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Calpain 12 function revealed through the study of an atypical case of autosomal recessive congenital ichthyosis

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ABSTRACT

Congenital erythroderma is a rare and often life-threatening condition, which has been shown to result from mutations in several genes encoding important components of the epidermal differentiation program. Using whole exome sequencing, we identified in a child with congenital exfoliative erythroderma, hypotrichosis, severe nail dystrophy and failure to thrive, two heterozygous mutations in *ABCA12* (c.2956C>T, p.R986W; c.5778+2T>C, p. G1900Mfs*16), a gene known to be associated with 2 forms of ichthyosis, autosomal recessive congenital ichthyosis and harlequin ichthyosis. Because the patient displayed an atypical phenotype, including severe hair and nail manifestations, we scrutinized the exome sequencing data for additional potentially deleterious genetic variations in genes of relevance to the cornification process. Two mutations were identified in *CAPN12*, encoding a member of the calpain proteases: a paternal missense mutation (c.1511C>A; p.P504Q) and a maternal deletion due to activation of a cryptic splice site in exon 9 of the gene (c.1090_1129del ; p.Val364Lysfs*11). The calpain 12 protein was found to be expressed in both the epidermis and hair follicle of normal skin but its expression was dramatically reduced in the patient's skin. Down-regulation of capn12 expression in zebrafish was associated with abnormal epidermal morphogenesis. siRNA knockdown of *CAPN12* in three-dimensional human skin models was associated with acanthosis, disorganized epidermal architecture and down-regulation of several differentiation markers, including filaggrin. Accordingly, filaggrin expression was almost absent in the patient skin. Using *ex vivo* live imaging, siRNA knockdown of calpain 12 in skin from *K14-H2B GFP* mice led to significant hair follicle catagen transformation compared to controls. In summary, our results indicate that calpain 12 plays an essential role during epidermal ontogenesis and normal hair

follicle cycling and that its absence may aggravate the clinical manifestations of *ABCA12* mutations.

INTRODUCTION

Congenital erythroderma refers to a genetically and phenotypically heterogeneous group of disorders of cornification featuring generalized redness and scaling present at birth (Pruszkowski *et al.*, 2000). Although congenital erythroderma has been associated with a number of acquired and hereditary disorders, the vast majority of cases are due to inherited mutations in genes encoding important components of the epidermal differentiation program. Among the various etiologies associated with congenital erythroderma, autosomal recessive congenital ichthyoses (ARCIs) rank first (Pruszkowski *et al.*, 2000). ARCIs are caused by mutations in genes encoding proteins involved in the formation of the epidermal barrier, such as *ABCA12*, encoding a transporter involved in lipid loading within epidermal lamellar granules (Oji *et al.*, 2010). Congenital erythroderma is not only characterized by extensive genetic heterogeneity, it is also characterized by striking clinical variability, which has suggested the existence of modifying genetic variants responsible for aggravating or attenuating disease phenotypes (Cooper *et al.*, 2013). The identification of modifier variants can sometimes reveal important biological functions. In the present study, the delineation of the molecular basis of an atypical case of congenital erythroderma revealed a hitherto unknown role for calpain 12 (CAPN12) during epidermal differentiation and hair follicle cycling.

RESULTS

Phenotype delineation

A 6-week old boy was referred for investigation because of congenital erythroderma and failure to thrive. The patient was the second child of healthy unrelated parents of Palestinian Armenian

and Palestinian Catholic origin. He had a healthy brother and there was no family history of skin or hereditary diseases. The patient was born after 34 weeks of gestation. At birth, his skin was reddish and partly covered with thick grayish secretions, but his hair was normal. He was hospitalized in the pediatric intensive care unit, where he was treated with intravenous antibiotics during three episodes of sepsis associated with hypoglycemia and hypocalcemia. Over the first two weeks of life, the skin became red and scaly (Fig. 1a), and he progressively lost most of his hair. General and neurological examinations were normal. Apart from ectropion, ophthalmological examination was normal. Complete blood count, blood chemistry and IgE levels were normal. Blood smear was normal with no evidence of Jordan's anomaly. Ultrasound examination of the abdomen and pelvis was unrevealing. Repeated echocardiograms and BERA hearing test were normal.

Over the first two years of life, the patient failed to grow (currently below the 3rd percentile) but did not experience any additional infectious complications. Although his skin condition remained stable and some hair growth was noticed (Fig. 1b), massive overgrowth of his nail plates (Fig. 1c) became evident at the age of one year, markedly interfering with daily function.

A skin biopsy revealed a perivascular, psoriasiform dermatitis with parakeratosis and a relatively thin stratum corneum showing intracorneal splitting (Fig. 1d). Hair microscopy was unremarkable (not shown).

Mutation analysis

Because the patient's clinical features (congenital erythroderma, skin peeling, hypotrichosis) were reminiscent of Netherton syndrome or related syndromes (Samuelov and Sprecher, 2014), we initially excluded mutations in *SPINK5*, *CDSN* and *DSG1* by direct sequencing (not shown).

A DNA sample from the patient was then subjected to whole exome sequencing and data were initially scrutinized for mutations in genes known to be associated with disorders of cornification (Oji *et al.*, 2010), leading to the identification of two heterozygous mutations in *ABCA12* (Fig. 2a): c.2956C>T, predicted to result in p.R986W substitution, and c.5778+2T>C, predicted to abolish a conserved donor splice site located in intron 38 and to result in p. G1900Mfs*16. Despite the fact that only one of the two mutations (p.R986W) has been published previously (Fukuda *et al*, 2012), a number of facts support the possibility that they are pathogenic. Both mutations were found to co-segregate with the disease phenotype (Fig. 2b). Mutation c.2956C>T was found in three out of 134,322 alleles deposited in public databases including NCBI, HGMD, UCSC, ENSEMBL, 1000 Genomes Project, ExAc and the NHLBI Grand Opportunity Exome Sequencing Project, while mutation c.5778+2T>C was not found in any of the public databases mentioned above. Both mutations were excluded using PCR-RFLP assays from a cohort of 274 and 207 population-matched non-affected individuals, respectively. Mutation p.R986W affects a highly conserved residue (Conseq = 9, range 1-9; http://conseq.tau.ac.il/) and is predicted to be damaging by Polyphen-2 (score = 1; http://genetics.bwh.harvard.edu/pph2/) and SIFT (Score = 0; http://sift.jcvi.org/) software. To assess the consequences of mutation c.5778+2T>C, we sequenced cDNA derived from a patient skin biopsy with a primer pair encompassing exons 37-39. As shown in Fig.2c, the mutation was found to result in an 88-bp deletion, due to exon 38 skipping, which in turn, is likely to result in mRNA decay as demonstrated in Fig. 2c.

As mentioned above, *ABCA12* mutations have been shown to cause both ARCI and harlequin ichthyosis. Our patient clearly did not display a phenotype resembling harlequin ichthyosis, and hypotrichosis with exuberant nail growth is not typical of ARCI.

We therefore re-ascertained the whole exome sequencing data for additional deleterious sequence alterations of potential relevance to the skin phenotype displayed by the patient. We looked for rare and functionally relevant changes and therefore applied the following filtration criteria (Isakov *et al.*, 2013a; Isakov *et al.*, 2013b): MAF <0.01 (and no more than 25 carriers reported); high conservation as predicted by four bioinformatics software: Conseq (Berezin *et al.*, 2004) > 8, Phylop (Siepel *et al.*, 2006) > 0.85, GERP (Pollard *et al.*, 2010) > 1.5 and phast-Cons-Elements-46way (Siepel *et al.*, 2005); and deleterious effects on protein function as predicted by PolyPhen2 (Adzhubei *et al.*, 2010) > 0.95 and SIFT (Kumar *et al.*, 2009) < 0.05. Only one heterozygous transversion, c.1511C>A in *CAPN12*, passed all functional filters and is , predicted to result in p.P504Q substitution.

CAPN12 encodes calpain 12, a calcium-activated cysteine protease, which belongs to a group of proteins which have been shown to play an important role in epidermal differentiation (Campbell and Davies, 2012; Ono and Sorimachi, 2012) and have recently been implicated in the pathogenesis of a human skin disease (Lin *et al.*, 2015). *Capn12* was shown in mice to be exclusively expressed in the skin (Dear *et al.*, 2000). Accordingly, we found out that it is mainly expressed in human skin with weaker to almost absent expression in other tissues (Fig. S1).

The mutation was identified by direct sequencing (Fig. 2d) and a PCR-RFLP assay (Fig. 2e) in the patient, his father and his brother, but not in the patient's mother. The mutation was found in two out of 25,126 alleles deposited in public databases including NCBI, HGMD, UCSC, ENSEMBL, 1000 Genomes Project, ExAc and the NHLBI Grand Opportunity Exome Sequencing Project. Using the PCR-RFLP assay described above, we excluded the mutation from a panel of 176 population-matched healthy controls. The mutation affects a highly conserved amino acid residue (Conseq = 9, range 1-9) and is likely to be damaging to the protein

function (SIFT score = 0, Polyphen-2 score = 0.997). The sequence of CAPN12 (NP_653292.2) was submitted to the Phyre2 Protein Fold recognition server (http://www.sbg.bio.ic.ac.uk/phyre2/html /page.cgi?id=index) to obtain a molecular model. A large number of Calpain and Calpain-like structures have been experimentally determined and deposited in the Protein Data Bank (http://www.rcsb.org/pdb/home/home.do). CAPN12 was modeled according to the human m-calpain large subunit structure (PDB code 1KFX:L) with a statistical confidence of 100% (residues 1-719). The site of the mutation P504 is located within the central domain of the protein, which includes a large β-sandwich motif (Fig. S2). This domain is surrounded by the N-terminal and C-terminal domains and likely stabilizes the entire protein fold. The proline pyrrolidine side chain faces into the sandwich interface directly facing and participating in a highly hydrophobic core that includes W363, F359 and F385. Mutation of the polar and bulky glutamine residue is predicted to have a disrupting effect on this hydrophobic core, destabilizing the sandwich interactions.

Full sequencing of the entire coding and non-coding (including the promoter region) sequence of *CAPN12* in the proband and his mother failed to reveal a second pathogenic sequence alteration. However, direct sequencing of a cDNA sample derived from a skin biopsy obtained from the patient revealed a 40 bp deletion, presumably due to activation of a cryptic splice site within exon 9 of the gene (Fig. 2f). The heterozygous mutation c.1090_1129del is predicted to result in frameshift, premature protein termination (p.Val364Lysfs*11) and most likely, nonsense-mediated mRNA decay. To confirm segregation of the mutation with the disease phenotype, we obtained skin biopsies from the patient's parents. Using direct sequencing of biopsy-derived cDNA, we demonstrated the presence of the c.1090_1129del mutation in the cDNA generated from the patient's mother, but not in the cDNA generated from the patient's father (not shown).

Because we failed to identify any DNA or RNA sequence alteration which could have caused activation of the cryptic splice site in exon 9 of the *CAPN12* gene, we hypothesized that epigenetic changes may possibly underlie this phenomenon. Indeed, a steadily growing body of evidence suggests that exonic DNA methylation is associated with increased alternative splicing (Lev Maor *et al.*, 2015; Malousi and Kouidou, 2012; Maunakea *et al.*, 2013). We therefore compared DNA methylation pattern in the patient, his parents and control individuals. Both the patient and his mother demonstrated significantly increased DNA methylation in exon 9 (Fig. 2g), as compared with all other DNA samples. Of interest, these differences in methylation status were noticed in keratinocytes but not in peripheral blood lymphocytes (not shown), suggesting that hypermethylation-associated alternative splicing within *CAPN12* may be tissue-specific, as previously shown for other genes (Gutierrez-Arcelus *et al.*, 2015). To assess the functional consequences of the presence of the two deleterious *CAPN12* mutations that were identified in the patient, we compared the pattern of expression of calpain 12 in the skin of the patient and in normal skin. As shown in Fig. 3a, calpain 12 is strongly expressed in normal epidermis, as previously shown in murine skin, but was absent in the patient skin. Using qRT-PCR, we found out that *CAPN12* RNA levels were 8-fold lower in the patient skin as compared with normal skin (Fig. 3b).

Delineation of CAPN12 function in the epidermis

Little is currently known about the function of calpain 12 in the epidermis (Dear *et al.*, 2000). Over the past few years, the zebrafish has become recognized as a useful model to ascertain the importance of mediators of human epidermal development (Li and Uitto, 2013). We therefore down-regulated the expression of *capn12* in zebrafish using a gene-specific morpholino (Fig.

S3). Scanning electron microscopy of morphant larvae at 3 dpf revealed abnormally large KCs with aberrant microridge formation as compared with normal keratinocytes in control larvae (Fig. 4 a-b). In addition, transmission electron microscopy analysis demonstrated almost complete absence of microridges in the morphant larvae (Fig. 4 c-d) which also developed pericardial edema and curled tail phenotype (Fig.4 e-f), associated with death at 6-8 dpf.

We then used two *in vitro* models to determine whether calpain 12 is involved in epidermal differentiation and hair cycling. First, using siRNA, we down-regulated *CAPN12* expression in primary keratinocytes and used these keratinocytes to generate three-dimensional skin equivalents (Fig. S4). As shown in Fig. 5a and Fig. S5, *CAPN12* down-regulation resulted in a disorganized epidermal architecture associated with mild acanthosis, suggesting abnormal differentiation. Keratin gene expression was also consistent with abnormal epidermal differentiation (Fig. 6S). We therefore analyzed the pattern of expression of filaggrin, which has been shown to be a substrate of calpains[38] and is a marker of terminal differentiation in the epidermis. Filaggrin was almost absent and mislocalized in organotypic skin equivalents down-regulated for *CAPN12*, as well as in the skin of the patient (Fig. 5 b-c). Of note, *ABCA12* gene expression, as assessed by qRT-PCR, was elevated in organotypic skin equivalents down-regulated for *CAPN12* while protein expression was not significantly different (not shown).

We considered the possibility that filaggrin deficiency in the patient skin may have been the result of bi-allelic mutations in the *FLG* gene. To rule out this possibility, the *FLG* gene was fully sequenced, which revealed no loss-of-function mutations (not shown). We also assessed the possibility that filaggrin deficiency may be related to ABCA12 dysfunction. However, as previously shown (Akiyama *et al.*, 1996), we found normal to increased filaggrin expression in the skin of patients carrying bi-allelic null mutations in the *ABCA12* gene (Fig. 5d). We also

attempted to generate skin equivalents deficient for both calpain 12 and ABCA12, but were unable to obtain epidermal differentiation in these double knock down skin models (not shown). Second, we used *K14 H2B-GFP* mice to assess by *ex vivo* live imaging the effect of *Capn12* down-regulation on hair cycling (Fig. S7, Supplementary movies 1-3). As shown in Fig. S8, we achieved efficient down regulation of calpain 12 expression in skin strips derived from those mice. Live imaging revealed that calpain 12 down-regulation induced catagen-like transformation in hair follicles (Fig. 6). Many hair follicles treated with *Capn12* siRNA showed evidence of increased apoptotic activity (Fig. S9), suggesting that calpain 12 expression is essential for normal hair follicle cycling.

DISCUSSION

Disorders of cornification, and ARCI more specially, are known to be characterized by clinical heterogeneity (Oji *et al.*, 2010), which in turn very much complicates both the diagnosis and the genetic counseling of families at risk for these disorders. Apart from the effect of filaggrin deficiency on the clinical manifestations of X-linked recessive ichthyosis (Liao *et al.*, 2007) and pachyonychia congenital (Gruber *et al.*, 2009), very little is currently known about genetic modifiers of clinical phenotypes in disorders of cornification. Here, we identified bi-allelic deleterious mutations in *CAPN12* in a child with exfoliative erythroderma carrying 2 mutations in *ABCA12*. Although congenital erythroderma can be a consequence of mutations in *ABCA12*, several clinical (severe hypotrichosis and exuberant nail plate growth) and pathological (loss of epidermal architecture and lack of hyperkeratosis) features were deemed atypical of ARCI. Severe alopecia has been reported in the context of harlequin ichthyosis but ARCI is usually

associated with no or a mild hair phenotype as previously shown in a patient carrying p.R986W and a splice sire mutation (Fukuda *et al.*, 2012).

Calpains form a large family of 14 distinct calcium-dependent cysteine proteinases which share a similar protease domain (Goll *et al.*, 2003). They have been shown to regulate major cellular functions including apoptosis and cell motility and have been implicated in the pathogenesis of human diseases including cancer, cardiovascular and neurodegenerative disorders (Miyazaki *et al.*, 2013; Momeni, 2011; Ono and Sorimachi, 2012; Sorimachi *et al.*, 2012; Storr *et al.*, 2011; Vosler *et al.*, 2008). Several lines of evidence suggest that calpains may play an important role in cutaneous biology. Calpain inhibition was found to be associated with abnormal cornification (Kim and Bae, 1998) and delayed wound healing (Nassar *et al.*, 2012) while calpain activity was also found to be necessary for staphylococci to break through the epidermal barrier (Soong *et al.*, 2012). More recently, calpastin, a calpain inhibitor, was found to be absent in the skin of patients with PLACK syndrome (MIM 616295), which is characterized by peeling skin, leukonychia, acral keratosis, cheilitis, and keratoderma as well as by loss of epidermal cell-cell adhesion (Lin *et al.*, 2015). Although most calpains are distributed ubiquitously, a minority of these proteinases are expressed in a tissue-specific fashion, including calpain 12 which is predominantly expressed in the skin (this study and (Dear *et al.*, 2000)).

Here we show that calpain 12 plays a pivotal role in epidermal differentiation and hair follicle cycling. In agreement with our data, a recent paper identified capn12 as essential for skin integrity in zebrafish (Westcot *et al.*, 2015). Although the exact mechanism underlying calpain 12 mode of action during epidermal differentiation remains to be determined, the histopathological findings secondary to CAPN12 down-regulation in three-dimensional models suggest that calpain 12 deficiency may interfere with the normal processing and/or activation of

critical components of the cornified cell envelope. This hypothesis is in line with earlier studies which suggested that calpains contribute to epidermal maturation by activating transglutaminase 1 and promoting the processing of filaggrin (Kim and Bae, 1998; Resing *et al.*, 1993; Yamazaki *et al.*, 1997). In fact, filaggrin expression was markedly reduced and mislocalized in the skin of the patient. The fact that down-regulation of *CAPN12* in three-dimensional skin equivalents was also associated with filaggrin deficiency argues for a direct role of calpain 12 in the regulation of filaggrin expression in the differentiating epidermis. Filaggrin deficiency could in part underlie the unusual severity of this patient's condition as *FLG* mutations have been associated with both epidermal and follicular phenotypes (Meng *et al.*, 2014), although it is possible that other abnormalities (e.g. abnormal expression of adhesion molecules, which are known to play an important role in the regulation of cornification(Harmon *et al.*, 2013)) are also responsible for the overall severe phenotype displayed by the patient.

Recently, another atypical case of ARCI caused by mutations in *NIPAL4* was also shown to be associated with modifying genetic variations in genes modulating epidermal differentiation (Kiritsi *et al.*, 2015), substantiating the notion that the phenotypic variability typical of ARCI could often be attributable to minor genetic variants in genes encoding elements of the epidermal differentiation program.

In summary, we have shown that calpain 12 plays a crucial role in interfollicular and follicular epidermal differentiation. In addition, calpain 12 deficiency may modify the clinical consequences of *ABCA12* mutations, although its role as a genetic modifier remains to be confirmed in additional cases. Given initial studies showing involvement of calpains in the pathogenesis of both inherited (Lin *et al.*, 2015) and acquired (Gutowska-Owsiak *et al.*, 2012;

Meephansan *et al.*, 2012) cutaneous disorders, the present observations warrant investigating calpain 12 as a potential therapeutic target for some of these conditions.

MATERIALS AND METHODS

Patients

All affected and healthy family members or their legal guardian provided written and informed consent according to a protocol approved by our institutional review board and by the Israel National Committee for Human Genetic Studies in adherence with the Helsinki principles.

Exome sequencing

Details regarding exome sequencing can be found in Supplementary materials and methods.

Mutation analysis

Technical details regarding mutation analysis and direct sequencing can be found in Supplementary materials and methods.

PCR-restriction fragment length polymorphism (RFLP)

Technical details regarding the design and execution of these assays can be found in Supplementary materials and methods.

Bisulfite sequencing

Technical details regarding the design and execution of bisulfite sequencing can be found in Supplementary materials and methods.

Quantitative RT-PCR

Technical details regarding quantitative RT-PCR can be found in Supplementary materials and methods.

Cell cultures and reagents

Primary KCs and fibroblasts were isolated from adult skin obtained from plastic surgery specimens after having received written informed consent from the donors according to a protocol reviewed and approved by our institutional review board as previously described (Samuelov *et al.*, 2013). Primary KCs were maintained in Keratinocytes Growth Medium (KGM) (Lonza, Walkersville, MD). Fibroblasts were cultured in Dulbecco's Modified Essential Medium (DMEM) supplemented with 20% fetal calf serum (FCS) (Biological Industries).

siRNA transfection

Primary KCs and fibroblasts were cultured in 100-mm culture plates at 37°C in 5% CO_2 in a humidified incubator and were harvested at 60% confluence. To down regulate *CAPN12* expression, we used human *CAPN12* small interference RNAs (siRNA) (Santa Cruz; sc-62060) (5`-GAACAGCGGAAUGAGUUCUtt-3`, 5`-CAAUCCUCAGUUCCGUUUAtt-3` and 5`-CGUACUCCUCACUCAGAAAtt-3`). As control siRNA, we used Stealth™ RNAi Negative Control Duplex (Invitrogen, Carlsbad, CA). One hundred eighty pmol of siRNAs were

transfected into primary KCs and fibroblasts using Lipofectamine RNAiMax (Invitrogen, Carlsbad, CA).The transfection medium was replaced after 6 hours with KGM (for KCs) or DMEM (for fibroblasts). Seventy two hours following transfection, the transfected cells were trypsinized and used for organotypic cell cultures as described below.

Preparation of organotypic cell cultures

Experimental details regarding the generation of organotypic cell cultures can be found in Supplementary materials and methods.

Immunostaining

Details regarding immunostaining techniques can be found in Supplementary materials and methods.

Capn12 knock down in zebrafish and morphant analysis

Details of the generation of capn12-deficient zebrafish embryos can be found in Supplementary materials and methods.

Ex vivo hair follicle live imaging

Details regarding the generation and use of *K14 H2B-GFP*$^{+/+}$ mice to ascertain the effect of Capn12 deficiency on hair follicle can be found in Supplementary materials and methods.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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Legends to figures

Figure 1

Clinical and pathological features

The proband demonstrates (a) exfoliative erythroderma, (b) hypotrichosis and thick yellowish scales over the scalp and (c) hypertrophic nails. (e) On histology, parakeratosis, acanthosis and mild perivascular dermatitis are seen (hematoxylin and eosin; scale bar = 100µm).

Figure 2

Mutations` analysis

(a) Direct sequencing of *ABCA12* revealed two heterozygous mutations: a C>T transition at position c.2956 of the DNA sequence (upper left panel) and a T>C transition at position c.5778+2 (upper right panel). The wild type (WT) sequences are given for comparison (lower panels); (b) A PCR-RFLP assay was used to assess co-segregation of the two *ABCA12* mutations with the disease phenotype. The c.2956C>T and c.5778+2T>C mutations are associated with the presence of 594-bp and 186-bp fragments, respectively; (c) cDNA was reverse transcribed from RNA extracted from the skin of the patient and of a healthy individual (WT). cDNA was PCR-amplified using primers spanning *ABCA12* exons 37-39 (left panel). Direct sequencing of the resulting amplicons revealed a wild type mRNA sequence in both the patient and the healthy individual (upper right panel) as well as a shorter isoform due to exon 38 skipping, in the patient only (lower right panel); (d) Direct sequencing of *CAPN12* revealed a heterozygous C>A transversion at position c.1511 of the cDNA sequence (upper panel). The wild-type (WT) sequence is given for comparison; (e) A PCR-RFLP assay was used to assess co-segregation of

the c.1511C>A mutation in *CAPN12* with the disease phenotype. The mutation is associated with the presence of a 195-bp fragment; (f) cDNA was reverse transcribed from RNA extracted from the skin of the patient and PCR-amplified using primers spanning *CAPN12* exon 8-10. Direct sequencing of the resulting amplicons revealed wild type mRNA sequence (upper panel) as well as a shorter sequence lacking the last 40 bp of exon 9 (lower panel); (g) DNA methylation analysis of *CAPN12* exon 9 was performed using DNA extracted from keratinocytes derived from skin biopsies of the patient, his mother and father as well as two unrelated control individuals. For details see supplementary materials and methods. Results represent the mean results derived from the analysis of 10-15 clones per sample $\pm$ SE (** $p<0.05$; *** $p<0.01$, two sided t-test).

Figure 3

CAPN12 expression in the skin.

(a) A skin biopsy obtained from a healthy individual demonstrates by immunostaining cytoplasmic expression of calpain 12 in the epidermis (more pronounced in the lower epidermal layers) (left panel). In contrast, calpain 12 expression is markedly reduced in the skin of the patient (right panel) (dermo-epidermal junction is marked with a dotted line; scale bars = 100 µm); (b) Quantitative RT-PCR analysis was used to assess *CAPN12* RNA expression in cDNA samples derived from healthy individual skin and patient skin. Results represent the mean of three replicates and are provided as percentage of expression relative to gene expression in 4 controls $\pm$ SE normalized to *ACTB* mRNA levels.

Figure 4

Capn12 knockdown in a zebrafish model.

Zebrafish embryos were injected with a global standard control morpholino (scMO) (a,c,e) or with a *Capn12*-specific morpholino (b,d,f). Scanning electron microscopy analysis of the skin of the tail of a control larvae injected with scMO shows the presence of keratinocytes with well-demarcated cell-cell borders containing well-defined microridges (a), while the morphant larvae injected with a splice site morpholino for *Capn12* demonstrates perturbed microridge formation in the center of the keratinocytes with cracks and sloughing (b) (scale bar = 30 μm); Transmission electron microscopy analysis demonstrates normal microridge formation (arrows) in the control larva (c), while the formation of microridges in the morphant fish (d) is markedly perturbed (arrows). Asterisks mark the basement membrane. e, epidermis; d, dermis. Scale bar = 500 nm; In contrast with the normal morphology of the control fish (e), the morphant fish developed pericardial edema and a curled tail phenotype associated with death at around 6-8 days post-fertilization (f).

Figure 5

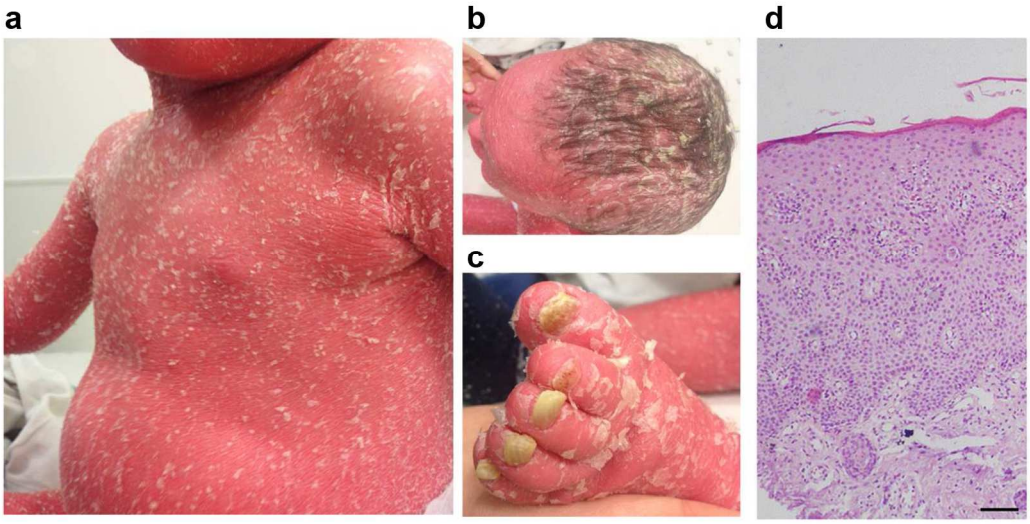
***CAPN12* knockdown in organotypic cell cultures**

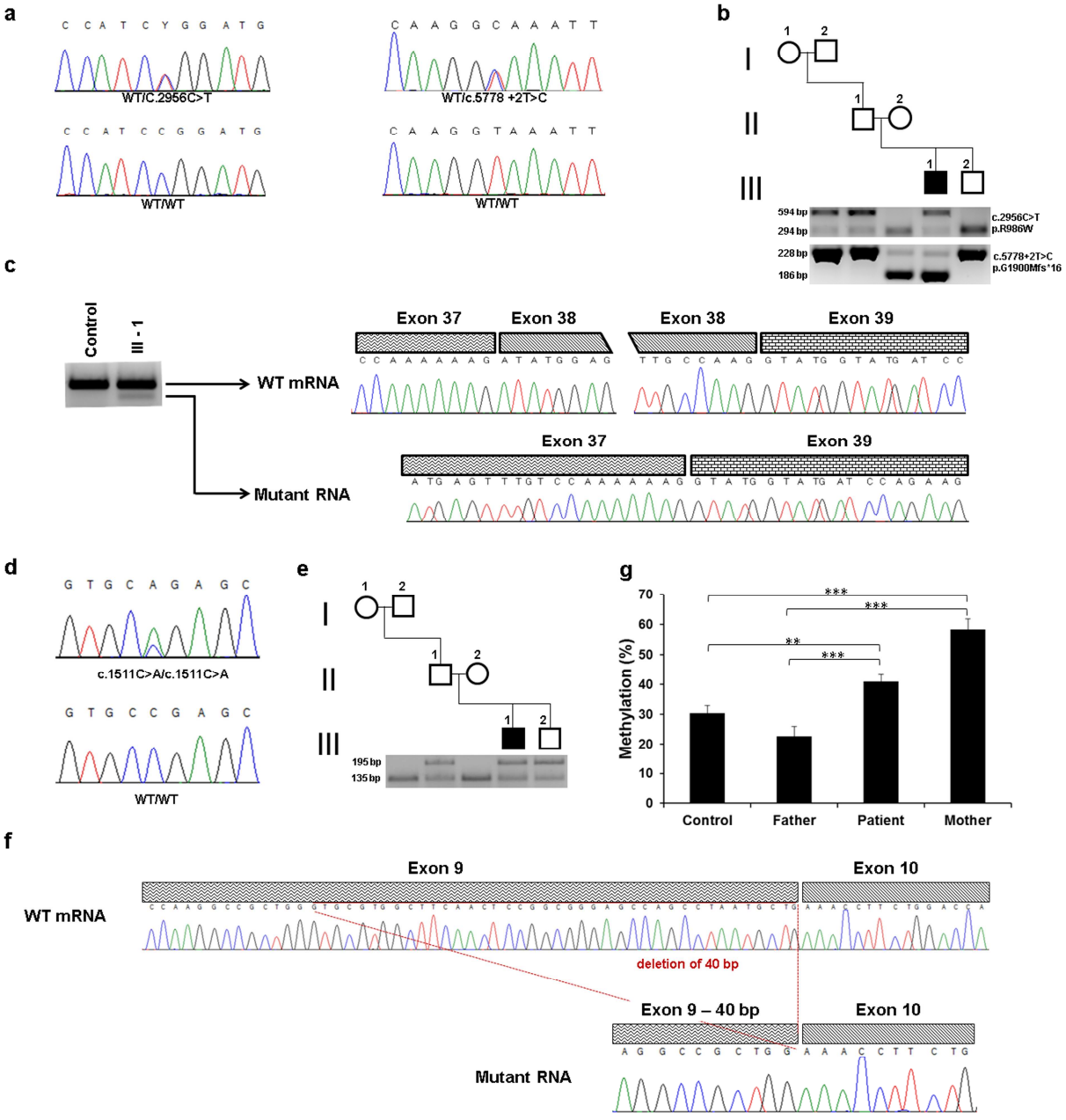
(a-b) Human primary KCs transfected with *CAPN12* siRNA (siCAPN12) or control siRNA (siControl) were used to generate skin equivalents. Punch biopsies were obtained from skin equivalents at day10 and stained for (a) hematoxylin and eosin or (b) filaggrin (bar = 100 µm); (c) Punch biopsies were obtained from control skin and from the patient (III-1) skin and stained for filaggrin (bar = 100µm). (d) Punch biopsies were obtained from control skin and from the skin of a patient with harlequin ichthyosis and biallelic null mutations in *ABCA12* gene and stained for filaggrin (bar = 100µm).

Figure 6

Effect of *Capn12* down-regulation on hair follicle cycle

(a-f) Z stacks optical sections of *K14-H2B-GFP* mouse anagen (a-c) and catagen (d-f) hair follicles treated with control siRNA and *Capn12* siRNA, respectively. The anagen hair follicles show stationary location of epithelial nuclei (see supplementary movie 1) while the catagen hair follicles demonstrate upward movement of the epithelial nuclei (see supplementary movie 3) during 9:20 hours of imaging. Epithelial nuclei are marked with K14-H2BGFP. Three nuclei in anagen (a-c) and four nuclei in catagen (d-f) hair follicles are circled in colors. Scale bar = 100 µm; (g) Anagen and catagen were ascertained as previously described (Foitzik *et al.*, 2005; Samuelov *et al.*, 2012). In brief, hair follicles were categorized as "alive" or "dead" based upon the presence of any movement of cells in the follicle. "Alive" hair follicles were further categorized as anagen or catagen hair follicles based upon the following defining parameters for catagen transformation: 1) hair bulb shrinkage and upward displacement of the bulb region; 2) upward displacement of epithelial nuclei in the bulb region; 3) apoptosis of epithelial cells with evidence of nuclear fragmentation. Three independent experiments were done with 3 different mice. In each mouse, 3 skin samples from each of the two treatment groups (*Capn12*-siRNA vs. control-siRNA) were used for *ex vivo* live imaging and in each sample the mean hair cycle score (HCS) was calculated as previously described (Foitzik *et al.*, 2005; Samuelov *et al.*, 2012). Results were pooled and are expressed as the mean HCS of all hair follicles per treatment group (*** $p<0.001$, *t*-test).

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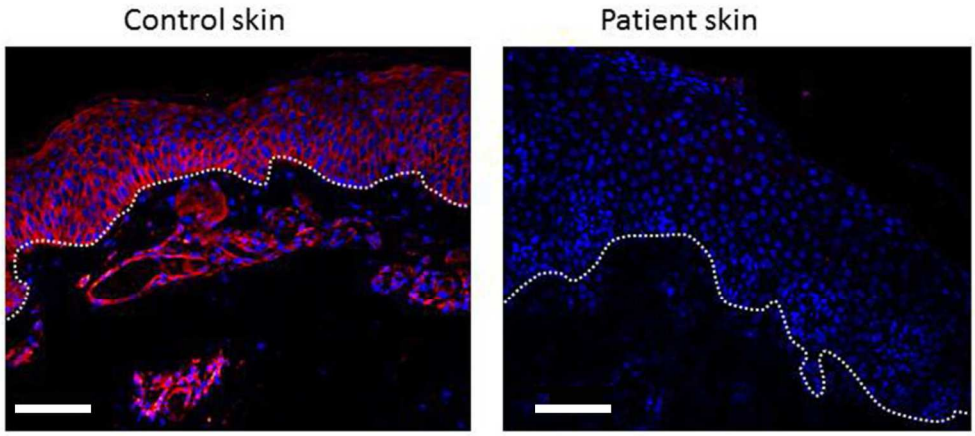
WT/C.2956C>T
C A A G G C A A A T T
WT/c.5778 +2T>C
C C A T C C G G A T G
WT/WT
C A A G G T A A A T T
WT/WT
b
I
II
III
1
2
594 bp
294 bp
c.2956C>T
p.R986W
228 bp
186 bp
c.5778+2T>C
p.G1900Mfs*16
c
Control
III - 1
WT mRNA
Mutant RNA
Exon 37
Exon 38
Exon 38
Exon 39
Exon 37
Exon 39
d
G T G C A G A G C
c.1511C>A/c.1511C>A
G T G C C G A G C
WT/WT
e
195 bp
135 bp
g
Methylation (%)
Control
Father
Patient
Mother
f
WT mRNA
Exon 9
Exon 10
deletion of 40 bp
Exon 9 – 40 bp
Exon 10
Mutant RNA

a



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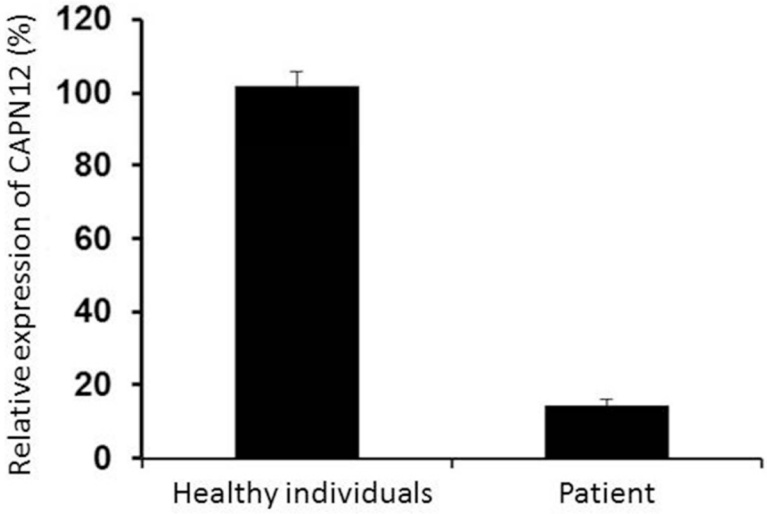

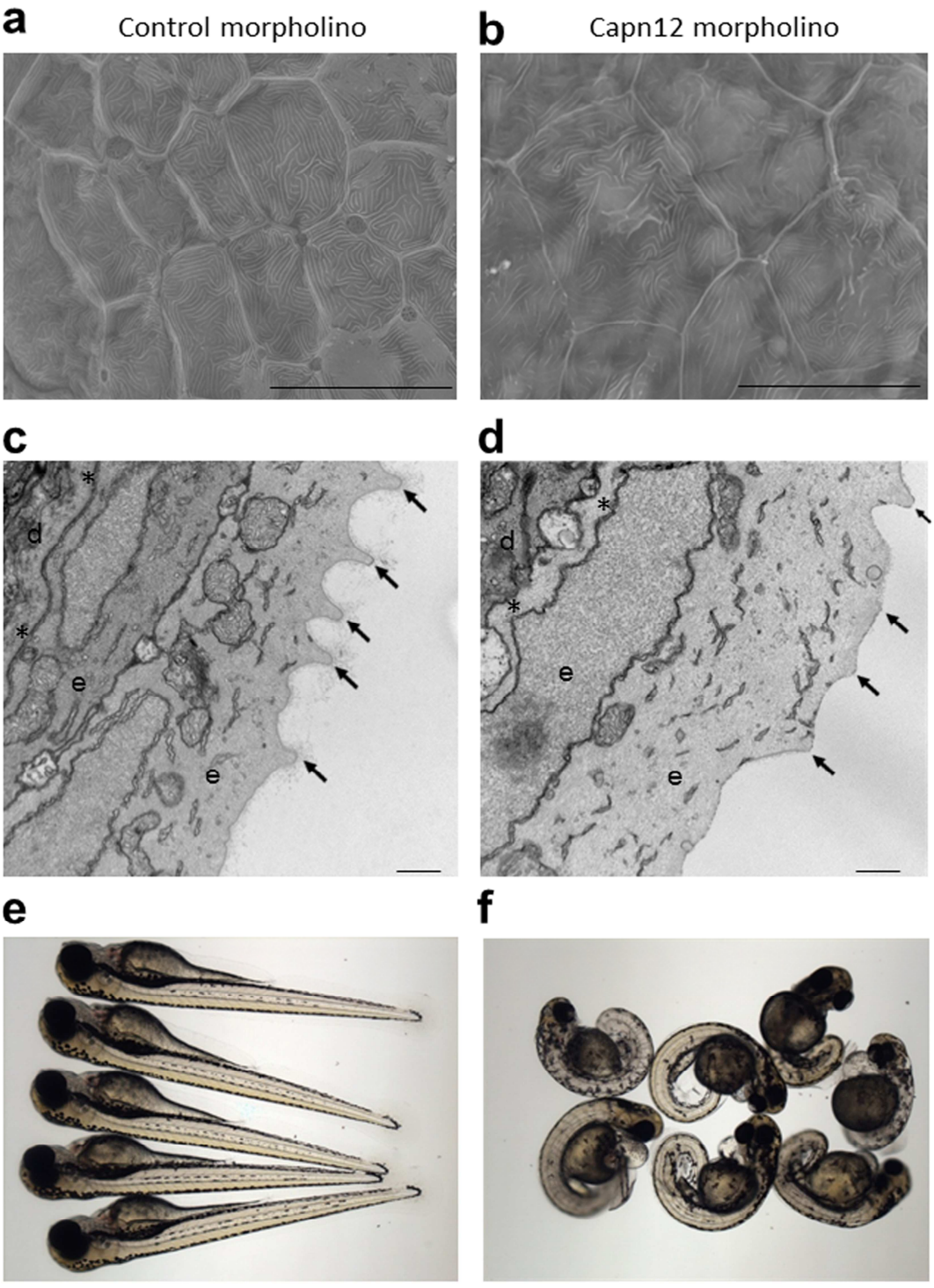
a
Control morpholino
b
Capn12 morpholino
c
d
e
f
d
e
*