Alopecia in Harlequin mutant mice is associated with reduced AIF protein levels and expression of retroviral elements

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Abstract
We investigated the contribution of apoptosis-inducing factor (AIF), a key regulator of mitochondrial biogenesis, in supporting hair growth. We report that pelage abnormalities developed during hair follicle (HF) morphogenesis in Harlequin (Hq) mutant mice. Fragility of the hair cortex was associated with decreased expression of genes encoding structural hair proteins, though key transcriptional regulators of HF development were expressed at normal levels. Notably, Aifm1 (R200 del) knockin males and Aifm1 (R200 del)/Hq females showed minor hair defects, despite substantially reduced AIF levels. Furthermore, we cloned the integrated ecotropic provirus of the Aifm1Hq allele. We found that its overexpression in wild-type keratinocyte cell lines led to down-regulation of HF-specific Krt84 and Krtap3-3 genes without altering Aifm1 or epidermal Krt5 expression. Together, our findings imply that pelage paucity in Hq mutant mice is mechanistically linked to severe AIF deficiency and is associated with the expression of retroviral elements that might potentially influence the transcriptional regulation of structural hair proteins.

Introduction

As an appendage of the skin, hair follicles (HF) represent a complex miniorgan that consists of several concentric layers of epithelial cells forming the outer root sheath (ORS) in continuity with the epidermis, the inner root sheath (IRS), and the hair shaft. The latter comprises the cuticle surrounding the cortex and a central medulla. Throughout the life of an organism, HFs constantly undergo phases of growth (anagen), regression (catagen), and rest (telogen) (Schneider et al. 2009; Fuchs 2018). Several transcription factors (Jave-Suarez et al. 2002; Rogers 2004; Cai et al. 2009) control the layer-specific expression of keratins and keratin-associated proteins, which provide rigidity to the terminally differentiated cells (Langbein et al. 2004; Langbein and Schweizer 2005). The biochemical and genetic complexity of hair follicle morphogenesis is reflected by the vast number of mutant mouse lines in which pelage development is affected by mutations of various genes (Sundberg 1994; Nakamura et al. 2013). Among these determinants, recent observations indicate that aberrant mitochondrial function undermines HF development, leading to fur abnormalities (Kloeppe et al. 2015; Singh et al. 2018; Stout and Birch-Machin 2019).

In this regard, the spontaneously arisen Harlequin (Hq) mutation was first described as an X-linked mutation causing complete alopecia in hemizygous males (Barber 1971), which was subsequently found to be associated with an endogenous ecotropic retrovirus (ERV) integration into the Aifm1 gene (Klein et al. 2002). Preliminary observations on the histological defects of the pelage in Aifm1Hq/Y hemizygous males revealed a fragility of hair shafts (Sundberg 1994).

Apoptosis-inducing factor (AIF) is a NADH- and FAD-containing oxidoreductase primarily anchored to the inner mitochondrial membrane (IMM) and facing the

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impaired mitochondrial function, may contribute to the phenotypes associated with the Hq mutation.

The bulk of the research involving the Hq mouse mutant mice has focused on AIF role in energy metabolism and cell death. The much more obvious pelage defect of the “Harlequin” mutant has been so far only described in few reports (Barber 1971; Sundberg 1994).

Here we report that the Hq allele is associated with the decreased transcription of genes specifically expressed in the hair cortex. The resulting decreased expression of hair cortex structural proteins caused mechanical weakness and finally breaking of the hair shafts below the epidermal surface level, leading to shedding of the hairs and hence baldness. Conversely, a reduction of AIF protein in the skin of Aifm1 (R200 del) KI mice did not result in detectable histological changes in the pelage. Notably, overexpression of the Hq-associated ERV in wild-type Hoxc13-expressing keratinocyte cell lines reduced the transcription of Krt84 and Krtap3-3, both of which are normally expressed in the hair cortex. Together, our findings indicate that the mutant pelage phenotype of Hq mutant mice strongly correlates with the expression level of AIF protein. As a proof-of-principle evidence, we additionally report that the exogenous expression of Hq proviral sequences is sufficient to alter the transcriptional profile of cultured keratinocytes.

Results

Reduced AIF expression is linked to hair loss in Harlequin mutant mice

The pelage defect of hemizygous Aifm1Hq/Y mice is apparent as early as postnatal day 9 (P9), when the tips of the hair shafts became visible on the skin surface, but did not elongate further in contrast to wild-type (wt) littermates (Fig. 1A.a). During the following days, the pelage was progressively lost in the mutant across the whole body surface, beginning on the head by P10 (Fig. 1A.b). By P14, Aifm1Hq/Y mice appeared bald, except for some hairs in the facial region (Fig. 1A.c). The gray color of P14 Aifm1Hq/Y skin was caused by active melanogenesis, indicating that Aifm1Hq/Y hair follicles (HF) remained in the anagen growth phase. Heterozygous Aifm1Hq/X females (Fig. 1A.c) showed some dark stripes in their agouti coat due to the darker underfur becoming visible in areas with reduced pelage. After HF morphogenesis and the first hair cycle, Aifm1Hq/Y mice developed some normal appearing, albeit shorter pelage regionally, especially in the facial region and around the root of the tail. Although variable among individual animals, pelage patches of various sizes remained on different regions of the body surface in older (P120) Aifm1Hq/Y phenotypic mutants (Fig. 1A.d).
Since the Hq allele affects AIF expression at the mRNA level (Klein et al. 2002; Wischhof et al. 2018), we performed immunoblot analysis to assess AIF protein in Aifm1Hq/Y skin. As in the brain, AIF was reduced to a similar degree in skin of Aifm1Hq/Y mice compared to wt littermates (Fig. 1b). Using immunohistochemistry on P10 skin tissue sections, we observed strong AIF immunoreactivity in the hair matrix and the sebaceous glands of wt skin, which is noticeably reduced in Aifm1Hq/Y skin (Fig. 1c).

**Hair follicle morphogenesis is altered in Hq mutant mice**

We performed histological examinations of dorsal skin biopsies in Aifm1Hq/Y and wt littermates. At P5, HFs were undistinguishable in number and shape between Aifm1Hq/Y HF (Hq) and wt littermates (Fig. 2a, g). At this stage, the tips of the Hq hair shafts reached the preformed pilary canals (Fig. 2g'). Around P9, the earliest detectable histological difference between mutant and wt hair follicles were subapically bended hair shafts in the mutant (Fig. 2h, h'), even though the tips of mutant hair shafts could pierce through the hair canals (insert in Fig. 2h, inset). Such bending of hair shafts never occurred in age-matched wt specimens (Fig. 2b). By P14, the curled-up hair shaft material caused a widening of pilary canals in the mutants (Fig. 2i, i'). Moreover, Hq HF s were slenderer and showed less active melanogenesis compared to wt HFs (Fig. 2c), indicating the beginning of HF regression in the mutants. By P17, when wt HFs reached catagen with a concomitant reduction in length (Fig. 2d), Hq HFs still remained long with enlarged sebaceous glands (Fig. 2j). Although the openings of the pilary canals were competent, the Hq mutant hair shafts continued to curl up inside the pilary canals (Fig. 2j'). Thinned fragile hair shafts and enlarged sebaceous glands (Meibom gland) were also observed in the cilia of the eyelids of Hq mutant mice (not shown). On P19, when wt HFs had entered telogen (Fig. 2e), the Hq HF s still showed some epithelial trailing strand (Fig. 2k) and residues of the hair shafts in the pilary canals (Fig. 2k'). On P24, both wt and the Hq mutant HFs initiated the first cyclic anagen (Fig. 2f, l). Remaining hair shaft material of Hq mutant HFs was finally exteriorized onto the skin surface (Fig. 2l').

Sections of P9 skin showed thinner hair shaft diameter and enlarged sebaceous glands in Hq mutants (Fig. 2n) compared to the wt (Fig. 2m). Electron microscopic examinations of Hq HFs revealed that the pilary canal was open and cuticles of hair shaft and inner root sheath were both intact (Fig. 2o, p). However, the hair cortex contained filamentous material indicating incomplete keratinization, in contrast to the adjacent hair cuticle which appeared homogeneously electron dense and thus fully keratinized (Fig. 2q, r).

Our morphological observations indicate that in Hq hemizygous mutant males, HFs produce hair shafts that are mechanically less rigid compared to wt controls, possibly because of keratinization defects in the subapical hair cortex.

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**Fig. 1** Decreased AIF level in the skin of Hq mutant mice is associated with hair loss. A Macroscopic aspect of Hq mutant mice showed normal hair growth until P9 (A.a). Hair loss of hemizygous Hq mutant males started from the head at P10 (A.b) until they appeared bald by P14, while heterozygous Hq/X females showed only mild pelage defects (A.c). Older Hq mutant males exhibited patches of pelage in various regions (A.d). B Western blot analysis demonstrated similar AIF reduction in brain and skin of Hq mutants at P12. C Confocal analysis of P10 skins from wt and Hq mutant mice. AIF immunoreactivity was most strongly detected in sebaceous glands (SG) and hair follicle matrix (Mx) as well as skeletal muscle (Mu, Panniculus carnosus) of wt skin, and was noticeably reduced in Hq mutant skin. Antibody control shows the absence of fluorescence except autofluorescence signal in the hair shafts (right panel, arrowheads)
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Fig. 2  Histological analysis of Hq mutant hair follicle development. a–l′. H.E. stained sections of wt and Aifm1<sup>Hq</sup> skin revealed proper initiation of hair follicle morphogenesis in the mutant, although hair bulbs appeared slenderer in the mutant at P9 (compare arrow heads in i to arrow heads in e). Starting at P9 when wt hair shafts penetrated the epidermis (white arrow head in b), subapical regions of Hq mutant hair shafts curled up or broke subapically (arrow heads in h′ and i′), after the tip had penetrated the epidermis (arrow head in h). Curled up hair shafts became exteriorized between P17 and P24 (arrow heads in j′–l′). Hq mutant sebaceous glands appeared slightly enlarged compared to wt controls (arrows in g′, j′, k′, l′). Scale bar in a applies also to b–l. Scale bar in g′ applies also to h′–l′. m–r  Ultrastructural analysis of wt and mutant hair shafts. m, n Semi-thin sections showed smaller hair shaft diameter with less developed hair cortex (Co) in the mutant, while sebaceous glands (SG) were enlarged. Scale bar in m applies also to n, o–r Electron micrographs of Hq mutant HFs show properly developed and competent pilary canals (PC). q, r Deficient compaction of hair cortex (Co, bracket) is indicated by filamentous material, in contrast to the properly compacted hair cuticle (Cu, bracket) as indicated by homogeneous electron density. m Melanosomes. Scale bar in q applies also to r
**Hq** mutant HF s exhibit a decreased expression of genes involved in hair cortex-specific keratinization

Using Northern blot analysis, we investigated the expression of genes encoding regulatory and structural hair proteins in postnatal pups (i.e., P5-P12), within a time window in which the hair shaft fragility became histologically evident in Hq mutant HF s. The transcription factors Foxn1 and Msx2, essential regulators of HF morphogenesis, were similarly expressed in Hq mutant and wt skin at P5, P8, P11, and P12 (Fig. 3a, gray labels). Because the layer-specific transcriptional regulation of structural genes within the hair follicle is not well understood (Rogers 2004), we next compared the

![Fig. 3 Dysregulated hair cortex-specific structural gene expression in Hq mutant hair follicles.](image-url)

**Fig. 3** Dysregulated hair cortex-specific structural gene expression in Hq mutant hair follicles. **a** Northern blot analyses of genes associated with hair and skin development demonstrated stable expression of hair cortex-specific regulators Foxn1 and Msx2 in Hq mutant skin during anagen between P5-P12 (shown in gray). Structural genes fall into four categories with respect to expression level changes in Hq mutant skin compared to wt littermates: some were not altered (black), some were partially lost (orange), and some were completely lost in the mutant (red), while Krt5 and Krt16 were up-regulated in the mutant (light blue). Line graph illustrates expression levels in Hq mutant skin relative to age-matched wt littermate samples, Northern blot results and respective 28S rRNA bands of the gel images as loading controls are shown below. **b** In-situ hybridization revealed that expression of the hair cortex-specific regulator Foxn1 was maintained in Hq mutant HF s. However, hair cortex-specific structural genes were lost in the mutant, while expression of genes in other layers of the hair follicle was not affected. Co, hair cortex; Cu, hair cuticle; IRS, inner root sheath; ORS, outer root sheath.
transcription levels of multiple genes encoding hair keratins and keratin-associated proteins (Krtaps) between Hq mutant and wt skin. We observed that the analyzed genes fall into four categories, while Krt71 and Krt82 remained unchanged (Fig. 3a, black labels), over time other hair-specific structural genes were partially (unchanged (Fig. 3a, black labels), over time other hair-specific structural genes were partially (Krt33, Krt86, Krtap3-3, Krtap11-1; Fig. 3a, orange labels) or completely lost (Krt84, Krtap4-7, Krtap8-2, Krtap9-1; Fig. 3a, red labels) in Hq mutant skin. By contrast, epidermal Krt5 and wound healing-related Krt16 appeared to be up-regulated in Hq mutant skin (Fig. 3a, light blue labels), consistent with the reactive thickening of the epidermis in Hq mutant mice compared to wt littermates.

To more precisely localize the dysregulated genes within the mutant HFs, we performed in situ hybridization and found that the down-regulation of hair structural genes specifically occurred in the hair cortex (Fig. 3b), consistent with the deficient hair cortex keratinization. Krt84, Krtap4-7, Krtap8-2, and Krtap9-1 genes were detectable only in the hair cortex of wt HFs and were all completely lost in Hq mutant HFs. Krt86 and Krtap3-3 genes were reduced, although they were still detectable due to residual expression in the cuticle of Hq mutant HFs, while their cortical expression domain was lost. Krt5, Krt71, Krt82, and Krtap5-2 genes, which are normally expressed in other layers of the HF, as well as the hair cortex-specific regulatory gene Foxn1, were comparably expressed in Hq mutant and wt HFs.

These results suggest that the Hq mutation causes the progressive down-regulation of distinct Krt and Krtap genes specifically in the hair cortex, although normal expression levels and localization of hair cortex-specific regulatory genes Foxn1 and Msx2 in Hq mutant skin indicate that the cells forming the hair cortex are not lost in mutant HFs.

**Reduced AIF protein expression is not associated with alopecia in Aifm1 (R200 del) KI mice**

The Aifm1Hq mutation is caused by the insertion of an endogenous retrovirus (ERV) genome into the Aifm1 locus, leading to reduced Aifm1 transcription and protein expression (Klein et al. 2002). To dissociate the in vivo consequence of AIF deficiency from the Hq-linked ERV, we employed Aifm1 (R200 del) KI mice. As previously described (Wischhof et al. 2018), these mice are a tractable model of an inherited form of human encephalomyopathy in which a single amino acid is lost in the AIF protein. We performed immunoblot analyses (Fig. 4b, c) in the skin of Aifm1 (R200 del) KI males compared to age-matched wt littermates. We found an AIF protein reduction (~49 ± 8%, n = 3) which was milder compared to Aifm1Hq/Hq males (12 ± 2% of wt levels, n = 4). More importantly, we did not notice macroscopic or histological pelage defects in Aifm1 (R200 del) KI males (Fig. 4a). Thus, we crossed Aifm1 (R200 del) KI males with Aifm1Hq/Hq females to generate a small cohort of double-heterozygous Aifm1HqR200 del/Hq females in which we histologically investigated the skin during anagen. In principle, the combination of the two alleles would lead to a more severe reduction of AIF protein levels than in Aifm1Hq/Hq females, further approximating the uniformly low levels detected in Aifm1Hq/Y hemizygous males. We hypothesized that this might lead to exacerbated alopecia in Aifm1HqR200 del/Hq females compared to Aifm1Hq/Hq heterozygous females. Interestingly, we found that double-heterozygous Aifm1HqR200 del/Hq females did not exhibit more frequent or more severe hair follicle defects at P10 than heterozygous Aifm1Hq/Hq mice (Fig. 4d).

We were very intrigued by the mild pelage phenotype in heterozygous Hq females. Thus, we investigated the expression of hair cortex-specific Krtap8-2 and Krtap9-1 genes using in situ hybridizations. Each HF is of polyclonal origin and random X-chromosome inactivation in each cell clone is inherited by its progeny (Legué and Nicolas 2005). Hence, each HF of heterozygous female Aifm1Hq/Hq mice is predicted to contain a mosaic of cells expressing wt and mutant alleles. On P14, when all HFs of hemizygous Aifm1Hq/Y males exhibited structural defects and enlarged sebaceous glands (see Fig. 2i), only few HFs of heterozygous Aifm1Hq/Hq females showed deformed hair shafts, though many more had noticeably enlarged sebaceous glands (data not shown). In situ hybridizations revealed that the hair cortex-specific Krtap9-1 and Krtap8-2 genes in the Aifm1Hq/Hq heterozygous females exhibit discontinuous expression domains (Fig. 4e), resembling clonally derived segments of the hair shaft (Sequeira and Nicolas 2012). Such pattern of structural hair cortex gene expression seems to be sufficient to provide for hair shaft rigidity in most HFs.

Taken together, reduction of AIF protein levels in Aifm1 (R200 del) KI mutant animals to as low as 50% of wt levels is not sufficient to impair HF development. Moreover, the mild pelage defect of heterozygous Aifm1Hq/Hq females is explained because each HF contains a mix of cell populations in which hair gene expression was either lost or completely preserved, rather than evenly reduced hair cortex gene expression in all cells of every HF. This mixed gene expression pattern in heterozygous Aifm1Hq/Hq HFs suggests a cell-autonomous mechanism for the impairment of hair gene expression in the mutant.

**Retroviral elements are detectable in Hq mutant skin**

RNA blots of P5 and P10 wt and Hq mutant skin hybridized with a probe specific for the 3’ end of the Aifm1 transcript showed a dramatic decrease of Aifm1 transcription at P5 and P10 (i.e., before and after the observed histological changes in the mutant HF; Fig. 5a). We also analyzed
Fig. 4 Mild hair defects in heterozygous Aifm1<sup>Hq</sup>/X and Aifm1<sup>(R200 del)Hq</sup> mice. a H.E. stained skin sections demonstrated no broken hair shafts at P10 in Aifm1<sup>(R200 del)Y</sup> skin, while almost all hair shafts were broken in Aifm1<sup>Hq</sup>/Y skin (white arrow heads). b Western blot showed reduced AIF protein levels in Aifm1<sup>(R200 del)Y</sup> skin, although not as dramatically as in Aifm1<sup>Hq</sup>/Y skin. c Quantification of B. d Only very few broken hair shafts (white arrow heads) were found in H.E. stained skin sections in Aifm1<sup>Hq</sup>/X skin at P10, which was not exacerbated in Aifm1<sup>(R200 del)Hq</sup> skin. e In-situ hybridizations revealed interrupted expression domains of hair cortex-specific genes Krtap9-1 and Krtap8-2 (expression domains indicated by brackets). White asterisks indicate melanin granules in the hair medulla, demonstrating longitudinal sections through the middle of the hair shafts.
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Using primers specific for the 5′ end of the Aifm1 full-length and the Aifsh isoform (Delettre et al. 2006a, b), we performed RT-PCRs on skin cDNA preparations of Hq mutant and wt mice at P5, P7 and P10. At the three tested time points, expression of full-length Aifm1 and Aifsh transcripts in Hq mutant skin was not detected using our PCR conditions (Fig. 5c). RNA blots from P10 Hq mutant and wt skin hybridized with two Aifm1-specific probes derived from either the 5′ or the 3′ end (as indicated in the sketch in Fig. 5c) also showed no detectable expression of any other alternative Aifm1 transcripts in Hq mutant skin (Fig. 5d). Based on this line of evidence, no known or uncharacterized Aifm1 splice variants are expressed in the skin of Hq mutant mutant mice.

We next hybridized the same wt and Hq mutant skin RNA samples using a probe specific for ecotropic env (envelope) sequences. We detected abundant expression of a full-length endogenous ecotropic retrovirus transcript (ERV, appr. 8 kb) and a spliced env transcript (approx. 2.5 kb) in the skin of Hq mutant mice, but not in skin RNA of wt littermates (Fig. 5b). We compared the expression of retroviral transcripts in cDNA samples of skin and cerebellum using semi-quantitative RT-PCR. We found strong expression of env and moderate expression of the gag-pol transcript in both Aifm1Hq/Y hemizygous males and Aifm1Hq/X heterozygous females, while both transcripts were barely detectable in wt littermates (Supplementary Fig. S1A). To confirm that the detected transcripts originated from the Aifm1Hq allele-associated ERV, we sequenced the transcripts and found...

Fig. 5 Expression of Aifm1 and retroviral elements in Hq mutant skin. 

a Northern blot analysis showed reduced expression of Aifm1 transcript in Hq mutant skin. 
b Retroviral elements are detectable by Northern blot analysis in Hq mutant skin but not in wt controls. 
c Aifm1 full-length or Aifsh transcripts are detected by RT-PCR in wt skin, but not in Hq mutant skin. Location of PCR primers relative to Aifm1 full-length transcript is indicated below. 
d Northern blot analysis using probes against the 5′-end (left panel) or the 3′-end (right panel) of the Aifm1 transcript showed no detectable expression of uncharacterized transcripts in Hq mutant skin. Et indicates 28S rRNA bands of the respective gel images as loading controls.
100% identity to the Hq-ERV genome (Supplementary Fig. S1B) inserted into the Aifm1Hq allele (see below). These results indicate that endogenous ecotropic retroviral transcripts are abundantly expressed in skin and cerebellum of hemizygous and heterozygous Hq mutants, but not in wt littermate controls.

**Overexpression of Hq-associated ERV represses Krtap3-3 and Krt84 in cultured cells**

To assess the putative influence of the Hq-associated ERV on the expression of hair cortex-specific genes, we cloned and sequenced the entire endogenous retroviral genome, including some flanking sequences derived from the first intron of the Aifm1 locus (Supplementary sequence). The Hq-associated ERV sequence is highly similar to the published sequence of the Emv30-like endogenous retrovirus (Triviali et al. 2014), differing only in 12 nucleotides (Fig. 6a), including 2 non-synonymous variations each in the gag-pol region (glyco-gag P50S; gag-pro-pol Q1719K), and in the env region (R282Q and T66I) as well as 4 synonymous mutations (gag-pol E179, V648; env P207). We also identified two single-nucleotide changes within a span of 12 nucleotides at the 5′ start of the U3 regions in the viral 5′ and 3′ LTRs.

To investigate the effect of Hq-associated retroviral expression, we established a permanent keratinocyte cell line from C3H/FeB wt skin using a retroviral vector transducing a temperature-labile SV40 T gene (Jat et al. 1986; Jat and Sharp 1989). Transcriptional profiling of the wt keratinocyte cell line Kera2 revealed that these cells expressed most of the epidermal genes, but no hair follicle-specific genes (Fig. 6b). We therefore derived clones of Kera2 cells stably transfected with a Hoxc13 expression vector to induce the expression of HF-specific genes (Jave-Suarez et al. 2002), knowing that the expression of hair shaft-specific genes depends on a delicate balance of Hoxc13 expression levels (Godwin and Capecchi 1998, 1999; Tkatchenko et al. 2001). We chose one clone of Kera2-Hoxc13-expressing cells that showed detectable levels of hair cortex-specific Krt84 and Krtap3-3 gene expression (Fig. 6b, arrowheads), which are down-regulated in Hq mutant skin (see Fig. 3). Immunoblot analysis demonstrated that decreased AIF protein was persistent in a keratinocyte cell line Hq-Kera which was obtained from Hq mutant skin (Fig. 5c). Since these immortalized cells still exhibit AIF loss, we used Kera2-Hoxc13 cells to investigate whether changes of HF-specific genes would occur in the presence of Hq-associated ERV sequences.

Kera2-Hoxc13 cells were co-transfected with the entire cloned Hq-ERV (including some flanking sequences from the Aifm1Hq genomic insertion site), together with a puromycin resistance gene for the selection of transfected cells. The transfected Hq-ERV genome was detected in three of the selected puromycin-resistant clones, while three other puromycin-resistant clones lacked the Hq-ERV proviral genome and were henceforth used as negative controls (Fig. 6d). ERV expression in all three Hq-ERV-positive clones was confirmed by RT-PCR using primers specific for ecotropic env sequences (Fig. 6d). Further expression analyses demonstrated that ecotropic env expression did not affect the expression of Aifm1 and Krt5, which is normally expressed in the epidermis and the outer root sheath (Fig. 3). Conversely, two of three Kera2-Hoxc13 clones carrying the Hq-ERV sequences showed reduced expression of hair-specific Krt84 gene compared to the Hq-ERV-negative clones, and all three Kera2-Hoxc13 clones carrying the Hq-ERV sequences lacked expression of Krtap3-3, which was readily detectable in the Hq-ERV-negative controls (Fig. 6d).

We quantified the expression levels of Krt5, hair cortex-specific Krt84 and Krtap3-3, as well as Aifm1 and ecotropic env sequences by qRT-PCR in Kera2-Hoxc13 clones that did or did not carry at least one genomic copy of the Hq-ERV genome. We found that Aifm1 as well as Krt5 expression levels were not reduced in Hq-ERV transfected clones regardless whether they expressed env or not (Fig. 6e). In contrast, Krt84 and Krtap3-3 expression were reduced in Hq-ERV-positive, but not in the control clones, thereby reflecting their dysregulation in vivo in the skin. Based on the data obtained using these cultured cells, down-regulation of hair cortex genes Krtap3-3 and Krt84 was associated to the expression of ERV-derived sequences. Our data suggest that the proviral insertion in the Aifm1 gene of the Hq mutant mice suppresses Aifm1 expression and might interfere with transcriptional programs involved in hair shaft development, possibly contributing to Hq-associated alopecia.

**Discussion**

The exploration of mouse mutations causing alterations of hair texture and alopecia has elucidated many molecular aspects of hair biology (Nakamura et al. 2013). This study shows that the pelage defect of the “Harlequin” (Hq) mutant is caused by the fragility of subapical parts of the hair shafts. We showed that the transcription of genes encoding structural proteins is specifically lost in the hair cortex of Hq mutant male HF, causing the hair shafts to curl up inside the pilary canal.

The X chromosome-linked Hq mutant arose spontaneously in 1971 and was identified by its pelage defect (Barber 1971). The pelage defect was associated with fragility of subapical parts of the hair shafts, resulting in complete baldness of male hemizygotes (Sundberg 1994). In this respect it differs from the nude (Foxn1nu) mutants, in which the hair shafts are fragile in their entirety and never appear on the skin surface (Mecklenburg et al. 2001). Moreover, scanning
electron microscopy revealed that the twisted parts of the *nude* hair shafts lacked a cuticle (Mecklenburg et al. 2001), while in *Hq* mutant males the cuticle was morphologically preserved and fully keratinized, even where it was bent in the pilary canal. Thus, *Aifm1* down-regulation by the *Hq* mutation in the hair follicle causes alopecia by increasing the fragility of the subapical hair cortex and hence is distinct from the *nude* and related phenotypes. This distinction is corroborated by the normal expression of *Foxn1* in *Aifm1*<sup>Hq/Y</sup> mice during hair follicle morphogenesis.

The *nude* phenotype is caused by mutations of the transcription factor gene *Foxn1* (Schlake et al. 1997; Mecklenburg et al. 2001), which regulates the expression of genes encoding structural proteins in the hair shaft (Schlake et al.
The expression of the Hq allele-related retrovirus suppresses structural hair gene expression in cultured keratinocytes. a Sequencing of the Hq allele-related retrovirus revealed a complete endogenous retroviral genome highly homologous to Emv30. Single-nucleotide changes within the Hq-ERV genome with respect to Emv30 are indicated (black arrows: non-conservative nucleotide changes within coding regions; white arrows: nucleotide changes in non-coding regions; white arrow heads: conservative nucleotide changes within coding regions). b RT-PCR analysis of HF-related genes in Kera2 and Kera2-Hoxc13 keratinocytic cell lines. Transcript names indicated above are color-coded by category (black: regulators of HF development; red: epidermal keratins; blue: HF-specific keratins; pink: HF-specific keratin-associated protein genes). Epidermal keratins were down-regulated, while HF-specific Krt84 and Krtap3-3 were up-regulated in Kera2-Hoxc13 cells (white arrow heads). c Western blot demonstrated AIF deficiency in Hq mutant keratinocytic cell line HqKera, similar to AIF reduction in skin (see Fig. 1B). d Semi-quantitative RT-PCR analysis revealed reduced expression of structural hair genes Krt84 and Krtap3-3 in Hq-ERV-transfected Kera2-Hoxc13 clones compared to clones lacking Hq-ERV, while Aifm1 expression was not changed. e qRT-PCR analysis demonstrated reduction of Krt83 and Krtap3-3 in Hq-ERV-transfected keratinocytic cells, similar to Hq mutant skin. Aifm1 was not reduced in Hq-ERV-transfected keratinocytic cells, in contrast to Hq mutant skin where Aifm1 expression level was reduced by almost 90% compared to wt controls.

2000; Mecklenburg et al. 2001; Schweizer et al. 2007). Other genes influencing the transcriptional activation by Foxn1 such as Msx2 and Hoxc13 produce similar effects on hair shaft morphology and hair cycling (Ma et al. 2003; Potter et al. 2011). Here we show that, in Hq mutant mice, the transcription of Foxn1 and Msx2 is not altered during the period of hair follicle morphogenesis when hair fragility is apparent, while some of their target genes, specifically hair keratin genes and genes encoding keratin-associated proteins, are down-regulated. Thus, the reduced expression of AIF by the Hq mutation interferes with the expression of structural genes specifically in the subapical hair cortex, without involvement of known upstream regulatory genes.

The keratin-associated proteins (KRTAPs) form a family of cysteine-rich and glycine-tyrosine repeat-rich proteins. Unique to mammals, they are a critical determinant of hair shaft and cuticle rigidity (Rogers et al. 2006; Jones et al. 2010; Fraser and Parry 2018). In our study, we found down-regulation of Krtap genes in the hair cortex, but not in the cuticle of Hq mutant mice. The pattern of down-regulation of murine hair keratin and Krtap genes in Hoxc13 null mice (Potter et al. 2011) differs from the one observed in Hq mutant mice in two aspects: first, in Hq mutant mice the expression of genes encoding structural proteins gradually decreases between P5 and P12, while in Hoxc13 null mice expression of structural genes is almost completely lost already on P5. Second, while the reduction of Krt82 was 40-fold in Hoxc13 null mice (Potter et al. 2011), the expression was preserved in Hq mutant mice. Thus, while Foxn1 and Hoxc13 mutations target hair cortex and cuticle alike, the Hq mutant allele causes alopecia by affecting specifically the hair cortex.

In humans, AIFM1 mutations have been identified in patients suffering from severe X-linked mitochondrial encephalomyopathy (Ghezzi et al. 2010) and Cowchock syndrome (Rinaldi et al. 2012), both conditions affecting the nervous system and skeletal muscle. A mouse model of the R201 deletion identified in humans exhibit early defects primarily in the skeletal muscle (Wischhof et al. 2018). In this mouse model, we showed that aberrant AIF expression had no effect on the hair follicle, while the gradual loss of ETC in the TfamEKO mutant mouse does affect hair follicles (Bodemére et al. 1999). Similarly, the assembly and function of human mitochondrial complex III depends on the BCS1L protein, and mutations of BCS1L can cause Björnstad syndrome (Bénit et al. 2009), which is a combination of sensorineural hearing loss and pili torti, a condition in which the hair shafts are thin and broad, and twisted along their long axis (Rogers 1995). There is also other evidence that dysfunctional mitochondrial complex I may contribute to the Aifm1HqWT pelage phenotype (Vahsen et al. 2004; Bénit et al. 2008; Kruse et al. 2008), since the alopecia in Aifm1HqWT mice resembles the alopecia in mice with functional inactivation of the complex I subunit NDUF54 (Kruse et al. 2008). Moreover, hair follicles engage in aerobic glycolysis, at least in vitro (Philpott and Kealey 1991; Kealey et al. 1994) and also require a controlled level of reactive oxygen species (ROS) generated by mitochondria for their normal development in vivo (Hamanaka et al. 2013). Thus, further studies will have to show by which mechanism the reduction of AIF protein contributes to the pelage defect in Hq mutant mice in vivo.

The pelage of Aifm1HqX heterozygous female mice exhibited a surprisingly mild defect, although theoretically half of all cells in each Aifm1HqX HF should suffer the same genetic deficit as all cells in Aifm1HqWT hemizygous male HFs. The deformation of each individual Aifm1HqX hair shaft seems to follow an all-or-none rule being either phenotypically normal or completely deformed like the hair shafts of the Hq mutant males. During anagen, each layer of the growing IRS and the hair shaft is replenished by lineage-restricted precursor cells originating from the HF matrix that form small clones of differentiated cells (Legué and Nicolas 2005; Legué et al. 2010). Hence, each layer of the hair follicle in Aifm1HqX heterozygous female mice is a mosaic of cell clusters with normal and impaired mitochondria.

We found that Aifm1 (R200 del) caused a roughly 55% decrease of AIF protein in skin, presumably due to decreased protein stability, which is a milder reduction compared to the AIF loss in other tissues (Wischhof et al. 2018). The decrease of AIF in the skin of Aifm1HqWT hemizygous males was roughly 80%, confirming previous reports (Klein et al. 2002; Wischhof et al. 2018). These results suggest that there
may be a critical threshold between a 55 and 80% reduction of AIF in the hair follicle to result in an all-or-none phenotype with respect to hair fragility. Apart from reduced AIF levels, we reported that the Aifm\textsubscript{1\textsuperscript{Hq}} mutation is associated with an ecotropic proviral integration that expresses a full-length proviral genome and a spliced env transcript. Thus, we wanted to investigate the possibility that the expression of Hq mutation-associated retroviral elements might play a role in the development of the mutant hair phenotype. Proviral integrations are associated with other pelage phenotypes, such as dilute (Jenkins et al. 1981; Copeland et al. 1983a), lethal yellow (Copeland et al. 1983b), or the Plcd3\textsuperscript{mNab/del9(Pas)}-associated alopecia (Runkel et al. 2012), even though the expression of viral elements may not be a contributory factor in these mutants. Given our observation that Hq mutation-associated retroviral elements are strongly expressed in Hq skin, we established an in vitro system to examine the role of the Aifm\textsubscript{1\textsuperscript{Hq}}-associated ERV for the dysregulation of hair follicle Krt and Krtap genes. Even though our cell line Kera2 showed a predominantly epidermal gene expression profile, the forced expression of Hoxc13 caused an elevated expression of Krt84 and Krtap33 genes, which are both expressed in the hair cortex in vivo. After transfection of the genomic sequence of the Hq-associated ERV, which lead to detectable ERV env expression, some clones specifically down-regulated the expression of hair cortex-specific Krt84 and Krtap33, but not of epidermal Krt5 or Aifm1 genes. These data suggest that the ecotropic ERV in the Aifm1 genomic locus might influence the expression of hair-associated structural genes on its own without altering Aifm1 expression.

Repeatedly during mammalian evolution, retroviruses have integrated into the genomes and functioned as mutagenic mobile genetic elements. Upon infection of mammalian cells, retroviruses reverse transcribe their RNA genomes and subsequently integrate as DNA proviruses into the host genome. When proviruses integrate into the genomes of germ cells, they become novel genomic elements of the host that will be transmitted to the following generations as endogenous retroviruses, reviewed in Mak-sakova et al. (2006); Meyer et al. (2017); and Gagnier et al. (2019). Endogenous retroviruses (ERV) in mice make up about 8 to 10% of the host genome. They may be active or become inactive due to the continuous acquisition of mutations or deletions after genomic recombination (Buzdin et al. 2017; Meyer et al. 2017) and their transposition may be the cause of approximately 10% of new mutations (Mak-sakova et al. 2006). In this report, we have characterized the Aifm\textsubscript{1\textsuperscript{Hq}}-associated provirus as an ecotropic full-length retrovirus almost identical in sequence to EMV30 (Trivai et al. 2014). ERVs can alter the their host’s transcription profile by viral promoter-mediated lateral activation of adjacent host genes, by viral enhancer-mediated cis-activation of cryptic promoters (Morishita et al. 1988), or by providing splice signals and polyA signals (Kapitonov and Jurka 1999). While such mechanisms may explain the down-regulation of Aifm1 expression in the Hq mutant, they cannot explain the dysregulation of hair cortex genes which are located on autosomes at a remote genomic distance from the X-chromosomal Aifm1 locus. Alternatively, ERVs may influence host gene expression by viral non-coding RNAs (Zhang et al. 2018) or by providing novel binding sites for transcription factors (Tsuruyama et al. 2011). ERV-derived proteins or protein fragments can also cause specific biological effects in the human placenta, the immune system, and the pathogenesis of neurodegenerative disease in mice (Apte and Sanders 2010; Lokossou et al. 2014). Our data obtained from Kera2 cells transfected with the full-length Hq-ERV provide evidence that expression of endogenous retroviral elements can interfere with hair keratins in vitro, which may be a contributory factor to the hair phenotype elicited in the mutant. Using this tool, future experiments with mutated and/or truncated forms of the viral genome will allow us to gain a more detailed understanding of the underlying molecular processes.

Materials and methods

Ethics statement

Animals were sacrificed according to §14.3 of the German law for the protection of animals (Tierschutzgesetz), with Thomas Franz and Daniele Bano holding the permission to sacrifice mice (file number 50.203.2-BN 6/02, 84-02.04.2014.A521, 84-02.04.2015.A007). Mice were killed by cervical dislocation avoiding unnecessary pain. A permission to hold and breed mice based on §11(1) of the relevant animal welfare law was granted on 21st of March, 2014 to Thomas Franz. All procedures were followed as requested by the competent authorities.

Mouse work

B6CBACa\textsuperscript{A\textsuperscript{w/J-Aifm1\textsuperscript{Hq/I}} mice were purchased from The Jackson Laboratory (Bar harbor, Maine, USA) (JAX stock number: 000501). Mice were crossed once with C57BL/6 J mice and the offspring maintained by minimal inbreeding as one agouti and one non-agouti mouse line. Animals were kept in a 12-h light/dark cycle with food and water ad libitum. Mouse genotypes were determined by PCR on genomic DNA obtained from tail or ear biopsies. Mutant Aifm1 (R200 del) KI mice have been previously described (Wischhof et al. 2018). Throughout all experiments, Aifm1\textsuperscript{Hq/\textsuperscript{I}} samples were compared to samples from age-matched Aifm1\textsuperscript{X/Y}}
Animals were sacrificed by cervical dislocation and rupture of cervical blood vessels, before tissues were removed for fixation.

**Histology, immunohistochemistry, and in situ hybridization**

Histology and immunofluorescence (IF) of paraffin-embedded tissue fixed in Bouin’s solution were performed as previously described (Runkel et al. 2008). For IF, heat-mediated antigen retrieval was performed using 1 mM EDTA (pH 9.0). Monoclonal rabbit antibody D39D2 against AIF (Cell Signaling Technology) was used at a dilution of 1:100, followed by Cy3-conjugated goat anti rabbit IgG F(ab)2 (Dianova, 111-165-006) at a dilution of 1:500.

Gene-specific cRNA probes used in Northern blot analyses and in situ hybridizations were generated by RT-PCR as described (Runkel et al. 2008). In situ hybridizations were performed as described (Runkel et al. 2004). Oligonucleotides used in this work are listed in Table 1.

Transmitted light microscopy images were captured on a Leica DMRB microscope. Confocal fluorescence images were captured on a Nikon A1R confocal microscope using a 20× objective.

Ultrastructural investigations using transmission electron microscopy (TEM) were performed as previously described (Runkel et al. 2008), using a Leo910 TEM.

**Western blot analyses**

Western blot analyses were carried out as described (Runkel et al. 2008). Goat antibody against AIF (Santa Cruz, sc-9416) was used at a dilution of 1:300. Goat antibody against actin (Santa Cruz, sc-1615) was used at a dilution of 1:30,000. Mouse monoclonal antibody against GAPDH (Hytest, 5G4MAb6C5) was used at a dilution of 1:500. POD-conjugated rabbit anti-goat IgG (Dianova, 305-036-003) or goat anti-mouse F(ab)2 (Dianova, 115-036-062) were used at 1:30,000 or 1:25,000 dilutions, respectively.

**Northern blot analyses**

RNA blot hybridizations were performed and gene-specific cRNA probes were generated as previously described (Runkel et al. 2008). The list of gene-specific primers used to generate DIG-labeled cRNA probes is reported in Table 1. Hq-ERV-specific cRNA probes were generated from PCR products with 100% sequence identity to our cloned Hq-ERV genomic sequence.

**qRT-PCR analysis**

Dorsal mouse skin biopsies were snap-frozen in liquid nitrogen and ground in a mortar for total RNA extraction using the NucleoSpin RNA midi kit (Macherey & Nagel) according to the manufacturer’s recommended protocol. RNA from Kera2 cells was isolated from a cell pellet collected by trypsin dissociation and centrifugation. mRNA was purified from 1 µg total RNA using the Oligotex mRNA mini kit (Qiagen) following the manufacturer’s recommended protocol. cDNA was synthesized from the resulting mRNA. Approximately 1 µl of skin cDNA or 2 µl of Kera2 cDNA were used for quantitative RT-PCR on a LightCycler™ 96 using the universal probe library protocol (both Roche), with primers and probes as listed in the table. Experiments were performed as biological triplicates and technical duplicates, expression levels were normalized to the respective wt samples, except for the Hq-Env expression, which was normalized to Hq/Env-containing samples (Hq/Y skin or Hq-ERV-transfected Kera2 cells, as applicable). Gene-specific primers and probes are listed in Table 2.

**Molecular cloning**

To generate a viral vector for cell immortalization, we sequentially cloned a blasticidin resistance gene (BlaR) followed by a modified T2A linker (PCR amplification from pDEST51 using primers FR2572 and FR2574) and a truncated temperature-labile SV40 tsA58T (PCR from pZipneotsA58 using FR2575 and FR2571; pZipneotsA58 was a kind gift from P. Jat. (Jat and Sharp 1989)) into the p50-M-Neo retroviral plasmid (Laker et al. 1998). In the resulting vector p50MBla-tsA58T, the viral MeSV LTR drives the expression of BlaR-T2A-SV40tsA58T (Primers for cloning are listed in Table 3).

The murine HoxC13 expression vector expressing a neomycin resistance was purchased from Origene, BioCat (MR226373-OR).

To clone the Hq allele-linked ERV genome, a 9.3-kbp-long DNA fragment was amplified from genomic DNA of Aifm1Hq/Y mice using primers FR470 and FR471 derived from the first intron of the Aifm1 gene as described in Klein et al. (2002). The amplified PCR fragment was then ligated into the TOPO XL2 cloning vector (ThermoFisher). The resulting plasmid pTOPO-Hq-ERV (Addgene #164088) was amplified in SblI3 bacteria and fully sequenced (GenBank MW030280).

**Tissue culture**

GP + E-86 ecotropic helper cells (Markowitz et al. 1990) were transfected with p50MBla-tsA58T and clones selected in 1 µg/ml blasticidin. Blasticidin-resistant clones were
### Table 1: List of primers used for RT-PCR and cRNA probe synthesis

| Gene   | Product length spliced/genomic | For primer | Rev primer | Comment/Gene function | Rev primer + T7 (where applicable) |
|--------|--------------------------------|------------|------------|-----------------------|-----------------------------------|
| FoxN1  | 800                            | CTGGGCTCACCTCACTATCC | AGGTCACTGCCCAAGGCTCT | Regulator               | GGATCCTAAATAGCAGACTCA               |
|         |                                |            | CC         |                       | CAGGTCAGCTCCCAAG                 |
|         |                                |            |            |                       | GTCTCC                            |
| Hoxc13 | 540                            | GGTGACAGCAGCTGCTCTCCAG | TTCCGGGTTGAGTTCCGTTAAGGA | Regulator               | GGATCCTAAATACGACTCA               |
|         |                                |            | AG         |                       | CAGGTCAGCTCCCAAG                 |
|         |                                |            |            |                       | GTCTCC                            |
| Msx2   | 350                            | GCAAGGATGTTGGACTTGCT | TCACTCTGACTCGGTTGTAGGA | Regulator               | GGATCCTAAATACGACTCA               |
|         |                                |            | CT         |                       | CAGGTCAGCTCCCAAG                 |
|         |                                |            |            |                       | GTCTCC                            |
| Msx2   | 530                            | GCCGCGTACTGTTTCGTC | CCTTACGCTCCCTCGCTCTTCG | Regulator               | GGATCCTAAATACGACTCA               |
|         |                                |            | TC         |                       | CAGGTCAGCTCCCAAG                 |
|         |                                |            |            |                       | GTCTCC                            |
| β-Catenin | 150                       | TACGAGACACATCGAGAAGCCA   | CTGCACAACCAACATGGATAAGG | Regulator               | GGATCCTAAATACGACTCA               |
| Krt5   | 550                            | TGAGGTTGAGAAGAGGAGTTGGA | GATGGGTTTGCTCTTTGTCGAGG | Regulator               | GGATCCTAAATACGACTCA               |
|         |                                |            | GA         |                       | CAGGTCAGCTCCCAAG                 |
| Msx2   | 260/900                        | TGGCGTAGCTATGGAGGAGGAAG | CAGGACATGGGATGGTCTACGAGGA | Regulator               | GGATCCTAAATACGACTCA               |
|         |                                |            | AG         |                       | CAGGTCAGCTCCCAAG                 |
|         |                                |            |            |                       | GTCTCC                            |
| Krt10  | 260/900                        | TGGCGTAGCTATGGAGGAGGAAG | CAGGACATGGGATGGTCTACGAGGA | Regulator               | GGATCCTAAATACGACTCA               |
|         |                                |            | AG         |                       | CAGGTCAGCTCCCAAG                 |
|         |                                |            |            |                       | GTCTCC                            |
| Krt16  |                                | CTGGGATGCGAGAAGAATACCA | ACCACCATGAGGGAGTAGAAGG | Regulator               | GGATCCTAAATACGACTCA               |
|         |                                |            | CA         |                       | CAGGTCAGCTCCCAAG                 |
| Krt31  | 240                            | CCAGCAAGGCAAGAGAGAGAGAAG | TGGTGACGCTAGAAGCTGGTGAGGA | Regulator               | GGATCCTAAATACGACTCA               |
|         |                                |            | GC         |                       | CAGGTCAGCTCCCAAG                 |
| Krt32  | 400                            | TGGAAACACCTGACTGACTCCACTG | AATTTAAGGCTAGAGCAAGCCAAGA | Regulator               | GGATCCTAAATACGACTCA               |
|         |                                |            | CC         |                       | CAGGTCAGCTCCCAAG                 |
| Krt33  | 380                            | CTGGCGTGGAGAGCGAGGACTG | GCAGAAAGAGGGAGAAGAGAAGG | Regulator               | GGATCCTAAATACGACTCA               |
|         |                                |            | TG         |                       | CAGGTCAGCTCCCAAG                 |
| Krt34  | 300                            | TTTAGTTCAATAGCAGAGAAGAAGG | TCGGGAGTTGATGAGAAGAGG | Regulator               | GGATCCTAAATACGACTCA               |
|         |                                |            | ACG        |                       | CAGGTCAGCTCCCAAG                 |
| Krt35  | 470                            | GGGAGAAGGGCATCCCTCAGGGAG | GGAACAGCAATCCACCTCAAGGAGG | Regulator               | GGATCCTAAATACGACTCA               |
|         |                                |            | GG         |                       | CAGGTCAGCTCCCAAG                 |
| Krt36  | 260                            | TCAAGTCAGACCCAGAGAGAGAGG | GGAATATTTCCAGGGAGAGAGGA | Regulator               | GGATCCTAAATACGACTCA               |
|         |                                |            | CC         |                       | CAGGTCAGCTCCCAAG                 |
| Krt71  | 390                            | TCCAAAGTGCTCTGAGGCT | AAAAGATACAAAAAGAGGAGAGAAGG | Regulator               | GGATCCTAAATACGACTCA               |
|         |                                |            | GGGTGAC | CAAGG | CAGGTCAGCTCCCAAG |
| Krt72  | 400                            | ACATGAGGAAGGAGTTGGAGAGG | TACCCGGATAGGCTTCCCTGACGAGGA | Regulator               | GGATCCTAAATACGACTCA               |
|         |                                |            | AG         |                       | CAGGTCAGCTCCCAAG                 |
| Krt73  | 600                            | ATCCGCACTCTGGATGAGAGGAGG | CTCCTCTGAGGAGCAGAGAGAAGGAAGG | Regulator               | GGATCCTAAATACGACTCA               |
|         |                                |            | GC         |                       | CAGGTCAGCTCCCAAG                 |
| Krt75  | 800                            | TACAGGAAGCTGCTGGAAAGGAGG | ATGCAGACATTGCAACAGAAGGGAG | Regulator               | GGATCCTAAATACGACTCA               |
|         |                                |            | GG         |                       | CAGGTCAGCTCCCAAG                 |
| Krt82  | 400                            | GGGCCAGCGGTAGACTCTAGGAGAAGG | CTCACCGTGGAGGAGAAGGAGG | Regulator               | GGATCCTAAATACGACTCA               |
|         |                                |            | CA         |                       | CAGGTCAGCTCCCAAG                 |
| Krt84  | 420/1600                       | CTCACCCCGAGATAGCAAGGATG | TCCACGGGCACATTTCCGCTG | Hair Krt                | GGATCCTAAATACGACTCA               |
|         |                                |            | TG         |                       | CAGGTCAGCTCCCAAG                 |
| Krt86  | 400                            | CTGGTACTGCTCCCTGCTGGTC | GCTCTGAGCTACCTGTTGTAGGAG | Hair Krt                | GGATCCTAAATACGACTCA               |
|         |                                |            | CT         |                       | CAGGTCAGCTCCCAAG                 |
| Krtap3-2 | 480                        | AGGGTTTGGTTATGAGGAGGGAAGGGAATAGG | CAGGGTTAAAATTTCTGCTGCTGCTTCTT | Hair Krtap                  | GGATCCTAAATACGACTCA               |
|         |                                |            | GCCGGA   | CAGGTCAGCTCCCAAG | CAGGTCAGCTCCCAAG |
| Krtap3-3 | 300                        | CTGGTCTGGCCAGAGGAGGAGGAGGGAAGG | CAGGGTTAAAATTTCTGCTGCTGCTTCTT | Hair Krtap                  | GGATCCTAAATACGACTCA               |
|         |                                |            | TG         |                       | CAGGTCAGCTCCCAAG                 |

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| Gene  | Product length | For primer Rev primer | Comment/Gene function | Rev primer + T7 (where applicable) |
|-------|----------------|-----------------------|-----------------------|----------------------------------|
| Krtap4-7 | 460 | TGGAGGGCTGAGCAGCAAG | CAGCTGGTAGCACAGGATCAG | Hair Krtap |
| Krtap5-2 | 400 | ATGATGCTGAGCTGGAGT | CAGCTGGTAGCACAGGATCAG | Hair Krtap |
| Krtap6-1 | 360 | AACCTCAACACCAAAG | TATTCCTGACTCAAGATCAGCA | Hair Krtap |
| Krtap8-2 | 130 | ACCGGAGCTACTAGGAGG | TACCTGGAGAATCCATAA | Hair Krtap |
| Aifm1 5' | 390 | GAGAGTGTAGCTGGGAAA | AGTACCCCTCACAATCAGCA | Aifm1 5' probe |
| Aifm1 3' | 380 | GGGGATGCTGATCGGCAAAG | TCCCGACCACACACTTGCTCC | Aifm1 3' probe |
| Aifsh | 470 | TGTCTTCCTTGGTGGGGAT | ACCCGTCTGACCAACTGAG | Aifsh variant |
| Hq-ERV | 509 | GTAGAGCGGCGATCTCTGCTGAAT | TTTTGGGAGATGAGCTGACTT | Hq-ERV probe |
| Hq-ERV gag | 1582 | GGCTGACTGGCCGCAAGAA | CCACATGGCGGGGTGGGCTTCC | Hq-ERV gag |
| ERV env | 1030 | GTTCAGGAGGCGCGACTCC | CAACAGCTGACCAGGACAAG | Hq-ERV env |

| Gene | Gene ID | For primer Rev primer | Description |
|------|---------|-----------------------|-------------|
| Krt5-001 | ENSMUST00000023709.5 | CAGACCTTGGTGAGGACTA | Aifm1 intron 1–2 For (Klein et al. 2002) |
| Krt84-201 | ENSMUSG00000044294.7 | CACCTGGACAGACATGTTGA | Aifm1 intron 1–2 Rev (Klein et al. 2002) |
| Krtap3-3-001 | ENSMUSG00000069722.4 | TGAAGAGATCAACCGCACAACCA | Hq-ERV U3 LTR For (Klein et al. 2002) |
| Aifm1-001 | ENSMUSG00000036932.14 | TCCAGAGGGCTTCTAGATCAGT | SV40tsA58 Rev with BamHI site |
| Hq-Env n.a. | | GGTTCACACAGCTGGGGAAGAAA | BlaR-For with NotI site |
| Gapdh-001 | ENSMUST000000118875.7 | GGTTTCCTTATAGGAGGACTGCC | SV40tsA58 For + partial T2A with EcoRI site |
| FR470 | | AGTTTGCACTGAAATGTCG | Aifm1 intron 1–2 For (Klein et al. 2002) |
| FR471 | | CATGCTCCTCTCTAGTATTT | Aifm1 intron 1–2 Rev (Klein et al. 2002) |
| FR472 | | CCAGAAACTGCTTCTAGGGGATT | Hq-ERV U3 LTR For (Klein et al. 2002) |
| FR2571 | | TTGGATCTTATGTTTCAGTCAGG | SV40tsA58 Rev with BamHI site |
| FR2572 | | AAAAGGCGCGCAGCTGGCAGACGTCG | BlaR-Rev with NotI site |
| FR2574 | | TTAGCTTACGCTAGGAAGAG | BlaR-Rev + partial T2A with EcoRI site |
| FR2575 | | TGGATCTTACGCTAGGAAGAG | SV40tsA58 For + partial T2A with EcoRI site |
| FR3134 | | GGTTCCTATTTATCTAGG | Aifm1 intron 5–6 For (Wischhof et al. 2018) |
| FR3135 | | CATTGCTCCTGGGAAAGG | Aifm1 intron 5–6 For (Wischhof et al. 2018) |
growth arrested by treatment with mitomycin C for 4h. Primary keratinocytes were isolated from the skin of 2-day-old C57BL/6 J mice and co-cultured with growth-arrested p50MBla-tsA58T-transfected producer cells for immortalization. The keratinocytic cell line Kera2 showed sustained growth at the permissive temperature of 32 °C in CaCl2-free DMEM. In the same way, a keratinocytic cell line Hq-Kera was established from Hq mutant males.

To generate Hq-ERV transfected Kera2 clones, the entire insert was excised from pTOPO-Hq-ERV using Pmel and NorI. The excised insert was gel-purified and co-transfected into mammalian Kera2 cells using Xfect transfection reagent (Takara) together with vector pPur (Clontec). After transfection a single-cell suspension was prepared using trypsin and the cells were plated at limiting dilutions in 96-well plates. The transfected cells were selected in 1 µg/ml puromycin and clones examined by genomic PCR for the presence of the Hq-ERV.

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Author contributions MH and TF: designed and carried out research, and analyzed data; SG, MM, BB, and LW: carried out research; DH: carried out research and analyzed data; MH, TF, and DB: wrote the manuscript.

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Availability of data and material Materials used in this study will be sent to interested researchers upon request.

Compliance with ethical standards

Conflict of interest The authors declare that no conflicts of interest exist.

Ethical approval Animals were sacrificed according to §14.3 of the German law for the protection of animals (Tierschutzgesetz), with Thomas Franz and Daniele Bano holding the permission to sacrifice mice (file numbers 50.203.2-BN 6/02, 84-02.04.2014.A521, 84-02.04.2015.A007). Mice were killed by cervical dislocation avoiding unnecessary pain. A permission to hold and breed mice based on §11(1) of the relevant animal welfare law was granted on 21st March, 2014 to Thomas Franz. All procedures were followed as requested by the competent authorities.

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