LETTER

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Identification and Characterization of Small-Molecule Inducers of Epidermal Keratinocyte Differentiation

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ABSTRACT An essential function of the human epidermis is the maintenance of a protective barrier against the environment. As a consequence, keratinocytes, which make up this layer of the skin, undergo an elaborate process of self-renewal, terminal differentiation, and cell death. Misregulation of these processes can lead to several human diseases, including psoriasis and basal cell and squamous cell carcinomas. To identify novel regulators of keratinocyte differentiation, a cell-based screen of small-molecule libraries was carried out for molecules that induce terminal differentiation of normal human epidermal keratinocytes. One class of molecules was identified, the 2-(3,4,5-trimethoxyphenylamino)-pyrrolo[2,3-*d*]pyrimidines, which were shown to induce differentiation of epidermal progenitor cells to terminally differentiated keratinocytes. These molecules serve as useful mechanistic probes of the cellular differentiation programs that regulate the formation and homeostasis of the epidermis and may lead to novel therapeutic approaches for the treatment of skin hyperproliferative disorders.

Normal human skin is an organ renewed throughout adult life by proliferation of stem cells and differentiation of their progeny (*1*). It is covered by a multilayered epidermis in which keratinocytes undergo a continuous process of proliferation, differentiation, and apoptosis. Tissue homeostasis requires a delicate balance of epidermal cells entering the proliferating pool and cells in various stages of differentiation. The switch between keratinocyte growth and differentiation is controlled by several signaling pathways, including protein kinase C (PKC), phospholipase C (PLC), mitogen-activated protein kinase (MAPK), nuclear factor kappa B, and Janus kinase/signal transducer and activator of transcription (JAK/STAT) (*2*). Under certain pathological conditions such as in hyperproliferative diseases (*3, 4*), this balance is disturbed. Therefore, detailed characterization of the cellular differentiation programs that regulate the formation and homeostasis of the epidermis not only is important for understanding normal organ morphogenesis and homeostasis but also may lead to new therapeutic approaches to human proliferative diseases of the skin.

Cell-based phenotypic assays and, more recently, pathway screens of natural products and synthetic small molecules have provided useful chemical tools to modulate and/or study complex cellular processes both *in vitro* and *in vivo* (*5–10*). Despite extensive studies of keratinocyte growth and differentiation, only a relatively small number of endogenous (*e.g.*, epidermal growth factor (EGF) (*11*), transforming growth factor (TGF)-α (*11*), and extracellular calcium (*12*) levels) and synthetic small-molecule modulators (*e.g.*, staurosporine (*13*) and the antiseptic CCT002080 (*14*)) have been described to date. Herein, we report the identification of small molecules that induce differentiation of epidermal progenitor cells to terminally differentiated keratinocytes.

Primary normal human epidermal keratinocytes (NHEKs) were used as an experimental model system to study epithelial growth/differentiation. To screen small-molecule libraries for compounds that induce differentiation of keratinocytes, NHEKs (30−40% confluent) grown in KGM-2 medium were transiently transfected (transfection efficiency is ~25%) with an involucrin (IVL)-specific luciferase reporter, pGL3/3.7 kbp-IVL-Luc (*15*), and the expression of IVL (*16*) was measured. IVL is a keratinocyte differentiation marker expressed in the suprabasal epidermal layers. The reporter afforded a 4- to 17-fold increase in luciferase signal upon standard keratinocyte differentiation by either 1.2 mM $CaCl_2$, 100 ng/mL phorbol myristate acetate (PMA) (*17*), or a combination of $CaCl_2$ and PMA (relative to cells grown in 0.03 mM $CaCl_2$). For the primary compound screen, transiently trans-

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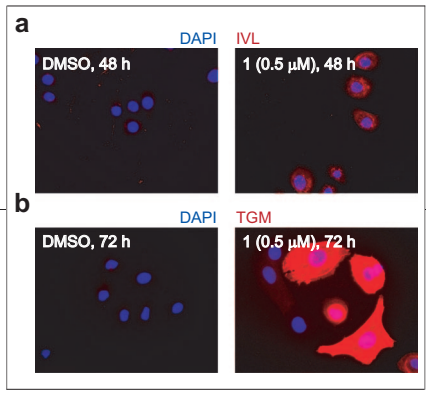

Figure 1. Induction of keratinocyte differentiation in NHEKs (40% confluent) by 1: a) immunofluorescence staining with anti-IVL (red) and DAPI (blue) after treatment with DMSO and 1 (0.5 μM) for 48 h; b) immunofluorescence staining with anti-TGM (red) and DAPI (blue) after treatment with DMSO and 1 (0.5 μM) for 72 h.

fected NHEKs were treated with 5 μM (final concentration) of ~13,000 compounds, including natural products, known drugs, and kinase-directed small-molecule libraries, which include phenylaminopyrimidines, trisubstituted purines, aminopyrazolopyrimidines, and phthalazines. After treatment with a compound for 48 h, luciferase activity was assayed.

Primary hits (88) were confirmed using the IVL-luc reporter, and compound efficacy was determined for a subset of structures (Supplementary Figure 1). To verify differentiation-promoting activity, selected compounds were characterized by direct immunostaining with keratinocyte differentiation markers and the observation of a differentiated morphology. Cells were treated with 1–5 μM of compound, fixed, and immunostained. A number of compounds afforded a strong IVL signal with mouse antihuman IVL antibody compared to DMSO-treated cells (Figure 1, panel a). At longer incubation times (up to 72 h) a high proporation of cells stained positively for transglutaminase (TGM) (*18*), a keratinocyte protein that catalyzes the cross-linking of structural proteins involved in the formation of

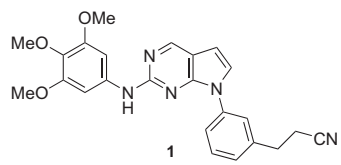

Figure 2. Structure of 2-(3,4,5-trimethoxyphenylamino)-pyrrolo[2,3-*d*]pyrimidine (1).

the cornified envelope during terminal differentiation (Figure 1, panel b). NHEKs treated with these compounds also exhibited an enlarged and flattened morphology similar to the cellular morphology observed in cells grown in 1.2 mM $CaCl_2$ at early time points (up to 12 h). However, compound-treated cells tended to be separate with increased IVL levels in a majority of cells, whereas cells grown in 1.2 mM $CaCl_2$ clustered tightly with separate groups of cells expressing IVL at later time points in a smaller percentage of cells (Supplementary Table 1).

These secondary assays identified a number of 7-aryl-substituted 2-(3,4,5-trimethoxyphenylamino)-pyrrolo[2,3-*d*]pyrimidines (*19*) (see Supporting Information) as the most potent inducers of NHEK differentiation (EC_{50} values of 0.1–5 μM). These pyrrolopyrimidines showed little cytotoxicity against BaF_3, U87, and A549 cells at the same concentrations and were therefore chosen for further study. A preliminary structure–activity relationship analysis revealed that the 3,4,5-trimethoxyphenylamino group is essential, while R can be a carboxylic acid, ester, amide, or nitrile without significant loss in activity (see Supplementary Figure 1). The most potent compound was **1** (Figure 2) with an EC_{50} value of 0.1 μM.

To confirm that the 7-aryl-substituted 2-(3,4,5-trimethoxyphenylamino)-pyrrolo [2,3-*d*]pyrimidines induce differentiation of epidermal progenitor cells to terminally differentiated keratinocytes, the time course for the expression of early- and late-stage keratinocyte differentiation markers was analyzed (Figure 3). NHEKs were treated with DMSO, 1.2 mM Ca^{2+}, or 0.5 μM **1**; incubated; and harvested for immunoblot analysis. Cells treated with 1.2 mM Ca^{2+}, a well-known inducer of keratinocyte differentiation, expressed IVL at 48 h as reported previously (*12*). Treatment of NHEKs with **1** led to an increase in the early differentiation markers keratins 1 and 10 at 48 h. Increased levels of IVL and TGM were observed in cells treated with the compound at 72 h and persisted at 96 h. In addition, NHEKs treated with the compound expressed loricrin, a terminal differentiation marker, at later time points. Similar results were observed by messenger RNA (mRNA) expression analysis of NHEKs treated with **1**. NHEKs were incubated with DMSO, 1.2 mM Ca^{2+}, or 0.5 μM **1** for 12, 24, 36, and 48 h; mRNAs were then isolated; and complementary RNAs (cRNAs) were then prepared, fragmented, and subsequently hybridized to Affymetrix HG_U113A GeneChip arrays. At early time points (12 and 24 h), treatment with **1** led to the upregulation of keratinocyte early differentiation marker genes, including keratin 1 (5.4-fold) and keratin 10 (5.4-fold). Terminal differentiation marker genes, including IVL (1.8-fold), TGM 1 (4.8-fold), loricrin (3.3-fold), and filaggrin (5.6-fold), were upregulated at late time points (36 and 48 h). These results are again consistent with the ability of **1** to induce differentiation of epidermal progenitor cells into terminally differentiated keratinocytes.

In addition, a pathway analysis of the genes whose expression was specifically altered by **1** with the extensive knowledge-based Ingenuity Systems database revealed that several members of the cell-cycle-related p38 MAPK signaling pathways were altered (36 genes out of 164 in the canonical pathway were differentially expressed at 48 h, see Supporting Information). Previous studies of keratinocyte differentiation have implicated p38δ as playing an important role in the differentiation process (*20*). Trends in the transcriptional analysis after treatment with **1** are similar, because mRNA levels of p38δ are 3.2- and 4.5-fold higher than DMSO-treated cells at the 36 and 48 h time points; moreover, there is no appreciable change in the low expression levels of p38α and p38β. In addition, several transcription factors downstream of p38 MAPK were also found to

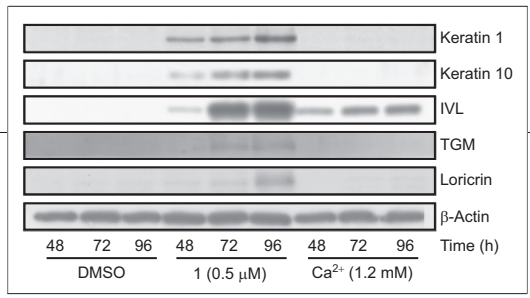

Figure 3. Western blot analysis of keratinocyte differentiation markers (keratin 1, keratin 10, IVL, TGM, and loricrin) in NHEKs (30% confluent) treated with DMSO, 1 (0.5 μM), or Ca^{2+} (1.2 mM) for 48–96 h.

change in expression levels, including the proto-oncogene c-myc. c-Myc expression at 48 h is 2.6-fold lower in cells treated with **1** compared to DMSO controls, in agreement with earlier work that shows reduction in c-myc mRNA levels upon terminal keratinocyte differentiation (*21*). Gene chip analysis also showed that several components of integrin-mediated signaling were downregulated during differentiation (15 genes out of 61 in the canonical pathway were differentially expressed at 48 h, see Supporting Information). This observation is in agreement with a literature report that shows high levels of β1 integrin family expression in epidermal stem cells (*22*). Further study is required to determine if **1** directly affects integrin signaling or whether reduced integrin levels are a byproduct of differentiation induced by **1**.

To further investigate the mechanism by which **1** induces keratinocyte differentiation, we attempted to identify the cellular targets of this compound by affinity chromatography ((*23*), Figure 4). The affinity matrix (**3**) was prepared by coupling **2** to Affi-Gel 10 by amide bond formation under mildly basic conditions. The affinity matrix was treated with NHEK cell extracts, and the proteins retained by the affinity matrix were separated by SDS-PAGE and visualized by silver staining. To identify those proteins that bind specifically to pyrrolopyrimidine **1**, an amide derivative of **2** (**4**, 120 μM final concentration) was added to the cell lysates during affinity chromatography. This compound effectively blocked the binding of 41 kDa and 45 kDa proteins to the affinity matrix (Figure 4, panel b); mass spectral analysis identified these two proteins as casein kinase 2, α′ subunit (CSNK2A2), and casein kinase 2, α subunit (CSNK2A1), respectively (see Supporting Information). This result was independently confirmed by Western blotting with anti-CSNK2A1 and anti-CSNK2A2 antibodies (Figure 4, panel c). An *in vitro* kinase assay using radiometric filter binding showed that the *N*-methyl amide derivative of **1** is a potent competitive inhibitor of ATP binding to casein kinase 2 (CK2) (IC_{50} = 9 nM).

Protein kinase CK2 is a ubiquitous and highly conserved serine/threonine kinase that is typically found in tetrameric complexes consisting of two catalytic (α and/or α′) subunits and two regulatory β subunits (*24*). CK2 has a number of physiological substrates and participates in a complex series of cellular functions, including cell cycle progression, control of cellular shape and architecture, apoptosis, regulation of transcription, and various steps of development (*24*). However, CK2 involvement in keratinocyte differentiation has not been previously demonstrated.

To confirm the involvement of CK2 in keratinocyte differentiation, CSNK2A1 and CSNK2A2 protein levels were individually knocked down with si*GENOME SMART*pool small interfering RNAs (siRNAs). NHEKs were transfected with siRNAs at a final concentration of 100 nM and harvested at 24, 48, 72, 96, and 120 h for immunoblot analysis. Semiquantitative reverse transcription polymerase chain reaction analysis indicated that the siRNAs caused a 72–87% reduction in CSNK2A1 and CSNK2A2 levels by 72 h, which persisted at 120 h after transfection; expression levels of CSNK2A1 and

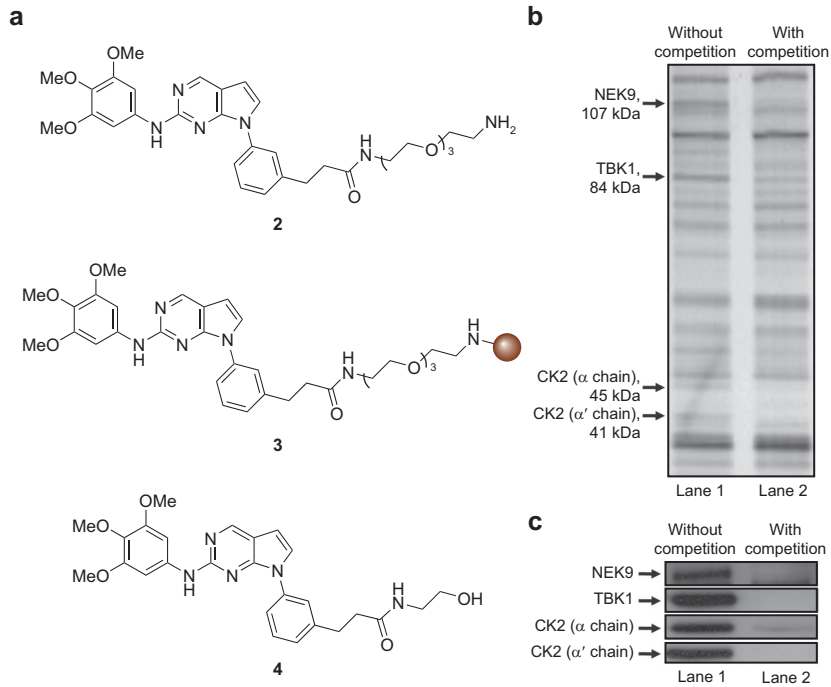

Figure 4. Affinity chromatography: a) structures of affinity matrix (3) and 2-(3,4,5-trimethoxyphenylamino)-pyrrolo[2,3-*d*]pyrimidine (4) used in a competition experiment; b) silver-stained SDS gel showing CSNK2A1 (CK2 (α chain)), CSNK2A2 (CK2 (α′ chain)), TBK1, and NEK9 proteins pulled down by 3 in an affinity chromatography experiment; c) confirmation of CSNK2A1, CSNK2A2, TBK1, and NEK9 in an affinity chromatography experiment by Western blot analysis.

CSNK2A2 were measured by Western blot analysis. The reduced expression level of CK2 caused by the siRNA resulted in a corresponding 2- to 2.3-fold increase in IVL levels, while the non-targeting siRNA-transfected cells showed little increase in IVL levels (see Supporting Information). However, reduction of CK2 *via* RNA interference (RNAi) did not result in an increase in TMG, loricrin, or keratin 1/10 protein levels (not shown). Therefore, it is likely that **1** has additional cellular targets and/or activities.

TANK-binding kinase 1 (TBK1) (*25*) and NIMA-related kinase 9 (NEK9) (*26*) also selectively bound the affinity matrix **3** (Figure 4, panels b and c). However, reduction in TBK1 and NEK9 protein levels by siRNAs had no effect on the expression of keratinocyte differentiation markers, including IVL (not shown). Additional studies are ongoing to identify other possible targets of compound **1** and to further characterize the full mechanism of action of compound **1**. In addition, the activity of **1** in *in vivo* models of skin hyperproliferative disease will be explored.

METHODS

Molecular Biology and Cell Culture. Plasmid pGL3/3.7 kbp-IVL-Luc plasmid was kindly provided by D. Bikle (University of California, San Francisco). NHEK cultures were purchased from Cambrex and expanded in the KGM-2 medium according to the method described in the manufacturer's protocol. Cells from the third passage were used in all experiments.

Transfection, Reporter Assay, and Compound Screens. For reporter gene assays with transiently transfected cells, the cells were typically transfected in 150 mm-diam dishes when 30–40% confluent. A reporter plasmid, pGL3/3.7 kbp-IVL-Luc plasmid, was transfected into the NHEKs using FuGENE 6 (Roche) following the manufacturer's protocol. Transfection efficiencies under these conditions were similar to those previously reported (*27*). After 24 h, the transfected cells were plated into 96-well assay plates and treated with compound to a final concentration of 5 μM. After incubation for 2 d, reporter gene activity was measured using the Bright-Glo luciferase assay system (Promega) and an Analyst AD system (Molecular Devices). The induction of luciferase activity of transfected NHEKs grown in 1.2 mM $CaCl_2$ was used as a positive control, while the activity of DMSO-treated NHEKs cultured in 0.03 mM $CaCl_2$ was used as a baseline measurement. Z-factors were calculated as described (*28*) using between 8 and 36 replicates per condition (1.2 mM $CaCl_2$ or DMSO).

Synthesis of 2-(3,4,5-Trimethoxyphenylamino)-pyrrolo[2,3-*d*]pyrimidines. Full experimental procedures and compound characterization data are provided (see Supporting Information).

Immunofluorescence. Cells in 96-well plates were washed with phosphate-buffered saline (PBS, pH 7.2), fixed with 4% paraformaldehyde in PBS for 20 min, rinsed, permeabilized with 0.2% Triton X-100 for 20 min, and blocked with 5% donkey serum (Jackson ImmunoResearch) in PBS for 30 min. For immunostains, the following antibodies were used: mouse monoclonal antihuman IVL (1:100, Sigma), mouse monoclonal antihuman TGM (1:50, Biomedical Technologies), and Cy3-conjugated antimouse (1:1000, Jackson ImmunoResearch).

Immunoblotting. Cells were washed with ice-cold PBS and lysed with RIPA buffer (Upstate) containing protease inhibitor cocktail (Sigma). Total protein concentration in the supernatant was determined with a BCA protein assay kit (Pierce Biotechnology), and samples were loaded and separated on a Criterion XT Bis-Tris Gel, 4–12% (Bio-Rad). For Western blot analysis, samples were electroblotted onto a nitrocellulose membrane and the membrane was blocked at RT for 1 h with 5% nonfat milk in Tris-buffered saline with 0.1% Tween-20 (TBST) and then incubated at 4 °C overnight with the following antibodies: antihuman keratin 1 (1:1000, Covance), anticytokeratin (1:100, Covance), anti-IVL (1:5000, Sigma), anti-TGM (1:100, Biomedical Technologies), antiloricrin (1:1000, Covance), and antiactin (1:1000, Santa Cruz Biotechnology) antibodies diluted in 1.5% bovine serum albumin (BSA) in TBST. The membrane was washed three times for 5 min each with TBST, incubated for 1 h at RT with horseradish peroxidase (HRP)-conjugated secondary antibody (1:2000, Sigma), washed three times for 5 min each with TBST and once for 5 min with TBS, and visualized by ECL Western Blotting Substrate (Pierce Biotechnology).

Microarray Gene Expression Analysis. NHEKs were treated with DMSO, $CaCl_2$ (1.2 mM), or **1** (0.5 μM) and harvested at 12, 24, 36, and 48 h. Total RNA from each sample was isolated and purified with the RNeasy Mini Kit (Qiagen). Total RNA was examined on an Agilent Bioanalyzer, and the 28S/18S ratio exceeded 2.0 for all samples. Five micrograms total RNA was used to synthesize cRNA, which was fragmented and hybridized to HG_U133A GeneChip arrays (Affymetrix, Santa Clara, CA) according to standard Affymetrix protocols (Affymetrix Expression Analysis Technical Manual, www.affymetrix.com/support/technical/manuals.affx). Duplicate amplifications/hybridizations were performed from the same total RNA. Arrays were washed and stained with standard Affymetrix reagents with a custom fluidics machine (GNF). Arrays were scanned with an Affymetrix scanner (model GCS3000). Probes were condensed from CEL files with gcRMA (Wu *et al*., 2004, www.bepress.com/jhubiostat/paper1). Differential expression cutoffs were taken prior to input to Ingenuity Pathways Analysis (IPA) software (Ingenuity Systems, Redwood City, CA). Expression cutoffs were (i) fold change values for replicate averages of ±2, (ii) expression values for the average of one group across the comparison needed to exceed twice the median intensity value across the entire array, and (iii) coefficient of variation of the replicates needed to be <0.8 in both groups being compared. All differential expression measurements were relative to a DMSO control at the same time point. Lists of genes and raw data will be deposited in the Gene Expression Omnibus database (www.ncbi.nlm.nih.gov/geo). Overlap of the lists with canonical pathways in IPA was examined and displayed for integrin and MAPK signaling pathways.

Affinity Matrix Synthesis and Chromatography. Syntheses of the linker derivative (**2**) and the affinity matrix (**3**) are described (see Supporting Information). NHEKs were cultured in the KGM-2 medium, harvested by centrifugation (200*g*, 5 min), washed with ice-cold PBS, and lysed with homogenization buffer (60 mM β-glycerophosphate, 15 mM *p*-nitrophenyl phosphate, 25 mM MOPS (pH 7.2), 15 mM EGTA, 15 mM $MgCl_2$, 1 mM DTT, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 mM phenyl phosphate, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 10 μg/mL soybean trypsin inhibitor, 100 μM benzamidine, and 0.5% Nonidet P-40). Cell lysates were centrifuged at 20,000*g* for 20 min at 4 °C (Eppendorf), and the supernatant was collected. The total protein concentration in the supernatant was determined by using a BCA protein assay kit (Pierce Biotechnology). Thirty microliters of packed affinity matrix (**3**) was washed twice with bead buffer (50 mM Tris (pH 7.4), 5 mM sodium fluoride, 250 mM sodium chloride, 5 mM EDTA, 5 mM EGTA, 0.1% Nonidet P-40, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 10 μg/mL soybean trypsin inhibitor, and 100 μM benzamidine). Protein extracts (1 mg), which were preincubated with ethanolamine-capped control resin at 4 °C for 1.5 h, were added to affinity matrix, and bead buffer was added up to final volume of 1 mL (for the competition experiment, **4** was added to a final concentration of 120 μM). After rotation at 4 °C for 1 h, the affinity matrix was centrifuged at 20,000*g* for 1 min at 4 °C (Eppendorf). The supernatant was removed by aspiration, and the affinity matrix was washed six times with ice-cold bead buffer. After the final wash, the supernatant was removed, and Laemmli sample buffer was added to the resin and heated at 95 °C for 3 min. Samples were loaded and separated on a Criterion XT Bis-Tris Gel, 4–12% (Bio-Rad), and silver staining was used to visualize protein bands. For Western blot analysis, samples were electroblotted onto a nitrocellulose membrane and the membrane was blocked at RT for 1 h with 5% nonfat milk in TBST and then incubated at 4 °C overnight with rabbit anti-CSNK2A1 (1:1000, Upstate) and anti-CSNK2A2 (1:5000, Bethyl Laboratories) antibodies diluted in 5% BSA in TBST. The membrane was washed three times for 5 min each with TBST, incubated for 45 min at RT with HRP-conjugated secondary antibody (1:4000, Bio-Rad), washed three times for 5 min each with TBST and

once for 5 min with TBS, and visualized by ECL Plus Western Blotting Detection Kit (Amersham Biosciences).

LC-MS/MS Analysis. Each gel band was destained (1:1 mixure of 30 mM $K_3Fe(CN)_6$ and 100 mM $Na_2S_2O_3$), dehydrated (acetonitrile), reduced (10 mM DTT), alkylated (55 mM iodoacetamide), and digested with sequencing grade trypsin (6 μg/mL) overnight (*29*). The resulting peptides were extracted with 30 μL of 1% formic acid, and automated nanoscale LC-MS/MS was performed using a ThermoFinnigan Surveyor HPLC and LCQ XP+ ion trap mass spectrometer along with a variation of the "vented column" approach described by Licklider (*30*). Twenty micrograms of a tryptic digest extract was loaded onto a 5 cm long × 75 μm i.d. precolumn packed with 5 μm C-18 silica (Monitor 100 Å) retained by a Kaisel frit. After being washed thoroughly, the vent was closed and the sample was transferred to a 12 cm long × 75 μm i.d. column with a pulled 5 μm tip packed with the same material by starting the reversed-phase run. The chromatographic profile was from 100% solvent A (0.1% acetic acid) to 50% solvent B (0.1% acetic acid in acetonitrile) in 30 min at ~200 nL/min (manual split from 300 μL/min) as well as appropriate times for column washing and reequilibration. An angiotensin I blank was run between each sample to eliminate carry-over as well as monitor overall system performance. The LCQ acquisition method involved one MS precursor ion scan followed by three data-dependent MS/MS scans. Tandem MS data were used to search the entire human database with no enzyme specification using SEQUEST. Each protein listed was identified by a minimum of five unique, fully tryptic peptides, and each tandem MS spectrum was manually validated. The list of peptides detected for each protein and a representative tandem MS spectrum for each protein identified can be found (see Supporting Information).

RNAi Experiments. Pooled synthetic siRNA duplexes (si*GENOME SMART*pool reagent) specific for CSNK2A1 (M-003475-00) and CSNK2A2 (M-004752-00) and nontargeting siRNA pool (D-001206-13-05) were purchased from Dharmacon. For siRNA transfection, NHEKs were seeded into six-well tissue culture plates at a density of 1×10^5 cells/well 24 h before transfection. Cells were transfected with siRNAs at a final concentration of 100 nM using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol. Cells were harvested at 24, 48, 72, 96, and 120 h after transfection and lysed in RIPA buffer for immunoblot analysis. For Western blots, the following antibodies were used: mouse monoclonal antihuman IVL (1:2000, Sigma), rabbit anti-CSNK2A1 (1:1000, Upstate), rabbit anti-CSNK2A2 (1:5000, Bethyl Laboratories), and appropriate HRP-conjugated secondary antibodies (1:4000).

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Supporting Information Available: This material is available free of charge *via* the internet.

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