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# Notch1 and Notch2 Receptors Influence Progressive Hair Graying in a Dose-Dependent Manner

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**The Notch signaling pathway is involved in diverse biological processes such as cell fate decisions or stem cell maintenance. In this study, we assessed the role of this pathway for melanocyte development and hair pigmentation using *RBP-Jκ*, *Notch1*, and *Notch2* conditional knockout mice. Disruption of the Notch pathway by inactivating RBP-Jκ in the melanocyte lineage using *Tyr::Cre* mice led to a severe coat color dilution. Similarly, hair graying was observed when Notch1 and/or Notch2 receptors were ablated in melanocytes. This phenotype was proportional to the number of floxed *Notch* alleles, with the most pronounced effect seen in *Tyr::Cre/°*; *Notch1*$^{flox/flox}$; *Notch2*$^{flox/flox}$ mice. Deletion of *Notch1* and/or *Notch2* in melanoblasts did not induce a congenital defect. The number of *Dct*-expressing cells at embryonic stages was not affected, but melanocytes located within the hair matrix progressively disappeared during the first regeneration of the hair follicle. In contrast, non-follicular melanocytes and pigmentation in the dermis and in the choroid were not affected. We suggest that both Notch1 and Notch2 receptors contribute to the maintenance of melanoblasts and melanocyte stem cells, and are essential for proper hair pigmentation.** ***Developmental Dynamics 236:282–289, 2007.*** **© 2006 Wiley-Liss, Inc.**

**Key words:** melanocyte; notch; pigmentation; coat color; transgenic; knockout; hair greying; hair graying; stem cell

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## INTRODUCTION

Melanocytes are neural crest–derived pigment cells that migrate into skin and hair follicles during embryogenesis. Additionally, melanocytes are also found in the eye (choroid, ciliary body, iris), in the inner ear (cochlea), and in leptomeninges. In mouse hair follicles, differentiated and transient amplifying melanocytes are mainly found in the bulb, while the bulge region is believed to contain melanocyte stem cells (Nishimura et al., 2002; Reya and Clevers, 2005). Indeed, hair follicles are in a continuous cycle, alternating periods of growth (anagen), regression (catagen), and rest (telogen). During the anagen phase, melanocytes arising from the bulge migrate along the ORS (outer root sheath) to colonize the hair matrix. Hair graying has been demonstrated to be caused by defective self-maintenance of melanocyte stem cells in the bulge (reviewed in Steingrimsson et al., 2005). Recently, Nishimura et al. (2005) described two mechanisms that mediate this melanocyte precursor depletion. In *Bcl2*$^{-/-}$ mice, abrupt loss of melanoblasts between postnatal day (P)6.5 and P8.5 is due to selective apoptosis of melanocyte stem cells in the bulge at their entry into the dormant state. In contrast, premature differentiation at early-mid-anagen of the third hair cycle causes a gradual decrease of me-

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lanocyte stem cells in *Mitf*$^{vit/vit}$ mutants (Nishimura et al., 2005).

While the Notch signaling pathway has been described to play a role in the development of most tissues (Nam et al., 2002; Nickoloff et al., 2003), only little is known about its role in the pigmentary system. Notch signaling is a conserved cell-interaction mechanism where both receptors and ligands are single-pass transmembrane proteins (Greenwald, 1998). In mammals, four Notch receptors (Notch1–4) and five ligands (Jagged-1 and 2, and Delta-like [Dll] 1, 3, and 4) have been described (reviewed in Radtke et al., 2005). Ligand binding to the Notch receptor results in two proteolytic cleavages. The first one occurs extracellularly, and is mediated by a metalloprotease of the ADAM family (TACE, tumor necrosis factor-α-converting enzyme). This renders the transmembrane domain susceptible to the second cleavage, which is achieved by a protein complex with γ-secretase activity (presenilin, nicastrin, APH1, and PEN2). The released intracellular domain of Notch (NIC) then translocates to the nucleus, where it binds to CSL transcription factors (CBF1 in human, Suppressor of Hairless in *Drosophila* and LAG in *C. elegans*, also known as RBP-Jκ in mouse) and thus activates the transcription of bHLH (basic helix-loop-helix) repressors of the HES (Hairy/Enhancer of Split) family. In addition to apoptosis and cell proliferation (Artavanis-Tsakonas et al., 1999), the Notch signaling pathway plays an important role in self-renewal of stem cells (reviewed in Radtke et al., 2005). Moreover, it is involved in cell differentiation in diverse tissues, by either influencing binary cell fate decisions of progenitor cells or inducing terminal differentiation of a particular cell lineage (Lai, 2004; Hansson et al., 2004; Lundkvist and Lendahl, 2001; Gaiano and Fishell, 2002; Ge et al., 2002).

In the pigmentary system, members of the Notch signaling pathway are expressed in melanocytes and seem to be upregulated in melanoma cell lines (Nickoloff et al., 2005; Hoek et al., 2004). In addition, expression of *Notch1* and *Notch2* as well as of several ligands has been detected in vibrissa follicles from embryonic to adult stages (Powell et al., 1998; Favier et al., 2000). During follicle morphogenesis, *Notch1* is first expressed in the epithelial cells of the hair plug, in the dermal condensation, as well as in the interfollicular epidermis. Later on, while it is absent in the inner root sheath (IRS) and the dermal papilla, *Notch1* expression can be detected in the epidermis and the suprabasal layers of the outer root sheath (ORS). As for *Delta1* and *Notch2*, their expression is complementary at embryonic stages, with the ligand being expressed in the dermal condensation, and the receptor in the interfollicular dermis. Then, while no specific expression pattern can be detected for *Notch2*, adult vibrissa follicles in anagen express *Notch1* in epithelial cells of the ORS and the hair matrix, with the exception of cells above the dermal papilla. In contrast, all cells above the papilla and of the hair matrix express *Notch1* in catagen.

Recently, conditional deletion of RBP-Jκ in melanocytes has been shown to cause elimination of melanoblasts and melanocyte stem cells, and to result in hair graying (Moriyama et al., 2006). Additional experiments suggested that Notch signaling via *Hes1* plays a critical role in the maintenance of melanoblasts by preventing apoptosis. In the present study, we now dissected the Notch signaling pathway by specifically deleting *Notch1* and/or *Notch2* in melanocytes, and demonstrated the indispensable and dose-dependent effect of both receptors in the maintenance of melanocyte-mediated hair pigmentation. In addition, non-follicular melanocytes that are found in the dermis as well as in the choroid of the eye were not affected by the absence of Notch1 and Notch2 receptors.

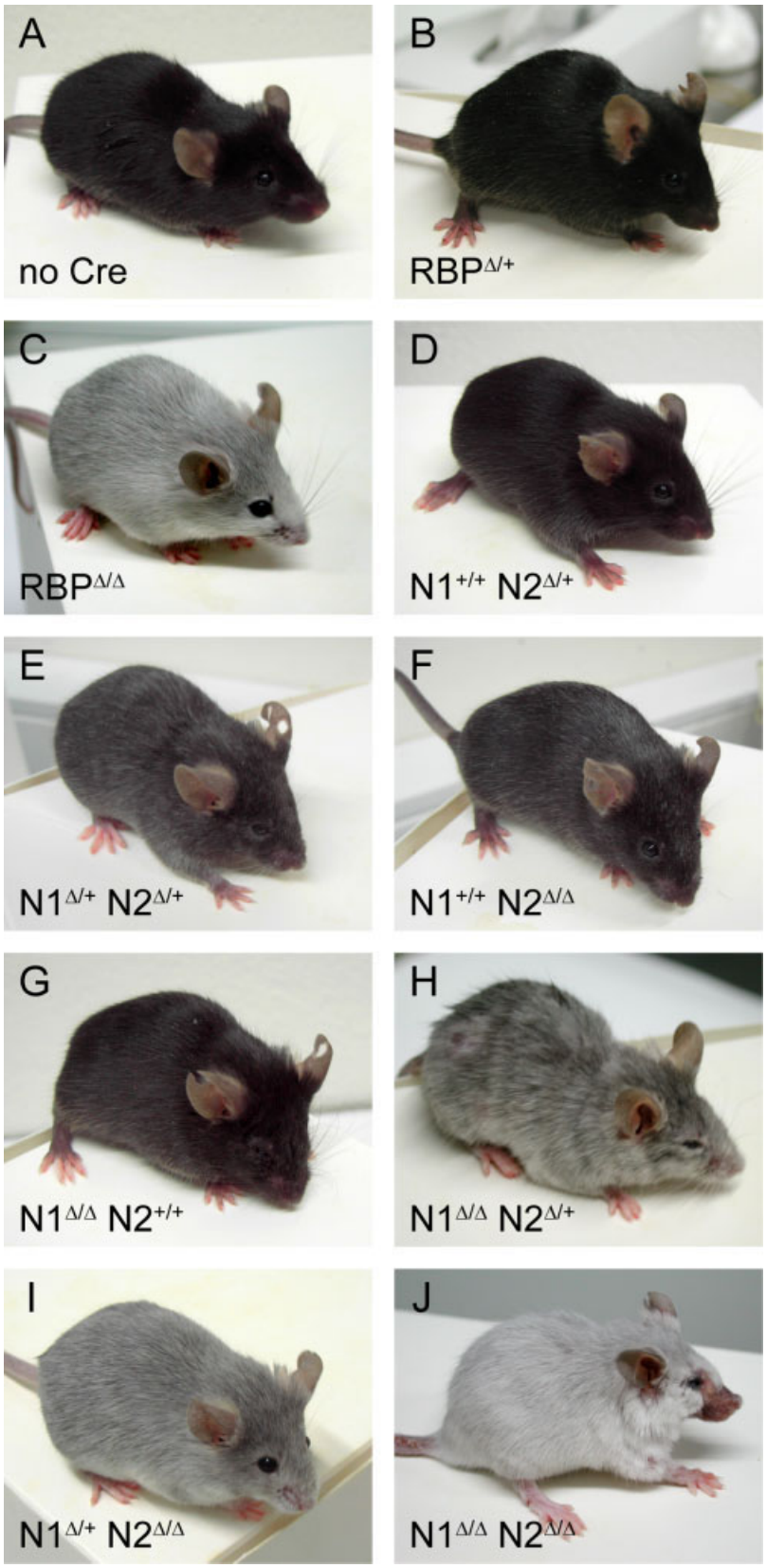
## RESULTS

### Conditional Deletion of *Notch1* and *Notch2* Alleles in the Melanocyte Lineage Results in a Dose-Dependent Hair Graying

Since constitutive deletions of RBP-Jκ, Notch1, or Notch2 lead to embryonic lethality (reviewed in Louvi et al., 2006), mice carrying floxed *RBP-Jκ* (Tanigaki et al., 2002), *Notch1* (Radtke et al., 1999), and *Notch2* (Besseyrias et al., unpublished data) alleles were mated to mice expressing Cre recombinase specifically in the melanocyte lineage (*Tyr::Cre*, Delmas et al., 2003). The artificial *tyrosinase* promoter used in *Tyr::Cre* mice is active in melanoblasts from embryonic day (E)10.5. In the skin, expression is not only found in hair follicles (bulb) or epidermis, but equally in melanocytes/melanoblasts in the bulge region (Yajima et al., 2006). Expression in the eye is confined to the choroidal melanocytes, but rather absent in the retinal pigment epithelium (Camacho-Hubner and Beermann, 2001; Porter et al., 1999).

Melanocyte-specific deletion of RBP-Jκ, which mediates Notch signaling of all Notch receptors, resulted in a gradual coat color dilution that finally led to almost complete hair whitening compared to control (Fig. 1A and C; Moriyama et al., 2006). However, recombination of one *RBP-Jκ* allele was not sufficient to induce this phenotype (Fig. 1B). Similarly, Cre/loxP-mediated recombination of the *Notch1* and *Notch2* alleles caused an obvious hair graying, which, in addition, appeared to be dose-dependent since it was influenced by the number of intact *Notch1* and *Notch2* alleles. At the same age (8 weeks), no hair graying was observed when only one *Notch* allele was absent in melanocytes (Fig. 1D). In contrast, dispersed gray hairs were discernible when two *Notch* alleles are floxed in *Tyr::Cre/°; Notch1*$^{flox/+}$*; Notch2*$^{flox/+}$ (Fig. 1E) and *Tyr::Cre/°; Notch1*$^{+/+}$*; Notch2*$^{flox/flox}$ mice (Fig. 1F). *Tyr::Cre/°; Notch1*$^{flox/flox}$*; Notch2*$^{+/+}$ mice (Fig. 1G) equally developed a hair-graying phenotype with the first gray hairs only visible at 12 weeks (not shown). Recombination of an additional *Notch* allele, in *Tyr::Cre/°; Notch1*$^{flox/flox}$; Notch2$^{flox/+}$ or *Tyr::Cre/°; Notch1*$^{flox/+}$*; Notch2*$^{flox/flox}$ mice, led to a more pronounced effect, characterized by a completely gray coat (Fig. 1H and I). Hair graying was only slightly different between the two genotypes, with *Tyr::Cre/°; Notch1*$^{flox/+}$*; Notch2*$^{flox/flox}$ mice being a little lighter than *Tyr::Cre/°; Notch1*$^{flox/flox}$*; Notch2*$^{flox/+}$ mice at the same age. Pigmentation of hairs was even more reduced in the

absence of both Notch1 and Notch2 (*Tyr::Cre/°; Notch1$^{flox/flox}$; Notch2$^{flox/flox}$*, Fig. 1J). Here, the coat was nearly white, resembling mice with melanocyte-specific deletion of RBP-Jκ (Fig. 1C).




## The Coat Color Dilution Caused by the Absence of Notch1 and Notch2 in Melanocytes Increases With the Age of Mice

Time-dependence of hair graying was equally observed in the different genotypes. This is illustrated in Figure 2 for *Tyr::Cre/°; Notch1$^{flox/+}$; Notch2$^{flox/flox}$* mice where variations of the phenotype were easily observable. The color of the first coat was slightly affected by the deletion of Notch1 and Notch2. While mutant newborns were not distinguishable from controls at 7 days (Fig. 2A), a weak coat color dilution could be observed at 3 weeks (Fig. 2B). Then, graying increased with each hair cycle, as observed at 7 weeks (Fig. 2C), and 16 and 23 weeks (Fig. 2D and E). After 7 months (31 weeks), the coat was completely white (Fig. 2F). Such hair graying was characterized by the appearance of an increasing amount of gray and white hairs (Fig. 2G). This progressive dilution of coat color was reflected by the relative melanin con-

**Fig. 1.** Notch signaling by RBP-Jκ in melanocytes is crucial for the maintenance of hair pigmentation. Melanocyte-specific disruption of the Notch signaling pathway by removing RBP-Jκ leads to hair graying (**C**, *Tyr::Cre/°; RBP-Jκ$^{flox/flox}$*). Nevertheless, Cre/loxP-mediated recombination of one *RBP-Jκ* allele is not sufficient to affect black hairs (**B**, *Tyr::Cre/°; RBP-Jκ$^{flox/+}$*). The coat color is equally affected by the deletion of Notch1 and Notch2 receptors in melanocytes. Moreover, this effect of Notch signaling is dose-dependent since three intact alleles of *Notch1* and *Notch2* are required for proper hair pigmentation (**D**, *Tyr::Cre/°; Notch2$^{flox/+}$*). As compared with control (**A**), conditional deletion of two *Notch* alleles in *Tyr::Cre/°; Notch1$^{flox/+}$; Notch2$^{flox/+}$* (**E**) and *Tyr::Cre/°; Notch1$^{+/+}$; Notch2$^{flox/flox}$* (**F**) mice results in a dispersed hair graying. In *Tyr::Cre/°; Notch1$^{flox/flox}$*; Notch2$^{+/+}$ mice, hair graying is retarded and not yet visible at 8 weeks (**G**). Mice with three deleted (floxed) *Notch* alleles (**H**, *Tyr::Cre/°; Notch1$^{flox/flox}$; Notch2$^{flox/+}$*; **I**, *Tyr::Cre/°; Notch1$^{flox/+}$; Notch2$^{flox/flox}$*) show a more intense hair graying. A coat color phenotype resembling deletion of RBP-Jκ (C) is obtained following deletion of all four alleles (**J**, *Tyr::Cre/°; Notch1$^{flox/flox}$; Notch2$^{flox/flox}$*). Note that the skin phenotype of mice with conditional deletion of both *Notch1* alleles (G, H, J) is not related to *Cre* expression in melanocytes. All mice are 8 weeks old. N1$^{\Delta}$ = *Tyr::Cre/°; Notch1$^{flox}$*, N2$^{\Delta}$ = *Tyr::Cre/°; Notch2$^{flox}$*, RBP$^{\Delta}$ = *Tyr::Cre/°; RBP-Jκ $^{flox}$*.

tent (Fig. 2H). While the amount of melanin in wild-type hairs remained quite constant with age, the quantity of melanin in *Tyr::Cre/°; Notch1$^{flox/+}$; Notch2$^{flox/flox}$* and *Tyr::Cre/°; Notch1$^{flox/flox}$; Notch2$^{flox/+}$* hairs decreased to the level of albino (*Tyr$^{c}$*) mice. We thus conclude that deletion of Notch1 and Notch2 leads to progressive hair graying, which is influenced by the number of intact alleles that remain.

### Deletion of Notch1 and Notch2 Affects the Maintenance of *Dct*-Expressing Melanocytes in the Hair Follicles

To analyze the number and the location of melanoblasts and melanocytes, we used *Dct::LacZ* mice (MacKenzie et al., 1997) that carry the reporter gene *LacZ* under the control of the *Dct* promoter. The embryonic expression of this promoter, from E9.5, allows visualization of melanoblasts and melanocytes before the production of pigment starts. In adult mice, this *Dct::LacZ* mouse line is convenient to mark pigment cells located in the epidermis and hair follicles, but not for detection of dermal melanocytes. When *Dct::LacZ* transgenic embryos (E13.5) were compared to *ROSA26R* (*R26R*) embryos (Soriano, 1999) expressing the *Tyr::Cre* transgene, an equal amount of melanoblasts was *LacZ*-positive, indicating that efficient Cre-mediated recombination occurs in *Dct*-expressing melanocytes (Fig. 3A). In *Dct::LacZ/°; Tyr::Cre/°; Notch1$^{flox/+}$; Notch2$^{flox/flox}$* embryos, deletion of Notch1 and Notch2 receptors did not affect the number of *Dct*-expressing cells at embryonic stages. At E13.5 and E16.5, an equal density of melanoblasts was found in control (Fig. 3B and D), *Dct::LacZ/°; Tyr::Cre/°; Notch1$^{flox/+}$; Notch2$^{flox/flox}$* (Fig. 3C and E) and *Dct::LacZ/°; Tyr::Cre/°; Notch1$^{flox/flox}$; Notch2$^{flox/flox}$* (not shown) embryos. We next analyzed the presence of melanocytes after birth according to the hair cycle (Fig. 4A). At 9 days, when hair morphogenesis is completed, histological analysis of *Dct::LacZ/°; Tyr::Cre/°; Notch1$^{flox/+}$; Notch2$^{flox/flox}$* dorsal skin revealed that both control and mutant hair matrix contained pigment and *Dct*-expressing melanocytes (Fig. 4B and C). However, at this stage (P9), a decrease in the number of melanocytes in the lower permanent portion (LPP) of mutant hair follicle could be observed. Nevertheless, an effect of Notch deletion on mature melanocytes in the bulb cannot be excluded since a slight difference in the coat color was already observed at the age of 3 weeks (Fig. 2B). During the following telogen phase (3 weeks, Fig. 4D and E), and anagen phase (4.5 weeks, Fig. 4F and G), differences became more obvious, as most mutant hair follicles completely lacked *Dct*-expressing cells. In addition, although mice retained a significant amount of hair pigment at this age (Fig. 2C and D), no pigmentation was found in the mutant hair bulbs after their first regeneration (Fig. 4G). Loss of pigmentation was progressive as hairs might still contain melanin while already lacking melanocytes in the bulb. Moreover, a few pigmented hairs might be sufficient to provide gray color to the coat. These results thus underline the requirement of signaling mediated by Notch1 and Notch2 in the maintenance of melanoblasts and melanocyte stem cells after birth.

### *Tyr::Cre*-Mediated Deletion of Notch1 and Notch2 Does Not Affect Non-Follicular Melanocytes

In adult mice, neural crest–derived melanocytes are also found in the dermis, especially in the ear, and in the eye (choroid, ciliary body, and iris). We, therefore, analyzed the presence of melanin on ear and eye sections of control and mutant mice knowing that the *Tyr::Cre* transgene is expressed in these non-follicular melanocytes. At 3 weeks, *Tyr::Cre/°; Notch1$^{flox/+}$; Notch2$^{flox/flox}$* ears (Fig. 5B) displayed wild-type pigmentation as in control mice (Fig. 5A). In contrast to hair follicles, ears from adult *Tyr::Cre/°; RBP-Jκ$^{flox/flox}$* (Fig. 5F), *Tyr::Cre/°; Notch1$^{flox/+}$; Notch2$^{flox/flox}$* (Fig. 5D) and *Tyr::Cre/°; Notch1$^{flox/flox}$; Notch2$^{flox/+}$* (Fig. 5E) mice are pigmented as controls (Fig. 5C). Similarly, pigmentation of the choroid layer was not influenced by the melanocyte-specific deletion of Notch1 and Notch2 in *Tyr::Cre/°; Notch1$^{flox/+}$; Notch2$^{flox/flox}$* eyes (Fig. 5G–L). As expected, the retinal pigment epithelium of mutant eyes was equally not affected since the artificial *tyrosinase* promoter used to drive expression of Cre recombinase is not active in this tissue (Camacho-Hubner and Beermann, 2001; Murisier and Beermann, 2006; unpublished observation).

## DISCUSSION

The Notch signaling pathway plays a critical role in biological processes such as apoptosis, proliferation, cell fate decisions (Artavanis-Tsakonas et al., 1999), and self-renewal of stem cells (reviewed in Radtke et al., 2005). *RBP-Jκ*-dependent Notch signaling via its target gene *Hes1* has been recently shown to be implicated in the maintenance of melanoblasts and melanocyte stem cells (Moriyama et al., 2006). Although these studies clearly demonstrate that Notch signaling is important for survival of melanoblast stem or progenitor cells, it is not clear which of the four Notch receptors mediates this function. The Notch3 and Notch4 receptors seem to be dispensable for pigmentation since *Notch3$^{-/-}$* and *Notch4$^{-/-}$* mutant mice are viable and do not display any coat color phenotype (Krebs et al., 2000, 2003; reviewed in Louvi et al., 2006). We, therefore, addressed the role of Notch1 and/or Notch2 using conditional gene ablation strategies. Our results reveal that both Notch1 and Notch2 receptors are required for proper hair pigmentation since their targeted deletion in melanocytes leads to inactivation of the *RBP-Jκ* gene, which mediates Notch signaling of all receptors.

The coat color phenotype was dose-dependent, and three intact alleles of *Notch1* and *Notch2* were required for preventing precocious hair graying. The expression pattern of activated Notch1 in *Dct*-positive melanoblasts suggests an important role for Notch1 signaling during melanoblast development (Moriyama et al., 2006). Surprisingly, inactivation of *Notch1* in melanoblasts revealed a slightly weaker phenotype compared to inactivation of the *Notch2* gene. This result suggests a more significant contribution of Notch2 in the maintenance of melano-

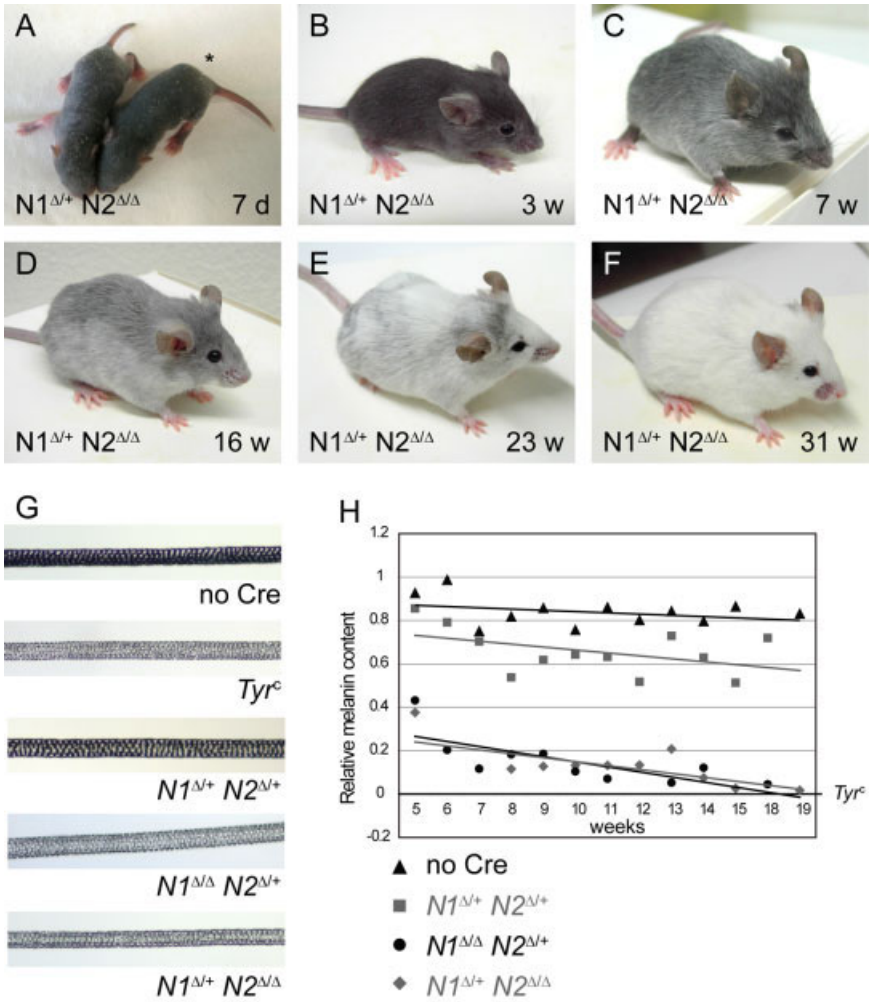



**Fig. 2.** Increasing hair graying follows deletion of Notch in melanocytes. First coat in *Tyr::Cre/°; Notch1$^{flox/+}$; Notch2$^{flox/flox}$* mice is only slightly affected by the targeted deletion of Notch receptors in melanocytes (**A**, 7 days, control newborn is indicated by *; **B**, 3 weeks). Later, at 7 weeks (**C**), the coat is completely gray and becomes progressively lighter with increasing age (**D**, 16 weeks; **E**, 23 weeks). After 7 months (31 weeks), the coat is white (**F**) and hairs are undistinguishable from albino (*Tyr$^c$*) hairs. The age of mice in days (d) and weeks (w) is indicated. **G:** As compared with albino (*Tyr$^c$*) hairs, the loss of pigmentation is evident in enlarged (63×) gray and white hairs of 12.5-week-old mutant mice. A wild-type hair of a C57BL/6 mouse is shown as a control. **H:** Spectrophotometric measurement indicates a progressive decrease of the melanin content to the level of albino (*Tyr$^c$*) hairs. Note the reduced melanin content in mice heterozygous for *Notch1* and *Notch2*. N1$^{\Delta}$ = *Tyr::Cre/°; Notch1$^{flox}$*, N2$^{\Delta}$ = *Tyr::Cre/°; Notch2$^{flox}$*.

cyte stem cells and pigmentation in hair follicles.

Nevertheless, our observations reveal that Notch1 and Notch2 have mostly overlapping functions in the pigmentary system. Similarly to the described redundancy between Notch1 and Notch4 during embryogenesis (Krebs et al., 2000), mice with combined deletion of *Notch1* and *Notch2* (Fig. 1I and J) display a more severe phenotype than mice lacking only Notch2 in melanocytes. This is in contrast with other systems where Notch1 and Notch2, although expressed in the same cells, have nonredundant functions. For example, in the skin, conditional deletion of *Notch1* in the embryonic ectoderm (*Msx2::Cre/°; Notch1$^{flox/flox}$*) results in a mosaic pattern of hair growth, whereas *Msx2::Cre/°; Notch2$^{flox/flox}$* mice are undistinguishable from wild-type (Pan et al., 2004). Other examples include the hematopoietic system, in which Notch1 plays a critical role in intrathymic T-cell development, whereas Notch2 signaling is essential for development of marginal zone B-cells (Robey et al., 2004; Wu, 2006).

Inactivation of RBP-Jκ in the melanocyte lineage leads to a decrease in the number of melanoblasts already at E16.5, and these cells almost completely disappear from mutant hair follicles at P4 (Moriyama et al., 2006). Similarly, deletion of Notch1 and Notch2 results in the elimination of pigment cell precursors after birth without, however, affecting the number of melanoblasts at embryonic

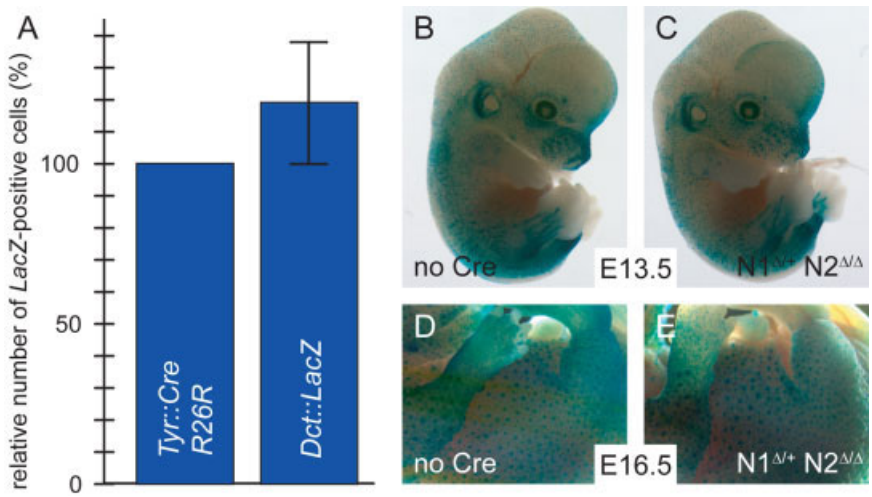



**Fig. 3.** Number and migration of *Dct*-expressing cells are not influenced by targeted deletion of Notch1 and Notch2 in melanocytes. Removal of Notch1 and Notch2 receptors does not affect the melanoblast population at embryonic stages. **A:** *Dct::LacZ* staining in melanoblasts reflects the Cre-mediated recombination by *Tyr::Cre*. Equal areas of LacZ staining were counted between somites 13 and 24 in *Tyr::Cre/°; R26R/+* and *Dct::LacZ/°* embryos at E13.5 (n = 10). The number of melanoblasts in *Tyr::Cre/°; R26R/+* embryos was set to 100%. **B–E:** As compared with control embryos (B and D), a normal density of melanoblasts is observed in *Dct::LacZ/°; Tyr::Cre/°; Notch1$^{flox/+}$; Notch2$^{flox/flox}$* embryos (C and E) at E13.5 and E16.5. N1$^{\Delta}$ = *Dct::LacZ/°; Tyr::Cre/°; Notch1$^{flox}$*, N2$^{\Delta}$ = *Dct::LacZ/°; Tyr::Cre/°; Notch2$^{flox}$*.

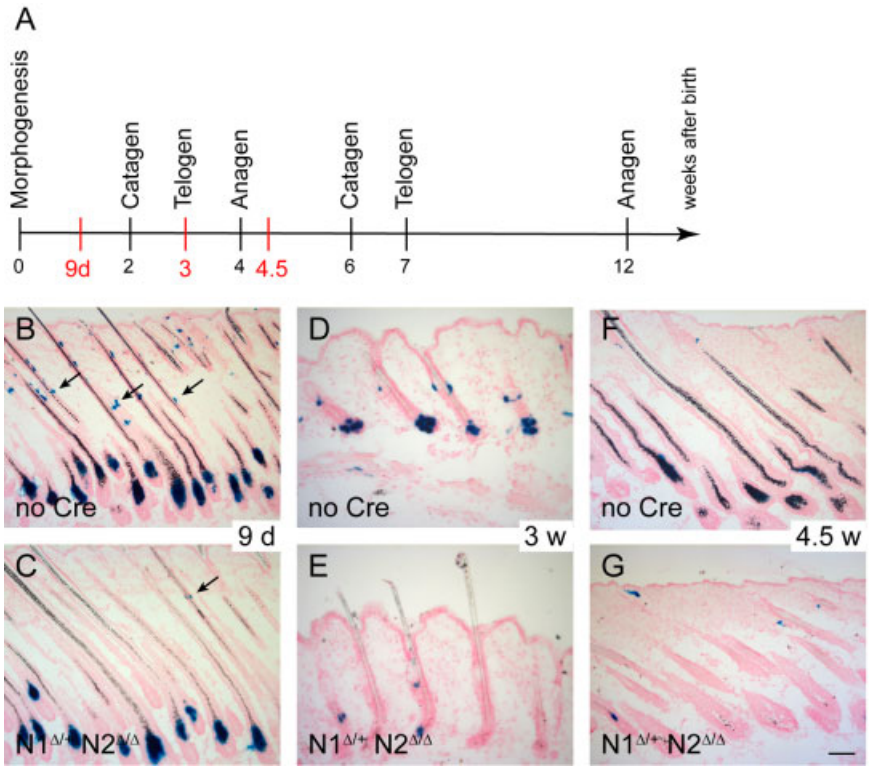



**Fig. 4.**

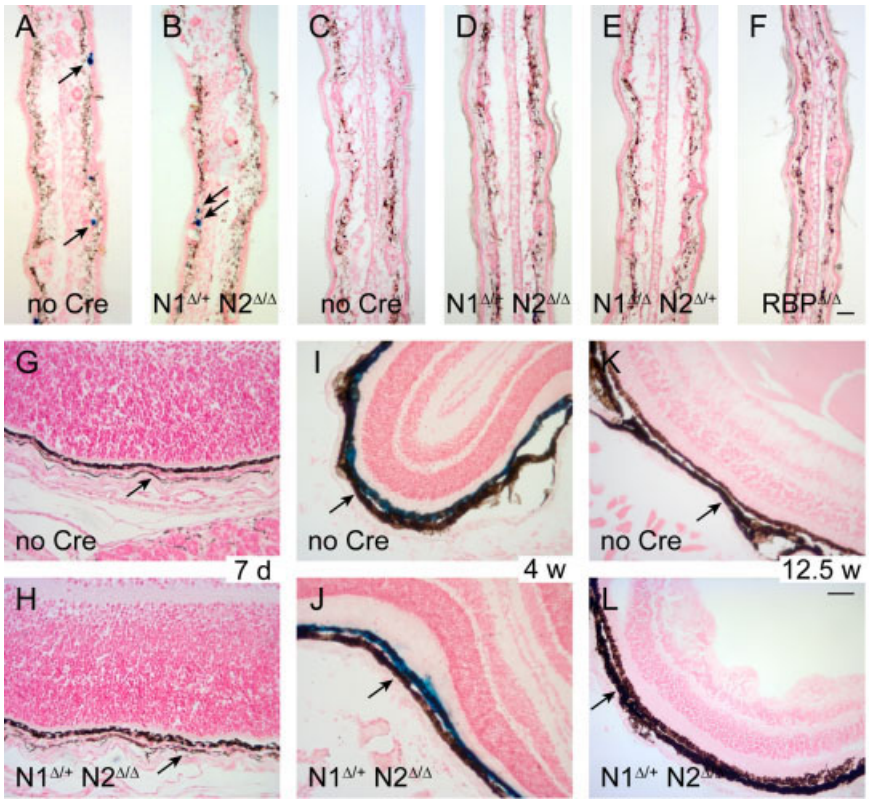



**Fig. 5.**

stages. The loss of melanocytes due to the deletion of *Notch1* and *Notch2* alleles could be caused by an apoptotic mechanism as described for the elimination of melanoblasts and melanocyte stem cells in *Tyr::Cre/°; RBP-Jκ$^{flox/flox}$* mice (Moriyama et al., 2006). Another well-documented function of

**Fig. 4.** Deletion of Notch1 and Notch2 receptors results in the elimination of pigment and melanocytes in hair follicles. Histological analysis of *Dct::LacZ/°; Tyr::Cre/°; Notch1$^{flox/+}$; Notch2$^{flox/flox}$* and control hair follicles at different stages of the hair cycle (**A**) reveals a depletion of melanocyte precursors and thus the absence of pigmentation after the first regeneration of hair follicles. **B,C:** At 9 days, *Tyr::Cre/°; Notch1$^{flox/+}$; Notch2$^{flox/flox}$* hair follicles (C) contain a normal amount of pigment as compared with control (B). However, while *Dct*-expressing cells are still detectable in mutant bulbs, they almost completely disappear from the bulge region and the ORS (arrows) in mutant hair follicles. **D,E:** In the telogen (3 weeks), mutant hair follicles lack *Dct*-expressing cells in the LPP, while they are still present in control follicles (D, control; E, *Dct::LacZ/°; Tyr::Cre/°; Notch1$^{flox/+}$; Notch2$^{flox/flox}$*). Only a few *Dct*-expressing cells are seen in mutant hair bulbs. **F,G:** Melanocyte-specific deletion of Notch1 and Notch2 results in a complete absence of pigment and *Dct*-positive cells during anagen (4.5 weeks; F, control; G, *Dct::LacZ/°; Tyr::Cre/°; Notch1$^{flox/+}$; Notch2$^{flox/flox}$*). The age of mice in days (d) and weeks (w) is indicated. Scale bars = 100 μm in B, C, F, and G, and = 50 μm in D and E. N1$^{\Delta}$ = *Dct::LacZ/°; Tyr::Cre/°; Notch1$^{flox}$*, N2$^{\Delta}$ = *Dct::LacZ/°; Tyr::Cre/°; Notch2$^{flox}$*.

**Fig. 5.** Targeted deletion of Notch1 and Notch2 does not affect pigmentation in non-follicular melanocytes in the dermis and the choroid. **A,B:** Dermal pigmentation in ears of 3-week-old mice is not affected by the deletion of Notch1 and Notch2 in melanocytes. Moreover, LacZ staining reveals the presence of *Dct*-expressing melanocytes (arrows) in both control and mutant ears (A, control; B, *Dct::LacZ/°; Tyr::Cre/°; Notch1$^{flox/+}$; Notch2$^{flox/flox}$*). **C–F:** In contrast to hair follicles, dermis of mutant ears still contains a normal amount of pigment at 9 months (C, control; D, *Tyr::Cre/°; Notch1$^{flox/+}$; Notch2$^{flox/flox}$*; E, *Tyr::Cre/°; Notch1$^{flox/flox}$; Notch2$^{flox/+}$*; F, *Tyr::Cre/°; RBP-Jκ$^{flox/flox}$*). **G–L:** Histological analysis of the eye reveals that the choroidal layer (arrows) remains normally pigmented when *Notch1* and *Notch2* alleles are deleted in melanocytes (G, I, and K, controls; H, J, and L, *Tyr::Cre/°; Notch1$^{flox/+}$; Notch2$^{flox/flox}$*). The age of mice in days (d) and weeks (w) is indicated. In addition, *Dct*-expressing cells are detectable in the retinal pigment epithelium by LacZ staining on eye sections of control and *Dct::LacZ/°; Tyr::Cre/°; Notch1$^{flox/+}$; Notch2$^{flox/flox}$* mice (I and J). Scale bars = 50 μm. N1$^{\Delta}$ = *Tyr::Cre/°; Notch1$^{flox}$*, N2$^{\Delta}$ = *Tyr::Cre/°; Notch2$^{flox}$*, RBP$^{\Delta}$ = *Tyr::Cre/°; RBP-Jκ$^{flox}$*.

the Notch cascade is its ability to influence cell fate decisions at developmental branch points. Examples include the nervous system, the hematopoietic system, or the pancreas (Hansson et al., 2004). In particular, Notch signaling has been shown to inhibit neurogenesis while promoting glial cell fate (Lai, 2004; Lundkvist and Lendahl, 2001; Gaiano and Fishell, 2002; Ge et al., 2002). Since pluripotent neural crest cells can give rise to multiple cell fates such as melanocytes, neurons, and glial cells (reviewed in Christiansen et al., 2000), it cannot be ruled out, even though unlikely, that deletion of Notch1 and Notch2 favors the development of another cell fate at the expense of the melanocyte lineage.

Finally, pigmentation mediated by non-follicular melanocytes seemed not to be affected by the absence of Notch1, Notch2, or RBP-Jκ as melanin was still observed in the dermis and choroid at late developmental stages. This could be explained by a lack of Cre expression in non-follicular melanocytes in *Tyr::Cre* mice. However, this is rather unlikely, since the tyrosinase regulatory elements are known to target expression to all melanocytes (Camacho-Hubner and Beermann, 2001). Moreover, when used in combination with other target genes (V.D. and L.L., personal communication), the same *Tyr::Cre* transgenic line leads to melanocyte-specific recombination as evident by unpigmented ears or malformed choroid. In consequence, our results might suggest that maintenance of melanoblasts in hair follicles and in the choroid or ear is regulated by distinct molecular mechanisms. Alternatively, self-renewal of the melanocyte population in the dermis and the choroid might be much slower compared to melanocytes in the hair follicle, which would preclude the observation of a phenotype.

In conclusion, our results demonstrate that, although deletion of Notch2 in melanocytes resulted in a slightly stronger phenotype, both Notch1 and Notch2 receptors have mostly redundant functions in the pigmentary system. Notch1 and Notch2 signaling maintains survival of melanocyte stem cells in hair follicles through a RBP-Jκ-dependent mechanism. In contrast, maintenance of pigmentation in the dermis and the choroid might be regulated through a distinct mechanism since it is not affected by the disruption of the Notch signaling pathway.

## EXPERIMENTAL PROCEDURES

### Mice and Genotyping

*Tyr::Cre* transgenic mice were mated to *RBP-Jκ*$^{flox/flox}$ mice (Tanigaki et al., 2002), *Notch1*$^{flox/flox}$ mice (Radtke et al., 1999) and *Notch2*$^{flox/flox}$ mice (Besseyrias et al., unpublished data). *Tyr::Cre/°; RBP-Jκ*$^{flox/flox}$ mice and *Tyr::Cre* transgenic mice carrying floxed alleles of *Notch1* and *Notch2* were kept on a mixed genetic background with >75% contribution of C57BL/6. All mice used for breeding and analyses were pigmented (*Tyr*$^{+}$), nonagouti (a) and black (*Tyrp1*$^{+}$). In the experimental analyses, littermates not expressing the *Tyr::Cre* transgene were used as control mice. Genotyping of mice was performed on DNA isolated from tail biopsies using standard PCR buffer composition and reaction (Porret et al., 2006). PCR reactions were terminated by a 10-min final incubation at 72°C. The *Tyr::Cre* transgene (0.4 kb fragment) was detected by PCR (30 sec at 94°C, 1 min at 57°C, 1 min at 72°C, 30 cycles) using primers 5′-CCT GGA AAA TGC TTC TGT CCG-3′ and 5′-CAG GGT GTT ATA AGC AAT CCC-3′. For detection of the floxed (0.35 kb) and wild-type (0.3 kb) alleles of *Notch1*, PCR amplification (1 min at 93°C, 1 min at 56°C, and 1 min at 72°C, 40 cycles) was done using primers 5′-CTG ACT TAG TAG GGG GAA AAC-3′ and 5′-AGT GGT CCA GGG TGT GAG TGT-3′. For *Notch2*, a 0.25-kb (wild-type) and a 0.3-kb (floxed) fragment were obtained (primers 5′-GAG AAG CAG AGA TGA GCA GAT G-3′ and 5′-GTG AGA TGT GAC ACT TCT GAG C-3′). Wild-type (0.45 kb) and floxed (0.55 kb) alleles of *RBP-Jκ* were detected using primers 5′-GTT CTT AAC CTG TTG GTC GGA ACC-3′ and 5′-GCT TGA GGC TTG ATG TTC TGT ATT GC-3′ (wild-type) and primers 5′-GAA GGT CGG TTG ACA CCA GAT AGC-3′ and 5′-GCA ATC CAT CTT GTT CAA TGG CC-3′ (floxed). The *Dct::LacZ* transgene (0.45 kb) was identified by PCR (45 sec at 94°C, 30 sec at 61°C, and 45 sec at 72°C, 30 cycles) using *LacZ*-specific primers (5′-TCG TCT GCT CAT CCA TGA CC-3′ and 5′-GAT TTC CAT GTT GCC ACT CG-3′). Primers and conditions for detection of *R26R* mice have been published previously (Soriano, 1999).

### Relative Melanin Content

Melanin was extracted from dorsal hairs (1.5 mg) by alkali treatment (1.5 ml of 1M NaOH, 4 hr at 85°C). Relative melanin content was determined by spectrophotometric measurement at 475 nm. Each hair sample was measured in triplicate and normalized with values obtained for albino (*Tyr*$^{c}$) hairs. The time-dependent decrease of melanin was depicted by linear regression.

### Whole Mount Embryo LacZ Staining

Embryonic day (E)0.5 was determined at noon of the day of detection of a vaginal plug. LacZ staining was essentially done as previously described (Porret et al., 2006; Schmidt et al., 1998). Embryos at E13.5 and E16.5 were dissected free of extraembryonic tissues, washed in PBS, and fixed in 4% paraformaldehyde for 1 hr on ice. After two washes with PBS, the embryos were incubated in permeabilization solution (0.1 M phosphate buffer pH 7.3, 2 mM $MgCl_2$, 0.01% desoxycholate, 0.02% NP40) twice for 1 hr at RT. They were then incubated in staining solution (3.33 mM potassium ferricyanid, 3.33 mM ferrocyanid, 20 mM Tris HCl, pH 7.4, 0.66 mg/ml X-Gal in permeabilization solution) for 4–6 hr at 37°C. After two washes in PBS, the embryos were post-fixed for 4–8 hr in 4% paraformaldehyde at 4°C.

### Histological Analysis

Dorsal skin was harvested from the right side of the spinal cord, in the region between the two limbs, and washed in PBS. It was then embedded in OCT and frozen on dry ice and kept at −70°C. Cryosections (8 μm) were fixed (3 min, 2% paraformaldehyde,

0.125% glutaraldehyde in PBS), washed three times (2 mM $MgCl_2$ in PBS), and treated two times in permeabilization solution (2 min, 0.1 M phosphate buffer, pH 7.3, 2 mM $MgCl_2$, 0.01% desoxycholate, 0.02% NP40). Sections were then stained (3.33 mM potassium ferricyanid, 3.33 mM ferrocyanid, 20 mM Tris HCl, pH 7.4, 0.66 mg/ml X-Gal in permeabilization solution) overnight at RT.

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## REFERENCES

Artavanis-Tsakonas S, Rand MD, Lake RJ. 1999. Notch signaling: cell fate control and signal integration in development. Science 284:770–776.

Camacho-Hubner A, Beermann F. 2001. Increased transgene expression by the mouse tyrosinase enhancer is restricted to neural crest-derived pigment cells. Genesis 29:180–187.

Christiansen JH, Coles EG, Wilkinson DG. 2000. Molecular control of neural crest formation, migration and differentiation. Curr Opin Cell Biol 12:719–724.

Delmas V, Martinozzi S, Bourgeois Y, Holzenberger M, Larue L. 2003. Cre-mediated recombination in the skin melanocyte lineage. Genesis 36:73–80.

Favier B, Fliniaux I, Thelu J, Viallet JP, Demarchez M, Jahoda CA, Dhouailly D. 2000. Localisation of members of the notch system and the differentiation of vibrissa hair follicles: receptors, ligands, and fringe modulators. Dev Dyn 218:426–437.

Gaiano N, Fishell G. 2002. The role of notch in promoting glial and neural stem cell fates. Annu Rev Neurosci 25:471–490.

Ge W, Martinowich K, Wu X, He F, Miyamoto A, Fan G, Weinmaster G, Sun YE. 2002. Notch signaling promotes astrogliogenesis via direct CSL-mediated glial gene activation. J Neurosci Res 69: 848–860.

Greenwald I. 1998. LIN-12/Notch signaling: lessons from worms and flies. Genes Dev 12:1751–1762.

Hansson EM, Lendahl U, Chapman G. 2004. Notch signaling in development and disease. Semin Cancer Biol 14:320–328.

Hoek K, Rimm DL, Williams KR, Zhao H, Ariyan S, Lin A, Kluger HM, Berger AJ, Cheng E, Trombetta ES, Wu T, Niinobe M, Yoshikawa K, Hannigan GE, Halaban R. 2004. Expression profiling reveals novel pathways in the transformation of melanocytes to melanomas. Cancer Res 64:5270–5282.

Krebs LT, Xue Y, Norton CR, Shutter JR, Maguire M, Sundberg JP, Gallahan D, Closson V, Kitajewski J, Callahan R, Smith GH, Stark KL, Gridley T. 2000. Notch signaling is essential for vascular morphogenesis in mice. Genes Dev 14: 1343–1352.

Krebs LT, Xue Y, Norton CR, Sundberg JP, Beatus P, Lendahl U, Joutel A, Gridley T. 2003. Characterization of Notch3-deficient mice: normal embryonic development and absence of genetic interactions with a Notch1 mutation. Genesis 37:139–143.

Lai EC. 2004. Notch signaling: control of cell communication and cell fate. Development 131:965–973.

Louvi A, Artavanis-Tsakonas S. 2006. Notch signalling in vertebrate neural development. Nat Rev Neurosci 7:93–102.

Lundkvist J, Lendahl U. 2001. Notch and the birth of glial cells. Trends Neurosci 24:492–494.

Mackenzie MA, Jordan SA, Budd PS, Jackson IJ. 1997. Activation of the receptor tyrosine kinase Kit is required for the proliferation of melanoblasts in the mouse embryo. Dev Biol 192:99–107.

Moriyama M, Osawa M, Mak SS, Ohtsuka T, Yamamoto N, Han H, Delmas V, Kageyama R, Beermann F, Larue L, Nishikawa S. 2006. Notch signaling via Hes1 transcription factor maintains survival of melanoblasts and melanocyte stem cells. J Cell Biol 173:333–339.

Murisier F, Beermann F. 2006. Genetics of pigment cells: lessons from the tyrosinase gene family. Histol Histopathol 21: 567–578.

Nam Y, Aster JC, Blacklow SC. 2002. Notch signaling as a therapeutic target. Curr Opin Chem Biol 6:501–509.

Nickoloff BJ, Osborne BA, Miele L. 2003. Notch signaling as a therapeutic target in cancer: a new approach to the development of cell fate modifying agents. Oncogene 22:6598–6608.

Nickoloff BJ, Hendrix MJ, Pollock PM, Trent JM, Miele L, Qin JZ. 2005. Notch and NOXA-related pathways in melanoma cells. J Invest Dermatol Symp Proc 10:95–104.

Nishimura EK, Jordan SA, Oshima H, Yoshida H, Osawa M, Moriyama M, Jackson IJ, Barrandon Y, Miyachi Y, Nishikawa S. 2002. Dominant role of the niche in melanocyte stem-cell fate determination. Nature 416:854–860.

Nishimura EK, Granter SR, Fisher DE. 2005. Mechanisms of hair graying: incomplete melanocyte stem cell maintenance in the niche. Science 307:720–724.

Pan Y, Lin MH, Tian X, Cheng HT, Gridley T, Shen J, Kopan R. 2004. gamma-secretase functions through Notch signaling to maintain skin appendages but is not required for their patterning or initial morphogenesis. Dev Cell 7:731–743.

Porret A, Mérillat AM, Guichard S, Beermann F, Hummler E. 2006. Tissue-specific transgenic and knockout mice. Methods Mol Biol 337:185-205.

Porter SD, Hu J, Gilks CB. 1999. Distal upstream tyrosinase S/MAR-containing sequence has regulatory properties specific to subsets of melanocytes. Dev Genet 25:40–48.

Powell BC, Passmore EA, Nesci A, Dunn SM. 1998. The Notch signalling pathway in hair growth. Mech Dev 78:189–192.

Radtke F, Wilson A, Stark G, Bauer M, van Meerwijk J, MacDonald HR, Aguet M. 1999. Deficient T cell fate specification in mice with an induced inactivation of Notch1. Immunity 10:547–558.

Radtke F, Schweisguth F, Pear W. 2005. The Notch 'gospel'. EMBO Rep 6:1120–1125.

Reya T, Clevers H. 2005. Wnt signalling in stem cells and cancer. Nature 434:843–850.

Robey EA, Bluestone JA. 2004. Notch signaling in lymphocyte development and function. Curr Opin Immunol 16:360–366.

Schmidt A, Tief K, Foletti A, Hunziker A, Penna D, Hummler E, Beermann F. 1998. LacZ transgenic mice to monitor gene expression in embryo and adult. Brain Res Brain Res Protoc 3:54–60.

Soriano P. 1999. Generalized lacZ expression with the ROSA26 Cre reporter strain. Nat Genet 21:70–71.

Steingrimsson E, Copeland NG, Jenkins NA. 2005. Melanocyte stem cell maintenance and hair graying. Cell 121:9–12.

Tanigaki K, Han H, Yamamoto N, Tashiro K, Ikegawa M, Kuroda K, Suzuki A, Nakano T, Honjo T. 2002. Notch-RBP-J signaling is involved in cell fate determination of marginal zone B cells. Nat Immunol 3:443–450.

Wu L. 2006. T lineage progenitors: the earliest steps en route to T lymphocytes. Curr Opin Immunol 18:121–126.

Yajima I, Belloir E, Bourgeois Y, Kumasaka M, Delmas V, Larue L. 2006. Spatiotemporal gene control by the CreERT2 system in melanocytes. Genesis 44: 34–43.