Online Methods

Tissue culture

Primary human keratinocytes were derived from fresh foreskin. Cells were grown in KSF-M (GIBCO-BRL) supplemented with epidermal growth factor (EGF) and bovine pituitary extract (BPE). Cells were induced to differentiate by the addition of 1.2 mM calcium for 1 and 3 days in full confluence. Amphotrophic phoenix cells were maintained in DMEM and 10% fetal bovine serum. Primary human myoblasts were purchased from Cell Applications and maintained in muscle growth medium (Cell Applications: 151-500). Myoblasts were induced to differentiate in muscle differentiation medium (Cell Applications: 151D-250) for 24 or 72 hours.

Gene transfer

Amphotrophic phoenix cells were transfected with 3 ug of each retroviral expression construct. Transfections were done in 6 well plates using Lipofectamine 2000 (Invitrogen). Viral supernatants were collected 48 hours post transfection and polybrene added (5ug/ml). These supernatants were placed on primary human keratinocytes and centrifuged for 1 hour. Cells were transduced 2 times and selected using puromycin (1ug/ml) after the last transduction.

Retroviral constructs

ShRNA retroviral constructs were generated by cloning oligos into the pSuper Retro vector. The oligo sequences targeting DNMT1, UHRF1, and GADD45A are as follows: DNMT1i (A): GGAACCAAGCAAGAAGTGA; DNMT1i (B): GGAAGTGAATGGACGTCTA; UHRF1i(A): GGTCAATGAGTACGTCGAT; UHRF1i (B): GAGGACCGCGGAACAGTCT; GADD45Ai(A): GGCTGAGTGAGTTCAACTA; GADD45Ai(B): GCTGGTGACGAATCCACAT . The shRNA sequence targeting the 3'UTR of DNMT1 is: GTATGAGTGGAAATTAAGA. The control shRNA used was a nonfunctional sequence targeting DNMT1. The control sequence is GCTTCAATTCGCGCACCTA. Functional shRNA constructs targeting GADD45B were purchased from Openbiosystems with the following catalog numbers: RHS4917-99748642 and RHS4917-99753120. Constructs for overexpressing GADD45A and GADD45B were made by cloning the GADD45A and GADD45B ORF into the LZRS retroviral vector. The cloning primers used for GADD45A and GADD45B were: GADD45A for: ACGCAAAGCTTGCCACCATGACTTTGGAGGAATTCTCGGCTGGAGAGC; GADD45A rev: ACGCAGCGGCCGCTCACCGTTCAGGGAGATTAATCACTGGAACC; GADD45B for: ACGCAAAGCTTGCCACCATGACGCTGGAAGAGCTCGTGGCGTGCGACA; GADD45B rev: ACGCAGCGGCCGCTCAGCGTTCCTGAAGAGAGATGTAGGGGA.The reporter construct HAK14
was cloned by fusing 3x HA tag to keratin 14 and cloned into the LZRS retroviral vector. The

wt DNMT1 construct was generated by purchasing the full length cDNA from Openbiosystems (MHS1768-98980929) and cloning into the LZRS retroviral vector. The catalytically inactive DNMT1 mutant construct was generated by using the Stratagene QuikChange mutagenesis kit using the following primers: 5' DNMT mut A: gctgtgcggcgggccgccc AGC cagggcttcagcggcatg and 3' DNMT mutA: catgccgctgaagccctg GCT gggcggcccgccgcacagc.

Western blotting and immunofluorescence

40 ug of the cell lysates were used for immunoblotting and resolved on 10% SDS-PAGE and transferred to PVDF membranes. Membranes were incubated in primary and secondary antibodies for 1 hour each. Primary antibodies used include B-actin (Sigma:A2228) at 1:2500, DNMT1 (BD Pharmingen:612618) at 1:500, UHRF1 (Abcam: ab46187) at 1:100 and Involucrin (Sigma:I9018) at 1:250. Sheep anti mouse or rabbit HRP (Amersham Biosciences) secondary antibodies were used at 1:4000. For immunofluorescence experiments, 7 _m thick epidermal sections from adult human skin or xenografts were fixed in 4% paraformaldehyde for 15 minutes followed by blocking in PBS with 2.5% normal goat serum, 0.3% triton X100, and 2% bovine serum albumin for 30 minutes. Sections were incubated in primary antibodies for 1 hour. Primary antibodies include Keratin 1 (Covance:PRB-149P) at 1:2000, human specific DSG3 (Zymed:32-6300) at 1:350, HA (Covance: MMS-101P) at 1:300, Keratin 10 (Neomarkers: MS-611-P) at 1:350, Ki67 (Neomarkers: RM-9106-S) at 1:200, p63(Santa Cruz: SC-8431) at 1:200, and DNMT1 (BD Pharmingen:612618) at 1:200. The secondary antibodies used were Alexa 555 conjugated goat anti-mouse IgG (Molecular Probes) (1:1000), and Alexa 488 conjugated goat anti-rabbit IgG(Molecular Probes)(1:1000). Nuclear dye, Hoechst 33342, was used at 1:1000(Molecular Probes).

Grafts of regenerated human skin onto immune deficient mice

For xenografts, 1 million genetically modified keratinocytes were seeded onto devitalized human dermis for 48 hours and then grafted onto female 10-12 week old CB17 *scid/scid* mice for up to 3 weeks. 3 animals were used for each time point per experimental group.

In vivo epidermal progenitor cell tracking system

Marked epidermal cells were generated by transducing the cells with the HA tagged retroviral constructs. Cells were then transduced with retroviral constructs to knockdown DNMT1 or control. Transduced cells were selected for knockdown constructs using puromycin. These modified cell were mixed at a 50:50 ratio with nonmarked cells and grafted onto immune deficient mice to regenerate human epidermis.

Quantitative reverse transcriptase-PCR analysis

Total RNA from cells was extracted using the Rneasy mini kit (Qiagen) and quantified by Nanodrop. One ug of total RNA was reverse transcribed using the 1st Strand cDNA Synthesis Kit

for RT-PCR from Roche. Quantitative PCR was performed using the Mx3000P (Stratagene) thermocycler. Samples were run in triplicate and normalized to GAPDH. Primer sequences for GAPDH, S100A8, IVL, and SPRR3 were the same as previously published1. Sequences for P15, P16, UHRF1, POU2F3, DNMT1, S100P, LCE3D, GADD45A, and GADD45B are as follows: P15 for: AAAGCCCGGAGCTAACGAC, P15 rev: CTCCTCGGCCAAGTCCAC; P16 for: CCCAACGCACCGAATAGT, P16 rev: GCAGCACCACCAGCGTGTC; UHRF1 for: GTGGGATGAGACGGAATTG, UHRF1 rev: CGGGTAGTCGTCGTATTTC; POU2F3 for: CAGACCACCATCTCACGAT, POU2F3 rev: GGATGTTGGTCTCGATGC; DNMT1 for: CTGAGGCCTTCACGTTCA, DNMT1 rev: CTCGCTGGAGTGGACTTGT; S100P for: GATGGAGAAGGAGCTACC, S100P rev: CTCTGCCAGGAATCTGT; LCE3D for: GCTGCTTCCTGAACCAC, LCE3D rev: GGGAACTCATGCATCAAG; GADD45A for: AGCAGAAGACCGAAAGGA, GADD45A rev: GCCACATCTCTGTCGTCGT; GADD45B for: TCAACATCGTGCGGGTGTC, GADD45B rev: CCCGGCTTTCTTCGCAGTA. The following primer sequences for muscle differentiation genes were used: TPM1 for: GCTGAGAAGTACTCGCAG, TPM1 rev: ATCGTTGAGAGCGTGGT: MYH8 for: GTCAAGGTTGGCAATGAG, MYH8 rev: CAAACATGTGGTGGTTGAA; ACTA1 for: CCTGGAAAAGAGCTACGA, ACTA1 rev: GGCGATGATCTTGATCTTC

Gene expression profiling

Microarray analysis was performed on biological duplicate samples. Labeling of cDNA and hybridization to Affymetrix HG-U133 2.0 plus arrays were performed at Stanford's P.A.N. facility. For gene expression analysis, arrays were RMA normalized and differential expression was defined using the following filters: Significance Analysis of Microarrays 3.02 with a false discovery rate less than 5%, an average fold change _ 2 in any group, and an average raw expression intensity _ 100 in any group. Hierarchical clustering and heat map generation were performed using GeneSpring GX software (Agilent). For future analyses, Affymetrix probe IDs were converted to unique Entrez IDs. P-values indicating the significance of the overlap between various gene sets were calculated using Fisher's Exact test. GO term enrichment was performed using DAVID with the total set of genes on the appropriate microarray as the background (full genome for the MeDIP profiling), p-values represent a Bonferroni-corrected modified Fisher's Exact test3.

Apoptosis assays

Apoptotic assays in cultured cells were performed by using the Annexin V-FITC apoptosis detection kit (BD Pharmingen: 556547). Assays were performed according to manufacturers protocol using the FACSCalibur (Becton Dickinson) flow cytometer. Epidermal tissue was stained for apoptosis using the In Situ Cell Death Detection kit (Roche: 12156792910) according to

manufacturers protocol.

Cell cycle analysis

Cell cycle profiles were determined by analyzing propidium iodide stained DNA using flow cytometry as previously described4.

Clonogenic assays

For clonogenic assays with primary human keratinocytes, 100 cells were plated onto mitomycin C treated (15ug/ml) 3T3 feeder cells in a 30 mm Petri dish. Colonies were fixed with cold methanol/acetone, stained using crystal violet (Fisher Scientific: S71262-1) and counted 2 weeks after plating.

Methylated DNA immunoprecipitation (MeDIP) and DNA methylation profiling

Genomic DNA was isolated from cells using the DNeasy kit (Qiagen). The DNA was sonicated to produce fragments ranging between 300 to 1,000 bp and subject to immunoprecipitation using a monoclonal antibody against 5-methylcytidine (Eurogentec) according to published protocols5,6. DNA enriched for 5-methylcytidine was subject to amplification using the Whole Genome Amplification Kit (Sigma) according to manufacturer's protocol. Amplified DNA was labeled and hybridized to NimbleGen's HG18 tiled promoter arrays which includes all known RefSeq genes, annotated splice variants, the mammalian gene collection and UCSC known genes. On average the promoters were tiled ~3500 bp upstream and ~750bp downstream of the gene. The median probe spacing is ~100bp with probe length being 50-75 nucleotides. Labeling, hybridization and data analysis were all performed by NimbleGen using standard protocols. Significantly methylated peak regions were identified by NimbleScan software using default parameters for the one-sided Kolmogorov-Smirnov (KS) test. Details of the software and peak calling algorithm are available on the NimbleGen website (http://www.nimblegen.com/products/software/). NimbleScan was also used to map the called peaks to unique RefSeq IDs. To determine methylated regions specific to the undifferentiated state, we used Galaxy software7 (http://www.g2.bx.psu.edu) to identify peaks that overlap between undifferentiated (-Ca2+) and differentiated (+Ca2+) conditions.

This overlapping set was then subtracted from the total set of peaks identified in the -Ca2+ sample. To identify genes that may be inhibited by methylation in -Ca2+ conditions, we overlapped

genes containing -Ca2+-specific methylation with those determined to be transcriptionally induced during differentiation.

MeDIP Data Visualization

Genomic coordinates in which significantly methylated peak regions in undifferentiated (-Ca2+) and differentiated (+Ca2+) conditions were submitted to the UCSC Genome Browser as custom

tracks and visualized relative to annotated tracks of CpG Islands and RefSeq genes. The resulting visualizations of selected genes were adapted to include regions tiled on the Nimblegen Promoter Tiling Array within an average viewing window of -3500bp to +1000bp relative to the gene transcription start site.

Quantitative PCR on MeDIP samples

Real time PCR was carried out using 50 ng of input as well as amplified MeDIP samples. For QPCR detection, the SYBR Green PCR master mix (Invitrogen) was used with the Mx3000P (Stratagene) machine. To determine the relative enrichment of target sequences, the ratio of the signals in the MeDIP samples were compared to input DNA. The resulting values were standardized against the input, which was given a value of 1. The primers used for the promoter regions are as follows: mS100P for: CCAGCCAAGGAACGGACC, mS100P rev: CAGCCCTGGATTGCATTC; mKCNQ1 for: GGCCTGGATTTACTCAG, mKCNQ1 rev: CCCTTCCTCTGCCTATCT; mLCE3D FOR: CTGAAGCTAGGTTTGAAC, mLCE3D REV: GGGTTTCAGCACTGAATA; mPOU2f3 for: CTGGGGCAGAGGCGAGG, and mPOU2f3 rev: AACCCGCTATCCACACCG.

Bisulfite Sequencing

DNA was isolated from cells using Qiagen's DNAeasy kit. 1 ug of DNA from each sample was treated with bisulfite using the EpiTect Bisulfite kit (Qiagen). Region 95-489 of the S100P gene was amplified using the following primers : S100P for: GAAGTTGGGTTTTTGAAGGATATAG; S100P rev: CCCAAAATATAAACTACCTCCAAAC.

CpG island analysis

To determine the presence of CpG islands upstream of 232 genes exhibiting both -Ca2+ specific methylation and induction during differentiation, we used Galaxy to (1) convert the Gene Symbols into corresponding RefSeq IDs, (2) determine the transcription start site (TSS) for each RefSeq ID, and (3) find CpG island annotations8 from the UCSC Table Browser9 within 2kb upstream of the TSS. Finally, we also used Galaxy to determine if any of the 256 unique MeDIP peaks identified in basal growth conditions (corresponding to the 232 genes) overlapped with the annotated chromosomal positions of any of the 28,226 annotated CpG islands in the genome. Note that 19 genes in Supplementary Table 7 have CpG islands that do not fall 2kB upstream of TSS, but do overlap with identified methylation peaks. For each of these analyses, we utilized the March 2006 human reference sequence (NCBI Build 36.1).

References for online methods

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