SUPPLEMENTAL INFORMATION

SUPPLEMENTAL METHODS

PCR primers
Pre-designed primer sets were used for mouse DGKα (mDGKα), mDGKβ, mDGKγ, mDGKδ, mDGKη, mDGKε, mDGKζ, mDGKε, mDGKθ, human DGKα (hDGKα), hDGKβ, hDGKγ, hDGKδ, hDGKε, hDGKζ, hDGKθ and human tyrosinase (TAKARA BIO Inc., Otsu, Japan). Other primers used were for hGAPDH (Satoh et al., 1999): forward primer 5'-GAA GGT GAGT GGA GGT AGT-3', reverse primer 5'-GAA GAT GGT GAT GGG ATT TC-3'; hDGKη: forward primer: 5'- TGACGCAGCCACAATTTCAC-3', reverse primer: 5'- CGCCTTCACATTGATGGCTA-3'; hDGKζ: forward primer: 5'- TCCTGTGCATCTCAGTCAC-3', reverse primer: 5'- ATGTTGCTGGAGCCAAGTC-3'; hDGKθ: forward primer 5'-CTC ACC AAG CCC ACC TTC T-3', reverse primer 5'-ACG TGC TTC AGG CAC TTC TC-3'; hMITF (Dynek et al., 2008): forward primer 5'-TCT CTT TGC CAG TCC TTC ATC A-3', reverse primer 5'-CTG CAC CTG ATA GTG ATT ATA TTC TAG CA-3'.

RNA Extraction, RT-PCR and Real-Time Quantitative RT-PCR
Total RNA was isolated using TRIzol (Invitrogen) from cultured cells. Contaminating residual genomic DNA was removed by digestion with RNase free DNase (Invitrogen) and total RNA was purified with an RNeasy Mini kit (Qiagen). The extracted RNA was diluted with double-distilled water.

RT-PCR was performed using a OneStep RT-PCR kit (Qiagen, Chatsworth, CA) according to standard protocols. Fifty ng total RNA was used in each reaction. The RT-PCR conditions were: one cycle of 50°C for 30 min, 95°C for 15 min, 40 or 50 cycles of 94°C for 60 sec, 56°C for 60 sec and 72°C for 60 sec, followed by one cycle of 72°C for 10 min. PCR products were separated on 2% agarose gels and stained with ethidium bromide.

Real-time RT-PCR was performed using the LightCycler thermal cycler System (Roche Diagnostics, Indianapolis, IN). RT-PCR was performed with a QuantiTect™ SYBR Green RT-PCR kit (Qiagen). Amplification was performed using an initial denaturation at 95°C for 15 min, 40 cycles of denaturation at 94°C for 15 sec, annealing at 53°C (GAPDH), or 58°C for 20 sec, and a final extension at 72°C for 20 sec.

Data were analyzed using LightCycler software (Roche Diagnostics). The noise level was defined following background subtraction to normalize fluorescence between various samples. The threshold cycle (Ct), defined as the fractional cycle at which the fluorescence signal significantly increases above the baseline signal, was manually determined. Sample concentrations were quantified from a standard curve that was generated by plotting Ct values against the logarithm of the calculated initial volume of total RNA using dilutions from cell extracts. Amplification was performed on each serially diluted sample of total RNA. The relative
amount of target molecule in the samples was estimated from the standard curve. The results were expressed as ratios of target molecule transcripts to GAPDH transcripts.

**Western blotting analysis**

Cell extracts were prepared using the M-PER mammalian protein extraction reagent (Pierce Biotechnology, Rockford, IL) containing a protease inhibitor mixture (Roche, Mannheim, Germany). The protein concentration of each extract was measured using a BCA Protein Assay kit (Pierce). Cell extracts were mixed with 2×Tris-glycine SDS sample buffer (Invitrogen) supplemented with 2.5% 2-mercaptoethanol and were boiled for 5 min. Samples were then separated on 4-20% gradient SDS polyacrylamide gels (Invitrogen), after which proteins were transferred to PVDF membranes (Invitrogen). Membranes were blocked for 1 h in PBS or Tris buffered saline (TBS) containing 0.1% Tween 20 and 5% (w/v) nonfat dry milk powder and were incubated 90 min at RT or overnight at 4°C with the primary antibody in PBS or TBS containing 0.1% Tween 20 with 5% (w/v) nonfat dry milk powder, depending on the antibody. Membranes were then incubated with appropriate HRP-linked anti-rabbit or anti-mouse antibodies (GE Healthcare) at 1:10,000 dilution or with an anti-goat antibody (Santa Cruz) at 1:2,000 dilution at room temperature for 1 h. Antigen-antibody complexes were detected using an ECL Plus Western Blotting Detection System (GE Healthcare) according to the manufacturer’s instructions.

**Melanin content and Tyrosinase assays**

Melanin content was measured as described previously (Tsuboi *et al.*, 1998) with slight modifications. Briefly, cells were washed twice with PBS and lysed in the presence of protease inhibitors (Roche). Cell extracts were centrifuged at 14,000 g for 10 min at 4°C. The supernatants were removed and protein contents were determined. Each pellet was dissolved in 1 N NaOH at 95°C for 1 h, and melanin concentrations were quantitated by absorbance at 405 nm using a standard curve generated from synthetic melanin. The melanin content was normalized to protein content.

Tyrosinase activity was determined by the method described previously (Tomita *et al.*, 1992) with slight modifications. Briefly, cells were cultured in 6-well plates. After incubating with the DGK inhibitor where indicated, the cells were washed with PBS and lysed with 1% Triton-X/PBS and then frozen at -80°C. After thawing, lysates were clarified by centrifugation at 10,000 g for 5 min. After quantifying protein levels to adjust protein concentrations, 90 μl of each lysate was placed in a 96-well plate, and 10 μl 10 mM L-DOPA was then added to each well. Control wells contained 90 μl lysis buffer and 10 μl 10 mM L-DOPA. Following incubation at 37°C, the absorbance was measured every 30 min for at least 3 h at 475 nm. Tyrosinase activity is expressed as a percentage of the control.

**Targeted inhibition using siRNA**

The following small interfering RNA (siRNA) oligonucleotides were used to knockdown the expression of endogenous human DGKα, DGKδ, DGKη and DGKζ (Qiagen, catalog numbers
SI00605199 and SI00605206, designated DGKα siRNA1 and 2, SI00287476 and SI00287483, designated DGKδ siRNA1 and 2, SI00155680 and SI02648639, designated DGKη 1 siRNA1 and 2, SI02223347 and SI02665390, designated DGKζ siRNA1 and 2, respectively). Two different siRNAs directed against DGK isoforms were tested. The Fast-Forward siRNA Protocol using HiPerfect Transfection Reagent (Qiagen), which allows simultaneous cell seeding and transfection by siRNA, was performed on the cells according to manufacturer’s instructions. Cells were transfected with transfection reagent and 20 nM siRNA or the scrambled control (Qiagen) in each experiment. The efficiency of RNA interference was evaluated by real-time RT-PCR 48 h after transfection. Four or 6 days after the initial transfection, cells were harvested for further analysis.

**Recombinant adenoviral vectors construction and infection**

Replication-incompetent adenoviruses expressing human DGKα and DGKζ were created using the Virapower adenovirus expression system (Invitrogen) according to the manufacturer’s instructions. Briefly, full-length human DGKα and DGKζ (Invitrogen) were subcloned into the adenoviral destination vector by LR clonase II (Invitrogen). The adenoviral expression vector was transfected into 293A cells using Lipofectamine 2000 (Invitrogen). Cells were grown until an 80% cytopathic effect was seen, and then were harvested for preparation of stock recombinant adenovirus. The titer of adenoviral stock was determined by plaque assay using MTT solution. NHEMs were infected with adenovirus vectors in melanocyte growth medium for 24 h, then replenished with fresh medium and further incubated for 2-3 days.
SUPPLEMENTAL FIGURES AND FIGURE LEGENDS

Suppl Figure 1 – NHEMs and B16F10 cells were plated and treated with the DGK inhibitor for 48 h, then were counted using a cellometer auto-counter. Results are averages of 3 independent experiments ± SD.

Suppl Figure 2 – Effect of the DGK inhibitor on melanogenesis in SK-Mel-23 cells. SK-Mel-23 cells were treated with the DGK inhibitor at the concentration noted, after which melanin content, tyrosinase activity, and tyrosinase protein levels were measured; results are average of 3 independent experiments ± S.D. **P<0.01 compared to the control.
Suppl Figure 3 - Verification of transgene expression in NHEMs. 72 h after transduction with the indicated multiplicity of infection (MOI), Western blotting analysis indicates successful overexpression of DGKα and DGKζ protein, respectively.