Differential Effects of Neurofibromin Gene Dosage on Melanocyte Development

Mugdha Deo[1], Jenny Li-Ying Huang[1], Helmut Fuchs[2], Martin Hrabe de Angelis[2] and Catherine D. Van Raamsdonk[1]

Mutations in neurofibromin (*NF1*) cause the dominant genetic disorder neurofibromatosis type 1. Neurofibromatosis is characterized by Schwann cell–based tumors and skin hyperpigmentation, resulting from both haploinsufficiency and loss of heterozygosity. The fact that some pigment cells (melanocytes) arise from Schwann cell precursors suggests that neurofibromin could be required during the common precursor stage. In this study, we found a missense mutation in neurofibromin in *Dark skin 9* (*Dsk9*) mutant mice, revealing that *Nf1* mutations cause skin hyperpigmentation in mice, as they do in humans. Using tissue-specific knockouts, we found that haploinsufficiency of neurofibromin in melanocytes via *Mitf-cre* is insufficient to cause darker skin, whereas haploinsufficiency in bipotential Schwann cell-melanoblast precursors via *Plp1-creER* is sufficient. These findings suggest that there is a narrow developmental window during which *Nf1* haploinsufficiency acts on pigment cells. Using fate mapping, we discovered differences in the colonization of the dermis and epidermis by melanocytes that arise from Schwann cell precursors, an unexpected complexity of melanocyte development. As homozygous knockout of *Nf1* via *Mitf-cre* is sufficient to cause darker skin, we conclude that reduced gene dosage can act by a mechanism different from complete gene loss, even when the end result of both is very similar.

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INTRODUCTION

Neurofibromatosis type 1 is an autosomal dominant genetic disorder that affects multiple organs with variable expressivity (Crowe *et al.*, 1956). Patients with neurofibromatosis develop neurofibromas, which are tumors composed of spindle-shaped Schwann cells, mast cells, and perineural cells, combined with collagen and extracellular matrix. Plexiform neurofibromas grow along nerves and can interfere with function and give rise to malignant nerve sheath tumors (Evans *et al.*, 2002). Abnormalities are also found in the skeletal, cardiovascular, endocrine, gastrointestinal, and neurological systems of neurofibromatosis patients (Boyd *et al.*, 2009).

[1]*Department of Medical Genetics, University of British Columbia, Vancouver, British Columbia, Canada and* [2]*Institute of Experimental Genetics, Helmholtz Zentrum München, Neuherberg, Germany*

Correspondence: Catherine D. Van Raamsdonk, Department of Medical Genetics, University of British Columbia, 2350 Health Sciences Mall, Vancouver, British Columbia, Canada V6T1Z3. E-mail: cvr@mail.ubc.ca

Abbreviations: ACTB, beta-actin; Asn, asparagine; CALMs, cafe au lait macules; Dct, dopachrome tautomerase; Dsk9, dark skin 9; EYFP, enhanced yellow fluorescent protein; GRD, GTPase-activating protein-related domain; KO, knock out; LacZ, beta-galactosidase; Lys, lysine; Mitf, microphthalmia; Nf1, neurofibromin; Plp1, proteolipid protein 1; Ras, rat sarcoma virus oncogene; SPRED1, sprouty-related, EVH1 domain containing 1; SSLP, simple sequence length polymorphism; Tuj1, tubulin, beta 3 class III; Vav1, vav 1 oncogene; X-gal, 5-bromo-4-chloro-3-indolyl-B-D-galactosidase

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Neurofibromatosis type 1 has a number of pigmentary manifestations, including generalized skin hyperpigmentation (Maertens *et al.*, 2007), Café au lait macules (CALMs), and skin-fold freckling (De Schepper *et al.*, 2005). Neurofibromatosis type 1 is caused by inherited or sporadic mutations in the tumor suppressor gene, *NF1*, which encodes neurofibromin (Fountain *et al.*, 1989; Wallace *et al.*, 1990). Neurofibromin is a positive regulator of rat sarcoma virus oncogene (RAS) inactivation (Cichowski and Jacks, 2001). Second-hit mutations in *NF1* are found in the Schwann cells of neurofibromas and the melanocytes of CALMs (Maertens *et al.*, 2006; De Schepper *et al.*, 2008). It has been recently shown that, in mice, Schwann cells and some melanocytes arise from a bipotential precursor expressing Proteolipid protein 1 (*Plp1*), a component of the Schwann cell myelin sheath (Adameyko *et al.*, 2009). Other melanocytes arise directly from the neural crest and migrate along the dorsolateral pathway. Melanoblasts, immature melanocytes, migrate through the dermis before invading the epidermis and its appendages, the hair follicles (Mayer, 1973; Hirobe, 1984; Kunisada *et al.*, 1996; Yoshida *et al.*, 1996). In this report, we identify a missense mutation in *Nf1* in a mouse with dominantly inherited dark skin and study the role of neurofibromin in pigmentation.

RESULTS

Positional cloning of *Dsk9*

The *Dark Skin 9* (*Dsk9*) mutant was recovered during a dominant ENU mutagenesis screen performed on a C3HeB/FeJ

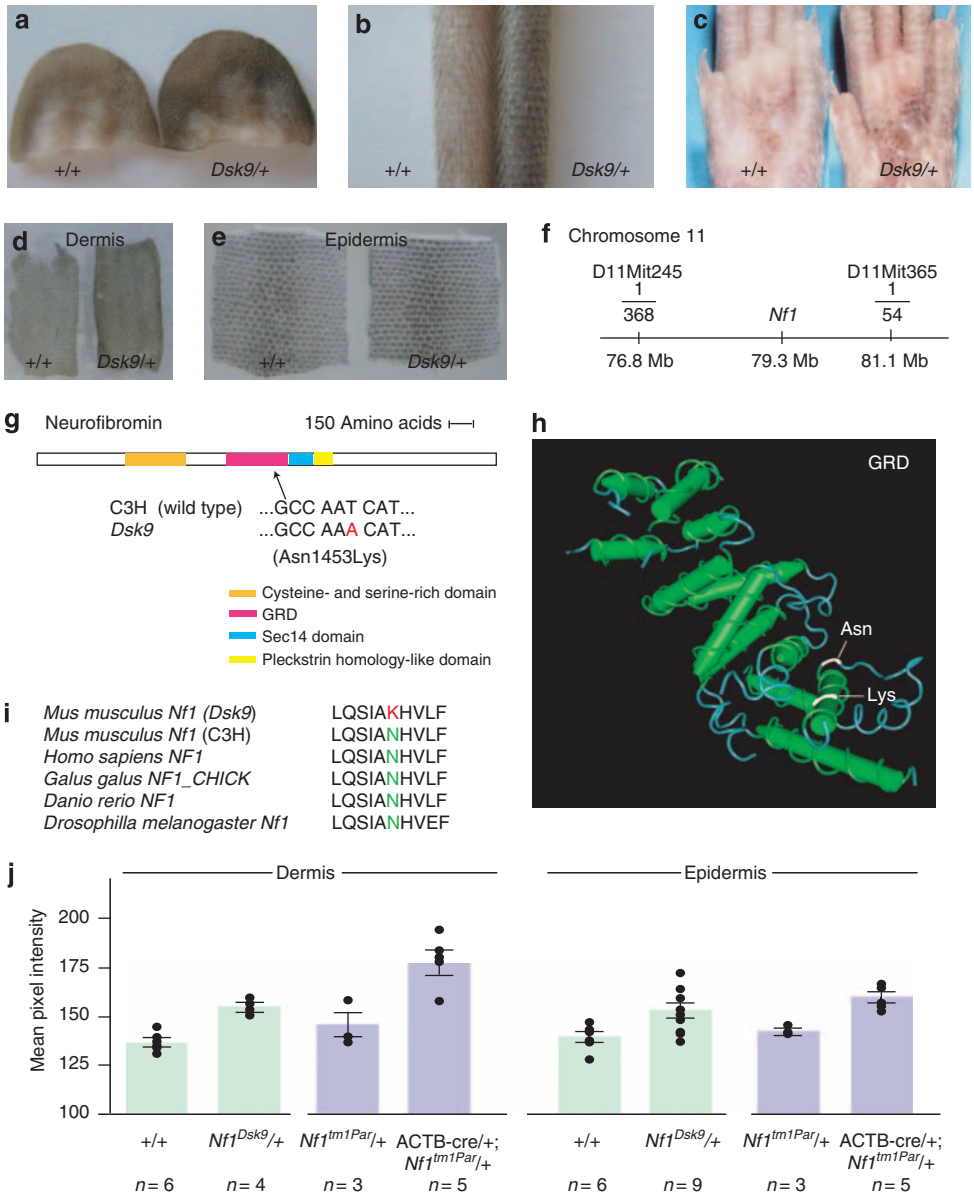

Figure 1. *Dsk9* phenotype and positional cloning. (**a**) Ears , (**b**) footpads, and (**c**) hind feet. (**d**) Split tail dermis and (**e**) epidermis. (**f**) Genetic and physical map of the *Dsk9* interval on chromosome 11. (**g**) The position and sequence alteration of the $Nf1^{Dsk9}$ mutation. (**h**) The GRD of human neurofibromin, highlighting Asn1451 (analogous to mouse Asn1453) and Lys1444 (ENSP00000351015) (Scheffzek *et al.*, 1998). (**i**) Predicted protein sequence for $Nf1^{Dsk9}$ aligned with homologous sequences in other species. (**j**) Pixel intensity of split dermis and epidermis of adult tails (mean ± SEM). Individual dots represent the value from each skin sample examined. Statistical analysis: +/+ versus $Nf1^{Dsk9}$/+ dermis, $P=0.000165$. $Nf1^{tm1Par}$/+ versus *ACTB-cre*/+; $Nf1^{tm1Par}$/+ dermis, $P=0.0139$. +/+ versus $Nf1^{Dsk9}$/+ epidermis, $P=0.0279$. $Nf1^{tm1Par}$/+ versus *ACTB-cre*/+; $Nf1^{tm1Par}$/+ epidermis, $P=0.0032$. *Dsk9*, *Dark skin 9*; GRD, GTPase-activating protein–related domain.

genetic background (Hrabe de Angelis *et al.*, 2000). A mild hyperpigmentation of the glabrous skin (ears, tails, and footpads) is visible in adult *Dsk9*/+ animals (Figure 1a–c). *Dsk9* darkens both the dermis and the epidermis (Figure 1d and e). In an outcross–backcross mapping strategy with C57BL/6J, we used darker skin to link *Dsk9* to polymorphic SSLP markers on chromosome 11 (Fitch *et al.*, 2003). Subsequently, an outcross–intercross mapping strategy was used when it was determined that homozygosity of the dark skin–linked region of chromosome 11 bearing *Dsk9* is homozygous lethal (46 *Dsk9/Dsk9* expected, 0 observed).

From these mapping crosses, *Dsk9* was localized to a 4.3-Mb physical interval on chromosome 11, which contains 83 candidate genes, including neurofibromin (*Nf1*; Figure 1f). We sequenced all exons of *Nf1* in DNA extracted from embryonic day (E) 11.5 *Dsk9/Dsk9* embryos and identified a single nucleotide substitution in exon 33 (ENSMUST00000071325, NCBI m37), which was not present in the strain of origin (Figure 1g). The nucleotide change predicts that asparagine 1453 in the C-terminal end of $\alpha 7_c$ of the GTPase-activating protein–related domain (GRD) is replaced with lysine (ENSMUSP00000071289, NCBI m37;

Table 1. Skin hyperpigmentation in various *Nf1* knockouts (KO)

Genotype	Cell type(s) targeted	Increase in average pixel intensity of the dermis compared with wild-type littermates	Increase in average pixel intensity of the epidermis compared with wild-type littermates
$Nf1^{Dsk9}/+$	All	13% (±2%)	9% (±4%)
$Nf1^{tm1Tyj}/+$	All	24% (±7%)	[1]
$Nf1^{ACTB-cre\ KO}/+$	All	22% (±8%)	13% (±3%)
$Nf1^{Mitf-cre\ KO}/Nf1^{Mitf-cre\ KO}$	Committed melanocytes	70% (±8%)	14% (±6%)
$Nf1^{Plp1-cre\ KO}/+$ (E11.5 tamoxifen injection)	Bipotential Schwann cell-melanoblast precursors expressing Plp1-creER at E11.5	8% (±3%)	None
$Nf1^{Mitf-cre\ KO}/+$	Committed melanocytes	None	None
$Nf1^{Mitf-cre\ KO\ +\ S100a4-cre\ KO}/+$	Committed melanocytes, keratinocytes, and fibroblasts	None	None
$Nf1^{Mitf-cre\ KO\ +\ Vav1-cre\ KO}/+$	Committed melanocytes and hematopoietic cells	None	None

Quantification of the percent increases in the mean pixel intensity of different *Nf1* mutant tail dermis and epidermis.
[1]The epidermis was not examined in these mice. At the time of this experiment, the epidermal hyperpigmentation of $Nf1^{Dsk9}$ mice had not been identified. It was missed in the initial survey of the *Dsk* mice (Fitch *et al.*, 2003).

Figure 1h). The GRD of neurofibromin accelerates the conversion of active RAS–GTP to inactive RAS–GDP, within the mitogen-activated protein kinase signaling pathway. Asn1453 is conserved through *Drosophila* (Figure 1i), and mutational analysis screening has shown that it is important for interactions with RAS (Morcos *et al.*, 1996). Missense mutations in the human *NF1* GRD have been previously reported in neurofibromatosis type 1 patients (Scheffzek *et al.*, 1998). The most frequently altered human residue in the GRD is also located on $\alpha 7_c$ (Lys1444, Figure 1h; Scheffzek *et al.*, 1998).

Several other lines of evidence suggest that activation of the mitogen activated protein kinase pathway can cause darker skin. In addition to neurofibromatosis type 1, multiple CALMs are a feature of Legius syndrome, which is caused by loss-of-function mutations in *SPRED1*, another negative regulator of mitogen-activated protein kinase signaling (Brems *et al.*, 2007). Transgenic mice expressing activated *HA-RAS* in melanocytes exhibit both epidermal and dermal hyperpigmentation, the only darker-skinned mouse transgenic known to do so (Powell *et al.*, 1995).

Knockout alleles of *Nf1* have been studied by other groups and have not been reported to have a darker skin phenotype. We analyzed a *Nf1* knockout allele (Jacks *et al.*, 1994) ($Nf1^{tm1Tyj}$) and a *Nf1* floxed allele (Zhu *et al.*, 2001) ($Nf1^{tm1Par}$) crossed to a ubiquitously expressed Cre line (Lewandoski *et al.*, 1997) (Tg(*ACTB-cre*)*2Mrt*). We group photographed split tail samples and quantified the darkness of the dermis and epidermis in terms of mean pixel intensity (Figure 1j, Supplementary Figure S1 online and Table 1). We found that both $Nf1^{tm1Par}/+$; *ACTB-cre*/+ (hereafter referred to as $Nf1^{ACTB\text{-}cre\ KO}/+$) and $Nf1^{tm1Tyj}/+$ mice have darker skin.

The $Nf1^{tm1Tyj}$ allele targets exon 40, whereas the $Nf1^{tm1Par}$ allele targets exons 40 and 41, all of which are downstream of the GRD, mutated in $Nf1^{Dsk9}$. However, neurofibromin protein was not detected in whole homozygous $Nf1^{tm1Tyj}$ embryos by western blotting, and thus the $Nf1^{tm1Tyj}$ allele is likely a complete null (Jacks *et al.*, 1994). The darker skin phenotype in these targeted *Nf1* alleles indicates that loss-of-function mutations in *Nf1* cause skin hyperpigmentation in mice, as they do in humans (De Schepper *et al.*, 2005).

Effects of homozygous loss of neurofibromin on melanocytes

Homozygosity of $Nf1^{tm1Tyj}$ is lethal at E13.5 owing to cardiac defects (Jacks *et al.*, 1994). Similar to $Nf1^{tm1Tyj}$, $Nf1^{Dsk9}/Nf1^{Dsk9}$ embryos dissected at E12.5 exhibit edema, hemorrhage, and small, hypopigmented eyes (Figure 2a and b). To be able to study the effects of homozygous loss of *Nf1* in melanocytes in adult mice, we used *Tg*(*Mitf-cre*)*7114Gsb*, which expresses Cre in melanoblasts under the control of the melanocyte-specific 1 M promoter of *Microphthalmia*, to knock out the floxed $Nf1^{tm1Par}$ allele beginning at E11.5 (Alizadeh *et al.*, 2008). The resulting *Mitf-cre*/+; $Nf1^{tm1Par}/Nf1^{tm1Par}$ animals (hereafter referred to as $Nf1^{Mitf\text{-}cre\ KO}/Nf1^{Mitf\text{-}cre\ KO}$) exhibited a very dark glabrous skin dermis and a darker epidermis (Figure 2c and d, quantified in Supplementary Figure S2 online and Table 1).

In histological sections, adult $Nf1^{Mitf\text{-}cre\ KO}/Nf1^{Mitf\text{-}cre\ KO}$ animals exhibit an increase in the amount of melanin in the tail dermis and epidermis compared with wild type animals (Figure 2e and f). The trunk skin dermis typically bears only a few pigmented cells in mice (example in Figure 2g); however, they are frequently observed in $Nf1^{Mitf\text{-}cre\ KO}/Nf1^{Mitf\text{-}cre\ KO}$ animals (Figure 2h). Over time, patches of darker dermis form in the neck region of both $Nf1^{Mitf\text{-}cre\ KO}/Nf1^{Mitf\text{-}cre\ KO}$ and $Nf1^{Mitf\text{-}cre\ KO}/Nf1^{Dsk9}$ mice, but never in $Nf1^{Mitf\text{-}cre\ KO}/+$ or $Nf1^{Dsk9}/+$ littermates (Supplementary Figure S3 online). The trunk skin epidermis of $Nf1^{Mitf\text{-}cre\ KO}/Nf1^{Mitf\text{-}cre\ KO}$ mice is unpigmented, as it is in normal mice (Figure 2g and h).

We next used two melanocyte reporters to study the melanoblasts in *Nf1* homozygous mutant embryos. First,

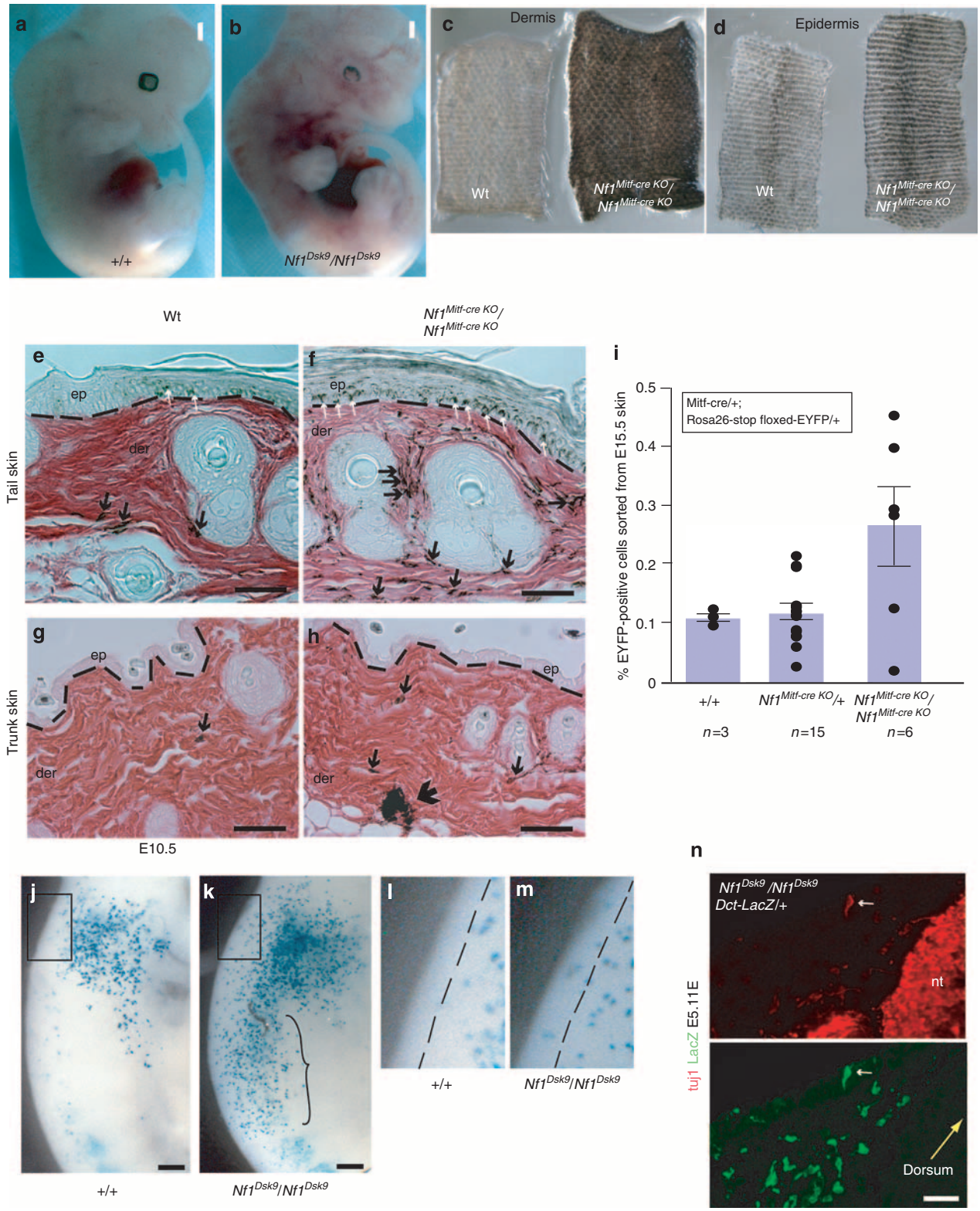

Figure 2. Homozygous knockout (KO) of *Nf1*. (**a**, **b**) E12.5 embryos. (**c**, **d**) Split tail dermis and epidermis. (**e**–**h**) Hematoxylin- and eosin-stained sections. Melanin in the epidermis (ep, white arrows) and dermis (der, black arrows). Clusters of melanin-containing cells (arrowhead). (**i**) Percentage of cells sorted from E15.5 trunk skin (mean ± SEM). (**j**–**m**) E10.5 *Dct-Lacz*/+ embryos. Boxed areas are shown in higher magnification. LacZ-positive cells are observed to the left of the line in **m**. (**n**) Immunofluorescence for Dct-LacZ (green) and Tuj1 (red) at E11.5. White arrow indicates a double-positive cell; yellow arrow points toward the dorsal aspect. Neural tube (nt). Bars: **a** and **b**, 500 μm; **e**–**h**, 50 μm; **j** and **k**, 200 μm; **n**, 30 μm. Statistical analysis: +/+ versus $Nf1^{Mitf\text{-}cre\ KO}/Nf1^{Mitf\text{-}cre\ KO}$ in **i**, $P=0.0238$. *Dsk9, Dark skin 9*; Wt, wild type.

we crossed the *Rosa26-LoxP-Stop-LoxP-EYFP* reporter line (Srinivas *et al.*, 2001) (*Gt(ROSA)26Sor*$^{tm1(EYFP)Cos}$/J) to *Mitf-cre* and *Nf1*^{tm1Par}, and FACS-sorted the EYFP-positive cells in *Nf1*$^{Mitf\text{-}cre\ KO}$/*Nf1*$^{Mitf\text{-}cre\ KO}$, *Nf1*$^{Mitf\text{-}cre\ KO}$/+, and +/+ embryos. We found a 2.4-fold increase in the percentage of EYFP-positive cells in the trunk skin of *Nf1*$^{Mitf\text{-}cre\ KO}$/*Nf1*$^{Mitf\text{-}cre\ KO}$ embryos at E15.5 as compared with wild type (Figure 2i).

Next, we crossed *Nf1*^{Dsk9} to the melanocyte reporter transgenic line, *Dct-LacZ* (*Tg*(*Dct-LacZ*)*A12Jkn*) (Mackenzie *et al.*, 1997). *Dct* encodes dopachrome tautomerase, a melanogenic enzyme expressed beginning at E10.5 (Mackenzie *et al.*, 1997). At E10.5, *Nf1*^{Dsk9}/*Nf1*^{Dsk9} embryos expressing *Dct-LacZ* showed a marked increase in LacZ-positive cells in the head and neck region (Figure 2j and k). We noted that in *Nf1*^{Dsk9}/*Nf1*^{Dsk9} embryos, LacZ-positive cells were located at a position more dorsal to any in +/+ embryos (Figure 2l and m). At E11.5, we found colocalization of LacZ with a neuronal marker, Tuj1, in migratory cells situated at the most dorsal positions in *Nf1*^{Dsk9}/*Nf1*^{Dsk9} embryos (Figure 2n and quantified in Supplementary Figure S4 online). These cells could be melanoblasts, as Tuj1 has been reported to be expressed in some melanoblasts (Adameyko *et al.*, 2012), or these cells might be neuronal progenitors (Jiao *et al.*, 2006). The number of Dct-LacZ-positive, Tuj1-negative cells was increased 3.2-fold in *Nf1*^{Dsk9}/*Nf1*^{Dsk9} embryos as compared with wild type at E11.5 (Supplementary Figure S5 online).

One of the features of neurofibromatosis type 1 is the presence of multiple CALMs. CALMs are epidermal, tan-brown, clearly demarcated macules with smooth borders and an increased melanocyte density, and they usually develop during early childhood (De Schepper *et al.*, 2006). Melanocytes within CALMs have a second hit in the *NF1* gene (Maertens *et al.*, 2007; De Schepper *et al.*, 2008), and thus the *Nf1*$^{Mitf\text{-}cre\ KO}$/*Nf1*$^{Mitf\text{-}cre\ KO}$ mice might serve as an animal model for this lesion. As with CALMs, which are typically benign, we found no evidence that homozygous loss of *Nf1* in melanocytes is tumorigenic in mice. We conclude that there is a mechanism by which homozygous loss of neurofibromin in melanoblasts increases melanocyte numbers beginning early during development.

Dermal hyperpigmentation in *Nf1* heterozygotes

In contrast to *Nf1*^{Dsk9}/+, *Nf1*$^{ACTB\text{-}creKO}$/+, and *Nf1*$^{Mitf\text{-}cre\ KO}$/*Nf1*$^{Mitf\text{-}cre\ KO}$ mice, *Nf1*$^{Mitf\text{-}cre\ KO}$/+ mice do not have a darker dermis (Figure 3a). Consistent with this, *Nf1*$^{Mitf\text{-}cre\ KO}$/+ embryos carrying the *Rosa26-LoxP-Stop-LoxP-EYFP* reporter do not exhibit an increase in the percentage of EYFP-positive cells at E15.5 (Figure 2i). There are several possible explanations for the observed lack of a pigmentary phenotype in *Nf1*$^{Mitf\text{-}cre\ KO}$ heterozygotes. For example, neurofibromin haploinsufficiency might be required in another cell type besides melanocytes in order to produce a darker dermis, or *Mitf-cre* might not recombine the *Nf1*^{tm1Par} allele at the correct stage of development.

We first addressed whether *Nf1* haploinsufficiency alters paracrine signaling initiated by other cells of the dermis, such as fibroblasts (Shishido *et al.*, 2001) and mast cells (Yang *et al.*, 2006, 2008), which could affect melanocytes. To recombine *Nf1*^{tmPar1} in the skin, we reproduced a previously described transgenic line that expresses Cre in fibroblasts and keratinocytes, under the *S100a4* EF-hand calcium-binding protein promoter (Supplementary Figures S6 and S7 online; (Shrestha *et al.*, 1998; Bhowmick *et al.*, 2004). We also obtained a transgenic line expressing Cre under the *Vav1* Dbl family guanine nucleotide exchange factor promoter to recombine *Nf1*^{tmPar1} in hematopoietic cells, including mast cells of the skin (*Tg*(*Vav1-cre*)*A2Kio/J*) (de Boer *et al.*, 2003). We did not detect any alteration in dermis pigmentation in either *Nf1*^{tmPar1}/+; *Mitf-cre*/+; *S100a4-cre*/+ (*Nf1*$^{S100a4\text{-}cre+Mitf\text{-}cre\ KO}$/+) or *Nf1*^{tmPar1}/+; *Mitf-cre*/+; *Vav1-cre*/+ (*Nf1*$^{Vav1\text{-}cre+Mitf\text{-}cre\ KO}$/+) mice (Figure 3a).

We then compared the fraction of cells that are *Dct-LacZ*-positive or EYFP-positive in *Dct-LacZ*/+ or *Mitf-cre*/+; *Rosa26-LoxP-Stop-LoxP-EYFP*/+ E15.5 trunk skin, respectively. From this, we estimate that the *Mitf-cre* transgene induces recombination at the *Rosa26* locus in about a quarter of melanoblasts by E15.5 (24% ± 8%, SEM). In adult *Nf1*$^{Mitf\text{-}cre\ KO}$/+ mice, we detected the recombined *Nf1* allele by PCR in ear skin DNA samples, where a minority of melanocytes is mixed with more abundant keratinocytes and fibroblasts (Supplementary Figure S6 online). As *Mitf-cre* is sufficient to induce a phenotype in *Nf1*$^{Mitf\text{-}cre\ KO}$/*Nf1*$^{Mitf\text{-}cre\ KO}$ embryos, but not in *Nf1*$^{Mitf\text{-}cre\ KO}$/+ embryos by E15.5 (Figure 2i), we wondered whether *Nf1* haploinsufficiency might be required before E15.5 to cause darker skin. Perhaps *Mitf-cre* is not expressed in a sufficient number of melanoblasts during some critical early melanoblast stage, or perhaps knockout must occur within melanoblast precursors.

Thus, we used a tamoxifen-inducible, *Plp1* promoter–driven Cre line (Doerflinger *et al.*, 2003; Adameyko *et al.*, 2009), *Tg*(*Plp1-cre/ERT*)*3Pop*, which is expressed in bipotential Schwann cell-melanoblast precursors, to recombine the *Nf1*^{tm1par} allele. A single injection of tamoxifen at E11.5 of development induced a darker tail dermis in *Nf1*^{tmPar1}/+; *Plp1-creER*/+ (*Nf1*$^{Plp1\text{-}creER\ KO}$/+) animals, as compared with their littermates without *Plp1-creER* (Figure 3b, Table 1). Injection of tamoxifen at E9.5 did not darken the dermis (Figure 3c). Consistent with the timing of these two tamoxifen injections (E11.5 vs. E9.5), we observed an increased number of LacZ-positive cells in *Nf1*^{Dsk9}/+ embryos carrying the *Dct-LacZ* reporter at E12.5, but not at E10.5 (Figure 3d). At E12.5, we quantified the number of LacZ-positive cells in the skin overlying the dorsal root ganglia in the trunk and found a significant increase in *Nf1*^{Dsk9}/+ embryos (Figure 3e). This is a site where melanoblasts are known to differentiate from Schwann cell precursors at E11.5 (Adameyko *et al.*, 2009).

These experiments indicate that neurofibromin haploinsufficiency acts during a narrow window of time during development to cause darker skin.

Epidermal hyperpigmentation in *Nf1* heterozygotes

Unlike the dermis, which is fairly uniform in pigmentation, the epidermis of the mouse tail is pigmented in a highly organized pattern (Figure 4a). Rows of scales, each associated

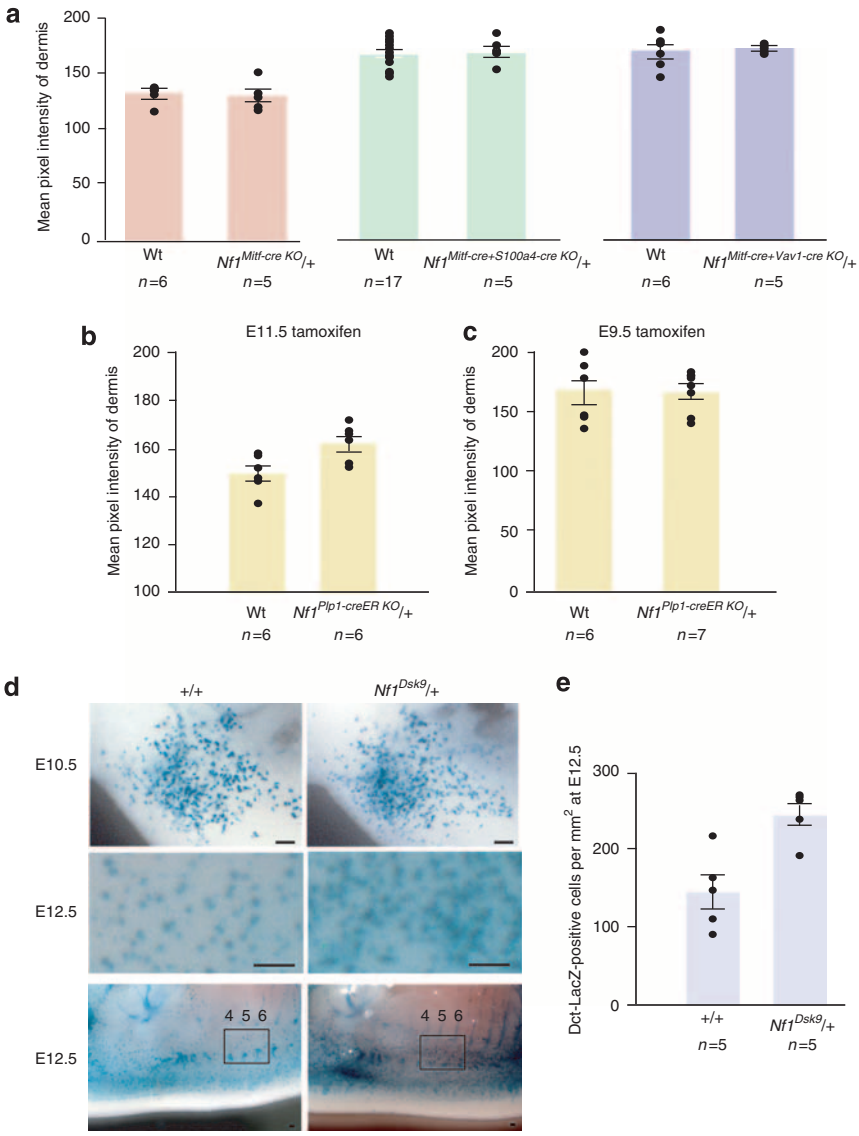

Figure 3. Effects of heterozygous knockout (KO) of *Nf1* in various cell types. (**a**–**c**) Pixel intensity of dermis from adult tails (mean ± SEM) using different Cre lines: (**a**, left) *Mitf-cre* alone, (**a**, middle) *Mitf-cre* plus *S100a4-cre*, (**a**, right) *Mitf-cre* plus *Vav1-cre*, and (**b**) *Plp1-cre* with an injection of tamoxifen at either E11.5 or (**c**) E9.5. (**d**) *Dct-LacZ*/+ embryos dissected at E10.5 (upper panel) or E12.5 (middle and lower panels). (**e**) Number of *Dct-LacZ*-positive cells per mm^2 in the skin overlying the 4th through 6th dorsal root ganglia of the trunk at E12.5, labeled in the lowest panel of **d** (mean ± SEM). Bars: **d**, 100 μm. Statistical analysis: wild type (Wt) versus $Nf1^{Plp1\text{-}creER\ KO}/+$ in **b**, $P=0.0218$. +/+ versus $Nf1^{Dsk9}/+$ in **e**, $P=0.0052$. *Dsk9, Dark skin 9.*

with a few hairs, ring the tail from base to tip (Schweizer and Marks, 1977). Only an oval in the center of each scale is pigmented, where melanocytes are concentrated. The dorsum of the tail is darker than the ventrum.

The darker epidermis in $Nf1^{Dsk9}/+$ mice can be appreciated by examining the whole epidermis split from the dermis (Figure 1e), as well as by comparing individual scales at a specific dorsal–ventral position under magnification (Figure 4a and b). To determine whether melanocyte numbers are increased in $Nf1^{Dsk9}/+$ epidermis, we removed the tail skin of adult $Nf1^{Dsk9}/+$ and +/+ animals carrying *Dct-LacZ*, stained it with X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside), and then split the dermis and epidermis (Figure 4c and d). We observed a general relationship between the density of LacZ-positive cells and the darkness of the scales (Figure 4e) and found that $Nf1^{Dsk9}/+$ animals exhibit a 24% (±8%) increase in LacZ-positive cells per mm^2. We conclude that the increase in pigmentation in the $Nf1^{Dsk9}/+$ epidermis is caused, at least in part, by an increased number of melanocytes.

Similar to the dermis, the epidermis of $Nf1^{Mitf\text{-}cre\ KO}/+$ and $Nf1^{S100a4\text{-}cre\ KO}/+$ mice is not darker (Figure 4f). We examined the tails of $Nf1^{P1p1\text{-}creERKO}/+$ animals produced by injecting 1 mg tamoxifen at E11.5 and found that, despite the darker dermis (Figure 3b), the epidermis is unaffected (Figure 4f). To examine this intriguing finding further, we used *Rosa26-LoxP-Stop-LoxP-LacZ* reporter mice (Soriano, 1999) (*Gt(Rosa)26Sor*^{tm1Sor}*/J*) to fate map melanocytes expressing *Plp1-creER* at E11.5. Previously, Adameyko *et al.* (2009) reported that the same injection of tamoxifen induced

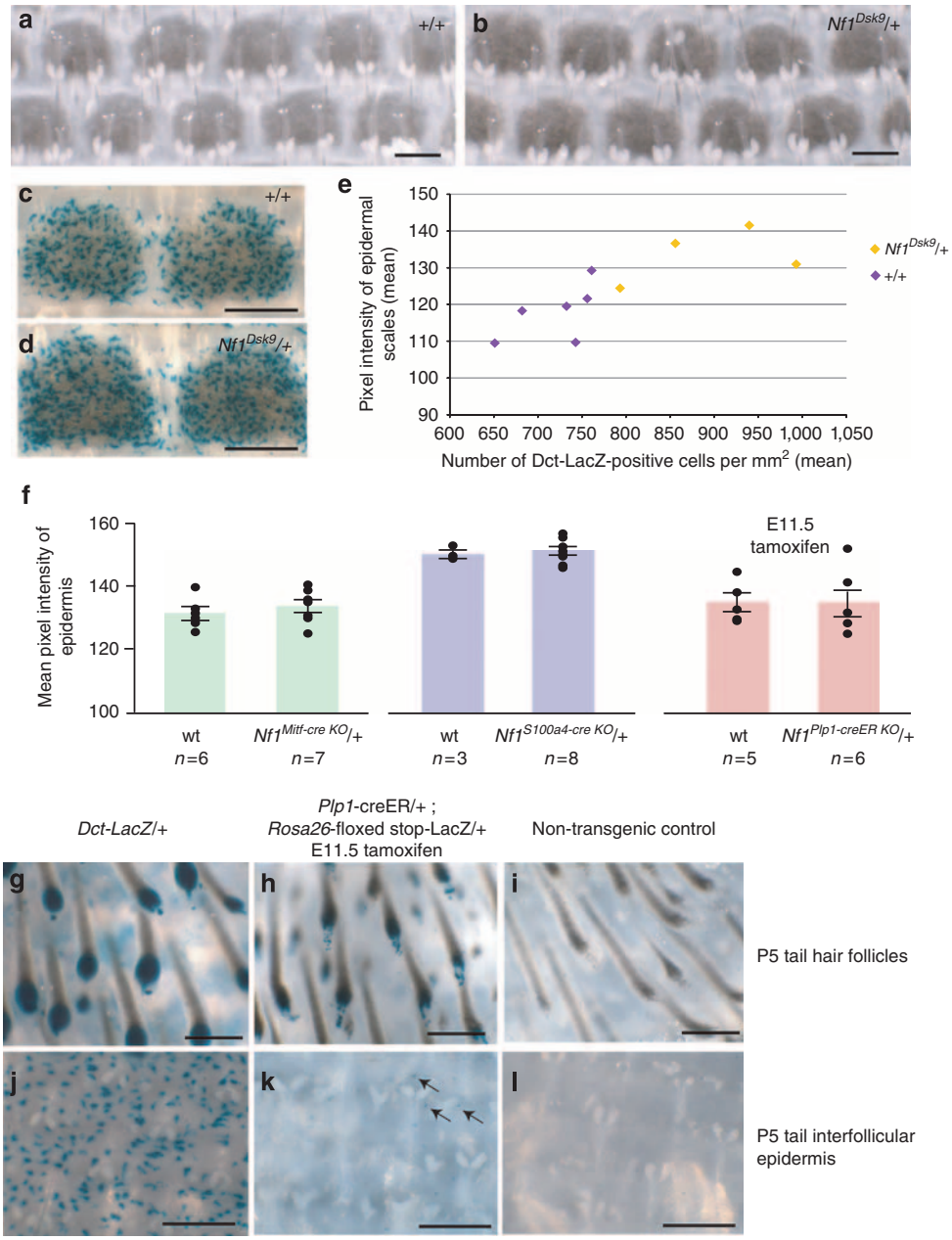

Figure 4. Epidermal pigmentation in *Nf1* heterozygous mice. (**a**–**d**) Epidermal scales with (**c**, **d**) and without (**a**, **b**) X-gal staining, from the center dorsum of adult tails. (**e**) Scatter plot of scale pixel intensity versus the number of LacZ-positive cells per mm^2 of scale. (**f**) Pixel intensity of the epidermis from adult tails (mean ± SEM) using different Cre lines: *Mitf-cre* (left), *S100a4-cre* (middle), and *Plp1-creER* with tamoxifen injection at E11.5 (right). (**g**–**l**) X-gal staining of representative P5 tail hair follicles (top) and P5 tail interfollicular epidermis (bottom). The three arrows in **k** point to the rare occurrence of faintly blue-stained cells in these samples. Bars: **a**–**d** and **g**–**l**, 200 μM. Statistical analysis: +/+ versus *Nf1*^{Dsk9}/+ melanocyte density in **e**, $P = 0.003$. *Dsk9, Dark skin 9*; KO, knockout; Wt, wild type; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactoside.

Plp1-creER-mediated recombination of *Rosa26-LoxP-Stop-LoxP-EYFP* in 66% of P11 trunk hair follicle melanocytes (Adameyko *et al.*, 2009). We analyzed the tail skin and found many LacZ-positive cells in the hair follicles (Figure 4h), but almost none in the interfollicular epidermis (Figure 4k). We conclude that it was not possible for the tail epidermis to be darker in the *Nf1*$^{Plp1\text{-}creER\ KO}$/+ mice, as the interfollicular epidermis mostly lacks melanocytes that express *Plp1-creER* at E11.5. This experiment shows that distinct groups of melanocytes populate the dermis/hair follicles and the interfollicular epidermis, a previously unappreciated complexity of melanoblast development. We speculate that an as yet unmapped lineage of melanoblasts requires *Nf1* haploinsufficiency before cell fate commitment to generate a darker tail epidermis. As migrating melanoblasts seem to be attracted to hair follicles (Jordan and Jackson, 2000; Peters *et al.*, 2002), it could be that the first wave of epidermal melanoblasts mostly end up in hair follicles,

leaving later waves of melanoblasts to populate the interfollicular epidermis.

DISCUSSION

We have found that mutations in neurofibromin (*Nf1*) cause dark skin in mice, as they do in humans. Among the genes known to cause skin hyperpigmentation in mice, *Nf1* is unique in that it darkens both the dermis and the epidermis (Fitch *et al.*, 2003). As homozygous loss of *Nf1* is lethal, we decided to make a homozygous knockout of *Nf1* specifically in melanocytes to study the effects on pigmentation in adults. To our surprise, the heterozygous knockout mice produced as a by-product of this cross had no skin darkening. Thus, our focus turned to determining which cell type is affected by *Nf1* haploinsufficiency, with the interesting conclusion that heterozygous and homozygous *Nf1* knockout apparently act by different mechanisms. Further experiments revealed that heterozygous knockout of *Nf1* in bipotential Schwann cell-melanoblast precursors produces darker skin.

Essential to our analysis is the comparison of the knockout in melanocytes (by *Mitf-cre*) with the knockout in Schwann cell precursors (by *Plp1-creER*). *Mitf* is a master regulator of melanocyte development, and it is upregulated immediately following the differentiation of melanoblasts from Schwann cell precursors, with a concurrent downregulation of *Plp1* (Widlund and Fisher, 2003; Adameyko *et al.*, 2009). *Mitf-cre*, which should be expressed in all melanoblasts, only induced recombination in 24% by E15.5, and thus it is possible that the $Nf1^{Mitf\text{-}cre\ KO}/+$ animals had no phenotype because not enough melanoblasts underwent recombination. However, we found no increase in melanoblast number in $Nf1^{Mitf\text{-}cre\ KO}/+$ embryos at E15.5, which could have been observed even if the increase was too small to cause a visible change in adult skin color. In addition, it seems unlikely that there is an ongoing increase in proliferation/survival in *Nf1* haploinsufficient melanocytes, as this would eventually have caused darker skin in the $Nf1^{Mitf\text{-}cre\ KO}/+$ adults. Thus, if *Nf1* haploinsufficiency in already committed melanoblasts has an effect, we think that it is for a limited amount of time early during development.

Plp1-creER expresses a fusion protein of Cre recombinase with the estrogen receptor. To reside in the nucleus, CreER must be bound to tamoxifen. Because tamoxifen interferes with pregnancy, we could only inject it on a single day. We chose E11.5, which had been previously shown to be a useful time point in which to target *Plp1*-expressing cells in the trunk (Adameyko *et al.*, 2009). However, the efficiency in Schwann cell precursors surrounding the dorsal root ganglia is only 61%. In addition, melanoblasts born from Schwann cell precursors before E11.5 do not undergo recombination (Adameyko *et al.*, 2012). This may explain why the dermis exhibited an 8% increase in darkening in $Nf1^{Plp1\text{-}creER\ KO}/+$ animals, compared with a 22% increase in $Nf1^{ACTB\text{-}cre\ KO}/+$ animals (Table 1).

From these findings, we suggest two possible roles for *Nf1* gene dosage in pigmentation: (1) *Nf1* haploinsufficiency increases the production of melanoblasts from Schwann cell precursors, but does not alter melanoblast survival/proliferation, or (2) *Nf1* haploinsufficiency increases melanoblast survival/proliferation, but only for a short period of time. We favor the first possibility, because our studies of the $Nf1^{Dsk9}/+$ phenotype show that the effects of *Nf1* haploinsufficiency are visible immediately after melanoblasts differentiate from Schwann cell precursors along the dorsal root ganglia (with a 1.7-fold increase in cell number by E12.5; Figure 5).

The interconnectedness of melanocytes and Schwann cells is apparent in the features of neurofibromatosis type 1. In addition to generalized epidermal hyperpigmentation and CALMs, skin hyperpigmentation can specifically overlie

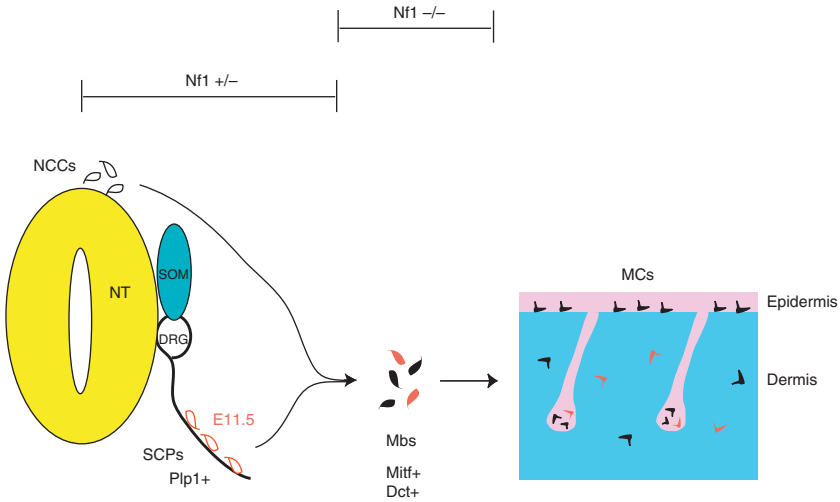

Figure 5. Requirement for *Nf1* during melanocyte development. Homozygous *Nf1* loss in committed melanoblasts (Mbs) darkened the skin. Haploinsufficiency of neurofibromin in committed melanoblasts via *Mitf-cre* did not alter skin pigmentation; however, haploinsufficiency in E11.5 Schwann cell-melanoblast precursors (SCPs) via *Plp1-creER* darkened the dermis. SCPs that express *Plp1* at E11.5 (red fate mapped cells) contribute to melanocytes (MCs) in the dermis and hair follicles, but not the interfollicular epidermis. DRG, dorsal root ganglia; NCCs, neural crest cells; NT, neural tube; SOM, somite.

superficial plexiform neurofibromas, resembling giant congenital melanocytic nevi (Boyd *et al.*, 2009). Sometimes neurofibromas are laden with melanin-containing cells (Fetsch *et al.*, 2000). Similar phenotypes are seen in mice. Wu *et al.* (2008) knocked out both copies of *Nf1* in Schwann cell precursors using a *Desert hedgehog-cre*-expressing transgene, and found that 39% of the mice exhibited hyperpigmentation overlying the plexiform neurofibromas/spinal cord. This was not reported for neurofibromas in *Plp1-creER* homozygous knockout mice, when tamoxifen was administered either perinatally or in adulthood, suggesting that perhaps *Plp1-creER*-expressing cells in adults do not produce melanocytes (Mayes *et al.*, 2011).

Recently described skin-derived precursors are neural crest–like stem cells present in the postnatal dermis that can differentiate along neuronal and glial lineages, and thus may serve as a reservoir for melanocytes in adult skin (Zabierowski *et al.*, 2011). *Nf1*-null skin-derived precursors develop into dermal neurofibromas when implanted in adult mice (Le *et al.*, 2009). It is possible that skin-derived precursors or dermal stem cells are playing a role in maintaining the generalized skin hyperpigmentation and/or CALMs of neurofibromatosis throughout adulthood. Study of tail pigmentation in mice implanted with *Nf1* null skin-derived precursors could address this.

Neurofibromatosis is one of the most common genetic diseases, affecting ~1:3,500 individuals. Although the pigmentary alterations of neurofibromatosis are tolerable, the developmental connection between melanocytes and Schwann cells requires that we understand the role of neurofibromin in both. We find that neurofibromin protein levels have an early role in the glial lineage of melanoblasts, different from the effects of complete neurofibromin loss. Future steps will be to determine whether immature Schwann cell numbers are altered in *Nf1* heterozygous embryos, to determine whether homozygous *Nf1* loss in adulthood can cause hyperpigmentation, and to pinpoint the developmental origin of interfollicular melanocytes.

MATERIALS AND METHODS

See the Supplementary data online section for more detailed information.

Mice

Nf1^{Dsk9}, *S100A4-cre*, *Dct-LacZ*, and *Mitf-cre* mice were maintained on a C3HeB/FeJ background. *Plp1-creER*, *Nf1*^{tm1Tyj}, *Tg*(*Vav1-cre*)*A2Kio/J*, *Gt*(*ROSA*)*26Sor*$^{tm1(EYFP)Cos}$/J, *Gt*(*Rosa*)*26Sor*^{tm1Sor}/J, and *Nf1*^{tm1Par} mice were obtained on an inbred C57BL6/J background. For embryo collection and tamoxifen injection, the noon of the day the copulatory plug was found was marked as day 0.5 of gestation. *Dsk9* was mapped as previously described (Fitch *et al.*, 2003). *S100a4-cre* mice were made by subcloning 3.44 kb of the *S100a4* promoter from a positively identified C57BL/6 BAC clone (RP23: 284L9) into pSP72 upstream of Cre. Purified targeting vector was digested by *Not*I and *Sph*I and microinjected into hybrid (129; C57 F1) pronuclear stage embryos to generate two founder lines. Experiments were performed under the approval of the CACC at University of British Columbia.

Whole-mount staining

Embryos, trunk skin, and tail skin samples were incubated with X-gal staining solution as previously described (Van Raamsdonk *et al.*, 2004). The tail dermis and epidermis were split using sodium bromide.

Tamoxifen

Tamoxifen was dissolved in a sunflower oil/ethanol (10:1) mixture at a concentration of 10 mg ml^{-1}. Pregnant females were injected intraperitoneally with 1 mg.

Immunofluorescence

Frozen embryo sections (8 μM) were blocked in 4% donkey serum and incubated with 1:1,000 anti-Tuj1 antibody (Covance, Princeton, NJ) and 1:500 anti-β-galactosidase antibody (Promega, Madison, WI). The following day, the sections were incubated with Alexa594-conjugated anti-rabbit antibody and Alexa488-conjugated anti-mouse antibody (Invitrogen, Grand Island, NY).

FACS analysis

The trunk skin of E11.5 embryos was removed and dissociated in trypsin for 15 minutes. Cells were sorted for EYFP expression and counted using a FACS LSRII cell sorter (Franklin Lakes, NJ).

Statistics

Jump was used to determine whether all data were distributed normally using the Shapiro–Wilk W test. Individual data points were plotted to indicate the range, given that some of the crosses were made on a mixed background of C3H and C57. *P*-values of statistical significance were calculated using either Student's *t*-test or analysis of variance and are indicated in the figure legends.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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