

miRNAs, 'stemness' and skin

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The epidermis and its appendages provide organisms with protection from the environment, keeping pathogens out and preventing the loss of essential body fluids. To perform both functions, the skin has elaborated a complex differentiation process known as cornification. The renewal capacity of the skin, which is responsible for maintaining tissue homeostasis, regenerating hair and repairing the epidermis after injury, resides in the basal proliferating compartment in which epidermal stem cells are located. These cells possess the remarkable capacity to both self-perpetuate and give rise to the differentiating cells that form mature tissues. Recent findings indicate that microRNAs have an essential role in orchestrating the formation of epidermis and skin appendages, in particular, at the interface between stemness and differentiation.

Are miRNAs expressed in skin?

MicroRNAs (miRNAs; see [Glossary](#)) are a class of non-coding RNA genes, the products of which are small single-stranded RNAs of 19–22 nucleotides (nts) that can suppress the expression of protein-coding genes by targeting the 3'UTR region of messenger RNAs (mRNAs) [1–4] ([Box 1](#)). Studies in animal models have revealed essential functions for miRNAs in developmental control. Indeed, many miRNAs exhibit a temporal or tissue-specific expression pattern, indicating that they might have a crucial role in tissue and organ development, function and maintenance. The main evidence that miRNAs are important for the development of the whole organism came from the observation that mutations in *Dicer*, which globally disrupts miRNA processing, cause a range of developmental defects [5]. Loss of function of mouse *Dicer* results in early embryonic lethality and inducible deletion of *Dicer* in mouse embryonic stem (ES) cells causes defects in their proliferation, indicating that, among other functions, *Dicer* might be essential for stem-cell expansion in gastrulating embryos [6,7]. To date, functions have been delineated for only a few of the several hundred identified miRNAs. In mammals, miRNAs regulate lineage differentiation in several tissue types. For example, miRNA-181 modulates haematopoietic lineage differentiation [8]; miRNA-223 regulates human granulopoiesis [9]; miRNA-1 and miRNA-133 promote myogenesis and myoblast proliferation [10]; miRNA-124a and miRNA-9 regulate neuronal lineage differentiation in ES cells [11]; and miRNA-143 is involved in human adipocyte differentiation [12].

The epidermis is a multilayered, stratified epithelium that forms a physical interface between the organism and its external environment, protecting it from pathogen invasion and limiting fluid loss. Epidermal homeostasis relies upon the strictly regulated control of proliferation and differentiation programs within keratinocytes. Indeed, the epidermis is continuously regenerated by the proliferative basal layer, which gives rise to terminally differentiated keratinocytes in a process termed cornification [13]. Indeed, basal keratinocytes periodically withdraw from the cell cycle and commit to differentiation by moving toward the skin surface. These keratinocytes progress through three layers, which correspond to different differentiation stages: spinous layer, granular layer and stratum corneum ([Figure 1a](#)). Basal-layer keratinocytes specifically express keratin (K) 5 and K14, whereas the suprabasal layer (spinous) cells express K1 and K10. Filaggrin, loricrin and transglutaminase 3 (TG3) are produced in the granular layer [13]. The renewal capacity of the epidermis resides in adult stem cells located within the innermost basal layer and in the hair follicle. In the hair follicle, stem cells reside in the bulge that is located at the base of the follicle and is established during morphogenesis. During

Glossary

Adult (or somatic) stem cells: undifferentiated cells found within a differentiated tissue that can renew themselves and differentiate (with certain limitations) to give rise to all the specialized cell types of the tissue from which it originated.

Dicer: a member of the RNase III family of nucleases that specifically cleaves double-stranded RNAs. Dicer processes long dsRNA into siRNAs of 21–23 nt.

Differentiation: the general process whereby an undifferentiated embryonic cell acquires the features of a specialized cell such as a heart, liver or muscle cell.

Drosha: a Class 2 RNase III enzyme that is responsible for initiating the processing of microRNAs, or short RNA molecules naturally expressed by the cell that regulate a wide variety of other genes by interacting with the RNA-induced silencing complex (RISC) to induce cleavage of complementary mRNA.

Embryonic stem cells: undifferentiated cells derived from day 5 pre-implantation embryos that have the potential to become a wide variety of specialized cell types.

MicroRNAs (miRNAs): tiny pieces of RNA, about 21–23 bases in length, which bind complementary stretches of mRNA, thereby decreasing the production of the corresponding protein. MicroRNAs were first discovered in the roundworm *C. elegans* in the early 1990s and have now been identified in many species, including humans.

Niche: the microenvironment in which stem cells are found.

p63: a transcription factor that belongs to the p53 family of tumour suppressors. p63 expression is crucial for the development of stratified epithelial tissues such as epidermis, breast and prostate and is responsible for maintaining the proliferative potential of the epithelial stem cells. *TP63* encodes multiple protein isoforms that possess both transactivating and transcriptional repressor activities and which regulate a wide spectrum of target genes. p63 is also implicated in tumour formation and progression in stratified epithelia.

Box 1. miRNA formation

miRNAs are generated by the sequential processing of long RNA polymerase II transcripts into a larger primary transcript (pri-miRNA) of several kilobases in length. These transcripts are capped, polyadenylated and cleaved in the nucleus by the nuclear RNase III endonucleases, Drosha [3], to generate a second set of precursors of ~70 nucleotides in length (pre-miRNA). The pre-miRNA is exported to the cytoplasm by means of the exportin 5 pathway and is further processed by the cytosolic enzyme Dicer, to yield a miRNA duplex of 19–24 nucleotides. One strand, the 'mature' miRNA, is incorporated into an effector complex termed the RNA-induced silencing complex (RISC). Here, it engages with specific sequences in target mRNA 3'UTRs. By associating with the 3'UTR region of target mRNAs, miRNAs direct mRNA cleavage or translational repression based on sequence complementarity [4]. According to this model, perfect sequence homology leads to endonucleolytic cleavage and degradation of the mRNA, whereas less strict complementarity results in translational repression. Approximately 50% of all miRNAs are embedded within introns of protein coding genes, indicating that a large number of miRNAs might be transcriptionally linked to the expression of their host gene promoters. Other miRNAs are clustered in polycistronic transcripts and undergo coordinated expression. To date, several hundred miRNAs have been identified, but the total number could be close to a thousand. It is predicted that up to 30% of human genes are regulated by miRNAs. In addition, each miRNA can target several hundred mRNA 3'UTRs, making miRNAs a large family of 'regulatory' molecules. Although bioinformatics is extremely valuable for predicting a given target sequence, experimental validation is always required. The conditional *Dicer1* knockout, which eliminates all miRNA formation, highlights the role of miRNAs in specific cellular processes (Figure 1b).

the hair cycle, bulge stem cells are stimulated to exit the niche, proliferate and differentiate to form the various cell types of the mature follicles. In addition, the bulge stem cells are a reservoir of multipotent adult stem cells that are recruited during wound healing to facilitate epidermal repair [14]. Clonogenicity studies have also identified

the presence of interfollicular epidermal stem cells with self renewal capacity [15]. Careful analyses of human skin keratinocytes revealed three different groups of cells based on the size of the clones that they generate in single plating experiments [15]: holoclones, meroclones and paraclones. Holoclones have the greatest proliferative potential: 95% of their cells continue forming proliferative colonies on passaging. Meroclones have intermediate proliferative potential and paraclone cells differentiate after only a few passages. In addition, holoclone cells can become meroclone and paraclone cells, but movement in the opposite direction has never been observed. As a consequence, the high proliferating holoclone-generating cells isolated *in vitro* are considered to be adult slow-cycling stem cells [16]. Recent studies estimate that ~0.01% of basal cells are epidermal stem cells, at least in mouse skin [17].

Specific markers for these interfollicular adult stem cells remain to be identified, but the intensity of some markers are proposed to enrich the epidermal stem-cell population *in vitro*. In particular, high surface levels of integrin $\beta 1$, denoted by $\beta 1^{\text{high}}$, in human skin [18] and integrin $\alpha 6^{\text{high}}$ and antibody-recognizing transferring receptor (CD71) $^{\text{low}}$ or Hoechst dye exclusion in mouse skin [19,20] are used to enrich epidermal stem-cell populations. Recent results indicate that the transcription factor p63 is highly expressed in holoclone cells; moreover, p63 is required to maintain the proliferative potential of these cells [16]. Other cells, derived from interfollicular stem cells, but with a more limited proliferative capacity, are regarded as transient amplifying (TA) cells. The epidermal differentiation process requires coordination of complex molecular events that lead to generation of a stratified cornified epithelium. Many molecules control keratinocyte proliferation and differentiation programs, including c-myc, Notch, activator protein 1 (AP1), activating enhancer

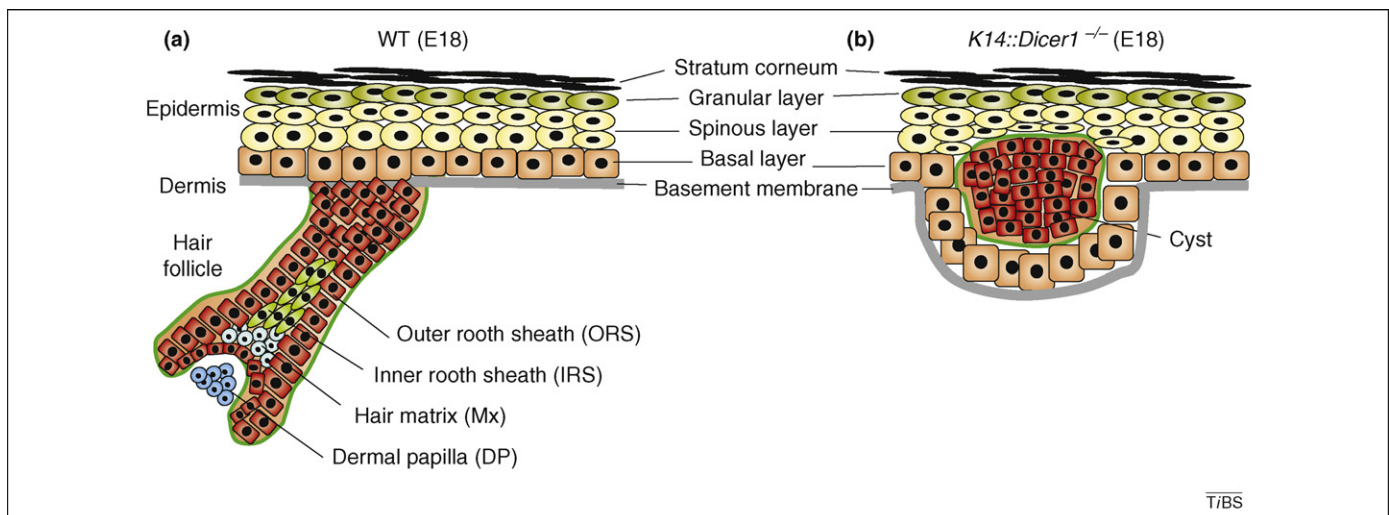


Figure 1. Skin epithelium and morphological abnormalities in *Dicer1* conditional null skin. This figure depicts mouse skin epithelium at developmental stage E18. **(a)** Keratinocytes within the basal layer (tan) adhere to the underlying basement membrane (gray). Keratinocytes are proliferating cells, but they lose their proliferative capacity when they detach from the basement membrane and embark on a migration process toward the skin surface (spinous layer, yellow; granular layer, lime green; stratum corneum, black). It is widely accepted that the epidermal interfollicular proliferative unit (basal layer) is composed of stem cells and transiently amplifying cells, which divide a few times and then leave the basal layer to execute their differentiation program. The hair follicle (outlined in green) proliferative unit is located in the bulge, a permanent portion of the hair follicle, during cycles of hair cycling, which are still not well defined at E18. Spinous and granular layers represent intermediate differentiation steps. The stratum corneum represents the final phase of terminal differentiation. During embryonic development, some of the undifferentiated basal cells are instructed to migrate downwards into the dermis to form a hair follicle. The dermal papilla (the dermal part of the hair follicle; blue) is formed and signals to the developing hair to grow and form different cell lineages of the hair follicle (inner root sheath, companion layer, medulla, cortex and cuticle of the hair shaft). **(b)** In *Dicer1* conditional skin knockout mice, the interfollicular epidermis remains normal. Abnormal hair follicles are generated in *Dicer1* conditional knockout skin, in which the hair shaft does not extend beyond the level of the epidermis and hair germs seem to evaginate in the epidermis rather than invaginating into the dermis, thereby resulting in cyst formation within the epidermis.

Review

binding protein 2 α (AP2 α), CCAAT-enhancer binding proteins (C-EBP) and p63 [21–26]. Skin appendages such as teeth, hair and several glands including the mammary gland, originate from adjacent epithelial (ectodermal) and mesenchymal (mesodermal or neural crest-derived) tissues. The early developmental stages of different skin appendages are similar and, in each case, the placode

forms a bud structure [27] that migrates outwards or inwards depending on the type of appendage. Hair follicles develop from the rapidly proliferating matrix, which is sustained by the dermal papilla. The proliferating matrix cells give rise to several differentiation specific lineages [28], including the inner root sheath, companion layer, medulla, cortex and cuticle of the hair shaft [29].

Table 1. miRNAs expressed in epidermis and hair follicle^a

miRNAs ^b	Expression ^c	Expression in other tissues and/or in diseases	Targets ^d	Refs ^e
miRNA-15a	hf	Leukaemia	Unknown	
miRNA-15b	hf, epi	Leukaemia, antigen-induced T-cell differentiation	<i>BCL2</i>	[47]
miRNA-16	hf, epi	Leukaemia, antigen-induced T-cell differentiation	<i>CCND1, CCND3, CDK6</i>	[48]
miRNA-17–5p	hf, epi	T-cell and monocyte differentiation	<i>AML1</i>	[49]
miRNA-18	hf, epi	Unknown	Unknown	
miRNA-19b	epi	T cells, leukaemia	<i>MYLIP, RBP1-like</i>	[50]
miRNA-20	hf, epi	Megakaryocytopoiesis	Unknown	
miRNA-21	hf, epi	Invasion, metastasis, squamous cell carcinoma, antigen-induced T-cell differentiation	<i>TPM1, PDCD4, MASP1N</i>	[51]
miRNA-24	hf, epi	Myogenic differentiation	Unknown	
miRNA-27a	epi	Breast cancer	<i>MYT1, ZBTB10</i>	[52]
miRNA-27b	hf, epi	Angiogenesis	<i>CYP1B1</i>	[53]
miRNA-30b	epi	Unknown	Unknown	
miRNA-34a	epi	Apoptosis in cancer cells	<i>BCL-2</i>	[54]
miRNA-92	hf, epi	Lymphoma, B cell development	<i>PTEN, BIM</i>	[55]
miRNA-93	epi	Gastric cancer	<i>CDKN1A, E2F1, BIM</i>	[56]
miRNA-99b	hf, epi	Megakaryocytopoiesis	Unknown	
miRNA-106b	hf, epi	Gastric cancer	<i>CDKN1A, E2F1, BIM</i>	[56]
miRNA-125a	epi	Breast cancer	<i>ERBB2, ERBB3</i>	[57]
miRNA-125b	hf, epi	Breast cancer, ovarian cancer	<i>ERBB2, ERBB3</i>	[57]
miRNA-126	hf	Endothelial cell, leukocyte adherence, breast cancer	<i>VCAM1, SOX4, VCRK</i>	[58,59]
miRNA-127	hf, epi	Tumour suppression	<i>BCL6</i>	[60]
miRNA-130a	hf, epi	Angiogenesis, Megakaryocytopoiesis	<i>GAX, HOXA5</i>	[61]
miRNA-133b	epi	Colorectal cancer	Unknown	
miRNA-141	epi	Placenta, EMT	<i>ZEB1, ZEB2</i>	[62]
miRNA-143	hf	Adipocytes differentiation, B-cell cancer	<i>ERK5</i>	[63,12]
miRNA-152	hf	Breast cancer	Unknown	
miRNA-191	epi	Leukaemia	Unknown	
miRNA-196	hf	Limb development, myeloid differentiation	<i>HOXB8</i>	[64]
miRNA-199a	hf	Leukaemia, ovarian cancer	Unknown	
miRNA-199b	hf	Unknown	Unknown	
miRNA-200a	epi	Ovarian cancer, EMT	<i>ZEB1, ZEB2</i>	[62]
miRNA-200b	epi	Ovarian cancer, brain, EMT	<i>Zfhx1b</i>	[65]
miRNA-200c	epi	Ovarian cancer, leukaemia	<i>ZEB1, ZEB2</i>	[62,66]
miRNA-203	epi	Skin, hair, ovary, squamous cell carcinoma	<i>TP73L, ZEB2, SOCS3, ABL1</i>	[31,32,39,42]
miRNA-205	epi	Ovarian cancer, squamous cell carcinoma	Unknown	
miRNA-206	hf	Skeletal muscle	<i>POLA, CX43, ESR1, FTL1</i>	[67]
miRNA-214	hf	Ovarian cancer	<i>PTEN</i>	[68]
miRNA-351	hf	Unknown	Unknown	
miRNA-429	epi	EMT	<i>ZEB1, ZEB2</i>	[62,66]
Let-7a	hf, epi	Lymphoma	<i>MYC</i>	[69]
Let-7b	hf, epi	Acute lymphoblastic leukaemia	Unknown	
Let-7c	hf, epi	Unknown	Unknown	
Let-7d	hf	Unknown	Unknown	
Let-7f	hf, epi	Angiogenesis, antigen-induced T-cell differentiation	Unknown	
Let-7 g	hf, epi	Unknown	Unknown	
Let-7i	hf, epi	Immune response	<i>TLR4</i>	[70]

^aAbbreviations for all mRNAs: *AML1*, acute myeloid leukaemia 1; *BCL2*, B-cell chronic lymphoid leukaemia (CLL) and lymphoma 2; *BCL6*, B-cell CLL and lymphoma 6; *BIM*, BCL2-like 11; *CCND1*, cyclin D1; *CCND3*, cyclin D3; *CDK6*, cyclin-dependent kinase 6; *CDKN1A*, cyclin-dependent kinase inhibitor 1A; *CX43*, connexin 43; *CYP1B1*, cytochrome P450, family 1, subfamily B, polypeptide 1; EMT, epithelial-to-mesenchymal transitions; *ERBB2*, v-erb-b2 erythroblastic leukaemia viral oncogene homolog 2; *ERBB3*, v-erb-b2 erythroblastic leukaemia viral oncogene homolog 3; *ERK5*, extracellular-signal-regulated kinase 5; *ESR1*, estrogen receptor 1; *E2F1*, E2F transcription factor 1; *FTL1*, ferritin light chain 1; *GAX*, growth arrest homeobox transcription factor; *HOXA5*, homeobox A5; *HOXB8*, homeobox B8; *MYC*, v-myc myelocytomatosis viral oncogene homolog; *MYT1*, myelin transcription factor 1; *PDCD4*, programmed cell death 4; *POLA*, polymerase (DNA directed); *PTEN*, phosphatase and tensin homolog; *RBP1-like*, retinoblastoma-binding protein; *SOX4*, SRY (sex determining region Y)-box 4; *TLRs*, toll-like receptor; *TPM1*, tropomyosin 1; *VCAM1*, vascular cell adhesion molecule; *VCRK*, v-crck sarcoma virus CT10 oncogene homolog (avian)-like; *ZBTB10*, zinc finger and BTB domain containing 10; *ZEB1*, zinc finger E-box binding homeobox 1; *ZEB2*, zinc finger E-box binding homeobox 2; *Zfhx1b*, zinc finger homeobox 1b.

^bmiRNAs detected with high signal intensity in microarray analyses of control mouse skin and hair [27,28]. miRNAs are not listed in order of expression frequency.

^cmiRNA detected in hair follicle (hf) and/or epidermis (epi).

^dUnknown: targets not yet identified.

^eReferences are related to the identification of the target mRNAs.

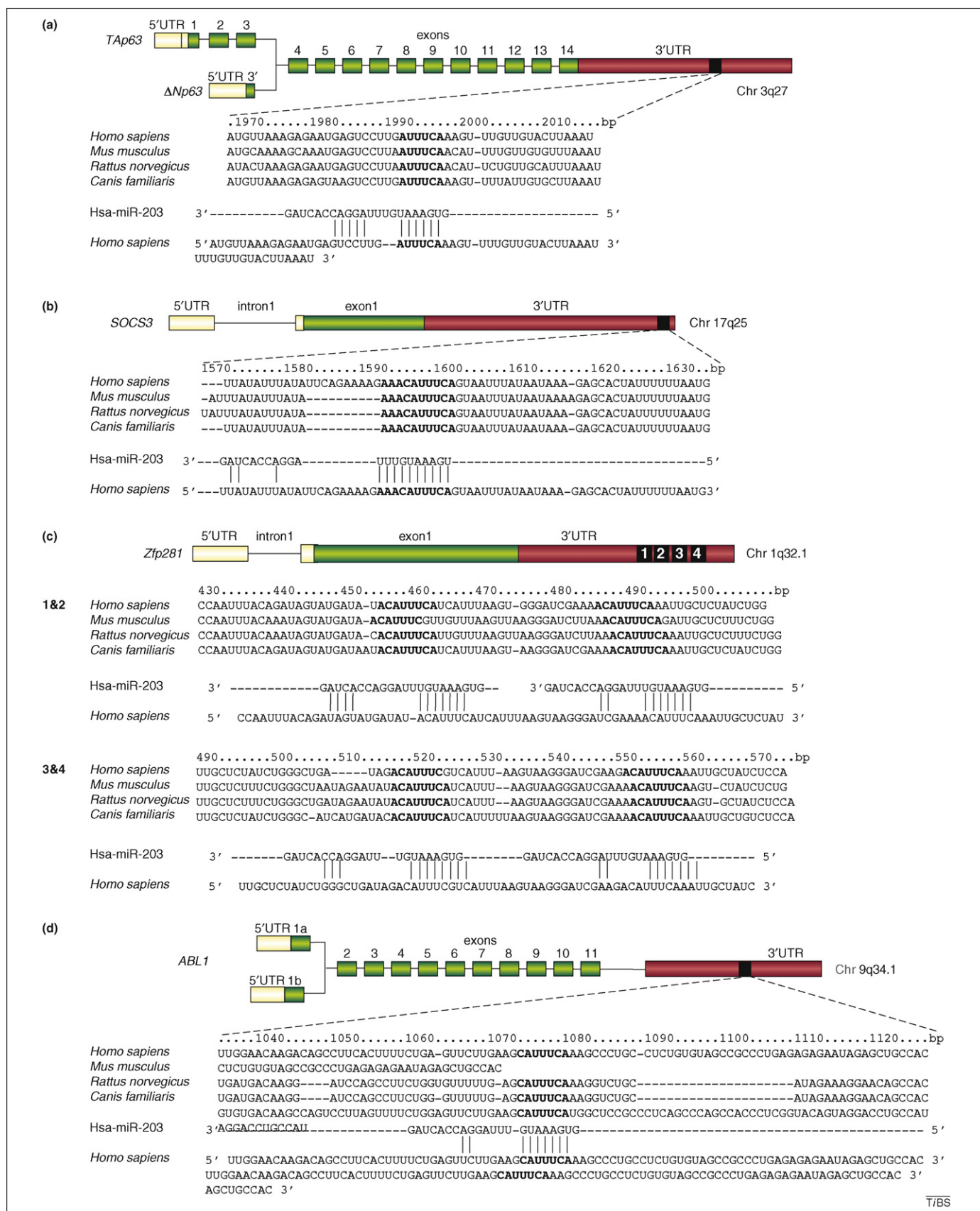


Figure 2. miRNA-203 targets *p63*, *Zfp281*, *SOCS3* and *ABL1* mRNAs within their 3' UTRs. The miRNA-203 recognition site in the 3' UTRs (red) of different targets is highly conserved. (a) The miRNA-203 recognition sequence is present in the *Tp63* 3'UTRs of human, mouse, rat and dog (indicated in bold). The miRNA-203 target site within the *Tp63* 3'UTR (black box; expanded underneath) was identified by TargetScan 4.1 software (<http://www.targetscan.org>). The human *Tp63* 3'UTR contains an additional heptamer sequence that matches the miRNA-203 binding consensus sequence [25]; however, this sequence is not conserved in other vertebrates and, therefore, we have not considered it further. Exons (green) and introns (black lines) are not drawn to scale; the 5'UTR is indicated in yellow. (b) The *SOCS3* 3'UTR contains a miRNA-203 recognition sequence in human, mouse, rat and dog. The 8mer seed sequence is indicated (bold). *SOCS3* mRNA is, therefore, a putative

Different stages of the differentiation of both the epidermis and the appendages are associated with a distinct protein expression signature. The differential gain or loss of expression of particular proteins can be regulated, for example, at the transcriptional level or by selective protein degradation. Recently, however, considerable interest has arisen regarding the role of several classes of small RNA molecules in the regulation of protein expression. The role of miRNAs in epidermal differentiation has received particular attention in recent years [30–33]. In this review, we discuss the current understanding of how miRNAs help to orchestrate epidermal development and regulate adult stem-cell maintenance in stratified epithelial tissue such as the epidermis.

miRNAs are important for skin development and homeostasis

To determine whether miRNAs are globally important for epidermal and hair follicle development, *Dicer* was specifically deleted in the epidermis [32,33]. *Dicer* is expressed in epidermis and in developing hair follicles both in mouse embryos (at the embryonic [E] 14.5 stage in which primary hair follicle development is initiated) and postnatally, as shown by *in situ* hybridization [33]. Conditional ablation in embryonic skin progenitors was achieved using Cre recombinase driven by the K14 promoter, which is specifically expressed in the basal layer. These mice were generated by two independent laboratories, using similar strategies, and displayed similar phenotypes [32,33]. Although epidermal-specific *Dicer* null mice were indistinguishable from control littermates as newborns, they had a life span of only a few days (4–7 days postnatal). Indeed, they began to lose weight 1–2 days after birth and neonatal epidermal-specific *Dicer* null mice seemed dehydrated [33]. A few days after birth, histological abnormalities began to appear (Figure 1b). Although the interfollicular epidermis looked normal, the granular layer displayed fewer granules and the dermal papilla, instead of invaginating downwards into the dermis, seemed to evaginate (Figure 1b). Epidermal-specific *Dicer* null mice developed large germ-like cysts, which disrupted the epidermis, resulting in apoptosis induction in both the hair bulb cells and the interfollicular epidermis. Hair-shaft and inner-root-sheath differentiation was initiated, but the mutant hair follicles were mis-orientated. The physical abnormalities caused by these cysts probably compromise the barrier function of the epidermis. Analyses of the hair follicular cells within the cysts indicated that cyst formation most probably arose from altered signalling from hair follicle epithelium to the dermal papilla [32,33]. Andl *et al.* [33] also described the absence of hair-follicular stem-cell marker expression (K15 and CD34). In addition, hyperproliferation was detected in the epidermis of older epidermal-specific *Dicer* null mice. The absence of significantly increased apoptosis in older (2 month) epidermal-specific *Dicer* null mice epidermis indicates that, during aging, these mice might be prone

to developing skin tumours [32]. These results indicate the functional importance of miRNAs in epidermis and in the morphogenesis of epithelial appendages.

miRNA expression in the epidermis and hair follicle

Using two different microarray approaches, the Fuchs and Millar laboratories identified specific miRNAs expressed in developing mouse skin and hair follicles [32,33]. The Fuchs laboratory isolated and cloned small RNAs from E17.5 epidermis and hair follicles [32] (Table 1). By contrast, the Millar laboratory [33] identified miRNAs in full-thickness skin samples at postnatal day 1 and compared them with the miRNAs expressed in full-thickness skin of K14::DKK1 (Dickkopf 1) transgenic mice (*DKK1* is a potent Wntless [WNT] inhibitor; these mice do not develop hair). Considerable overlap is observed between the miRNAs expressed in epidermis and hair follicles (Table 1). Many skin miRNAs can be classified into families on the basis of their 5' seed sequences, but others cannot and represent orphan sequences. Some miRNAs showed selective expression between epidermis and hair follicles. In particular, the miRNA-200 family (i.e. miRNA-200a, miRNA-200b, miRNA-200c, miRNA-141 and miRNA-149) and the miRNA-19 and miRNA-20 family (i.e. miRNA-19b, miRNA-20, miRNA-17-5p and miRNA-93) are preferentially expressed in the epidermis, whereas the miRNA-199 family (miRNA-199a and miRNA-199b) is exclusively expressed in hair follicles [32]. Among the orphan miRNAs, miRNA-203 is highly and specifically expressed in epidermis. miRNA-203 is also upregulated in psoriatic plaques (see later), indicating that miRNA deregulation could be involved in epidermal pathogenesis.

miRNA-203 regulates the exit from 'stemness'

miRNA-203 is abundantly expressed in the epidermis and in hair follicles between the E13.5 and E15.5 developmental stages. Its expression is absent in single-layered epidermal progenitors during early developmental stages but appears at E14.5 upon the induction of stratification [30]. In adult epidermis, miRNA-203 is not expressed in proliferating basal-layer keratinocytes but is detectable in the upper layers. Moreover, miRNA-203 is rapidly upregulated when primary keratinocytes isolated from newborn mouse skin are induced to differentiate *in vitro* by calcium addition [31].

The transcription factor p63 is essential for the formation of the epidermis, skin appendages and other stratified epithelia; mice lacking p63 show profound defects in epithelial and appendage formation [23,24]. In addition, p63 expression is required to maintain the proliferative potential of epidermal stem cells [16]. The p63 protein, encoded by *TP63*, is the progenitor of the *TP53* gene family, which includes the tumour suppressor p53 [16,34–36]. All three members of this family, p53, p63 and p73, have two distinct promoters coding for a full length protein (TA isoforms) or for an amino-truncated isoform (ΔN isoforms). In addition, extensive 3' splicing generates a large number of C-terminal isoforms, the physiological roles of which

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have not yet been fully elucidated. Mutations in the *TP63* sequence are responsible for several genetic diseases which cause limb and skin defects [37]. Because the expression of Δ Np63, the principal p63 isoform found in the epidermis, is largely confined to the basal layer and disappears in the upper layers, it is possible that the inverse correlation between miRNA-203 and Δ Np63 expression in the epidermis provides evidence for a functional relationship. The 3'UTR of Δ Np63 mRNA contains a heptamer that perfectly matches the miRNA-203 consensus sequence (Figure 2a). miRNA-203 expression reduces the activity of a luciferase reporter gene that contains the Δ Np63 3'UTR [31] and deletion of the Δ Np63 3'UTR heptamer abolishes miRNA-203-mediated repression under the same conditions. Similarly, Δ Np63 protein expression is markedly diminished in proliferating mouse keratinocytes upon miRNA-203 overexpression [31]. This miRNA-203 recognition sequence is conserved in the Δ Np63 3'UTR of several species [31,32] (Figure 2a), indicating that this miRNA-203-dependent mechanism for regulating Δ Np63 is conserved among species.

The association between miRNA-203 and Δ Np63 has also been elegantly addressed *in vivo* [30]. Transgenic mice that overexpress miRNA-203 in the basal layer under the control of the K14 promoter (K14::miRNA-203) die shortly after birth, probably owing to dehydration. Moreover, the epidermis of these newborn mice lacks basal progenitor cells. Indeed, Δ Np63 is detected in the skin of these mice only sporadically and its expression is associated with a reduction of proliferating cells *in vivo* [30]. Injection of antago-miR-203 (the complementary miRNA-203 sequence; the miRNA equivalent of knockdown of coding RNA sequences) into newborn mice repressed miRNA-203 expression; as a consequence, Δ Np63 expression persists longer in the epidermis, which results in increased keratinocyte proliferation *in vivo* [30]. Likewise, whereas the proliferative capacity of primary keratinocytes in a colony-forming assay *in vitro* is reduced by miRNA-203 expression, antago-miRNA-203 expression increases cell growth [31]. Furthermore, primary keratinocytes isolated from transgenic mice (K14::miRNA-303) produced mostly paraclones in an *in vitro* clonogenic assay; by contrast, wild-type keratinocytes also formed typical holoclones, as expected [30]. miRNA-203 overexpression is not, by itself, sufficient to trigger epidermal differentiation, indicating that processes, in addition to inhibition of proliferation, are involved. These results, therefore, indicate that miRNA-203 impacts epidermal proliferation predominantly by restricting the proliferative potential of basal-cell precursors as they migrate from the basal layer to the suprabasal layers (Figure 3).

An *in vitro* ES cell model that mimics the early embryonic steps of epidermal development shows that endogenous Δ Np63 enables ectodermal progenitor K8 and K18⁺ cells to become K5 and K14⁺ stratified epithelial cells, whereas Δ Np63 loss-of-function drastically impairs commitment to the epithelial fate [38]. These findings indicate that, consistent with the well-demonstrated role of p63 in epithelial-cell proliferation [16], Δ Np63 could be essential for embryonic epidermal fate. Interestingly, Δ Np63 mRNA upregulation during ES-driven embryonic epidermal fate

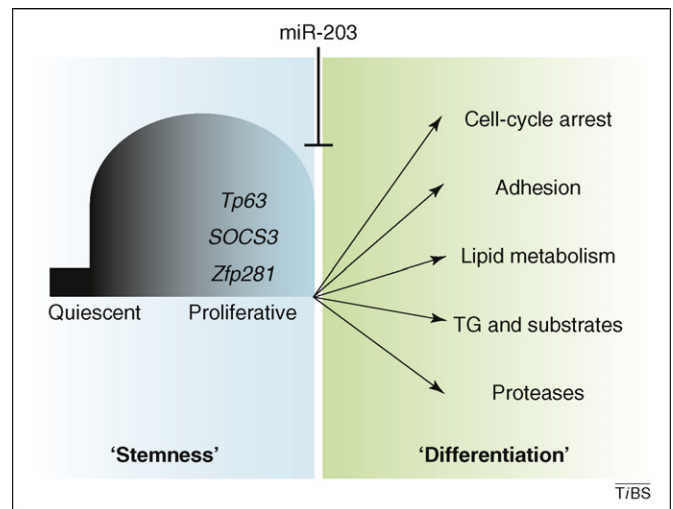


Figure 3. miRNA-203 functions as a switch between epidermal proliferation and differentiation. The epidermal 'stemness' compartment includes quiescent (stem cells) and proliferative basal progenitor (transient amplifying) cells. miRNA-203 has an important role in regulating the transition between progenitor cells with high proliferative potential (cells belonging to the basal proliferative layer of the epidermis, also termed the proliferative compartment) and terminal differentiating suprabasal cells. The keratinocyte differentiation process includes several changes in cells, such as cell-cycle arrest, change of adhesion molecule expression, changes in lipid metabolism, the expression of specific markers such as TG and their substrates, and the expression of specific proteases. miRNA-203 participates in this transition by repressing Δ Np63 expression [30,31], whereas Δ Np63 expression regulates 'stemness' maintenance in stratified epithelial tissues [16]. *SOCS3* [31] and *Zfp281* are additional potential miRNA-203 targets, which might contribute in the basal-basal-suprabasal cell transition. *Zfp281* is involved in maintaining ES cell pluripotency and proliferative capacity. According to this model, miRNA-203 contributes to the switch between proliferation and differentiation in epithelia.

occurs concomitantly with miRNA-203 downregulation [31]. These data indicate that miRNA-203 might be absent or only expressed at low levels in multipotent proliferating cells to enable p63-dependent epidermal commitment, whereas its expression increases as the multipotent epidermal cells exit the proliferating basal layer and terminally differentiate. Functional genomics using both miRNAs and antago-miRNAs will be required to confirm this hypothesis.

The mechanism(s) by which miRNA-203 can regulate the transition of keratinocytes from the basal layer to the upper layers is probably more complex. Indeed, miRNA-203 can also target the mRNA encoding Zinc finger protein 281 (*Zfp281*) [31] (Figure 2c) and, possibly, other still unknown mRNAs. It is important to note, however, that although *Zfp281* is also expressed in basal-layer keratinocytes, its expression has not yet been studied in the context of skin development or epidermal stem-cell proliferative potential.

miRNAs are deregulated in epidermal diseases and tumours

miRNA expression profiles differ between healthy human skin and two chronic inflammatory diseases: psoriasis and atopic eczema [39] (Table 2). The differences include miRNA-21 upregulation in both diseases and specific miRNA-203 upregulation in psoriasis. miRNA-122 is downregulated in both psoriasis and atopic eczema, whereas miRNA-99b downregulation is observed specifically in psoriasis. The significant miRNA-203 upregulation

Table 2. miRNA expression in psoriasis and atopic eczema^a

Psoriasis		Atopic eczema	
Upregulated miRNAs ^b	Downregulated miRNAs ^b	Upregulated miRNAs ^b	Downregulated miRNAs ^b
miRNA-17-5p	miRNA-10a	miRNA-17-5p	miRNA-122a
miRNA-20a	miRNA-22	miRNA-20a	miRNA-133a
miRNA-21	miRNA-30c	miRNA-21	miRNA-133b
miRNA-30e-5p	miRNA-99b	miRNA-24	miRNA-215
miRNA-31	miRNA-100	miRNA-27	miRNA-326
miRNA-106a	miRNA-122a	miRNA-29a	miRNA-335
miRNA-142-3p	miRNA-125b	miRNA-106b	miRNA-483
miRNA-146b	miRNA-133a	miRNA-146a	miRNA-515-5p
miRNA-146a	miRNA-133b	miRNA-193a	miRNA-519
miRNA-200a	miRNA-197	miRNA-199a	
miRNA-141	miRNA-215	miRNA-146	
miRNA-203	miRNA-326		
	miRNA-365		
	miRNA-381		
	miRNA-518b		
	miRNA-524		
	let-7e		

^aData are derived from whole skin analyses and include different cell types, not only keratinocytes.
^bmiRNAs that show a >1.7-fold change in expression between normal skin and psoriasis and normal skin and atopic eczema [39]. miRNAs are not listed in order of expression frequency.

in psoriatic plaques coincides with the downregulation of a conserved miRNA-203 potential target, suppressor of cytokine signalling 3 (*SOCS3*; **Figure 2b**), the product of which is involved in both inflammatory responses and keratinocyte functions [40,41]. This finding might indicate that miRNAs contribute to psoriatic pathogenesis by acting on the crosstalk between keratinocytes and infiltrating cells involved during the inflammatory response. However, *SOCS3* has not been shown to be a bona fide miRNA-203 target in psoriatic skin [39] and, in addition, miRNA-203 overexpression in normal mouse keratinocytes does not repress *SOCS-3* protein expression [31]. This finding indicates that the role of increased miRNA-203 expression in psoriasis *in vivo* is complex and further studies on the interplay between miRNA-203, *SOCS3* and other miRNA-203 targets in psoriasis are clearly needed.

The relationship between miRNA-203 and $\Delta Np63$ might be relevant, not only to normal and benign pathological keratinocyte biology, but also to cancer biology. $\Delta Np63$ lacks the major transactivation (TA) domain of TAp63 and can function as a dominant-negative inhibitor of the ability of TAp63, the related protein TAp73, and of p53 to induce cell-cycle arrest and DNA-damage-induced expression of pro-apoptotic genes [41–44]. miRNA-203 expression increases after UV-C irradiation in squamous cell carcinoma cells, thus, repressing $\Delta Np63$. Consequently, TAp73 isoforms can induce cell death [31] in response to DNA damage. Recent evidence indicates that miRNA-203 also has an important role as a tumour suppressor miRNA in other tumour types, including chronic myelogenous leukaemias (CML) and lymphoblastic leukaemias [42]. miRNA-203 is lost in these hematopoietic tumours owing to a 7-Mb chromosomal region deletion or via promoter CpG (cytosine and guanine separated by a phosphate, which links the two nucleosides together in DNA) hypermethylation. miRNA-203 controls the expression levels of Abelson murine leukaemia viral oncogene homolog 1 (*ABL1* or *c-abl*; **Figure 2d**), a classic oncoprotein, and the expression levels of breakpoint cluster region (BCR)–*ABL1* translocation protein produced by the Phila-

delphia chromosome t(9;22), present in CML [42]. The reintroduction of miRNA-203 in these tumour cells decreases their proliferation, thus, offering the potential for novel therapeutic options.

Concluding remarks and future perspectives

These new findings add epidermal development to the extensive role of miRNAs in overall animal development (as shown by the global *Dicer* disruption) and demonstrate that specific miRNAs participate in lineage differentiation in a wide variety of tissue types. The further investigation and elucidation of the molecular pathways that induce miRNA expression in skin will increase our understanding of the molecular mechanisms underlying skin diseases. Although studies of miRNAs in the epidermis have, so far, concentrated on miRNA-203, much remains to be learned about the role of the other epidermally-expressed miRNAs (**Table 1**). Further exploration of these miRNAs could facilitate the identification of new target genes involved in skin pathology. This knowledge, in turn, could be translated into new tools for diagnosis, prognosis and therapy. In particular, the differences in miRNA expression patterns between normal, psoriatic and eczematous skin (again, not just involving miRNA-203) should be informative. Could we approach new forms of anti-psoriatic therapy by topical application of antago-miRNA-203? How do the other miRNAs in which expression is altered in psoriasis and eczema contribute to the pathology? What are the miRNA profiles in other hyperproliferative skin disorders?

Furthermore, because single miRNAs can simultaneously regulate dozens of target genes, it will be important to identify additional miRNA-203-target genes and to evaluate their relevance to skin physiopathology. For instance, the identification of *ABL1* as a miRNA-203 target opens up interesting questions regarding its possible role in skin. Although it is conceivable that *ABL1* is important to protect basal highly proliferating cells from DNA damage, this function is not required for differentiating keratinocytes; therefore, it is tempting to speculate that

the induction of miRNA-203 (Figure 3) explains the absence of apoptotic cells after UV irradiation (also named sunburn cells) in the upper layer of the epidermis. Conversely, given that an individual promoter could be regulated by distinct miRNAs, it will be important to investigate the miRNAs that are involved in the same modes of epidermal regulation. It is clear from these initial studies that miRNAs regulate the epidermal 'stemness' compartment, which includes quiescent (i.e. bulge stem cells) and interfollicular stem cells (holoclone cells) (Figure 3). In addition, miRNAs regulate the interface between 'stemness' versus differentiation, rather than an individual linear pathway (Figure 3). The importance of miRNAs in maintaining the self-renewal capacity of different epithelial progenitor cells also has been addressed for mammary and lung epithelial-cell progenitors [45] confirming their crucial role in contributing to stemness. Do miRNAs only regulate cellular proliferation, or can they also induce differentiation? Are they also implicated in other compartmental transitions? For example, is the move from the quiescent stem cell to the proliferating 'staminal' cell regulated by miRNAs? Can miRNAs direct the formation of the cornified envelope, thereby ensuring an intact epidermal barrier function?

We might also consider an alternative treatment strategy of knocking down oncogenes (or inducing tumour suppressors) after skin cancer diagnoses. It remains to be seen if this approach could be useful in combination with conventional chemotherapy. Because oncoproteins often confer resistance to cytotoxic drugs, the application of 'anti oncogenic miRNAs' to tumour cells might restore their sensitivity to chemotherapeutic drugs. Head and neck squamous carcinomas (HNSCC) usually carry a poor prognosis and Δ Np63 expression levels correlate with the response to cisplatin-based therapies [46]. Δ Np63 levels are reduced after DNA damage in positive responders, raising the possibility that response rates might be improved if existing therapies were complemented with the local application of miRNA-203. A similar strategy could also be a therapeutic opportunity for BCR-ABL1 positive malignancy and in ABL1 overexpressing tumours. The finding that many other miRNAs are involved in normal epidermal and follicular development indicates that their therapeutic expression or inhibition might also be relevant to epidermal pathology. Indeed, exciting times lie ahead in this rapidly expanding field.

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Spotlight on p63

TAp63 and Δ Np63 in Cancer and Epidermal Development

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p63, IKK α , epidermis, cornification, skin, development

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ABSTRACT

The epidermis is a multilayered stratified epithelium, continuously regenerated by differentiating keratinocytes, that requires the transcription factor p63 for its development and maintenance. The TP63 gene encodes two major protein isoforms, TAp63 and Δ Np63, which have both transactivating and transcriptional repressing activities and regulate a wide range of target genes. TAp63 shows clear pro-apoptotic activity, mediated both by death receptors (CD95, TNF, TRAIL) and mitochondrial (bax, puma) pathways. Conversely, Δ Np63 protects from apoptosis by directly competing for TAp63 target promoters or sequestering it, forming inactive tetramers. Accordingly, p63 is expressed in epithelial tumors, contributing to both tumorigenesis and chemoresistance. However, the predominant physiological role of p63 is in epithelial development, as demonstrated by the lack of epidermis and other epithelia in p63-deficient mice. The specific role of TAp63 and Δ Np63 isoforms in epithelial development remains mostly unclear. Nevertheless, recent work utilizing in vivo genetic complementation of TAp63 and/or Δ Np63 into a p63 null background has shed new light into the specific functions of the two isoforms and allowed the in vivo validation of several p63 transcriptional targets, originally identified by microarray analysis in in vitro systems. However, despite concerted efforts to address the role of p63 isoforms, several questions remain unanswered. The main aim of this review is to critically discuss the data available in the literature and thoroughly analyze the models proposed.

ABBREVIATIONS

H&E, haematoxylin and eosin; K, keratin; p63^{-/-}, mouse knockout for p63; p63^{-/-};TA, TAp63 transgenic mice complemented into p63^{-/-}; p63^{-/-};TA; Δ N, Δ Np63 transgenic mice complemented into p63^{-/-}; IKK α , I κ B kinase- α ; TA, transactivation domain; Δ N, amino-terminal truncated protein

INTRODUCTION

As recently demonstrated also for other members of the p53 family, p63 is expressed from two different promoters that generate two classes of proteins, TAp63, which contains the N-terminal transactivation (TA) domain, and the N-terminal truncated (Δ Np63) isoform, which lacks the transactivation domain.¹⁻³ In addition, alternative splicing at the 3' end of the transcripts generates three different C-termini: α , β and γ . However, information about the protein expression levels of splice variants is not currently available due to the lack of isoform-specific antibodies, thus hampering the identification of specific functions.

It is emerging that p63 is involved in tumorigenesis and in controlling chemosensitivity. Indeed, it is highly expressed in several epithelial cancers, and can regulate apoptosis and sensitivity to drug treatments at least in vitro. However, a large body of evidence indicates that the main role of p63 lies in the regulation of epithelial development and in particular in the formation of the epidermis.

The epidermis is a multilayered, stratified epithelium that provides a physical barrier for the organism, protecting it from pathogens and dehydration. The epidermis is continuously regenerated by terminally differentiated keratinocytes that migrate from the inner basal layer (proliferative compartment) to the outer cornified layer (terminal differentiated compartment): this process is called cornification.⁴ The notion that the transcription factor p63 is essential for the formation of the epidermis and other stratified epithelia arises from two facts. First, mice lacking p63 show profound defects in

formation of the epidermis.^{1,5,6} In addition to the defect in the skin and other stratified epithelia, p63^{-/-} mice lack epithelial appendages, such as mammary, salivary, lachrymal glands, hair follicles and teeth. p63^{-/-} mice also show truncated limbs and abnormal craniofacial development, which are due to failure to maintain or differentiate the apical ectodermal ridge important for coordination of epithelial-mesenchymal interactions.^{5,6} Remarkably, genetic mutations of p63 in man are causatively linked to ectodermal dysplastic syndromes.^{7,8}

In this review, we will focus first on the involvement of p63 and its TAp63 and ΔNp63 isoforms in cancer and induction of apoptosis, and then on their role in the development of the epidermis.

p53 IN CANCER AND APOPTOSIS REGULATION

Expression of p63 in cancer. The identification of the p53-p63-p73 network has opened a new chapter in cancer research. In particular, it emphasizes that there is a tight link between developmental processes and tumorigenesis.⁹ Indeed, there is mounting evidence that p63 and p73 play an important role in DNA damage controlling cell cycle arrest and apoptosis (Fig. 1) and in human cancer,¹⁰⁻¹⁸ although their precise roles in tumorigenesis remain to be clarified.

Unlike p53, p63 is rarely mutated in human cancers, thus indicating it is not a canonical tumor suppressor. The majority of tumors maintain p63 expression, and in many cases either p63 appears to

Another work showed that Tap63 proteins have also been described to be expressed in the nuclei of a subpopulation of lymphoid cells and in most malignant lymphomas, whereas Δ Np63 proteins are not expressed.^{27,28} However, the functional implications of Tap63 expression in this context have not been investigated. Despite the above mentioned attempts, the role of specific p63 isoforms in cancer is still unclear.

The analysis of p63 deficient mice has led to often conflicting results with regard to its role in tumorigenesis. As mentioned above, p63-deficient mice have several developmental abnormalities, whereas p53-deficient mice are viable and predispose to malignancy. Interestingly, a number of p63^{+/-} mice are cancer-prone.²⁹ Nevertheless, this appears to be linked to the genetic background, as p63^{+/-} mice on a different inbred strain show premature aging but no cancer.^{30,31} Furthermore, p63 heterozygosity does not accelerate tumorigenesis when p53 is compromised.³¹ Although these conflicting results appear as difficult to conciliate, more work is needed to understand how the different genetic background could influence tumorigenesis. In particular, it is not clear what is the ratio between different p63 isoforms in the mouse strains used in the above mentioned studies. In summary, our understanding of the role of p63 in tumorigenesis is still preliminary. Clearly, future studies are needed to study the complex interactions between different p63 isoforms, in addition to determining how these proteins impact other members of the p53 protein family. In this respect, a recent work has shown that p63 and p73 are required for full p53 activation, thus suggesting that

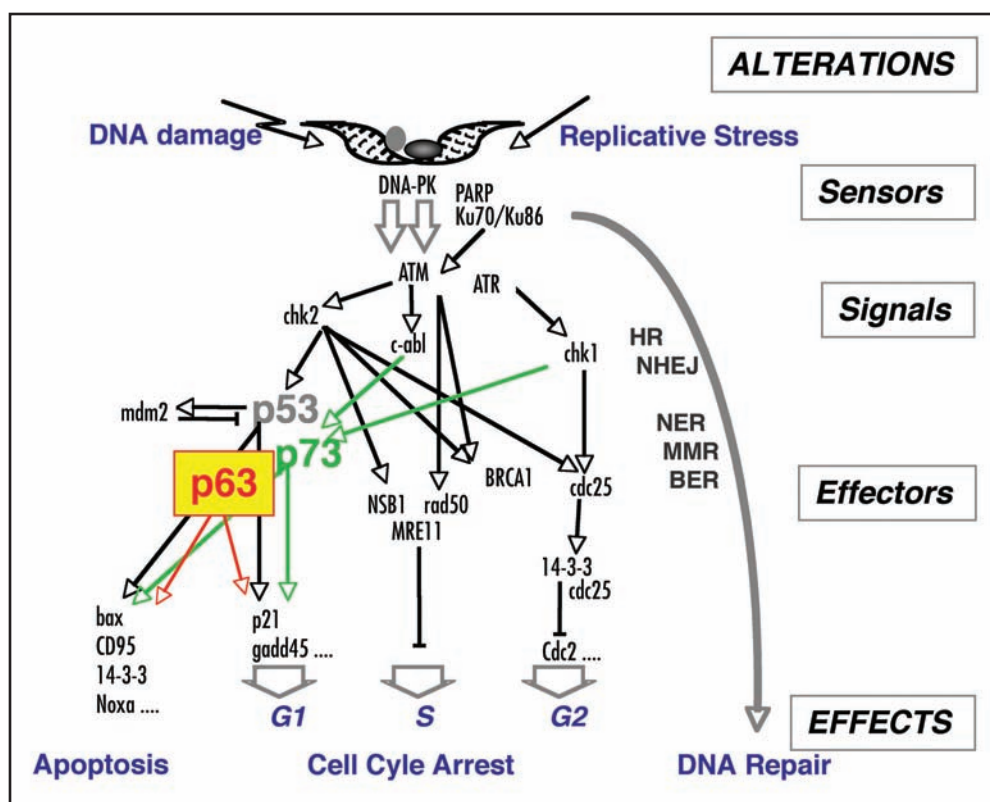


Figure 1. p53 and its involvement in DNA damage. The p53 family is involved in the regulation of DNA damage response, controlling both cell cycle arrest and apoptosis. A tightly controlled cascade originating at the site of DNA damage aims at controlling the activation of the tumour suppressor p53, which, depending on the type of insult or the regulatory component involved, can induce either apoptosis or cell cycle arrest through upregulation of its target genes. p73 is regulated in a similar manner, although its upstream activators appear to be different (c-Abl, Chk1). Much less is known about p63 regulators upon DNA damage. One regulator is p53 itself, although it is not clear at what extent this happens in vivo.

Table 1 **Expression of p63 in cancer**

Tissue/organ site	Variant mRNA expressed	Comments	Refs.
Breast cancer	Δ Np63	Protein expression	24; 94; 95; 96; 97
Bladder cancer	TAp63 and Δ Np63	Protein expression	24; 25; 98
Urothelial cancer	TAp63 and Δ Np63	Protein expression	99
Cervical cancer	Not determined	Protein expression	100; 101; 102; 103; 104
Uterine cancer	Not determined	Protein expression	105
Head and neck cancer	TAp63 and Δ Np63	Protein expression	20; 106; 107; 108
Lung cancer	TAp63 and Δ Np63	Protein expression	20; 21; 109
Prostate cancer	Δ Np63	Protein expression	110
Esophageal cancer	Δ Np63	Squamous cell carcinoma, protein expression	111
Gastric carcinoma	TAp63 and Δ Np63	Protein expression, mRNA expression	112
Pancreatic cancer	Not determined	Protein expression	113

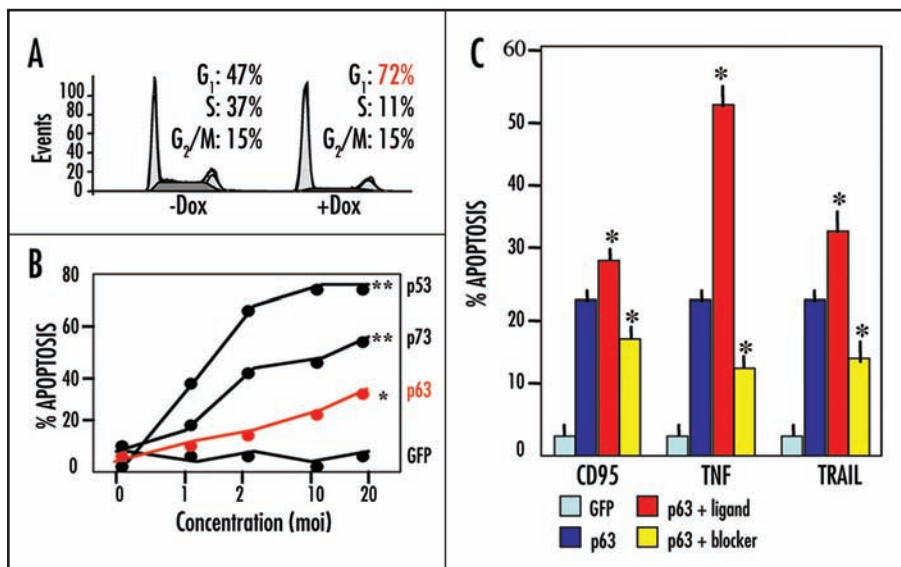


Figure 2. Mechanism of apoptosis elicited by p63. Induction of TAp63 in a Doxycycline-dependent inducible model is sufficient to elicit cell cycle arrest (A) and apoptosis (B). These effects are the molecular basis for the involvement of p63 in tumorigenesis and chemosensitivity, see Figure 1. (A) shows a significant arrest in the G₁ cell cycle phase. (B) shows that even though TAp63 can trigger cell death, its efficacy and kinetics is less efficient than the other family members, possibly suggesting that its major function is different, and that it could become significant in tumors where p53 has been inactivated. (C), the molecular mechanisms to trigger apoptosis involve the upregulation of several death receptors. The panel shows that overexpression of TAp63 sensitizes cells to die by CD95, TNF or TRAIL ligation. Conversely, receptor blockers reduce the ability of TAp63 to kill. The data are modified from.³²

the interplay between members of the family may have a big impact on tumorigenesis and response to therapy.¹¹

p63 and apoptosis. We have recently described the downstream mechanisms of TAp63 α -induced apoptosis in different cellular systems. TAp63 α activates major apoptosis pathways by triggering signaling via death receptors and mitochondria (Fig. 2 and Fig. 3) and thus sensitizes cancer cells towards chemotherapy. We demonstrated that TAp63 α , like p53, is involved in more than one apoptotic

operation, being capable of transactivating genes encoding death receptors, for example, CD95, TNF-R1, and TRAIL-R1 and -R2 (Fig. 2) as well as genes encoding mitochondrial proteins, e.g. Bax, BCL2L11 and the genes encoding RAD9, DAP3 and APAF1³² (Table 2).

p63 is similar to p53 with regards to its ability to modulate specific genes that mediate cell cycle arrest and apoptosis. TAp63 isoforms are able to bind DNA through p53-responsive elements and activate transcription of many p53 target genes. Thus, these p63 isoforms are described as 'p53-like'. Recent studies indicate that p63 proteins can bind DNA through response elements (p63RE), which are slightly different from p53RE thus conferring specific responsiveness to p63 but not p53^{16,33,34} (a more detailed discussion on this subject can be found in this issue; see ref. 35). The Δ Np63 isoforms can bind DNA through p53RE and can exert dominant-negative effects over p53, p73 and p63 activities by either competing for DNA binding sites or by direct protein-protein interaction.^{16,36} HSP70, an anti-apoptotic stress response protein associated with malignancy, is induced by Δ Np63 α consistent with the proposed oncogenic role of Δ Np63. By contrast, the pro-apoptotic gene IGFBP-3 is transcriptionally repressed by Δ Np63 α .³⁷

An intense debate is ongoing on whether the interaction between p53 family members plays a role in apoptosis and tumor suppression.³⁸ The p53/p63/p73 family members are capable of interacting in many ways that involve direct or indirect protein interactions, regulation of common target genes and regulation of each other's promoters. The p53 family members and their isoforms can bind differentially to promoters and it may well be that the ratio of the isoforms is an important cell fate determinant for cell cycling, senescence or the onset of tumor formation. As mentioned above, an example of cooperativity among the three p53 family members has been reported in E1A-expressing mouse embryo fibroblasts and in primary neuronal cultures.³⁹ However, results of a more recent study indicate, that at least in thymocytes, p53-dependent apoptosis occurs independently of p63 and p73.³⁹ This is also observed in oocytes in which TAp63 is specifically expressed during meiotic arrest. Indeed, in female germ cells TAp63 is essential in a process of DNA damage-induced oocyte death not involving p53.⁴⁰

In summary, TAp63 activates genes exerting roles in different steps of the apoptosis program (Fig. 3). However, the interaction with other isoforms or other members of the p53 family could tightly regulate the proapoptotic activity of TAp63. What also remains to be addressed is whether tissue distribution of different p53 family members and respective isoforms could determine stress response, tumorigenesis and chemoresistance. This multifaceted scenario may

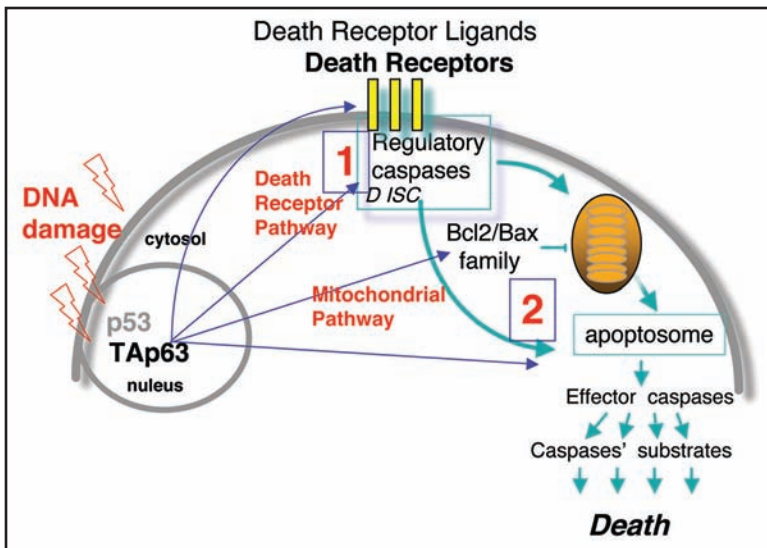


Figure 3. Apoptosis elicited by p63. Overexpression of TAp63 triggers apoptosis via two main pathways, death receptors (pathway 1), and mitochondria (pathway 2). While the former is outlined in Figure 2, the latter involves a direct transactivation of BH3-only proteins (e.g. PUMA) and bax. The relative contribution to death might therefore vary depending upon the specific inducer and tissue.

Table 2 Selected p63 target genes involved in tumorigenicity

	TAp63 (isoform)	ΔNp63	Ref.
CASP1	α ↑	-	32
CASP3	α ↑	-	32
CASP4	α ↑	-	32
CASP5	α ↑	-	32
CASP8	α ↑	-	32
CASP9	α ↑	-	32
TNF	α ↑	-	32
TNFRSF1A/TNF-R1	α ↑	-	32
TNFRSF6/CD95	α ↑	-	32
TNFRSF10A/TRAIL-R1	α ↑	-	32
TNFRSF10B/TRAIL-R2	α ↑	-	32
TRIP	α ↑	-	32
APAF1	α ↑	-	32
BCL2L11	α ↑	-	32
BAX	α, γ ↑	-	32; 114
DAP3	α ↑	-	32
RAD9	α ↑	-	32
p21	γ ↑	-	114
IGFBP3	-	α ↑	37
HSP70	-	α ↓	18

(↑)=upregulation, (↓)=downregulation, (-)=no effect

help to explain why p53 status is not a universal predictor of response to chemotherapy.

p63 and chemosensitivity. We have recently shown a relevant role for the TAp63α isoform in chemosensitivity of hepatoma cells. TAp63α activates both, death receptor- and mitochondria-mediated apoptosis pathways, and both mechanisms are clearly reinforced by concomitant

treatment with chemotherapeutic drugs. Of clinical importance, we found that endogenous TAp63α is induced by a variety of chemotherapeutic agents and that blocking TAp63α function leads to enhanced chemoresistance.³² These data are consistent with recent observations that p63 participates in p53-mediated DNA damage responses.^{11,41} There have been several reports which have demonstrated that p73 is essential for apoptosis induced by many cytotoxic agents and that inactivation of p73 by a dominant negative mutation or RNA interference leads to resistance of cells to apoptosis induced by genotoxic agents.⁴²⁻⁴⁹ To our knowledge our study is the first to link chemosensitivity to TAp63α function. Our in vitro data have been confirmed by a recent report showing that ΔNp63α expression directly correlates with a poor clinical response to cisplatin in patients with head and neck tumors.⁵⁰ A critical role of p63 in cell death following DNA damage is further substantiated by a study in head and neck squamous cell carcinoma (HNSCC) cells which demonstrated that ΔNp63α is an essential survival factor in HNSCC. This report suggested that p73-mediated cell death following DNA damage may represent the cumulative effect of increased p73 levels in addition to decreased ΔNp63-mediated transcriptional inhibition.⁵¹

In sum, it appears that chemosensitivity may be determined not only by p53, but also by p73- and p63 function, and that dominant negative isoforms could play a crucial role in this scenario. The potential synergistic effects of different p53 family members in the response to chemotherapeutic drugs should be taken into consideration for the development of future anticancer strategies.

p63 IN EPITHELIAL DEVELOPMENT

Expression of p63 isoforms in epidermal development and in adult epidermis. During mouse embryogenesis, the epidermis develops from the surface ectoderm, which is originally a single-layered epithelium. At this stage, the epithelium express K18, which is still present in different single-layer epithelia in the adult organism.⁵² Stratification occurs with the formation of the periderm that appears around the upper limb buds at embryonic day E9.5, and is coincident with the expression of K5 and K14, which are the first stratification markers. At this stage the developing outer layer of the embryo exhibits barrier function.⁵³ At embryonic day E15.5 the spinous and granular layers are formed, as demonstrated by the expression of two other differentiation markers, respectively K1 and loricrin.⁵⁴ At E19.5, epidermal stratification and maturation is complete, although periderm is no longer present at this stage (Fig. 4A).

A very detailed investigation of p63 isoforms expression during epidermal development has been carried out in the mouse by different investigators (see reviews in this issue).^{55,56} p63 is expressed before stratification occurs at embryonic day E8.5.^{57,58} RT-PCR analysis using specific probes to discriminate between TAp63 and ΔNp63 isoforms demonstrated that the latter is the main isoform expressed at all embryonic stages during epidermal, tooth and hair development, accounting for 100% of all p63 isoforms expressed up to E9 and 99% at E13. TAp63 isoforms expression starts at E13, and only accounts for 1% of total p63 mRNA expressed at this embryonic stage. These results

were also confirmed by in situ hybridization, confirming that Δ Np63 is the most abundant transcript from embryonic stage E10 to day 3 post-birth; with this method the TAp63 isoform is not detected.^{55,57} However, as the abovementioned studies lack an analysis of isoforms expression at the protein levels.

Other studies have studied protein levels of p63 in the mature epidermis. This has been mainly performed using antibodies directed against the two N-terminal isoforms (TAp63 and Δ Np63), the C-terminal region of the p63 α proteins and the DNA-binding domain.^{1,27} Again, Δ Np63 is the main isoform detected and is expressed mainly in the basal layers; its expression is down regulated in more differentiated layers.^{1,27,59} Expression of the full-length TAp63 mRNA is also seen,^{58,59} and TAp63 proteins are found in epidermis.^{27,61}

Differential function of p63 isoforms in epidermal formation. Since the publication of the two p63^{-/-} studies, it has become clear that p63 is a "master gene" for epidermal development,^{5,6,62} see fig. 5. The two mouse models showed a very similar phenotype, but their molecular interpretation led to different conclusions (Fig. 4D). Yang et al. found that p63^{-/-} mice had some patches of skin expressing markers of differentiation, so they argued that differentiation was not impaired by p63, but probably that the mice failed to develop skin due to an impaired proliferation potential of stem cells (Fig. 4D, left). Conversely, Mills et al. did not detect any marker of skin differentiation, concluding that skin development is arrested at an early stage because of a lack of pluristratification (Fig. 4D, center).

An attempt to answer the unsolved question of p63 function in epithelial development and to dissect the role of the different isoforms was provided first by Koster et al.^{56,63} Their work started from the observation that TAp63 was expressed earlier than Δ Np63 (E7.5 and 9.5 respectively, although this observation is in contrast with other reports, see section above), and its expression was just upstream to the commitment to pluristratification. To investigate the function of TAp63, the authors ectopically expressed the isoform in a WT background by using "gene-switch" mice. As a result, they found that TAp63 was able to induce pluristratification of the single layered lung epithelium and to block skin differentiation delaying K1 expression. Furthermore the skin overexpressing TAp63 in the basal layer was hyperproliferative. These results were specific for TA, as over expression of Δ Np63 had no effect. In this context TAp63, but not Δ Np63, was able to block keratinocyte differentiation in vitro and to induce the expression of K14, a marker of the basal layer of the skin. In conclusion the authors propose that TAp63 is the molecular switch responsible for epithelial

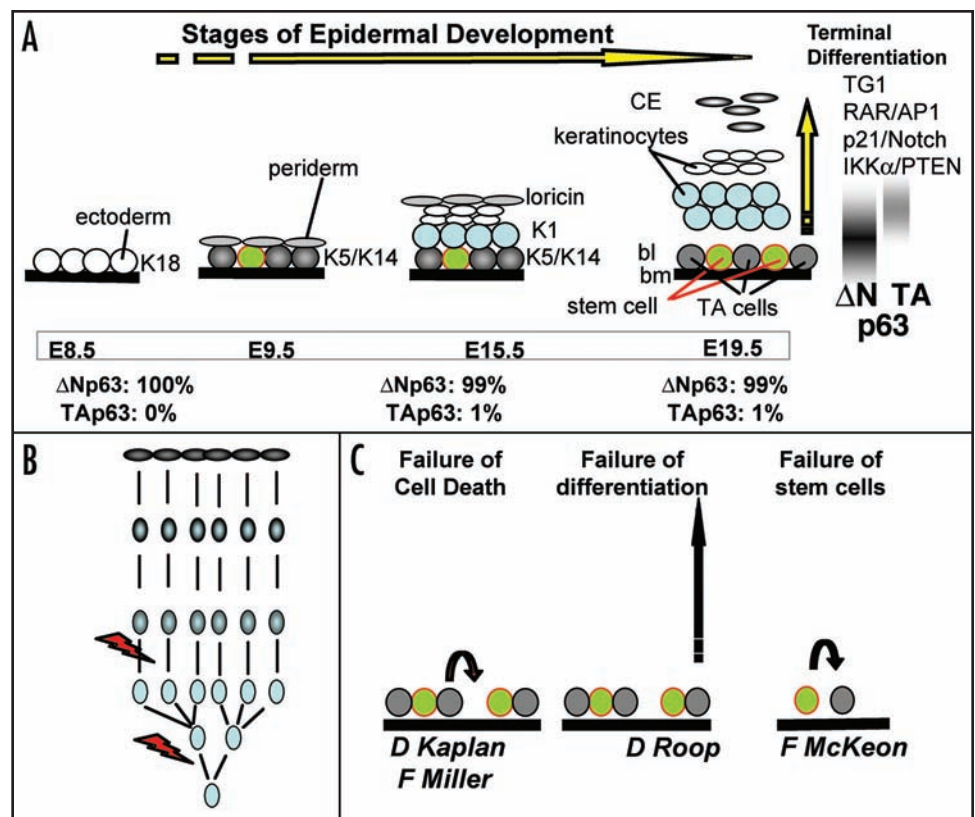


Figure 4. Hypothesis on the function of p63. The epidermis is formed by layers of proliferating and differentiating keratinocytes (A), which have to be constantly renewed by the action of epidermal stem cells that are located at the basal layer of the epidermis. During mouse embryogenesis, the epidermis develops from the surface ectoderm, single-layered epithelium. At this stage, the epithelium express K18. Stratification occurs with the formation of the periderm that appears around the upper limb buds at embryonic day E9.5, here K5 and K14, which are the first stratification markers, are expressed. At embryonic day E15.5 the spinous and granular layers are formed (expression of two other differentiation markers: K1 and loricrin). At E19.5, epidermal stratification and maturation is complete. Beside p63, other genes are necessary to achieve terminal differentiation, some are indicated in the right side of (A). The p63 proteins are mainly expressed in the lower layers, the relative expression of Δ Np63 and TAp63 during development is indicated.⁵⁷ The current data do not indicate whether p63 controls the stem cell or the differentiation compartment (B). Indeed, the current hypothesis (C) states that p63 is required to regulate cell death,⁹³ to regulate the differentiation program of squamous epithelia cells,⁶ or to maintain the proliferative potential of the stem cells,⁵ according to Kaplan/Miller, Roop or McKeon respectively. bm, basal membrane; bl, basal layer of keratinocytes; CE, cornified envelope; TA cells, transient amplifying cells.

pluristratification and that the main function of Δ Np63 is to counteract the TAp63 isoform allowing cell differentiation.⁶³ These data and their interpretation is in contrast with evidence that Δ Np63 is the only isoform expressed in epidermal development until E13^{55,57} and that Δ Np63 is expressed in the basal undifferentiated layer of the skin, in particular in the stem cell compartment, and is rapidly degraded when keratinocytes are induced to differentiate.^{1,64-66} The reason for this profound discrepancy is not understood at the moment. More recently we carried in vivo genetic complementation in p63 null mice (Fig. 5A).⁶⁷ This method avoided the presence of endogenous p63 isoforms. The mice were obtained by crossing p63^{-/-} mice with transgenic mice over-expressing TAp63, Δ Np63 or both in the basal layer of the epidermis using the basal layer specific K5 promoter. Interestingly, even if we were unable to obtain a full reversal of the phenotype of the null mice, probably due to technical limitations of the system, a major role for Δ Np63 in epidermal development emerged, with a possible cooperative activity between

the two isoforms. The $p63^{-/-}$;TA (mice knock-out for p63, expressing TAp63 α) complemented mice show no signs of epithelialisation, like $p63^{-/-}$ mice. In contrast, the $p63^{-/-}$; Δ N transgenic mice showed greater, but still incomplete signs of re-epithelialisation, along with rescue of the expression of markers of the basal layer, such as K14/K5 and no significant increase of differentiation markers, such as K1 and filaggrin (Fig. 5B).⁶⁷ Reintroduction of both isoforms resulted in a higher level of rescue of the epidermal phenotype. The $p63^{-/-}$;TA; Δ N mice showed a greater degree of re-epithelialisation and an expanded number of keratinocytes. Indeed, the epidermis of these mice appeared to be formed by many layers of keratinocytes which expressed both basal and supra-basal markers (K14, K1, filaggrin). These data⁶⁷ suggest that Δ Np63 is important for maintaining the proliferative potential of the basal layer, whereas TAp63 contributes by acting synergistically and/or subsequently to Δ Np63 to allow differentiation. Furthermore, these results are consistent with a recent study based on siRNAs directed against p63 isoforms, in which the authors demonstrated that Δ Np63 isoforms are the main mediators of p63 effects and that TAp63 isoforms contribute to late stage of differentiation in mature keratinocytes.⁶⁸

How to reconcile these apparently conflicting results? First, experiments should be conducted in the same genetic background to exclude the possible interference of other genetic components. Second, the promoter used in the two studies is different (K5 vs K14), and this could also be another critical factor. Finally, expression levels achieved using in the two transgenic models could be different. Apart from these differences, the two studies clearly demonstrate that different isoforms can play different functions during the development of the epidermis.

Ultrastructural features of genetically complemented mice. Transmission electron microscopy of the surface layer of $p63^{-/-}$ mice (Fig. 6A) showed the absence of a stratified epithelium. This layer was dominated by fibroblast-like cells, which were interspersed by irregular cell profiles that contained a few randomly arranged keratin filaments. These irregular cells were not restricted to the outer surface but showed localised cornification of their cell envelopes. This morphology was in marked contrast with that of wild-type mice (Fig. 6B), which was characterised by fully keratinised squamous corneocytes, intercellular lipid and corneodesmosomes.

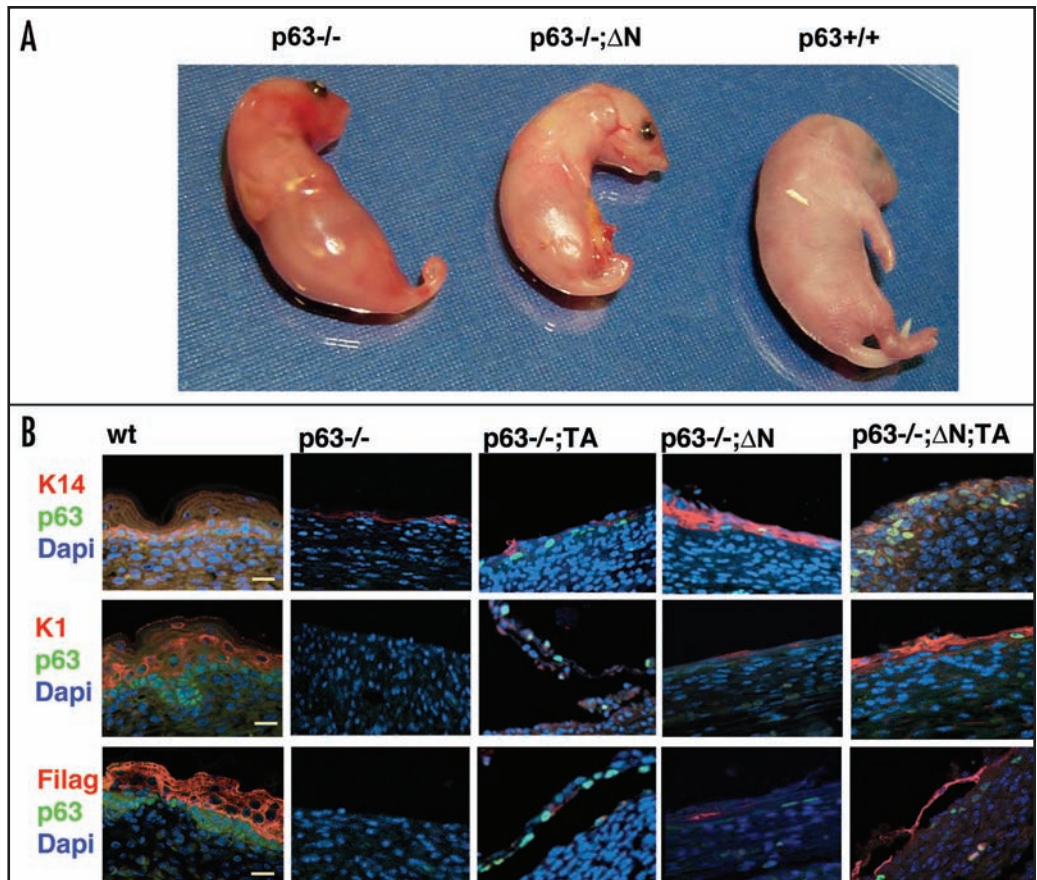


Figure 5. Epidermal development in the absence of p63 protein and expression of epidermal differentiation markers in genetic complemented $p63^{-/-}$ mice. The absence of all p63 proteins (knockout mice) does not allow the formation of the epidermis and related annexes. (A) $p63^{-/-}$ mice show absence of epidermis and limbs, as compared to wild type mice ($p63^{+/+}$). Genetic in vivo complementation by the Δ Np63 α isoform (under control of the keratin 5 promoter) into a p63 null background, middle mouse, shows a limited but significant reversion of the phenotype, see also Figure 6. (B) Histological expression of K14 (marker of the basal layer), K1 and filaggrin (differentiation markers) and p63 in wild type (WT), $p63^{-/-}$, $p63^{-/-};TA$ ($p63^{-/-}$ mice re-expressing TAp63 α); $p63^{-/-};\Delta N$ ($p63^{-/-}$ mice re-expressing Δ Np63 α) and $p63^{-/-};TA;\Delta N$ ($p63^{-/-}$ mice re-expressing both TAp63 α and Δ Np63 α) mice. The $p63^{-/-};TA$ complemented mice show no signs of epithelialisation, like $p63^{-/-}$ mice. In contrast, the $p63^{-/-};\Delta N$ transgenic mice showed greater, but limited signs of re-epithelialisation, with a rescue in the expression of markers of the basal layer, as K14 and not significant increase of differentiation markers, such as K1 and filaggrin. Reintroduction of both isoforms resulted in a higher level of rescue of the epidermal phenotype. The $p63^{-/-};TA;\Delta N$ mice showed a greater degree of re-epithelialisation and an expanded number of keratinocytes. The epidermis of these mice is formed by many layers of keratinocytes, which express both basal and suprabasal markers (K14, K1, filaggrin). Nuclei are stained with Dapi. Bars, 50 μ m.

Re-introduction of TAp63 α in $p63^{-/-}$ mice, by genetic complementation, resulted in the formation of small epithelial patches consisting of irregular or rounded cornified cell envelopes containing enlarged loricrin granules and accumulations of a moderately electron-dense material (Fig. 6C). The re-introduction of Δ Np63 α resulted in the development of larger patches of epithelium composed of a much more orderly arrangement of outer cells, which tended to be more flattened than rounded (Fig. 6D). These patches contained many cells with traces of randomly dispersed keratin filaments and signs of nuclear disintegration or cornification of the cell envelope but these cells were usually overlaid by non-cornified cells. Re-introduction of both TAp63 α and Δ Np63 α into the $p63^{-/-}$ background did not result in a completely normal epithelium but there was a greater degree of reepithelialisation than in the selective Δ Np63 α genetic complementation and much greater than in the $p63^{-/-}$ or $p63^{-/-};TA$ mice. The patches of epithelium in the $p63^{-/-};\Delta N;TA$ mice had a greater degree

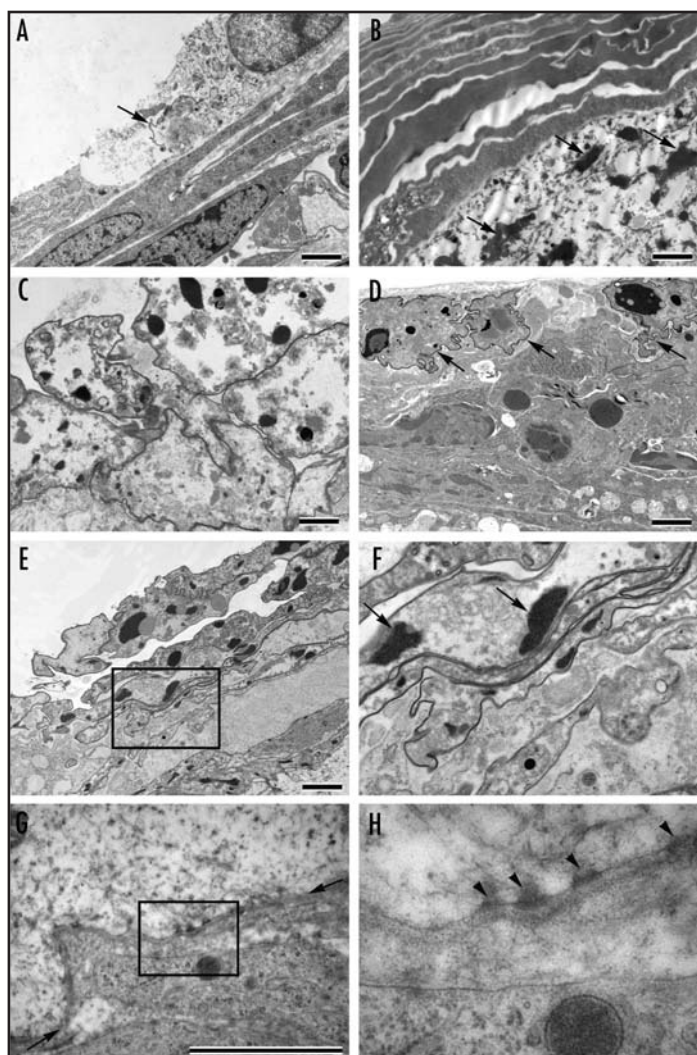


Figure 6. Ultrastructural characteristics of genetically complemented mice. (A) Fibroblast-like cells predominate in the superficial layers of p63-null mice and only a few, isolated cells show some localised cornification of the cell envelope (arrow). (B) The skin of a WT mouse is characterised by layers of cornified squamous cells (top left) overlying the granular layer where numerous filaggrin granules (arrows) are associated with keratin fibrils. (C) Patch of cornified epithelial cell envelopes in a p63^{-/-} transgenic mouse following reintroduction of TAp63 α contain enlarged loricrin granules and accumulations of a moderately electron-dense material but fail to form an intact epithelium. (D) Epithelial patch in a p63^{-/-} transgenic mouse with reintroduction of Δ Np63 α comprises a range of cell-types, including some showing signs of cornification (arrows). (E) Partially cornified squamous cells predominate in the superficial layers of the epithelium of a mouse with re-introduction of both TAp63 α and Δ Np63 α . (F) detail of panel (E) Keratin filaments are present in most of these cells, often associated with filaggrin granules (arrows). (G) the basal cells of this epithelium are separated from the underlying connective tissue by a basement membrane (arrows). (H) detail of panel (G) Numerous hemidesmosomes are also present at the base of the epithelial cells (arrow-heads). All bars, 2 μ m

of organization and were more differentiated than in the other genetically complemented mice (Fig. 6E). Definite flattening and layering of the superficial cells was observed in many of these patches but cornification was invariably incomplete and no corneodesmosomes or traces of intercellular lipid were found (Fig. 6F). Keratin filaments were, however, common throughout the epidermis; they were usually organized into fibrils and these were associated with filaggrin

granules in the more superficial layers. Underlying some of the skin patches there were traces of a basement membrane, together with numerous hemidesmosomes (Fig. 6G and H). These structures were not found in any of the other genetically complemented mice. This is consistent with a role of p63 for targeting β 4 integrin to hemidesmosomes, where it forms a link with the cytoskeleton necessary for cell adhesion and orientation of developing tissue.⁶⁹ In sum, these findings helped to understand the contribution of different isoforms to skin differentiation at the ultrastructural level.

TRANSCRIPTIONAL REGULATION BY p63

Transcriptional activities of p63 isoforms. The idea that TAp63 and Δ Np63 isoforms work in competition, as TAp73 and Δ Np73 do, represented the first “dogma” regarding the function of p63 isoforms. This concept arose from the observation that since Δ Np63 lacked a transactivation domain, it acted as a dominant negative towards the TAp63 isoforms; indeed it could effectively repress TAp63 transactivation of luciferase reporter genes.¹ This view has now significantly changed as more recent papers showed the presence of an additional transactivation domain at the Δ Np63 C-terminal end⁷⁰⁻⁷³ (for review see 35; 74; 75). This observation was also confirmed by microarray analysis, showing that both isoforms were able to induce gene transcription.^{68,71,76,77} Δ Np63 transcriptional activity is further enhanced by its interaction with other transcription factors, as shown for HSP70 transcriptional regulation.¹⁸ In this case, Δ Np63 interacts with the NF-Y transcription factor to form a complex with the CCAAT box located in the HSP70 promoter and enhance HSP70 expression.¹⁸ In addition, Δ Np63 α is able to repress transcription of cell cycle related genes such as cyclin B2, Cdc2 and topoisomerase II α , in these cases being recruited to the respective CCAAT boxes by its interaction with the CCAAT binding factor, NF-Y.⁷⁸

p63 target genes relevant in epidermal formation and differentiation. The animal models analysed so far suggested that p63 isoforms have different roles in epidermal formation and in mature epidermis. However, a still open question is: what are the molecular mechanisms through which p63 isoforms regulate the development and the maintenance of the epidermis?

Recently, Vigano' et al.^{74,77} identified high-confidence target genes for Δ Np63 using a genome-wide approach. They identified new categories of genes regulated by p63, among which are developmental, morphogenetic genes and those involved in tissue regeneration. However, few of p63 target genes identified so far have a documented impact on epidermal formation and differentiation. Table 3 shows a list of selected and validated p63 target genes that have a defined impact on epidermal formation and differentiation (a more detailed discussion on this issue.^{74,75}

One of the mechanisms by which p63 could regulate epidermal proliferation, at least in part, could be its ability to affect the FGFR2b receptor and its associated signalling, since it has been shown that there is a reduction in keratinocyte proliferation in the epidermis of Fgfr2b^{-/-} embryos.⁷⁹ In addition the proliferative capacity of p63^{-/-} mice keratinocytes could be also reduced as a consequence of increased expression of p21, a direct transcriptional repression target of Δ Np63⁸⁰ (see also Fig. 2). Notch signalling is also very important in epidermal stratification and in keratinocyte differentiation.^{81,82} p63 functions as a selective modulator of Notch1-dependent transcription and function, and also directly transactivates the Notch ligand, JAGG-1.⁸³ Thus, a complex crosstalk between Notch and p63 could regulate the balance between keratinocyte self-renewal and differentiation.⁸⁴

Very recently, our laboratory performed a microarray analysis for p63 target genes, which was then validated in the genetically complemented mice (Fig. 7A). Our work indicates that the function of p63 in epithelial development is at least in part mediated by IKKα (IκB kinase-α) and GATA-3 (Fig. 7B).⁸⁵ IKKα is required for epidermal formation and has both kinase-dependent and -independent functions, the latter being crucial for epidermal keratinocyte differentiation and for skeletal and cranio-facial morphogenesis as clearly demonstrated in the IKKα^{-/-} mice.⁸⁶⁻⁸⁸ Expression of IKKα, under control of the basal layer promoter K14 in IKKα^{-/-} mice, produced efficient rescue of the major morphological abnormalities.⁸⁷ p63 regulation of IKKα is both direct and indirect, as well as isoform-specific (Fig. 7B). Indeed, TAp63 directly transactivates IKKα by binding a p53-like consensus sequence on the IKKα promoter. In addition TAp63 also transactivates IKKα indirectly through the induction of Ets-1 and GATA-3, for which consensus binding sites are present in the IKKα promoter^{67,85,89} (Fig. 7B). However, ΔNp63 can only transactivate IKKα indirectly via GATA-3 (Fig. 7B). GATA-3 is an important mediator of skin and hair development,⁸⁸ although its specific function in epidermal development and differentiation has not yet been established.

Several additional targets involved in maintaining the structural integrity of the epidermis have been identified by others and our laboratory (see parallel reviews in this issue^{67,73}). These include keratin 14⁶⁷ (Fig. 7C) *perp*,⁹¹ *envoplakin*,⁶⁹ and *BPAG-1*.⁹² Fig. 8 shows a simplified scheme of the actions of p63 in epidermal development, with particular emphasis on IKKα and K14, although the table does not aim at undermining the importance of other targets.

FUTURE PERSPECTIVES

Despite the strong and continuing emphasis on the involvement of the p53 family in tumorigenesis because of the tumor suppressive role of the founding member p53, it now seems evident that the remaining family members p63 and p73 are heavily involved in the regulation of development of different tissues, thus unveiling their specialized functions. Evidence is accumulating that the balance between TA and ΔN isoforms can modulate the function of p53 family members with respect to DNA damage (a role primarily developed by p53; Fig. 8, right cartoon) and development.

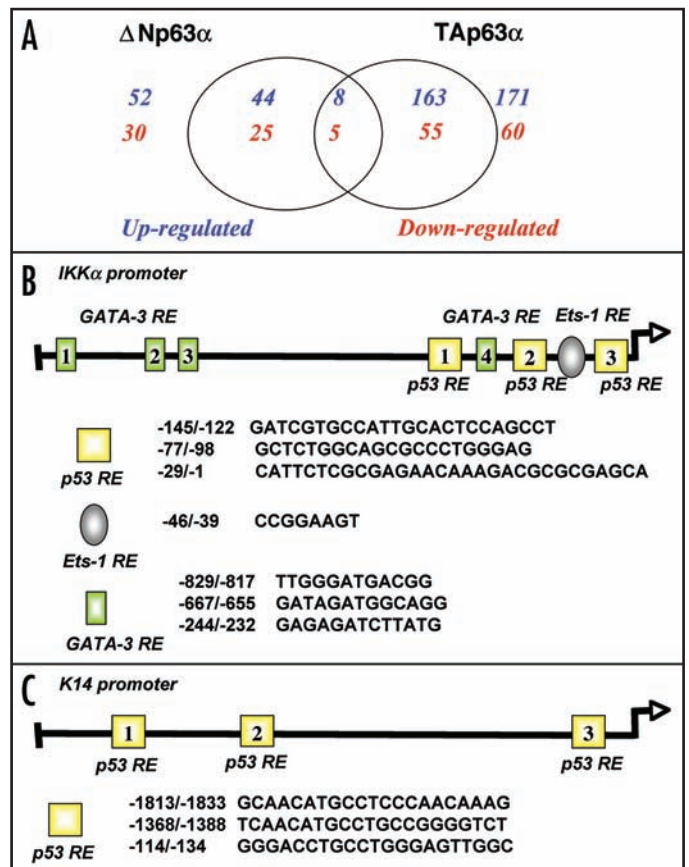
Figure 7. Target genes of p63 in epithelial development. Several studies have been performed to identify the target promoters of p63, see related papers in this issue. Using a microarray analysis in tet-on Saos2 cells (panel A) we identified several targets that have been confirmed in vivo using our genetically complemented mice. Panel B shows that p63 acts directly upstream of IKKα in epidermal formation. TAp63 (but not ΔNp63) isoforms directly induce IKKα expression through p53-like responsive elements in its promoter. Furthermore, p63 also drives IKKα indirectly, via Ets-1 (driven by TAp63) and GATA-3 (driven both TAp63 and ΔNp63), two additional transcription factors that regulate the IKKα promoter. Consequently, genetic complementation with TAp63 and ΔNp63 increases IKKα expression in p63^{-/-} mice. This is a clear demonstration that p63 acts upstream of IKKα in epithelial development. Panel C shows the promoter of keratin 14, a marker of the basal layer. ΔNp63 (but not TAp63) directly induces K14 expression, both in vitro and in vivo, further demonstrating a role for ΔNp63 in the formation of the basal layer. Panel D shows some of the genes transactivated by p63, involved both in cell death, cell cycle and epidermal development. A deeper analysis of the targets of p63 is discussed in related papers in this issue.

Table 3 Selected p63 target genes with a defined impact in epidermal development

	TAp63 (isoforms)		ΔNp63 (isoforms)		KO mice with epidermal phenotype	Ref.
IKKα	all	↑	all	-	Yes	85
PERP	α and γ	↑	α and γ	↑	Yes	91
EGFR	γ	↓	α	↑	Yes	69; 115
FGFR2	α	↑	α	↓	Yes	76
AP2-γ	α	↑	α	-		116
K14	α	↑	α	↑	Yes	63; 67
Envoplakin	γ	↑			Yes	33
p21	all	↑	α	↓		80
Jagged-1 & 2	γ	↑			Yes	83
Hes-1	α	↓	α	↓		84
Hey-1,2	α	↑	α	↑		84
GATA-3	α		α		Yes	85
BPAG-1	β and γ	↑	β	↑	Yes	92
Ets-1	α	↑	α	-	no skin phenotype	85

(↑)=Upregulation by p63, (↓)=downregulation by p63 (-) = no effect

p63, which is probably the more ancestral member of the family, is essential for the development of several different epithelia. Nevertheless, p63 is also involved in pathological conditions (cancer, DNA damage). With respect to cancer and the response to therapy, the balance between TAp63 and ΔNp63 isoforms appears to dictate the different cellular endpoints, survival and transformation versus



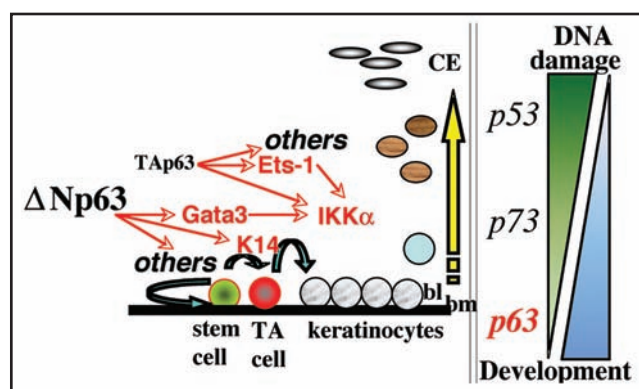


Figure 8. Evolution of function in the p53 family. According to the data presented here, ΔNp63 and TAp63 have distinct roles in epidermal development. We present here a model for the role of p63 isoforms in epidermal development, although we do not want to diminish other interpretations that have been proposed based on the evidence available in the literature. Moreover, only some of the target genes, those discussed above, are indicated. The right panel suggest that the function of the p53 family ranges from development (more pronounced for p63) to DNA damage (more pronounced for p53).

cell death. In contrast, the role of p63 isoforms in the development of epithelia, and in particular of the epidermis, is less clear. One interesting aspect is that ΔNp63 isoforms have an independent role as a transcriptional factors, consequently, TAp63 and ΔNp63 control the selective transactivation of specific target genes (as shown for IKKα, Ets-1 and other genes). We believe that an essential step toward an increased understanding of the role of specific isoforms will be achieved only through the generation of isoform-specific p63 knockout mice.

Finally, the interaction between different p53 family members has been proposed to exert an important regulatory function, thus further complicating the picture. This opens the way to completely new avenues of investigation aimed at solving this complex puzzle.

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THE CORNIFIED ENVELOPE: A MODEL OF CELL DEATH IN THE SKIN

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Abstract | The epidermis functions as a barrier against the environment by means of several layers of terminally differentiated, dead keratinocytes — the cornified layer [AU:OK?], which forms the endpoint of epidermal differentiation and death. The cornified envelope replaces the plasma membrane of keratinocytes in the cornified layer [AU:OK?] and consists of keratins that are enclosed within an insoluble amalgam of proteins, which are crosslinked by transglutaminases and surrounded by a lipid envelope. New insights into the molecular mechanisms and the physiological endpoints of cornification are increasing our understanding of the pathological defects of this unique form of programmed cell death, which is associated with barrier malfunctions and ichthyosis.

CORNIFICATION

The process of terminal keratinocyte differentiation, also known as keratinization or cornified-envelope formation, which allows the formation of the epidermal barrier (cornified layer) in the skin.

The biology of mammalian skin involves two distinct models of cell death, apoptosis and CORNIFICATION (or keratinisation), which occur in the lower and upper layers of the skin, respectively. In this review we focus on cornification.

The epidermis, which forms the uppermost, multi-layered compartment of the skin, has evolved to provide a physical and permeability barrier, which is essential for survival as an adaptation to terrestrial life in mammals^{1–3}. This barrier against the environment — which excludes foreign substances and organisms and prevents the loss of vital fluids — is provided, and continuously regenerated, by terminally differentiating keratinocytes. This process is known as cornification and is highly organized, both in space and in time. Keratinocytes move from a proliferative cell type in the basal cell layer of the EPIDERMIS through the granular layer where the CORNIFIED ENVELOPE is formed, to an association of flattened, dead cell remnants in the uppermost layer of the skin, the CORNIFIED LAYER (also known as the stratum corneum) [AU: OK?]⁴. At the cellular level, cornification begins with the synthesis of an immature type of envelope underneath the plasma membrane, and this envelope then undergoes maturation by the covalent attachment of preformed dedicated molecules to produce a rigid structure that fulfils its main physiological functions — that is, providing physical resistance and acting as a water barrier^{5,6}.

The aim of this review is to describe the mechanisms that are involved in the keratinocyte terminal-differentiation pathway, which is a unique form of programmed cell death, and its related diseases.

The cornified envelope [AU:OK?]

Epidermal differentiation begins with the migration of keratinocytes from the basal layer, and ends with the formation of the cornified layer (FIG. 1a). Cell proliferation, differentiation and death occur sequentially, and each process is characterized by the expression of specific proteins (see FIG. 2 and TABLE 1), and specific diseases can result from abnormalities in these proteins. In normal epidermis, the proliferation rate in the basal layer is precisely balanced by DESQUAMATION of the cornified layer [AU:OK?] at the skin surface. This epidermal homeostasis constantly rejuvenates the epidermis.

Keratins K5 and K14 are the main structural protein products in proliferating basal keratinocytes. They assemble into 10-nm KERATIN INTERMEDIATE FILAMENTS (KIFs; reviewed in REFS 7–9), which extend from the DESMOSOMES towards the NUCLEAR LAMINA and, along with microtubules (tubulin) and microfilaments (actin), form the cytoskeleton of epithelial cells. Triggered by poorly understood signals, certain basal keratinocytes migrate from the basal into the spinous layers, lose their mitotic activity, and begin to synthesize a new set of structural proteins and enzymes that are characteristic of cornification.

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EPIDERMIS

The external, uppermost multilayered compartment of the skin, which has evolved in mammals to provide a physical and permeability barrier for the organism. It is separated by a basal membrane from the dermis.

CORNIFIED ENVELOPE

An insoluble protein structure that is assembled by TGMs to replace the plasma membrane in corneocytes where it functions as a scaffold for lipid attachment. Corneocytes [AU:OK?] reside in the uppermost layer of the skin and constitute a barrier for the organism against the external environment.

CORNIFIED LAYER

The uppermost layer of the epidermis, previously known as the stratum corneum. It is formed by flattened dead cell remnants to create a physical barrier for the skin.

DESQUAMATION

The physiological process of shedding of dead corneocytes from the uppermost layer of the epidermis. Desquamation counterbalances regeneration, to maintain epidermal homeostasis.

KERATIN INTERMEDIATE FILAMENT

(KIF). A keratin structure that forms the cytoskeleton of all cells. KIFs are grouped into six types: I (acidic keratins); II (neutral-basic keratins); III (desmin, vimentin, peripherin and glial filament proteins); IV (neurofilaments including α -internexin); V (nuclear lamins); and VI (nestin).

DESMOSOME

A structure that contains integrins and connects the keratin-filament cytoskeletons of adjacent cells, and through which the basal layer adheres to the basement membrane. During terminal keratinocyte differentiation, TGMs crosslink specific proteins onto desmosomes, forming corneodesmosomes.

NUCLEAR LAMINA

A nuclear-membrane-associated protein structure that is made up of type-V KIFs.

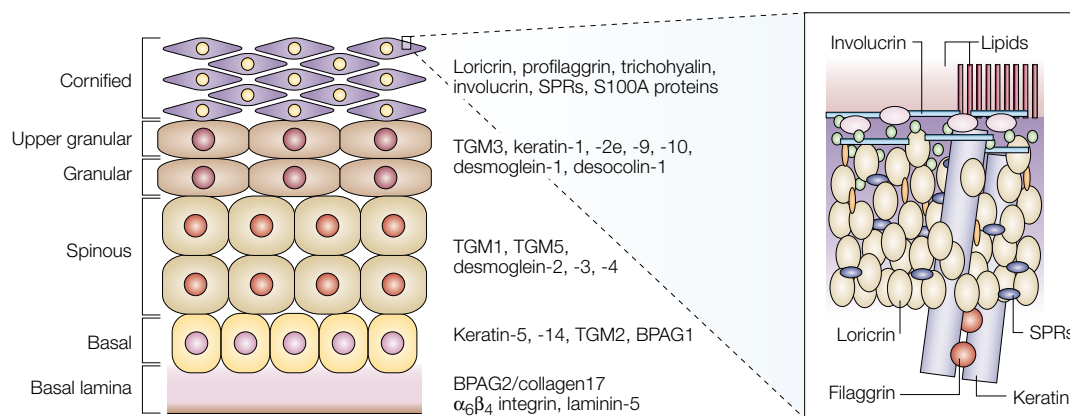


Figure 1 | Terminal differentiation and apoptosis in the epidermis. The proteins that are expressed in particular locations in the epidermis during skin differentiation are shown. Apoptosis is restricted to the basal layer, whereas cornification occurs in the supra-basal layers, to form a cornified envelope (see inset). At the molecular level, the cornified envelope is formed by proteins that are highly crosslinked by transglutaminases, with specific lipids on the outside, to guarantee specific physical properties (TABLE 1). BPAG, bullous pemphigoid antigen; SPR, small proline-rich proteins; TGM, transglutaminase. [AU: please label beige, yellow and light-green structures]

K1 and **K10** are among the first proteins to be expressed during cornification. K1 and K10 filaments replace the pre-existing K5 and K14 KIF network. In specific locations, significant amounts of other keratins are expressed, such as **K9** in palms and soles, and **K2e** in thickened sites. At a more advanced stage, the cells acquire keratohyalin granules, which contain **profilaggrin** — the precursor of the interfilamentous protein filaggrin. Filaggrin aggregates the keratin filaments into tight bundles. This promotes the collapse of the cell into a flattened shape, which is characteristic of **CORNEOCYTES** in the cornified layer. Together, KIFs and filaggrin constitute 80–90% of the protein mass of mammalian epidermis and therefore, they form a uniform layer that functions as a template or scaffold for the subsequent maturation or reinforcement steps of cornified-envelope assembly. Concurrently, a series of other structural proteins, including **involucrin**, **loricrin**, **trichohyalin** (THH) and the class of small proline-rich proteins (SPRs), are synthesized, and subsequently crosslinked by several **TRANSGLUTAMINASES** (TGMs [AU: Use TGM throughout as is most common name OK?]; see BOX 1) to reinforce the cornified envelope just beneath the plasma membrane^{1,3}. The proteins of the cornified envelope (TABLE 2) constitute about 7–10% of the mass of the epidermis.

Besides the cornified-envelope proteins, a complex series of lipids (for example, ceramides) are synthesized, some of which become covalently attached to proteins of the cornified envelope, and most of which form intercellular lamellae [AU: do the lipids themselves or the lipidated proteins form the lamellae?] that help produce a complete barrier [AU:OK?]. So, the cornified layer [AU:OK?] is composed of terminally differentiated, dead, cornified, flattened cells that are known as corneocytes. These corneocytes [AU: or 'The cornified envelope of these corneocytes?'] mostly consist of KIFs embedded in a filaggrin matrix and surrounded by insoluble lipids [AU:OK?]. Cornified envelopes [AU: Corneocytes?] are

tightly attached to each other by **CORNEODESMOSOMES** — modified desmosomal structures, which are proteolytically degraded in the uppermost layers of the cornified layer [AU:OK?] to allow desquamation¹⁰.

Similar structures are formed in many other types of stratified squamous epithelia, including hair and nail cuticles, as well as within the alimentary canal and urogenital tract. The reinforcement process varies between epithelia¹¹. The relative amino-acid composition of the reinforcement proteins that are used (such as loricrin or SPRs) confers different properties (such as elasticity, mechanical resistance and water impermeability) on distinct epithelia (for example, palm skin, dorsal skin or oral epithelia)¹² [AU:OK?].

Transglutaminase substrates in cornified-envelope formation [AU: please cut to 1 line]

The characteristic resistance and insolubility of the cornified envelope is based on the formation of very stable isopeptide bonds that are catalysed by transglutaminases (see FIG. 2 and BOX 1). **TGM1**, **TGM3** and **TGM5** are involved in cornified-envelope formation (for a review, see REF. 13). Despite a certain redundancy in this system — which is mainly due to [AU: insert 'the overlapping functions of?'] the TGM substrates, as suggested by loricrin^{−/−} (REF. 14) and involucrin^{−/−} (REF. 15) [AU:OK?] knockout mice — the TGM1^{−/−} mouse confirms that this enzyme is essential for correct cornified-envelope assembly¹⁶. The fact that these mice die at birth due to impaired barrier function is the strongest validation of the importance of TGM1 for cornified-envelope formation.

The type-II keratin chains (K1, K2e and K5) are crosslinked by TGMs at a specific Lys residue that is located in a conserved region of the V1 subdomain of the head domain^{2,17}. This results in the coordination and stabilization of the KIF–filaggrin network in the cornified cell and the periphery of the cornified envelope [AU:layer?]. Mutation of this Lys residue results in gross shape malformations, which are largely manifest as

CORNEOCYTE

A terminally differentiated keratinocyte that resides in the cornified layer (or stratum corneum). Corneocytes are dead, but still carry out functions such as forming the mechanical and water barrier that protects the skin. [AU:OK?]

TRANSGLUTAMINASE

A Ca^{2+} -dependent enzyme that catalyses the formation of N^{ϵ} -(γ -glutamyl)lysine bonds between proteins. Three different transglutaminases are involved in the formation of the cornified envelope in the skin.

thickening of the palms and soles¹⁸. Similarly, abnormal differentiation of skin in benign and malignant tumours correlates well with the aberrant expression of the differentiation-specific K1 and K10 keratins¹⁹.

Several proteins are present in the cornified envelope, and new components are still being discovered. For example, S100 proteins are a family of 10–14-kDa, EF-HAND-containing, Ca^{2+} -binding proteins, which transmit Ca^{2+} -dependent cell-regulatory signals. **S100A7**, **S100A10** and **S100A11** are expressed in the basal and spinous layers²⁰ and are substrates of TGM1 and TGM2 (REF. 21). S100A7 is present in the cytosol and in the endoplasmic reticulum (ER)²⁰. When the intracellular Ca^{2+} concentration increases, cytoplasmic S100A7 redistributes to α -actinin- and paxillin-containing peripheral complexes in FOCAL-ADHESION-like structures²². THH²³, a significant component of the THH [AU: or should this be keratohyalin granules?] granules of the hair follicle, is also expressed in the granular layer of the

epidermis, as well as in other specialized epithelial tissues such as the mouse hard palate and the filiform ridges of the tongue²⁴. THH is post-translationally modified by TGMs and peptidylarginine deiminase (PAD; see BOX 2). A new family of 18 proteins that are also TGM substrates [AU:OK?] are encoded by genes that are located in the epidermal-differentiation complex (human chromosome 1q21); these proteins are known as the late envelope proteins (LEPs), and are expressed after SPRs in the keratinocyte differentiation pathway [AU:OK?]²⁵. This gene cluster encodes involucrin, loricrin, SPRs and profilaggrin in addition to LEPs. The main substrates that are crosslinked by TGMs, are discussed below.

Involucrin. Involucrin²⁶ is a common, if not obligatory, component of the cornified envelope. Human involucrin is rich in Gly and Asp residues. Like other cornified-envelope structural proteins, it consists of repeat-

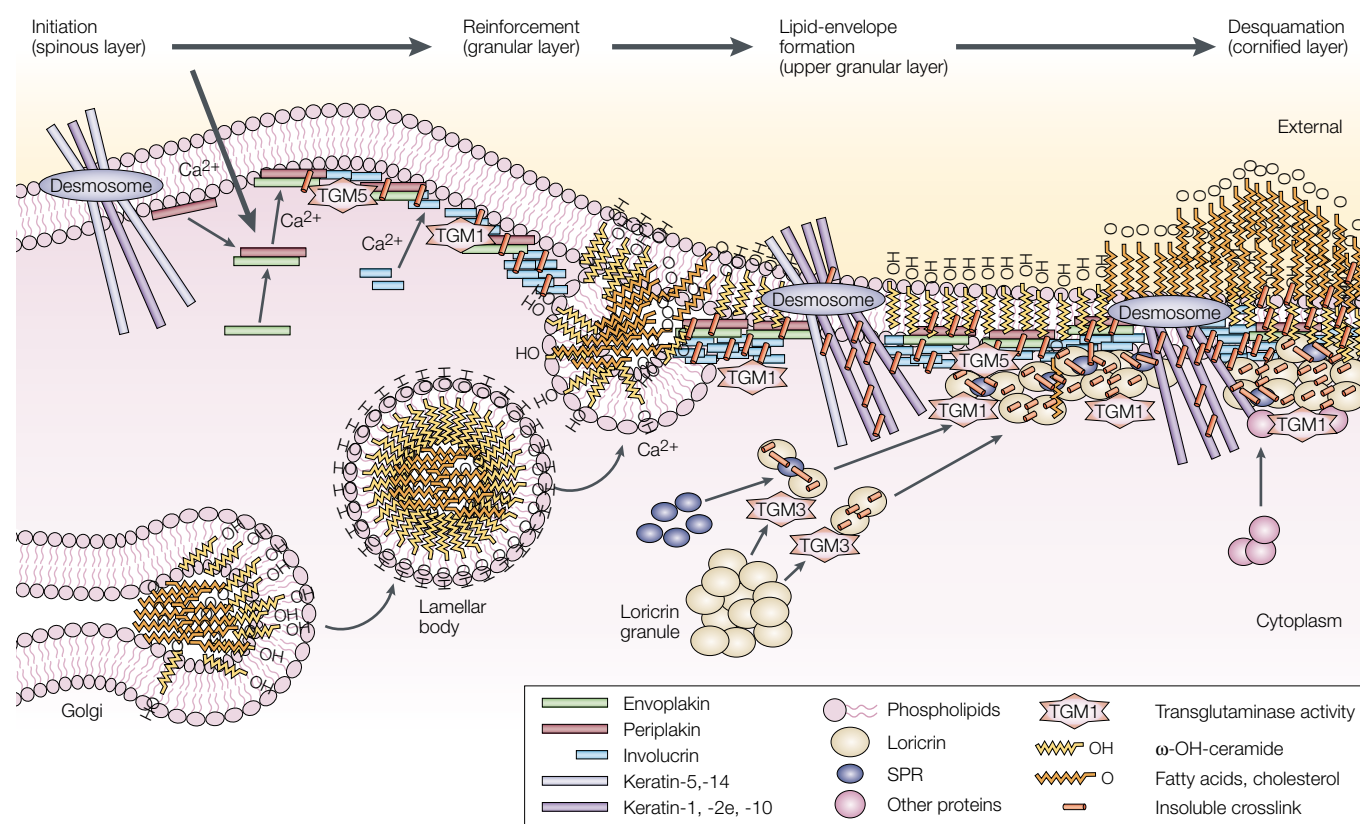


Figure 2 | Progressive steps in the formation of the cornified envelope. The first step is the initiation stage, which takes place in the spinous layer, and involves the synthesis of the cornified-envelope structural proteins (TABLE 2) and the synthesis and extrusion into the intercellular space of specific lipids. Transglutaminase (TGM)1 and TGM5 crosslink envoplakin and periplakin under the cell membrane, thereby anchoring them to the desmosome. The second step is the reinforcement phase, which takes place in the granular layer, and entails the covalent attachment of some lipids to the cornified-envelope proteins, and the crosslinking of loricrin to small proline-rich proteins (SPRs) by TGM3 and TGM1. Heavy crosslinking occurs on the desmosome, where these proteins function as substrates for TGMs. Next, during the formation of the lipid envelope, also in the granular layer, lipids from the lamellar body, which are derived from the Golgi, are attached and crosslinked by TGM5 and TGM1 on the already crosslinked proteins (envoplakin, periplakin, involucrin) and are exposed on the outside of the membrane. The reinforcement and lipid-envelope-formation steps take place concomitantly. Finally, the desquamation phase, which takes place in the cornified layer, involves further crosslinking of loricrin and other proteins by TGM1 on the protein scaffold, and the extrusion of ω -OH-ceramides, fatty acids and cholesterol. The physical properties of the cornified envelope depend on the nature of the substrates and on the crosslinks, as well as the lipid deposition. The location of the epidermal layers in which these progressive steps take place is indicated in FIG. 1.

Table 1 | **Formation of the cornified envelope: effectors and biological consequences**

[AU: Titles changed to accommodate lipids OK?]

Properties	Effectors	Diseases	References
Structural stability	Crosslinking enzymes: TG1	Lamellar ichthyosis	98,99
Elasticity	TG substrates: loricrin, involucrin and SPRs	Vohwinkel syndrome	104,105
	Lipids		
Mechanical resistance	Cytoskeleton: keratins	WSN (K4, K13), EPPK (K9) PC (K6, K16, K17), EHK (K1, K10), IBS (K2e), NCIE (K1)*	[AU: add refs?]
Water repellence	Lipids: FALDH	Sjögren–Larsson syndrome	114
	Lipoxygenases -3, -12R	NCIE-1/LI-5	113
	Cholesterol sulphatase	X-linked ichthyosis	111
	β-glucocerebrosidase	Gaucher disease type 2	[AU: add ref?]
	Phytanoyl-CoA hydroxylase	Refsum’s disease	137
	Peroxin-7 receptor	Refsum’s disease	138
	3β-hydroxysterol-Δ8,Δ7-isomerase	Conradi–Hunermann syndrome	139
	3β-hydroxysterol-Δ8,Δ7-isomerase	CHILD syndrome	140
	3β-hydroxysteroid dehydrogenase	CHILD syndrome	141
	sphingomyelinase	Niemann–Pick disease	86
	sterol-Δ7-reductase	Smith–Lemli–Opits syndrome	142

*For a more complete survey, see: <http://www.interfil.org>. CHILD, congenital hemidysplasia with ichthyosiform erythroderma; EHK, epidermolytic hyperkeratosis; EPPK, epidermolytic palmoplantar keratoderma; FALDH, fatty aldehyde dehydrogenase; IBS, ichthyosis bullosa of Siemens; K, keratin; LI-5, lamellar ichthyosis type 5; NCIE, non-bullous congenital ichthiosiform erythroderma; PC, pachyonychia congenital; SPR, small proline-rich protein; TG, transglutaminase; WSN, white sponge nevus.

ing peptide units, which have undergone extensive rearrangements during recent evolution²⁷. Involucrin is highly α-helical²⁸, and TGM1 preferentially crosslinks residues Gln495 and Gln496 (REF. 29). Involucrin becomes promiscuously crosslinked to various other structural proteins, which is consistent with the idea that involucrin is an early component in the assembly of cornified envelopes, and provides a scaffold to which other proteins subsequently become crosslinked. In the cornified-envelope structure, involucrin is adjacent to the cell membrane. And as the cell membrane is subsequently replaced, involucrin might be the preferred substrate to which the externalized lipids, mainly ceramides, are covalently attached to form the exterior surface of the cornified envelope³⁰. [AU: edit ok?]

Loricrin. Human loricrin is expressed in the granular layer during cornification, and is unusually enriched in Gly, Ser and Cys residues (TABLE 2). Computer modelling³¹ predicts that the protein has a non-organized structure with discrete domains (FIG. 3a): three (Gly, Ser, Cys)-rich domains, which are interspersed with short regions that are enriched in Gln, and flanked at the amino and carboxy termini by sequences that are rich in Gln and Lys. These (Gln, Lys)-rich regions are highly conserved in loricrin between species, whereas the (Gly, Ser, Cys) domains vary. The Gly residues are configured in inexact tandem peptide repeats and might fold into a unique protein conformation that is known as the Gly loop³². The Gly residues are interspersed by occasional long-chain aliphatic or aromatic residues, which might associate by hydrophobic interactions, thereby displacing the Gly sequences into an Ω-loop-

like configuration. This structural motif might also exist in at least two other protein families: the amino- and carboxy-terminal Gly-rich end-domains of certain KIF chains, and the single-stranded-RNA-binding proteins that include human hnRNP1A. Detailed structural analyses of this motif will prove difficult because of the extreme flexibility and essentially anisotropic nature of the Gly sequences.

Loricrin is the main component of the epidermal cornified envelope, and comprises 70–85% of the total protein mass of the conified layer [AU:OK?]^{2,3,11}. Limited proteolysis and sequencing experiments on the cornified envelope from foreskin cells [AU:OK?] shows that most of the crosslinks are loricrin–loricrin through isopeptide bonds. But smaller amounts of loricrin are also crosslinked to SPR1 and SPR2 [AU: do you want to refer to specific isoforms here?], which seem to function as cross-bridging proteins between the loricrin molecules. Loricrin seems to function as a main reinforcement protein for the cornified envelope on its cytoplasmic face. Presumably, loricrin admixed with the SPRs is deposited on a scaffold of *elafin*, cystatin α [AU: is this cystatin A? I can’t find a cystatin α?], involucrin, desmosomal proteins and possibly other Ca²⁺-binding proteins³³ (FIG. 2). In addition, crosslinked loricrin–keratin and loricrin–filaggrin were recovered, which further indicates that portions of the subjacent KIF–matrix-protein complex of cornified epidermal cells are covalently attached to the cornified envelope. This anchorage might therefore provide a means for coordinating the cell structure^{17,31,34}.

Loricrin functions as a key structural cornified-envelope protein³⁵. It is a substrate for TGM1, TGM2, TGM3

CORNEODESMOSOME
An adhesive structure within the cornified layer that resides between corneocytes and is generated by modifications (TGMs that crosslink desmoglein-1, desmocollin-1, corneodesmin) of desmosomes during terminal keratinocyte differentiation.

EF HAND
A protein motif that potentially binds Ca²⁺.

FOCAL ADHESION
A cell-to-substrate adhesion structure that anchors the ends of actin microfilaments (stress fibres) and mediates strong attachment to substrates.

and TGM5 *in vitro*, and undergoes different types of crosslinking. The TGM1, TGM5 and TGM3 enzymes crosslink residues sequentially *in vitro*, and they follow a similar sequence *in vivo*, [AU:OK?] thereby raising the intriguing possibility that multiple TGMs are required for loricrin crosslinking in the epidermis. In addition, loricrin was shown to be a highly favoured substrate protein [AU: Simplified sentence and deleted fig citation as seemed incorrect. Is this OK?]. Together, these data suggest that the accretion of loricrin by its crosslinking onto the cornified envelope *in vivo* might involve at least two steps: the initial attachment by TGM1 and TGM5, which oligomerize loricrin by way of interchain crosslinks, and a reinforcement process that

involves compaction by the TGM3 enzyme (FIG. 2). The lack of ordered structure contributes to the elasticity of the cornified envelope, whereas the TGM crosslinks are responsible for the mechanical resistance of the protein net (FIG. 3c–d).

Human loricrin is initially deposited in the granular layer of the epidermis in keratohyalin granules and is intermixed with profilaggrin³⁶. Newborn rodent epidermis contains morphologically distinct profilaggrin and loricrin granules (F and L granules, respectively)³⁷. Direct proof of loricrin phosphorylation *in vivo* has not yet been obtained, because loricrin is essentially insoluble in physiological solutions and is rapidly integrated into the insoluble cornified envelope by TGMs.

Box 1 | Skin transglutaminases

Transglutaminases (TGMs) are Ca^{2+} -dependent enzymes that catalyse the formation of N^ϵ -(γ -glutamyl)lysine bonds between proteins (see figure, panel a). They also catalyse the covalent incorporation of biogenic polyamines into proteins through N,N -bis(γ -glutamyl) bonds, which function as bridges between molecules¹³. Four of the nine mammalian TGMs are expressed in the epidermis.

TGM1 is expressed primarily in keratinocytes. In proliferating or basal keratinocytes, TGM1 is almost entirely bound to membranes through multiple N - and S -myristate adducts (M in panel c of the figure) on an amino-terminal anchorage segment, and is of low specific activity¹²⁰. During terminal differentiation, TGM1 is overexpressed and is attached to membranes primarily through palmitate linkages (P in panel c of the figure; other abbreviations: N, Asn; S, Ser)¹²¹. A significant portion of this protein is proteolytically processed to form a complex of 10-, 33- and 67-kDa chains (the full-length enzyme is 106 kDa). This complex is a membrane-anchored form of TGM1 [AU:OK?] and has a high specific activity and is responsible for most of the TGM1 activity in epithelial cells^{120–122}. TGM1 shows mutations along the entire primary protein sequence, although they are mostly located at the interface between the catalytic and N-terminal domains [AU:OK?] or around the catalytic site (see figure, panel b). The protein comprises four domains: the N-terminal, catalytic and β -barrel-1 and -2 domains. The coloured circles represent single amino acids. TGM1 is activated by an N-terminal cleavage event, which requires the positioning of the 97–102 hexapeptide (purple). In addition to the N-terminal proteolytic activation, catalysis is regulated by myristoylation and palmitoylation as well as the cleavage of the entire N-terminal domain, the two β -barrels, and the 10-, 67- and 33-kDa fragments (see figure, panel c; the relative enzymatic activities are indicated by the number of '+' symbols) [AU:OK?]. Membrane-anchored TGM1 can covalently esterify ω -hydroxyceramides with long chain (>C30) fatty acids onto Gln residues of the scaffold proteins³⁰, which allows a direct link between the protein (inner) and lipid (outer) component of the cornified envelope.

TGM2 is only detected in the basal layer of the epidermis, it is not involved in cornification, and its involvement in apoptosis^{123,124} is debated^{125,126}. TGM3, which is expressed in hair follicles and in terminally differentiating keratinocytes, is involved in cornification^{35,39,127}. TGM5, which is present in the upper epidermal layers, also has a role in cornified-envelope assembly, as it is induced during the early stages of keratinocyte differentiation *in vitro*^{128,129}. [AU: are R314 and L268 labelled correctly in the fig? Should the unlabelled amino acids be labelled?]

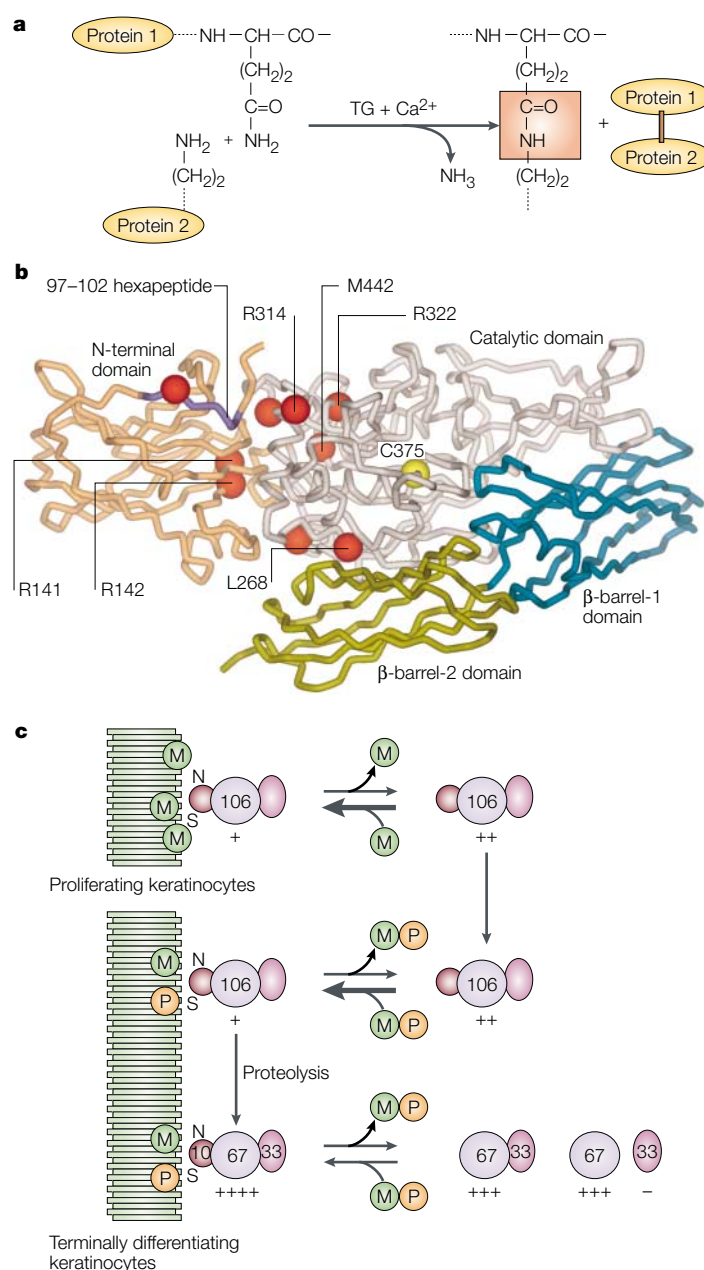


Table 2 | Amino-acid and protein composition of the cornified envelope*

Cornified-envelope protein [‡]	% Gly	% Ser	% Lys	% Gln	% Pro	% protein found in the epidermis	% protein found in the forestomach
Loricrin [§]	59.4	24.1	2.0	3.4	1.8	82	65
Keratins	23.8	13.9	4.8	12.7	0.8	0 [AU:OK?]	10
Filaggrin	15.7	19.8	0.0	21.4	2.8	6	5
Involucrin	5.0	3.2	7.7	25.6	9.1	0 [AU:OK?]	0 [AU:OK?]
SPRs [§]	0.4	3.8	12.4	21.6	43.6	8	18
Overall amino-acid composition of the cornified envelope	50.3	21.8	3.4	5.9	5.1	–	–

*Composition is expressed as % in w/w. [‡]The most abundant amino acids in the cornified envelope are Gly, Ser, Lys, Gln and Pro residues. The unusually high content of these residues implies the lack of ordered structure (Gly, Ser, Pro) and the ability to function as a substrates for transglutaminases (Gln, Lys). [§]It is interesting to note that 83.5% of loricrin is composed of Gly and Ser and 77.6% of SPRs is composed of Lys, Gln and Pro. SPRs, small proline-rich proteins.

However, phosphorylation might be the crucial event in targeting loricrin to keratohyalin granules, as has been shown for another cornified-envelope structural protein that is stored in granules, cystatin α ³⁸. Phosphorylated cystatin α is detected *in vivo* in keratohyalin granules, but treatment with protein-kinase-C inhibitors markedly suppresses its incorporation into these granules. Further studies will be necessary to evaluate the mechanisms that mediate loricrin storage in, and release from, keratohyalin granules during cornification.

Small proline-rich proteins. SPRs consist of a family of related small (6–18 kDa) proline-rich proteins, which is subdivided into SPR1 (2 proteins), SPR2 (10 proteins) and SPR3 (1 protein) classes. They contain head and tail domains that are rich in Gln and Lys residues, as well as a central domain that consists of variable numbers (2–20) of repeating peptide units of eight (SPR1 and SPR3) or nine (SPR2) residues that are highly enriched in prolines.

SPRs have little organized structure in solution, and what structure there is seems to be associated with the proline-rich peptide repeats (FIG. 3b)^{39–41}. *In vitro*, SPRs function as TGM substrates, with a preference for TGM3. Notably, Gln and Lys residues that are located on the head and tail domains are used for crosslinking. The residues used *in vitro* are almost identical to those *in vivo*. This indicates that the model that has been suggested for loricrin, where the disorganized structure is responsible for elasticity and the TGM crosslinks are responsible for mechanical resistance, also applies to SPRs (FIG. 3b–d).

The exclusive use of head- and tail-domain sequences for crosslinking and the nature of the crosslinked peptides that have been recovered from cornified envelopes indicates that the SPRs function as cross-bridging proteins among the more abundant loricrins of the cornified envelope^{11,12,42}. The content of loricrin and SPRs in the epidermis of different body sites and skin thicknesses is always ~85% of the total protein content, but the ratio of SPRs to loricrin varies from <1:100 in the thinner trunk epidermis to about 1:10 in lip, palm and sole epidermis, to >1:3 in rodent forestomach (TABLE 2; see also REFS 11,43). This composition is reminiscent of a composite material. The flexibility, rigidity or toughness of a composite material can be altered by varying the amounts of a minor crosslinking

component (in this case, the SPRs) that are admixed with a quantitatively major ground substance (loricrin). We postulate that the content of SPRs can markedly alter the physical properties of the cornified envelope with significant consequences for the epidermal structure and barrier function.

The central SPR repeats consist largely of β -turn motifs⁴¹. We postulated that the repeats of SPR2 proteins are more rigid than those of SPR1 proteins, which, in turn, are more rigid than those of the SPR3 proteins. Expression of SPR genes is modulated *in vitro* during keratinocyte differentiation, senescence and in response to differentiation-inducing agents such as phorbol ester, retinoids, cyclic AMP and interferon- γ . SPR genes, and in particular the SPR2 genes, are also induced *in vivo* in human skin during ageing or after ultraviolet irradiation.

The mechanical properties of the cornified envelope, and thereby the cornified layer, [AU: OK?] and respective epithelia vary depending on the type and amount of SPRs that are expressed in the tissue, which thereby adapts the tissues to their specific function. Likewise, the mechanical properties of cornified envelopes might be altered in human skin diseases, wound healing, ageing or under various types of environmental stress.

Profilaggrin. Human profilaggrin is a large (~500 kDa) complex protein. Its product, filaggrin, becomes crosslinked to the epidermal cornified envelope and participates in this way in coordinating the structure of the cornified cells². The bulk of filaggrin protein sequences consist of a tandem array of repeating filaggrin units of ~35 kDa, separated by a short 7–10-amino-acid linker peptide^{44,45}. Profilaggrin is initially highly phosphorylated and is accumulated in keratohyalin granules. During cornification it is dephosphorylated and proteolytically cleaved to release individual filaggrin molecules^{46,47}.

In vitro, filaggrin has the remarkable property of aggregating KIFs and other types of intermediate filaments into tight bundles in which the individual filaments are closely aligned in regular arrays⁴⁸. We believe that filaggrin functions in the same way *in vivo*, by organizing KIFs into bundles in the cells. The effect of this would be to collapse the KIF cytoskeleton, which in turn would promote a significant change in shape from an ellipsoid to a flat cell in which KIFs are aligned parallel

HEMIDESMOSOME

A specialized junction between epithelial cells and the extracellular matrix that is mediated by integrins and is associated with keratin intermediate filaments.

ADHERENS JUNCTION

An actin-filament-associated, epithelial cell–cell junction that has classic cadherins as its core component.

XEROSIS

Abnormal dryness, especially of the skin or the eye.

to the outer surface of the epidermis. The half life of filaggrin, which is 6 hours, indicates that these events must occur rapidly. In the cornified cell, filaggrin is degraded mostly into free amino acids. This high concentration of hydrophilic amino acids (in the 100 mM range) is essential for the retention of water and contributes to the osmolarity and, thereby the flexibility, of the cornified layer. So, the profilaggrin system has at least three critical roles in epidermal differentiation: the alignment of KIFs, the control of significant changes in cell shape [AU:OK?] and, subsequently, the maintenance of epidermal texture.

The numerous protein turns of filaggrin are positively charged with Arg and His residues, which have been postulated to interact by the formation of ionic salt bridges with the many negative charges on the KIF (and other intermediate filament) rod domains⁴⁹. The first 80 amino acids contain two perfectly formed and functional EF-hand, Ca²⁺-binding domains that are typical of many small Ca²⁺-binding proteins and proteases⁵⁰, and in this regard, profilaggrin is similar to THH. Profilaggrin is polymorphic in that there are three alleles in the human population, which encode 10, 11 or 12 filaggrin repeats⁴⁵. Moreover, the exact amino-acid sequences of neighbouring filaggrin repeats have not been conserved, as there is as much as 40% variation in the amino-acid sequence between them. However, the secondary structure has been conserved, as filaggrins consist almost entirely of repeating quasi-tetrapeptide repeats that form a β -turn configuration⁴⁹. So, filaggrin does not contain an organized higher-order structure;

indeed, this is what might be expected of a protein that functions in an important way as a KIF 'glue'.

Cell adhesion: from desmosomes to corneodesmosomes [Au: please cut to 1 line]

The epidermis is separated from the underlying dermis by the basal lamina, which is composed of extracellular-matrix proteins. The basal layer adheres to the basement membrane through HEMIDESMOSOMES, which contain integrins (for a review, see REF. 51).

Within the epidermis, two types of junctions are responsible for intercellular adhesion and for cohesion: the ADHERENS JUNCTIONS (which connect the actin cytoskeletons of neighbouring cells) and the desmosomes (which connect the keratin-filament cytoskeletons of adjacent cells). Certain proteins that constitute the adhesion complexes are specifically expressed during cornification. These include **desmoglein-1**, **desmocollin-1**, **envoplakin**, **periplakin**, **plakophilin-1** and **corneodesmosin**. At the transition between the granular layer and the cornified layer, profound changes are observed in desmosome morphology, namely the integration of the cytoplasmic plaque into the cornified envelope and the formation of a homogeneous electron-dense plaque in the extracellular core^{10,52}. Corneocyte cohesion within the cornified layer depends on these modified desmosomes, which are known as corneodesmosomes.

Two main components of the corneodesmosomes are desmoglein-1 and desmocollin-1, which are two glycoproteins that belong to the cadherins, a family of Ca²⁺-dependent cell-adhesion molecules. Another constituent of the corneodesmosomes is corneodesmosin, which is located in the core of the extracellular compartments. Corneodesmosin is expressed in the upper spinous and granular layers [AU: insert 'of the epidermis'?], and is a phosphorylated and glycosylated protein. It is a Gly- and Ser-rich protein, and these sequences are believed to form Ω -loop-related domains in a similar manner to loricrin and the keratins, as described above. The suggested function of these structural motifs is to interact with identical loops on the same or neighbouring proteins. Corneodesmosome degradation is of importance during the desquamation process.

In several pathological conditions (for example, psoriasis and XEROSIS), a large increase in the number of persisting corneodesmosomes at the surface of the cornified layer is observed. Several serine proteases, including the stratum corneum chymotryptic enzyme (SCCE) and the stratum corneum tryptic enzyme (SCTE), are thought to be involved in corneodesmosome proteolysis (see below and REFS 53,54). Presumably, proteolysis during cornified-layer maturation results in the suppression of the glycine-loop-related domains, thereby eliminating the adhesive parts of the corneodesmosin and allowing desquamation^{55,56}.

Deregulation of desmosome formation is observed in many degenerative cutaneous diseases. Pemphigus is an autoimmune blistering disease that is caused by autoantibodies against components of the desmosome, desmoglein-1 in pemphigus foliaceus and desmoglein-

Box 2 | Protein deimination in the epidermis

Peptidylarginine deiminases (PADs) are Ca²⁺-dependent enzymes that convert protein-bound arginine to citrulline by elimination of the imino group. This process, which is known as deimination or citrullination, is an important post-translational modification at the granular/cornified interface¹³⁰.

Deimination of a single Arg residue significantly changes the structure and function of target proteins. Unfortunately, little is known about the biochemical effect of deimination in the epidermis^{131,132}. Keratin-1 (K1), filaggrin and trichohyalin (THH) are principal substrates for PAD. The amino-acid composition of filaggrin is 16% Arg residues, 95% of which can be converted *in vitro*, which results in the loss of the ordered protein structure. In addition, the protein becomes more soluble, increases its electrophoretic mobility, and becomes a better substrate for transglutaminases (TGMs).

Two preferred deimination sites were identified in mouse and human K1, in the V1-2 subdomains^{133,134}. Here, Arg deimination probably modulates their interactions with epidermal proteins other than keratins and filaggrin during the terminal stage of epidermal differentiation; K1 deimination is also modulated in developing human epidermis¹³⁵.

THH, which is involved in hair keratinization and contains 22.8% Arg residues, is postsynthetically modified by different Ca²⁺-dependent enzymes, PADs and TGMs, *in vitro*^{127,136}. This results in the loss of organized protein structure and increased solubility^{127,136}, which are essential processes for THH to function as an interfilamentous protein. PAD-modified THH becomes a much better substrate for TGM3 with which it is co-expressed in hair-follicle cells¹²⁷. It is also a crosslinked component of the cornified envelope of rodent forestomach epithelium and guinea pig inner root sheath [AU: please clarify inner root sheath?] cells¹¹, by crosslinking to loricrin, small proline-rich proteins (SPRs) and keratins. THH might contribute to the rigid structures in the hair-follicle cells and in other highly specialized epithelia with particular requirements for toughness and durability.

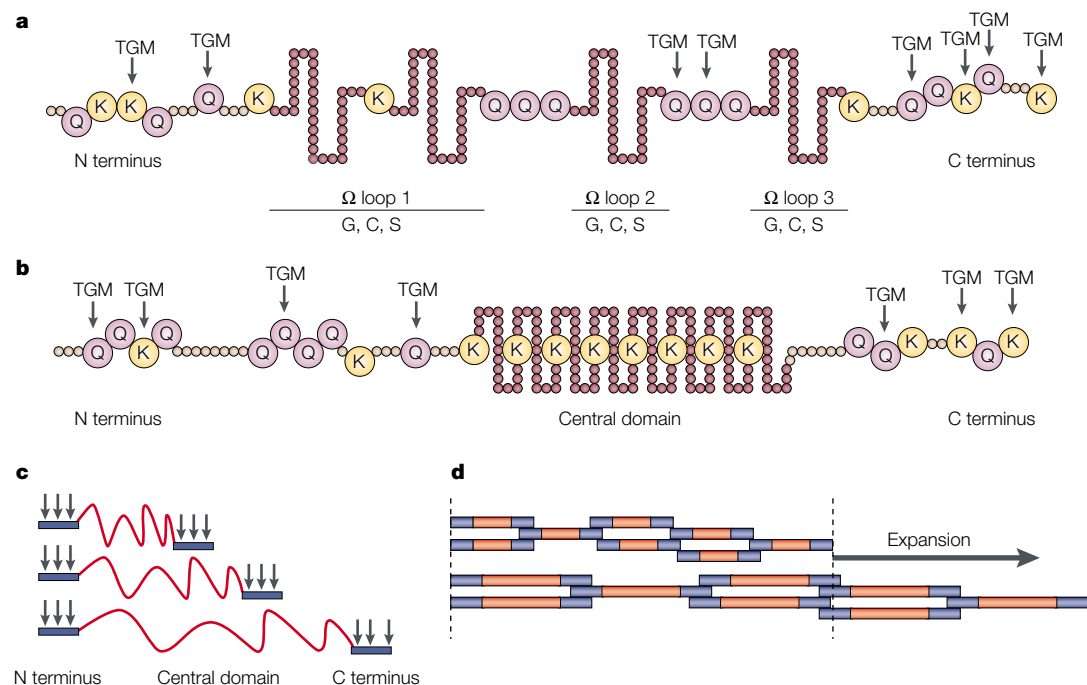


Figure 3 | Lack of ordered structure in the substrates of skin transglutaminases. a | Loricrin has three Ω loops, which are non-structured domains with high mobility that are formed by Gly, Cys and Ser residues. The principal transglutaminase (TGM) crosslinking sites (Gln (Q) and Lys (K)) are indicated by arrows. These are intra- or inter-chain crosslinks, which form a highly insoluble structure that is essential for the barrier function of the epidermis. **b** | Small proline-rich proteins (SPRs) comprise three domains: the N-terminal, central (8–16 repeats of 8 residues) and C-terminal domains. Like the Ω loops in loricrin, the central domain does not have an ordered structure and shows high mobility. The residues that are used by TGMs for crosslinking (arrows) are mainly localized in the N- and C-terminal domains; their quantum yield is crucial. First, multiple TGMs are required for efficient crosslinking. Second, the order of TGM action is essential. For example, in human SPR3 10% of the residues Gln17–19 yield crosslinks if TGM1 acts before TGM3, but the yield raises to 90% when the order of action is reversed. The same is true for other residues. **c–d** | Due to their peculiar amino-acid composition, the structures of loricrin and SPR are predicted to be ‘spring-like’: the N- and C-terminal domains contain areas that are rich in Gln and Lys residues (shown in blue), which are used by TGMs as crosslinking substrates, and which confer mechanical resistance by creating a net of heavily crosslinked proteins (each cornified-envelope protein has about 10 crosslinks). By contrast, the central domain, which is rich in Gly and Ser residues (shown in red), forms a flexible, non-ordered structure that contributes to the expansion and elasticity of the cornified envelope.

3 in pemphigus vulgaris (reviewed in REF. 57). Mutations in plakophilin-1 are responsible for ectodermal dysplasia, whereas mutations in desmoplakin are found in patients that are affected by palmoplantar keratoderma^{58,59}. Ca^{2+} is also necessary for desmosomal integrity, as reflected in Hailey–Hailey disease and Darier’s disease, which are both characterized by desmosomal dysfunction that is caused by mutations in the ATP2A2 Ca^{2+} pumps^{60,61}.

Proteases in skin differentiation

Cornification requires a massive activation of epidermal proteases, but for most of these (for example, brain-specific serine protease, neuropsin and PACE4) their precise role remains elusive. Metalloproteases (such as matrix metalloproteases (MMPs) or gelatinases) are mainly involved in the migration of melanoblasts and Langerhans cells and in wound healing. Both cysteine-protease inhibitors (cystatin α , M and E) and serine-protease inhibitors (elafin, hurpin, maspin, plasminogen activator inhibitor-1/-2 and protease inhibitor-6/-9) are abundant and pivotal in controlling these mechanisms. Proteases are involved in at least three processes in skin

differentiation. First, certain cornified-envelope precursors require proteolytic processing before cornified-envelope formation occurs. Second, the loss of nuclei and mitochondria requires proteolytic processing. Third, desquamation requires proteolysis of the corneodesmosomes.

Crosslinking of substrates by TGMs during the assembly of the cornified envelope requires specific proteolytic activation of the TGMs^{62–64}, possibly by μ -calpain, **cathepsin D** or **furin**. A mutation in TGM1 that prevents its processing has been identified as a cause of lamellar **ICHTHYOSIS**⁶⁵ (BOX 1). Similarly, filaggrin is the substrate of many proteases such as PEP1, calpain [AU: μ -calpain?], furin and the transmembrane serine protease matriptase MT-SP1. Mice that lack MT-SP1 show aberrant profilaggrin processing, defects in cornified layer [AU:OK?] desquamation and epithelial lipid-matrix defects⁶⁶. The exact order of the different proteolytic events is not completely resolved, but it is believed that dephosphorylation precedes filaggrin processing.

There are at least three classes of intracellular proteases: lysosomal proteases, calpains and caspases. Lysosomes contain a large number of hydrolases. The

ICHTHYOSIS

A dermatological disorder in which the keratinocyte cornified envelope is abnormal, which results in a defective external layer (cornified layer [AU:OK?]). From the greek *Ichthys*, which means fish, to indicate the scaly skin like that of a fish.

LAMELLAR BODY

A small multilayer cytosolic organelle that is surrounded by a membrane.

signals for the release and activation of lysosomal enzymes during cornification are unclear, as is the mechanism by which they degrade the cellular organelles. The large cathepsin family includes the lysosomal cysteine, aspartic-acid [AU:OK?] and serine proteases, which are all present in keratinocytes^{67,68}. Cathepsin D, E and L2 are found in the cornified layer, whereas cathepsin L is required for normal epidermal homeostasis and hair-follicle morphology^{69,70}. Recently, it has been shown that cathepsin-D-deficient mice have reduced TGM1 activity and reduced cornified-envelope proteins, which indicates a functional link between the activation of cathepsin-D and TGM1 (REF. 64).

The increase in the Ca^{2+} gradient with terminal differentiation, both in the intra- and extracellular compartments, regulates Ca^{2+} -dependent proteases. μ -Calpain (I) and m-calpain (II), which require micro- or millimolar concentrations of Ca^{2+} for their activation, are present in the suprabasal layers⁷¹. The addition of synthetic calpain inhibitors results in the blockage of profilaggrin and TGM1 processing^{62,72}. However, mice that are deficient in μ -calpain (I) [AU:OK?] show no abnormalities in skin formation, although mice that are deficient in the small calpain subunit [AU: small calpain subunit 1 or 2?] die very early in embryonic development, thereby preventing an evaluation of its importance in skin differentiation⁷³. Other Ca^{2+} -dependent serine proteases are the subtilisin-like protein convertases (SPCs) that are involved in the secretory pathway; four of them are found in the epidermis. Furin (SPC1) becomes activated in the later stages of cornification and can remove the profilaggrin amino terminus *in vitro*. Caspase-14 is expressed in the suprabasal layers, where it is not involved in apoptosis, but is processed at the transition from the granular to the cornified layer during keratinocyte differentiation⁷⁴.

Extracellular proteases are abundant in the cornified layer and have been correlated with desquamation. Corneodesmosomal proteins that are degraded during desquamation are desmoglein-1, desmocollin-1, plakoglobin and corneodesmosin^{52,56}. The enzymes that are responsible for their processing and degradation are not yet known, although possible candidates include SCCE, SCTE and stratum corneum cathepsin-L-like enzyme (SCCL). Other proteases that have been identified in the cornified layer are cathepsin D and cornified layer thiol protease (SCTP) or cathepsin L2. SCCE and SCTE are expressed in the granular layer and belong to the serine-protease kallikrein family. The SCCE precursor is present in the LAMELLAR BODIES that extrude their content at the transition between the granular and cornified layers into the extracellular space. The activation occurs by a tryptic-like cleavage, removing the amino terminus^{75,76}. SCTE is also confined to the differentiating suprabasal layers of the epidermis. Both SCCE and SCTE can degrade corneodesmosin *in vitro*⁵².

The role of proteolysis in desquamation is evident in the *Spink5*^{-/-} mice, which mimic severe Netherton syndrome (see below, and REF. 77). The *Spink5* gene encodes the Kazal-type-5 protease inhibitor (LEKTI), which causes desmosomal fragility that is associated with pre-

mature proteolysis of an extracellular desmosomal component (corneodesmosin).

Skin lipids and the lipid envelope

The cornified envelope is embedded in the lipid envelope. Skin lipids are essential for the barrier function of the epidermis that helps avoid transepidermal water loss. Most of these lipids are ceramides, cholesterol, fatty acids and cholesterol esters that form lamellae between the corneocytes of the cornified layer. Intercellular skin lipids are synthesized and stored in granules that are known as lamellar granules (or Odland bodies), which are extruded (probably by fusion) into the extracellular space in the upper granular layer during cornification^{78,79} (FIG. 2). The lamellar bodies are composed of lipid lamellae that contain glucosylceramides, phospholipids and cholesterol, which are the precursors of the intercellular skin lipids^{80,81}. Complex changes in lipid composition occur after the extrusion of the contents of the lamellar granules by the enzymatic action of a group of acid hydrolases, which are also secreted into the extracellular space^{80,82,83}. Enzymes such as β -glucocerebrosidase (which is involved in Gaucher disease, see below), acid sphingomyelinase and phospholipase A₂, are involved in the metabolic modification of the extracellular lipids^{84–86}. Even though several animal models show a defined role for lipids in the epidermal barrier^{87–90}, the physical structural organization of the extracellular lipids of the cornified envelope is not known at the molecular level. Electron-micrographic studies show that the lipids are organized as stacked membrane sheets, which occupy all the extracellular space^{91,92}. Biophysical studies indicate that the free fatty acids and the amide-linked fatty-acid chains in the ceramides are tightly packed to form gel-phase membrane domains, which coexist with liquid crystalline domains. The experimental difficulties in studying these lipids has led to the formulation of different models: the 'domain mosaic' model⁹³, the more recent 'sandwich' model and the 'single gel-phase' models⁹⁴.

An important, although still unresolved, area is the mechanism of interaction of the extracellular lipids and the cornified envelope. Long-chain ω -hydroxyceramides are present on the extracellular surface of the cornified envelope where they form an external layer or coating — the 'lipid envelope' — and some of them are covalently attached through ester linkages to the outer surface of the cornified envelope^{95,96}. Indeed, ω -hydroxyceramides are linked to involucrin through an ester bond by TGM1, at least *in vitro*^{6,30,97}. Possibly, other cornified-envelope proteins could also be covalently linked to ω -hydroxyceramides, as the involucrin-knock-out mice show normal cornified envelopes and a normal cornified layer¹⁵.

The components of the lipid envelope have particularly long fatty-acid chains, which can cross the plasma membrane bilayer of the corneocytes and become ester-linked to cornified-envelope proteins. On the other side of the molecules, the sphingosine chains can interact freely with the unbound extracellular lipid lamellae. This interaction could help establish the lamellar orga-

nization that is seen in the extracellular lipids. The ω -hydroxyceramide coating of the cornified envelope contributes significantly to the establishment of [AU: insert 'epithelial?'] elasticity and barrier function by impermeabilising the cornified envelope, and by participating in the organization of the intercellular lipid lamellae.

Cornified-envelope-related diseases

Transglutaminase-related defects. About 50% of lamellar ichthyosis (LI) patients carry mutations in the *TGM1* gene^{98,99}. LI is an autosomal-recessive disorder, which causes severe abnormalities of the cornified layer [AU:OK?] and which is characterized by the presence of a collodion membrane (large, brown, plate-like scales with a random distribution) at birth. This membrane sheds in the first weeks of life leaving the patient with a disfiguring ichthyosis, which is often associated with scarring alopecia. The mutations that have been identified in *TGM1* in LI patients are often located at the interface between the amino-terminal and the catalytic domains (BOX 1), which indicates that this region is crucial for the enzymatic activity of the protein. Loss of *TGM1* function that results in inadequate or incomplete crosslinking of cornified envelope proteins therefore provides a molecular mechanism for the impaired barrier phenotype of the disease. However, some mutants retain enzyme activity, which implies that there must be a more complex explanation. The active mutants might stabilize the tertiary structure of the enzyme by interfering with, or actually preventing, proteolytic processing and therefore the formation of highly active forms of *TGM1* (BOX 1). This ultimately leads to the same pathological consequences as the inactive enzyme mutants. So far, no cornified-envelope defects have been correlated with genes that encode other TGMs. Other LI patients show a linkage to different chromosomal regions such as 3p21 and 19p12-q12 (REF. 100). Indeed, mutations have been identified in two lipoxygenase genes (*ALOXE3* and *ALOX12B*) on 17p13 (REF. 101), and on the ATP-binding-cassette transporter gene (*ABCA12*)¹⁰² on 2q33-35 (REF. 103). Even though the molecular mechanism of pathogenesis that is elicited by the proteins encoded by these three genes has not been identified, it is evident that defects in lipid metabolism result in similar abnormalities in epidermal homeostasis and cornified-envelope formation.

Defects of structural proteins of the cornified envelope.

Defects of the epidermal barrier might also result from mutations that lead to gross changes in, or complete loss of, structural proteins of the cornified envelope. However, no disease has so far been linked to an absent cornified-envelope structural protein. This might be due to the early lethality of such defects *in utero*, or more likely, to the presence of back-up systems in the epidermis to compensate for the defect or loss by the use of other proteins.

Two skin diseases are related to defects in loricrin: **Vohwinkel syndrome** (keratoderma hereditaria mutilans) and **progressive symmetric erythrodermatoderma** (PSEK)^{104,105}. Vohwinkel syndrome is characterized by hyperkeratosis of the palms and soles and constricting

bands that encircle the digits of the hands and feet, which can lead to auto-amputation, and is inherited as an autosomal-dominant trait. It is caused by mutations in the loricrin gene¹⁰⁴, in which a single nucleotide deletion causes a frameshift mutation. This mutant encodes an extended terminal sequence that is highly enriched in Arg instead of Gly residues and a number of important Gln and Lys crosslinking sites are also lost. It remains to be established whether the disease phenotype is indeed caused by competition between the products of the mutant and normal alleles, as is typical of dominant-negative effects in autosomal-dominant diseases, or whether it is due to a gain of function by the abnormal presence of the mutant basic protein in the nucleus instead of the cytoplasm. Patients that are affected by PSEK show erythematous plaques over the entire body and palmoplantar hyperkeratosis¹⁰⁵.

However, overexpression of human loricrin in transgenic mice yields a normal phenotype³⁶ and the absence of loricrin in null mice results in no obvious abnormality, although an early, temporary deficiency in water-barrier function is observed that reverts to normal within five days¹⁰⁶. This indicates that keratinocytes might compensate for the loss of one cornified-envelope component by using other available proteins to form a functional cornified envelope¹⁴. A mouse model for Vohwinkel syndrome and PSEK has been developed, which expresses a carboxy-terminal truncated mutant of loricrin, which is similar to the mutant found in the patients that are affected by these diseases¹⁰⁷.

Keratin diseases have been discussed in separate reviews^{7,9,108-110} (see also TABLE 1). Most of these defects are inherited as autosomal-dominant traits, as the altered keratin protein interferes with the formation and dynamic function of the KIFs in the cells in a dominant-negative way. The nucleotide substitutions that are found in these mutants often involve the residues of the rod-domain segments 1A and 2B, which is the region that is responsible for KIF assembly. In some cases, the mutation involves the same Lys residue¹⁸ that is critically involved in *TGM1* crosslinking of KIFs to the cornified envelope¹⁷.

Defects of lipid metabolism. Genetic ichthyosiform diseases can be caused by impaired lipid synthesis (TABLE 1). Even though the genetic defect is often known, the underlying molecular mechanism that leads to cornified-layer alteration with barrier malfunction is elusive.

The first lipid disease to be associated with a lipid metabolic defect was the autosomal-recessive disease **X-linked ichthyosis** (RXLI), which is characterized by thick dark scales (usually sparing the palms and soles) and corneal opacities, and is caused by a defect in the ubiquitous enzyme steroid sulphatase¹¹¹. The accumulation of cholesterol sulphate in the extracellular space of the cornified layer inhibits desquamation, the catalytic activity of *TGM1* and involucrin crosslinking to envelope proteins, as well as involucrin esterification to the lipid envelope¹¹². Among the ichthyoses, as discussed above, a subset of LI patients (LI-5 or **nonbullous congenital ichthiosiform erythroderma** (NCIE) type-1) carry muta-

ALOPECIA
The loss of hair.

NECROSIS [NOT FOUND]
Non-genetically programmed cell death, which is different from apoptosis and cornification, and is relevant in tissue damage.

tions in lipoxygenase-3 or -12R (REF. 113) that catalyse the oxygenation of free and esterified polyunsaturated fatty acids to produce the corresponding hydro-derivatives. The epithelial substrates of these enzymes are still unknown¹⁰². In addition, patients that are affected by LI type-2 carry mutations in the *ABCA12* gene¹⁰². The ABCA proteins are transporters of various substrates across extra- and intracellular membranes, and are associated with recessive disorders of lipid metabolism.

Sjögren–Larsson syndrome is characterized by the presence of three main symptoms: ichthyosis, spastic diplegia or tetraplegia and mental retardation. We have shown that this disease is correlated with mutations in the gene that encodes microsomal fatty-aldehyde dehydrogenase¹¹⁴, an enzyme that catalyses the oxidation of long-chain aliphatic fatty aldehydes to fatty acids. The pathogenesis is not yet clear, but in view of the massive synthesis of lipids in the skin, the particular involvement of the epidermis is not surprising.

Gaucher disease is an autosomal-recessive disorder that is due to mutation of the enzyme β -glucocerebrosidase, which catalyses the hydrolysis of glucosylceramides to ceramides. Ceramides are a critical component of the intercellular lamellae that mediate the epidermal permeability barrier. When the enzyme is defective, glucosylceramides accumulate in the cells, which causes damage in the splenic, hepatic, skeletal and central nervous systems. Moreover, in severe Gaucher disease type-2, patients display scaling skin ('collodion baby'). The relationship between glucocerebrosidase deficiency and epidermal permeability-barrier function has been studied in detail using both Gaucher mice and hairless mice that have been treated topically with inhibitors of glucocerebrosidase^{84,115}. These studies indicated that the skin changes that are observed in patients affected by Gaucher disease result from the formation of inadequate intercellular lamellar bilayers in the cornified layer.

Chanarin–Dorfman syndrome, which is caused by a mutation in the *CGI-58* gene, is a rare autosomal-recessive form of inherited lipid storage, which is character-

ized by ichthyosiform erythroderma and by the presence of intracellular lipid droplets in most tissues, and therefore also involves several internal organs. Ultrastructural observations show that [AU:OK?] abundant abnormal lamellar granules disturb the intercellular lamellar structure, creating lipid vacuoles in the epidermis^{116,117}. Even though the CGI-58 protein contains three sequence motifs that correspond to a catalytic triad found in the esterase/lipase/thioesterase subfamily, its function is unknown.

Protease-related defects. Defects in proteases also result in barrier-function abnormalities or skin diseases. **Netherton syndrome** is caused by mutations in the *SPINK5* gene, which encodes the serine protease inhibitor LEKTI¹¹⁸. It is a severe autosomal-recessive skin disorder that is characterized by congenital erythroderma, a specific hair-shaft abnormality, and atopic manifestations with high immunoglobulin (Ig)E levels. Mutations in the gene that encodes the protease cathepsin C causes the autosomal-recessive Papillon–Lefèvre syndrome¹¹⁹. This is characterized by hyperkeratosis of the palms and soles and severe early-onset periodontitis that results in the premature loss of both the primary and secondary dentitions. Both examples show abnormalities in the lipid structure of the cornified layer.

Concluding remarks

This review has concentrated on cornification, which is a sophisticated mechanism of programmed cell death that is also essential for life. The molecular events that are involved in cornification have been described, in particular the role of TGMs and their substrates, and the related diseases that are caused by abnormalities in these proteins. Many interesting, unresolved questions remain; for example, it is unknown as yet how the keratinocyte dies, or more specifically, what the precise suicide signal is, and how the nucleus and mitochondria are eliminated. Once the main components of cornification have become better known, we hope to identify more efficient and novel ways of treating skin disorders.

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Competing interests statement

The authors declare that they have no competing financial interests

Online links

DATABASES

The following terms in this article are linked online to:

Entrez: <http://www.ncbi.nlm.nih.gov/entrez>
ABCA12 | *ALOXE3* | *ALOX12B* | *Spink5* | *TGM1*
OMIM: <http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM>
 Chanarin–Dorfman syndrome | Gaucher disease | lamellar ichthyosis | Netherton syndrome | nonbullous congenital ichthyosiform erythroderma | progressive symmetric erythrokeratoderma | Sjögren–Larsson syndrome | Vohwinkel syndrome | X-linked ichthyosis
Swiss-Prot: <http://us.expasy.org/sprot/>
 μ -calpain (I) | cathepsin D | corneodesmosin | desmoglein-1 | desmocollin-1 | elafin | envoplakin | furin | K1 | K2e | K5 | K9 | K10 | K14 | lorincrin | involucrin | m-calpain (II) | periplakin | plakophilin-1 | profilaggrin | S100A7 | S100A10 | S100A11 | TGM1 | TGM2 | TGM3 | TGM5 | Trichohyalin

FURTHER INFORMATION

[AU: Any web sites to add?]

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Biographies

Eleonora Candi received her Ph.D. from Rome [AU: which university?] where she worked with Gerry Melino, before joining Peter Steinert's laboratory at the National Institutes of Health in Bethesda, USA, where she spent 6 years. She is now an assistant professor in biochemistry at the Faculty of Medicine at the University of Rome 'Tor Vergata'. Her work is fully dedicated to transglutaminases and their substrates.

Reiner Schmidt received his Ph.D. in Berlin, Germany. After being a research associate at McGill University in Montreal, Canada, he worked for 8 years as a senior scientist at the Centre International de Recherches Dermatologiques (CIRD) in Sophia Antipolis, France. Since 1992, Reiner Schmidt has been part of L'Oréal Life Sciences Research in Clichy, France, where he is in charge of International Scientific Liaisons.

Gerry Melino received his M.D. from Rome [AU: which university?] and his Ph.D. from London [AU: which university?], before becoming a professor of molecular biology at the Faculty of Medicine at the University of Rome 'Tor Vergata'. He is also director of the Apoptosis Laboratory at the Medical Research Council, Toxicology Unit in Leicester, UK, and Editor-in-Chief of the journal 'Cell Death and Differentiation' (<http://www.nature.com/cdd>). He has been working on cell death — both apoptosis and cornification — for the past 15 years.

Online summary

- Keratinocytes exert their functions after cell death to guarantee the stability, mechanical resistance, elasticity, physical-barrier and water-impermeability functions of the skin.
- These properties are conferred by the cornified envelope where specialized substrates are crosslinked by transglutaminases.
- Four out of the nine transglutaminases are expressed in the epidermis, where they exert their function in a coordinated way.
- At least ten distinct proteins function as substrates for epidermal transglutaminases. These proteins end up comprising the cornified envelope of the terminally differentiated corneocytes that make up the cornified layer. The cornified layer has a relative content (% of protein in dry weight) of ~80% loricrin, 8% small-proline rich proteins (SPRs) and 6% filaggrin. [AU:OK?]
- Loricrin and SPRs lack a significant ordered structure, which confers a considerable mobility and flexibility to these molecules. This is crucial for allowing a spring-like elasticity to the epidermis, whereas the intramolecular and intermolecular transglutaminase-crosslinked residues guarantee stability and mechanical resistance.
- Mutations of transglutaminases and their substrates cause severe skin diseases, such as lamellar ichthyosis.

Melino Links**Entrez:***ABCA12*

http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene&cmd=Retrieve&dopt=Graphics&list_uids=26154

ALOXE3

http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene&cmd=Retrieve&dopt=Graphics&list_uids=59344

ALOX12B

http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene&cmd=Retrieve&dopt=Graphics&list_uids=242

Spink5

http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene&cmd=Retrieve&dopt=Graphics&list_uids=72432

TGM1

http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene&cmd=Retrieve&dopt=Graphics&list_uids=7051

OMIM

Chanarin–Dorfman syndrome

<http://www.ncbi.nlm.nih.gov/entrez/dispomim.cgi?id=275630>

Gaucher disease

http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=OMIM&dopt=DocSum&list_uids=231000,230900,230800,231005,608013

Lamellar ichthyosis

http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=OMIM&dopt=DocSum&list_uids=242300,146750,601277,604777,606545

Netherton syndrome

<http://www.ncbi.nlm.nih.gov/entrez/dispomim.cgi?id=256500>

nonbullous congenital ichthiosiform erythroderma

<http://www.ncbi.nlm.nih.gov/entrez/dispomim.cgi?id=242100>

progressive symmetric erythrokeratoderma

<http://www.ncbi.nlm.nih.gov/entrez/dispomim.cgi?id=602036>

Sjögren–Larsson syndrome

<http://www.ncbi.nlm.nih.gov/entrez/dispomim.cgi?id=270200>

Vohwinkel syndrome

<http://www.ncbi.nlm.nih.gov/entrez/dispomim.cgi?id=604117>

X-linked ichthyosis

<http://www.ncbi.nlm.nih.gov/entrez/dispomim.cgi?id=308100>

Swiss-Prot

μ -calpain (I)
<http://us.expasy.org/cgi-bin/niceprot.pl?P07384>

cathepsin D
<http://us.expasy.org/cgi-bin/niceprot.pl?P07339>

corneodesmosin
<http://us.expasy.org/cgi-bin/niceprot.pl?Q15517>

desmoglein-1
<http://us.expasy.org/cgi-bin/niceprot.pl?Q02413>

desmocollin-1
<http://us.expasy.org/cgi-bin/niceprot.pl?Q08554>

elafin
<http://us.expasy.org/cgi-bin/niceprot.pl?P19957>

envoplakin
<http://us.expasy.org/cgi-bin/niceprot.pl?Q92817>

furin
<http://us.expasy.org/cgi-bin/niceprot.pl?P09958>

K1
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K2e
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K5
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loricrin
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plakophilin-1
<http://us.expasy.org/cgi-bin/niceprot.pl?Q13835>

profilaggrin
<http://us.expasy.org/cgi-bin/niceprot.pl?P20930>

S100A7
<http://us.expasy.org/cgi-bin/niceprot.pl?P31151>

S100A10
<http://us.expasy.org/cgi-bin/niceprot.pl?P60903>

S100A11
<http://us.expasy.org/cgi-bin/niceprot.pl?P31949>

TGM1
<http://us.expasy.org/cgi-bin/niceprot.pl?P22735>

TGM2
<http://us.expasy.org/cgi-bin/niceprot.pl?P21980>

TGM3
<http://us.expasy.org/cgi-bin/niceprot.pl?Q08188>

TGM5
<http://us.expasy.org/cgi-bin/niceprot.pl?O43548>

Tricohyalin
<http://us.expasy.org/cgi-bin/niceprot.pl?Q07283>