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Title:

Extracellular cleavage of collagen XVII is essential for correct cutaneous basement membrane formation

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Abstract

In skin, basal keratinocytes in the epidermis are tightly attached to the underlying dermis by the basement membrane (BM). The correct expression of hemidesmosomal and extracellular matrix (ECM) proteins is essential for BM formation, and the null-expression of one molecule may induce blistering diseases associated with immature BM formation in humans. However, little is known about the significance of post-translational processing of hemidesmosomal or ECM proteins in BM formation. Here we show that the C-terminal cleavage of hemidesmosomal transmembrane collagen XVII (COL17) is essential for correct BM formation. The homozygous p.R1303Q mutation in COL17 induces BM duplication and blistering in humans. Although laminin 332, a major ECM protein, interacts with COL17 around p.R1303, the mutation leaves the binding of both molecules unchanged. Instead, the mutation hampers the physiological C-terminal cleavage of COL17 in the ECM. Consequently, non-cleaved COL17 ectodomain remnants induce the aberrant deposition of laminin 332 in the ECM, which is thought to be the major pathogenesis of the BM duplication that results from this mutation. As an example of impaired cleavage of COL17, this study shows that regulated processing of hemidesmosomal proteins is essential for correct BM organization in skin.

Introduction

The skin, the largest organ in the body, protects us from mechanical stresses and invasion by pathogens. To achieve these functions, epidermal keratinocytes continuously detach from basement membrane (BM) and dermal-epidermal junctions are firmly attached via various extracellular matrix (ECM) proteins underlying dermis (1). The basement membrane (BM) is a specialized layer in the ECM, connecting keratinocyte to dermal connective tissue. Keratin intermediate filaments and actin microfilaments respectively bind hemidesmosomes and focal contacts at the cell membrane of basal keratinocytes, whereby keratinocyte adhesion and detachment are tightly regulated (2). Hemidesmosomes are electron-dense adhesion devices composed of integrin α6 and β4, collagen XVII (COL17), tetraspanin CD151 and two plakin family members (plectin and BP230) (1, 3). The vital role of hemidesmosomal proteins are evident from epidermolysis bullosa (EB), a blistering skin disorder that is induced by mutations in genes encoding molecules associated with dermal-epidermal adhesion, including hemidesmosomal proteins (4-6).

COL17 is a type II-oriented hemidesmosomal transmembrane protein, whose amino terminus is located in the cytoplasm and whose carboxyl (C)-terminus is located in the

ECM (7, 8). Gene mutations in *COL17A1* (NM_000494) result in a blistering phenotype that is known in humans as junctional EB, generalized intermediate (4) and that is observed also in mice (9). In addition, autoimmunity to COL17 induces bullous pemphigoid, an acquired autoimmune blistering skin disease (10, 11). These genetic and autoimmune blistering diseases indicate that COL17 is a vital molecule for maintaining stable adhesion between the epidermis and the dermis. In contrast to its adhesive function, the 120-kD extracellular domain of COL17 can be cleaved within the juxtamembranous non-collagenous (NC) 16A domain by ADAM9/10/17 (10, 12, 13). In addition, a site 23-kD upstream from the C-terminal end of this molecule may be cleaved (14), yielding a further processed 97-kD ectodomain. Although the physiological role of the cleavage of the COL17 ectodomain remains uncertain, it may be involved in the migration of basal keratinocytes (15).

In contrast to patients with junctional EB, generalized intermediate who lack COL17 expression in the skin, patients with junctional EB, late onset who have homozygous c.3908 G>A, p.R1303Q mutations in *COL17A1* (designated as R1303Q) show distinct clinical and histopathological features (16-19). In addition to blister formation, R1303Q patients clinically show progressive poikilodermic and atrophic skin, and sclerotic

fingers. Interestingly, duplicated disorganized BM is a characteristic histopathological feature of R1303Q patients, indicating that COL17 also plays vital roles in BM organization. The Arg1303 is located within the extracellular C-terminal NC4 domain of COL17, whereby the molecule is thought to interact with other ECM molecules, including laminin 332 (17, 20). Thus, the R1303Q mutation suggests that COL17 plays a vital role in the organization of the skin BM, probably through binding with laminin 332 in the ECM. However, the precise pathomechanism underlying the R1303Q mutation has remained uncertain.

Duplication of the BM can be induced by dysfunctions of various molecules including kindlin-1 (21) and integrin α3 (22); however, little is known about the underlying pathogenesis. In this study, we focused on EB patients with R1303Q mutation in COL17, since genetic diseases are helpful for understanding the physiological roles of proteins with unknown functions. Unexpectedly, we found that the presence of R1303Q does not alter the interactions between COL17 and laminin 332; rather, the mutation hampers C-terminal cleavage of COL17. The increase in remnants of non-cleaved COL17 ectodomain in the ECM induces aberrant laminin 332 deposition in the ECM, which may be associated with disorganized BM formation. Furthermore, increased

expression of collagen V (COL5) was found in R1303Q primary keratinocytes as well as in *in vivo* skin obtained from the R1303Q patients, a finding that may be associated with the pathomechanism of sclerotic fingers, a characteristic clinical feature of patients with R1303Q mutation in COL17 (17).

Results

Homozygous R1303Q mutation in COL17A1 results in the duplication of cutaneous BM components. The clinical and histopathological findings of two cases with R1303Q mutations in *COL17A1* are shown in **Figure 1**. Both patients intermarried among relatives, as shown in the family tree of case 1 (**Fig. 1A**) and of case 2 (**Fig. 1B**). Case 1 is a 32-year-old Japanese female. She was initially diagnosed with atopic dermatitis due to erythema on the dorsal hands and extremities. When she was in her early teens, photosensitivity, blister formation around the fingers and toes, and nail deformity appeared. Currently, in addition to focal mechanical blister formation, poikiloderma around her neck and sclerotic fingers associated with nail loss were observed **(Fig. 1C**). Case 2 is a 46-year-old Japanese female. When she was 6 years old, focal blisters started to develop due to mechanical friction. Sclerosis of fingers with nail deformity and

photosensitivity were observed around her mid teens. At present, in addition to focal mechanical blister formation and poikiloderma around her neck, her nails have been completely lost and her fingers are too sclerotic to be flexed and extended (**Fig. 1D**). Immunofluorescence antigen mapping of skin samples resulted in duplication of BM proteins, including collagen IV, VII, XVII and laminin 332 in both cases (**Fig. 1E and Supplementary Material, Fig. S1**); in addition, electron microscopy findings showed duplicated lamina densa (**Fig. 1F and 1G**). Hemidesmosomal components, including plectin, integrin α6 and integrin β4, are normal, whereas BP230 was diffusely distributed in the cytoplasm of basal keratinocytes (**Supplementary Material, Fig. S1**).

The R1303Q mutation does not attenuate the interaction between COL17 and laminin 332. *In silico* prediction showed that R1303Q may influence the affinity between COL17 and laminin 332 (17). To assess whether the R1303Q mutation affects the binding of COL17 to laminin 332, we produced full-length normal recombinant COL17 protein (designated as normal-COL17) and a recombinant full-length COL17 with R1303Q mutation (designated as R1303Q-COL17) (**Fig. 2A**). Both the normal-COL17 and the R1303Q-COL17 show a homotrimer form with three 180-kDa chains (**Fig. 2B and 2C**). Unexpectedly, COL17-lamnin 332 binding assay revealed that the

R1303Q-COL17 binds to laminin 332 in a manner similar to that of normal-COL17 (**Fig. 2D**).

C-terminal cleavage of R1303Q-COL17 is hampered. To address the pathomechanism of R1303Q mutation in COL17, we focused on the processing of the extracellular domain of the molecule. The 120-kD shed COL17 ectodomain can be further processed into a 97-kD polypeptide by cleaving of the C-terminus of the molecule (14, 23, 24). Based on the molecular weight, the C-terminal cleavage site is predicted to be within the NC4 domain of COL17, in which R1303 is located (**Fig. 2A**), indicating that R1303Q mutation may affect the C-terminal cleavage. Plasmin, a serine protease that preferentially cleaves the carboxyl side of arginine (25), is known to cleave COL17 into 120-kD and 97-kD polypeptides (14). When purified normal-COL17 and R1303Q-COL17 were treated with plasmin, the 97-kD ectodomain was markedly reduced in the R1303Q-COL17, revealing that the R1303Q mutation hinders the C-terminal cleavage (**Fig. 2E**). In contrast, an increased 120-kD ectodomain was produced in R1303Q-COL17, indicating that the R1303Q mutation does not affect the cleavage within NC16A domain. At high plasmin concentrations, the 97-kD COL17 ectodomain started to appear, and it gradually increased dose-dependently on plasmin

(**Fig. 2F**). Thus, the R1303Q mutation does not completely suppress the C-terminal cleavage of COL17. However, these results suggest that R1303Q gives partial but substantial resistance to the C-terminal cleavage of COL17.

Impaired C-terminal cleavage of COL17 in the ECM of R1303Q primary keratinocytes. To assess whether the R1303Q mutation impairs C-terminal cleavage of COL17 in the ECM *in vitro*, normal human epidermal keratinocytes (NHEKs) and keratinocytes from a patient with the mutation (**Case 2, 46 years old, Fig. 1B and 1D**) were studied. In line with *in vitro* limited digestion experiments using plasmin, the 97-kD ectodomain was not present in ECM proteins of R1303Q keratinocytes that had been deposited on the plastic dish at 48 hours after seeding. In contrast, the 97-kD ectodomain was observed in culture medium of R1303Q keratinocytes (**Fig. 3A**). These findings suggest that the C-terminus of immobilized COL17 in the ECM *in vitro* may be less susceptible to digestion enzymes than non-immobilized COL17 on the cell surface of NHEKs or culture medium.

R1303Q keratinocytes deposit increased amounts of laminin332 in the ECM. *In vitro* studies suggest that increased amounts of COL17 with native C-terminus are present in

the ECM of R1303Q keratinocytes. Since the C-terminal region of COL17 is essential for the binding of COL17 and laminin 332 (20, 26), we hypothesized that the non-cleaved native COL17 ectodomain may bind greater amounts of laminin 332 from the ECM of R1303Q keratinocytes. To address this, we investigated ECM proteins of R1303Q keratinocytes. As expected, R1303Q keratinocytes were found to deposit increased amounts of laminin 332 in the ECM **(Fig. 3B)**. However, the laminin 332 gene expression of R1303Q keratinocytes was reduced compared with that of NHEKs **(Fig. 3C)**, indicating that laminin 332 deposition of R1303Q keratinocytes in the ECM may be attributed to posttranslational processes.

The R1303Q mutation altered the morphology of keratinocytes in vitro. Laminin 332, as well as COL17, has been shown to regulate cell adhesion and motility *in vitro* (27-30). R1303Q keratinocytes did not show reduced attachment compared with that of NHEKs (**Fig. 4A**). Regarding morphology, R1303Q keratinocytes showed a greater spreading phenotype and much greater cell size compared with NHEKs (**Fig. 4B and 4C**). Since focal adhesion-associated and hemidesmosome-associated proteins also influence migration ability, we next the assessed expression of these proteins in migrating R1303Q keratinocytes *in vitro*. COL17 and integrin α1 were widely

expressed not only at the leading edges but also at the extended trailing ridge around the cells and the migration tracks in the ECM. These results are in sharp contrast to those of NHEKs (**Fig. 4D and 4E**). These findings may reflect the possibility that the hindered C-terminal cleavage of COL17 prevents keratinocytes from detaching from the ECM.

The R1303Q mutation reduces the motility of keratinocytes in vitro. The above data indicate the altered motility of R1303Q keratinocytes. In line with this, R1303Q keratinocytes showed reduced motility (**Fig. 5A**). Indeed, cumulative migration distance, the total cell migration length from the start point to the end points, was significantly reduced in R1303Q keratinocytes compared with that of NHEKs (**Fig. 5B**). In contrast, gene expression of integrin α3 and β1, which play major roles in the attachment and migration of keratinocytes as components of focal contact (31), had no significant differences between NHEKs and R1303Q (**Fig. 5C and 5D**). To assess whether metalloproteinases (MMPs) and growth factors affect migration, we performed RT-PCR and antibody arrays of these proteins. Gene expression of MMPs from R1303Q keratinocytes tended to be reduced compared with those of NHEKs **(Supplementary Material, Fig. S2A and Table S1)**; however, the protein expression of MMPs in the cultured medium were not solely changed by the mutation **(Supplementary Material,**

Fig. S2B). These findings support the idea that impaired migration of R1303Q keratinocytes is not associated with unregulated MMP expression nor with integrin-mediated keratinocyte motility.

Increased expression of collagen V in the dermis of patients with p.R1303Q. The patients with the homozygous missense mutation p.R1303Q in *COL17A1* show progressive skin atrophy, scarring, and nail abnormalities **(Fig. 1C and 1D)** (16, 17, 19). In addition, sclerotic fingers are a characteristic clinical feature, although the pathomechanisms of this feature remains unclear. We focused on COL5, which is known to be abundantly expressed in the dermis of the patients with systemic sclerosis in the early stage (32). Interestingly, it has been shown that transgenic mice overexpressing the pro α1 (V) chain driven under keratin 5 promotor show duplications of the BMZ (33). Gene expression profiles of ECM proteins showed that *COL5A1* encoding COL5 is markedly increased in R1303Q keratinocytes (**Fig. 6A**). In line with this observation, immunofluorescence studies of skin samples show aberrant deposition of COL5 in the dermis beneath the epidermis (**Fig. 6B**). These observations suggest that increased COL5 might be associated with sclerosis, as well as being associated with BM duplication. Reduced COL1A1 RNA expression in mutant keratinocytes (Fig.6A) is

not involved in the pathogenesis of the present case, because collagen I in the dermis is mainly supplied by dermal fibroblasts rather than by keratinocytes.

Discussion

COL17 is a vital hemidesmosomal transmembrane protein that tightly links basal keratinocytes to the underlying dermis. The essential role of COL17 has been revealed through investigations on the genetic blistering skin diseases (EB (4, 34-36)) and autoimmune blistering skin disease (bullous pemphigoid (9, 11, 37)), in which COL17 is genetically diminished or targeted by autoimmunity. In contrast, the physiological role of COL17 cleavage has not been fully elucidated. Our group and others have recently shown that cleavage of COL17 within the juxtamembranous extracellular NC16A domain is associated with the migration of keratinocytes, which is probably required for these cells to detach from the BM (20, 38, 39). However the physiological significance and the pathological roles of C-terminal cleavage of the COL17 ectodomain has never been revealed. In this study, we have revealed that the homozygous R1303Q mutation hampers the C-terminal cleavage of COL17. Protein-binding studies and analysis using primary keratinocytes from a patient with R1303Q mutation suggested that the impaired C-terminal cleavage of COL17 is

associated with aberrant deposition of laminin 332 in the ECM, which is probably the main pathogenesis of BM disorganization.

Laminin 332, a pivotal molecule in the BM, interacts epidermal receptors with other ECM proteins (40-42). In skin, α3β1 and α6β4 integrins which are respectively expressed as a focal adhesion and a hemidesmosomal molecules interact with laminin 332 as a supramolecular bridge (43). *In vitro*, migrating keratinocytes assemble laminin 332, which is deposited in the ECM (44-47). It is well known that laminin 332 influences cell motility and adhesion (27-29) and that polymerized laminin 332 in the matrix induces stable cell adhesion and suppresses the migration of primary NHEKs (48). In line with this observation, increased deposition of laminin 332 in R1303Q keratinocytes was found to result in a spreading morphology associated with reduced motility.

Unexpectedly, the R1303Q mutation in the NC4 domain of COL17 did not impair binding with laminin 332. Rather, laminin 332 deposition in the ECM was markedly increased, while gene expression of laminin 332 was significantly reduced in primary keratinocytes with the mutation. To address the pathomechanism of the increased

laminin 332 deposition in the ECM due to R1303Q mutation in COL17, we carefully studied the stability of normal and mutant COL17 recombinant proteins. Previous studies have shown that laminin 332 interacts with COL17 via its C-terminal domains (17, 20, 26). In addition, a recent study has shown that the NC4 domain of COL17 is a vital region for the binding of laminin 332 (17). Thus, COL17 whose C-terminus has been cleaved within the NC4 domain is expected to have less binding ability with laminin 332 than that of full-length COL17. The limited digestion of normal and R1303Q COL17 recombinant proteins by plasmin revealed that the mutation attenuates C-terminal cleavage within the NC4 domain. This finding suggests that impaired C-terminal cleavage of COL17 due to R1303Q mutation may be a pathogenesis of increased laminin 332 deposition in the ECM.

The protease(s) responsible for cleaving the C-terminus of COL17, which yields a 97-kD cleaved ectodomain, is poorly defined, although a previous study has shown that a serine protease plasmin can cleave the C-terminus of COL17 to produce a 97-kD ectodomain (14). Plasmin-mediated cleavage sites on COL17 have never been reported; however, the NC4 domain is expected to be targeted, based on the molecular weights of the digested polypeptides. The NC4 domain of COL17 contains four arginines that are

candidate cleavage sites of plasmin, and two of these arginines are Arg1302 and Arg1303. The present study showed that homozygous R1303Q mutation hampers the C-terminal cleavage of this molecule; however, treatment with increased plasmin cleaved the C-terminus domain of recombinant COL17 with the homozygous R1303Q mutation. These results may indicate that cleavage within the NC4 domain of COL17 is biologically important and that such cleavage can be compensated by other cleavage sites such as R1302.

BM duplication can be induced by the impaired functioning of various proteins in basal keratinocytes. Regarding EB patients, COL17 with R1303Q mutation (16, 17, 19, 49), kindler-1 in Kindler syndrome (21) and null expression of integrin α3 (22) all result in BM duplication. In addition, in mice, the null expression of integrin α3 (50) and collagen VI (51), and the overexpression of COL5 (33) in basal keratinocytes, are also associated with BM duplication. These facts indicate that various molecules, including not only intracellular ones but also molecules present in the ECM, are associated with BM formation, although the pathomechanisms of BM duplication have yet to be elucidated. The present study showed that aberrant laminin 332 deposition associated with attenuated C-terminal cleavage of COL17 may induce BM duplication. This

observation argues for the importance of the regulated processing of proteins in the ECM for correct BM formation.

It is of interest that patients with the p.R1303Q mutation show sclerotic fingers**,** which is not observed in Kindler syndrome (21) or in integrin α3-deficient mice (50) or humans (22), all of which show BM duplication that is caused by dysfunction of focal contact components. Fibrosing connective tissue disorders have been known to be caused by activated fibroblasts that lead to the excessive production and deposition of ECM proteins (52). Although the main source of the ECM proteins is fibroblasts, keratinocytes also regulate ECM molecules *in vitro* (53-57). The present study showed that there is increased COL5 expression in R1303Q keratinocytes *in vitro* as well as in the dermis beneath the epidermis of affected skin *in vivo*, although skin biopsy specimens were not obtained from sclerotic fingers lesions. COL5 is a minor fibrillar collagen among ECM components that has important roles in controlling fibrogenesis and regulating fiber size (58, 59). Importantly, the overexpression of COL5 in basal keratinocytes induces BM duplication in mice (33). Taken together with the result of the present study, this suggests that increased COL5 might have, at least in part, an important role in the pathogenesis of fibrosis in the early stage and the duplication of

BM associated with p.R1303Q mutation in COL17.

A limitation of the current study is that it is impossible to observe pathological events due to R1303Q mutation in COL17 for the long term. R1303Q mutation in COL17 is associated with a late-onset phenotype, and skin fragility usually appears around the age of 5 to 17 years (17). The BM of the patients may be mostly normal for several years after birth. Thus, to elucidate the complete pathomechanism underlying the R1303Q mutation, further studies need to address *in vivo* skin.

The current study mainly focuses on the aberrant interaction of COL17 and laminin 332 in the ECM, although other molecules may be involved in the duplication of the BM due to R1303Q mutation in COL17. One candidate molecule is BP230, which is known to interact with COL17, and disorganized BP230 expression in basal keratinocytes has been reported in the skin of COL17-null EB patients (60). In the basal keratinocytes of both cases with R1303Q mutation in COL17, diffuse cytoplasmic expression of BP230 was observed. Another candidate molecule is the major BM molecule collagen IV (COL4), whose RNA expression was slightly increased in the R1303Q keratinocytes. The pathological and physiological roles of these molecules need to be elucidated by

future studies.

In summary, this study is the first to demonstrate the physiological significance of C-terminal cleavage of COL17, which is necessary for the correct migration of basal keratinocytes and BM formation in the ECM. Our results suggest that regulated processing of ECM proteins is essential for cutaneous BM formation.

Materials and Methods

Generation of recombinant normal and mutant COL17. Full-length human *COL17A1* cDNA expressing a DDDDK-tag on the N-terminus (a gift from Professor Kim B. Yancey) was introduced into NotI site of pcDNA5/FRT (designated as COL17-pcDNA5) (Invitrogen). To generate R1303Q mutant COL17, a 2073-bp DNA fragment with the c.3908 G>A mutation in the C-terminal region of COL17 **(Supplementary Material, Fig. S3)** was synthesized in a vector plasmid pUC57 (Genscript). The ClaI and NotI digested mutant 2073-bp fragment was introduced into COL17-pcDNA5, which was digested by ClaI and NotI. For recombinant protein expression, the Flp-In-293 cell line was grown in Dulbecco's Eagle's medium (DMEM) containing 10% fetal calf serum. To establish a stably expressing cell line,

pcDNA5/FRT plasmid with normal or mutant *COL17A1* cDNA was co-transfected with pOG44 into the Flp-In-293 cells by Lipofectamine 2000 (Invitrogen). Stably expressing cells were selected under 200 μg/ml hygromycin B (Invitrogen), as described previously (61). Stably transfected Flp-In 293 cells were incubated in DMEM, and ascorbic acid was added to the culture medium at a concentration of 50 μg/ml for 24 h prior to harvesting. Then, the cells were lysed for 30 min on ice in a buffer containing 1% Nonident P-40, protein inhibitor cocktail and 10mM EDTA in 25 mM Tris, pH 7.2. The normal and mutant COL17 recombinant proteins were immunoprecipitated by DDDDK magnet beads (MBL), following elution by DDDDK peptide (Sigma-Aldrich) (Izumi K, et al. manuscript under preparation). To remove the DDDDK peptide, dialysis was performed overnight against PBS containing 0.1% Nonident P-40.

Plasmin digestion of normal and mutant COL17 recombinant proteins. COL17 recombinant protein was digested with 0.0001 to 0.02 mg/ml human plasmin (ab90928, Abcam) for 1 hour at 37°C. The samples were separated on SDS-PAGE on 7% polyacrylamide gels, after which Coomassie blue staining and immunoblotting were performed.

Protein-protein binding assay. 96-well plates were coated with laminin 332 (0.5 µg/well) (a gift from Dr. Amano at Shiseido, Japan (62)) by incubation overnight at 4°C. After nonspecific binding sites were blocked with 2% bovine serum albumin, the plates were incubated with normal and mutant COL17 diluted in PBS. COL17 bound with immortalized laminin 332 was detected by ELISA using 1:250 diluted rabbit polyclonal antibodies against the human NC16A domain of human COL17 (63).

Mutation analysis for COL17A1 and FERMT1. Genomic DNA was extracted from the patient's peripheral blood cells by DNeasy Blood & Tissue Kit (Invitrogen). The mutation detection strategy was implemented after polymerase chain reaction (PCR) amplification of all exons and the intron–exon border of *COL17A1* and *FERMT1* (NM_017671), followed by direct automated sequencing using the 3130 Genetic Analyzer (Applied Biosystems). Oligonucleotide primers and PCR conditions used in this study are described elsewhere (4, 64).

Cell culture. Primary NHEKs were isolated from normal skin tissue samples obtained from 3 healthy, age-matched volunteers from whom full informed consent was obtained. R1303Q keratinocytes were also isolated from skin samples from patient 2. NHEKs and

R1303Q keratinocytes were cultured in serum-free keratinocyte medium supplemented with bovine pituitary extract and epidermal growth factor (Cnt-57, CELLnTEC). Cells up to the 4th passage were used for this study. Cell attachment ability onto plastic was assessed as previously described (65). To assess migration of basal keratinocytes, $1x10^4$/ml cells were grown on μ-dishes with culture inserts (Ibidi). Eighteen hours after seeding, the cells were incubated further in culture medium under 5% CO2 at 37°C equipped in a FV100 confocal laser scanning microscope (Olympus), and phase-contrast images were captured every 5 minutes for 2 hours. The migration parameters, migration tracks and sizes of cells were analyzed with the Image J software (Manual tracking) and Chemotaxis and Migration Tool software (Ibidi). These experiments were performed at least 3 times.

Immunohistochemistry and Immunoblotting. For immunohistochemistry, skin specimens were mounted and snap-frozen in OCT-compound, and 5-μm cryosections were prepared. Cells cultured on cover slips were fixed with 4% paraformaldehyde and then permeabilized with 1% TritonX-100 in PBS. The cryosections and fixed cells on cover slips were then incubated with primary antibodies for 1 hr at room temperature and detected with secondary FITC or Alexa488-conjugated antibodies. Following

primary antibodies were used to detect COL17 (D20, a gift from Prof. Owaribe and NC16A-1, a gift from Prof. Bruckner-Tuderman (14)): BP230 (s1193, a gift from Prof. J. R. Stanley), plectin (HD1-121, a gift from Dr K. Owaribe), laminin 332 (GB3, Abcam), collagen IV (NeoMarkers, Fremont), collagen VII (LH7.2, Chemicon), COL5 (LS-C 119460, LifeSpan BioSciences), integrin α6 (GoH3, a gift from A. Sonnenberg), integrin β1 (4B7R, Abcam) and integrin β4 (3E1, Chemicon). To stain cytoskeletal actin filaments and nuclei, rhodamine phalloidin (R415, Invitrogen) and propidium iodide were used, respectively. For immunoblotting, subconfluent NHEKs or R1303Q keratinocytes were lysed in a lysis buffer as described (15). The medium proteins were concentrated by Amicon ultrafiltration cassette (30 kDa, Millipore). Preparation of ECM proteins was performed as previously described (66) with some modifications. Briefly, 48 hours after seeding, the cells were incubated at room temperature with 20 mM NH_4OH solution. After all cells were detached, the ECM proteins were thoroughly washed with PBS and then directly lysed with sample buffer. The samples were separated on SDS-PAGE on 7% polyacrylamide gels, followed by transfer onto nitrocellulose membrane. For immunoblotting, following primary antibodies were used to detect COL17 (NC16A-3, a gift from Prof. Bruckner-Tuderman (14)): laminin 332 polyclonal antibody (ab14509, Abcam), laminin α3 (BM2, also termed BM165 (67)),

laminin β3 (sc-20775, Santa Cruz), laminin γ2 (D4B5, Millipore) and b-tubulin (ab6046, Abcam). After incubation with an HRP-conjugated secondary Ab, signals were visualized by ECL-plus (GE Healthcare). Electron microscopy was performed as described elsewhere (9).

Antibody arrays for MMPs and growth factors. Twenty-four hours after cultivation, the culture medium of NHEKs and R1303Q keratinocytes was collected. Ray Bio Human Matrix Metalloproteinase Antibody Array 1 Map and Human Growth Factor Antibody Array 1 Map were used according to the manufacturer's protocols (RayBiotech Inc).

Quantitative RT-PCR. mRNA was extracted from cultured NHEKs or R1303Q keratinocytes 24 after plating by RNeasy Mini Kit (Invitrogen). Single-strand cDNA was synthesized using RT2 First Strand Kit (Qiagen). According to the manufacturer's instructions, assays were performed using RT^2 SYBR GREEN/ROX PCR Master Mix (Qiagen) and Step-OnePlus (Applied Biosystems). The RT^2 Profiler^{TM} PCR Array System with Human Extracellular Matrix & Adhesion Molecules (Qiagen) was used three times, and the results were analyzed using the RT^2 Profiler program (Qiagen). Data were normalized to Hypoxanthine phosphoribosyltransferase 1 housekeeping gene.

Statistical analysis. Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software, CA). Data were expressed as mean ± standard error of means. P values were analyzed using parametric Student's unpaired *t*-test, or non-parametric Mann-Whitney U-test. We regarded *P* values of less than 0.05 as significant. P-values are indicated with *0.01<p<0.05, **0.01<p<0.001, ***0.0001<p<0.001, ****p<0.0001.

Ethics. The medical ethics committee of Hokkaido University approved all described studies. The study was conducted according to T*he Declaration of Helsinki Principles*. The patients gave their written informed consent.

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Conflict of Interest Statement

None declared.

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Legends to Figures

Figure 1. Clinical and histopathological findings of a 32-year-old (case 1) and a 46-year-old (case 2) with R1303Q mutations in *COL17A1*. (A, B) The family tree of case 1 **(A)** and of case 2 **(B)**. **(C)** Clinical findings of case 1. Focal mechanical blister formation on the little finger (arrow), poikiloderma around the neck and sclerotic fingers associated with nail loss are observed. **(D)** The clinical findings of case 2. In addition to focal mechanical blister formation on the right knee (arrows) and poikiloderma around the neck, the nails have been completely lost and the fingers are too sclerotic to flex and extend. **(E)** Immunofluorescent studies on skin samples from case 1 (upper) and case 2 (lower) found the duplication of collagen VII, XVII and laminin 332. Stars: blisters. Red: propidium iodide. Scale bar: 50 μm. **(F, G)** Electron microscopy findings of case 1 **(F)** and case 2 **(G)** show duplicated lamina densa (arrows). Scale bar: 5.0 μm. Arrowheads: hemidesmosomes.

Figure 2. *In vitro* limited digestion experiment by plasmin and COL17-laminin 332 binding assay. (A) A schema of COL17. The 120-kD COL17 ectodomain can be further processed into a 97-kD polypeptide by cleaving the C-terminus of the molecule. Based on the molecular weight, the C-terminal cleavage site is predicted to be within the NC4

domain of COL17, in which Arg1303 (red colored) is present. **(B, C)** Full-length normal COL17 recombinant protein (designated as normal-COL17) and full-length COL17 with R1303Q mutant recombinant protein (designated as R1303Q-COL17). Without boiling before SDS-PAGE (non-denaturing conditions), a 540-kDa band corresponding to a homotrimer of three 180 k-Da chains is seen. After boiling (denaturing conditions), a 180-kDa band corresponding to a monomeric form appears. Coomassie blue staining is shown in **(B)** and immunoblotting using antibody NC16A-3 is shown in **(C)**. **(D)** COL17-lamnin 332 binding assay reveals that the full-length R1303Q-COL17 binds to immobilized laminin 332 in a manner similar to that of full-length normal-COL17. Immunoblotting findings using antibody NC16A-3 for the normal and R1303Q-COL17 recombinant proteins used in this study are shown. **(E)** Normal and R1303Q-COL17 recombinant proteins were treated with 0.01mg/ml of plasmin. Note that the 97-kD ectodomain (arrow) is markedly reduced in R1303Q-COL17, indicating that the R1303Q mutation inhibits C-terminal cleavage. **(F)** Treatment of normal and R1303Q-COL17 recombinant proteins with different concentrations of plasmin. At high concentrations (>0.005 mg/ml of plasmin), the 97-kD ectodomain begins to appear.

Figure 3. ECM proteins and gene expression of R1303Q keratinocytes. (A) In the

ECM of NHEKs, in addition to full-length 180-kD COL17 (star), we can observe 120-kD (arrowhead) and 97-kD (arrow) polypeptides, whereas the 97-kD ectodomain is not present in the R1303Q keratinocytes. In culture medium, 120-kDa and 97-kDa fragments are also seen in NHEKs and R1303Q keratinocytes. **(B)** R1303Q keratinocytes deposited increased amounts of laminin 332 in the ECM, although their processing pattern has not been changed. **(C)** The gene expression of *LAMA3* coding α3 chains of laminin 332 and *LAMB3* coding β3 chains of laminin 332 in the R1303Q keratinocytes is significantly reduced compared with those in the NHEKs. (**p*=0.0165, ****p*=0.0002 respectively.)

Figure 4. Morphology of R1303Q keratinocytes *in vitro*. **(A)** R1303Q keratinocytes do not show reduced attachment compared with NHEKs (n=3, *p*=0.47). **(B)** The cell size of the R1303Q keratinocytes (n=229) is significantly greater than that of the NHEKs (n=205) (****p*=0.0002). **(C)** The gross appearance of the R1303Q keratinocytes is not different from that of the NHEKs, but the former tend to show a spreading morphology, especially when they accumulate together (arrows). Scale bar: 50 μm. **(D, E)** Morphology and immunolocalization of focal adhesion-associated and hemidesmosome-associated proteins in R1303Q keratinocytes. **(D)** COL17 (NC16A-1,

green) is widely expressed not only at the leading edges but also at the extended trailing ridge and the migration tracks in the ECM (arrows) in the R1303Q keratinocytes. Red: phalloidin rhodamine. Scale bar: 40 μm. **(E)** Integrin β1 (green) shows similar to those of COL17, and elongated migration tracks and numerous trailing ridges around the R1303Q keratinocytes (arrows) are detected. Red: phalloidin rhodamine. Scale bar: 40μm.

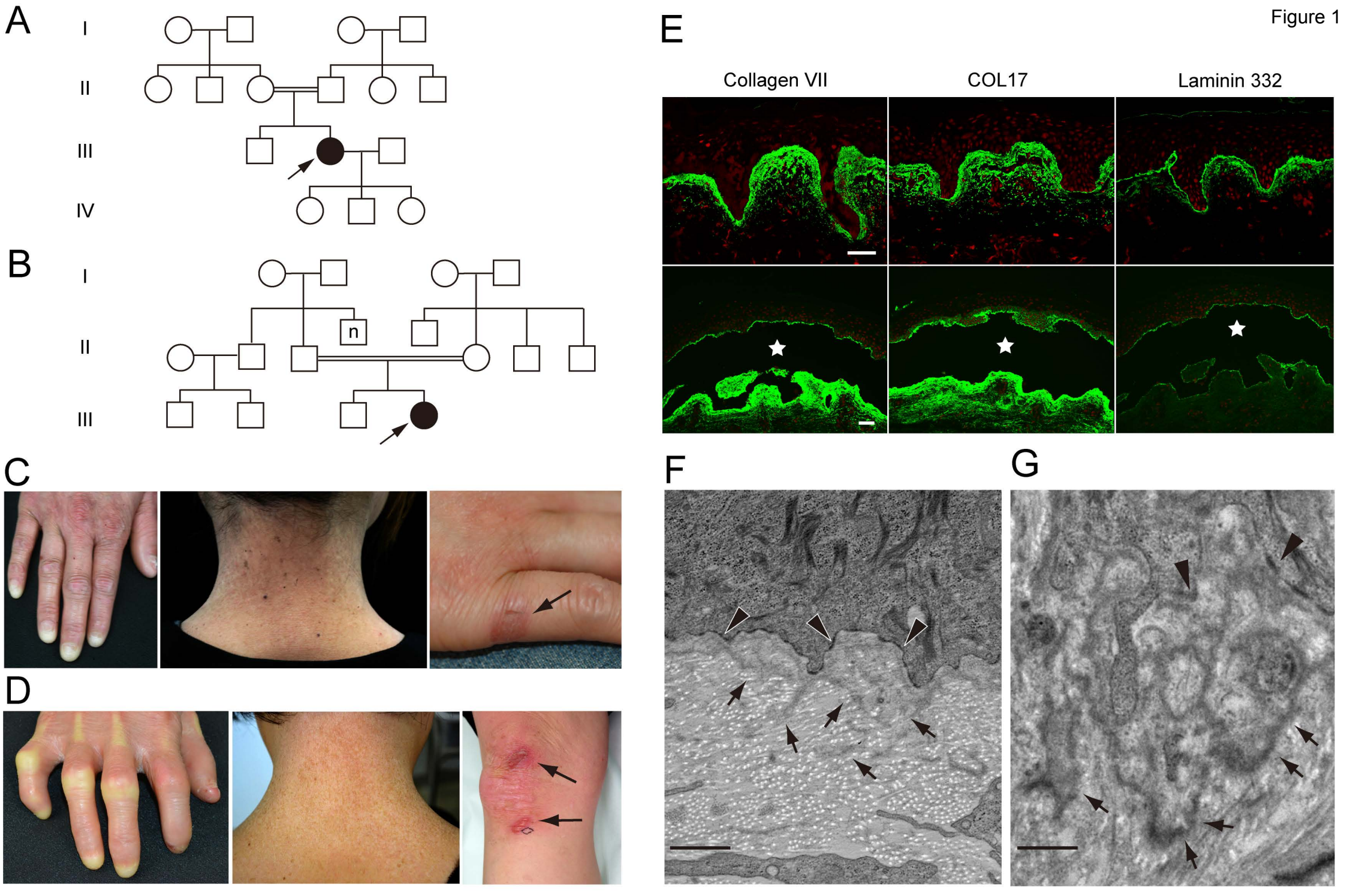
Figure 5. Motility of R1303Q keratinocytes *in vitro*. (A) Time-lapse imaging captured every 5 minutes for 2 hours. Compared with NHEKs, R1303Q keratinocytes showed reduced motility. Scale bar: 200μm. **(B)** Cumulative distance of R1303Q keratinocytes for 1 hour was significantly reduced compared with that of NHEKs (****p=0.0001). **(C, D)** Gene expression levels of integrin α3 and β1 show no significant differences between NHEKs and R1303Q keratinocytes (n=3). **(C)** p=0.3095, **(D)** p=0.0952

Figure 6. Gene expression of ECM proteins in R1303Q keratinocytes. (A) Gene expression of collagen super-family molecules in the NHEKs and R1303Q keratinocytes is shown. *COL5A1* is markedly increased, and *COL1A1*, *COL6A1* and *COL6A2* were reduced. **(B)** Immunofluorescent studies in low magnification (upper)

and high magnification (lower). In the patients with the p.R1303Q mutation, COL5 deposition at the upper dermis is increased compared with that of normal human skin. E: epidermis. Scale bar: 100 μm

Abbreviations

basement membrane, BM; extracellular matrix, ECM; collagen XVII, COL17; epidermolysis bullosa, EB); juxtamembranous non-collagenous, NC; collagen V, COL5; normal human epidermal keratinocyte, NHEK; metalloproteinases, MMP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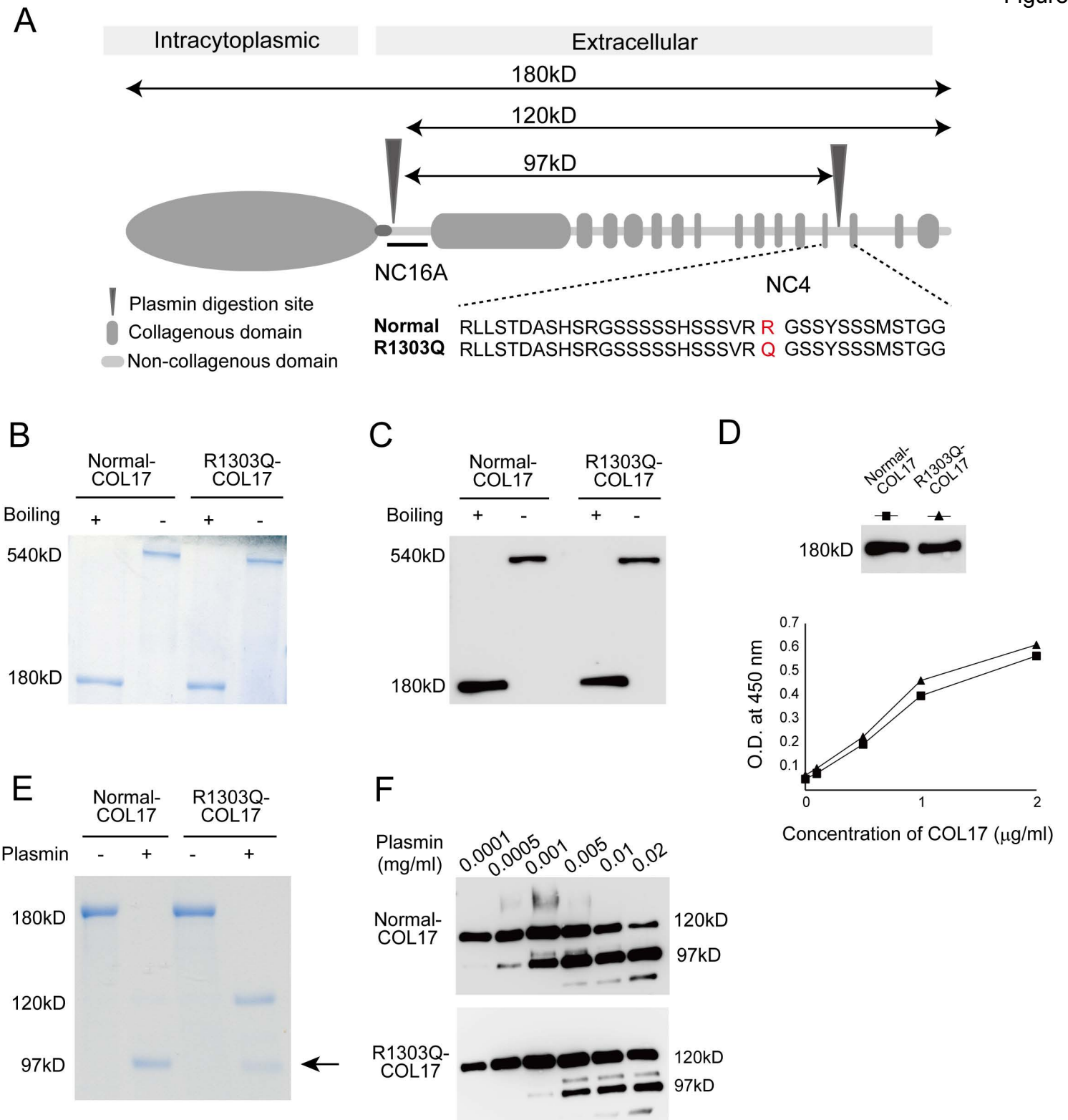
A
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180kD
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Collagenous domain
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Normal RLLSTDASHSRGSSSSSHSSSVR R GSSYSSSMSTGG
R1303Q RLLSTDASHSRGSSSSSHSSSVR Q GSSYSSSMSTGG
B
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R1303Q-COL17
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97kD
F
Plasmin (mg/ml)
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0.0005
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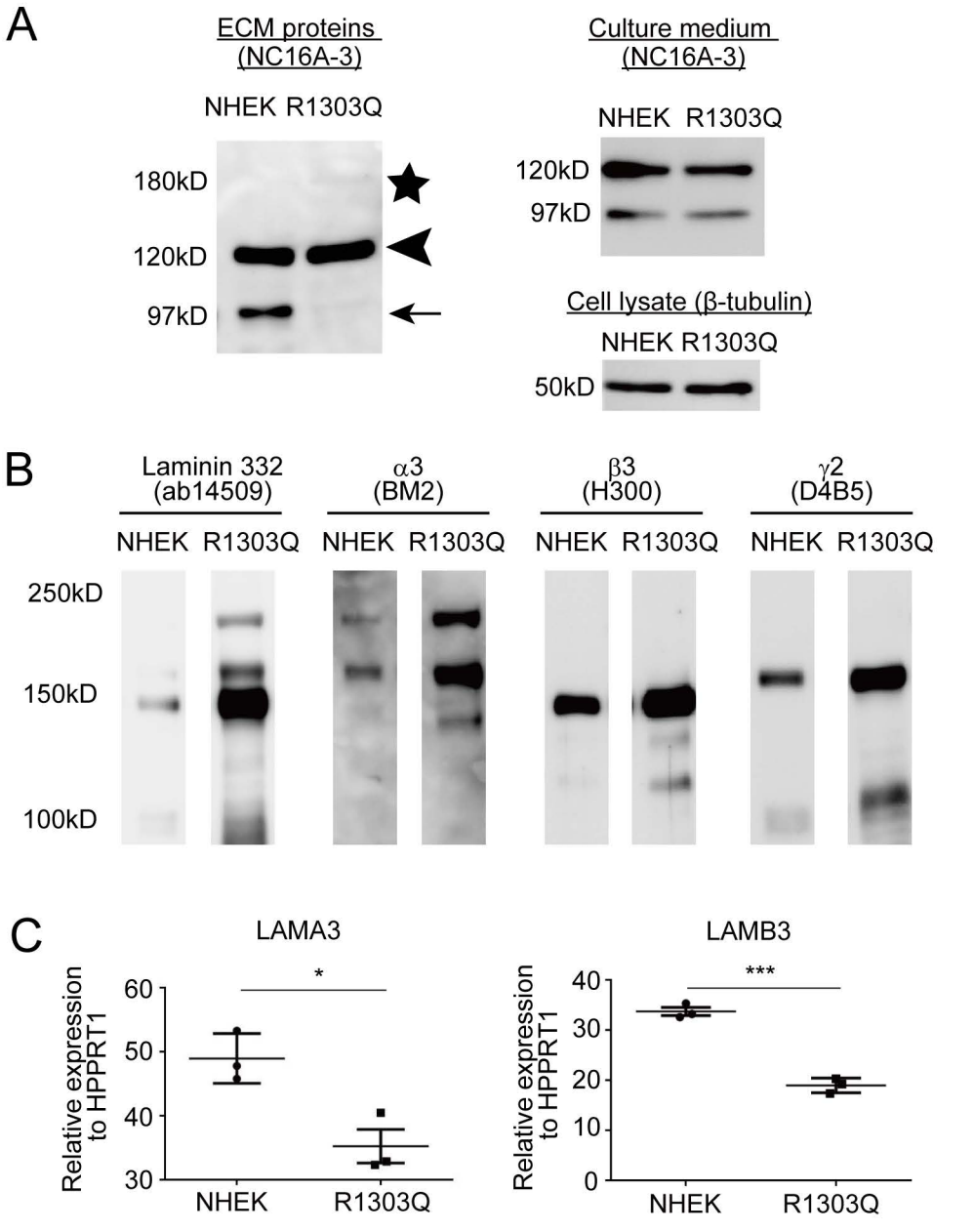
Normal-COL17
120kD
97kD
R1303Q-COL17
120kD
97kD


A
ECM proteins (NC16A-3)
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NHEK R1303Q
120kD
97kD
Cell lysate (β-tubulin)
NHEK R1303Q
50kD
B
Laminin 332 (ab14509)
α3 (BM2)
β3 (H300)
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Relative expression to HPPRT1
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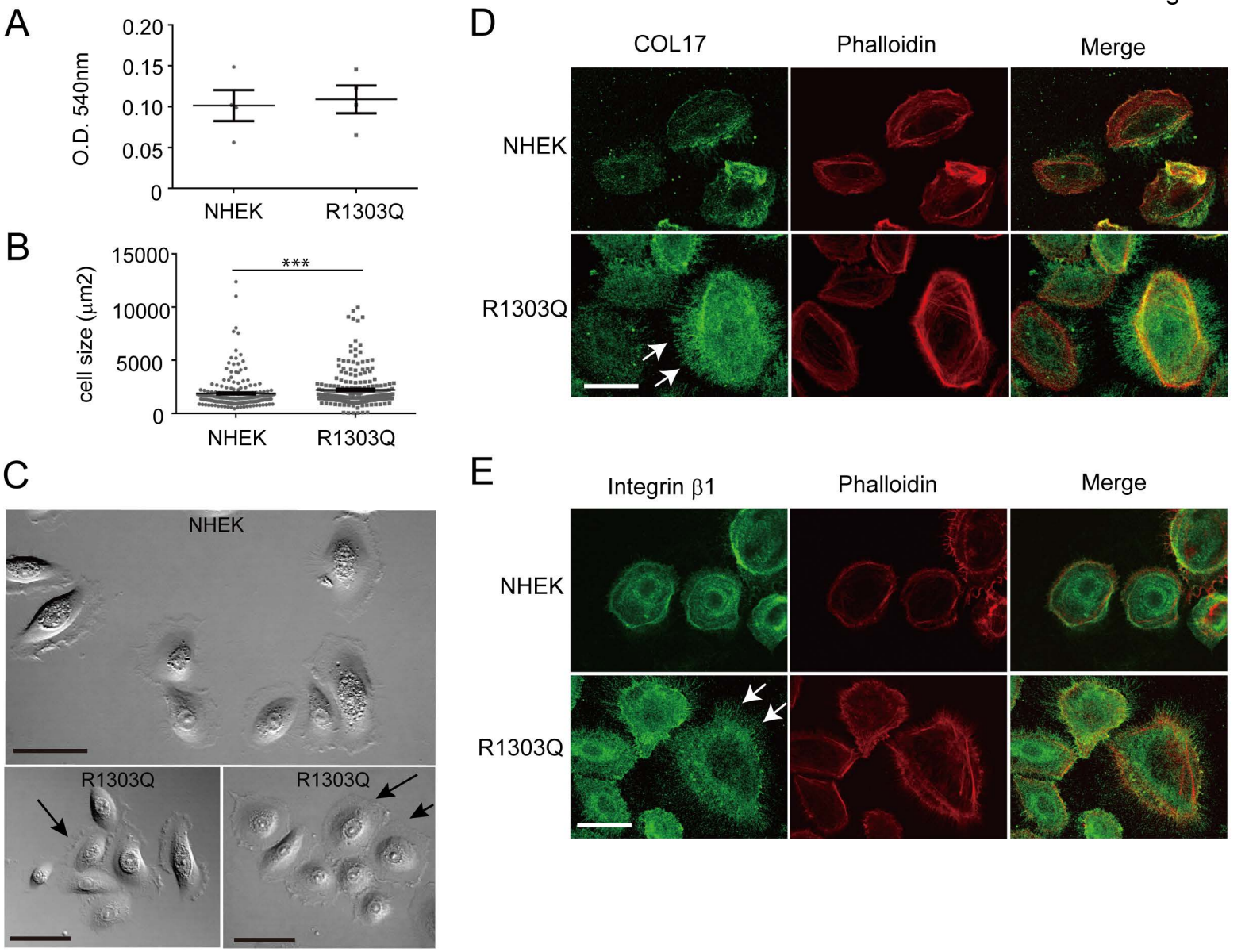
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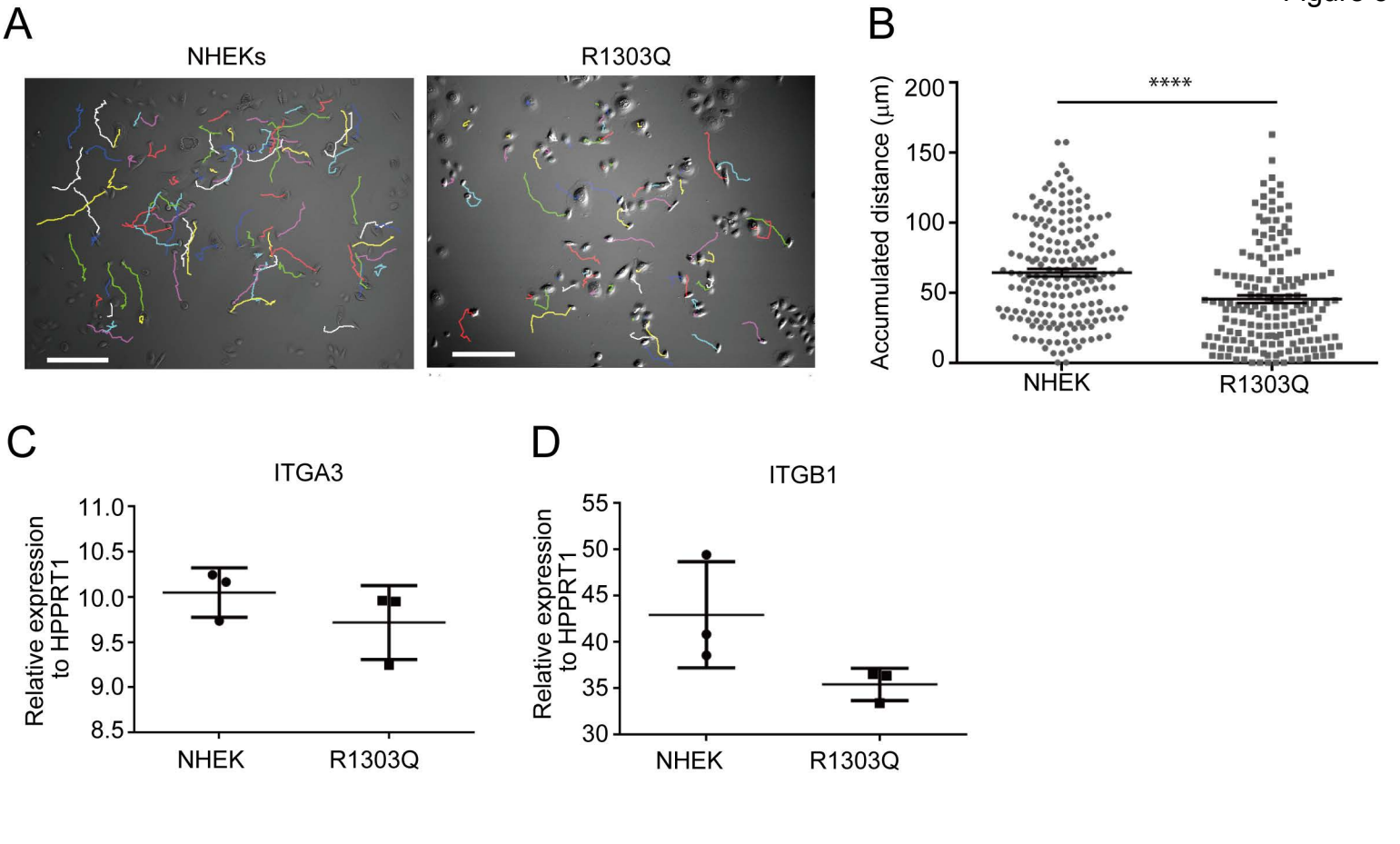
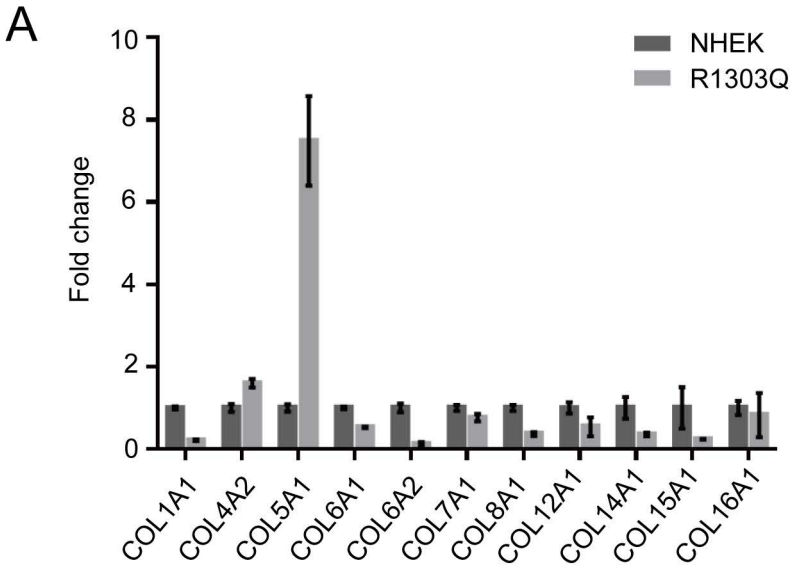
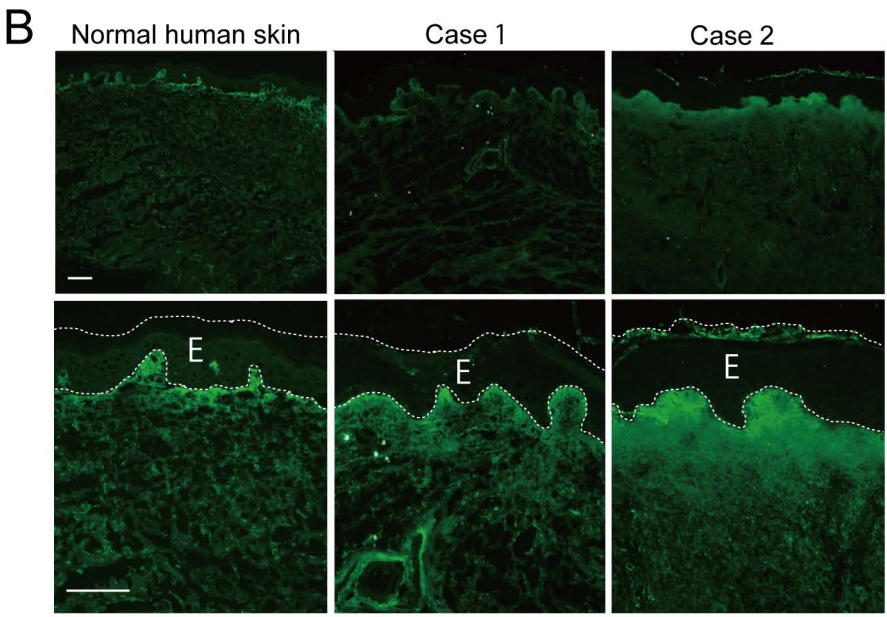
Phalloidin
Merge
NHEK
R1303Q
E
Integrin β1
Phalloidin
Merge
NHEK
R1303Q


A
NHEKs
R1303Q
B
Accumulated distance (μm)
200
150
100
50
0
****
NHEK
R1303Q
C
ITGA3
Relative expression to HPPRT1
11.0
10.5
10.0
9.5
9.0
8.5
NHEK
R1303Q
D
ITGB1
Relative expression to HPPRT1
55
50
45
40
35
30
NHEK
R1303Q


A
NHEK
R1303Q
Fold change
10
8
6
4
2
0
COL1A1
COL4A2
COL5A1
COL6A1
COL6A2
COL7A1
COL8A1
COL12A1
COL14A1
COL15A1
COL16A1


B
Normal human skin
Case 1
Case 2
E
E
E