miRNAs, ‘stemness’ and skin

Daniel Aberdam1, Eleonora Candi2, Richard A. Knight3 and Gerry Melino2,3

1 Institut national de la santé et de la recherche médicale U898, University of Nice-Sophia Antipolis, 28 Avenue Valombrose, 06107 Nice, France
2 Biochemistry Laboratory IDI-IRCCS and University of Rome ‘Tor Vergata’, Via Montpellier 1, 00133 Rome, Italy
3 Medical Research Council, Toxicology Unit, Leicester University, Lancaster Road, Leicester LE1 9HN, UK

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 disruption 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 transcriptional activator 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.

Corresponding author: Melino, G. (melino@uniroma2.it).
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, meroclines and paraclines. Holoclones have the greatest proliferative potential: 95% of their cells continue forming proliferative colonies on passaging. Meroclines have intermediate proliferative potential and paracline cells differentiate after only a few passages. In addition, holoclone cells can become meroclone and paracline 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 β1, denoted by β1high, in human skin [18] and integrin α6high and antibody-recognizing transferring receptor (CD71)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 (API), activating enhancer

![Figure 1. Skin epithelium and morphological abnormalities in Dicer1 conditional null skin. This figure depicts mouse skin epithelium at developmental stage E18.](image-url)
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

<table>
<thead>
<tr>
<th>miRNAsb</th>
<th>Expressionc</th>
<th>Expression in other tissues and/or in diseases</th>
<th>Targetsd</th>
<th>Refse</th>
</tr>
</thead>
<tbody>
<tr>
<td>miRNA-15a</td>
<td>hf</td>
<td>Leukaemia</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>miRNA-15b</td>
<td>hf, epi</td>
<td>Leukaemia, antigen-induced T-cell differentiation</td>
<td>BCL2</td>
<td>(47)</td>
</tr>
<tr>
<td>miRNA-16</td>
<td>hf</td>
<td>Leukaemia, antigen-induced T-cell differentiation</td>
<td>CCND1, CCND3, CDK6</td>
<td>(48)</td>
</tr>
<tr>
<td>miRNA-17-5p</td>
<td>hf, epi</td>
<td>T-cell and monocyte differentiation</td>
<td>AML1</td>
<td>(49)</td>
</tr>
<tr>
<td>miRNA-18</td>
<td>hf, epi</td>
<td>Unknown</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>miRNA-19b</td>
<td>epi</td>
<td>T cells, leukaemia</td>
<td>MYLIP, RBP1-like</td>
<td>(50)</td>
</tr>
<tr>
<td>miRNA-20</td>
<td>h, epi</td>
<td>Megakaryocytopoiesis</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>miRNA-21</td>
<td>hf, epi</td>
<td>Invasion, metastasis, squamous cell carcinoma, antigen-induced T-cell differentiation</td>
<td>TPM1, PDCD4, MASPIN</td>
<td>(51)</td>
</tr>
<tr>
<td>miRNA-24</td>
<td>hf, epi</td>
<td>Myogenic differentiation</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>miRNA-27a</td>
<td>epi</td>
<td>Breast cancer</td>
<td>MYT1, ZBTB10</td>
<td>(52)</td>
</tr>
<tr>
<td>miRNA-27b</td>
<td>hf, epi</td>
<td>Angiogenesis</td>
<td>CYP1B1</td>
<td>(53)</td>
</tr>
<tr>
<td>miRNA-30b</td>
<td>epi</td>
<td>Unknown</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>miRNA-34a</td>
<td>epi</td>
<td>Apoptosis in cancer cells</td>
<td>BCL-2</td>
<td>(54)</td>
</tr>
<tr>
<td>miRNA-35</td>
<td>hf, epi</td>
<td>Lymphoma, B cell development</td>
<td>PTEN, BIM</td>
<td>(55)</td>
</tr>
<tr>
<td>miRNA-93</td>
<td>epi</td>
<td>Gastric cancer</td>
<td>CDKN1A, E2F1, BIM</td>
<td>(56)</td>
</tr>
<tr>
<td>miRNA-99b</td>
<td>hf, epi</td>
<td>Megakaryocytopoiesis</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>miRNA-106b</td>
<td>hf, epi</td>
<td>Gastric cancer</td>
<td>CDKN1A, E2F1, BIM</td>
<td>(56)</td>
</tr>
<tr>
<td>miRNA-125a</td>
<td>hf</td>
<td>Breast cancer</td>
<td>ERBB2, ERBB3</td>
<td>(57)</td>
</tr>
<tr>
<td>miRNA-125b</td>
<td>hf, epi</td>
<td>Breast cancer, ovarian cancer</td>
<td>ERBB2, ERBB3</td>
<td>(57)</td>
</tr>
<tr>
<td>miRNA-126</td>
<td>hf</td>
<td>Endothelial cell, leukocyte adherence, breast cancer</td>
<td>VCA1M, SOX4, VCRK</td>
<td>(58,59)</td>
</tr>
<tr>
<td>miRNA-127</td>
<td>hf, epi</td>
<td>Tumour suppression</td>
<td>BCL6</td>
<td>(60)</td>
</tr>
<tr>
<td>miRNA-130a</td>
<td>epi, hf</td>
<td>Angiogenesis, Megakaryocytopoiesis</td>
<td>GAX, HOXA5</td>
<td>(61)</td>
</tr>
<tr>
<td>miRNA-133b</td>
<td>epi</td>
<td>Colorectal cancer</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>miRNA-141</td>
<td>epi</td>
<td>Placenta, EMT</td>
<td>ZEB1, ZEB2</td>
<td>(62)</td>
</tr>
<tr>
<td>miRNA-143</td>
<td>hf</td>
<td>Adipocytes differentiation, B-cell cancer</td>
<td>ERK5</td>
<td>(63,12)</td>
</tr>
<tr>
<td>miRNA-152</td>
<td>hf</td>
<td>Breast cancer</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>miRNA-191</td>
<td>epi</td>
<td>Leukaemia</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>miRNA-196</td>
<td>hf</td>
<td>Limb development, myeloid differentiation</td>
<td>HOX8B</td>
<td>(64)</td>
</tr>
<tr>
<td>miRNA-199a</td>
<td>hf</td>
<td>Leukaemia, ovarian cancer</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>miRNA-199b</td>
<td>hf</td>
<td>Leukaemia, ovarian cancer</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>miRNA-200a</td>
<td>epi</td>
<td>Ovarian cancer, EMT</td>
<td>ZEB1, ZEB2</td>
<td>(62)</td>
</tr>
<tr>
<td>miRNA-200b</td>
<td>epi, hf</td>
<td>Ovarian cancer, brain, EMT</td>
<td>Zfhx1b</td>
<td>(65)</td>
</tr>
<tr>
<td>miRNA-200c</td>
<td>epi</td>
<td>Ovarian cancer, leukaemia</td>
<td>ZEB1, ZEB2</td>
<td>(62,66)</td>
</tr>
<tr>
<td>miRNA-203</td>
<td>epi</td>
<td>Skin, hair, ovary, squamous cell carcinoma</td>
<td>TP73L, ZEB2, SOCS3</td>
<td>(31,32,39,42)</td>
</tr>
<tr>
<td>miRNA-205</td>
<td>epi</td>
<td>Ovarian cancer, squamous cell carcinoma</td>
<td>ABL1</td>
<td></td>
</tr>
<tr>
<td>miRNA-206</td>
<td>hf</td>
<td>Skeletal muscle</td>
<td>POLA, CX43, ESR1, FTL1</td>
<td>(67)</td>
</tr>
<tr>
<td>miRNA-214</td>
<td>hf</td>
<td>Ovarian cancer</td>
<td>PTEN</td>
<td>(68)</td>
</tr>
<tr>
<td>miRNA-351</td>
<td>hf</td>
<td>Unknown</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>miRNA-429</td>
<td>epi</td>
<td>EMT</td>
<td>ZEB1, ZEB2</td>
<td>(62,66)</td>
</tr>
<tr>
<td>Let-7a</td>
<td>hf, epi</td>
<td>Lymphoma</td>
<td>MYC</td>
<td>(69)</td>
</tr>
<tr>
<td>Let-7b</td>
<td>hf, epi</td>
<td>Acute lymphoblastic leukaemia</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>Let-7c</td>
<td>hf, epi</td>
<td>Unknown</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>Let-7d</td>
<td>hf</td>
<td>Leukaemia</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>Let-7f</td>
<td>hf, epi</td>
<td>Angiogenesis, antigen-induced T-cell differentiation</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>Let-7g</td>
<td>hf, epi</td>
<td>Unknown</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>Let-7i</td>
<td>hf, epi</td>
<td>Immune response</td>
<td>TLR4</td>
<td>(70)</td>
</tr>
</tbody>
</table>

*Abbreviations 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; BLM, 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 erythroblast leukaemia viral oncogene homolog 2; ERBB3, v-erb-b2 erythroblast leukaemia viral oncogene homolog 3; ERK5, extracellular-signal-regulated kinase 5; ESR1, estrogen receptor 1; EZH2, EZF transcription factor 1; FTL1, ferritin light chain 1; GAX, growth arrest homeobox transcription factor; HOXA5, homeobox A5; HOX8B, homeobox B8; MYC, v-myc myelocytomatosis viral oncogene homolog; MYT1, myelin transcription factor 1; PDCC4, 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; TTP1, tropomyosin 1; VCA1M, vascular cell adhesion molecule; VCRK, v-crk 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.

*miRNAs 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.

*miRNA detected in hair follicle (hf) and/or epidermis (epi). *Unknown: targets not yet identified.

*References are related to the identification of the target mRNAs.
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 miR-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. (c) The ABL1 3′ UTR contains a miRNA-203 recognition sequence in human, mouse, rat and dog.

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 miR-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. (c) The ABL1 3′ UTR contains a miRNA-203 recognition sequence in human, mouse, rat and dog.
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::DKK 1 (Dickkopf1) transgenic mice (DKKI is a potent Wingless [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

---

**miRNA-203 target. Exons and introns are not drawn to scale. (c) The Zfp281 3’UTR contains four putative miRNA-203 target sites (in bold). (d) The miRNA-203 recognition site in the 3’UTR of ABL1 mRNA is also highly conserved in four different species.**
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 ΔNp63, 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 ΔNp63 expression in the epidermis provides evidence for a functional relationship. The 3’UTR of ΔNp63 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 ΔNp63 3’UTR [31] and deletion of the ΔNp63 3’UTR heptamer abolishes miRNA-203-mediated repression under the same conditions. Similarly, ΔNp63 protein expression is markedly diminished in proliferating mouse keratinocytes upon miRNA-203 overexpression [31]. This miRNA-203 recognition sequence is conserved in the ΔNp63 3’UTR of several species [31,32] (Figure 2a), indicating that this miRNA-203-dependent mechanism for regulating ΔNp63 is conserved among species.

The association between miRNA-203 and ΔNp63 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, ΔNp63 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, ΔNp63 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-203) produced mostly paracorns 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 ΔNp63 enables ectodermal progenitor K8 and K18+ cells to become K5 and K14+ stratified epithelial cells, whereas ΔNp63 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], ΔNp63 could be essential for embryonic epidermal fate. Interestingly, ΔNp63 mRNA upregulation during ES-driven embryonic epidermal fate 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 epithelial 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...
Subsequently, TAp73 isoforms can induce cell death in cell carcinoma cells, thus, repressing expression increases after UV-C irradiation in squamous p53 to induce cell-cycle arrest and DNA-damage-induced of the ability of TAp63, the related protein TAp73, and of TAp63 and can function as a dominant-negative inhibitor.

The relationship between miRNA-203 and ΔNp63 might be relevant, not only to normal and benign pathological keratinocyte biology, but also to cancer biology. ΔNp63 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. miRNA-203 expression increases after UV-C irradiation in squamous cell carcinoma cells, thus, repressing ΔNp63. Consequently, TAp73 isoforms can induce cell death in response to DNA damage. Recent evidence indicates that miRNA-203 also has an important role as a tumour suppressor in other tumour types, including chronic myelogenous leukaemias (CML) and lymphoblastic leukaemias. 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 Philadelphian chromosome t(9;22), present in CML.

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

### Table 2. miRNA expression in psoriasis and atopic eczema

<table>
<thead>
<tr>
<th>Psoriasis</th>
<th>Downregulated miRNAs</th>
<th>Upregulated miRNAs</th>
<th>Downregulated miRNAs</th>
<th>Upregulated miRNAs</th>
</tr>
</thead>
<tbody>
<tr>
<td>miRNA-17-5p</td>
<td>miRNA-10a</td>
<td>miRNA-17-5p</td>
<td>miRNA-122a</td>
<td></td>
</tr>
<tr>
<td>miRNA-20a</td>
<td>miRNA-22</td>
<td>miRNA-20a</td>
<td>miRNA-133a</td>
<td></td>
</tr>
<tr>
<td>miRNA-21</td>
<td>miRNA-30c</td>
<td>miRNA-21</td>
<td>miRNA-133b</td>
<td></td>
</tr>
<tr>
<td>miRNA-30e-5p</td>
<td>miRNA-99b</td>
<td>miRNA-24</td>
<td>miRNA-215</td>
<td></td>
</tr>
<tr>
<td>miRNA-31</td>
<td>miRNA-100</td>
<td>miRNA-27</td>
<td>miRNA-326</td>
<td></td>
</tr>
<tr>
<td>miRNA-106a</td>
<td>miRNA-122a</td>
<td>miRNA-29a</td>
<td>miRNA-335</td>
<td></td>
</tr>
<tr>
<td>miRNA-142-3p</td>
<td>miRNA-125b</td>
<td>miRNA-106b</td>
<td>miRNA-483</td>
<td></td>
</tr>
<tr>
<td>miRNA-146b</td>
<td>miRNA-133a</td>
<td>miRNA-146a</td>
<td>miRNA-515–5p</td>
<td></td>
</tr>
<tr>
<td>miRNA-146a</td>
<td>miRNA-133b</td>
<td>miRNA-193a</td>
<td>miRNA-519</td>
<td></td>
</tr>
<tr>
<td>miRNA-200a</td>
<td>miRNA-197</td>
<td>miRNA-199a</td>
<td>miRNA-146</td>
<td></td>
</tr>
<tr>
<td>miRNA-141</td>
<td>miRNA-215</td>
<td>miRNA-142</td>
<td>miRNA-133a</td>
<td></td>
</tr>
<tr>
<td>miRNA-203</td>
<td>miRNA-326</td>
<td>miRNA-146b</td>
<td>miRNA-133a</td>
<td></td>
</tr>
<tr>
<td>miRNA-365</td>
<td>miRNA-125a</td>
<td>miRNA-215</td>
<td>miRNA-133a</td>
<td></td>
</tr>
<tr>
<td>miRNA-381</td>
<td>miRNA-222</td>
<td>miRNA-215</td>
<td>miRNA-133a</td>
<td></td>
</tr>
<tr>
<td>miRNA-518b</td>
<td>miRNA-125a</td>
<td>miRNA-215</td>
<td>miRNA-133a</td>
<td></td>
</tr>
<tr>
<td>miRNA-524</td>
<td>let-7e</td>
<td>miRNA-326</td>
<td>miRNA-133a</td>
<td></td>
</tr>
</tbody>
</table>

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.
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 ΔNp63 expression levels correlate with the response to cisplatin-based therapies [46]. ΔNp63 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.

Acknowledgements
We thank Anna Maria Lena, Pia Rivetti di Val Cervo and Alessandro Terrinoni for scientific discussion. Work in our laboratory was supported by the Medical Research Council (to G.M.) and by grants from Telethon (GGP02251 to E.C.), AIRC (2743 to G.M.), EU (LSGBH-2005–019067-Epistem; LSHC-CT-2004–503576-Active p53) to G.M., MIUR to G.M., PRIN 06 to E.C., PRIN 06 to G.M., MinSan to G.M., ISS ‘Programma Italia–USA’ NS26DS to G.M., Philip Morris USA Inc. to G.M., ACC12 to G.M. and E.C. and by ANR (ANR-06-BLAN-0387) and EU (LSGBH-2005–019067-Epistem) to D.A.

References
25 Rangarajan, A. et al. (2001) Notch signaling is a direct determinant of keratinocyte growth arrest and entry into differentiation. EMBO J. 20, 3427–3436
53 Sano, S. et al. (2008) miR-16 family induces cell cycle arrest by transcriptional repression of ZFHX1B. RNA 14, 1172–1178
55 Scott, G.K. et al. (2007) Coordinate suppression of ERBB2 and ERBB3 by enforced expression of microRNA miR-125a or miR-125b. J. Biol. Chem. 282, 1479–1486
60 Chen, Y. and Gorski, D.H. (2008) Regulation of angiogenesis through a microRNA (miR-130a) that down-regulates angiogenic homeobox genes GAX and HOXAS. Blood 111, 1217–1226
66 Hurteau, G.J. et al. (2007) Overexpression of the microRNA hsa-miR-200c leads to reduced expression of transcription factor 8 and increased expression of E-cadherin. Cancer Res. 67, 7972–7976
68 Andreason, K. et al. (2005) The microRNA miR-196 acts upstream of the microRNAs hsa-miR-195, hsa-miR-196a and hsa-miR-196b. This study uses bioinformatic approaches to identify potential targets for the microRNA miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1. Nat. Cell Biol. 10, 593–601
Spotlight on p63

TAp63 and ΔNp63 in Cancer and Epidermal Development

Eleonora Candi1,*
David Dinsdale2
Alessandro Rufini1
Paolo Salomoni2
Richard A. Knight2
Martina Mueller2
Peter H. Krammer2
Gerry Melino2

1University of Rome Tor Vergata; Rome, Italy
2Medical Research Council, Toxicology Unit; Leicester University, Leicester, UK

*Correspondence to: Eleonora Candi; University of Rome Tor Vergata; Via Montpellier 1; Rome 00133 Italy. Tel.: +39.06.7259.6487; Fax: +39.06.2042.7290; Email: candi@uniroma2.it

Original manuscript submitted: 12/01/06
Manuscript accepted: 01/03/07

Previously published online as a Cell Cycle E-publication:
http://www.landesbioscience.com/journals/cc/abstract.php?id=3797

KEY WORDS
p63, IKKα, epidermis, cornification, skin, development

ACKNOWLEDGEMENTS

The work discussed in this manuscript has been supported by the Medical Research Council (G.M., D.D., P.S.) and by grants from Telethon (GGP02251 to E.C. & GGP04110 to G.M.), AIRC (2743), EU (QLK-CT-2002-01956-Impaled; LSGH-2005-019067-Epistem; Blandino-2004-LSHC-CT-2004-503576-Active p53), Genomica Funzionale COMETA, FIRB-2001- RBNE01KHT-004 (Marino) & RBNE01WNCH-008 (Rotilio), MIUR, MinSan to G.M. Tumorzentrum Heidelberg/Mannheim to P.H.K. and M.M., and the Forschungsschwerpunktprogramm Baden-Württemberg to M.M.

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 ΔNp63, 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, ΔNp63 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 ΔNp63 isoforms in epithelial development remains mostly unclear. Nevertheless, recent work utilizing in vivo genetic complementation of TAp63 and/or ΔNp63 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 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;ΔN, ΔNp63 transgenic mice complemented into p63−/−; IKKα, IκB kinase-α; TA, transactivation domain; ΔN, 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 (ΔNp63) isoform, which lacks the transactivation domain.1,2 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.3 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
The identification of the p53-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 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.

Formation of the epidermis, 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. Remarkably, genetic mutations of p63 in man are causatively linked to ectodermal dysplastic syndromes. 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.

**p63 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. 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, 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 be over expressed or its locus is amplified, consistent with a potential p63 pro proliferative or oncogenic role. A potential role for p63 in tumorigenesis is supported by the finding that p63 is a target of genomic amplification and/or over expression in > 80% of primary head and neck squamous cell carcinomas (HNSCC) as well as other squamous epithelial malignancies (Table 1). A genome-wide micro array screen of non-small cell lung cancer revealed that the 3q26-29 locus encompassing p63 is frequently amplified in squamous cell carcinomas of the lung, suggesting that over expression of p63 may promote or facilitate tumorigenesis. Nevertheless, these studies did not address which are the p63 isoforms expressed in cancer.

A recent study conducted on a cohort of 245 esophageal tumors demonstrated that both TAp63 and ΔNp63 isoforms are specifically upregulated at the transcript level in squamous cell carcinoma, and ΔNp63 was the predominant isoform expressed at the protein level. Some tumor types have been reported to lose p63 expression, suggesting that p63 loss accelerates tumorigenesis. This is supported by in vitro data which reveal that disruption of p63 in squamous cell lines resulted in up-regulation of genes associated with increased capacity for invasion and metastasis in tumors. 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 ΔNp63 proteins are not expressed. 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. Nevertheless, this appears to be linked to the genetic background, as p63 mice on a different inbred strain show premature aging but no cancer. Furthermore, p63 heterozygosity does not accelerate tumorigenesis when p53 is compromised. 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
We have recently described the downstream signaling via death receptors and mitochondria (Fig. 2 and Fig. 3) and thus sensitizes cancer cells towards chemotherapy. We demonstrated that TAp63, like p53, is involved in more than one apoptotic pathway, 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 APAF1.\(^2\) (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 ΔNp63 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.\(^16,33,36\) HSP70, an anti-apoptotic stress response protein associated with malignancy, is induced by ΔNp63α consistent with the proposed oncogenic role of ΔNp63. By contrast, the pro-apoptotic gene IGFBP-3 is transcriptionally repressed by ΔNp63α.\(^37\)

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 pro-apoptotic 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

---

**Table 1** Expression of p63 in cancer

<table>
<thead>
<tr>
<th>Tissue/organ site</th>
<th>Variant mRNA expressed</th>
<th>Comments</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast cancer</td>
<td>ΔNp63</td>
<td>Protein expression</td>
<td>24; 94; 95; 96; 97</td>
</tr>
<tr>
<td>Bladder cancer</td>
<td>TAp63 and ΔNp63</td>
<td>Protein expression</td>
<td>24; 25; 98</td>
</tr>
<tr>
<td>Urothelial cancer</td>
<td>TAp63 and ΔNp63</td>
<td>Protein expression</td>
<td>99</td>
</tr>
<tr>
<td>Cervical cancer</td>
<td>Not determined</td>
<td>Protein expression</td>
<td>100; 101; 102; 103; 104</td>
</tr>
<tr>
<td>Uterine cancer</td>
<td>Not determined</td>
<td>Protein expression</td>
<td>105</td>
</tr>
<tr>
<td>Head and neck cancer</td>
<td>TAp63 and ΔNp63</td>
<td>Protein expression</td>
<td>20; 106; 107; 108</td>
</tr>
<tr>
<td>Lung cancer</td>
<td>TAp63 and ΔNp63</td>
<td>Protein expression</td>
<td>20; 21;109</td>
</tr>
<tr>
<td>Prostate cancer</td>
<td>ΔNp63</td>
<td>Protein expression</td>
<td>110</td>
</tr>
<tr>
<td>Esophageal cancer</td>
<td>ΔNp63</td>
<td>Squamous cell carcinoma, protein expression</td>
<td>111</td>
</tr>
<tr>
<td>Gastric carcinoma</td>
<td>TAp63 and ΔNp63</td>
<td>Protein expression, mRNA expression</td>
<td>112</td>
</tr>
<tr>
<td>Pancreatic cancer</td>
<td>Not determined</td>
<td>Protein expression</td>
<td>113</td>
</tr>
</tbody>
</table>

---

**Figure 2.** Mechanism of apoptosis elicited by p63. Induction of TAp63 in a Doxicycline-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 chemoresistance, 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.\(^32\)
We have recently shown a relevant DD-development, accounting for - 32 During mouse embryogenesis, the pathways, and both mechanisms are clearly reinforced by concomitant T Ap63 role for the TAp63 to chemotherapy. 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

---

Table 2  **Selected p63 target genes involved in tumorigenicity**

<table>
<thead>
<tr>
<th>Gene</th>
<th>TAp63 (isom)</th>
<th>ΔNp63</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CASP1</td>
<td>α ↑</td>
<td>-</td>
<td>32</td>
</tr>
<tr>
<td>CASP3</td>
<td>α ↑</td>
<td>-</td>
<td>32</td>
</tr>
<tr>
<td>CASP4</td>
<td>α ↑</td>
<td>-</td>
<td>32</td>
</tr>
<tr>
<td>CASP5</td>
<td>α ↑</td>
<td>-</td>
<td>32</td>
</tr>
<tr>
<td>CASP8</td>
<td>α ↑</td>
<td>-</td>
<td>32</td>
</tr>
<tr>
<td>CASP9</td>
<td>α ↑</td>
<td>-</td>
<td>32</td>
</tr>
<tr>
<td>TNF</td>
<td>α ↑</td>
<td>-</td>
<td>32</td>
</tr>
<tr>
<td>TNFRSF1A/TFNFR1</td>
<td>α ↑</td>
<td>-</td>
<td>32</td>
</tr>
<tr>
<td>TNFRSF6/CD95</td>
<td>α ↑</td>
<td>-</td>
<td>32</td>
</tr>
<tr>
<td>TNFRSF10A/TRAIL-R1</td>
<td>α ↑</td>
<td>-</td>
<td>32</td>
</tr>
<tr>
<td>TNFRSF1B/TRAIR2</td>
<td>α ↑</td>
<td>-</td>
<td>32</td>
</tr>
<tr>
<td>TRIP</td>
<td>α ↑</td>
<td>-</td>
<td>32</td>
</tr>
<tr>
<td>APAF1</td>
<td>α ↑</td>
<td>-</td>
<td>32</td>
</tr>
<tr>
<td>BCL2L11</td>
<td>α ↑</td>
<td>-</td>
<td>32</td>
</tr>
<tr>
<td>BAX</td>
<td>α, γ ↑</td>
<td>-</td>
<td>32, 114</td>
</tr>
<tr>
<td>DAP3</td>
<td>α ↑</td>
<td>-</td>
<td>32</td>
</tr>
<tr>
<td>RAD9</td>
<td>α ↑</td>
<td>-</td>
<td>32</td>
</tr>
<tr>
<td>p21</td>
<td>γ ↑</td>
<td>-</td>
<td>114</td>
</tr>
<tr>
<td>IGFBP3</td>
<td>-</td>
<td>α ↑</td>
<td>37</td>
</tr>
<tr>
<td>HSP70</td>
<td>-</td>
<td>α ↓</td>
<td>18</td>
</tr>
</tbody>
</table>

(↑) upregulation, (↓) downregulation, (¬) no effect

---

![Death Receptor Ligands and Death Receptors](image-url)

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.
were also confirmed by in situ hybridization, confirming that ΔNp63 is the most abundant transcript from embryonic stage E10 to day 3 post-birth; with this method the TAp63 isofrom 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 ΔNp63), the C-terminal region of the p63α proteins and the DNA-binding domain.1,27 Again, ΔNp63 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 ΔNp63 (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 ΔNp63 had no effect. In this context TAp63, but not ΔNp63, 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 pluristratification and that the main function of ΔNp63 is to counteract the TAp63 isoform allowing cell differentiation.53 These data and their interpretation is in contrast with evidence that ΔNp63 is the only isoform expressed in epidermal development until E13,55,57 and that ΔNp63 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, ΔNp63 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 reversion of the phenotype of the null mice, probably due to technical limitations of the system, a major role for ΔNp63 in epidermal development emerged, with a possible cooperative activity between...
the two isoforms. The p63\(^{-/-}\);TA (mice knock-out for p63, expressing TAp63\(\alpha\)) complemented mice show no signs of epithelialisation, like p63\(^{-/-}\) mice. In contrast, the p63\(^{-/-}\);\(\Delta N\) 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;\(\Delta N\) 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 suprabasal markers (K14, K1, filaggrin). These data\(^{67}\) suggest that \(\Delta N\)p63 is important for maintaining the proliferative potential of the basal layer, whereas TAp63 contributes by acting synergistically and/or subsequently to \(\Delta N\)p63 to allow differentiation. Furthermore, these results are consistent with a recent study based on siRNAs direct against p63 isoforms, in which the authors demonstrated that \(\Delta N\)p63 isoforms are the main mediators of p63 effects and that TAp63 isoforms contribute to late stage of differentiation in mature keratinocytes.\(^{58}\)

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.

Re-introduction of TAp63\(\alpha\) 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 \(\Delta N\)p63\(\alpha\) 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\(\alpha\) and \(\Delta N\)p63\(\alpha\) into the p63\(^{-/-}\) background did not result in a completely normal epithelium but there was a greater degree of reepithelialisation than in the selective \(\Delta N\)p63\(\alpha\) 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
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 β4 integrin to hemidesmosomes, where it forms a link with the cytoskeleton necessary for cell adhesion and orientation of developing tissue. 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 ΔNp63 isoforms work in competition, as TAp73 and ΔNp73 do, represented the first “dogma” regarding the function of p63 isoforms. This concept arose from the observation that since ΔNp63 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. This view has now significantly changed as more recent papers showed the presence of an additional transactivation domain at the ΔNp63 C-terminal end (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 ΔNp63 transcriptional activity is further enhanced by its interaction with other transcription factors, as shown for HSF70 transcriptional regulation. 18 In this case, ΔNp63 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, ΔNp63α is able to repress transcription of cell cycle related genes such as cyclin B2, Cdc2 and topoisomerase IIα, 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 ΔNp63 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).

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 ΔNp63 80 (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. 83,84 Thus, a complex crosstalk between Notch and p63 could regulate the balance between keratinocyte self-renewal and differentiation. 84

**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 predominate in genetically complemented mice (Fig. 6E) Keratin filaments are present 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. (C) Patch of cornified epithelial cell envelopes in a p63+/− transgenic mouse following reintroduction of TAp63α 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 ΔNp63α 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 TAp63α and ΔNp63α (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 (arrowheads). All bars, 2μm.
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 (IKB kinase-α) and GATA-3 (Fig. 7B). IKKα is required for epithelial formation and has both kinase-dependent and -independent functions, the latter being crucial for epithelial keratinocyte differentiation and for skeletal and cranio-facial morphogenesis as clearly demonstrated in the IKKα−/− mice. Expression of IKKα, under control of the basal layer promoter K14 in IKKα−/− mice, produced efficient rescue of the major morphological abnormalities. 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. (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, although its specific function in epithelial 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). These include keratin 14 (Fig. 7C) perp, envelopakin, and BPAG-1. Fig. 8 shows a simplified scheme of the actions of p63 in epithelial 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 AN 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 epithelial 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 (dropped by TAp63) and GATA-3 (dropped 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 epithelial development. A deeper analysis of the targets of p63 is discussed in related papers in this issue.

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Selected p63 target genes with a defined impact in epithelial development</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAp63</td>
<td>ΔNp63</td>
</tr>
<tr>
<td>(isoforms)</td>
<td>(isoforms)</td>
</tr>
<tr>
<td>IKKα</td>
<td>all</td>
</tr>
<tr>
<td>PERP</td>
<td>α and γ</td>
</tr>
<tr>
<td>EGFR</td>
<td>γ</td>
</tr>
<tr>
<td>FGFR2</td>
<td>α</td>
</tr>
<tr>
<td>AP2γ</td>
<td>α</td>
</tr>
<tr>
<td>K14</td>
<td>α</td>
</tr>
<tr>
<td>Envopakin</td>
<td>γ</td>
</tr>
<tr>
<td>p21</td>
<td>all</td>
</tr>
<tr>
<td>Jagged-1 &amp; 2</td>
<td>γ</td>
</tr>
<tr>
<td>Hes-1</td>
<td>α</td>
</tr>
<tr>
<td>Hey-1,2</td>
<td>α</td>
</tr>
<tr>
<td>GATA-3</td>
<td>α</td>
</tr>
<tr>
<td>BPAG-1</td>
<td>β and γ</td>
</tr>
<tr>
<td>Ets-1</td>
<td>α</td>
</tr>
</tbody>
</table>

(+) = Upregulation by p63, (−) = downregulation by p63, (−) = no effect
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.

References


The expression of TA and p63 are regulated by different mechanisms in liver cells. Oncogene 2005; 24:512-19.


Westfall MD, Myers D, Snieczek JC, Petepol JA. The p63α phosphoprotein binds the p21 and 14-3-3 proteins in vivo and has transcriptional repression activity that is reduced by Hay-Wells syndrome-derived mutations. Mol Cell Biol 2003; 23:2264-76.


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. 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. At the cellular level, cornification begins with the synthesis of an immature type of envelope underneath the plasma membrane of keratinocytes in the cornified layer 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.

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

Epidermal differentiation begins with the migration of keratinocytes from the basal layer, and ends with the formation of the cornified layer. Cell proliferation, differentiation and death occur sequentially, and each process is characterized by the expression of specific proteins. In normal epidermis, the proliferation rate in the basal layer is balanced by desquamation of the cornified layer 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, which extend from the desmosomes towards the nuclear lamina and, along with microtubules and microfilaments, 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.

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

Eleonora Candi*, Rainer Schmidt‡ and Gerry Melino*§

Abstract | The epidermis functions as a barrier against the environment by means of several layers of terminally differentiated, dead keratinocytes — the cornified layer, which forms the endpoint of epidermal differentiation and death. The cornified envelope replaces the plasma membrane of keratinocytes in the cornified layer 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.
KERATIN-FILAGGRIN
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 reside in the uppermost layer of the skin and constitute a barrier for the skin.

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 reside in the uppermost layer of the skin and constitute a 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, β, γ-interneurin); V (nuclear lamins); and VI (nuclein).

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.

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 profilagrin — 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. 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 is composed of terminally differentiated, dead, cornified, flattened cells that are known as corneocytes. These corneocytes are 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 to allow desquamation.

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 epithelia. The relative amino-acid composition of the reinforcement proteins that are used (such as loricin 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). 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 loricin-/- (REF. 14) and involucrin-/- (REF. 15) knockout mice — the TGM1-/- mouse confirms that this enzyme is essential for correct cornified-envelope assembly. 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. This results in the coordination and stabilization of the KIF-filaggrin network in the cornified cell and the periphery of the cornified envelope. Mutation of this Lys residue results in gross shape malformations, which are largely manifest as...
thickening of the palms and soles. Similarly, abnormal differentiation of skin in benign and malignant tumours correlates well with the aberrant expression of the differentiation-specific K1 and K10 keratins.

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 layers and are substrates of TGM1 and TGM2 (REF. 20). When the intracellular Ca\(^{2+}\) concentration increases, cytoplasmic S100A7 redistributes to \(\alpha\)-actinin- and paxillin-containing peripheral complexes in focal-adhesion-like structures. THH\(^{25}\), 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 tongue. 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 profilagrin in addition to LEPs. The main substrates that are crosslinked by TGMs, are discussed below.

**Involucrin.** Involucrin\(^{26}\) 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-

![Diagram of Progression Steps](image-url)
REVIEWS

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

<table>
<thead>
<tr>
<th>Properties</th>
<th>Effectors</th>
<th>Diseases</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Structural stability</td>
<td>Crosslinking enzymes: TG1</td>
<td>Lamellar ichthyosis</td>
<td>98, 99</td>
</tr>
<tr>
<td>Elasticity</td>
<td>TG substrates: loricrin, involucrin and SPRs</td>
<td>Vohwinkel syndrome</td>
<td>104, 105</td>
</tr>
<tr>
<td>Lipids</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mechanical resistance</td>
<td>Cytoskeleton: keratins</td>
<td>WSN (K4, K13), EPPK (K9)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>PC (K6, K16, K17), EHK (K1, K10), IBS (K29), NCIE (K1)</td>
<td></td>
</tr>
<tr>
<td>Water repellence</td>
<td>Lipids: FALDH</td>
<td>Sjögren–Larsson syndrome</td>
<td>114</td>
</tr>
<tr>
<td></td>
<td>Lipoxigenases -3, -12R</td>
<td>NCIE-1/LJ-5</td>
<td>113</td>
</tr>
<tr>
<td></td>
<td>Cholesterol sulphatase</td>
<td>X-linked ichthyosis</td>
<td>111</td>
</tr>
<tr>
<td></td>
<td>β-glucocerebrosidase</td>
<td>Gaucher disease type 2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Phytanoyl-CoA hydroxylase</td>
<td>Refsum’s disease</td>
<td>137</td>
</tr>
<tr>
<td></td>
<td>Peroxin-7 receptor</td>
<td>Refsum’s disease</td>
<td>138</td>
</tr>
<tr>
<td></td>
<td>3β-hydroxysteroid-Δ8,Δ7-isomerase</td>
<td>Conradi–Hunermann syndrome</td>
<td>139</td>
</tr>
<tr>
<td></td>
<td>3β-hydroxysteroid-Δ8,Δ7-isomerase</td>
<td>CHILD syndrome</td>
<td>140</td>
</tr>
<tr>
<td></td>
<td>3β-hydroxysteroid dehydrogenase</td>
<td>CHILD syndrome</td>
<td>141</td>
</tr>
<tr>
<td></td>
<td>sphinogomylinase</td>
<td>Niemann–Pick disease</td>
<td>86</td>
</tr>
<tr>
<td></td>
<td>sterol-Δ7-reductase</td>
<td>Smith–Lemli–Opitz syndrome</td>
<td>142</td>
</tr>
</tbody>
</table>

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

[AU: Tities changed to accommodate lipids 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 modelling 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 loop. The Gly residues are interspersed by occasional long-alphatic or aromatic residues, which might associate by hydrophobic interactions, thereby displacing the Gly sequences into an Ω-loop-like configuration. This structural motif might 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 cornified layer. Limited proteolysis and sequencing experiments on the cornified envelope from foreskin cells shows that most of the crosslinks are loricrin–loricrin through isopeptide bonds. But smaller amounts of loricrin are also crosslinked to SPR1 and SPR2. Limited structural analyses of this motif will prove difficult because of the extreme flexibility and essentially anisotropic nature of the Gly sequences.

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

ing peptide units, which have undergone extensive rearrangements during recent evolution. Involucrin is highly α-helical, and TGM1 preferentially crosslinks residues Gln495 and Gln496 (REF. 29). Involucrin might be the preferred protein mass of the cornified layer, and is unusually enriched in Gly, Ser and Cys residues (TABLE 2). Computer modelling 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 loop. The Gly residues are interspersed by occasional long-alphatic or aromatic residues, which might associate by hydrophobic interactions, thereby displacing the Gly sequences into an Ω-loop-like configuration. This structural motif might 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. Human loricrin is expressed in the granular layer during cornification, and is unusually enriched in Gly, Ser and Cys residues (TABLE 2). Computer modelling 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 loop. The Gly residues are interspersed by occasional long-alphatic or aromatic residues, which might associate by hydrophobic interactions, thereby displacing the Gly sequences into an Ω-loop-like configuration. This structural motif might 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 functions as a key structural cornified-envelope protein. 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, cornesodesmosin) 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.

endnote
1. For a more complete survey, see: http://www.interfil.org. CHILD, congenital hemidysplasia with ichthyosiform erythroderma; EHK, epidermolytic hyperkeratosis; EPPK, epidermolytic palmoplantar keratodema; FALDH, fatty aldehyde dehydrogenase; IBS, ichthyosis bullosa of Siemens; K, keratin; LI-5, lamellar ichthyosis type 5; NCIE, non-bullous congenital ichthyosiform erythroderma; PC, pachyonychia congenital; SPR, small proline-rich protein; TG, transglutaminase; WSN, white sponge nevus.
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 profilaggrin [46]. Newborn rodent epidermis contains morphologically distinct profilaggrin and loricrin granules (F and L granules, respectively) [35]. 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 Ca²⁺-dependent enzymes that catalyse the formation of N-(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 molecules [41]. 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 activity [38]. 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) [42]. 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 [38]. 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-hydroxy-ceramides with long chain (>C30) fatty acids onto Gln residues of the scaffold proteins [43], 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 is debated [39,44,45]. TGM3, which is expressed in hair follicles and in terminally differentiating keratinocytes, is involved in cornification [38,39,42]. 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 keratinoctye differentiation in *vitro* [38,42].

[AU: are R314 and L268 labelled correctly in the fig? Should the unlabelled amino acids be labelled?]
REVIEWS

6 | APRIL 2005 | VOLUME 6

www.nature.com/reviews/molcellbio

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. 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–d). 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 (1,12,14). 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 to 10 in 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 motifs. 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 cells. 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. Profilaggrin is initially highly phosphorylated and is accumulated in keratohyalin granules. During cornification it is dephosphorylated and proteolytically cleaved to release individual filaggrin molecules.

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 arrays. 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...

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

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

aComposition is expressed as % in w/w. iThe 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.
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 osmolality 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 domains. The first 80 amino acids contain two perfectly formed and functional EF-hand, Ca$^{2+}$-binding domains that are typical of many small Ca$^{2+}$-binding proteins and proteases, 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 repeats. 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 $\beta$-turn configuration. 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 REFS 31).

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. 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 Ca$^{2+}$-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 $\gamma$- and Ser-rich protein, and these sequences are believed to form $\Omega$-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. Corneodesmosomal 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 striatum corneum chymotryptic enzyme (SCCE) and the striatum corneum tryptic enzyme (SCTE), are thought to be involved in corneodesmosome proteolysis (see below and REFS 33,54). Presumably, proteolysis during cornified-layer maturation results in the suppression of the glyceine-loop-related domains, thereby eliminating the adhesive parts of the corneodesmosin and allowing desquamation.

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-
Ichthyosis
A dermatological disorder in which the keratinocyte cornified envelope is abnormal, which results in a defective external layer (cornified layer [AU:OK1]). 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.

3 in pemphigus vulgaris (reviewed in REF. 57). Mutations in plakophilin-1 are responsible for epidermal dysplasia, whereas mutations in desmoplakin are found in patients that are affected by palmoplantar keratodermast, Ca²⁺ 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 Ca²⁺ pumpst.

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 TGMsut, 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 (BOX 1). Similarly, filaggrin is the substrate of many proteases such as PEP1, calpain [AU:calpain], furin and the transmembrane serine protease matriptase MT-SP1. Mice that lack MT-SP1 show aberrant profilaggrin processing, defects in cornified layer [AU:OK7] desquamation and epithelial lipid-matrix defectsw. The exact order of the different proteolytic events is not completely resolved, but it is observed 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
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 keratinocytes67,68. Cathepsin D, E and L2 are found in the cornified layer, whereas cathepsin L is required for normal epidermal homeostasis and hair-follicle morphology69,70. 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 Ca2+ gradient with terminal differentiation, both in the intra- and extracellular compartments, regulates Ca2+-dependent proteases. µ-Calpain (I) and m-calpain (II), which require micro- or millimolar concentrations of Ca2+ for their activation, are present in the suprabasal layers71. The addition of synthetic calpain inhibitors results in the blockage of profilaggrin and TGM1 processing72,73. 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 differentiation74. Other Ca2+-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 differentiation75.

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 corneodesmosin76. 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 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 terminus77,78. SCTE is also confined to the differentiating suprabasal layers of the epidermis. Both SCCE and SCTE can degrade corneodesmosin in vitro79.

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 cornification80,81. The lamellar bodies are composed of lipid lamellae that contain glucosylerceramides, phospholipids and cholesterol, which are the precursors of the intercellular skin lipids82,83. 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 space84,85,86. 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 lipid87,88. Even though several animal models show a defined role for lipids in the epidermal barrier89,90, 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 space89,90. 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’ model91, the more recent ‘sandwich’ model and the ‘single gel-phase’ model92.

An important, although still unresolved, area is the mechanism of interaction of the extracellular lipids and the cornified envelope. Long-chain ω-hydroxyoceramides 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 envelope93,94. Indeed, ω-hydroxyoceramides are linked to involucrin through an ester bond by TGM1, at least in vitro89,90,95. Possibly, other cornified-envelope proteins could also be covalently linked to ω-hydroxyoceramides, as the involucrin-knockout mouse shows normal cornified envelopes and a normal cornified layer96.

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 o-hydroxyeceramide coating of the cornified envelope contributes significantly to the establishment of the 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\(^9\). LI is an autosomal-recessive disorder, which causes severe abnormalities of the cornified layer [AU: insert 'epithelial'?] and 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 lipoygenase genes (ALOX12 and ALOX12B) on 17p13 (REF. 101), and on the ATP-binding-cassette transporter gene (ABCA12) on 2q33-35 (REF. 102). 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 multians) and progressive symmetric erythrokeratodermia (PSEK)\(^10\). 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 gene\(^10\), 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 hyperkeratosis\(^10\).

However, overexpression of human loricrin in transgenic mice yields a normal phenotype\(^7\) 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 days\(^10\). This indicates that keratinocytes might compensate for the loss of one cornified-envelope component by using other available proteins to form a functional cornified envelope\(^4\). 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 diseases\(^10\).

**Keratin diseases** have been discussed in separate reviews\(^9,108–110\) (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 residue\(^10\) that is critically involved in TGM1 crosslinking of KIFs to the cornified envelope\(^10\).

**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-envelope 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 sulphatase\(^11\). 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 envelope\(^11\). Among the ichthyoses, as discussed above, a subset of LI patients (LI-5 or nonbullous congenital ichthyosiform erythroderma (NCIE) type-1) carry muta-
tions in lipoygenase-3 or -12R (1994) 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 unknown. In addition, patients that are affected by LI type-2 carry mutations in the ABCA12 gene (1998). 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-alkalde dehydrogenase (1994), 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 (1994). 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 characterized 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 (AUOK) abundant abnormal lamellar granules disturb the intercellular lamellar structure, creating lipid vacuoles in the epidermis (1998). 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 LEKTI (1998). 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 Papp–Lerdon–Levine syndrome (1998). 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 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.


Acknowledgments
We wish to dedicate this manuscript to Peter M. Steinert, whose sudden and premature loss deprived us all of a great master and teacher. The first draft of this manuscript was written by Peter and as pupils and friends of his, we would like to express our deepest appreciation for his teaching. We would also like to thank D. Bernardi, A. Serpolini and R. A. Knight for helpful discussions and criticism. Some of the work from which this review originated was supported by grants from the National Institutes of Health to P. M. Steinert, L’Oréal to R.S., Telethon to E.C., and by grants from the Medical Research Council, AIRC [AU: Please define], Telethon, the European Union, MIRU [AU: Please define] and MiRIN to G.M.

Competing interests statement
The authors declare that they have no competing financial interests.

Online links
DATABASES
The following forms in this article are linked online to:

ABCA12 | ALOXE3 | ALOX12B | Sprink | TGM2
Chanarin-Dortmann syndrome | Gaucher disease | lamellar ichthyosis | Netherton syndrome | nonbullous congenital ichthyosiform erythroderma | progressive symmetric erythrokeratodermia | Sitigren-Larsson syndrome | Vohwinkel syndrome | X-linked ichthyosis

Swiss-Prot: http://us.expasy.org/sprot/
κ-catenin | c-myc | c-myc | c-myc | c-myc | c-myc | c-myc | c-myc | c-myc | c-myc | c-myc | c-myc | c-myc | c-myc | c-myc | c-myc | c-myc | c-myc | c-myc | c-myc | c-myc | c-myc | c-myc | c-myc | c-myc | c-myc |

Further information
[AU: Any web sites to add?]

Access to this links box is available online.
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’Oreal 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

ALOXE3

ALOX12B

Spink5

TGM1

OMIM
Chanarin–Dorfman syndrome

Gaucher disease

Lamellar ichthyosis

Netherton syndrome

nonbullous congenital ichthiosiform erythroderma

progressive symmetric erythrokeratoderma

Sjögren–Larsson syndrome

Vohwinkel syndrome

X-linked ichthyosis
<table>
<thead>
<tr>
<th>Protein Name</th>
<th>Swiss-Prot ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>µ-calpain (I)</td>
<td><a href="http://us.expasy.org/cgi-bin/niceprot.pl?P07384">http://us.expasy.org/cgi-bin/niceprot.pl?P07384</a></td>
</tr>
<tr>
<td>corneodesmosin</td>
<td><a href="http://us.expasy.org/cgi-bin/niceprot.pl?P02413">http://us.expasy.org/cgi-bin/niceprot.pl?P02413</a></td>
</tr>
<tr>
<td>desmoglein-1</td>
<td><a href="http://us.expasy.org/cgi-bin/niceprot.pl?P08554">http://us.expasy.org/cgi-bin/niceprot.pl?P08554</a></td>
</tr>
<tr>
<td>desmocollin-1</td>
<td><a href="http://us.expasy.org/cgi-bin/niceprot.pl?P19957">http://us.expasy.org/cgi-bin/niceprot.pl?P19957</a></td>
</tr>
<tr>
<td>elafin</td>
<td><a href="http://us.expasy.org/cgi-bin/niceprot.pl?Q02817">http://us.expasy.org/cgi-bin/niceprot.pl?Q02817</a></td>
</tr>
<tr>
<td>envoplakin</td>
<td><a href="http://us.expasy.org/cgi-bin/niceprot.pl?Q09580">http://us.expasy.org/cgi-bin/niceprot.pl?Q09580</a></td>
</tr>
<tr>
<td>K1</td>
<td><a href="http://us.expasy.org/cgi-bin/niceprot.pl?P04264">http://us.expasy.org/cgi-bin/niceprot.pl?P04264</a></td>
</tr>
<tr>
<td>K5</td>
<td><a href="http://us.expasy.org/cgi-bin/niceprot.pl?P13647">http://us.expasy.org/cgi-bin/niceprot.pl?P13647</a></td>
</tr>
<tr>
<td>K9</td>
<td><a href="http://us.expasy.org/cgi-bin/niceprot.pl?P35527">http://us.expasy.org/cgi-bin/niceprot.pl?P35527</a></td>
</tr>
<tr>
<td>K10</td>
<td><a href="http://us.expasy.org/cgi-bin/niceprot.pl?P13645">http://us.expasy.org/cgi-bin/niceprot.pl?P13645</a></td>
</tr>
<tr>
<td>loricrin</td>
<td><a href="http://us.expasy.org/cgi-bin/niceprot.pl?P23490">http://us.expasy.org/cgi-bin/niceprot.pl?P23490</a></td>
</tr>
<tr>
<td>involucrin</td>
<td><a href="http://us.expasy.org/cgi-bin/niceprot.pl?P07476">http://us.expasy.org/cgi-bin/niceprot.pl?P07476</a></td>
</tr>
<tr>
<td>m-calpain (II)</td>
<td><a href="http://us.expasy.org/cgi-bin/niceprot.pl?P17655">http://us.expasy.org/cgi-bin/niceprot.pl?P17655</a></td>
</tr>
<tr>
<td>periplakin</td>
<td><a href="http://us.expasy.org/cgi-bin/niceprot.pl?P060437">http://us.expasy.org/cgi-bin/niceprot.pl?P060437</a></td>
</tr>
<tr>
<td>plakophilin-1</td>
<td><a href="http://us.expasy.org/cgi-bin/niceprot.pl?Q13835">http://us.expasy.org/cgi-bin/niceprot.pl?Q13835</a></td>
</tr>
<tr>
<td>S100A7</td>
<td><a href="http://us.expasy.org/cgi-bin/niceprot.pl?P31151">http://us.expasy.org/cgi-bin/niceprot.pl?P31151</a></td>
</tr>
<tr>
<td>S100A10</td>
<td><a href="http://us.expasy.org/cgi-bin/niceprot.pl?P60903">http://us.expasy.org/cgi-bin/niceprot.pl?P60903</a></td>
</tr>
<tr>
<td>S100A11</td>
<td><a href="http://us.expasy.org/cgi-bin/niceprot.pl?P31949">http://us.expasy.org/cgi-bin/niceprot.pl?P31949</a></td>
</tr>
<tr>
<td>TGM1</td>
<td><a href="http://us.expasy.org/cgi-bin/niceprot.pl?P22735">http://us.expasy.org/cgi-bin/niceprot.pl?P22735</a></td>
</tr>
<tr>
<td>TGM2</td>
<td><a href="http://us.expasy.org/cgi-bin/niceprot.pl?P21980">http://us.expasy.org/cgi-bin/niceprot.pl?P21980</a></td>
</tr>
<tr>
<td>TGM3</td>
<td><a href="http://us.expasy.org/cgi-bin/niceprot.pl?Q08188">http://us.expasy.org/cgi-bin/niceprot.pl?Q08188</a></td>
</tr>
<tr>
<td>TGM5</td>
<td><a href="http://us.expasy.org/cgi-bin/niceprot.pl?Q07283">http://us.expasy.org/cgi-bin/niceprot.pl?Q07283</a></td>
</tr>
</tbody>
</table>