

Protein Arginine Methyltransferase 5 (PRMT5) Signaling Suppresses Protein Kinase C δ - and p38 δ -dependent Signaling and Keratinocyte Differentiation*

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Background: MAPK signaling is an important mechanism controlling keratinocyte differentiation.

Results: PRMT5 and p38 δ interact as part of a multiprotein signaling complex, and PRMT5 and p38 δ produce opposing actions in regulating differentiation.

Conclusion: PRMT5 modulates p38 δ MAPK kinase phosphorylation and signaling.

Significance: This is a novel mechanism that links p38 δ MAPK signaling and PRMT5 signaling.

PKC δ is a key regulator of keratinocyte differentiation that activates p38 δ phosphorylation leading to increased differentiation as measured by an increased expression of the structural protein involucrin. Our previous studies suggest that p38 δ exists in association with protein partners. A major goal is to identify these partners and understand their role in regulating keratinocyte differentiation. In this study we use affinity purification and mass spectrometry to identify protein arginine methyltransferase 5 (PRMT5) as part of the p38 δ signaling complex. PRMT5 is an arginine methyltransferase that symmetrically dimethylates arginine residues on target proteins to alter target protein function. We show that PRMT5 knockdown is associated with increased p38 δ phosphorylation, suggesting that PRMT5 impacts the p38 δ signaling complex. At a functional level we show that PRMT5 inhibits the PKC δ - or 12-*O*-tetradecanoylphorbol-13-acetate-dependent increase in human involucrin expression, and PRMT5 dimethylates proteins in the p38 δ complex. Moreover, PKC δ expression reduces the PRMT5 level, suggesting that PKC δ activates differentiation in part by reducing PRMT5 level. These studies indicate antagonism between the PKC δ and PRMT5 signaling in control of keratinocyte differentiation.

Mitogen-activated protein kinases (MAPK) are dual-specificity serine/threonine kinases that drive intracellular signal transduction (1). MAPK family kinases share sequence similarity and conserved structural domains and include the extracellular signal-regulated kinases (ERK), Jun N-terminal kinases (JNK), and p38 MAPK. ERK is activated by mitogens and growth factors (2), whereas JNK and p38 kinases are typically activated in response to cellular stress (3). MAPKs play a central role in control of keratinocyte cell fate, and the balance between

ERK and p38 δ activity is a key determiner of keratinocyte survival status. Enhanced ERK activity is associated with survival, whereas enhanced p38 δ activity is associated with differentiation and apoptosis (4–10). Three p38 isoforms, p38 α , p38 β , and p38 δ , are expressed in keratinocytes (5, 8). Among these, p38 δ has a key role as a positive regulator of keratinocyte differentiation (7). p38 δ mediates the response to differentiating agents including phorbol ester, calcium, okadaic acid, and green tea polyphenol (7,10–15).

It is clear that MAPK signaling is regulated by cross-talk from other signaling cascades; however, this regulation is not well understood. This is because the key cascades that cross-talk with MAPK signaling are not well defined, are likely cell type- and context-specific, and the impact of this cross-talk on biological outcome is not well understood. In this study we demonstrate a role for protein arginine methyltransferase five (PRMT5)² in modulating MAPK signaling in keratinocytes. PRMT5 is an enzyme that dimethylates protein-bound arginine residues (16). Protein methylation is receiving increasing attention as an important post-translational modification. Protein arginine methyl transferases (PRMTs) are evolutionarily conserved enzymes that catalyze transfer of methyl groups from *S*-adenosyl methionine to the guanidino nitrogen of protein-bound arginine. Eight functional PRMT proteins are encoded in the mammalian genome (17). These enzymes mono- and dimethylate arginine residues in proteins and are classified as type I (PRMT1, 2, 3, 4, 6, and 8) and type II (PRMT5 and 7) enzymes. Type I enzymes catalyze formation of asymmetrically dimethylated arginine (16, 18).

PRMT5 is the sole type II member of the PRMT family that catalyzes formation of symmetrically dimethylated arginine (SDMA) (16). PRMT5 was discovered by yeast two-hybrid screening as Janus kinase-interacting protein 1 (16). PRMT5 dimethylates a variety of histone and non-histone proteins. Histone targets include histones H3 and H4 (19, 20), whereas non-

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² The abbreviations used are: PRMT, protein arginine methyltransferase; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; KERN, normal human keratinocyte; SDMA, symmetric dimethylarginine; hINV, involucrin; KSMF, keratinocyte serum-free medium.

PRMT5 and Keratinocyte Differentiation

histone targets include small heterodimer partner (21), myelin basic protein (22), and a host of others. PRMT5 interacts in a number of protein complexes that regulate RNA processing, signal transduction, and transcription (19, 23–29). *PRMT5* is a critical determinant of circadian period in *Arabidopsis* (30), and as a component of the androgen receptor cofactor complex, PRMT5 positively modulates androgen receptor-driven transcription independent of its methyltransferase activity (31, 32). PRMT5 modulates enhanced GFR-mediated ERK activation (33) and is required for p53 expression and induction of p53 targets (34). PRMT5 also binds to death receptor 4 (35). An important study shows that PRMT1 modulates p38 MAPK regulation of differentiation in megakaryocytes (36). In addition to these functions in signal transduction, PRMT5 also participates in the assembly of the transcriptional repressor complex on various eukaryotic promoters (37). Thus, PRMT5 and protein arginine dimethylation are emerging as important regulators of cell function.

Involucrin is a keratinocyte structural protein that functions as a precursor of the cornified envelope and is expressed in the suprabasal layers of epidermis (38, 39). Regulation of involucrin gene expression has been extensively studied as a model for understanding regulation of differentiation-dependent gene expression in epidermis (5). A PKC, Ras, MEKK1, MEK3/MEK6 signaling cascade has been implicated as a key control pathway in regulating involucrin expression (5). In this study we use this system to study cross-talk between PRMT5 and MAPK signaling in regulating keratinocyte differentiation. We show that PRMT5 reduces involucrin expression in normal human keratinocytes (KERN). These studies further show that PRMT5 is part of a p38 δ -ERK signaling complex and that PRMT5 modification of proteins in this complex is associated with reduced p38 δ phosphorylation. The net impact is that PRMT5 antagonizes p38 δ -dependent keratinocyte differentiation.

EXPERIMENTAL PROCEDURES

Reagents—Keratinocyte serum-free medium (KSFM), trypsin and Hanks' balanced salt solution were purchased from Invitrogen. Rabbit polyclonal antibodies specific for ERK1/2 (sc-94), protein kinase C δ (PKC δ , sc-213), PKC α (sc-208), p38 δ (sc-7587), p38 α (sc-7572), p38 β (sc-6176), PRMT5 (sc-22132), PRMT1 (sc-59648), and peroxidase-conjugated anti-goat IgG (sc-2033) were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti- β -actin (A5441), anti-FLAG M2 mouse monoclonal antibody (F-3165), anti-FLAG M2 affinity gel (A2220), and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) were obtained from Sigma. Peroxidase-conjugated anti-mouse IgG (NXA931) and peroxidase-conjugated anti-rabbit IgG (NA934V) were purchased from GE Healthcare. The antibody for detection of symmetric dimethyl arginine (07-412) was from Millipore (Bedford, MA). Knockdown of PKC δ and PRMT5 was achieved using siRNA. Pooled PKC δ -siRNA was from Santa Cruz (sc-36253) and from Dharmacon (J-003524-08). We also used individual siRNAs that target PKC δ mRNA sequences 5'-AG-AAGAAGCCGACCAUGUAAU (PKC δ -1) and 5'-GUUGAUGUCUGUUCAGUAUUU (PKC δ -2) (40). All were equally effective at reducing PKC δ level. Pooled PRMT5-siRNA

(NM_001039619) was from Santa Cruz (sc-41073) and Origene (SR307063). In addition, we used individual siRNA that target the PRMT5 mRNA sequences 5'-CAGCCACUGAUGGACA-AUCUGGAAU (PRMT5-1) and 5'-CCGGCUACUUGAGACUGUGCUUUA (PRMT5-2) (35). All were equally effective at reducing PRMT5 levels. Rabbit anti-human involucrin was previously described (42, 43). Rabbit anti-phospho-p38 (9211S) was from Cell Signaling (Danvers, MA), and mouse monoclonal anti-phospho-ERK1/2 (M8159) was from Sigma. The involucrin promoter luciferase reporter construct, pINV-2473, was previously described (44).

Primary Keratinocyte Culture and Adenovirus Production—Newborn foreskin epidermis was separated from dermis with dispase, and the keratinocytes were dispersed with trypsin and cultured in KSFM supplemented with epidermal growth factor and pituitary extract (44, 45). Adenoviruses encoding wild-type FLAG-p38 δ , PKC δ , and empty control adenovirus (Ad5-FLAG-p38 δ , Ad5-PKC δ , Ad5-EV) were prepared as previously described by propagation in 293 cells and purification by cesium chloride centrifugation (9, 46). For infection, keratinocytes were treated with multiplicity of infection 10 of adenovirus in the presence of 2.5 μ g/ml Polybrene for 4 h before the addition of fresh KSFM.

Affinity Purification of FLAG-p38 δ and Mass Spectrometry—Keratinocytes were infected with Ad5-FLAG-p38 δ and after 24 h extracts were prepared in lysis buffer (20 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β -mercaptoethanol, 1 mM Na₃VO₄, 1 μ g/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and proteinase inhibitor mixture). Anti-FLAG M2 affinity gel was washed with a large volume of lysis buffer followed by two bed volumes of 20 mM glycine-HCl, pH 2.8, and then re-equilibrated with lysis buffer. Cell lysate (120 mg of total protein) was incubated with M2 gel for 12 h at 4 °C with shaking and then packed in to PD10 column and washed with lysis buffer until A₂₈₀ < 0.02 to remove unbound protein. FLAG-p38 δ -associated proteins were eluted with 20 mM glycine-HCl, pH 2.8, and the collected fractions (200 μ l) were neutralized by the addition of 1 M Tris before storage at -20 °C.

Affinity-purified FLAG-p38 δ -associated proteins, prepared as outlined above, were concentrated by trichloroacetic acid precipitation, and samples were separated by SDS-PAGE followed by Coomassie Blue staining. Gel bands were excised and digested with trypsin, and the tryptic peptides were analyzed by liquid chromatography-tandem mass spectrometry using a Bruker Omniflex benchtop MALDI-TOF MS/MS. For protein identification, the monoisotopic mass maps of tryptic peptides were searched in databases using the MASCOT search engine.

Immunoprecipitation and Immunoblot—Total extracts in lysis buffer were incubated with appropriate primary antibody and protein A/G-agarose overnight at 4 °C and washed 4 times with Tris-buffered saline, pH 7.4. After the final wash the beads were collected, boiled with sample buffer, and centrifuged, and supernatant was electrophoresed and transferred to nitrocellulose membrane. The blots were blocked with 5% skimmed milk in Tris-buffered saline containing 0.1% Tween 20 and incubated with appropriate primary and horse-

radish peroxidase-conjugated secondary antibody, and antibody binding was visualized by chemiluminescent detection (Amersham Biosciences).

Keratinocyte Electroporation—Keratinocytes were electroporated with plasmids using an Amaxa electroporator and the VPD-1002 nucleofection kit (Amaxa, Cologne, Germany). Keratinocytes were harvested with trypsin and replated 1 day before use. On the day of electroporation, 1.2 million replated cells were harvested with trypsin and resuspended in KSFM. The cells were collected at $2000 \times g$, washed with 1 ml of sterile phosphate-buffered saline, and suspended in 100 μ l of keratinocyte nucleofection solution. The cell suspension, which included 3 μ g of plasmid or siRNA, was mixed by gentle up and down pipetting and electroporated using the T-018 program. KSFM (500 μ l) was added, and the suspension was transferred to a 60-mm cell culture dish.

Construction of pcDNA3-myc-hPRMT5—The pOTB7-hPRMT5 (GenBankTM BC025979) plasmid (MHS1011-7508890) was obtained from Open Biosystems (Huntsville, AL). hPRMT5 was amplified to create a BamHI/NotI fragment using forward (5'-GATCGAATTCGGATCCATGGAACAAAACTTATTTCTGAAGAAGATCTGATGGCGGCG-ATGGCGGTCCGGG3) and reverse (5'-GATCTCTAGAGCG-GCCGCCTAGAGGCAATGGTATATGAGCGGCCTGT3) primers. The ATG start codon (*double underline*) and myc epitope (*underlined*) are indicated. The insert was then cloned into pcDNA3 to create pcDNA3-myc-hPRMT5.

Quantitative PCR—RNA was isolated using RNeasy Mini kit (25-0500-71) (GE Healthcare). RNA (1 μ g) was used for cDNA synthesis with Moloney murine leukemia virus transcriptase (Invitrogen). Gene expression was measured by real time PCR using Light Cycler 480 SYBR Green I Master mix (04-707 516 001) from Roche Diagnostics and specific primers for hPRMT5 (forward, 5'-TGAGGCCAGTTTGGAGATGCC-TTA; reverse, 5'-AGTAGCCGGCAAAGCCATGTAGTA3), involucrin (forward, 5'-CCTCAGCCTTACTGTGAG; reverse, 5'-GGGAGGCAGTGGAGTTGG), PKC δ (forward, 5'-GGC-CACATCAAGATTGCCGACTTT; reverse, 5'-ACTGGCCA-ATGAGCATCTCGTACA), and GAPDH (forward, 5'-TCCA-CTGGCGTCTTACC; reverse, 5'-GGCAGAGATGATG-ACCCTTT).

RESULTS

Identification of FLAG-p38 δ -associated Proteins—Our previous studies describe a p38 δ -ERK signaling complex in keratinocytes that regulates keratinocyte differentiation (6). Although these studies show that p38 δ interacts with ERK1/2, it is likely that p38 δ also interacts with other proteins and that these proteins influence function. To identify additional interaction partners, extracts were prepared from FLAG-p38 δ -expressing keratinocytes, and p38 δ -associated proteins were purified by FLAG affinity column chromatography. The column was loaded with 120 mg of total cell lysate and washed with loading buffer, and affinity-bound proteins were eluted with glycine buffer. Fig. 1A shows a Coomassie-stained gel illustrating the affinity purification of FLAG-p38 δ . The fractions show the lysate (L) that was loaded onto the column, the column wash (W), and column elution fractions (F1, etc.). The Co-

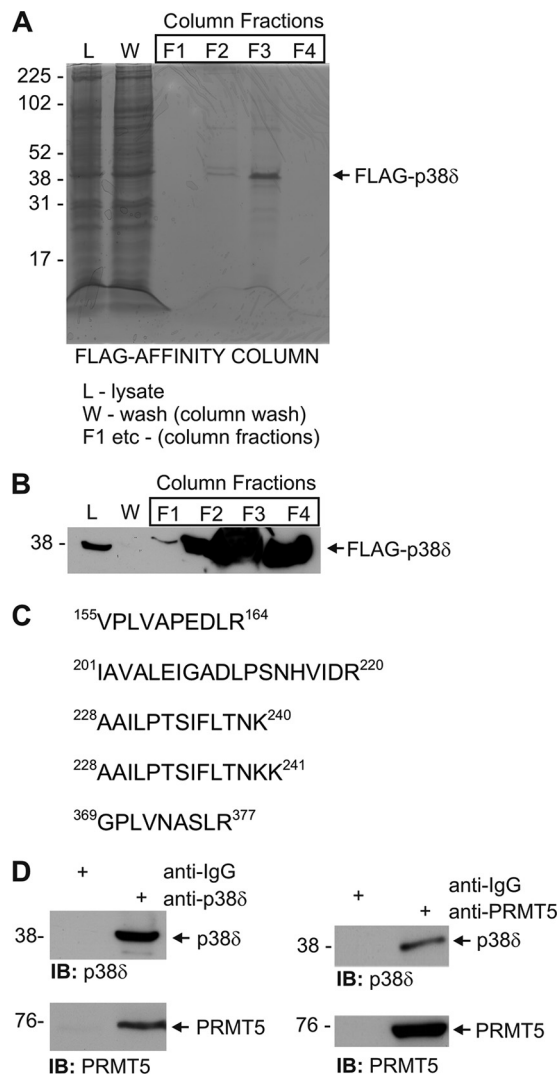


FIGURE 1. FLAG-p38 δ complex includes PRMT5. A, KERn were infected with multiplicity of infection 10 of Ad5-FLAG-p38 δ , and after 24 h extracts were prepared for FLAG affinity chromatography. Total cell lysate (L), column wash (W), and fractions eluted from the FLAG affinity column (F1, etc.) were electrophoresed, and the gel was Coomassie-stained. Molecular mass markers are indicated. B, shown is detection of FLAG-p38 δ . Samples were electrophoresed and immunoblotted with anti-FLAG. FLAG-p38 δ was absent from the wash fraction. F3 contains the highest concentration of FLAG-p38 δ . C, amino acid sequence of PRMT5 tryptic fragments were identified by mass spectrometry analysis. D, co-precipitation of p38 δ and PRMT5 is shown. Total cell extracts were prepared from KERn and then immunoprecipitated (IB) with anti-IgG (control), anti-p38 δ , or anti-PRMT5. The precipitates were then electrophoresed for immunoblot detection of p38 δ and PRMT5. Similar results were observed in each of three experiments.

massie-stained gel shows the presence of FLAG-p38 δ in elution fractions F2, F3, and F4 but most prominently in F3 (Fig. 1A). Anti-FLAG immunoblot of proteins derived from the total lysate and elution fractions reveals FLAG-p38 δ in elution fractions F2, F3, and F4 (Fig. 1B). These fractions were pooled and analyzed by matrix-assisted laser desorption ionization mass spectrometry, which revealed the presence of several proteins. Among these was PRMT5. Fig. 1C indicates that PRMT5-derived tryptic peptides identified by mass spectrometry. PRMT5 is a protein arginine methyltransferase that modifies arginine in target proteins to form SDMA (16). This covalent post-translational modification acts to alter target protein function (16).

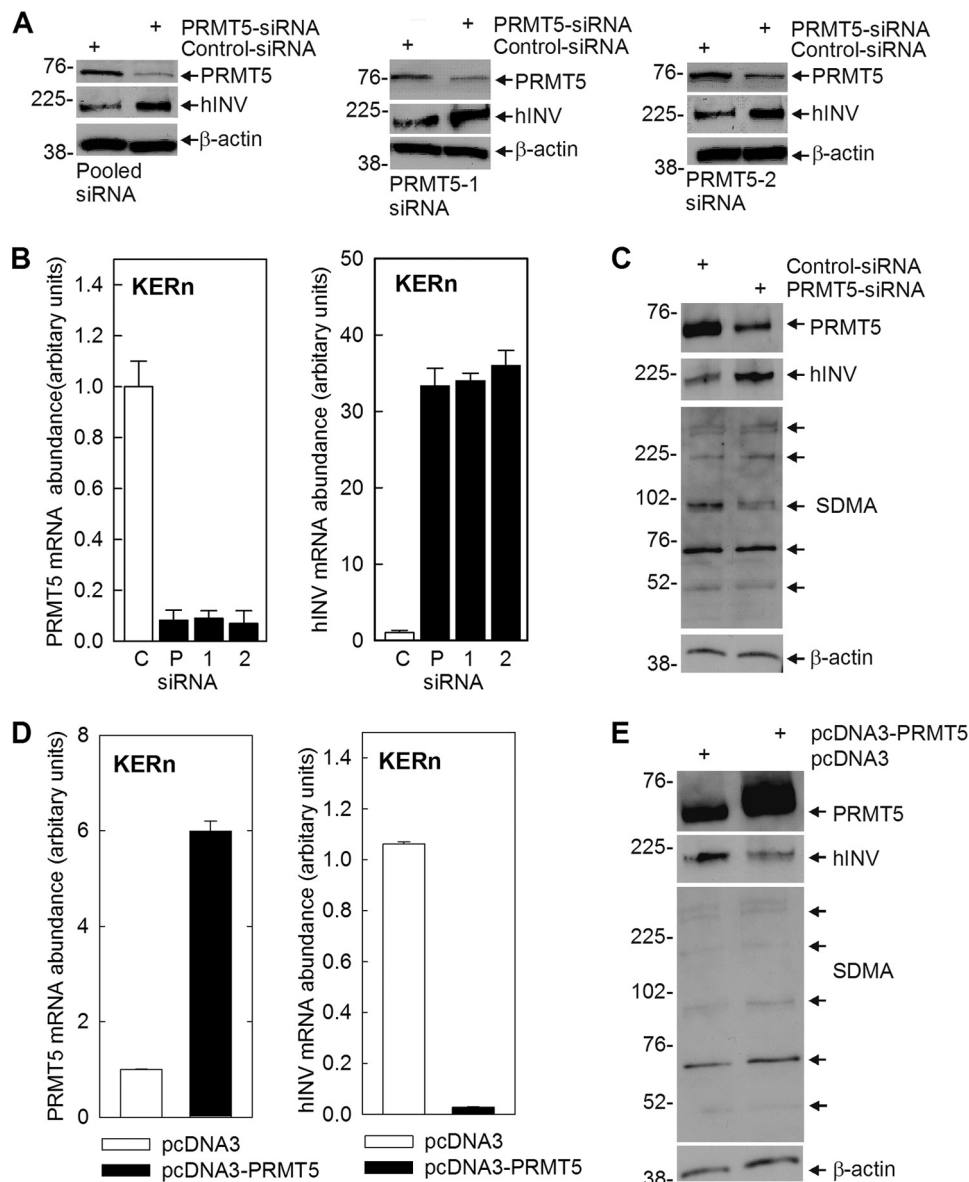


FIGURE 2. PRMT5 regulates hINV mRNA and protein level. *A*, KERNs were electroporated with 3 μ g of control, PRMT5-pooled (Santa Cruz), PRMT5-1, or PRMT5-2 siRNA (35), and after 48 h, PRMT5 and hINV protein levels were assessed by immunoblot. *B*, KERNs were electroporated with 3 μ g of the control (C), PRMT5-pooled (P, Santa Cruz), or individual (PRMT5-1, 1) or PRMT5-2, 2) siRNA and after 48 h PRMT5 and hINV mRNA was measured, by qPCR. Bars indicate relative levels of PRMT5 and hINV RNA normalized relative to GAPDH RNA levels and are the mean \pm S.D. for triplicate samples in a single experiment. Similar results were obtained in three independent experiments. *C*, KERNs were electroporated with 3 μ g of control or PRMT5-pooled (Santa Cruz) siRNA. hINV, PRMT5, and SDMA were measured in total cell extracts prepared at 48 h post-electroporation. The arrows in the SDMA blot indicate the major SDMA-modified proteins. β -Actin is the control to normalize protein loading. *D* and *E*, KERNs were electroporated with 3 μ g of pcDNA3 or pcDNA3-hPRMT5, PRMT5 and hINV mRNA levels were measured after 24 h, and protein levels were measured at 48 h post-exposure. The values are the mean \pm S.D. of triplicate samples in a single experiment. Similar results were observed in each of three experiments. The arrows in the SDMA blot indicate the major SDMA-modified proteins.

These findings suggest that PRMT5 is a binding partner for p38 δ . To confirm this, we prepared total cell extracts from human keratinocytes and immunoprecipitated endogenous p38 δ with anti-p38 δ . The precipitate was electrophoresed, and the gel was stained with anti-PRMT5. Fig. 1D shows that PRMT5 co-precipitates with p38 δ whether the precipitation is performed with anti-p38 δ or anti-PRMT5, thereby providing additional evidence for p38 δ /PRMT5 intracellular interaction.

Role of PRMT5 in Regulating Keratinocyte Differentiation—Our previous studies demonstrate that a PKC δ signaling cascade increases p38 δ activity, which leads to increased cell differentiation (5, 6, 10, 47, 48). Because PRMT5 interacts with

p38 δ , we hypothesized that it may regulate differentiation-associated responses that depend upon p38 δ activity. To assess this we monitored the impact of PRMT5 on involucrin (hINV) expression. hINV is a marker protein that is increased during keratinocyte differentiation and in response to differentiation stimuli (5, 6, 10, 47, 48). We began by assessing the impact of PRMT5 knockdown and overexpression on hINV mRNA and protein levels. Fig. 2A shows that treatment with PRMT5-pooled (Santa Cruz, sc-41073) or individual (PRMT5-1 or PRMT5-2) siRNA (35) reduces PRMT5 levels, and this is associated with a marked increase in hINV protein levels. Fig. 2B shows this is due to changes in mRNA levels. Treatment

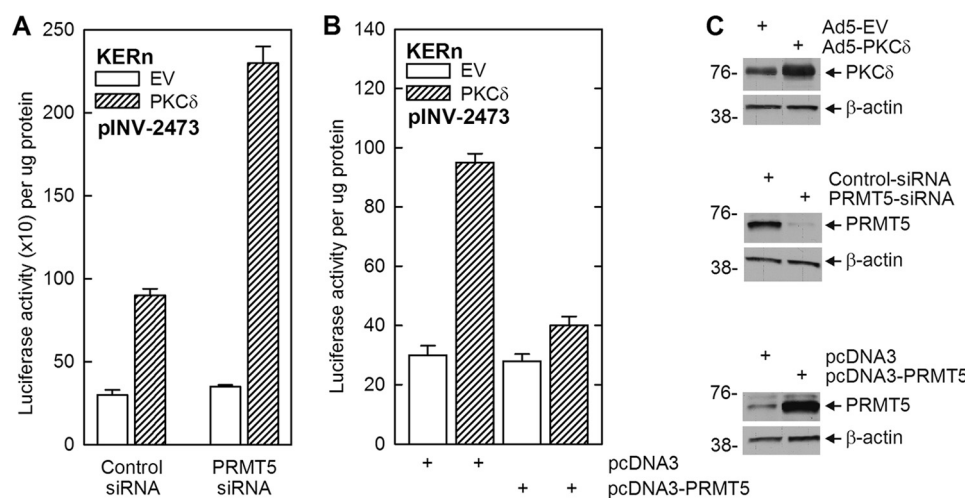


FIGURE 3. Impact of PRMT5 on PKC δ -dependent hINV promoter activity. *A*, KERN were electroporated with 3 μ g of control- or PRMT5-pooled (Santa Cruz) siRNA, and after 48 h the cells were trypsinized and electroporated with 1.5 μ g of pINV-2473 (involucrin promoter luciferase reporter) in the presence of 1.5 μ g of empty vector (pEGFN1) or PKC δ -encoding plasmid (pPKC δ -EGFN1). After an additional 18 h, cell extract was prepared for assay of luciferase activity. Values are the mean \pm S.E., $n = 3$. The control-siRNA EV and PKC δ values are significantly different, $p < 0.001$; the PRMT5-siRNA EV and PKC δ values are significantly different, $p < 0.001$; the Control-siRNA PKC δ and PRMT5-siRNA PKC δ values are significantly different, $p < 0.001$. *B*, KERN were electroporated with 1.5 μ g of pcDNA3 or pcDNA3-PRMT5 expression vector, and after 48 h cells were re-electroporated with 1.5 μ g of pINV-2473 and 0.5 μ g of pEGFN1 or pPKC δ -EGFN1. After an additional 18 h, extracts were prepared for luciferase assay. The values are the mean \pm S.E., $n = 3$. The pcDNA3 EV and PKC δ values are significantly different, $p < 0.001$. *C*, KERN were incubated with the indicated expression vectors or siRNA, and after 24 h extracts were prepared for detection of the indicated proteins by immunoblot. Similar results were observed at 48 and 72 h (not shown).

with PRMT5-pooled (*P*), PRMT5-1 (*I*) or PRMT5-2 (*2*) siRNA reduces PRMT5 mRNA, and this is associated with increased hINV mRNA. Consistent with these findings, the converse is also observed, as overexpression of PRMT5 reduces hINV mRNA and protein level (Fig. 2, *D* and *E*).

PRMT5 is a protein arginine methyltransferase that catalyzes modification of arginine residues in target proteins to form SDMA, a modification that modulates target protein function (16, 18). We, therefore, examined the impact of PRMT5 knock-down and overexpression on SDMA formation. Extracts from PRMT5-siRNA-treated and PRMT5-overexpressing cells were prepared for anti-SDMA immunoblot. Proteins ranging in molecular mass from 50 to 300 kDa are detected (Fig. 2, *C* and *E*). Immunostaining of a limited number of proteins covering a broad range of molecular weights is typical of anti-SDMA reagents (49). Using this as a crude assay of PRMT5 activity, we observe a 10–15% reduction in average SDMA intensity in PRMT5-siRNA-treated cells and a similar 10–15% increase in extracts derived from PRMT5-overexpressing cells. We do not presently know which proteins are identified in this assay, but these findings suggest that increased PRMT5-dependent SDMA formation is associated with suppression of involucrin gene expression.

Regulation of hINV Expression by PKC δ and PRMT5—The above studies indicate that PRMT5 suppresses hINV mRNA and protein levels. We next compared the action of PRMT5 against two treatments that increase differentiation, PKC δ overexpression and TPA treatment. PKC δ promotes keratinocyte differentiation and increases involucrin gene expression via activation of p38 δ (4, 5, 47). pINV-2473 encodes the full-length hINV promoter linked to a luciferase reporter gene such that increased promoter activity is reflected in increased luciferase activity (44). Keratinocytes were transfected with pINV-2473 in the presence of control-siRNA or PRMT5-siRNA and

empty or PKC δ -encoding expression vector. In the presence of control-siRNA, PKC δ increases hINV promoter activity 3-fold (Fig. 3*A*). In contrast, when PRMT5-siRNA is present and PRMT5 is reduced (Fig. 3*C*), the induction is 6.5-fold (Fig. 3*A*). Moreover, the PKC δ induction of hINV promoter activity is suppressed (Fig. 3*B*) in PRMT5 overexpressing (Fig. 3*C*) cells.

TPA is a pharmacological agent that increases PKC activity and stimulates differentiation (44). We used TPA treatment as a second method of PKC δ activation. Keratinocytes were transfected with pINV-2473 and treated with TPA in the presence of control- or PRMT5-siRNA. TPA at 5 and 10 ng/ml increases hINV promoter activity, and a further 2-fold increase in promoter activity is observed in PRMT5 knockdown cells (Fig. 4*A*). In the converse experiment in which PRMT5 is overexpressed, the TPA-dependent increase in hINV promoter activity is suppressed by 2-fold (Fig. 4*B*). These studies suggest that PRMT5 opposes the pro-differentiation action of TPA and PKC δ .

It is well established that PKC δ enhances keratinocyte differentiation (4–6, 47). We wondered whether part of this mechanism may involve PKC δ suppression of PRMT5 expression. To test this we manipulated PKC δ level and monitored the impact on PRMT5 expression. Fig. 5*A* shows that PRMT5 RNA level is increased 4-fold when PKC δ mRNA levels is reduced by treating KERN with PKC δ -pooled (Santa Cruz) or individual (PKC δ -1 or PKC δ -2) (40) siRNA. Moreover, the reduction in PKC δ level is associated with increased PRMT5 protein expression and SDMA formation (Fig. 5*B*). In the inverse experiment, PKC δ overexpression reduces PRMT5 RNA and protein level (Fig. 5, *C* and *D*), and this is associated with reduced SDMA formation (Fig. 5*D*). As noted in Fig. 2, the anti-SDMA blots shown in Fig. 5, *B* and *D*, provide a generally index of PRMT5 activity (49).

PRMT5 Knockdown Enhances Total p38 Phosphorylation—PKC δ or TPA stimulation of differentiation is associated with

PRMT5 and Keratinocyte Differentiation

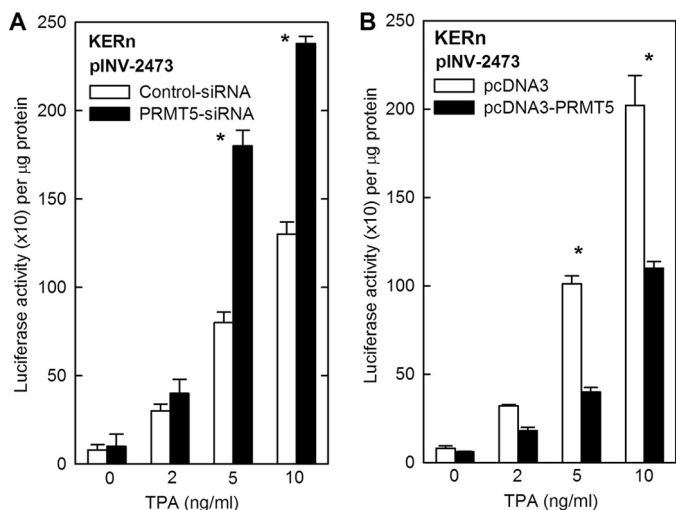


FIGURE 4. Impact of PRMT5 on TPA-dependent hINV promoter activity. *A*, KERN were electroporated with 3 μ g of control- or PRMT5-pooled (Santa Cruz) siRNA. After 48 h cells were re-electroporated with 3 μ g of pINV-2473 and involucrin promoter luciferase reporter construct. After an additional 6 h, TPA was added, and after an additional 18 h cells were lysed for luciferase activity assay. The values are the mean \pm S.E., $n = 3$. The asterisks indicate a significant difference as determined by Student's t test, $p < 0.001$, between control-siRNA and PRMT5-siRNA at 5 and 10 ng/ml TPA. *B*, KERN were electroporated with 3 μ g of pcDNA3 or pcDNA-PRMT5 and after 48 h re-electroporated with 3 μ g of pINV-2473. After an additional 6 h the cells were treated with TPA for 18 h, and then extracts were prepared for luciferase activity assay. The results are presented as the mean \pm S.E., $n = 3$. The asterisks indicate a significant difference as determined by Student's t test, $p < 0.001$, between pcDNA3 and pcDNA3-PRMT5 at 5 and 10 ng/ml TPA.

increased p38 MAPK activity as evidenced by increased p38 phosphorylation (4, 6, 10, 15). We hypothesized that PRMT5 may regulate differentiation by modulating the extent of this activation. Because no antibody is available that specifically detects phosphorylation of the individual p38 α , - β , and - δ isoforms, we began by monitoring total p38 phosphorylation (*i.e.* phosphorylation of p38 α , - β , and - δ) (9, 46). Keratinocytes were electroporated with control-siRNA or PRMT5-siRNA, and the level of total phosphorylated p38 was measured. Fig. 6 shows that PRMT5 knockdown increases total p38 phosphorylation (p38T-P). We also monitored the impact of PRMT5 knockdown on the level of other key regulators. p38 α , - β , and - δ are expressed in keratinocytes (9, 46), but p38 δ is the major form phosphorylated by stimuli that drive differentiation (4, 10, 47). ERK1/2 is also an important regulator, and ERK1/2 phosphorylation enhances keratinocyte survival (4). Fig. 6 shows that PKC δ , p38 δ , and ERK1/2 levels and ERK1/2 phosphorylation are not altered by PRMT5 knockdown. p38 α and β levels were also not altered by PRMT5 knockdown (not shown). Thus, a major impact of PRMT5 knockdown is to enhance total p38 phosphorylation.

PRMT5-dependent Covalent Modification of Proteins in p38 δ Complex Is Associated with Increased p38 δ Phosphorylation—Given that PRMT5 knockdown enhances total p38 phosphorylation, a key question is whether PRMT5 specifically modulates phosphorylation of the p38 δ isoform. This could be expected, as previous studies indicate that p38 δ is the key p38 isoform involved in regulation of differentiation (6, 7, 9). A second key issue is whether any such ability of PRMT5 to regulate p38 δ phosphorylation is associated with altered SDMA modification

of proteins in the p38 δ signaling complex. To assess this, we performed two types of experiments. We first treated cells with control or PRMT5-pooled PRMT5-1 or PRMT5-2 siRNA for 48 h and then immunoprecipitated p38 δ and assessed phosphorylation status. In Fig. 7A shows that p38 δ is phosphorylated in PRMT5 knockdown but not control cells. As a second method to confirm this result, we monitored the phosphorylation state of vector-expressed FLAG-p38 δ . Cells were treated with control- or PRMT5-siRNA and then infected with Ad5-EV or Ad5-FLAG-p38 δ . At 24 h post-infection, FLAG-p38 δ was immunoprecipitated with anti-FLAG, and the p38 δ phosphorylation state was measured. Fig. 7B confirms that PRMT5 knockdown is associated with increased p38 δ phosphorylation.

We next monitored whether the p38 δ complex contains SDMA modified proteins. We infected cells with Ad5-EV or Ad5-FLAG-p38 δ and after 24 h immunoprecipitated with anti-FLAG and monitored the level of SDMA in the precipitate. Fig. 7C shows that SDMA modified proteins are present in the p38 δ complex. We next assessed whether this SDMA modification requires PRMT5. We treated cells with control- or PRMT5-siRNA and after 48 h prepared extracts for immunoprecipitation with anti-p38 δ . Fig. 7D shows that a SDMA-modified 60-kDa protein is associated with the p38 δ complex. Moreover, PRMT5 knockdown reduced the level of this protein or the extent of its SDMA modification. This protein has a molecular mass of 60 kDa, suggesting that it is a protein other than p38 δ . As a control for assay integrity, we confirm appropriate immunoprecipitation of p38 δ and PRMT5. Taken together, these findings suggest that PRMT5 suppresses p38 δ phosphorylation and that this is associated with increased SDMA modification of a 60-kDa protein present in the p38 δ complex.

DISCUSSION

Epidermal Differentiation—The epidermal keratinocyte, the major cell type present in the epidermis, undergoes a complex and choreographed program of differentiation. This process requires a balance among keratinocyte proliferation, differentiation, and apoptosis. Ultimately, this process leads to the formation of a multilayered epidermis that contains a proliferative basal zone beneath several layers of cells that are at various stages in the differentiation process (50). Involucrin is a marker of this process that is increased in differentiated cells (5) and is an important component of the scaffolding of the cornified envelope (43, 51–53). Understanding how this process is regulated to produce a multilayered tissue is a major goal in epidermal biology, as disorders in this process are observed in a host of epidermal disease states and also in cancer.

p38 δ -ERK Signaling Complex—Previous studies show that a p38 δ -ERK complex regulates expression of key apoptosis, differentiation, and proliferation-associated genes in keratinocytes (4–6, 10, 11). p38 δ activity is increased, and ERK1/2 activity is reduced in differentiated keratinocytes (6). The importance of interaction within this complex is illustrated by the role of p38 δ and ERK1/2. p38 δ and ERK1/2 are associated either directly or indirectly in this complex, and increased p38 δ phosphorylation and reduced ERK1/2 activity is observed in

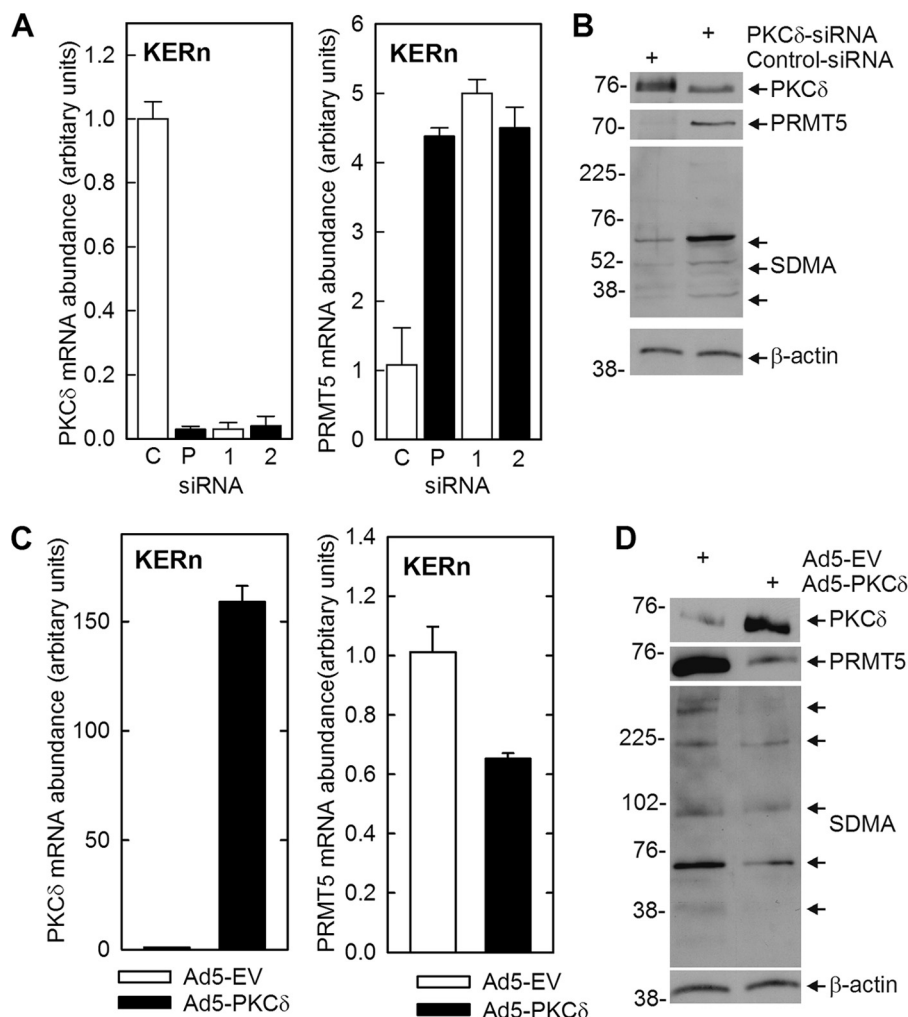


FIGURE 5. **PKC δ regulates PRMT5 level.** *A*, KERns were electroporated with 3 μ g of control- and PKC δ -pooled (*P*, Santa Cruz) or individual (PKC δ -1, PKC δ -2) (40) siRNA, and after 24 h, PKC δ and PRMT5 mRNA levels were monitored as outlined in Fig. 2. *B* KERns were electroporated with 3 μ g of control- or PKC δ -pooled (Santa Cruz) siRNA, and after 24 h extracts were prepared for detection of PKC δ , PRMT5, and SDMA by immunoblot. The values are the mean \pm S.D. of three replicates, and similar results were observed in each of three experiments. *C* and *D*, KERn were infected with the indicated adenovirus, and after 24 h mRNA and protein extracts were prepared for detection of PKC δ and PRMT5 RNA and protein. SDMA was also monitored by immunoblot. The arrows in the SDMA blot indicate the major SDMA-modified proteins. The bar graph values are the mean \pm S.D. of three replicated samples. Similar results were observed in each of three experiments.

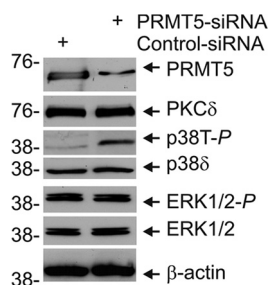


FIGURE 6. **PRMT5 suppresses total p38 phosphorylation.** Keratinocytes were electroporated with 3 μ g of control- or PRMT5-siRNA, and after 48 h cell extracts were prepared and electrophoresed for immunoblot detection of the indicated proteins. Similar results were observed in each of two experiments.

response to treatment with differentiation stimuli (4–7). We propose that this complex serves to integrate incoming signals and that the net output then determines cell fate. For example, activation of PKC δ increases p38 δ activity and cell differentiation and death, whereas activation of ERK1/2 by delivery of

constitutively active Raf antagonizes this response and promotes cell survival (5). The fact that a p38 δ -ERK complex is involved in this regulation suggests that other proteins may also be partners in this regulatory complex.

An important goal is identification of additional partners that are part of this complex and influence the signaling activity. To achieve this we expressed FLAG-p38 δ in keratinocytes and identified proteins associated with p38 δ by FLAG-affinity chromatography and mass spectrometry. One of the proteins that co-purified with p38 δ was PRMT5. This interaction was confirmed by co-immunoprecipitation of PRMT5 and p38 δ from keratinocyte extracts using a p38 δ -specific antibody. This confirmation is important, as it has been reported in one study that PRMT5 can interact with anti-FLAG (16). Protein arginine methyltransferases are a family of proteins that dimethylate arginine residues in target proteins (16). Arginine modification in this context influences target protein structure, function, and activity (16). PRMTs have a role in numerous cellular processes including regulation of cell signaling and gene expression (17,

PRMT5 and Keratinocyte Differentiation

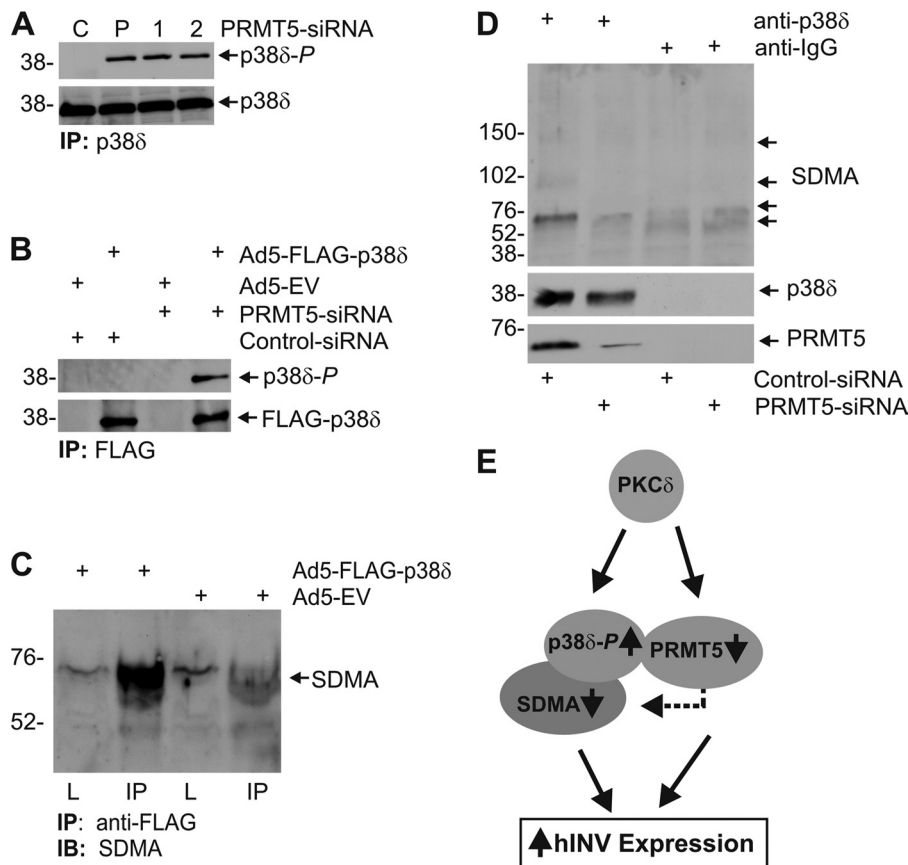


FIGURE 7. PRMT5 regulates p38 δ phosphorylation and SDMA modification of p38 δ -associated proteins. *A*, KERN were electroporated with 3 μ g of control, PRMT5-pooled, PRMT5-1, or PRMT5-2 siRNA, and after 48 h extracts were prepared for immunoprecipitation (IP) with anti-p38 δ . The precipitated material was then monitored by immunoblot to detect p38 δ and phosphorylated p38 δ . *B*, KERNs were electroporated with 3 μ g of control- or PRMT5-pooled (Santa Cruz) siRNA and after 24 h infected with multiplicity of infection 10 of Ad5-EV or Ad5-p38 δ and after an additional 24 h extracts were prepared for immunoprecipitation with anti-FLAG. The precipitated proteins were electrophoresed, and the level of FLAG-p38 δ (anti-FLAG) and phosphorylated p38 δ was monitored by immunoblot. *C*, KERN (70% confluent) was infected with multiplicity of infection 10 Ad5-EV or Ad5-FLAG-p38 δ , and after 24 h lysate was prepared, and 200 μ g of protein was immunoprecipitated with anti-FLAG antibody. The resulting pellet was electrophoresed for immunoblot (IB) with anti-SDMA. 20 μ g of total lysate (L) was electrophoresed in a parallel lane. The blot was then incubated with anti-SDMA. *D*, KERN were electroporated with 3 μ g of control- or PRMT5-pooled (Santa Cruz) siRNA, and after 48 h extracts were prepared for immunoprecipitation with anti-IgG or anti-p38 δ . The precipitates were then electrophoresed for immunodetection of p38 δ , PRMT5, and SDMA. The arrows indicate the major SDMA modified bands. *E*, PKC δ and PRMT5 regulation of p38 δ activity; a working model. The model is explained in the "Discussion." We realize that multiple complexes may exist and that some may lack one or more of the components, and that it is likely that other proteins are also present in this complex. We also note that we do not know whether there is direct interaction between the indicated components. The arrows indicate relative change in level. The dashed arrow indicates PRMT5 SDMA modification of target proteins.

32, 33, 54–57). However, additional studies are needed to explore the physiological mechanisms whereby PRMTs regulate cells survival, differentiation, and apoptosis. Despite the recognized importance of PRMT-dependent modification in a host of cell types, the role of PRMT in regulating keratinocyte function has not been studied.

PRMT5 Regulates Basal Involucrin Expression—Our previous studies show that keratinocyte differentiation requires activity in a PKC δ , Ras, MEKK1, MEK3 cascade that triggers changes in activity in a p38 δ -ERK complex to increase p38 δ activity relative to ERK activity (4–7, 9–11, 15, 47, 48, 58). Activation of this cascade is associated with cessation of cell proliferation and increased morphological differentiation (8, 10, 15), and prolonged stimulation can cause apoptosis (4, 8). Stimulation of MAPK activity by this cascade is not associated with changes in expression of p38 δ or ERK1/2 level but is associated with increased p38 δ activity and reduced ERK1/2 activity (6). Involucrin is a marker of differentiation that is increased in differentiated cells and is expressed at a fixed basal level in rest-

ing keratinocytes (5). An interesting and unexpected finding is that PRMT5 knockdown increases basal involucrin expression including increasing hINV mRNA and protein levels and promoter activity. Moreover, the converse is also observed in that PRMT5 overexpression reduces basal involucrin mRNA and protein levels.

PRMT5 Influences Stimulus-dependent Involucrin Expression—We also monitored the impact of PRMT5 on response to agents that activate p38 δ signaling to increase differentiation. Overexpression of PKC δ and treatment with TPA enhance p38 δ phosphorylation and increase involucrin expression (4, 6). TPA is a diacylglycerol analog that enhances PKC δ activity and induces differentiation (44, 48). We show that PRMT5 knockdown enhances the differentiation promoting ability of these agents and the PRMT5 overexpression suppresses the differentiation promoting response. These findings suggest that PRMT5 is a negative regulator of keratinocyte differentiation. Thus, these studies identify an important new PRMT5-mediated regulatory circuit in keratinocytes that functions to sup-

press differentiation. In addition to an important role in normal cells, these findings suggest a role for PRMT5 in skin cancer. PRMT5 levels are increased (29), and PKC δ levels are decreased in cancer (59, 60). Thus, the balance between these signaling inputs is likely to be important in disease development, and we anticipate that PRMT5 may serve to enhance skin cancer cell survival. The possibility that PRMT5-related regulation is increased is an important area for further investigation in skin cancer. PRMT5 may also link the p38 δ complex to other signaling cascades, as PRMT5 associates with Janus kinases and STAT (54, 56). Thus, the knowledge that PRMT5 is part of the p38 δ complex helps us in our effort to assemble the signaling network that controls keratinocyte differentiation.

Suppression of PRMT5 Level by PKC δ —There are numerous situations where increased pro-differentiation signaling is associated with reduced pro-survival signaling and vice versa. An example in keratinocytes is the coupling of increased p38 δ and decreased ERK1/2 activity in differentiation agent-stimulated cells (5). PKC δ and PRMT5 appear to share such a relationship, as PKC δ overexpression is associated with reduced PRMT5, and PKC δ knockdown is associated with increased PRMT5. Thus, it is possible that suppression of the PRMT5 level and activity may be a mechanism whereby PKC δ drives keratinocyte differentiation. We know that PKC δ -dependent reduction in PRMT5 is associated with reduced PRMT5 mRNA and protein levels. This suggests a potential impact of PKC δ -associated signaling on transcription of the PRMT5 gene or an impact on PRMT5 RNA stability; however, further studies will be necessary to fully understand this regulation.

Impact of PRMT5 on p38 δ Signaling Complex—To gain insight regarding the role of PRMT5 in p38 δ MAPK signaling, we examined the impact of PRMT5 on the complex. These studies are extremely interesting, as they suggest that PRMT5 influences the p38 δ phosphorylation state. First, we show that PRMT5 is a partner in the p38 δ complex. PRMT5 is found associated with FLAG-p38 δ when analyzed by mass spectrometry and also co-immunoprecipitates with p38 δ present in total cell extracts. That this interaction is real is supported by functional studies. At a functional level, PRMT5 influences molecular events in this complex. Overexpression of PRMT5 results in reduced p38 δ phosphorylation of p38 δ , and this is associated with increased SDMA modification of a 60-kDa protein that also co-precipitates with p38 δ . Moreover, this regulation is reversed in PRMT5 knockdown cells.

The identity of the 60-kDa protein is not presently known, but it appears to be a *bona fide* target of PRMT5, as PRMT5 knockdown results in reduced anti-SDMA detection of this band. This is likely due to decreased SDMA labeling of this protein but could also be due to a reduction in protein level. It is interesting that this 60-kDa band is also a major SDMA-modified protein in total keratinocyte cell extracts, suggesting that it may be a major PRMT5 target that is involved in a range of processes. It should also be noted that we do not intend to imply that all p38 δ complexes included all of these partners (ERK1/2, PRMT5, 60-kDa protein, etc.), as it is likely that multiple sub-complexes of varying composition exist and that these complexes may have unique intracellular functions. It is also worth noting that no previous reports described an impact of PRMT5

on MAPK signaling. The only available report relates an impact of PRMT1, a type I PRMT that asymmetrically dimethylates arginine (16, 18), on p38 α function in megakaryocyte differentiation (36).

Working Model—Our previous studies show that PKC δ activates involucrin gene expression (keratinocyte differentiation) by stimulating a cascade that regulates a p38 δ and ERK1/2-containing complex to increase p38 δ and decrease ERK1/2 phosphorylation (4–6, 10, 48). This change in the balance of p38 δ and ERK1/2 activity ultimately leads to increased nuclear AP1 factor levels, increased AP1 factor interaction with specific sites on the hINV promoter, and increased transcription (41, 44, 61, 62).

Based on the studies presented in this proposal, we propose that PRMT5 is a new component of this signaling complex. The role of PRMT5 in this complex is to inhibit p38 δ activity and suppress differentiation by catalyzing SDMA modification at the arginine residue(s) of a 60-kDa protein (and perhaps other proteins) that are present in this complex. We propose that SDMA modification of these targets alters the complex in a way that leads to reduced p38 δ phosphorylation. Thus, when PRMT5 levels are high and PRMT5 is active, differentiation-dependent gene (hINV) expression is suppressed. Under conditions where cells are exposed to a differentiation stimulus, PKC δ activity triggers signaling events that drive differentiation. We propose that PKC δ acts to increase p38 δ phosphorylation by two potential mechanisms (Fig. 7E). One mechanism is stimulating signaling events that lead to increased p38 δ phosphorylation (6). Our present studies suggest that a second mechanism is suppression of PRMT5 mRNA and protein levels, thereby reducing PRMT5 modification of proteins in the p38 δ complex. Our studies show that reduced PRMT5 levels are associated with increased p38 δ phosphorylation. Thus, the present studies identify a new signaling protein present in the p38 δ complex that suppresses differentiation-associated p38 δ activity.

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