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Temporal control of neural crest lineage generation by Wnt/β-catenin signaling

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SUMMARY

Wnt/β-catenin signaling controls multiple steps of neural crest development, ranging from neural crest induction, lineage decisions, to differentiation. In mice, conditional β-catenin inactivation in premigratory neural crest cells abolishes both sensory neuron and melanocyte formation. Intriguingly, the generation of melanocytes is also prevented by activation of β-catenin in the premigratory neural crest, which promotes sensory neurogenesis at the expense of other neural crest derivatives. This raises the question of how Wnt/β-catenin signaling regulates the formation of distinct lineages from the neural crest. Using various *Cre* lines to conditionally activate β-catenin in neural crest cells at different developmental stages, we show that neural crest cell fate decisions in vivo are subject to temporal control by Wnt/β-catenin. Unlike in premigratory neural crest, β-catenin activation in migratory neural crest cells promotes the formation of ectopic melanoblasts, while the production of most other lineages is suppressed. Ectopic melanoblasts emerge at sites of neural crest target structures and in many tissues usually devoid of neural crest-derived cells. β-catenin activation at later stages in glial progenitors or in melanoblasts does not lead to surplus melanoblasts, indicating a narrow time window of Wnt/β-catenin responsiveness during neural crest cell migration. Thus, neural crest cells appear to be multipotent in vivo both before and after emigration from the neural tube but adapt their response to extracellular signals in a temporally controlled manner.

KEY WORDS: Wnt, β-catenin, Neural crest stem cells, Sensory neurons, Melanocytes, Mouse

INTRODUCTION

Many neural and non-neural cell types in vertebrates are produced by the neural crest, a transient embryonic structure that emerges during neurulation in the dorsal part of the neural tube (Le Douarin et al., 2008). Among the neural crest derivatives are the pigment cells in the skin that are reported to originate either directly from neural crest cells migrating from the neural tube on a dorsolateral pathway or from nerves innervating the skin (Sommer, 2011). Melanocyte lineage specification of dorsolaterally migrating cells is thought to occur at an early stage of neural crest development. Cells fated for the melanocyte lineage stall in the migration staging area (MSA) adjacent to the neural tube before continuing their dorsolateral migration (Weston, 1991). In the MSA, melanocyte specification is indicated by expression of the microphthalmia-associated transcription factor (Mitf), which controls melanoblast development and survival (Opdecamp et al., 1997). Both in cell culture and in vivo, expression of *Mitf* is regulated by canonical Wnt signaling, indicating a central role of this signal transduction pathway in melanocyte development (Dorsky et al., 2000; Takeda et al., 2000; Hari et al., 2002; Widlund et al., 2002).

Canonical Wnt signaling involves the intracellular signaling component β-catenin, which, upon activation, translocates to the nucleus and induces specific transcriptional responses (Gordon and Nusse, 2006). In cultures of mouse and quail neural crest cells, treatment with Wnt or activation of β-catenin enhances melanoblast proliferation and differentiation (Dunn et al., 2000; Jin et al., 2001). More strikingly, constitutive activation of β-catenin in zebrafish in vivo promotes the formation of melanocytes while repressing neural cell lineages (Dorsky et al., 1998). By contrast, upon inhibition of Wnt signaling, neural crest cells adopt a neural rather than a pigment cell fate. Similarly, in the mouse, conditional deletion of β-catenin (*Ctnnb1*) in premigratory neural crest cells also prevents the generation of melanocytes (Hari et al., 2002). However, unlike in zebrafish, inactivation of *Ctnnb1* in the mouse neural crest not only prevents melanocyte formation but also sensory neurogenesis (Hari et al., 2002). Moreover, in contrast to zebrafish, *Ctnnb1* signal activation in the mouse neural crest in vivo does not enhance melanocyte formation, but rather promotes the widespread formation of sensory neurons at the expense of virtually all other possible neural crest cell fates, including melanocytes (Lee et al., 2004).

In the present study we offer an explanation for these inconsistent findings, suggesting that in mouse embryos Wnt/β-catenin signaling controls sensory fate acquisition before regulating melanocyte lineage formation. To analyze the temporal control of neural crest lineage generation by Wnt/β-catenin signaling, we genetically manipulated *Ctnnb1* at different stages of neural crest and early melanocyte development. Our results are in agreement with the idea that sensory neuronal and melanocyte lineages are successively generated from neural crest cells by sequential β-catenin signaling.

MATERIALS AND METHODS

Animals and genotyping

The *Cre-loxP* system was used to conditionally express a stabilized form of β-catenin as described (Lee et al., 2004). Mutant embryos were heterozygous for *Cre* and for $Ctnnb1^{\Delta ex3}$. Littermates inheriting an

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incomplete combination of alleles, i.e. either *Cre* or *Ctnnb1*$^{\Delta ex3}$, served as controls. For in vivo fate mapping experiments, either *ROSA26* reporter (*R26R*) (Soriano, 1999) or *Z/EG* (Novak et al., 2000) mouse lines were used. Embryos carrying one allele for lineage tracing are indicated as *R26R* or *Z/EG*. The following *Cre* lines were used: *Wnt1-Cre* (Danielian et al., 1998), *Sox10-Cre* (Matsuoka et al., 2005), *Dhh-Cre* (Jaegle et al., 2003; Joseph et al., 2004), *Plp-CreERT2* (Leone et al., 2003) and *Tyr-CreERT2* (Bosenberg et al., 2006). Genotyping for all *Cre* alleles was performed by PCR with primers Cre-up (5′-ACCAGGTTCGTTCACTCATGG-3′) and Cre-lo (5′-AGGCTAAGTGCCTTCTCTACAC-3′) and 35 cycles of 94°C for 45 seconds, 58°C for 30 seconds and 72°C for 1 minute, to amplify a 217 bp fragment within the *Cre* ORF.

Inducible Cre expression

For activation of the CreERT2 protein, tamoxifen (Sigma T5648) was dissolved in 1:10 ethanol:sunflower oil at 10 mg/ml. Tamoxifen (1 mg) was injected intraperitoneally into the pregnant mother on 1 day for *Plp-CreERT2* mice at the indicated stages and on 2 consecutive days at E11.5 and E12.5 for *Tyr-CreERT2* mice.

In situ hybridization, X-Gal staining and immunohistochemistry

In situ hybridization on cryosections with digoxigenin-labeled riboprobes was performed as described (Hari et al., 2002). *Mitf*- and *Dct*-specific riboprobes were used as described (Hari et al., 2002). The 1.2 kb *Mitf* riboprobe corresponded to position 177-1399 of the published mouse *Mitf* sequence (GenBank accession no. NM_008601) (Bondurand et al., 2000).

lacZ reporter gene expression was detected using X-Gal staining. Cryosections were fixed for 5 minutes on ice in PBS containing 2% formaldehyde, 0.2% glutaraldehyde, 0.02% NP40 and stained for 4-16 hours at 37°C in PBS containing 1 mg/ml X-Gal, 10 mM $K_3Fe(CN)_6$, 10 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$, 0.02% NP40.

For immunohistochemistry, cryosections were fixed for 5 minutes in 4% formaldehyde at room temperature and treated with blocking buffer (1% BSA, 0.3% Triton X-100, in PBS) for 30 minutes. Primary antibodies were used as follows: mouse anti-Neurog2 (1:10; gift from D. J. Anderson, California Institute of Technology, Pasadena, CA, USA), goat anti-Dct (1:200; Santa Cruz sc-10451), mouse anti-Mitf (1:200; Abcam ab12039, C5 clone), rat anti-c-Kit (1:200; eBioscience 14-1171), mouse anti-PHH3 (1:200; Cell Signaling 9706), goat anti-NeuroD (1:200; Santa Cruz sc-1084), mouse anti-Cre (1:150; Abcam ab24607), chicken anti-β-galactosidase (1:2000; Abcam ab9361), mouse anti-TH (1:200; Sigma T1299), goat anti-Sox10 (1:200; Santa Cruz sc-17342), rabbit anti-GFP (1:500; Abcam ab290) and rabbit anti-Brn3a (1:2000; gift from E. E. Turner, Seattle Children's Research Institute, Seattle, WA, USA). Secondary antibodies were from Jackson ImmunoResearch or Invitrogen.

Statistical analysis

All quantifications were performed on 18-μm transverse sections. Recombination efficiency of the *Wnt1-Cre* line was assessed by counting β-galactosidase/Sox10 double-positive cells among total Sox10-positive cells in three different *Wnt1-Cre/R26R* E10.5 embryos at the level of the hindlimbs, counting five sections per embryo. Recombination efficiency of *Sox10-Cre* was quantified by counting Cre/Sox10 double-positive cells among total Sox10-positive cells in three different *Sox10-Cre* embryos at the level of the forelimbs, counting 11 sections per embryo. Fig. 3U represents the average numbers of *Dct*-positive cells per MSA at E11.5 and per mm length of skin at E16.5. At E11.5, *Dct*-positive cells were counted in 19 MSAs on ten sections per embryo. At E16.5, *Dct*-positive cells were counted in the skin of six entire lateral sides (dorsal to ventral) on six sections. Quantification for both stages was at the level of the forelimbs of three control and three *Sox10-Cre/Ctnnb1*$^{\Delta ex3}$ embryos. Proliferation for Fig. 3V was analyzed by counting PHH3/Sox10 double-positive cells among total Sox10-positive cells. At E9.5, quantification was performed on every fourth section between the forelimbs and the hindlimbs; at E10.5, quantification was on six sections at the level of the forelimbs. Three control and three *Sox10-Cre/Ctnnb1*$^{\Delta ex3}$ embryos were quantified for each stage. Brn3a- and *Mitf*-positive cells for Fig. 5K were counted on at least seven sections of the sympathetic ganglia at the level of the forelimbs. Quantifications were performed on four *Wnt1-Cre/Ctnnb1*$^{\Delta ex3}$ embryos and on four *Sox10-Cre/Ctnnb1*$^{\Delta ex3}$ embryos. Quantification of *Mitf*- and Brn3a-positive cells was on adjacent or near-adjacent sections of the same embryos. All results are shown as mean ± s.d. Statistical analyses (two-tailed unpaired Student's *t*-test; calculation of s.d.) were performed with Microsoft Excel.

RESULTS

β-catenin signal activation in premigratory but not in migratory neural crest cells suppresses melanocyte specification

Although, in mice, neural crest cells concomitantly engage in the ventral and dorsolateral migratory pathways (Serbedzija et al., 1990), neural and melanocyte cell type specification might occur sequentially. This is reflected by the sequential expression of transcription factors associated with lineage specification. Neurogenin 2 (Neurog2), a basic helix-loop-helix (bHLH) transcription factor required for sensory neurogenesis (Ma et al., 1996), is already expressed in neural crest cells as they begin to delaminate from the neural tube (Sommer et al., 1996). By contrast, the bHLH leucine zipper transcription factor Mitf, which promotes melanocyte development, is only expressed from E10.5 onwards (Opdecamp et al., 1997). Accordingly, Neurog2 was prominently expressed in a subset of migrating neural crest cells and in forming dorsal root ganglia (DRG) in caudal regions of mouse embryos at embryonic day (E) 10.5. By contrast, dopachrome tautomerase (Dct), an enzyme of the pigment synthesis pathway and an early marker of the melanocyte lineage, was not expressed in these cells (Fig. 1A). Similarly, at this early stage, neural crest cells marked by the transcription factor Sox10 (Kuhlbrodt et al., 1998; Paratore et al., 2001; Kleber et al., 2005) expressed neither Mitf protein, *Mitf* mRNA nor c-Kit, another early marker of the melanocyte lineage (Fig. 1B-D). At E11.5, however, Neurog2 expression was weak, whereas Mitf was strongly expressed in Sox10-positive cells found in the MSA (Fig. 1E,F). Expression of Dct, c-Kit and *Mitf*

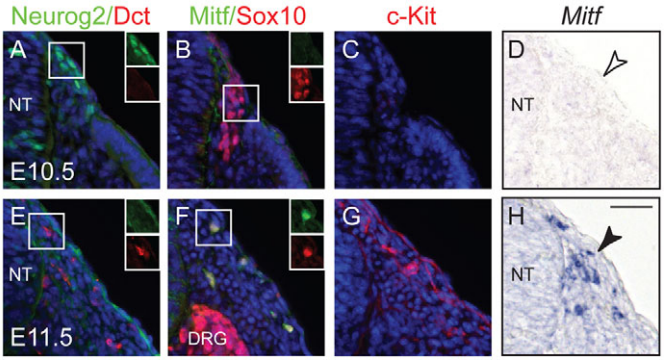

Fig. 1. Sequential expression of sensory and melanocyte markers in neural crest cells. (**A-H**) Immunohistochemistry of Neurog2, Dct, Mitf, Sox10, c-Kit (A-C,E-G) and in situ hybridization analyses of *Mitf* expression (D,H) on transverse sections of mouse embryos at E10.5 (A-D) and E11.5 (E-H). Presumptive neural crest cells next to the caudal neural tube express the sensory marker Neurog2 at E10.5 (A), whereas they are still negative for Dct, Mitf protein, c-Kit and *Mitf* mRNA (A-D, arrowhead). Dct, Mitf protein, c-Kit and *Mitf* mRNA are expressed in migrating neural crest cells only 1 day later in caudal regions at E11.5 (E-H, arrowhead). Note that Sox10 is expressed in migratory neural crest cells (B), in the dorsal root ganglia (DRG) (F), as well as in melanoblasts together with Mitf (F). Insets show single fluorescent channels of the boxed areas. A-C and E-G show adjacent sections; D and H show sections of different embryos. NT, neural tube. Scale bar: 50 μm.

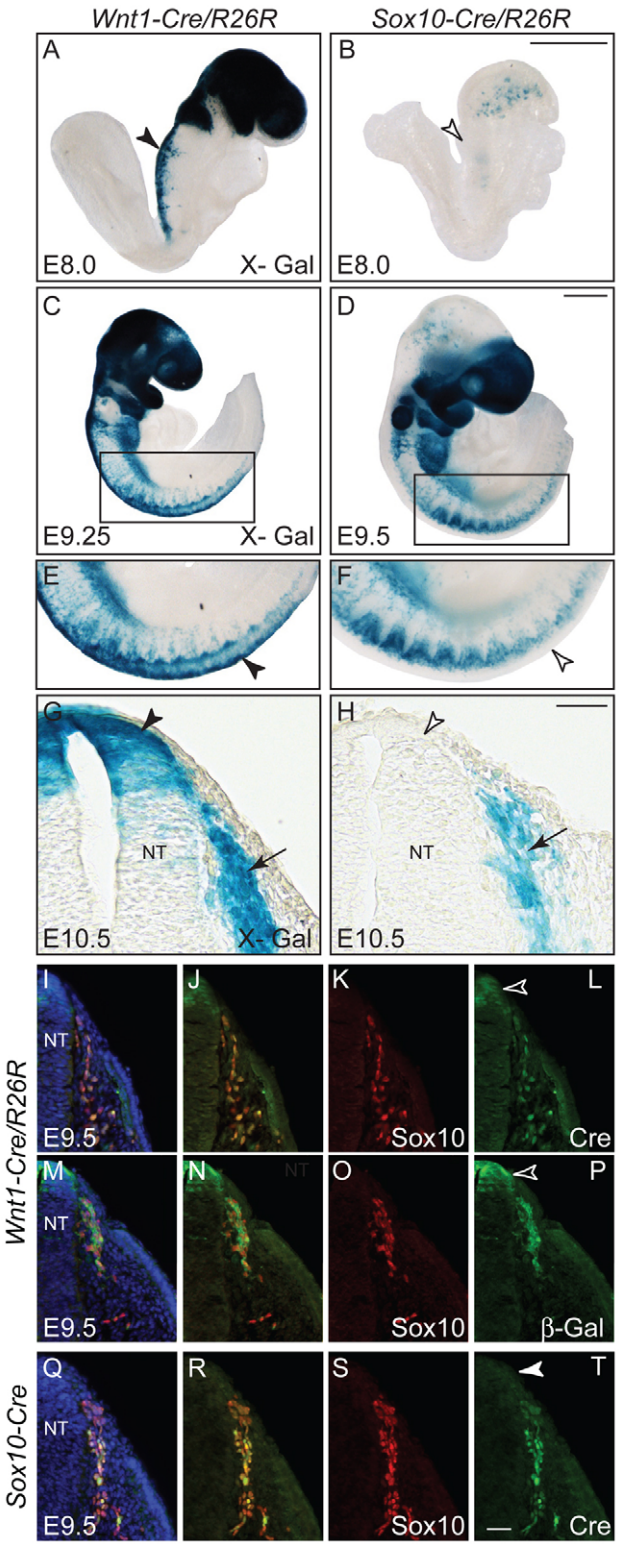

Fig. 2. Neural crest cells can be manipulated in a temporally controlled manner using the *Cre-loxP* system. (**A-H**) In vivo fate mapping of neural crest cells by *Wnt1-Cre* or *Sox10-Cre* using the Cre reporter line *R26R*. X-Gal staining on whole-mounts and on sections demonstrates recombination in the dorsal neural tube (A,C,E,G, arrowheads) and in migratory neural crest cells (G, arrow) in *Wnt1-Cre/R26R* mouse embryos. By contrast, recombination in *Sox10-Cre/R26R* embryos only occurs in migratory neural crest cells (H, arrow), but never in the neural tube (B,D,F,H, open arrowheads). E and F are higher magnifications of the boxed areas in C and D, respectively. (**I-T**) Double immunofluorescent staining for Sox10 and either Cre or β-galactosidase on transverse sections of *Wnt1-Cre/R26R* and *Sox10-Cre* embryos. Cre and β-galactosidase are expressed in the dorsal neural tube and in many Sox10-positive migratory neural crest cells of E9.5 *Wnt1-Cre/R26R* embryos (I-L,M-P, arrowheads). By contrast, Cre protein is detected in the majority of Sox10-positive migratory neural crest cells but never in the dorsal neural tube of *Sox10-Cre* embryos (T, arrowhead). I-L, M-P and Q-T show overlays and corresponding single fluorescent channels of the same sections. NT, neural tube. Scale bars: 500 μm in A-D; 50 μm in G-T.

mRNA at this stage further demonstrates that traits of the melanocyte lineage become apparent after expression of the sensory lineage marker Neurog2 (Fig. 1A-H).

To address whether the sequential appearance of sensory and melanocyte lineage markers might involve reiterative β-catenin signaling, we aimed to genetically manipulate β-catenin activation in a temporally controlled manner using the *Cre-loxP* system. In *Wnt1-Cre* transgenic mice, *Cre* is expressed in the dorsal neural tube at stages before neural crest delamination (Danielian et al., 1998; Jiang et al., 2000; Hari et al., 2002). Therefore, Cre recombinase in these mice is active at least in a subset of the premigratory neural crest population and its derivatives, as demonstrated for the stages E8.0 and later by Cre-induced β-galactosidase expression in *Wnt1-Cre* embryos crossed to the *ROSA26* (*R26R*) *Cre* reporter line (Soriano, 1999) (Fig. 2A,C,E,G). Immunohistochemistry for Cre and Sox10 in *Wnt1-Cre* mice at E9.5 confirmed prominent expression of Cre already at premigratory stages of neural crest development in the dorsal neural tube, whereas Sox10 was only expressed in Cre-positive cells after their delamination from the neural tube (Fig. 2I-L). Importantly, quantification of Cre-dependent β-galactosidase expression in *Wnt1-Cre/R26R* mice at E10.5 (Fig. 2M-P) demonstrated that 96±0.4% of all Sox10-expressing migratory neural crest cells had been subject to *Wnt1-Cre*-mediated recombination. Given that Wnt activity drops after neural crest cells have emigrated from the neural tube (Kleber et al., 2005), our data indicate that the vast majority of neural crest cells are very efficiently targeted by *Wnt1-Cre* activity before, or at the time of, their emigration from the neural tube.

As in *Wnt1-Cre/R26R* mice, Cre reporter activity was shown to occur in all neural crest-derived tissues in *Sox10-Cre/R26R* animals (Matsuoka et al., 2005) (Fig. 2D). Unlike *Wnt1-Cre*, *Sox10-Cre* marked neural crest cells only after their emigration from the neural tube, as shown by staining for Cre reporter-driven β-galactosidase expression both on whole-mount embryos and on transverse sections at E8.0, E9.5 and E10.5 of *Sox10-Cre/R26R* embryos (Fig. 2B,D,F,H). Furthermore, Cre protein was detected in 78±1.7% of all Sox10-positive migratory neural crest cells, but not in the neural tube of E9.5 *Sox10-Cre/R26R* embryos (Fig. 2Q-T, arrowhead). The combined data demonstrate that *Wnt1-Cre* and *Sox10-Cre* are expressed in a large population of lineage-related neural crest cells, albeit at different stages of their development.

The sequential Cre expression in the neural crest population of *Wnt1-Cre* and *Sox10-Cre* animals allowed us to address a potential stage-dependent role of canonical Wnt signaling during neural crest

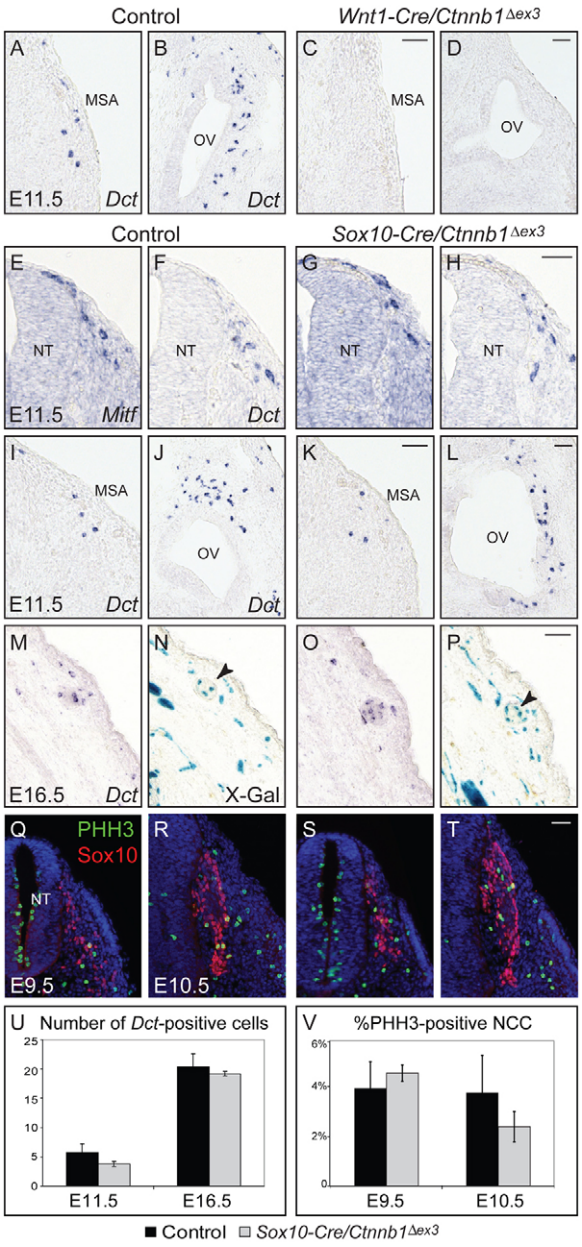

Fig. 3. Formation of orthotopic *Dct*- and *Mitf*-positive melanoblasts is not altered in *Sox10-Cre/Ctnnb1$^{\Delta ex3}$* embryos. (**A-P**) In situ hybridization analyses on transverse sections of E11.5 and E16.5 mouse embryos with melanoblast-specific *Mitf* and *Dct* riboprobes and X-Gal staining. Whereas *Dct*-positive cells are readily found in the migration staging area (MSA) and around the otic vesicle (OV) of control embryos, they are absent in *Wnt1-Cre/Ctnnb1$^{\Delta ex3}$* embryos (A-D). By contrast, *Mitf*- and *Dct*- positive melanoblasts in *Sox10-Cre/Ctnnb1$^{\Delta ex3}$* embryos are found in normal numbers emigrating from the neural tube in caudal regions (E-H), in the MSA and OV (I-L) at E11.5, and in the skin and hair follicle primordia at E16.5 (M-P). X-Gal staining on *Sox10-Cre/R26R* and *Sox10-Cre/Ctnnb1$^{\Delta ex3}$/R26R* embryos shows recombined cells in hair follicle primordia (N,P, arrowheads). E,F, G,H, M,N and O,P are adjacent sections. (**Q-T**) Double immunohistochemistry for Sox10 and phospho-histone H3 (PHH3) shows comparable numbers of proliferating cells in *Sox10-Cre/Ctnnb1$^{\Delta ex3}$* and control embryos at E9.5 and E10.5. (**U**) Quantification of *Dct*-positive cells per MSA at E11.5 and per mm skin at E16.5 reveals no significant difference in numbers of *Dct*-positive cells between *Sox10-Cre/Ctnnb1$^{\Delta ex3}$* and control embryos. (**V**) Quantification of PHH3/Sox10 double-positive cells among total Sox10-expressing cells demonstrates no significant change in proliferation between *Sox10-Cre/Ctnnb1$^{\Delta ex3}$* and control embryos. mean ± s.d. NT, neural tube; NCC, neural crest cells. Scale bars: 50 μm.

development. Previously, we have demonstrated that expression of a constitutively active stabilized form of β-catenin (*Ctnnb1$^{\Delta ex}$*) in the premigratory neural crest of *Wnt1-Cre/Ctnnb1$^{\Delta ex3}$* embryos promotes sensory neurogenesis at the expense of virtually all other neural crest derivatives, including melanocytes (Lee et al., 2004). Accordingly, presumptive melanoblasts expressing *Mitf* or *Dct* were absent in the MSA and around the otic vesicle (OV) of *Wnt1-Cre/Ctnnb1$^{\Delta ex3}$* embryos at E11.5 (Fig. 3A-D). By contrast, *Mitf*- and *Dct*-positive cells were present next to the neural tube, in the MSA, and around the OV of *Sox10-Cre/Ctnnb1$^{\Delta ex3}$* embryos at E11.5 (Fig. 3E-L). *Dct*-positive cells were able to colonize the skin and hair follicle primordia of *Sox10-Cre/Ctnnb1$^{\Delta ex3}$* embryos at E16.5 (Fig. 3M,O). The number of recombined cells found in the hair follicle primordia was comparable in *Sox10-Cre/Ctnnb1$^{\Delta ex3}$/R26R* and control embryos (Fig. 3N,P, arrowheads). Quantification of *Dct*-positive cells at E11.5 in the MSA and at E16.5 in the skin further demonstrated the presence of similar numbers of *Dct*-positive melanoblasts in *Sox10-Cre/Ctnnb1$^{\Delta ex3}$* and control embryos (Fig. 3U).

These data are in agreement with an earlier study (Delmas et al., 2007) reporting that constitutive activation of the Wnt/β-catenin pathway in the melanocyte lineage neither increased proliferation nor the number of melanoblasts. Similarly, proliferation of neural crest cells was not altered in *Sox10-Cre/Ctnnb1$^{\Delta ex3}$* animals, as demonstrated by quantifying the frequency of mitotic phospho-histone H3 (PHH3)/Sox10 double-positive cells among neural crest cells at E9.5 and E10.5 (Fig. 3Q-T,V). Likewise, BrdU incorporation experiments performed previously in our laboratory revealed no change in proliferation in cultures of neural crest cells isolated from *Wnt1-Cre/Ctnnb1$^{\Delta ex3}$* embryos (Lee et al., 2004). Thus, although β-catenin signal activation in premigratory neural crest using the *Wnt1-Cre* transgenic mouse line prevents formation of *Dct*- and *Mitf*-positive melanoblasts, it does not interfere with melanocyte lineage specification and localization of appropriate numbers of *Dct*-positive cells to the OV and the skin, if activated after emigration of neural crest cells from the neural tube using the *Sox10-Cre* mouse line.

β-catenin signal activation in migratory neural crest cells prevents formation of multiple neural and non-neural derivatives of the neural crest

The normal generation of orthotopic *Dct*- and *Mitf*-positive melanoblasts in *Sox10-Cre/Ctnnb1$^{\Delta ex3}$* embryos raised the question of whether constitutive β-catenin activity affects neural crest cell fates only at developmental stages of premigratory, but not migratory, neural crest cells. To address this issue, we further analyzed the phenotype of *Sox10-Cre/Ctnnb1$^{\Delta ex3}$* embryos, focusing on various neural crest derivatives that fail to properly form in *Wnt1-Cre/Ctnnb1$^{\Delta ex3}$* animals (Lee et al., 2004). In the head, *Sox10-Cre/Ctnnb1$^{\Delta ex3}$* displayed gross morphological anomalies from E13.5 onwards, with varying degrees of

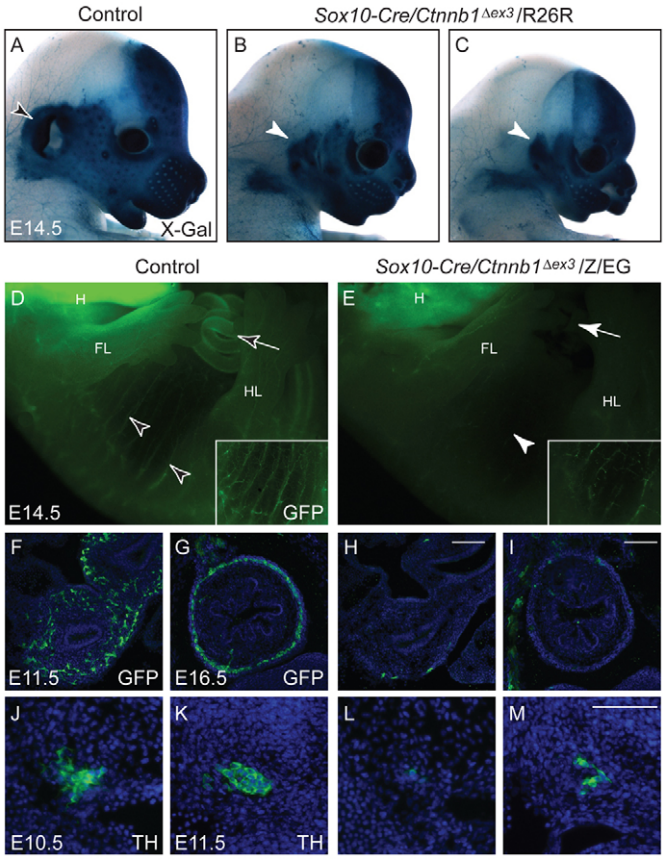

Fig. 4. Absence of multiple neural and non-neural derivatives of the neural crest in *Sox10-Cre/Ctnnb1$^{\Delta ex3}$* embryos. (**A-C**) In vivo fate mapping of crest-derived craniofacial structures on whole-mount heads of E14.5 mouse embryos carrying the *R26R* reporter allele. Malformations of craniofacial structures in *Sox10-Cre/Ctnnb1$^{\Delta ex3}$/R26R* embryos include, most prominently, lack of external ears (arrowheads) and truncated snouts frequently associated with facial clefts. (**D-I**) Lineage tracing of neural crest derivatives using *Z/EG* reporter mice visualized directly by GFP fluorescence (D,E) or with anti-GFP staining (F-I). Whole-mounts of E14.5 embryos demonstrate reduction of nerves (arrowheads, enlarged image in insets) and of the enteric nervous system (arrows) in *Sox10-Cre/Ctnnb1$^{\Delta ex3}$/Z/EG* embryos (E) as compared with *Sox10-Cre/Z/EG* control embryos (D). Absence of the enteric nervous system of *Sox10-Cre/Ctnnb1$^{\Delta ex3}$/Z/EG* embryos is confirmed on transverse sections at E11.5 and E16.5 (F-I). (**J-M**) Immunohistochemistry on transverse sections for the autonomic neuronal marker tyrosine hydroxylase (TH) reveals a drastic reduction of autonomic neurons in the sympathetic ganglia of *Sox10-Cre/Ctnnb1$^{\Delta ex3}$* embryos. H, head; FL, forelimb; HL, hindlimb. Scale bars: 100 μm.

malformations and reduced population of craniofacial structures by neural crest-derived cells, as shown by in vivo fate mapping at E14.5 (Fig. 4A-C). Fate mapping experiments using *Z/EG* reporter mice (Novak et al., 2000) revealed a strong reduction of peripheral nerves and of the enteric nervous system in *Sox10-Cre/Ctnnb1$^{\Delta ex3}$/Z/EG* embryos (Fig. 4D-I). Furthermore, the number of autonomic neurons expressing tyrosine hydroxylase (TH) was drastically reduced in the sympathetic ganglia of *Sox10-Cre/Ctnnb1$^{\Delta ex3}$* animals (Fig. 4J-M). These data demonstrate that β-catenin signaling interferes with the generation of several neural crest-derived cells types, even if activated only after neural crest cell emigration.

β-catenin signal activation in migratory neural crest cells leads to the formation of ectopic *Mitf*- and *Dct*-positive melanoblasts and melanocytes

In *Wnt1-Cre/Ctnnb1$^{\Delta ex3}$* mice, activation of β-catenin signaling in premigratory neural crest cells results in the generation of ectopic, often enlarged sensory ganglia (Lee et al., 2004). At E10.5, such oversized ganglia can be found, for instance, at the location of normal sympathetic ganglia and contain undifferentiated Sox10-positive cells, *Neurog2*-positive sensory progenitors, and Brn3a-expressing (Pou4f1 – Mouse Genome Informatics) differentiated sensory neurons (Fig. 5G,H) (Lee et al., 2004). By contrast, although ectopic Brn3a-positive cells were still present at the sites of sympathetic ganglia, the ganglia were smaller in *Sox10-Cre/Ctnnb1$^{\Delta ex3}$* than in *Wnt1-Cre/Ctnnb1$^{\Delta ex3}$* embryos (*P<0.05) (Fig. 5G-J). Intriguingly, whereas in *Wnt1-Cre/Ctnnb1$^{\Delta ex3}$* mice only a few *Mitf*-positive cells were present at sites where sympathetic ganglia usually reside, *Sox10-Cre/Ctnnb1$^{\Delta ex3}$* embryos exhibited significantly more *Mitf*-positive cells in these structures at E10.5 (Fig. 5B-E,K). The relative number of Brn3a-expressing cells was reduced accordingly in ganglia of *Sox10-Cre/Ctnnb1$^{\Delta ex3}$* embryos (Fig. 5K). Neither Brn3a- nor *Mitf*-positive cells were detected in control sympathetic ganglia (Fig. 5A,F). In addition to *Mitf*, some of the ectopic cells found in *Sox10-Cre/Ctnnb1$^{\Delta ex3}$* embryos already expressed *Dct* (data not shown). Double immunohistochemistry for Mitf and Brn3a, Mitf and NeuroD, and c-Kit and Brn3a, revealed exclusive expression of ectopic melanocyte and sensory neuronal markers in *Sox10-Cre/Ctnnb1$^{\Delta ex3}$* embryos at E10.5 (Fig. 5L-W). These experiments indicate that migratory neural crest cells in *Sox10-Cre/Ctnnb1$^{\Delta ex3}$* embryos undergo proper sensory and melanocyte lineage formation and lineage segregation, but that a considerable fraction of these lineages is found at an ectopic site. Moreover, the concurrent suppression of autonomic neurogenesis and other neural crest cell lineages in *Sox10-Cre/Ctnnb1$^{\Delta ex3}$* embryos (Fig. 4) suggests that β-catenin signaling influences neural crest cell fate decisions after their emigration from the neural tube.

To investigate whether ectopic *Dct*- and *Mitf*-positive cells in *Sox10-Cre/Ctnnb1$^{\Delta ex3}$* embryos are restricted to sympathetic ganglia or more broadly found, we systematically analyzed various mutant embryo structures for the presence of cells expressing Mitf and Dct. At E10.5, *Mitf*-positive cells were found not just within sympathetic ganglionic anlagen, but also dispersed around these structures (Fig. 6A,B). At subsequent developmental stages, *Dct*-positive cells were detected at several ectopic places in *Sox10-Cre/Ctnnb1$^{\Delta ex3}$* embryos, including in many tissues that usually do not harbor neural crest derivatives. For instance, at E12.5, the kidney primordium comprised *Dct*-expressing cells, which were shown by in vivo fate mapping to be of neural crest origin (Fig. 5C-F). Additionally, neural crest-derived *Dct*-positive cells were detected in the diaphragm, in the spleen and in the urogenital tract (Fig. 6G-R). Ectopic *Dct*-positive cells were also found at further locations, for example in reproductive organs (testes and ovaries) and around the dorsal aorta (data not shown), and were sometimes even integrated into epithelial structures. As confirmed by Cre-mediated genetic cell tracking, all these organs were devoid of neural crest-derived cells in the control. Of note, many cells marked by *Dct* expression or by Cre-induced β-galactosidase expression in different organs of *Sox10-Cre/Ctnnb1$^{\Delta ex3}$/R26R* embryos also displayed pigmentation (Fig. 6S-X). Thus, expression of a constitutively active form of β-catenin in migratory neural crest cells in *Sox10-Cre/Ctnnb1$^{\Delta ex}$* embryos leads to the ectopic

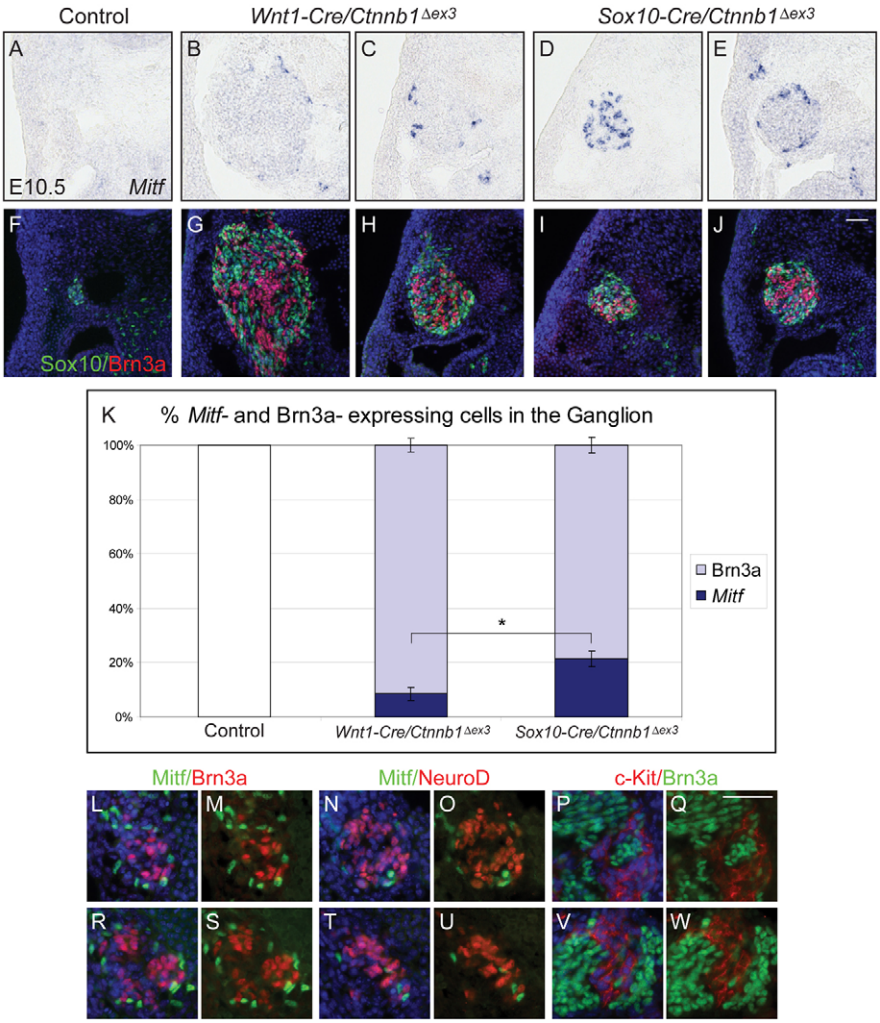

Fig. 5. Formation of ectopic *Mitf*-positive cells at the location of sympathetic ganglia in *Sox10-Cre/Ctnnb1$^{\Delta ex3}$* embryos. (**A-J**) In situ hybridization for *Mitf* and immunohistochemistry for Sox10 and Brn3a on adjacent transverse sections at E10.5. Ectopic Brn3a-positive and ectopic *Mitf*-positive cells are detected at the location of the sympathetic ganglia in *Wnt1-Cre/Ctnnb1$^{\Delta ex3}$* and in *Sox10-Cre/Ctnnb1$^{\Delta ex3}$* but never in control mouse embryos (A-J). However, the number of ectopic *Mitf*-positive cells is increased in *Sox10-Cre/Ctnnb1$^{\Delta ex3}$* embryos as compared with *Wnt1-Cre/Ctnnb1$^{\Delta ex3}$* embryos (B-E,K). Owing to variations in the phenotype, examples of two different embryos are shown for each genotype. (**K**) Percentages of *Mitf*- and Brn3a-positive cells at the location of the sympathetic ganglia quantified on transverse sections. The relative increase in *Mitf*-positive cells among total ectopic cells in *Sox10-Cre/Ctnnb1$^{\Delta ex3}$* embryos is statistically significant (*P<0.05) and is accompanied by a relative decrease in numbers of Brn3a-positive cells. (**L-W**) Double immunohistochemistry for markers of the melanocyte lineage such as Mitf and c-Kit, and for markers of the sensory lineage such as Brn3a and NeuroD. Absence of double-positive cells demonstrates that the ectopic cells observed at the location of the sympathetic ganglia of *Sox10-Cre/Ctnnb1$^{\Delta ex3}$* embryos belong to distinct lineages, expressing either sensory or melanocyte markers. Exclusive expression of Mitf and Brn3a is shown in L,M and R,S, exclusive expression of Mitf and NeuroD is shown in N,O and T,U, and exclusive expression of c-Kit and Brn3a is depicted in P,Q and V,W. For every double staining, two examples of sympathetic ganglia are shown. L,M, N,O, P,Q, R,S, T,U and V,W represent the same pictures with and without the DAPI channel for clearer visibility of marker exclusiveness. Scale bars: 50 μm.

localization of neural crest-derived *Dct*- and *Mitf*-positive melanoblasts and differentiated bone fide melanocytes in various embryonic organs.

Ectopic melanoblast formation is due to β-catenin signal activation in migratory neural crest cells rather than in committed melanoblasts or glial progenitors

The emergence of extra melanoblasts, concomitant with the loss of other neural crest derivatives in *Sox10-Cre/Ctnnb1$^{\Delta ex3}$* embryos, is consistent with the idea that β-catenin signal activation regulates cell fate choices in multipotent migratory neural crest cells, promoting melanoblast formation at the expense of other fates. Alternatively, β-catenin signal activation might cause an expansion of melanoblasts on their way from the MSA to the skin, leading to the ectopic localization of excessively produced cells. To exclude this alternative explanation for the presence of ectopic melanoblasts, we made use of the inducible *Tyr-CreERT2* allele that drives Cre recombinase in neural crest cells only after dorsolateral migration and melanocyte fate specification (Bosenberg et al., 2006) (Fig. 7A). Induction of *Ctnnb1$^{\Delta ex3}$* expression by treatment of the pregnant females with tamoxifen at E11.5 and E12.5 resulted in recombination in melanoblasts in the skin and in forming hair follicle primordia at E16.5 (Fig. 7C).

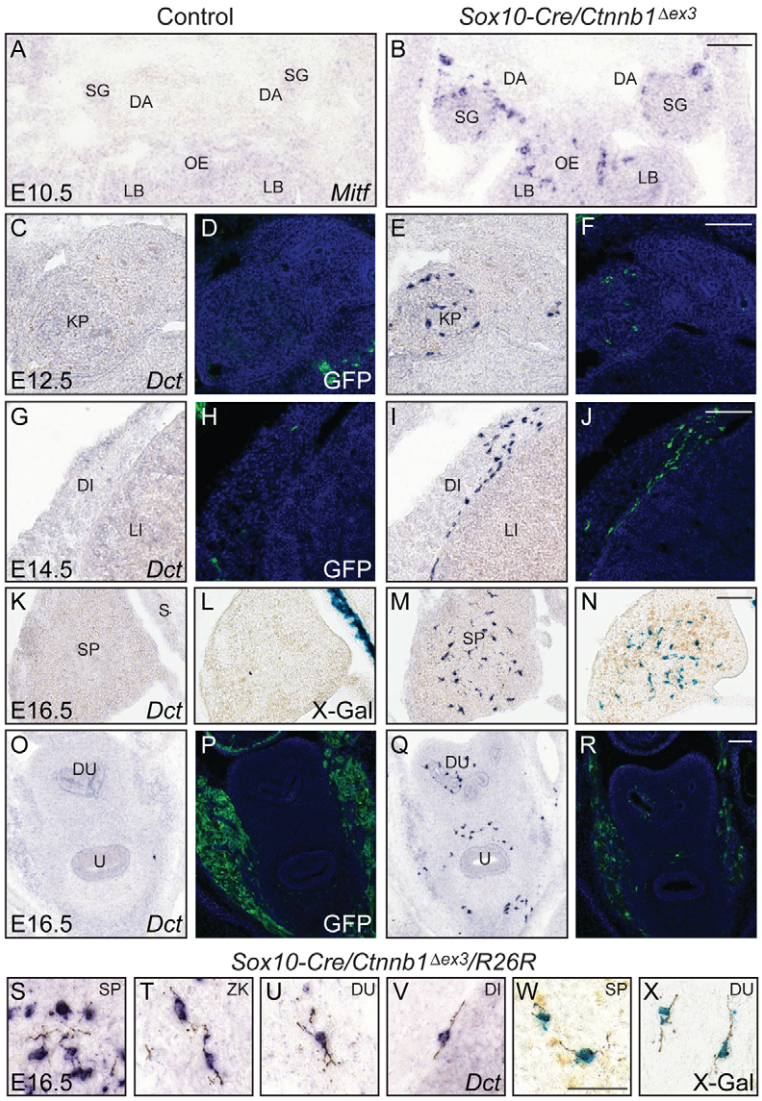

Fig. 6. Formation of ectopic melanoblasts at various locations in *Sox10-Cre/Ctnnb1$^{\Delta ex3}$* embryos. Ectopic *Dct*- and *Mitf*-positive melanoblasts are detected by in situ hybridization on transverse sections using *Mitf* (A,B) and *Dct* (C-V) riboprobes. The neural crest origin of the *Dct*- and *Mitf*-positive melanoblasts is illustrated by anti-GFP or X-Gal staining using mouse embryos carrying the *Z/EG* or *R26R* reporter lines, respectively. (**A-R**) The following examples of organs populated by *Dct*- and *Mitf*-positive melanoblasts in *Sox10-Cre/Ctnnb1$^{\Delta ex3}$* embryos are shown: tissue surrounding the sympathetic ganglia (SG) and the lung buds (LB) at E10.5 (A,B); the kidney primordium (KP, C-F) at E12.5; the diaphragm (DI, G-J) at E14.5; the spleen (SP, K-N) at E16.5; the urogenital tract, around the urethra (U) and the reproductive ducts (DU, O-R) at E16.5. (**S-V**) Pigmentation of *Dct*-positive ectopic cells in *Sox10-Cre/Ctnnb1$^{\Delta ex3}$* embryos demonstrates their melanocyte identity. Examples of pigmented cells are shown in the spleen (S), in the ganglion of Zuckerkandl (ZK, T), around the reproductive ducts (U) and in the diaphragm (V). (**W**,**X**) X-Gal staining shows recombination in these pigmented melanocytes; cells in the spleen (W) and around the reproductive ducts (X) are shown as examples. Note the absence of neural crest-derived cells in all locations of ectopic *Dct*-positive cells in control embryos, as revealed by X-Gal and anti-GFP staining (D,H,L,P). DA, dorsal aorta; OE, oesophagus; LI, liver; S, stomach. Scale bars: 100 μm in A-R; 50 μm in S-X.

Consistent with a previous report (Delmas et al., 2007), β-catenin signal activation did not cause an overt increase in melanoblast cell numbers in the skin of *Tyr-CreERT2/Ctnnb1$^{\Delta ex3}$* embryos (data not shown), disfavoring a role of β-catenin signaling in promoting melanoblast proliferation. Of note, we did not observe any ectopic melanoblasts in *Tyr-CreERT2/Ctnnb1$^{\Delta ex3}$/R26R* embryos, as demonstrated by in vivo fate mapping and in situ hybridization analysis of *Dct* expression (Fig. 7D,E, the spleen is shown as an example). These data are consistent with the idea that ectopic melanoblasts in *Sox10-Cre/Ctnnb1$^{\Delta ex3}$* animals are not a consequence of increased orthotopic melanoblast proliferation.

Recently, it has been reported that melanocytes not only arise from neural crest cells migrating along the dorsolateral pathway, but also from Schwann cell precursors (SCPs) located in nerves innervating the skin (Adameyko et al., 2009). To address whether melanocyte fate acquisition at ectopic sites results from elevated β-catenin signaling in nerves, we expressed *Ctnnb1$^{\Delta ex3}$* specifically in peripheral glia by means of *Dhh-Cre*-mediated recombination (Jaegle et al., 2003) (Fig. 7A). As previously shown, *Dhh-Cre* is active in neural crest-derived cells present along peripheral nerves (Jaegle et al., 2003; Joseph et al., 2004). In agreement with this, immunostaining for Cre protein confirmed that *Dhh-Cre* is not yet active in migratory neural crest cells at E9.5 (data not shown). Although recombination in nerves appeared to be efficient in *Dhh-Cre/Ctnnb1$^{\Delta ex3}$/R26R* embryos from E12 onwards (Jaegle et al., 2003) (data not shown), we observed neither recombined orthotopic melanoblasts (Fig. 7G, arrowhead) nor melanoblasts at ectopic locations in these animals (Fig. 7H,I; data not shown). These findings do not support a role of β-catenin in promoting the generation of melanocytes from nerve cells.

Based on fate mapping experiments with an inducible *Plp-CreERT2* line, it has been suggested that the SCP-to-melanocyte transition occurs within a narrow time window during murine development at ~E11 (Ernfors, 2010). To exclude the possibility that *Dhh-Cre* activity might be too late to track and influence a SCP-to-melanocyte lineage switch, we induced expression of *Ctnnb1$^{\Delta ex3}$* at various early and late time points using the *Plp-CreERT2* line (Leone et al., 2003). At E9.5, Cre is broadly expressed in migratory Sox10-positive neural crest cells of *Plp-CreERT2/R26R* embryos (Fig. 8B-E). Accordingly, tamoxifen treatment at this early stage led to Cre-mediated recombination and β-galactosidase expression in the majority of neural crest-derived cells at E10.5 (Fig. 8F-I) and in multiple neural crest derivatives at E16.5, such as neuronal cells in the DRG and in the enteric nervous system, nerves and presumptive melanoblasts in the skin (Fig. 8J,K; data not shown). This indicates that at this early

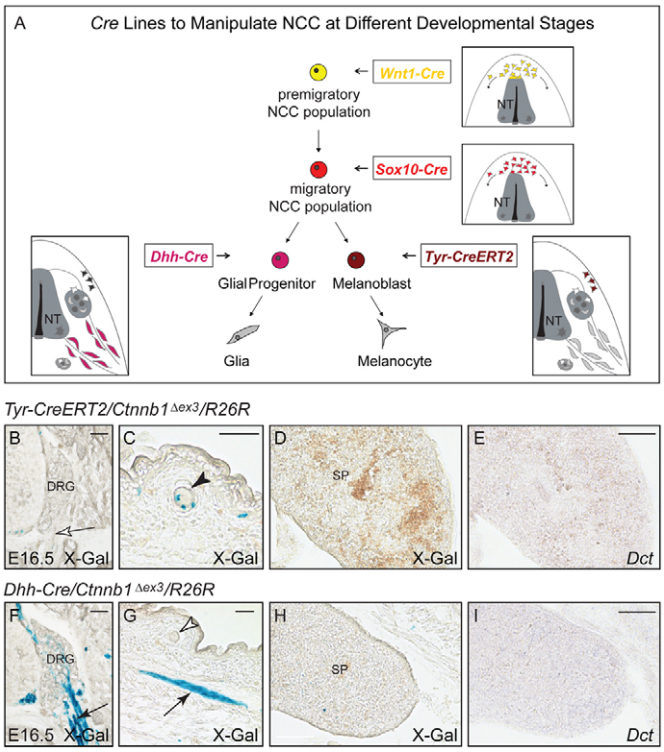

Fig. 7. Ectopic melanoblasts are derived from migratory neural crest cells rather than from melanoblasts or glial progenitors. (**A**) Illustration of the different Cre lines used in this study and their recombination pattern in neural crest cells and their lineage descendants. (**B-I**) X-Gal staining on transverse sections of E16.5 mouse embryos to identify recombined cells carrying the *R26R* reporter (B-D,F-H), and in situ hybridization analysis on adjacent sections for *Dct* to visualize ectopic melanoblasts (E,I). No ectopic *Dct*-positive melanoblasts are formed in *Tyr-CreERT2/Ctnnb1*$^{\Delta ex3}$*/R26R* embryos; the spleen (SP) is shown as an example (D,E). Recombined cells are detected in hair follicle primordia (C, arrowhead), but not in the in the dorsal root ganglia (DRG) and nerves (B, arrow). Likewise, the spleen of *Dhh-Cre/Ctnnb1*$^{\Delta ex3}$*/R26R* embryos is devoid of ectopic *Dct*-positive melanoblasts (H,I). Recombination is apparent in nerves (F,G, arrows), but in neither neuronal cells of the DRG nor in melanoblasts in hair follicle primordia (F,G, arrowhead). NT, neural tube; NCC, neural crest cells. Scale bars: 100 μm in B,D-F,H,I; 50 μm in C,G.

developmental stage, *Plp-CreERT2* expression occurs in unspecified neural crest cells and is not restricted to SCPs. In agreement with the results obtained with *Sox10-Cre/Ctnnb1*$^{\Delta ex3}$ animals, β-catenin signal activation in *Plp-CreERT2/Ctnnb1*$^{\Delta ex3}$ embryos at E9.5 led to the appearance of ectopic *Dct*-positive melanoblasts at E16.5 (Fig. 8L,M, the spleen is shown as an example). Cre activity in *Plp-CreERT2/Ctnnb1*$^{\Delta ex3}$ embryos became more restricted, however, upon induction at E11.5 and later stages (Fig. 8N-U). In contrast to *Dhh-Cre*, *Plp-CreERT2* efficiently marked nerves as well as melanoblasts in the skin when induced at E11.5, E12.5, E14.5, or at postnatal stages (Fig. 8N,O,R,S; data not shown). When activated from E11.5 onwards, β-catenin signaling failed to promote ectopic melanoblast formation in *Plp-CreERT2/Ctnnb1*$^{\Delta ex3}$ animals (Fig. 8P,Q,T,U). These data indicate that β-catenin-mediated generation of melanoblasts at ectopic sites is due to its activation in neural crest cells before they have localized to nerves or to the skin.

DISCUSSION

Canonical Wnt/β-catenin signaling has been shown to play reiterative roles during neural crest development, sequentially controlling neural crest induction, neural crest cell delamination, lineage specification, and differentiation of neural crest derivatives (Garcia-Castro et al., 2002; Burstyn-Cohen et al., 2004; Lewis et al., 2004; Kleber and Sommer, 2004; Steventon et al., 2009; Li et al., 2011). In the present study, we add a new twist to this theme, demonstrating that the generation of distinct neural crest cell fates in the mouse is also subject to sequential Wnt/β-catenin signaling. Activation of β-catenin in the premigratory neural crest promotes sensory neurogenesis while suppressing virtually all other possible lineages, including melanocytes (Lee et al., 2004). If β-catenin signal activation occurs in migratory rather than premigratory neural crest cells, however, melanocytes represent a major cell type generated, while most other neural crest cell lineages still fail to develop. These data suggest that neural crest cell fate decisions are regulated by stage-dependent differential responsiveness to Wnt/β-catenin signal activation.

Wnt/β-catenin signaling regulates sensory and melanocyte lineage decisions in neural crest cells

Wnt/β-catenin signal activation in neural crest cells has previously been associated either with expansion of pigment cell numbers or with increased sensory neurogenesis (Dorsky et al., 1998; Jin et al., 2001; Lee et al., 2004). Differences in the outcomes of these studies have been attributed, among others, to possible species-specific mechanisms controlling neural crest lineage decisions (Sommer, 2011). Our work provides an additional explanation for how to reconcile the divergent reports: the exact timing of Wnt/β-catenin signal manipulation appears to have an influence on whether neural crest cells choose to produce sensory neurons or melanoblasts. The use of mouse lines expressing Cre recombinase at different stages of neural crest development allowed us to temporally control conditional *Ctnnb1* activation. As demonstrated by in vivo fate mapping, the use of *Wnt1-Cre* results in recombination in the dorsal neural tube at stages when this structure comprises the premigratory neural crest cell population. By contrast, *Sox10-Cre* is not active in premigratory neural crest cells in the dorsal neural tube. Hence, genetic tracking of *Sox10-Cre*-mediated recombination fails to mark cells of the dorsal neural tube at any developmental stage. However, *Sox10-Cre* efficiently labels neural crest cells after their emigration from the neural tube. As shown by immunostaining for Cre expression and in vivo fate mapping by means of Cre-mediated β-galactosidase expression, *Wnt1-Cre* and *Sox10-Cre* are sequentially active in a large fraction of lineage-related neural crest cells. Therefore, the differential outcome of *Wnt1-Cre*-driven versus *Sox10-Cre*-driven *Ctnnb1* activation is consistent with the idea that canonical Wnt signaling regulates sensory neurogenesis in neural crest cells before or during their emigration, when the cells are still localized in or close to the dorsal neural tube. By contrast, according to this idea, the melanocyte lineage is promoted after neural crest cell emigration. This view is also compatible with our previous finding that conditional inactivation of *Ctnnb1* in the premigratory neural crest reveals a requirement of the Wnt pathway for both sensory neuron as well as melanocyte formation (Hari et al., 2002).

Although we show that β-catenin signal activation is sufficient to influence neural crest cell lineage decisions in a stage-dependent manner, we were unable for technical reasons to demonstrate a stage-dependent requirement for β-catenin. Using Cre lines active in specified melanoblasts and/or in glial progenitors, we have

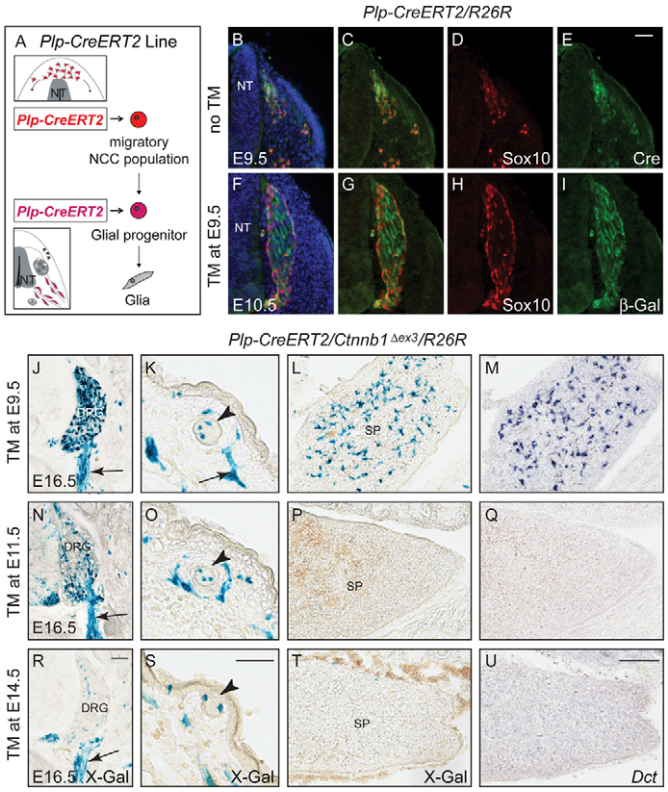

Fig. 8. Formation of ectopic melanoblasts in *Plp-CreERT2/Ctnnb1$^{\Delta ex3}$* embryos is induced only during a narrow time window. (**A**) Illustration of recombination patterns obtained by *Plp-CreERT2*-mediated Cre activation. (**B-I**) Double immunohistochemistry for Sox10 and either Cre or β-galactosidase on transverse sections of *Plp-CreERT2/R26R* mouse embryos. At E9.5, Cre is expressed in many migratory Sox10-positive neural crest cells of *Plp-CreERT2/R26R* embryos (B-E). Consequently, tamoxifen (TM) treatment at this early stage results in Cre-mediated recombination and β-galactosidase expression in the majority of neural crest-derived cells at E10.5 (F-I). B-E and F-I are overlays and corresponding single fluorescent channels of the same sections. (**J-U**) Expression of stabilized β-catenin in *Plp-CreERT2/Ctnnb1$^{\Delta ex3}$/R26R* induced at different developmental stages. Activation of *Plp-CreERT2* by injection of tamoxifen at E9.5 results in efficient recombination of cells in the DRG, nerves (J,K, arrows) and of melanoblasts localized to forming hair follicles (K, arrowhead). Numerous ectopic X-Gal-stained and *Dct*-positive melanoblasts are found in the spleen (L,M) and in other organs. Activation of *Plp-CreERT2* at E11.5 and at E14.5 results in staining of nerves (N,R, arrows) and of melanoblasts (O,S, arrowheads). The spleen of these embryos is devoid of ectopic *Dct*-positive or X-Gal-stained melanoblasts (P,Q,T,U). NT, neural tube; NCC, neural crest cells. Scale bars: 100 μm in J,L-N,P-R,T,U; 50 μm in B-I,K,O,S.

defined a relatively narrow time window during which neural crest cells respond to β-catenin activation by increased melanocyte formation (see below for further discussion): after neural crest cell emigration, but before the cells have reached the dorsolateral migratory pathway or the nerves. Since loss of protein lags behind conditional gene deletion, *Sox10-Cre*-dependent ablation of *Ctnnb1* did not abolish canonical Wnt signaling during this narrow time window of signal responsiveness, and neural crest cells were found, therefore, to adopt a melanocytic fate in *Sox10-Cre/Ctnnb1$^{flox/flox}$* animals (L.H. and L.S., unpublished). However, the combined in vivo loss- and gain-of-function data, together with the observation that the Wnt/β-catenin pathway is active in a fraction of migratory neural crest cells in vivo (data not shown), point to a role of canonical Wnt signaling in controlling melanocyte fate in migratory neural crest cells after their delamination from the neural tube.

Wnt/β-catenin signaling regulates melanocyte lineage decisions in migrating neural crest cells during a narrow time window

The virtual loss of several neural crest derivatives in *Sox10-Cre/Ctnnb1$^{\Delta ex3}$* animals, concomitant with the generation of surplus melanoblasts in these mice, is consistent with the hypothesis that migratory neural crest cells can adopt multiple fates in vivo and that neural crest lineage segregations in these cells can be influenced after their emigration from the neural tube. Alternatively, Wnt signaling might induce expansion of committed melanoblasts, as has been suggested previously based on cell culture experiments (Dunn et al., 2000). However, constitutive activation of β-catenin in melanoblasts using *Tyr-CreERT2*-driven gene expression led neither to cell expansion in the skin, confirming work by others (Delmas et al., 2007), nor to the appearance of melanoblasts at ectopic sites. Therefore, altered proliferation of melanoblasts cannot explain the phenotype described in the present study.

Apart from dorsolaterally migrating neural crest cells, neural crest-derived cells along nerves innervating the skin have been reported to contribute to pigment cell formation (Adameyko et al., 2009). We used two different Cre-expressing mouse lines to exclude the possibility that β-catenin activation promotes melanoblast generation from nerve cells rather than from migratory neural crest cells. In a first mouse line, *Cre* is expressed from *Dhh* promoter elements, which leads to Cre recombinase activity specifically in nerves from early stages onwards (Jaegle et al., 2003). Notably, we found no history of recombination in melanoblasts and melanocytes in these mice, and *Dhh-Cre*-driven β-catenin activation neither promoted extra melanoblast generation nor the suppression of other fates. In a second mouse line, inducible *Plp-CreERT2* expression has been used by others to investigate a transition from SCPs to melanocytes (Leone et al., 2003; Adameyko et al., 2009). In the present study, we show that Cre-dependent recombination in these mice marks multiple neural crest derivatives, including neurons, glia and melanocytes, when induced at early stages of neural crest development. Induction at later stages revealed a more restricted expression pattern of Cre, with recombination being detectable in both glia and melanocytes at all stages examined, including postnatally. Given the reported SCP-melanocyte transition observed in *Plp-CreERT2* mice, it is unclear why *Dhh-Cre*-mediated recombination does not label melanoblasts. Possibly, some melanocytic cells are marked independently of glia in *Plp-CreERT2* animals. Alternatively, *Dhh-Cre* and *Plp-CreERT2* might be expressed in distinct nerve cell subpopulations (Sommer, 2011). In any case, the dynamic activity of *Plp-CreERT2* during development allowed us to confirm that ectopic melanoblasts found in *Sox10-Cre/Ctnnb1$^{\Delta ex3}$* animals are due neither to enhanced SCP-to-melanoblast transition nor to expansion of melanoblasts: surplus melanoblasts were only seen in *Plp-CreERT2/Ctnnb1$^{\Delta ex3}$* when Cre recombinase was induced at early stages to mark multiple neural crest derivatives, but not when recombination in these mice was restricted to nerve cells and melanocytes at stages E11.5 or later. These data demonstrate that β-catenin activation can promote the generation of melanoblasts only during a narrow time window of

neural crest development, after emigration of neural crest cells and before their association with nerves or engagement in the dorsolateral pathway.

The proposed relatively narrow time window of β-catenin responsiveness in migratory neural crest cells might also explain why surplus melanoblasts are found at ectopic sites within the embryo, rather than at their normal location in the skin. Normally, neural crest cells destined for a melanocytic fate localize to the MSA and then migrate dorsolaterally to populate the skin. Conceivably, β-catenin activation in *Sox10-Cre/Ctnnb1*$^{\Delta ex3}$ embryos imposes a melanocytic fate on neural crest cells before they have reached their destination in the skin. This causes ventrally migrating neural crest cells to generate melanoblasts at the expense of other derivatives. Intriguingly, in *Sox10-Cre/Ctnnb1*$^{\Delta ex3}$ mice, ectopic melanoblasts and melanocytes are not only found in neural crest target structures, such as the autonomic ganglia, but also in tissues that are normally devoid of neural crest derivatives, such as the spleen, urogenital tract and diaphragm. Although this needs to be addressed, the extensive migration of β-catenin-overexpressing melanocytic cells might reflect processes that are also relevant to the high migratory capacity of cells present in aggressive melanoma.

Multipotency and lineage restrictions in avian and mouse neural crest cells

Earlier clonal assays in avian embryos indicated that at least some neural crest cells are multipotent in vivo, generating clones composed of both pigment and neural cell types (Bronner-Fraser and Fraser, 1988; Bronner-Fraser and Fraser, 1989; Frank and Sanes, 1991). A more recent study suggested, however, that neural crest cells are already lineage restricted prior to their emigration from the neural tube: at early stages, cells migrate ventrally to form neural structures only, whereas at later stages cells migrate dorsolaterally to exclusively produce melanocytes (Krispin et al., 2010a; Krispin et al., 2010b). According to these data, multifated neural crest cells (i.e. cells adopting multiple fates) would be rare in avian embryos, and the majority of neural crest cells would consist of discrete cell subpopulations generating single rather than multiple cell types (Krispin et al., 2010b). In contrast to avian embryos, there is no evidence for a temporal switch from ventral to dorsolateral migration in mouse embryos, as mouse neural crest cells migrate dorsolaterally at the onset of neural crest migration (Serbedzija et al., 1990). In addition, single-cell labeling in mouse embryos identified many clones composed of multiple neural crest derivatives, including neural cells and presumptive melanocytic cells in the dorsolateral pathway (Serbedzija et al., 1994).

Despite these data, our findings support the idea that the generation of distinct neural crest lineages in the mouse is also subject to temporal control mechanisms, as in avian embryos. Because most, but not all, neural crest cells are targeted by Cre in the mouse lines used in this study, we cannot exclude a certain heterogeneity in the neural crest cell population with respect to Wnt/β-catenin responsiveness at a given time point. Nonetheless, a large fraction of neural crest cells appears to be multipotent rather than lineage determined at premigratory as well as migratory stages, and their fate can be influenced in vivo by modulation of cues controlling lineage choices. However, our findings do not exclude the possibility that cells with the potential to respond to β-catenin signaling and to adopt multiple fates upon signal manipulation might actually be lineage restricted during normal development. Thus, whether multipotent cells are also multifated at different stages of neural crest development in vivo needs to be elucidated in future studies.

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Competing interests statement
The authors declare no competing financial interests.

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