Nrf2 establishes a glutathione-mediated gradient of UVB cytoprotection in the epidermis

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Ultraviolet (UV) B irradiation can severely damage the skin and even induce tumorigenesis. It exerts its effects by direct DNA modification and by formation of reactive oxygen species (ROS). We developed a strategy to genetically activate target gene expression of the transcription factor NF-E2-related factor 2 (Nrf2) in keratinocytes *in vivo* based on expression of a constitutively active Nrf2 mutant. Activation of Nrf2 target genes strongly reduced UVB cytotoxicity through enhancement of ROS detoxification. Remarkably, the protective effect was extended to neighboring cells. Using different combinations of genetically modified mice, we demonstrate that Nrf2 activates the production, recycling, and release of glutathione and cysteine by suprabasal keratinocytes, resulting in protection of basal cells in a paracrine, glutathione/cysteine-dependent manner. Most importantly, we found that endogenous Nrf2 controls selective protection of suprabasal keratinocytes from UVB-induced apoptosis through activation of cytoprotective genes. This finding explains the preferential UVB-induced apoptosis of basal cells, which is important for elimination of mutated stem cells as well as for preservation of skin integrity. Taken together, our results identify Nrf2 as a key regulator in the UV response of the skin.

[**Keywords:** Apoptosis; Nrf2; UVB; reactive oxygen species; glutathione]

Supplemental material is available at <http://www.genesdev.org>.

Received November 18, 2009; revised version accepted March 30, 2010.

Ultraviolet (UV) B irradiation is one of the most dangerous insults for the skin, and causes sunburn, erythema, and photoaging. Most importantly, UV-induced DNA damage is a major risk factor for skin carcinogenesis. The highly energetic short-wavelength UVB light (280–320 nm) has the strongest carcinogenic potency (Bowden 2004). UVB damages DNA directly, resulting in formation of dimeric photoproducts, and indirectly by reactive oxygen species (ROS) formation, causing strand breaks and hydroxylation of DNA bases. If repaired inefficiently, these DNA modifications result in deleterious mutations (Ichihashi et al. 2003). The mutagenic potential of ROS is enhanced further by oxidation of proteins and lipids, which can lead to altered signaling and cell proliferation (Bickers and Athar 2006).

Low levels of DNA damage initiate cell cycle arrest and removal of DNA lesions by nucleotide excision repair (Ichihashi et al. 2003). If DNA damage exceeds the repair

capacity, apoptosis is initiated. In addition, apoptosis is induced by high levels of ROS and by death receptor signaling (Kulms et al. 2002). This prevents the persistence and spreading of DNA mutations and thus cancer formation, but, on the other hand, can lead to severe tissue damage.

As ROS contribute to UVB-induced photocarcinogenesis and apoptosis, it is of major importance to unravel the mechanisms of ROS detoxification. One of the most important players in the defense of cells against oxidative stress is the transcriptional regulator NF-E2-related factor 2 (Nrf2), which activates the expression of a battery of ROS-detoxifying enzymes and stimulates the production of antioxidants, including the tripeptide glutathione (GSH). GSH is one of the most versatile cellular antioxidants, and is produced in a two-step reaction from cysteine, glutamic acid, and glycine (Supplemental Fig. S1A). Interestingly, all enzymes involved in its biosynthesis are under the control of Nrf2 (Lu 2009). Due to its antioxidant properties, GSH is likely to be important for the prevention of UV-induced oxidative damage (Tyrrell and Pidoux 1988). This hypothesis is supported by the severe GSH depletion of keratinocytes in response to UVB

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Article is online at <http://www.genesdev.org/cgi/doi/10.1101/gad.568810>.

irradiation (Zhu and Bowden 2004), which in turn enhances the susceptibility to UV-induced damage (Hanada et al. 1991).

We showed previously that Nrf2 is constitutively expressed in keratinocytes of normal skin and is up-regulated during wound healing (Braun et al. 2002). Using *Nrf2* knockout mice as well as mice expressing a dominant-negative Nrf2 mutant in the epidermis, we found that the constitutive, basal activity of Nrf2 in the skin is crucial for normal wound repair and for prevention of skin carcinogenesis (Braun et al. 2002; auf dem Keller et al. 2006). Nrf2 can be further activated by electrophiles, and possibly also by ROS (Beyer et al. 2007). In response to low doses of UVB or long-wave UVA irradiation of a mouse keratinocyte cancer cell line and of human fibroblasts and keratinocytes, increased Nrf2 activity was detected (Kannan and Jaiswal 2006; Gruber et al. 2010). Physiological doses of UVB, in contrast, did not activate Nrf2 in primary mouse keratinocytes or in murine back skin epidermis (Durchdewald et al. 2007). Recent results even indicated repression of Nrf2 activity by high UVB doses in normal human keratinocytes (Marrot et al. 2008; Kokot et al. 2009). The basal activity of Nrf2 seems to be required for protection of keratinocytes from UV cytotoxicity, since *Nrf2* knockout mice showed a higher rate of apoptosis and oxidative damage in ear skin keratinocytes after UVB irradiation (Kawachi et al. 2008). Vice versa, topical treatment of SKH-1 mouse back skin with broccoli sprout extracts that contain the Nrf2 activator sulforaphane protected against UVB-induced photocarcinogenesis (Dinkova-Kostova et al. 2006). Since sulforaphane has multiple anti-carcinogenic effects independent of Nrf2 (Fimognari and Hrelia 2007; Dickinson et al. 2009), the effect of Nrf2 activation in the UV response needs to be studied using genetic approaches. This will also allow the identification of the molecular and cellular mechanisms underlying the protective effect of Nrf2 in the UVB response, which are as yet unknown. Activation of Nrf2 by ablation of its inhibitor kelch-like ECH-associated protein 1 (Keap1) causes postnatal lethality (Wakabayashi et al. 2003). Therefore, *Keap1*-deficient mice cannot be used to study the role of Nrf2 activation in UVB protection. Furthermore, Keap1 has additional binding partners besides Nrf2, and Nrf2-independent functions therefore cannot be excluded (Lee et al. 2009).

To circumvent this problem, we established a novel strategy to determine the consequences of Nrf2 target gene activation in keratinocytes in vivo by targeted expression of a constitutively active (ca) Nrf2 mutant. Our results show that Nrf2 activation is a potent strategy to protect the skin from UV damage and unravel a gradient of UVB protection in the skin that is controlled by Nrf2.

Results

Generation of transgenic mice expressing a caNrf2 mutant in keratinocytes

To specifically activate Nrf2 in the epidermis, we generated transgenic mice expressing a caNrf2 mutant in

keratinocytes. A caNrf2 cDNA was cloned, which lacks the N-terminal Neh2 domain responsible for binding to the inhibitory protein Keap1 (Fig. 1A; Itoh et al. 1999). Transfection of COS-1 cells with a *caNrf2* expression vector revealed nuclear localization of caNrf2 (Fig. 1B). Luciferase reporter assays were performed after cotransfection of HEK293T cells with a *CMV-caNrf2* expression plasmid and a luciferase reporter plasmid containing the 51-base-pair (bp) antioxidant response element (ARE) of the rat NAD(P)H dehydrogenase quinone-1 (*Nqo1*) gene. AREs are *cis*-acting elements in the promoters of Nrf2 target genes. They are the binding sites for Nrf2, and mediate Nrf2-induced gene expression (Wasserman and Fahl 1997). Strong luciferase expression was found in cotransfected cells compared with cells transfected with the *ARE(Nqo1)-Luc* reporter alone (Fig. 1C).

The *caNrf2* cDNA was cloned into an expression cassette with a β -actin promoter, a transcription/translation STOP cassette flanked by *loxP* sites, an internal ribosomal entry site (*IRES*), and the EGFP cDNA (Fig. 1D). Five transgenic founder mice (named β ACTcaNrf2; FVB/N background) with different integration sites were generated by pronucleus injection. To induce caNrf2 expression in the epidermis and hair follicles, β ACTcaNrf2 mice were mated with transgenic mice expressing Cre recombinase under control of the *keratin 5* (*K5*) promoter (C57BL/6 background) (Ramirez et al. 2004). RNase protection assays (RPAs) using skin RNA showed that double-transgenic mice (named K5cre-caNrf2 mice) express the *caNrf2* transgene at similar or lower levels compared with the endogenous *Nrf2* gene (Fig. 1E). Proportional to the expression level of *caNrf2*, up-regulation of the established Nrf2 target genes *Nqo1*, *Gsta3* (*glutathione S-transferase 3*), *Gclc* (*glutamate cysteine ligase, catalytic subunit*), and *Srxn1* (*sulfiredoxin1*) (Kensler et al. 2007; Soriano et al. 2008) was detected by RPA or quantitative RT-PCR (qRT-PCR) (Fig. 1F,G). PCR amplification of the STOP cassette and adjacent nucleotides identified a 424-bp fragment in the epidermis of K5cre-caNrf2 transgenic mice, demonstrating excision of the STOP cassette (Fig. 1H). Since the STOP cassette is excised in the basal layer of the epidermis and hair follicles, caNrf2 is expressed in all layers in the course of epidermal self-renewal. This was verified by mating of K5cre-caNrf2 mice with AREhPAP reporter mice (Johnson et al. 2002). They carry a transgene with the ARE of the rat *Nqo1* gene, an initiator element containing a minimal promoter and the human placental alkaline phosphatase (hPAP) cDNA. Strong hPAP activity was seen in all viable layers of the epidermis and hair follicles of triple-transgenic mice, demonstrating expression and activity of caNrf2 in keratinocytes in vivo (Fig. 1I).

Mild hyperkeratosis in K5cre-caNrf2 transgenic mice

K5cre-caNrf2 transgenic mice have normal growth and body weight. Between postnatal day 5 (P5) and P8, enhanced scaling was observed (Fig. 1J) that was no longer visible at later stages due to the dense fur. Histological analysis showed a similar thickness of the viable layers of the epidermis, but a slightly thicker cornified layer

Figure 1. Generation of K5cre-caNrf2 transgenic mice. (A) Functional domains of Nrf2 and caNrf2 (Δ Neh2 mutant). (B) Immunofluorescence staining for Nrf2 in COS-1 cells transfected with a CMV-caNrf2 plasmid. Arrows point to nuclear caNrf2. (C) Luciferase reporter assay with a CMV-caNrf2 vector. The control is set to 1. (D) Scheme of the transgene used for generation of K5cre-caNrf2 mice. (E,F) RPA for *caNrf2*/*Nrf2* (E) and *Nqo1* (F) using RNAs from skin of transgenic/wild-type (tg/wt) and transgenic/transgenic (tg/tg) mice of lines 1 and 4. tRNA served as negative control (labeled "tRNA"); 10^4 counts per minute (cpm) of the hybridization probes were used as marker (labeled "probe"). Note up-regulation of *Nqo1* proportional to the *caNrf2* expression level. (G) qRT-PCR of the Nrf2 target genes *Nqo1*, *Gclc*, *Srxn1*, and *Gsta3* using epidermal RNAs of tg/tg and tg/wild-type mice (set to 1). (H) PCR amplification of the STOP cassette (424 bp, top lane) and the Cre recombinase cDNA (419 bp, bottom lane) using DNA from mice of transgenic lines 1 and 4. (I) Staining for hPAP activity in back skin of double-transgenic AREhPAP/K5cre mice (tg/tg/wt) (top panel) and triple-transgenic AREhPAP/K5cre-caNrf2 mice (tg/tg/tg) (middle panel), and tail skin of triple-transgenic AREhPAP/K5cre-caNrf2 mice (tg/tg/tg) (bottom panel). Bars: top panel, 50 μ m; bottom panel, 30 μ m. (J) P2.5 tg/wild-type and tg/tg K5cre-caNrf2 mice in overview (left picture) and close-up (right picture) of lower back. Note enhanced scaling in tg/tg mice. (K) Longitudinal sections of back skin of 8-wk-old (8w) tg/wild-type and tg/tg mice. The arrow points to the area with the thicker cornified layer. Bar, 100 μ m. (BL) Basal layer; (CL) cornified layer; (D) dermis; (E) epidermis; (HF) hair follicle; (SL) suprabasal layer.

postnatally and throughout adulthood (Fig. 1K; data not shown). Morphometric analysis of 2- μ m paraffin sections of K5cre-caNrf2 mice confirmed the hyperkeratosis (Supplemental Fig. S1B). This phenotype is similar but less severe compared with *Keap1* knockout mice (Wakabayashi et al. 2003), which further demonstrates the expected activity of the *caNrf2* transgene in vivo. *Keap1* knockout mice die postnatally from malnutrition due to severe hyperkeratosis in the upper digestive tract (Wakabayashi et al. 2003). K5cre-caNrf2 transgenic mice, in contrast, showed no hyperscaling in the esophagus and oral epithelium, due to low K5 promoter activity and consequent low caNrf2 expression levels in these epithelia (data not shown). Thus, mice expressing *caNrf2* in keratinocytes are viable and can be used to investigate the consequences of enhanced Nrf2 activity in adult mice.

caNrf2 protects keratinocytes from UVB cytotoxicity through enhanced ROS detoxification

Primary keratinocytes from K5cre-caNrf2 mice showed enhanced Nrf2 activity, as verified by hPAP staining of keratinocytes from K5cre-caNrf2/AREhPAP triple-transgenic mice (Fig. 2A). Their rate of apoptosis was significantly lower compared with controls. This was already observed under normal culture conditions, reflecting the protection from stress that is imposed on culturing. Most importantly, caNrf2 protected keratinocytes from the toxicity of the ROS-producing agent menadione (Fig. 2B).

Upon irradiation of adult mice with a physiological dose of UVB (100 mJ/cm²), we found a significant reduction in the number of apoptotic keratinocytes in the epidermis of K5cre-caNrf2 transgenic mice compared with control mice 24 h post-irradiation (hpi), as determined by staining for cleaved caspase-3 (Fig. 2C). To determine the mechanisms underlying this UV-protective effect, intracellular ROS levels were determined in UVB-irradiated cultured keratinocytes. For this purpose, cells were treated with H₂DCF-DA (2',7'-dichlorodihydrofluorescein diacetate), and DCF fluorescence was analyzed by flow cytometry. ROS levels were significantly lower in immortalized (Fig. 2D) as well as primary keratinocytes (data not shown) from K5cre-caNrf2 transgenic mice compared with keratinocytes from control mice after irradiation with 10–40 mJ/cm² UVB. The effect of caNrf2 on direct UVB-induced DNA damage and its repair was analyzed by staining for cyclobutyl pyrimidine dimers (CPDs), which are the most predominant lesions induced by direct interference of UVB light with the DNA (Verschooten et al. 2006). Five minutes post-irradiation (mpi) and 24 hpi with 100 mJ/cm² UVB, no difference in the number of CPD-positive keratinocytes was found between control and K5cre-caNrf2 transgenic mice (Fig. 2E). Immunohistochemical detection of p53, which is stabilized by DNA damage and ROS (Liu et al. 2008), showed a reduced number of positive cells in K5cre-caNrf2 mice (Fig. 2F). These findings strongly suggest that caNrf2 protects keratinocytes from UVB-induced apoptosis by reduction of intracellular ROS and subsequent ROS-induced DNA damage, but has no influence on formation

and repair of lesions resulting from direct DNA modification by UVB.

A gradient of UVB-induced apoptosis in the murine epidermis: correlation with Nrf2 expression and activity

Histological analysis of hematoxylin/eosin (H&E)-stained 2- μ m paraffin sections from UVB-irradiated wild-type mouse back skin showed that 85% of (apoptotic) sunburn cells are located in the basal layer, and only 15% are located in the suprabasal layers ($N = 11$) (Fig. 3A). Sunburn cells are apoptotic keratinocytes, which can be identified by their pyknotic nuclei and the eosinophilic cytoplasm (Young 1987). In the much thicker tail skin epidermis, 63% of the sunburn cells were in the basal layer, 26% were in the lower suprabasal layers, and 11% were in the upper suprabasal layers ($N = 6$) (Fig. 3B). This was confirmed by staining for cleaved caspase-3 (58% basal layer, 37% lower suprabasal layers, 5% upper suprabasal layers, $N = 6$) (Fig. 3C; data not shown) and p53 (data not shown). Thus, apoptotic keratinocytes are distributed in a basal-to-suprabasal gradient (see below; Fig. 3J).

To determine if the reduced apoptosis of suprabasal keratinocytes results from enhanced Nrf2 activity and subsequent ROS detoxification, we compared the expression levels of Nrf2 and established Nrf2 target genes between suprabasal and basal keratinocytes. Suprabasal cell layers of tail skin were separated from the basal layer by chymotrypsin treatment, as described for human skin (Sasai et al. 1979). Successful separation was verified histologically, by K10 immunostaining (Fig. 3D), and by qRT-PCR for the late differentiation marker *Sprr2a* (Fig. 3E; Song et al. 1999). In the samples obtained from the first separation (19 $\times$ *Sprr2a* suprabasal vs. basal keratinocytes), qRT-PCR showed a much higher expression of *Nrf2* and its target genes *Nqo1*, *Gclc*, *Gclm* (glutamate cysteine ligase, modifier subunit), *Gsr* (glutathione reductase), and *Srxn1* in suprabasal compared with basal keratinocytes (Fig. 3E). *Gss* (glutathione synthetase) was expressed at similar levels in basal and suprabasal keratinocytes, while *Gsta3* had a lower expression level in suprabasal cells. In a more efficient separation (588 $\times$ *Sprr2a* suprabasal vs. basal keratinocytes), an even higher enrichment of *Nrf2*, *Nqo1*, *Gclc*, *Gclm*, *Gsr*, and *Srxn1* was observed in suprabasal keratinocytes. In this experiment, *Gss* and *Gsta3* showed equal expression levels in suprabasal and basal keratinocytes (Fig. 3E). For *Nqo1*, a suprabasal-to-basal gradient was confirmed on the protein level by immunofluorescence staining (Fig. 3F).

Studies with *Nrf2* knockout mice revealed that the differential expression of *Nqo1*, *Gclc*, *Gclm*, and *Gsr* is indeed Nrf2-dependent, since expression levels of these genes were similar in basal and suprabasal keratinocytes in the absence of Nrf2 (Fig. 3G). Only *Srxn1* showed a higher expression in suprabasal compared with basal cells of *Nrf2* knockout animals. Most importantly, the steep gradient in the distribution of sunburn cells that is seen in wild-type mice 24 hpi with UVB was much less pronounced in *Nrf2* knockout mice. This was due predominantly to a strong

Paracrine control of UVB-induced cell death by Nrf2

Figure 2. ROS detoxification and protection from UVB-induced apoptosis in K5cre-caNrf2 transgenic mice. (A) hPAP activity in primary keratinocytes of double-transgenic AREhPAP/K5cre mice (tg/tg/wt) and triple-transgenic AREhPAP/K5cre-caNrf2 mice (tg/tg/tg). Bar, 100 μ m. (B, left panel) TUNEL staining of primary keratinocytes (green), nuclei of tg/wild-type mice (left), and tg/tg K5cre-caNrf2 mice (right) stained with Hoechst (blue). (Right graph) Percentage of TUNEL-positive keratinocytes of tg/wild-type and tg/tg mice without (control) ($P = 0.0012$) and after 8 h of treatment with 25 μ M menadione ($P = 0.0350$). (C, left) Immunofluorescence for cleaved caspase-3 (red), counterstained with Hoechst (blue), in nonirradiated (control) skin and in UVB-irradiated back skin (UVB) (24 hpi, 100 mJ/cm²) of wild-type mice. Bar, 50 μ m. (Right) Number of cleaved caspase-3-positive cells per length epidermis in tg/wild-type and tg/tg mice, 24 hpi, 100 mJ/cm² UVB ($N = 9$, $P = 0.019$). (D) FACS analysis of DCF in control (nonirradiated) and 20 mJ/cm² UVB-irradiated immortalized keratinocytes of tg/wild-type (blue) and tg/tg (red) K5cre-caNrf2 mice. (E, left) Staining for CPD-positive cells in the epidermis of nonirradiated (control) and UVB-irradiated (UVB) wild-type mice. Bar, 50 μ m. (Right) CPD-positive cells per length epidermis, 5 min (left side) and 24 hpi (right side) with 100 mJ/cm² UVB in tg/wild-type and tg/tg K5cre-caNrf2 transgenic mice ($N = 6$). (F, left) Staining for p53-positive cells in the epidermis of nonirradiated (control) and UVB-irradiated (UVB) wild-type mice. Bar, 25 μ m. (Right) p53-positive cells per length epidermis in tg/wild-type and tg/tg K5cre-caNrf2 transgenic mice, 24 hpi, 100 mJ/cm² UVB ($N = 11$, $P = 0.0039$).

increase in the number of apoptotic keratinocytes in the suprabasal layers of the knockout mice (Fig. 3H). This finding is consistent with the decrease in the expression of Nrf2 target genes in suprabasal relative to basal keratinocytes (see Fig. 3G). Surprisingly, a minor decrease in the

number of apoptotic basal cells was observed in Nrf2 knockout mice compared with wild-type controls (Fig. 3H), possibly due to activation of compensatory mechanisms in basal cells that may involve other Nrf family members (Braun et al. 2002).

Figure 3. UVB-induced apoptosis and Nrf2-mediated cytoprotection in the murine epidermis. (A,B, left) H&E staining of wild-type mouse back skin (A) and tail skin (B), 24 hpi, 100 mJ/cm² UVB. Arrows point to sunburn cells in the basal (A,B) and lower suprabasal (B) layers. Bars: A,B, 10 μ m. (Right side) Distribution of sunburn cells in back skin (A) ($N = 11$) and tail skin (B) ($N = 6$) epidermis in percent. (C) Cleaved caspase-3 staining of wild-type mouse tail skin, 24 hpi, 100 mJ/cm² UVB. The arrow points to a positive keratinocyte. Bar, 15 μ m. (D) Tail skin after chymotrypsin treatment stained with rapid differential hematology staining (Haem, left panel) or immunostained for K10 (red), costained with Hoechst (blue) (right panel). (Top picture) Suprabasal layers. (Bottom picture) Basal layer and dermis. The arrow points to remaining K10-positive suprabasal keratinocytes. Bars, 50 μ m. (E) *Nrf2*, *Nqo1*, *Gclc*, *Gclm*, *Gss*, *Gsr*, *Srxn1*, and *Gsta3* expression in suprabasal relative to basal keratinocytes of tail epidermis from wild-type mice shown for two different separations, analyzed by qRT-PCR. Expression levels in basal keratinocytes were set to 1, indicated by dashed line. (F) Immunofluorescence analysis of *Nqo1* (red) in tail skin, costained with Hoechst (blue). Note the staining gradient from basal to suprabasal. Bar, 25 μ m. (G) *Nqo1*, *Gclc*, *Gclm*, *Gss*, *Gsr*, *Srxn1*, and *Gsta3* expression in suprabasal relative to basal keratinocytes of tail epidermis from *Nrf2* knockout (ko) mice, analyzed by qRT-PCR. Note reduced expression of *Nqo1*, *Gclc*, *Gclm*, and *Gss*, but elevated expression of *Srxn1* in *Nrf2* knockout compared with wild-type mice (left graph in E). (H,I) Distribution of sunburn cells per length epidermis in *Nrf2* knockout

mice (H) ($N = 8/9$) and K5cre-caNrf2 mice (I) ($N = 8$) in back skin epidermis, 24 hpi with 100 mJ/cm² UVB. Note the significant difference between basal and suprabasal keratinocytes in wild-type mice ($P = 0.0012$ for H; $P = 0.0002$ for I), the strong increase in suprabasal sunburn cells ($P = 0.059$) but the slight decrease in basal sunburn cells in *Nrf2* knockout mice (H), and the strong decrease in basal sunburn cells in K5cre-caNrf2 mice (I) ($P = 0.0499$). (J) Scheme demonstrating the gradient of ROS detoxification and UVB-induced apoptosis in wild-type and *Nrf2* mutant mice. In wild-type mice, suprabasal keratinocytes express higher levels of Nrf2 and its cytoprotective target genes compared with basal keratinocytes. This results in preferential UVB-induced apoptosis of basal cells. In K5cre-caNrf2 transgenic mice, basal cells are protected from apoptosis, resulting in reduced numbers of mainly basal apoptotic cells and reduction of the apoptotic gradient. In *Nrf2* knockout mice, the gradient is also abolished, mainly due to strongly enhanced apoptosis of suprabasal cells but slightly reduced apoptosis of basal cells. (BL) Basal layer; (SL) spinous layer; (GL) granular layer; (CL) cornified layer.

These findings revealed that endogenous Nrf2 establishes a suprabasal-to-basal expression gradient of many ROS-detoxifying genes, which controls at least in part the basal-to-suprabasal gradient of UVB-induced apoptosis (shown schematically in Fig. 3J).

In support of this hypothesis, pan-epidermal activation of Nrf2 in K5cre-caNrf2 transgenic mice, which results in similar activity of Nrf2 in all epidermal layers, also reduced the gradient of apoptosis due to a preferential reduction in the number of apoptotic cells in the basal layer (Fig. 3I,J).

Generation of mice expressing caNrf2 in suprabasal keratinocytes

To enforce the basal-to-suprabasal gradient of cytoprotection in the epidermis, we next generated transgenic mice expressing caNrf2 only in suprabasal keratinocytes using the bovine keratin 10 (K10) promoter and a previously described expression cassette (Fig. 4A; Werner et al. 1993). Three transgenic founder mice were generated with lower or similar expression of caNrf2 compared with endogenous *Nrf2* (Supplemental Fig. S1C). They showed

Figure 4. Generation of K10caNrf2 transgenic mice and protection from UVB-induced apoptosis. (A) Scheme of the transgene used for generation of transgenic mice. (B) NBT/BCIP staining for hPAP activity in back skin of AREhPAP transgenic mice (left panel) and AREhPAP/K10caNrf2 double-transgenic mice (middle panel). Bar, 50 μ m. (Right panel) Fluorescence staining for hPAP activity (red), immunofluorescence for K14 (green), and counterstaining with Hoechst (blue) on back skin sections of AREhPAP/K10caNrf2 mice. Note nonoverlapping localization of hPAP staining in suprabasal and K14 staining in basal keratinocytes. (C) Wild-type (wt) and K10caNrf2 transgenic mice at P2.5 in overview (top picture) and close-up (bottom picture) of lower back. Note enhanced scaling in K10caNrf2 mice. (D) Longitudinal back skin sections of wild-type (wt) and K10caNrf2 (tg) mice at 8 wk (8w). The arrow points to the area with the thicker cornified layer. Bar, 100 μ m. (E, top panel) H&E staining of 2- μ m sections of skin from wild-type and K10caNrf2 mice. Bar, 2 μ m. (Graph) Morphometric measurement of the thickness of the cornified layer ($N = 5$, $P = 0.0025$). (F) Number of cleaved caspase-3-positive cells per length epidermis in 8- to 10-wk wild-type and K10caNrf2 mice, 24 hpi, 100 mJ/cm² UVB ($N = 8$, $P = 0.003$). (G) Position of p53-positive keratinocytes per length epidermis in 8- to 10-wk wild-type (left) and K10caNrf2 mice (right) ($N = 5$, $P = 0.0317$). (H) Scheme of caNrf2 expression in the epidermis of K10caNrf2 mice and proposed paracrine cytoprotection of basal keratinocytes by suprabasal keratinocytes, indicated by arrows.

strong up-regulation of the Nrf2 target genes *Nqo1*, *Gclc*, and *Gst π* (Supplemental Fig. S1D). Mating of K10caNrf2 mice with AREhPAP mice showed a strong hPAP activity in the epidermis of double-transgenic mice (Fig. 4B). Fluorescence analysis of hPAP activity and K14 immunofluorescence showed selective hPAP expression in suprabasal keratinocytes (Fig. 4B).

Similar to K5cre-caNrf2 mice, K10caNrf2 transgenic mice showed mild hyperkeratosis at P2.5 (Fig. 4C), and a slightly thickened cornified layer (Fig. 4D). This was confirmed by morphometric analysis of 2- μ m paraffin sections of K10caNrf2 mice ($N = 5/7$, $P = 0.0025$, Fig. 4E). The extent of hyperkeratosis was dependent on the genetic background: It was hardly detectable in K10caNrf2 trans-

genic mice in a pure B6D2F1 background, but was very pronounced in a B6D2F1, C57BL/6J mixed background (Supplemental Fig. S1E). In spite of the hyperkeratosis, keratinocyte differentiation was not obviously altered, as determined by normal expression of K14, K10, K6, and loricrin (Supplemental Fig. S1F,G). This is consistent with the normal thickness of the viable part of the epidermis.

Enhanced Nrf2 activity in suprabasal keratinocytes protects basal cells from UVB cytotoxicity

When K10caNrf2 transgenic mice were irradiated with 100 mJ/cm² UVB, the number of apoptotic keratinocytes was significantly lower 24 hpi compared with wild-type

mice, as determined by cleaved caspase-3 staining (Fig. 4F) and TUNEL assay (Supplemental Fig. S2A). The number of p53-positive keratinocytes was also significantly lower (Supplemental Fig. S2B). The anti-apoptotic effect was dose-dependent: While 20 mJ/cm² UVB caused a 60% reduction in the number of apoptotic cells in K10caNrf2 transgenic compared with wild-type mice, 100 mJ/cm² UVB led to only 28% reduction (Supplemental Fig. S2A). Surprisingly, the decrease in apoptosis was similar in K10caNrf2 and K5cre-caNrf2 transgenic mice (see Fig. 2C), in spite of the exclusive expression of caNrf2 in suprabasal keratinocytes of K10caNrf2 mice. Remarkably, a significant UVB protection—as shown by counting of p53-positive cells—was observed in the basal layer, where caNrf2 is not expressed (Fig. 4G; data not shown). This was not due to UVB-induced premature differentiation (Jiang et al. 2007) and consequent caNrf2 expression in the basal layer, since hPAP activity was still restricted to suprabasal keratinocytes after UVB irradiation of K10caNrf2/AREhPAP double-transgenic mice (Supplemental Fig. S2C). Moreover, it is unlikely to result from enhanced absorption of UVB by the thicker cornified layer in K10caNrf2 mice, since the number of apoptotic cells was similar after UVB irradiation of K10caNrf2 mice in the B6D2F1 background and the B6D2F1, C57BL/6 mixed background (Supplemental Fig. S2D), which show a different severity of hyperkeratosis (see Supplemental Fig. S1E). Finally, no difference in the inflammatory response was detected, as the number of neutrophils and mast cells and the expression of the proinflammatory cytokine *IL-1β* were not changed (Supplemental Fig. S2E,F; data not shown).

caNrf2-mediated protection of neighboring keratinocytes is dependent on GSH

Based on these findings, we hypothesized that the protection of basal cells from UVB-induced apoptosis in K10caNrf2 mice is mediated by a paracrine-acting factor (Fig. 4H), which is activated by Nrf2 in suprabasal keratinocytes. A possible candidate is GSH. To functionally test this hypothesis, K10caNrf2 transgenic mice were mated with mice lacking *Gclm*, the modifier subunit of the glutamate cysteine ligase (Yang et al. 2002). These mice can still produce GSH, since they still express the *Gclc*. However, *Gclm* knockout mice (C57BL/6 background) have an 84%–91% reduction in the levels of GSH in a variety of tissues (Yang et al. 2002). *Gclm*-deficient mice showed only a minor increase in the number of apoptotic cells after UVB irradiation compared with wild-type mice, as determined by staining for cleaved caspase-3 (Fig. 5A) and by TUNEL staining (Supplemental Fig. S3A). Apparently, the residual level of GSH is sufficient to protect keratinocytes from UVB-induced apoptosis. In K10caNrf2/*Gclm* double-mutant mice, however, the number of apoptotic cells was not reduced significantly compared with wild-type/*Gclm* knockout mice (Fig. 5A; Supplemental Fig. S3A). Thus the protective effect of caNrf2 was abolished in a *Gclm*-deficient mouse background.

Nrf2 controls a GSH biosynthesis/recycling pathway in the murine epidermis

One possible mechanism for how basal keratinocytes are protected by suprabasal keratinocytes expressing caNrf2 is secretion and import of the antioxidant GSH. So far there is little evidence for a direct cellular import of GSH. Therefore, cells need to import substrates for intracellular GSH synthesis—in particular, the rate-limiting amino acid cysteine (Dalton et al. 2004).

One mechanism to provide extracellular cysteine involves the formation of γ -glutamyl cystine conjugates and the dipeptide cysteine–glycine from GSH by the ectoenzyme γ -glutamyltransferase (γ GT) (Supplemental Fig. S3B; Kinlough et al. 2005). However, γ GT activity was not detectable in the interfollicular epidermis of wild-type and K10caNrf2 transgenic mice before and after UVB irradiation using the chromogenic substrate γ -glutamyl-4-methoxy-2-naphthylamide (GMNA) (Fig. 5B; Rutenburg et al. 1969; De Young et al. 1978). Hair follicles, which are known to have active γ GT in the anagen phase, served as internal positive control (Chiba and Jimbow 1986).

Another mechanism to provide extracellular cysteine involves the glutamate/cystine antiporter x_c^- , which mediates the uptake of cystine. The latter is intracellularly reduced to cysteine, which can be secreted to the extracellular space (see Fig. 6; Lo et al. 2008). qRT-PCR showed that K10caNrf2 transgenic mice express higher levels of the light subunit *xCT* of the x_c^- antiporter compared with wild-type mice (Fig. 5C). Furthermore, a higher expression of this gene was seen in suprabasal compared with basal keratinocytes of wild-type mice (see below; Fig. 5I), consistent with their stronger expression of Nrf2. This most likely leads to increased secretion of cysteine by suprabasal cells in wild-type mice, and particularly in K10caNrf2 transgenic mice (see Fig. 6). Extracellular cysteine is taken up by the ubiquitous alanine–serine–cysteine (Asc) transporter (Christensen 1990). Indeed, expression of *Asct1* (*Slc1a4*) was also increased in the epidermis of K10caNrf2 transgenic mice (Fig. 5C). Interestingly, this gene is expressed at higher levels in basal compared with suprabasal layers of wild-type mice (see below; Fig. 5I), consistent with the requirement of these cells to import cysteine.

Due to its high reactivity, extracellular cysteine is oxidized rapidly to cystine, which cannot be imported by Asc transporters (Toohey 1975). Extracellular cystine, however, can undergo a thiol/disulfide exchange reaction with GSH, resulting in formation of a GSH–cysteine conjugate and free cysteine (see Fig. 6; Wang and Cynader 2000). We investigated whether Nrf2 enhances the levels of extracellular GSH by GSH synthesis, recycling, and export from suprabasal keratinocytes. qRT-PCR revealed a strong increase in the mRNA levels of both *Gcl* subunits (*Gclc* and *Gclm*), as well as of *Gss* and *Gsr*, in nonirradiated K10caNrf2 transgenic mice and 24 hpi with 100 mJ/cm² UVB (Fig. 5D; data not shown). Although the caNrf2-positive suprabasal cells comprise only a minor percentage of the epidermal cells, GSH levels were reproducibly

Figure 5. GSH metabolism in K10caNrf2 transgenic mice. (A) Cleaved caspase-3-positive cells per length epidermis, 24 hpi, 100 mJ/cm² in wild-type and K10caNrf2 transgenic mice crossed with wild-type (*left*) ($N = 15$, $P = 0.0003$) and *Gclm* knockout (*right*) mice ($N = 14$, $P = 0.23$). (B) γ GT activity staining (auburn) in back skin of wild-type and tg mice, nonirradiated (control) and 24 hpi, 100 mJ/cm² UVB-irradiated (UVB) mice. Arrows point to staining in lower hair follicle (HF). No staining is visible in the interfollicular epidermis. Bar, 50 μ m. (C) qRT-PCR of *xCT* and *Asct1* in epidermis of tg versus wild-type mice (set to 1, indicated by dashed line). (D) qRT-PCR analysis of *Gclc*, *Gclm*, *Gss*, and *Gsr* using RNAs from epidermis of wild-type and tg mice. The expression level in wild-type mice was set to 1, indicated by dashed line. The qRT-PCR data shown in C and D were obtained from pooled epidermal samples, and were reproduced with a different set of RNAs from different pools of mice. (E) GSH levels normalized to total protein in keratinocytes of wild-type and tg mice ($N = 5$, $P = 0.0571$). (F) RT-PCR of *Mrp1*, *Mrp3*, *Mrp4*, *Mrp5* in wild-type epidermis with (+) and without (-) RT. (G) qRT-PCR of *Mrp3* in wild-type and tg mice. (H) Immunofluorescence analysis of Mrp3 (red), counterstained with Hoechst (blue) in wild-type and tg mouse back skin. Bars: *left* panel, 15 μ m; *right* panel, 30 μ m. (I) *xCT*, *Asct1*, and *Mrp3* expression in suprabasal relative to basal keratinocytes of wild-type mouse tail epidermis (average of two different separations), analyzed by qRT-PCR. (n.s.) Not significant.

higher in the epidermis of K10caNrf2 transgenic mice compared with wild-type mice (Fig. 5E). Consistent with previous observations (Li et al. 2006), semiquantitative RT-PCR revealed expression of the GSH exporters *Mrp1*, *Mrp3*, *Mrp4*, and *Mrp5* in the murine epidermis (Fig. 5F). *Mrp3* and *Mrp4* were even up-regulated in K10caNrf2 transgenic mice (Fig. 5F,G; data not shown). Immunofluorescence analysis localized Mrp3 in the plasma membrane and cytoplasm of keratinocytes of wild-type and K10caNrf2 transgenic mice (Fig. 5H). In wild-type mice, it was expressed at much higher levels by suprabasal cells compared with basal cells (Fig. 5I), consistent with the enhanced expression of Nrf2 and in accordance with its postulated role in the export of GSH by suprabasal cells. In

contrast, Mrp4 was detected only in the basal membrane of basal keratinocytes and therefore cannot be responsible for the export of GSH from suprabasal keratinocytes (data not shown).

These findings strongly suggest that suprabasal elevation of Nrf2 activity enhances production and secretion of GSH, possibly via Mrp3. In addition, cystine uptake and subsequent release of cysteine is increased. Together, this leads to increased availability of extracellular cysteine and its uptake by basal cells. This allows the production of GSH by these cells, which protects them from UVB-induced apoptosis. This pathway, as well as the role of Nrf2 and its regulation, are shown schematically in Figure 6.

Figure 6. Model describing biosynthesis and recycling of GSH in the epidermis. (*Middle*) Scheme of the murine epidermis with basal, spinous, granular, and cornified layers. Arrows indicate a postulated paracrine cytoprotection of basal keratinocytes by suprabasal keratinocytes. (*Left*) Paracrine action of GSH and cysteine (Cys) involving suprabasal and basal layers. GSH is synthesized in a two-step reaction catalyzed by the enzymes Gcl and Gss. Extracellular cystine (Cys₂), the oxidized (predominant extracellular) form of cysteine, is imported into suprabasal cells by the antiporter X_c⁻. Intracellular cystine is reduced to cysteine, which is the rate-limiting amino acid for GSH synthesis. Cysteine as well as GSH are subsequently exported by suprabasal keratinocytes. GSH export occurs via MRP transporters. Extracellular GSH provides cysteine via a thiol exchange reaction with cystine. Extracellular cysteine can be imported directly by basal keratinocytes via the importer Asc to serve as substrate for GSH synthesis in basal keratinocytes. Genes activated by Nrf2 in keratinocytes are encircled and highlighted with blue. (*Right*) A suprabasal-to-basal gradient of Nrf2 and its target genes coding for ROS-detoxifying enzymes and antioxidant proteins (in red) control UVB-induced apoptosis in the murine epidermis, resulting in an antagonistic basal-to-suprabasal gradient of apoptosis (in blue).

change reaction with cystine. Extracellular cysteine can be imported directly by basal keratinocytes via the importer Asc to serve as substrate for GSH synthesis in basal keratinocytes. Genes activated by Nrf2 in keratinocytes are encircled and highlighted with blue. (*Right*) A suprabasal-to-basal gradient of Nrf2 and its target genes coding for ROS-detoxifying enzymes and antioxidant proteins (in red) control UVB-induced apoptosis in the murine epidermis, resulting in an antagonistic basal-to-suprabasal gradient of apoptosis (in blue).

Most of the genes involved in this pathway are likely to be direct targets of Nrf2, since they were also up-regulated in primary keratinocytes of K5cre-caNrf2 mice (Supplemental Fig. S3C) and down-regulated in the epidermis of Nrf2 knockout mice (Supplemental Fig. S3D), and since they have been described as direct Nrf2 targets in other cells (Supplemental Table 1). The much higher expression of xCT and Mrp3 (Fig. 5I), as well as of Nrf2, Gclc, Gclm, and Gsr (see Fig. 3E), in suprabasal versus basal keratinocytes of wild-type mice strongly suggests that a GSH- and cysteine-mediated paracrine cytoprotection of basal keratinocytes by suprabasal keratinocytes also exists in the epidermis of wild-type mice.

Discussion

We established a novel strategy to study the function of Nrf2 in vivo through expression of a caNrf2 mutant in keratinocytes. Increased Nrf2-mediated gene expression in all layers of the epidermis strongly reduced the rate of UVB-induced apoptosis. This is in line with the UV-protective effect of broccoli sprout extracts (Dinkova-Kostova et al. 2006), and with the enhanced UVB-induced apoptosis in ear skin keratinocytes of Nrf2 knockout mice (Kawachi et al. 2008). As the mechanism underlying the UVB-protective effect of caNrf2, we identified Nrf2-mediated reduction of oxidative stress through enhanced expression of ROS-detoxifying enzymes and increased synthesis of GSH. Enhanced Nrf2 activity did not affect formation and repair of direct UVB-induced DNA lesions (CPDs). Consistent with this finding, CPD formation was also not altered in UVB-irradiated ear skin of Nrf2 knockout mice compared with wild-type controls (Kawachi et al. 2008). We even observed a decrease in the number of cells positive for p53 in caNrf2 transgenic mice, which is stabilized in response to DNA damage and ROS (Liu

et al. 2008). Thus, Nrf2-mediated suppression of apoptosis is unlikely to result in an accumulation of keratinocytes with damaged DNA. Consistent with this finding, treatment of hairless mice with broccoli sprout extract containing sulforaphane, which among other effects activates Nrf2, reduced UVB-induced skin carcinogenesis (Dinkova-Kostova et al. 2006). Thus, activation of Nrf2 seems to be a useful strategy to reduce UVB-induced damage of keratinocytes. This is of major medical importance due to the strong increase in the incidence of epidermal skin cancer.

Interestingly, we noticed a basal-to-suprabasal gradient in UVB-induced apoptosis in the murine epidermis. This is surprising, since UVB is strongly absorbed by keratinocytes and should therefore target predominantly suprabasal keratinocytes (Anderson and Parrish 1981). On the other hand, this finding is biologically important, since prevention of apoptosis in suprabasal cells is essential for the maintenance of skin integrity after UVB irradiation. In contrast, damaged stem and transit-amplifying cells in the basal layer need to be eliminated to avoid malignant transformation. Our analysis in wild-type, caNrf2 transgenic, and Nrf2 knockout mice demonstrates that the remarkably selective protection of suprabasal, differentiated cells against apoptosis is achieved by the predominant expression of Nrf2 and its cytoprotective target genes suprabasally. The gradient of UVB-induced apoptosis is consistent with findings obtained with cultured murine and human keratinocytes, which showed enhanced UVB resistance upon differentiation (Chaturvedi et al. 2004; Sasaki et al. 2005; Mandinova et al. 2008). It may be that Nrf2 induces keratinocyte differentiation, but the normal differentiation pattern of keratinocytes that we observed in K10caNrf2 transgenic mice (see Supplemental Fig. S1F,G) does not support this hypothesis. Differentiation may also be affected by the levels of ROS.

However, the normal differentiation of keratinocytes in mice overexpressing or lacking the ROS-detoxifying enzyme peroxiredoxin 6 in keratinocytes also argues against this possibility (Kumin et al. 2006, 2007). On the other hand, the enhanced UVB resistance of cultured keratinocytes upon differentiation may result from enhanced expression/activity of Nrf2 under these conditions. It will be interesting to address this question and to determine if the increased UVB resistance of in vitro differentiated keratinocytes is reduced or even abolished in keratinocytes from *Nrf2* knockout mice.

In human skin, UVB-induced apoptosis is very limited in basal keratinocytes (Supplemental Fig. S4A,B). This may be due to the higher absorption of UVB light by the thick human epidermis, resulting in reduced exposure of basal keratinocytes to UVB. However, basal keratinocytes of mouse tail skin were also susceptible to UVB-induced apoptosis, although the tail epidermis also comprises multiple layers. Therefore, the most likely explanation for the different finding obtained with human compared with mouse skin is the existence of additional protective mechanisms, such as pigmentation (Supplemental Fig. S4C; Agar and Young 2005). In addition, cytoprotection seems to be increased in the basal layer of human skin, as no significant difference between suprabasal and basal expression of *NRF2*, *NQO1*, *GCLC*, and *GCLM* and only a mild up-regulation of *SRXN* and *GSTA3* in suprabasal layers were observed in human epidermis ($N = 4$) (Supplemental Fig. S4E). However, a basal-to-suprabasal gradient of apoptosis was observed within the suprabasal epidermis (Supplemental Fig. S4A,B). Whether the intra-suprabasal gradient of apoptosis is controlled by an Nrf2-mediated cytoprotective gradient within the suprabasal layers remains to be determined. In favor of such a hypothesis are results from in vitro experiments showing that progression of human keratinocyte differentiation results in increased activities of GST, GPX (GSH peroxidase), GSR, and total SOD. As mentioned above, this was accompanied by enhanced resistance to oxidative stress and reduced UVB-induced apoptosis of differentiated keratinocytes (Vessey et al. 1995; Sasaki et al. 2005; Mandinova et al. 2008).

Finally, our results demonstrate that Nrf2-mediated protection from UVB cytotoxicity in the murine epidermis is not only cell-autonomous. It has been shown in vitro that astrocytes protect neurons from ROS damage by secretion of cysteine and GSH (Desagher et al. 1996; Wang and Cynader 2000; Shih et al. 2003). Using biochemical and genetic approaches, we provide strong evidence for the existence of a similar paracrine mechanism in the epidermis of K10caNrf2 mice in vivo and most likely also in wild-type mice. The model that we propose is summarized in Figure 6. It is based on (1) the observed protective effect of caNrf2-expressing suprabasal keratinocytes for the underlying basal cells; (2) the dependency of this protection on GSH synthesis; (3) the up-regulation of genes involved in GSH production, secretion, and recycling by Nrf2 in keratinocytes in vivo and in vitro; and (4) the predominant expression of *xCT* and *Mrp3* by suprabasal keratinocytes and of *Asct1* by

basal keratinocytes. In addition, a first experiment with primary keratinocytes from wild-type and K5cre-caNrf2 transgenic mice revealed that mouse keratinocytes can indeed take up ^{14}C -cystine, and that the import is enhanced by caNrf2 (data not shown).

While the role of GSH in the protection of basal cells was confirmed by the loss of the protection in a Gclm-deficient background, we cannot fully exclude the possibility that another paracrine-acting factor stimulates GSH production in basal keratinocytes. A potential candidate is Notch-1, which was shown recently to protect keratinocytes from UVB cytotoxicity (Mandinova et al. 2008). However, expression of the Notch-1 target genes *Hes1*, *p21*, $\Delta\text{Np}63$, and *Hey1* was not up-regulated in caNrf2 transgenic mice (data not shown), indicating that Notch-1 is not involved. This further strengthens our hypothesis that the paracrine-acting factor is the low-molecular-weight antioxidant GSH and its precursor, cysteine.

The control of basal keratinocyte survival by suprabasal keratinocytes is likely to be of major biological importance. Under normal conditions, this mechanism could be required to maintain the redox balance and survival of basal keratinocytes, which express low levels of cytoprotective Nrf2 target genes. Upon UVB-induced damage of suprabasal keratinocytes, their protective function is reduced, as demonstrated by the inhibition of cystine import by keratinocytes in response to UVB irradiation and subsequent GSH depletion (Zhu and Bowden 2004). Concomitantly, secretion of GSH and cysteine by suprabasal cells is diminished, resulting in apoptosis of basal keratinocytes. These findings highlight the importance of paracrine interactions and unravel a novel function of antioxidants in the cross-talk between cells at different stages of differentiation within a single tissue.

Materials and methods

Generation of mice transgenic for caNrf2

The cDNA encoding caNrf2 was amplified by PCR from cDNA of mouse skin wounds using oligonucleotides 5'-TCCCCCGG GCCACCATGCAGCACATCCAG-3' and 5'-CGGGATCCATC CTCCCGAAC-3', subcloned, and completely sequenced. It encodes amino acids 89–597 of the murine Nrf2 protein (NCBI NP_035032) (Fig. 1A). Transgenic mice were generated by pronucleus injection (FVB/N background for $\beta\text{ACTcaNrf2}$, and B6D2F1 background for K10caNrf2). Genotyping of founder mice was performed by PCR with the following primers: for $\beta\text{ACTcaNrf2}$, 5'-CGGCTCAGCACCTTGAT-3' (down) and 5'-CT TATCCCAAGCGGCTTCGGC-3' (up); for K10caNrf2, the same down and 5'-CGGAATTCAGCTTGTTCCCG-3' (up). All animal experiments were approved by the local veterinary authorities of Zurich, Switzerland (Kantonales Veterinäramt Zürich).

Human skin biopsies

Biopsies from abdominal human skin from four different female patients were provided by Dr. Gertrude Beer and Dr. Reinhard Dummer (University of Zurich) in the context of the Biobank project (approved by the local and cantonal institution review

boards; EK no. 647). None of the patients claimed having taken a sunbath 1 wk before the surgery, or to have excessive sunbathing habits (more than once a week on average).

Separation of basal and suprabasal keratinocytes, and of epidermis from dermis

For separation of basal and suprabasal keratinocytes, human abdominal skin or murine tail skin was incubated for 40 min at 37°C in 225 U/mL α -chymotrypsin A4 (Boehringer Mannheim) as described in Sasai et al. (1979), or in a less efficient α -chymotrypsin Type IV (Sigma Aldrich). Afterward, the skin was washed in PBS, and the suprabasal layers were removed manually and snap-frozen in liquid nitrogen. Remaining basal keratinocytes were scraped off from the dermis in lysis buffer and were also snap-frozen. For separation of epidermis from dermis, murine back skin was incubated for 90 min at 37°C in 2.5 U/mL dispase (Invitrogen), and the epidermis was scraped off from the dermis and snap-frozen in liquid nitrogen.

UVB irradiation

UVB irradiation of mice was described previously (Kumin et al. 2007). Explants of human abdominal skin (~ 4 cm²) were irradiated within 1 d post-operation with 100 mJ/cm² UVB and were cultured together with control explants for 24 h in DMEM with high glucose (Sigma) containing 10 mL/L penicillin/streptomycin (Sigma) and 3% FCS (Biocconcept).

Morphometric analysis of stratum corneum thickness

Paraffin-embedded skin was cut from apical to basal to obtain 2- μ m-thick sections. After H&E staining, pictures were taken at 1000 $\times$ magnification. Only areas where all layers of the stratum corneum were tightly together were morphometrically analyzed.

Measurement of ROS and GSH levels, and of γ GT activity

Intracellular levels of ROS were determined in primary and immortalized keratinocytes using H₂DCF-DA (Invitrogen) as described previously (Durchdewald et al. 2007). For measurement of GSH levels, the epidermis was isolated from adult murine back skin. For this purpose, shaved back skin was incubated in 0.8% trypsin/PBS for 1 h at 37°C, and the epidermis was scraped off and shaken for 1 h in defined K-SFM (Invitrogen) containing supplements, 10 ng/mL epidermal growth factor, and 10⁻¹⁰ M cholera toxin (Sigma). Consequently, the suspension was filtered, centrifuged, and resuspended in 5% 5-sulfosalicylic acid. After 15 min of incubation on ice and subsequent centrifugation, the supernatant was used for measurement of total GSH using the GSH assay kit (Sigma-Aldrich). The pellet was resuspended in 0.1 M NaOH, and protein measurement was performed using BCA assay.

γ GT activity was analyzed on cryosections of murine back skin as described (Rutenburg et al. 1969).

Morphometry, replicate experiments, and statistical analyses

Morphometrical analysis was performed using ImageJ 1.410 (NIH) or Openlab 3.1.5 (Improvision). RPA and qRT-PCR were performed with RNA pools from two to four animals from one litter and were repeated with independent samples. Statistical analyses were performed using Prism version 5.0 software (GraphPad Software). All data were analyzed using the non-parametric Mann-Whitney *U*-test for non-Gaussian distribution. Error bars represent standard deviation.

Acknowledgments

We thank Christiane Born-Berclaz and Nicole Hallschmid for excellent technical assistance; Dr. Christina Rycken, Claudia Defila, Ann-Helen Willrodt, and Jörg Renkawitz for help with the characterization of the transgenic mice and cell culture experiments; Drs. Francois Verrey (University of Zurich, Switzerland), Simone Camargo (University of Zurich, Switzerland), and Tamara Ramadan (ETH Zurich, Switzerland) for valuable help with the ¹⁴C cystine experiment; Drs. Gertrude Beer and Reinhard Dummer (University of Zurich) for providing human skin samples; Dr. Dietrich Keppler (DKFZ, Heidelberg, Germany) for the Mrp3 and Mrp4 antibodies; Dr. José L. Jorcano (CIEMAT, Madrid, Spain) for the K5-Cre transgenic mice; Dr. Timothy Dalton (University of Cincinnati, Cincinnati, OH) for *Gclm* knockout mice; Dr. Yuet Wai Kan (University of California at San Francisco, San Francisco, CA) for *Nrf2* knockout mice; and Dr. Manfred Blessing (University of Leipzig, Germany) for the K10 promoter. This work was supported by the Swiss National Science Foundation (grant 3100A0-109340 to S.W.); the Promedica Foundation, Chur, Switzerland (to S.W.); and the CERES award (to S.W.). M.S. was supported by an EMBO post-doctoral fellowship and U.a.d.K. was supported by a Boehringer Ingelheim predoctoral fellowship.

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Genes Dev. 2010, **24**:

Access the most recent version at doi:[10.1101/gad.568810](https://doi.org/10.1101/gad.568810)

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