**SUPPORTING INFORMATION related to**

# Nrf2 links epidermal barrier function with antioxidant defense

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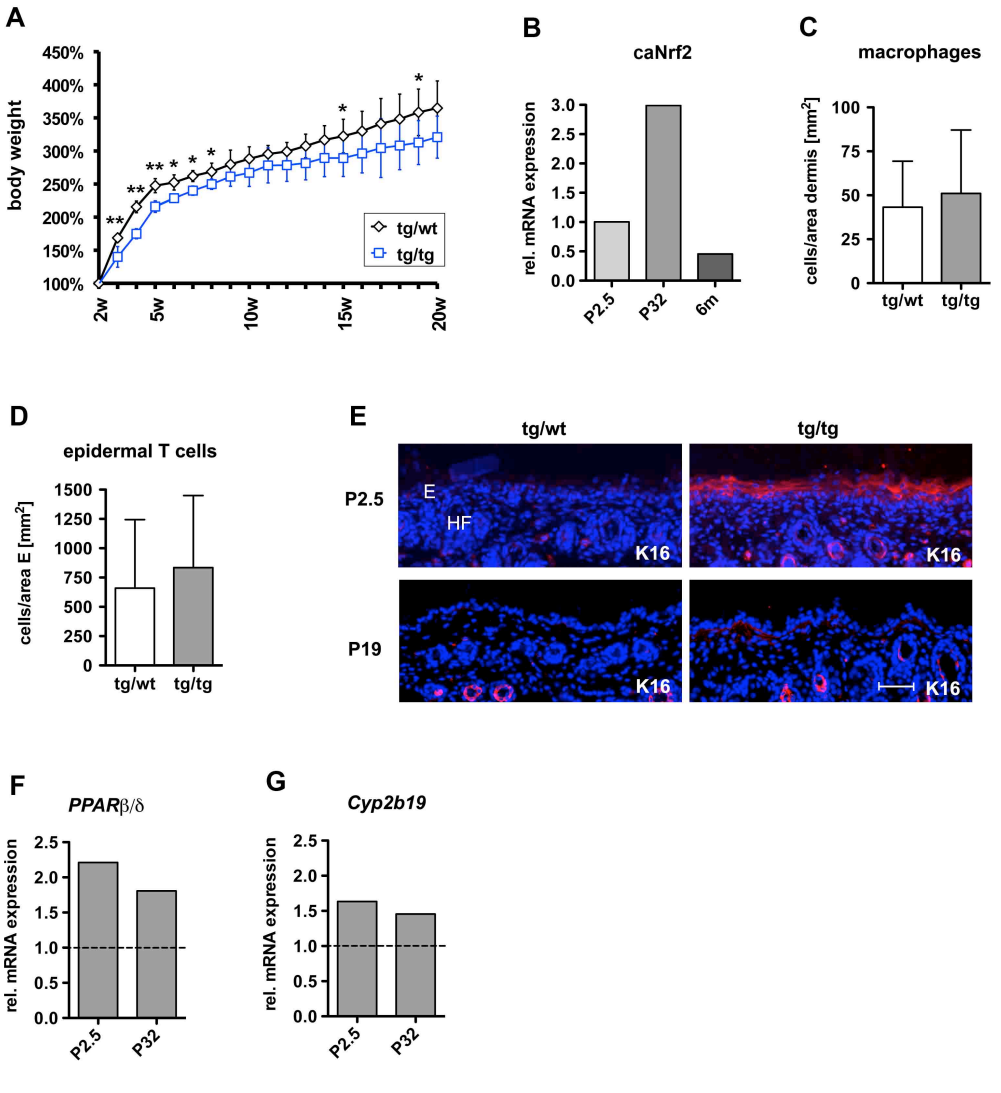
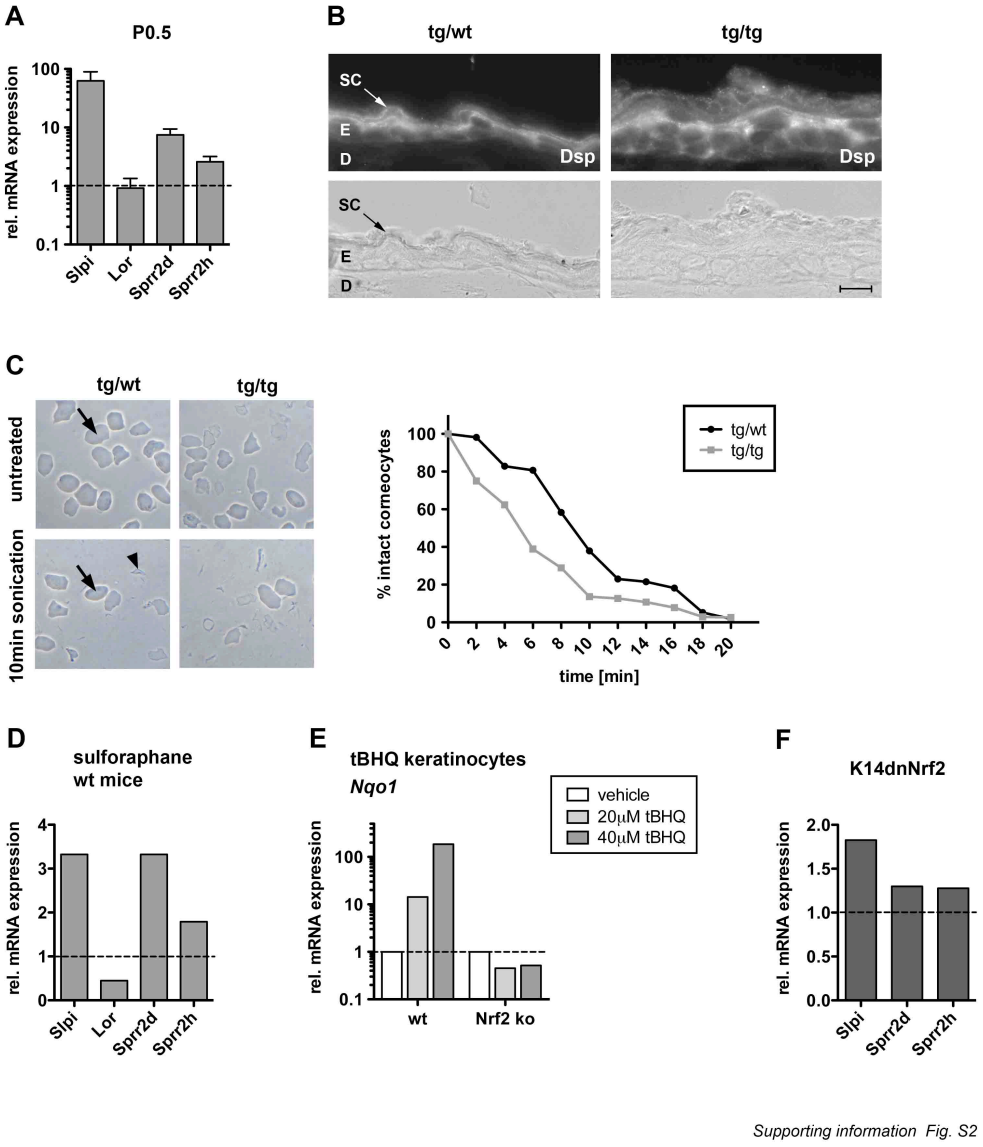



*Supporting information Fig. S1*

**Supporting information Fig. S1: Analysis of K5cre-CMVcaNrf2 transgenic mice**

(**A**) Body weight of female K5cre-CMVcaNrf2 (tg/tg) mice with mild phenotype relative to control (tg/wt) mice (set 100% at 2w postnatal). (**B**) qRT-PCR analysis of *caNrf2* relative to *Gapdh* using RNAs from back skin of P2.5 (N=3), P32 (N=3) and 6m (N=3) K5cre-CMVcaNrf2 mice. Expression in P2.5 K5cre-CMVcaNrf2 mice was arbitrarily set as 1. (**C,D**) Number of macrophage-specific lectin positive macrophages per area dermis (N=8/6, P=0.662) (C) and number of CD3 positive T cells per area epidermis (N=7/5, P=0.7551) (D) at P32. (**E**) Immunofluorescence staining of K16, counterstained with Hoechst using sections from back skin of P2.5 and P32 K5cre-CMVcaNrf2 and control

mice. Scale bar, 50μm. E, epidermis; HF, hair follicles (**F,G**) qRT-PCR analysis of *Pparβ/δ* (F) and *Cyp2b19* (G) relative to *Gapdh* using RNAs from skin of P2.5 (N=2/3) and P32 (N=3) K5cre-CMVcaNrf2 relative to control mice. Expression levels in control mice were arbitrarily set to 1.

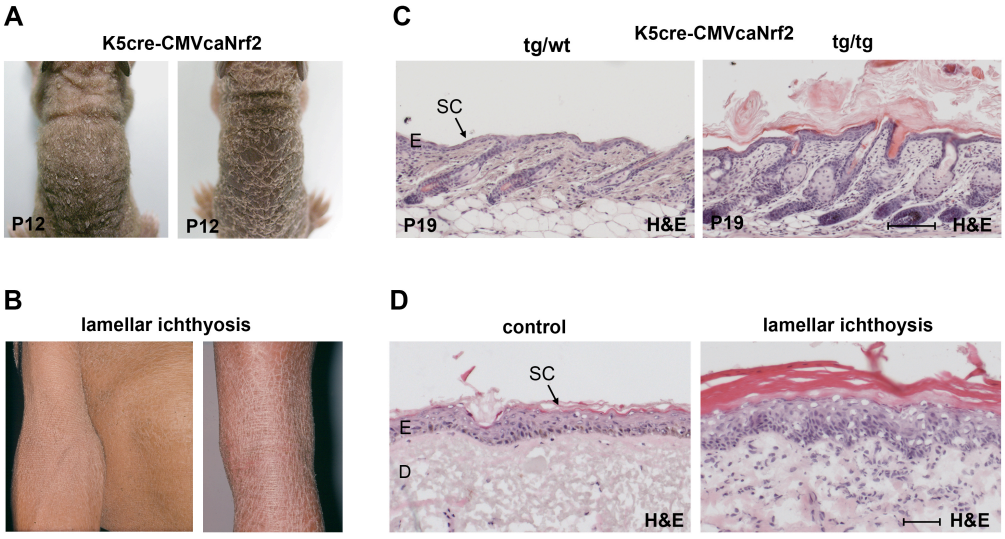



*Supporting information Fig. S2*

**Supporting information Fig. S2: Gene expression in the skin and in cultured murine keratinocytes treated with tBHQ**

(**A**) qRT-PCR analysis of *Slpi, Lor, Sprr2d* and *Sprr2h* relative to *Gapdh* using RNAs from skin of P0.5 K5cre-CMVcaNrf2 (N=4) relative to control mice (N=4). (**B**) Immunofluorescence analysis of desmoplakin (Dsp) (upper panel) and bright field image

of the same microscopic field (lower panel) of tg/wt and tg/tg back skin. Note Dsp staining in the most upper layers of the SC in tg/wt and tg/tg mice. Scale bar, 10μm. SC, stratum corneum; E, epidermis; D, dermis. (**C**) Ultrasound treatment of dissociated corneocytes from tail skin of P32 K5cre-CMVcaNrf2 and control mice. Upper panel: representative picture of untreated corneocytes (left) and corneocytes 10min after sonication (right). Graph: Percentage of intact corneocytes after 2-20min sonication relative to untreated corneocytes. Note the irregular shape and transparency of untreated corneocytes from K5cre-CMVcaNrf2 mice and the faster destruction of K5cre-CMVcaNrf2 corneocytes compared to control corneocytes. Arrows point to intact corneocytes, arrowheads to cell debris of damaged corneocytes. (**D**) qRT-PCR of *Slpi*, *Lor, Sprr2d* and *Sprr2h* relative to *Gapdh* using RNAs from skin of wild-type mice topically treated with tBHQ (N=4) relative to vehicle (N=4) treated mice. Expression levels in control mice were arbitrarily set to 1. (**E**) qRT-PCR of *Nqo1* relative to *Gapdh* using RNAs from primary keratinocytes of wild-type and *Nrf2* ko mice treated with vehicle or tBHQ as indicated. (**F**) qRT-PCR of *Slpi*, *Sprr2d* and *Sprr2h* relative to *Gapdh* using RNAs from skin of P2.5 wild-type (N=2) and K14dnNrf2 mice (N=3).




*Supporting information Fig. S3*

**Supporting information Fig. S3: Parallels between the phenotype of K5cre-CMVcaNrf2 mice and patients with lamellar ichthyosis**

(**A**) Dorsal view of the trunk of K5cre-CMVcaNrf2 mice with scaly skin. Same picture as shown in Fig. 1D. (**B**) Adult lamellar ichthyosis patient with scaly skin on arm, abdomen (left) and knee flexure (right). (**C**) H&E staining of longitudinal sections from the back skin of tg/wt (left) and tg/tg K5cre-CMVcaNrf2 mice (right). Same picture as shown in Fig. 1E. Scale bar, 100μm. E, epidermis; SC, stratum corneum. (**D**) H&E staining of skin sections from a healthy patient (left) and from a patient with lamellar ichthyosis (right). Note acanthosis and severe hyperkeratosis, but only moderate dermal infiltrate in (C) and (D). Scale bar, 50μm. D, dermis; E, epidermis; SC, stratum corneum.

**Supporting information Table SI: Validated Nrf2 target genes involved in ROS or xenobiotics detoxification**

| Symbol | Gene name | Fold Change |
|---|---|---|
| *Gsta3* | Glutathione S-transferase, alpha 3 | 71.9/6.1 |
| *Gpx2* | Glutathione peroxidase 2 | 22.8 |
| *Srxn1* | Sulfiredoxin 1 homolog | 21.2/20.1/15.1 |
| *Nqo1* | NAD(P)H dehydrogenase, quinone 1 | 21.0 |
| *Slc7a11* | Solute carrier family 7, member 11 | 10.7/8.6 |
| *Gsta4* | Glutathione S-transferase, alpha 4 | 8.4 |
| *Gstm1* | Glutathione S-transferase, mu 1 | 6.1/5.8/4.6/3.5 |
| *Abcc4* | ATP-binding cassette, sub-family C, member 4 | 6.0 |
| *Cbr3* | Carbonyl reductase 3 | 5.6 |
| *Abcc3* | ATP-binding cassette, sub-family C, member 3 | 5.1 |
| *Gsta2* | Glutathione S-transferase, alpha 2 | 4.5/2.8 |
| *Gstm6* | Glutathione S-transferase, mu 6 | 4.1 |
| *Gss* | Glutathione synthetase | 3.7 |
| *Gclc* | Glutamate-cysteine ligase, catalytic subunit | 3.1 |
| *Gclm* | Glutamate-cysteine ligase, modifier subunit | 2.8 |
| *Gstm3* | Glutathione S-transferase, mu 3 | 2.6 |
| *Txnrd1* | Thioredoxin reductase 1 | 2.5/2.3 |
| *Hmox1* | Heme oxygenase (decycling) 1 | 2.3 |
| *Gsr* | Glutathione reductase 1 | 2.3 |
| *Cat* | Catalase | 2.3/2.1 |

Genes with ≥2 fold change ($P < 0.005$) detected in the microarray analysis of back skin RNA from P2.5 K5cre-CMVcaNrf2 mice compared to control mice are listed. Multiple values of fold change indicate the results obtained with different gene fragments spotted on the array.

## Supporting information Materials and Methods

### RNA isolation, RPA and qRT-PCR

Isolation of RNA from murine skin and RPA were performed as previously described (Chomczynski & Sacchi, 1987) (Werner et al, 1993). The template for synthesis of a probe detecting caNrf2 and endogenous Nrf2 is described in (Schäfer et al, 2010). For qRT-PCR, RNA from murine skin was further purified with the Nucleospin RNAII kit (Machery-Nagel, Oensingen, Switzerland), followed by ethanol precipitation.

RNA from cultured cells was isolated using the GenElute Mammalian Total RNA Miniprep kit (Sigma-Aldrich, Buchs, Switzerland). qRT-PCR was performed as described previously (Koegel et al, 2009). Primers used for qRT-PCR are listed in Supplementary Table SII.

### Histological and immunohistological staining techniques

Histological analyses and immunofluorescence were previously described (Schäfer et al, 2010). Primary and secondary antibodies are listed in Supplementary Table SIII.

TUNEL assays were performed on 4% PFA-fixed skin sections using the in situ cell death detection kit (Roche, Rotkreuz, Switzerland).

Toluidine blue staining was performed by incubation of 4% PFA-fixed sections in 0.5% toluidine blue (Sigma-Aldrich) in 0.5 N HCl, pH 2.3, for 30min. Subsequently, slides were washed 3 times in PBS, dehydrated, incubated in xylene and mounted with Eukitt quick hardening mounting medium (Sigma-Aldrich).

OIL RED O lipid staining was performed on skin cryosections after fixation with 4% PFA for 20min and dehydration in 60% isopropanol. Staining was performed for 15min using OIL RED O solution (Sigma-Aldrich; 3g/ml in 60% isopropanol). Subsequently, slides were rinsed in 60% isopropanol, rehydrated and mounted in Mowiol (Hoechst, Frankfurt, Germany).

For BrdU analysis, mice were injected intraperitoneally with BrdU (Sigma-Aldrich; 250mg/kg in 0.9%NaCl) and sacrificed 2h later. Cultured cells were incubated for 2h with 100μM BrdU. Incorporated BrdU was detected on skin sections that had been fixed with

95% ethanol/1% acetic acid or on 4% PFA-fixed cells by immunohistochemistry using a peroxidase-coupled anti-BrdU antibody and subsequent staining with AEC peroxidase substrate (Vector Laboratories, Burlingame, CA).

Immunohistochemistry stainings were photographed using a Zeiss Axioskop 2 microscope with an Axiocam HRc camera and the Axiovision 4.2 software. Immunofluorescence stainings were photographed using a Zeiss Axio Imager.A1 microscope with an Axiocam MRm camera and the Axiovision 4.6 software (all from Zeiss, Oberkochen, Germany). Electron microscopy was preformed as recently described (Yang et al, 2010) using a 902A electron microscope (Zeiss) with Megaview III digital image acquisition system and iTEM software, version 5.0.

**Westernblot analysis**

Snap-frozen skin samples were homogenized using an Ultra Turrax homogenizer (IKA, Staufen, Germany) in either urea lysis buffer (10mM Tris/HCl pH8, 8M urea and 2mM EDTA) or T-PER lysis buffer (Pierce, Rockford, IL) with protease and phosphatase inhibitors. Protein lysates were cleared by sonication and centrifugation. SDS page and Westernblot were performed according to standard procedures. Antibodies were incubated in 5% non-fat dry milk or 5% BSA in TBS + 0.01% Tween-20. Bound secondary antibodies were detected using NBT/BCIP (Promega, Madison, WI).

**Cell Culture**

Isolation and cultivation of keratinocytes was performed as described previously (Yang et al, 2010). Cells were cultured in defined keratinocyte SFM (Invitrogen) or keratinocyte growth medium 2 (Promocell, Heidelberg, Germany).

**Supporting information Table SII: List of Primers**

| Name | Forward | Reverse |
|---|---|---|
| **PCR primers** | | |
| *CMVcaNrf2* | CGGCTCAGCACCTTGTAT | CT TATTCCAAGCGGCTTCGGC |
| **qRT-PCR primers** | | |
| *caNrf2* | TGTTCCTTGTTCCCAAAAGC | CCGGAATATTAATAGGGACGGTA |
| *Csf2* | TCATTTTTGGCCTGGTTTTT | TATTCGAGCAGGGTCTACGG |
| *Csf3* | TGCACTATGGTCAGGACGAG | GGGGTGACACAGCTTGTAGG |
| *Cyp2b19* | CTCGGTCCCAGGTGTACTGT | CTCTTGCAGATGGACAGACG |
| *Elovl1* | CCTCCATGTCTTCCACCACT | CAAGGGCAGACAATCCATAG |
| *Elovl3* | CCTTTTTGGAGGAGTACTGGG | ATCCGTGTAGATGGCAAAGC |
| *Elovl4* | GTTCTATCGCTGGACCTGGA | CAGCCACACGAACAGGAGAT |
| *Elovl5* | CTTGCACATCCTCCTGCTC | TGAGTGACGCATCGAAATGT |
| *Elovl6* | ACAATGGACCTGTCAGCAAA | GGTACCAGTGCAGGAAGATCA |
| *Elovl7* | GGAAAATGGCGTTCAGTGAT | GACATAGAGGCCCAGGATGA |
| *Gapdh* | TCGTGGATCTGACGTGCCGCCTG | CACCACCCTGTTGCTGTAGCCGTAT |
| *Gclc* | AACAAGAAACATCCGGCATC | CGTAGCCTCGGTAAAATGGA |
| *Gclm* | TCCCATGCAGTGGAGAAGAT | AGCTGTGCAACTCCAAGGAC |
| *Gsta3* | TACTTTGATGGCAGGGGAAG | GCACTTGCTGGAACATCAGA |
| *Hgf* | TTTGCTTTAGGGATCAGTCTG | GGCCTTGCAAGTGAAC |
| *Ifn$\gamma$* | TTTTAACTCAAGTGGCATAGATGTG | ATGAATGCATCCTTTTTCGC |
| *Il1$\beta$* | GGACAGAATATCAACCAACAAGTG | TGCTGATGTACCAGTTGGGG |
| *Il6* | CCGGAGAGGAGACTTCACAG | TTCTGCAAGTGCATCATCGT |
| *Lor* | CACTCATCTTCCCTGGTGCT | TCCACCAGAGGTCTTTCCAC |
| *Nqo1* | CTGGCCCATTCAGAGAAGAC | GTCTGCAGCTTCCAGCTTCT |
| *PPAR$\beta/\delta$* | AGATCCGATCGCACTTCTCATAC | CGGCAGCCTCAACATGG |
| *Slpi* | CGGCAAATACAAGTGCTGTG | CCTGGGAGCAGGGAAGTAGT |
| *Sprr1a* | CAGAGAACCTGCTCTTCTCTGAGT | CAGGGATCCTTGGTTTTGG |
| *Sprr2d* | CTGGTACTCAAGGCCGAGAC | CAGGGCACTTTGGTGGAG |
| *Sprr2h* | GACACTTGGTACTCAAGCTCTGG | TGCACTGCTGCTGTTGGTAA |
| *Srxn1* | CGGTGCACAACGTACCAAT | TTGATCCAGAGGACGTCGAT |
| *Tnf$\alpha$* | GACCCTCACACTCAGATCATCTTCT | CCACTTGGTGGTTTGCTACGA |
| *Tslp* | CCAGGCTACCCTGAAACTGA | TCTGGAGATTGCATGAAGGA |

**Supporting information Table SIII: List of antibodies**

| Name | Company |
|---|---|
| **Primary antibodies** | |
| Anti-β-actin | Sigma-Aldrich, Buchs, Switzerland |
| Anti-BrdU POD | Roche, Rotkreuz, Switzerland |
| Anti-CD3 | Dako, Baar, Switzerland |
| Anti-Desmoplakin 1&2 | Fitzgerald, Acton, MA |
| Anti-Filaggrin | Covance, Berkeley, CA |
| Anti-Involucrin | |
| Anti-Keratin 6 | |
| Anti-Keratin 10 | |
| Anti-Keratin 14 | |
| Anti-Keratin 16 | kindly provided by Dr. Pierre Coulombe, Johns Hopkins University, Baltimore, MA |
| Anti-Loricrin | Covance |
| Anti-Ly6G | BD Bioscience Pharmingen, Basel, Switzerland |
| Anti-Macrophage specific lectin | Abcam, Cambridge, UK |
| Anti-Sprr2 | Dr. Daniel Hohl, University Hospital Lausanne, Switzerland |
| **Secondary antibodies** | |
| Goat anti-mouse AP | Promega, Madison, WI |
| Goat anti-mouse Biotin | Vector Laboratories, Burlingame, CA |
| Goat anti-rabbit Biotin | Jackson ImmunoResearch |
| Goat anti-rabbit Cy3 | |
| Goat anti-rabbit AP | Promega |
| Goat anti-rat Biotin | Vector Laboratories |

**Supporting information References**

Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162**:** 156-159

Koegel H, von Tobel L, Schafer M, Alberti S, Kremmer E, Mauch C, Hohl D, Wang XJ, Beer HD, Bloch W et al (2009) Loss of serum response factor in keratinocytes results in hyperproliferative skin disease in mice. J Clin Invest 119**:** 899-910

Schäfer M, Dütsch S, auf dem Keller U, Navid F, Schwarz A, Johnson DA, Johnson JA, Werner S (2010) Nrf2 establishes a glutathione-mediated gradient of UVB cytoprotection in the epidermis. Genes Dev 24**:** 1045-1058

Werner S, Weinberg W, Liao X, Peters KG, Blessing M, Yuspa SH, Weiner RL, Williams LT (1993) Targeted expression of a dominant-negative FGF receptor mutant in the epidermis of transgenic mice reveals a role of FGF in keratinocyte organization and differentiation. Embo J 12**:** 2635-2643

Yang J, Meyer M, Muller AK, Bohm F, Grose R, Dauwalder T, Verrey F, Kopf M, Partanen J, Bloch W et al (2010) Fibroblast growth factor receptors 1 and 2 in keratinocytes control the epidermal barrier and cutaneous homeostasis. J Cell Biol 188**:** 935-952