A function for Rac1 in the terminal differentiation and pigmentation of hair

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Summary

The small GTPase Rac1 is ubiquitously expressed in proliferating and differentiating layers of the epidermis and hair follicles. Previously, Rac1 was shown to regulate stem cell behaviour in these compartments. We have asked whether Rac1 has, in addition, a specific, stem-cell-independent function in the regulation of terminal hair follicle differentiation. To address this, we have expressed a constitutively active mutant of Rac1, L61Rac1, only in the basal epidermal layer and outer root sheath of mice possessing an epidermis-specific deletion of endogenous Rac1, which experience severe hair loss. The resulting 'rescue' mice exhibited a hair coat throughout their lives. Therefore, expression of Rac1 activity in the keratin-14-positive compartment of the skin is sufficient for the formation of hair follicles and hair in normal quantities. The quality of hair formed in rescue mice was, however, not normal. Rescue mice showed a grey, dull hair coat, whereas that of wild-type and L61Rac1-transgenic mice was black and shiny. Hair analysis in rescue mice revealed altered structures of the hair shaft and the cuticle and disturbed organization of medulla cells and pigment distribution. Disorganization of medulla cells correlates with the absence of cortical, keratin-filled spikes that normally protrude from the cortex into the medulla. The desmosomal cadherin Dsc2, which normally decorates these protrusions, was found to be reduced or absent in the hair of rescue mice. Our study demonstrates regulatory functions for Rac1 in the formation of hair structure and pigmentation and thereby identifies, for the first time, a role for Rac1 in terminal differentiation.

Key words: Rac1, Hair follicle, Pigmentation, Terminal differentiation

Introduction

Hair is the product of a complex differentiation process of epidermal keratinocytes that occurs in the hair follicle. The innermost part of the hair is the medulla, which consists of one or more rows of cells that are arranged in a ladder-like fashion and divided by air-filled cavities (Chernova, 2003). Medulla cells are nucleated and carry pigment granules in pigmented hair. They are tethered to the cells of the surrounding cortex by keratin-filled, hoop-like structures that protrude from cortex cells, thereby constricting the medulla cells. In electron-microscope images of longitudinal sections of hair shafts, this structure appears as spikes that extend from the cortex into the medulla cell on both sides (Morioka, 2005) (see Fig. 3D). Formation of these cortical protrusions during hair differentiation is probably initiated by medullar keratinocytes as it is preceded by the occurrence of pseudopodia in restricted areas of medulla cells. These pseudopodia are subsequently shed as cytoplasm-containing vesicles, resulting in the formation of a groove in medulla cells that is filled with tonofilament bundles originating from cortex cells (Morioka, 2005). The border between cortex and medulla cells is decorated with the desmosomal cadherin desmocollin 2 (Dsc2 protein) (Johns et al., 2005).

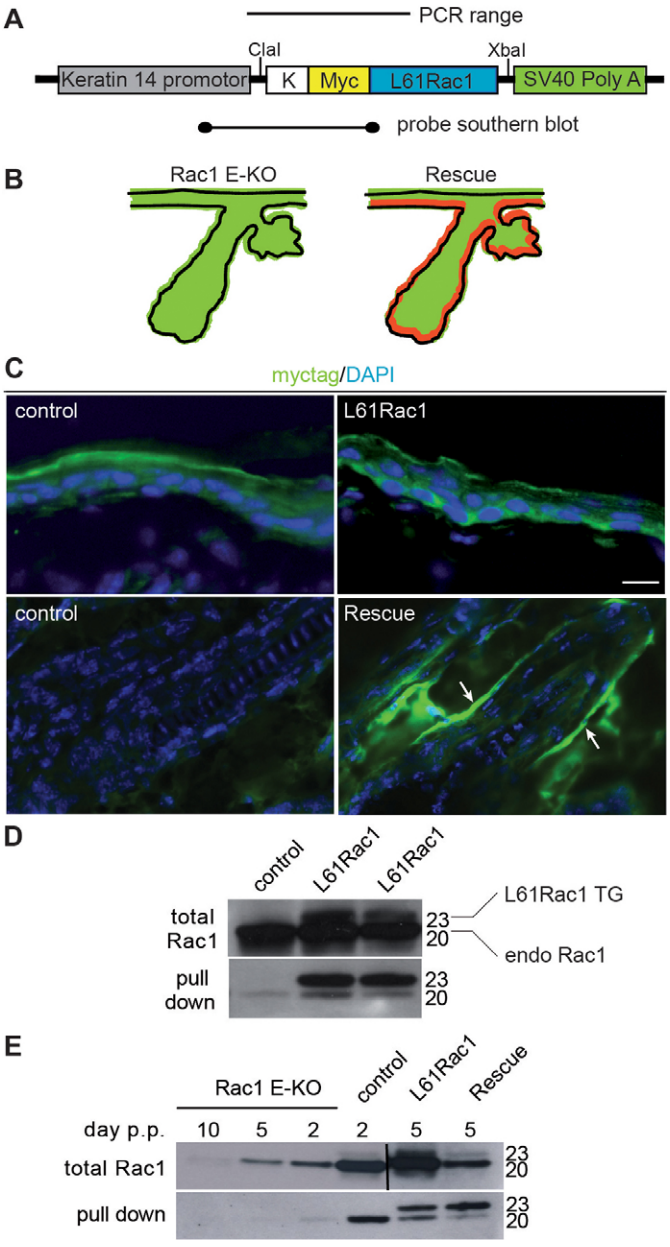
Pigmentation of the hair shaft is the result of complex interactions between hair follicle melanocytes and cortical and medullar keratinocytes (reviewed by Slominski et al., 2005). The transfer of pigment granules from follicular melanocytes to hair matrix keratinocytes is actively regulated by keratinocytes through expression of the transcription factor Foxn1 and secretion of fibroblast growth factor 2 (Fgf2) (Weiner et al., 2007). Hair shaft keratinocytes containing melanosomes are transported towards the skin surface while they differentiate into pigmented cortex and medulla cells, thus giving the hair shaft its characteristic pigmentation pattern.

Functional characterization of the small GTP-binding protein ras-related C3 botulinum toxin substrate (Rac1) identified it as an actin-polymerization-modulating factor that regulates the formation of cell protrusions called lamellipodia (Nobes and Hall, 1995). Gene-inactivation studies in epithelia expressing keratin 5 and keratin 14 have shown that Rac1 is essential for the maintenance of the epidermal and hair follicle stem cell compartments (Benitah et al., 2005; Chrostek et al., 2006; Castilho et al., 2007) and plays an important role in epidermal and mucosal wound healing (Tscharntke et al., 2007; Castilho et al., 2010). In these studies, gene deletion occurred in keratinocytes of the basal

Journal of Cell Science

layer and the outer root sheath (ORS) and all their progeny, whereas dominant inhibition through expression of N17Rac1 was restricted to the basal layer of the interfollicular epidermis (IFE) and the ORS. Therefore, these models do not allow for the investigation of Rac1 functions specifically in the differentiated compartments of the hair follicle.

We have previously generated transgenic mice expressing an activating mutant of Rac1, L61Rac1, under the control of the promoter for the gene encoding keratin 14 (Stachelscheid et al., 2008). By crossing these mice to mice with epidermis-specific deletion of Rac1, we generated a model in which Rac activity is absent only in the differentiating, keratin-14-negative compartments of the epidermis and hair follicles. This gives us the unique opportunity to address, for the first time, functions of Rac in a terminal differentiation process of an epidermal appendage, namely in hair shaft formation.



Results

Reconstitution of Rac1 activity by expression of the K14–L61Rac1 transgene

Within the hair bulb, Rac1 is expressed by all keratinocytes (supplementary material Fig. S1A). To reconstitute Rac1 activity specifically in the keratin-14-expressing compartments of mice with epidermis-specific deletion of Rac1 (Rac1 E-KO mice), we first expressed the constitutively active mutant L61Rac1 (Lamarche et al., 1996) as a transgene under the control of the keratin 14 promoter (Fig. 1A) in wild-type C57/Bl6 mice. The resulting L61Rac1-transgenic mice had no overt phenotype (Fig. 2A, and data not shown). Macroscopically, the hair coat appeared normal and histological examination and immunostaining against keratin 14, keratin 10 and keratin 15 revealed no pathological changes (supplementary material Fig. S1C and Fig. S4D). We then bred L61Rac1-transgenic mice with mice homozygous for a floxed *Rac1* allele (Chrostek et al., 2006) that also expressed Cre recombinase under the control of the keratin 14 promoter (Hafner et al., 2004). The breedings yielded mice with epidermis-specific deletion of endogenous Rac1 in the epidermis and hair follicles. Simultaneously, basal epidermal keratinocytes and ORS cells of hair follicles expressed the L61 transgene (see Fig. 1B,C). These mice with deletion of endogenous Rac1 from the epidermis and hair follicles and simultaneous expression of L61Rac1 in the basal compartment of the epidermis and the ORS are referred to as rescue mice. Both L61Rac1-transgenic mice and rescue mice showed expression and activity of the L61Rac1 construct in lysates of epidermis samples (Fig. 1D,E). Pulldown analysis revealed that the levels of active Rac1 in L61Rac1 and rescue mice were similar to those in control mice at day 2 p.p. Activity and expression of endogenous Rac1 protein in the epidermis of Rac1 E-KO mice were already decreased at day 2 p.p. and further diminished until day 10 p.p., when they became undetectable (Fig. 1E).

Fig. 1. Generation of rescue mice. (**A**) Illustration of the transgenic construct used to express myc-tagged L61Rac1 under the control of a truncated keratin 14 promoter. Sequences corresponding to the Southern blot probe and the amplified PCR fragment are indicated above and below, respectively. K, Kozak sequence; Myc, Myc-tag. (**B**) Effect of breeding of L61Rac1-transgenic mice to mice with epidermis-specific deletion of Rac1 (Rac1 E-KO). In Rac1 E-KO mice, the entire epidermis and its appendages are devoid of Rac1 activity (green). L61Rac1 Rac1 E-KO mice (rescue mice) have Rac1 activity in the keratin-14-expressing compartment (shown in red), whereas all other parts of the epithelium are Rac1 negative. (**C**) Myc-tag staining (green) of interfollicular epidermis (IFE) of L61Rac1-transgenic mice and control mice (upper panel) and hair follicles of rescue mice and control mice (lower panel) at day 10 p.p. Nuclei were counterstained with DAPI and appear blue. Arrows show the outer root sheath (ORS). Scale bar: 10 µm. (**D**) Rac1 pulldown analysis in epidermal lysates from newborn L61Rac1-transgenic mice (L61Rac1) and wild-type controls. The upper panel shows total Rac1 protein; the lower panel shows the pulldown fraction of GTP-loaded Rac1. Transgenic L61Rac migrates at 23 kDa (L61Rac1 TG), endogenous Rac1 (endo Rac1) at 21 kDa. (**E**) Pulldown analysis in epidermal lysates at 2, 5 and 10 days p.p. in Rac1 E-KO and control mice as well as mice with floxed *Rac1* alleles without Cre expression but transgenic for L61Rac1 (L61Rac1) and rescue mice. Note that GTP-loaded endogenous Rac1 is undetectable in Rac1 E-KO mice at day 5 and 10 p.p., whereas transgenic GTP-loaded L61Rac1 is present in rescue mice at day 5 p.p.

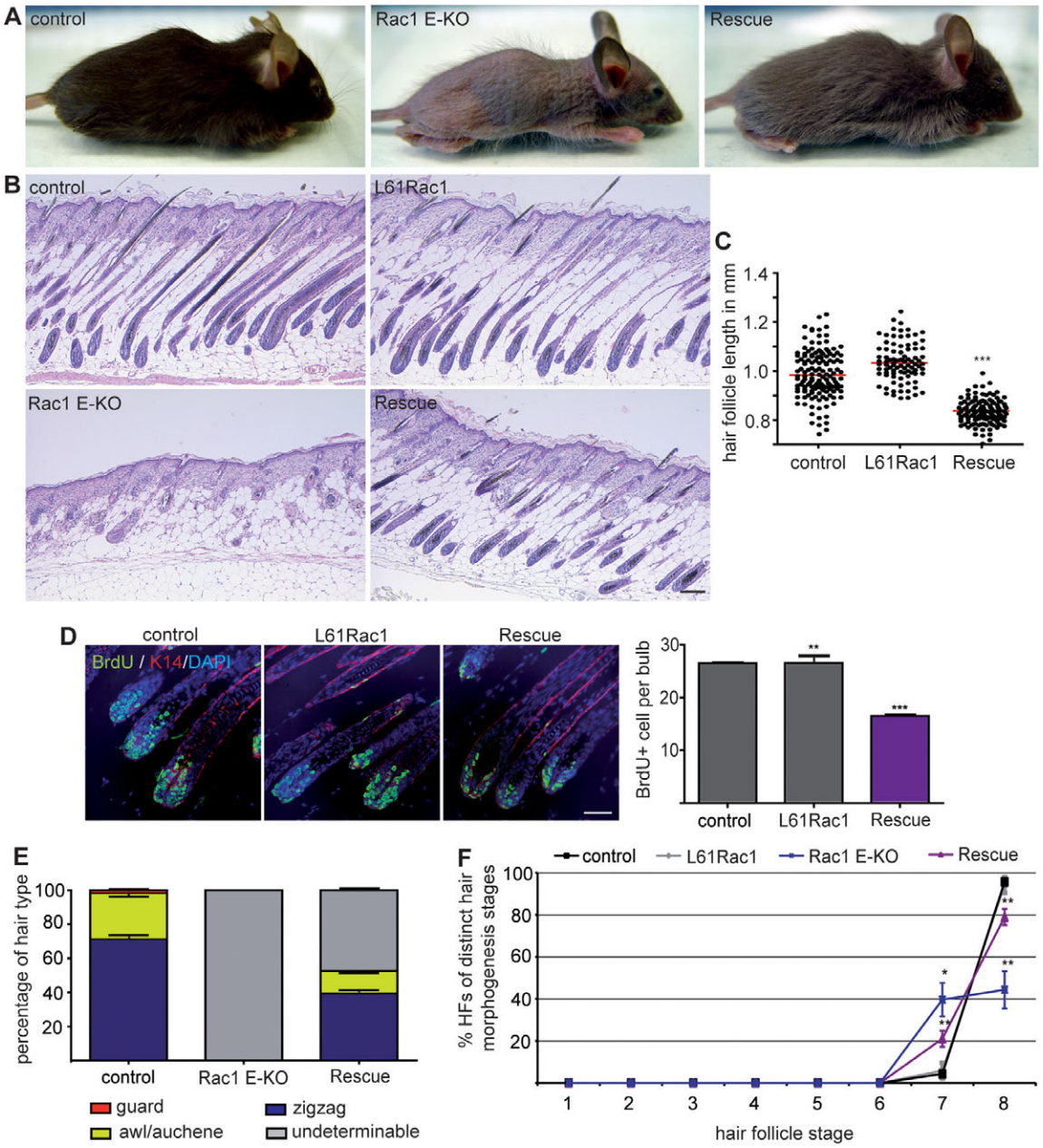


Fig. 2. Characterization of hair growth in rescue mice. (**A**) Appearance of a wild-type mouse (control), Rac1 E-KO mouse and rescue mouse at p.p. day 28. Note the growth of a grey and dull hair coat in the rescue mouse. (**B**) Microscope pictures of hair follicles of control, L61Rac1, Rac1 E-KO and rescue mice at postnatal day 10 in H&E-stained sections. Scale bar: 50 µm. (**C**) Quantification of hair follicle length at day 10 p.p. The length of individual hair follicles, including the calculated mean value (red bar), is shown. ***$P<0.001$ control, L61Rac1 versus rescue (Student's t test). (**D**) Left: Immunostaining of sections of paraffin-embedded skin for incorporated BrdU (green) in hair follicle keratinocytes at day 9 p.p. Red staining shows keratin 14; blue staining shows nuclei. Scale bar: 50 µm. Right: BrdU-positive cells in ten hair bulbs cut in the same plane of three control, L61Rac1 and rescue mice each were counted and plotted as bar graphs showing the mean number of positive cells (±s.e.m.). ***$P<0.001$ control versus rescue mice (Student's t test). (**E**) Hair type analysis of at least 89 hairs of three control, Rac1 E-KO and rescue mice each at postnatal day 28. Bar graphs show the mean±s.e.m. of the fractions of individual hair types expressed as a percentage. Note that rescue mice, in contrast to Rac1 E-KO mice, have zigzag and awl or auchene hairs. (**F**) Classification of hair follicles reflecting their stage of morphogenesis according to Paus et al. (Paus et al., 1999). The graph shows the mean percentage (±s.e.m.) of hair follicles at day 10 p.p. for the different genotypes. *$P<0.05$, **$P<0.01$ control versus Rac1 E-KO, rescue (Mann–Whitney U test).

Hair growth in rescue mice

Rac1 E-KO mice suffered from progressive loss of hair follicles and hair until day 28 p.p., when they were bald, except for a population of thin, long, grey hair scattered over the body surface. N17 rescue mice, which we generated as a control by expressing a dominant-negative mutant of Rac1 under the control of the keratin 14 promoter in the Rac1 E-KO background, had a phenotype that was identical to that of Rac1 E-KO mice (supplementary material Fig. S4A–C). Rescue mice, by contrast, showed abundant hair growth and retained a hair coat

throughout life. Quantitative analysis of hair follicle density in histological sections at day 10 p.p. revealed normal hair densities in L61Rac1 mice and rescue mice, whereas hair follicle density was reduced in Rac1 E-KO mice (supplementary material Fig. S1B). When we stained histological sections of back skin with antibodies against keratin 15 and CD34, markers for hair follicle stem cells, skin of Rac1 E-KO mice showed reduced staining. By contrast, rescue mice exhibited a keratin-15-positive population of epithelial cells in the bulge region, similar to that of L61Rac1-transgenic mice and wild-type mice (supplementary material Fig. S4D). This indicates that Rac1 activity in the basal epidermal layer and the ORS is sufficient to maintain the hair follicle stem cell pool. To further investigate the role of Rac1 in the maintenance of epidermal stem cells, we performed colony forming efficiency (CFE) assays in which the formation of large colonies in clonogenic cultures is a readout for a high proliferative potential of individual cells within a keratinocyte population (Barrandon and Green, 1987; Morris and Potten, 1994; Jensen et al., 2010). While primary epidermal keratinocytes isolated from wild-type and L61Rac1-transgenic mice showed similar CFEs, the fractions of large- and medium-sized colonies were dramatically reduced in Rac1 E-KO mice. In keratinocytes isolated from rescue mice, the percentage of large- and medium-sized colonies was higher than in Rac1 E-KO mice but lower than in wild-type or L61Rac1-transgenic mice (supplementary material Fig. S4E). Thus, expression of L61Rac1 in keratinocytes deficient in endogenous Rac1 partially rescued their proliferative potential.

Although rescue mice had pelage hair, there was an obvious macroscopic difference between their hair coats and those of L61Rac1-transgenic or wild-type mice as observed in a mixed background and in the C57/Bl6 background. In the C57/Bl6 strain, L61Rac1-transgenic mice and wild-type mice exhibited a black and shiny coat, whereas the coat of rescue mice was matt and scruffy. In addition, the coat colour was grey instead of black (Fig. 2A). In histological sections, the hair of rescue mice appeared shorter than that of L61Rac1-transgenic mice or wild-type controls (Fig. 2B). We therefore measured the length of at least 89 back skin hair follicles in five rescue mice as well as six L61Rac1-transgenic mice and nine wild-type controls each. This analysis revealed a significant reduction in average hair follicle length in rescue mice (Fig. 2C). We hypothesized that the reduced length of rescue hair follicles might be caused by decreased proliferation of hair matrix keratinocytes and therefore stained skin sections of nine-day-old mice that had been injected with BrdU 2 hours before killing with an antibody against BrdU. In rescue mice, significantly fewer hair matrix keratinocytes incorporated BrdU as compared with L61Rac1-transgenic mice or wild-type controls (Fig. 2D).

We conclude that expression of Rac1 activity in the keratin-14-positive compartments of hair follicles deficient for endogenous Rac1 is, on the one hand, sufficient to maintain the hair follicle stem cell pool. On the other, rescue of Rac1 activity in the keratin-14-expressing compartment only is not sufficient for the formation of normal hair. The reduced length of hair follicles in rescue mice is caused by decreased proliferation of Rac1-deficient hair matrix keratinocytes.

Delayed hair follicle morphogenesis in rescue mice

Deletion of Rac1 from the epidermis and hair follicles of mice has been reported to lead to severe disturbances in hair follicle morphogenesis (Chrostek et al., 2006). The normal hair coat of mice has four different types of hair: guard, zigzag, awl and auchene, with zigzag hairs being the most frequent hair type. When we analyzed the sparse hair of three Rac1 E-KO mice we were not able to classify their hair according to these four described hair types (Sundberg, 1994; Duverger and Morasso, 2009). These changes in hair type morphology were only partially normalized in three rescue mice at day 28 p.p., which had fewer zigzag and awl or auchene hairs, whereas guard hairs were not identifiable. To investigate whether this abnormal hair phenotype in rescue mice was the consequence of aberrantly regulated hair morphogenesis, we classified hair follicles of 21 mice at day 10 p.p. according to their stage of morphogenesis. This detailed analysis showed a dramatic reduction of the numbers of hair follicles in morphogenesis stages 7 and 8 in Rac1 E-KO mice. While expression of L61Rac1 in a wild-type background had no influence on hair morphogenesis, rescue mice showed a higher proportion of follicles at stages 7 and 8, although the number of hair follicles did not reach the values that were present in control mice. We conclude that Rac1 activity is essential both in the ORS as well as in the differentiated compartments to regulate hair follicle morphogenesis.

We therefore investigated the expression of components of several signaling pathways known to be involved in hair follicle morphogenesis and differentiation. The transcriptional repressor CCAAT displacement protein (CDP) is required for the formation of a normal inner root sheath (IRS) (Ellis et al., 2001). The transcription factor Foxn1 is essential for hair differentiation and hair shaft formation as well as hair pigmentation (Mecklenburg, 2001; Mecklenburg et al., 2005; Weiner et al., 2007). The wnt signaling pathway involving β-catenin and Lef1 is another important pathway regulating hair formation (Niemann and Watt, 2002). However, staining with antibodies against these signaling proteins did not reveal obvious differences in their staining patterns between rescue mice and L61Rac1-transgenic mice or wild-type controls (supplementary material Fig. S2A), suggesting that the observed phenotype is not due to altered function of these pathways.

Abnormal hair structure and medulla cell organization in rescue mice

Macroscopically, the hair of rescue mice appeared grey and dull. We therefore analyzed the ultrastructure of hair in rescue and control mice by using SEM. The hair shaft of L61Rac1-transgenic mice and wild-type controls was round or oval, whereas the hair of rescue mice showed frequently deep indentations. Moreover, the surface of the individual cuticle scales was smooth in the controls but showed longitudinal grooves in the hair of rescue mice (Fig. 3A). These changes reflect aberrant structure formation of hairs in rescue mice and could explain the dull appearance of their coat.

The hair of normal C57/Bl6 mice shows a characteristic pattern of pigment bands. This banding pattern evolves through the restricted distribution of pigment granules to the apical poles of hair medulla cells. The medulla cells therefore appear dark, whereas the wide and air-filled intercellular spaces appear bright (Chernova, 2003). When analyzing native hair and also semi-thin sections of hair shafts, we noticed that the pigment granule banding pattern was completely disrupted in the hair of rescue mice, whereas L61Rac1-transgenic mice and wild-type mice showed a normal banding pattern (Fig. 3B,C). In rescue mice, the

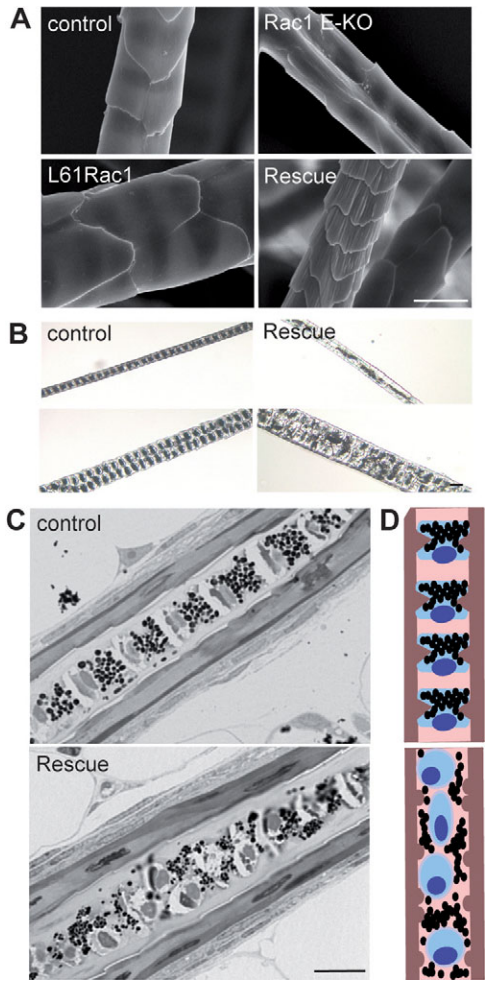


Fig. 3. Disturbed hair surface structure and hair shaft pigmentation in rescue mice. (**A**) Scanning electron microscopy pictures of hair of wild-type (control) and mutant mice at day 17 p.p. Note indentations and longitudinal grooves in Rac1 E-KO and rescue hair. (**B**,**C**) Microscope pictures of zigzag (B, upper panel and C) and awl or auchene (B, lower panel) hairs. Note disruption of the normal pigmentation pattern. (B) Pictures taken from plucked native hair at day 17 p.p. (C) Pictures taken from semi-thin sections at postnatal day 10. Scale bars: 10 μm. (**D**) Cartoon illustrating the structural and pigmentation changes seen in hair of rescue mice (lower) versus controls (upper).

distribution of pigment granules was obviously not restricted to medulla cells. Indeed, analysis of semi-thin sections and TEM revealed pigment granules in extracellular spaces of the medulla and also of the hair bulb. Furthermore, the medulla cells appeared less well organized in rescue mice in comparison with those of controls (Fig. 3C,D; Fig, 5A,B; and data not shown).

Medulla cells are tethered to the surrounding cells of the hair cortex by cell–cell contacts. Although the nature of these contacts has not been thoroughly investigated, it is possible that they have characteristics of desmosomes as desmosomal cadherins are expressed by cells of the hair cortex and medulla. Medulla cells express desmocollin 2 (Dsc2), whereas cortex cells express desmoglein 4 (Dsg4) (Kurzen et al., 2001; Bazzi et al., 2006; Bazzi et al., 2009). We therefore stained skin sections with an antibody against desmocollin 2 (Dsc2), a desmosomal cadherin that is expressed by medulla cells and is localized to the so-called A-ridges (Trigg, 1972; Johns et al., 2005), and with the antibody AE-13 that stains keratins of the hair cortex. As has been described previously (Lynch et al., 1986), staining of normal hair with AE-13 detected small hair-keratin-containing spikes of cortex cells that protrude into the medulla space. This was different in rescue mice, where cortical AE-13-positive spikes were mostly lacking (Fig. 4A). The absence of cortical spike-like structures was also apparent when we performed TEM analysis of hair shafts (Fig. 4B). In addition, in L61Rac1-transgenic mice and wild-type controls, Dsc2 localized to these spikes and formed a regular, zipper-like pattern, whereas in rescue mice Dsc2 did not show the characteristic zipper-like pattern but seemed to be reduced or even lacking completely in most areas (Fig. 4A). As Rac1 is known as a principal regulator of actin polymerization, we used TRITC-labeled phalloidin to stain for structures containing F-actin in the hair shaft. We found that the cortical spikes protruding into the hair medulla that are decorated by the Dsc2 staining in normal hair were strongly positive when stained with TRITC–phalloidin. This actin staining of cortical spikes was strongly reduced in the hair shafts of rescue mice (Fig. 4C).

As Dsc2 expression was altered in the hair follicles of rescue mice, we asked whether the absence of Rac1 activity in differentiated parts of the epithelium and hair follicles had a general effect on the expression of cell–cell adhesion molecules. We therefore stained skin of wild-type mice, L61Rac1-transgenic mice and rescue mice with antibodies against desmoglein 1 and 2, desmoglein 4, plakoglobin and β-catenin. These stainings did not show changes in the expression of these proteins in hair follicles and IFE of L61Rac1-transgenic and rescue mice in comparison with that of the controls (supplementary material Fig. S3), indicating a specific role for Rac1 in the establishment of cell–cell contacts in cortical spikes.

Disturbance of pigment granule size and deposition in rescue mice

When analyzing the pigmentation of hair of rescue mice in H&E-stained histological sections, we noticed that the pigment present in the hair bulb appeared more compact and sometimes clumpy in comparison with that of L61Rac1-transgenic controls. Microscopic investigation of semi-thin sections revealed that pigment granules were present inside the hair matrix and medulla cells in control mice. By contrast, hair bulbs of rescue mice showed numerous pigment granules in extracellular spaces between hair matrix and medulla cells (Fig. 5A). In addition, pigment granules were present in the dermal papilla of rescue but not control mice (Fig. 5A, middle panel). Furthermore, morphometric analysis of pigment granule size using EM images revealed that pigment granules in rescue mice were, on average, smaller than those in control mice (Fig. 5B).

Phagocytosis of pigment granules by epidermal keratinocytes has been proposed to be an important mechanism of pigment transfer from melanocytes to keratinocytes (Cardinali et al., 2005). To analyze the phagocytic capacity of keratinocytes with inhibited Rac1 activity, we determined the uptake of fluorescently labeled polystyrene microparticles (fluorospheres) into primary keratinocytes, as described previously (Cardinali et al., 2005). As Rac1-deficient keratinocytes do not grow in culture, three different populations each of primary keratinocytes isolated from wild-type mice and from mice expressing the dominant-negative mutant N17Rac1 under the control of the keratin 14 promoter (Tscharntke et al., 2007) were incubated with

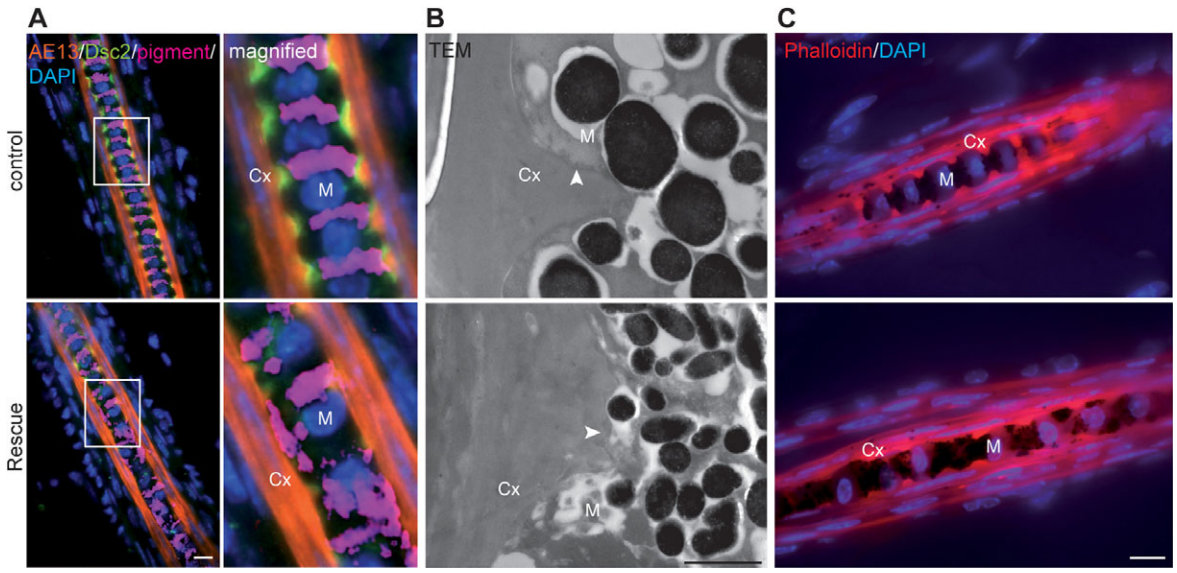


Fig. 4. Altered Dsc2 expression and impaired formation of cortical protrusions in the hair shaft of rescue mice. (**A**) Skin sections from control and rescue mice taken at postnatal day 10 were immunostained for the desmosomal cadherin desmocollin 2 (Dsc2) (green), hair cortex keratin with clone AE13 (red) and DAPI (blue). Hair shaft pigment was visualized by light microscopy and subsequently inverted and pseudo-coloured (pink). Pictures on the right show a digital magnification corresponding to the areas marked on the left. Note the absence of the regular Dsc2 expression pattern in rescue mice. (**B**) Transmission electron microscopy (TEM) images of sections of hair shaft in skin samples taken from day 10 p.p. control and rescue mice. White arrowheads indicate protrusions of cortex cells into the medulla. (**C**) Polymerized actin in the hair shaft was visualized by phalloidin staining (red) in sections from day 10 p.p. control and rescue mice. DAPI (blue) stains nuclei. Cx, cortex; M, medulla. Scale bars: 10 μm.

fluorospheres for 1, 2, 4 and 6 hours. We did not observe significant differences in the numbers of ingested fluorospheres between the different keratinocyte populations (data not shown). We then asked whether the abnormal pigment distribution in rescue mice was the result of disturbed recruitment of melanocytes and melanocyte precursors into the hair bulb. We therefore stained sections of paraffin-embedded skin of rescue and control mice with antibodies against 5,6-dihydroxyindole-2-carboxylic acid oxidase (tyrosinase-related protein 1, Tyrp1), which is expressed by mature melanocytes (Virador et al., 2001) and against L-dopachrome tautomerase (tyrosinase-related protein 2, Trp2), which is expressed in early melanoblasts (Steel et al., 1992). These stainings did not show any differences between rescue and control mice (Fig. 5C). Furthermore, analysis of tyrosinase activity using a tyramide-based tyrosinase assay (Han et al., 2002) suggested that the activity of melanogenesis was comparable in rescue and control mice (supplementary material Fig. S2B).

We conclude that the aberrant deposition of melanin granules in extracellular spaces of the hair matrix and medulla as well as in the dermal papilla of rescue mice is probably not caused by a decrease in the phagocytic capability of hair matrix keratinocytes and is also not caused by a recruitment deficiency of melanocytic cells to the hair bulb.

Discussion

We present here a new mouse model for the investigation of Rac1 functions in different compartments of the epidermis and hair follicles. Expression of the transgene encoding L61Rac1 under the control of the K14 promoter confines Rac1 activity to the basal, keratin-14-expressing portions of the epithelium. All differentiated parts are devoid of Rac1, which enabled us to study its functions in these compartments. For reconstitution of Rac1 activity, we used a K14–L61Rac1 transgene coding for a constitutively active mutant of Rac1. The magnitude of Rac1 activity achieved by expression of this mutant is comparable to that in the epidermis of control mice. L61Rac1-transgenic mice, which were always used as controls in the analysis of rescue mice, do not exhibit an abnormal phenotype, indicating that expression of the L61Rac1 transgene alone does not disturb epidermal homeostasis and hair formation. The fact that N17 rescue mice show a phenotype that is identical to the phenotype of Rac1 E-KO mice demonstrates that the growth of hair in rescue mice is a consequence of reconstituted Rac1 activity.

Although our analyses of rescue mice suggest a similar Rac1 activity in their epidermis as compared to that of wild-type mice, the functional equality of Rac1 in the basal layer of the IFE and the ORS of rescue epidermis and wild-type epidermis remains a matter of debate. One could therefore argue that it is altered Rac1 activity in the basal compartment, but not in the differentiated parts of the epidermis and hair follicles, that results in changes in the differentiation program that could be responsible for the observed hair phenotype. In our view, this is, however, very unlikely for two reasons. First, hair differentiation in L61Rac1-transgenic mice is normal, although the L61Rac1 transgene is expressed in the basal IFE and ORS. Second, all major differentiated compartments of the hair follicle are formed in rescue mice, and it is only very late during the differentiation process that specific functions of the communication between medulla and cortex cells are inhibited. It would be difficult to envisage how this specific change could be caused by altered Rac1 activity in the basal IFE or ORS. Rac1 has been shown to exert non-redundant functions in the formation and maintenance of hair follicles (Chrostek et al., 2006; Castilho et al., 2007) and the IFE (Benitah et al., 2005). It has been suggested that Rac1 essentially contributes to the maintenance of the keratin-14-positive stem cell pools for these skin compartments. Our results

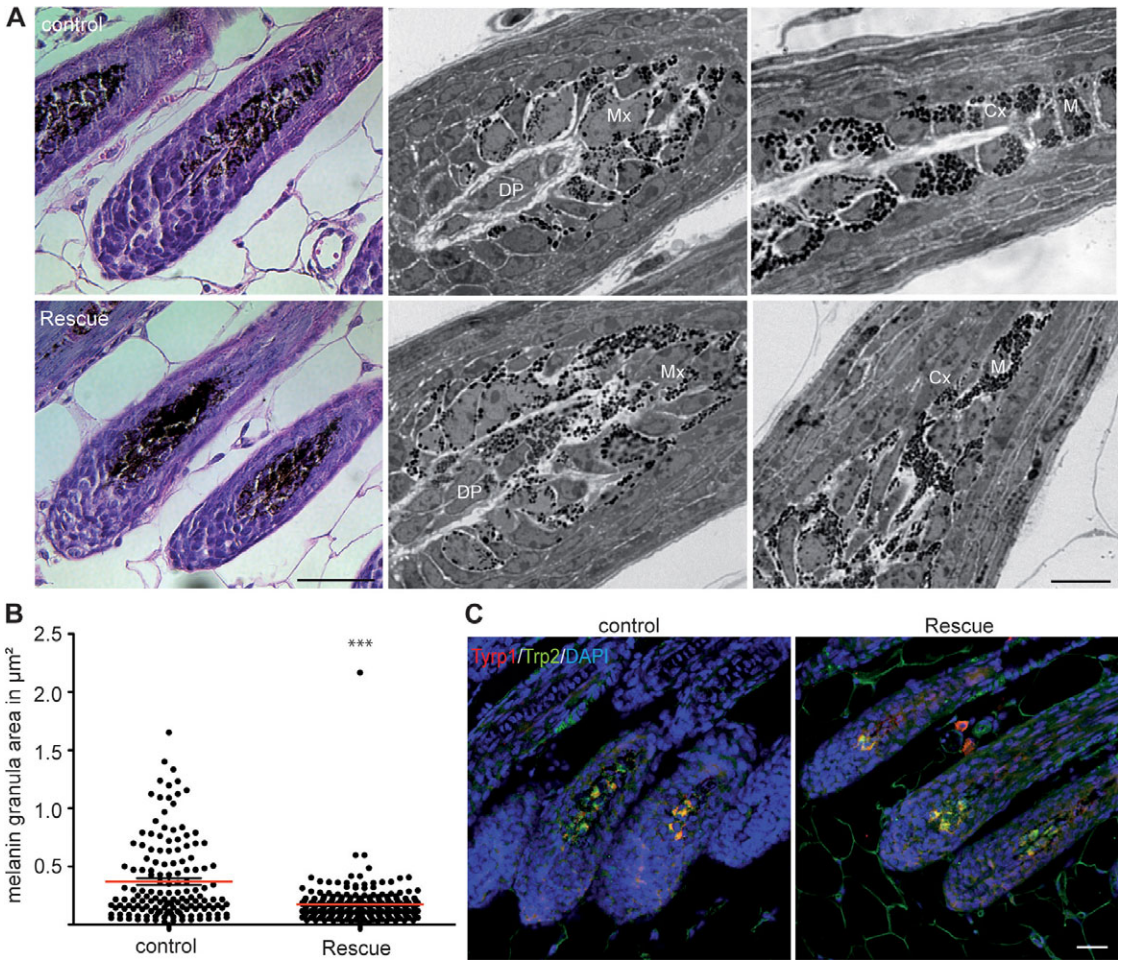


Fig. 5. Disturbed pigment distribution and pigment granule size in hair bulbs of rescue mice. (**A**) Light microscopy pictures of H&E-stained sections of paraffin-embedded skin (left) and of Methylene-Blue-stained semi-thin sections (right) of control and rescue mice. Scale bars: 50 µm (left), 10 µm (right). Note the clumpy appearance of pigment granules as well as deposition of pigment granules in extracellular spaces and in the dermal papilla. (**B**) Dot plot showing melanin granule size measured in TEM images of two control (n=147) and three rescue (n=239) mice. Red lines indicate the respective mean. ***P<0.001 control versus rescue (Student's t test). (**C**) Fluorescence microscopy pictures of sections of paraffin-embedded skin of control and rescue mice at day 10 p.p. stained with antibodies against Tyrp1 for mature melanocytes (red) and Trp2 for melanocyte precursors (green). Scale bar: 100 µm.

show that Rac1 activity in the keratin-14-expressing layers is not only required but also sufficient for the formation and maintenance of hair follicles. Indeed, the fact that the rescue of hair follicle formation correlates with increased keratin 15 and CD34 expression in the bulge region in vivo and with a partial reconstitution of the proliferative potential of keratinocytes in vitro would be well in accordance with a role for Rac1 in the maintenance of hair follicle stem cells. The formation of hair follicles with normal length, as well as of fully differentiated and normally pigmented hair, however, needs additional activity of Rac1 in keratin-14-negative compartments of the stratified epithelium of the skin. Although it is most likely that this Rac1 activity is required in differentiating keratinocytes of the hair bulb – that is, in the hair matrix, precortex and cortex – we cannot completely exclude a contribution from the keratinocytes of the suprabasal IFE. The deficiency of Rac1 in the differentiated compartments of the hair follicle leads to a delay in hair morphogenesis. In particular, late stages of hair morphogenesis are affected, in which hair elongation as well as hair shaft differentiation and pigmentation are most prominent (Paus et al., 1999). This shows that Rac1 activity in the K14-positive layers of the epidermis and hair follicle is not sufficient for normal hair morphogenesis. How could deficiency of Rac1 from the differentiated portions of the hair follicle lead to the observed delay in hair follicle morphogenesis? Obviously, Rac1 is required for the proliferation of hair matrix keratinocytes during hair follicle elongation. In agreement with that, incorporation of BrdU was reduced in hair bulbs of rescue mice, indicating less proliferative activity. Rac1 could influence proliferation of hair matrix keratinocytes in different ways: either by directly activating signaling pathways that regulate cell cycle progression (Sharov et al., 2006) or by regulating the activation and/ or migration of hair follicle stem cells that give rise to proliferating hair matrix keratinocytes (Cotsarelis et al., 1990; Sun et al., 1991; Wilson et al., 1994; Tumbar et al., 2004). Alternatively, it is conceivable that the migration of hair follicle keratinocytes is inhibited in the absence of Rac1 (Langbein et al., 2003) as Rac1 is well known to be required for migration in various cell types, including epidermal keratinocytes (Tscharntke et al., 2007).

Apart from determining proliferation and migration of keratinocytes within the hair follicle, Rac1 is essential for

structure formation of the hair shaft, as shown, for the first time, by our results. In the absence of Rac1 from all differentiating hair follicle compartments, the hair has an abnormal composition, with obvious disturbances of the surface and medulla structure. How could Rac1 regulate structure formation of the hair shaft? It is known that cells of the medulla are tethered to the cortex by cell–cell contacts that contain Dsc2 (Johns et al., 2005; Cai et al., 2009). Considering the role of Rac1 in cell–cell contact formation in vitro (Braga et al., 1997; Takaishi et al., 1997), it is possible that the absence of Rac1 prevents the formation of these cortex–medulla contacts. This could result in the observed chaotic arrangement of medulla cells.

Contacts between cortex and medulla cells form during hair shaft development and are initiated by the formation of pseudopodia in certain areas of medulla cells that are subsequently shed as cytoplasm-containing vesicles (Morioka, 2005). As Rac1 has a known function in the formation of lamellipodia in various cell types, including keratinocytes (Nobes and Hall, 1995; Ridley, 2006; Tscharntke et al., 2007), it is well conceivable that Rac1 is also required for the formation of these pseudopodia in medulla cells. The failure to form these membrane structures could result in the disturbance of subsequent steps of cell–cell contact formation between cortex and medulla. It remains open here whether the failure to form stable cortex–medulla contacts in the hair shafts of rescue mice is the cause or the consequence of altered Dsc2 distribution. Considering the normal distribution of other desmosomal components in rescue epidermis, it seems more likely that the absence of Dsc2 from cortical spikes is an indirect consequence of disruption of cortex–medulla cell contacts.

Although the arrangement of medulla cells in the hair shafts of rescue mice shows severe disturbances, this is unlikely to be the sole cause for the changed appearance of their fur coat. Other mutants with disturbed medulla structure following manipulation of signaling pathways that influence hair shaft differentiation show an abnormally shiny coat (Hong et al., 2001; Petiot et al., 2003; Schlake, 2005); this is in contrast to rescue mice in which we observed a dull appearance of the fur. It is therefore likely that the altered surface structure that we additionally observed in rescue hair contributes to the appearance of the coat. Similarly, changes in the coat appearance correlating with abnormalities of the hair shaft surface have been described in mice with forced activation of Notch1 signaling in the hair cortex (Lin et al., 2000; Vauclair et al., 2005). The abnormal indentations and longitudinal grooves seen on the surface of rescue hair suggest a function for Rac1 in the differentiation of the hair cortex and cuticle. We found that the absence of Rac1 does not influence expression of CDP, Foxn1, Lef1 and β-catenin, which are known regulators of hair differentiation. Other signaling proteins such as Notch1, Sonic hedgehog, Dkk1, Krox20, IGFBP5, FGFR2-IIIb, Hoxc13 and Foxq1 could be involved (Hong et al., 2001; Schlake, 2005; Vauclair et al., 2005; Weger and Schlake, 2005; Schlake, 2006; Hammerschmidt and Schlake, 2007).

The most prominent feature of rescue mice was their grey coat colour in a C57/Bl6 background, which normally shows black hair. We have considered several mechanisms through which loss of Rac1 from differentiated portions of the hair follicle could lead to pigmentation disturbances. Immunostaining for melanocytes and melanocyte precursors as well as analysis of tyrosinase activity in situ excluded the possibility that recruitment of melanocytic cells or their melanogenic activity were impaired. Obviously, the changes observed in pigment granule distribution and size are either due to disturbed transfer of melanosomes from melanocytes to hair matrix keratinocytes or due to an incapability of keratinocytes to localize and maintain pigment granules within the cytoplasm. Although the transfer of melanosomes from melanocytes to keratinocytes is, in general, poorly understood, recent research has shown that it requires instructions from dedicated hair follicle keratinocytes that depend on the transcription factor Foxn1. While this is true for hair matrix keratinocytes that give rise to the hair cortex, pigment acquisition by medulla cell precursors is independent of Foxn1 (Weiner et al., 2007). Thus, there are two reasons why Foxn1 is unlikely to be involved in the pigmentation disturbances observed in rescue mice: first, immunostaining of hair follicles did not show changes in the expression of Foxn1 and, second, pigment distribution was particularly disturbed in medulla cells that do not express Foxn1.

Apart from Foxn1, Rac1 itself has been hypothesized to be of potential importance for melanosome transfer to keratinocytes by regulating their phagocytosis. In our experiments using primary cultured keratinocytes, we did not find a dependence of fluorosphere uptake upon Rac1 activity. This suggests that Rac1 might not be directly involved in this process; it is, however, important to stress that (i) in the cells we used, Rac1 activity was only inhibited but not abolished (Tscharntke et al., 2007) and (ii) primary cultured keratinocytes probably do not represent hair follicle keratinocytes in all of their functions. In summary, we here demonstrate, for the first time, a function for Rac1 in the differentiated compartments of the hair follicle that is independent of its known role in the regulation of hair follicle stem cells. Our study shows that Rac1, in addition to maintaining the hair follicle stem cell pool, regulates the formation of hair, the terminally differentiated end-product of the hair follicle. To our knowledge, this is the first study to show such a role for Rac1 in the regulation of a terminal differentiation process.

Materials and Methods

Generation of rescue mice

Mice with conditionally targeted *Rac1* alleles have been described previously (Chrostek et al., 2006). To generate epidermis-specific Rac1 knockout mice (‘Rac1 E-KO’), these mice were crossed from a mixed background with mice expressing Cre recombinase under the control of the keratin 14 promoter in a C57/Bl6 background (Hafner et al., 2004). Mice with reconstituted Rac1 activity in the basal epidermal layer and the ORS (referred to as ‘rescue mice’) were created by crossing the resulting Rac1 E-KO mice to mice transgenic for a constitutively active mutant of Rac1, L61Rac1, under the control of the K14 promoter in a C57/Bl6 background. N17 rescue mice, which expressed the N17Rac1 transgene (Tscharntke et al., 2007) instead of the L61Rac1 transgene, were generated in an analogous way. Mice were then further crossed into the C57/Bl6 background for several generations. L61Rac1-transgenic mice were generated as described for N17Rac1 transgenic mice previously (Tscharntke et al., 2007). The Rac1 c-DNA with the point mutation Q→L in codon 61 and containing a C-terminal myc tag (Lamarche et al., 1996) was inserted via Sac1–Xba1 sites into a keratin 14 expression cassette harbouring an SV40 intron and an SV40 poly-A signal sequence at the N-terminus (Hafner et al., 2004). Correct insertion was confirmed by direct DNA sequencing. After digestion of the plasmid with BssH2, the transgene was separated by agarose gel electrophoresis and isolated from the gel using the MiniElute gel extraction kit (Qiagen, Hilden, Germany). Purification was performed using an Elutip mini column (Schleicher and Schüll, Dassel, Germany) and subsequent precipitation with ethanol. For pronucleus injection, the DNA was dissolved in microinjection buffer and adjusted to a final concentration of 10 ng/µl. Transgenic mice were generated by injection of the DNA construct into the pronucleus of fertilized oocytes. For screening of transgene insertion, genomic DNA was isolated from mouse tails and analyzed by means of polymerase chain reaction (PCR) using the following primers – SF3-25: 5′-TTGGTTGTGTAA-CTGATCAGTAGGC-3′ and SF5-23: 5′-TGGAGAGCTAGCAGGAAACTAGG-3′. Insertion was confirmed by Southern blot analysis using a 600 bp fragment as probe. Copy numbers of the inserted transgene were estimated using the interleukin 1 (IL1) receptor gene as a single-copy gene for comparison (Carroll

et al., 1995). The presence of the L61 mutation in the transgenic Rac1 cDNA was verified by sequencing of PCR fragments obtained from the screening of transgenic mice. Founder mice were backcrossed into a wild-type C57/Bl6 background for five generations.

Rac1 pulldown assay

Rac1 activity was assayed with a biotinylated peptide corresponding to the CRIB motive of the Rac1 effector Pak, as described previously (Price et al., 2003) and adequately modified for analysis in epidermis samples. Briefly, epidermis was separated from the dermis with forceps after incubation with 0.5 M ammonium thiocyanate (NH_4SCN) in phosphate buffer, pH 6.8 (0.1 M Na_2HPO_4, 0.1 M KH_2PO_4) for 40 minutes on ice. Epidermal sheets were homogenized for 2 minutes in a Mixermill MM 400 tissue homogenizer (Retsch, Haan, Germany) in 350 µl lysis buffer (Tris–HCl pH 7.4, 100 mM NaCl, 10 mM $MgCl_2$) containing 5 µl of the CRIB peptide (2 mg/ml), PMSF and a protease inhibitor mix. After adding 350 µl lysis buffer with 2% NP-40 and rotation mixing of the lysates for 45 minutes at 4℃, samples were centrifuged at 16,000 ***g*** for 15 minutes at 4℃. An aliquot of total lysate was taken after which supernatants were rotated for an additional 45 minutes at 4℃. Rac1–GTP complexes were precipitated by incubating lysates with streptavidin–agarose (Novagen, Merck, Darmstadt, Germany) for 30 minutes at 4℃. Beads were washed three times with lysis buffer and then transferred to SDS sample buffer. Bead supernatants and equal amounts of total lysate were separated by SDS PAGE. Detection of Rac1 was performed by standard western blotting protocols described previously (Tscharntke et al., 2007) using an antibody against Rac1 (clone 23A8; Sigma-Aldrich, Taufkirchen, Germany).

BrdU labeling

For visualization of S-phase keratinocytes in skin sections, mice were injected with 1.6 mg bromodeoxyuridine (BrdU) intraperitoneally 2 hours prior to killing. Detection of BrdU was performed on paraffin sections, which were de-paraffinized in xylene, rehydrated in graded alcohols and washed in PBS. Target retrieval was performed by boiling sections in citrate buffer for 20 minutes. After washing for 5 minutes in PBS, sections were blocked with 10% goat serum for 1 hour at room temperature and incubated with an anti-BrdU antibody (clone B44, BD Biosciences, Heidelberg, Germany) at 1:10 dilution in antibody diluent solution (Dako, Hamburg, Germany) overnight at 4℃. The following day, stainings with an anti-keratin 14 antibody (Covance, Princeton, NJ) at 1:2000 dilution in antibody diluent solution (Dako) and with secondary antibodies coupled to Alexa Fluor 488 and Alexa Fluor 555 (Molecular Probes, Eugene, OR) were performed.

Histological analysis and immunostaining

Tissue samples were fixed in 4% paraformaldehyde overnight or embedded in OCT compound and frozen immediately. Further processing, paraffin embedding of fixed tissue and hematoxylin-and-eosin (H&E) staining were performed according to standard histological procedures. Immunostainings of keratin 14 and keratin 10 were performed as described previously (Stratis et al., 2006). Antibodies were purchased from Covance, Princeton, NJ. For immunostaining of β-catenin, CDP, Dsg1 and Dsg2, Dsg4, Foxn1, Lef1, plakoglobin, keratin 15 and CD34, the following antibodies and procedures were applied on paraffin sections according to standard protocols: β-catenin (clone 14, BD Biosciences, Heidelberg, Germany), 1:250 in 1% milk, 1% BSA, 10% goat serum, target retrieval with EDTA, pH 9 (Dako); CDP (kind gift from M. Busslinger, Vienna), 1:100 in 10% goat serum, citrate, pH 6 (Dako); Dsg1 and Dsg2 (clone DG 3.10, Progen, Heidelberg, Germany), 1:50 in 1% milk, 1% BSA, 10% goat serum, EDTA; Dsg4 (clone 18G8, kind gift from A.M. Christiano, Department of Dermatology, Columbia University, New York, NY), 1:10 in 1% milk, 1% BSA, 10% goat serum, EDTA; Foxn1 (G-20, Santa Cruz Biotechnology, Santa Cruz, CA), 1:100 in antibody diluent solution (Dako), 1% trypsin–PBS; Lef1 (clone C12A5, Cell Signaling Technology, Danvers, MA), 1:100 in 10% goat serum, citrate; plakoglobin (clone PG 5.1, Progen Biotechnik, Heidelberg, Germany), 1:10 in 1% milk, 1% BSA, 10% goat serum, EDTA, keratin 15 (Covance, Princeton, NJ), 1:7000 in 10% goat serum, 0.1% trypsin–PBS; CD34 (EBiosciences San Diego, CA, clone RAM34), 1:50. Dsc2 and AE13 double-staining on paraffin sections was performed after EDTA target retrieval with 1:50 Dsc2 (K-20, Santa Cruz Biotechnology) and 1:50 pan-cytokeratin AE13 antibodies (Santa Cruz Biotechnology). Antibodies were diluted in 1% milk, 1% BSA and 10% goat serum. Double staining for Tyrp1 and Trp2 on paraffin sections was performed after EDTA target retrieval with 1:2000 Tyrp1 (kind gift from V.J. Hearing, Laboratory of Cell Biology, NCI, NIH, Bethesda, MD) and 1:100 Trp2 antibodies (D-18, Santa Cruz Biotechnology). Antibodies were diluted in antibody diluent solution (Dako). For staining of the Myc-tag on frozen sections, a monoclonal antibody against Myc (clone 9B11, Cell Signaling Technology) was applied at 1:250 dilution in 3% BSA–TBS on 4% paraformaldehyde-fixed and 0.3% Triton X-100-permeabilized sections. Polymerized actin was stained using TRITC-labeled phalloidin (Sigma-Aldrich, Taufkirchen, Germany) at a dilution of 1:200. For staining of human and murine hair follicles on sections of paraffin-embedded skin, the antibody clone 23A8 (Sigma-Aldrich, cat. # R2650) against Rac1 was used at 1:3000 dilution after target retrieval with pH 9 target retrieval solution (Dako). Secondary antibodies coupled to Alexa Fluor 488 or Alexa Fluor 546 (Molecular Probes) at a dilution of 1:500 or 1:2000 were used for visualization.

Scanning EM

To analyse the structure of the hair surface, scanning electron microscopy (SEM) was performed of hair samples plucked from the dorsal region of rescue and control mice at day 17 post partum (p.p.). SEM analysis was performed by Elmiservice, Erftstadt, Germany. Hair fibers were mounted on an aluminum stub, sputter-coated with a 5 nm gold layer using a sputter coater (Bio-Rad SC 650m) and examined on a Leitz AMR 1200 scanning electron microscope.

Tyramide-based tyrosinase assay

The tyramide-based tyrosinase assay (TTA) assay was used for fluorimetric detection of melanogenic tyrosinase activity. The assay was performed on frozen sections according to a protocol described previously (Han et al., 2002) using a commercial TSA Kit from Perkin Elmer, Waltham, MA.

Transmission electron microscopy and semi-thin sections

Skin samples were immersion-fixed for ~8 hours in 2% paraformaldehyde, 2% glutaraldehyde in 0.1 M cacodylate buffer pH 7.2 at room temperature, rinsed in 0.1 M cacodylate buffer and stored in PBS. After washing in 0.1 M cacodylate buffer pH 7.2 at 40℃, the specimens were directly treated with 2% OsO_4 for 2 hours. After washing, they were stained in 1% uranyl acetate, dehydrated through a series of graded ethanol solutions and embedded in araldite resin. Semi-thin sections (500 nm) were cut with a glass knife using an ultramicrotome (Reichert, Bensheim, Germany) and stained with Methylene Blue. Ultra-thin sections (30–60 nm) were processed on the same microtome with a diamond knife and placed on copper grids. Transmission electron microscopy (TEM) was performed using a Zeiss 902A electron microscope (Zeiss, Oberkochen, Germany).

Light and fluorescence microscopy

Fluorescence images were acquired on a DeltaVision microscope system, equipped with a CoolSnap hQ2 camera by using softWoRx-Suite (Applied Precision, Issaquah, WA). Images of H&E-stained sections and single hairs were taken with a Leica DM4000B light microscope (Leica, Wetzlar, Germany), equipped with a KY-F75U digital camera (JVC Professional, Frankfurt, Germany) using the Diskus software (Carl H. Hilgers, Königswinter, Germany). Pictures of Methylene-Blue-stained semi-thin sections were acquired on an Olympus IX81 microscope system with OBS Cell R software (Olympus, Hamburg, Germany). Measurements were performed using either Diskus software or Image J freeware.

Fluorosphere uptake experiments and colony forming efficiency (CFE) assays

Primary murine keratinocytes were isolated from wild-type, Rac1 E-KO, L61Rac1-transgenic, N17Rac1-transgenic and rescue mice and cultured as described therein (Tscharntke et al., 2007). For fluorosphere uptake experiments, cells were irradiated with 100 mJ cm^{-2} UVB using a Waldmann TP4 UV lamp and then incubated with 36×10^7 FITC-labeled FluoroSpheres (Invitrogen, cat. # F8813, particle size 0.5 µm) for 1, 2, 4 and 6 hours. Next, cells were washed in FCS and PBS and fixed with paraformaldehyde. After washing, cells were permeabilized using 0.1% Triton and stained for 10 minutes with TRITC-labeled phalloidin. The number of ingested fluorospheres per cell were counted using a fluorescence microscope. At least 100 cells were counted per group.

For CFE assays, cells were seeded at clonal density (3000 cells per well) onto a feeder layer of mitomycin-C-treated NIH 3T3 fibroblasts as described previously (Stachelscheid et al., 2008). Cells were cultured for 2 weeks, then fixed and stained with 0.1% Crystal Violet, and 30–60 colonies per well were counted and classified according to their colony area in arbitrary units (a.u.) as: small (≤3 a.u.), medium-sized (≤6 a.u.) and large (> 6 a.u.).

Statistics

Determination of significance was performed as indicated by using either a Student's *t*-test or Mann–Whitney *U* test (GraphPad Prism software, GraphPad Software, La Jolla, CA, USA). For all statistical tests, the 0.05 level of the confidence interval was accepted as statistically significant.

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References

Barrandon, Y. and Green, H. (1987). Three clonal types of keratinocyte with different capacities for multiplication. *Proc. Natl. Acad. Sci. USA* **84**, 2302-2306.

Bazzi, H., Getz, A., Mahoney, M. G., Ishida-Yamamoto, A., Langbein, L., Wahl, J. K. and Christiano, A. M. (2006). Desmoglein 4 is expressed in highly differentiated keratinocytes and trichocytes in human epidermis and hair follicle. *Differentiation* **74**, 129-140.

Bazzi, H., Demehri, S., Potter, C. S., Barber, A. G., Awgulewitsch, A., Kopan, R. and Christiano, A. M. (2009). Desmoglein 4 is regulated by transcription factors implicated in hair shaft differentiation. *Differentiation* **78**, 292-300.

Benitah, S. A., Frye, M., Glogauer, M. and Watt, F. M. (2005). Stem cell depletion through epidermal deletion of Rac1. *Science* **309**, 933-935.

Braga, V. M., Machesky, L. M., Hall, A. and Hotchin, N. A. (1997). The small GTPases Rho and Rac are required for the establishment of cadherin-dependent cell-cell contacts. *J. Cell Biol.* **137**, 1421-1431.

Cai, J., Lee, J., Kopan, R. and Ma, L. (2009). Genetic interplays between Msx2 and Foxn1 are required for Notch1 expression and hair shaft differentiation. *Dev. Biol.* **326**, 420-430.

Cardinali, G., Ceccarelli, S., Kovacs, D., Aspite, N., Lotti, L. V., Torrisi, M. R. and Picardo, M. (2005). Keratinocyte growth factor promotes melanosome transfer to keratinocytes. *J. Invest. Dermatol.* **125**, 1190-1199.

Carroll, J. M., Romero, M. R. and Watt, F. M. (1995). Suprabasal integrin expression in the epidermis of transgenic mice results in developmental defects and a phenotype resembling psoriasis. *Cell* **83**, 957-968.

Castilho, R. M., Squarize, C. H., Patel, V., Millar, S. E., Zheng, Y., Molinolo, A. and Gutkind, J. S. (2007). Requirement of Rac1 distinguishes follicular from interfollicular epithelial stem cells. *Oncogene* **26**, 5078-5085.

Castilho, R. M., Squarize, C. H., Leelahavanichkul, K., Zheng, Y., Bugge, T. and Gutkind, J. S. (2010). Rac1 is required for epithelial stem cell function during dermal and oral mucosal wound healing but not for tissue homeostasis in mice. *PLoS ONE* **5**, e10503.

Chernova, O. F. (2003). Architectonic and diagnostic significance of hair cortex and medulla. *Izv. Akad. Nauk. Ser. Biol.*, **2003**, 63-73.

Chrostek, A., Wu, X., Quondamatteo, F., Hu, R., Sanecka, A., Niemann, C., Langbein, L., Haase, I. and Brakebusch, C. (2006). Rac1 is crucial for hair follicle integrity but is not essential for maintenance of the epidermis. *Mol. Cell. Biol.* **26**, 6957-6970.

Cotsarelis, G., Sun, T. T. and Lavker, R. M. (1990). Label-retaining cells reside in the bulge area of pilosebaceous unit: implications for follicular stem cells, hair cycle, and skin carcinogenesis. *Cell* **61**, 1329-1337.

Duverger, O. and Morasso, M. I. (2009). Epidermal patterning and induction of different hair types during mouse embryonic development. *Birth Defect Res. C* **87**, 263-272.

Ellis, T., Gambardella, L., Horcher, M., Tschanz, S., Capol, J., Bertram, P., Jochum, W., Barrandon, Y. and Busslinger, M. (2001). The transcriptional repressor CDP (Cutl1) is essential for epithelial cell differentiation of the lung and the hair follicle. *Genes Dev.* **15**, 2307-2319.

Hafner, M., Wenk, J., Nenci, A., Pasparakis, M., Scharffetter-Kochanek, K., Smyth, N., Peters, T., Kess, D., Holtkotter, O., Shephard, P. et al. (2004). Keratin 14 Cre transgenic mice authenticate keratin 14 as an oocyte-expressed protein. *Genesis* **38**, 176-181.

Hammerschmidt, B. and Schlake, T. (2007). Localization of Shh expression by Wnt and Eda affects axial polarity and shape of hairs. *Dev. Biol.* **305**, 246-261.

Han, R., Baden, H. P., Brissette, J. L. and Weiner, L. (2002). Redefining the skin's pigmentary system with a novel tyrosinase assay. *Pigment Cell Res.* **15**, 290-297.

Hong, H. K., Noveroske, J. K., Headon, D. J., Liu, T., Sy, M. S., Justice, M. J. and Chakravarti, A. (2001). The winged helix/forkhead transcription factor Foxq1 regulates differentiation of hair in satin mice. *Genesis* **29**, 163-171.

Jensen, K. B., Driskell, R. R. and Watt, F. M. (2010). Assaying proliferation and differentiation capacity of stem cells using disaggregated adult mouse epidermis. *Nat. Protoc.* **5**, 898-911.

Johns, S. A., Soullier, S., Rashbass, P. and Cunliffe, V. T. (2005). Foxn1 is required for tissue assembly and desmosomal cadherin expression in the hair shaft. *Dev. Dyn.* **232**, 1062-1068.

Kurzen, H., Schäfer, S., Franke, W. W., Moll, I., Moll, R., Simics, E., Amagai, M. and Wheelock, M. J. (2001). Compositionally different desmosomes in the various compartments of the human hair follicle. *Differentiation* **63**, 295-304.

Lamarche, N., Tapon, N., Stowers, L., Burbelo, P. D., Aspenstrom, P., Bridges, T., Chant, J. and Hall, A. (1996). Rac and Cdc42 induce actin polymerization and G1 cell cycle progression independently of p65PAK and the JNK/SAPK MAP kinase cascade. *Cell* **87**, 519-529.

Langbein, L., Rogers, M. A., Praetzel, S., Winter, H. and Schweizer, J. (2003). K6irs1, K6irs2, K6irs3, and K6irs4 represent the inner-root-sheath-specific type II epithelial keratins of the human hair follicle. *J. Invest. Dermatol.* **120**, 512-522.

Lin, M. H., Leimeister, C., Gessler, M. and Kopan, R. (2000). Activation of the Notch pathway in the hair cortex leads to aberrant differentiation of the adjacent hair-shaft layers. *Development* **127**, 2421-2432.

Lynch, M. H., O'Guin, W. M., Hardy, C., Mak, L. and Sun, T. T. (1986). Acidic and basic hair/nail ("hard") keratins: their colocalization in upper cortical and cuticle cells of the human hair follicle and their relationship to "soft" keratins. *J. Cell Biol.* **103**, 2593-2606.

Mecklenburg, L. (2001). The nude mouse skin phenotype: the role of Foxn1 in hair follicle development and cycling. *Exp. Mol. Pathol.* **71**, 171-178.

Mecklenburg, L., Tychsen, B. and Paus, R. (2005). Learning from nudity: lessons from the nude phenotype. *Exp. Dermatol.* **14**, 797-810.

Morioka, K. (2005). Hair Follicle Differentiation Under Electron Microscope: an Atlas. Tokyo; New York: Springer.

Morris, R. J. and Potten, C. S. (1994). Slowly cycling (label-retaining) epidermal cells behave like clonogenic stem cells in vitro. *Cell Prolif.* **27**, 279-289.

Niemann, C. and Watt, F. M. (2002). Designer skin: lineage commitment in postnatal epidermis. *Trends Cell Biol.* **12**, 185-192.

Nobes, C. D. and Hall, A. (1995). Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. *Cell* **81**, 53-62.

Paus, R., Müller-Röver, S., Van Der Veen, C., Maurer, M., Eichmüller, S., Ling, G., Hofmann, U., Foitzik, K., Mecklenburg, L. and Handjiski, B. (1999). A comprehensive guide for the recognition and classification of distinct stages of hair follicle morphogenesis. *J. Invest. Dermatol.* **113**, 523-532.

Petiot, A., Conti, F. J. A., Grose, R., Revest, J.-M., Hodivala-Dilke, K. M. and Dickson, C. (2003). A crucial role for Fgfr2-IIIb signalling in epidermal development and hair follicle patterning. *Development* **130**, 5493-5501.

Price, L. S., Langeslag, M., ten Klooster, J. P., Hordijk, P. L., Jalink, K. and Collard, J. G. (2003). Calcium signaling regulates translocation and activation of Rac. *J. Biol. Chem.* **278**, 39413-39421.

Ridley, A. J. (2006). Rho GTPases and actin dynamics in membrane protrusions and vesicle trafficking. *Trends Cell Biol.* **16**, 522-529.

Schlake, T. (2005). FGF signals specifically regulate the structure of hair shaft medulla via IGF-binding protein 5. *Development* **132**, 2981-2990.

Schlake, T. (2006). Krox20, a novel candidate for the regulatory hierarchy that controls hair shaft bending. *Mech. Dev.* **123**, 641-648.

Sharov, A. A., Sharova, T. Y., Mardaryev, A. N., Tommasi di Vignano, A., Atoyan, R., Weiner, L., Yang, S., Brissette, J. L., Dotto, G. P. and Botchkarev, V. A. (2006). Bone morphogenetic protein signaling regulates the size of hair follicles and modulates the expression of cell cycle-associated genes. *Proc. Natl. Acad. Sci. USA* **103**, 18166-18171.

Slominski, A., Wortsman, J., Plonka, P. M., Schallreuter, K. U., Paus, R. and Tobin, D. J. (2005). Hair follicle pigmentation. *J. Invest. Dermatol.* **124**, 13-21.

Stachelscheid, H., Ibrahim, H., Koch, L., Schmitz, A., Tscharntke, M., Wunderlich, F. T., Scott, J., Michels, C., Wickenhauser, C., Haase, I. et al. (2008). Epidermal insulin/IGF-1 signalling control interfollicular morphogenesis and proliferative potential through Rac activation. *EMBO J.* **27**, 2091-2101.

Steel, K. P., Davidson, D. R. and Jackson, I. J. (1992). TRP-2/DT, a new early melanoblast marker, shows that steel growth factor (c-kit ligand) is a survival factor. *Development* **115**, 1111-1119.

Stratis, A., Pasparakis, M., Rupec, R. A., Markur, D., Hartmann, K., Scharffetter-Kochanek, K., Peters, T., van Rooijen, N., Krieg, T. and Haase, I. (2006). Pathogenic role for skin macrophages in a mouse model of keratinocyte-induced psoriasis-like skin inflammation. *J. Clin. Invest.* **116**, 2094-2104.

Sun, T. T., Cotsarelis, G. and Lavker, R. M. (1991). Hair follicular stem cells: the bulge-activation hypothesis. *J. Invest. Dermatol.* **96**, 77S-78S.

Sundberg, J. P. (1994). *Handbook of Mouse Mutations with Skin and Hair Abnormalities: Animal Models and Biomedical Tools*. New York: CRC Press LLC.

Takaishi, K., Sasaki, T., Kotani, H., Nishioka, H. and Takai, Y. (1997). Regulation of cell-cell adhesion by rac and rho small G proteins in MDCK cells. *J. Cell Biol.* **139**, 1047-1059.

Trigg, M. J. (1972). Hair growth in mouse mutants affecting coat texture. *J. Zool.* **168**, 165-198.

Tscharntke, M., Pofahl, R., Chrostek-Grashoff, A., Smyth, N., Niessen, C., Niemann, C., Hartwig, B., Herzog, V., Klein, H. W., Krieg, T. et al. (2007). Impaired epidermal wound healing in vivo upon inhibition or deletion of Rac1. *J. Cell Sci.* **120**, 1480-1490.

Tumbar, T., Guasch, G., Greco, V., Blanpain, C., Lowry, W. E., Rendl, M. and Fuchs, E. (2004). Defining the epithelial stem cell niche in skin. *Science* **303**, 359-363.

Vauclair, S., Nicolas, M., Barrandon, Y. and Radtke, F. (2005). Notch1 is essential for postnatal hair follicle development and homeostasis. *Dev. Biol.* **284**, 184-193.

Virador, V., Matsunaga, N., Matsunaga, J., Valencia, J., Oldham, R. J., Kameyama, K., Peck, G. L., Ferrans, V. J., Vieira, W. D., Abdel-Malek, Z. A. et al. (2001). Production of melanocyte-specific antibodies to human melanosomal proteins: expression patterns in normal human skin and in cutaneous pigmented lesions. *Pigment Cell Res.* **14**, 289-297.

Weger, N. and Schlake, T. (2005). Igf-I signalling controls the hair growth cycle and the differentiation of hair shafts. *J. Invest. Dermatol.* **125**, 873-882.

Weiner, L., Han, R., Scicchitano, B. M., Li, J., Hasegawa, K., Grossi, M., Lee, D. and Brissette, J. L. (2007). Dedicated epithelial recipient cells determine pigmentation patterns. *Cell* **130**, 932-942.

Wilson, C., Cotsarelis, G., Wei, Z. G., Fryer, E., Margolis-Fryer, J., Ostead, M., Tokarek, R., Sun, T. T. and Lavker, R. M. (1994). Cells within the bulge region of mouse hair follicle transiently proliferate during early anagen: heterogeneity and functional differences of various hair cycles. *Differentiation* **55**, 127-136.

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