Methods

1. Cell culture.

HaCaT - the immortalized human skin keratinocyte cell line

HaCaT keratinocytes were cultured in RPMI 1640 containing 2mM GLUTAMAX™-I (substituted on a molar equivalent basis for L-glutamine) and 0.424mM Calcium. RPMI Medium was supplemented with antibiotics (100 µg/ml streptomycin, 100 U/ml penicillin from Gibco) and 10% fetal calf serum (FCS). Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2. HaCaT were subcultivated with a split ratio of 1:10 every other day. To mobilize the cells medium was removed, the cells rinsed with D-PBS without calcium and magnesium, than Trypsin-EDTA solution added and the cells incubated at 37°C for 10-15 min. Subsequently trypsin was inactivated with fresh culture medium.

For Ca-Free Medium experiments HaCaT were seeded at $1x10^6$ cells per $57cm^2$ CELLSTAR® Cell Culture Dish and incubated overnight in Keratinocyte Growth Medium 2 (KGM-2) without Ca^{2+}. KGM-2 medium was prepared according to manufacturers instruction by supplementing Keratinocyte Basal Medium 2 (KBM-2) without calcium (Cambrex, Cat.-No.: CC-3158) with KBM-2 SingleQuot Kit (Cambrex, Cat.-No.: CC-4152) containing BPE (Bovine Pitutary Extract), hEGF, Insulin (bovine) Hydrocortisone, GA-1000 (Gentamicin, Amphotericin-B), Epinephrine and Transferrin.

HeLa - human epithelial carcinoma cell line

HeLa cells were cultured in RPMI 1640 containing 2mM GLUTAMAX™-I (substituted on a molar equivalent basis for L-glutamine) and 0.424mM Calcium. RPMI Medium was supplemented with antibiotics (100 µg/ml streptomycin, 100 U/ml penicillin from Gibco) and 10% FCS. Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2. HeLa cells were subcultivated with a split ratio of 1:10 every other day. To mobilize the cells medium was removed, the cells rinsed with D-PBS without calcium and magnesium, than Trypsin-EDTA solution added and the cells incubated at 37°C for 7-10 min. Subsequently trypsin was inactivated with fresh culture medium. For the virus titration $8x10^4$ cells/well was seeded on 6-well plates and incubated overnight at 37°C in a humidified atmosphere of 5% CO_2.

<u>HEK 293T/17 - human embryonic kidney-293T cell line</u>

HEK 293T/17 cells were purchased from ATCC, expanded to a maximum of 5 passages and frozen until use. HEK 293T/17 were cultured in D-MEM, 4500 mg/L Glucose containing 2mM GLUTAMAX™-I (substituted on a molar equivalent basis for L-glutamine) D-MEM Medium was supplemented with antibiotics (100 μg/ml streptomycin, 100 U/ml penicillin from Gibco) and 10% FCS. Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2. HEK 293T/17 cells were subcultivated with a split ratio of 1:5 every second day. To mobilize the cells medium was removed, the cells rinsed with D-PBS without calcium and magnesium, than Trypsin-EDTA solution added and the cells incubated at 37°C for 2-4 min. Subsequently trypsin was inactivated with fresh culture medium. For the virus production 5,6-5,8x10^6 cells were seeded on 57 cm² CELLSTAR® cell culture dish and incubated overnight at 37°C in a humidified atmosphere of 5% CO_2.

Cell lines.

Cell line name	Description	ATCC number
HaCaT	immortalized human skin keratinocytes	-
HEK 293T/17	human embryonic kidney - 293T	CRL-11268
HeLa	human epithelial carcinoma	CCL-2

Reagents for cell culture.

500 ml HeLa/HaCaT medium, store at +4°C
500ml RPMI Medium 1640 (with GlutaMAX™ I) Gibco, Cat.-No.: 361870-044
+10%FCS (50ml FBS Biochrom AG, Cat.-No.: S 0115)
+ Pen/Strep (100U of penicillin, 100ug of streptomycin)
6 ml Penicillin-Streptomycin Invitrogen, Cat.-No.: 15140-163

500 ml HEK 293T/17 medium, store at +4°C
500ml D-MEM high glucose (with GlutaMAX™ I, 4500 mg/L D-Glucose, without Sodium Pyruvate) Gibco, Cat.-No.: 61965-059
+10%FCS (50ml FBS Biochrom AG, Cat.-No.: S 0115)
+ Pen/Strep (100U of penicillin, 100ug of streptomycin)
6 ml Penicillin-Streptomycin Invitrogen, Cat.-No.: 15140-163

2. Culture of primary adult keratinocytes.

Expansion of adult epidermal keratinocytes on feeder layer was performed modified according to (Rheinwald & Green, 1975). All experiments were performed with primary keratinocytes expanded up to passage 2.

Protocol for isolation and culture of primary adult keratinocytes.

1st Day – Preparation of the 6-well plates with feeder layer

Adult fibroblast derived from mamma reduction surgery are expanded to a maximum of 5 passages and frozen until use. Following thawing, cells are plated in 75cm^2 standard tissue culture flask with filter (TPP) in basal fibroblast medium. The following day, cells are split 1:3 and incubated for an additional 24h.

75cm^2 cell culture flask with 90% confluent human fibroblasts in 12,4 ml basal fibroblast medium

+ 125 µl Mitomycin C 0,4mg/ml stock solution (working concentration: 0,4µg/ml)

➢➢ incubate: 2h-3h, 37°C, 5% CO_2

remove and discard

+1 ml D-PBS

remove and discard

+1ml Trypsin-EDTA

➢➢ incubate: 5min, 37°C, 5% CO_2

+10ml basal fibroblast medium

↺ 5min, 1500 RPM

Resuspend the cell pellet with 10 ml of fresh basal fibroblast medium

Plate the fibroblasts on 5-6 6-Well plates (6x9.5 cm² each) or 5-6 cell culture dishes (55 cm² each).

➢➢ incubate: overnight, 37°C, 5% CO_2

2nd Day – Dispase I digestion of a skin fragment

Full thickness 4 mm punch biopsy is performed on a skin fragment derived from surgery. Subcutaneous fatty tissue is removed by gentle scraping.

+ 2-3 ml dispase I solution (freshly prepared at 0,2 mg/ml in PBS)

➢➢ incubate: overnight, 4°C

3rd Day – Isolation of primary adult keratinocytes

Carefully remove epidermal sheets with the forceps and fragment the epidermal part with a scalpel, transfer the fragments to a new 100mmx20mm cell culture dish

+5ml Trypsin-EDTA

➢➢ incubate: 10 min, 37°C, 5% CO_2

Mechanically disrupt the sheets with a 1ml pipette tip cut at the end

➢➢ incubate: 5-10 min, 37°C, 5% CO_2 (check under the microscope)

When most of the cells are mobilized

+10ml keratinocyte medium

↺ 10min, RT, 1500 RPM

Resuspend the cell pellet with the fresh keratinocyte medium:

use 2 ml of keratinocyte medium per biopsy/well

➢➢ incubate: 48h, 37°C, 5% CO_2

6th Day – Medium change

Change medium for the first time at least 2 days after plating the keratinocytes.

Primary keratinocytes can be expanded on 10 cm cell culture dish only after the confluency of 70% has been reached.

10th -14th Day – Fractionated trypsination

Wash with 1 ml D-PBS

remove and discard

+2ml Trypsin-EDTA (4ml Trypsin-EDTA for 10 cm cell culture dish)

➢➢incubate: 5 min, 37°C, 5% CO_2

Check under the microscope every minute. When the feeder layer is detached, remove the trypsin solution including fibroblasts.

+2ml Trypsin-EDTA (5ml Trypsin-EDTA for 10 cm cell culture dish)

➢➢ incubate: 5-10 min, 37°C, 5% CO_2

Check under the microscope every minute, starting with 5th minute of incubation. When the keratinocytes are detached, inactivate trypsin with keratinocyte medium.

+4ml keratinocyte medium (10ml keratinocyte medium for 10 cm cell culture dish)

↺ 5min, RT, 1500 RPM

Resuspend the cells in 6 ml fresh keratinocyte medium and plate on 10 cm cell culture dish with a new feeder layer.

Reagents for culture of primary adult keratinocytes.

0.2mg/ml Dispase I in PBS, prepare fresh, do not store

Dispase I (neutral protease, grade I) Roche Cat.-No.: 04942086 001

Working concentration: 0.2 mg/ml ≈ 3,5U/ml

To prepare stock solution to 1 vial of dispase I (~2mg ≈ 34U) add 10 ml D-PBS

0,4mg/ml Mitomycin C in H_2O, store at -20°C

Mitomycin C from Streptomyces caespitosus, powder Sigma, Cat.-No.: MO503-2MG

Stock solution: 0,4mg/ml in H_2O

Working concentration: 0,4µg/ml (1:1000)

To prepare stock solution to 1 vial of Mitomycin C (2mg) add 5 ml H_2O

500 ml Basal fibroblast medium, store at +4°C

500ml DMEM low glucose (+1g/L D-Glucose, +L-Glutamine +Pyruvate)

Gibco, Cat.-No.: 31885-049

+10%FCS (50ml FBS Biochrom AG, Cat.-No.: S 0115)

+ Pen/Strep (100U of penicillin, 100ug of streptomycin) 6 ml Penicillin-Streptomycin Invitrogen, Cat.-No.: 15140-163

50 ml Keratinocyte medium, store at +8°C up to 7 days

50 ml Basal Keratinocytes Medium

+ 60 µl IHE Cocktail : **I**nsulin (Working concentration: 5µg/ml)

Hydrocortisone (Working concentration: 0,5µg/ml)

EGF (Working concentration: 10ng/ml)

+0,02µg/ml Cholera Toxin GENTAUR, Cat.-No.: 100 Inaba 569 B

(+1µl Cholera Toxin stock solution)

1mg/ml Cholera Toxin, store at +8°C

Cholera Toxin (azide-Free) from *Vibrio cholerae* Inaba 569B

(GENTAUR, Cat.-No.: 100 Inaba 569 B)

To prepare stock solution to 1vial of Cholera Toxin (1mg) add 1 ml D-PBS

500 ml Basal Keratinocytes Medium, store at +8°C
333 ml DMEM low glucose (+1g/L D-Glucose, +L-Glutamine +Pyruvate),
Gibco, Cat.-No.: 31885-049
+167 ml Nutrient Mixture F-12 Ham (With sodium bicarbonate, without L-glutamine,
Sigma, Cat.-No.: N4888-6X500ML
+0.024 mg/ml Adenine (1ml of adenine stock solution)
Adenine Sigma, Cat.-No.: A2786-5G
+10%FCS (50ml FBS Biochrom AG, Cat.-No.: S 0115)
+ Pen/Strep (100U of penicillin, 100ug of streptomycin) 6 ml Penicillin-Streptomycin
Invitrogen, Cat.-No.: 15140-163

12,1 mg/ml Adenine in 1M NaOH stock solution, store at -20°C:
Adenine (Sigma, Cat.-No.: A2786-5G)
End concentration: 0.024 mg/ml
To prepare stock solution dilute 121mg Adenine pulver with 10 ml 1M NaOH
Filtrate with 0,2 µm filter
Aliquot: 1 ml

IHE Cocktail, store at -20°C
1ml of 5mg/ml **I**nsulin in 5mM HCL
100µl of 5mg/ml **H**ydrocortisone in 100% Ethanol
100µl of 100µg/ml **E**GF in Basal Keratinocytes Medium
Aliquot: 60 µl

5mg/ml **I**nsulin in 5mM HCL, store at -20°C
Insulin from bovine pancreas (Sigma, Cat.-No.: I5500-50MG)
To prepare stock solution dilute 50mg of Insulin with 10ml of 5mM HCL
Stock solution: 5mg/ml in
Working concentration: 5µg/ml
Filtrate with 0,2 µm filter
Aliquot: 1ml

5mg/ml **H**ydrocortisone in 100% Ethanol, store at +4°C
Hydrocortisone, Chromatographic Standard (CALBIOCHEM, Cat.-No.: 386698)
To prepare stock solution dilute 25mg Hydrocortisone with 5ml of 100% Ethanol
Stock solution: 5mg/ml in 100% Ethanol
Working concentration: 0,5µg/ml
Filtrate with 0,2 µm filter

100µg/ml **E**GF in Basal Keratinocytes Medium, store at -80°C
Epidermal Growth Factor human recombinant, expressed in Escherichia coli, lyophilized powder, cell culture tested (Sigma, Cat.-No.: E9644-.2MG)
To prepare stock solution dilute 0,2mg of Epidermal Growth Factor with 2ml of Basal Keratinocytes Medium
Stock solution: 100µg/ml
Working concentration: 10ng/ml
Filtrate with 0,2 µm filter
Aliquot: 100µl

3. Culture of primary neonatal mouse keratinocytes.

Isolation and expansion of a neonatal mouse keratinocytes was performed modified according to (Tan *et al.*, 2001). All experiments were performed with primary keratinocytes expanded up to passage 2.

Protocol for culture of primary neonatal mouse keratinocytes.

1st Day – Dispase I digestion of a skin fragment

Perform a full thickness 4 mm punch biopsy on a skin fragment derived from 1 to 3-day-old pups.
Place all 4mm biopsies (approx. 12 per skin) and fragmented rest of the skin in separate 15 ml tubes

+ 7 ml Dispase I solution (freshly prepared at 2,5U/ml in PBS)

➢➢ incubate: overnight, 4°C

2nd Day – Isolation of primary neonatal mouse keratinocytes

Place the skin fragments of biopsies in a fresh 50 ml sterile tube

+33 ml of KBM-2 medium with 0,05mM Ca^{2+} (for rest of skin)

OR

+4 ml of KBM-2 medium with 0,05mM Ca^{2+} (per 1 biopsy: 4 ml + 2 ml direct to well)

(per 12 biopsies: 41 ml + 2 ml direct to well)

➢➢ firmly, at least 50 times shake the tubes

When most of the cells are mobilized seed 20 ml of skin fragments suspension per Corning® CellBIND® Surface Culture Dish (55 cm²) or 4 ml of cell suspension per well of Corning® CellBIND® 6 Well Clear Microplate which corresponds to 1 biopsy, than add 2 ml of KBM-2 medium per well.

➢➢ incubate: 48h, 37°C, 5% CO_2

5th Day – Medium change

Change medium for the first time at least 2 days after plating the keratinocytes.

Primary keratinocytes can be expanded on 10 cm cell culture dish only after the confluency of 70% has been reached.

10th Day – Lentivirus infection

Remove medium.

+8ml virus supernatant per 10 cm cell culture dish correspond to multiplicity of infection (MOI) of 10

+8µl polybrene (8 mg/ml polybrene stock solution)

➢➢ incubate: 12h-16h, 37°C, 5% CO_2

11th Day – Change medium, Dox stimulation

Wash the plates with D-PBS

+10 ml fresh KBM-2 medium with 0,05mM Ca^{2+}

+10 µl Dox stock solution (1mg/ml Dox stock solution)

➢➢ incubate: 30h, 37°C, 5% CO_2

Cells may be detached with trypsin-EDTA solution and protein or RNA extracted.

Reagents for culture of primary neonatal mouse keratinocytes.

2,5U Dispase I in PBS, prepare fresh, do not store

Dispase I (neutral protease, grade I) Roche Cat.-No.: 04942086 001

Working concentration: 2,5U/ml

To prepare stock solution to 1 vial of dispase I (~2mg ≈ 34U) add 13,6 ml D-PBS

8 mg/ml polybrene stock solution, store at -20°C

Hexadimethrine bromide (Polybrene) Sigma, Cat.-No.: 107689-10G

working solution - 8 µg/ml (1:1000)

2,5 M $CaCl_2$ stock solution, store at -20°C

184 g $CaCl_2 \cdot 2\, H_2O$ (Calcium chloride dihydrate Sigma, Cat.-No.: 21098-500G-F)

add H_2O bidest. to 500 ml

Filtrate with 0,2 µm filter

1mg/ml Doxycycline stock solution, store at -20°C

10mg Dox (Doxycycline hyclate Sigma, Cat.-No.: D9891-25G)

add H_2O bidest. to 10 ml

Filtrate with 0,2 µm filter

50 ml KBM-2 medium, 0,05mM Ca^{2+} store at +8°C up to 7 days

50 ml KBM-2 medium with supplements

+1µl 2,5 M $CaCl_2$ stock solution

4. Freezing and thawing of cells.

To freeze the cell lines as well as primary cells 70% confluent cells, which received fresh growth medium the day before, were washed with 10 ml D-PBS and trypsinized as described in respective methods section for each cell type. The cells were then pelleted at 200 x g for 5 minutes. After aspiration of the supernatant, the cells were resuspended in a freezing medium containing 10% DMSO and 90% FCS. 1,5 ml of the cell suspension was then transferred into 2 ml Cryo.s™ Cryogenic Storage Vials, (Greiner, Cat.-No.: 122 263). The presence of DMSO in the freezing solution prevents the formation of ice crystals within cells. Freezing of cells has to be a slow process, therefore the freezing tubes were deposited in a freezing container (NALGENE, Cat.-No.: 5100-0001) and kept overnight in a refrigerator at -80°C. The day after, stock tubes were transferred to a low-temperature freezer (Hereaus) (-140°C) for a long term storage.

In contrast, thawing of the cells should be fast. Therefore, cells were thawed in a 37°C water bath and resuspended in 10 ml of warm cell culture medium appropriate for the cell type. The cells were then pelleted by centrifugation at 200 x g for 5 minutes. After aspiration of the supernatant, a cell pellet was resuspended in 12 ml culture medium and was then redistributed in a standard tissue culture flask with filter (75cm^2 or 150cm^2) or a Corning® CellBIND® Surface Culture Dish (55 cm^2) for a primary keratinocytes.

5. Stimulation of the cells.

For synthetic ligand stimulation HaCaT cells were seeded 5x10^5 or 1x10^6 (Ca-Free medium) cells per 57cm^2 dish and incubate overnight at 37°C in a humidified atmosphere of 5% CO_2. HaCaT cells as well as primary keratinocytes were stimulated with 1µM L-165041 or 5µM GW501516, for a time period specified in results. Respective cell culture medium with fresh stimulant was changed every 24h. Control cells received a similar volume of vehicle (DMSO).

Selective PPARδ agonist L-165041 (Calbiochem, Cat.-No.: 422175) or GW501516 (AXXORA, Cat.-No.: ALX-420-032-M001) was diluted in DMSO under

N_2 atmosphere, aliquoted and stored up to 3 months at -20°C. Cycloheximide was purchased from Sigma (Cat.-No.: C4859-1ML) as a ready made 100mg/ml stock solution in DMSO and diluted to working concentration of 5 µg/ml directly before use.

Reagents for keratinocytes stimulation.

Reagent	Working concentration	Stock solution concentration	Dilution
L-165041	1µM	2mM 5mg in 5ml DMSO	1:2000
GW501516	5µM	10mM 1mg in 220µl DMSO	1:2000
Cycloheximide	5µg/ml	100mg/ml	1:20 000
DMSO	0,05%	100%	1:2000

6. SDS-polyacrylamide-gel electrophoresis (SDS-PAGE).

Preparation of nuclear and cytoplasmic protein extracts from primary human keratinocytes or HaCaT keratinocytes was performed with NE-PER Nuclear and Cytoplasmic Extraction Reagents (PIERCE, Cat.-No.: 78833) according to manufacturer instructions. Protein concentrations were determined using BCA Protein Assay Kit (PIERCE, Cat.-No.: 23225). Protein extracts were aliquoted, shock-frozen in liquid nitrogen and stored at -80ºC prior to SDS-PAGE.

For the proteins separation according to their molecular weights, polyacrylamide gels were prepared using a Mini-PROTEAN 3 electrophoresis system (BioRad, Cat.-No.: 165-3301). Separating gels were poured in between a short plate and a spacer plate, covered with bidest H_2O and allowed to polymerize for 45 min at RT. Afterwards, the water was removed with Whatman filter paper. The stacking gel was then added on top of the separating gel, and a comb was incorporated in between the 2 glass plates to form the wells. The stacking gel was allowed to polymerize for 30 min. Protein samples were mixed with Tricine Sample Buffer (BioRad, Cat.-No.: 161-0739) supplemented with 2% (v/v) β-Mercaptoethanol (Sigma, Cat.-No.: M7154-25ML) and denatured for 10 min

at 95°C before loading. 20µg of protein or 0,5µl of MagicMark™ XP Western Protein Standard (Invitrogen, Cat.-No.: LC5602) was loaded per line and separated in 7,5% acrylamide-SDS minigel for ~30min at 10mA, than for ~1 h, 15 mA.

Reagents and solutions for SDS-PAGE.

10% APS, store at -20°C

Ammonium persulfate for electrophoresis, ≥98% Sigma, Cat.-No.: A3678-100G

To prepare stock solution dissolve 5g APS in 50 ml H_2O.

Aliquot: 1 ml

Separating Buffer	**(500ml)**
1,5 M Tris-HCl,	(90,85 g TRIS Trizma base Sigma, Cat.-No.: T1503-1KG)
Adjust: pH = 8,8	
add H_2O bidest. to 500 ml	

Stacking Buffer	**(500ml)**
0,5 M Tris-HCl,	(30,28 g TRIS Trizma base Sigma, Cat.-No.: T1503-1KG)
Adjust: pH = 6,8	
add H_2O bidest. to 500 ml	

10 x Electrophoresis buffer	**(1 litre)**
230 mM TRIS-HCl	(28 g TRIS Trizma base Sigma, Cat.-No.: T1503-1KG)
1,9 M Glycine	(142,6 g Glycine Sigma, Cat.-No.: G8898-1KG)
2%SDS	(100 ml 20% SDS Solution Serva, Cat.-No.: 20767.03)
pH=8,3 without adjustment	
add H_2O bidest. to 1000 ml	

Gels for SDS-PAGE.

	Separating Gel 15 ml	
	7,5%	**10%**
H_2O	7,8 ml	7 ml
Separating Buffer (1,5 M Tris-HCl, pH = 8,8)	4 ml	4 ml
40% Acrylamid 37:1 ratio	2,8 ml	3,7ml
10% SDS	150 μl	150μl
TEMED	10 μl	10 μl
10% APS	200 μl	200 μl

	Stacking Gel ~ 3,5%		
	~ 5 ml	**~ 10 ml**	**~ 20 ml**
H_2O	3 ml	6 ml	12 ml
Stacking Buffer (0,5 M Tris-HCl, pH = 6,8)	1,2 ml	2 ml	5 ml
40% Acrylamid 37:1 ratio	0,5 ml	1 ml	2 ml
10% SDS	50 μl	100 μl	200 μl
TEMED	5 μl	10 μl	20 μl
10% APS	100 μl	200 μl	400 μl

7. Western blotting.

The resolved proteins were transferred from the SDS gel by elcetroblotting. Semi-dry blotting on cellulose nitrate membrane (Protran BA 85, Schleicher&Schuell, Cat.-No.: 10 402 594) was committed by 1 mA/cm^2 for 1 hour. Protein transfer was confirmed with Ponceau S staining. After washing twice with H_2O, the membrane was incubated with gentle agitation throughout all steps. Subsequently, the membrane was blocked for 30 min. with 4% non-fat milk in TBST (Tris-buffered saline with 0,05% Tween 20) at RT, to reduce unspecific binding. Next, the membrane was incubated at 4°C overnight with primary antibody, diluted in blocking buffer as specified in the table "Antibodies". For a peptide competition experiment the anti-PPARδ antibody was preincubated for 30 min with PPARδ blocking peptide (Cayman Chemical, Cat.-No.:

10006247) in a 1:2 (v/v) ratio with occasional mixing prior to further dilution and application on a membrane.

After the overnight incubation the membrane was removed to a clean container, washed 4 times for 10 min with TBST and incubated with appropriate horseradish peroxidase conjugated secondary antibodies diluted in blocking buffer. Incubation with appropriate secondary antibody was carried for 1 hour at room temperature, than the membrane was washed as previously and the protein bands were detected with ECL Plus Western Blotting Detection System from Amersham Biosciences (Cat.-No.: RPN2 132) and analyzed with Luminescent Image Analyzer (Fujifilm).

Reagents and solutions for Western blotting.

1xTBST **(1 litre)**

1xTBS (1l TBS)

0,05% (v/v) Tween 20 (0,5 ml Tween 20 Serva, Cat.-No.: 37470)

10xTris-Buffered Saline (TBS) **(2 litres)**

100mM TRIS-HCl (24,23g TRIS Trizma base Sigma, Cat.-No.: T1503-1KG)

1500mM NaCl (175,32g Sodium chloride (NaCl),Sigma, Cat.-No.: S7653-1KG)

Adjust: pH=7,6

add H_2O bidest. to 2000 ml

10xPonceau S Staining Solution **(100 ml)**

2% (w/v) Ponceau S (2g Ponceau S Sigma, Cat.-No.: P 3504-10G)

30% (v/v) TCA (30g Trichloroacetic acid Sigma, Cat.-No.: T9159-500G)

30% sulfosalicylic acid (30g 5-Sulfosalicylic acid hydrate Sigma, Cat.-No.: 390275-500G)

add H_2O bidest. to 100 ml

Blocking Buffer

4% Non-Fat Dry Milk in TBST

4 g Non-Fat Dry Milk **(**BioRad, Cat.-No.: 170-6404)

dissolve in 100 ml TBST

1xBlot Buffer	**(1 litre)**
25mM TRIS-HCl	(3g TRIS Trizma base Sigma, Cat.-No.: T1503-1KG)
150mM Glycine	(11,3g Glycine Sigma, Cat.-No.: G8898-1KG)
10% (v/v) Methanol	(100 ml Methanol Baker, Cat.-No.: 8045)
pH=8,3 without adjustment	
add H_2O bidest. to 1000 ml	

Antibodies.

Primary antibodies				
Antigen	Source/Isotype	Manufaturer	Catalog number	Dilution
β-Catenin	goat anti-human, polyclonal affinity purified IgG	Santa Cruz Biotechnology, Inc	sc-1496	1:1000
HB-EGF	goat anti-human, polyclonal affinity purified IgG	R&D Systems, Inc.	AF-259-NA	1:500
p65/RelA	goat anti-human, polyclonal affinity purified IgG	Santa Cruz Biotechnology, Inc	sc-109G	1:1000
PPARδ	rabbit anti-human, polyclonal purified IgG	Cayman Chemical	101720	1:300
Secondary antibodies				
Antigen	Source/Isotype	Manufaturer	Catalog number	Dilution
anti-goat	horseradish peroxidase conjugated IgG	Santa Cruz Biotechnology, Inc	SC-2922	1:6000
anti-rabbit	horseradish peroxidase conjugated IgG	Amersham Biosciences	NA934	1:2000

8. Lipofectamine transfection of siRNA.

The c-jun and junB knock down in HaCaT keratinocytes was facilitated by direct transfection of specific siRNA oligonucleotides into the cells. In parallel with every experiment the control transfection with BLOCK-iT™ fluorescent oligo (Invitrogen, Cat.-No.: 2013) was performed to ensure high transfection efficiency. Similar experiment were also performed using siRNA oligonucleotides inserted into lentiviral construct to control knock down efficiency in cell line prior to primary keratinocytes experiments.

Protocol for lipofectamine transfection of siRNA.

1st Day – Seeding HaCaT keratinocytes
18h before transfection (16:00)

Set the $5x10^4$ cells per well, on the 6-well plate. Use RPMI 1640 medium **without antibiotics**

2nd Day – Lipofectamine mediated transfection.
2-4h before transfection (8:00)

Wash with PBS
+1,5ml RPMI 1640 medium **without antibiotics**

Prepare siRNA/Lipofectamine 2000 mix

- **8 µl Lipofectamine 2000** + 250 µl Optimem
  mix gently, 5 min, RT
- **2,5 µl siRNA** oligo (100 pMol/µl) + 250 µl Optimem
- Mix Optimem/Lipofectamine 2000 and Optimem/ siRNA
  mix gently, 20 min, RT

TRANSFECTION (10:00)

Add slowly, drop wise siRNA/lipofectamine mix to cell culture medium

4h after transfection (14:00)

Wash with PBS

+2 ml RPMI 1640 medium **without antibiotics**

Stimulation. If necessary add synthetic PPARδ agonist or CHX.

3rd Day - RNA or/and protein extraction.

30h after transfection (16:00)

26h after stimulation (16:00)

RNA or/and protein extraction

siRNA oligonucleotides sequence.

Number	Name	Sequence
-	β-Catenin-siRNA	gcugaaacaugcaguuguatt
169	cJun-siRNA-	gucaugaaccacguuaaca
170	JunB-siRNA	gacgacucauacacagcua
120	PPAR	CCACUACGGUGUUCAUGCATT

9. Reverse transcription-polymerase chain reaction (RT-PCR).

RNA extraction was performed with NucleoSpin® RNA II Kit **(**MACHEREY-NAGEL, Cat.-No.: MN-740955/25) according to manufacturer instructions. All reagent used for cDNA synthesis (SuperScript II Reverse Transcriptase, dNTP mix, RnaseOut, Oligo(dT)$_{12\text{-}18}$ Primer, DTT) were products of Invitrogen. For each cDNA synthesis a control reaction with no reverse transcriptase present was executed in parallel. For PCR reaction GoTaq DNA Polymerase (Promega Corporation, Cat.-No.: M3005) was used at the concentration 1U/25µl reaction. The annealing temperature for all PCR reactions was 55ºC, except for PPARδ where it was 54ºC.

Protocol for First-Strand cDNA Synthesis.

2x (40µl)	3x (60µl)
0,5-1 µg RNA	2-3 µg RNA
in 20µl DEPEC-H_2O	in 30µl DEPEC-H_2O

+2µl Oligo(dT)$_{12-18}$ Primer	+2µl Oligo(dT)$_{12-18}$ Primer
+2µl 10mM dNTP Mix	+3µl 10mM dNTP Mix

➢➢ 5 min, 65°C

On ice

+8µl 5x First-Strand Buffer	+12µl 5x First-Strand Buffer
+4µl 0,1 M DTT	+6µl 0,1 M DTT
+2µl RNaseOUT	+2µl RNaseOUT

➢➢ 2 min, 24°C

(-9 µl Mix for Contorol)

+1µl SuperScript™ II RT	+2µl SuperScript™ II RT

➢➢42°C for 50 min

➢➢70°C for 15 min

Store cDNA at -20°C

Protocol for Polymerase Chain Reaction (PCR).

Pre-Mix

(1 sample)

17,5µl	H_2O
5µl	5X Green GoTaq Buffer
0,5µl	10mM dNTP Mix
0,25µl	GoTaq DNA Polymerase

23,5µl	Pre-Mix
+0,5 µl	PrimerMix (50pmol/µl each primer)
+1 µl	cDNA (~ 100 ng)

Cyclig conditions:

Initial denaturation	1. 95,0°C for 2:00	
Denaturation	2. 95,0°C for 0:30	
Annealing	3. 55,0°C for 0:30	(54°C for PPARδ primer)
Elongation	4. 72,0°C for 1:30	
Cycling	5. go to 2 for 29 times	(cycle number is primer specific)
Final elongation	6. 72,0°C for 7:00	
Storage	7. 4,0°C forever	

Reagents for RT-PCR.

Reagent	Manufaturer	Catalog number
100 mM dNTP Set, PCR Grade	Invitrogen	10297-018
GoTaq DNA Polymerase	Promega Corporation	M3005
Oligo(dT)$_{12-18}$ Primer	Invitrogen	18418-012
RNaseOUT™ Ribonuclease (RNase) Inhibitor	Invitrogen	10777019
SuperScript™ II Reverse Transcriptase	Invitrogen	18064-071

Primer sets for RT-PCR.

Name	Forward primer	Reverse primer	Number of cycles	Amplicon length
ACADVL	ggaagcaagctttggatcag	ccctggtccatgttagcact	30	488 bp
β-Catenin	gcttggttcaccagtggatt	gagtcccaaggagaccttcc	20	484 bp
c-Jun	ggtatcctgcccagtgttgt	gacttctcagtgggctgtcc	25	440 bp
CIDE-A	actctggtgctggaggaaga	ccctatccacacgtgaacct	30	443 bp
GAPDH	gtcagtggtggacctgacct	aggggtctacatggcaactg	20	420 bp
HB-EGF	ggtggtgctgaagctctttc	cccatgacacctctctccat	25	418 bp
JunB	cccagctcaaacagaaggtc	ttccacagtacggtgcagag	25	466bp
PPARδ	aactgcagatgggctgtaac	gtctcgatgtcgtggatcac	30	483 bp

10. Quantitative real-time polymerase chain reaction (qRT-PCR).

For quantitative real-time PCR, cDNA synthesis was performed using Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV-RT) from EPICENTRE, Cat.-No.: M6125H. Amplification was performed in the ABI PRISM® 7700 Sequence Detection System (Applied Biosystems) using TaqMan® Gene Expression Assays (Applied Biosystems) according to manufacturer instructions. Reactions were performed in triplicate and expression levels normalized to HPRT.

Primer sets for qRT-PCR.

Name	Exon Boundary	Assay Location	Catalog number
CIDE-A	3 - 4	461	Hs00154455_m1
HB-EGF	3 - 4	660	Hs00181813_m1
HPRT	6 - 7	564	Hs99999909_m1
PPARδ	3 - 4	385	Hs00602622_m1

11. Fluorescence-activated cell sorting (FACS) analyses.

Fluorescence Activated Cell Sorting (FACS) analysis was used to determine infection ratio for lentiviral-based knock out experiments. Enhanced Green Fluorescent Protein (E-GFP) reporter expression was used also for the virus titration. See "Virus production" section of this thesis. Briefly, the adherent cells we mobilized with trypsin and after trypsin inactivation washed with D-PBS. The cell pellet was than suspended with 400 µl of PBS and stored on ice prior to FACS analysis, which was performed immediately (less than an hour after cell trypsinization). The instrument's voltage and gain settings were set according to the GFP-negative control. Each experiment represents 50 000 total events counted. The mean fluorescence index and the percentage of GFP-positive cells were calculated by using CellQuestTM software (Becton Dickinson).

12. Lentiviral-based RNAi.

Knock down of β-Catenin or PPARδ in primary keratinocytes or HaCaT keratinocytes was facilitated by lentivirus infection. Lentiviral constructs harboring a β-catenin-specific siRNA sequence or two alternative PPARδ-specific siRNA target sequences were engineered using pLL3.7 or pLVTHM backbone. For details see the table "Lentiviral transfer vectors" below. Parent plasmid served as control in each experiment. Packaging vectors: pPAX2 and pMD2.G for 2nd generation packaging was

kindly provided by Didier Trono. Production of VSV-G pseudotyped replication-deficient lentivirus was carried out according to (al Yacoub, 2007). Lentivirus infection was performed at a multiplicity of infection (M.O.I.) of three for 12h at 37°C as detailed in section "Culture of primary neonatal mouse keratinocytes – Lentivirus infection" of this thesis.

Lentiviral transfer vectors.

Plasmid name		RNAi sequence	Reference
Insert	Backbone		
PPARδ	pLL3.7	**GTCGTACGATCCGCATGAA**	(Rubinson *et al.*, 2003)
PPARδ	pLVTHM	**CCACTACGGTGTTCATGCA**	(Szulc *et al.*, 2006)
β-Catenin	pLL3.7	**GCTGAAACATGCAGTTGTA**	(Rubinson *et al.*, 2003)

Cloning of shRNAi sequence into lentiviral transfer vectors.

shRNAi sequence specific to β-Catenin or PPARδ were design according to a scheme (see below), synthesized in both strands (5'-Phosphorylated), annealed, and cloned immediately downstream of the U6 promoter using HpaI/XhoI restriction sites of pLL3.7 transfer vector. An alternative shRNAi sequence for PPARδ (CCACTACGGTGTTCATGC) was synthesized analogously with MluI and ClaI restriction sites and cloned between under the control of H1 promoter of pLVTH.

Design shRNA according the scheme:

pLVTHM

```
 MluI          19nt sense siRNA         Loop     19nt anti-sense siRNA   STOP     ClaI
CGCGTCCCCNNNNNNNNNNNNNNNNNNNTTCAAGAGANNNNNNNNNNNNNNNNNNNTTTTGGAAAT
    AGGGGNNNNNNNNNNNNNNNNNNNAAGTTCTCTNNNNNNNNNNNNNNNNNNNAAAACCTTTAGC
```

pLL3.7

```
HpaI   19nt sense siRNA      Loop      19nt anti-sense siRNA   STOP    XhoI
 TNNNNNNNNNNNNNNNNNNNTTCAAGAGANNNNNNNNNNNNNNNNNNNTTTTTTGGAAG
 ANNNNNNNNNNNNNNNNNNNAAGTTCTCTNNNNNNNNNNNNNNNNNNNAAAAAACCTTCATTG
```

Annealing:

1 µl sense primer (100pmol/µl)

1 µl anti-sense primer (100pmol/µl)

4 µl 5x annealing buffer

14µl H_2O

Annealing cycling conditions:

1. 95,0°C for 4:00
2. 70,0°C for 10:00
3. 69,0°C for 1:00
   -1,0°C per cycle
4. go to 3 for 65 times
5. 4°C for 10:00
6. END

5X siRNA Annealing Buffer (100ml)

30 mM HEPES	0,71g HEPES Sigma, Cat.-No.: H3375-1KG
100 mM KAc	0,98g Potassium Acetate Sigma, Cat.-No.: P1190-500G
2 mM MgAc	43mg Magnesium acetate tetrahydrate $(CH_3COO)_2Mg•4H_2O)$ Sigma, Cat.-No.: M5661-250G

Adjust: pH=7,4

add H_2O bidest. to 100 ml

After preparative digestion, the vector has to be dephosphorylated with alkaline phosphatase to prevent its recircularization.

Dephosphorylation

50µl DNA

+38µl H_2O

+10µl 10X Reaction Buffer for SAP

+2µl SAP (Shrimp Alkaline Phosphatase (SAP), Fermentas Cat.-No.: EF0511)

➢➢ 30 min, 37°C

➢➢ 15 min, 65°C

Ligation

Insert + Vector ≤ 0,2 µg total DNA

Insert + Vector ≤ 17µl

x µl Vector

x µl Insert (10 fold more than Vector - molar ratio

+ H_2O to 17µl

➢➢ 5 min, 45°C

➢➢ On ice

+2µl 10X T4 DNA Ligase Reaction Buffer

+1µl T4 DNA Ligase **(**T4 DNA Ligase New England BioLabs, Cat.-No.: M0202T)

➢➢ 16°C over night (16-20 h)

13. Virus production.

All vectors used for virus production were purified with the Jetstar kit (Genomed) and resuspended in TE buffer. Plasmid preparations with absorbance (260/280) ratios below 1.8 were not used for transfection. Production of VSV-G pseudotyped replication-deficient lentivirus was carried out according to (Mitta et al., 2005) modified as detailed in (al Yacoub, 2007).

Protocol for virus production.

1st Day - Plate the cells

Afternoon: plate 5,6-5,8 x10^6 cells/plate (100/20 mm Cellstar plate) producer HEK cells **293FT/17** (passage below 15!) in 6 ml normal medium (D-MEM, 4500 mg/L Glucose with GlutaMaxI)

Take HEPES-H_2O, 2xHBS Buffer, 2,5 M $CaCl_2$ out of -20°C and let it unfreeze overnight at RT

When required, unfreeze HeLa cells.

2nd Day – Transfection

8:00 Change medium 1-4 h before transfection

remove and discard

+ 6 ml Advanced DMEM with supplements pro plate

9:00 Prepare 1200 µl of transfection mix:

transfer plasmid ≥ 10kb	transfer plasmid ≤ 10kb
pIGETOn	pLL3.7
+36 µg transfer plasmid	18 µg transfer plasmid
+18 µg psPAX2	9 µg psPAX2
+18 µg P4 (pMD2.G)	9 µg P4 (pMD2.G)
+ HEPES-H_2O to 540 µl	
+ 600 µl 2xHBS Buffer	

➢ intensely mix

60 µl 2,5 M $CaCl_2$ **add slowly, dropwise**

➢ gently mix

➢➢ 15-25 min, RT

+ 3,6 µl 50 mM Chloroquine (1:2000)

➢ mix and add dropwise to the plate

➢➢ incubate the plates : 5h, 37°C, 5% CO_2

15:00 Change medium 5-6 h after transfection

remove and discard

+ 8 ml Advanced DMEM with supplements pro plate

4th Day – Collect the 1st Supernatant, Plate HeLa cells

Collect the supernatant from each virus type in separate falcon tube
+ 6 ml Advanced DMEM with supplements pro plate

↺ 5min, 1500 RPM, RT

Filtrate with 0,45 µm filter (Stericup and Sreritop 250 ml, 73 mm/.45 µm MILLIPORE, Cat.-No.: SCHV U02 RE or Disposable Filter Unit 0,45µm Whatman Cat.-No.: 10 462 100)

Make aliquots ca. 3 ml in Cryo tubs
(NAME of the virus, date of **2nd Day – Transfection**), store at -80°C
Make 1 aliquot ca. 50 µl of each virus type for titration, store at -20°C

Plate $8x10^4$ HeLa cells pro 1 well in 6 well plate (4 well pro virus + 2 well controls)

5th Day – Collect the 2nd Supernatant, Infecion of HeLa cells

Collect the supernatant from each virus type in separate falcon tube

↺ 5min, 1500 RPM, RT

Filtrate with 0,45 µm filter

Make aliquots ca. 3 ml in Cryo tubs

(NAME of the virus, date of **2nd Day – Transfection**), store at -80°C

Change medium in 6-well plates with HeLa, add 2 ml of fresh RPMI

Count 1 well of HeLa cells :

remove and discard

+1 ml PBS

remove and discard

+500 µl Trypsin

➢➢ incubate the plates : ca.7-10 min, 37°C, 5% CO_2

+1500 µl RPMI

count cells in 4 squares

$\frac{cells}{4}$ x10^4 = cells/ml x2 = **HeLa cell number in the day of infection (write down!)**

+2µl of 8 mg/ml polybrene to each well (1:1000)

+2 µl of **1st Supernatant** 1:1000 well

+4 µl of **1st Supernatant** 1:500 well

+ 10 µl of **2nd Supernatant** 1:200 well

+ 20 µl of **2nd Supernatant** 1:100 well

➢➢ incubate the plates : 48h, 37°C, 5% CO_2

7th Day –Virus titration, FACS

Trypsynize HeLa cells, suspend in 400 µl of FACS buffer

FACS

Count the number of virus/ml

Ultracentrifugation.

Concentration of lentiviral stocks was performed both to increase titers and to remove impurities for sensitive *in vivo* applications. Ultracentrifugation was carried out in a Beckman Optima LE-80 K ultracentrifuge using a SW28 rotor. 5 ml of sterile 20% (w/v) sucrose in TNE buffer was filled in polyallomer conical tubes (Beckman Cat.-No.: 358126) and gently overlayed with 25 ml virus supernatant. Centrifugation was carried at 12000 RPM (25700 x g RCF) for 4 hours. The initial acceleration mode was inactivated and "no breaks" mode activated .

Reagents for virus production.

20% (w/v) sucrose in TNE buffer
20g sucrose (Sucrose Sigma, Cat.-No.: S9378-1KG)
add TNE buffer to 100 ml
Filtrate with 0,2 µm filter

TNE buffer, store at 4ºC	**(500ml)**
50mM Tris-HCl, pH= 7,4	25ml 1 M Trizma® hydrochloride solution pH 7.4 (Sigma Cat.-No.: T 2663-1L)
100mM NaCl	50 ml 1M NaCl stock solution
0,5 mM EDTA	500µl 0,5M EDTA stock solution (Sigma Cat.-No.: E7889-100ML)

Autoclave, store at 4ºC

1M NaCl stock solution (500ml)
29,22 g NaCl (Sodium chloride (NaCl), Sigma, Cat.-No.: S7653-1KG)
add H_2O bidest. to 500 ml

Producer cells: **FT293/T17** - all cells should be healthy, low passage count (passage below 15!)

500ml **Advanced DMEM**: GIBCO, Case order nr. 12491-023 (10x500ml)

Supplements:

+0,01 mM Egg lecithin (1 ml of 5 mM stock solution: 0,2g lecithin)
(Lecithin from egg yolk Serva, Cat.-No.: 27608) in 50 ml PBS)

+4 mM GlutaMAX (10ml of 200 mM stock solution)
(GIBCO Cat.-No.: 35050-038)

+2% FCS (10 ml FBS Biochrom AG, Cat.-No.: S 0115)

+1x chemically defined lipid concentrate
(5 ml of GIBCO Cat.-No.: 11905-031, **keep under N_2**)

+0,01 mM Cholesterol (500 µl of 10 mM stock solution: 30mg pack
(Sigma Cat.-No.: C4951-30mg) + 7,5 ml sterile PBS)

TRANSFECTION mix solutions

All transfection mix solutions should be stored at -20°C, and transfered on Day 1st into RT

HEPES-H_2O

2,5mM HEPES, pH = 7,3
50 ml H_2O bidest.
+125 µl 1M HEPES, pH = 7,3

1M HEPES, pH = 7,3 (500ml)
154,15g HEPES (HEPES Sigma, Cat.-No.: H3375-1KG)
400 ml H_2O bidest.
Adjust: pH= 7,3
add H_2O bidest. to 500 ml

2,5 M $CaCl_2$

184 g $CaCl_2 \cdot 2\ H_2O$
(Calcium chloride dihydrate ($CaCl_2 \cdot 2\ H_2O$) Sigma Cat.-No.: 21098-500G-F)
add H_2O bidest. to 500 ml
Filtrate with 0,2 µm filter

2xHBS Buffer	**(500 ml)**
0,27 M NaCl	8g NaCl
	(Sodium chloride (NaCl), Sigma, Cat.-No.: S7653-1KG)
10 mM KCl	0,38g KCl
	(Potassium chloride (KCl) Sigma, Cat.-No.: P9333-1KG)
1,4 mM Na_2HPO_4	0,1g Na_2HPO_4
	(Sodium phosphate dibasic (Na_2HPO_4) Sigma, Cat.-No.: S7907-1KG)
42mM HEPES	5g HEPES (HEPES Sigma, Cat.-No.: H3375-1KG)
10 mM Glucose	1g Glucose (D-(+)-Glucose Sigma, Cat.-No.: G8270-1KG)

Adjust very carefully: pH= 7,05

add H_2O bidest. to 500 ml

Filtrate with 0,2 µm filter

50 mM Chloroquine stock solution, store at 4°C, in dark

Chloroquine diphosphate salt (Sigma Cat.-No.: C6628-25G)

working solution - 25µM (1:2000)

8 mg/ml polybrene stock solution, store at -20°C

Hexadimethrine bromid (Sigma-Aldrich Cat.-No.: 10,768-9, 10g)

working solution - 8 µg/ml (1:1000)

14. Expression profiling.

Primary keratinocyte culture and stimulation with L-165041, was performed as detailed above. Total RNA was preparatcd and purified with NucleoSpin® RNA II Kit (MACHEREY-NAGEL, Cat.-No.: MN-740955/250). Samples were labelled either with Cy3 or Cy5, hybridized to Piquor SkinPatho microarrays (Memorec), and scanned by the Charité in-house microarray service facility. Dye swab experiments were performed to rule out uneven green/red intensities. Data with at least twice background intensity and the percentage coefficient of variation of less than 30% between the four replicas on each array were further analyzed by applying threshold of three standard – deviations of the mean change across all genes.

15. Cloning strategy for 5LN-33 CAM-PPARδ-pIGETOn.

Backbone: pTK272, from Dr. Tal Kafri,

Synthetic tetracycline-controlled transactivator: rtTA2^{S}-M2, from Dr. Christian Berens

Name	Date	Concentration	Remarks
pTK 272	29.08.2005	1,245 μg/μl	From Dr. Tal Kafri SEQUENCED 01.09.2005
pUHrT62-1	04.12.2004	0,197 μg/μl	From Dr. Christian Berens

VP16: the transactivation domain (78 amino acid residues, corresponding to 413–490 bp) of VP16 amplified from Herpes simplex virus cDNA

2AN. 2AN **AgeI**-kozak-**start**-VP16-**PacI**

Amplify VP16 fragment form HSV cDNA

Template: VP16 DNA

Primers: 291+292

Primers with AgeI-kozak – start-vp16-**PacI-**

2A AgeI-kozak–start-vp16-**PacI- = 253bp insert**

Number	Name	Sequence
291	VP16 for	ccggtGcCACCatggcccccccgaccgatgtcagc
292	VP16 rev	taacccaccgtactcgtcaattcc

Cyclig conditions:

1. 95,0°C for 2:00
2. 95,0°C for 0:30
3. 55,0°C for 0:30
4. 72,0°C for 1:00
5. go to 2 for 40 times
6. 72,0°C for 10:00
7. 4,0°C forever

~250bp PCR fragment extracted from 2% agarose gel and digested with **AgeI**/**PacI** (Buffer1, BSA)

Mouse PPARδ: M-PPARD/pBluescript KS +, cDNA clone MGC:86084 IMAGE:5694282, complete cds

Name	Date	Concentration	Remarks
2/77 pm PPARd tsp Bcl-TAA	31.03.2005	1,05 µg/µl	**SEQUENCED** 25.04.2005 **OK**

EMCV IRES-EGFP: WNT5aNoSP-IRES EGFP/pLenti6 plasmid

2A. Mutagenesis: 2A pUHrT62-1 **BspEI**

Site-directed mutagenesis of pUHrT62-1 to introduce the **BspEI** restriction site upstream -hCMV-rtTA2^{S}-M2

Template: pUHrT62-1 MaxiPrep 04.12.2004 0,197 µg/µl

Primers: 19+20

Number	Name	Sequence
19	Xho I/BspE I For	cctttcgtc**tc**cgg**ga**gagcttggcccattgcatacgttg
20	Xho I/BspE I Rew	caacgtatgcaatgggccaagctc**tc**cgg**ga**gacgaaagg

Check point – restriction: 2A-6 pUHrT62-1 **BspEI**

BspEI/BamHI 1530bp, 2664bp (NEBuffer 3 + BSA at 37°C)

2BT. Mutagenesis: 2BT pUHrT62-1 **BspEI**-XhoI

Site directed mutagenesis of 2A-6 pUHrT62-1 **BspEI** to introduce the XhoI restriction site downstream rtTA2^{S}-M2

Template: 2A-6 pUHrT62-1 **BspEI** MaxiPrep 18.01.2005 0,25 µg/µl

Primers: 153+154

Number	Name	Sequence
153	2BT for	ccccgggtaactaagtaaggCtcGagacatgataagatac
154	2BT rev	gtatcttatcatgtct**CgaGcc**ttacttagttacccgggg

Check point – restriction: 2BT-1 pUHrT62-1 **BspEI**-XhoI

BspEI/XhoI 2682bp, 1512bp (NEBuffer 3 + BSA at 37°C)

3T. Preparative digestion: 3T **BspEI**-hCMV-rtTA2^{S}-M2- XhoI

Preparative digestion of 2BT-1 pUHrT62-1 **BspEI**-XhoI with **BspEI**/XhoI

2682bp + **1512bp** = 4194 bp

Template: 2BT-1 pUHrT62-1 BspEI-XhoI MaxiPrep 22.07.2005 0,641 µg/µl

Restriction: **BspEI**/XhoI (NEBuffer 3 + BSA at 37°C)

4A. Preparative digestion:4A pTK 272 cut **BspEI**/XhoI

Preparative digestion of pTK 272 with **BspEI**/XhoI

8808bp+1877bp = 10685 bp

Template: pTK 272 MaxiPrep 25.07.2005 0,413 µg/µl

Restriction: **BspEI**/XhoI (NEBuffer 3 + BSA at 37°C)

4LT. Ligation: 4LT EGFP/rtTA

vector: 4A pTK 272 cut **BspEI**/XhoI **(8808bp)**

insert: 3T **BspEI**-hCMV-rtTA2^{S}-M2- XhoI **(1512bp)**

Check point – restriction: 4LT-5 EGFP/rtTA **BspEI**/XhoI 8808bp, 1512bp (NEBuffer 3 + BSA at 37°C)

8BT. Preparative digestion: 8BT **AgeI**-rtTA-**BspEI** (**9609bp**)

Preparative digestion of 4LT-5 EGFP/rtTA with **BspEI** and **Age I** sequencial:

9609bp +730bp = 10339 bp

Template: 4LT-5 EGFP/rtTA, MaxiPrep 03.08.2005 1,093 µg/µl

Restriction: **Age I** (NEBuffer 1 + BSA at 37°C)

BspE I (NEBuffer 3 + BSA at 37°C)

16M. Mutagenesis:16M SpeI-M-PPARδ/pBluescript KS+

Site directed mutagenesis of M-PPARδ/pBluescript KS+ to introduce the SpeI restriction site upstream M-PPARδ.

Template: 5C 5ng 1 M-PPARD/pBluescript KS +AgeI-BspEI-NheI,
MaxiPrep 21.06.2005 0,59 µg/µl

Primers: 195+196

Number	Name	Sequence
195	SpeI -M-PPARδ F	ctgcgctcagaccca**ACtAgt**ggcagagctatgacc
196	SpeI -M-PPARδ R	ggtcatagctctgcc**acTaGT**tgggtctgagcgcag

Check point – restriction: 16M-7 SpeI-M-PPARδ/pBluescript KS+
SpeI/**NheI** 1382bp, 3245bp (NEBuffer 2 + BSA at 37°C)

17A. Preparative digestion: 17A SpeI-**AgeI**-M- PPARδ-**NheI**
Preparative digestion of 16M-7 SpeI-M-PPARδ/pBluescript KS+ with SpeI/**NheI**
3245bp + **1382bp** + 300bp = 4927bp

Template:16M-7 SpeI-**AgeI**-M-PPARδ-**NheI**-**BspEI**/pBluescript KS+
MiniPrep 23.01.2006

Restriction: SpeI/**NheI** (NEBuffer 2 + BSA at 37°C)

5A. Mutagenesis: 5A **AgeI**-M-PPARδ/pBluescript KS+

Site directed mutagenesis of M-PPARδ/pBluescript KS+ to introduce the **AgeI** restriction site upstream M-PPARδ.

Template: 2/77 pm PPARd tsp Bcl-TAA, MaxiPrep 31.03.2005 1,05 µg/µl

Primers: 21+31

Number	Name	Sequence
21	PPAR-Age I	cgccaagtgggg**ac**c**g**gtcatggaacagccacaggagg
31	PPAR-Age I Rev	cctcctgtggctgttccatgaccggtccccacttggcg

Check point – restriction: 5A5ng-2 **AgeI**-M-PPARδ/pBluescript KS+

AgeI/KpnI ~2900bp, ~1550bp (NEBuffer 1 + BSA at 37°C)

5B. Mutagenesis: 5B **AgeI**-M-PPARδ-**BspEI**/pBluescript KS+

Site directed mutagenesis of 5A5ng-2 **AgeI**-M-PPARδ/pBluescript KS+ to introduce the **BspEI** restriction site downstream **AgeI**-M-PPARδ

Template:5A 5ng-2 **AgeI**-M-PPARD/pBluescript KS +

MiniPrep 04.05.2005 0,298 µg/µl

Primers: 22+32

Number	Name	Sequence
22	RRAR-BspE I	gcagcccaggcctcccctc**c**gg**a**tctgctgggcc
32	RRAR-BspE I Rev	ggcccagcagatccggaggggaggcctgggctgc

Check point – restriction: 5B 5ng-3**AgeI**-M-PPARδ-**BspEI**/pBluescript KS+

AgeI/KpnI ~2900bp,~1550bp (NEBuffer 1 + BSA at 37°C)

Check point – sequencing

5BA. Mutagenesis: 5BA **AgeI**-M-PPARδ-**BspEI**/pBluescript KS+
Site directed mutagenesis of 5B 5ng-4**AgeI**-M-PPARδ-**BspEI**/pBluescript KS+ to repair deletion in the plasmid

Template: 5B 5ng-4**AgeI**-M-PPARδ-**BspEI**/pBluescript KS+
MiniPrep 26.05.2005 0,182 μg/μl

Primers: 21+31

Number	Name	Sequence
21	PPAR-Age I	cgccaagtgggg**acc**g**g**tcatggaacagccacaggagg
31	PPAR-Age I Rev	cctcctgtggctgttccatgaccggtccccacttggcg

Check point – restriction: 5BA-3 **AgeI**-M-PPARδ-**BspEI**/pBluescript KS+
AgeI/KpnI ~2900bp,~1550bp (NEBuffer 1 + BSA at 37°C)
Check point – sequencing

5C. Mutagenesis: 5C **AgeI**-M-PPARδ-**NheI**-**BspEI**/pBluescript KS+
Site directed mutagenesis of 5BA-3 **AgeI**-M-PPARδ-**BspEI**/pBluescript KS+ to introduce the **NheI** restriction site upstream **BspEI** restriction site.

Template: 5BA-3 **AgeI**-M-PPARδ-**BspEI**/pBluescript KS+
MiniPrep 10.06.2005 254 ng/μl

Primers:135+136

Number	Name	Sequence
135	M-PPAR-NheI for	ggacatgtac**taa**ggc**GCT**agcccaggcctcccctc
136	M-PPAR-NheI rev	gaggggaggcctgggct**AGC**gcc**tta**gtacatgtcc

Check point – restriction: 5C 5ng-1 **AgeI**-M-PPARδ-**NheI**-**BspEI**/pBluescript KS+
AgeI/**NheI** 3117bp, 1333bp (NEBuffer 1 + BSA at 37°C)

5D. Mutagenesis: 5D **NheI**-IRES-EGFP/pLenti6

Site directed mutagenesis of WNT5aNoSP-IRES EGFP/pLenti6 to introduce the **NheI** restriction site upstream IRES

Template: WNT5aNoSP-IRES EGFP/pLenti6 MaxiPrep 24.03.2005 0,182 µg/µl

Primers: 137+138

Number	Name	Sequence
137	IRES-NheI for	**catcatcatcattaGctAgCg**tggcggccg**ccccc**
138	IRES-NheI rev	gggggcggccgccac**G**c**T**ag**C**taatgatgatgatg

Check point – restriction: 5D-12 **NheI**-IRES-EGFP/pLenti6

NheI/SpeI 8233bp, 1109bp (NEBuffer 2 + BSA at 37°C)

5E. Mutagenesis: 5E **NheI**-IRES-EGFP-**BspEI**/pLenti6

Site directed mutagenesis of 5D-12 **NheI**-IRES-EGFP/pLenti6 to introduce the **BspEI** downstream EGFP.

Template: 5D-12 **NheI**-IRES-EGFP/pLenti6 MiniPrep 08.07.2005 0,31 µg/µl

Primers: 139+140

Number	Name	Sequence
139	IRES-BspEI for	cgaaggtaagccta**tccGGa**accctctcctcgg
140	IRES-BspEI rev	ccgaggagagggtt**CC**ggataggcttaccttcg

Check point – restriction: 5E-21 **NheI**-IRES-EGFP-**BspEI**/pLenti6

BspEI/EcoNI 5423bp, 3919bp (NEBuffer 3 + BSA at 37°C)

NheI/SpeI 8233bp, 1109bp (NEBuffer 3 + BSA at 37°C)

17B. Preparative digestion:17B IRES-EGFP/pLenti6 cut SpeI/**NheI**

Preparative digestion of 5E-21 WNT5aNoSP- IRES-EGFP/pLenti6 with SpeI/**NheI**

1113bp + **8271bp** = 9384 bp

Template: 5E-21 WNT5aNoSP-IRES-EGFP/pLenti6 MaxiPrep 17.07.2005 0,307 µg/µl

Restriction: SpeI/**NheI** (NEBuffer 2 + BSA at 37°C)

18L. Ligation:18L M-PPARδ-IRES-EGFP/pLenti6

vector:17B IRES-EGFP/pLenti6 cut SpeI/**NheI** **(8276 bp)**

insert: 17A SpeI-AgeI-M- PPARδ-**NheI** **(1382 bp)**

Check point – restriction: 18L-25 M-PPARδ-IRES-EGFP/pLenti6:

NheI/XhoI 8601 bp, 1014bp, (NEBuffer 2 + BSA at 37°C)

8C. Preparative digestion: 8C **AgeI**-M-PPARδ-IRES-EGFP-**BspEI**-**Age I**

Preparative digestion of 18L-25 M-PPARδ-IRES-EGFP/pLenti6 with **Age I**:

6901bp + **2714bp** = 9615 bp

Template: 18L-25 M-PPARδ-IRES-EGFP/pLenti6, MaxiPrep 27.02.2006 0,14 µg/µl

Restriction: **Age I** (NEBuffer 1 + BSA at 37°C)

8CA. Preparative digestion: 8CA **AgeI**-M-PPARδ-IRES-EGFP-**BspEI** (**2679bp**)

Preparative digestion of 8C **AgeI**-M-PPARδ-IRES-EGFP-**BspEI**-**Age I** with **BspE I**

2679bp + 35bp = 2714 bp

Template: 8C **AgeI**-M-PPARδ-IRES-EGFP-**BspEI**-**Age I**

Restriction: **BspE I** (NEBuffer 3 + BSA at 37°C)

88L. Ligation: 88L M-PPARδ/pIGETOn

vector: 8BT **AgeI**-rtTA-**BspEI** (**9609bp**)

insert: 8CA **AgeI**-M-PPARδ-IRES-EGFP-**BspEI** (**2679bp**)

Check point – restriction:

5LN-33 CAM-PPARδ/pIGETOn:

BspEI/XhoI 8397 bp, 2360bp, 1531bp (NEBuffer 3 + BSA at 37°C)

4M. Mutagenesis: 4M M-PPARδ/pIGETOn

Site directed mutagenesis of 88L-6 M-PPARδ/pIGETOn to introduce **PacI** upstream PPARδ

Template: 88L-6 M-PPARδ/pIGETOn, MaxiPrep 28.03.2006 0,134 µg/µl

Primers: 293+294

Number	Name	Sequence
293	4M PacI-For	cgaattcgatgctaccggtACGTTAATTaACgaacagccacagg
294	4M PacI-Rev	cctgtggctgttcgttaattaacgtaccggtagcatcgaattcg

Check point – restriction:

4M M-PPARδ/pIGETOn:

PacI/NheI 10965 bp, 1323bp (NEBuffer 1 + BSA at 37°C)

AgeI/XhoI 8078bp, 3891bp,319bp (NEBuffer 1 + BSA at 37°C)

5A. Preparative digestion: 5A M-PPARδ/pIGETOn cut **AgeI**/**PacI**

Preparative digestion of **4M** M-PPARδ/pIGETOn with **AgeI**/**PacI**

12283bp +13bp = 12286bp

Template: 4M-2 M-PPARδ/pIGETOn, MaxiPrep 26.02.2007 3,17 µg/µl

Restriction: **AgeI**/**PacI** (Digest in NEBuffer 1 + BSA at 37°C)

5LN. Ligation: 5LN CAM-PPARδ/pIGETOn

vector:5A M-PPARδ/pIGETOn cut AgeI/PacI dephosphorylated **(12283 bp)**

insert: 2AN **AgeI**-kozak-**start**-VP16-**PacI** **(253bp)**

Check point – restriction:

5LN-33 CAM-PPARδ/pIGETOn:

PacI/ **AgeI**	12283 bp, 253bp	(NEBuffer 1 + BSA at 37°C)
PstI	6980bp, 3949bp, 981bp, 581bp, 45bp	(NEBuffer 3 + BSA at 37°C)