Notch signaling via *Hes1* transcription factor maintains survival of melanoblasts and melanocyte stem cells

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Melanoblasts (Mbs) are thought to be strictly regulated by cell–cell interactions with epidermal keratinocytes, although the precise molecular mechanism of the regulation has been elusive. Notch signaling, whose activation is mediated by cell–cell interactions, is implicated in a broad range of developmental processes. We demonstrate the vital role of Notch signaling in the maintenance of Mbs, as well as melanocyte stem cells (MSCs). Conditional ablation of Notch signaling in the melanocyte lineage leads to a severe defect in hair pigmentation, followed by intensive hair graying. The defect is caused by a dramatic elimination of Mbs and MSCs. Furthermore, targeted overexpression of *Hes1* is sufficient to protect Mbs from the elimination by apoptosis. Thus, these data provide evidence that Notch signaling, acting through *Hes1*, plays a crucial role in the survival of immature Mbs by preventing initiation of apoptosis.

Introduction

Melanocytes (MCs) are pigment cells responsible for the pigmentation of animals. MCs synthesize pigment granules within a special organelle termed a melanosome, where several enzymes that are involved in melanin biosynthesis, such as tyrosinase (Tyr) and dopachrome tautomerase (Dct), are assembled and thereby transfer pigment granules into keratinocytes to form pigmented skin or hairs. MCs provide an attractive system to study the molecular basis of cell regulation, as genetic alterations involved in MC regulations are easily identifiable as coat-color mutants. In fact, >120 different loci have been identified as coat color mutants (Bennett and Lamoreux, 2003). Of these loci, mutations in genes that are particularly critical for MC development, such as *Pax3*, *Mitf*, and *Kit*, are characterized by severe hair-pigmentation defects.

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Abbreviations used in this paper: d2EGFP, destabilized EGFP; DAPT, (3,5-difluorophenylacetyl)-L-alanyl-L-2-phenylglycine t-butyl ester; Dct, dopachrome tautomerase; E, embryonic day; HF, hair follicle; HM, hair matrix; LPP, lower permanent portion; Mb, melanoblast; MC, melanocyte; MSC, melanocyte stem cell; NICD, Notch intracellular domain; P, postnatal day; Q-PCR, quantitative PCR; Tg, transgenic; Tyr, tyrosinase.

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In the hairy region of the skin, MCs are exclusively localized in hair follicles (HFs), where proliferation and maturation of MCs are strictly regulated by the hair cycle. MCs at the hair matrix (HM) appear and proliferate during the growth phase of the HF and thereafter differentiate into mature MCs to generate pigmented hairs. Subsequently, all differentiated MCs are eliminated by apoptosis during the regression phase of the HF. It has been demonstrated that this cyclic appearance of MCs in the HM is maintained by a small pool of undifferentiated MC stem cells (MSCs) localized at the lower permanent portion (LPP) of the HF (Nishimura et al., 2002).

During embryogenesis, MC precursors, melanoblasts (Mbs), arise from the neural crest and migrate through the epidermis toward newly developing HFs. Once in the follicles, they are segregated into two populations: MCs, which localize at the HM and contribute to the initial wave of melanogenesis during HF morphogenesis, and MSCs, which colonize at the LPP and constitute the MC system in subsequent hair cycles (Mak et al., 2006). In nonhairy regions, Mbs stay immature and remain on the basement membrane of the epidermis, where they undergo differentiation into mature MCs upon stimulation from keratinocytes. It has been proposed that the homeostatic

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regulation of Mbs is maintained by the keratinocytes through cell–cell interactions (Haass et al., 2005), although the precise molecular interactions are largely unknown.

Notch comprises a family of highly conserved receptors, whose activation is induced by their specific ligands, Delta and Jagged, through cell–cell interactions (Artavanis-Tsakonas et al., 1999). Once activated, the Notch intracellular domain (NICD) is cleaved by γ-secretase, which leads to translocation of the NICD into the nucleus. Subsequently, NICD is associated with the transcription factor RBP-J to generate the transactivation complex, which initiates transcription of target genes such as the *hairy/enhancer of split* (*Hes*) transcriptional repressors (Kageyama et al., 2000). Notch signaling is involved in various aspects of cellular regulation, including stem cell maintenance (Hitoshi et al., 2004; Duncan et al., 2005); however, the exact molecular mechanisms remain unclear.

In this study, we demonstrate the role of Notch signaling in the maintenance of Mbs and MSCs. In addition, we demonstrate the critical role of Hes1 in ensuring the survival of Mbs by preventing apoptosis. Thus, our data provide new insights to the molecular mechanisms underlying the homeostatic regulation of Mbs.

Results and discussion

During gene expression profiling of embryonic Mbs, we noted that several Notch-related genes were expressed in the Mb population (unpublished data). To examine the implication of Notch signaling in Mbs, we performed double immunostaining of embryonic skin using specific antibodies against activated Notch1 (NICD1) and Dct, which can mark all the subsets of MC lineage. Consistent with previous observations (Lin and Kopan, 2003; Okuyama et al., 2004), nuclear localization of NICD1 was observed in the suprabasal and basal layers of the epidermis that include Dct$^+$ Mbs (Fig. 1, A and B). In parallel with Notch1 activation, the Notch ligand *Jagged2* is expressed in the basal layer of the epidermis (Fig. 1, C and D). To identify Notch target genes in Mbs, we performed transcript analysis using highly enriched Mbs (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200509084/DC1). The initial RT-PCR screening detected *Hes1*, *Hes5*, and *Hey1* transcripts in Mbs (Fig. 1 E). Further quantitative analysis shows that, of these genes, *Hes1* expression is the most prominent, whereas expression of *Hes5* and *Hey1* is much less intensive (Fig. 1 F). These data suggest that, among *Hes* family genes, *Hes1* represents a predominant target of Notch signaling in Mbs. To further confirm *Hes1* transcription in Mbs, we used *pHes1-d2EGFP* (*destabilized EGFP*) mice, in which GFP expression mimics endogenous *Hes1* promoter activation (Ohtsuka et al., 2005). Parallel to Notch1 activation, the *Hes1* promoter is also activated in Dct$^+$ Mbs (Fig. 1 G). Hence, these data indicate that both Notch1 and Hes1 are activated in Mbs, implying that Notch signaling plays a role in the regulation of Mbs.

To assess the role of Notch signaling in Mbs, we conditionally ablated the *RBP-J* gene in an MC lineage. For this purpose, we crossed mice carrying floxed *RBP-J* alleles (*RBP-J^f*; Han et al., 2002) with transgenic (Tg) mice expressing *Cre* under the

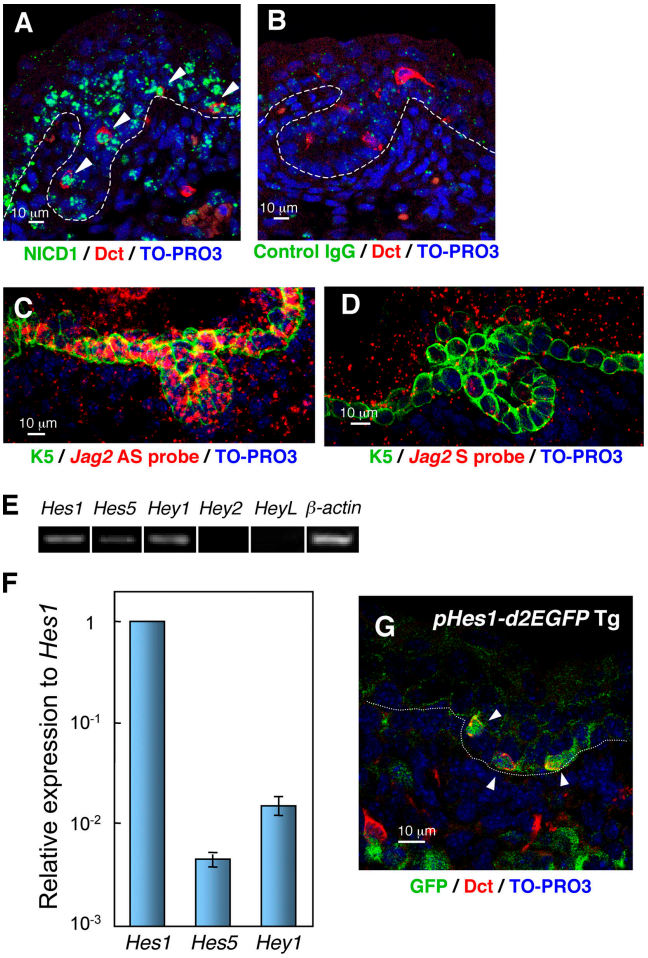

Figure 1. **Activation of Notch signaling in Mbs in the E16.5 epidermis.** (A and B) Activation of Notch1 in Mbs. (A) Immunofluorescence staining showing antibodies for NICD1 (green) and Dct (red). Arrowheads indicate Mbs in the epidermis. (B) Negative control staining for NICD1. (C and D) In situ hybridization staining of *Jagged2*. (C) *Jagged2* (red) and anti-Keratin5 (K5) antibody (green). (D) Negative control staining using *Jagged2* sense probe. (E and F) Expression of *Hes* family genes in Mbs. RT-PCR (E) and Q-PCR (F) analyses were performed using purified Mbs from the E16.5 epidermis. (F) Expression value of each transcript relative to *Hes1* was calculated. Data represent the mean of three data points. Error bars indicate SD. Note that *Hes1* expression is the most prominent among Hes family genes, whereas expression of *Hes5* and *Hey1* is much less intensive and might represent transcripts present in contaminating cells (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200509084/DC1). (G) Activation of *Hes1* promoter in Mbs. Immunofluorescent staining of E16.5 *pHes1-d2EGFP* epidermis is shown.

control of an MC-specific *Tyr* promoter (*Tyr-Cre*; Delmas et al., 2003), which is stringently activated in Mbs from embryonic day (E) 11.5 onward. By crossing *Tyr-Cre* mice with the *Rosa26R* Cre-reporter strain (Soriano, 1999), we also confirmed specific Cre-mediated recombination in Mbs in a postnatal day (P) 1 mouse (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200509084/DC1). *Tyr-Cre;RBP-J$^{f/f}$* mice showed severe coat-color dilution in the initial hairs, followed by loss of hair pigmentation after the first hair molting (Fig. 2, A–C). Detailed analysis of the hairs of *Tyr-Cre;RBP-J$^{f/f}$* mice revealed that the first hairs developed from the mice were a mixture of pigmented and unpigmented, whereas the hairs grown in the subsequent hair cycle were almost all unpigmented

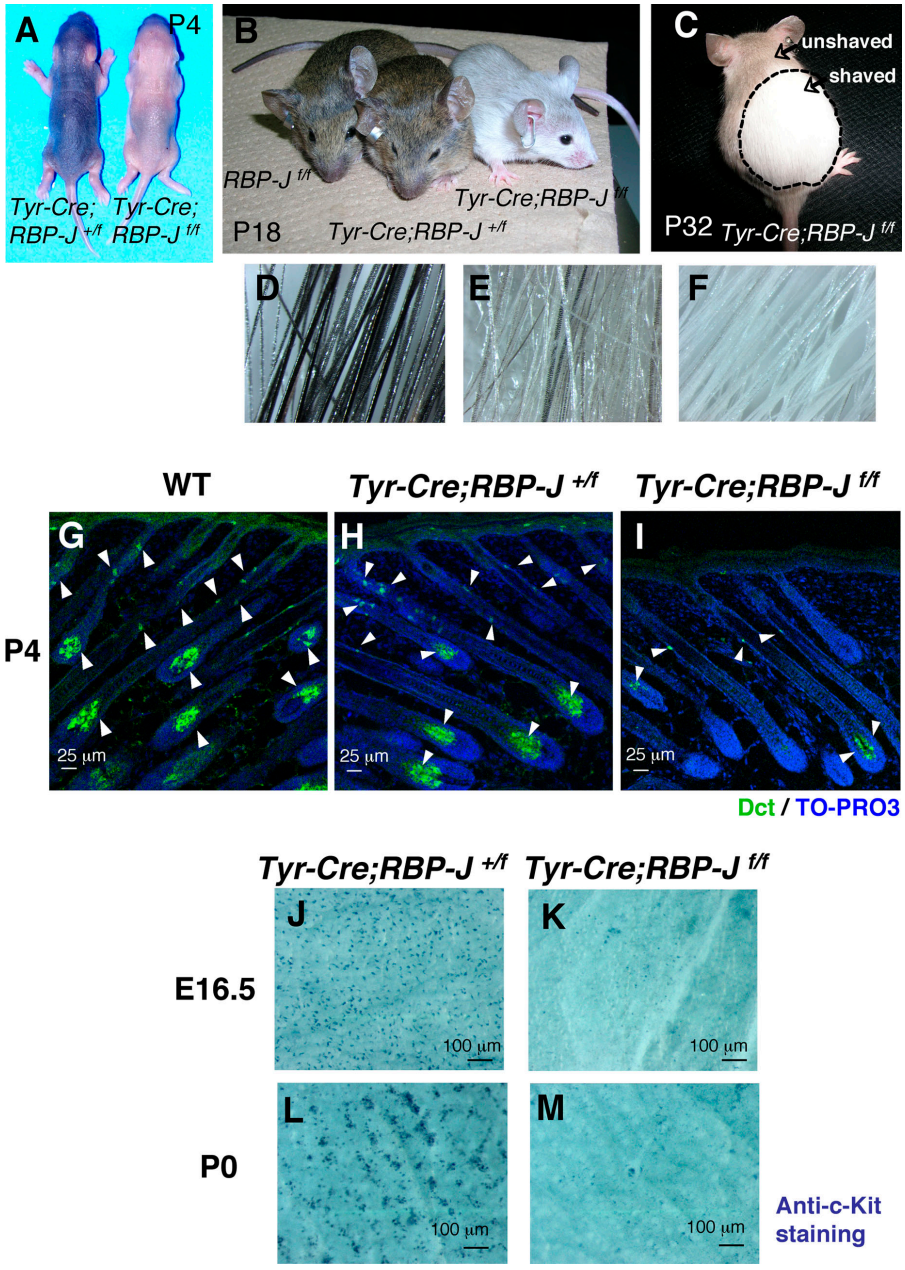

Figure 2. **The number of MC lineage cells is dramatically reduced by MC lineage–specific knock out of *RBP-J*.** (A–F) Coat-color phenotype of a *Tyr-Cre;RBP-J*$^{f/f}$ mouse. P4 (A), P18 (B), and P32 (C) *Tyr-Cre;RBP-J*$^{f/f}$ mice with their control littermates are shown. Hairs from a P18 *Tyr-Cre;RBP-J*$^{+/f}$ mouse (D) are fully pigmented, whereas hairs from a P18 *Tyr-Cre; RBP-J*$^{f/f}$ mouse (E) are a mixture of pigmented and unpigmented. Hairs grown in the next hair cycle from a P32 *Tyr-Cre;RBP-J*$^{f/f}$ mouse (C, shaved area; and F) are almost all unpigmented compared with the mixture of the primary and secondary hairs (C, unshaved area). (G–I) Immunofluorescent staining of P4 skin using anti-Dct (green) antibody. Skin from wild-type (WT; G), *Tyr-Cre;RBP-J*$^{+/f}$ (H), and *Tyr-Cre; RBP-J*$^{f/f}$ (I) mice are shown. Arrowheads indicate MCs in the HF. (J–M) Whole-mount staining of dorsal skin epidermis from E16.5 (J and K) and P0 (L and M) mice using anti–c-Kit antibody. Epidermis obtained from a *Tyr-Cre;RBP-J*$^{+/f}$ (J and L) or a *Tyr-Cre;RBP-J*$^{f/f}$ mouse (K and M) is shown.

(Fig. 2, D–F). In addition, a histological analysis of *Tyr-Cre;RBP-J*$^{f/f}$ skin showed a dramatic reduction of MCs in the HFs at P4 (Fig. 2, G–I) and the virtually complete absence of MCs in the HFs at P32 (see Fig. 5, M and N). These data indicate that melanogenesis is severely impaired in *Tyr-Cre;RBP-J*$^{f/f}$ mice, demonstrating the indispensable role of Notch signaling in the MC system. Given that the initial wave of melanogenesis during HF morphogenesis is directly derived from embryonic Mbs (Mak et al., 2006), we reasoned that the severe reduction of MCs in *Tyr-Cre;RBP-J*$^{f/f}$ mice is due to a defect in the maintenance of embryonic Mbs in which Notch signaling is shown to be activated.

To examine the effect of *RBP-J* disruption in Mbs, we performed whole-mount immunostaining of embryonic skin using an antibody against c-Kit (ACK4) that allows identification of embryonic Mbs (Yoshida et al., 1996). As compared with the control, numbers of Mbs were apparently decreased at E16.5 and P0 in *Tyr-Cre;RBP-J*$^{f/f}$ mice (Fig. 2, J–M). Hence, these data indicate that Notch signaling is critical for the maintenance of Mbs.

As an alternative avenue for analyzing the function of Notch signaling in Mbs, we adopted an embryonic skin organ culture system by blocking Notch activation with a pharmacological inhibitor for γ-secretase (Geling et al., 2002). Skin fragments obtained from E13.5 embryos were cultured for 4 d in the presence of the γ-secretase inhibitor DAPT ([3,5-difluorophenylacetyl]-L-alanyl-L-2-phenylglycine t-butyl ester) and then grafted onto immunodeficient (nude) mice to examine the effect on melanogenesis. In contrast to the control skin, which emerged with normal black hair, the hair grown from the DAPT-treated skin was unpigmented from the initial hairs and subsequently through several hair cycles (Fig. 3, A and B). In fact, histological

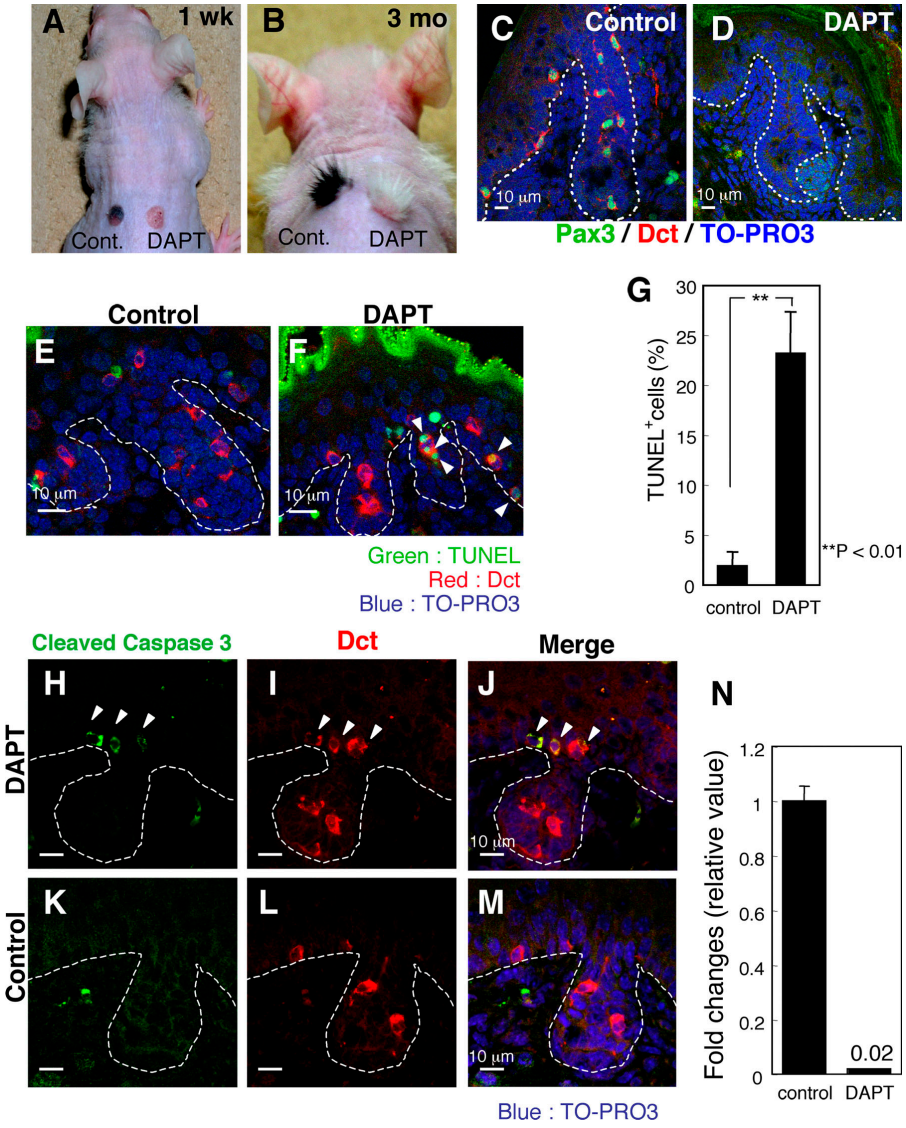

Figure 3. **Inhibition of Notch signaling in skin organ culture induces apoptosis of Mbs.** (A–D) Elimination of Mbs from DAPT-treated skin. The E13.5 dorsal skin was cultured with (A and B, right) or without (A and B, left) 1 μM DAPT for 4 d and transplanted onto nude mice (A and B) or processed for immunofluorescent staining using antibodies against the Mb-specific markers Pax3 and Dct (C and D). (E–M) Induction of apoptosis of Mbs in embryonic skin treated with DAPT. E15.5 dorsal skin was cultured in the presence or absence of DAPT for 24 h. After organ culture, skin sections were processed for either TUNEL staining (E–G) or anti–cleaved caspase 3 immunostaining (H–M). TUNEL-positive cells are shown in green, Dct-positive Mbs in red, and nuclear staining in blue (E and F). (G) Plotted percentages of TUNEL-positive Mbs, on average, from four sections of either control or DAPT-treated skin show a significant difference (**, $P < 0.01$). Cleaved caspase 3–positive cells are shown in green (H and K), Dct-positive Mbs in red (I and L), and nuclear staining in blue. Merged images are shown in J and M. Arrowheads indicate apoptotic Mbs in the epidermis. (N) Down-regulation of *Hes1* expression in Mbs after DAPT treatment for 24 h. Deference in *Hes1* expression in between DAPT-treated and control Mbs was calculated based on the ΔΔCt method by Q-PCR. Error bars indicate SD.

analysis of DAPT-treated skin demonstrated the virtually complete loss of Mbs in the epidermis (Fig. 3, C and D), indicating that all the subsets of Mbs that contribute to the postnatal melanogenesis were ablated by DAPT treatment. Shortly after the DAPT treatment (24 h), a proportion of Mbs were positive for TUNEL staining and activated caspase 3 (Fig. 3, E–M), demonstrating the induction of apoptosis in these Mbs. Thus, it is evident that Mbs are eliminated from DAPT-treated skin by apoptosis. By combining these results with the phenotype of *Tyr-Cre;RBP-J*$^{f/f}$ mice, our observations suggest a direct role for Notch signaling in the promotion of Mb survival by inhibiting the initiation of apoptosis; however, we cannot rule out the possibility that DAPT treatment indirectly affects the survival of Mbs.

Immunostaining of DAPT-treated skin with NICD1 indicated a down-regulation of Notch signaling (unpublished data). In addition, quantitative PCR (Q-PCR) demonstrated a dramatic reduction of *Hes1* transcript in DAPT-treated Mbs (Fig. 3 N). These data indicate that DAPT treatment abrogates Notch signaling through *Hes1* in Mbs.

To test whether the specific expression of *Hes1* in Mbs would be sufficient to rescue the loss of Mbs after DAPT treatment, we generated Tg mice in which the *Hes1* gene was expressed under the control of an MC-specific *Dct* promoter (*Dct-Hes1*; Mackenzie et al., 1997; Fig. 4 A). In the Tg mice, the majority of Mbs remained in the epidermis even after DAPT treatment (Fig. 4, B–E). In addition, hairs grown from the DAPT-treated *Dct-Hes1* skin showed hair pigmentation (Fig. 4 G), demonstrating that Mbs, which contribute to postnatal melanogenesis, are restored in *Dct-Hes1* skin. These observations provide strong evidence for the direct involvement of *Hes1* in the promotion of Mb survival at the downstream of Notch signaling. Because *Hes1* functions as a transcriptional repressor (Kageyama et al., 2000), the data suggest that *Hes1* represses the initiation of apoptosis by preventing the gene expression required for apoptosis in Mbs.

Immunohistochemical staining showed that Mbs at the LPP were positive for NICD1 (Fig. 5 A). Further, analysis of *pHes1-d2EGFP* follicles also indicated that *Hes1* transcription was activated in the Mb population at the LPP, whereas it was

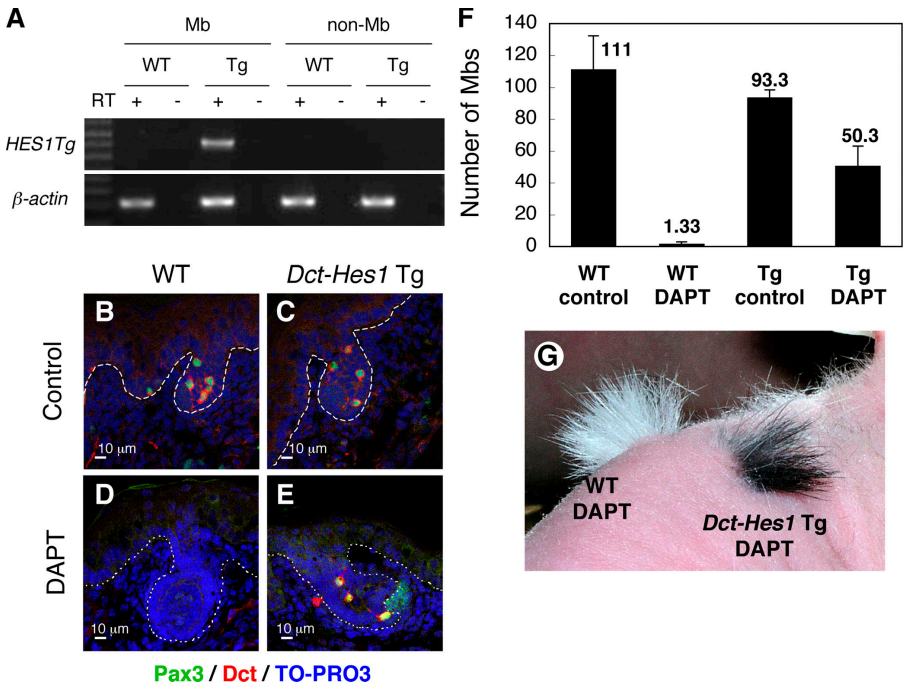

Figure 4. **Constitutive expression of *Hes1* promotes survival of Mbs in *Dct-Hes1* Tg mice.** (A) Specific expression of *Hes1* transgene in Mbs in *Dct-Hes1* Tg mice. RT-PCR analysis for either *Hes1* transgene or *β-actin* is shown. Mbs and non-Mbs (see supplemental text, available at http://www.jcb.org/cgi/content/full/jcb.200509084/DC1) were isolated from either wild-type (WT) or *Dct-Hes1* Tg mice. RT-PCR analysis was performed in the presence (RT+) or absence (RT−) of reverse transcriptase. (B–E) Attenuation of DAPT-induced apoptosis in *Dct-Hes1* skin. Dorsal skin from E13.5 wild-type mice (B and D) or *Dct-Hes1* Tg (C and E) was organ cultured without (B and C) or with (D and E) DAPT for 4 d and stained with anti-Pax3 and anti-Dct antibodies. (F) Numbers of Dct- and Pax3-positive Mbs were counted in each skin section and then plotted. Data represent the mean of three sections with SD. (G) E13.5 dorsal skin obtained from either wild-type (left) or *Dct-Hes1* Tg mice (right) was cultured in the presence of 1 μM DAPT and grafted onto a nude mouse.

inactive in the differentiated MCs at the HM (Fig. 5 B). These observations indicate that Notch signaling through *Hes1* is also activated in MSCs, supporting the notion that Notch signaling may function in MSCs.

To demonstrate this idea, we examined the distribution of immature Mbs in HFs during postnatal hair cycles in *Tyr-Cre;RBP-J$^{f/f}$* mice. Besides an apparent reduction of MCs at the HM (Fig. 5 E), some Dct-positive Mbs are identifiable at the LPP of HFs in *Tyr-Cre;RBP-J$^{f/f}$* mice at P4 (Fig. 5, E and F). However, at P12, loss of these Mbs at the LPP was evident (Fig. 5, I and J), whereas Mbs were retained at the LPP in control mice (Fig. 5, G and H). Looking at the next hair cycle at P32, *Tyr-Cre;RBP-J$^{f/f}$* HFs lack both immature Mbs at the LPP and differentiated MCs at the HM (Fig. 5, M and N), indicating the elimination of the MSCs that reconstitute the MC system in subsequent hair cycles after the initial HF morphogenesis. These data, along with the extensive graying phenotype, demonstrate a defect in the maintenance of MSCs in *Tyr-Cre;RBP-J$^{f/f}$* mice during the early stage of HF morphogenesis. Thus, it is shown that Notch signaling is also critical for the maintenance of MSCs.

We demonstrate that Notch signaling, acting through a Hes1 transcription factor, plays a predominant role in the maintenance of Mbs, including MSCs. Given *Jagged2* expression in the basal layer of the embryonic epidermis and the outer root sheath of the developing HFs (Powell et al., 1998), Notch signaling in Mbs is likely to be activated through interaction with the surrounding keratinocytes. Thus, our data suggest that Notch signaling represents a fundamental component of the homeostatic regulation of Mbs that may be mediated by Mb–keratinocyte interactions. Taking account of the intensive migration capacity of Mbs, one possible explanation for the physiological role of Notch signaling in the regulation of Mbs is that, in collaboration with c-Kit signaling (Das et al., 2004), it may play a role in confining Mbs to the epidermis by allowing their survival and proliferation only under the control of epidermal keratinocytes.

Notch signaling is implicated in the maintenance of the stem/progenitor cells of a variety of stem cell systems (Molofsky et al., 2004). In addition, recent reports also have evidenced that Notch signaling regulates stem cell fate in collaboration with other signaling pathways, such as Wnt and BMP (Dahlqvist et al., 2003; Duncan et al., 2005). In such a situation, it is important to define the exact molecular linkages at the downstream of Notch signaling under physiological conditions. Taking advantage of the MC system, where genetic alterations affecting stem/progenitor cell maintenance can be easily identified by coat-color defects, MCs would provide an avenue for elucidating the exact molecular networks involved in stem cell regulation by Notch signaling, as we have shown here that Hes1 acts at the downstream of Notch in Mbs.

Materials and methods

Mice

C57BL/6 mice and nude mice (BALB/C Slc-*nu*) were purchased from Japan SLC, Inc. *pHes1-d2EGFP*, *Tyr-Cre;RBP-J$^{f/f}$* mice were bred in our animal facility. All animal experiments were performed in accordance with the guidelines of the RIKEN Center for Developmental Biology for animal and recombinant DNA experiments.

Generation of Tg animals

Dct-Hes1 Tg mice were generated by injecting the Tg constructs that allow *Hes1* to be expressed under the control of the *Dct* promoter (provided by I. Jackson, Western General Hospital, Edinburgh, UK; Mackenzie et al., 1997) into fertilized eggs.

Skin organ culture and transplantation

Our organ culture system was adapted from Kashiwagi et al. (1997). Skin specimens were prepared from the dorsal coat of E13.5 embryos derived from wild-type or *Dct-Hes1* Tg mice and cultured in the presence or absence of 1 μM of the γ-secretase inhibitor DAPT (Peptide Institute, Inc.).

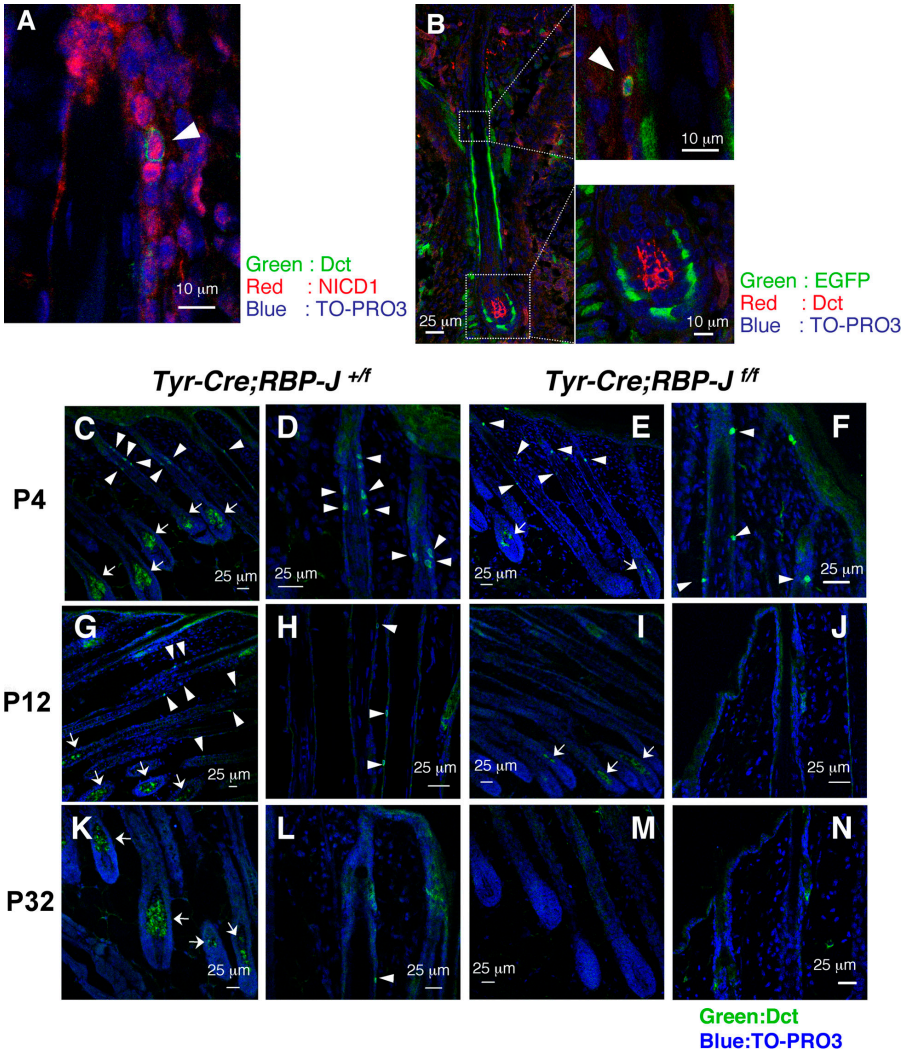

Figure 5. **Critical role of Notch signaling in the maintenance of MSCs.** (A and B) Activation of Notch signaling in MSC. (A) P4 skin section was stained with anti-Dct (green) and anti-NICD1 (red). (B) Skin section from a P4 *pHes1-d2EGFP* Tg mouse was stained with anti-GFP (green) and anti-Dct (red). Arrowheads indicate Mbs at the LPP. (C–N) Loss of MSCs in *Tyr-Cre;RBP-J$^{f/f}$* mice. Skin sections from P4 (C–F), P12 (G–J), and P32 (K–N) *Tyr-Cre;RBP-J$^{+/f}$* mice (C, D, G, H, K, and L) and *Tyr-Cre;RBP-J$^{f/f}$* mice (E, F, I, J, M, and N) were immunostained using anti-Dct antibody (green). Whole HF images (C, E, G, and I), LPP of HF images (D, F, H, J, L, and N), and HM images (K and M) are shown. Note that Dct$^+$ Mbs at the LPP of a HF exist at P4 (E and F) but have essentially disappeared at P12 (I and J), whereas mature MCs at the HM retain in *Tyr-Cre;RBP-J$^{f/f}$* mice (I). Arrowheads indicate Mbs at the LPP of the HF, and arrows indicate MCs at the HM.

After 4 d of skin organ culture, skin fragments were transplanted onto nude mice to assess the effect of DAPT treatment on melanogenesis.

RT- and Q-PCR

Total RNA was harvested from the FACS-isolated Mbs (Nishimura et al., 1999) by using an RNeasy mini kit (QIAGEN). Reverse transcription was performed with oligo dT primer using SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer's protocol. To examine expression of *Hes* family genes in the isolated Mbs, RT-PCR analysis was performed with 40 cycles of amplification. To confirm specific transgene expression in *Dct-Hes1* Tg mice, a specific primer pair was designed at the 3′ region of the *Dct* promoter and the 5′ region of *Hes1* cDNA, and PCR was performed with 35 cycles of amplification. Q-PCR was performed using a QuantiTect SYBR Green PCR kit (QIAGEN) according to the manufacturer's protocol. To present relative expression values to that of *Hes1*, the absolute expression value of each gene was calculated from each external calibration curve generated by serially diluted control vectors containing each gene. Then, each absolute expression value was divided with that of *Hes1*. PCR primers used in this study are shown in Table S1 (available at http://www.jcb.org/cgi/content/full/jcb.200509084/DC1).

Immunohistochemistry, in situ hybridization, and TUNEL staining

Immunohistochemistry using cryosections was performed as described previously (Nishimura et al., 2002). The following antibodies were used as primary antibodies: cleaved Notch1 (Cell Signaling), GFP (Invitrogen), cleaved caspase 3 (Cell Signaling), Pax3 (provided by G. Grosveld, St. Jude Children's Research Hospital, Memphis, TN; Hollenbach et al., 2002), Keratin 5 (Covance), and Dct (Santa Cruz Biotechnology, Inc.). Staining was performed using specific secondary antibodies conjugated to Alexa 488 or 546. TO-PRO3 iodide (Invitrogen) was used for nuclear staining. For whole-mount immunostaining, epidermis sheets were peeled off the dorsal skin of E16.5 and P0 mice. Immunostaining using ACK4 was then performed as described previously (Yoshida et al., 1996). In situ hybridization was performed as described previously (Wilkinson and Nieto, 1993). TUNEL staining was performed using an ApoAlert DNA fragmentation assay kit according to the manufacturer's protocol (BD Biosciences).

Microscopy, digital photography, and image processing

Gross images were acquired using a digital camera (model C-5050; Olympus). Light microscopy images were taken using a microscope (Axioplan 2; Carl Zeiss MicroImaging, Inc.) with a 20×/0.6 plan-APOCHROMAT objective by a cooled charge-coupled device camera (AxioCam HRc; Carl Zeiss MicroImaging, Inc.) controlled by AxioVision 3.0 software (Carl Zeiss MicroImaging, Inc.). Confocal imaging was performed using a microscope (Axiovert 200; Carl Zeiss MicroImaging, Inc.) with 40×/1.3 plan-NEOFLUAR and 63×/1.4 plan-APOCHROMAT objectives equipped with a confocal microscope system (Radiance 2100; Bio-Rad Laboratories) controlled by LaserSharp 2000 software (Bio-Rad Laboratories). Images were processed using Photoshop 7.0 (Adobe).

Online supplemental material

Fig. S1 shows flow cytometric isolation of Mbs from the E16.5 embryo epidermis. Fig. S2 shows the MC lineage–specific Cre-mediated recombination in *Tyr-Cre* mice. Table S1 shows PCR primers used in this study. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200509084/DC1.

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