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Defining keratin protein function in skin epithelia: Epidermolysis Bullosa Simplex and its aftermath

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Abstract

Epidermolysis bullosa simplex (EBS) is a rare genetic condition typified by superficial bullous lesions following incident frictional trauma to the skin. Most cases of EBS are due to dominantly-acting mutations in keratin 14 (K14) or K5, the type I and II intermediate filament (IF) proteins that co-polymerize to form a pan-cytoplasmic network of 10nm filaments in basal keratinocytes of epidermis and related epithelia. Defects in K5–K14 filament network architecture cause basal keratinocytes to become fragile, and account for their rupture upon exposure to mechanical trauma. The discovery of the etiology and pathophysiology of EBS was intimately linked to the quest for an understanding of the properties and function of keratin filaments in skin epithelia. Since then, continued cross-fertilization between basic science efforts and clinical endeavors has highlighted several additional functional roles for keratin proteins in the skin, suggested new avenues for effective therapies for keratin-based diseases, and expanded our understanding of the remarkable properties of skin as an organ system.

Discovery of the genetic basis for the inherited bullous disease epidermolysis bullosa simplex (EBS), made twenty years ago, provided a new and paradigm-shifting blueprint for the characterization and understanding of a large number of monogenic disorders with a significant impact on skin. Since then EBS as well as related genetic conditions have continued to provide a breeding ground for the cross-fertilization of ideas and successes between basic science endeavors and clinical findings. After a brief introduction to keratins, we here review the clinical features of EBS, the discovery of its genetic basis, and our current understanding of phenotype-genotype correlations in this genodermatosis. We briefly discuss the numerous roles assigned so far to keratin proteins and/or filaments in skin

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epithelia, address the challenge of devising effective therapies for this condition and, finally, identify a few important priorities for the future.

A primer on keratin intermediate filaments

Keratins are the most abundant structural proteins in the cytoplasm of epithelial cells, in which they form a network of 10–12 nm wide intermediate filaments (IFs) (Figure 1a–c) (Fuchs and Cleveland, 1998; Kim and Coulombe, 2007). Keratin proteins are encoded by a large family of conserved genes, numbering ~54 in the human genome (Schweizer *et al.*, 2006), that partition to the type I and II subgroupings of IF-encoding genes. There are 28 type I (K9–K28; K31–K40) and 26 type II (K1–K8; K71–K86) genes, each coding for one polypeptide chain (Schweizer *et al.*, 2006). Type I proteins tend to be smaller (40–64 kDa) and more acidic (pI ~4.7–6.1) than the larger (52–70 kDa) and basic-neutral (pI ~5.4–8.4) type II proteins (Moll *et al.*, 1982; Schweizer *et al.*, 2006). Keratin polymerization obligatorily begins with formation of coiled-coil heterodimers involving one type I and one type II protein (Fuchs and Cleveland, 1998; Kim and Coulombe, 2007; Steinert *et al.*, 1976). This requirement underlies the pairwise transcriptional regulation of keratin genes *in vivo* (Fuchs, 1995; Moll *et al.*, 1982; O’Guin *et al.*, 1990). Further, the regulation of keratin genes, individually or as pairs, depends upon the type of epithelia, stage of cellular differentiation, and context (such as disease) (Freedberg *et al.*, 2001; Fuchs, 1995; Moll *et al.*, 1982; O’Guin *et al.*, 1990). Keratin genes and proteins thus provide unparalleled molecular markers to track the status of epithelial cells in health and disease.

In *homo sapiens*, the functional type I and type II keratin genes are clustered on the long arms of chromosomes 17 and 12, respectively, the sole exception being the K18 locus (located next to its partner gene K8 on the type II cluster). Whole genome sequencing has confirmed that the genomic organization, sequence, and regulation of keratin genes are conserved among mammals (Coulombe *et al.*, 2004; Hesse *et al.*, 2004; Schweizer *et al.*, 2006). In addition to the large number of keratin genes (Schweizer *et al.*, 2006), the promiscuity with which type I and II keratin proteins pair up and polymerize with one another (Hatzfeld and Franke, 1985) generates a tremendous potential for diversity in the surface chemistry of keratin filaments in living cells. The latter remains underappreciated as there is yet no high-resolution structural insight into the core architecture of keratins filaments.

An astounding two-thirds of known keratin genes are expressed in skin epithelia alone. In normal interfollicular epidermis, progenitor basal cells express the K5–K14 pair, and small amounts of K15 protein (Lloyd *et al.*, 1995; Nelson and Sun, 1983; Porter *et al.*, 2000). Upon commitment to differentiation, keratinocytes rapidly turn on the expression of K1–K10 (Fuchs and Green, 1980; Woodcock-Mitchell *et al.*, 1982) as they switch off K5–K14 gene transcription (though the proteins persist) (Figure 1D–F) (Fuchs, 1995). At a later stage, i.e., in the granular layer, K2e gene expression occurs (Collin *et al.*, 1992). These specific switches in keratin gene expression have been highly conserved throughout evolution, and are paralleled by striking changes in the density and organization of keratin IFs in keratinocytes (Figure 1G). This basic blueprint can be altered to reflect regional specialization (e.g., palmo-plantar epidermis) or a departure from normal differentiation. For

instance, K9 is prominently expressed post-mitotically in the thick “primary epidermal ridges” of palmar and plantar epidermis, whereas K6, K16 and K17 occur in the thinner, “secondary ridges” connecting them (Swensson *et al.*, 1998). Otherwise, K6, K16 and K17 are induced, generally at the expense of the K1–K10 pair, in the post-mitotic layers of interfollicular epidermis under conditions of environmental challenges (e.g., tissue injury, UV exposure, viral infection) and in specific diseases (e.g., psoriasis, carcinoma) (Freedberg *et al.*, 2001; McGowan *et al.*, 1998; O’Guin *et al.*, 1990). Evidence in hand suggests that while keratin proteins do not impact the execution of differentiation, the maintenance of a specific complement of keratin proteins plays an important role towards cytoarchitecture and several cellular functions in differentiated or specialized epithelial states.

Epidermolysis Bullosa Simplex: A rare, devastating, but instructive genodermatosis

Epidermolysis Bullosa (EB) corresponds to a group of rare genetic conditions in which bullous lesions affecting primarily the skin arise after exposure to mechanical trauma. Three major forms of EB have been defined. The dystrophic, junctional, and simplex forms of EB are characterized by loss of tissue integrity in the upper dermis, at the dermo-epidermal interface, and within the epidermis, respectively (Figure 2A) (Fine *et al.*, 2008). EB Simplex (EBS) (Figure 2B) is the most frequently occurring, and least severe, form of EB disease (~1 case per 25,000 live births), and is inherited in an autosomal dominant fashion with rare exceptions (Coulombe *et al.*, 2009; Fine *et al.*, 2008; Szeverenyi *et al.*, 2008). In EBS, trauma-induced loss of tissue integrity occurs *within* the basal layer of epidermal keratinocytes (Figure 2A; Figure 3). The genetic defect renders basal keratinocytes fragile, causing them to “rupture” when the epidermis (and, in some cases, other stratified epithelia) is subjected to mechanical trauma. The rupture of basal keratinocytes often occurs in a specific area of the cell, between hemidesmosomes and the nucleus, in the epidermis of EBS individuals (Haneke and Anton-Lamprecht, 1982) and in relevant mouse models (Coulombe *et al.*, 1991a) (Figure 3), a phenomenon that remains unexplained. Skin pigmentation anomalies can occur in EBS (Fine *et al.*, 2008; Gu and Coulombe, 2007), but terminal keratinocyte differentiation and epidermal barrier function are reportedly normal in the untraumatized skin of EBS patients.

Several clinical variants of EBS have been described (note: the new nomenclature for EBS is being used here; Fine *et al.*, 2008). The most frequent, and better understood, variants are EBS-generalized (in which blistering is “generalized” over the body), EBS-localized (in which blistering is largely restricted to hands and feet), and EBS-Dowling-Meara (EBS-DM, in which blisters are generalized as well but show a distinct “herpetiform” or clustered pattern). The main clinical findings distinguishing these three variants are the distribution, frequency, and severity of skin blistering over the body (Table 1), the ultrastructural features of basal keratinocytes, involvement of other epithelia, and prognosis (Table 1). Additional forms of EBS are not only less frequent but also tend to exhibit additional clinical features (Table 1; see also Fine *et al.*, 2008). For instance, EBS-AR resembles the generalized form but is recessively inherited, EBS with mottled pigmentation (EBS-MP) feature anomalies in skin pigmentation, and EBS with muscular dystrophy (EBS-MD) is accompanied by a

progressive, limb-girdle type of muscular dystrophy (Table 1) (Fine *et al.*, 2008; Omary *et al.*, 2004).

Despite the degree to which clinical presentation varies (Fine *et al.*, 2008; Fine *et al.*, 2000; Horn *et al.*, 2000; Paller, 2004) (Table 1), all variants of EBS are caused by genetic mutations in intracellular proteins, compromising their ability to provide structural support in keratinocytes of the epidermis and related tissues (Coulombe *et al.*, 2009; Gu and Coulombe, 2007; Szeverenyi *et al.*, 2008). The vast majority of EBS cases are due to mutations affecting either *K14* or *K5* which, as mentioned above, are type I and II intermediate filament (IF) genes selectively expressed in basal keratinocytes in the epidermis and related complex epithelia (Fuchs, 1995; Nelson and Sun, 1983) (Figure 1). Because of the complex substructure of keratin filaments, the perturbations engendered by mutations altering the primary structure of either *K5* or *K14* proteins for the structure, organization and/or regulation of filaments can range from the very subtle to the very severe, thereby accounting for a substantial fraction of the broad clinical spectra typifying EBS (see below). EBS-MD is caused by mutations in plectin (Gache *et al.*, 1996; McLean *et al.*, 1996), a cyto-linker protein responsible for integrating various cytoskeletal and cell adhesive elements into a functionally unified network (Wiche, 1998; see Table 1). The presence of plectin in hemidesmosomes of basal epidermal keratinocytes and at the Z-lines of skeletal myocytes account for the involvement of both skin and muscle in EBS-MD (Wiche, 1998). Mutations in type XVII alpha 1 collagen (*COL17A1*), a hemidesmosomal plaque protein required for tight adherence of basal keratinocytes to the basal lamina, account for a subset of patients featuring elements typical of both EBS and EB junctional (Fontao *et al.*, 2004; Pfindner *et al.*, 2005) (Figure 2A). Additional examples of rare forms of EBS linked to genes other than *K5* and *K14* are discussed in Fine *et al.* (Fine *et al.*, 2008).

Early 90's: A function for keratin filaments and a promising lead for the etiology of EBS

By the late 1980's, several mechanisms had been proposed to account for the etiology of EBS and the fragility of basal keratinocytes that typifies this condition. These mechanisms could be assigned to either one of two broadly defined categories: a misregulated or defective enzyme, or a defect in a structural component (reviewed in Coulombe, 1993). With the notable exception of Dr. Anton-Lamprecht (Anton-Lamprecht, 1983), the literature suggests that no one was considering the possibility of defects in keratin protein or filaments in this condition. Given the heterogeneity in EBS clinical features (Table 1) and the absence of a clear understanding of keratin filament function at the time, there was no obvious reason to draw a link between the disorder and keratin filaments. In the early 90's, however, the combination of a reverse genetic approach with the transgenic mouse technology provided key clues about the etiology of EBS (and related genodermatoses) in addition to revealing a key function for keratin filaments in their natural setting (Coulombe *et al.*, 1991b; Fuchs *et al.*, 1992; Vassar *et al.*, 1991).

The cloning and sequencing of (human) keratin genes, and the resulting ability to better define the secondary structure of their protein products (e.g., Fuchs *et al.*, 1981; Hanukoglu *et al.*, 1982; Steinert *et al.*, 1983), set the stage for subsequent efforts aimed at defining the

key determinants in K14 and K5 co-assembly using systematic deletion mutagenesis. Initial studies of this kind revealed that K14 deletion mutants missing small portions of either end of the central rod domain had a significant, and biochemically dominant, impact on the organization of keratin filaments when expressed in cultured epithelial cells (Albers and Fuchs, 1987, 1989). Important details about the impact and mode of action of such K14 deletion mutants (Coulombe *et al.*, 1990) and, later on, K5 deletion mutants (Wilson *et al.*, 1992) were subsequently provided by in vitro polymerization assays making use of purified recombinant keratin proteins. Relevance to EBS, and to keratin function in vivo, was ultimately revealed when select K14 deletion mutants were tissue-specifically expressed in transgenic mice using the *K14* promoter (Coulombe *et al.*, 1991b; Vassar *et al.*, 1991). Mutant K14-expressing transgenic mice exhibited trauma-induced blistering in the epidermis and oral mucosa, beginning at or shortly after birth, in a manner that closely approximated the key features of EBS (Figure 3). The phenotype of these mice strongly hinted that EBS could indeed arise from mutations affecting the protein-coding sequence of either *K14* or *K5* (Vassar *et al.*, 1991). This possibility was further supported by publications that had described defects in keratin IF network architecture in basal keratinocytes from individuals with EBS, in the epidermis in situ (Anton-Lamprecht, 1983; Haneke and Anton-Lamprecht, 1982; Ito *et al.*, 1991) as well as in ex vivo culture (Kitajima *et al.*, 1989). Such mutations were indeed found shortly thereafter, initially in K14 (Bonifas *et al.*, 1991; Coulombe *et al.*, 1991a) and later on in K5 (Lane *et al.*, 1992), in the genome of individuals with EBS.

In addition to providing key clue about the genetic basis of EBS, the characterization of the first cohort of transgenic mouse lines with tissue-specific expression of K14 deletion mutants (Coulombe *et al.*, 1991b; Vassar *et al.*, 1991) suggested a biological framework for many heretofore misunderstood features of EBS (Coulombe *et al.*, 2009). First, mutations mapping to the α -helical rod domain of keratin can elicit the formation of aggregates of amorphous proteins in the cytoplasm in addition to dominantly disrupting filament structure (Coulombe *et al.*, 1991b; Fuchs *et al.*, 1992; Vassar *et al.*, 1991). Such aggregates are diagnostic for the most severe form of EBS, EBS-DM (see Table 1), and are immunopositive for K5 and K14 (Coulombe *et al.*, 1991a; Ishida-Yamamoto *et al.*, 1991). Second, onset of disease occurs at birth or shortly thereafter, reflecting in part the embryonic onset of *K5* and *K14* gene expression (Byrne *et al.*, 1994; Kopan and Fuchs, 1989) along with the protected environment experienced by the human fetus up until birth. Third, the broad range of clinical severity typifying EBS in humans can be mimicked in mouse skin tissue by expressing K14 mutants having either a mild, moderate, or severe impact on keratin IF assembly in vitro (Coulombe *et al.*, 1991b). This finding helped dismiss the idea that variants such as EBS-DM and EBS-localized (Table 1) had a distinct etiology. Fourth, the ability of blisters to heal leaving no scar distinguishes EBS from the junctional and dystrophic forms of EB (Fine *et al.*, 2008; Fine *et al.*, 2000; Paller, 2004). From the observation that the wound-inducible keratins 6, 16, and 17 (K6, K16, K17) are induced in keratinocytes populating the margins of epidermal lesions, it has been suggested these keratins could compensate for the cellular fragility fostered by *K5* or *K14* mutants, allowing for normal epithelialization to occur (Coulombe *et al.*, 1991b). Fifth, this initial set of mouse models suggested that similar defects in other keratin proteins should elicit analogous

deficiencies in the epithelial cell types. This was later shown via the tissue-specific expression of a deletion mutant of K10, a keratin expressed in terminally differentiating keratinocytes of the epidermis and related cornifying epithelia (Fuchs and Green, 1980; Woodcock-Mitchell *et al.*, 1982), in transgenic mice (Fuchs *et al.*, 1992). These mice exhibited trauma-induced cell fragility in the suprabasal portion of epidermis, in a fashion that proved analogous to the human condition epidermolytic hyperkeratosis. Mutations in the *K1* and *K10* genes were later found in patients with this condition (Cheng *et al.*, 1992; Chipev *et al.*, 1992; Rothnagel *et al.*, 1992).

The cell fragility that typifies EBS largely corresponds to a “loss-of-keratin-function” phenotype since mice homozygous null for *K14* (Figure 3), *K5*, or *plectin* display the key features of EBS (with accompanying muscle defects in the case of *plectin*; Table 1). The lesser degree to which the esophagus is affected relative to epidermis in newborn *K14* null mice correlates with higher levels of K15, a type I keratin related to K14, in this tissue (Lloyd *et al.*, 1995). The presence of small amounts of K15 likely accounts for the markedly more severe blistering of skin, and earlier death, in *K5* null relative to *K14* null mice (Kerns *et al.*, 2007; Lloyd *et al.*, 1995; Peters *et al.*, 2001). A compensatory role for K15 was also proposed based on studies of individuals with EBS whose genome bear the equivalent of a *K14* null mutation (Chan *et al.*, 1994a; Jonkman *et al.*, 1996). Later on, the targeted expression of K16, another type I keratin homologous to K14, in basal keratinocytes of the epidermis was shown to markedly attenuate skin blistering and prevent neonatal death in *K14* null mice (Paladini and Coulombe, 1999). Such findings helped foster the notion that highly homologous keratins are partially redundant in their structural support role *in vivo*, and paved the way for a search for small molecules that would, upon topical application, provide therapeutic relief as a result of the selective induction of a compensatory program of gene expression (see below).

Genotype-phenotype correlations in EBS: From the bench to the bedside, and back

A comprehensive and useful database of human keratin mutations, and associated diseases, is maintained at Singapore’s Bioinformatics Institute (see www.interfil.org and (Szevenyi *et al.*, 2008). *K5* and *K14* mutations account for > 39% of all keratin disease-associated mutations characterized thus far (450 out of 1143 reports), and together they are second only to mutations in *LMNA*, the gene encoding lamins A and C (nuclear IF proteins), for disease-causing mutations in an IF protein-encoding gene. Mutations in *K5* or *K14* account for the vast majority of EBS cases, with only a small (albeit biologically significant) fraction of cases involving mutations in genes encoding IF-associated proteins (plectin, integrins, collagen XVII; see Fine *et al.*, 2008). Most of the EBS-causing mutations occurring in *K5* (>86%) and *K14* (>87%) are dominantly-acting missense alleles, and they are distributed across much of the sequences of the corresponding protein products (Figure 4). This said, the majority of these mutations alter amino acid residues located within the central α -helical rod domain of K5 and K14 (Figure 4), known to play a crucial role towards the assembly and structure of keratin filaments (Coulombe *et al.*, 2009; Fuchs and Cleveland, 1998; Omary *et al.*, 2004).

Basic principles could readily be drawn as additional reports of EBS-causing mutations came to the fore (Coulombe, 1993; Epstein, 1992; Leigh and Lane, 1993), and are affirmed nowadays by surveying the Singapore database. For EBS-localized, EBS-generalized, and EBS-DM, a strong concordance exists between the position and nature of the mutation in the target keratin protein, the extent to which it disrupts keratin IF assembly and structure in established assays, and the severity of clinical presentation (Chan *et al.*, 1993; Chan *et al.*, 1994b; Gu and Coulombe, 2005; Letai *et al.*, 1993; Morley *et al.*, 2003; Morley *et al.*, 1995). Corollary to this notion, the nature of the mutation affecting either K5 or K14 usually defines the sensitivity threshold that the epithelium displays toward frictional trauma, thereby contributing to whether the symptoms are “local” (e.g., EBS-localized) or “generalized” (e.g., EBS-generalized and EBS-DM). Mutations altering highly-conserved residues located within the short and highly-conserved sequence segments located at the beginning and the end of the central rod domain, in either K5 or K14, are often associated with EBS-DM (Figure 4). Further, twice as many cases of EBS-DM have been traced to mutations in *K14* rather than *K5* (n=106 vs. n=43, respectively), reflecting the presence of a “mutational hot spot” in codon 125 in the highly conserved segment within subdomain 1A, which normally encodes an Arg residue (Coulombe *et al.*, 1991a; Fuchs and Coulombe, 1992; Stephens *et al.*, 1993) (Figure 4). The very high frequency of mutations at K14’s codon 125 (~29% of all reported mutations in *K14*), and the corresponding (conserved) codon in other type I keratins and even non-keratin IF proteins (Omary *et al.*, 2004; Szeverenyi *et al.*, 2008), likely results from deamination of methylated cytosine occurring in the context of a CpG dinucleotide. The latter represents the most frequent mechanism causing mutations in the human genome (Cooper, 1988). Several lines of evidence underscore the crucial role of this specific arginine residue in keratin IF assembly and function (Coulombe *et al.*, 1991a; Letai *et al.*, 1993; Ma *et al.*, 2001; Werner *et al.*, 2004).

Conversely, the mildest variant of EBS, EBS-localized, often involve missense mutations affecting amino acids located in the non-helical “head” and “linker” domains, particularly K5 (Figure 4). The primary structure of these domains is less well-conserved than that of the helix boundary motifs and other α -helical segments of the central rod domain (Parry, 2005). Mutations in these domains are generally less consequential for IF assembly and function (Chan *et al.*, 1993; Chan *et al.*, 1994b; Hatzfeld and Weber, 1991; Letai *et al.*, 1992; Letai *et al.*, 1993). Why the analogous head and linker regions of K14 are not as frequently targeted for mutation as they are in K5 is unclear (Figure 4). One possibility is that such mutations might behave recessively (Hovnanian *et al.*, 1993; Szeverenyi *et al.*, 2008). Another likely factor is the presence of K15 in basal keratinocytes (Lloyd *et al.*, 1995). Although not sufficient to prevent the consequences of severe K14 mutations, the presence of K15 might ameliorate subtle K14-generated defects in the IF network of basal keratinocytes.

EBS-generalized, which is intermediate in severity between EBS-localized and EBS-DM, is also frequently associated with mutations in the helix-initiation and helix-termination motifs but also shows a greater frequency of mutations mapping elsewhere in the rod domain, relative to EBS-DM. Another mutation bias worth mentioning is the strong association between mutations mapping to the non-helical head and tail domains of K5 protein with, respectively, the “non-classical” EBS-MP and EBS-Migr, variants (Table 1). While the

analysis of phenotype-genotype correlation reported here is heavily emphasized on “mutation location” in either K5 or K14, the nature of the mutation at any given site on these two proteins also can weigh very heavily on the consequences for overall biological function and disease presentation.

The presence of cytoplasmic aggregates containing mispolymerized mutant keratin proteins, a defining characteristic of EBS-DM (Table 1), may contribute to pathophysiology at the cellular and tissue levels. Mice made to express aggregation-prone K14 mutants (Cao *et al.*, 2001; Coulombe *et al.*, 1991b; Vassar *et al.*, 1991) exhibit an earlier-onset of disease, more severe blistering, and die earlier than *K14* null mice (Lloyd *et al.*, 1995). Akin to this, the EBS phenotype exhibited by individuals null for *K14* is milder than autosomal-dominant cases of EBS-DM (Chan *et al.*, 1994a; Jonkman *et al.*, 1996; Rugg *et al.*, 1994).

Conceivably, the failure of the misfolded-protein response to resolve these aggregates may lead to cellular and tissue stress (D’Alessandro *et al.*, 2002; Liovic *et al.*, 2008; Loffek *et al.*, 2010) and this could in turn impact the pathophysiology of EBS and related conditions. Such aggregates of mispolymerized IF proteins are characteristic of many additional disorders caused by mutations in genes encoding non-keratin IF proteins (Omary *et al.*, 2004; Szeverenyi *et al.*, 2008).

The post-EBS era: Keratin proteins fulfill multiple functions in skin epithelia

A wealth of evidence supports the view that the fragility state of basal keratinocytes accounts for the production of skin blisters in individuals with EBS, reflecting a loss of epidermal integrity following incipient mechanical trauma. This includes substantive pathophysiological findings obtained from the analysis of individuals with EBS or relevant mouse models of this condition (cf. above), along with a more detailed, and more recent, set of studies assessing the properties of mutant keratin-expressing epithelial cells in culture (Lulevich *et al.*, 2010; Russell *et al.*, 2004; also, see Beil *et al.*, 2003) and mutant-keratin containing filaments in vitro (Gu *et al.*, 2005; Ma *et al.*, 2001). This role, referred to as structural support (Table 2), has since then been extended to virtually all keratins expressed in other epithelial settings and to all major classes of IF polymers, including the nuclear lamins (Kreplak and Fudge, 2007; Lammerding *et al.*, 2004).

Further efforts towards the creation and characterization of transgenic mouse models alongside the study of individuals with keratin-based diseases together have (yet again) led to the discovery of several novel functions for keratin proteins in skin epithelia (Table 2). A novel function so far evidenced for K10 and K17 relates to the regulation of cell and tissue growth in the epidermis and hair follicles. Thus, dominantly-acting mutations in either *K10* or its partner *K1* cause epidermolytic hyperkeratosis, a condition typified by the fragility of suprabasal keratinocytes, hyperplasia in the basal compartment of epidermis, and hyperkeratosis (Omary *et al.*, 2004; Szeverenyi *et al.*, 2008). Nullifying the *K10* gene results in hyperproliferation in the basal layer of epidermis (Reichelt and Magin, 2002) and sebaceous glands (Reichelt *et al.*, 2004) in mouse, in the absence of any sign of fragility in suprabasal epidermal keratinocytes (Gu and Coulombe, 2007; Reichelt and Magin, 2002). Together these human and mouse findings point to a role for K10, which is expressed post-mitotically (Figure 1), in regulating the rate of proliferation in the basal layer. The

mechanism underlying this remarkable phenotype is not clear at present (Chen *et al.*, 2006; Paramio *et al.*, 2007; Reichelt and Magin, 2002).

K17 fosters epithelial growth in multiple settings in skin tissue, whether healthy, injured, or diseased (Table 2). Mediated in part by its ability to interact with and presumably sequester 14-3-3 σ (stratifin) in a phosphorylation-dependent fashion, K17 participates in the stimulation of protein synthesis and epithelial cell growth occurring in wound-proximal keratinocytes following injury to the epidermis (Kim and Coulombe, 2010; Kim *et al.*, 2006). In anagen-stage hair follicles, K17 promotes maintenance of growth in part by antagonizing TNF α signaling towards apoptosis (Tong and Coulombe, 2006), an effect that could involve the sequestration of the adaptor protein TRADD (Inada *et al.*, 2001). Finally, there is evidence that K17 accelerates the onset of basaloid skin tumors, in which it is faithfully expressed (Markey *et al.*, 1992), by promoting keratinocyte proliferation correlating with the expression of pro-inflammatory and/or mitogenic cytokines and chemokines in keratinocytes (DePianto *et al.*, 2010). The latter finding is captivating in light of the implication of specific SNPs affecting the coding sequence of K5 and enhancing the risk of developing basal cell carcinoma in human (Stacey *et al.*, 2009). Besides, a recent genome-wide screen has implicated *K6*, *K16*, and *K17* in a genetic network defining the key interrelationship between barrier function, inflammation, and tumor susceptibility in mouse skin (Quigley *et al.*, 2009).

In keeping with the theme of chemokines, Magin and his colleagues have reported on an increased density of Langerhans cells in mice null for *K5*, and in individuals with EBS resulting from mutations at the *K5* locus (Roth *et al.*, 2009). This phenomenon, which does not occur in *K14* null mouse skin or in *K14* mutation-related cases of EBS, correlates with an upregulation in chemokines (e.g., CCL2, CCL19, CCL20) known to attract Langerhans cell precursors to the skin (Roth *et al.*, 2009), and otherwise adds to the emerging concept that keratins may act as key immune modulators in the skin (DePianto *et al.*, 2010).

Keratin 6, which along with K16 and K17 is rapidly induced in wound-proximal, surviving keratinocytes following injury to the skin (Paladini *et al.*, 1996), was shown years ago to be essential to the maintenance of cell integrity in keratinocytes migrating into the wound (Wong and Coulombe, 2003). Though more fragile, K6 null mouse skin keratinocytes migrate faster than their wildtype counterpart in mechanically softer settings, such as skin explant culture and primary culture. This surprising property has been linked to K6's ability to directly bind to, and negatively regulate, Src kinase, a key driver of cell migration (J. Rotty and P.A.C., ms. in revision). Thus K6, and keratin-associated proteins such as plakoglobin and plectin (Osmanagic-Myers *et al.*, 2006; Yin *et al.*, 2005), may promote the maintenance of intercellular cohesiveness in migrating sheet of keratinocytes by scaling back the extent to which a potentially oncogenic signaling protein, namely Src, is activated during tissue repair.

Pigmentation represents yet another key property of the skin potentially impacted by keratin proteins. The evidence supporting this newly defined role has been reviewed (Gu and Coulombe, 2007) so that only three elements relevant to other sections of this text will be mentioned here. First, Uttam *et al.* (1996) discovered that a rather peculiar missense

mutation in the head domain of K5, Pro24->Leu, causes EBS with “mottled pigmentation” (EBS-MP; Table 1). Several additional cases of EBS-MP have since then been traced to the same K5 allele (Szeverenyi *et al.*, 2008), and to distinct mutations in K5 (1649delG; Horiguchi *et al.*, 2005) or K14 (Met119->Thr; Harel *et al.*, 2006). The 1649delG allele of K5 can also result in EBS-Migr (Table 1), and this too is characterized by the appearance of hyper- or hypo-pigmented skin patches in adults (Gu *et al.*, 2003). Second, a very rare condition typified by reticulate hyper-pigmentation and dark hyperkeratotic papules in skin flexural regions, Dowling-Degos disease (DDD) (Table 1), has been linked to K5 haploinsufficiency (Betz *et al.*, 2006). Third, dominantly-acting, missense alleles in K2 and K1 elicit a dark skin phenotype, as discovered in a random-mutagenesis screen designed to identify novel determinants of coat color in mouse (Fitch *et al.*, 2003; McGowan *et al.*, 2006). Expression of these K2 and K1 alleles in mouse skin reproduces the keratinocyte fragility and epidermolytic hyperkeratosis-like manifestations associated with similar mutations in the human orthologs. No mechanism has been established to account for these pigmentation phenotypes, though there is preliminary evidence pointing to an interaction between K5 and components of microtubules-dependent motors, which are involved in melanin pigment transport (Betz *et al.*, 2006; Gu *et al.*, 2007).

Finally, select clinical attributes of EBS still have no sound biological basis. Topping the list of such attributes are two frequent occurrences: enhanced frequency of skin blistering during the warm season, and age-related improvement in clinical symptoms (Fine *et al.*, 2008; Fine *et al.*, 2000; Paller, 2004). Although experimental evidence is still lacking, one possibility is that conditions that activate the heat shock/stress response may help to resolve the aggregates of misassembled keratins (Loffek *et al.*, 2010; Omary *et al.*, 2004).

Understanding the basis for these peculiar clinical features may lead to new insight into keratin regulation and function, epithelial biology, and innovative ideas for the treatment of EBS.

Devising an effective therapy for EB simplex: challenges and opportunities

Elucidating the etiology and gaining a better understanding of the pathophysiology of EBS has undoubtedly resulted in more extensive, and effective, means of pre- and post-natal genetic testing and counseling options (Holbrook *et al.*, 1992; Pfindner *et al.*, 2003; Rugg *et al.*, 2000). Therapeutic options available to patients suffering from this chronic debilitating disorder are still lacking, however. Notwithstanding its significantly milder nature relative to the other subtypes of EB diseases, and in spite of the age-related improvement in the severity of its symptoms, EBS imposes significant restrictions and affects lifestyle well into adulthood. Yet the standard of care for EBS, i.e., minimizing trauma and preventing infections in healing blisters, remains largely preventive and palliative in spite of two decades of very substantive progress about its genetic and biological underpinnings. The broad spectrum of mutations occurring in either K5 or K14 along with their dominant mode of action, the intrinsically high cell turnover rate taking place in the epidermis, and the low incidence of EBS, an orphan disease, in the human population all contribute to the challenge of devising safe, effective, and long-lasting therapies. The relatively few formalized efforts to test therapies for EBS in human subjects have yielded inconclusive results (Langan and Williams, 2008), or need to be scaled up.

There now is compelling experimental evidence, obtained in the setting of mouse skin tissue *in vivo*, confirming the prediction that the impact of strong dominant-negative mutant keratin proteins can be reversed as expression of the corresponding wild-type protein (or a functional equivalent) is titrated back in epidermal basal cells (Cao *et al.*, 2001). This is also generally consistent with a phenomenon of “revertant mosaicism” noted in the skin of individuals with EBS in which a “second hit” (e.g., a frameshift mutation) in the original mutant K14 allele generates the equivalent of a null allele (Smith *et al.*, 2004) or a “corrected” allele (Schuilenga-Hut *et al.*, 2002), thus negating the impact of an otherwise strong dominant-negative mutation (also, see D’Alessandro *et al.*, 2011). The elegant study by Cao *et al.* (2001) also showed that wild-type keratinocytes have a growth advantage over those expressing mutant keratin in skin epithelia *in vivo*, a finding with obvious implications for gene therapy.

Considerable progress has been made in recent years with regards to strategies that specifically target, or interfere with, disease-causing keratin alleles in keratinocytes (Lewin *et al.*, 2005; McLean and Moore, 2011). Various allele-specific strategies are being tested, including gene targeting (Petek *et al.*, 2010) and trans-splicing (Wally *et al.*, 2010; Wally *et al.*, 2008), which are directed at the relevant genomic DNA locus and thus offer the distinct advantage of being potentially curative, and alternatively gene silencing (Atkinson *et al.*, 2011), which targets the relevant mRNA and therefore would necessitate continuous treatment. A distinct strategy is to attempt to intervene at the protein level (Kerns *et al.*, 2010; Kerns *et al.*, 2007). Thus, topical application of the natural product sulforaphane significantly ameliorates the skin blistering and survival of K14 null mice, but not K5 null ones, correlating with its ability to induce the K16 and K17 expression (Kerns *et al.*, 2007). Besides, there is evidence that stimulation of protein homeostasis pathways lessens keratin mutant protein aggregation in EBS keratinocyte cell lines (Chamcheu *et al.*, 2011; Chamcheu *et al.*, 2010; Lee *et al.*, 2008; Loffek *et al.*, 2010). Finally, there are reports showing that injection of botulinum toxin (“Botox”) in plantar skin ameliorates the blistering and diminishes pain in individuals with EBS and pachyonychia congenita (Swartling *et al.*, 2010). These encouraging results notwithstanding, there is much work to be done as one attempts to export and translate encouraging results obtained in virally-transformed human keratinocyte cell lines or transgenic mouse models to human skin.

Prioritizing future efforts in this field

There are multiple issues of significant interest and importance in the triad formed by keratin genes and their proteins, skin epithelia, and genodermatoses. Some stand out, however, and they are as follows. First, we sorely need a reliable, atomic-resolution model for the structure of intermediate filaments. Second, we need to better understand the mechanisms regulating keratin filament assembly in their natural setting *in vivo*. Third, we need to better understand the relationship between keratin filament and mechanical resilience, and whether the cell fragility that is seen in conditions such as EBS is entirely the result of defects in cellular mechanics. Related to this, we need to devise reliable biomarkers reporting on cell fragility. Fourth, we need “actionable findings” that lead to a genuine clinical improvement in patients suffering from EBS and other keratin-based genodermatoses. As a final point, perhaps, we need to understand the rationale for the

remarkable diversity of keratin proteins, and the differentiation- and context-dependent nature of their expression in epithelia.

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Nonstandard abbreviations

EB	epidermolysis bullosa
EBS-DM	EBS Dowling-Meara
EBS-gen	EBS generalized
EBS-MCS	EBS with migratory circinate erythema
EBS-MP	EBS with mottled pigmentation
EBS-loc	EBS localized
IF	intermediate filament
K	keratin

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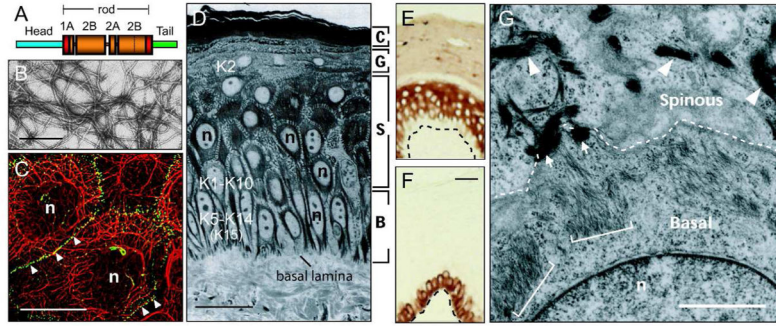
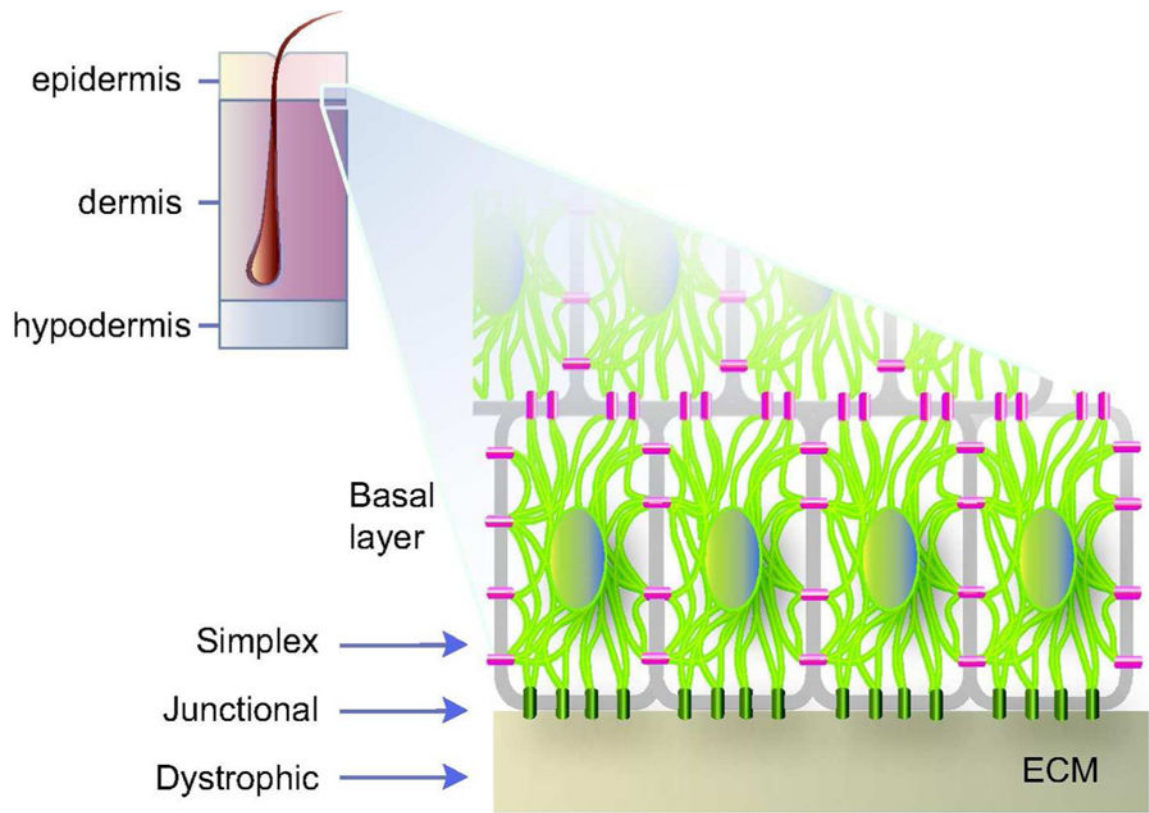


Figure 1. Keratin filaments: Structure, organization, and expression in the interfollicular epidermis

A) Schematic representation of the common domain structure shared by IF proteins including keratins. A central domain, comprised of α -helical coils 1A, 1B, 2A, and 2B separated by non-helical linkers L1, L12 and L2, is flanked by head and tail domains of unknown structure at its N- and C-termini. The boundaries of the rod domain (see red boxes) are highly conserved in primary structure. B) Visualization of filaments, reconstituted *in vitro* from purified human K5 and K14, by negative staining and electron microscopy. Bar: 150 nm. C) Double-labeling for keratin (red chromophore) and desmoplakin, a desmosome component (green chromophore), epitopes in human epidermal keratinocytes in culture. Keratin IFs are organized in a network that spans the entire cytoplasm and are attached at desmosomal cell-cell contacts between cells (arrowheads). n, nucleus. Bar: 50 μ m. Micrograph courtesy of Dr. K. Green (Northwestern Univ., Chicago, IL). D) Histological cross-section of resin-embedded human trunk epidermis, revealing the basal (B), spinous (S), granular (G), and cornified (C) cell layers. Bar: 50 μ m. n, nucleus. E, F) Differential distribution of keratin epitopes in the human epidermis. K14 occurs in the basal layer (E), where the epidermal progenitor cells reside. K10 occurs in the suprabasal, differentiating keratinocytes of epidermis (F). Basal lamina is depicted by a dashed line. Bar: 50 μ m. G) Ultrastructure of the boundary between the basal and suprabasal layer in mouse trunk epidermis, as seen in cross-section by routine transmission electron microscopy. Organization of keratin filaments as loose bundles correlates with the expression of K5–K14 in basal cells (see brackets), whereas the formation of denser, electron-dense filament bundles reflects the onset of K1–K10 expression in early differentiating cells (see arrowheads). Arrows point to desmosomes. Bar: 2 μ m. n, nucleus. Adapted from (Miller *et al.*, 2008).



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Figure 2. Introduction to Epidermolysis Bullosa Simplex

A) Schematic representation of skin tissue (left) and detailed view of the bottom portion of the epidermis (right), highlighting the cytoplasmic network of keratin IFs (light green) attached to hemidesmosome cell-ECM (green) and desmosome cell-cell (magenta) contacts in basal keratinocytes. Arrows depict the plane of tissue rupture seen in the “simplex”, “junctional”, and “dystrophic” forms of EB. B) Example of trauma-induced bullous skin lesions (arrows) in the feet of a 2-month old child diagnosed with EBS. Picture kindly provided by Dr. Bernard Cohen (Johns Hopkins School of Medicine; see <http://dermatlas.med.jhmi.edu>). Adapted from (Coulombe *et al.*, 2009).

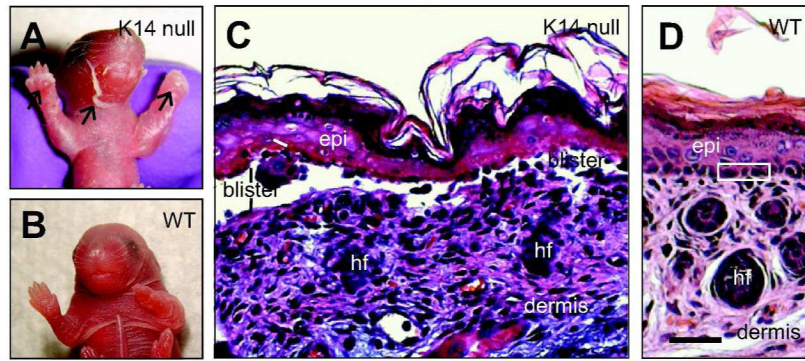


Figure 3. Loss of keratin 14 elicits EBS-like features in newborn mouse skin

A, B) Picture of newborn mouse littermates, comparing K14 null and wildtype. A) The K14 null neonate exhibits massive skin blistering. Front paws and facial area are severely affected (see arrows). B) A wildtype littermate exhibits intact skin. C, D) Micrographs from hematoxylin/eosin-stained histological sections prepared from front paws of 2-day old K14 null (C) and wildtype (D) mice. Epidermal cleavage is obvious in the K14 null sample. Loss of epidermal integrity occurs “near” the basal layer of keratinocytes (see “blister”), the defining characteristic of EBS. Three basal keratinocytes are boxed in frame D. Abbreviations: epi, epidermis; hf, hair follicles. Bar: 100 μ m. Adapted from (Coulombe *et al.*, 2009).

Table 1

Epidermolysis Bullosa Simplex and other diseases caused by mutations in Keratin 5 or Keratin 14

EBS Variant #	Distinguishing Features #	Onset	Target Genes	Orig. Clinical Description
EBS, localized	Formerly known as “Weber-Cockayne”-type EBS. Blistering usually limited to hands and feet, but can occur at sites of repeated trauma; often associated with palmo-plantar keratoderma; worsens in warmer months	Infancy, early childhood, adulthood	Most frequently K5; Less frequently, K14	F.P. Weber (1926) ¹ E.A. Cockayne (1938) ²
EBS, generalized	Formerly known as “Koebner”-type EBS. Blisters predominantly on hands and feet but blistering is often generalized; absence of large tonofilaments in basal keratinocytes on electron microscopy; worsens in warmer months	Birth or infancy	K5 (C-terminal region and L2 linker domain); K14	H. Koebner (1886) ³
EBS-DM (Dowling-Meara)	This form of EBS retained its original designation. Widespread and severe blistering; herpetiform and hemorrhagic blisters; frequent mucosal involvement; progressive palmo-plantar keratoderma; nail dystrophy and milia; hyper- and hypo-pigmentation may occur; may improve with heat or fever	Birth	K5, K14 (at N- or C-terminal ends of the rod domain)	G.B. Dowling & R.H. Meara (1954) ⁴
EBS-AR (autosomal recessive)	Very rare variant. Largely similar to the generalized form, though the frequency of blistering may be less.	Infancy	K14	Hovnanian et al., (1993) provided the first mutation report. ⁵
EBS-MP (with Mottled Pigmentation)	Skin blistering with mottled pigmentation of trunk and limbs; punctate palmo- plantar keratoderma; nail dystrophy.	Birth or infancy	K5 (Pro24->Leu mutation in head domain); K14	T. Fischer & T. Gedde-Dahl (1979) ⁶
EBS-MD (with Muscular Dystrophy)	EBS-like skin blistering with progressive limb-girdle muscular dystrophy. Very rare variant.	Infancy	Plectin	K.M Niemi et al. (1988) ⁷
EBS-Migr (Migratory)	Formerly known as EBS with migratory circinate erythema. Annular migratory multiple erythema circumatum; multiple vesicles on the hands, feet, and legs; lesions heal with brown pigmentation but no scarring. Very rare variant.	Birth	K5 (tail domain)	L.H. Gu et al. (2003) ⁸
DDD (Dowling-Degos Disease)	Reticular hyperpigmentation on flexure surfaces.	After puberty	K5 haploinsufficiency	G.B. Dowling and W. Freudenthal, (1938) ⁹
NEJS (Naegeli-Franceschetti- Jadassohn)	Reticular hyper-pigmentation that disappears over time; hypohydrosis; palmo-plantar keratoderma; absent fingerprint lines	Birth	K14 (head domain)	O. Naegeli (1927); A. Franceschetti & W. Jadassohn (1954) ¹⁰

[#]This table is adapted from Coulombe et al. (*J. Clin. Invest.* 119: 1784-93, 2009). Additional EBS subtypes, caused by mutations in genes other than K5 and K14, are more rare. See Fine et al. (*J. Amer. Acad. Dermatol.* 58:931-50, 2008) for a classification of all EBS variants.

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Table 2

Established roles of keratin proteins in skin epithelia

Description of functional role	Keratin(s)	Biological context	Comments #
Structural support	All	Epidermis & hair follicles	Proper structure and organization of intracellular keratin filaments is essential to withstanding frictional and mechanical stresses. ^{1,2}
Cell and tissue growth	K10	Epidermis	In mouse, loss of K10 results in enhanced proliferation in the epidermis, akin to what occurs in epidermolytic hyperkeratosis. The mechanism(s) involved remains to be defined, but well might be non-cell autonomous. ³
	K17	Hair follicle cycling	In mouse, loss of K17 results in premature entry into catagen, owing in part to enhanced TNF- α signaling and apoptosis. A potential mechanism is the interaction with, and negative regulation of, the adaptor protein TRADD. ^{4,5}
	K17	Wound edge	K17 participates in the increase occurring in the size of keratinocytes while in primary culture and at the wound edge in embryonic ectoderm by stimulating protein synthesis, an event that depends in part on its phosphorylation-dependent interaction with 14-3-3s (stratifin). ⁶
Skin pigmentation	K1, K2, K5	Epidermis	A heroic random mutagenesis screen revealed a role for K1 and K2 in skin pigmentation in mouse. Also, EBS-MP and Dowling-Degos disease (cf. Table 1) are caused by mutations at the K5 locus in humans. ^{7,8,9}
Keratinocyte migration	K6	Wound edge	In addition to providing structural support in keratinocytes migration into wound site, K6 negatively regulates their migration speed via a direct, and inhibitory, interaction with Src kinase. The latter is believed to foster the cohesive migration of keratinocytes as a cellular sheet. ¹⁰
Langerhans cell density	K5	Epidermis	In mouse, loss of K5 results in an increased Langerhans cell density in the epidermis. The same phenomenon occurs in EBS patients with K5 mutations, specifically. The mechanism is unknown, but likely involve elevated expression of select chemokines acting on DC precursors. ¹¹
Immunomodulation	K17	Skin cancer	In mouse, loss of K17 causes a delay in the onset of basaloid skin tumors and the extent of phorbol ester-induced dermatitis, correlating with reduced keratinocyte proliferation and a global switch from a Th1/Th17- dominated to a Th2-dominated immune cytokine/chemokine profile. Mechanism(s) unknown. ¹²

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