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Running head: *Agouti* and color pattern evolution

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 demonstrate that ventral-specific embryonic expression of *Agouti* establishes a pre-pattern 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 profoundly impact fitness of organisms in the wild (*1*). In vertebrates, several genes involved in pigment-type switching (*2, 3*) and those necessary for proper pigment patterning in mice (*4, 5*) and fish (*4, 6, 7*) have been described; however, this 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.

Here we take 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 (Fig. 1A). Beach mice (*P. p. leucocephalus*), which have recently colonized the light-colored sand dunes of the 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 a mainland and beach mouse subspecies by classifying hair into four distinct types (based on the distribution of pigments along individual hairs; *10*) and quantifying the proportion of each type along the dorso-ventral axis. 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 dorso-ventral boundary is shifted upwards) and their ventral region is comprised of hairs entirely lacking pigments compared to mainland mice, which have bi-colored hairs (i.e., melanic base, un-pigmented tip) (Fig. 1C, D). These subspecific differences in pigment pattern are visible at birth (Fig. 1E, 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 dorso-ventral differences in pigmentation (*5*, *12, 13, 14*). While 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 confirmed that *Agouti* is a causal gene responsible for color pattern differences between beach and mainland mice using a genetic approach (Fig. 2A; fig. S1; *10*). Because there were no differences in Agouti protein sequence between beach and mainland mice (*11*), we measured 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 compared to mainland mice (Fig. 2B). In F1 hybrids, the beach mouse (light) allele shows significantly higher expression than the mainland (dark) allele (~17 fold, p=0.01, one-tailed Student's T-test; Fig. 2B). This expression level difference is replicated but smaller in dorsal skin (~4 fold, 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 dorso-ventral boundary and white ventral hairs (Fig. 2D, E; 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. 3A, B) and to the ventral dermis and hair follicles at fetal stages (Fig. 3C, D), which 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 pre-pattern 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 addition, ventral *Agouti* expression was significantly higher in *Agouti LL* than in mainland embryos (~4.9 fold at E12 and ~4.4 fold at E14, $p=0.03$ and $p=0.003$, respectively, one-tailed Student's T-tests; Fig. 3I, J); these differences were allele-specific (fig. S2) and correlated with the presence or absence of adult pigmentation in the ventrum. These results suggest that modifications in the embryonic pre-pattern 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, 17, 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-positive ($Trp2^+$) melanocytes had colonized

the entire embryonic dermis (fig. S4), demonstrating that the formation of dorso-ventral color differences and the evolution 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. 3K, L; fig. S5), suggesting 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 both fewer in number and proliferated less (fig. S6), showing that 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. 3M, 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. 3N, 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 of 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 homogenously 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; 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), while Trp2 expression was no longer detectable (fig. S7). These results, consistent with previous *in vitro* studies (*18, 17*), 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 ectopically expressed Agouti in hair follicles of mainland embryos using ultrasound-assisted retroviral infection *in utero* (*22*). Embryos collected 10 days after injection (*10*) displayed a robust ectopic expression of Agouti in all neural derived GFP^+ cell lineages, including the melanocytes, and epidermal cells of the hair follicle wall (Fig. 4, G-L). GFP^+ melanocytes were detected in both the dorsal and ventral parts of the fetal skin (Fig. 4G, J), confirming that Agouti does not interfere with dorsal-ventral melanocyte migration. In the dorsum, while many $Trp1^+$ melanocytes were present in hair follicles infected with viruses containing control GFP only (Fig. 4H, O), their numbers decreased in mice infected with the virus expressing Agouti (Fig. 4K-O). This 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 (bi-colored) 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.

While *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 pre-pattern, which 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 (*25, 26*, *27, 28*), 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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29. We thank R. Kanadia, C. Punzo, E. McGlinn, J. Gros, A. Abzhanov and C. Tabin for technical assistance and G. Barsh for helpful discussions. *In situ* hybridizations on sections and *in utero* injections were conducted in the Abzhanov and Tabin/Cepko laboratories. V. Hearing provided the anti-Trp1/anti-Trp2 antibodies. C. Desplan, C. Tabin, A. Abzhanov, C. Linnen and J. Gros commented on the manuscript. V.S.D was supported by the Portuguese Foundation for Science & Technology. Research was funded by a grant from the National Science Foundation.

Supporting Online Material
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Materials and Methods
Figs. S1, S2, S3, S4, S5, S6, S7, S8

Figures Legends

Fig. 1. (**A**, **B**) Mainland and beach mice differ in coat-color pattern, which provides camouflage in their respective habitats (inset = local soil sample). (**C**, **D**) Position of the boundary between the dorsal region, comprised of banded and black hairs, and ventral region, comprised of bi-colored or white hairs, in mainland and beach mice (black dotted lines). (**E**, **F**) The position of the dorso-ventral boundary is established before birth (1 day old pups). Error bars indicate standard error of the mean (SEM).

Fig. 2. (**A**) Fine-scale mapping of the causal locus in *Peromyscus* by quantitative trait loci (QTL) (left) and recombinant-breakpoint analyses (right). (**B**, **C**) Quantitative PCR (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. (**D**) Coat-color pattern of *Agouti LL* mice. (**E**) Pigment of ventral hairs and position of dorso-ventral boundary in mainland, beach and *Agouti LL* mice. Error bars indicate SEM.

Figure 3. (**A** to **H**) *In situ* hybridization against *Agouti* in mainland (top) and *Agouti LL* (bottom) embryos. *Agouti* expression 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 area outlined by squares. (**I, 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 Trp1 (in white) along the dorso-ventral axis in transverse sections at E14 (schemes based on embryos in fig. S5) and E22 relative to the future position of the dorso-ventral pigment boundary (dotted lines). (**Q, 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; d, dermis; ep, epidermis.

Fig. 4. (A) Adult Non-Agouti (NA) *Peromyscus* mice have a homogeneously black coat. (**B**) Lack of dorso-ventral 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. (**G** to **L**) Transgenic expression of retroviruses 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). (**I**, **L**) Robust ectopic expression of *Agouti* detected in dorsal hair follicles infected with the GFP/Agouti virus, but absent from the control, GFP^+, dorsal hair follicles. (**M**, **N**) Dorsal and ventral hair follicles (Dapi in blue) containing typical numbers of $Trp1^+$ melanocytes (in green). (**O**) Percentage of GFP^+ hair follicles containing 0, 1, 2, or >3 $Trp1^+$ cells for the control (left) and the GFP/Agouti (right) viruses. d, dermis; ep, epidermis.