The Secreted Metalloprotease ADAMTS20 Is Required for Melanoblast Survival

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Abstract

ADAMTS20 (A disintegrin-like and metalloprotease domain with thrombospondin type-1 motifs) is a member of a family of secreted metalloproteases that can process a variety of extracellular matrix (ECM) components and secreted molecules. ADAMTS20 mutations in belted (bt) mice cause white spotting of the dorsal and ventral torso, indicative of defective neural crest (NC)-derived melanoblast development. The expression pattern of ADAMTS20 in dermal mesenchymal cells adjacent to migrating melanoblasts led us to initially propose that ADAMTS20 regulated melanoblast migration. However, using a ΔCt-LacZ transgene to track melanoblast development, we determined that melanoblasts were distributed normally in whole mount E12.5 bt/bt embryos, but were specifically reduced in the trunk of E13.5 bt/bt embryos due to a seven-fold higher rate of apoptosis. The melanoblast defect was exacerbated in newborn skin and embryos from bt/bt animals that were also haploinsufficient for ADAMTS9, a close homolog of ADAMTS20, indicating that these metalloproteases functionally overlap in melanoblast development. We identified two potential mechanisms by which ADAMTS20 may regulate melanoblast survival. First, skin explant cultures demonstrated that ADAMTS20 was required for melanoblasts to respond to soluble Kit ligand (sKitl). In support of this requirement, bt/bt;Kitl+/− and bt/bt;Kitl−/− mice exhibited synergistically increased spotting. Second, ADAMTS20 cleaved the aggregating proteoglycan versican in vitro and was necessary for versican processing in vivo, raising the possibility that versican can participate in melanoblast development. These findings reveal previously unrecognized roles for ADAMTS proteases in cell survival and in mediating Kit signaling during melanoblast colonization of the skin. Our results have implications not only for understanding mechanisms of NC-derived melanoblast development but also provide insights on novel biological functions of secreted metalloproteases.

Introduction

Δ disintegrin-like and metalloprotease with thrombospondin type-1 motifs (ADAMTS) metalloproteases constitute a large family of 19 zinc-dependent proteolytic enzymes that are distantly related to both the Δ disintegrin and metalloproteinase (ADAM) family, and to the matrix metalloproteinases (MMPs) [1,2]. In contrast to the ADAM proteases that are membrane anchored, ADAMTS proteases are secreted; however, some may be considered as operational cell-surface proteases as they bind to the cell surface. Some ADAMTS proteases, such as ADAMTS10 (GeneID: 224697), ADAMTS13 (GeneID: 279028), and the procollagen amino-propeptidases (e.g. ADAMTS2), are highly specialized; others process a variety of substrates within the extracellular matrix (ECM), including chondroitin sulfate proteoglycans (CSPGs), such as aggrecan (GeneID: 11595) and versican (GeneID: 13003). Mouse models harboring mutations in ADAMTS family members have demonstrated the importance of these proteases during development. In some cases mutant phenotypes can be attributed to a failure to cleave specific substrates [3–7]. Mutation or dysregulation of ADAMTS proteases is associated with inherited and acquired pathologies including Ehlers-Danlos syndrome VIIIC (OMIM#225410), thrombocytopenic purpura (OMIM#274150), Weill-Marchesani syndrome (OMIM#277600) and arthritis [5,8–10].

Among the animal mutants in ADAMTS proteases is a classical white-spotted mouse named belted (bt), so named because it contains white spots in the lumbar region creating the appearance of a belt [11–13]. Sequencing of three of the twelve known alleles of belted—btPat, btBelt, and bt (Mouse Genome Informatics, MGI: 2660628)—revealed nonsense or missense mutations in ADAMTS20, thus implicating metalloproteases in skin pigmentation [12]. Analyses of white spotting mutants suggest that such phenotypes are often due to defective development of neural crest (NC)-derived melanoblasts that produce pigment of the integument (skin, hair, feathers, and scales), inner ear, and eye [14,15].

Melanoblasts develop from a subset of NC that emigrate from the neural tube and overlying ectoderm and migrate dorso-laterally relative to the neural tube through prospective dermal mesenchyme (embryonic day (E) 8.5–E9.5) [15,16]. Subsequently, the melanoblasts differentiate and expand (E9.5-E13.5), migrate into the epidermis and hair follicle (E13.5-E15.5), and eventually produce
melanin (E15.5-P0). Several molecules, including the receptor tyrosine kinase Kit (GeneID: 16590) and its ligand, Kit ligand (Kitl, GeneID: 17311), regulate melanoblast development. Kit and Kitl act throughout melanoblast development, with independent requirements for melanoblast survival, proliferation and migration [17–23]. Kit is expressed on melanoblasts, and Kitl is expressed in the dermis and in dermal mesenchymal condensations and papillae [21,24–26]. Similar to Kitl, Adamts20 is expressed in dermal mesenchymal cells adjacent to and in advance of migrating melanoblasts throughout their development. This expression pattern led us to initially propose that the bt phenotype was caused by defective melanoblast migration [12]. This hypothesis was also based upon the observation that the Adams20 ortholog Gon-1 (GeneID: 177850) is essential for gonadal morphogenesis and distal tip cell migration in C.elegans [27–29]. The current study tests our hypothesis by performing extensive characterization of melanoblast development in bt/bt embryos. Our findings suggest that Adams20 mutant mice exhibit white spotting in a belted pattern due to a combination of regional variation of melanoblast number, increased apoptosis, and functional overlap with Adams9 (GeneID: 101401). Furthermore, our analyses of bt/bt embryos indicate that defective Kit signaling and versican processing may explain the failure of melanoblasts to develop properly.

Results

Genetic and Molecular Characterization of a New Belted Allele

Adams20 mutant mice exhibit white spotting in the lumbar region on both dorsal and ventral surfaces, frequently resulting in the appearance of a white belt in recessive animals (Figure 1A) [11–13]. For these studies an allele of bt on an inbred C57BL/6 background was used, as different genetic backgrounds can affect the expressivity of the bt phenotype [30,31]. Since an Adams20 mutation had not been specifically demonstrated in this particular bt allele, a complementation cross was performed between these bt mice and bt+/+ mice. Seven out of 10 animals born exhibited a bt phenotype, consistent with the expected Mendelian ratios (30% bt) and indicative of a recessive allele of bt. This allele has now been designated bt9J and is listed at MGI.

The Adams20 gene was sequenced from bt9J/bt9J genomic DNA in order to identify the molecular lesion. A single C to T point mutation was identified at nucleotide 2451, which is predicted to cause a missense mutation (Leu761Phe) in the spacer domain of ADAMTS20. The leucine residue mutated in bt9J mice is present in both mouse and human Adams20 genes and is highly conserved among all Adams mouse genes (present in 15 of the 19 genes). This suggests that Leu761 may be a critical residue for ADAMTS20 folding or for enzymatic function. We designed a Taqman™ assay to genotype bt9J mice (see Materials and Methods). We determined that bt9J is the same strain as Mutant Mouse Resource Center (MMRC) strain #183 (Figure 1B). Although the missense mutations in bt9J, btMri1, and bt affect different domains of ADAMTS20, these and other bt alleles are recessive and exhibit very similar phenotypes, strongly suggesting they act as functional null alleles.

Initial Specification and Migration of Melanoblasts Is not Disrupted in bt9J/bt9J Embryos

White spotting of mouse coats is typically caused by defective melanoblast development during embryogenesis. In order to elucidate when and how Adams20 affects melanoblast development, melanoblast distribution in bt9J/bt9J embryos was examined. We generated C57BL/6 mice containing a Dct-LacZ transgene, which marks specified melanoblasts, and crossed these onto a C57BL/6 bt9J/bt9J background [18,32]. Since there are no phenotypes associated with bt9J/+ adult animals, this heterozygous genotype served as a control for this study.

Figure 1. The bt9J/bt9J mouse contains a mutation in Adams20. (A) An image of a bt9J/bt9J mouse (left) and a C57BL/6 mouse (right). (B) A representative result from a genotyping assay for the following genotypes: bt9J/bt9J (red triangle), bt9J/+(green square), wild-type (C57BL/6) (blue diamond), and water control (grey circle).

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We compared melanoblast distribution in whole mount $bt^{9J}/+$;Dct-LacZ and $bt^{9J}/bt^{9J}$;Dct-LacZ embryos at E11.5 through E16.5 (Figure S1 and Figure 2). In E11.5 and E12.5 control embryos, melanoblasts had completed their initial migration from the neural tube and were distributed evenly across the dorsal and lateral surfaces of the embryo (Figures S1 and Figure 2A and 2C). A similar distribution pattern in E11.5 and E12.5 $bt^{9J}/bt^{9J}$ embryos was observed (Figures S1 and Figure 2B and 2D). Quantification of melanoblasts in the head and the presumptive belt region of the trunk of E12.5 whole mount embryos showed no difference in melanoblast number between control and $bt^{9J}/bt^{9J}$ embryos (Table 1, boxes in Figure 2A and 2C). These results show that initial specification and migration of NC-derived melanoblasts is not disrupted in $bt^{9J}/bt^{9J}$ animals.

Melanoblast Distribution Is Reduced only in the Trunk of $bt^{9J}/bt^{9J}$ Embryos

Since there was no defect in younger embryos, melanoblast distribution in $bt^{9J}/+$; Dct-LacZ and $bt^{9J}/bt^{9J}$;Dct-LacZ E13.5- E16.5 embryos was compared (Figure 2E-2P). In E13.5 and E14.5 $bt^{9J}/+$;Dct-LacZ embryos, the melanoblast population had expanded throughout the embryo and melanoblasts were distributed uniformly across dorsal and lateral surfaces of the trunk (Figure 2E, 2G, 2I, and 2K). In contrast, melanoblast distribution was specifically reduced in the trunk of E13.5 and E14.5 $bt^{9J}/bt^{9J}$;Dct-LacZ embryos (Figure 2F, 2H, 2J, and 2L). Quantitation of the head and trunk region of E13.5 and E14.5 $bt^{9J}/bt^{9J}$;Dct-LacZ embryos confirmed that the melanoblast defect was limited to the trunk (Table 1, boxes in Figure 2E, 2G, 2I, and 2K) ($p = 0.0000002$ at E13.5, $p = 0.000096$ at E14.5). By E15.5 and E16.5, melanoblasts were distributed evenly on both dorsal and ventral surfaces of $bt^{9J}/+$;Dct-LacZ embryos (Figure 2M and 2O). However, $bt^{9J}/bt^{9J}$;Dct-LacZ embryos displayed a notable absence of melanoblasts on both surfaces in the region corresponding to the future belt (Figure 2N and 2P). Close examination of the belt region in E16.5 $bt^{9J}/bt^{9J}$;Dct-LacZ embryos showed no accumulation of melanoblasts at the edges of the belt, as might be expected if migration into this region were impaired (Figure S2). Collectively, these analyses of the melanoblast distribution in $bt^{9J}/bt^{9J}$ animals reveal the following:

![Figure 2. Melanoblast distribution is defective in whole mount E13.5 and older $bt^{9J}/bt^{9J}$ embryos.](https://doi.org/10.1371/journal.pgen.1000003.g002)
The melanoblast defect is first observed at E13.5, coincident with the timing of melanoblast migration from the dermis into the epidermis, and with generalized dermal expression of Adamts20 [12,15]. Therefore we hypothesized that Adamts20 may be required for normal distribution of melanoblasts in dermal and epidermal compartments of the skin. To address this, melanoblasts were quantified in the dermis, epidermis, and dermal-epidermal border in E13.5 trunk sections (Figure 3A and Table 2) (see Materials and Methods). In control trunk sections (n = 178) we observed an average of 33.6 melanoblasts per section, whereas in bt9J/bt9J;Dct-LacZ sections (n = 179) there was an average two-fold reduction in melanoblast number (15.9 per section, p < 0.0001) (Table 2). This finding is consistent with the analyses of whole mount embryos (see Table 1 and Figure 2E–2H). However, the relative distribution of melanoblasts within each of the skin layers in bt9J/bt9J;Dct-LacZ embryos did not differ significantly from bt9J/+;Dct-LacZ control embryos (p = 0.056) (Figure 3B). These results show that while melanoblast number is significantly reduced in the lumbar region of bt9J/bt9J embryos, melanoblast migration from the dermis into the epidermis is unaffected.

**Table 1.** Quantitation of melanoblasts in whole mount embryos.

| Age   | Location | bt9J/+ | bt9J/bt9J | P value |
|-------|----------|--------|-----------|---------|
| E12.5 | Eye      | 162±85 (16) | 190±48 (19) | 0.13241 |
|       | Trunk    | 162±102 (16) | 197±59 (19) | 0.11472 |
| E13.5 | Eye      | 171±27 (10)  | 159±18 (10) | 0.25806 |
|       | Trunk    | 105±19 (10)  | 42±14 (10)  | 0.0000002 ** |
| E14.5 | Eye      | 128±36 (8)   | 134±30 (10) | 0.35728 |
|       | Trunk    | 220±69 (8)   | 56±23 (10)  | 0.000096 ** |

Shown are the average number of melanoblasts quantitated, standard deviations, and the number of samples in parentheses. P values are included with ** indicating highly significant values. The regions quantitated are represented in Figure 2 and described in the Materials and Methods. doi:10.1371/journal.pgen.1000003.t001

**Figure 3.** Melanoblast distribution in the skin layers of E13.5 bt9J/bt9J embryos is normal. (A) A cartoon representation of an E13.5 whole mount embryo showing the region between the fore- and hind-limbs from which 16 µm trunk sections were analyzed. (B) Pie charts representing the melanoblast distribution in the dermis (blue), epidermis (red), and dermal/epidermal border (yellow) from E13.5 bt9J/+ (top) and bt9J/bt9J (bottom) sections with the total number of melanoblasts indicated in parentheses (n = 2 embryos, each genotype). There was no significant difference in melanoblast distribution between bt9J/+ and bt9J/bt9J embryos (p = 0.056).

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**Table 2.** Quantitation of Melanoblasts in sections through the head and trunk at E13.5.

| Location | Genotype | Total sections analyzed | Average number Melanoblasts/section | Average % apoptotic Melanoblasts/section |
|----------|----------|-------------------------|--------------------------------------|-----------------------------------------|
| Head     | bt9J/+   | 40                      | 110 (±22)                            | 0.3 (±0.6)                              |
|          | bt9J/bt9J| 45                      | 117 (±36) NS                         | 1.2 (±2.2)**                            |
| Trunk    | bt9J/+   | 178                     | 33.6 (±20)                           | 1.2 (±2.6)                              |
|          | bt9J/bt9J| 179                     | 15.9 (±14)**                         | 8.4 (±16.9)**                           |

At least 3 embryos of each genotype were analyzed. For the head, only horizontal sections containing one or both eyes were included. For the trunk, horizontal sections corresponding to regions shown in Figure 3 were included. The standard deviations are included in parentheses. The results of statistical analyses comparing bt9J/+ and bt9J/bt9J are included (NS = not significant and ** = p<0.0001, which is highly significant).

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bt9J/bt9J Embryos Exhibit Normal Melanoblast Proliferation but Defective Melanoblast Survival

At E13.5, when the defect in melanoblast development in bt9J/bt9J embryos is first apparent, melanoblasts are undergoing dramatic increases in cell proliferation [18]. To assess if the bt phenotype is caused by reduced melanoblast proliferation in the trunk region of bt9J/bt9J embryos, the mitotic index of melanoblasts at E13.5 was examined in bt9J/+;Dct-LacZ and bt9J/bt9J;Dct-LacZ trunk sections (Figures 3A and 4). Melanoblasts undergoing mitosis were identified by co-expression of β-galactosidase and phospho-histoneH3 (PH3) (Figure 4A–4C). The average percentage of dividing melanoblasts in control and bt9J/bt9J sections was not significantly different (2.94% and 3.03% respectively, p = 0.9067) (Figure 4G), suggesting that the melanoblast reduction in bt9J/bt9J embryos is not a consequence of abnormal proliferation.

The reduced number of melanoblasts in the trunk region of E13.5 bt9J/bt9J;Dct-LacZ embryos could also be due to a failure of melanoblasts to survive [33]. To examine this, the apoptotic index of melanoblasts was quantitated from cells co-expressing bt9J/bt9J, galactosidase and cleaved-caspase3 (CC3) (Figures 3A and 4D–4F). The average percentage of apoptotic melanoblasts was significantly increased in bt9J/bt9J;Dct-LacZ sections relative to bt9J/+;Dct-LacZ sections (8.4% versus 1.2%, p = 0.00005) (Figure 4H, Table 2). Apoptotic melanoblasts were apparent in both dorsal and ventral skin in control and mutant embryos. In addition, no significant differences were seen in the distribution of apoptotic, CC3+melanoblasts in the three skin compartments, as follows: dermal (bt9J/bt9J = 77.8%; control = 82.4%), epidermal (bt9J/bt9J = 14.8%; control = 5.8%), and dermal/epidermal (bt9J/bt9J = 7.4%; control = 11.85%), (p = 0.6133). These results show that Adams20 is required for melanoblast survival in all cell layers of the trunk at E13.5.

Adams20 is expressed along the length of the embryo, yet the spotting phenotype in adult mice is evident only in the lumbar region. Therefore we examined melanoblast number and melanoblast apoptosis in a region outside of where the belt occurs, in the head (Table 2). In normal embryos, the density of melanoblasts is not the same in all regions, as previously described [34–37]. As expected, quantitation of melanoblasts in control embryos demonstrated that there were over three-fold more total melanoblasts in the head region than in the trunk (compare 110 versus 33.6, p = 0.0001). There was no difference in melanoblast number in the head of control and bt9J/bt9J;Dct-LacZ embryos (Table 2), similar to what was seen in whole mount analyses.

The apoptotic index of melanoblasts varied in different regions of control embryos as well as between control and mutant embryos (Table 2). In control embryos, melanoblast apoptosis was lower in the head than in the trunk. Comparing control and bt9J/bt9J embryos, we found that apoptosis was increased in the head although to a lesser extent than observed in the trunk. This result indicates that Adams20 is required for melanoblast survival throughout the embryo even though it does not result in white spotting in the head.

Melanoblasts in bt9J/bt9J Skin Fail To Respond to sKitl

Melanoblast survival is dependent upon Kit activation [20,38] by Kit ligand (Kit), which is expressed in the dermis in a similar temporal and spatial pattern to Adams20 [12,21,24–26]. We hypothesized that Adams20 regulates melanoblast survival through modulation of Kit signaling, and assessed the effects of altered Kit signaling upon the extent of white spotting in bt9J/bt9J animals harboring various mutant alleles of Kit (MGI: 966777) or Kitl (MGI: 960747). Kit+/−/+;LacZ and Kit+/−/+; bt9J/bt9J mice contain a null mutation in Kit and have primarily ventral spotting [39–41]. As depicted in Figure 5, bt9J/bt9J;KmA-w/+ mice exhibited dramatically wider dorsal and ventral belts in comparison to bt9J/bt9J mice or Kit+/−/+;LacZ mice alone (Figure 5A and 5B). The increased spotting was a synergistic effect rather than an additive one (compare 46.9% of dorsal and ventral surfaces in bt9J/bt9J;KmA-w/+ with 11.2% and 9.1% in Kit+/−/+ and bt9J/bt9J, respectively). Synergistic increases in spotting were also observed in bt9J/bt9J mice carrying mutant alleles of Kit. Heterozygosity for either Kitb, a null allele, or Kitb+/-, a deletion that generates short soluble Kit but not membrane-bound Kit, combined with homozygosity for bt9J resulted in synergistically increased spotting (Figure 5C and 5D) [42,43]. In contrast, bt9J/bt9J mice carrying a mutation in Mif (bt9J/bt9J;MifSl-d) (MGI: 104554) exhibited no synergistic spotting (Figure 5E). These results show that decreasing Kit signaling exacerbates the bt9J/bt9J spotting phenotype.

Heterozygous Kit+/-/+;LacZ knock-in at Klt locus, we monitored melanoblasts using β-galactosidase staining. There was no detectable difference in Kit-positive melanoblast distribution between bt9J/+;Klt+/−/+ and bt9J/bt9J;Klt+/−/+ embryos at E12.5. These results demonstrate that the synergistic defect in melanoblast development does not precede the onset of the bt phenotype at E13.5.

The synergistic interaction studies indicate that Kit signaling may be disrupted in Adams20 mutant animals. To test if melanoblasts in bt9J/bt9J embryos could respond to sKitl we used an ex vivo embryonic skin explant assay using dorsal trunk skin from E13.5 embryos [44–46]. Observation of bt9J/+;Dct-LacZ and bt9J/bt9J;Dct-LacZ skin after four days in culture demonstrated that...
ex vivo explant culture conditions could recapitulate both normal melanoblast colonization and the bt defect (Figure 6). Melanoblasts in the control skin entered hair follicles and were distributed evenly across the explant \((n = 5)\) (Figure 6A). While some melanoblasts survived and migrated into hair follicles in the \(bt^{9}/bt^{9};Dct^{-}/LacZ\) skin, in a large domain of the explant the melanoblasts were reduced \((n = 5)\) (Figure 6B), similar to the phenotype of whole mount E16.5 \(bt^{9}/bt^{9};Dct^{-}/LacZ\) embryos (Figure 2O and 2P).

Previous studies showed that melanoblasts within embryonic skin cultures respond to soluble Kitl \((sKitl)\) [44] and that sKitl promotes melanoblast survival in NC cultures [38]. ADAMTS20 could be required for proteolytic cleavage of Kit or Kitl, or for modifying additional molecules necessary for Kit signaling. To test if Kit signaling was defective in \(b^{9}/b^{9};Dct^{-}/LacZ\) skin \((n = 7)\) to sKitl did not restore melanoblasts to the presumptive belt (Figure 6D). Interestingly, melanoblasts at the rostral and caudal regions of the trunk did not increase in number, even though these are outside of the expected belt. These results show that in the absence of Adamts20, melanoblasts are unable to respond to sKitl, indicating that Kit signaling is disrupted in \(b^{9}/b^{9}\) mutants. Furthermore, since sKitl could not rescue the \(bt\) phenotype, it suggests that the melanoblast defect in \(b^{9}/b^{9}\) animals is not caused by defective cleavage of Kitl.

ADAMTS20 Cleaves Versican in vitro and Is Necessary for Versican Cleavage in vivo

We explored the possibility that Adamts20 could regulate melanoblast survival through additional pathways. One of the best-understood activities of ADAMTS proteases is processing of hair follicles was observed in control skin exposed to sKitl as compared to FBS alone (Figure 6C, \(n = 5\)). In contrast, exposure of \(b^{9}/b^{9};Dct^{-}/LacZ\) skin \((n = 7)\) to sKitl did not restore melanoblasts to the presumptive belt (Figure 6D).

Figure 4. Melanoblast proliferation and apoptosis in \(b^{9}/+\) and \(b^{9}/b^{9}\) embryos. (A–F) Representative immunofluorescence images depicting 16 \(\mu m\) trunk sections from \(b^{9}/b^{9}\) E13.5 embryos assayed for proliferation (A–C) and apoptosis (D–F). Proliferating melanoblasts were identified by staining sections for LacZ (cytoplasmic red, A) and PH3 (nuclear green, B), and their co-localization together with nuclear DAPI staining (blue, C). Apoptotic melanoblasts were identified by staining sections for LacZ (cytoplasmic red, D) and CC3 (cytoplasmic green, E), and their co-localization together with DAPI (blue, F). (G–H) The Y axis (%Dct+PH3+ and %Dct+CC3) indicates the average percentage of total melanoblasts per section that are PH3 positive (G) or CC3 positive (H), for \(b^{9}/+\) (blue) and \(b^{9}/b^{9}\) (red) E13.5 embryos \((n = 5\) embryos for each genotype). The total number of sections and melanoblasts analyzed are listed beneath each graph, as well as the standard deviations. Asterisks indicate highly significant \(P\) values. The scale bars are: (A–F) 50 \(\mu M\).
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various ECM substrates. Of note, ADAMTS20 is most closely related phylogenetically to ADAMTS metalloproteases that specifically process chondroitin sulfate proteoglycans (CSPGs) [1,2,47]. Although no substrates for ADAMTS20 have been identified, the CSPG versican is enriched in the skin and implicated in NC development, making it an excellent candidate substrate of ADAMTS20 [48–52].

We examined Versican and Adamts20 expression by whole mount in situ hybridization in wild-type embryos. At E12.5, when melanoblasts are widely distributed in the trunk, both Versican and Adamts20 are expressed broadly across the trunk, overlapping in many regions of the skin and the developing mammary ducts (data not shown). Since both versican and ADAMTS20 are secreted proteins, the expression patterns of their RNAs relative to each other and to melanoblasts support the possibility of a functional relationship in vivo.

We assessed the ability of ADAMTS20 to process versican by expressing ADAMTS20 in 293 cells, and subsequently incubating these cells with versican (Figure 7A). Versican is alternatively spliced to generate four isoforms, with VersicanV1 being the dominant variant in adult skin [53–56]. Versican cleavage was examined using anti-DPEAEE antibody, which specifically recognizes the neo-epitope generated by ADAMTS cleavage of versicanV1 (70 KDa) [57]. While secreted media from 293 cells alone exhibited little to no cleavage of versican, versican processing was evident in secreted media from 293 cells expressing Adamts20 (Figure 7A). As a positive control, cleaved versican was also evident in secreted media from cells expressing Adamts9, which has previously been shown to process versican [47]. These results show that in vitro, versican is a substrate for cleavage by ADAMTS20.

Analysis of skin extracts from the dorsal trunk of E15.5 b^{57}/+ and b^{57}/b^{57} embryos showed ADAMTS20 is also necessary for
ADAMTS20 Regulates Melanoblast Survival

Versican cleavage in vivo [Figure 7B]. The levels of the cleaved 70 KDa band were reduced in b/f/GAG bt9J/E13.5 control extracts compared to control extracts (n = 4 experiments). We also observed a reduction in levels of a 220 KDa band corresponding to the V0 isoform (data not shown). In contrast, there was no alteration in total versican, which was assessed using a GAG β antibody that recognizes the GAG β domains of full-length versicanV0 and V1 (Figure 7B). In (+/+ sections, cleaved versican was evident in the dermal mesenchyme and was enriched in the proliferating basal layer of the epidermis (arrow in Figure 7C) but was excluded from the outermost layer of the epidermis, in a similar pattern to that of intact versican in adult human skin [56]. In contrast to (+/+ sections, b/f/GAG bt9J/+ sections displayed a dramatic reduction in cleaved versican in the epidermis (Figure 7D). Inclusion of the DPEAEE peptide in the staining procedure demonstrated the specificity of the antibody for this peptide (Figure 7E and 7F). Total versican expression was similar in both (+/+ sections and b/f/GAG bt9J/+ sections, with high expression in the basement membrane, dermis, and dermal condensations, but lower levels in the epidermis (Figure 7G and 7H). Outside of the belt region, at the level of the forelimb, the levels of cleaved versican in b/f/GAG bt9J/+ sections were reduced relative to wild-type, but not to the same extent as seen in the trunk (Figure 7I and 7J). Together these results show that ADAMTS20cleaves versican in vitro and that it is required for versican cleavage in skin in vivo.

Immunofluorescence was performed to evaluate the spatial pattern of versican cleavage in embryonic skin. Versican expression was assessed on trunk sections of E15.5 C57BL/6 b/f/GAG bt9J/+/+;Dct bt9J/bt9J-LacZ (A,C) or b/f/GAG bt9J/+;Dct bt9J/bt9J-LacZ (B,D) embryos, and treated with either 5% FBS (A,B) or 5% FBS+Kitl (C,D). Collectively, these results show that ADAMTS20 cleaves versican in vivo and that it is required for versican cleavage in skin in vivo.

Figure 6. Melanoblasts in b/f/GAG bt9J/+ skin do not respond to soluble Kitl. (A–D) Images of b-galactosidase stained skin cultures grown from E15.5 b/f/GAG bt9J/+;Dct bt9J/+/+;b/f/GAG bt9J/bt9J-LacZ (B,D) embryos, and treated with either 5% FBS (A,B) or 5% FBS+Kitl (C,D). Brackets indicate the presumptive belt regions in b/f/GAG bt9J/+ skin. The scale bars are: (A–D) 0.5 mm. doi:10.1371/journal.pgen.1000003.g006

Adamts9 and Adamts20 Act Cooperatively To Regulate Melanoblast Development

Although Adamts20 is expressed across the length of the embryo with high expression in the presumptive belt regions in b/f/GAG bt9J/+ skin. The scale bars are: (A–D) 0.5 mm. doi:10.1371/journal.pgen.1000003.g006

Adamts9 and Adamts20 Act Cooperatively To Regulate Melanoblast Development

Although Adamts20 is expressed across the length of the embryo [12], only the lumbar region of the trunk in b/f/GAG bt9J/+/+;Dct bt9J/bt9J-LacZ (A,C) or b/f/GAG bt9J/+;Dct bt9J/bt9J-LacZ (B,D) embryos, and treated with either 5% FBS (A,B) or 5% FBS+Kitl (C,D). Brackets indicate the presumptive belt regions in b/f/GAG bt9J/+ skin. The scale bars are: (A–D) 0.5 mm. doi:10.1371/journal.pgen.1000003.g006

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between anterior and posterior boundaries of the skin samples (n = 4 each) (Figures 8B and 8C). In the 
\( b^{b97}/b^{b97} \) skin samples (n = 6), a partial de-pigmentation was observed on about 50% of the anterior-posterior length, consistent with the likely boundaries of ‘‘belted’’ and ‘‘non-belted’’ regions (Figure 8D). However, in the 
\( b^{b97}/b^{b97}; Adamts9^{ko}/+ \) skin samples (n = 5), de-pigmentation was apparent on 75% of the anterior-posterior length (Figure 8E). Our analyses showed a statistically significant increase in the severity of de-pigmentation in 
\( b^{b97}/b^{b97}; Adamts9^{ko}/+ \) skin compared to that seen in 
\( b^{b97}/b^{b97} \) skin (Figure 8F). Importantly, there was also a significant reduction of melanoblasts in regions outside of the trunk (Figure 8G and 8H). These results indicate that 
\( Adamts9 \) and 
\( Adamts20 \) are partially redundant during melanoblast development not only in the trunk but in the head as well.

**Figure 7. ADAMTS20 cleaves versican in vitro and in vivo.** (A,B) Western analyses probed with anti-DPEAEE that recognizes the 70 KDa versican cleavage product. (A) Protein extracts from 293 cells transfected with vector alone, 
\( Adamts20 \) (2 experiments) and 
\( Adamts9 \), and incubated with versican. (B) Protein extracts of E15.5 day old skin from six different 
\( b^{b97}/+ \) and 
\( b^{b97}/b^{b97} \) embryos. Anti-\( \alpha\)-Tubulin serves as a loading control and anti-GAG \( \beta \) indicates levels of total versican. (C–J) Immunofluorescence images of \(+/+\) (C,E,G,I) or 
\( b^{b97}/b^{b97} \) (D,F,H,J) E15.5 10 \( \mu \)m trunk (C–H) and forelimb (I,J) sections stained with anti-DPEAEE (C,D,I,J), anti-DPEAAE and DPEAEE peptide (E,F), and anti-GAG \( \beta \) (G,H). The anti-DPEAEE staining in the proliferating layer of the epidermis (indicated by arrows) is reduced dramatically in trunk sections and reduced to a lesser extent in forelimb sections from 
\( b^{b97}/b^{b97} \) embryos. The scale bars are: (C–F,I,J) 50 \( \mu \)M, (G,H) 100 \( \mu \)M.

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In order to assess melanoblast development in 
\( b^{b97}/+; Adamts9^{ko}/+ \) and 
\( b^{b97}/b^{b97}; Adamts9^{ko}/+ \) mice, E13.5 embryos were examined using in situ hybridization with 
\( Pmel17 \) (GeneID: 20431), a marker specific for melanoblasts. Similar to what was observed in newborn skin, there was a significant reduction in melanoblasts in the trunk region of 
\( b^{b97}/b^{b97}; Adamts9^{ko}/+ \) embryos compared to 
\( b^{b97}/+; Adamts9^{ko}/+ \) embryos (data not shown). Importantly, there was also a significant reduction of melanoblasts in regions outside of the trunk (Figure 8G and 8H). These results indicate that 
\( Adamts9 \) and 
\( Adamts20 \) are partially redundant during melanoblast development not only in the trunk but in the head as well.
Discussion

Analysis of mouse coat color mutants has yielded mechanistic insights into normal developmental pathways as well as human disease processes. In this paper we explored how mutation of Adamts20, a secreted metalloprotease that is not expressed in melanoblasts, can disrupt melanoblast development and cause white spotting. The expression pattern of Adamts20, along with studies performed on the C. elegans ortholog Gon-1, initially suggested that Adamts20 regulated melanoblast migration [12,27–29]. However, rather than this predicted requirement for melanoblast migration, we have identified an unexpected role for Adamts20 in melanoblast survival. Our results have implications not only for understanding mechanisms of melanoblast development and pigmentation-associated diseases but also for the biological functions of secreted metalloproteases.

Mutation of Adamts20 Increases Melanoblast Apoptosis

The most striking alteration observed in bt3/bt3 embryos was a reduction in trunk melanoblasts beginning at E13.5. Early events of melanoblast specification, migration, proliferation and survival were not affected, as the number and distribution of Dct-LacZ-positive melanoblasts was similar between control and bt3/bt3 embryos up to E12.5. This result contrasts many melanoblast mutants including KIt, Mixf, Pax3 (MGI: 97487), Ednrb (MGI: 102720), and Sox10 (MGI: 98358), in which melanoblast defects can be observed as early as E10.5–11.5 [15].

Multiple lines of evidence also indicate that later stages of melanoblast migration were not defective in bt3/bt3 embryos. Dct-LacZ-positive cells did not accumulate dorsally or in the dermis, suggesting that melanoblast migration along the dorso-lateral pathway and between skin layers was normal. It is also unlikely that melanoblasts undergo cell death as a result of or subsequent to failed migration, as apoptotic melanoblasts were apparent in both dorsal and ventral regions of mutant embryos and in similar proportions within dermal and epidermal compartments. Our results also exclude a defect in the second wave of melanoblast migration [37,60], since melanoblasts did not accumulate at the belt edges in E16.5 embryos. Instead, our results show that reduction of melanoblasts is due to the seven-fold increase in apoptosis observed in the trunk region of Adamts20 mutant embryos.

Why Do Mutations in Adamts20 Cause a Belt?

We propose that the white belt occurs in Adamts20 mutants for a combination of three factors: uneven densities of melanoblasts along the length of normal embryos, regional differences in apoptosis, and functional redundancy with other metalloproteases.

In wild-type animals there are fewer embryonic melanoblasts in the trunk relative to other areas of the body, as shown in this study and by others [17,34–37]. Related to this, many of the coat color mutants exhibit spotting in the lumbar level of the trunk suggesting that melanoblasts in this region are particularly sensitive to genetic perturbation, perhaps due to reduced numbers. This suggests that if melanoblast numbers were further reduced along the trunk in Adamts20 mutants, the resulting belt would be wider. Indeed this was the case since genetic interactions of bt3/bt3 with KIt mutations caused a wider belt as opposed to increased spotting in the head.

Second, Adamts20 is required for melanoblast survival throughout the embryo, consistent with the expression pattern of Adamts20 along the length of the embryo. In bt3/bt3 embryos, we found significant increases in apoptosis in both head and trunk regions. Importantly, the proportion of melanoblasts undergoing apoptosis was much more pronounced in the trunk than in the head. Thus a combination of increased apoptosis and lower initial melanoblast number in the trunk region of a bt3/bt3 mutant depletes melanoblast numbers to levels at which it cannot generate adequate pigmentation.

Third, Adamts20 mutant animals have limited white spotting due to functional redundancy with other metalloproteases, including Adamts9. In a bt3/bt3 background, loss of a single copy of Adamts9 results in reduced melanoblast numbers in the trunk as well as in the head. Thus, in bt3/bt3 mice, Adamts9 can compensate for Adamts20 deficiency in regions of higher melanoblast number such as the head. The early embryonic lethality of Adamts9+/Adamts9ko mice makes it impossible to currently assess the absolute requirement of Adamts9 in pigmentation. However, the widespread expression of Adamts9 in the skin paired with the dramatic defect in melanoblast development in bt3/bt3;Adamts9ko/+ animals predict an even greater pigmentation defect in a homozygous background. These future analyses will depend upon the generation of a conditional Adamts9 knock-out in the skin.

Adamts20 Modulates Kit Signaling

Our results suggest that Adamts20 is required for melanoblast survival at least in part through modulating Kit signaling. We show that bt3/bt3;Kitlt−/− mutants exhibit synergetic spotting, and melanoblasts in bt/bt;Kitlt−/− skin cultures are unable to respond to aKitl. This mechanism is consistent with the requirement of Kit signaling for melanoblast survival as well as the overlapping expression of Adamts20, Kit and Kitl in the dermis [12,17,20,24–26,61].

Kit signaling is essential for several events in melanoblast development beginning at E10.5 and including both migration and survival. Yet our findings indicate that mutation of Adamts20 modulates only a subset of Kit functions, namely survival, and only during a defined window of time, around E13.5, rather than throughout development. This is evidenced by the fact that bt3/bt3 embryos do not show altered melanoblast development prior to E13.5, such as seen in Kit−/+ embryos [17,18]. In addition, Kit heterozygous mutations do not exacerbate the onset of the melanoblast defect in E12.5 bt3/bt3 embryos, as would be expected if Adamts20 were required earlier. The stage at which Adamts20 disrupts melanoblast development may also explain why bt mice do not exhibit extensive ventral spotting like other white spotted mutants that impede earlier events in development.

Given these observations, it is notable that restricted disruption of Kit, during the embryonic period associated with onset of the
b^{DT}/b^{D} phenotype, results in mice with spotting phenotypes similar to b^{t} [23,62]. Pregnant mice injected with neutralizing Kit antibodies between E10.5 and E13.0 give birth to mice exhibiting white belts. In contrast, injection of these Kit antibodies into pregnant animals prior to E10.5 and after E13.0 results in mostly unpigmented mice.

A white belt/band is also evident in Kit^{W-blood}/+, Kit^{WAT2}/Kit^{WAT2}, and Kit^{WAT2}/Kit^{WAT2}+ mice that carry mutations in Kit regulatory sequences and cause altered Kit expression patterns [35,63,64]. In addition, Kimura and colleagues describe belted phenotypes in Kit^{mu/mu} and Kit^{mu/mu} mice containing targeted mutations of Kit at tyrosine residues 567 and 569, amino acids essential for proper phosphorylation and downstream signaling [65]. It is interesting that disruption of these specific amino acids results in spotting similar to that seen in belted mice, and we speculate that Adams20 may be required for efficient Kit phosphorylation at these sites in melanoblasts in vivo.

There are several possible mechanisms by which ADAMTS20 could regulate Kit signaling. ADAMTS20 could be required directly for cleavage of either Kit and/or Kitl to produce sKitl. For example, the ADAM family member ADAM17 (GeneID: 11491) cleaves Kit in vitro, and ADAM17, 19 (GeneID: 8728) and 33 (GeneID: 110751) cleave Kit [66–69]. However, sKitl did not rescue the melanoblast defect in b^{DT}/b^{D} skin cultures as would be expected if Kitl were an essential substrate of ADAMTS20. As further evidence that the b^{t} phenotype is not due to defective Kit cleavage, b^{DT}/b^{D} exhibited similar genetic interactions with both a Kit null allele and with Kit^{Lo/d+}/+ animals, which produce a short sKitl [42,43,70]. Kuniyashia et. al have also shown that transgenic mice over-expressing Kitl transgene, which greatly reduces the white spotting associated with Kit mutants, only slightly reduces the spotting in b^{DT}/b^{D} animals [71]. Taken together this suggests that the b^{t} phenotype is not due to defective cleavage of Kitl. Instead, Adams20 may regulate the interaction of Kit with Kit, the activation of Kit, or signaling downstream of the receptor. Future biochemical studies will be necessary to define the exact mechanism by which Adams20 modulates Kit signaling.

The observation that melanoblasts in b^{DT}/b^{D} trunk explants did not exhibit any response to soluble Kitl indicates that Kit signaling is defective, but it is important to note that this does not exclude the possibility that Adams20 may additionally regulate other factors essential for melanoblast development. For example, Adams20 could be required to activate other pathways that act synergistically with Kit to regulate melanoblast survival.

**ECM Alterations in b^{DT}/b^{D} Mice**

Another possible mechanism by which Adams20 could modulate survival is by altering the extracellular matrix in which melanoblasts are located. We show that b^{DT}/b^{D} mutants exhibit reduced cleavage of at least one ECM component, versican. Versican, Adams20, and Kitl are expressed in embryonic skin at appropriate times and sites to regulate melanoblast development [12,24,72]. In fact, sKitl enhances melanoblast proliferation to a greater extent when primary NC cells are cultured on chondroitin sulfate, suggesting that the ECM can modulate Kit signaling [73]. Taken in context of this study, it could be melanoblasts in b^{DT}/b^{D} explant cultures did not respond to sKitl because the ECM was defective.

Versican proteolysis could influence melanoblast behavior and Kit signaling by promoting direct interactions of Kit with Kit receptor. Such a scenario is analogous to heparin sulfate proteoglycans, which modulate growth factor signaling by sequestering ligand and promoting its receptor binding [74]. Studies of Weil-Marchesani syndrome strongly suggest ADAMTS10 (GeneID: 81794) regulates TGF β signaling through its interactions with the ECM component, fibulin-1 (GeneID: 2200) [9]. Interestingly, versican binds extracellular cytokines, including the secreted growth factor, midkine (GeneID: 17242) [75–77]. Future studies will reveal if versican, Kit, and Kitl physically interact and if versican regulates Kit signaling directly.

Versican and its proteolytic products could also promote melanoblast survival through a pathway that is independent of Kit signaling. Versican V1 promotes survival of NIH3T3 cells and down-regulates expression of the pro-apoptotic protein Bad (GeneID: 12015) [78]. Expression of the versican G1 domain protects sarcoma cells from apoptosis, and binding of the versican G3 domain to β-Integrin promotes survival of astrocytoma cells [79,80]. Since integrins are expressed by melanoblasts [81,82], versican-integrin interactions are another potential mechanism by which versican could influence melanoblast development. Since versican mutants are embryonic lethal around E10.5 [83], conditional models of versican as well as transgenic animals containing constitutively cleaved and uncleaved Versican molecules will be necessary to assess the requirement of versican for melanoblast development.

**ADAMTS20 and Human Disease**

Given that cell survival is an integral component of melanoma progression, ADAMTS9 and ADAMTS20 are excellent candidates for participating in melanoma. ADAMTS20 over-expression has not been carefully examined in melanoma, but has been observed in brain, colon and breast tumors [47,84]. The related metalloproteases ADAM9 (GeneID: 8754) and ADAMTS13 are upregulated in primary melanoma tumors and in melanoma cell lines, respectively [85,86]. Interestingly, VERSICAN and KITL overexpression are observed in primary melanomas and levels correlate with melanoma progression [87–91]. Given the requirement of Adams20 for Kit signaling and versican cleavage, it is intriguing to consider how dysregulation of signaling between ADAMTS20, Kit, and versican might contribute to melanoma progression.

**Materials and Methods**

**Mice and Genetic Interactions**

The following mouse stocks were used and kindly provided by: Adams20^{Bial} [David Beier, Harvard Medical School], Adams20^{Rex} [Lynn Lamoreux, Texas A&M University], Det-LacZ, Me^{Bial}, Kit^{mu/bi}, Kit^{D} (Heinz Arnheiter, NINDS), Kit^{Lo/d+} (Jackson Laboratories, Bar Harbor, ME). Adams20^{D} and Adams20^{S} knockout animals (both on a C57Bl/6 background) are described elsewhere (D. McCullough, H. Enomoto, S. Apte, unpublished). Quantification of white spotting was performed using Image J software [http://rsb.info.nih.gov/ij] (NIH, Bethesda, MD) and statistical significance of spotting calculated using ANOVA tests.

**Genotyping and Sequencing**

cDNA was sequenced by GeneDx (Gaithersburg, MD). Adams20^{Rex} animals were genotyped using standard conditions on an ABI Prism 7000 using a Taqman™ assay of genomic DNA. For this assay, a region containing the point mutation is amplified by PCR. Two fluorescently labeled single-stranded oligonucleotides (probes), one complementary to the wild-type product, and one complementary to the mutant product are included in the assay. The relative amount of wild-type and mutant product is measured by fluorescence quenched upon DNA synthesis. Allelic discrimination was performed to detect the total levels of each allele at the conclusion of the PCR reaction. The following cycling
conditions were used: 1. 95°C for 10 minutes 1x, 2. 92°C for 15 seconds, 3. 55°C for 60 seconds (2 and 3 repeated 40 times). The following primers and probes were used: TTCAGGAAGC-TATTCTGGAAGAC (forward primer), GCACCTGAGAG-ACATACAC (reverse primer), CTTACCAGATAAGTGGTC (VIC probe, C57BL/6 allele), and CTTCACCAGATAAGTGGTC (FAM probe, b97 allele) (designed using Applied Biosystems software, Foster City, CA).

Melanoblast Quantitation

For quantitation of whole mount embryos, melanoblasts were counted from both sides of each embryo within a 1 mm x 0.7 mm field surrounding the eye or the trunk. For quantitation of melanoblasts in E13.5 sections, Dct-LacZ expression was scored. The mitotic index was calculated using the average number of PHS+ melanoblasts per section divided by the average number of total melanoblasts per section and multiplied by 100. The apoptotic index was calculated using the average number of CC3+ melanoblasts per section divided by the average number of total melanoblasts per section and multiplied by 100. For quantification of melanoblasts in different skin compartments the following criteria were used: melanoblasts above the basal membrane were classified as epidermal, melanoblasts below the basal membrane were classified as dermal, and melanoblasts crossing the basal membrane and present in both regions were classified as dermal/epidermal region. The following statistical tests were used: for average number of melanoblasts in sections (two-tailed Student’s t-test), for proportion of total melanoblasts and apoptotic melanoblasts in sections (Chi-square test of independence), for proportion of total melanoblasts per section and multiply by 100. For apoptotic index was calculated using the average number of CC3+ melanoblasts per section divided by the average number of total melanoblasts per section.

Whole Mount β-Galactosidase Staining and In situ Hybridization

β-galactosidase activity staining was performed as previously described [41]. In situ hybridization was performed as previously described [92] using a 1 Kb mouse Adar1s20 probe [12], a 644 bp mouse Versican V1 probe made using mouse Versican V1 cDNA (a kind gift of Andrew Copp, UCL), a Pml17 probe made as previously described [92], and a 1 Kb mouse Adar1s9 probe [50].

Immunofluorescence

Frozen sections were prepared from embryos fixed overnight in 4% paraformaldehyde. Staining was performed with modifications to manufacturer’s protocol (Vector Laboratories, Burlingame, CA), and sections were mounted using Hardset Vectashield with DAPI (Vector Laboratories). The following antibodies were used at 1:200: mouse anti- β galactosidase (Promega, Madison, WI), rabbit anti-cleaved Caspase-3 (Cell Signaling, Danvers, MA), rabbit anti-phospho-Histone H3 (Upstate Biotechnology, Charlottesville, VA), rabbit anti-versican GAG β (Chemicon, Temecula, CA), rabbit anti-versican V0/V1 Neo (Affinity Bioreagents, Golden, CO), anti-mouse and anti-rabbit rhodamine-conjugated or FITC-conjugated secondary antibodies (Vector Laboratories).

Generation of Expression Plasmids and Versican Digestion

Adar1s20 short isoform cDNA was assembled by PCR from E17.5 mouse embryo mRNA, sequence verified, and cloned into pCDNAmyc-hisA+ plasmid (Invitrogen, Thousand Oaks, CA). The expression plasmid for full-length Adar1s9 was previously described [47]. Transfected or untransfected cells were incubated with versican as previously described [47]. Extracts of embryo skin were prepared using a RIPA lysis buffer containing protease inhibitor mixture (20 μl) and 2 mM phenylmethylsulphonyl fluoride (Pierce, Rockford, IL). For analysis of total versican, cell extracts were treated with 0.1 units chondroitinase ABC (Associates of Cape Cod, East Falmouth, MA) in 0.1 M Tris, 50 mM NaAc for 10 minutes at 37°C. Skin extracts and conditioned medium from transfected cells were run on 4-12% SDS-Polyacrylamide gels (Invitrogen) and analyzed by Western blotting using the following primary antibodies: mouse anti-myec 9E10 (Invitrogen), rabbit anti-versican GAG β at 1:1000, rabbit anti-versican V0/V1 Neo at 1:1000, and mouse anti-z-Tubulin at 1:1000 (Sigma, St.Louis, MO); and secondary antibodies: anti-rabbit HRP and anti-mouse HRP (Amersham, Piscataway, NJ).

Ex vivo Embryonic Skin Analysis

Dorsal skin between the forelimb and hindlimb was isolated from E13.5 bt+/+Dct-LacZ and bt/bt Dct-LacZ embryos with ventral bone, spleen and internal organs removed. The skin explants were placed epidermal side up onto polyethylene terephlate track-etched membranes in a cell culture insert (8.0 μm pore size, Becton-Dickinson, Franklin Lakes, NJ). Separate cultures were established for each explant. Explants were cultured for four days with 95% DMEM, 5% FBS (control medium) and as noted the medium was supplemented daily with 500 ng/ml Kitl (R&D Systems, Minneapolis, MN). After a culture period of four days, the explants were fixed in 4% paraformaldehyde in PBS (pH 7.4) for one hour and staining for β-galactosidase activity was performed as previously described [41].

Newborn Skin Analysis

Skin was removed from newborn pups, fixed in 4% paraformaldehyde overnight and washed 3 × in PBS and then analyzed on the dermal side for pigmentation. Pigmentation was scored as the extent of pigmentation between anterior and posterior edges of samples, using the following scale: 1 (no pigmentation), 2 (between 0 and 25%), 3 (between 25 and 50%), 4 (between 50 and 75%), 5 (between 75% and 100%). Statistical significance was calculated using a student’s t-test.

Supporting Information

Figure S1 Melanoblast distribution is normal in bt97/E11.5 embryos. (A-D) Images of β-galactosidase stained E11.5 bt97/+;Dct-LacZ, (A,C) and bt97/bt97;Dct-LacZ, (B,D) embryos. Shown are whole embryos (A,B) and trunks (C,D). The scale bars are: (A,B) 2mm, (C,D) 1mm.

Figure S2 Melanoblasts do not build up at the lateral edges of the belt in bt97/bt97 E16.5 embryos. (A-D) Images of the belt region of β-galactosidase stained E16.5 whole mount bt97/+;Dct-LacZ, (A,C) and bt97/bt97;Dct-LacZ, (B,D) embryos. Shown are low magnification (A,B) and high magnification (C,D) images from four different embryos. The scale bars are: (A,C) 1 mm, (B,D) 0.5 mm.

Figure S3 Kit heterozygous mutations do not exacerbate the bt phenotype at E12.5. Representative images of the trunks of E12.5 bt97/+;Kit+/+/+ (A) and bt97/bt97;Kit+/+/+ embryos (n = 3 each genotype) (B). Melanoblasts are marked using LacZ, which is targeted to the Kit locus. The scale bar is 1mm. Found at: doi:10.1371/journal.pgen.1000003.s003 (9.47 MB TIF)
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