Supplemental information

Materials and Methods

Generation and genotyping of conditional l MED1 null mice: To delete the expression of MED1 from keratinocytes, floxed MED1 null mice (previously named as floxed PBP) were mated with transgenic mice expressing Cre recombinase under the control of the K14 promoter (The Jackson Laboratory, C57/BL6 background). Heterozygous floxed MED1 were interbred to produce homozygous floxed mice. The whole skins were excised from null mice and control littermates. They were incubated with dispase (1 mg/ml) (BD Bioscience) overnight at 4°C, and the epidermis was separated from dermis. Genomic DNA was purified from whole skin, epidermis, dermis and other tissues excised. The excision of exons 8-10 was detected by PCR analysis of genomic DNAs isolated from tissues specified with primers designed from exon 7 (forward) and exon 11 (reverse) (see supplement Table SII for primer sequences), which amplified a ~1.5kb fragment from the MED1 gene allele. Mouse genotypes were determined by PCR of genomic DNAs from tail ships with primers for the Cre transgene which amplified a ~500 bp cDNA, and same primer set to span exons 8-10 to detect recombination of the MED1. All mice are maintained under standard conditions of protocols approved by the Animal Care Subcommittee, San Francisco Department of Veterans Affairs Medical Center.

Antibodies; Antibodies are used as follows; anti-K14 (PRB-155P-100, Covance. Berkeley, CA), anti-Ha1/KRT31 (GP-hHa1, Progen biotechnik, Germany), and anti-PCNA (93-1143, Invitrogen, Carlsbad, CA), anti-MED1 (TRAP220 C-19, Santa Cruz Biotechnology, Santa Cruz, CA), anti-K1 (Covance), anti-FLG (PRB-417P, Covance). Secondary antibodies of Alexa 594 (red) labeled anti rabbit IgG (goat), Alexa 488-(green) anti Guinea pig, horseradish peroxidase-conjugated anti rabbit IgG (GE Healthcare) were used.

Protein analysis by Western blot; Epidermis was separated from the whole skin by dispase treatment as mentioned above, and keratinocytes were isolated from epidermis by trypsinization
(0.25% trypsin, 37 °C, 1h). Nuclear extracts and cytoplasmic fractions were prepared by using NE-PER Nuclear Protein Extraction Kit (Pierce). MED1 protein was detected by western analysis as described (Oda et al., 2003; Tu et al., 2008). Briefly, 20 µg of nuclear extracts or cytoplasmic fractions were electrophoresed through SDS-PAGE and electroblotted onto a PVDF membrane. After blocking, the blots were incubated with antibodies raised against MED1 (TRAP220 C-19 Santa Cruz Biotechnology)(1:1000). Subsequently, the blot was incubated with secondary antibody conjugated with HRP (Amersham), and bound antibody was visualized using a chemiluminescence system (SuperSignal ULTRA) (Pierce) by exposing X-ray film.

**Histological analysis and K1/FLG immunostaining:** The lower portion of the dorsal skin was excised from the MED1 null and their littermate control mice, fixed with 4% paraformaldehyde overnight. The skin was embedded in paraffin, and cut into 5µm thick sections. H&E staining was performed as described previously (Bikle et al., 2006). Morphology of HF and IFE was evaluated in approximately 1cm lengths of dorsal skin of KO and CON (n=3) at different ages of mice, and reproducibility was confirmed by using at least two litters of mice for these analyses.

The K1 and FLG were detected by immuno-staining. After deparaffinization, antigen retrieval was performed using unmasking Solution (Vector Laboratories #H-3300), and the section was blocked by Block solution (sc-2051, Santa Cruz Biotechnology), and quenched with 3% H2O2 in methanol. The sections were then incubated with primary antibodies against K1 (1:1000) and FLG (1:500), and signals were detected by using InnunoCruz rabbit™ LSAB Staining System (sc-2051, Santa Cruz Biotechnology). They are counterstained by hematoxylin.

**Evaluation of keratinocyte proliferation:** Proliferative keratinocytes were quantitatively evaluated by both PCNA staining and epidermal thickness. The proliferating keratinocytes was detected by using PCNA staining kit (93-1143, Invitrogen, Carlsbad, CA) in MED1 null dorsal skin and equivalent skin of littermate control mice (n=3 each) at various ages. The number of PCNA positive cells was counted by image analysis software Bioquant (Nashville, TN)
(Supplement Fig. S1) and normalized by the length of the skin, and expressed as PCNA positive cells per mm. Epidermal thickness was also measured by by using Bioquant (Supplement Fig. S1), and average thickness (µm) within 0.5-1cm length of dorsal skin was calculated.

**Immunodetection of the MED1;** The MED1 expression in the skin was detected by immunostaining. The paraffin embedded section was pretreated with trypsin (Digest-AllZ™ Kit, Zymed Laboratorix Inc, South San Francisco, CA) for antigen retrieval, quenched by 3% H2O2/methanol, and blocked by Avidin/biotin blocking kit (SP-2001, Vector, Lab). The sections were incubated with primary antibody for MED1 (TRAP220 C-19 1:250), and signals were detected by Vectastain ABC kit (Vector Laboratories, Burlingame, CA). Sections were counterstained with hematoxylin.

**Hair keratin Immunofluorescence:** Paraffin embedded skin sections were incubated with trypsin (Digest-AllZ™ Kit, Zymed Inc), and blocked with PBS containing 1% BSA, 5% goat serum, 0.01% Tween 20. Sections were incubated with primary antibodies against Ha1 (Progen, made in guinea pig 1:1000) and K14 (Covance, made in rabbit 1:4000), and subsequently incubated with secondary antibodies, Alexa 594 (red) conjugated anti rabbit IgG and Alexa 488 (green) conjugated anti guinea pig IgG (10 mg/ml). Sections were counterstained with DAPI, and observed under fluorescence microscopy (Carl Zeiss Axioskop 40 FL Microscopes) and composite images of three colors, Ha1 green, K14 red, DAPI bue, were prepared by multidimensional analysis by using Axioskop software.

**Measurement of mRNA expression by RT-QPCR** Total RNAs were isolated from whole skin excised from lower dorsal part of the null mice and from equivalent skin from their littermate control mice using STAT 60 (Invitrogen) and cleaned up with RNeasy Mini RNA purification kit (74134, Qiagen Valencia CA). cDNA synthesis was performed using TaqMan Reverse Transcription Reagents kit using 50µM random hexamer provided (Applied Biosystems, N808-0127, Foster City, CA). Real-time quantitative PCR was performed using the synthesized cDNA
as a template in a 20µl reaction mixture using Power SYBR Green (Applied Biosystems, 1102115) with the 7300 Real Time PCR system (Applied Biosystems). Relative mRNA levels compared to the control gens GAPDH was determined by the ΔΔCt method. Primers for QPCR analysis were designed using Primer Express software (Applied Biosystem) to span the exon and intron boundaries, or were derived from Primer bank. Primer sets were verified by drawing dissociation curves. Primer sequences for QPCR are available in supplement Table SII. These experiments were repeated in more than two litters of knockout mice (n=3 or more in each litter) to confirm reproducibility.

**Statistical analysis** Values are expressed as the average ± standard deviation (SD). Unpaired Student t test was performed for determination of p-values using Excel or Graphpad Prism software (Graphpad Software). In case the p-values were smaller than 0.05 or 0.01, differences were considered to be statistically significant.
Supplement Table SI

Concentrations of serum calcium and calcium panels of the MED1 null (KO) and littermate control (CON) at 9 week-old mice. Average±standard deviation (SD), and statistical significance are shown (n=4-5), ALP; alkaline phosphatase; Bun, blood urea nitrogen.

| Genotype | Albumin (g/dL) | ALP (U/L) | Bun (mg/dL) | Calcium (mg/dL) | Creatinine (mg/dL) | Magnesium (mg/dL) | Phosphate (mg/dL) |
|----------|---------------|-----------|-------------|-----------------|-------------------|------------------|------------------|
| CON      | 2.40±0.16     | 103.4±22.1| 25.0±3.24   | 7.72±0.40       | 0.24±0.05         | 3.02±0.34        | 10.7±1.28        |
| KO       | 2.50±0.08     | 126.3±32.1| 23.8±2.98   | 7.70±0.68       | 0.23±0.05         | 3.30±0.85        | 11.8±3.73        |
| p-value  | 0.15          | 0.12      | 0.29        | 0.45            | 0.34              | 0.28             | 0.28             |

Supplement Table SII. The primers used for genotyping and QRT-PCR analyses

| Gene        | Forward Primer (5'–3') | Reverse Primer (5'–3') | Purpose       |
|-------------|------------------------|------------------------|---------------|
| GAPDH       | ACCTGCCAAGTATGATGACATCA| GGTCTCTGTTAGGCAAGAGAT | qRT-PCR       |
| PADI1       | TGTGGCCTGTGTTGAGTGT    | TCGAGGAGTCTGAGCATG    | qRT-PCR       |
| PADI3       | AGGATTGTTACGGTATCTCGGA| GGTGTCCTAGCATGCTCAA   | qRT-PCR       |
| S100A       | CAGTATCGCATCTTGTTG     | TACTCCCAAAAGGCTACCT   | qRT-PCR       |
| Tubb3       | TGGACCTGTTGGCTCTGG     | CTCCTGATATAGGCCCTTTG | qRT-PCR       |
| Dlx3        | TGGCTCAAGTGGAAATATCC  | TCCACCAGCAACCTCTCG   | qRT-PCR       |
| Knt15       | AGCTATGTGGAGGAAAACGT   | GGTCTCTACAGGCTACCT   | qRT-PCR       |
| TGFβ1       | CCACACACCCCATCTATG     | CTCTGCAAGGCAAGCAAT   | qRT-PCR       |
| TGFβ-1      | CTCTCCAGACATCGTCTTG   | ACAAAGCCCAATACATGAC  | qRT-PCR       |
| TGFβ-2      | TTGGATGCGTCTCCTACCA   | GCAAGACGACCTACATGAC  | qRT-PCR       |
| FGFR2       | GTATTCTCTAGTACGCGACA  | GACAGACAGGTTCTAAAGCAT| qRT-PCR       |
| FGFR5       | CAGCTATAATCTATGCTCCTG | GTTGGGGTTGACCCGGTG   | qRT-PCR       |
| Shh         | TGGAAAGAGTTGTCGACTTG  | CTCCGGGAACTGCTCTTCA  | qRT-PCR       |
| Patched     | GCCCAAGCCCTTAAACAAAT  | ACCCACAACTAATCTTC   | qRT-PCR       |
| Patched 2   | CTCCTTATGTTTGATTCCAGCTG| GTGACTCTGGAACCTTACTGCA| qRT-PCR       |
| Gli1        | CCAAGGCAACTTCTGACGGAG| ACGCAGCTCTGTGTAATGTA| qRT-PCR       |
| Gli2        | CAACGCCCTACTCTCCCAGAC | GACCTGTAGTGTGTTACCC  | qRT-PCR       |
| BMP1a       | TGCACCTGTATCTGAACTGAC| CAAGGTATCTTGAGTCTAAAG| qRT-PCR       |
| BMP2        | GGGACCCCGCTCTTCTAAGT  | TCAACTCAATGTGTCAGGAC | qRT-PCR       |
| BMP4        | GAGGAGATTTACGGCGTCC   | GTTGAGAAGAGAAGAAAAAGCAG| qRT-PCR       |
| Notch1      | CCCCCCTCTCTCGACTAAGCC | GAGTCTCTGCGACTTGTTG  | qRT-PCR       |
| Lef1        | TGTCTTACTCCTCACGCCCTT| CATGGAATGTCGCCCTGAC  | qRT-PCR       |
| H1          | GGTGCGATAGATTATACTGCAAG| AGCTACAGATCATCTCCGC| qRT-PCR       |
| H2          | GGCCTCAATGAGAATTCCTGAA| GCATCTCTGGTTAGGCAAGAG| qRT-PCR       |
| Kn2-16      | GGTAAGTGCAAGCATCAAC   | ATCACAGATCCAAAGGCCCAT| qRT-PCR       |
| MED1        | TCCATCTGATCTGGATGATAA | GGGTGTGACCCCATATT    | PCR (Genotyping) |
Supplement Table SIII
Keratinocyte proliferation in IFE evaluated by PCNA staining.

PCNA positive cells were counted and normalized by length of the skin of MED 1 null (KO) and their littermate control (WT) mice at P17, P25 and 10 weeks (n=3). Number of PCNA positive cells (#PCNA) and length of the skin (µm) were measured by Bioquant. PCNA positive cells were normalized by length of the skin and expressed as PCNA per mm skin (PCNA/mm). Average (Ave) and standard deviation (SD) of each group are shown. Statistical significance between control and null skins was evaluated by student t-test and p-values are shown.

| Age  | Genotype | Length (µm) | #PCNA | PCNA/mm | Genotype | Length (µm) | #PCNA | PCNA/mm | p-value |
|------|----------|-------------|-------|---------|----------|-------------|-------|---------|---------|
| P17  | WT1      | 5347        | 46    | 8.6     | KO1      | 3915        | 82    | 20.9    |         |
|      | WT2      | 7240        | 126   | 17.4    | KO2      | 3859        | 125   | 32.4    |         |
|      | WT3      | 7088        | 51    | 7.2     | KO3      | 4678        | 90    | 19.2    |         |
|      | Ave      |             | 11.1  |         |          |             |       |         | 0.033   |
|      | SD       |             | 5.5   |         |          |             |       |         | 7.1     |
| P25  | WT1      | 8097        | 111   | 13.7    | KO1      | 7716        | 190   | 24.6    |         |
|      | WT2      | 8024        | 116   | 14.5    | KO2      | 7736        | 186   | 24.0    |         |
|      | WT3      | 6091        | 92    | 15.1    | KO3      | 7326        | 169   | 23.1    |         |
|      | Ave      |             | 14.4  |         |          |             |       |         | 4.89E-05|
|      | SD       |             | 0.7   |         |          |             |       |         | 0.8     |
| 10 wk| WT1      | 15144       | 182   | 12.0    | KO1      | 13015       | 367   | 28.2    |         |
|      | WT2      | 10482       | 142   | 13.5    | KO2      | 8862        | 195   | 22.0    |         |
|      | WT3      | 6640        | 53    | 8.0     | KO3      | 4646        | 106   | 22.8    |         |
|      | Ave      |             | 11.2  |         |          |             |       |         | 24.3    | 0.0033  |
|      | SD       |             | 2.9   |         |          |             |       |         | 3.4     |
Supplement Fig S1

Strategies to evaluate keratinocyte proliferation by PCNA staining and epidermal thickness. The dorsal skin was excised from MED1 null mouse. The section was cut on the angle to show full length of hair follicles and stained by PCNA. (A) Representative images of PCNA staining lightly counter stained with hematoxylin (B) The dark brown PCNA positive cells were counted by using image analysis software Bioquant, which were labeled by red/yellow dots (shown by arrows). (B) The length of the skin (red line in C) was also measured by drawing red line in epidermis. The PCNA positive cells per mm epidermis were calculated (PCNA/mm) (Supplement Table SIII). (C) Epidermal thickness was measured as length of green bars, which spanned the epidermis by drawing top (red) and bottom (yellow) of the epidermis by using Bioquant. Average thickness was calculated by accumulating green bars in 0.3-1.5 cm length of the skin in each mouse.