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# Piebald lethal ($s^l$) acts early to disrupt the development of neural crest-derived melanocytes

(tyrosinase-related protein 2)

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**ABSTRACT** **Mice homozygous for the piebald lethal ($s^l$) mutation have a predominantly white coat due to the absence of neural crest-derived melanocytes in the hair follicles. To investigate the time in embryonic development when the $s^l$ gene affects the melanocyte lineage, we compared the distribution of melanocyte precursors in wild-type and mutant embryos, using an antibody specific for tyrosinase-related protein 2 (TRP-2). TRP-2 positive cells were first observed adjacent to the anterior cardinal vein in 10.5-day postcoitem wild-type embryos. From 11.5 to 13.5 days postcoitem, there was a nonuniform distribution of TRP-2 positive cells along the anterior–posterior axis, with the highest density of cells in the head and tail regions. Along the dorsal–ventral axis, the cells were restricted to positions lateral, but never dorsal, to the neural tube. In homozygous $s^l/s^l$ embryos TRP-2 staining was restricted to the non-neural crest-derived melanocytes of the pigmented retinal epithelium and the telencephalon. Few positive cells were seen in areas that will form neural crest-derived melanocytes in the inner ear, skin, hair follicles, leg musculature, or heart. We conclude that the piebald lethal mutation acts prior to the onset of TRP-2 expression to disrupt the development of neural crest-derived melanocytes. The non-uniform distribution of melanoblasts in wild-type mice suggests that piebald acts stochastically to affect melanocyte development.**

Melanocytes are specialized melanin-producing cells that are responsible for pigmentation of skin and hair. They arise in mice from neural crest cells that leave the apical ridge of the neural tube beginning at 8.5–9 days postcoitem (dpc) and migrate dorsal to the somites and through the mesenchymal layer below the ectoderm (1, 2). Pigmented melanocytes are not apparent in the skin of developing embryos until 16.5 dpc.

One powerful approach to understanding neural crest development has been to analyze mutant mouse strains that exhibit specific developmental defects in the neural crest and its derivatives. Some of the most interesting of these are the "spotting" mutants, such as Dominant spotting (*W*), Steel (*Sl*), and piebald (*s*), because these mice lack neural crest-derived melanocytes in only a portion of the skin, resulting in nonpigmented patches of hairs (spots) (reviewed in ref. 3). In addition to a spotted coat, mice carrying homozygous mutations in the piebald gene occasionally develop aganglionic megacolon due to an absence of enteric ganglion cells in the distal portion of the colon (4). Since both melanocytes and enteric ganglion cells are derived from the neural crest, the *s* gene product is likely to play a role in the development of these neural crest derivatives.

Mice with a more severe mutation at the piebald locus, piebald lethal ($s^l$), are almost completely white with pigmented hair restricted to small areas on the head and base of the tail. The deficiency of melanocytes in $s^l/s^l$ mice cannot be attributed to a defect in pigment production, as the retinal epithelium, which is not derived from the neural crest, produces melanin and hence the animals have black eyes. Rather, the absence of pigmented melanocytes could result from defects in any one or a combination of the stages involved in melanocyte development, including determination of the neural crest precursors to the melanocyte lineage, the proliferation and migration of melanoblasts to the epidermis, their differentiation into pigmented melanocytes, or their survival.

Study of the neural crest in mammals has been hampered by the lack of specific neural crest lineage markers. Recently, transcripts of the tyrosinase-related protein 2 gene (*tyrp-2*), an enzyme involved in melanin synthesis (5, 6), have been detected in presumptive melanoblasts in the head at 10.5 dpc, at least 6 days prior to melanin production, indicating that this gene product could serve as a marker for melanocyte precursors (7). In this study we used an antibody against tyrosinase-related protein 2 (TRP-2) (6) to fully characterize the development of melanoblasts in both the head and trunk of wild-type and piebald lethal embryos.

## MATERIALS AND METHODS

**Mice and Timed Pregnancies.** Wild-type mice (C57BL/6J) and $s/s^l$ SSL/Le mice were obtained from The Jackson Laboratory. Homozygous $s^l/s^l$ mice, generated by intercrossing $s/s^l$ SS/Le mice, were mated to C57BL/6J females to generate $s^l/+$ mice.

Mutant $s^l/s^l$ embryos were generated from timed pregnancies of intercrossed $s^l/+$ mice. The morning of a visible vaginal plug was scored as 0.5 dpc and, upon dissection, embryos were staged according to Kaufman (8). Pregnant mice were killed by cervical dislocation and the embryos were placed in OCT cryoembedding medium (Tissue-Tek, Miles) and snap frozen in liquid nitrogen.

**PCR Analysis.** Four hundred microliters of PK solution (10 mM Tris·HCl, pH 8/100 mM NaCl/100 mM $Na_2EDTA$, pH 8/0.5% SDS/0.2 mg of proteinase K per ml) was added to each extraembryonic tissue sample and the mixtures were incubated at 55°C overnight. Samples were extracted with 0.25 ml of buffered phenol/chloroform (3:1) and the aqueous phase was precipitated with 2 vol of 95% ethanol. The DNA was recovered by centrifugation and resuspended in 50 μl of $H_2O$. Five to 50 ng of DNA was amplified by the PCR using primers *D14MIT7f* and *r* (9) as described (10).

**Immunohistochemistry.** For embryonic ages 10.0–13.5 dpc, transverse sections were examined every 100 μm through the entire embryo. For ages 14.5 and 15.5 dpc, transverse sections were examined caudal to the hindlimbs, in the thoracic and lumbar regions between the limbs, dorsal to the forelimbs, and at various regions of the head. For ages 16.5 through 18.5 dpc, several transverse sections were examined

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Abbreviations: TRP-2, tyrosinase-related protein 2; dpc, day(s) postcoitem.

over the hindlimbs. At least two embryos of each genotype were examined for each time point.

Embryos were sectioned at 10 μm and transferred to SuperFrost Plus microscope slides (Fisher Scientific). Samples were dried for 5 min at 25°C and then treated as follows with phosphate-buffered saline (PBS) washes between solutions: 4% paraformaldehyde/PBS, 1 min at 25°C; 0.1% Tween 20/PBS, 3 min at 25°C; 0.23% periodic acid (Sigma)/PBS, 45 sec at 25°C; endogenous avidin and biotin block (50 μg/ml; Zymed), 10 min at 25°C; goat serum blocking solution [10% normal goat serum/10% dry milk/5% bovine serum albumin fraction IV (BSA, Sigma) in PBS], 10 min at 25°C.

Blotted sections were incubated with a 1:200 dilution of the rabbit polyclonal antiserum α-PEP8 (6) in 5% BSA/1% normal goat serum in PBS at 37°C for 75 min, washed with 0.1% Tween 20/PBS, then washed with PBS, and incubated with the biotinylated secondary antibody: goat anti-rabbit IgG F(ab')2-biotin (Zymed; 4 μg/ml in 5% BSA/1% normal goat serum/PBS) for 50 min at 25°C. Sections were washed and incubated according to streptavidin-peroxidase and aminoethyl carbazole red kits (Zymed). Slides were counterstained with Mayer's hematoxylin (Sigma) and mounted in GVA mounting solution (Zymed). Sections were viewed using bright-field microscopy.

## RESULTS

**TRP-2 Protein Is Present in Pigmented Melanocytes and Nonpigmented Melanoblasts.** The restricted mutant phenotypes of piebald mice suggest that only a subset of neural crest derivatives is affected. Therefore to study the role of the gene in melanocyte development a marker is required that distinguishes melanocyte precursors from other neural crest derivatives whose development would presumably not be perturbed. Transcripts of the mouse *tyrp-2* gene have been detected as early as 10.5 dpc in presumptive melanoblasts in the head region of mouse embryos (7). Therefore we explored the utility of a polyclonal antibody raised against a synthetic peptide of the TRP-2 protein (6) as a marker for early melanocyte precursors.

To verify that the antibody was identifying only those cells that express TRP-2 transcripts, we first examined its staining pattern in neonatal skin as well as in the embryonic head. As expected, TRP-2 was specifically expressed in pigmented melanocytes of hair follicles in 7-day-old mice (Figs. 1*a* and 2*i*) and in interfollicular pigmented melanocytes in the dermis of 18.5-dpc embryos (Fig. 1*b*). Nonpigmented melanoblasts also expressed TRP-2 as indicated by the staining of cells in the hair follicles and in the basal layer of the epidermis of 18.5-dpc embryos (Fig. 2*g*) and in ventral hair follicles of newborn mice (data not shown). No other cell types in the skin expressed TRP-2 protein.

In the head region of 13.5-dpc embryos, TRP-2-containing cells were detected in the inner ear around the cochlea and saccule, around the semicircular canals, and in the endolymphatic sac (Fig. 1*c*) and the eye (Fig. 1*d*), where staining was restricted to the pigmented retinal epithelium and future choroidal layers. Taken together, the sites of expression of TRP-2 protein are entirely consistent with the distribution of its mRNA (7).

**Distribution of TRP-2 Protein in Wild-Type Embryos.** The timing of the appearance of TRP-2 expression was examined in staged C57BL/6J embryos and wild-type embryos generated from $s^l/+$ intercrosses (see below). TRP-2 was first

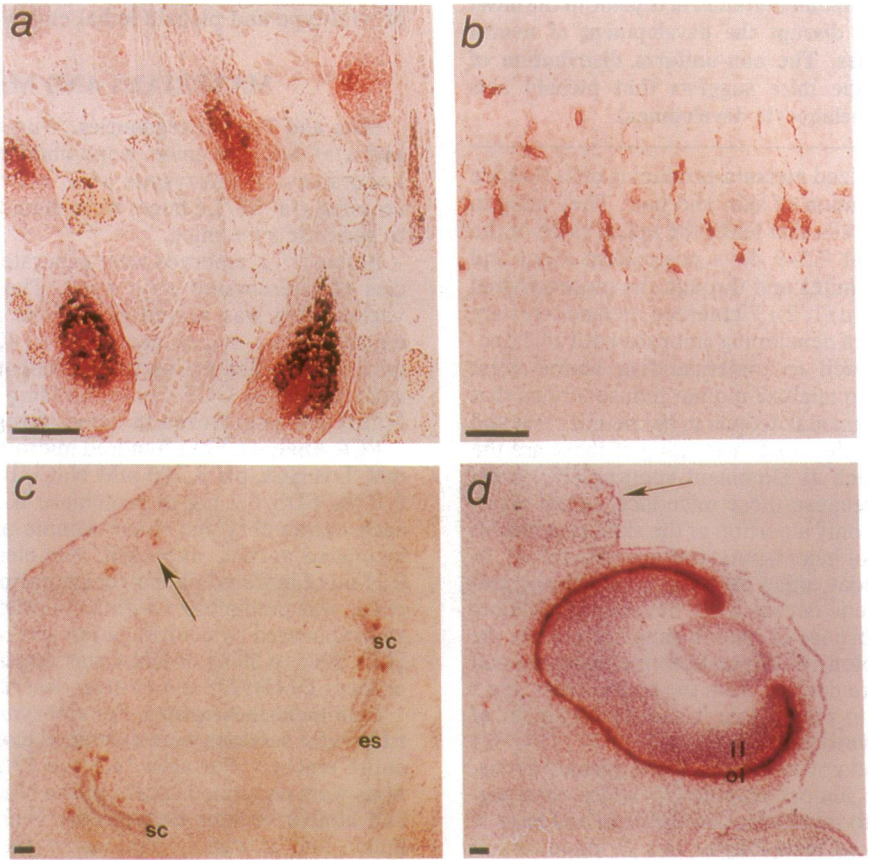


FIG. 1. TRP-2 protein expression in fetal and neonatal mice. Immunohistochemistry was performed on sections of wild-type C57BL/6J skin using an antibody directed against TRP-2. (*a*) Hair follicles in the dorsal skin of a mouse 7 days postnatal. (*b*) Interfollicular melanocytes between the hair follicles in the dermis of an 18.5-dpc embryo. (*c* and *d*) Inner ear (*c*) and eye (*d*) of a 13.5-dpc mouse embryo. il, Inner layer of the retina; ol, outer layer of the retina; sc, semicircular canal of the inner ear. Arrows indicate TRP-2 positive (red) cells. (Bars = 50 μm.)

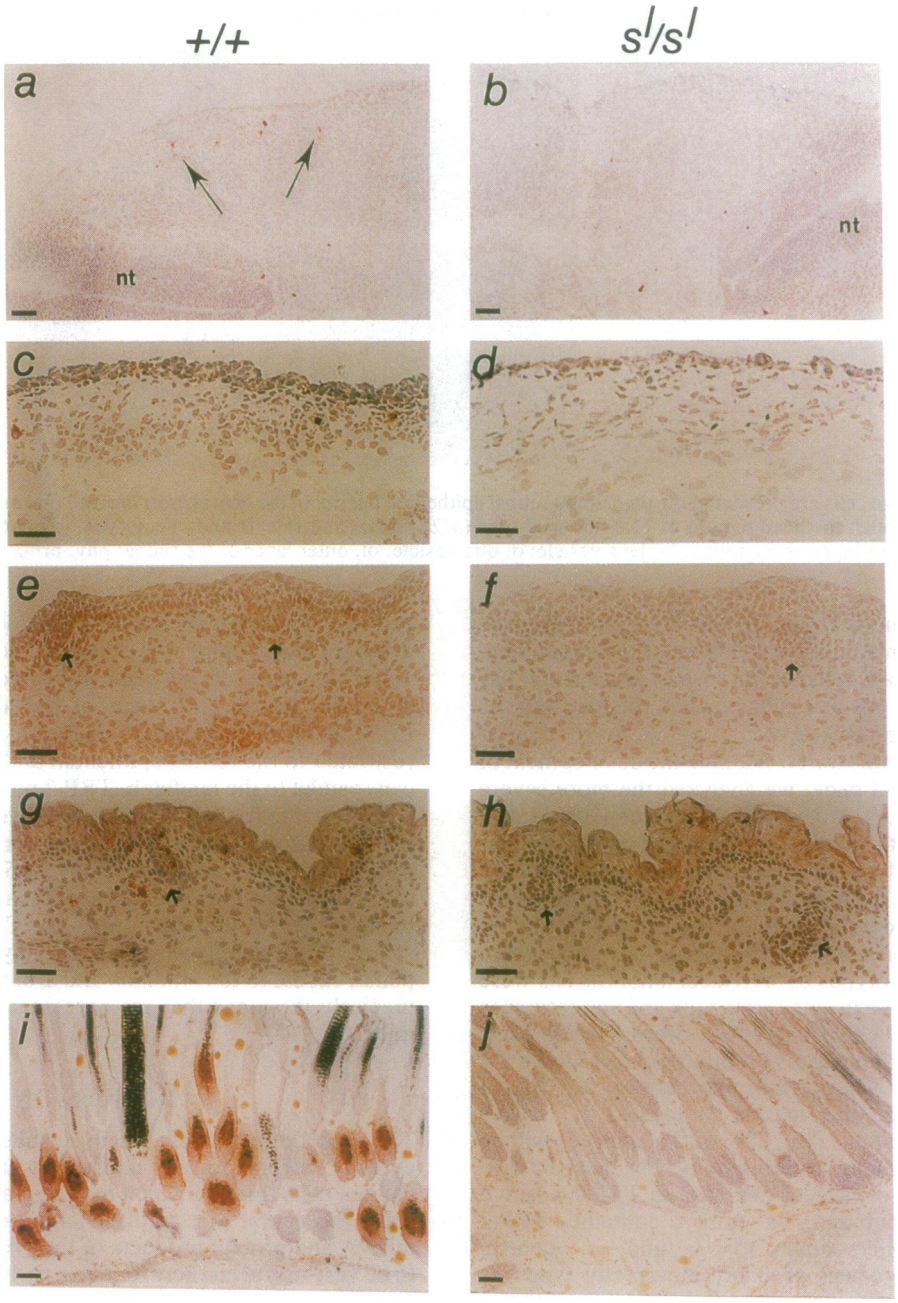


FIG. 2. Comparison of TRP-2 expression in wild-type and $s^l/s^l$ embryonic skin. Transverse sections through the trunk region of +/+ (*Left*) and $s^l/s^l$ (*Right*) embryos: 11.5 dpc (*a* and *b*), 13.5 dpc (*c* and *d*), 15.5 dpc (*e* and *f*), 18.5 dpc (*g* and *h*), and postnatal day 7 (*i* and *j*). Large arrows indicate TRP-2 positive (red) cells and small arrows denote developing hair follicles. (Bars = 50 μm.)

detected at 10 dpc in the outpocketing neural epithelium of the telencephalon, the presumptive optic cup (not shown). At 11.0 dpc the number of TRP-2 positive cells had increased in the head region, just below the ectoderm in the mesenchymal layer, mostly dorsal to the eyes and around the otic vesicle and anterior cardinal vein (Fig. 3). The preretinal epithelium and restricted cells in the telencephalon were also heavily stained.

At 11.5 dpc, TRP-2 positive cells were first observed in the trunk in a restricted position lateral and ventral to the neural tube, but never directly dorsal to the neural tube (Fig. 2*a*). The majority of the positive cells were detected at a frequency of two or three cells per section and located in the mesenchymal layer. A reduced number of positive cells was observed between the limb buds because only ≈50% of the sections contained any TRP-2-staining cells. The number of TRP-2 cells increased in 12.5-dpc embryos, with an average of four or five positive cells per section over the fore- and hindlimbs, whereas only one or two cells were observed in sections between the limbs. Once again, these cells were lateral to but never directly dorsal or very far ventral to the neural tube.

Between 12.5 and 13.5 dpc, TRP-2 positive cells increased in numbers and by 14.5 dpc, some lightly staining cells were observed in the muscle of the limbs. As in younger embryos, a relative overabundance of cells was still observed in the head and the posterior as compared to between the limbs, and many more positive cells were observed dorsally than ventrally. Finally, more rostrally localized positive cells were detected both in the basal layer of the epidermis and in the dermis, while more caudally, the majority of the cells were still in the dermis. By 15.5 dpc TRP-2 positive cells were observed around the perimeter of the entire embryo in every section examined, although fewer positive cells were still

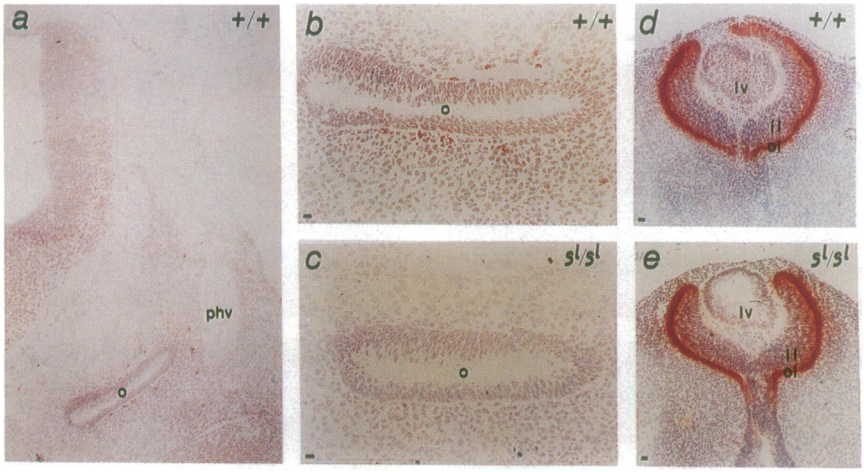


FIG. 3. TRP-2 positive cells are present in the pigmented retinal epithelium but not in the neural crest derived melanoblasts of $s^l/s^l$ embryos. Transverse sections through the head regions of 11.0-dpc wild-type (*a*, *b*, and *d*) and $s^l/s^l$ (*c* and *e*) embryos. (*b* is an enlargement of the otic vesicle seen in *a*.) il, Inner layer of the retina; lv, lens vesicle; o, otic vesicle; ol, outer layer of the retina; phv, primary head vein. (Bars = 50 μm.)

observed in the abdominal area. At this stage positive cells were seen in the dermis, in the basal layer of the epithelium, and in developing hair follicles in rostral areas only. By 16.5–18.5 dpc TRP-2 positive cells reached the hair follicles in the hindlimb as well (Fig. 2*g*).

**Genotyping $s^l/s^l$ Embryos.** To examine the development of melanocytes in piebald mice, we analyzed the more severe allele, piebald lethal ($s^l$). Mice homozygous for $s^l$ are almost completely white, with occasional pigmented patches of hair restricted to the head and tail regions. Therefore the majority of sections examined from $s^l/s^l$ embryos were derived from regions of the embryo expressing the piebald defect.

To generate $s^l/s^l$ embryos, heterozygous $s^l/+$ mice were intercrossed. The genotypes of individual embryos at *s* were determined from DNA extracted from yolk and amniotic sacs using the PCR. The PCR primers used were specific for a DNA marker closely linked to *s* on mouse chromosome 14, *D14Mit7* (10), which was polymorphic between C57BL/6J and $s^l/s^l$ mice. Since this marker is located 5.7 ± 2.9 centimorgans proximal to *s* (10), 88% of the embryos will be typed correctly. In the course of this study, 15 genotypic $s^l/s^l$ embryos exhibited a consistent decrease in TRP-2 positive cells, while a single putative $s^l/s^l$ embryo contained TRP-2 positive cells in numbers similar to $s^l/+$ and +/+ control embryos. This embryo was probably incorrectly typed as $s^l/s^l$ due to recombination between *s* and *D14Mit7*. Of the 31 embryos examined that were genotypically scored as $s^l/+$ or +/+, all contained TRP-2 positive cells.

**Distribution of TRP-2 Protein in $s^l/s^l$ Mutant Embryos.** All of the 10.5 to 11.5-dpc $s^l/s^l$ embryos examined expressed TRP-2 in the nonneural crest-derived pigmented retinal epithelium of the eye and the telencephalon, in a pattern indistinguishable from that observed in +/+ embryos (Fig. 3). However in the rest of the embryo the number of TRP-2-staining cells was drastically reduced and restricted only to areas of the embryo that are occasionally pigmented in $s^l/s^l$ mice. No positive cells were seen around the anterior cardinal vein at 10.5 dpc, and in the five embryos examined at 11.5 dpc positive cells were restricted to the region between the hindlimb and the base of the tail. In two of the 11.5-dpc $s^l/s^l$ embryos a few additional TRP-2 positive cells were observed: in one embryo two cells were observed just rostral to the forelimb; the other embryo had two positive cells around the inner ear, one just rostral to the forelimb and one in the head region dorsal to the eye. The remaining three 11.5-dpc $s^l/s^l$ embryos completely lacked any additional TRP-2 positive cells. Thus at the earliest time when TRP-2 cells are observed, there was a significant decrease in their numbers in $s^l/s^l$ embryos compared to +/+ embryos.

Differences seen between $s^l/s^l$ and +/+ embryos persisted through the later stages of development (Fig. 2 *Right*). In 12.5- to 13.5-dpc $s^l/s^l$ embryos, TRP-2 staining was restricted to the retinal epithelium and telencephalon and to cells caudal to the hindlimb. TRP-2 positive cells were rarely seen rostral to the hindlimb: one embryo contained three cells clustered around the inner ear and pinna. While hair follicle development proceeded normally 14.5 dpc to birth, no positive cells were ever seen in areas that were destined to be unpigmented in the adult (Fig. 2*j*). Consistent with earlier stages, positive cells were occasionally observed around the pinna, caudal to the hindlimb and around the base of the tail, precisely the areas that are occasionally pigmented in $s^l/s^l$ mice.

## DISCUSSION

The subset of neural crest cells destined to become melanocytes is morphologically indistinguishable from the mesenchymal cells through which they migrate from the time they leave the neural tube until melanin deposition begins. The mRNA and protein products of the *tyrp-2* gene have proven to be very useful markers for these early stages in melanocyte development (ref. 7 and this study). TRP-2 mRNA and protein are first detected in the head shortly after the neural crest cells leave the neural tube and are restricted thereafter almost exclusively to melanoblasts and melanocytes (7). We used this melanocyte lineage neural crest marker to characterize the development of melanocytes in wild-type and $s^l$ mutant mice.

**Distribution of Melanoblasts in Wild-Type Embryos.** The study revealed an uneven distribution of TRP-2 positive cells in wild-type embryos. Along the rostral to caudal axis, high numbers of positive cells were observed in the head, the hindlimbs, and at the base of the tail. In contrast, relatively few TRP-2-staining cells were observed between the limbs until 15.5 dpc. These observations are consistent with complementary studies on the distribution of TRP-2 mRNA in mouse embryos (J. Cable, I. Jackson, and K. Steel, personal communication). In addition we observed a nonuniform distribution of TRP-2 cells along the dorsal–ventral axis; TRP-2 positive cells in 11.5- to 13.5-dpc embryos were primarily positioned lateral and slightly ventral to the neural tube. Areas dorsal to the neural tube remained devoid of positive cells until 14.5 dpc.

We envision two possible explanations for the small number and nonrandom distribution of the TRP-2 positive melanoblasts in 11.5- to 13.5-dpc mouse embryos. First, neural crest cells destined to become melanocytes may be present throughout the entire embryo, but the TRP-2 gene product is expressed in only a subset of them. Alternatively, TRP-2 could reliably mark all melanoblast precursors, and during the 13.5-dpc to 15.5-dpc interval, a wave of proliferation and migration occurs from the relatively restricted region lateral to the neural tube both dorsally and ventrally. Distinguishing between these possibilities will require the fate mapping of individual neural crest cells.

**Piebald lethal Affects Early Melanocyte Development.** From the earliest time examined, there was a significant difference in the numbers of TRP-2 positive cells in wild-type and $s^l/s^l$ mice. No TRP-2 positive cells were ever observed in the mid-section of the $s^l/s^l$ embryos that gives rise to the nonpigmented region in $s^l/s^l$ adults. When positive cells were detected, they were generally localized in the head, on the back of the hindlimbs, or at the base of the tail, consistent with the areas that are often pigmented in $s^l/s^l$ mice.

The absence of TRP-2 expression does not necessarily reflect the absence of melanoblasts themselves, although this would be consistent with the absence of melanocytes in the adult hair follicle. It could be, for example, that the neural crest cells destined to become melanoblasts are present but, because of the mutation, have not activated the *tyrp-2* gene. We can conclude, however, that the product of the piebald gene has already executed at least one of its functions by this time. A decrease in the number of TRP-2 cells at 10.5 dpc represents the earliest manifestation of the mutation in the piebald gene and suggests that the wild-type gene must act very early in melanocyte development, before or coincident with neural crest migration.

TRP-2 has been used as a marker to follow melanocyte development in two other spotting mutants, *W* and *Sl*. In $W^v/W^v$, $W^{sh}/W^{sh}$, $/W^{41}/W^{41}$, and $Sl^d/Sl^d$ embryos, TRP-2 positive cells were present in reduced numbers in 10.5- and 11-dpc embryos, and by 12.5 dpc, the cells were no longer detected (ref. 7; J. Cable, I. Jackson, and K. Steel, personal communication). The detection of TRP-2 cells at a later stage in development in *W* and *Sl* mutant embryos than in $s^l$ homozygotes suggests piebald acts upstream of *W* and *Sl*.

**Models to Explain Spotting in Mice.** Of the >20 loci in the mouse that produce a spotting phenotype, many exhibit similar patterns of spotting to that seen in piebald mice, with melanocytes clustered in the head and tail, and lateral spotting between the limbs (3). To explain this pattern, Mintz (11) used the distribution of melanocyte clones in chimeric mice to propose that all melanocytes arise from 34 precursor stem cells, 17 along each side of the neural axis. Each clone contributes all of the melanocytes within a single dorsal to ventral stripe along one side of the embryo. According to Mintz, spotting arises from defects intrinsic to the neural crest such that two classes of precursor cells arise—ones that will give rise to viable melanocytes and ones in which cells are preprogramed to die. The selective death of clonal melanoblasts leads to gaps of melanocytes in the coat that cannot be filled by the viable cells because the cells destined to die occupy those positions in the skin until late in development.

Alternatively, Mayer (12) has suggested that the survival of melanoblasts in circumscribed regions of the coat reflects an enhanced ability of mutant stem cells to survive in specific environments. His model therefore requires that non-cell autonomous factors that are essential for melanoblast survival and/or differentiation be unevenly distributed along the rostral–caudal axis.

Schaible (13) proposed in a third theory that normal mice have only 14 stem cell precursors, and these cells migrate to specific "pigment centers" on the lateral surface of the mice where they proliferate and expand to cover the entire coat. Spotting, according to Schaible, results from either the elimination of one or more stem cells or their restricted proliferation within the pigment center. This theory predicts that early in development, the areas between the pigment centers will be devoid of melanoblasts.

Assuming that TRP-2 is an accurate reflection of the distribution of melanoblasts, our data clearly support one premise common to both Mintz's and Schaible's models: there are very few precursors to melanocytes early in development. However neither model can fully explain the rest of our observations. While the localization of melanoblasts lateral to the neural tube is consistent with the lateral location of Schaible's pigment centers, the cells are not restricted to a limited number of centers. In addition, we do not see a uniform distribution of precursors along the dorsal–ventral axis, as Mintz's model would predict.

Based upon our findings we propose that in piebald lethal mice, a stochastic event blocks the development of most of the neural crest cells destined to become melanoblasts prior to the onset of TRP-2 expression. A stochastic model predicts that those stem cells that survive do so in the areas with the largest numbers of precursors—that is, in the head and tail region. The proliferation of the few surviving clones results in the small amount of pigment observed in these areas of piebald lethal mice.

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