FOXN1 Is Critical for Onycholemmal Terminal Differentiation in Nude (*Foxn1^{nu}*) Mice

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Nude mice have a mutation in the transcription factor *Foxn1^{nu}*, resulting in downregulation of hair keratins. Although hair follicles develop normally, the hair fibers become structurally weak, curl, and break off at the surface. Nails in nude mice are deformed, based on alterations of the onychocyte differentiation process. Elemental microanalysis of the nail plate reveals marked decreases in sulfur concentrations in the nude mouse nail plates. Immunohistochemistry shows a lack of keratin 1 expression in terminally differentiating keratinocytes of the nail matrix. Instead, the typical differentiation process of the matrix is altered toward an epidermis-like differentiation pattern, comprising the production of filaggrin-containing keratohyalin granules in cells resembling those of the stratum granulosum, which are never observed in normally haired mice. The nail plate has diffuse basophilic stippling. It is thinner than normal, weak, and in most *Foxn1^{nu}*/*Foxn1^{nu}* mice breaks where it separates from the hyponychium. These studies indicate that the *Foxn1^{nu}* mutated gene has effects beyond downregulating keratin expression, including changes in filaggrin expression, and is critical for normal onycholemmal differentiation. The nails of nude mice provide new insights into the molecular controls of onychocyte differentiation, and they offer a useful model to investigate the pathogenesis of nail hypergranulosis, a common feature in human nail diseases.

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Since its discovery in 1966, the original nude mouse mutation has proven to be a valuable tool for biomedical research (Flanagan, 1966). The immunodeficiency in these animals, based on thymic aplasia, made nude mice valuable as hosts for xenografts, primarily for cancer research (Povlsen and Jacobsen, 1975; Schmidt and Good, 1975; Sundberg, 1994). The phenotype of nude mice is caused by a mutation in the *Whn* (winged-helix-nude) gene that encodes an evolutionarily highly conserved transcription factor of the winged-helix domain family, formerly called WHN or HFH11, and now called FOXN1 (Nehls *et al*, 1994; Segre *et al*, 1995; Schueddekopf *et al*, 1996; Schlake *et al*, 1997; Kaestner *et al*, 2000). There are eight known allelic mutations in the mouse *Foxn1^{nu}* gene and four transgenics, that all cause a non-functional FOXN1 protein (Sundberg, 1994; Kurooka *et al*, 1996; Prowse *et al*, 1999; Schlake *et al*, 2000; Mouse Genome Informatics, 2004).

The functions of FOXN1-signaling, however, have long remained unclear and are still not completely understood. There is some weight of evidence that FOXN1 plays a key role early in the process of keratinocyte differentiation, since expression of FOXN1 in mature skin is restricted to keratinocytes displaying early stages of terminal differentiation. It is likely that the mitogen-activated protein kinase (MAPK) pathway is inactivated as cells commit to the differentiation program and that subsequent FOXN1 expression is induced. The FOXN1 protein functions as a transcriptional activator, directly activating early parts of the differentiation program, such as the synthesis of the intermediate filament keratin 1. Consequently, transcription of late markers of keratinocyte differentiation such as profilaggrin and loricrin are inhibited by FOXN1 expression (Brissette *et al*, 1996; Lee *et al*, 1999; Prowse *et al*, 1999; Baxter and Brissette, 2002).

Evidence exists that FOXN1 is a major activator of several keratin genes. In nude mice, expression of *Krt3* (the orthologue of the human acidic hair keratin 3, hereafter called *mHA3*) is completely absent in pelage hair follicles, suggesting that the nude phenotype represents the first example of an inherited keratinization disorder that is caused by loss of expression rather than production of an abnormal protein (Meier *et al*, 1999; Schlake *et al*, 2000). Investigations in human HeLa cells suggest that *Foxn1* not only influences the transcription of the acidic hair keratin gene *mHA3*, but also that of the mouse orthologs of the

Abbreviations: *Foxn1^{nu}*, current accepted gene symbol for nude; *Hfh11^{nu}*, third and currently obsolete gene symbol for nude; *mHA3*, mouse orthologue of human acidic hair keratin 3; *nu*, original gene symbol for the nude mutant gene locus; PB, phosphate buffer; TBS, Tris-buffered saline; TEM, transmission electron microscopy; *Whn*, gene symbol for winged helix nude

human hair keratin genes *KRTHA1*, *KRTHA2*, *KRTHA4*, *KRTHB3*, *KRTHB4*, *KRTHB5*, and *KRTHB6* (Schorpp *et al*, 2000).

The loss of hair keratins might indeed explain the cutaneous phenotype of nude mice. Although they exhibit approximately the same number of hair follicles as wild-type animals (Koepf-Maier *et al*, 1990), and although the hair follicles are actively growing in a cyclic pattern (Flanagan, 1966; Eaton, 1976; Militzer, 2001), hair fibers lack a normal cuticle, and break (Flanagan, 1966; Rigdon and Packchanian, 1974; Koepf-Maier *et al*, 1990; Mecklenburg *et al*, 2001). Thus, altered keratinization appears to be a major mechanism in the pathogenesis of the nude phenotype. *Foxn1^{nu}* mRNA and *mHA3* are co-expressed not only in hair follicles but also in the nails and in the papillae of the tongue (Lee *et al*, 1999). It is therefore not surprising that abnormalities of the nails in nude mice were noticed by Flanagan in his original description in 1966 (Flanagan, 1966), who described the "toe-nails" as "frequently constricted and spirally malformed". The precise nature of nail abnormalities in nude mice was never investigated; however, this is clinically important since nail dystrophy is one feature of the human nude phenotype complementing a severe functional T cell immunodeficiency and congenital alopecia (Frank *et al*, 1999). Moreover, investigating the precise nature of nail abnormalities in nude mice is biologically interesting, since our understanding of nail physiology and the pathogenesis of nail diseases is still very limited (Fleckman, 1999; Baran and Kechijian, 2001; Paus and Peker, 2003).

Although many spontaneous mutant or transgenic mice exert nail abnormalities (Morita *et al*, 1995; Foley *et al*, 1998; Godwin and Capecchi, 1998; Sundberg and Boggess, 1998; Jumlongras *et al*, 2001; Bardos *et al*, 2002; Lin and Kopan, 2003), surprisingly little is known about the nature and the underlying pathomechanism of the observed onychodystrophy.

We report here a detailed evaluation of the nails in two inbred strains of mice homozygous for *Foxn1^{nu}* linking macroscopic, histologic, ultrastructural, and molecular observations. We propose that the nails of nude mice are a valuable model to study the molecular controls of onycholemmal differentiation and to investigate the mechanisms involved in nail hypergranulosis, a pathomorphological feature that is commonly observed in various diseases of the human nail such as psoriasis, Hallopeau acrodermatitis, lichen planus, and spongiotic trachyonychia (Fanti *et al*, 1994; Baran, 2002).

Results

No alterations were found in the tongue papillae, palate epithelium, olfactory epithelium, or teeth FOXN1 is expressed in the papillae of the tongue epithelium, in the epithelium of the palate, in the epithelium of the nasal cavity and in tooth premordia (Lee *et al*, 1999). It is likely that it regulates keratin expression in these tissues. Therefore, we examined these tissues with regard to subtle histologic abnormalities. Although a functional FOXN1 protein is lacking in *nu/nu* mice, however, we were not able to detect any histologic differences in these tissues (data not shown).

Nude mice exhibit a severe onychodystrophy The nails of *nu/nu* mice are severely shortened (brachyonychia). Compared with normal +/? (heterozygous, +/*nu*, or wild-type +/+; hereafter referred to as +/? mice) littermates, where nails exhibit a sharp tip, nails of nude mice (*nu/nu*) reveal a blunt, irregularly formed end. In most nude mice nails terminate at the junction of the nail plate and hyponychium. The mutant mouse nail plate is compact and narrows toward the tip of the nail. By contrast, all +/? control mice have nails that extend beyond the junction with the hyponychium forming a sharp tip approximately 800 μm from the separation (Fig 1*A*, *B*).

The nail plate of *nu/nu* mice is markedly thinned Morphometric analysis of sagittal sections of the nail plate demonstrates that the width of the nail plate at the proximal nail fold (i.e., where the skin of the dorsal surface reflects proximally) (Fig 1, *double arrow 1*) and the width of the nail plate at the tip of the nail (i.e., at the junction of the nail plate and the hyponychium) (Fig 1, *double arrow 2*) are significantly thinner in *nu/nu* compared to +/? normal mice (Fig 2).

By scanning electron microscopy, normal +/? nails are elongated and come to a sharp point (Fig 3*A*). By contrast, *nu/nu* mouse nails are short with blunt, broken, distal ends (Fig 3*B*, *C*) or split longitudinally at the tip (Fig 3*D*). Superficial squames on the nail plate surface are smooth, flat, and have regular smooth edges in +/? mice (Fig 3*E*). *Nu/nu* nail plates consist of smaller, elongated squames with a slightly irregular surface and serrated borders (Fig 3*F*).

Onychocyte differentiation in the nail matrix is altered Longitudinal sections of nails from *nu/nu* and +/? mice are markedly different. Similar alterations are found in *nu/nu* mice from both inbred strains (Han:NMRI, NU/J). These differences are described with respect to the three distinctive zones of nail epithelium, i.e., nail matrix, nail bed, and ventral digit epithelium

The *nail matrix* of +/? mice is characterized by a multilayered epithelium with distinctive keratinocyte differentiation (Figs 4 and 5). Basal keratinocytes display small nuclei and little cytoplasm. The more suprabasal the cells are located, the larger the cytoplasm becomes and the nuclei become flattened. Differentiating keratinocytes finally exhibit an eosinophilic cytoplasm and condensed nuclei. This layer is continuously merging into a homogenous zone of completely cornified cells that form the nail plate without any remnants of nuclei (Figs 4 and 5). A few dark structures are visible in the upper layers of the nail matrix that likely represent degenerating nuclei. There is no granular cell layer in the matrix zone, as is seen in interfollicular epidermis or ventral digit epithelium where keratohyalin granules are seen. In *nu/nu* mice, the matrix region is severely altered (Figs 4 and 5). Some suprabasal cells show increased cytoplasm, often associated with multiple intracytoplasmic vacuoles (Fig 5). Keratinocytes high in the spinous layer reveal striped cytoplasm, probably related to aggregated intermediate filaments (Fig 5*F*). Simultaneously, there is a prominent granular layer between the upper layers of the stratum spinosum and the nail plate (Figs 4 and 5), a feature

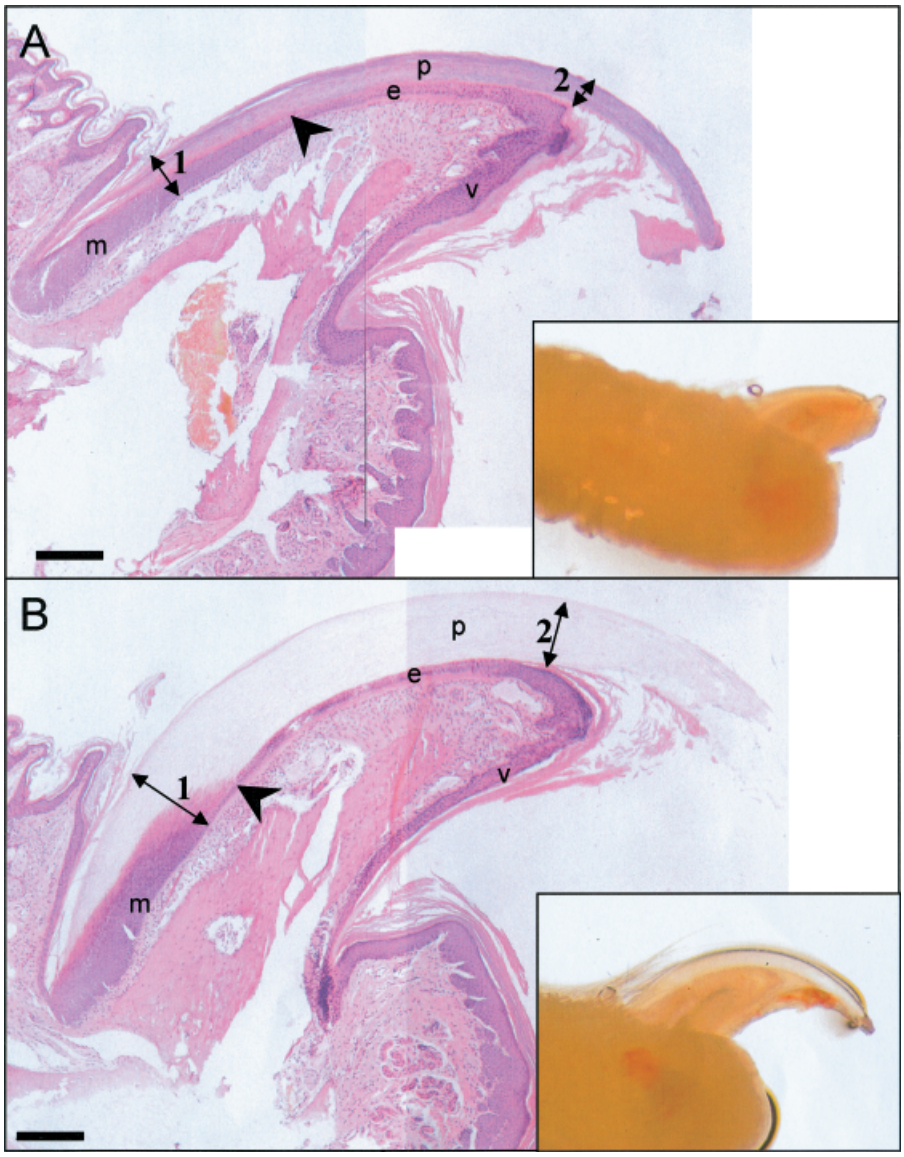

Figure 1
Severe onychodystrophy in *nu/nu* mice. Nail from phalanx IV of the hind leg in an adult *nu/nu* (*A*) and +/? control (*B*) NMRI mouse. In *nu/nu* mice (*A*), nails exhibit a blunt tip (insert). The nail plate (p) is compacted, basophilic, and thinned. The nail matrix (m) is thinner, extends farther distally, and does not exhibit a sharp border as it merges with the nail bed epithelium (e) (*arrowhead*). The ventral digit epithelium (v) is not altered. The nail plate is markedly thinner at its base (1) and at the tip of the nail (2). Paraffin sections. Hematoxylin–eosin stain. *Scale bar* = 200 μm. Insets: photograph of formalin-fixed phalanx used for histology.

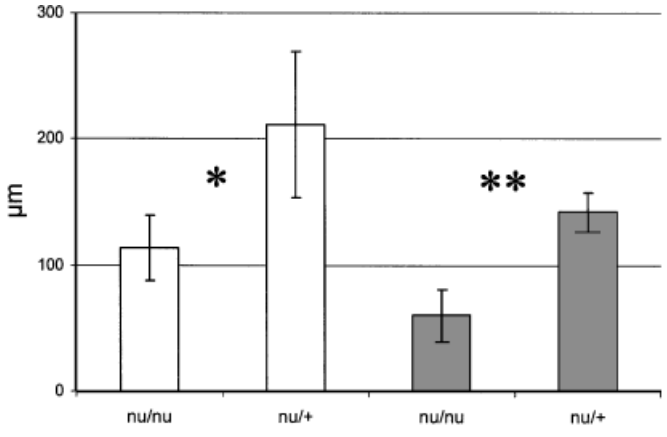

Figure 2
The nail plate is significantly thinner in *nu/nu* mice. The width was measured at the proximal nail fold (i.e., where the skin of the dorsal surface reflects proximally, white columns) and the width of the nail plate at the tip of the nail (i.e., at the junction of the nail plate and hyponychium, grey columns). Mean ± SEM. *$p<0.05$, **$p<0.01$. ($n=3$).

that is never observed in +/? mice. When compared with normally haired mice where cells of the nail plate lack remnants of cell granules and the nail plate is clear, the nail plate appears basophilic in *nu/nu* mice (Figs 1*A*, 4*B*, and 6*B*).

In normally haired +/? animals, there is a well-demarcated border between the nail matrix and the *nail bed* (Figs 1*B*, 5*C*, and 6*C*). The latter is characterized by a basal layer consisting of one row of pallisading keratinocytes and a suprabasal layer, consisting of only one or two rows of rounded keratinocytes. These cells merge into the nail plate abruptly without the occurrence of a granular layer and merge into a homogenous zone of tightly packed fully cornified cells (Fig 6*C*). The transition from the matrix into the nail bed can also be seen in *nu/nu* mice (Fig 6*D*). The matrix zone, however, is thinner and extends farther toward the distal tip of the nail (Fig 1*A*). The nail bed epithelium itself is not altered in *nu/nu* mice (Fig 6*C*, *D*).

At the tip of the nail, there is another transition from the nail bed into a multilayered epithelium with a marked granular layer, representing the *ventral digit epithelium*. The

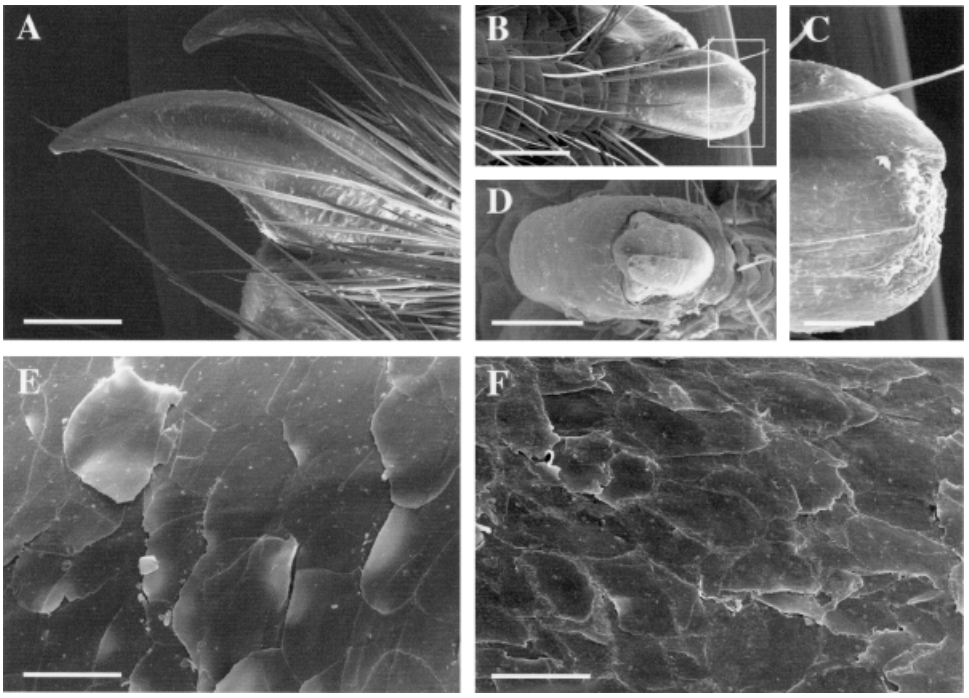

Figure 3
Scanning electron microscopic pictures of a nail from a NU/J *nu/nu* and +/? mice. Normal +/? nails are long and pointed (*A*, *scale bar* = 500 μm). By contrast, *nu/nu* nails are short and broken (*B*, *scale bar* = 500 μm; *C*, enlargement of boxed area in *B*, *scale bar* = 150 μm). Others are split longitudinally (*D*, scale bar = 500 μm). The normal +/? nail plate surface consists of uniform, smooth, flat plates of squames (*E*, *scale bar* = 25 μm) whereas that in *nu/nu* mice is irregular with smaller squames (*F*, *scale bar* = 25 μm).

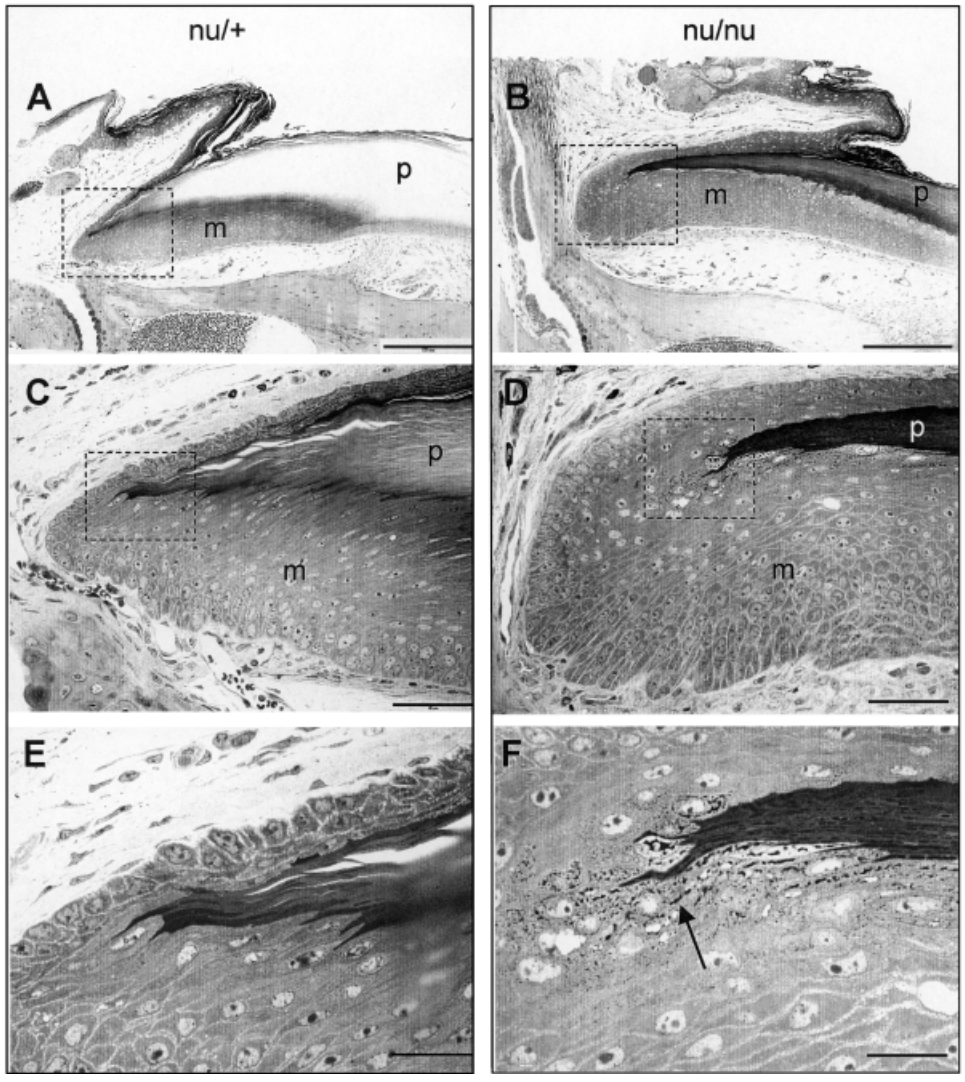

Figure 4
Abnormalities in the differentiation of the nail matrix. Nails from phalanx IV of the hind leg in an adult +/? (*A*, *C*, *E*,) and *nu/nu* (*B*, *D*, *F*) NMRI mouse. Epon-embedded, semi-thin sections, stained with toluidine blue/borax. (*A*, *B*) Overview. m = nail matrix. p = nail plate. Note the density of the nail plate and the accentuated distal extension of the matrix in *nu/nu* mice. (*C–F*) Higher magnification of the boxed areas indicated in *A–D*. Abrupt keratinization into a compacted nail plate (p) in *nu/+* mice without formation of keratohyalin granules. In nu/nu mice, keratinocytes higher in the spinous layer have keratohyalin granules (*arrow*). *Scale bar* = 200 μm (*A*, *B*), 100 μm (*C*, *D*) 50 μm (*E–F*).

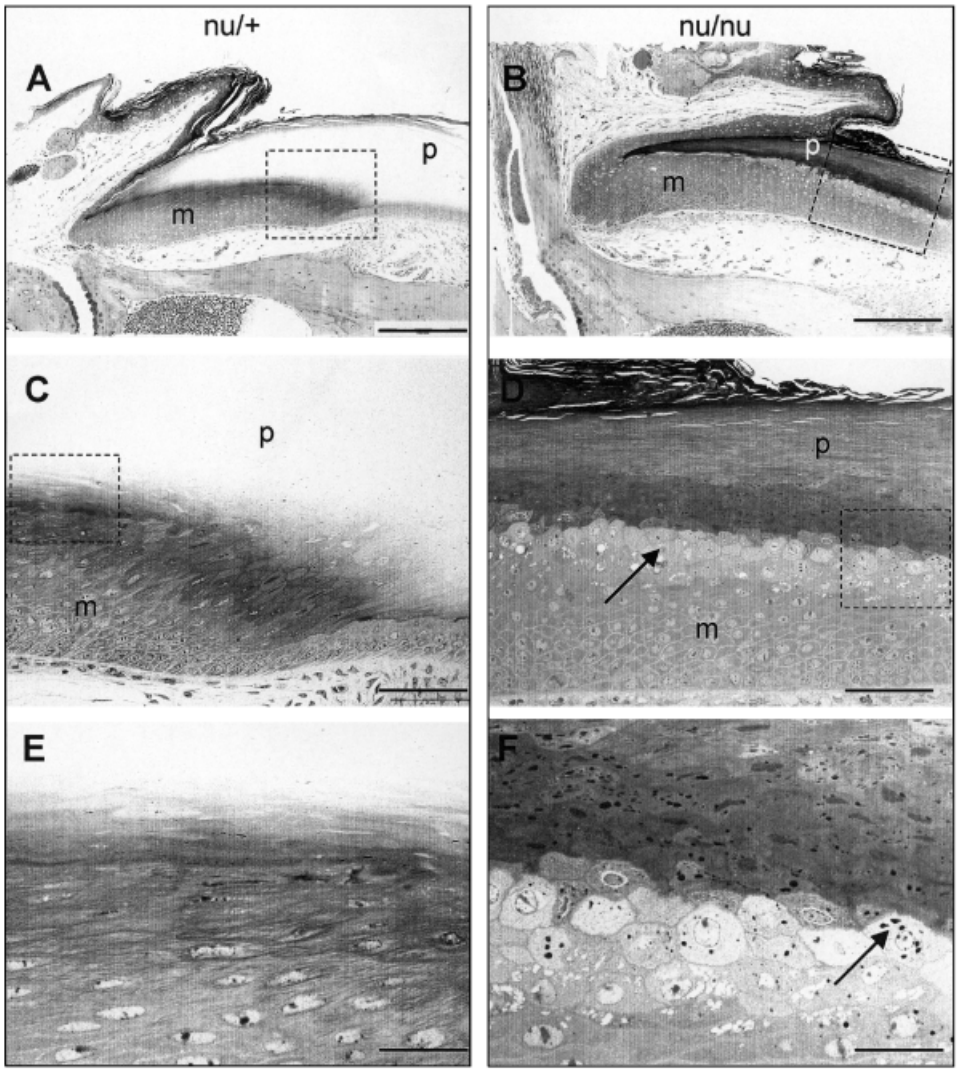

Figure 5
Abnormalities in the differentiation of the nail matrix. Nails from phalanx IV of the hind leg in an adult +/? (*A*, *C*, *E*,) and *nu/nu* (*B*, *D*, *F*) NMRI mouse. Epon-embedded, semi-thin sections, stained with toluidine blue/borax. (*A*, *B*) Overview. m = nail matrix. p = nail plate. Note the density of the nail plate and the accentuated distal extension of the matrix in *nu/nu* mice. (*C–F*) Higher magnification of the boxed areas indicated in *A–D*. Distal matrix thins abruptly into proximal nail bed in +/? mice (*C*, nail plate is above). In *nu/nu* mice, keratinocytes higher in the spinous layer are occasionally vacuolated, have abundant pale cytoplasm and keratohyalin granules (arrow), which are also visible within the naiil plate (p). *Scale bar* = 200 μm (*A*, *B*), 100 μm (*C*, *D*), 50 μm (*E–F*).

granular layer is especially prominent at the tip of the nail, where the nail plate separates from the underlying hyponychium (Fig 6*E*). The hyponychium marks the point where onycholemmal keratinization ends and volar epithelial keratinization begins (Zaias and Alvarez, 1968). In homozygous nude animals, there are no alterations with respect to the ventral nail epithelium. The dorsal nail plate separates from the hyponychium but bends toward the ventral side and finally breaks off (Figs 1 and 6*F*).

Nails of *nu/nu* mice exhibit a lower sulfur content Elemental analysis was done of the nail plate, foot pad, and hairs on the digits of *nu/nu* and +/? mice (Fig 7). Based on weight, the sulfur content of the soft keratins in foot pads for both *nu/nu* and +/? mice is consistently under 1.5%. By contrast, sulfur content of the hair and nail plates that contain hard keratins and the high and ultrahigh sulfur keratin associated proteins from +/+ control mice is over 3%. Sulfur content of *nu/nu* nail plates and hair fibers is consistently under 3% and usually at the level found in foot pads.

In *nu/nu* mice there is loss of keratin 1 protein and subsequent abnormal production of filaggrin protein in the nail matrix and the nail plate Using an antibody against the mouse filaggrin protein, it is shown that the basophilic granules within the suprabasal keratinocytes of the nail matrix in *nu/nu* mice contain filaggrin (Fig 8*B*). A thin layer of cells resembling those of the stratum granulosum directly under the nail plate along its entire length contains filaggrin positive granules. This is most prominent in the NU/J *nu*/nu mice. Simultaneously, Keratin 1 immunoreactivity is lost in the ventral epithelium of the proximal nail fold and the suprabasal cells of the nail matrix in homozygous mice (Fig 8*A*, *C*).

Expression patterns of keratin 5, several integrins, and Ki-67 are not altered in *nu/nu* mice Since expression of keratin 1 is altered in nails of *nu/nu* mice, the expression pattern of other keratins and of certain integrins, important for integrity of the nail, were investigated (Cameli *et al*, 1994; De Berker *et al*, 2000). Immunoreactivity patterns of keratin 5 (expressed basally in the nail matrix and panepithelially in the ventral digit epithelium), integrin α4 (expressed basally in the nail bed epithelium), and integrin α6 (expressed basally in the nail matrix and the ventral digit epithelium and panepithelially in the nail bed epithelium) are not altered in *nu/nu* mice (data not shown). Immunoreactivity against the Ki-67 protein, detecting proliferation of cells, showed that keratinocyte proliferation within the matrix and the basal

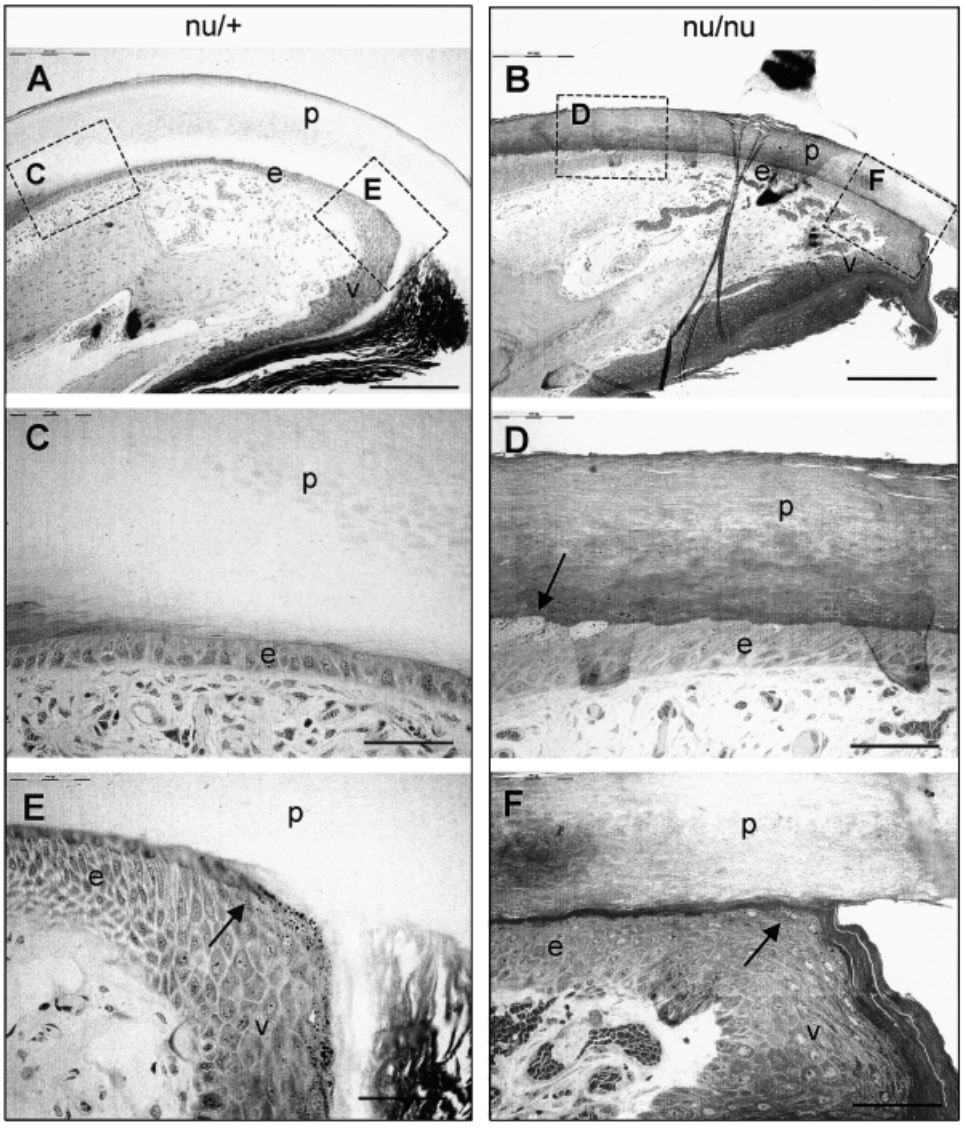

Figure 6
Thinning and breakage of the dorsal nail plate in *nu/nu* mice. Nails from phalanx IV of the hind leg in an adult +/? (*A*, *C*, *E*) and *nu/nu* (*B*, *D*, *F*) NMRI mouse. Epon-embedded, semi-thin sections, stained with toluidine blue/borax. (*A*, *B*) Overview. e = nail bed epithelium. p = nail plate. (*C–F*) Higher magnification of the boxed areas indicated in *A* and *B*. Note the granular layer (*indicated by arrows*) both in the nail matrix of *nu/nu* mice (*D*), and in the ventral digit epithelium of both *nu/+* (*E*) and *nu/nu* mice (*F*). A granular layer is absent from the nail bed epithelium in both genotypes (*C*, *D*). The dorsal nail plate separates from the hyponychium where the dorsal epithelium merges into the ventral epithelium (*E*, *F*). *Scale bar* = 200 μm (*A*, *B*), 50 μm (*C–F*).

layer of the nail bed are not altered by the *nu/nu* mouse phenotype (data not shown).

***Nu/nu* mice exhibit keratohyalin granules within the matrix epithelium and disturbed filament aggregation** Changes in the differentiation process within the nail matrix demonstrated by light microscopy were further investigated by transmission electron microscopy (TEM). The cytoplasm of suprabasal cells is more electron lucent than normal and the stripes within the cytoplasm, detected by light microscopy (Fig 5*F*), are aggregates of tonofilaments (Fig 9). The remaining cytoplasm appears to be rather lighter and free of intermediate filaments, likely due to this aggregation of tonofilaments. These keratinocytes have a round shape. Their nuclei also exhibit a round to oval shape, although evidence of nuclear disintegration already becomes detectable (nuclear membrane hyperchromasia, infoldings of the nuclear membrane, fragmentation of the nucleus) (Fig 9*A*). The latter can be considered normal, since these are features of the epidermal terminal differentiation (Fuchs, 1990). Simultaneously, keratohyalin granules of irregular size and shape become visible in the cytoplasm of suprabasal keratinocytes (Fig 9*A–C*). These keratohyalin granules insert into the aggregated tonofilaments, linking them to each other resulting in compaction of the tonofilaments, which become oriented parallel to the surface of the nail plate. Keratohyalin granules are still visible within the compacted layer of fully differentiated corneocytes of the nail plate (Fig 9*C*).

These ultrastructural characteristics contrast the process of onychocyte differentiation within the nail matrix of +/? mice. There, high spinous keratinocytes exhibit a flattened shape (Fig 9*D*). Nuclei within these cells are also flattened in the same plane. Simultaneously, the cytoplasm is filled with thick aggregates of intermediate filaments that are oriented longitudinally. These are interspersed by single tonofilaments in high spinous cells but become markedly compacted in more superficial cells, where homogenous aggregates of parallel tonofilaments represent the nail plate. Keratohyalin granules are not visible in the nail matrix.

Discussion

Nude mice have a mutation in the gene coding for the transcription factor FOXN1, resulting in downregulation of hair

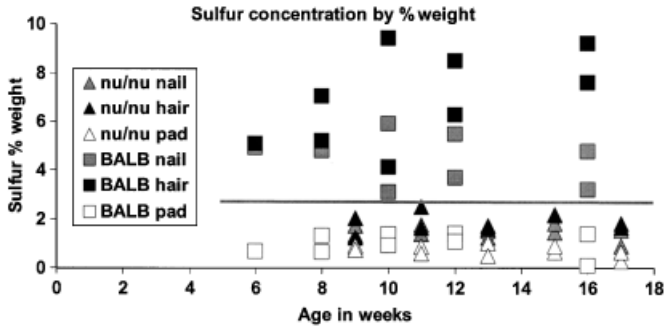

Figure 7
X-ray microanalysis for sulfur content by weight in the nail, hair fiber, and footpads. Normal hair and nails from BALB/cByJ +/+ mice were above 3% sulfur. By contrast, +/+ and *nu/nu* footpads were below 1.5% sulfur. *Nu/nu* nails and hair fibers contained less than 3% sulfur indicating a distinct dichotomy from controls.

keratin 2, 3, 4, 5, and 6 production. This results in a severe hair shaft dysplasia in which the hair fibers do not produce normal cuticles. Subsequently, the hair fibers become structurally weak, curl within the follicular infundibulum, and eventually break off at the surface resulting in alopecia (Koepf-Maier *et al*, 1990; Mecklenburg *et al*, 2001).

Since the pleiotropic phenotype of nude mice, i.e., loss of hair and athymia, are both related to abnormalities in epithelial cell differentiation (Baxter and Brissette, 2002; Cunliffe *et al*, 2002), it is conceivable that other ectoderm-derived tissues are also affected by the loss of a functional FOXN1 protein. FOXN1 is expressed not only in the epithelium of the hair follicle and the thymus but also in the epithelium of the nasal cavity, the palate, tongue, teeth, and nails (Lee *et al*, 1999). Therefore, it is not surprising that Flanagan had already noticed nail abnormalities of nude mice in 1966 (Flanagan, 1966). These changes, however, have not been investigated carefully up to now. This study analyses the abnormalities in nude mice that occur in tissues other than the thymus and the hair follicle, focusing on the nails.

No abnormalities were found in the tongue, palate, nasal cavity, or teeth of $Foxn1^{nu}/Foxn1^{nu}$ mice by light microscopy. This suggests that their morphology and probably their function is independent of FOXN1 signaling and of the expression of certain FOXN1 dependent keratin proteins.

In contrast to the above-mentioned tissues, *nu/nu* mice develop severe malformation of the nails. Therefore, in addition to its crucial roles in hair fiber formation and thymic development, the transcription factor FOXN1 is critical for normal onycholemmal differentiation. The fact that FOXN1 expression is crucial in some differentiating keratinocytes, i.e., those of hair and nail matrix and thymic epithelium, but not in others, i.e., tongue, palate, nasal cavity, and teeth, underlines the homogeneous nature of molecular controls of terminal differentiation on one hand and the heterogeneous nature of tissue responses on the other. Moreover, it demonstrates that it is of major importance to analyze a variety of epithelial compartments when investigating epithelial differentiation defects, especially in novel genetically modified mice (Ward *et al*, 2000).

Nails of *nu/nu* mice exhibit severe brachyonychia (short nails) and onychodystrophy (abnormal nail formation). The onychodystrophy results from abnormal differentiation of onychocytes within the matrix. The *nu/nu* mutation results in loss of keratin 1 protein within suprabasal matrix cells. The loss of keratin proteins was confirmed by immunohistochemistry and implied by X-ray microanalysis that demonstrated a lower sulfur content in the nails when compared with epidermis, supporting the downregulation of both hard keratin and keratin-associated protein production (Rogers *et al*, 2004). Subsequently, and possibly as a reponse to the loss of keratin proteins, filaggrin containing granules are produced in suprabasal cells. Keratohyalin granules within

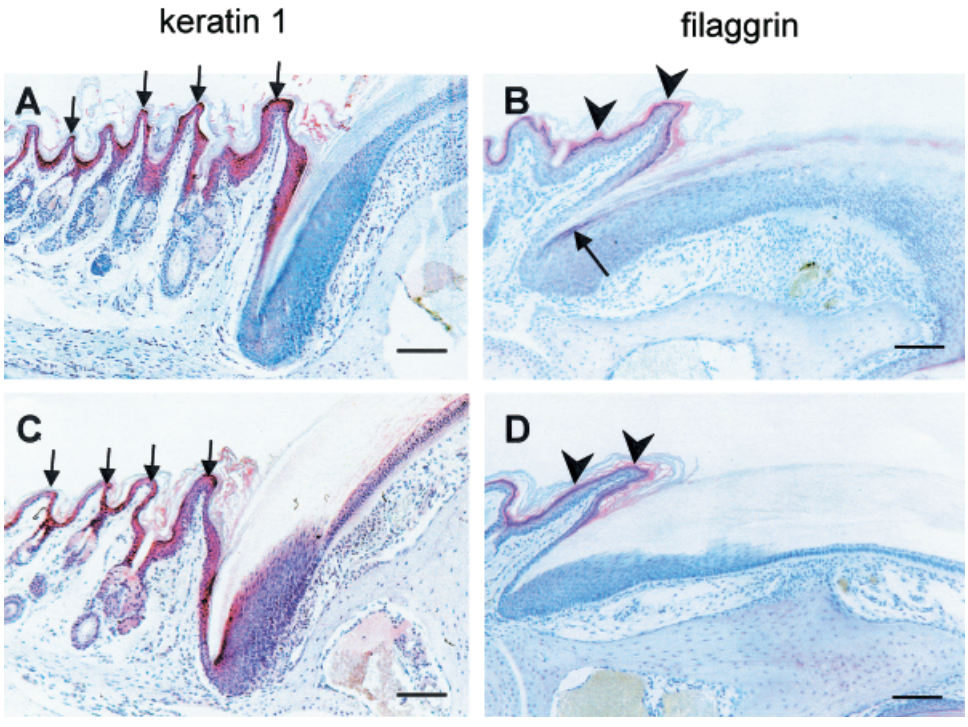

Figure 8
Loss of Keratin 1 protein and abnormal expression of filaggrin protein in terminally differentiating keratinocytes of the nail matrix. Nail from *nu/nu* mouse (*A*, *B*) and +/? control (*C*, *D*). Immunohistochemistry of mouse cytokeratin 1 (*A*, *C*) and mouse filaggrin (*B*, *D*). Note the presence of keratin 1 immunoreactivity in the interfollicular epidermis (*A*, *C*, *arrows*), and the presence of weak filaggrin immunoreactivity in the granular layer of the interfollicular epidermis from both animals (*B*, *D*, arrowheads). +/? mice lack filaggrin immunoreactivity in terminally differentiating keratinocytes of the nail matrix (*D*). Nails of *nu/nu* mice lack keratin 1 immunoreactivity in the nail matrix (*A*) and show filaggrin immunoreactivity in the nail matrix and the nail plate (*B*) arrow. Paraffin sections. Counterstained with hematoxylin. *Scale bar* = 100 μm.

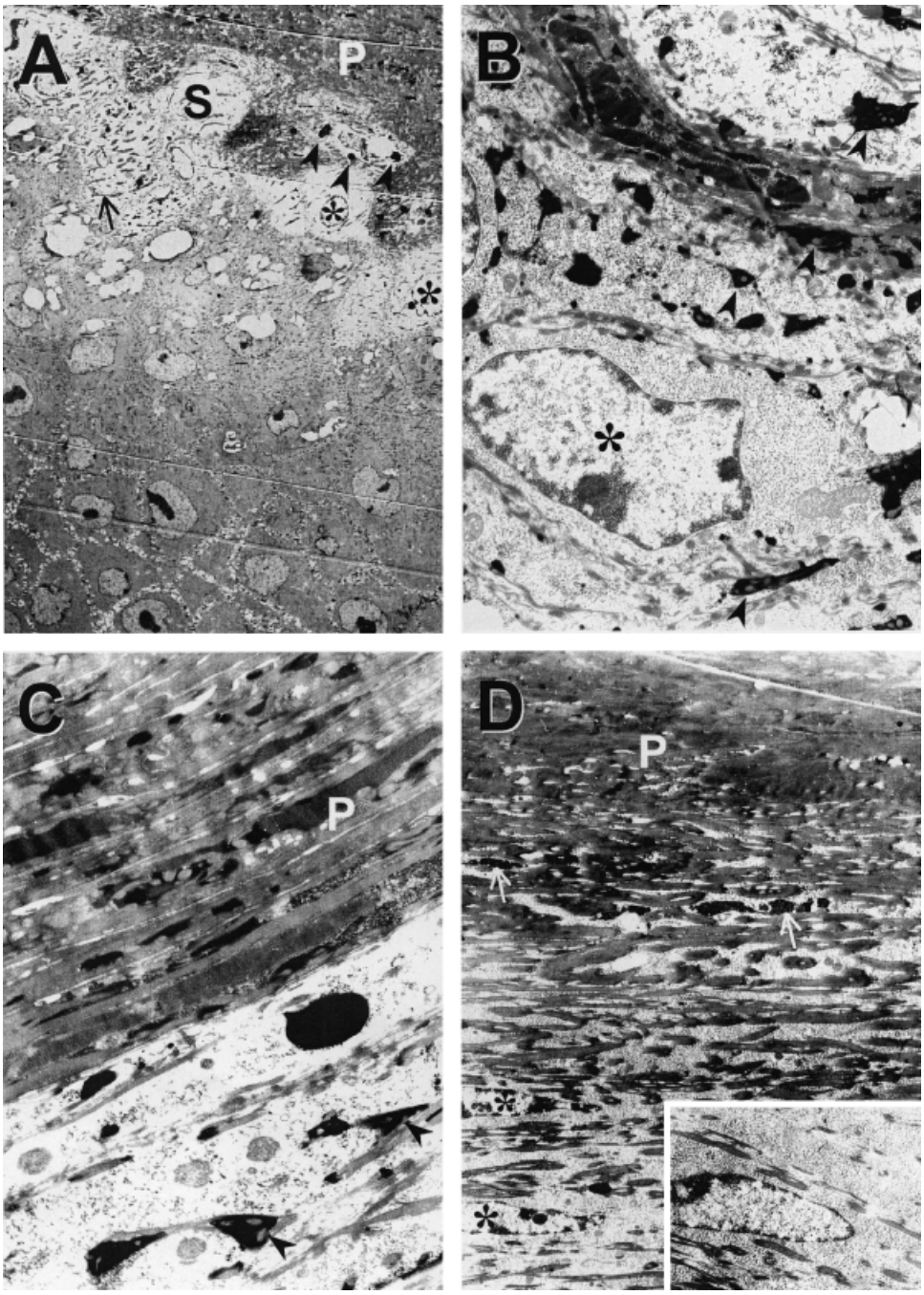

Figure 9
TEM of the transition zone from upper spinous layer to lower nail plate in a NMRI *nu/nu* mouse (*A–C*), compared to a +/? Control (*D*). (*A*) *nu/nu* mouse. Keratinocytes in the upper spinous layer of the matrix (S) have pale cytoplasm. Within the cytoplasm, aggregated tonofilaments (*arrows*) and keratohyalin granules (*arrowheads*) are visible. Nuclei of keratinocytes in this area (asterisks) show margination of chromatin and infolding of the nuclear membrane, representing normal features of terminal differentiation. P = nail plate. Original magnification: 2430 ×. (*B*) *nu/nu* mouse. Irregularly shaped keratohyalin granules (*arrowheads*) insert into aggregated tonofilaments and link them to each other. Note the margination of chromatin and the irregularity of the nuclear membrane (asterisk), suggesting early nuclear disintegration, features of keratinocyte terminal differentiation. Original magnification: 17,000 ×. (*C*) *nu/nu* mouse. Irregular keratohyalin granules insert into aggregated tonofilaments and link them to each other. The nail plate (P) is composed of compacted tonofilaments, interspersed by vacuoles and keratohyalin granules (*arrowheads*). Original magnification: 17,000 ×. (*D*) +/? control mouse. In keratinocytes in the upper spinous layer with elongated, flattened nulcei (asterisks) large aggregates of tonofilaments are oriented parallel to the nail surface and are dispersed equally within the cytoplasm. They are separated from each other by condensed ribosomes and debris of other cell organelles. The nail plate (P) is composed of very regular aggregates of tonofilaments, occasionally interspersed by condensed remnants of cell nuclei (arrow). No keratohyalin granules are detectable. Original magnification: 8,000 ×. Insert: TEM of a nail matrix keratinocyte from the upper spinous layer of a NMRI +/? control mouse. Note the large, regular aggregates of tonofilaments within the cytoplasm and the complete lack of keratohyalin granules. Original magnification: 31,400 ×.

the nail matrix and the nail plate represent a characteristic feature of the nude phenotype on at least two inbred mouse strain backgrounds. These keratohyalin granules do not occur in the normal matrix epithelium of +/? mice nor do they occur in normal human nail matrix (Zaias and Alvarez, 1968; Hashimoto, 1971). The lack of FOXN1-dependent keratin proteins and the presence of keratohyalin granules is associated with impaired structure of the nail plate. Retention of keratohyalin granules in nude mice may be responsible for the basophilic appearance of the nail plate by light microscopy. Altered intermediate filament aggregation may also account for the abnormal appearance of the nail plate. The presence of these granules in *nu/nu* nail matrix may also be responsible for the marked thinning of the nail plate in homozygous mice, since superficial layers of the nail plate separate, as is the case on the tip of the nail, where production of keratohyalin granules within the hyponychial epithelium is associated with cornification, loss of adhesion, and separation of the nail plate from the hyponychium.

In the case of absence of FOXN1 function, primary epidermal keratinocytes lose markers of early terminal differentation, e.g., keratin 1, and gain features of late differentiation (Baxter and Brissette, 2002). This affects the interfollicular epidermis and the hair follicles, although only the latter show impairment of function (Koepf-Maier *et al*, 1990). Our findings suggest that this process also affects onycholemmal differentiation, resulting in impaired nail formation. Similar to primary epidermal keratinocytes from

nu/nu mice, onychocytes of the nail matrix lack expression of keratin 1. Simultaneously, they gain production of filaggrin-containing keratohyalin granules.

The keratinization process of nails and hair cortex is largely similar to that of the epidermis. A striking difference, however, between keratinization of the nail and hair cortex and that of the epidermis is that in the nail and hair cortex, keratin fibrils form by accretion of cytoplasmic filaments without formation of keratohyalin granules (Hashimoto, 1971; Powell and Rogers, 1994). This might explain why loss of filaggrin expression, e.g., in flaky tail mice (*ft/ft*) does not impair nail formation (Presland *et al*, 2000). Filaggrin aids in packing of keratin filaments in interfollicular epidermis (Dale *et al*, 1997). Filaggrin is not involved in normal onycholemmal or tricholemmal keratinization. Although a similar, fused S-100 protein, trichohyalin, is present in inner root sheath and nail matrix (O'Keefe *et al*, 1993), aggregation of intermediate filaments in these appendages is poorly understood. Thus, aggregation of intermediate filaments by filaggrin may replace cross-linking by the missing hard keratins and keratin associated proteins in the nail matrix of *nu/nu* mice. Upregulation of keratohyalin production was also demonstrated in the interfollicular epidermis of mice that lack a functional keratin 10 protein (Reichelt *et al*, 2001). The abnormal profilaggrin expression might be an indirect effect, representing a common mechanism of adaptation to increased terminally differentiated keratinocyte fragility. Inhibition of profilaggrin expression by exogenous FOXN1, however, has been demonstrated in primary keratinocytes from transgenic mice expressing FOXN1 under control of the involucrin promoter (Baxter and Brissette, 2002), and therefore could also represent a direct effect of FOXN1 transcriptional activity. Thus, the presence or absence of a functional FOXN1 protein in certain keratinocytes may determine whether keratinocytes embark in an epidermal-like pattern of differentiation or undergo a specialized differentiation program that comprises expression of distinctive hard keratins.

We suggest that nails of nude mice represent a useful model for dissecting the effects of impaired keratinocyte stability. Moreover, nude mice represent an interesting biomedical tool to investigate the molecular controls of onycholemmal differentiation, and they are particularly useful for dissecting the pathogenesis of nail matrix hypergranulosis. The latter is a common feature in human nail diseases such as psoriasis, Hallopeau acrodermatitis, lichen planus, lichen striatus, punctate keratoderma, isolated congenital nail dysplasia, spongiotic trachyonychia, and other inflammatory or compressive insults. In these diseases, nail matrix hypergranulosis is associated with thinning of the nail plate and onychodystrophy (Kato and Ueno, 1993; Peluso *et al*, 1993; Tosti *et al*, 1993; Fanti *et al*, 1994; Tosti *et al*, 1997; Krebsova *et al*, 2000; Fleckman and Omura, 2001). Finally, these studies indicate that the nail unit is a useful model to study development of skin appendages and formation of normal anatomic structures.

Materials and Methods

Animals and tissue collection The experiments were approved by the institutions they were performed in (University Hospital Hamburg-Eppendorf and The Jackson Laboratory) and followed the institutional and national guides for the care and use of laboratory animals. Nude mice (*Foxn1^{nu}/Foxn1^{nu}*, hereafter refered to as *nu/nu*) on the NMRI (Han:NMRI^{nu}) and NU/J inbred backgrounds were obtained from the animal facility of the University Hospital Eppendorf (Hamburg, Germany) and The Jackson Laboratory (Bar Harbor, Maine), respectively. In the NMRI strain homozygous (*nu/nu*) mutant mice were compared with normal littermates (+/? mice). The NU/J colony is maintained homozygous for the nude mutation. The colony was derived from an inbred BALB/cByJ colony carrying the *nu* mutant gene. Wild-type (+/+) BALB/cByJ mice were used as controls.

Mice were maintained in polycarbonate boxes, five mice to a box, and kept on a 12 h light/dark cycle. They received autoclaved food pellets (NIH 31 modified with 6% fat, diet code 5K52, PMI, Richmond, Indiana, or complete diet for nude mice, ssniff Spezialdiaeten GmbH, Soest, Germany) and acidified water *ad libitum*.

For analysis of nails, three females and three males at 8, 10, 12, and 16 wk of age from each strain were euthanized by CO_2 asphyxation. Front and rear feet were fixed *in toto* by immersion. For histology and immunohistology, tissues were fixed in either Fekete's acid-alcohol-formalin solution or in 4% buffered formalin solution, decalcified, and transferred to 70% ethanol. They were processed routinely and embedded in paraffin. Serial longitudinal sections of 5–6 μm-thickness were cut and stained with hematoxylin & eosin or used for immunohistochemistry (Relyea *et al*, 2000).

Scanning electron microscopy and X-ray microanalysis Whole feet were fixed overnight at 4°C in 2.5% glutaraldehyde in 0.1M phosphate buffer (PB), pH 7.2. After rinsing the feet several times in PB, they were post-fixed in 1% osmium tetroxide in PB for 48 h at 4°C, dehydrated in a graded series of ethanol and critical point dried. The feet were mounted and sputter-coated with a 4 nm layer of gold.

For scanning electron microscopy and elemental analysis, the feet were examined at 20 kV at a working distance of approximately 15 mm on a Hitachi S3000N VP Scanning Electron Microscope (Hitachi Science Systems, Japan). Selected areas of the foot, including the middle digit nail and dorsal skin of the rear foot, the middle digit toe pad of the front foot, as well as two or three hairs, if hairs were present, from the rear foot, were analysed for elemental composition using an EDAX X-ray microanalysis system (Mahwah, New Jersey). Samples were examined for an average of at least 300 live seconds to ensure a comprehensive reading was obtained.

TEM Tissue samples were fixed in 6% glutaraldehyde in 0.05 M phosphate buffer and decalcified in EDTA for 4 wk. Following decalcification, small tissue blocks were post-fixed in 1% OsO_4 in 0.1 M sodium phosphate buffer with 1% sucrose at pH 7.4 for 2 h before embedding in glycidether (Epon). Serial semithin sections obtained from these blocks were stained with toluidine blue/borax and according to Laczko and Levai (1975). Selected blocks were cut into ultrathin sections and contrasted with 1% uranyl acetate and 1% lead citrate for electron microscopy (Reynolds, 1963).

Immunohistochemistry Paraffin sections were deparaffinized in xylene, rehydrated in graded alcohols and washed in 0.05 M Tris-buffered saline (TBS), pH 7.4 (Ausubel *et al*, 1999). Sections were incubated with normal rabbit serum (10% in TBS) to block unspecific binding of the secondary antibody. Sections were incubated overnight with the primary antibodies indicated in Table I. After washing in TBS, slides were incubated with a biotin-conjugated secondary antibody (Dianova, Hamburg, Germany) diluted 1:200 in TBS for 30 min at room temperature. After thorough washing, slides were incubated with an alkaline phosphatase-conjugated avidin–biotin complex (Vector, Burlingame, California) for 30 min at room temperature. Labeling with alkaline phosphatase was

Table I. Primary antibodies for immunohistochemistry

Antigen	Antibody	Supplier	Dilution
Keratin 1	Polyclonal rabbit antiserum	Covance Research Products, Berkeley, California	1:1000
Keratin 5	Polyclonal rabbit antiserum		1:2000
Filaggrin	Polyclonal rabbit antiserum		1:3000
Integrin α4	Monoclonal rat antibody	BD Biosciences Pharmingen, San Diego, California	1:50
Integrin α6	Monoclonal rat antibody		1:50
Ki-67 antigen	Monoclonal rat antibody	DakoCytomation, Hamburg, Germany	1:100

developed using the alkaline phosphatase substrate kit I (Vector). Sections were counterstained with hematoxylin (DAKO, Hamburg, Germany), dehydrated, and mounted (Relyea *et al*, 2000). For negative controls, sections were incubated with an irrelevant antibody from the same species.

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