Europe PMC Funders Group
Author Manuscript
J Invest Dermatol. Author manuscript; available in PMC 2014 December 10.

Published in final edited form as:
J Invest Dermatol. 2014 November ; 134(11): 2685–2692. doi:10.1038/jid.2014.204.

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

Trichohyalin-like proteins have evolutionarily conserved roles in the morphogenesis of skin appendages

Veronika Mlitz[1], **Bettina Strasser**[1], **Karin Jaeger**[1], **Marcela Hermann**[2], **Minoo Ghannadan**[1], **Maria Buchberger**[1], **Lorenzo Alibardi**[3], **Erwin Tschachler**[1], and **Leopold Eckhart**[1,*]

[1]Department of Dermatology, Medical University of Vienna, Vienna, Austria

[2]Department of Medical Biochemistry, Max F. Perutz Laboratories, Medical University of Vienna, Vienna, Austria

[3]Dipartimento di Scienze Biologiche, Geologiche ed Ambientali (BiGeA), Università di Bologna, Bologna, Italy

Abstract

S100 fused-type proteins (SFTPs) such as filaggrin, trichohyalin and cornulin are differentially expressed in cornifying keratinocytes of the epidermis and various skin appendages. To determine evolutionarily conserved, and thus presumably important, features of SFTPs, we characterized non-mammalian SFTPs and compared their amino acid sequences and expression patterns to those of mammalian SFTPs. We identified an ortholog of cornulin and a previously unknown SFTP, termed scaffoldin, in reptiles and birds, whereas filaggrin was confined to mammals. In contrast to mammalian SFTPs, both cornulin and scaffoldin of the chicken are expressed in the embryonic periderm. However, scaffoldin resembles mammalian trichohyalin with regard to its expression in the filiform papillae of the tongue and in the epithelium underneath the forming tips of the claws. Furthermore, scaffoldin is expressed in the epithelial sheath around growing feathers, reminiscent of trichohyalin expression in the inner root sheath of hair. The results of this study show that SFTP-positive epithelia function as scaffolds for the growth of diverse skin appendages such as claws, nails, hair and feathers, indicating of a common evolutionary origin.

Introduction

S100 fused-type proteins (SFTPs) are a family of epidermal proteins which comprises seven members in human and mouse, namely cornulin (encoded by the gene, *Crnn*), filaggrin (*Flg*), filaggrin 2 (*Flg2*), hornerin (*Hrnr*), repetin (*Rptn*), trichohyalin (*Tchh*), and trichohyalin-like 1 (*Tchhl1*) (Henry et al., 2012; Kypriotou et al., 2012). The SFTP genes are located in the epidermal differentiation complex (EDC) on human chromosome 1q21 and mouse chromosome 3q. SFTPs consist of an amino-terminal S100 domain that is followed by a segment of 200 to more than 4000 amino acid residues of highly diverse sequences among the various SFTPs. Many SFTPs contain repetitive sequences with a high abundance

*Corresponding author: Leopold Eckhart, Department of Dermatology, Medical University of Vienna, Waehringer Guertel 18-20, 1090 Vienna, Austria, Tel. +43-1-40400-73735; Fax: +43-1-40400-73584; leopold.eckhart@meduniwien.ac.at.

Conflict of Interests: The authors state no conflict of interest.

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

of arginine, glutamic acid, glutamine, glycine, serine, and/or histidine residues (Henry et al., 2012). In this regard, the SFTPs resemble proteins that are encoded by EDC genes in close vicinity to SFTPs (Henry et al., 2012; Kypriotou et al., 2012). This apparent similarity of protein domains has led to the hypothesis that SFTP genes have arisen by the fusion of a S100 gene with a flanking gene within the EDC (Markova et al., 1993; Krieg et al., 1997; Contzler et al., 2005).

Mammalian SFTPs have distinct expression patterns and functions which are best characterized for filaggrin, trichohyalin, and cornulin (Henry et al., 2012; Kypriotou et al., 2012). Filaggrin is expressed in differentiating keratinocytes of the epidermis where it supposedly aggregates keratin intermediate filaments (Sandilands et al., 2009). In addition, filaggrin is a major source of histidine that is converted to an endogenous protectant against ultraviolet radiation, urocanic acid (Barresi et al., 2011). Mutations in the *FLG* gene are associated with ichthyosis vulgaris and atopic dermatitis (Sandilands et al., 2009; Brown and McLean, 2012). Trichohyalin is expressed in the inner root sheath of the hair and other sites of hard cornification (Rothnagel and Rogers, 1986; O'Keefe et al., 1993; Rogers, 2004). It has a very high content of glutamic acid and arginine residues, and many of the latter are deiminated to citrulline (Tarcsa et al., 1996). Polymorphisms in the *TCHH* gene determine the shape of human hair (Medland et al., 2009). Both filaggrin and trichohyalin accumulate in the form of characteristic intracellular granules known as keratohyalin and trichohyalin granules, respectively. Cornulin is predominantly expressed in the epithelium of the esophagus but also in the inner root sheath of the hair follicles and in the upper epidermis (Xu et al., 2000; Henry et al., 2012). Cornulin has been implicated in esophageal squamous cell carcinoma (Chen et al., 2013) and atopic eczema (Liedén et al., 2009).

Recent advances in genome sequencing have facilitated the identification of homologs of human skin proteins in non-model species, leading to new concepts of the evolution of hair and other skin appendages (Eckhart et al., 2008). Here, we apply comparative genomics in the investigation of SFTPs and define evolutionarily conserved roles of SFTPs. We identify SFTPs in sauropsids, i.e. reptiles and birds, and suggest an origin of SFTPs in early amniotes. Comparative gene expression analyses in chicken and human tissues point to conserved principles of morphogenesis of skin appendages. An improved understanding of the evolution of SFTPs will provide a framework for further investigations of the biological functions and dermatological relevance of proteins such as trichohyalin and filaggrin.

Results

Identification of non-mammalian S100 fused-type proteins

Publicly available genome sequences of nonmammalian vertebrates were screened for the presence or absence of SFTP genes. Indeed, two genes encoding proteins with an amino-terminal S100 domain, a long domain of low sequence complexity, and conserved sequence elements at the carboxy terminus were identified at reptilian and avian genome loci homologous to those of the human SFTP gene cluster (Fig. 1a and b; Suppl. Fig. S1). Similar to their human homologs, the SFTP genes of sauropsids were arranged in tandem and with the same orientiation as the next gene downstream, *S100A11* (Fig. 1a). On the 5′-side, the sauropsidian SFTP genes were flanked by genes apparently similar to cornified

envelope genes of the EDC (Fig. 1a; Suppl. Fig. S2). By contrast, the genome of an amphibian, the clawed frog, *Xenopus tropicalis*, contained an *S100A11* gene but no genes encoding SFTPs. Likewise, no homologs of SFTPs were found in fish genomes. Thus, SFTP genes appear to be present in amniotes (mammals and sauropsids) but not in other vertebrates.

A phylogenetic analysis showed that the SFTPs of different sauropsidian species represented orthologs of 2 proteins, i.e. a homolog of mammalian cornulin and a newly identified SFTP that we tentatively named scaffoldin (proposed gene name *Scfn*; see below). Genes encoding cornulin (*Crnn*) were identified in chicken, budgerigar, alligator, and python but not in the green anole lizard (*Anolis carolinensis*) (Fig. 1a; Suppl. Table S1; Suppl. Fig. S1a). Amplification and sequencing of the corresponding chromosomal locus of the lizard confirmed the absence of a *Crnn* gene (not shown). *Scfn* genes were identified in all the above-mentioned species including the lizard (Fig. 1a; Suppl. Table S1; Suppl. Fig. S1b). Mammalian and non-mammalian SFTPs formed 4 phylogenetic clades (Suppl. Fig. S3) of which only the cornulin clade comprised both mammalian and sauropsidian homologs. By contrast, the scaffoldin clade was sauropsid-specific and the clades of filaggrin-type proteins (filaggrin, filaggrin 2 and hornerin) and trichohyalin-type proteins (trichohyalin, trichohyalin like-1 and repetin) contained only mammalian proteins.

Like their mammalian counterparts, the sauropsidian SFTP genes consisted of 3 exons with the coding sequence being located in exons 2 and 3 (Suppl. Table S1). Sequence alignment showed conservation of putative binding sites for transcription factors AP-1 (Jang et al., 1996) and KLF4 (Patel et al., 2006) in the proximal promoters of several sauropsidian and mammalian SFTP genes (Suppl. Fig. S4). Most likely due to the enormous length of up to 10 kbp and the presence of highly repetitive sequence elements (see below), the third exon of the *Scfn* gene of several species was not completely covered by the available genome sequences. However, the complete coding sequence (8037 nucleotides), with the exception of 7 unidentified nucleotides, was available for the alligator (Suppl. Fig. S1). Moreover, we sequenced approximately 2500 nucleotides on both ends of the *Scfn* gene of the chicken strain, Tetra SL, that we used for further investigations of SFTPs (Accession number KC700629). There were several differences in the nucleotide sequence of *Scfn* in Tetra SL chicken and in the red jungle fowl genome sequence deposited in the GenBank (International Chicken Genome Sequencing Consortium, 2004), most likely reflecting differences in the chicken strains and errors in the assembly of the whole genome sequence. Moreover, we performed long range PCR on chicken genomic DNA and determined the size of the product by agarose gel electrophoresis in comparison to DNA length standards. We estimate the length of the coding sequence of the *Scfn* gene to be approximately 9600 nucleotides. Accordingly, the protein encoded by the chicken *Scfn* gene has approximately 3200 amino acids and a molecular weight of 385 kDa. The lengths of scaffoldins of the chicken and alligator species (2,678 aa) are comparable to that of human trichohyalin (1,943 aa), whereas all cornulin proteins of the same three species were in the size range of 380 to 500 amino acids (Fig. 1b).

Europe PMC Funders Author Manuscripts
Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

SFTPs of sauropsids are most similar to mammalian cornulin and trichohyalin

The protein domains of sauropsid SFTPs contained several features that are also present in mammalian SFTPs, indicating inheritance from common ancestral molecules. In the S100 domains of sauropsid cornulin and scaffoldin proteins, the amino acid residues implicated in calcium binding (Zimmer et al., 2013) as well as many residues at other positions were conserved (Suppl. Fig. S5). Likewise, sequence elements at the carboxy-terminus of SFTPs were conserved (Suppl. Fig. S6). In particular, the motif LYxY(L/I/V)QxQ(K/R) was present in diverse, though not in all, SFTPs. Between the S100 domain and the conserved carboxy-terminus, several sauropsidian scaffoldin proteins contained sequence repeats (Suppl. Fig. S1c). Alligator scaffoldin contained 40 moderately conserved sequence repeats of 45 amino acids, and a sequence unit of 37-39 amino acids was repeated approximately 65 times in chicken scaffoldin (Suppl. Fig. S1c). The amino acid composition of the central segment of sauropsidian SFTPs, irrespective of the presence or absence of repeats, showed a clear bias to the presence of a small subset of amino acid residues that are also enriched in the corresponding region of mammalian SFTPs (Fig. 1c) (Henry et al., 2012). This domain of scaffoldin proteins was particularly rich in arginine (17% of amino acid residues in alligator, 18% in chicken) and glutamic acid (17% in alligator, 19% in chicken), being most similar to trichohyalin among human SFTPs (Fig. 1c). The cornulin proteins of alligator, chicken and humans were rich in glutamine (14%) (Fig. 1c). For comparison, the proteins encoded by the entire human genome contain, on average, 5.8% arginine, 6.8% glutamic acid, and 4.6% glutamine (Scherer, 2011). Notably, high contents of serine, glycine and histidine residues were found only in mammalian SFTPs, i.e. filaggrin, filaggrin 2 and hornerin.

Scaffoldin and cornulin are expressed in the periderm and in skin appendages of the chicken

Next, we determined the expression patterns of SFTP genes in the chicken and in the green anole lizard. Reverse transcription-PCR showed that cornulin was expressed in the esophagus of chicken, thus resembling its human ortholog (Xu et al., 2000), whereas scaffoldin was not present in this tissue (Suppl. Fig. S7a). Both cornulin and scaffoldin were expressed in the tongue and, at lower levels, in the skin and in the claw-forming part of the toes. By contrast, tissues that did not contain keratinocytes lacked expression of *Crnn* and *Scfn* (Suppl. Fig. S7a). In the lizard, scaffoldin mRNA was detected only in the tips of the toes which mostly contain the claw-forming epidermis (Suppl. Fig. S7b).

To localize the expression of SFTP genes in individual cells within tissues, we subjected a set of epithelial tissues of the chicken to mRNA *in situ* hybridization with antisense sequence probes specific to the 3′-untranslated regions of *Crnn* and *Scfn*. Cornulin was expressed in the suprabasal epithelial layers of the esophagus (Fig. 2a) whereas scaffoldin mRNA was not detected there (Fig. 2b). Suprabasal epidermal keratinocytes of chicken skin did not express SFTPs, however, both cornulin and scaffoldin mRNAs were detected in the embryonic periderm (Fig. 2c, d). Moreover, the mRNAs of both SFTPs were present in the differentiating epidermal cells of the ventral part of the claw apparatus, the subunguis (Fig. 2e, f). In the dorsal part of the claw, the unguis, SFTP expression was confined to the periderm whereas cells giving rise to the mature claw did not express cornulin nor scaffoldin

(Fig. 2e, f, and data not shown). The corresponding controls with sense probes did not yield signals, confirming the specificity of the method (Suppl. Fig. S8).

Scaffoldin is expressed in epithelial scaffoldings of hard skin appendages

To determine the expression pattern of chicken scaffoldin at the protein level, we generated a specific antiserum and performed immunochemical analyses (Fig. 3). Western blot analysis revealed a band compatible with the predicted size of 385 kDa in embryonic beak, toe, skin and tongue (Fig. 3a), whereas adult skin as well as muscle were negative (data not shown). In a preparation of the beak of E18 and, to a lesser extent, also in other samples at least one additional band with an apparently higher molecular weight was detected, a feature that is reminiscent of the immunoblot pattern reported for mammalian trichohyalin (Méchin et al., 2005). Consistently, low molecular weight proteins, indicative of filaggrin-type proteolytic processing (Sandilands et al., 2009) were not detected (Fig. 3a). Preadsorption of the antiserum with the peptide used for immunization abolished the high molecular weight signals, confirming the specificity of the method (not shown).

The *in situ* distribution of scaffoldin protein was determined by immunohistochemical analysis of chicken embryonic tissues. Scaffoldin was expressed at high amounts in the peridermal compartment of the egg tooth on embryonic day E10 (Fig. 3b), in the periderm of embryonic epidermis (Fig. 3c), in the feather sheath at E18 (Fig. 3d), at the borders of the lingual papillae (Fig. 3e), and in the epithelial cells below the forming tip of the claw (Fig. 3f). In the periderm above the egg tooth (Fig. 3b, inset), in the feather sheath (Fig. 3d) and in the subunguis of the claw (Fig. 3f, inset), scaffoldin accumulated in distinct granules, most likely identical to the so-called periderm granules (Sawyer et al., 1974; Kuraitis and Bowers, 1978). The specificity of the immunoreaction was confirmed by preadsorption of the antibody with the immunogenic peptide which abolished the staining whereas preadsorption with an unrelated peptide had no effect (Suppl. Fig. S9).

The human nail unit contains trichohyalin at a site homologous to the expression site of chicken scaffoldin

As the investigation of sauropsidian SFTPs revealed expression in the periderm and in the epithelium flanking the claw tips on the ventral side, we wondered whether the corresponding sites in mammals also contained SFTPs. Immunohistochemical investigations suggested absence of cornulin from the periderm but expression in the upper layers of the epidermis of both embryonic and adult mouse skin (Suppl. Fig. S10 and data not shown). Cornulin was also expressed in the hyponychium of the nail unit which is the mammalian homolog of the sauropsidian subunguis (data not shown). Trichohyalin was previously reported to be absent from the periderm but expressed transiently in the suprabasal embryonic epidermis (Lee et al., 1999). Immunohistochemical analysis of the human nail unit using an antibody against the granular form of trichohyalin (O'Guin et al., 1992) demonstrated that trichohyalin was strictly confined to epithelial cells in a segment of the hyponychium, beneath the forming nail tip (Fig. 4). In contrast to a previous report (O'Keefe et al., 1993), no expression was detected in the nail matrix and in the epidermis. These results were consistently obtained in the nail samples from 4 different donors. Thus, trichohyalin is specifically expressed in the epithelium beneath the tip of the nail but not in

Europe PMC Funders Author Manuscripts
Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

the epithelial cells that form the nail itself. The conserved expression patterns of scaffoldin and trichohyalin in claws and nails, feathers and hair, as well as in filiform papillae of the tongue of chicken and humans suggest evolutionary ancient and important roles of scaffoldin and trichohyalin in epithelial scaffoldings of growing skin appendages.

Discussion

The results of this study uncover SFTPs in sauropsids and, thereby, imply an evolutionary origin of SFTPs pior to the divergence of mammals and sauropsids approximately 310 million years ago (Donoghue and Benton, 2007). The comparison of nucleotide sequences of SFTP gene promoters (Suppl. Fig. S4) and the comparison of amino acid sequences of SFTPs from sauropsids and mammals (Suppl. Figs. S5 and S6) revealed sequence elements that have been conserved up to the present. The conserved features of human SFTP genes and proteins are likely to be relevant in skin biology and dermatology, and therefore should be further investigated in future studies.

Besides the S100 domain and the sequence motif in the carboxy-terminal domain, a high content of glutamine in the central domain have apparently been inherited from an ancestral SFTP. Glutamine is the target of transglutamination during keratinocyte cornification. The content of other amino acids has greatly diversified during the evolution of SFTPs resulting in glutamic acid- and arginine-rich proteins such as trichohyalin and scaffoldin on one end and glycine- and serine-rich proteins such as filaggrin on the other end. Most likely, the primordial structure of SFTPs was similar to that of cornulin which has an intermediate content of glutamic acid, arginine, glycine and serine in its central domain (Fig. 1c). According to this hypothesis, cornulin appears to have retained many features of the ancestral SFTP, from which other SFTPs evolved by gene duplication and more pronounced sequence modification. Notably, cornulin is expressed in a wider variety of human and murine epithelia than other SFTPs, including the esophagus, the interfollicular epidermis, the tongue, the inner root sheath of the hair and nail apparatus (Contzler et al., 2005; Suppl. Fig. S10; Suppl. Fig. S11, and our unpublished data). Likewise, the ancestral cornulin protein may have had a common role in epithelia of several body sites, and additional SFTPs with specialized functions and restricted expression patterns in the skin and its appendages have evolved later.

Scaffoldin is a newly identified sauropsidian SFTP that has an amino acid composition similar to that of mammalian trichohyalin. A phyologenetic analysis separated the scaffoldin proteins of sauropsidian species from the clade of mammalian trichohyalin-type proteins (trichohyalin, trichohyalin like-1, repetin) as well as from other SFTPs (Suppl. Fig. S3). As the amino acid sequences of the central domains of SFTPs are highly divergent (Suppl. Fig. S1) and carboxy-terminal sequences are incompletely conserved (Suppl. Fig. S6), the phylogenetic analysis had to be restricted to the S100 domains of SFTPs. Considering this limitation, it remains uncertain whether the *Scfn* and *Tchh* genes have originated by independent duplications of the primordial SFTP gene, or whether there was a single gene duplication that yielded a gene from which *Scfn* and *Tchh* evolved. The origin of the ancestral *Scfn* gene may have occurred either before or after the divergence of the evolutionary lineages that have led to the formation of sauropsidians and mammals and,

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

accordingly, the most recent ancestor of sauropsids and mammals may have had one or two SFTP genes (Fig. 5). The confinement of filaggrin-type SFTPs to mammals is likely to be caused by mammal-specific gene duplications with subsequent sequence modifications. However, the origin of an ancestral filaggrin-type gene in stem amniotes (prior to separation of mammalian and sauropsidian lineages) and the subsequent loss of this gene in sauropsids cannot be excluded. The hypothesis of SFTP loss is exemplified by the presence of cornulin in both mammals and many sauropsids, indicative of an origin in stem amniotes, and absence of cornulin in the lizard (Fig. 1a). Further phylogenetic analyses, including molecular dating using SFTP genes from a large panel of species, may help to define the times of origin of individual SFTP genes.

The expression patterns of cornulin and scaffoldin in the chicken share important features with those of cornulin and trichohyalin in mammals, respectively. Our *in situ* hybridizations of chicken cornulin and scaffoldin as well as the immunostaining of chicken scaffoldin and human trichohyalin demonstrate that these SFTPs are strongly expressed in the epithelium beneath the forming tip of the claw/nail. This epithelium is known as subunguis in the chicken and appears to be homologous to the proximal hyponychium or nail isthmus in humans (Perrin, 2007). In the tongue, mammalian trichohyalin and cornulin are confined to the anterior side of filiform papillae, which in many species (including the chicken and mouse) form a nail-like tip. Interestingly, scaffoldin is also expressed in the feather sheath while the mammalian SFTPs, cornulin and trichohyalin, are expressed in the inner root sheath of the hair (Contzler et al., 2005; Rothnagel and Rogers, 1986). Feathers and hair are structurally different skin appendages which, however, have in common the requirement for mechanical stabilization or scaffolding by an ensheathing epithelium (Wu et al., 2004; Alibardi, 2003). The comprehensive characterization of the possible roles of scaffoldin in sculpting the complex structures of feathers (Chang et al., 2004) will require further studies. Interestingly, cornulin and scaffoldin are expressed in the periderm which transiently covers the embryonic epidermis of the chicken (Alibardi, 2009), with particularly high levels of scaffoldin being present above the forming egg tooth (Fig. 3b), whereas SFTPs have not been detected yet in the periderm of mammals. Taken together, SFTPs of sauropsids and some SFTPs of mammals are expressed in diverse epithelial structures that support the formation of hard skin appendages such as the egg tooth, the filiform papillae of the tongue, nails, feathers and hair. We propose that these SFTP-expressing cells function as scaffolds during the outgrowth of appendages from the skin. Comparative investigations of SFTPs in phylogenetically diverse species may help to further define the mechanistic roles of cornulin, scaffoldin and trichohyalin in epithelial scaffoldings and to identify the ancestral skin appendage from which filiform papillae, nails, feathers and hair have evolved.

Materials and Methods

Ethics statement

All animal procedures were approved by the Animal Care and Use Committee of the Medical University of Vienna and conducted according to the guidelines established by the Animal Care and Use Committee of the Medical University of Vienna. Human toe samples were obtained from cadavers of individuals who had died of diseases not associated with

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

dystrophies of the nails, as described previously (Jäger et al., 2007). The study was approved by the Ethics Committee of the Medical University of Vienna and complied with the principles of the Declaration of Helsinki.

Sequence queries, alignments and phylogenetic analyses

Mammalian SFTP sequences were used as queries in tBLASTn searches in all vertebrate genome sequences available in the GenBank (http://www.ncbi.nlm.nih.gov/). Amino acid sequences of S100 domains were aligned using CLUSTALW, and a phylogenetic tree was built with PHYLIP using the Jones-Taylor-Thornton matrix as distance algorithm and the neighbor-joining method for clustering. Trees were displayed using the TreeView program (Page, 1996).

Nucleic acid preparation, polymerase chain reactions and sequence analysis

Genomic DNA was prepared from chicken and anole lizard samples according to a standard protocol (Strauss, 2001). A region spanning the repeat-rich region of exon 3 of the chicken *Scfn* gene was amplified using Expand Long Range enzyme mix (Roche Applied Science, Basel, Switzerland) using the primers 5′-AGGAAGGCACAATCAACCAC-3′ and 5′-CACGACAAACCTCTGCTTCA-3′. PCRs of shorter gene segments were performed using DreamTaq™ DNA polymerase (Thermo Scientific, Waltham, MA). The amplicons were purified, sequenced, and resulting sequences were submitted to the GenBank (Accession numbers: KC700629 for chicken scaffoldin and KC700628 for anole lizard scaffoldin).

RNA was extracted from homogenized tissues with peqGOLD TriFast™ (Peqlab, Erlangen, Germany) and reverse transcribed using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA) according to the manufacturers' instructions. Reverse transcription PCR was performed using DreamTaq™ DNA polymerase (Thermo Scientific) with the primers listed in Suppl. Table S2. The sequence of a cornulin cDNA derived from the embryonic tongue of the chicken (Accession numbers GQ149127 and KC700625) and the sequence of scaffoldin cDNA derived from the embryonic skin of the chicken (Accession number KC700626) as well as the adult toe of the lizard (Accession number KC700627) were submitted to the GenBank.

mRNA in situ hybridization

The primers Crnn-s1 (5′-ACCAGGAGGAACCACAGACA-3′), Crnn-a1 (5′-GGCAGAGGCAGCCTAATACA-3′), Scfn-s1 (5′-CCACTGCAATATTTAGTCACTTTCC-3′) and Scfn-a1 (5′-TGAAGAAGAAACCCCAATGC-3′) were used for PCR amplifications of 3′-untranslated regions of *Crnn* and *Scfn* from chicken genomic DNA. The amplicons were cloned in sense and antisense orientation into pCR®2.1-TOPO® plasmids (Life Technologies, Paisley, UK) and transcribed *in vitro* using the DIG RNA labeling kit (Roche Applied Science) which introduces digoxigenin (DIG)-UTP into RNA probes. Agarose gel electrophoreses and dot blot assays validated antisense and sense probes. Hybridization experiments were performed according to a previously published protocol (Weninger et al., 1996) omitting the RNA digestion step. Antisense and sense probes were applied at concentrations of 5 and 10 ng/ml, respectively, at hybridization temperatures of 55°C (cornulin) and 50°C (scaffoldin). Only

those stainings obtained with antisense probes for which the corresponding sense probes did not yield to staining were considered specific.

Generation of antibodies and immunochemical assays

The oligopeptide RYERTREDIAAEAE, corresponding to 14 amino acid residues of the consensus sequence of the repeat unit in the central domain of chicken scaffoldin, was synthesized by Genecust Europe, Dudelange, Luxembourg and coupled to keyhole limpet haemocyanin (KLH). Mice received six injections of 100 μg of the oligopeptide to generate antisera. Western blot and immunohistochemistry with antiserum dilutions of 1:2000 gave specific signals which were not obtained with pre-immune sera. The specific immunoreactivity was blocked by preincubation with antigenic peptide (4 μg peptide per 1 μl antiserum).

For Western blot experiments, proteins were extracted from tissues with the Precellys homogenizer (Peqlab) using the NuPAGE® LDS Sample buffer (Life Technologies) and electrophoresed through ExcelGel SDS Gradient 8-18 (GE Healthcare Life Sciences, Buckinghamshire, UK). For immunohistochemical analyses, tissue samples were fixed with 7.5% formaldehyde and embedded in paraffin. Antigen retrieval was done by incubation in citrate buffer (pH 6.0) for all antigens investigated. In addition, some nail sections were treated with protease K (20μg/ml) at 37°C for 20 min before trichohyalin immunohistochemistry, which yielded essentially the same results as the protocol involving antigen retrieval with citrate buffer. The sections were incubated with mouse anti-scaffoldin antibody (1:2000), mouse anti-trichohyalin antibody clone AE15 (1:500; Santa Cruz Biotechnology, Dallas, Texas), rabbit anti-cornulin antibody (1:100; Abcam, Cambridge, UK) or rabbit anti-keratin K6 antibody (1:500; Covance, Princeton, NJ). Biotinylated sheep anti-mouse immunoglobulin (1:200; GE, Chalfont, UK) and goat anti-rabbit immunoglobulin (1:100; Vector Laboratories, Burlingame, CA) were used as secondary antibodies. Sheep or goat serum (10%) was added to the secondary antibody to prevent unspecific binding. The sections were then incubated with streptavidin-biotin-horseradish peroxidase complex and 3-amino-9-ethylcarbazole (DakoCytomation, Glostrup, Denmark), and counterstained with hematoxylin.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Michael Mildner, Heinz Fischer, Wolfgang Sipos and Lutz Langbein for helpful discussions and Susanne Karner for excellent technical support. The authors acknowledge all providers of public genome sequences that were used in the present study. This work was supported by the Austrian Science Fund (FWF): P23801 grant to LE, by the European Cooperation in Science and Technology (COST) Action BM0903 (Skin Barrier in Atopic Diseases, SkinBAD) and by a research grant from CE.R.I.E.S., Neuilly, France.

Abbreviations

aa amino acid residues

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

AP-1	activator protein 1
Crnn	cornulin
EDC	epidermal differentiation complex
Flg	filaggrin
Flg2	filaggrin 2
Hrnr	hornerin
kDa	kilodalton
KLF4	Kruppel-like factor 4
KLH	keyhole limpet haemocyanin
RT-PCR	reverse transcription-PCR
Rptn	repetin
Scfn	scaffoldin
SFTP	S100 fused-type proteins
Tchh	trichohyalin
Tchhl-1	trichohyalin like-1

References

Alibardi L. Adaptation to the land: The skin of reptiles in comparison to that of amphibians and endotherm amniotes. J Exp Zool B Mol Dev Evol. 2003; 298:12–41. [PubMed: 12949767]

Alibardi L. Embryonic keratinization in vertebrates in relation to land colonization. Acta Zool. 2009; 90:1–17.

Barresi C, Stremnitzer C, Mlitz V, et al. Increased sensitivity of histidinemic mice to ultraviolet B radiation suggests a crucial role of endogenous urocanic acid in photoprotection. J Invest Dermatol. 2011; 131:188–194. [PubMed: 20686493]

Brown SJ, McLean WH. One remarkable molecule: filaggrin. J Invest Dermatol. 2012; 132:751–762. [PubMed: 22158554]

Chang CH, Yu M, Wu P, et al. Sculpting skin appendages out of epidermal layers via temporally and spatially regulated apoptotic events. J Invest Dermatol. 2004; 122:1348–1355. [PubMed: 15175023]

Chen K, Li Y, Dai Y, et al. Characterization of tumor suppressive function of cornulin in esophageal squamous cell carcinoma. PLoS One. 2013; 8:e68838. [PubMed: 23894350]

Contzler R, Favre B, Huber M, et al. Cornulin, a new member of the “fused gene” family, is expressed during epidermal differentiation. J Invest Dermatol. 2005; 124:990–997. [PubMed: 15854041]

Donoghue PC, Benton MJ. Rocks and clocks: Calibrating the tree of life using fossils and molecules. Trends Ecol Evol. 2007; 22:424–441. [PubMed: 17573149]

Eckhart L, Valle LD, Jaeger K, et al. Identification of reptilian genes encoding hair keratin-like proteins suggests a new scenario for the evolutionary origin of hair. Proc Natl Acad Sci U S A. 2008; 105:18419–18423. [PubMed: 19001262]

Henry J, Toulza E, Hsu CY, et al. Update on the epidermal differentiation complex. Front Biosci. 2012; 17:1517–1532.

International Chicken Genome Sequencing Consortium. Sequence and comparative analysis of the chicken genome provide unique perspectives on vertebrate evolution. Nature. 2004; 432:695–716. [PubMed: 15592404]

Jang SI, Steinert PM, Markova NG. Activator protein 1 activity is involved in the regulation of the cell type-specific expression from the proximal promoter of the human profilaggrin gene. J Biol Chem. 1996; 271:24105–24114. [PubMed: 8798649]

Jäger K, Fischer H, Tschachler E, et al. Terminal differentiation of nail matrix keratinocytes involves upregulation of DNase1L2 but is independent of caspase-14 expression. Differentiation. 2007; 75:939–946. [PubMed: 17490414]

Krieg P, Schuppler M, Koesters R, et al. Repetin (Rptn), a new member of the "fused gene" subgroup within the S100 gene family encoding a murine epidermal differentiation protein. Genomics. 1997; 43:339–348. [PubMed: 9268637]

Kuraitis KV, Bowers RR. An ultrastructural study of periderm granules in the regenerating feather of the jungle fowl. Cell Tissue Res. 1978; 192:319–326. [PubMed: 699020]

Kypriotou M, Huber M, Hohl D. The human epidermal differentiation complex:cornified envelope precursors, S100 proteins and the 'fused genes' family. Exp Dermatol. 2012; 21:643–649. [PubMed: 22507538]

Lee SC, Lee JB, Kook JP, et al. Expression of differentiation markers during fetal skin development in humans: immunohistochemical studies on the precursor proteins forming the cornified cell envelope. J Invest Dermatol. 1999; 112:882–886. [PubMed: 10383733]

Liedén A, Ekelund E, Kuo IC, et al. Cornulin, a marker of late epidermal differentiation, is down-regulated in eczema. Allergy. 2009; 64:304–311. [PubMed: 19133922]

Markova NG, Marekov LN, Chipev CC, et al. Profilaggrin is a major epidermal calcium-binding protein. Mol Cell Biol. 1993; 13:613–625. [PubMed: 8417356]

Medland SE, Nyholt DR, Painter JN, et al. Common variants in the trichohyalin gene are associated with straight hair in Europeans. Am J Hum Genet. 2009; 85:750–755. [PubMed: 19896111]

Méchin MC, Enji M, Nachat R, et al. The peptidylarginine deiminases expressed in human epidermis differ in their substrate specificities and subcellular locations. Cell Mol Life Sci. 2005; 62:1984–1995. [PubMed: 16091842]

O'Guin WM, Sun TT, Manabe M. Interaction of trichohyalin with intermediate filaments: three immunologically defined stages of trichohyalin maturation. J Invest Dermatol. 1992; 98:24–32. [PubMed: 1728637]

O'Keefe EJ, Hamilton EH, Lee SC, et al. Trichohyalin: a structural protein of hair, tongue, nail, and epidermis. J Invest Dermatol. 1993; 101:65S–71S. [PubMed: 7686953]

Page RD. TreeView: an application to display phylogenetic trees on personal computers. Comput Appl Biosci. 1996; 12:357–358. [PubMed: 8902363]

Patel S, Xi ZF, Seo EY, et al. Klf4 and corticosteroids activate an overlapping set of transcriptional targets to accelerate in utero epidermal barrier acquisition. Proc Natl Acad Sci U S A. 2006; 103:18668–18673. [PubMed: 17130451]

Perrin C. Expression of follicular sheath keratins in the normal nail with special reference to the morphological analysis of the distal nail unit. Am J Dermatopathol. 2007; 29:543–550. [PubMed: 18032949]

Rogers GE. Hair follicle differentiation and regulation. Int J Dev Biol. 2004; 48:163–170. [PubMed: 15272381]

Rothnagel JA, Rogers GE. Trichohyalin, an intermediate filament-associated protein of the hair follicle. J Cell Biol. 1986; 102:1419–1429. [PubMed: 3958055]

Sandilands A, Sutherland C, Irvine AD, et al. Filaggrin in the frontline: role in skin barrier function and disease. J Cell Sci. 2009; 122:1285–1294. [PubMed: 19386895]

Sawyer RH, Abbot UK, Fry GN. Avian scale development. III. Ultrastructure of the keratinizing cells of the outer and inner epidermal surfaces of the scale ridge. J Exp Zool. 1974; 190:57–70. [PubMed: 4436621]

Scherer, S. Guide to the Human Genome. Cold Spring Harbor Laboratory Press; New York: 2011. p. 1008

Strauss, WM. Preparation of genomic DNA from mammalian tissue. In: Ausubel, FM.; Brent, R.; Kingston, RE.; Moore, DD.; Seidman, JG.; Smith, JA.; Struhl, K., editors. Current protocols in molecular biology. Wiley; New York: 2001. p. 2.2.1-2.2.3.

Europe PMC Funders Author Manuscripts
Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

Tarcsa E, Marekov LN, Mei G, et al. Protein unfolding by peptidylarginine deiminase. Substrate specificity and structural relationships of the natural substrates trichohyalin and filaggrin. J Biol Chem. 1996; 271:30709–30716. [PubMed: 8940048]

Weninger W, Uthman A, Pammer J, et al. Vascular endothelial growth factor production in normal epidermis and in benign and malignant epithelial skin tumors. Lab Invest. 1996; 75:647–657. [PubMed: 8941211]

Wu P, Hou L, Plikus M, et al. Evo-Devo of amniote integuments and appendages. Int J Dev Biol. 2004; 48:249–270. [PubMed: 15272390]

Xu Z, Wang MR, Xu X, et al. Novel human esophagus-specific gene c1orf10:cDNA cloning, gene structure, and frequent loss of expression in esophageal cancer. Genomics. 2000; 69:322–330. [PubMed: 11056050]

Zimmer DB, Eubanks JO, Ramakrishnan D, et al. Evolution of the S100 family of calcium sensor proteins. Cell Calcium. 2013; 53:170–179. [PubMed: 23246155]

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

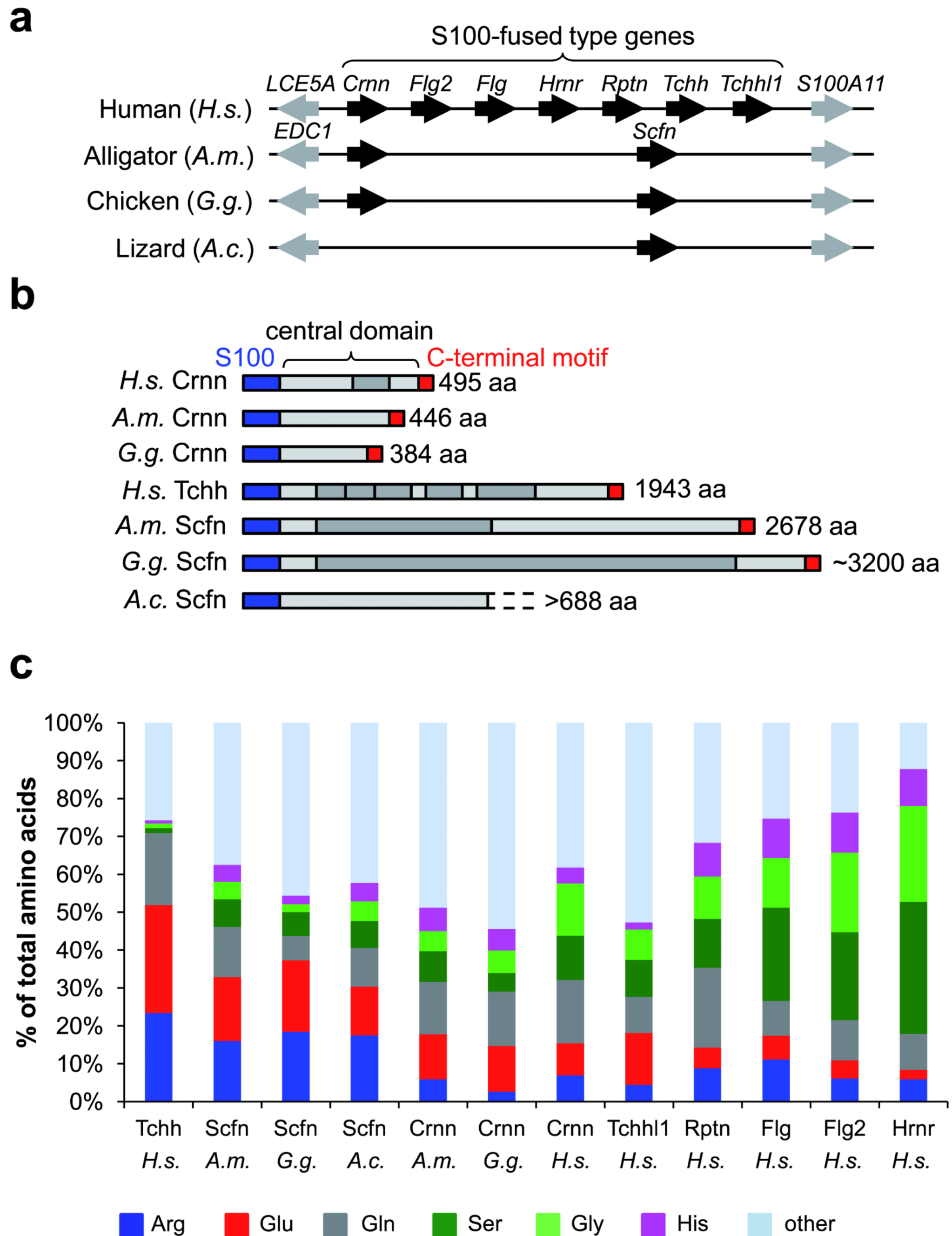

Fig. 1. Identification and phylogenetic analysis non-mammalian genes encoding S100 fused-type proteins (SFTPs)

(**a**) The chromosomal loci containing the SFTP genes of different species are depicted. Genes are represented by arrows pointing from the 5′ to the 3′-end. The genes flanking the SFTPs on the left side have not been assigned a name yet with the exception of LCE5A (late cornified envelope 5A); they are provisionally denoted here as EDC1 (epidermal differentiation complex gene 1). (**b**) Scheme of protein domains and sizes. Protein segments containing sequence repeats are indicated in dark grey. Lengths are indicated by numbers of amino acid residues (aa). The proteins are not drawn to scale. (**c**) Amino acid composition of

the central domain of SFTPs. Differently colored portions of each column indicate the percent of total amino acid residues present in the central domain. *A.c.*, *Anolis carolinensis*; *A.m.*, *Alligator mississippiensis*; *G.g.*, *Gallus gallus*; *H.s.*, *Homo sapiens*.

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

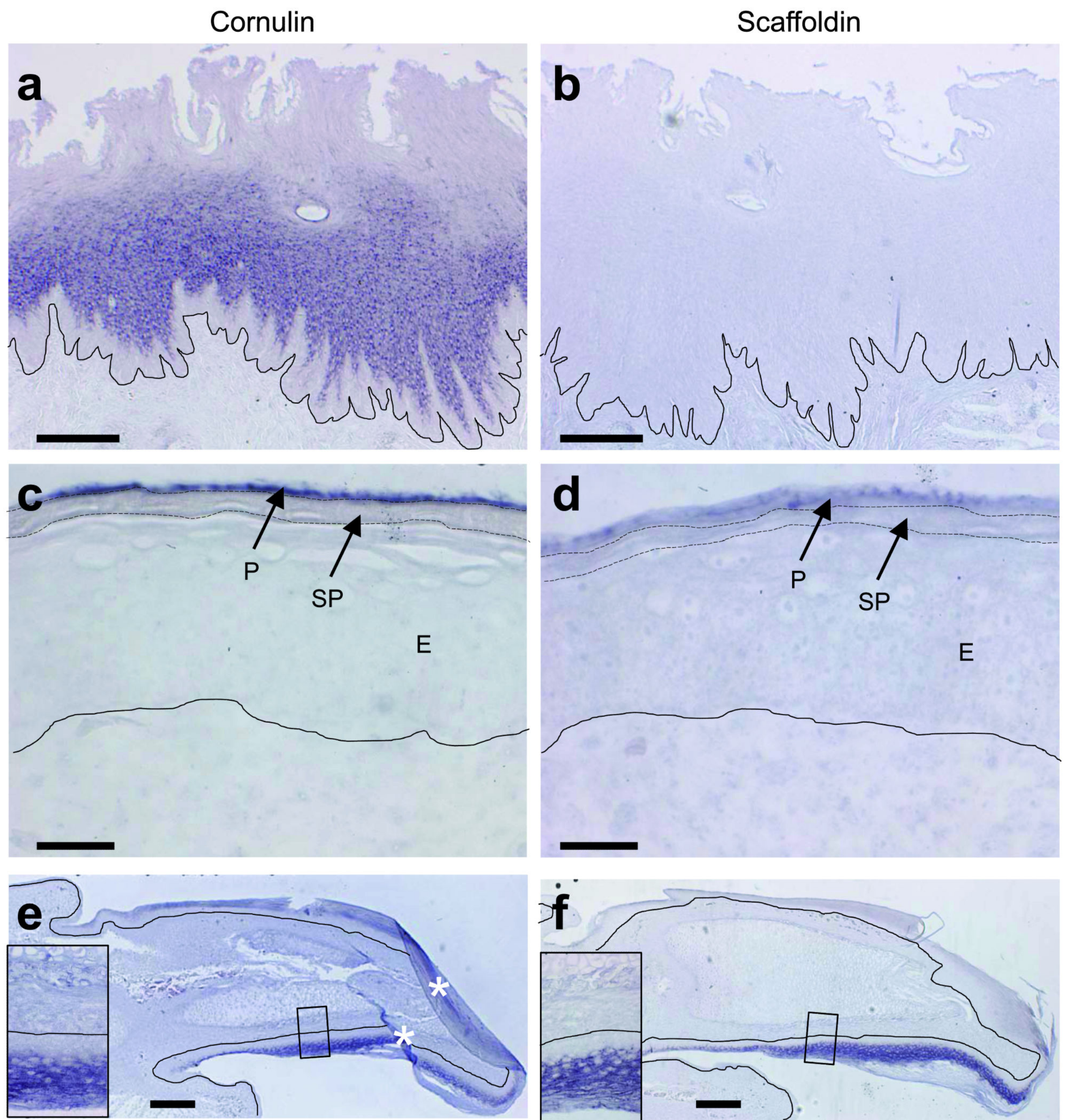

Fig. 2. *In situ* hybridization of cornulin and scaffoldin mRNAs in the chicken

The expression of mRNAs of cornulin (**a**, **c**, **e**) and scaffoldin (**b**, **d**, **f**) was analyzed by *in situ* hybridization of sections through the esophagus of adult chicken (**a**, **b**), skin including the periderm of chicken embryos at embryonic day E18 (**c**, **d**) and the claw unit of chicken embryos at embryonic day E14 (**e**, **f**). Bars: 20 µm (**a**-**d**), 200 µm (**e**, **f**). E, epidermis; P, periderm; SP, subperiderm. Folded areas of the section in **e** are indicated by asterisks. The position of the dermo-epidermal junction is marked by a line. The broken lines indicate the

borders of the subperiderm. Control stainings with sense probes were negative (Suppl. Figure S8).

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

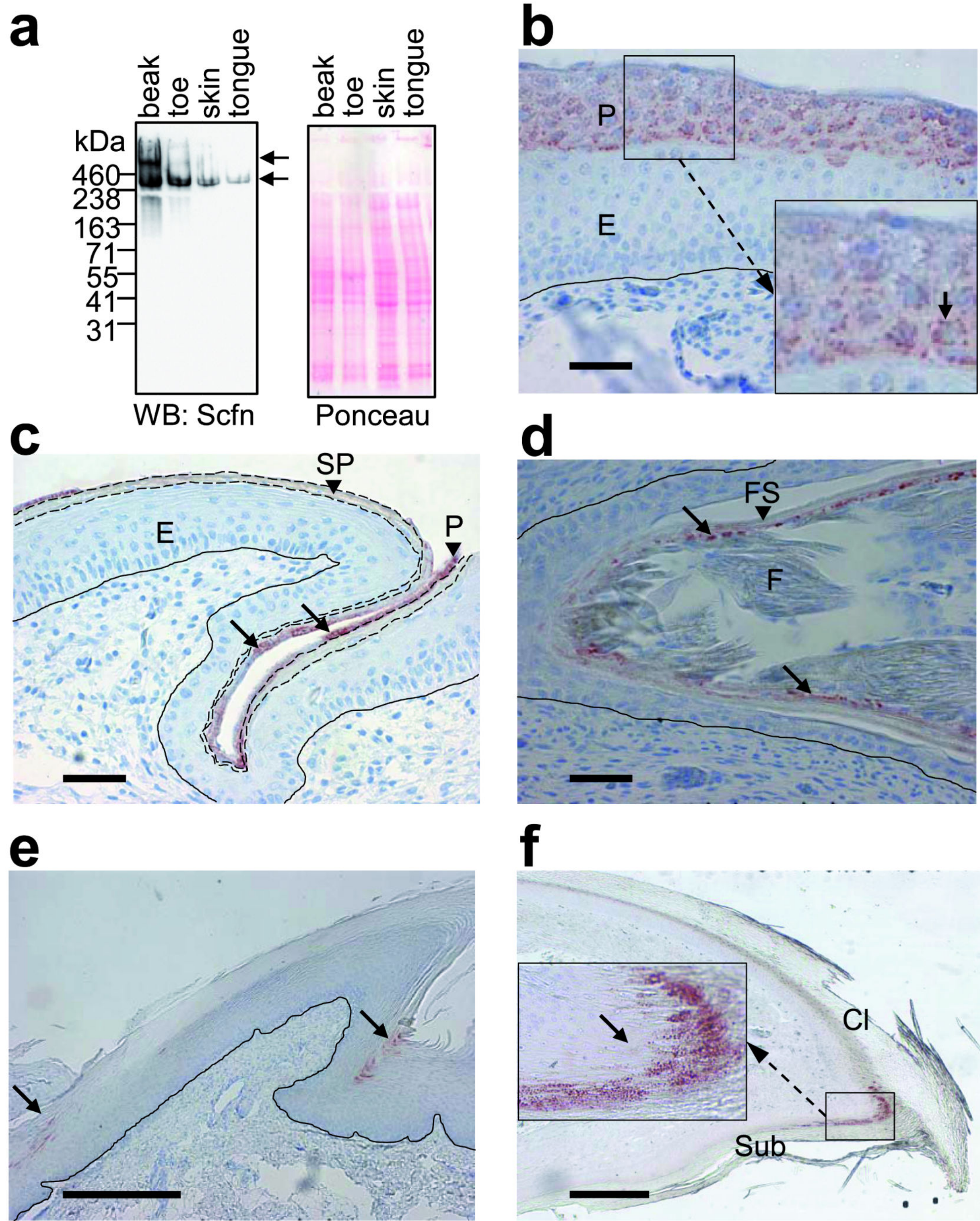

Fig. 3. Immunochemical analysis of scaffoldin expression in the chicken

Western blot analysis of scaffoldin and Ponceau staining of proteins blotted onto the membrane (**a**). (**b-f**) Immunohistochemical staining of scaffoldin (red), indicated by arrows, in the periderm of the egg tooth at E10 (**b**, inset: periderm granules), in the periderm of leg skin at E18 (**c**), in the feather sheath at E18 (**d**), in the tongue of an adult chicken (**e**) and in the subunguis of the claw apparatus of a 3-day old chick (**f**). The position of the dermo-epidermal junction is marked by a line. Broken lines indicate the borders of the subperiderm.

Bars: 40 μm (**b-d**). 300 μm (**e** and **f**). Cl, claw; E, epidermis; F, feather; FS, feather sheath; kDa, kilodalton; P, periderm; SP, subperiderm; Sub, subunguis; WB, Western blot.

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

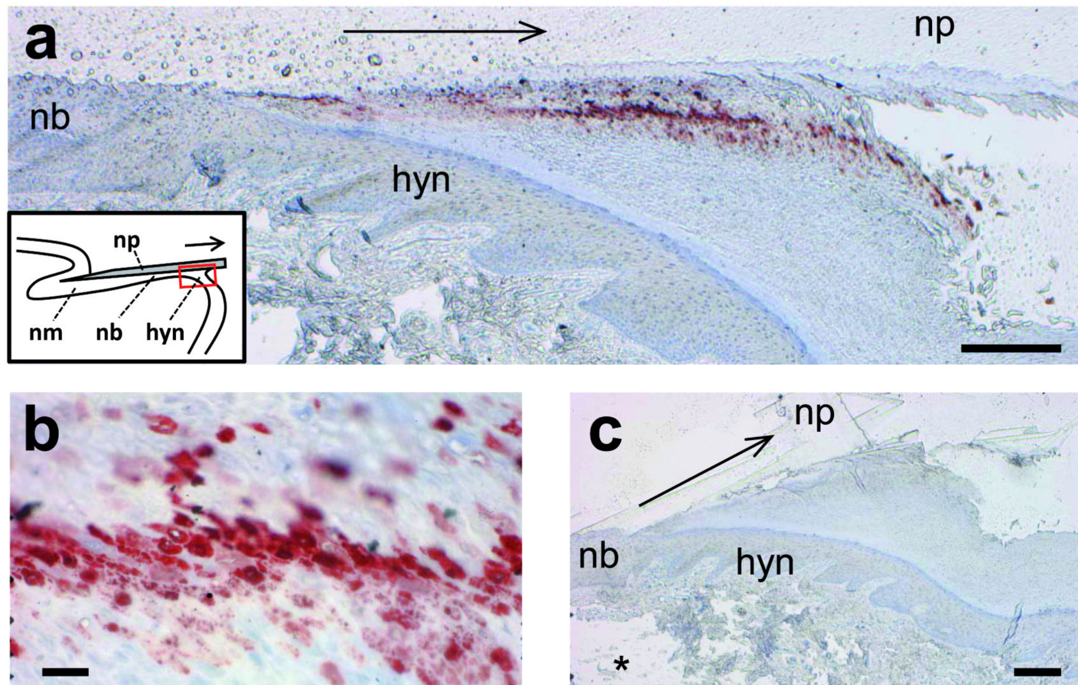

Fig. 4. Immunohistochemical detection of trichohyalin in the human nail unit

Longitudinal sections of the human nail unit were immunostained with an antibody against human trichohyalin. The immunostaining (red) was restricted to distinct cells of the hyponychium (**a**, **b**). A schematic overview of the nail unit is depicted in the inset of panel (**a**). The red box in the inset corresponds to the area shown in panel (**a**). Panel (**b**) shows a detail at higher magnification. A negative control reaction with an antibody isotype yielded no signal (**c**). An asterisk marks an artefactual discontinuity caused by the sectioning of the nail. The growth direction of the nail is indicated by arrows (**a** and **c**). Bars: 200 μm (**a**, **c**), 20 μm (**b**). hyn, hyponychium; nb, nail bed; nm, nail matrix; np, nail plate.

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

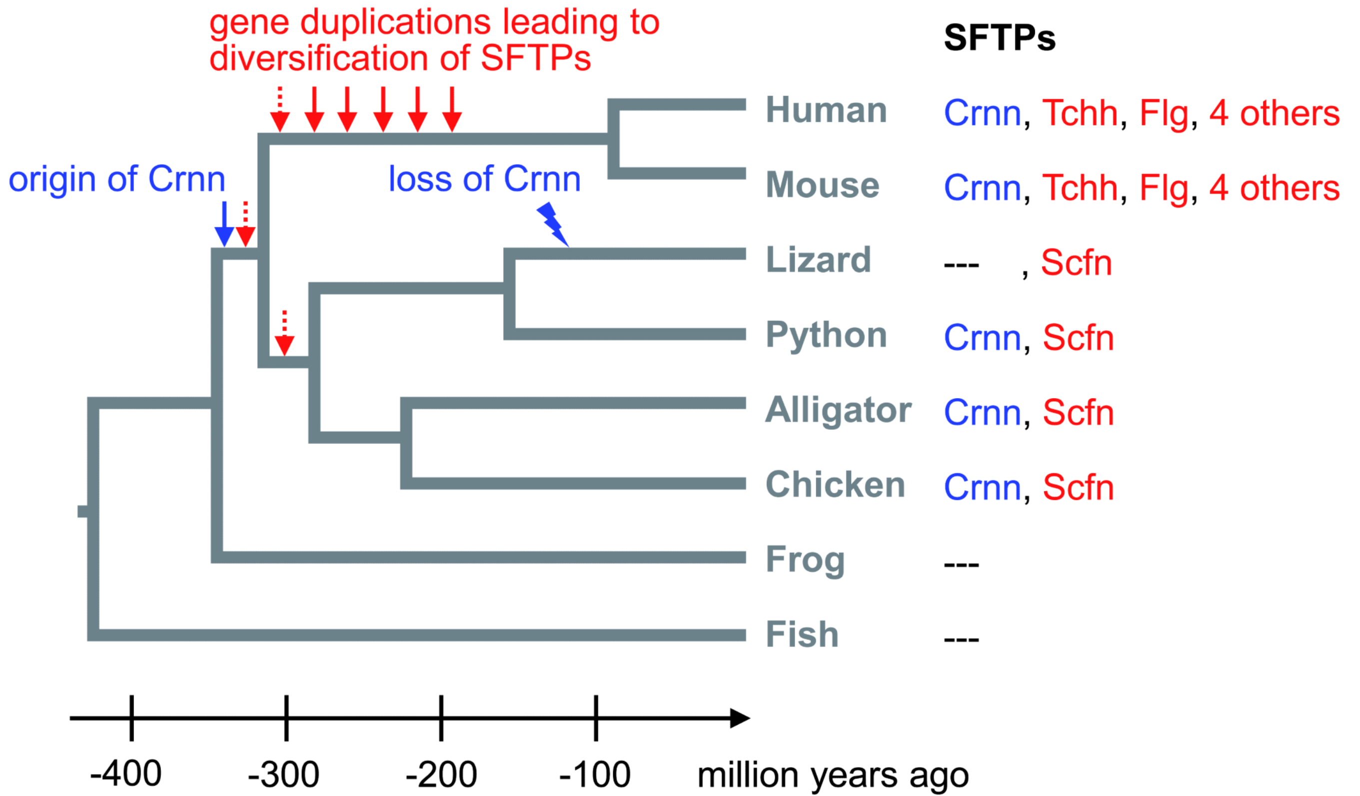

Fig. 5. Schematic overview of S100 fused-type protein (SFTP) evolution

The distribution of SFTPs among extant vertebrates and the inferred gene origin and loss events are shown on a phylogenetic tree. The divergence times of evolutionary lineages are indicated on the timescale at the bottom. Arrows with dotted lines indicate possible origins of the second SFTP gene either before or after the evolutionary divergence of sauropsids and mammals. Additional SFTP gene duplications have occurred in the mammalian lineage.