Supplementary Information

Experimental

Isolation of primary keratinocytes and fibroblasts from human foreskin

Primary human keratinocytes and fibroblasts were isolated from the foreskin of circumcised 4 year old and 9 year old healthy males. The local ethical committee approved the study, and informed consent was obtained from the patients. After excess fat and blood was removed from the tissue, it was rinsed three times with PBS w/o Ca^{2+}, Mg^{2+} (Invitrogen, Karlsruhe, Germany) supplemented with 0.5% Ciprobay (Bayer, Leverkusen, Germany), and transferred to a sterile Petri-dish. For the isolation of primary keratinocytes the sample was cut into 5x5 mm pieces, and incubated in Trypsin/EDTA solution (0.05/0.02% w/v, Pan Biotech, Aidenbach, Germany) at 37°C. After 60 min the samples were transferred into a solution of PBS with 10% FCS (PAA, Coelbe, Germany) to inhibit the trypsin. The epidermis was separated from the dermis, and torn into small pieces with forceps. The cell suspension was pelleted at 1000 xg, resuspended in keratinocyte serum free medium (SFM) (Invitrogen), supplemented with pituitary gland extract, EGF (Invitrogen), and 0.5% Ciprobay (10mg/L) and plated into a T25 primary tissue culture flask. For the isolation of primary fibroblast the tissue sample was chopped into 3x3 mm pieces and gently pressed on the bottom of a T75 cell culture flask (BD Biosciences, Heidelberg, Germany). After incubation for 2 hours at 37°C and 5% CO_2 Dulbecco's Modified Eagle Medium (DMEM,high glucose) (Invitrogen) supplemented with 20% FCS (Biochrom, Berlin, Germany) , 1% Antibiotic-Antimycotic (100 x, Invitrogen) and 1% L-Glutamin (200mM, PAN Biotech) was added to the cell culture flask. After one week, the medium was removed every other day until primary fibroblasts emerge from the tissue samples. To generate cell lines fibroblasts and keratinocytes were immortalized with the HPV18 E6 and E7 genes as described.[1-4]

Stable Isotope labeling in cell culture

Primary human keratinocytes were cultured in keratinocyte SFM (-arginine, -lysins), supplemented with bovine pituitary gland extract, EGF, 0.5% Ciprobay (10mg/L), 210 mg/L L-arginine (Sigma-Aldrich), and 63 mg/L L-lysine (Sigma-Aldrich) for the control population (6 cm cell culture dishes). Cell populations were labeled for 14 days with 210 mg/L L-arginine-$^{13}C_6$-$^{15}N_4$ (Arg_{10}; Sigma-Aldrich) and 63 mg/L L-lysine-$^{13}C_6$-$^{15}N_2$ (Lys_8; Silantes, Munich, Germany).[5, 6] Cells were harvested by incubation in trypsin/EDTA for 5 min at 37°C, spun down at 300 x g for 5min, and resuspended in PBS containing 10% FCS to inhibit the trypsin. Cells were washed two times with PBS, counted six times using the CASY Cell Counter (Modell TT, Innovatis, Reutlingen, Germany), pelleted, and frozen at -80°C.

Primary human Fibroblasts were cultured in SILAC-DMEM (Thermo Fisher, Langenselbold, Germany), supplemented with with 10% FCS (PAA, Coelbe, Germany), 1% Antibiotic-Antimycotic (100 x, Invitrogen), 1% L-Glutamin (PAN Biotech), 42 mg/L L-Arginine (Sigma-Aldrich), 73 mg/L L-Lysine (Sigma-Aldrich) and 82 mg/L Prolin (Sigma-Aldrich) for the control population (6-well cell culture plate, BD Bioscience).

Cell populations were labeled up to 4 months with 42 mg/L L-Arginine-$^{13}C_6$-$^{15}N_4$ (Arg_{10}; Sigma-Aldrich), 73 mg/L L-Lysine-$^{13}C_6$-$^{15}N_2$ (Lys_8; Silantes, Munich, Germany) and 82 mg/L Prolin (Sigma-Aldrich). Cells were harvested by incubation in trypsin/EDTA for 5 min at 37°C and resuspended in DMEM containing 10% FCS to inhibit the trypsin. Cells were washed three times with PBS, counted using a neubauer counting chamber, pelleted, and frozen at -80°C.

MS sample preparation

Harvested cells were lysed in SDS-loading buffer. Benzonase (Merck, Darmstadt, Germany) was added before samples were reduced by DTT (1 mM) (Sigma-Aldrich) for 30min at 75°C and alkylated using iodacetamide (5.5 mM) (Sigma-Aldrich) for 30 min at 25°C. Protein mixtures were separated by SDS-PAGE (4-12% Bis-Tris mini gradient gel, NuPAGE (Invitrogen)), gel lanes were cut into 10 equal slices,

samples in-gel digested using trypsin (Promega, Mannheim, Germany),[7] and resulting peptide mixtures were processed on STAGE tipps as described.[8] Phosphopeptides were further enriched using TiO_2 stage-tips essentially as described.[9]

Mass spectrometry

Mass spectrometric measurements were performed on LTQ Orbitrap XL mass spectrometers (Thermo Fisher Scientific, Bremen, Germany) coupled to an Agilent 1200 nanoflow-HPLC (Agilent Technologies GmbH, Waldbronn, Germany). HPLC-column tips (fused silica) with 75 µm inner diameter (New Objective, Woburn, MA, USA) were self packed [10] with Reprosil-Pur 120 ODS-3 (Dr. Maisch, Ammerbuch, Germany) to a length of 20 cm. Samples were applied directly onto the column without pre-column. A gradient of A [0.5% acetic acid (high purity, LGC Promochem, Wesel, Germany) in water (HPLC gradient grade, Mallinckrodt Baker B.V., Deventer, Netherl, ands)] and B [0.5% acetic acid in 80% ACN (LC-MS grade, Wako, Germany)] with increasing organic proportion was used for peptide separation (loading of sample with 2% B; separation ramp: from 10% to 30% B within 80 min). The flow rate was 250 nl/min and for sample application 500 nl/min. The mass spectrometers were operated in the data-dependent mode and switched automatically between MS (max. of 1 $x10^6$ ions) and MS/MS. Each MS scan was followed by a maximum of five MS/MS scans in the linear ion trap using collision energy of 35% and a target value of 30000. Parent Ions with a charge states from z = 1 and unassigned charge states were excluded for fragmentation. The mass range for MS was m/z = 370 to 1800. The resolution was set to 60.000. Mass-spectrometric parameters were as follows: spray voltage 2.3 kV; no sheath and auxiliary gas flow; ion-transfer tube temperature 125°C.

Identification of proteins, and protein ratio assignment using MaxQuant

The MS raw data files were uploaded into the MaxQuant software [11] which performs peak detection,

SILAC-pair detection, and generates peak lists of mass error corrected peptides using the following parameters: IPI human decoy database containing common contaminants such as keratins and enzymes used for in-gel digestion (based on IPI human version 3.59, 160820 entries) was used, carbamidomethylcysteine was set as fixed modification, methionine oxidation and protein amino-terminal acetylation were set as variable modifications. Double SILAC were chosen as quantitation mode. Three miss cleavages were allowed, enzyme specificity was trypsin/P, and the MS/MS tolerance was set to 0.5 Da. Peak lists were searched by MASCOT 2.2 (Matrix Science, London, UK) for peptide identification. The average mass precision of identified peptides was in general less than 1 ppm after recalibration. Peptide lists were further used by MaxQuant to identify and relatively quantify proteins using the following parameters: peptide, and protein false discovery rates (FDR) were set to 0.01, maximum peptide posterior error probability (PEP) was set to 1, minimum peptide length was set to 6, the PEP was based on Mascot score, minimum number peptides for identification and quantitation of proteins was set to two of which one must be unique, and identified proteins have been re-quantified.

Phosphorylation sites were also assigned and quantified using MaxQuant. For further analysis only sites with a posttranslational modification localization score >0.75 (class I),[12] were considered.

Data analysis

As ratios showed a non-normal distribution (Kolmogorov-Smirnov test), identified proteins were divided into three quantiles. DAVID 6.7 BETA (Bioinformatic Database, NIAID)[13] was used to investigate protein enrichment in each quantile using respective GO terms.[14] Annotations were functionally clustered from biological processes level 4, cellular compartments level 4 and from the KEGG pathway database. In general, GO terms had to be observed for a minimum of 5 proteins with a minimum significance of $p<0.01$. Resulting p-values were clustered by one-way hierarchical clustering using “Euclidian distance”

as distance function and "Average Linkage Clustering" method available in Multiple Experiment Viewer v4.5.1[15] and a heat map was generated.

Supplementary References

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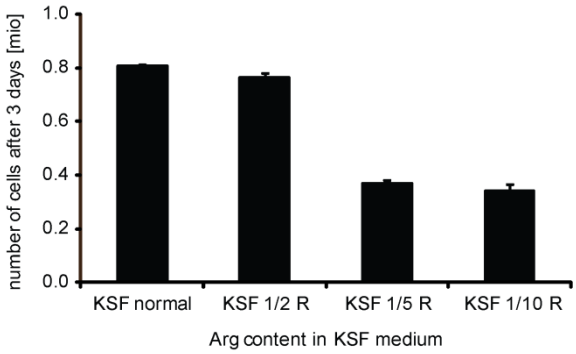
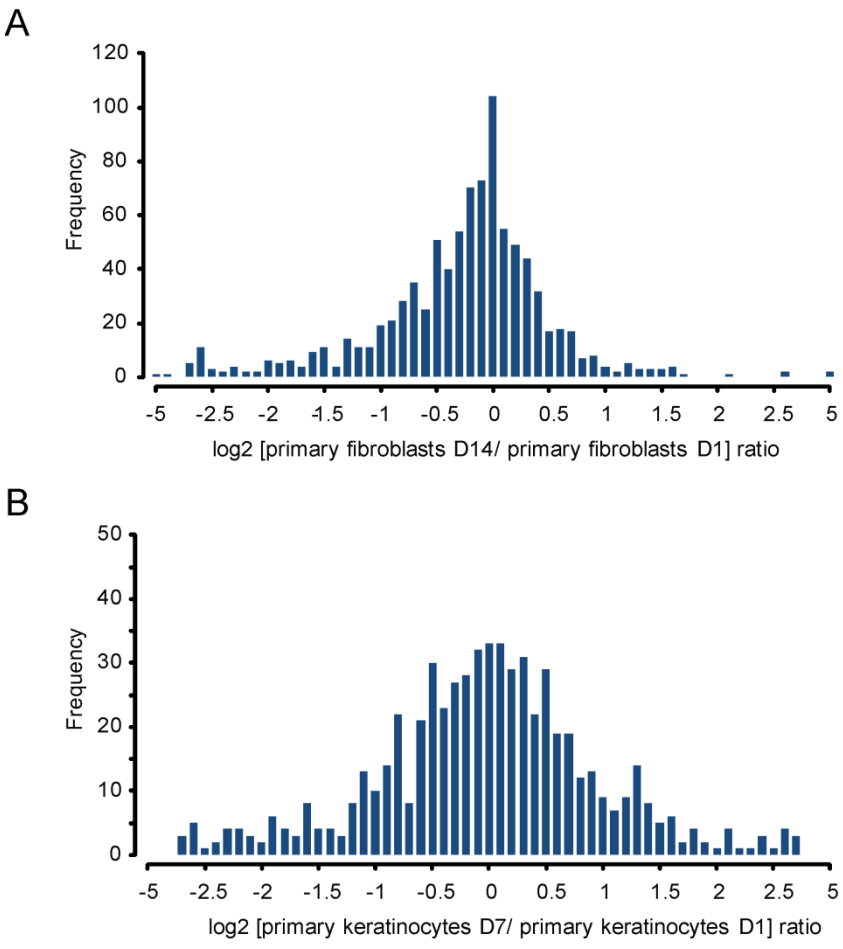
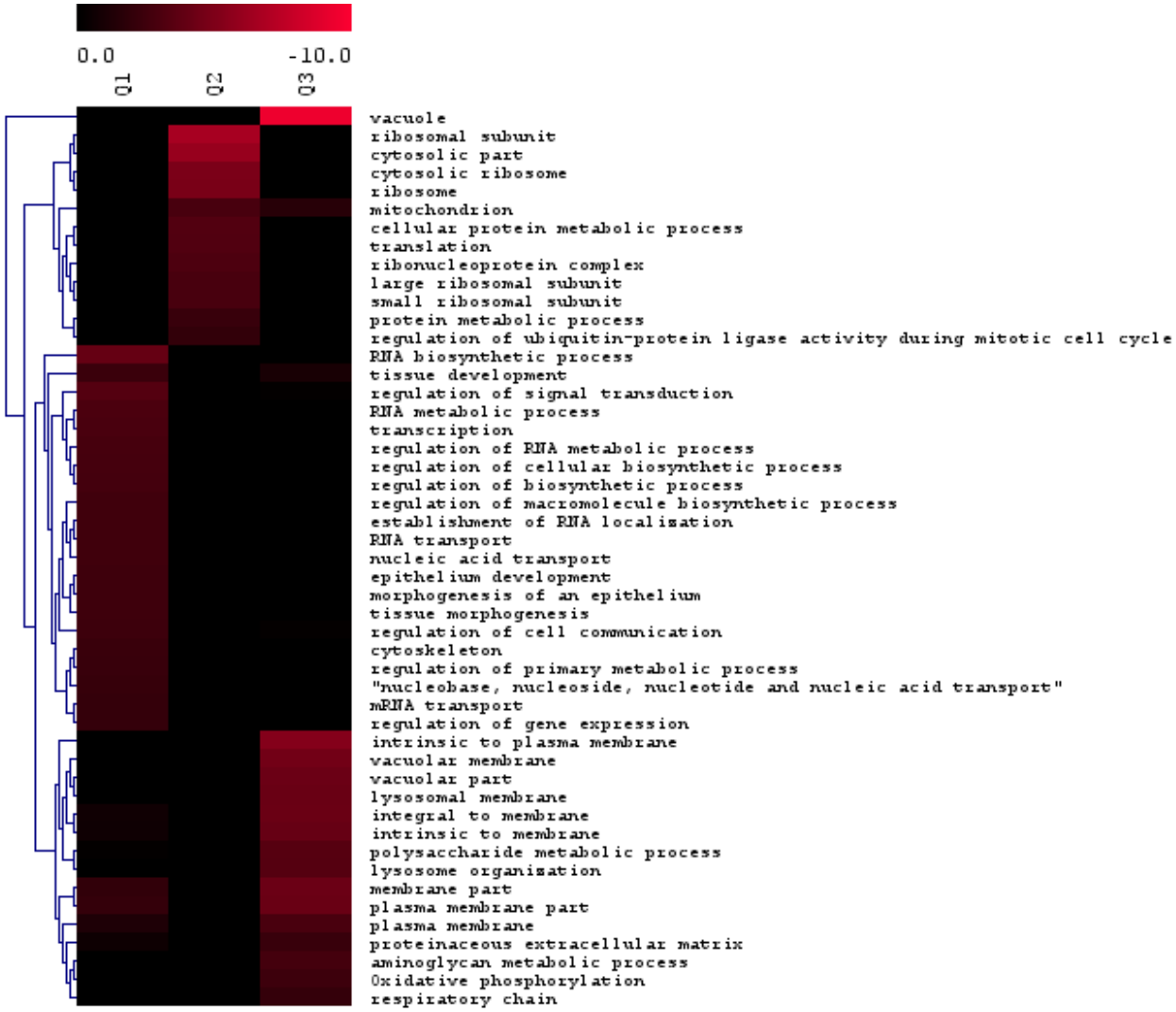
Supplementary figure 1: Arginine-dependent keratinocyte growth. Primary human keratinocytes were cultured for 3 days in custom-made KSF medium containing different amounts of arginine (Arg, R; normal represents 420 mg/l). Depicted is the average of two measurements with respective error bars.



Supplementary figure 2: Phospho site dynamics during cell culture. (A) Phosphorylation changes of primary human fibroblasts during 14 cell doublings. 909 phosphorylation sites were detected and quantified by SILAC-based MS. Ratios show only minor changes (>82% less than 2-fold change) and follow protein ratio differences depicted in figure 2 (see supplementary table 5). **(B) Phosphorylation changes of primary human keratinocytes during 7 cell doublings.** 616 phosphorylation sites were detected and quantified by SILAC-based MS. Ratios show only minor changes (>73% less than 2-fold change) and follow protein ratio differences depicted in figure 2 (see supplementary table 6).

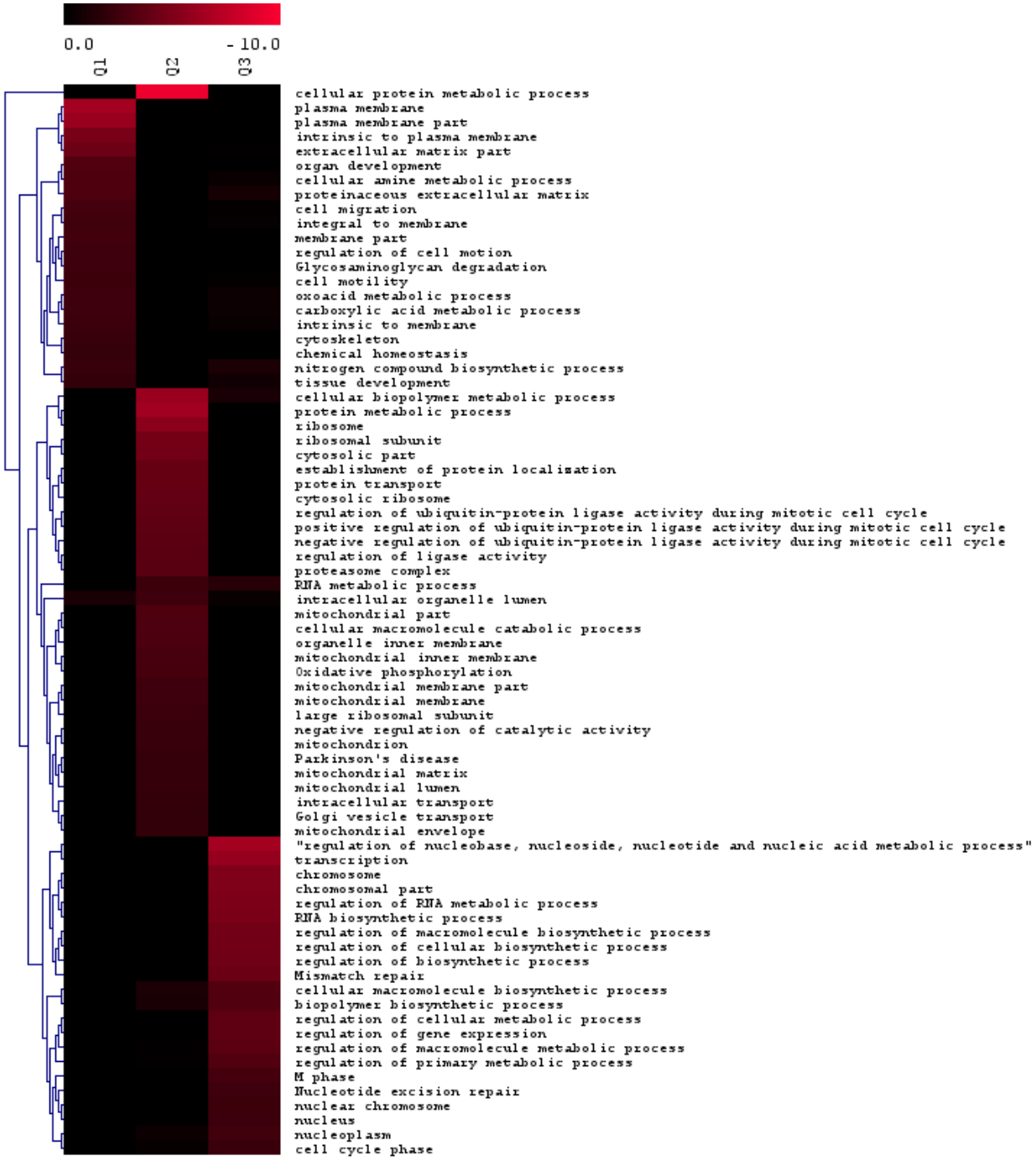


Supplementary figure 3: Heat map of cultured fibroblasts (D14/D1). Proteins in quantiles 1-3 from Figure 2A were separately loaded into DAVID and analyzed on enriched GO terms (level 4) and KEGG pathways. Enriched terms were clustered according to their p values using the TM4 MultiExperiment Viewer (see experimental for details). Color bar represents p values from 0 to 1^{-10}.



Supplementary figure 4: Heat map of cultured keratinocytes (D7/D1). Proteins in quantiles 1-3 from Figure 2B were separately loaded into DAVID and analyzed on enriched GO terms (level 4) and KEGG pathways. Enriched terms were clustered according to their p values using the TM4 MultiExperiment Viewer (see experimental for details). Color bar represents p values from 0 to 1^{-10}.

Supplementary figure 5: Heat map of fibroblast cell line (line/D1). Proteins in quantiles 1-3 from Figure 2E were separately loaded into DAVID and analyzed on enriched GO terms (level 4) and KEGG pathways. Enriched terms were clustered according to their p values using the TM4 MultiExperiment Viewer (see experimental for details). Color bar represents p values from 0 to 1^{-10}.



Supplementary figure 6: Heat map of keratinocyte cell line (line/D1). Proteins in quantiles 1-3 from Figure 2F were separately loaded into DAVID and analyzed on enriched GO terms (level 4) and KEGG pathways. Enriched terms were clustered according to their p values using the TM4 MultiExperiment Viewer (see experimental for details). Color bar represents p values from 0 to 1^{-10}.