Alopecia in a Novel Mouse Model RCO3 Is Caused by mK6irs1 Deficiency

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Reduced coat 3 (*Rco3*) is a new spontaneous autosomal recessive mutation with defects in hair structure and progressive alopecia. Here we describe chromosomal mapping and molecular identification of the *Rco3* mutation. The murine *Rco3* locus maps to a 2-Mb interval on chromosome 15 encompassing the keratin type II gene cluster. Recently, mK6irs1 was described as a type II keratin expressed in Henle's and Huxley's layer of the murine inner root sheath. Genomic sequencing revealed a 10-bp deletion in exon 1 of mK6irs1 resulting in a frameshift after 58 amino acid residues and, therefore, the absence of 422 carboxy-terminal amino acid residues containing the complete α-helical rod domain. Henle's and Huxley's layers show no immunoreactivity with mK6irs1-specific antibodies and the absence of intermediate filament formation in electron microscopic images. These results indicate that the expression of functional mK6irs1 is indispensable for intermediate filament formation in the inner root sheath and highlights the importance of the keratinization of the inner root sheath in the normal formation of the hair shaft.

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The major structural proteins of the hair follicle have been identified as keratins. The keratin multigene family comprises more than 40 individual but structurally related members (Hesse *et al*, 2001) that are divided into cytokeratins and hair-type (trichocyte) keratins. Based on their charge properties, keratins are further classified as acidic type I keratins (cytokeratins K9–K20; trichocyte keratins Ha1–8) and more basic type II keratins (cytokeratins K1–K8; trichocyte keratins Hb1–6). Keratins form obligate heterodimers of type I/type II pairs that assemble to form the intermediate filament cytoskeleton of all epithelial cells (Hatzfeld and Weber, 1990; Steinert, 1990).

Since 1991, mutations in many cytokeratin genes have been identified in a variety of human diseases affecting the epidermis and other epithelial structures (Lane, 1993; Fuchs and Cleveland, 1998; Irvine and McLean, 1999; Corden and McLean, 2001). Several mutations in trichocyte keratins have also been observed underlying the hair fragility disorder monilethrix (Winter *et al*, 1997a, b). Recently, novel type I and type II keratins of the inner root sheath (IRS) have been described (Aoki *et al*, 2001; Porter *et al*, 2001; Langbein *et al*, 2002) that may be associated with other hair fragility disorders. In humans there are four type II keratins, hK6irs1–4 (Langbein *et al*, 2003), and in the mouse at least one, mK6irs1 (former mouse gene nomenclature Krt2–6g), but there is evidence for three more murine K6irs proteins, XP_128271, XP_128282, and XP_139603. Several expressed sequence tags support the existence of XP_128271 and XP_128282, whereas no murine expressed sequence tag information is available for XP_139603. Most likely, mK6irs1 and hK6irs1 are orthologs (Langbein *et al*, 2002). In the hair follicle, hK6irs1 is expressed in Henle's and Huxley's layers and in the cuticle of the IRS. hK6irs2 and 3 are sequentially expressed in the IRS cuticle, hK6irs4 expression is strictly restricted to Huxley's layer (Langbein *et al*, 2003). In northern blots, in the mouse, it has been shown that expression of mK6irs1 is restricted to skin tissue (Aoki *et al*, 2001). No hybridization signal was observed in stratified epithelia of tongue, esophagus, or fore stomach tissue. *In situ* hybridization and immunohistochemical studies of mouse anagen hair follicles confirmed expression of this cytokeratin in the IRS in anagen III and in Henle's and Huxley's layers during anagen VI (Aoki *et al*, 2001; Porter *et al*, 2001; Langbein *et al*, 2002). Four type I keratins have been localized to the IRS in the sheep; IRSa1, IRSa2, IRSa3.1, and IRSa3.2 and at least three predicted orthologs with high sequence homology exist in both mouse and human (Bawden *et al*, 2001; Porter *et al*, 2003).

Inbred laboratory mice develop many of the same diseases as humans and have become important *in vivo* models for studying biology, pathology, genetics, and molecular mechanisms. Mice are useful tools to help understand human diseases of skin and hair (Sundberg, 1994). Although there are over 100 mutations in the mouse causing morphologic abnormalities of the hair, or hair follicles, only a few have been studied at the molecular level (Nakamura *et al*, 2001). Nevertheless, mouse mutations such as hairless, Tabby, or nude helped reveal the cause of several disorders associated with alopecia in humans (Nehls *et al*, 1994; Panteleyev *et al*, 1998; Frank *et al*, 1999; Monreal *et al*, 1999).

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Abbreviations: IRS, inner root sheath; RCO3, reduced coat 3; SNP, single nucleotide polymorphism.

Here we describe the phenotype of a new mouse mutation *Rco3* (*reduced coat 3*) located on chromosome 15. We performed a positional cloning approach to determine the location of the mouse gene and discovered a mutation in mK6irs1.

MATERIALS AND METHODS

Mice The *Rco3* mutation was originally found on C3HeB/FeJ background in the course of the ENU-Mouse Mutagenesis Screen Project within the German Human Genome Project (DHGP). C57BL/6J, BALB/c, and AKR inbred mice used for generating outcross progenies were obtained from the Jackson Laboratory (Bar Harbor, ME).

Morphologic studies Dorsal skin biopsies were fixed by immersion in Bouin's solution for 6 h. The fixed tissues were kept in three changes of 70% ethanol for another 6 h before being embedded in paraffin wax after clearing in methylbenzoate. Tissues were processed for histology in semithin sections and electron microscopy as previously described (Reichelt *et al*, 2001).

Immunohistochemistry of keratins was carried out on frozen sections of vibrissae and pelage follicles from 8-wk-old control *Rco3*+/– and *Rco3*–/– mice. Frozen sections were fixed in methanol/acetone 1:1 at –20°C for 5 min. Primary antibodies were rabbit antisera raised to murine IRSa2 (RPmIRSa2) (Porter *et al*, 2003) and mK6irs1 (gift from W.H.I. McLean) (Porter *et al*, 2001) diluted 1:500. The secondary antibody was anti-rabbit IgG conjugated to Alexa 488 diluted 1:500 (Molecular Probes, Eugene, OR). Sections were counterstained with DAPI and mounted in Hydromount/2.5% DABCO.

DNA analyses Mouse genomic DNA was purified from tail tips of F2 individuals by using the DNeasy 96 tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Pyrosequencing was performed on a PSQ96 pyrosequencing device according to manufacturer's recommendations. Linkage analysis on the individual level was performed using fluorescent-labeled simple-sequence-length polymorphism and single-nucleotide polymorphism (SNP) markers. PCR products for genotyping and sequencing were separated on ABI 3700 automated sequencing devices (Applied Biosystems, Foster City, CA). Simple-sequence-length polymorphism markers were analyzed using the ABI Genotyper program; SNPs were analyzed by BigDye terminator (v3.0) sequencing. For mapping of *Rco3* the following new polymorphic markers were established: D15Ing32 sense GGGAGGTGAGACTTCG-CTAC and antisense TGGGAATTTCCAGAACTAGGG; and D15Ing18, sense TCATCTTGGGAGACTCAGTGTT and antisense GTGCACCACT-ATACCCAGCA. Length polymorphisms were D15Ing32, C3H, C57BL/6J, 123 bp; BALB/c, AKR, approximately 150 bp; D15Ing18, C3H, AKR, 160 bp; and C57BL/6J, BALB/c, 164 bp. For mutational analyses of candidate genes, exons and adjacent intronic segments were amplified from three affected F2*(C3HeB/FeJ × BALB/c)-*Rco3/Rco3* individuals, and their sequence was compared with both parental strains and heterozygous controls.

Chromosomal mapping For chromosomal mapping of the *Rco3* locus, homozygous C3HeB/FeJ-*Rco3/Rco3* individuals were outcrossed to C57BL/6J, BALB/c, and AKR genetic background. Heterozygous F1 hybrids were intercrossed and affected F2 individuals were identified by their characteristic phenotype. A total of 874 meioses were investigated to map the Rco3 mutation. For initial mapping F2*(C3HeB/FeJ × C57BL/6J)-*Rco3/Rco3* individuals were used. Comparable DNA amounts of 48 individuals were pooled, and 100 ng of pool DNA per PCR reaction was subjected to pyrosequencing of 86 SNPs equally distributed over the genome. Linkage to chromosome 15 was judged by basecallings identical to inbred C3H situations. High-resolution mapping on affected and unaffected (C3HeB/FeJ × BALB/c) and (C3HeB/FeJ × AKR) hybrids on an individual basis was carried out by microsatellite analysis with dye-labeled primers and by SNP sequencing.

RESULTS

Identification of a new mouse model of inherited alopecia *Rco3* is a novel spontaneous autosomal recessive mutation of the mouse characterized by severe alopecia. *Rco3* heterozygotes are phenotypically normal and indistinguishable from wild-type littermates. *Rco3* homozygotes are vital and fertile and have a normal life expectancy. The obvious phenotype of RCO3 mutants is a progressive hair loss detectable in the pelage. RCO3 homozygotes can be identified around day 9 after birth by the curled form of their whiskers, which remain curly throughout life (**Fig 1*c***). When the first coat is fully grown by day 16 postpartum, the body pelage appears curly everywhere (not shown). By the time the second generation of hair follicles is formed and the hair cycle begins, the alopecia becomes apparent. The coat is sparse on the trunk (**Fig 1*a***), but less so in the facial region (**Fig 1*c***). The dorsal alopecia appears patchy with the distribution of the patches differing from individual to individual. These differences are most likely due to the grooming activity of the animals, which causes epilation of the fragile mutant hairs (**Fig 1*d***). All types of hairs in homozygous animals are malformed showing kinks and twists and can be plucked without force (**Fig 1*d***). Ventrally, the coat of homozygotes is also reduced with only sparse pelage remaining on the hind leg and around the genital area (**Fig 1*b***). Thus, the *Rco3* mutation causes alopecia by altering the strength and shape of all types of hairs in the murine pelage.

Positional cloning of the Rco3 mutation A total of 874 meioses (437 individuals) were investigated to map the *Rco3* mutation. The initial chromosomal mapping to chromosome 15 was performed on 48 F2*(C3HeB/FeJ × C57BL/6 J)-*Rco3/Rco3* individuals. For high-resolution mapping we bred the *Rco3* mutation on C3HeB/FeJ × AKR (n = 109, 32 affected, 77 unaffected) and C3HeB/FeJ × BALB/c backgrounds (n = 280, 65 affected, 215 unaffected) with a penetrance of the pathologic phenotype of 29.3 and 23.2% for AKR and BALB/c background, respectively. In total, we obtained 24.9% affected individuals for both strain combinations. The penetrance on C3HeB/FeJ × C57BL/6J background was not recorded. No obvious variation in expression of the RCO3 phenotype was observed, however, in all three strain combinations investigated. The *Rco3* locus is flanked by informative microsatellite markers D15Ing32 (proximal) and D15Ing18 (distal) on Mb position 102–104. This chromosomal region contains the type II keratin gene cluster. In affected F2*(C3HeB/FeJ × BALB/c)-*Rco3/Rco3* individuals we sequenced two candidates by function, Krt2–6b and the recently discovered mK6irs1 (former nomenclature Krt2–6g).

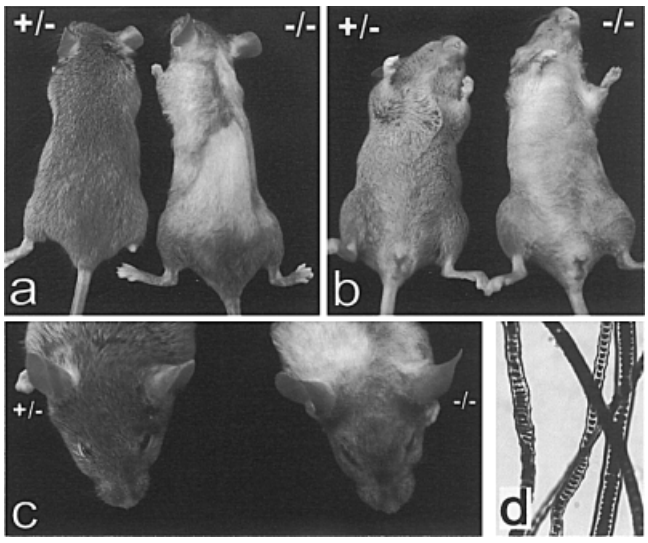

Figure 1. The development of the coat phenotype in RCO3 homozygotes and wild-type littermates. Dorsal (*a*) and ventral (*b*) view of a RCO3 heterozygous (*left*) and homozygous (*right*) mouse. Note that the heterozygote has a normal pelage, whereas the homozygote shows a patchy alopecia. In the homozygote the vibrissae are not straight but are bent and curl distally (*c*). Plucked dorsal hairs of a homozygote show kinks in the cortex (*d*).

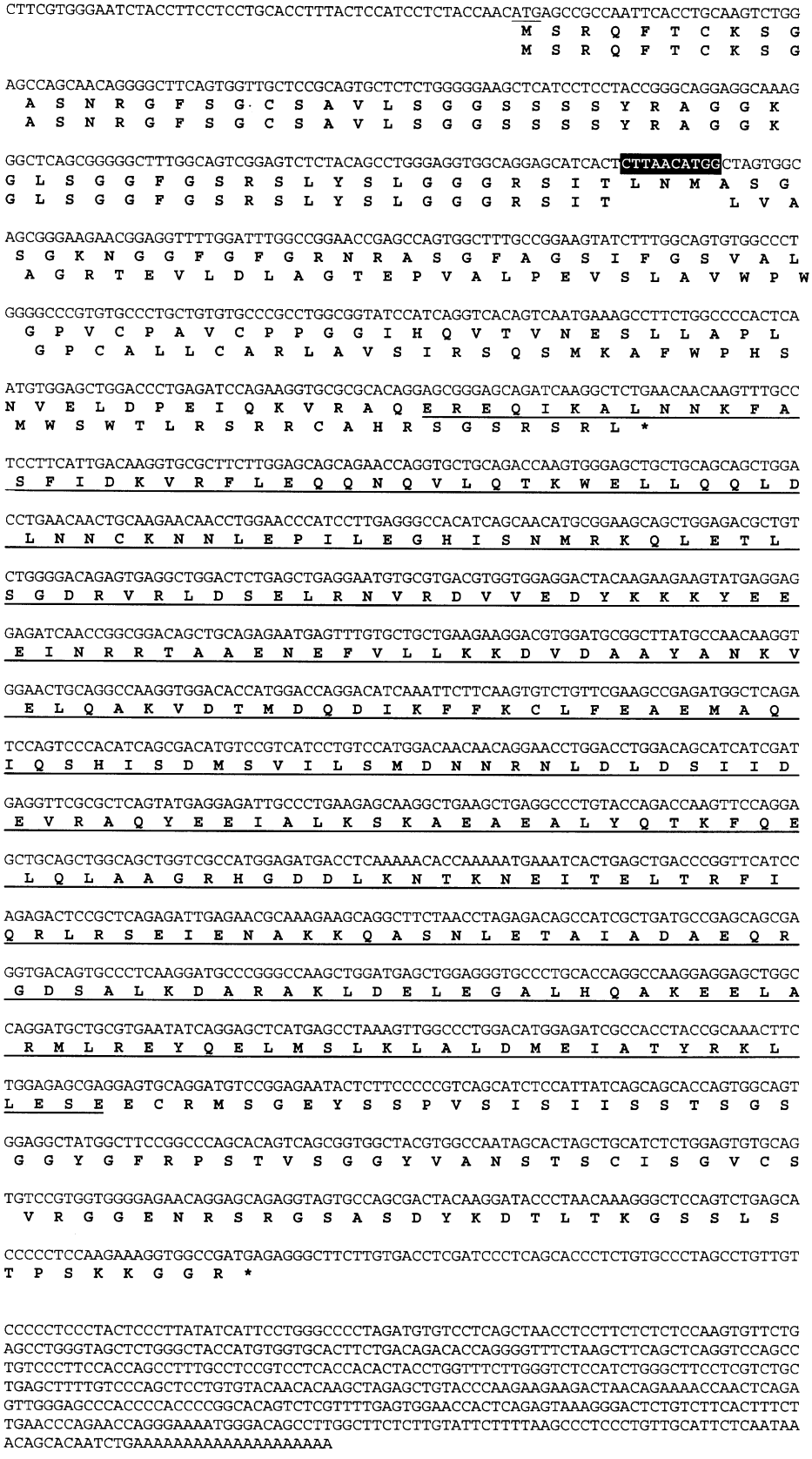

Figure 2. mK6irs1^{Rco3} cDNA sequence and derived amino acid sequence. Highlighted nucleotides indicate the 10-bp (CTTAACATGG) deletion diagnostic for the *Rco3* mutation; mK6irs1 nucleotide sequence, AB033744. The deduced amino acid sequence of RCO3 is given below the mK6irs1 wild-type sequence. Start codon and central α-helical rod domain of the wild-type protein are underlined. The 10-bp deletion results in a frameshift after 58 amino acids and a truncated protein lacking the complete α-helical rod domain.

No mutation was found in Krt2–6b. A 10-bp deletion of nucleotides 223–232 (CTTAACATGG, numbering according to mK6irs1 wild-type sequence; Accession No. AB033744) was detected in exon 1 of mK6irs1 (**Fig 2**). The segregation of the deletion was investigated in our F2*(C3HeB/FeJ × BALB/c) progeny and all affected individuals analyzed showed homozygosity for the deletion. Additionally, three polymorphisms were detected in the 3′-untranslated region between strains C3H and BALB/c (corresponding to nucleotide positions 1641 A/G; 1960, 1961A,G deleted in BALB/c; and 2070 C/A), which confirmed the C3H origin of the mutated allele and excluded possible recombination events. The deletion causes a frameshift in the mK6irs1*RCO3 protein after 58 amino acid residues. The truncated RCO3 protein has a length of 134 amino acids, whereas residues 59–134 show no similarity to any known or predicted protein sequence. Keratins share a central α-helical rod domain of approximately 310 amino acids, a basic molecular structure common to all intermediate filaments responsible for dimerization and higher order polymerization (Albers and Fuchs, 1992; Cary and Klymkowsky, 1992; Okabe *et al,* 1993; Stewart, 1993; Irvine and McLean, 1999). The RCO3 keratin completely lacks this domain; thus, *Rco3* can be expected as a loss of function allele of mK6irs1.

Morphologic analysis of the RCO3 coat phenotype Microscopic examination of hairs plucked from the dorsal trunk of RCO3 mutant mice 34 d postpartum revealed the pelage to comprise solely of hairs showing little variation in diameter, a round cross-sectional profile, and frequent kinks and twists of the cortex (**Fig 1*d***). To identify the origin of this hair defect, we investigated histologically the anagen hair follicles of mutant mice and phenotypically normal littermates during the first hair cycle using semithin sections (**Fig 3*a–i***). Comparison of distal cross-sections of wild-type and mutant hair follicles usually showed two zigzag hair follicles flanking one with a larger shaft. In the wild type, the latter had a bean-shaped profile (**Fig 3*a***), but in the mutant it was somewhat smaller and oval (**Fig 3*b***). This indicates that the *Rco3* mutation affects structures of the hair follicle that mold the developing hair shafts and thus alters the shape of the hair.

Histologic analysis further revealed that in the mutant, keratinization of Henle's and Huxley's layer of the IRS was defective. In proximal cross-sections of wild-type anagen hair follicles, keratinization of Henle's layer of the IRS could be detected by the intense toluidine staining (**Fig 3*c,d***). In the RCO3 mutant follicle keratinization of Henle's layer was either discontinuous (**Fig 3*e***) or absent (**Fig 3*f***). Longitudinal sections showed that the mutant hair shafts were bent and twisted in the middle of the anagen follicle where keratinization of Henle's layer was defective (**Fig 3*h–i***). Given that mK6irs1 is expressed in the IRS but not in the cortex, these observations suggest that in the RCO3 mutant, malformation of the hair shaft is secondary to the IRS defect, and that in the wild-type, the IRS, especially Henle's layer, may serve as a splint for the growing hair shaft.

Comparison of electron micrographs of day 32 postpartum hair follicles from the wild type (**Fig 4*a,c***) with those from the mutant (**Fig 4*b,d***) revealed abnormalities of keratinization of

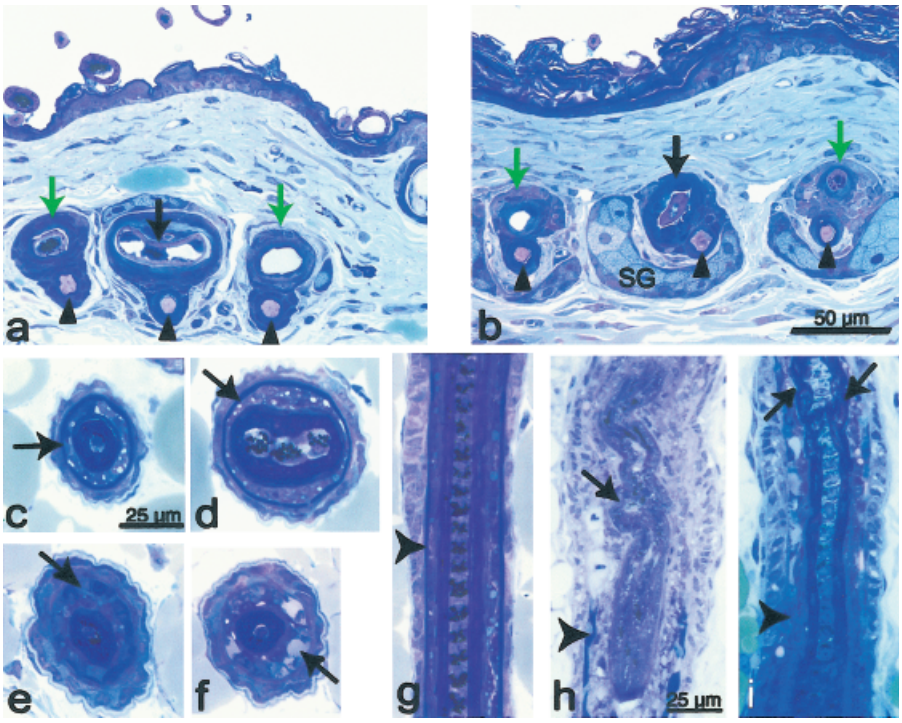

Figure 3. Morphologic features of RCO3 mutant dorsal pelage during anagen of the first hair cycle. Semithin sections stained with toluidine blue and pyronine red (*a–i*). RCO3 heterozygous mouse (phenotypically normal) on day 34 postpartum (*a,c,d,g*) and RCO3 homozygous littermate (*b,e,f,h,i*). Same magnification in *a* and *b* (*bar* in *b*), *c–f* (*bar* in *c*), and *g–I* (*bar* in *h*). (*a*) In the dermis of normal murine dorsal skin, the follicle of a large hair type (awl or auchene) with a convex-shaped cross-section (*black arrow*) is flanked by two small zigzag follicles showing round cross-sections (*green arrows*), both in close association with the proximal ends of the telogen hair follicles of the first coat that was formed during hair follicle morphogenesis (*black arrowheads*). (*b*) Shows that this arrangement of hair follicles is preserved in the RCO3 mutant, but the large hair type positioned in the middle (*black arrow*) shows an abnormal irregular cross-section and a comparatively small medulla. (*c–f*) Show cross-sections through anagen hair follicles of normal (*c,d*) and RCO3 mutant (*e,f*) mice at the level of the subcutis. At this level, normal hair follicles show intense staining of Henle's layer of the IRS indicating keratinization (*arrows in c,d*). This keratinization in mutant samples is either incomplete (*arrow in e*) or absent (*arrows in f*). Note the large pale cells in Henle's layer of the IRS (*f*). Longitudinal sections of proximal normal hair follicles show the keratinized Henle's layer of the IRS as a straight line (*arrowhead in g*). In mutant samples, the defective or absent keratinization of Henle's layer of the IRS (*arrowheads in h and i*) is associated with kinks and spiral deformations of the hair shaft (*arrows in h and i*). *SG,* sebaceous gland.

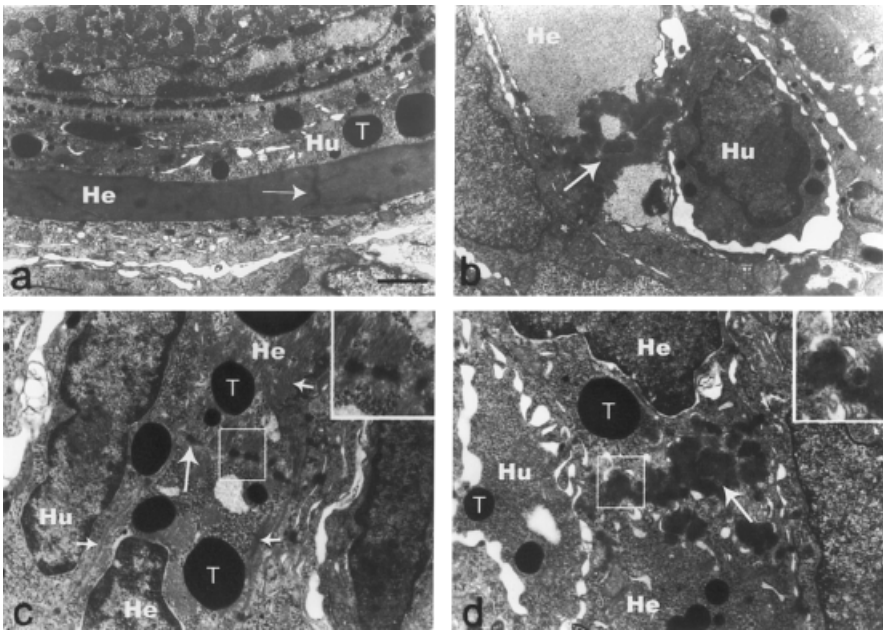

Figure 4. Electron microscopic examination of mutant RCO3 hair follicles. Anagen hair follicles of wild-type (*a,c*) and RCO3 homozygous littermate (*b,d*). Sections *a* and *b* were taken at the level of the follicle where the cuticule begins to keratinize, whereas sections *c* and *d* were taken more proximally. Same magnification in *a–d* (*bar in a,* 1.2 μm); *insets in c and d* indicate 2.5-fold magnification of framed regions. Henle's layer of the wild type is keratinized, and trichohyalin granules occur only in Huxley's layer at this level (*a*). *Arrow,* border between two adjacent Henle's layer cells. In the mutant (*b*), electron-dense material accumulates near the cell borders of neighboring Henle's layer cells (*arrow*). Before keratinization, i.e., at more proximal sections levels as in *a* and *b,* in both normal (*c*) and mutant (*d*) Henle's layer cells, trichohyalin granules (*T*) can be seen. Whereas filament bundles can be seen in cells of both Huxley's and Henle's layer in the wild type (*short white arrows in c*), such are absent from the mutant. The desmosomes in the normal specimen show associated filament bundles (*long white arrow in c;* see also *inset*), whereas in the mutant, electron-dense material is deposited near the cell border (*white arrow in d;* see also *inset*). *He,* Henle's layer of the IRS; *Hu,* Huxley's layer of the IRS; *T,* trichohyalin granula.

Henle's and Huxley's layers of the IRS. Where the normal Henle's layer showed keratinization (**Fig 4*c***), that of the mutant showed accumulation of homogenous, electron-dense materials and an absence of filament bundles (**Fig 4*b***).

Abnormal keratinization in the RCO3 mutant hair follicle is also evident at more proximal section levels. In the controls, the cells of Henle's layer undergoing keratinization show some remaining trichohyalin granules and filament bundles associated with desmosomes (**Fig 4*c***), whereas the mutant Henle's and Huxley's layers lack filament bundles (**Fig 4*d***). The failure of keratinization in Henle's layer detected by electron microscopy (**Fig 4*c,d***) is also seen in semithin sections where mutant cells are larger and less densely stained in comparison with controls (**Fig 3*e–i***). The absence of normal intermediate filament bundles in mutant Henle's and Huxley's layers of the mutant IRS suggests that no other type II keratin compensates for the loss of mK6irs1. In the mutant, however, both the bundles and the intercellular connections seem to be grossly normal in the cuticle and companion layer (not shown).

To determine whether the electron dense material associated with the membranes in the micrographs of *Rco3*–/– mice is keratinaceous, we tested for expression of keratins of the IRS. As expected, no immunoreactivity with an antibody to murine K6irs1 was observed in RCO3–/– animals (**Fig 5*b***), whereas there was normal expression of this keratin in the Henle's and Huxley's layers in the control RCO3+/– animals (**Fig 5*a***). In control vibrissae reactivity to antibodies to type I keratin, mIRSa2 was associated with filament bundles in all cell layers of the IRS. Surprisingly, despite the absence of mK6irs1, mIRSa2 was expressed normally in the IRS of RCO3–/– mice (**Fig 6*a,b***). Whereas keratin filaments were visible only in the cuticle of IRS, in Henle's and Huxley's layers large aggregates of keratin were visible (**Fig 6*c,d***). Pelage follicles showed similar aggregations (**Fig 6*e***).

DISCUSSION

The *Rco3* mutation arose spontaneously in the C3HeB/FeJ breeding colony maintained for the ENU mouse mutagenesis screen project within the DHGP. Here we describe a 10-bp deletion in the mK6irs1 gene that is responsible for the phenotype. The resulting protein consists of 58 amino acids of the head domain plus 76 amino acids with no sequence homology to any keratin. This protein is unlikely to directly participate keratin filament assembly.

Ultrastructural examination of the RCO3 mutant demonstrated that intermediate filament bundles are absent and keratinization is abrogated in both Henle's and Huxley's layers. The structure of the IRS is severely irregular with a very uneven edge (see **Fig 6**). In wild-type follicles the Huxley's layer is thought to communicate with the companion layer via "Flügelzellen," processes that cross the Henle's layer (Langbein *et al,* 2002, 2003). These Flügelzellen are clearly present in wild-type follicles but are likely to be disrupted in the irregularly formed IRS of the RCO3 mutants (**Figs 5, 6**).

We have shown that the RCO3 mutant produces only structurally altered hairs with kinks and twists and none that show the typical features of guard hairs, awls, auchenes, or zigzags. Because the mK6irs1 protein has been shown to be specifically expressed in the IRS (Aoki *et al,* 2001; Porter *et al*, 2001; Langbein *et al*, 2002), and not in the cortex, all alterations in hair shaft morphology must be secondary to the observed keratinization defects in Henle's and Huxley's layers. Thus, the analysis of the RCO3 mutant highlights the role of the IRS in molding the shape of the various hair types and providing a splint for the straight orientation of the shaft. It appears that loss of mK6irs1 protein from these cells cannot be compensated for by the expression of other type II keratins, implying that it is indispensable for normal IRS keratinization. A compensation was described for mK6a/b knock-

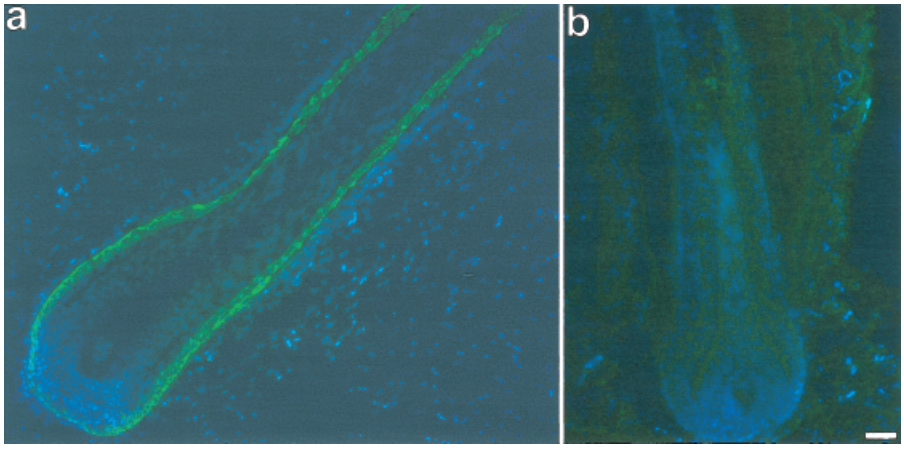

Figure 5. Immunohistochemistry of type II keratin mK6irs1 in the vibrissae and pelage follicles. Immunofluorescence microscopy of vibrissae from control RCO3+/– animals (*a*) shows that mK6irs1 is present in the Henle's and Huxley's layers of the IRS but is completely absent in the vibrissae from RCO3–/– animals (*b*). Same magnification in *a* and *b* (*bar in b,* 100 μm).

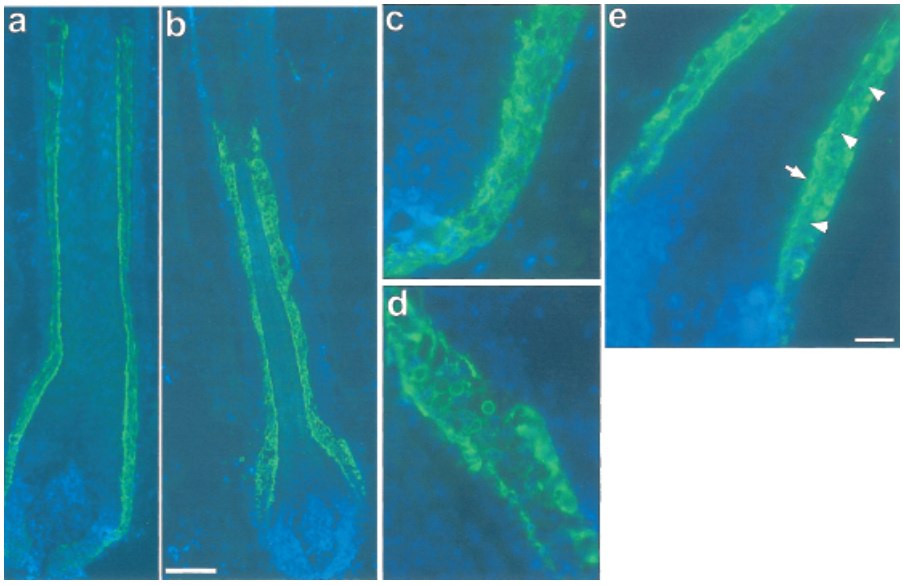

Figure 6. Immunohistochemistry of type I keratins in the vibrissae and pelage follicles. Vibrissae from control (*a,c*) and RCO3 (*b,d*) animals show similar localization of the type I keratin, IRSa2, in all the IRS layers. At higher magnification, immunostaining with the IRSa2 antibody appears very different in the mutant (*d*) to controls (*c*), suggesting aggregation of keratin in the Henle's and Huxley's layers. In mutant pelage follicles (*e*), similar aggregates of keratin can be seen in those layers (*arrowheads*), but normal filaments are visible in the cuticle (*arrow*). Same magnification in *a* and *b* (*bar in b,* 100 μm) and *c–e* (*bar in e,* 20 μm).

out mice in which a novel K6 isoform, mK6hf, can replace the mK6a/b function in the companion layer (Wojcik *et al*, 2001). In the human, hK6irs4 is also present in the Huxley's layer and would be expected to compensate for the lack of K6irs1 (Langbein *et al*, 2003). This suggests that the mouse equivalent of hK6irs4 (presumably murine XP_139603), if it exists is not able to compensate for the absence of mK6irs1. The formation of filament bundles and intercellular connections appears normal in the mutant IRS cuticle. Most likely the reason for this is that the murine orthologs of hK6irs2 and hK6irs3 are expressed in this layer (see XP_128271 and XP_128282 for potential murine orthologs of hK6irs2 and hK6irs3, respectively). In humans hK6irs2 and 3 are sequentially expressed in the cuticle (Langbein *et al*, 2003).

Normally, in the absence of a functional type II partner it would be expected that a type I partner would be degraded. Here we show by immunohistochemistry that type I keratins of the IRS are stable but incapable of forming filaments. Instead they form aggregates in the RCO3 mutant IRS. This suggests that the type I keratins are binding to something that prevents their degradation. Although IRS keratins are known to cross-link to trichohyalin (O'Guin *et al,* 1992) aggregation of the filaments before cross-linking to trichohyalin would be expected to occur (Tarcsa *et al*, 1997). It is probable that the aggregates observed in the immunofluorescent images may represent the electron-dense aggregates observed in the micrographs in association with cell–cell junctions in Henle's layer of the IRS. Whether association of keratin with the membrane is capable of stabilizing them remains to be determined.

Because the mutation in RCO3 may allow the formation of a truncated form of mK6irs1 that is not detected by the antiserum against the protein's C-terminal domain, we cannot exclude the possibility that truncated mK6irs1 exerts a concentration-dependent effect on keratin filament formation. Such an effect has been observed in mutants expressing a truncated form of K10, K10T, which causes filament aggregation and hence fragility in the suprabasal layer of the epidermis (Porter *et al*, 1996; Reichelt *et al*, 1997), but not following a complete knockout of the K10 gene (Reichelt *et al*, 2001). The RCO3* mK6irs1 protein lacks the helix initiation motif that exists in the truncated K10 created by Porter *et al* (1996). Taken together with the recessive inheritance of the RCO3 phenotype in contrast to the dominant inheritance of K10T, we favor the theory of *Rco3* representing a loss of function owing to mK6irs1 deficiency rather than a truncated protein impairing keratin filament formation.

As suggested previously (Porter *et al,* 2001), human K6irs proteins are good candidates for dominant forms of alopecia or other structural hair defects such as woolly hair (Ormerod *et al*, 1987), uncombable hair (Rogers, 1996), or loose anagen hair syndrome (Price and Gummer, 1989; Tosti *et al*, 1997; Sinclair *et al*, 1999; Tosti,

2002). In particular, in loose anagen hair syndrome, abnormal keratinization of the IRS (Price and Gummer, 1989) as well as fragility of the IRS epithelial cells (Tosti *et al*, 1997) have been reported. In this disorder anagen hair follicles are easily pulled from the scalp by mild mechanical trauma. It can be speculated that dominant-negative mutations in any of the IRS keratins could disrupt keratin structures of the IRS resulting in structural defects similar to RCO3. We would therefore suggest these keratins as candidates for human hair fragility syndromes.

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