

The serine protease Corin is a novel modifier of the agouti pathway

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The hair follicle is a model system for studying epithelial-mesenchymal interactions during organogenesis. Although analysis of the epithelial contribution to these interactions has progressed rapidly, the lack of tools to manipulate gene expression in the mesenchymal component, the dermal papilla, has hampered progress towards understanding the contribution of these cells. In this work, *Corin* was identified in a screen to detect genes specifically expressed in the dermal papilla. It is expressed in the dermal papilla of all pelage hair follicle types from the earliest stages of their formation, but is not expressed elsewhere in the skin. Mutation of the *Corin* gene reveals that it is not required for morphogenesis of the hair follicle. However, analysis of the 'dirty blonde' phenotype of these mice reveals that the transmembrane protease encoded by *Corin* plays a critical role in specifying coat color and acts downstream of *agouti* gene expression as a suppressor of the agouti pathway.

KEY WORDS: Corin, Dermal papilla, Agouti, Pigmentation

INTRODUCTION

The hair follicle is an important model system for the study of organogenesis: in part, because of the attributes that make it amenable to study during follicular neogenesis in the embryo and newborn animal; and, in part, because the lower portion of the hair follicle is regenerated from comparatively well-defined keratinocyte stem cells in the adult. In both neogenesis and regeneration, inductive signaling between epithelium and mesenchyme directs morphogenesis of the follicle. In the embryo, pluripotent keratinocytes in the basal epidermis initiate follicular development by forming a thickened epidermal placode in response to inductive signals from the dermis. Signals from the placode recruit dermal cells to aggregate beneath it and form the dermal condensate, which in turn acquires additional inductive properties. These include signals required for the growth of the placodal keratinocytes and their invagination to form the hair peg that extends into the dermis. The dermal condensate further differentiates into the dermal papilla (DP), which is engulfed by the extending hair peg and comes to lie in the center of the hair bulb at the base of the mature follicle.

During the active growth phase, the mature hair follicle is composed primarily of keratinocytes arranged in concentric layers of differentiated cell types that comprise the hair shaft (HS), inner root sheath (IRS) and outer root sheath (ORS). Growth of the hair occurs as proliferating cells in the hair matrix at the base of the follicle generate additional constituents of the inner layers of the follicle that are organized into the IRS and HS and extruded through the ORS towards the surface of the skin. The DP plays a central role in this process. The keratinocytes abutting the DP act as stem cells of the hair bulb, undergoing asymmetrical divisions to generate transit amplifying (TA) progeny, which then undergo a few divisions before terminally differentiating (Legue and Nicolas, 2005). Ablation and grafting studies have demonstrated that the DP is required to maintain the growth of the hair follicle

and suggest that it plays an instructive role in driving morphogenesis of the hair (Jahoda et al., 2001; Oliver and Jahoda, 1988).

This period of sustained hair growth lasts for a few weeks in the mouse. At the end of this growth or anagen phase, proliferation in the hair bulb ceases. During the degeneration or catagen phase, the matrix cells either terminally differentiate or apoptose, and the basal end of the hair shaft becomes anchored in the upper follicle. The outer root sheath from the lower two-thirds of the follicle degenerates and the DP is drawn to the base of the permanent portion of the follicle. A quiescent or telogen phase ensues that may last from a few days during the first hair cycle to many weeks in mature animals. At the end of the telogen phase, keratinocyte stem cells resident in the bulge region of the permanent follicle are activated and regenerate the lower portions of the anagen follicle (Morris et al., 2004). It is thought that the DP plays a central role in the activation of these keratinocytes and the subsequent guidance of their proliferation and differentiation to regenerate the follicle, although the nature of that role remains to be empirically defined (Sun et al., 1991).

In addition to its functions in follicle morphogenesis and cycling, the DP also regulates pigmentation of the hair. Pigment is synthesized by melanocytes resident in the hair bulb and deposited in the keratinocytes of the hair medulla and cortex. Pigment production in melanocytes is regulated in part by the activity of the Mc1r receptor expressed on their surface. When this receptor is active, the black pigment eumelanin is produced and deposited in the hair shaft. Mc1r has a basal level of constitutive activity in the absence of its agonists melanocortins that is further stimulated by the binding of these ligands (Barsh, 1999; Smart and Low, 2003). Although the DP is likely to play a role in generating an environment that attracts and maintains melanocytes in the hair bulb (Yoshida et al., 1996), it also regulates hair pigmentation more directly by expressing the agouti signaling protein, hereafter referred to as agouti (Millar et al., 1995). Binding of agouti to Mc1r reduces its activity and results in a shift from the production of eumelanin to the synthesis of pheomelanin, a yellow pigment (Barsh et al., 2000; Chai et al., 2003). *Agouti* (also known as *nonagouti* or *ASIP*) is expressed transiently in the DP of the dorsal pelage during the early growth phase of the hair cycle. The resultant provisional switch to pheomelanin deposition generates a subapical yellow band in the

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otherwise black hair that defines the agouti coat color. Despite the predominance of black pigment in the hair of agouti mice, the presence of lighter pigment in the hair tip creates the overall appearance of a mottled brown hair coat that provides adaptive coloration in the natural environment. Modest variations in the length of this apical pheomelanin band can dramatically alter coat appearance and represent one mechanism by which adaptive changes in coat color can occur (Hoekstra, 2006).

Partly as a result of the development of mouse strains that allow the manipulation of gene expression in the embryonic precursors of the epidermis and follicular epithelium (Byrne et al., 1994; Indra et al., 1999), the epidermal placode (Levy et al., 2005) or the stem cells of the adult hair follicle (Ito et al., 2005), significant progress has been made in understanding the genetic mechanisms that direct follicle formation in its keratinocyte constituents. Despite its functions in maintaining the niche for matrix stem cells and in organizing both the morphogenesis and pigmentation of the hair follicle, the molecular genetics of DP formation and function have remained less accessible to study because of a comparative lack of tools to purify these cells or manipulate gene expression in vivo. To address this need, we evaluated genes preferentially expressed in the DP of the hair follicle to identify those whose expression in the skin is restricted to this population. Here we report the identification of *Corin* as a gene specifically expressed in the DP of the hair follicle. Ablation of *Corin* activity in the DP reveals that it is not required for hair follicle morphogenesis and confirms that the *Corin* gene is well suited to serve as a platform for the manipulation of gene expression in the DP in vivo. This analysis also reveals an unexpected function for this transmembrane serine protease in the regulation of hair shaft pigmentation and identifies a novel mechanism by which the DP directs the pattern of pigmentation in the hair shaft.

MATERIALS AND METHODS

Mice

Using a 129SvJ ES-cell line, 64bp downstream of the ATG in exon1 of *Corin* was replaced by a YFP-Neo cassette. Chimeric mice (A^W/A^W), derived from correctly targeted ES clones, were crossed with C57BL6J (*a/a*) to generate F0 mice heterozygous for both *Corin* and *agouti* (A^W/a). F0 mice were crossed with FVB (*A/A*) to generate F1 mice (either A^W/A or *A/a*). Phenotypic analysis was performed on offspring from matings of F1 *Corin* heterozygous mice. Qualitatively similar results were obtained when the *Corin* allele was first moved to FVB, C57BL6J or 129 backgrounds for at least six generations.

In-situ hybridization and immunohistochemistry

Non-radioactive in situ hybridization of sections from embryos and dorsal skins were performed with probes corresponding to nts 682-1252 of *Corin* (GenBank Acc. No. NM_016869) and nts 126-613 of *Agouti* (NM_015770). Anti-*Corin* antibodies (1:800) raised in rabbits (Fig. 2A), were detected with FITC-conjugated secondary antibody (1:250) (Jackson) in the presence of TO-PRO-3 (1:40,000).

Hair shaft analysis

The analysis of the length of the subapical yellow band was performed on six wild-type and seven mutant postnatal day (P)20 mice. At P20, hairs derived from the first hair cycle are fully grown and are the only hairs present in the dorsal pelage. For each mouse, approximately 400 hairs from mid-dorsal pelage were randomly mounted on slides in a thin layer of Gelvatol. Awl hairs represent approximately 10% of the total hair population. Therefore, about 80 awl hairs per mouse were specifically collected for analysis. Two-tailed unpaired *t*-tests were performed.

Hair shafts were photographed in bright field and in green fluorescent channel at 100 \times magnification. Lack of black pigment in the hair shaft results in autofluorescence that corresponds with the deposition of yellow pigment. The green channel of the fluorescent image was duplicated in

Table 1. Primers used in this study

Primer name	Sequence
SC5	5'-TTGGTCTCTCAAGCAGTGTCAG-3'
SC3	5'-TGTTGTTGCGACTGGACACAGTG-3'
WT51	5'-GCAGGGTTTCCTTCAGCGTTTCG-3'
WT31	5'-AGTCACGGAGCTAGTAGCAAGG-3'
SCNEO5	5'-TGTCGATCAGGATGATCTGGAC-3'
SEQFP1	5'-GTCGTCCTGAAGAAGATGGTG-3'
KILY5	5'-GAGTCCTGGTGACTTCAGAGTC-3'

the red channel to generate a yellow color. The modified fluorescent image was overlaid on the bright field image in PhotoShop and reduced to 45% opacity.

Real-time PCR

Mid-dorsal skins from P0-P9 mice were collected and used to prepare RNA with Trizol solution (Invitrogen). The total RNA was further purified using RNeasy mini kit (Qiagen) and a DNase I digestion step. Normalized RNA was reverse transcribed using random hexamer primers. Primer pairs (Superarray) for *Actb* (PPM02945A), *Corin* (PPM41062A), *Agouti* (PPM24722A), *Mclr* (PPM04903A), *Pomc* (PPM37114A), *Atrn* (PPM30947A) and *Mgrn1* (PPM02945A) and CYBR Green-Fluorescein PCR Mater Mix were used with an iCycler (Bio-Rad), MyiQ Single-color Detection system, MyiQ Optical System Software. Differences between samples were quantified based on the $\Delta\Delta C_t$ method. The number of mice per genotype (WT/Mut) per stage used in this expression profile was as follows: P0(5/3); P1(9/10); P2(13/10); P3(8/7); P4(13/9); P5(7/11); P6(5/6); P7(3/4); P8(2/2); P9(4/5). The analysis was performed individually for each mouse and the average values were calculated for each group of mice. Thus, the s.d. reflects the variation in gene expression between individual mice in the same group.

RESULTS

Corin is preferentially expressed in DP cells

As an initial step towards identifying genes that might serve as platforms for the study of DP function, we used a mouse expressing EGFP under the control of a short segment of the human versican promoter (Kishimoto et al., 2000). A population enriched in DP cells was purified from newborn skin on the basis of GFP expression (Shimizu and Morgan, 2004). Gene expression in these cells immediately after isolation was compared with that of cells maintained in culture with the expectation that genes expressed in the DP as a consequence of inductive signaling from the follicular keratinocytes would be preferentially lost from the cultured cell expression profile. Among the genes identified in this way was the *Corin* locus. *Corin* transcripts were readily detected in freshly isolated cells but dropped below the level of detection within 24 hours in culture (see Fig. S1 in the supplementary material). *Corin* encodes a transmembrane protease that is expressed in the heart and participates in blood pressure regulation by cleaving the prohormone proANP to its active form (Chan et al., 2005; Yan et al., 2000). EST expression profiles and available expression data suggested that *Corin* expression was largely restricted to cardiomyocytes (see also Yan et al., 1999). This restricted expression profile and the expression of the *Corin* protein on the surface of the cell made this gene an attractive candidate for further investigation of its expression and function in the skin. Although mice lacking *Corin* have the altered blood pressure regulation and cardiovascular defects predicted by its role in cleaving ANP, no skin or hair phenotypes were reported in these mice (Chan et al., 2005).

Corin expression in the hair follicle

Hair follicle formation occurs in three waves during embryogenesis, which initiate at E14, E16 and between E18 to birth. These waves give rise to follicles that generate distinct hair types. Follicles that

give rise to the long, straight guard hairs arise in the first wave, whereas the intermediate-sized awl and smaller zigzag and auchene hair types are thought to arise in the second and third waves, respectively. *Corin* expression at the RNA level was examined in embryonic skin and in the dorsal pelage of post-natal animals by in situ hybridization (Fig. 1). It is first detected in the skin in the nascent dermal condensate at embryonic day (E)15, as the first wave of follicles form (Fig. 1A). Its expression persists in the DP throughout the development of these follicles and is also observed in the dermal condensate and DP of the second and third waves of follicles throughout their morphogenesis (Fig. 1B,C). *Corin* transcripts were not detected elsewhere in the skin at any stage examined. *Corin* expression persists and remains restricted to the DP throughout the anagen (growth) phase of the hair cycle (Fig. 1D-G and data not shown). Expression is not detected during the catagen (regression) or telogen (resting) phases but returns with the onset of a new anagen phase (data not shown). *Corin* is expressed in a similar pattern in all follicle types of the dorsal pelage.

Corin protein is expressed on the surface of DP cells

Antisera were generated to evaluate *Corin* protein distribution in the skin. A schematic representation of the inferred protein structure is shown in Fig. 2A. In addition to the C-terminal protease domain, this type II transmembrane protein has two cysteine-rich ‘frizzled homology’ domains, two series of LDLR-like repeats and a scavenger receptor conserved region. A segment corresponding to LDLR repeats 1-5 was expressed in bacteria and used to immunize rabbits. The resultant antisera revealed *Corin* expression in the DP of the hair follicle (Fig. 2B-F). *Corin* protein levels mirror the expression pattern of *Corin* at the RNA level, suggesting that any post-transcriptional events controlling *Corin* activity and function do not include the participation of translational regulation. Furthermore, consistent with the predicted protein structure, the majority of *Corin* protein detected remains associated with DP cells (Fig. 2E,F). Although detailed analysis was restricted to dorsal pelage, *Corin* was also detected in the DP of vibrissae and pelage follicles of the ventral skin.

Corin is not required for hair growth or cycling

A null allele of *Corin* was generated by inserting a YFP-Neo cassette into the first exon of *Corin* that harbors the initiation codon and encodes most of the intracellular N-terminal domain (Fig. 3A). Mice homozygous for this allele (*Corin*^{-/-}) were recovered at expected frequencies and were viable and fertile (Fig. 3B-D). Immunostaining with anti-*Corin* antibodies confirmed both the absence of detectable *Corin* protein in these mice and the specificity of the anti-sera used in this study (Fig. 3E,F). In P3 skin, the three waves of follicle formation are represented by follicles in different stages of development. All three stages of hair follicles are present in mutant skin with frequencies similar to that of the wild type, suggesting that hair follicle initiation is normal in homozygous mutants (Fig. 3E,F, see Fig. S2 in the supplementary material). Indeed, the hair coat in homozygous mutant animals appears normal with respect to numbers, frequency, structure and growth rate at all stages examined (Fig. 4 and see Fig. S3 in the supplementary material). *Corin* activity is not required for the normal morphogenesis or cycling of the hair follicle.

Corin modifies agouti activity

However, there is a striking difference between the wild-type and mutant pelage. Homozygous mice exhibit a distinctly lighter coat color that is most pronounced in juveniles but persists through adulthood (Fig. 4A,B). This phenotype is dependent on the presence

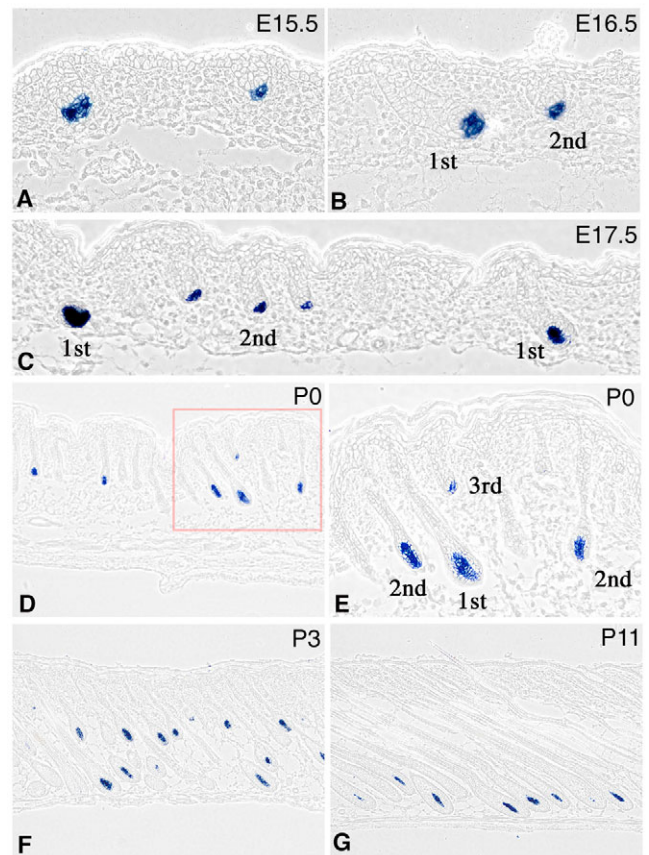


Fig. 1. *Corin* expression is confined to the DP. In situ hybridization to detect *Corin* transcripts (blue) in FVB mice. *Corin* is first detected when the dermal condensate segregates from surrounding dermis during the first (A, E15.5) and second (B, E16.5) waves of follicle formation. Follicles derived from the first, second and third waves of follicle formation are indicated. Throughout the growth phase of the hair cycle, *Corin* transcripts are expressed in the DP and not detected elsewhere in the skin. Samples from E17.5 (C) P0 (D,E), P3 (F) and P11 (G) mice are shown. The field enclosed by the red square in D is shown at higher magnification in E to reveal that *Corin* is expressed throughout the DP and not in the surrounding hair matrix.

of a functional allele (A) of *agouti*. *Corin* mutants homozygous for a null allele of the *agouti* gene (*a/a*) are black and indistinguishable from the wild type (Fig. 4C), whereas *Corin* mutants on *A/a* or *A/A* backgrounds are progressively lighter than corresponding mice with a wild-type *Corin* allele (Fig. 4D). The yellow appearance is a result of pheomelanin production and not alteration of the diffractive properties of the hair, because tryosinase mutants incapable of synthesizing melanin have white fur irrespective of the presence or absence of *Corin* (Fig. 4C). Heterozygotes for *Corin* are indistinguishable from the wild type on all *agouti* backgrounds tested (data not shown).

To gain more insight on the mechanism underlying the coat color phenotype, hair shafts were plucked from the back skin of wild-type and mutant mice at the end of the first hair cycle. The four types of hair, guards, awls, zigzags and auchenes, differ in the extent of pheomelanin content. Changes in hair type could in principle explain the lighter coat color. However, all four types of hair were present at similar frequencies in both wild-type and mutant mice (see Fig. S3 in the supplementary material). *Corin* is not required to specify hair type.

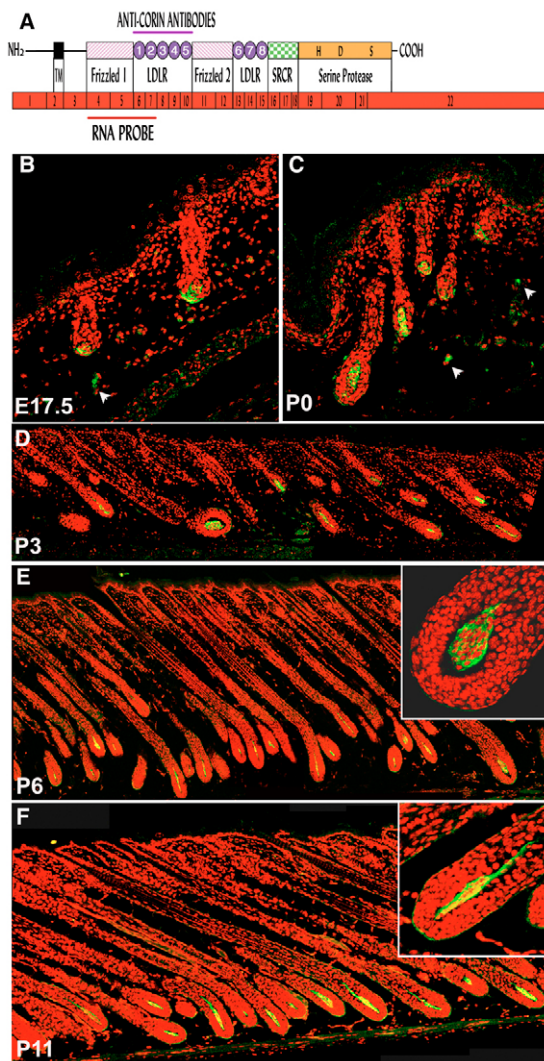


Fig. 2. Expression of Corin protein coincides with *Corin* transcript accumulation. (A) Schematic representation of *Corin* mRNA and protein. The mRNA of *Corin* (shown in red) comprises 22 exons encoding a type-II transmembrane serine protease. The single-pass transmembrane domain (TM) of *Corin* resides in close proximity to the N-terminus, and the large extracellular portion includes two frizzled-like cysteine-rich motifs (designated Frizzled 1 and 2), eight LDL receptor repeats (LDLR1-8), a macrophage scavenger receptor-like domain (SRCR) and a catalytic domain of trypsin-like serine protease at the C-terminus. The active site residues of the catalytic triad (H, D and S) are shown. The regions corresponding to the RNA probe and the fragment of *Corin* protein used to immunize rabbits are indicated. (B-F) Immunohistochemical detection of *Corin* (green) in DP at E17.5 (B), P0 (C), P3 (D), P6 (E) and P11 (F). Red stain highlights nuclei. Occasional staining was observed outside the DP (white arrowheads) but this was also detected in controls in which only the secondary antibody was used (data not shown and supplementary material Fig. S2). In E and F, higher magnification of a bulb region is shown (upper right) to reveal that the majority of the protein is localized at the periphery of DP cells.

The length of the subapical yellow band was analyzed separately for each hair type. Zigzag hairs were the most abundant hair type and have a prominent yellow band. Regardless of *Corin* genotype, all zigzag hairs exhibited a yellow band that was restricted to the apical segment of the hair. In mutant zigzag hairs, the length of the yellow

band was increased. Fig. 5A shows extreme examples of wild-type and mutant zigzag hairs: in the absence of *Corin* the yellow band extended for the length of the terminal segment, whereas in the wild type it extended less than half of that length. Both the length of the terminal segment and the length of the yellow band vary between different zigzag hairs on the same mouse. To quantify the differences between wild-type and mutant zigzag hairs, the ratio (R) between the length of the yellow band (Y) and the length of the apical segment (Z) was calculated (Fig. 5B). Hairs were scored for the presence or absence of a black tip and assigned to one of three categories: $R \leq 0.5$, $0.5 < R \leq 0.75$, or $0.75 < R \leq 1$ (Fig. 5C-H). This analysis revealed two major differences (see Fig. 5I). First, the basal extension of the yellow band increased substantially in the mutant. The number of zigzag hairs with $R > 0.5$ increased significantly from 33% in the wild type to 85% in mice lacking *Corin*, and the category exhibiting $R > 0.75$ increases from 0 to 32%. Second, the yellow band was also extended in the apical direction in most hairs. Although virtually all of the wild-type hairs had a terminal black tip, 70% of the mutant hairs did not (see Fig. S4 in the supplementary material). It is striking that the extension of the yellow band in the apical and basal directions are to some extent independent events. Hairs with the longest yellow bands may nevertheless have a black tip, whereas hairs in the shortest category may have yellow tips. Despite the breadth of the categories used in this analysis, the pigmentation pattern of a minimum of 85% of the zigzag hairs is altered in the *Corin* mutant.

Similar analysis with some minor modifications was performed for the awl hairs (Fig. 6). The ratio (R) between the length of the yellow band (Y) and the length of the whole hair (A) was calculated (Fig. 6B). In contrast to zigzag hairs, many of the wild-type awl hairs were completely black ($R=0$, Fig. 6C). Furthermore, regardless of *Corin* genotype, all awl hairs terminated with a black tip. Two additional categories were defined: $R \leq 0.25$ and $0.25 < R \leq 0.5$. Representative examples of all three categories are shown in Fig. 6A. A striking reduction in the frequency of completely black awl hairs from 61% in the wild type to 28% in the mutant was observed (Fig. 6C). In the absence of *Corin*, many awl hairs that otherwise would be completely black were transformed to include a subapical yellow band. Furthermore, the percentage of awl hairs with $0.25 < R \leq 0.5$ increased from 7% in the wild type to 54% in mutant mice. This increase (47%) exceeds the proportion that could be contributed by awl hairs that normally exhibit a yellow band in wild type (39%) and demonstrates that hairs that would normally lack any discernible agouti activity show pheomelanin deposition over a quarter of their length in the absence of *Corin*.

A similar elongation of the yellow band was observed in auchene hairs as a result of *Corin* ablation (see Fig. S5 in the supplementary material). As observed in zigzag hairs, the extension of the pheomelanin band in the apical and basal directions were independent events. Although substantial extension in the basal direction was observed, all mutant auchene hairs end with black tips as observed in wild-type mice. In contrast to zigzags, awls and auchenes, all guard hairs were completely black in both wild-type and mutant mice, suggesting that agouti signaling is completely inactive in this type of hair, regardless of the presence or absence of *Corin* (data not shown).

The expression of agouti pathway genes is not altered in *Corin* mutants

The extent of the yellow band is regulated in part by the limited window in which the *agouti* gene is expressed in dorsal skin (Millar et al., 1995; Vrieling et al., 1994), and *Corin* could in principle modulate signals that impinge on DP cells to regulate the expression of *agouti*. Detailed analysis of agouti expression by real-time PCR

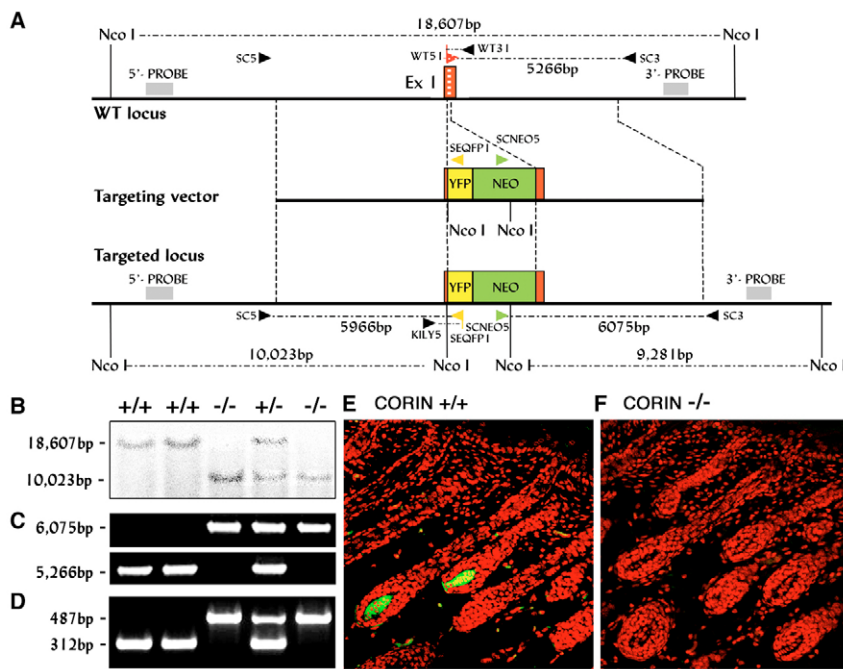


Fig. 3. The generation of *Corin* knockout mice.

(A) Gene targeting approach used for *Corin* ablation. A YFP-Neo cassette replaced 64 bp downstream of the ATG in exon 1 (red box). Primers (arrowheads, see Table 1) and expected PCR fragments are indicated. Primer WT51 binds in the deleted 64 bp segment and the primers SC3 and SC5 bind outside the targeting construct. The *NcoI* fragments used for Southern analysis are indicated. The FRT flanked pgkNeo^R cassette was oriented in parallel to *Corin* transcription. (B,C,D) Genomic analysis of wild-type, heterozygous and homozygous *Corin* mice. (B) Southern analysis of *NcoI*-digested genomic DNA. The 5'-probe reveals 18.6 kb and 10 kb bands from wild-type and mutant alleles, respectively. (C) PCR with primers SCNEO5 and SC3 generates a 6075 bp band from the targeted allele (upper panel) whereas WT51 and SC3 generate a 5266 bp fragment from the wild type. (D) PCR with WT51, WT31, KILY5 and SEQFP1 detects both wild-type (312 bp) and mutant (487 bp) alleles for routine genotyping. (E,F) Immunostaining of frozen P3 skin-sections from wild-type and mutant mice with anti-*Corin* antibodies. *Corin*, green; Nuclei, red.

during the hair cycle in wild-type mice was performed to define the pattern of *agouti* expression (Fig. 7B). *Agouti* transcript levels were extremely low at P0 but rose rapidly from P1 to P3 and then dropped off dramatically to baseline levels by P7. No changes in the levels or timing of *agouti* transcript accumulation were detected in *Corin* mutant mice. This whole skin analysis preferentially detected the expression of *agouti* in zigzag hairs that comprise 70-80% of the dorsal pelage. Therefore, *agouti* expression was also evaluated by in situ hybridization to score expression in guard and awl hair follicles (Fig. 7G). As predicted by the lack of a pheomelanin band in *Corin* mutant guard hairs, *agouti* expression was not detected in guard hair follicles in either wild-type or mutant mice at any of the time points evaluated, which included P0-P6 (Fig. 7G and see Fig. S6 in the supplementary material). However, *agouti* transcripts were readily detected in the DP of some, but not all awl hair follicles at P2 and later stages. No change in the pattern, timing or apparent levels of *agouti* transcript accumulation was observed in *Corin*^{-/-} skin, and expression in both second and third wave follicles of wild-type and mutant mice was largely extinguished between P6 and P7.

Alternatively, *Corin* might exert its function by modifying the levels of another component in this signaling pathway downstream of *agouti*. A decrease in *Mc1r* expression would be expected to render melanocytes more sensitive to *agouti* inhibition and expand the 'agouti band'. *Mc1r* mRNA levels in wild-type mice increased to a peak at P3 as the third wave of hair follicles develop and then declined to a stable baseline level of expression that persisted throughout the anagen phase (Fig. 7C). No change in *Mc1r* expression was detected in the absence of *Corin*. In addition to directly augmenting *Mc1r* activity, binding of the agonist α -melanocyte stimulating hormone (α -MSH) to *Mc1r* competitively inhibits *agouti* binding (Ollmann et al., 1998). By either mechanism, a decrease in α -MSH levels would be expected to enhance pheomelanin production on an *Agouti* background. Although *Pomc* (the gene encoding the precursor of α -MSH) is mainly expressed in the brain, it is also expressed at low levels in the skin and in cultured keratinocytes (Slominski and Paus, 1993). Therefore, *Corin* may act on the environment surrounding the DP to control mRNA levels of

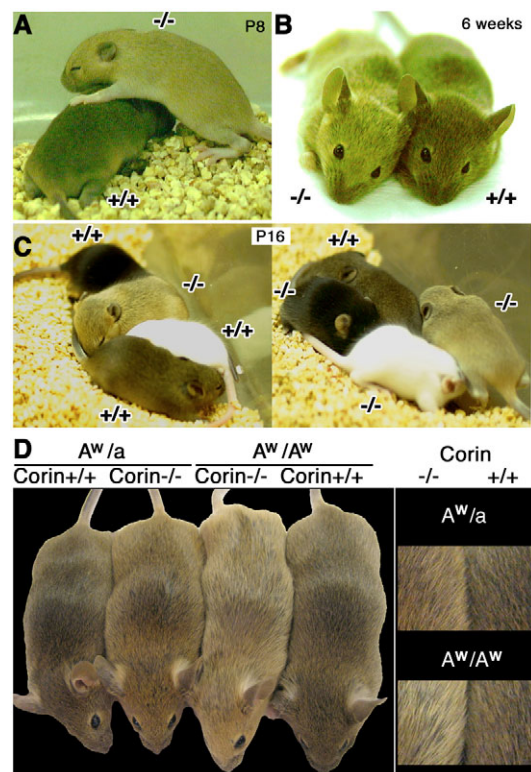


Fig. 4. Coat color phenotype of *Corin* mutants. Mice lacking *Corin* (-/-) exhibit lighter coat-color than corresponding wild type (+/+) on an *Agouti* background. (A,B) Mice during the first (A) and second (B) hair cycles are shown. (C) Two litters at P16 are shown each containing one *a/a* (black), one *tyrC/tyrC* (white) and two *A/A* with *Corin* genotypes indicated. (D) Pairs of mice homozygous and heterozygous for a functional *agouti* allele and wild-type or mutant for *Corin* are shown with higher magnification views at right. On both *Agouti* genotypes lack of *Corin* leads to a lighter coat color and in the absence of *Corin*, *A^W/A^W* are lighter than *A^W/a*.

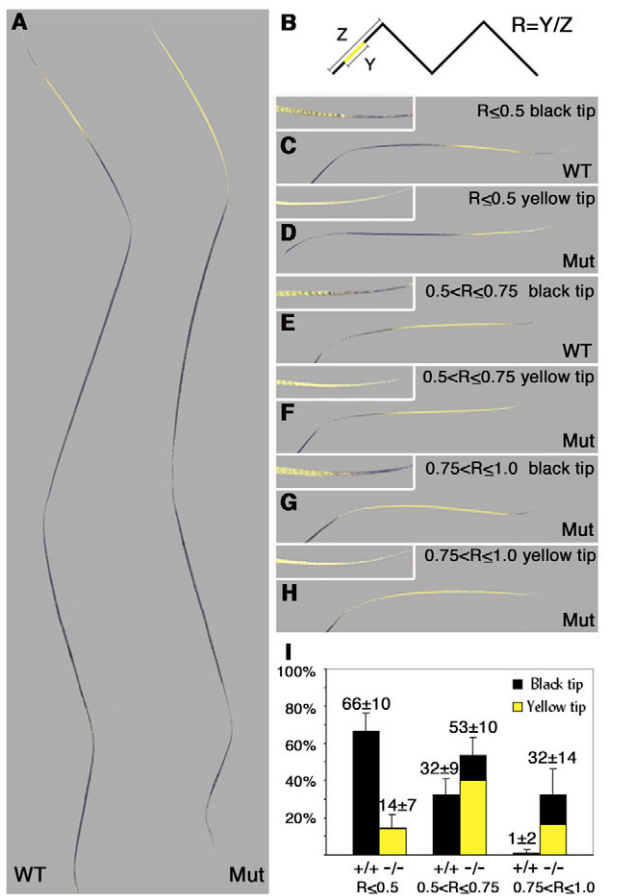


Fig. 5. Yellow band extension in *Corin* mutant zigzag hairs.

(A) The longest yellow band found in zigzag hairs lacking *Corin* (Mut) and the shortest subapical yellow band found in wild-type zigzag hairs (WT) are shown. (B) The approach used to quantify the differences between wild-type and mutant zigzag hairs. The ratio (R) between the length of the yellow band (Y) and the length of the apical segment (Z) was scored and assigned to three categories: $R \leq 0.5$ (C,D), $0.5 < R \leq 0.75$ (E and F), $0.75 < R \leq 1$ (G,H). (C-H) The apical segment of representative hairs in these categories is shown. The tip of each hair is shown in higher magnification in the upper left corner. Both the length of the yellow band and the length of the apical segment vary in both wild-type and mutant mice (compare D with F). (I) The distribution of zigzag hairs in wild-type and mutant mice among the above categories is shown, including the proportion of black versus yellow tips in each category (black vs yellow shading, respectively). All wild-type zigzag hairs end with a black tip (C,E), whereas 70% of mutant zigzag hairs exhibit a yellow tip (D,F,H). The hair population of $R \leq 0.5$ with black tips that predominates in the wild type is almost completely absent in mice lacking *Corin*. The category of $0.75 < R \leq 1$ is essentially unique to mice that lack *Corin*. The P values for $R \leq 0.5$, $0.5 < R \leq 0.75$ and $0.75 < R \leq 1$ are $P < 0.0001$, $P = 0.0017$ and $P = 0.0002$, respectively. Data are mean \pm s.d.

Pomc. However, similar low levels of *Pomc* expression were detected in the skin of wild-type and *Corin* mutant mice throughout anagen (Fig. 7D). In addition to *Mc1r*, the activity of *agouti* is dependent on at least two other proteins expressed by the melanocyte, attractin (*Atrn*) and mahogunin (*Mgrn1*) (Gunn et al., 1999; He et al., 2003a; He et al., 2003b; Phan et al., 2002). *Atrn* is a single-pass transmembrane protein that binds *agouti*, and *Mgrn1* is an E3 ubiquitin ligase. Although the mechanism by which these proteins modify pigment production remains unknown, the prevailing model is that the simultaneous binding of *agouti* to *Atrn*

and *Mc1r* activates *Mgrn1* to catalyze ubiquitylation events targeting *Mc1r* for modifications that decrease its activity, possibly by promoting its degradation or internalization (He et al., 2003a). In contrast to *agouti* and *Mc1r*, which are predominantly expressed in the DP and the melanocyte respectively, *Atrn* and *Mgrn1* are more widely expressed. The mRNA levels of *Atrn* and *Mgrn1* were stable throughout the anagen phase of the hair cycle and remained unchanged regardless of *Corin* genotype (Fig. 7E,F).

Although the lack of *Corin* did not modify the expression of genes involved in *agouti* signaling, this analysis does confirm that *Corin* acts downstream of *agouti* transcript accumulation to modify pigment production. In wild-type mice, follicles expressing *agouti* in the DP but nevertheless producing eumelanin in the hair bulb were readily detected at P3 and P5 (Fig. 7G and data not shown). By contrast, all follicles expressing *agouti* in the DP at levels detected by in situ hybridization produce pheomelanin in the hair bulb of *Corin* mutant mice (Fig. 7G and data not shown). Thus in the absence of *Corin*, the expression of *agouti* is sufficient to dictate pheomelanin production, whereas in the presence of *Corin*, eumelanin production can occur despite *agouti* mRNA expression.

DISCUSSION

The *Corin* gene as a tool to study the DP

These results reveal that *Corin* is quite specifically expressed within the skin in the DP of hair follicles during the anagen phase of the hair cycle. It was not detected elsewhere in the dermis at any other time in development that we analyzed. This specificity, coupled with a dearth of expression elsewhere, other than in cardiomyocytes, makes the *Corin* locus an attractive platform for strategies to manipulate gene expression in the DP. The fact that a functional *Corin* gene is not required for the morphogenesis or cycling of the hair follicle extends the range of such strategies to include knock-ins to this locus. The inability to manipulate gene expression in the DP has been an unfortunate impediment to progress in hair follicle biology and the identification of *Corin* locus as a prime candidate for such approaches is a significant advance.

Corin ablation reveals cryptic *agouti* expression

These results also demonstrate that *Corin* plays an unexpected role in modifying the balance between eumelanogenesis and pheomelanogenesis. This activity is exerted in concert with the *agouti* pathway. In the absence of a functional allele of *agouti* there is no discernible effect on pigment production. It is noteworthy that *Corin* expression precedes *agouti* signaling activity and persists long after the decline of detectable *agouti* activity, whereas the phenotypic effects of *Corin* ablation are observed at the start and/or the end of *agouti* signaling period. As *Corin* expression at the RNA level coincides with protein detection of *Corin* throughout the anagen phase of the hair cycle (compare Figs 1 and 2), translational regulation does not explain the failure of *Corin* to suppress pheomelanogenesis during the entire period of *agouti* expression. Therefore, *Corin* is only able to effectively counteract *agouti* activity when the levels of *agouti* are low, at the peripheries of its bell-shaped curve of expression. This conclusion is supported by the observation that coat color is sensitive to the dosage of *agouti* in the presence of *Corin*. The length of the pheomelanin band is reduced when *agouti* dosage is halved, presumably because a level of *agouti* sufficient to overcome *Corin*-mediated inhibition is only reached closer to the peak period of *agouti* expression. The fact that coat color is also sensitive to the dose of *agouti* in the absence of *Corin* further demonstrates that *agouti* levels are limiting for pheomelanin production at the edges of its period of expression.

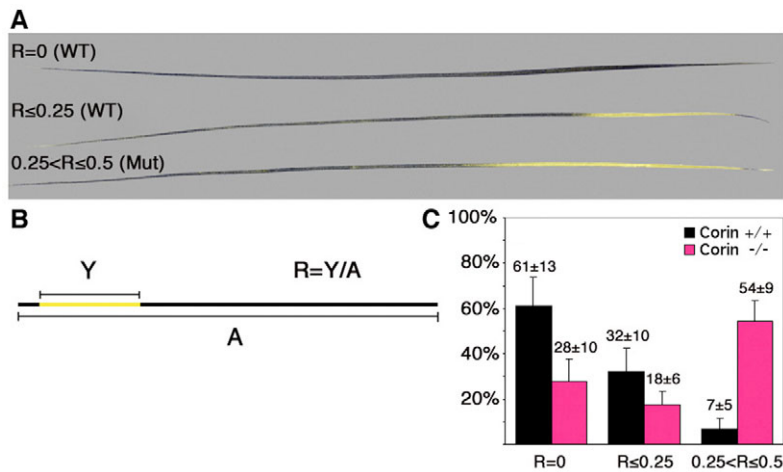


Fig. 6. Yellow band extension in *Corin* mutant awl hairs. (A) Representative examples of three categories of awl hairs used in this analysis. In contrast to zigzag hairs, awl hairs may lack a subapical yellow band. (B) The ratio (R) between the length of the yellow band (Y) and the length of the whole hair (A) was calculated and assigned to the categories: R=0, R≤0.25, 0.25<R≤0.5. (C) The distribution of awl hairs among these categories in wild-type (black) and mutant mice (pink). Although a small proportion of 0.25<R≤0.5 is present in wild-type mice, most of these fall close to R=0.25 (data not shown). The *P* values for R=0, R≤0.25 and 0.25<R≤0.5 are *P*=0.0002, *P*=0.01 and *P*<0.0001, respectively. Data are means ± s.d.

The ablation of *Corin* does not alter the timing or level of *agouti* expression, or the expression of other known components of the *agouti* signaling pathway. Instead, ablation of *Corin* unmasks cryptic *agouti* activity. This cryptic activity is revealed in in situ analysis of *agouti* expression in wild-type skin, where hair follicles containing both *agouti* transcripts in the DP and melanocytes producing eumelanin in the adjacent hair bulb are readily detected. By contrast, the deposition of pheomelanin and the expression of *agouti* transcripts in the DP are well correlated

in *Corin*^{-/-} skin. In the mutant mice, all DP with detectable *agouti* expression are embedded in hair bulbs that do not exhibit eumelanin synthesis.

The strong correlation between pheomelanin deposition and the detection of *agouti* expression in the DP in the absence of *Corin* suggests that the DP is the source of physiologically relevant *agouti* in the regulation of pigment production in the dorsal pelage. This observation further suggests that there are no other functionally significant suppressors of *agouti* protein activity in the mouse strains

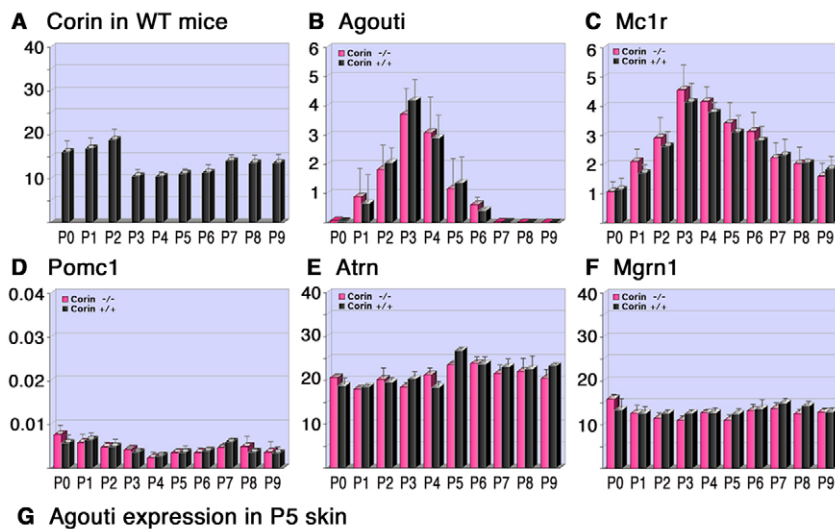


Fig. 7. Analysis of the RNA levels of *Corin*, *agouti*, *Mc1r*, *Pomc1*, *Atrn* and *Mgrn1* throughout the anagen phase of the hair cycle. (A-F) The relative RNA levels determined by real-time PCR (y-axis) of *Corin* (A), *agouti* (B), *Mc1r* (C), *Pomc1* (D), *Atrn* (E) and *Mgrn1* (F) are shown from birth to P9 (x-axis) for *Corin*^{+/+} (black) and *Corin*^{-/-} (pink) mice. The RNA levels of all genes tested were normalized to the same units, but different scales used in A,E,F vs. B,C vs. D. Thus the level of *Atrn* mRNA is about fivefold higher than the RNA levels of *agouti* at its peak (B,E), whereas *Pomc1* levels are similar to those of *agouti* at its baseline levels from P7 onwards (B,D). Data are means ± s.d. (G) In situ hybridization of *agouti* in P5 skin. Asterisks indicate hair follicle bulbs with *agouti* expression in the DP and eumelanin deposition. Note that in the absence of *Corin*, eumelanin deposition has not been detected in the bulb region surrounding *agouti*-expressing DPs. In the right upper corner, higher magnification of the region enclosed by the red square is shown.

studied that contribute to this pattern of pigment deposition, although modifiers of pigment production that act at the level of *agouti* gene expression or independently of *agouti* may also contribute to coat color in these strains. A final corollary to this conclusion is that unlike other hair types, *agouti* expression levels in guard hairs remain below biologically significant levels during the elaboration of hair shafts, which remain black despite the absence of *Corin* activity. This is substantiated by in situ analysis. *Agouti* expression was not detected in the DP of guard hair follicles from P0-P7 under conditions that detected *agouti* transcripts in awl and zigzag follicles (Fig. 7, see Fig. S6 in the supplementary material; data not shown).

Corin acts downstream of *agouti* mRNA expression

These results demonstrate that *Corin* acts downstream of *agouti* gene transcript accumulation to functionally counteract *agouti* protein activity. As a protease tethered to the surface of the DP cell, the *Corin* protein is well positioned to regulate *agouti* protein activity in several ways. One known *Corin* substrate is the prohormone *Nppa*, which when cleaved to its active form, contributes to blood pressure regulation by activating the natriuretic peptide pathway (Chan et al., 2005; Wu et al., 2002; Yan et al., 1999; Yan et al., 2000). Although we can detect the expression of the *Nppb* and *Nppc* genes that encode related natriuretic peptide precursors in the skin at low levels by PCR, neither these genes nor the signaling receptors *Npr1* and *Npr2* are detectable in the hair follicle by in situ hybridization under conditions that readily detect the robust expression of *Nppa* and *Nppb* in the heart (data not shown). Although we cannot rule out the *Npp* family as substrates that mediate this effect, alternative mechanisms seem more likely.

Among these, a direct protein-protein interaction with a component of the *agouti* pathway is a strong possibility. As secreted or transmembrane proteins, *agouti*, α -MSH (*Pomc1*), *Atrn* and *Mc1r* are all potential targets whose activity could be attenuated (*agouti* or *Atrn*) or augmented (*Mc1r* or α -MSH) by the extracellular *Corin* protease activity. Of these, α -MSH is unlikely to be the target of *Corin*. Although *Pomc1*-null mice have lighter coat color, particularly in the ventral skin, they do not resemble the dramatic effect on coat color observed in *Corin*-deficient mice (Challis et al., 2004; Yaswen et al., 1999). By contrast, the *agouti* protein is clearly limiting for the pheomelanin switch in wild-type animals (Miller et al., 1997). The fact that it is secreted from the DP, where *Corin* levels are high, also makes *agouti* an attractive candidate for modification by this protease.

The fact that the N-terminal and C-terminal domains of *agouti* bind separately and independently to *Atrn* and *Mc1r*, respectively, and the failure of these two domains to rescue in trans the activity of full-length *agouti* (He et al., 2001; Ollmann and Barsh, 1999), suggest that binding of *agouti* to both *Atrn* and *Mc1r* forms a ternary complex that is required for *agouti* signaling. If so, a proteolytic cleavage that physically separates the N-terminal domain from the C-terminal domain would not only render the *agouti* protein inactive but could also produce competitive antagonists that interfere with *agouti* binding to both *Mc1r* and *Atrn*.

Agouti binding to *Mc1r* is conformation dependent. Cis and trans conformers with markedly different abilities to compete for *Mc1r* binding are generated when the C-terminal domain of *agouti* is synthesized in vitro (McNulty et al., 2005). The fact that *agouti* protein can assume different conformations with distinct binding capabilities suggests that activity of *agouti* protein could also be modified by a proteolytic cleavage that promotes a conformational change that reduces or abolishes the affinity of *agouti* protein for either *Mc1r*, *Atrn* or both.

In ventral skin, where *agouti* levels remain high and promote pheomelanin production throughout the hair cycle, the majority of *agouti* protein detected by western analysis migrates at the position expected of the full-length form (Ollmann and Barsh, 1999). Unfortunately, the low and variable level of *agouti* protein in dorsal skin, the limited sensitivity of available reagents, and the partial nature of the predicted cleavage events prevent direct assessment of these potential cleavage models in dorsal skin in vivo.

Although mechanisms in which *Corin* acts directly on *agouti* might limit the impact of these observations on human pigmentation, where a postulated role for *agouti* in pigmentation based on linkage analysis remains controversial, they might still have other implications for human health. Ectopic expression of *agouti* in the brain induces obesity by acting on the *Mc4r* that is normally inhibited by the *agouti*-related protein (*Agrp*) (Duhl et al., 1994; Manne et al., 1995). Aspects of these two signaling pathways are distinct. First, *agouti* activity in the brain is dependent on attractin and mahogunin (He et al., 2001) whereas *Agrp* activity is not (He et al., 2003a; Ollmann et al., 1997). Second, although the C- and N-terminal domains of *agouti* must act in concert to inhibit *Mc1r* activity in vivo, binding of the C-terminal domain of *Agrp* to its receptor is sufficient for function (Ollmann et al., 1997). Furthermore, the N-terminal domain of *Agrp* inhibits this function and must be removed by proteolytic cleavage to activate *Agrp* (Creemers et al., 2006; Jackson et al., 2006). Thus, cleavage between the N- and C-terminal domains is likely to have opposite effects on *Agouti* and *Agrp* activity, but other possible proteolytic modifications may have similar effects on both pathways. Although *Corin* is not expressed in the CNS (Yan et al., 1999), it is possible that an analogous serine protease modulates the activity of *Agrp* signaling and thus participates in the process of weight control and energy balance. Whether *Corin* acts by modifying some component of *agouti* signaling or by a natriuretic peptide pathway-related mechanism that antagonizes *Mc1r* responses in melanocytes, these results identify a novel pathway to modulate *Mc1r* activity. Identifying the postulated analogous serine protease in the brain would increase the repertoire of targets for drug development and allow the exploration of new therapeutics against obesity.

Finally, variations in coat color have played a prominent role in discussions of adaptation to the environment and the mechanisms that drive evolution. Although a large number of genes contribute to pigmentation either through functions in the generation, migration or maintenance of melanocytes, biosynthesis of eumelanin, or transfer of pigment to recipient cells, comparatively few have been identified that contribute to the pheomelanin/eumelanin switch that plays such a significant role in coat appearance (<http://www.cbc.umn.edu/ifpes/micemut.htm>). We note that the *Corin* mutation reproduces the described differences in *agouti* band length seen in deer mouse populations adapted to lighter colored environments than their forest-dwelling counterparts (Hoekstra, 2006). The observations that levels of *agouti* signaling are limiting in the switch to pheomelanin production and are effectively regulated by *Corin* only when *agouti* activity is marginal identifies a novel route to the phenotypic changes associated with adaptive coloration changes. Loss-of-function mutations in *Pomc* cause a similar albeit less dramatic lightening of the dorsal pelage, but also cause obesity and adrenal insufficiency (Krude et al., 1998; Yaswen et al., 1999). Loss of *Corin* function may also incur other fitness costs, as both modest hypertension and cardiac hypertrophy are observed in mutant mice, but these costs are less dramatic (Chan et al., 2005). Furthermore, if pigmentation and cardiovascular regulation use different substrates, these two processes can be uncoupled by substrate-specificity mutations (Knappe et al., 2004). These attributes, and the lack of other genetic modifiers

downstream of agouti expression, suggest that an analysis of the relative contribution of *Corin* mutations to adaptive changes in pigmentation in wild populations will be informative.

In conclusion, these studies identify the transmembrane serine protease Corin as a novel modifier of the agouti signaling pathway that acts downstream of agouti gene expression to suppress agouti protein activity on the pheomelanin/eumelanin switch. The tight restriction of *Corin* expression to the DP during the anagen phase of the hair cycle within the skin, its relative lack of expression elsewhere and the apparent restriction of its activity to the regulation of pigment patterning, all suggest that this gene will serve as a useful platform to manipulate gene expression in the DP. This will broaden its contribution to the understanding of hair follicle biology beyond these important insights into the regulation of coat color.

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Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/135/2/217/DC1>

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