# Colocalization of Cystatin M/E and its Target Proteases Suggests a Role in Terminal Differentiation of Human Hair Follicle and Nail

Tsing Cheng[1], Ivonne M.J.J. van Vlijmen-Willems[1], Kiyotaka Hitomi[2], Marcel C. Pasch[1], Piet E.J. van Erp[1], Joost Schalkwijk[1] and Patrick L.J.M. Zeeuwen[1]

The cysteine protease inhibitor cystatin M/E is a key regulator of a biochemical pathway that leads to epidermal terminal differentiation by inhibition of its target proteases cathepsin L, cathepsin V, and legumain. Inhibition of cathepsin L is important in the cornification process of the skin, as we have recently demonstrated that cathepsin L is the elusive processing and activating protease for transglutaminase 3, an enzyme that is responsible for crosslinking of structural proteins in cornified envelope formation. Here, we study the localization of all players of this pathway in the human hair follicle and nail unit in order to elucidate their possible role in the biology of these epidermal appendages. We found that cathepsin L and transglutaminase 3 specifically colocalize in the hair bulb and the nail matrix, the regions that provide cells that terminally differentiate to the hair fiber and the nail plate, respectively. Furthermore, transglutaminase 3 also colocalizes with the structural proteins loricrin and involucrin, which are established transglutaminase substrates. These findings suggest that cathepsin L and transglutaminase 3 could be involved in the pathway that leads to terminal differentiation, not only in the epidermis but also in the human hair follicle and nail unit.

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## INTRODUCTION

Recent work of our group has revealed a previously unreported biochemical pathway that controls skin barrier formation by regulation of both crosslinking and desquamation of the stratum corneum. The cysteine protease inhibitor cystatin M/E, which is a key molecule in this pathway, has two important regulatory functions in epidermal differentiation and hair follicle morphogenesis. First, cystatin M/E regulates crosslinking of structural proteins by transglutaminase 3 (TGase 3) in the cornification process of the epidermis and the hair follicle by controlling cathepsin L (CTSL) and legumain activities (Zeeuwen *et al.*, 2004; Cheng *et al.*, 2006). We provide evidence for this assumption when we demonstrated that human CTSL is the elusive enzyme that is able to process and activate human TGase 3, an epidermis-specific enzyme responsible for the crosslinking of loricrin and small proline-rich proteins (SPRs) in the cornification process of the skin (Cheng *et al.*, 2006). During keratinocyte differentiation, TGase 3 is activated by limited proteolysis (Hitomi *et al.*, 1999; Ahvazi *et al.*, 2002), a process that is apparently under control of cystatin M/E, at least during skin morphogenesis in the neonatal phase (Zeeuwen *et al.*, 2004). We suppose that processing and activation of TGase 3 in terminally differentiating keratinocytes is regulated by the inhibitory activity of cytoplasmic cystatin M/E against CTSL. Legumain inhibition by cystatin M/E could have a regulatory role in CTSL activity as legumain is involved in CTSL processing (Maehr *et al.*, 2005). As a second regulatory function, cystatin M/E could have a function in desquamation by the regulation of cathepsin V (CTSV) protease activity, which is involved in the degradation of corneodesmosomal components. We recently showed that cystatin M/E and CTSV are separately transported within lamellar granules and that both proteins are secreted in the extracellular space of the stratum corneum where they associate with corneodesmosomes as determined at the ultrastructural level (Zeeuwen *et al.*, 2007).

Misregulation of this pathway by unrestrained protease activity, as seen in cystatin M/E-deficient mice, leads to abnormal stratum corneum and hair follicle formation, disturbance of skin barrier function, and neonatal death (Sundberg *et al.*, 1997; Zeeuwen *et al.*, 2002, 2004). The importance of regulated proteolysis in epithelia is well demonstrated by the recent identification of the *SPINK5*

[1]*Department of Dermatology, Nijmegen Centre for Molecular Life Sciences, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands and* [2]*Department of Applied Molecular Biosciences, Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya, Japan*

*Correspondence: Professor Joost Schalkwijk or Dr Patrick L.J.M. Zeeuwen, Department of Dermatology, Nijmegen Centre for Molecular Life Sciences, Radboud University Nijmegen Medical Centre, P.O. Box 9101, 6500 HB, Nijmegen, The Netherlands. E-mails: j.schalkwijk@ncmls.ru.nl or p.zeeuwen@ncmls.ru.nl*

*Abbreviations: CTSL, cathepsin L; CTSV, cathepsin V; IRS, inner root sheath; ORS, outer root sheath; TGase, transglutaminase; THH, trichohyalin*

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serine protease inhibitor as the defective gene in the Netherton syndrome, cathepsin C mutations in the Papillon-Lefevre syndrome, ichthyosis because of matriptase deficiency, and targeted ablation of CTSL and cathepsin D in mice (for a comprehensive review see Zeeuwen, 2004).

The hair follicle and nail unit are epidermal appendages that also show processes of terminal differentiation. Hair follicles undergo cycles of growth, regression, and rest, which are, respectively, named anagen, catagen, and telogen phase (Paus and Foitzik, 2004; Alonso and Fuchs, 2006). During the anagen phase the entire hair from tip to root is produced when matrix cells in the hair bulb proliferate and differentiate into the several layers of the hair follicle (Figure 1): the medulla, cortex, and cuticle layer of the hair shaft (the actual hair fiber); the Huxley's and Henle's layers, and the cuticle of the inner root sheath (IRS), and the outer root sheath (ORS) (Niemann and Watt, 2002; Fuchs, 2007). Keratinization of hair follicles occurs in specific compartments, that is the hair shaft, cuticle, and IRS, through crosslinking of structural precursor proteins by TGases (Commo and Bernard, 1997; Thibaut *et al.*, 2005). The degeneration of the IRS in the infundibulum of the hair follicle near the opening of the sebaceous duct requires desquamation, a process in which proteolytic enzymes are involved (Ekholm and Egelrud, 1998).

Contrary to hairs, nails show continuous growth of the hard nail plate out of the nail matrix, sliding over the underlying nail bed (Dawber *et al.*, 2001; Baran *et al.*, 2005). Keratinization of the nail starts in the nail matrix, but the relative contribution of TGases to the formation of nail corneocytes which end up in the nail plate is not exactly known. Nevertheless, it is known that lack of TGase 1 in lamellar ichthyosis patients could be reflected in structural nail alterations (Rice *et al.*, 2003).

In the present study we sought to address the possible role of cystatin M/E and its physiological target proteases in the biology of the human nail unit and hair follicle. We have examined the localization of cystatin M/E, CTSV, CTSL, TGase 3, and legumain in (longitudinal and/or transversal) sections of the human hair follicle and nail unit by immunofluoresence microscopy. We also examined the localization of known TGase substrates like the structural proteins involucrin, loricrin, and trichohyalin (THH). We found colocalization of CTSL, TGase 3, involucrin, and loricrin in the hair bulb and the nail matrix, suggesting that these proteins are key players in the pathway that initiate biochemical changes in the terminal differentiation of these epidermal appendages.

## RESULTS

### Hair follicle

Immunofluorescence microscopy was performed on both longitudinal and transversal sections of human hair follicle in order to examine the localization of cystatin M/E and its physiological target proteases in the different layers of the hair follicle. For a comprehensive biochemical and kinetic analysis of the interaction between cystatin M/E and its target proteases (see Cheng *et al.*, 2006). Double staining was performed for cystatin M/E with its target proteases, for TGase 3 with its activating protease CTSL, and for TGase 3 with its presumed substrates (Figures 2–4).

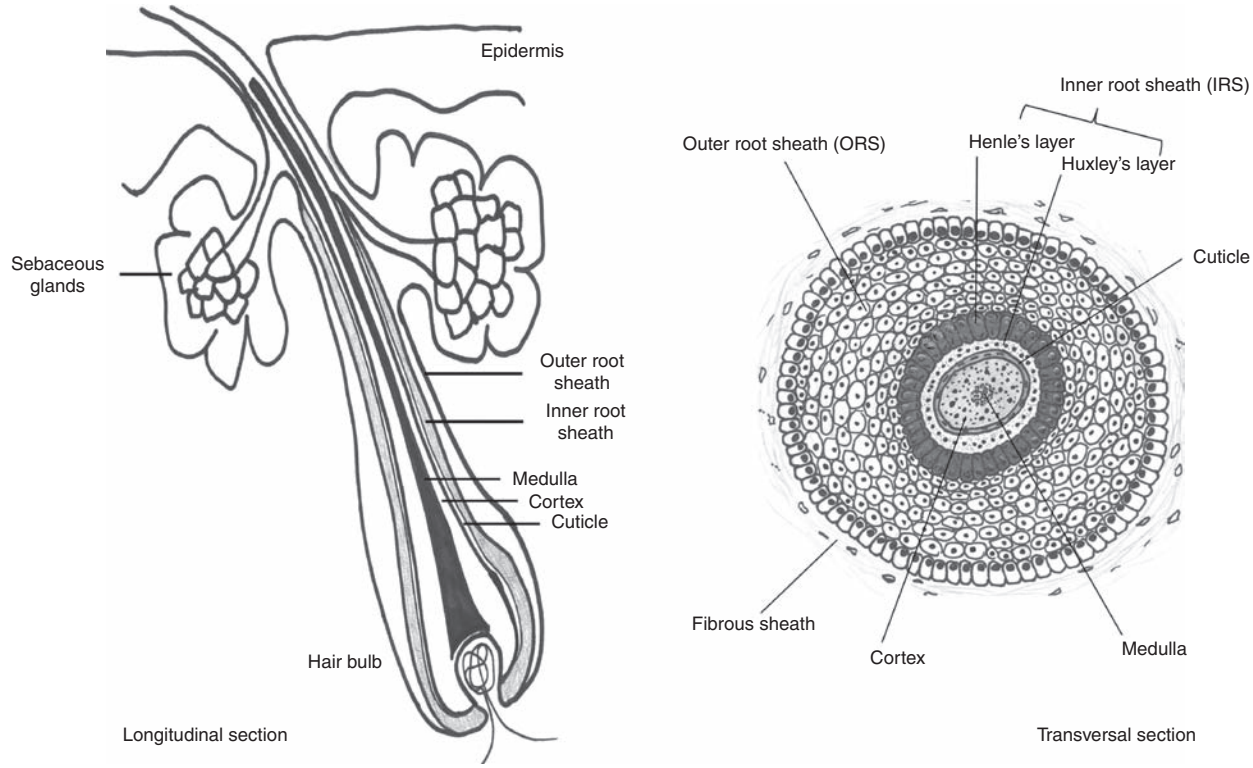


**Figure 1. Schematic overview of longitudinal and transversal sections demonstrating the various components of the hair follicle.**

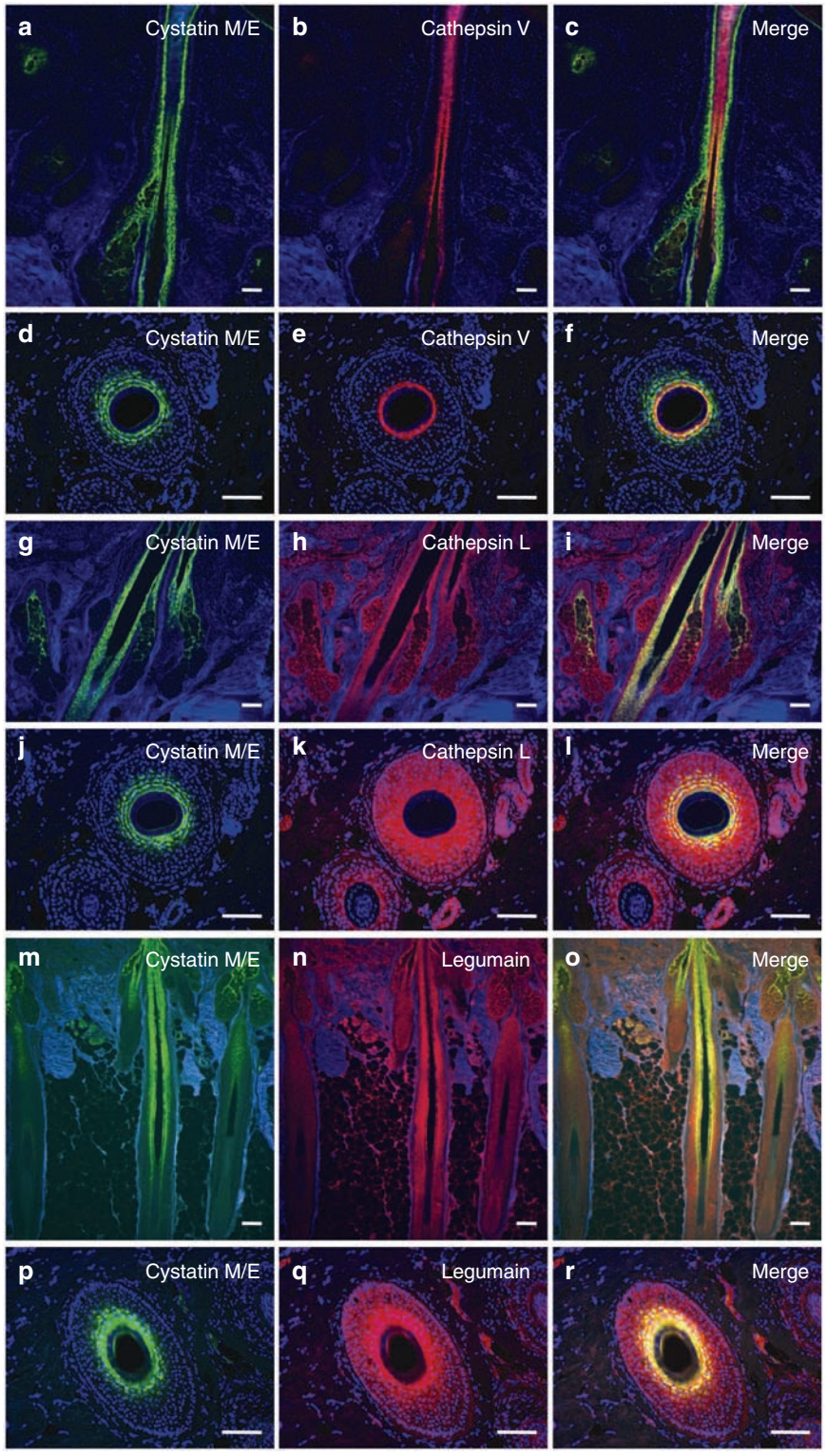


**Figure 2. Colocalization of cystatin M/E and its target proteases CTSV, CTSL, and legumain in human hair follicle.** Immunofluorescence staining of (**a**, **d**, **g**, **j**, **m**, and **p**) cystatin M/E (green color), and (**b**, **e**, **h**, **k**, **n**, and **q**) CTSV, CTSL, or legumain (red color), and (**c**, **f**, **i**, **l**, **o**, and **r**) double staining (yellow–orange merge color) for both proteins on (**c**, **i**, and **o**) longitudinal and (**f**, **l**, and **r**) transversal sections of the hair follicle. Nuclei are blue (DNA staining by 4′, 6-diamine-2′-phenylindole dihydrochloride (DAPI)). Bar = 100 μm.

Cystatin M/E is expressed in the IRS, the inner half of the ORS, and in the sebaceous glands of the hair follicle. However, cystatin M/E is only expressed in the distal part and infundibulum of the hair follicle, but is absent in the proximal part, the hair bulb (Figures 2a, g, m, and 3g). Furthermore, staining of the IRS is only strong in the Henle's layer;

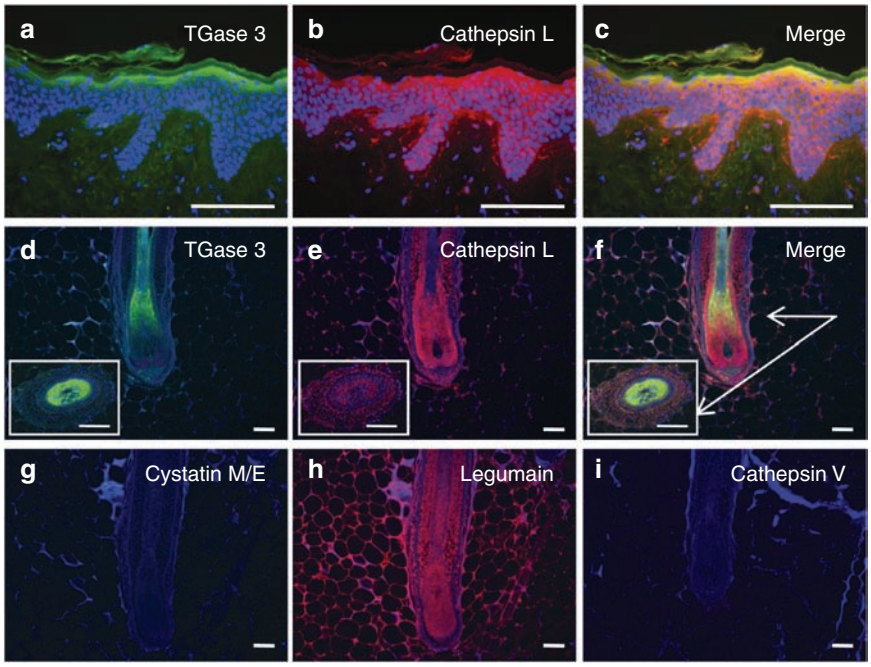


**Figure 3. Colocalization of TGase 3 and CTSL in human epidermis and hair follicle.** Immunofluorescence staining of (**a** and **d**) TGase 3 (green color), and (**b** and **e**) CTSL (red color), and (**c** and **f**) double staining (yellow–orange merge color) for both proteins on epidermis (**a**–**c**), and both longitudinal (**d**–**f**) and transversal (insets **d**–**f**) sections of the hair follicle. The arrows (**f**) show the position of the transversal section (inset). Immunofluorescence staining of legumain was found in the (**h**) hair bulb, whereas no positive staining could be detected for (**g**) cystatin M/E or (**i**) CTSV in this part of the follicle. Nuclei are blue by DAPI staining for DNA. Bar = 100 μm.

expression of cystatin M/E is weak or absent in the cuticle and Huxley's layer (Figure 2d, j, and p). Colocalization of cystatin M/E and CTSV can be found specifically in the Henle's layer of the IRS, as CTSV expression is restricted to this layer of cells of the hair follicle (Figure 2e–f). Interestingly, colocalization of cystatin M/E and CTSV is only seen in the infundibulum of the hair follicle near the opening of the sebaceous ducts into the hair canal (Figure 2c). CTSL shows a less restricted expression pattern and is expressed in IRS (but not in the Huxley's layer), ORS, and sebaceous glands (Figure 2h and k). Coexpression of cystatin M/E and CTSL can be seen in the distal part and infundibulum of the hair follicle and is located in the Henle's layer of the IRS, the inner half of the ORS, and the sebaceous glands (Figure 2i and l). CTSL is also expressed in the medulla and cortex of the hair shaft, but only in the lower hair bulb (inset Figure 3e). Legumain expression can be found throughout the entire hair follicle resulting in a colocalization with cystatin M/E in the IRS, the inner layers of the ORS, and the sebaceous glands (Figure 2o and r), but again only in the distal part and infundibulum of the hair follicle.

TGases are enzymes involved in protein crosslinking of structural proteins in cornified envelope formation. In human epidermis TGase 3 is expressed in the upper layers of the stratum granulosum and the stratum corneum, where the keratinocytes undergo cornification (Figure 3a). Previously, we have demonstrated that CTSL is the elusive enzyme that processes and activates TGase 3. Here, we show that in human epidermis TGase 3 and CTSL colocalize in the stratum granulosum (Figure 3c). Note that the yellow merge staining forms a transition zone between the red CTSL staining in the spinous and granular layers and the green TGase 3 staining in the upper granular layers and the stratum corneum, which suggests that first the activating protease is expressed followed by its substrate. Double staining of TGase 3 and CTSL in the hair follicle shows a similar overlapping colocalization in the hair bulb (Figure 3f). First, CTSL is expressed at the bottom of the hair bulb followed by the expression of TGase 3 in the upper part of the hair bulb. TGase 3 expression is restricted to the medulla and cortex of the hair shaft, whereas CTSL is also expressed in the root sheaths (see insets Figure 3d–f). Subsequently, CTSL expression disappears from the hair shaft and only TGase 3 expression remains (Figure 3f). However, staining of TGase 3 in the hair shaft was only seen in the proximal part of the hair follicle; no expression could be detected in the distal part and infundibulum of the hair follicle (data not shown). Remarkably, cystatin M/E is not present in the hair bulb, as is CTSV, whereas legumain staining was positive for this section (Figure 3g–i). During epidermal cornification TGase 3 is responsible for crosslinking of loricrin and small proline-rich proteins into the cornified envelope. Loricrin is abundantly expressed in the hair shaft; a weak signal can be detected in the Henle's layer of the IRS, but not in Huxley's layer (Figure 4b). This expression pattern results in a colocalization with TGase 3 in the medulla, cortex, and cuticle of the hair shaft (Figure 4c). We also found colocalization of TGase 3 with involucrin, another structural protein of cornified envelopes,

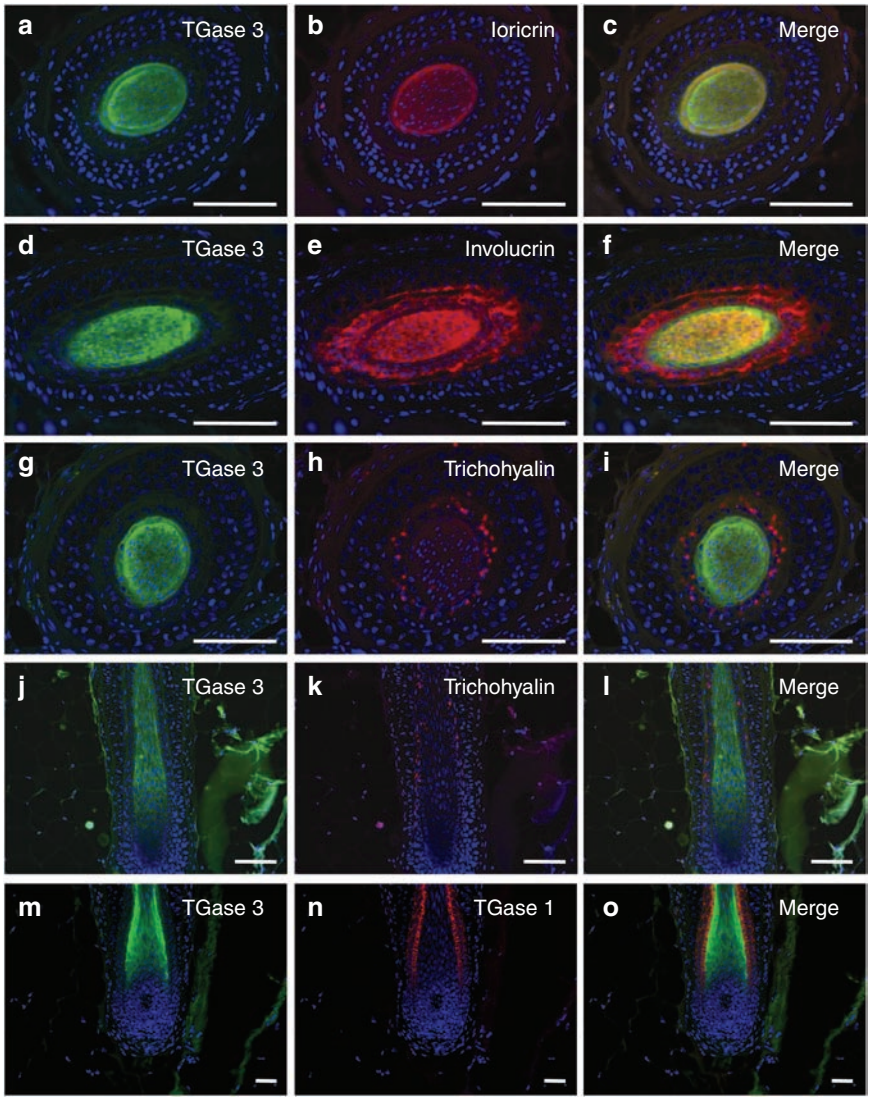


**Figure 4. TGase 3 and its presumed substrates in human hair follicle.** Immunofluorescence staining of (**a**, **d**, **g**, **j**, and **m**) TGase 3 (green color), and (**b**, **e**, **h**, **k**, and **n**) loricrin, involucrin, THH, or TGase 1 (red color), and (**c**, **f**, **i**, **l**, and **o**) double staining (yellow–orange merge color) for these proteins on transversal (**a**–**i**) and longitudinal (**j**–**o**) sections of the hair follicle. Nuclei are blue by DAPI staining for DNA. Bar = 100 μm.

as involucrin is expressed in the hair shaft and the IRS (Figure 4e). However, colocalization of these proteins is only detected in the medulla and cortex, but not the cuticle, as involucrin seems to be absent in this layer as indicated by green staining surrounding the medulla and cortex (Figure 4f). Immunofluorescence detection of THH, the major structural component of hair follicles and a presumed substrate for TGase 3, shows positive staining of granules in the IRS (Figure 4h and k). However, TGase 3 and THH show no colocalization in this part of the hair follicle (Figure 4i and l). Finally, we studied the distribution of TGase 1 in the hair follicle as TGase 3 expression is restricted to the hair shaft. Interestingly, TGase 1 is specifically expressed in the IRS at the same location where THH is expressed (Figure 4k and n). Double staining of TGase 1 and TGase 3 shows no overlap between both enzymes in the hair follicle (Figure 4o).

**Nail unit**

Five specific regions of the nail unit were studied in close-up, namely the epidermis (at the proximal side of the nail), the proximal nail fold, the nail matrix, the nail bed, and the hyponychium (Figure 5). We performed immunofluorescence microscopy for double staining of cystatin M/E and its target protease CTSL, and for TGase 3 with its activating protease CTSL (Figures 6 and 7).

Cystatin M/E is expressed in the upper granular layers of epidermis and proximal nail fold (Figure 6a and d), but expression diminishes towards the nail matrix where staining of cystatin M/E is faint and located in the cell layers bordering the nail plate (Figure 6g). Cystatin M/E is not present in the nail bed, but reappears at the hyponychium (Figure 6j and m). Positive staining of CTSL can be seen throughout the entire nail unit and is suprabasal with the exception of the stratum corneum of the epidermis and hyponychium

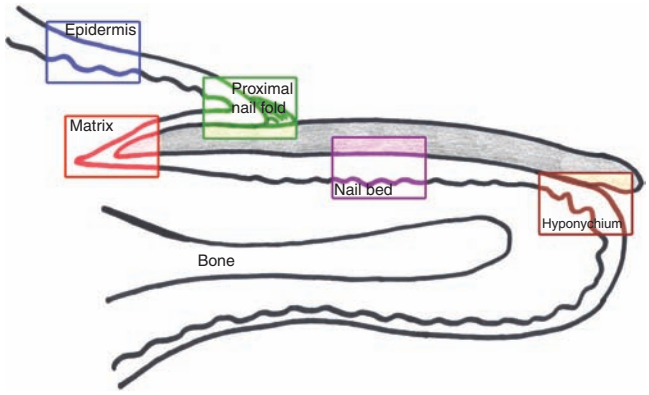


**Figure 5. Cross-section through the finger demonstrating the various components of the nail unit.** Different parts of the nail unit (epidermis, proximal nail fold, nail matrix, nail bed, and hyponychium) that were examined for immunohistochemistry in this study are marked in a colored box.

(Figure 6b, e, h, k, and n), resulting in a colocalization with cystatin M/E in all regions of the nail unit that express cystatin M/E (Figure 6c, f, i, l, and o). The double staining of cystatin M/E and legumain shows a similar picture as legumain is also expressed in the entire nail unit (data not shown). No colocalization of cystatin M/E with CTSV can be found as CTSV seems to be absent in the nail unit, except for the epidermis and the hyponychium (data not shown). TGase 3 is expressed in all regions of the nail unit except the nail bed, which is completely negative for TGase 3 (Figure 7a, d, g, j, and m). The expression of TGase 3 in the epidermis, proximal nail fold, and hyponychium is suprabasal, but most intense in the upper granular layers, although the distal part of the proximal nail fold shows a weaker expression of TGase 3. However, TGase 3 staining is very strong in the nail matrix, but restricted to only the upper cell layers bordering the nail plate (Figure 7g). Colocalization of TGase 3 and CTSL is seen in the granular layers of the nail unit, that is epidermis,

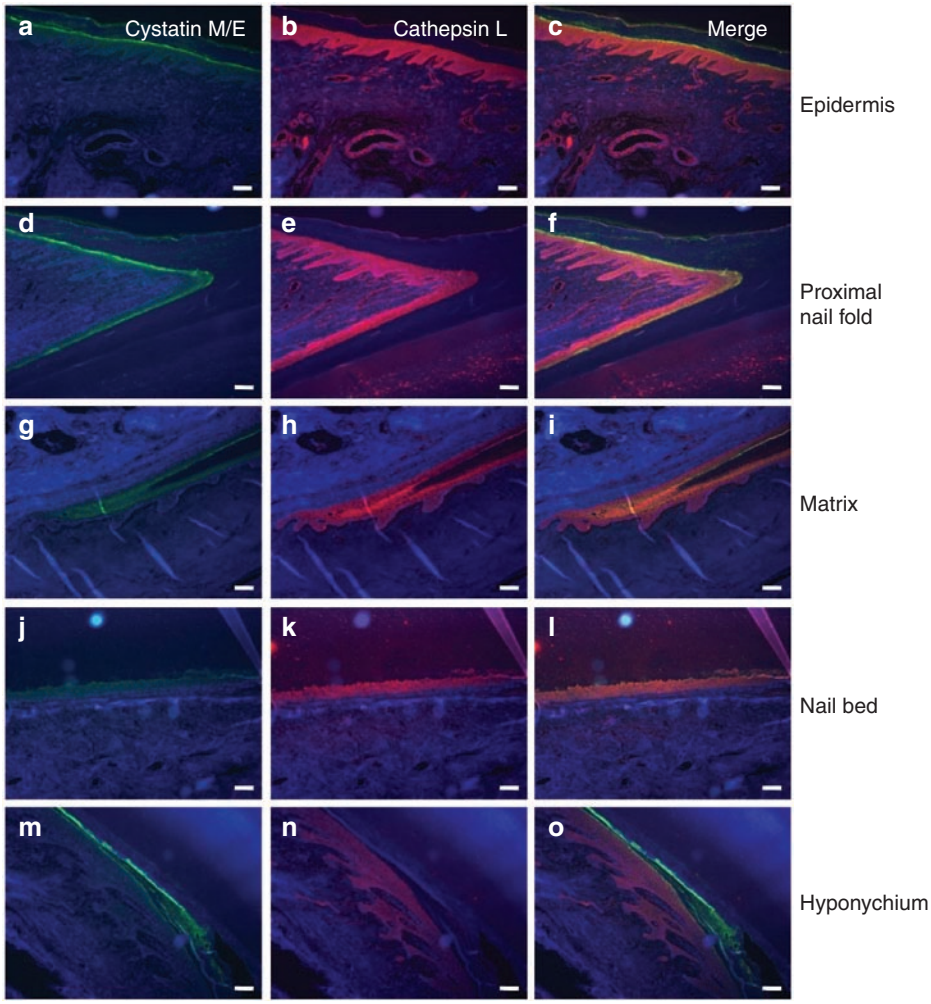


**Figure 6. Colocalization of cystatin M/E and CTSL in the human nail unit.** Immunofluorescence staining of (**a**, **d**, **g**, **j**, and **m**) cystatin M/E (green color), and (**b**, **e**, **h**, **k**, and **n**) CTSL (red color), and (**c**, **f**, **i**, **l**, and **o**) double staining (yellow–orange merge color) for both proteins in epidermis (**a**–**c**), proximal nail fold (**d**–**f**), nail matrix (**g**–**i**), nail bed (**j**–**l**), and hyponychium (**m**–**o**). Nuclei are blue by DAPI staining for DNA. Bar = 100 μm.

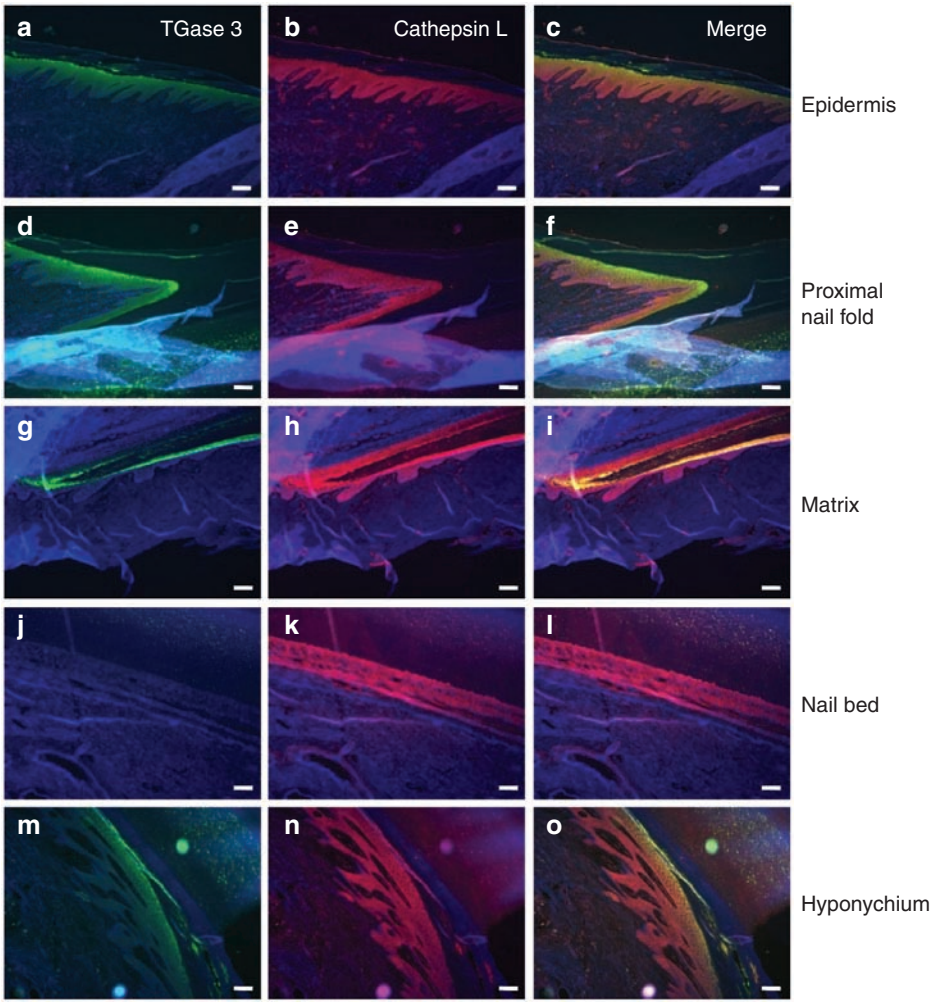


**Figure 7. Colocalization of TGase 3 and CTSL in the human nail unit.** Immunofluorescence staining of (**a**, **d**, **g**, **j**, and **m**) TGase 3 (green color), and (**b**, **e**, **h**, **k**, and **n**) CTSL (red color), and (**c**, **f**, **i**, **l**, **o**) double staining (yellow–orange merge color) for both proteins in epidermis (**a**–**c**), proximal nail fold (**d**–**f**), nail matrix (**g**–**i**), nail bed (**j**–**l**), and hyponychium (**m**–**o**). Nuclei are blue by DAPI staining for DNA. Bar = 100 μm.

proximal nail fold, and hyponychium (Figure 7c, f, and o), but is most prominent in the nail matrix (Figure 7i).

## DISCUSSION

Cystatin M/E is a key regulator of a previously unreported biochemical pathway that leads to epidermal terminal differentiation through its inhibitory activities against its target proteases CSTL, CTSV, and legumain. Inhibition of CTSL is important in the cornification process as CTSL processes and activates TGase 3. In this study we analyzed the localization of the key molecules of this pathway in the human hair follicle and nail unit. CTSL and TGase 3 specifically colocalize in the hair bulb and the nail matrix, the regions that provide cells that terminally differentiate into the hair fiber and the nail plate, respectively. Moreover, the structural proteins loricrin and involucrin are also expressed at these sites suggesting an involvement in the pathway that leads to terminal differentiation, not only in the epidermis but also in the human hair follicle and nail unit.

Our results show that CTSL and TGase 3 are coordinately expressed in the epidermis and the developing human hair follicle. CTSL expression appears in the lower (suprabasal) layers of the epidermis where terminal differentiation of the cells starts, followed by the expression of TGase 3 in the upper granular layer where processing and activation of the crosslinking enzyme takes place (Figure 3). Likewise, CTSL is expressed in the bottom of the hair bulb, which contains matrix cells that produce progeny cells that terminally differentiate to form the growing hair shaft. Just above this area, TGase 3 appears in the hair bulb at a location where hair shaft precursors are normally expressed (Rogers, 2004). The colocalization of CTSL and TGase 3 in the hair bulb fits well with our previous discovery that CTSL is the elusive protease that processes and activates TGase 3 (Cheng *et al.*, 2006). The nail matrix does also show a gradual transition from CTSL to TGase 3 expression (Figure 7i). Terminally differentiated cells comprising the nail bed originate from the nail matrix, which suggests that activation of TGase 3 takes place at a location that is analogous to the human hair bulb, the location where the matrix cells of the hair reside. Remarkably, cystatin M/E is absent in the hair bulb and almost not detectable in the nail matrix, which could mean

that full, uninhibited activity of CTSL is necessary for activating sufficient amounts of TGase 3. It is also possible that another cysteine protease inhibitor regulates CTSL activity in these regions. Cystatin A is able to inhibit CTSL and has been detected in hair shaft and nail extracts (Tsushima *et al.*, 1992; Tsushima, 1993). Expression of cystatin M/E starts above the hair bulb and is further continuously present in the distal part and the infundibulum, up to the epidermis where the hair exits the skin. One might speculate that in these parts of the hair follicle CTSL has other functions than processing TGase 3 (which is not expressed at these sites), and that at these locations CTSL activity has to be regulated by cystatin M/E. Nonetheless, the colocalization of CTSL and TGase 3 supports the proposed role of CTSL in the processing and activation of TGase 3 and the ensuing keratinization of the hair shaft and nail matrix.

In the epidermis TGases are responsible for crosslinking of loricrin, involucrin, and other structural barrier proteins into the cornified envelope of keratinocytes (Candi *et al.*, 2005). To our knowledge we showed for the first time colocalization of TGase 3 and loricrin in the hair follicle suggesting that TGase 3 is involved in cornified envelope formation of this epidermal appendage. We also found expression of loricrin and involucrin in the nail matrix at the same location were TGase 3 is expressed (not shown). The colocalization of TGase 3 and involucrin in the hair follicle was surprising as a previous study, also based on immunofluorescence microscopy, reported that these proteins did not colocalize in the hair follicle. It was found that involucrin was abundantly expressed only in the IRS and the internal layer of the ORS of the hair follicle, whereas TGase 3 expression was restricted to the hair shaft and the hair cuticle (Thibaut *et al.*, 2005). We could confirm the involucrin expression in the IRS, but in contrast to the study of Thibaut *et al.*, we also found high expression of involucrin in the medulla and the cortex of the hair shaft at the level of the hair bulb. We suppose that the transversal sections of the hair follicle in our study (Figure 4) originate from a more proximal part of the hair follicle, whereas the transversal sections in the study of Thibaut *et al.* were taken at a higher level of the follicle. Nevertheless, as TGase 3 is not expressed in the IRS other TGases such as TGase 1 and TGase 5, which are both expressed in the IRS and the internal layer of the ORS, could be responsible for involucrin crosslinking in the more distal parts of the hair follicle.

Surprisingly, our results showed no colocalization of TGase 3 and THH in the hair follicle. THH could be a substrate of TGase 3 in the hair shaft as a biochemical *in vitro* study has revealed that TGase 3 is the most efficient crosslinking enzyme of THH and also showed colocalization of TGase 3 and THH in mouse skin (Tarcsa *et al.*, 1997). THH is a major structural hair follicle protein that is crosslinked to cornified envelope proteins and keratin intermediate filaments in cells of the hair shaft and the IRS providing mechanical strength to the cells (Lee *et al.*, 1993; Steinert *et al.*, 2003). In our study THH is specifically expressed in the Huxley's layer of the IRS, whereas previous studies generally reported localization of THH in the IRS and also in the medulla (Rothnagel and Rogers, 1986; Hamilton *et al.*, 1991; Tarcsa *et al.*, 1997; Steinert *et al.*, 2003). However, none of these studies did use human material, and it is clear that THH expression and also the expression of TGase 3 is different between mice and men. Furthermore, in these studies a polyclonal antibody was used to detect TGase 3, which could possibly crossreact with other TGases resulting in the positive staining of the IRS. As TGase 3 does not show staining in the IRS in our study, other TGases could be responsible for THH crosslinking at this location. We found that TGase 1 is specifically expressed in the IRS (Figure 4n) suggesting that not TGase 3 but TGase 1 is the crosslinking enzyme of THH in human hair follicle. This colocalization of THH and TGase 1 in the IRS of the human hair follicle was previously also found by another group (Commo and Bernard, 1997). In our study THH could not be detected in the nail unit, whereas a previous study showed staining of the nail matrix for THH. Interestingly, the matrix cells showed an apparent absence of THH granules and only cytoplasmic THH was detected (O'Keefe *et al.*, 1993). This could explain our results as the used THH antibody of our choice, AE15, only stains THH in granules and not when THH is associated with keratin intermediate filament (O'Guin *et al.*, 1992; Takahashi *et al.*, 2007).

We showed colocalization of cystatin M/E and CTSV in the IRS of the hair follicle, especially in Henle's layer (Figure 2a–f). This colocalization is restricted to the infundibulum of the hair follicle around the opening of the sebaceous duct into the hair canal. At this point the IRS is degenerated allowing the secretion of sebum into the hair canal and movement of the hair fiber (Stenn and Paus, 2001; Alonso and Fuchs, 2003). The expression pattern of cystatin M/E and CTSV suggests that both proteins are involved in the degeneration of the IRS, possibly through desquamation of the IRS cells. Epidermal desquamation requires the proteolysis of corneodesmosomes, specialized junctions that keep epidermal cells together. In normal human skin the kallikreins KLK5 and KLK7 (two epidermis-specific serine proteases) are able to degrade corneodesmosomal proteins desmoglein-1, desmocollin-1, and corneodesmosin (Caubet *et al.*, 2004) and it is presumed that cysteine proteases such as CTSV could have a similar function (Elias, 2005; Zeeuwen *et al.*, 2007). Desquamation in the hair follicle could result from proteolytic activity of both serine and cysteine proteases. Desmoglein-1 and corneodesmosin are expressed in the IRS as well as KLK7 (Ekholm and Egelrud, 1998; Nachat *et al.*, 2005), which both show an identical expression pattern as observed for CTSV in this study. Altogether these findings provide a plausible explanation for the degeneration of the IRS at the opening of the sebaceous duct. Furthermore, we found no expression of CTSV in the ventral part of the proximal nail fold (bordering the nail plate), the nail matrix, and the nail bed. As desquamation does not occur in the regions bordering the nail plate, CTSV is not expected to be expressed at this location.

It is likely that regulation of protease activity by cystatin M/E is important in terminal differentiation of the hair follicle and nail unit, and that disturbance could lead to abnormal development of these skin appendages. Cystatin

M/E-deficient mice show a severe skin and hair phenotype characterized by defects in cornification processes, although the nails on digits of both front and rear paws were normal (Sundberg *et al.*, 1997). It must be noted however, that these mice die shortly after birth, and nail abnormalities cannot be studied beyond day 10. CTSL-deficient mice show abnormal hair follicle cycling and morphogenesis including faulty cornification and incomplete desquamation of the IRS (Roth *et al.*, 2000; Benavides *et al.*, 2002; Tobin *et al.*, 2002). The latter observation is interesting as murine CTSL is likely to harbour the specific enzymatic activities of both human CTSL and CTSV as no murine ortholog of CTSV appears to exist (Brömme *et al.*, 1999; Hagemann *et al.*, 2004). Moreover, other proteases and inhibitors, as well as TGases, may also be involved in terminal differentiation of the hair follicle and nail unit. Mutations in the human cathepsin C gene have been identified as the cause of Haim-Munk syndrome (MIM245010), a rare disorder resembling Papillon-Lefevre syndrome but with the addition of nail abnormalities (Hart *et al.*, 2000). Recently, a human autosomal recessive ichthyosis with hypotrichosis syndrome (MIM610765) was linked to a specific mutation in the *ST14* gene, which encodes the serine protease matriptase (Basel-Vanagaite *et al.*, 2007; List *et al.*, 2007). Furthermore, the Netherton syndrome (MIM256500) features ichthyosis and hair shaft defects caused by mutations in the *SPINK5* gene resulting in a reduced activity of the serine protease inhibitor lympho-epithelial Kazal type inhibitor (LEKTI) (Chavanas *et al.*, 2000). Concerning TGases, it is known that lack of TGase 1 in lamellar ichthyosis (MIM242300) patients could cause nail and hair shaft abnormalities (Rice *et al.*, 2003, 2005), whereas patients with acral peeling skin syndrome (MIM609796), due to a missense mutation in the *TGase 5* gene, do not show nail nor hair defects (Cassidy *et al.*, 2005). So far no patients are known that are deficient for TGase 3.

We conclude that the previously unreported biochemical pathway regulated by cystatin M/E, could also be involved in terminal differentiation of the hair follicle and nail unit. The localization of all key molecules, from protease and protease inhibitor to structural components, suggests a role for these proteins in both cornification and desquamation. Terminal differentiation could start in the hair bulb and the nail matrix where CTSL is able to process and activate TGase 3, followed by crosslinking of loricrin, involucrin, and other structural proteins. One should keep in mind that other TGases such as TGase 1 and TGase 5 contribute to this process as well. In the hair follicle, CTSV could be involved in the proteolysis of corneodesmosomal proteins, which is required for desquamation of the IRS cells. Altogether, this study provides more insight in the molecular processes that underlie terminal differentiation of the hair follicle and nail unit, and could lead to further understanding of human skin disorders with impaired barrier function and hair and nail abnormalities.

## MATERIALS AND METHODS

### Tissue samples

Human hair follicles ($n=3$) were obtained from surgically removed scalp tissue from patients with basal-cell carcinoma on the head. Nail specimens ($n=2$) were obtained from toes of patients after distal limb amputations. All samples were collected with written, informed consent using protocols approved by the local ethics committee, Commissie Mensgebonden Onderzoek Arnhem-Nijmegen, that complies with the Declaration of Helsinki principles.

### Antibodies

Primary antibodies that were used in this study: affinity-purified polyclonal rabbit anti-human cystatin M/E antibody (Zeeuwen *et al.*, 2001), affinity-purified polyclonal sheep anti-human legumain antibody (Li *et al.*, 2003), monoclonal mouse anti-human CTSV antibody, monoclonal rat anti-human/mouse CTSL antibody (both from R&D Systems, Minneapolis, MN), monoclonal mouse anti-human TGase 3 antibodies C2D and C9D (Hitomi *et al.*, 2003), monoclonal mouse anti-human involucrin antibody MON150 (Sanbio, Uden, The Netherlands), polyclonal rabbit anti-human/mouse loricrin antibody (BAbCO, Richmond, CA), and mouse anti-human THH antibody AE15 (Santa Cruz Biotechnology, Santa Cruz, CA). For immunofluorescence analysis, the following secondary reagents were used: Alexa-Fluor 488 goat anti-rabbit IgG highly cross-absorbed, Alexa-Fluor 594 goat anti-rat, goat anti-mouse, and donkey anti-sheep IgG highly cross-absorbed (Molecular Probes, Eugene, OR).

### Immunofluorescence analysis

Tissues were rinsed in phosphate-buffered saline, fixed for 4 hours in buffered 4% formalin, and embedded in paraffin wax. All material was cut in 7-μm sections, mounted on SuperFrost slides (Menzel, Braunschweig, Germany), deparaffinized and rehydrated. The sections were subsequently incubated with primary antibodies diluted in 1% BSA/phosphate-buffered saline for 1 hour at room temperature. After washing in phosphate-buffered saline, fluorescent secondary antibodies were applied for 30 minutes at room temperature. For double labeling with antibodies raised in different animals, a mixture of primary antibodies was applied and this was followed by incubation with a mixture of secondary antibodies conjugated with different fluorescent dyes. Nuclei were stained with 4′,6-diamine-2′-phenylindole dihydrochloride (DakoCytomation, Copenhagen, Denmark), and the slides were mounted using Prolong Gold Antifade reagent (Molecular Probes). The slides were subsequently examined using an immunofluorescence microscope (Axioskop 2 MOT; Zeiss, Sliedrecht, The Netherlands) and images were photographed using a digital camera (Sony DXC-390P 3 CCD; Scanalytics, Fairfax, VA) and edited using Axiovision Software (Zeiss).

### Immunohistochemistry

Immunohistochemical staining was performed using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA) as previously described (Latijnhouwers *et al.*, 1996). Sections stained for involucrin received a pretreatment with citrate buffer and needed antigen retrieval in the microwave oven.

## CONFLICT OF INTEREST

The authors state no conflict of interest.

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