Neural crest and Schwann cell progenitor-derived melanocytes are two spatially segregated populations similarly regulated by Foxd3

Erez Nitzan*, Elise R. Palfitzgraff†, Patricia A. Laboskyb,1, and Chaya Kalcheima,2

*Department of Medical Neurobiology, IMRIC and ELSC, Hebrew University-Hadassah Medical School, Jerusalem 91120, Israel; and †Department of Cell and Developmental Biology, Vanderbilt University Medical Center, Nashville, TN 37232-0494

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Skin melanocytes arise from two sources: either directly from neural crest progenitors or indirectly from neural crest-derived Schwann cell precursors after colonization of peripheral nerves. The relationship between these two melanocyte populations and the factors controlling their specification remains poorly understood. Direct lineage tracing reveals that neural crest and Schwann cell progenitor-derived melanocytes are differentially restricted to the epaxial and hypaxial body domains, respectively. Furthermore, although both populations are initially part of the Foxd3 lineage, hypaxial melanocytes lose Foxd3 at late stages upon separation from the nerve, whereas we recently found that epaxial melanocytes segregate earlier from Foxd3-positive neural progenitors while still residing in the dorsal neural tube. Gain- and loss-of-function experiments in avians and mice, respectively, reveal that Foxd3 is both sufficient and necessary for regulating the balance between melanocyte and Schwann cell development. In addition, Foxd3 is also sufficient to regulate the switch between neuronal and glial fates in sensory ganglia. Together, we propose that differential fate acquisition of neural crest-derived cells depends on their progressive segregation from the Foxd3-positive lineage.

DRG | Ednrb2 | MITF | pigment

All body melanocytes except the retinal pigment derive from the embryonic neural crest (NC), which in addition generates components of the peripheral nervous system, including sensory and autonomic neurons, satellite glia and Schwann cells (SCs), endocrine cells, and cranial mesectoderm (1, 2). The origins of pigment cells, their ancestral relationship to additional NC derivatives, and the mechanisms by which they segregate from each other remain poorly understood. Research in these areas is of fundamental importance for understanding aspects of pigment cell development as well as of adult physiology and pathology (3).

In the trunk of avian embryos, neural and melanocyte derivatives are sequentially produced after NC emigration from the dorsal neural tube (NT). The early emigrating cells migrate in a dorsoventral direction to generate first the sympatho-adrenal primordium, then SC of the peripheral nerves; this is closely followed by cells that generate the dorsal root ganglia (DRG) (4–6). A day later, the last delaminating cells switch their pathway to migrate dorsolaterally through the nascent dermis. These cells give rise to melanocytes (4, 5, 7). In the NT, this continuous cell delamination is compensated for by a corresponding relocation of progenitors toward the dorsal area that constitutes a transition zone for the progressive influx and departure of cells. This dynamic behavior of dorsal NT progenitors is also reflected by the differential expression of at least three transcription factors, Snail2, Sox9, and Foxd3, in early neural progenitors and their down-regulation in the late subset of prospective melanoblasts, hence suggesting an early segregation between the pre-delineages (4, 5). Along this line, gain of Foxd3 activity in vivo induced the development of glial cells while inhibiting melanocytes, and conversely, genetic ablation of Foxd3 function enhanced ectopic melanogenesis in the NT and later in DRG at the expense of both neurons and glia (8).

Adameyk et al. (9) found that SC progenitors (SCPs) lining elongating spinal nerves also constitute a source of pigment cells. These melanocytes differentiate at late stages relative to pigment cells directly generated from the NC. Thus, in addition to late emigrating/early differentiating NC-derived melanocytes, a subset of early delaminating NC cells that migrate dorsally and later become SCPs in association with the growing nerves can develop either into mature SCs if they remain in contact with nerve fibers or generate melanocytes if they detach from the nerve environment (3, 9, 10). Except for their common origin from the NC, the relationship between these two melanocyte subsets, at both cellular and molecular levels, remains to be elucidated. Not less intriguing is uncovering the downstream factors responsible for the switch between glial and melanocyte lineages. In vitro clonal analysis revealed the existence of single NC cells with melanocyte–glial potential (11), and further in vitro manipulations showed the plasticity of the differentiated melanocyte state (12). The mechanisms underlying this phenotypic conversion in the developing embryo are still obscure.

To begin investigating these questions, we asked whether the embryonic territories colonized by each melanocyte population are distinct or overlapping. Next we examined whether the nerve-derived SCP–melanocyte switch is regulated by Foxd3 as previously observed for melanocytes directly issued from the NC (8, 13, 14). Separate lineage tracing of each population revealed that NC and SCP-derived melanocytes are differentially restricted to the epaxial and hypaxial body domains, respectively, respecting the boundaries of the somite vs. lateral plate mesoderm-derived dermis through which they migrate. Furthermore, although both populations are initially part of the Foxd3 lineage, epaxial melanocytes segregate from Foxd3-positive neural progenitors already in the dorsal NT, whereas hypaxial melanocytes lose Foxd3 at late stages upon separation from the nerve. Gain- and loss-of-function experiments in avians and mice, respectively, reveal that Foxd3 is both sufficient and necessary for regulating the balance between melanocyte and SC development, similar to its role in epaxial melanocytes (8). Foxd3 is thus a negative regulator of both melanocyte populations. Because Foxd3 is also down-regulated in differentiating sensory neurons (15), we asked whether it plays a similar role during DRG development.
this normal down-regulation is prevented by conditional Foxd3 overexpression in nascent DRG, neurogenesis is inhibited. Hence, a timely down-regulation of Foxd3 is required for proper differentiation of both populations of NC-derived melanocytes as well as of NC-derived sensory neurons.

Results and Discussion

Dorsal Melanocytes Are Restricted to the Epaxial Domain of the Embryo, Whereas Ventral Melanocytes Primarily Colonize the Hypaxial Territory.

We characterized the relative domains colonized by “late emigrating, early differentiating” melanocytes directly issued from the NC and “early emigrating, late differentiating” melanocytes issued from NC-derived SCPs. To this end, a GFP-encoding DNA was electroporated into hemiblts of 35 somite stage (ss) embryos, a time when in the trunk, all ventrally migrating SCPs have already delaminated and only late-emigrating melanocytes are yet to leave the NT (5). Twenty-four hours later, at the limb level, labeled cells were present along the dorsolateral pathway, with the farthest cells reaching less than half-way between the dorsal midline and the ectodermal notch, an ectodermal/dermal indentation that subdivides epaxial from hypaxial domains of the embryonic body and serves as the limit between the somite and lateral plate-derived dermis, respectively (16–18) (n=9; Fig. 1A, arrowhead). By 48 h, dorsal melanocytes reached the level of the ectodermal notch (Fig. 1B), which remained their ventral limit of migration even at 72 h, both at limb and flank levels of the axis (n=7; Fig. 1 C and D). To further examine whether the ectodermal notch is the ventral-most limit attained by NC-derived melanocytes in the flank, where this structure is less conspicuous, sections were examined for expression of Sim1, a marker of somite-derived dermis, whose ventral limit of expression reaches the notch (17, 18). Indeed, GFP-labeled dorsal melanocytes, whether localized in dermis or ectoderm, did not migrate beyond the lateral-most Sim1-positive, epaxial domain of the body (n=4; Fig. 1 D–D’). Consistent with the precedent results, the majority of Ednrb2 and MC/1+ cells (5, 8) were apparent in the epaxial domain of the body at flank regions of embryonic day (E)5 embryos with very few pigment cells in the abdominal area at this stage (Fig. 1 E, G, and I). In contrast to flank, at wing and hindlimb levels, total Ednrb2 and MC/1+ melanocytes were similarly distributed in both epaxial and hypaxial domains comprising the ectoderm and dermis of wings and limbs (n=5 for each axial level; Fig. 1 F, H, and I, P < .05 for hypaxial melanocytes in flank compared with limbs). To compensate for differences in surface area, we also determined the density of melanocytes in each domain; this measurement similarly showed that hypaxial melanocyte density is higher at limb compared with flank levels (Fig. 1 F, P < .05). Together, this raised the questions of the origin of the hypaxial melanocytes and the precise embryonic territories they colonize.

Previously, SCP-derived melanocytes were reported to populate the limbs (3). Because our results show that NC-derived melanocytes are restricted to the epaxial body domain, we asked whether SCP-derived pigment is limited to the hypaxial domain or alternatively distributes throughout the dorsolateral wall. To examine this issue, the dorsal NT was removed by cauterization at 35 ss at the hindlimb level after emigration of SCP but before delamination of NC-derived melanocytes (5), thus selectively eliminating the latter population. A day later, no dorsolaterally migrating melanocytes were apparent in the cauterized embryos compared with intact controls, and only few pigment cells were present close to the nerves in both intact and cauterized cases (n=6 and 6; Fig. 2A and B). At E5.5, Ednrb2+ SCP-derived pigment cells populated the hindlimb region but were virtually absent from the epaxial domain located dorsal to the ectodermal notch (n=5 and 11 for controls vs. cauterized; Fig. 2 C–E, *P < .05). This suggests that at least until E5.5, NC and SCP-derived melanocytes are topographically segregated to epaxial and hypaxial body domains, respectively.

To further challenge this conclusion, we selectively and stably labeled the early ventrally migrating NC cells (i.e., prospective SCP population) with GFP using Tol2 transposon-mediated genomic integration (19). Electroporation was performed at the hindlimb level of embryos aged 23 ss, before the onset of NC emigration. To selectively label the early emigrating cells, electric current was focally applied in a ventral to dorsal direction (Fig. 2F). Sixteen hours later, live embryos were inspected under a fluorescent microscope to ensure that all GFP+ cells emigrated (Fig. 2G) and that no residual labeling remained in the NT that at this stage still contained presumptive late-emigrating, NC-derived melanoblasts (5). Final analysis at E7 revealed that GFP+ cells were restricted to the hindlimbs where a subset coexpressed MC/1 (n=5; Fig. 2 H and I–I’ and Fig. S1). In
Fig. 2. Early-emigrating, SCP-derived melanocytes colonize the hypaxial domain. (A–D) Indirect evaluation of the territory colonized by SCP-derived melanocytes. ISH for Ednrb2 of intact embryos (A and C) and embryos whose dorsal NT was cauterized at 35 ss at the hindlimb level (B and D). (A and B) Twenty-four hours after cauterity, no Ednrb2* cells are found in the dorsolateral pathway compared with intact controls. In contrast, note in both cases the presence of few ventral Ednrb2* cells adjacent to the nerves (arrowheads). Black dotted lines in A and B delineate the spinal nerves. (C–E) At E5.5, Ednrb2* melanocytes are found in both epaxial and hypaxial domains of control embryos but are missing in the epaxial domain of cauterized embryos. Dotted black line separates between epaxial and hypaxial (limb) domains. White dotted lines delimit the embryo surface and the spinal cord. (E) Quantification of the number of Ednrb2* cells in the epaxial domain of E5.5 embryos, *P < 0.05. (F–J) Direct visualization of SCP-derived melanocytes colonizing only the hypaxial limb territory. Stable and focal labeling of early emigrating NC cells that migrate along the ventral pathway to populate spinal nerves and other neural derivatives. (F) Experimental scheme. Ventral to dorsal coelectroporation of pCAGG-T2TP and pT2K-EGFP was performed at E2. Sixteen hours later, embryos were inspected to ensure that no residual GFP* cells remained in the NT (i.e., that the prospective late emigrating melanocytes are unlabeled; see also dorsal view of a living embryo in G). At E6–E7, the localization of GFP* nerve-derived melanocytes cells was monitored (H–J), same embryo as in G). I and J are higher magnifications of the boxed areas in H. (F) Hypaxial limb (I–J) regions separated in H by a white line. Note that GFP*/MC1* SCP-derived melanocytes (arrowheads in I–J) are restricted to the hypaxial domain. In contrast, NC-derived MC1* epaxial melanocytes are GFP-negative (U and J). Fig. S1 shows transverse sections. EN, epidermal notch; ON, overnight; SC, spinal cord. (Scale bar, 50 μm in A and B; 40 μm in C and D.)

Together, MC1+ pigment cells in the epaxial domain were GFP-negative (Fig. 2 H and J–I). Altogether, NC-derived melanocytes migrate through the dermis and later invade the ectoderm as far ventral as the ectodermal notch, which represents the border between the epaxial and hypaxial body domains. Reciprocally, SCP-derived melanocytes are initially restricted to the hypaxial domain at wing and hindlimb regions, at least until E7. We cannot rule out the possibility that few melanocytes could derive from the dorsal ramus of the peripheral nerve and spread into the epaxial area. Even if this were the case, this does not undermine the significance of an epaxial vs. hypaxial segregation of melanocytes during the patterning phase, when these two subsets are first established. Previous experiments in chicken embryos in which GFP-labeled NC-derived melanocytes are unlabeled; not discriminate between a direct NC vs. a dorsal ramus origin because labeling concerned the entire hemi-NT (9). Hence, during formation of the body plan, establishment of the epaxial/hypaxial border is relevant to the formation of distinct muscles (18, 20, 21) to the development of the epaxial dermis from the dermomyotome vs. the hypaxial dermis from the somatic layer of the lateral plate mesoderms (17, 22) and also to the restricted distribution of the two melanocyte subpopulations. The association between these processes is sensible because the dissociating dermal mesenchyme serves as substrate for melanoblast migration (23).

**Foxd3 Acts as a Fate Switch Between SCPS and Hypaxial Melanocytes.** Foxd3 expression characterizes premigratory neural progenitors of the NC and early ventrally migrating cells (5, 14, 15), yet it is down-regulated in presumptive epaxial melanocytes before their exit from the NT (5, 8); at later organogenetic stages, Foxd3 is down-regulated in DRG and SG neurons yet persists in glia and SCP for a few more days, until their final differentiation (refs. 14 and 15 and see below). Thus, we asked whether SCP-derived melanocytes express Foxd3. Cross-sections of E5 hindlimb segments showed expression of Foxd3 in cells along the spinal nerve (Fig. 34). However, MC1* cells surrounding the nerve were Foxd3-negative (Fig. 3 B and C, arrowheads), suggesting that SCP-derived melanocytes down-regulate Foxd3. To examine whether hypaxial melanocytes derive from Foxd3+ SCPS, a previously characterized Foxd3 enhancer element was cloned upstream of Cre recombinase and focally electroporated into the dorsal NT of avian embryos aged 22 ss along with a Cre-dependent GFP plasmid (Foxd3::Cre + pCAGG::LoxP-STOP-LoxP-GFP) (8), using the method described in Fig. 2F. This approach enabled stable lineage tracing of the enhancer expressing cells (8, 24, 25). Six hours after ventral to dorsal electroporation of NTs, GFP* cells were restricted to the dorsal NT, while cells expressing endogenous Foxd3 are located (8). Further analysis at E5 revealed the presence of GFP expression in both SCP along the nerve, as well as in MC1+ melanocytes in the limb (n = 5 of 6; Fig. 3 D–F), corroborating the notion that SCP-derived hypaxial melanocytes, similar to their epaxial counterparts, stem from a Foxd3+ lineage.

Previous results demonstrated that misexpression of Foxd3 in presumptive epaxial melanocytes inhibits their proper dorsolateral migration and differentiation while promoting instead a glial fate (8). Here we asked whether the SCP–melanocyte switch is similarly sensitive to Foxd3. To test this possibility, we prevented the normal down-regulation of Foxd3 in SCP after they reached their final sites. To this end, full-length Foxd3 under regulation of the tetracycline-responsive promoter (Foxd3-TRE) was electroporated into hindlimb-level hemitubes at 25 ss, along with rtTA2s-M2 (19). Two days later, when the transfected cells had reached the spinal nerve, doxycycline was injected underneath the blastoderm to induce Foxd3 activity, and embryos were reincubated for an additional 24 h (Fig. 3G). In control GFP-TRE embryos, a subset of labeled cells lost contact with the nerve and up-regulated expression of MC1 and Ednrb2 (n = 10; Fig. 3 H–H**, arrows, and Fig. S2 A and B). In contrast, all Foxd3-TRE-GFP–transfected cells remained associated with the
nerve and were MC1 and Ednrb2-negative; reciprocally, the Ednrb2 and MC1 Foxd3 melanocytes in the hypaxial domain were Foxd3-GFP negative (n = 7, Fig. 3I–I'''), arrowheads, and Fig. S2 C and D). Quantifications revealed the presence of 3.1 ± 0.5 and 0.12 ± 0.1 GFP+/MC1 Foxd3 melanocytes per section in control vs. Foxd3-treated conditions (n = 4 embryos and 18 sections counted for each treatment, P < 0.01). We conclude that both early migrating SCP as well as late-emigrating epaxial NC cells must down-regulate Foxd3 for proper development into melanocytes.

Consistent with this notion, attenuation of Foxd3 in vitro with antisense morpholinos enhanced melanogenesis (13, 26), and loss of Foxd3 gene function in the mouse NC promoted melanocyte development at the expense of neural fates in DRG (8). To examine whether Foxd3 similarly affects the balance between SCP and melanocytes in the mouse, we analyzed mouse embryos carrying a conditional deletion of Foxd3 in the NC. We compared Foxd3Δflx/+; Wnt1-Cre; R26R (mutant) embryos with Foxd3Δflx/+; Wnt1-Cre; R26R (control) embryos. In the trunk of 12.5 days postcoitum (dpc) control embryos, growing nerves expressed TuJ1, and Mitf1 melanocytes were observed in the neighboring hypaxial dermis and ectoderm (Fig. 4A and A'). In striking contrast, many Mitf1 cells were apparent along the mutant nerves in addition to their expected dermal/ectodermal distribution (Fig. 4B and B'). Likewise, an ectopic up-regulation of Mitf was observed at 11.5 dpc along mutant cranial nerves compared with controls (Fig. 4C and D). Together, these genetic data suggest that Foxd3 is necessary for the maintenance of NC-derived SCP by repressing ectopic melanogenesis. Hence, both NC-derived epaxial and SCPtrived hypaxial melanoblasts require that Foxd3 be down-regulated to correctly migrate and differentiate (Fig. 4E).

Because enhanced melanogenesis was also reported to occur in mouse embryos lacking the neuregulin receptor Erbb3 (9), we examined expression of Erbb3 in Foxd3 mutants. No apparent change in the pattern of Erbb3 expression could be detected in SCPs along mutant compared with control nerves (Fig. S3). Reciprocally, no dependency of Foxd3 expression on Erbb3 was detected (10), altogether indicating that each gene independently affects the SCP–melanocyte balance in the nerve environment. Nevertheless, less Erbb3 expression was observed in mutant compared with control DRG at 11.5 and 12.5 dpc (Fig. S3), likely reflecting the previously observed reduction in glial cell number associated with a glial-to-melanocyte switch (8).

**Foxd3 Acts as a Fate Switch Between Neurons and Glia in the DRG.** Next, we examined whether Foxd3 activity is involved in fate decisions of additional NC derivatives. Although ventrally migrating NC progenitors transcribe Foxd3 (5, 14, 15), differentiating sensory neurons in nascent DRG, marked by expression of ISLET1, down-regulate Foxd3 mRNA (Fig. 5A). However, many Fabp7 glial cells as well as undifferentiated progenitors (ISLET1/Fabp7) still maintain Foxd3 expression (Fig. 5B–B'', (15)). We therefore hypothesized that preventing Foxd3 down-regulation in differentiating DRG will inhibit neuronal differentiation while rescoping them into glia as previously shown (see above and ref. 8) and/or maintaining them in a progenitor state (15, 27, 28).

Foxd3-TRE was electroporated into the E2 chick NT as shown in Fig. 3G. Thirty hours later, when migrating NC cells have begun colonizing the DRG, doxycyclin was supplied and embryos reincubated for another day. At E4, control GFP-TRE+ cells were distributed throughout the ganglion where they coexpressed CN, cranial nerve; OV, otic vesicle; SC, spinal cord. (Scale bar, 50 μm in A–C, 70 μm in D; 30 μm in E and F; 80 μm in H and I; 40 μm in H''–I''–).
the sensory neuronal markers ISLET1, BRN3a, and Hmx1 (n = 5; Fig. 5 C, D, and I and Fig. S4) and the glial markers Fabp7 and P0 (Fig. 5 E and E' and Fig. S5). In contrast, Foxd3-TRE+ cells populated only the ganglion periphery and were negative for all neuronal markers (n = 6; Fig. 5 F, G, and I, P < 0.05 for ISLET1; Fig. S4). Nevertheless, a subset of Foxd3+ cells coexpressed Fabp7 or P0, whereas others remained negative for the above, the latter likely representing undifferentiated progenitors (Fig. 5 H and H' and Fig. S5). These data suggest that, in DRG, Foxd3 both maintains NC progenitors in an undifferentiated state (29) and is also compatible with glial development (8, 14). However, Foxd3 down-regulation is required for their differentiation into sensory neurons. These multiple effects indicate that Foxd3 acts differentially on DRG progenitors with various degrees of fate restriction.

**Progressive Exit of NC-Derived Fates from the Foxd3+ Lineage.**

Analysis of the dynamics of Foxd3 expression (4, 5, 15) suggested that this transcription factor is gradually lost in NC lineages as they commit to a specific fate. However, functional evidence for this notion was missing. Using temporally regulated conditional misexpression of Foxd3 in either dorsal NT, DRG, or SCPs along peripheral nerves, we show that the progressive exit from the Foxd3 lineage is pivotal for proper fate selection and differentiation of most NC-derived cells in the trunk (ref. 8 and this study; Fig. S7). Hence, progressive down-regulation of Foxd3 mediates restrictions on NC derivatives. The question remains open whether Foxd3 acts on multipotent NC progenitors to maintain this state or on fate-restricted precursors. At the beginning of development, Foxd3 is expressed in all NC progenitors in the trunk (8), where it might act to maintain their multilineage characteristics (29). However, recent functional data suggest that already before cell emigration from the NT, fate-restricted NC progenitors also exist (5). As part of this mechanism, while still residing in the dorsal NT, the late-emigrating prospective epaxial melanocytes down-regulate Foxd3 as well as additional transcription factors and become committed to melanogenic differentiation (8) (Fig. 5J).

Later, the early ventrally migrating cells that begin colonizing the DRG still transcribe Foxd3 (14, 15). Upon DRG formation, nascent sensory neurons down-regulate Foxd3 and differentiate (Fig. 5J). Notch-dependent lateral inhibition within the ganglion environment affects the balance between neuronal and glial development (30). It remains to be tested whether Notch signaling acts via Foxd3 to regulate these fate decisions.

Approximately 1 d after DRG colonization, SCPs that home to the spinal nerve maintain Foxd3 expression (this study and refs. 3 and 9). At this point, SCPs can be considered as bipotent cells that chose their final fate depending on interactions with the growing nerves. Nerve-derived Neuregulin1 (3, 9) or other yet unknown signals could stimulate gliogenesis via Foxd3, a possibility that remains to be tested. In contrast to presumptive SCs, hypaxial melanocytes stemming from the SCP population down-regulate Foxd3 (Fig. 5J), lose contact with the nerve, up-regulate Ednrh2, and migrate to the ectoderm, where differentiation occurs. Only days later, terminally differentiated SCs also down-regulate Foxd3 (15).

Taken together, our functional in vivo data lend support to the notion that the timely loss of Foxd3 activity is a general prerequisite for melanocyte and neural development issued from the NC and its derivatives. Because in addition Foxd3 plays a positive role on gliogenesis in DRG and nerves and was also shown to maintain early multipotent progenitors in an undifferentiated state, Foxd3 is likely to play different roles in progenitors exhibiting different states of commitment.
Embryos. Avian embryos. Chick (Gallus gallus) eggs were obtained from commercial sources. Experiments were conducted either in forelimb, hindlimb, or midtrunk level as indicated.

Mouse embryos. The Foxd3 conditional and null alleles (Foxd3lox/lox and Foxd3flox/flox) were described previously (28, 31). The Wnt1Cre transgenic line was used to delete Foxd3lox in the NC (Foxd3flox/flox; Wnt1Cre mutant embryos), and to lineage map NC, the R26ERT2 reporter strain was used (29).

Expression Vectors. The following vectors were used: pCAGG-APF, pCAGG-RFP, the Foxd3 enhancer element 168-Cre, pCAGG::LoxP-STOP-LoxP-GFP (8), pT2K-CAGGS-EGFP and pCAGGS-2T2P (19), and pBI-TRE-GFP and pBI-TRE-FOX3 along with pCAGGS-rtTA2s-M2 (see below and ref. 32).

Lineage Analysis of Foxd3-Expressing Cells Along Spinal Nerves. A plasmid containing the #168 enhancer from the Vista enhancer browser (http://enhancer.lbl.gov) driving expression of Cre recombinase was coelectroporated into dorsal NT along with a reporter plasmid in which a floxed transcriptional STOP module was inserted between the CAGG enhancer/promoter and the GFP gene (pCAGG::LoxP-STOP-LoxP-GFP) (25, 33).

Tetracyclin-Induced Expression of Foxd3. Full-length chick Fox3 was cloned into pBl (pET) and coelectroporated along with pCAGGS-rtTA2s-M2 into hemi-NTs at the hindlimb level of 25 ss embryos. Embryos were inspected under epifluorescence to monitor for possible signal leakage at 24 or 48 h after electroporation; doxycycline was then injected underneath the blastoderm, as previously described (32). Embryos were reincubated for additional 12–24 h before fixation.

Embryo Manipulations. Electroporation. Plasmid DNA (2–5 mg/mL) was microinjected into the lumen of the chick NT. For hemi-NT electroporations, 0.5-mm after electroporation; doxycycline was then injected underneath the blastoderm, as previously described (32). Cells nuclei were visualized with Hoechst.

In situ hybridization (ISH) was performed as previously described (36). The following probes were used: chick Fox3 (14, 15), Sim1 (17), Ednrb2 (8), Fabp7 (37), Hmx1 (9), and mouse Mitf (38) and Erbb3 (39).

Data Analysis and Statistics. The relative percentages of epaxial vs. hypaxial melanocytes expressing either MCT1 or ErbB2, the number of melanocytes per unit area, and the percentage of ISLET-1 of total GFP+ neurons in DRG were monitored. Results represent mean ± SEM of 5–10 sections per embryo counted in 4–8 embryos per experimental treatment. An average of 4–15 melanocytes per section was counted in the different domains. Data were subjected to statistical analysis using the parametric Mann–Whitney and Kruskal-Wallis tests. All tests applied were two-tailed with P < 0.05.

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Fig. S1. Schwann cell progenitor (SCP)-derived melanocytes colonize the hypaxial domain. (A–B”) Transverse sections through the hindlimb region of the embryo presented in Fig. 2 F–I, showing restricted localization of GFP+/MC1+ cells to the hypaxial limb domain with no crossing toward the epaxial region. Dotted line separates epaxial from hypaxial areas. (B–B”) High magnifications of a hypaxial region in A showing colocalization of the GFP lineage tracer with the melanocyte marker MC1 (arrowheads). Methods: ventral to dorsal coelectroporation of pCAGG-T2TP and pT2K-EGFP was performed at embryonic day (E)2 to label SCPs that reach and associate with spinal nerves. Sixteen hours later, embryos were inspected to ensure that no residual GFP+ cells remained in the NT (i.e., that the prospective late-emigrating, neural crest (NC)-derived melanocytes are unlabeled). At E6–E7, the localization of GFP+ nerve-derived melanocytes cells was monitored (details in Fig.2). DRG, dorsal root ganglion; SC, spinal cord. (Scale bar, 40 μm in A; 20 μm in B–B").

Fig. S2. Continuous expression of Foxd3 in SCP inhibits melanocyte development. (A–D) Transverse sections through E5.5 embryos electroporated at E2 (hindlimb level) with pCAGGS-rtTA2s-M2 together with either pBI-TRE-GFP (A–B”) or pBI-TRE-Foxd3 (C–D’). Doxycyclin was added 2 d later (see below for details and also legend to Fig. 3). In control embryos, GFP+ cells are attached to the nerve, where they coexpress HNK-1 (A). Because HNK-1 is also expressed by the spinal nerve fibers, A’ and A” show GFP+ staining surrounding Hoechst+ nuclei [i.e., corresponding to nerve-associated SCPs (arrowheads)]. In addition, control GFP+ cells are distributed in the dermis and in the vicinity of nerve fibers, where many coexpress Ednrb2 (arrows in B’ and B”; arrowheads point to few GFP+/Ednrb2- cells). In contrast, Foxd3+ cells are absent from the dermis/epidermis. Instead they are only found in HNK-1–expressing SCPs associated with nerve fibers (arrowheads in C and C’), where they fail to up-regulate Ednrb2 (arrowheads in D and D’). Arrows in D’ represent Ednrb2+ melanocytes that are negative for Foxd3-TRE. HL, hindlimb; NT, neural tube; SN, spinal nerve. In all panels, lateral is to the left. (Scale bar, 50 μm in A–D; 40 μm in B and B’; 30 μm in A’, A”, and D’; 20 μm in C’).
Fig. S3. Expression of *Erbb3* in control and FoxD3 mutants. Transverse sections showing expression of *Erbb3* mRNA in control (A, C, and C') and FoxD3 mutant (B, D, and D') embryos at 11.5 and 12.5 days post-coitum (dpc). A strong signal is apparent in SCPs lining the spinal nerves in all cases (nerves delimited by dotted white lines). In contrast, mutant DRG exhibit reduced *Erbb3* expression that is mainly localized to cells in the ganglion periphery; in controls, *Erbb3*-positive cells are distributed throughout the DRG. Note that overall DRG size is slightly reduced in mutants. (C' and D') Higher magnifications of DRG in C and D, respectively. *Erbb3* is also transcribed in skeletal muscle (M). (Scale bar, 90 μm in A; 80 μm in B; 60 μm in C and D; 30 μm in C' and D'.)

Fig. S4. Conditional FoxD3 misexpression in DRG inhibits sensory neurogenesis. Expression of the sensory neuron markers Brn3a and *Hmx1* in E4 DRG, electroporated with pCAGGS-rtTA2s-M2 along with either pBI-TRE-GFP (control; A, A', C, and C') or pBI-TRE-Foxd3 (B, B', D, and D') at E2, and injected with doxycyclin at E3 (details in legend to Fig. 5). Note homogeneous localization of control GFP+ cells throughout the DRG (A and C), contrasting with the restricted distribution of Foxd3/GFP+ cells to the ganglion periphery (B and D). Arrowheads in A and C point to neurons coexpressing control GFP and Brn3a or *Hmx1*. No such double-positive cells were detected in Foxd3-treated ganglia (arrows in A–D'). (Scale bar, 30 μm.)
Fig. S5. Sustained Foxd3 activity in DRG is compatible with glial development. Electroporation of control GFP (A and C) or conditional misexpression of Foxd3 in DRG (B and D). pCAGGS-rtTA2s-M2 together with either pBI-TRE-GFP or pBI-TRE-Foxd3 were electroporated into hemitubes at E2. Doxycyclin was added 32 h after electroporation when NC cells already formed DRG, and analysis performed 24 h later. (A and B) Costaining of GFP with Fabp7. (C and D) Costaining of GFP with melyn protein zero (P0). Arrowheads point to double-positive glia cells and arrows to GFP+/Fabp7 or P0-negative progenitors. (A–A''', B–B''', C–C''', and D–D'') Higher magnifications of the boxed areas in A–D, respectively. (Scale bar, 60 μm in A, C, and D; 50 μm in B; 20 μm in A''–A'''' and B–B''''; 25 μm C–C'''; 30 μm in D–D''.)