2007). Moreover, treating keratinocytes with agents that activate this cascade, including TPA and calcium, increase hINV gene expression (Adhikary, Crish et al. 2004, Chew, Adhikary et al. 2013, Deucher, Efimova et al. 2002, Eckert, Crish et al. 2004). This pathway includes PKC $\delta$ , Ras, MEKK1, and MEK3 and activation results in increased p38 $\delta$  and reduced ERK1/2 activity (**Fig. 4-7**) (Efimova, LaCelle et al. 1998, Efimova, Broome et al. 2003, Efimova, Broome et al. 2004b, Efimova, Eckert 2000, Kraft, Efimova et al. 2007). p38 $\delta$  activation drives nuclear accumulation of AP1 transcription factors which interacts with the AP1-5 transcription factor binding site in the hINV promoter DRR to increase hINV gene transcription (Efimova, LaCelle et al. 1998, Welter, Eckert 1995, Welter, Crish et al. 1995b). The DRR also includes a Sp1 (GC-rich) transcription factor binding site that is essential for optimal activity (Banks, Crish et al. 1998, Banks, Crish et al. 1999). Studies in transgenic mice, wherein the AP1-5 site is inactivated by mutation, reveal that this site is essential for differentiation-associated

involucrin expression *in vivo* (Crish, Howard et al. 1993a, Crish, Bone et al. 2002, Crish, Gopalakrishnan et al. 2006).

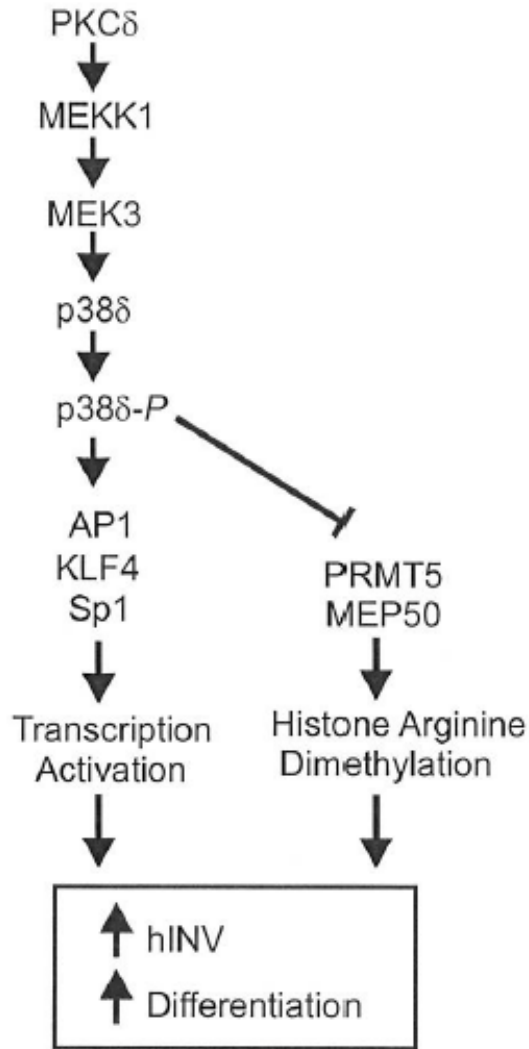
### **1. Epigenetic silencing of involucrin gene expression - MEP50:**

Understanding molecular events that occur at the AP1-5 element is important for understanding control of differentiation. Although substantial knowledge is available regarding pathways that positively regulate differentiation-associated gene expression (i.e., differentiation), less is known about pathways that prevent or suppress expression. This is important, as suppression of gene expression, in epidermis, prevents premature activation of differentiation. Epigenetic mechanisms often inhibit differentiation-associated gene expression (Gilbert, Gore et al. 2004, Saha, Hornyak et al. 2013) and we have shown that these mechanisms are important in controlling keratinocyte differentiation (Kanade, Eckert 2012, Eckert, Adhikary et al. 2011). PRMT5 is the major type II arginine methyltransferase (Branscombe, Frankel et al. 2001), and its first identified biological role was that of a transcriptional repressor (Fabrizio, Messaoudi et al. 2002). We recently reported that PRMT5 interacts with and antagonizes PKC $\delta$ /p38 $\delta$  MAPK activation of hINV gene expression (Kanade, Eckert 2012). We showed that PRMT5 interacts with a multiprotein regulatory complex that includes p38 $\delta$  to antagonize PKC $\delta$ /p38 $\delta$  signaling and suppress differentiation (Kanade, Eckert 2012). However, PRMT5 also modifies histones and chromatin that contains highly arginine dimethylated histones silences gene expression (Karkhanis, Hu et al. 2011). PRMT5 acts to symmetrical dimethylate arginine eight of H3 and arginine 3 of histone 4 to form, respectively, H3R8me2s and H4R3me2s, to silence gene expression (Pal, Vishwanath et al. 2004, Pal, Sif 2007, Yang, Bedford 2013). In this context, PRMT5 interacts with

MEP50, which has recently been shown to form an octamer with PRMT5 including four PRMT5 and four MEP50 subunits (Antonysamy, Bonday et al. 2012, Ho, Wilczek et al. 2013, Hosohata, Li et al. 2003, Kanade, Eckert 2012). MEP50 is required for optimal PRMT5 activity.

## **2. Regulation of MEP50 and PRMT5 level:**

We show that MEP50 and PRMT5 exist as a complex in keratinocytes, and that MEP50 and PRMT5 are co-regulated such that a forced change in MEP50 level results in a parallel change in PRMT5 level and vice versa. These findings are consistent with recent information showing that PRMT5 and MEP50 form an octamer which requires that the cell maintain approximately equal amount of each (Antonysamy, Bonday et al. 2012, Ho, Wilczek et al. 2013). Translational regulation, dependent upon specific miRNAs, has been described as a mechanism to control PRMT5 level (Karkhanis, Hu et al. 2011, Yang, Bedford 2013). However our studies suggest that regulation of gene transcription or mRNA stability may also control PRMT5 and MEP50 levels, since knockdown of MEP50 causes a marked reduction in PRMT5 mRNA level (**Fig. 4-1**), suggesting that MEP50 directly or indirectly controls PRMT5 mRNA stability or PRMT5 gene transcription. Additional future studies will be required to understand this mechanism.



**Figure 4-7: Proposed regulatory model - transcriptional activation and epigenetic de-repression.**

PKC $\delta$  activates the indicated p38 $\delta$  MAPK cascade that triggers AP1, KLF4 and Sp1 transcription factor movement to the nucleus which activates hINV gene expression and other differentiation-related events. The PKC $\delta$ /p38 $\delta$  cascade also suppresses PRMT5 and MEP50 level, leading to reduced histone arginine dimethylation of histone associated with the hINV promoter leading to de-repression of expression. This cascade can be triggered by expression of PKC $\delta$ , p38 $\delta$  or by treatment with pro-differentiation agents (e.g., TPA).

### **3. MEP50 suppresses hINV expression:**

Functional studies indicate an inverse relationship between MEP50 level and hINV gene promoter activity and mRNA level. MEP50 suppresses transcription of hINV promoter luciferase reporter plasmids encoding nucleotides -2218/-2055, -2473/-1, -241/-1 or -41/-1 of the hINV promoter. This suggests that arginine dimethylation of chromatin, occurring at various regions in the promoter, can reduce gene expression. This is anticipated, as PRMT5 activity need not only impact a single target element in a promoter, but can impact large tracks of chromatin. To study the details of this regulation, we focused on the DRR region, located within nucleotides -2218/-2055, which encodes key transcription factor binding sites that are required for hINV gene expression (Banks, Crish et al. 1998, Crish, Howard et al. 1993b, Crish, Zaim et al. 1998, Welter, Eckert 1995). We observed a reduction in MEP50 interaction with DRR chromatin following MEP50 knockdown. Consistent with an obligatory interaction between PRMT5 and MEP50, MEP50 knockdown also reduced PRMT5 levels in chromatin, and these reductions were associated with reduced H3R8me2s and H4R3me2s formation. Thus, histone arginine dimethylation is associated with reduced hINV gene expression and demethylation is required for increased expression. The idea that PRMT5 and MEP50 act as anti-differentiation/pro-survival regulators is consistent with current reports showing that PRMT5 negatively controls transcription of growth inhibitory genes (Gu, Li et al. 2012, Wang, Pal et al. 2008a).

### **4. PKC $\delta$ /p38 $\delta$ versus PRMT5/MEP50 signaling - a productive balance:**

We also show that activation of a classic PKC $\delta$ /p38 $\delta$  cascade, which increases differentiation (Eckert et al., 2004; Efimova et al., 2002; Efimova et al., 2003; Efimova et

al., 2004), is associated with the reduction in MEP50 and PRMT5 level and PRMT5-dependent chromatin modification. Reduced PRMT5 and MEP50 level and activity was observed when PKC $\delta$  or p38 $\delta$  were overexpressed, or when activity of these kinases was stimulated by phorbol ester, a known inducer of keratinocyte differentiation (Eckert et al., 2004; Efimova et al., 2002; Efimova et al., 2003; Efimova et al., 2004). This novel observation suggests that pro-differentiation signaling inhibits PRMT5/MEP50 epigenetic silencing as a component of the process that drives differentiation. We also observed that PRMT5/MEP50 feedback regulates PKC $\delta$  and p38 $\delta$  activity, suggesting that a balance between these regulatory mechanisms controls cell status. For example, MEP50 overexpression reduces PKC $\delta$ -Y311 phosphorylation, an indicator of reduced PKC $\delta$  activity (not shown). This finding is consistent with our recent report showing that PRMT5 arginine dimethylates proteins in the p38 $\delta$  MAPK complex, that this part of the PKC $\delta$ /p38 $\delta$  signaling cascade, to reduce p38 $\delta$  phosphorylation and activity (Kanade, Eckert 2012). This suggests that proper control of keratinocyte fate requires a balance between PKC $\delta$ /p38 $\delta$  pro-differentiation signaling and PRMT5/MEP50 anti-differentiation/pro-survival signaling.

Regulation of PRMT5 level has been extensively studied at the translational and post-translation level. An important study showed that mutant JAK kinase, in myeloproliferative neoplasms, phosphorylates PRMT5 (Liu, Zhao et al. 2011) which reduced PRMT5 association with MEP50 leading to reduced PRMT5 activity, but no change in PRMT5 level (Liu, Zhao et al. 2011). Additional studies show that specific miRNAs control translation of PRMT5 and MEP50 protein (Yang, Bedford 2013). Moreover, another study suggests that MEP50 is phosphorylated by CDK4 and that this

leads to increased PRMT5/MEP50 activity (Aggarwal, Vaites et al. 2010). Our present studies suggest a third option - that PRMT5 and MEP50 levels can be controlled by transcriptional mechanisms or by altered mRNA stability. We show that stimulation of PKC $\delta$ /p38 $\delta$  signaling reduces PRMT5 and MEP50 mRNA level, leading to reduced production of these proteins, as a mechanism to reduce PRMT5 activity and arginine methylation of the hINV promoter (**Fig. 4-7**). We have previously shown that a PKC $\delta$ , MEKK1, MEK3, p38 $\delta$  cascade increases AP1/Sp1 and KLF4 transcription factor binding to the DRR element to activate transcription (Eckert et al., 2004; Efimova et al., 2002; Efimova et al., 2003; Efimova et al., 2004). We now propose that this same pathway acts to suppress PRMT5/MEP50 expression, leading to reduced histone arginine dimethylation of the involucrin DRR. It is a particular interesting feature that PKC $\delta$ /MAPK signaling converges via two mechanisms, transcriptional activation of hINV gene expression and inhibition of hINV promoter histone arginine dimethylation, to increase hINV promoter activity (**Fig. 4-7**). We propose that this may be a general mechanism to regulate differentiation-associated gene expression in keratinocytes.

## CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTIONS

### A. CONCLUSIONS:

A key aspect contributing towards efficient barrier formation is a perfect balance between proliferation and differentiation (Eckert, Crish et al. 1997). Deciphering the mechanisms of epidermal homeostasis is an important goal. The novel PKC isoforms act as critical regulators of this epidermal homeostasis. PKC $\delta$  activates the MAPK pathway and controls transcription of genes involved in keratinocyte differentiation (Denning, Dlugosz et al. 2000, Denning 2004, Adhikary, Chew et al. 2010, Sonkoly, Wei et al. 2009, Papp, Czifra et al. 2004, Balasubramanian, Efimova et al. 2002, Kamioka, Akahane et al. 2010, Efimova, Eckert 2000). The role of PKC $\delta$  in keratinocyte proliferation has not been heavily studied. Our current studies identify p38 $\delta$  as a key mediator in PKC $\delta$  regulated keratinocyte proliferation. We show that PKC $\delta$  activates the MEK3-p38 $\delta$  cascade to increase p21<sup>Cip1</sup> transcription in a p53 dependent manner. Our studies identify p53 as a novel target of p38 $\delta$  (Saha, Adhikary et al. 2014). Furthermore, these studies are physiologically relevant as PKC $\delta$ /p38 $\delta$  knockdown rafts are significantly thicker displaying additional layers than the control. These rafts also have reduced cornified layer formation indicating that absence of PKC $\delta$ /p38 $\delta$  drives proliferation. Additionally, both p21<sup>Cip1</sup> and p53 mRNA and protein levels were found to be reduced in the p38 $\delta$  knockdown rafts as compared to the controls (Saha, Adhikary et al. 2014). Our studies have identified the novel role of p38 $\delta$  in keratinocyte proliferation and have identified PKC $\delta$ -MEK3-p38 $\delta$ -p53 as an additional cascade contributing to p21<sup>Cip1</sup> transcription.

The p38 $\delta$ /ERK complex is a crucial signaling control point in the novel PKC activated cascade wherein stimulus dependent activation of p38 $\delta$  or ERK1/2 directs keratinocyte cell fate (Efimova, Broome et al. 2003). Given the key function of this complex, it is logical to assume that p38 $\delta$  and ERK1/2 exist as part of a multiprotein complex. An interesting finding was the presence of PRMT5 as a novel component of this complex (Kanade, Eckert 2012). PRMT5, a type II protein arginine methyltransferase is a highly versatile protein resulting in symmetric methylation of arginine residues in proteins. PRMT5 was shown to inhibit differentiation by methylating proteins in the p38 $\delta$  complex and inhibiting p38 $\delta$  activity. MEP50, a WD40 protein is an important binding partner of PRMT5 and essential for its activity. Characterizing the role of MEP50 in keratinocytes is the focus of our recent work.

We began by staining the foreskin epidermis, fixing and labeling the tissue with anti-MEP50 antibody. We observed that MEP50 is expressed in all layers of the epidermis with the basal layers displaying a cytoplasmic localization and the granular layers exhibiting a nuclear localization. We also expressed MEP50 exogenously and tested for localization with the endogenous MEP50. These experiments revealed that MEP50 is abundantly expressed in keratinocytes and localized to both the nucleus and cytoplasm (Saha, Eckert 2015). Similar localization patterns have also been reported in lung epithelial cells (Wei, Hsia et al. 2014). MEP50 and PRMT5 knockdown resulted in significant decrease in keratinocyte cell proliferation (Saha, Eckert 2015). Additionally, MEP50 was found to decrease p21<sup>Cip1</sup> and involucrin mRNA and protein levels. p21<sup>Cip1</sup> acts as a negative regulator of the cell cycle by inhibiting the activation of the cyclin dependent kinases, CDK1 and CDK2. p21<sup>Cip1</sup> expression is associated with suppression

of keratinocyte proliferation (Wong, Pickard et al. 2010, Cheng, McLaughlin et al. 2009, Aliouat-Denis, Dendouga et al. 2005, Devgan, Mammucari et al. 2005, Okuyama, LeFort et al. 2004, Hauser, Ma et al. 2004). The pro-proliferation role of MEP50 and PRMT5 was further confirmed in the organotypic raft cultures. These epidermal equivalents mimic *in vivo* differentiation and are valuable tools to study gene function. We observed that thickness of the MEP50 and PRMT5 knockdown rafts was significantly less than the control rafts. Furthermore, the MEP50/PRMT5 knockdown rafts contained fewer Ki67-positive cells suggesting reduced proliferation rates (Saha, Eckert 2015). PKC $\delta$ /p38 $\delta$  is the classic pathway which regulates epidermal homeostasis by controlling the dual processes of proliferation and differentiation (Adhikary, Chew et al. 2010, Chew, Adhikary et al. 2011, Efimova, Deucher et al. 2002). A significant finding of our current study was that pro-differentiation stimulus of PKC $\delta$ /p38 $\delta$  expression lead to reduction in MEP50/PRMT5 level and downstream methylated histone level. This correlated with reduced enrichment of MEP50, PRMT5 and methylated histones at the p21<sup>Cip1</sup> and involucrin promoter indicating increased gene transcription (Saha, Adhikary et al. 2015, Saha, Eckert 2015). This novel finding highlights the importance of divergent pathways necessary to mediate target gene expression. Our studies are significant as they:

- a) Identify a novel link between the MAPK pathway and arginine methylation in mediating keratinocyte differentiation.
- b) Identify involucrin as a MEP50 target.
- c) Establish the role of MEP50 and PRMT5 as key regulators of keratinocyte proliferation.
- d) Identify p38 $\delta$  as a common kinase regulating proliferation and differentiation.

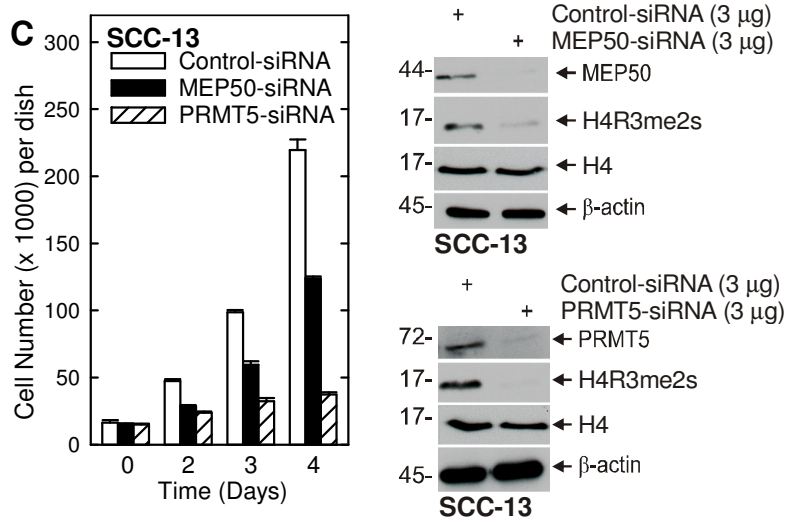
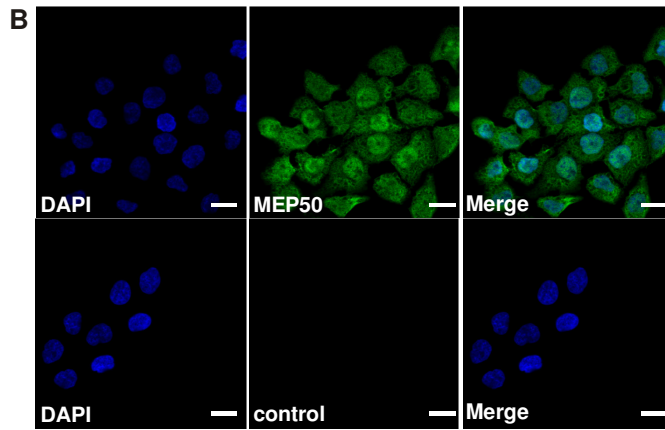
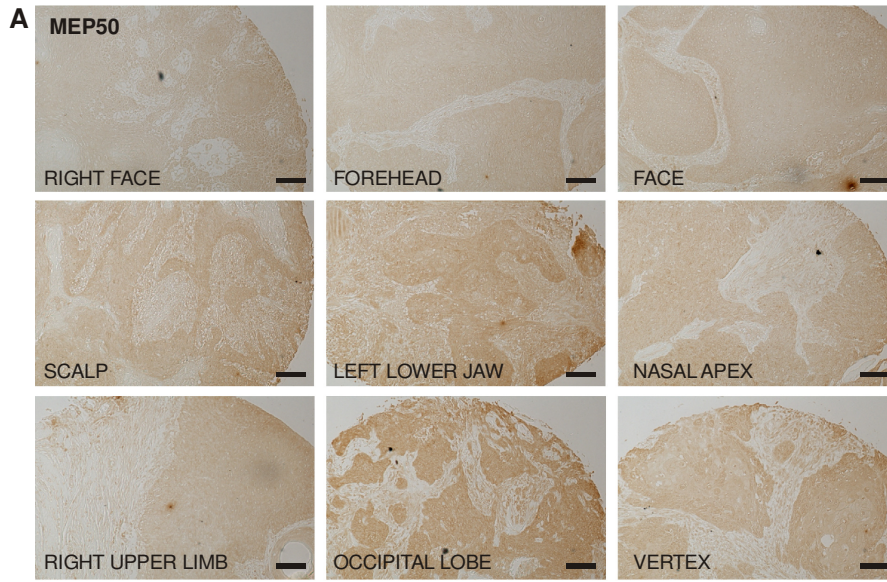
## ***B. FUTURE DIRECTIONS:***

MEP50 and PRMT5 are new players in the keratinocyte biology arena. While these studies have enhanced our understanding of the role of MEP50 and PRMT5 as negative regulators of differentiation in keratinocytes, several questions remain to be answered. A current area of interest is to investigate the role of MEP50 in tethering PRMT5 to the p38 $\delta$ /ERK complex. This will be investigated by testing the effect of MEP50 knockdown on PRMT5 association with the complex. The experiment will involve electroporation of the keratinocytes with control-siRNA or MEP50-siRNA for 72 hours. This will be followed by IP with p38 $\delta$  and levels of MEP50 and PRMT5 will be measured in the precipitate. Based on our previous results, we anticipate that MEP50 knockdown will impact complex formation between p38 $\delta$  and PRMT5. Decreased or no interaction between p38 $\delta$  and PRMT5 will be observed in the MEP50 knockdown cultures. Another important goal is to identify the effect of MEP50 knockdown on PRMT5 methyltransferase activity. We aim to study changes in global SDMA as well as SDMA levels of the proteins involved in the p38 $\delta$ -PRMT5 complex. We expect that MEP50 knockdown will result in the reduction of global SDMA levels as well as decrease the methylation status of the proteins in the p38 $\delta$ -ERK1/2 complex.

Another area of interest is the mechanism of cross-regulation of PRMT5 by MEP50. Our data suggests that MEP50 regulates PRMT5 mRNA and protein levels however the mechanism is unknown. We predict that MEP50 knockdown disrupts the interaction between PRMT5 and MEP50 resulting in degradation of PRMT5 protein. We will test this by first electroporating the cells with control-siRNA or MEP50-siRNA for 48 hours followed by treatment with a proteasome inhibitor for 24 hours. These lysates

will be used for immunoblot and monitored for stabilized PRMT5 protein in the MEP50-siRNA and proteasome treatment group. Another possibility is that MEP50 regulates the rate of PRMT5 transcription or mRNA stability. We can test this by nuclear run-on assay or actinomycin D assay respectively.

Additionally, we aim to study the role of MEP50 and PRMT5 in skin cancer cells. Our studies have indicated that MEP50 is expressed in squamous cell carcinoma tissue sections and in the squamous carcinoma SCC-13 cell line (**Fig. 5-1 A/B**). Additionally, MEP50 and PRMT5 siRNA resulted in substantial reduction in proliferation in the SCC-13 cells as compared to the control-siRNA transfected cells (**Fig. 5-1 C**). These indicate that MEP50 and PRMT5 act as pro-proliferation factors in the SCC-13 cells. We aim to identify the pathways in which MEP50 and PRMT5 contribute to cell proliferation.



**Figure 5-1:MEP50 is expressed in squamous cell carcinoma and negatively impacts proliferation.**

**A** The skin cancer tissue microarray (US Biomax BC21014) were stained with anti-MEP50 and binding was visualized using a peroxidase-conjugated secondary antibody (upper panels). Bar = 125  $\mu$ m. **B** SCC-13 cells were fixed and stained with anti-MEP50 (green). Similar results were observed in each of three experiments. The staining indicates MEP50 distribution in the nucleus and cytoplasm. **C** KERN were electroporated with 3  $\mu$ g of control, MEP50-siRNA or PRMT5-siRNA. The cells were allowed to seed overnight. Next, 15,000 cells from each group were seeded in each well of a 6 well plate and day 0 counts were taken the same day. Cells were counted on Day 2, Day 3 and Day 4. Simultaneously, protein lysates were tested by immunoblot using anti-MEP50, anti-PRMT5, anti-H4R3me2s or histone H4 antibody.  $\beta$ -actin was used as the loading control.

Another area of interest is the contribution of PRMT5 and MEP50 to tumor growth using a xenograft model. In order to study this, we aim to generate a stable cell line which is able to reduce PRMT5 or MEP50 expression in an inducible manner (tetracycline dependent). These cells will be injected in nude mice. The role of MEP50 and PRMT5 as essential contributors for tumor growth will be studied in two experiments. In the first setting, the mice will be given control or the tetracycline analog, doxycycline, immediately after injection of the cancer cells. This will enable us to monitor the role of PRMT5 and MEP50 in tumor formation. We predict that the control-treated mice will form tumors while the doxycycline treated mice will be unable to form tumors or form fewer tumors as compared to the control. The second experiment will involve feeding the mice doxycycline once tumors reach an appropriate size. This will be significant from the chemotherapeutic standpoint and allow us to test if PRMT5 or MEP50 depletion results in reduction in tumor numbers and tumor load. We predict that tumors in the control-treated mice will continue to proliferate while the tumors in doxycycline-treated mice will reduce in size or disappear. The number and size of tumors will be monitored weekly. At the end of the study; mRNA, protein and tumor sections will be collected to monitor for pro-proliferation markers. These experiments are important from the clinical standpoint as they will test the possibility of MEP50/PRMT5 as therapeutic targets in squamous cell carcinoma treatment.

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