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The Developmental Role of Agouti in Color Pattern Evolution

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Animal color patterns can affect fitness in the wild; however, little is known about the mechanisms that control their formation and subsequent evolution. We took advantage of two locally camouflaged populations of *Peromyscus* mice to show that the negative regulator of adult pigmentation, *Agouti*, also plays a key developmental role in color pattern evolution. Genetic and functional analyses showed that ventral-specific embryonic expression of *Agouti* establishes a prepattern by delaying the terminal differentiation of ventral melanocytes. Moreover, a skin-specific increase in both the level and spatial domain of *Agouti* expression prevents melanocyte maturation in a regionalized manner, resulting in a novel and adaptive color pattern. Thus, natural selection favors late-acting, tissue-specific changes in embryonic *Agouti* expression to produce large changes in adult color pattern.

Variation in pigment type (i.e., color) and distribution (i.e., color pattern) can have a profound impact on the fitness of or-

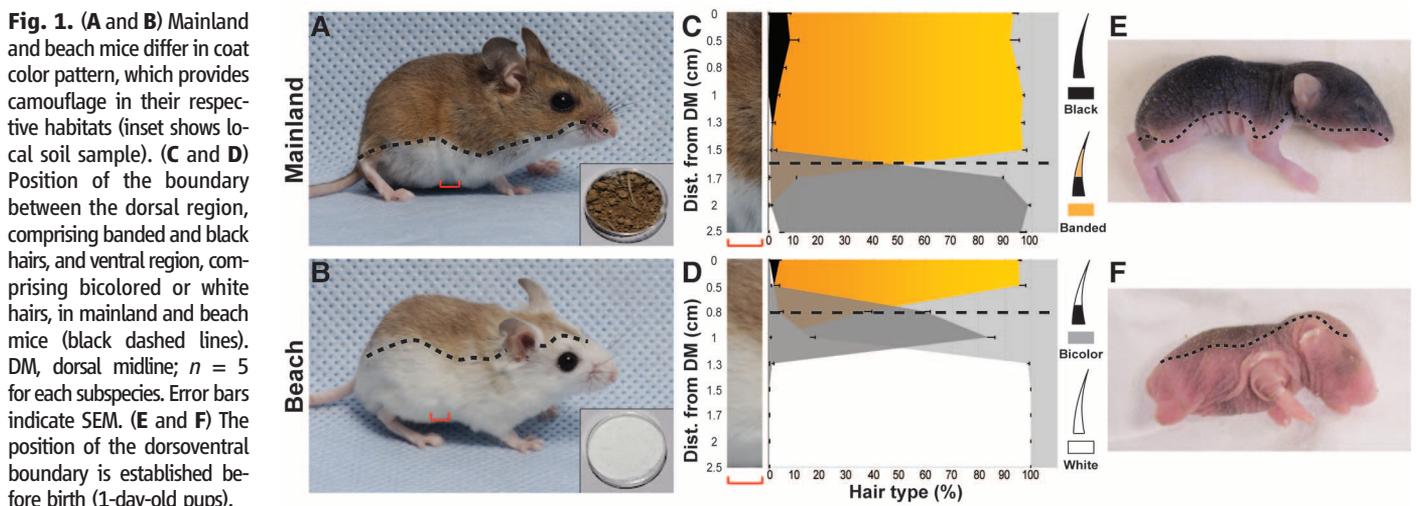
ganisms in the wild (1). In vertebrates, several genes involved in pigment type switching (2, 3) and those necessary for proper pigment pattern-

ing in mice (4, 5) and fish (4, 6, 7) have been described; however, such work has focused on laboratory mutants rather than natural variation. Therefore, the molecular factors responsible for color pattern formation and evolution (i.e., the genes and developmental processes targeted by selection) remain poorly understood in wild vertebrates.

We took advantage of the striking color pattern variation in natural populations of deer mice (genus *Peromyscus*). Mainland mice (*P. polionotus subgriseus*) inhabit oldfields with dark soil and have the most common color pattern observed in vertebrates: a dark dorsum and light ventrum

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(Fig. 1A). Beach mice (*P. p. leucocephalus*), which have recently colonized the light-colored sand dunes of Florida's Gulf Coast, have evolved adaptive differences in color (i.e., lighter overall pigmentation) and pattern (i.e., absence of pigmentation on the face, flanks, and tail) relative to their mainland ancestors (Fig. 1B) (8, 9).

We characterized these differences in adult pigment pattern of mainland and beach mouse subspecies by classifying hair into four distinct types according to the distribution of pigments

along individual hairs and quantifying the proportion of each type along the dorsoventral axis (10). Although both subspecies have all types of hair, their distribution differs: The dorsal region, which has black and banded hairs, is reduced in beach mice (i.e., the dorsoventral boundary is shifted upward) and the hairs in their ventral region entirely lack pigments, whereas mainland mice have bicolored ventral hairs (i.e., melanic base, unpigmented tip) (Fig. 1, C and D). These subspecific differences in pigment pattern are visible at

birth (Fig. 1, E and F), which indicates that they are established during embryonic development.

Mutations in three genetic loci explain most of the pigment variation in adult pelage between beach and mainland mice (11). We focused on the locus containing the candidate pigmentation gene *Agouti* because in laboratory mice, ventral *Agouti* expression is necessary for the establishment of dorsoventral differences in pigmentation (5, 12–14). Although the developmental mechanism through which *Agouti* acts to establish these color differences remains unclear, it may contribute to color pattern evolution in natural populations.

We used a genetic approach to confirm that *Agouti* is a causal gene responsible for color pattern differences between beach and mainland mice (Fig. 2A and fig. S1) (10). Because there were no differences in *Agouti* protein sequence between beach and mainland mice (11), we measured the allele-specific expression of *Agouti* in the two tissues, skin and testis, where it is expressed in *Mus* (15). We found that *Agouti* expression is higher in the ventral skin of beach mice relative to mainland mice (Fig. 2B). In F₁ hybrids, the beach mouse (light) allele shows significantly higher expression than the mainland (dark) allele (factor of ~17, *P* = 0.01, one-tailed Student's *t* test; Fig. 2B). This expression level difference is replicated but smaller in dorsal skin (factor of ~4, *P* = 0.015, one-tailed Student's *t* test; fig. S2). By contrast, no *Agouti* expression differences were detected in the testes (Fig. 2C). These data show that mutation(s) in *Agouti* are cis-acting and likely involve a skin-specific regulatory element.

To determine the specific effects of these *Agouti* expression differences on color pattern, we generated *Peromyscus* individuals homozygous for the light allele of *Agouti* (*Agouti LL*) and dark alleles at the two other implicated pigment loci (10). Adult *Agouti LL* mice displayed both an upward shift in the dorsoventral boundary and white ventral hairs (Fig. 2, D and E, and fig. S2), thereby partly recapitulating the derived color pattern of wild beach mice. Because these differences are apparent at birth (fig. S2), changes in *Agouti* expression pattern contribute to changes in pigment pattern through developmental modifications.

We next described typical stages of *Peromyscus* development (fig. S3) and compared the embryonic expression patterns of dark and light *Agouti* alleles. In embryos from mainland mice, *Agouti*'s expression was restricted to the ventral half of the dermis in early developmental stages (Fig. 3, A and B) and to the ventral dermis and hair follicles at fetal stages (Fig. 3, C and D). Thus, *Agouti*'s expression domain is tightly correlated with the light-colored ventrum in adult skin. This suggests that the color pattern is spatially determined early in embryonic development by a prepattern established by *Agouti*. By comparison, in *Agouti LL* embryos, the ventral expression of *Agouti* showed an upward shift (Fig. 3F) that corresponds to the dorsal displacement of the pigment boundary observed in adult mice. In

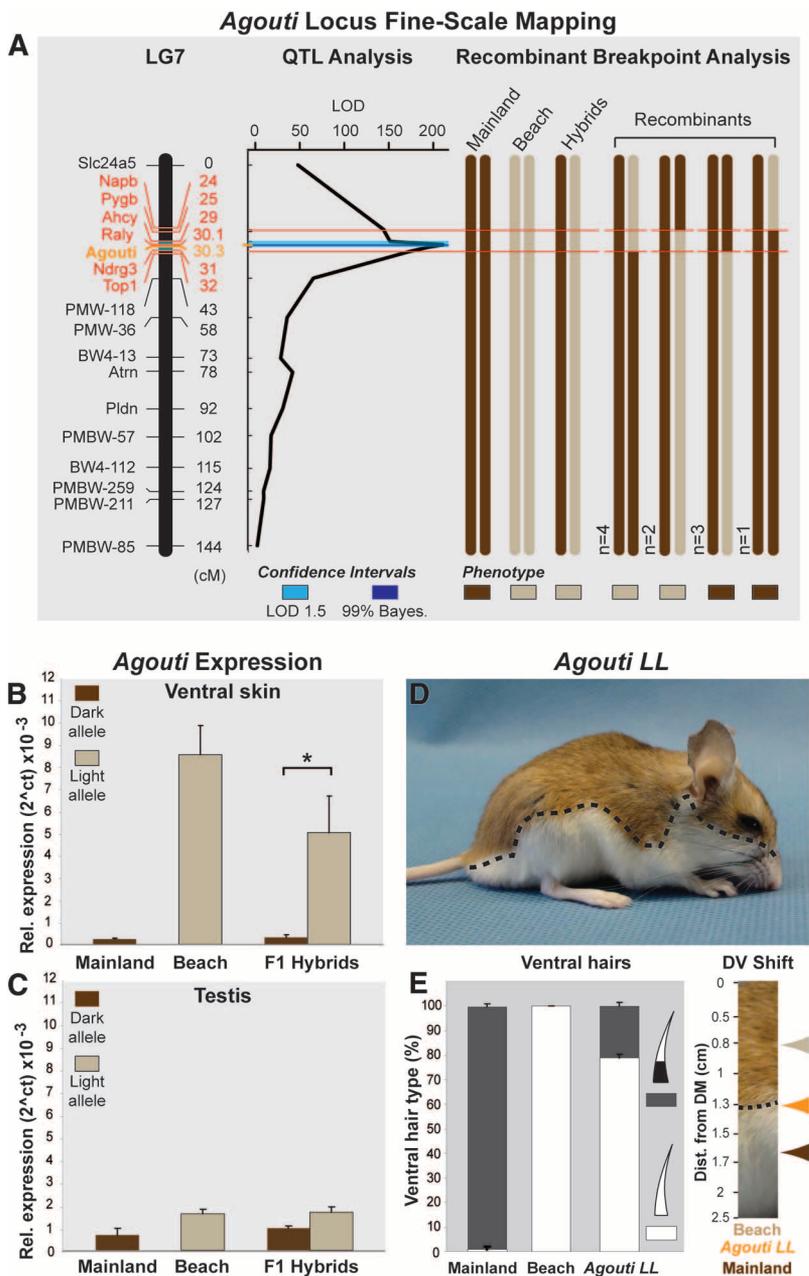


Fig. 2. (A) Fine-scale mapping of the causal locus in *Peromyscus* by quantitative trait loci (QTL) (left) and recombinant breakpoint analyses (right). (B and C) Quantitative polymerase chain reaction (qPCR) analyses of *Agouti* mainland (dark) and beach (light) allele transcript levels in the ventral skin and testes of mainland mice, beach mice, and their F₁ hybrids (*n* = 3 to 6 for each strain) (2^{-Δct} is the inferred difference in transcript level of *Agouti* relative to the control gene β-actin). (D) Coat color pattern of *Agouti LL* mice. (E) Pigment of ventral hairs and position of dorsoventral (DV) boundary in mainland, beach, and *Agouti LL* mice. DM, dorsal midline; *n* = 5 for each strain. Error bars indicate SEM.

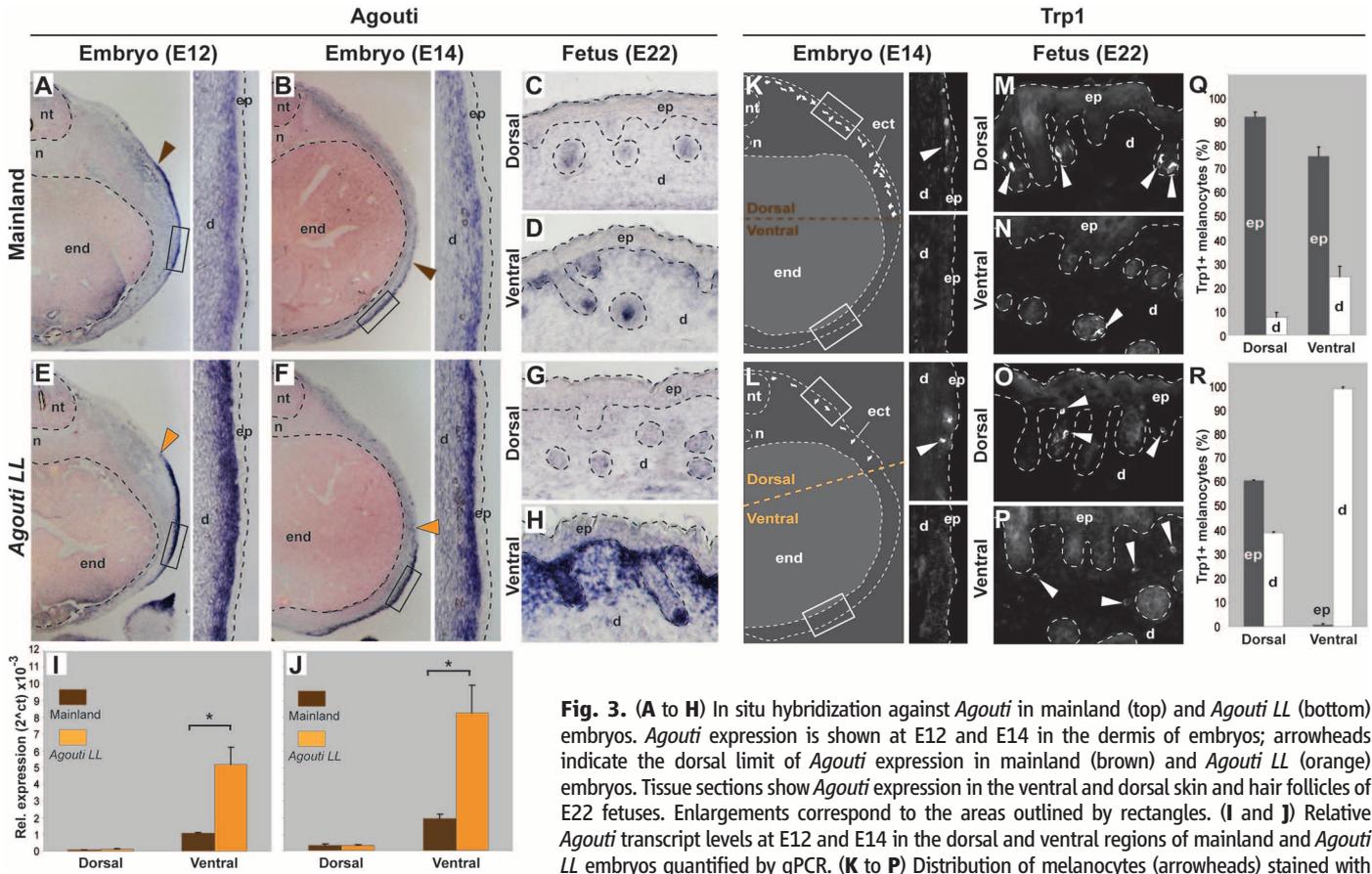


Fig. 3. (A to H) In situ hybridization against *Agouti* in mainland (top) and *Agouti LL* (bottom) embryos. *Agouti* expression is shown at E12 and E14 in the dermis of embryos; arrowheads indicate the dorsal limit of *Agouti* expression in mainland (brown) and *Agouti LL* (orange) embryos. Tissue sections show *Agouti* expression in the ventral and dorsal skin and hair follicles of E22 fetuses. Enlargements correspond to the areas outlined by rectangles. (I and J) Relative *Agouti* transcript levels at E12 and E14 in the dorsal and ventral regions of mainland and *Agouti LL* embryos quantified by qPCR. (K to P) Distribution of melanocytes (arrowheads) stained with antibody to Trp1 (in white) along the dorsoventral axis in transverse sections at E14 (schemes (K and L) and (M and O) show the dorsal/ventral pigment boundary (dotted lines)). (Q and R) Relative proportions of Trp1⁺ melanocytes within the dermal or the epidermal compartments at E22. Error bars indicate SEM. nt, neural tube; n, notochord; end, endoderm; ect, ectoderm; d, dermis; ep, epidermis.

based on embryos in fig. S5) and E22 relative to the future position of the dorsoventral pigment boundary (dotted lines). (Q and R) Relative proportions of Trp1⁺ melanocytes within the dermal or the epidermal compartments at E22. Error bars indicate SEM. nt, neural tube; n, notochord; end, endoderm; ect, ectoderm; d, dermis; ep, epidermis.

addition, ventral *Agouti* expression was significantly higher in *Agouti LL* than in mainland embryos [by a factor of ~4.9 at embryonic day 12 (E12) and by a factor of ~4.4 at E14, $P=0.03$ and $P=0.003$, respectively; one-tailed Student's *t* tests] (Fig. 3, I and J); these differences were allele-specific (fig. S2) and correlated with the presence or absence of adult pigmentation in the ventrum. These findings suggest that modifications in the embryonic prepattern defined by *Agouti* contribute to color pattern evolution in beach mice.

In vitro studies suggested that *Agouti* may also cause melanocyte dedifferentiation by down-regulating pigment cell-specific genes (16–18). We tested how *Agouti* expression changes affected melanocyte behavior in vivo by comparing the distribution and maturation of melanocytes during *Peromyscus* embryogenesis. We used Trp2 (also known as Dct) and Trp1, two enzymes consecutively expressed in melanocytes during both their migration in the dermis and maturation in hair follicles, as markers of early and late differentiation, respectively (19). In both mainland and *Agouti LL* E14 embryos, Trp2⁺ melanocytes had colonized the entire embryonic dermis (fig. S4), which demonstrates that the formation of dorsoventral color differences and the evolu-

tion of the novel color pattern are not caused by changes in melanocyte migration. By contrast, fully differentiated (Trp1⁺) melanocytes were restricted to a dorsal region complementary to the ventral domain of *Agouti* expression (Fig. 3, K and L, and fig. S5), which suggests that their distribution early in development is restricted by the extent of *Agouti* expression.

During late fetal stages, Trp2⁺ cells successfully colonized hair follicles in the dorsum, but in the ventrum they were confined to the dermis (fig. S4) and were fewer in number and proliferated less (fig. S6); therefore, melanocyte differentiation and proliferation were impaired in this region. Dorsal Trp1⁺ melanocyte behavior in *Agouti LL* fetuses was similar to that observed in mainland mice (Fig. 3, M and O). However, in the ventrum, Trp1⁺ melanocytes were present but did not reach the epidermal compartment or hair follicles, as they did in mainland fetuses (Fig. 3, N and P), and thus remained similar in distribution to less mature (Trp2⁺) melanocytes (fig. S4). These results suggest that increased ventral expression levels of *Agouti* repress the terminal differentiation of ventral melanocytes and their colonization into the epidermis, and that this is the developmental mechanism by which the

absence of pigmentation in the beach mouse ventrum and flanks evolved.

To functionally test *Agouti*'s embryonic role in vivo, we took advantage of a natural strain of *Peromyscus* ("non-*Agouti*," NA) in which a large deletion in the *Agouti* locus results in a loss of function (20). NA mice, as in *Mus musculus Agouti* mutants (21), displayed no visible patterning, with a homogeneously black color (Fig. 4A) present at birth (Fig. 4B). This observation confirms that *Agouti* is necessary for establishing color pattern in *Peromyscus*. The melanocytes in NA embryos expressed both Trp2 and Trp1 in the ventral dermis (Fig. 4D and fig. S7), and, at fetal stages, Trp1⁺ cells localized in the hair follicles (Fig. 4F) to produce pigments (fig. S8) similar to dorsal melanocytes (Fig. 4E), whereas Trp2 expression was no longer detectable (fig. S7). These results, consistent with previous in vitro studies (17, 18), clearly demonstrate in vivo that *Agouti* represses the terminal maturation of Trp1⁺/Trp2⁺ melanocytes in the ventral embryonic skin.

To further understand *Agouti*'s function during development, we used ultrasound-assisted retroviral infection in utero to ectopically express *Agouti* in the hair follicles of mainland embryos (22). Embryos collected 10 days after injection

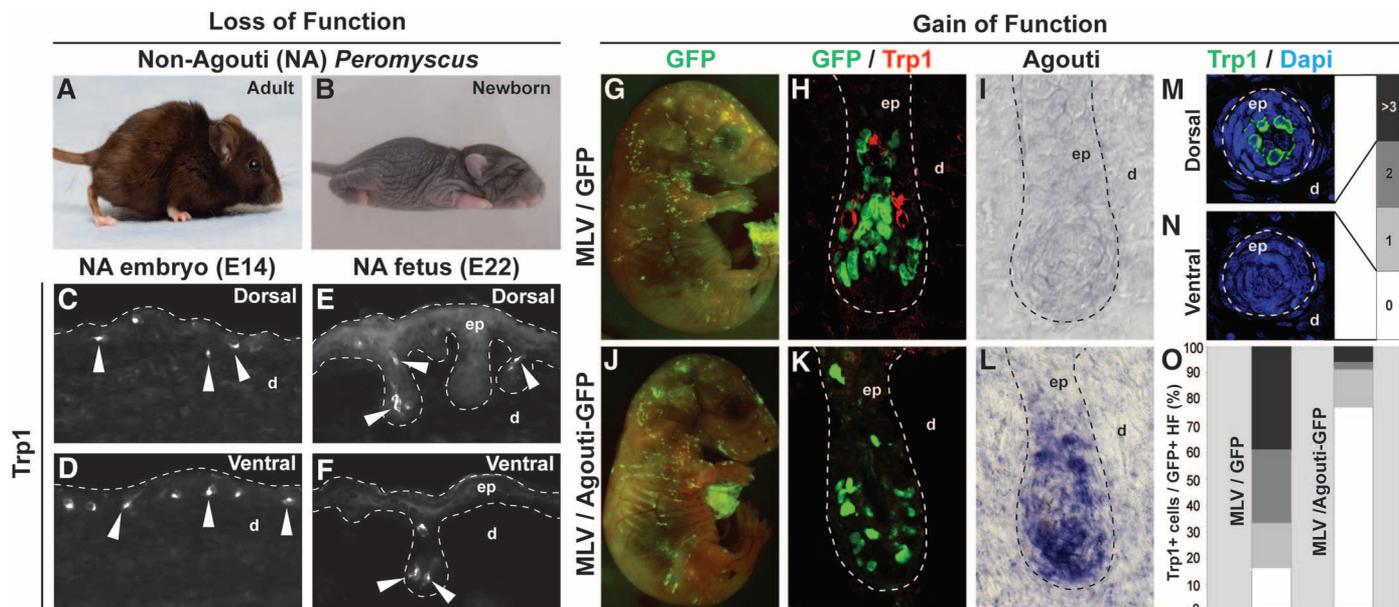


Fig. 4. (A) Adult non-Agouti (NA) *Peromyscus* mice have a homogeneously black coat. (B) Lack of dorsoventral color difference is visible at birth. (C to F) Dorsal and ventral views of NA skins at E14 and E22 stained with a Trp1 antibody (arrowheads). (G to L) Transgenic expression of murine leukemia retroviruses (MLVs) coding for the nuclear GFP-only or the *Peromyscus Agouti* gene with the nuclear GFP are shown in whole-mount embryos or transverse views of dorsal GFP⁺ hair follicles stained with GFP (in green) and Trp1 (in red).

In (I) and (L), robust ectopic expression of *Agouti* is detected in dorsal hair follicles infected with the GFP/*Agouti* virus but is absent from the control, GFP⁺, dorsal hair follicles. (M and N) Dorsal and ventral hair follicles (stained with the nuclei marker Dapi in blue) containing typical numbers of Trp1⁺ melanocytes (in green). (O) Percentage of GFP⁺ hair follicles (HF) containing 0, 1, 2, or >3 Trp1⁺ cells for the control (left) and the GFP/*Agouti* (right) viruses. d, dermis; ep, epidermis.

(10) displayed a robust ectopic expression of *Agouti* in all neural-derived GFP⁺ (green fluorescent protein-positive) cell lineages, including melanocytes and epidermal cells of the hair follicle wall (Fig. 4, G to L). GFP⁺ melanocytes were detected in both the dorsal and ventral parts of the fetal skin (Fig. 4, G and J), confirming that *Agouti* does not interfere with dorsal-ventral melanocyte migration. In the dorsum, many Trp1⁺ melanocytes were present in hair follicles infected with viruses containing control GFP only (Fig. 4, H and O), whereas their numbers decreased in mice infected with the virus expressing *Agouti* (Fig. 4, K to O). This finding confirms that higher expression of *Agouti* prevents melanocytes from undergoing terminal differentiation in the epidermis.

Our results indicate that the level and extent of *Agouti* expression during development affects adult color pattern by modulating the degree of repression of a terminal step in melanocyte differentiation. In mainland mice, where *Agouti* is expressed at low levels in the ventrum, ventral melanocyte differentiation is delayed, which leads to the formation of partially pigmented (bicolored) hairs (fig. S8). In beach mice, changes in *Agouti* expression contribute to the evolution of their novel and adaptive color pattern. Specifically, in *Agouti LL* individuals, the expression of *Agouti* in a new spatial domain causes an upward shift in the pigment boundary, and an increase in its expression level completely prevents ventral melanocyte maturation, leading to an absence of pigment production in ventral hairs.

Although *Agouti*'s role in adult pigmentation and its pleiotropic effects on obesity (2, 3, 23) have been well described, our study has identified a developmental mechanism through which the region-specific expression of *Agouti* controls the distribution of pigments across the body. Here, *Agouti* establishes an embryonic prepattern that subsequently evolved through skin-specific changes to *Agouti* expression, which in turn affect the late stages of pigment cell differentiation, thereby minimizing pleiotropy in two ways. Because some minimally pleiotropic developmental loci might constitute "hotspots" for morphological evolution (24–27), one may speculate that even small changes in *Agouti* expression during embryogenesis contribute to the establishment of more complex vertebrate pigment patterns.

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Supporting Online Material

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