SUPPORTING INFORMATION

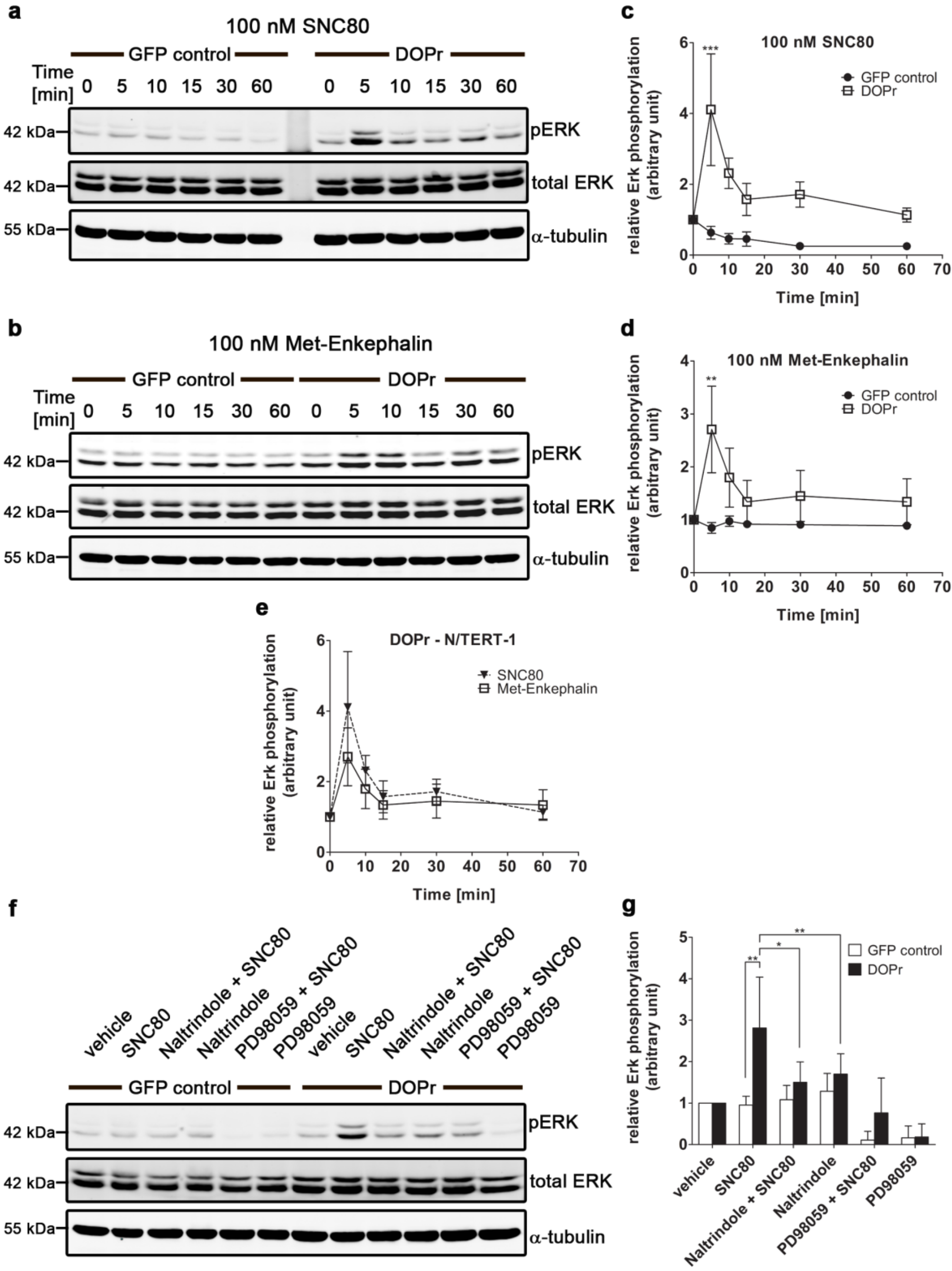

Figure S1. DOPr activation induces ERK 1/2 signaling in keratinocytes

Previous studies in HEK293 and NG108-15 hybrid cells showed that the MAPK ERK 1/2 pathway can be activated by DOPr signaling (Audet *et al.*, 2005; Eisinger and Ammer, 2008a;

Eisinger and Ammer, 2008b). Therefore, regulation of DOPr-mediated ERK 1/2 signaling in keratinocytes was assessed in keratinocyte cell lines N/TERT-1 (Fig. S1) and HaCaT (Fig. S2) after DOPr-overexpression. 80% confluent cells were starved overnight, treated, and analyzed by western blot and fluorography for threonine 202 and tyrosine 204 phosphorylation of ERK 1/2. Fig. S1 shows the immunoblot for pErk, total Erk, and α-tubulin after **(a)** 100 nM SNC80 or **(b)** 100 nM Met-enkephalin incubation for indicated times. Within 5 minutes a rapid increase in ERK 1/2 phosphorylation was detected **(a)**, which declined back to basal levels within 30 minutes after stimulation. A similar phosphorylation of ERK could be detected after activation of the DOPr by the endogenous agonist Met-enkephalin **(b)**. Semi-quantification of these western blots using densitometry indicated significantly more activated ERK 1/2 in DOPr-expressing cells treated with the DOPr ligand SNC80, compared to control cells **(c)**. Displayed is the ratio of pERK/total ERK after **(c)** SNC80 and **(d)** Met-enkephalin treatment, normalized to respective basal control. Mean +/- SEM, n=3. Two-way ANOVA, ***$p<0.001$, **$p<0.01$. The course of activation was the same for both, exogenous and endogenous ligand and both ligands had the same potency to activate ERK 1/2 in DOPr-overexpressing cells **(e)** but the amount of phospho-ERK 2 (p42) detected was higher than that for phospho-ERK 1 (p44) (Fig. S1a,c).

ERK 1/2 activation could specifically be inhibited in cells pre-treated with the selective DOPr antagonist naltrindole (NTI), while the antagonist itself had no effect on ERK phosphorylation **(f)** and similar results were obtained following pre-treatment with the MEK 1 inhibitor PD98059 **(g)** as control, which prevents ERK 1/2 phosphorylation further downstream in the DOPr signaling cascade than NTI. 0.1% DMSO vehicle control or 100 nM SNC80 were added and incubated for 10 min. 10 μM NTI was added 5 min prior to SNC80, 20 μM of ERK inhibitor PD98059 30 min before SNC80. The quantification graph shows a reduction of ERK phosphorylation to a

minimum with PD98059 treatment and residual basal activity in DOR-overexpressing cells following co-treatment with both inhibitor and agonist **(g)**. These results demonstrated that in DOPr-overexpressing keratinocytes, DOPr activation induced ERK 1/2 MAPK pathway signaling. Shown is the ratio of pERK/total ERK normalized to respective basal control. Mean +/- SD, n=3. One-way ANOVA, **$p<0.01$, *$p<0.05$.

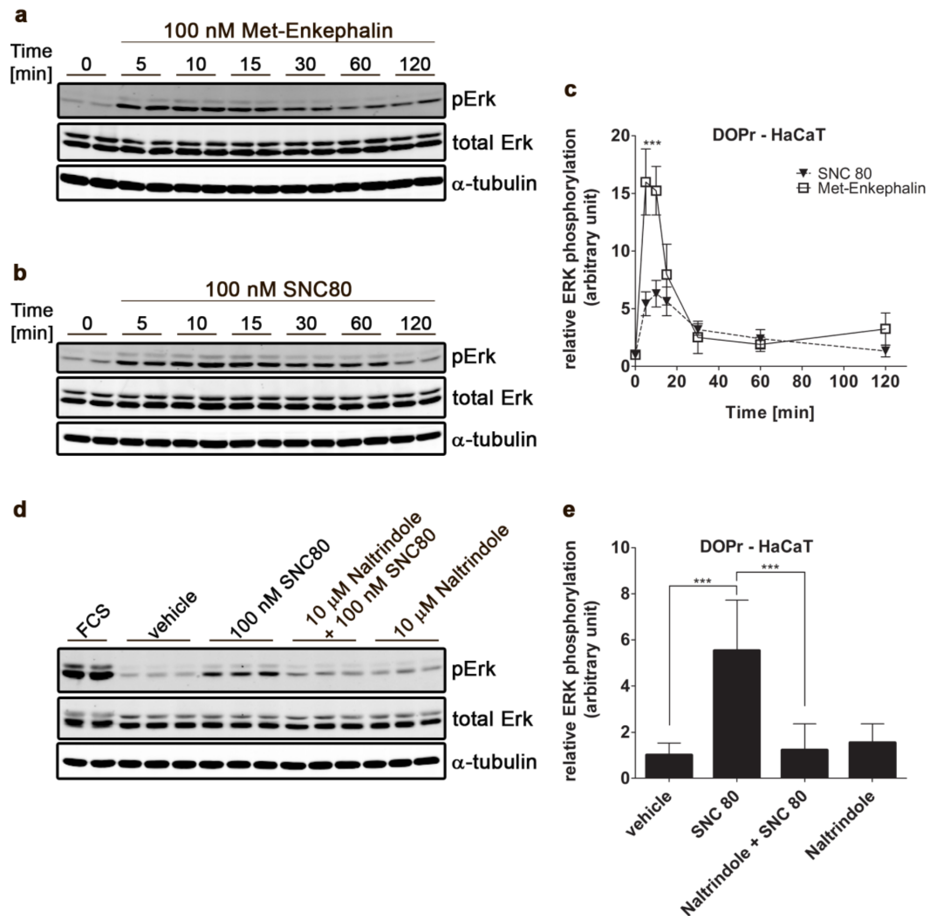

Figure S2. Confirmation of DOPr-specific modulation of the ERK 1/2 MAPK pathway in a second keratinocyte cell line

(a) HaCaT cells overexpressing DOPr and starved for 6 hours in growth supplement-free DMEM, were incubated with the endogenous DOPr agonist Met-enkephalin at a concentration of 100 nM over a time course of 120 min followed by western blot analysis of the obtained cell lysates. Immunoblot for phosphorylated ERK shows strong activation of ERK 2 within 5 min of addition of ligand and rapid decrease of the phosphorylation within 30 min. **(b)** HaCaT cells overexpressing the DOPr were incubated with 100 nM of the exogenous DOPr agonist SNC80 over a time course of 120 min followed by western blot analysis of the obtained cell lysates. Immunoblot for phosphorylated ERK shows similar strong activation of ERK 2 as in **(a)** within a

maximal response observed after 10 min of addition of ligand and rapid decrease of the phosphorylation within 30 min. **(c)** Western blot fluorescence signals were quantified by densitometry. The ratio of phospho-ERK relative to total ERK normalized to the respective basal control is displayed in the graph showing higher amplitude of ERK phosphorylation for Met-enkephalin. The graph represents the mean of two independent experiments each with three biological replicates. Error bars represent standard deviation. Two-way ANOVA reveals significant difference in ERK phosphorylation at time point 5 and 10 min, ***$p<0.001$. **(d)** HaCaT cells overexpressing the DOPr were incubated with DMSO vehicle control, 100 nM of the exogenous DOPr agonist SNC80 for 10 min alone, SNC80 after 5 min pre-incubation with 10 μM of the DOPr specific antagonist naltrindole (NTI), and 10 μM of NTI alone. Cell lysates were subjected to western blot using the phospho-specific ERK antibody and signals quantified by densitometry. Foetal bovine serum (FBS) served as a positive control for ERK activation. **(e)** The graphical representation of the phospho-ERK to total ERK ratio shows significant activation of ERK by SNC80 and specific blockage of activation by the antagonist NTI demonstrating specific DOPr-mediated ERK activation in keratinocytes. The data represent three independent experiments, each performed in three biological replicates. Error bars represent standard deviation. One-way ANOVA reveals ***$p<0.001$.

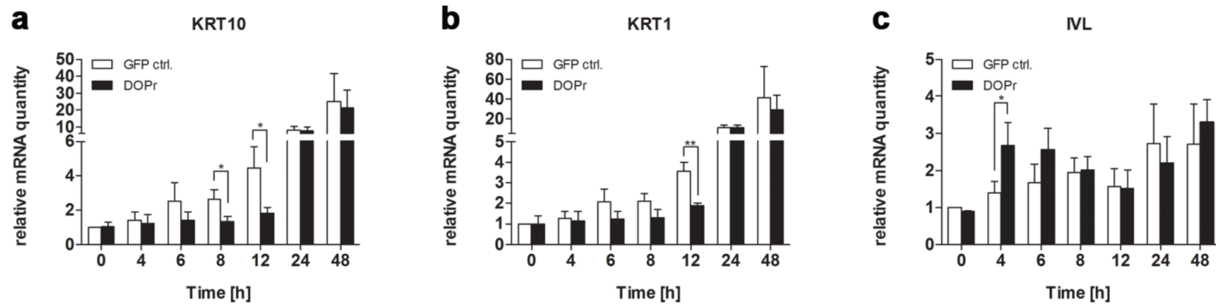

Figure S3. DOPr overexpression delays expression of keratinocyte differentiation related genes in HaCaT cell model

HaCaT cells were grown to confluence and subjected to growth factor withdrawal-induced differentiation. RNA was extracted at indicated time points and subjected to real-time PCR quantification. **(a)** In the presence of 100 nM SNC80 GFP control cells rapidly induce KRT10 expression while DOPr-overexpressing cells do not induce the expression within 12 h of incubation. 24 h after addition of ligand and induction of differentiation, no difference in KRT10 expression level between DOPr-overexpressing and control cells can be detected. **(b)** Similar to KRT10 expression, in the presence of 100 nM SNC80 GFP control cells rapidly induce KRT1 expression while DOPr-overexpressing cells start expression after about 12 h of incubation. 24 h after addition of ligand and induced differentiation no difference in KRT1 expression level between DOPr-overexpressing and control cells can be detected. **(c)** The analysis of mRNA expression of IVL in the presence of SNC80 after growth factor withdrawal induced differentiation reveals a rapid induction in DOPr-overexpressing cells after 4 h of incubation before the expression level is equalized with that of the control cells, which show a delayed expression of IVL in this experiment. The graphs represent the mean of three independent experiments. Error bars indicate standard deviation. t-test results: $^{**}p<0.01$, $^{*}p<0.05$.

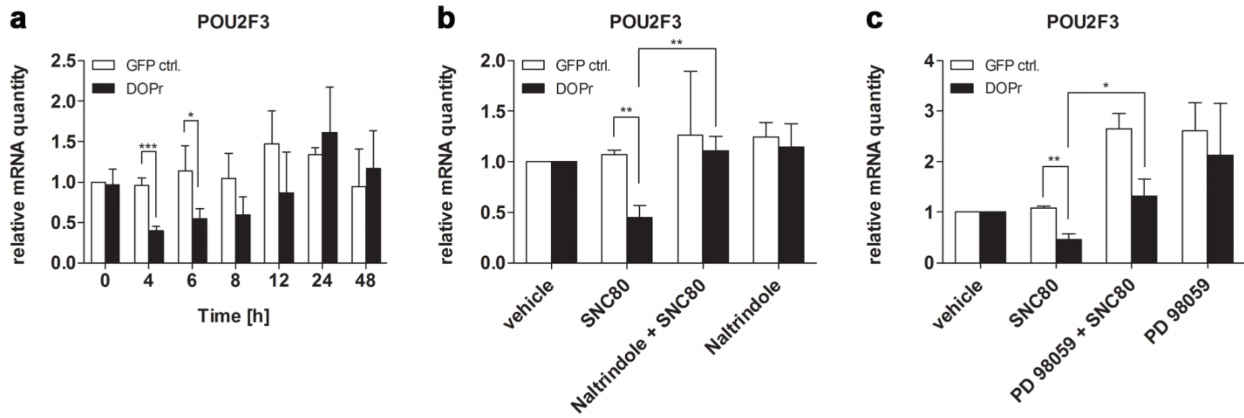

Figure S4. POU2F3 is a target gene of DOPr-mediated ERK activation in HaCaT cells

(a) HaCaT cells were grown to confluence and subjected to growth factor withdrawal induced differentiation. RNA was extracted at indicated time points and subjected to real-time PCR quantification. Within 4 h after addition of the DOPr-specific ligand SNC80 to the cultures a strong downregulation of POU2F3 can be observed in DOPr-overexpressing HaCaT cells while expression in control cells remains unchanged. Within 12 h of incubation the expression of POU2F3 goes back to the initial state and increases during further incubation time while differentiation progresses. The expression pattern correlates well with observed pattern for KRT10 (Fig. S3a), which is a POU2F3 target gene. **(b)** HaCaT cells were subjected to differentiation in the presence of 100 nM DOR agonist SNC80, 5 min pre-incubated with 10 μM of the antagonist NTI prior to SNC80 addition, the vehicle control or antagonist alone. Cells were incubated for 4 h before RNA was extracted and subjected to quantitative real-time PCR. NTI could effectively block the SNC80 mediated downregulation of POU2F3 and confirms that the regulation of POU2F3 by SNC80 is DOPr-specific. **(c)** HaCaT cells were subjected to differentiation in the presence of 100 nM of the DOPr agonist SNC80, after 30 min pre-incubation with 20 μM of the MEK 1 inhibitor PD98059 prior to SNC80 addition, the vehicle control or PD98059 alone. Cells were incubated for 4 h before RNA was extracted and subjected

to quantitative real-time PCR. The inhibition of ERK could effectively reverse DOPr-mediated POU2F3 downregulation, indicating an ERK-dependent, DOPr-mediated mechanism. At the same time control cells subjected to double treatment and DOPr-overexpressing cells with inhibitor treatment alone show a 2.5 fold induction of POU2F3 mRNA expression. This indicates a basal regulation mechanism for POU2F3 by ERK mediated pathways. The quantification after pre-incubation of DOPr-overexpressing cells with PD98059 prior to SNC80 addition, displays a slightly higher level than the vehicle POU2F3 expression. This indicates that a complex network of inhibitory and activating signals controls POU2F3 expression and more cellular signaling pathways might be involved. The graphs represent the mean of three independent experiments. Error bars indicate standard deviation. t-test results: ***$p<0.001$, **$p<0.01$, *$p<0.05$.

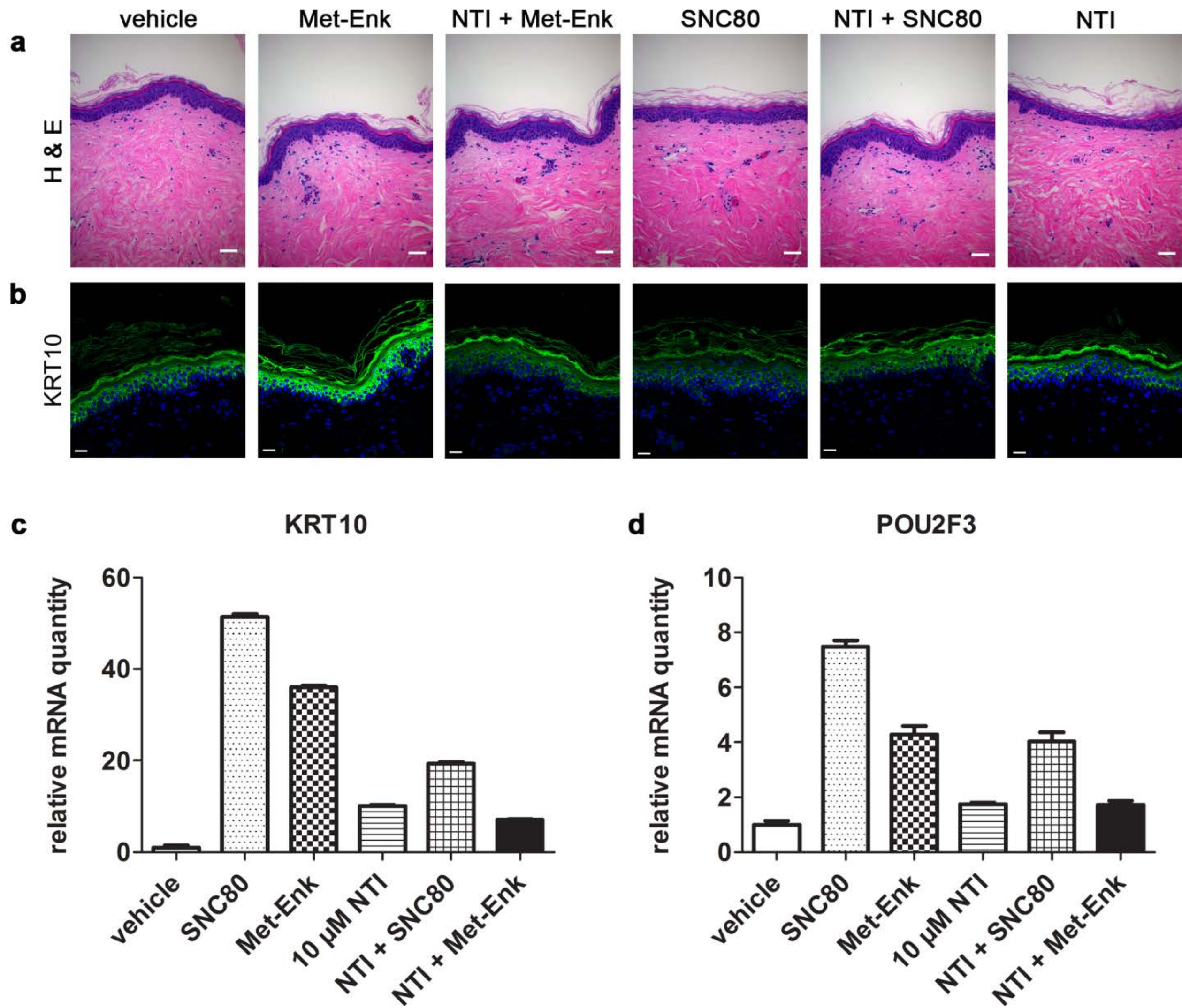

Figure S5. Opioids affect skin homeostasis *in situ*

Skin organ cultures were kept for 7 days in medium containing either vehicle (0.001% DMSO), 100 nM agonists SNC80 or Met-enkephalin or 10 µM antagonist NTI or both. Cultures were maintained submerged for the whole time of incubation. **(a)** H&E staining of skin organ cultures do not show obvious morphological changes caused by the different ligands. A small number of pyknotic nuclei indicates low apoptotic rates in the cultures and does not reveal differences between culture conditions. **(b)** KRT10 labeling revealed a trend of higher expression for SNC80

and Met-enkephalin treated cultures but quantification showed no significantly enhanced expression of KRT10 upon opioid activation. **(c)** and **(d)** quantitative real time PCR show strong upregulation of differentiation-related genes KRT10 and POU2F3. NTI was able to block this upregulation indicating a DOPr-specific effect. SNC80 thereby seemed to be a more potent ligand than Met-enkephalin indicating a possible involvement of heteromer formation of MOPr and DOPr (M.D. Metcalf *et al.*, 2012). These skin organ cultures clearly show that DOPr signaling is involved in epidermal homeostasis.

MATERIALS AND METHODS

Cell Culture

HaCaT cells (Boukamp *et al.*, 1988) were maintained in DMEM (Life Technologies, Singapore) containing 10% FBS (J R Scientific, PAA, Singapore), 100 U/ml penicillin, and 100 μg/ml streptomycin (PAA) at 37°C and 5% CO_2.

DNA construct and lentiviral particle production

The human DOPr-GFP plasmid was constructed by PCR amplifying hDOPr (nucleotides 1–1359 of human DOPr mRNA, NCBI entry NM_000911) from the hDOPr-FLAG pcDNA3 plasmid (a gift from B.L. Kieffer, IGBMC, Institut de Génétique et de Biologie Moléculaire et Cellulaire, Neurobiology and Genetic Department, Illkirch F-67400, France) with the oligonucleotide hDOR-GFP_BamHI_fwd [TTT (feeder sequences) GGATCC (BamHI site) ATGGAACCGGCCCCCT (sequences complementary to human DOR from first amino acid onwards] and oligonucleotide hDOR-stop_MluI_rev [TTT (feeder sequences) ACGCGT (MluI site) A (filling sequence) AGGCGGCAGCGCCA (nucleotides 1346–1359 of human DOR mRNA)]. The resulting 1136 bp PCR fragment was cut with BamHI and MluI and sub-cloned into SIN-PGK-GFP-WHV, opened with the same enzymes. The generated DOPr-GFP fusion fragment was then sub-cloned into TRIP-PGK-IRESNEO-WHV, which is a lentiviral vector bearing the neomycin resistance gene (Yang *et al.*, 2004). DOPr-GFP was amplified by PCR with the oligonucleotide hDOPr-GFP_BamHI_fwd (see above) and EGFP_SalI_rev [GGA (feeder sequence) GTCGAC (SalI site) TTA (filling sequence) CTTGTACAGCTCGTCC (nucleotides 702-717 of eGFP from plasmid SIN-PGK-WHV)]. The resulting 1876 bp fragment was cut with

BamHI and SalI and sub-cloned into TRIP-PGK-IRESNEO-WHV, opened with the same enzymes. This construct was used in all HaCaT experiments

Production of recombinant lentiviral particles was modified from the method described by J.Y. Yang *et al.* (2004). In brief, HEK 293FT cells were co-transfected using the Ca^{2+} phosphate DNA precipitation method (Jordan *et al.*, 1996) with 20 µg of the lentiviral vector (TRIP-PGK-IRESNEO-WHV), containing the cDNA of interest (hDOPr-GFP), 6 µg of the envelope protein-coding plasmid (pMD2.G), and 15 µg of the packaging construct (psPAX2) per 10 cm culture dish. Viral particle containing medium was harvested and concentrated as described before.

Reagents

The antibody detecting p44/42 MAPK (ERK 1/2) was purchased from Cell Signaling (137F5; n°4695) as was the phospho-p44/42 MAPK (ERK 1/2) (Thr202/Tyr204) rabbit monoclonal antibody (197G2; n°4377). The monoclonal anti-α-tubulin antibody (DM 1A; n°T9026) was from Sigma-Aldrich.

Immunoblot

Cells were seeded in 6-well plates and subjected to treatments as indicated in the figures before lysis in RIPA-like lysis buffer, comprising 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 1% IGEPAL CA-630, 0.1% SDS, 0.5% Sodium deoxycholic acid at pH 7.4, supplemented with 1 mM DTT, 1 mM PMSF, 10 mM Na_3VO_4, and 1x protease inhibitor (Roche). 20 µg of proteins were separated by SDS-PAGE. After transfer and blocking blots were incubated in TBS containing 0.1% Tween-20 and 5% BSA overnight at 4°C with the ERK specific primary antibodies at 1:1000 dilution. Quantification of fluorescence signal was done

using the Odyssey software and normalized to the background signal. Obtained values of integrated intensity were exported to EXCEL, and relative ERK phosphorylation was calculated by normalization to the total ERK signal and the respective vehicle control as reference for each group.

Skin organ culture

Skin organ culture was obtained from a routine surgery for cosmetic purposes after written informed consent of the patient approved under the NUS-IRB Reference Code 13-25-IE of the University of Singapore. Subcutaneous fat tissue was removed and 4 mm punch biopsies were taken. Skin was cultured in serum-free Wiliam's E medium (Sigma-Aldrich) supplemented with 2 mmol/l L-glutamin (Life Technologies), 10 ng/ml hydrocortisone (Sigma-Aldrich) and antibiotics (penicillin and streptomycin, PAA). Medium was changed every two days. Opioid ligands Met-enkphalin and SNC80 were added at a concentration of 100 nM and antagonist NTI at 10 µM concentration. Controls were incubated with 0.01% DMSO vehicle control. After 7 days in culture , skin was either embedded in O.C.T embedding compound (TissueTek), snap frozen in liquid nitrogen or fixed in 4% paraformaldehyde and processed for paraffin sections at the Institute of Molecular and Cell Biology Core Histopathology Laboratory, Singapore or the epidermis was separated from the dermis using dispase treatment for 1 h at 37 ºC and stored in RNAlater (Qiagen) for RNA extraction and quantitative real-time PCR analysis. RNA was extracted after homogenization in buffer RLT using the Qiagen RNeasy kit according to manufacturer protocol. 700 ng of RNA extracted from three pooled biopsies were reverse transcribed and analyzed for KRT10 and POU2F3 expression.