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Genetic evidence does not support direct regulation of EDNRB by SOX10 in migratory neural crest and the melanocyte lineage

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Abstract

Mutations in the transcription factor *Sox10* or Endothelin Receptor B (*Ednrb*) result in Waardenburg Syndrome Type IV (WS-IV), which presents with deficiencies of neural crest derived melanocytes (hypopigmentation) and enteric ganglia (hypoganglionosis). As *Sox10* and *Ednrb* are expressed in mouse migratory neural crest cells and melanoblasts, we investigated the possibility that SOX10 and EDNRB function through a hierarchical relationship during melanocyte development. However, our results support a distinct rather than hierarchical relationship. First, SOX10 expression continues in *Ednrb* null melanoblasts, demonstrating that SOX10 expression is not dependent on EDNRB function. Second, *Ednrb* expression persists in E10.5 *Sox10* null embryos, demonstrating that *Ednrb* is not dependent on SOX10 for expression in migratory neural crest cells. Third, over-expression of SOX10 in melanoblasts of mice that harbor null or hypomorphic *Ednrb* alleles does not rescue hypopigmentation, suggesting that SOX10 overexpression can neither complement a lack of EDNRB function nor increase *Ednrb* expression. Fourth, mice that are double heterozygous for loss-of-function mutations in *Sox10* and *Ednrb* do not demonstrate synergistically increased hypopigmentation compared to mice that are single heterozygotes for either mutation alone, suggesting a lack of direct genetic interaction between these genes. Our results suggest that SOX10 does not directly activate *Ednrb* transcription in the melanocyte lineage. Given that SOX10 directly activates *Ednrb* in the enteric nervous system, our results suggest that SOX10 may differentially activate target genes based on the particular cellular context.

INTRODUCTION

A number of human diseases are associated with improper neural crest development (Gammill and Bronner-Fraser, 2003; Le Douarin et al., 2004). Among these, Waardenburg syndrome (WS) is characterized by neurosensory deafness and pigmentation anomalies in the skin, hair and iris (Baxter et al., 2004; Read, 2000; Tachibana et al., 2003). Four types of WS have been classified which share the common features of hereditary auditory-pigmentary abnormalities. Different subtypes are distinguished from one another based on additional symptoms. Individuals with WS type 4 (WS4) present with aganglionosis of the distal colon (referred to as Hirschsprung Disease) in addition to neurosensory deafness and pigment cell loss. WS4 has been associated with mutations in the HMG-box transcription factor *Sox10* (Kuhlbrodt et al., 1998; Southard-Smith et al., 1998), the seven transmembrane G protein-coupled receptor endothelin receptor B (*Ednrb*) (Hosoda et al., 1994), and EDNRB ligand, endothelin 3

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(*Edn3*) (Baynash et al., 1994). This study focuses on the genetic pathways that underlie the pigmentation defects in WS4.

SOX10 is important for both the survival of NC-derived precursor cells and their differentiation to melanocytes and glia (Mollaaghababa and Pavan, 2003). Thus, mice heterozygous for a spontaneous *Sox10* mutation *(Dominant megacolon*, $Sox10^{Dom}$) or a targeted mutation ($Sox10^{LacZ}$) present with regional hypopigmentation (head and belly spots) and enteric aganglionosis (Britsch et al., 2001; Southard-Smith et al., 1998). A disruption of melanocyte development can be detected by E10.5 in $Sox10^{Dom}$ homozygous embryos, indicated by the absence of melanoblast markers *Dopachrome tautomerase* (*Dct*) and *Microphthalmia-associated transcription factor* (*Mitf*) (Potterf et al., 2001; Southard-Smith et al., 1998), both direct targets of SOX10 transcriptional regulation (Bondurand et al., 2000; Britsch et al., 2001; Jiao et al., 2004; Lee et al., 2000; Ludwig et al., 2004; Potterf et al., 2000; Potterf et al., 2001; Verastegui et al., 2000). Consistent with this, *Dct* expression is significantly reduced in SOX10 haploinsufficent embryos, suggesting that SOX10 target genes are sensitive to its expression levels.

EDNRB is a G-protein coupled receptor that responds to EDN3 and endothelin-1 (EDN1) ligands (McCallion and Chakravarti, 2001). *Ednrb* exhibits overlapping embryonic expression patterns with *Sox10* (Lee et al., 2003; Southard-Smith et al., 1998), and, similar to *Sox10*, is important for the development of neural crest-derived melanocytes and enteric ganglia (Pla and Larue, 2003). A number of *Ednrb* alleles have been characterized in mouse (Hosoda et al., 1994; Matsushima et al., 2002; Shin et al., 1997). $Ednrb^{s-l}$ (*piebald-lethal*) is a null allele carrying a deletion of the *Ednrb* coding sequence and flanking genomic DNA (Hosoda et al., 1994). Mice homozygous for $Ednrb^{s-l}$ are almost completely white and develop megacolon. $Ednrb^{s}$ *(piebald)* is a hypomorphic allele, encompassing a wild type coding sequence and with the ability to produce structurally normal mRNA (Hosoda et al., 1994). However, the *Ednrb* mRNA expression level is reduced to about 28% of the wild type level in $Ednrb^{s/s}$ animals (Hosoda et al., 1994). Reportedly, the *piebald* mutation corresponds to a retrotransposon insertion within the first intron of *Ednrb*, introducing a cryptic splice site between the first exon and the insert that leads to formation of abnormal transcripts at the expense of appropriately spliced mRNA (Ohtani S et al., 2004). Unlike the $Ednrb^{s-l}$ allele, mice homozygous for the $Ednrb^{s}$ allele rarely present with megacolon (Hosoda et al., 1994). While $Ednrb^{s-l}$ heterozygotes ($Ednrb^{s-l/+}$) manifest haploinsufficiency as a white belly spot with variable penetrance, compound heterozygous $Ednrb^{s-l/s}$ mice show a further reduction in *Ednrb* expression level and demonstrate a vastly hypopigmented ventral surface and extensive dorsal white spotting.

It has been shown that EDNRB is transiently required between E10.5 and E12.5 for proper melanocyte development (Pavan and Tilghman, 1994; Shin et al., 1999), overlapping the time when a requirement for SOX10 in melanocyte development first becomes evident. However, MITF- and DCT-positive melanoblasts are present in *Ednrb* null embryos, albeit at reduced numbers (Hou et al., 2004b; Lee et al., 2003). Therefore, unlike SOX10, EDNRB is not required for the initial specification of melanoblasts. In the gut, a role for EDNRB in both the generation and migration of NCSCs has been established (Kruger et al., 2003). Recently SOX10 has also been reported to directly bind to and activate *Ednrb* in the enteric nervous system (Zhu et al., 2004); however, an interaction in melanocyte development was not investigated. Here, we present evidence that while EDNRB is essential for the development of two distinct neural crest derivatives, enteric ganglia and melanocytes, its transcriptional activation by SOX10 may be differentially regulated in these two cell types.

RESULTS

Ednrb and *Sox10* have similar expression patterns, but *Ednrb* expression in migratory neural crest is not dependent on *Sox10* function

In situ hybridization was used to examine *Ednrb* expression in the neural crest of mouse embryos at E10.5 and E11.5 (Figure 1). In E10.5 wild type embryos, *Ednrb* transcripts can be observed in migratory neural crest along the rostral to caudal axis (Figure 1A). This distribution is similar to that of *Sox10* visualized by in situ hybridization (Southard-Smith et al., 1999) and by activity of β-galactosidase expressed from the endogenous *Sox10* promoter ($Sox10^{LacZ}$) in a *Sox10* knock-in allele (Britsch et al., 2001) (Figure 1C). In E11.5 wild type embryos, a subset of *Ednrb* expression is observed in neural crest derivatives which also express *Sox10* (Figure 1E and F versus 1G and H). These include a subset of the cranial nerves, the dorsal root ganglia, the sympathetic chain and melanoblasts. Notably, *Ednrb*+ cells were located rostral and dorsal to the eye, and dorsal to the hindlimbs, locations similar to that observed for *Sox10*+ melanoblasts (Figure 1G and H, and Figure 3D).

To examine if the expression of *Ednrb* in neural crest is dependent on *Sox10* function, in situ hybridization was used to examine *Ednrb* transcripts in *Sox10* deficient embryos (Figure 1). At E10.5, *Sox10*-deficient neural crest cells are still present in *Sox10* null embryos (Southard-Smith et al., 1999) and can be visualized by the expression of β-galactosidase (Figure 1D). *Ednrb* transcripts were clearly present in migratory neural crest cells of *Sox10* null embryos (Figure 1B) and *Sox10* heterozygous embryos (not shown). The observation of *Ednrb* transcripts in neural crest cells that are null for *Sox10* indicates that functional SOX10 is not required for *Ednrb* expression in migratory neural crest at E10.5. At E11.5, *Ednrb* expression in *Sox10*-null embryos is still detectable in neural crest derivatives (such as the dorsal root ganglia), but the in situ hybridization signal is greatly reduced (Figure 1I and J). Assessment of *Ednrb* expression in *Sox10*-null melanoblasts is not feasible because two lines of evidence indicate that these cells are missing. First, the melanoblast markers *Dct, Si (Pmel17) and Mitf* are not observed in locations corresponding to melanoblasts in E11.5 *Sox10* null embryos (data not shown and (Potterf et al., 2001)). Secondly, in $Sox10^{LacZ/LacZ}$ embryos, *Sox10* null melanoblasts, which would be identified by activity of β-galactosidase, are not observed in these regions (Figure 1L). The reduced *Ednrb* in situ signal in neural crest derivatives is consistent with the neural crest cells undergoing apoptosis at this time of embryonic development (Kapur, 1999;Sonnenberg-Riethmacher et al., 2001). While a few *Ednrb* expressing cells can be observed in the ectoderm of the forebrain in *Sox10* null embryos (Figure 1I), these cells are not in locations typical for *pMel17*- or *Dct*-expressing melanoblasts (Baxter and Pavan, 2003). Moreover, *Ednrb*+ cells are observed in a similar location in E11.5 *Mitf* deficient embryos (not shown), suggesting that these forebrain *Ednrb*+ cells are not presumptive melanoblasts. In summary, our data demonstrate that *Ednrb* expression is not dependent on *Sox10* function in several neural crest derivatives, including migratory neural crest cells at E10.5, a time point at which *Ednrb* function is essential for melanocyte development (Shin et al., 1999).

Sox10 and *Ednrb* are co-expressed in neural crest cells, but *Sox10* expression is not dependent on EDNRB function

The expression patterns of *Sox10 and Ednrb* are similar in early neural crest and melanoblast development; however, it has not been determined if the two genes are expressed in the same cells. To determine if *Sox10* and *Ednrb* are co-expressed in neural crest cells, we examined SOX10 expression in neural tube explant cultures isolated from embryos where *Ednrb* expression is marked by expression of beta-galactosidease from an $Ednrb^{LacZ}$ knock-in allele (Lee et al., 2003). Neural tube explants from $Ednrb^{LacZ/+}$ embryos were cultured for 48 hours and double-stained for β-galactosidase and SOX10 (Figure 2A). Analysis of these cultures

demonstrated co-localization of the two signals in a majority of the cells, indicating the co-expression of *Sox10* and *Ednrb* in neural crest-derived cells.

To assess if SOX10 and EDNRB are co-expressed in melanoblasts, MITF expression was assessed in *Sox10* and *Ednrb* expressing neural crest. Five neural tube explant cultures from wild-type embryos were established and in all cultures 100% of MITF-positive cells were SOX10-positve. Also, four neural tube explant cultures from $Ednrb^{LacZ/+}$ embryos were established and in all cultures 100% of MITF-positive cells were β-galactosidase-positive. Therefore, combining these results, we can conclude that SOX10 and EDNRB are co-expressed in neural crest derived MITF+ melanoblasts.

SOX10 expression was also assessed in 48-hour neural tube explant cultures derived from $Ednrb^{LacZ/LacZ}$ homozygous embryos to determine if SOX10 expression is dependent on EDNRB function. In these cultures, robust SOX10 expression continues in β-galactosidase-positive neural crest cells that lack EDNRB (Figure 2B). Therefore, SOX10 expression is not dependent on EDNRB function in cultured neural crest-derived cells.

Over-expression of SOX10 from the *Dct* promoter can correct melanocyte defects associated with mutation in *Sox10* but not *Ednrb*

Transgenic mice were generated that over-express SOX10 during melanoblast development using a minimal *Dct* promoter (Figure 3A). We generated *Dct-Sox10* transgenic mice to achieve SOX10 overexpression in melanoblasts throughout the embryonic time window in which EDNRB function is critical (Shin et al., 1999). *Sox10* expression from *Tg(Dct-Sox10)* mice was determined by embryonic whole mount *in situ* hybridization, using a probe that recognizes endogenous and *Sox10* transgene mRNA. Expression patterns in transgenic embryos were compared with endogenous *Sox10* expression in non-transgenic littermates. Consistent with previously characterized *Tg(Dct-LacZ)* mice, we observe that the *Tg*(*Dct-Sox10*) transgene expression has commenced by E10.5 (data not shown). As expected, the transgenic embryos exhibit endogenous *Sox10* expression plus ectopic *Sox10* expression in the optic vesicle and in the dorsal telencephelon (Figure 3E). In addition, transgenic animals show increased *Sox10* expression in the melanoblast population (Compare Figure 3D with Figure 3F). These data show that the *Sox10* transgene recapitulates the expression pattern of the minimal *Dct* promoter. Therefore, in *Tg*(*Dct-Sox10*) transgenic mice, *Sox10* is over-expressed during the developmental time window that is critical for EDNRB function in melanoblasts (Figure 3B).

A total of 7 founders showed appropriate *Dct* transgene expression. Four lines were selected for further analysis. Among these, 3 lines demonstrated normal coat pigmentation when maintained in the heterozygous state on a C57/BL6JXFVB/N mixed background (CF1-10, 2272, and 2245). A low percentage of the heterozygous mice from a fourth line (2274) showed a small amount of ventral hypopigmentation (small belly spot).

Heterozygous *Tg(Dct-Sox10)* mice from the CF1-10 line were crossed with $Sox10^{LacZ/+}$ animals to assess if over-expression of SOX10 in DCT+ melanoblasts can complement the defects in melanocyte development due to *Sox10* haploinsufficiency (reduced *Dct* expression and belly spot). We first investigated whether expression of the SOX10 transgene in DCT-positive melanoblasts could rescue the transient down-regulation of *Dct* expression observed in *Sox10* mutant embryos (Figure 4). Similar to previous observations for $Sox10^{Dom/+}$ embryos at E11.5 (Potterf et al., 2001), a severe down-regulation of *Dct* expression is observed in $Sox10^{LacZ/+}$ embryos in comparison to their wild type littermates (Figures 4A versus 4C). However, *Dct* expression is substantially restored in the presence of the SOX10 transgene (compare Figures 4C and 4D), demonstrating that the transgene is functional in early melanoblasts and that overexpression of SOX10 can increase transcription of a direct target gene of SOX10 in the melanocyte lineage.

We next assessed if SOX10 overexpression could rescue the belly spot phenotype associated with heterozygosity for the *Sox10*^{LacZ} mutation. The results in Table 1 demonstrate that the SOX10 transgene can rescue this haploinsufficiency phenotype of the *Sox10*$^{LacZ/+}$ mice ($p < 0.005$). These results, together with the complementation of *Dct* expression, indicate that the SOX10 transgene is functionally expressed in the melanocyte lineage and during the time in which EDNRB function is critical.

Next, it was determined if SOX10 over-expression can rescue the hypopigmentation of *Ednrb* mutant mice. Complementation was tested in mice null for *Ednrb (Ednrb*$^{s-l/s-l}$*)*, or haploinsuffcient for *Ednrb* (*Ednrb*$^{s-l/+}$*)*, or carrying a hypomorphic allele (*piebald)* that has an intact coding sequence but gives reduced levels of wild type mRNA (Table 1; Figure 5). In all three cases, the SOX10 transgene did not rescue the hypopigmentation phenotypes in *Ednrb* mutant animals.

Hypopigmentation is not increased in mice that are double heterozygous for *Sox10* and *Ednrb* mutations

We also assessed if reducing both SOX10 and EDNRB expression levels could exacerbate hypopigmention. For this, hypopigmentation of *Sox10*$^{Dom/+}$; *Ednrb*$^{s-l/+}$ double heterozygous mice was compared with *Sox10*$^{Dom/+}$ and Ed*nrb*$^{s-l/+}$ single heterozygotes. Mice were generated using two independent genetic schemes involving different genetic background contributions to randomize modifier strain variations. Regardless of the cross or background strain used, none of the *Sox10*$^{Dom/+}$*; Ednrb*$^{s-l/+}$ double heterozygous animals showed increased hypopigmentation when compared to either *Sox10*$^{Dom/+}$ or *Ednrb*$^{s-l/+}$ single heterozygous siblings (Table 1; Figure 6), showing a lack of synergistic interaction between these genes in the manifestation of hypopigmentation. This is in stark contrast with the observation made in enteric neurons where *Ednrb* $^{tm1Ywa/+}$, *Sox10*$^{Dom/+}$ double heterozygous animals show dramatic increase in aganglionosis compared to *Ednrb* $^{tm1Ywa/+}$ or *Sox10*$^{Dom/+}$ single heterozygous siblings (Cantrell et al., 2004). Taken together with the non-complementation experiments described above, these data are consistent with a genetic model where *Ednrb* expression is not altered by varying SOX10 expression levels in the melanocyte lineage in vivo.

DISCUSSION

Mutations in *Ednrb* and *Sox10* have been associated with pigmentary defects, congenital neurosensory deafness and megacolon in several species. In addition to showing similar loss-of-function phenotypes, *Sox10* and *Ednrb* show similar embryonic expression patterns in mouse migratory neural crest cells in vivo and are co-expressed in individual neural crest cells, including melanoblasts, in vitro (Figure 2). The possibility of *Ednrb* acting downstream of *Sox10* was previously investigated by examining *Ednrb* expression in *Sox10*^{Dom} mutant embryos (Southard-Smith et al., 1998). However, the results did not lend themselves to a straightforward interpretation. While there was an apparent lowering of *Ednrb* expression in the absence of proper SOX10 function, there was also a significant reduction in the number of *Ednrb*-positive cells, presumably because of the increased apoptosis in the NC-derived population seen in these embryos. Consequently, the apparent lowering of *Ednrb* expression may have been an indirect consequence of the significant reduction in the number of *Ednrb*-positive cells. Consistent with a direct hierarchical relationship between these two genes, SOX10 was recently shown to directly bind a defined enhancer element within the *Ednrb* locus and activate transcription in the neural crest-derived enteric ganglia (Zhu et al., 2004). Transgenic mice harboring an *Ednrb* BAC containing these SOX10 binding sites show rescued megacolon without any rescue of pigmentation (Zhu et al., 2004), suggesting that either additional SOX10 binding sites are needed for *Ednrb* expression in melanocytes or that

EDNRB and SOX10 act independently in melanocytes. In this study, we tested for a genetic link between *Sox10* and *Ednrb* in neural crest-derived melanocyte development. Our results suggest that unlike the direct regulation of *Ednrb* by SOX10 in the enteric nervous system these genes may not share a hierarchical relationship in the melanocyte lineage.

Our results do not support *Sox10* acting downstream of *Ednrb*. First, expression analysis demonstrates that SOX10 continues to be robustly expressed in neural crest progenitors that lack *Ednrb* function. Second, while SOX10 over-expression in the melanocyte lineage can correct the ventral hypopigmentation in $Sox10^{LacZ/+}$ mice it doesn't complement the melanocyte deficiency of *Ednrb* null melanoblasts in vivo. These results are consistent with the finding that SOX10 is required before EDNRB during melanocyte development. Specifically, the expression of MITF and DCT are vastly reduced in *Sox10* mutant embryos at E10.5 (Potterf et al., 2001), consistent with SOX10 requirement for direct transcriptional regulation of these two genes (Bondurand et al., 2000; Jiao et al., 2004; Lee et al., 2000; Ludwig et al., 2004; Potterf et al., 2000; Verastegui et al., 2000). However, DCT-expressing melanoblasts are present in *Ednrb* mutant embryos at E10.5, although they do not expand to fill in the white areas of the coat (Lee et al., 2003; Pavan and Tilghman, 1994). Taken together, these results strongly suggest that *Sox10* does not function directly downstream of *Ednrb*.

Our results also do not support a role for *Sox10* acting directly upstream of *Ednrb* to activate its transcription in migrating neural crest or during melanocyte development. In situ analysis showed similar patterns of *Ednrb* expression in both wild type and *Sox10* null embryos at E10.5. At E11.5, *Ednrb* signal was greatly reduced but present in multiple cell types, including dorsal root ganglia. These data clearly show that *Ednrb* expression in migratory neural crest is not dependent on SOX10 function, although we were unable to directly assess expression of *Ednrb* in *Sox10* null melanoblasts. In *Sox10* null embryos, the expression of the melanoblast marker *Dct* is missing from regions where putative melanoblasts should be located. This could be because *Dct* is dependent on SOX10 for its expression or because the melanoblasts are absent. Consistent with the latter hypothesis, *Sox10-LacZ*-positive cells are also absent from regions where melanoblasts are expected in E11.5 *Sox10* null embryos. The latter hypothesis is also consistent with the increased level of apoptosis observed in *Sox10* mutant neural crest cells at this age. It remains a possibility that SOX10 is involved in the maintenance of *Ednrb* expression in the melanocyte lineage as seen with the regulation of KIT by MITF (Opdecamp et al., 1997).

To further examine if *Ednrb* expression might be dependent on SOX10 function, we examined if varying SOX10 expression levels could alter hypopigmentation in *Ednrb* mutant mice. First, the *Tg(Dct-Sox10)* transgenic mice were used to determine if SOX10 over-expression can rescue hypopigmentation in mutant mice that either carry the $Ednrb^{s}$ allele or are heterozygous for the $Ednrb^{s-l}$ mutation. $Ednrb^{s}$ is a hypomorphic allele that can produce wild type mRNA, albeit at lower levels. If *Sox10* acts upstream of *Ednrb* in the melanocyte lineage, proper spatiotemporal over-expression of SOX10 should in principle induce the $Ednrb^{s}$ allele to produce higher levels of EDNRB and partially rescue hypopigmentation. Or, it could increase the wild type transcript in $Ednrb^{s-l}$ heterozygous mice in which the EDNRB levels are reduced by half, thus rescuing their belly spot phenotype. However, *Dct-Sox10* transgenic mice do not rescue hypopigmentation in either $Ednrb^{s-l/+}$ or in $Ednrb^{s/s}$ mice. We also assayed for possible synergistic effects of lowering SOX10 levels on *Ednrb*-associated hypopigmentation. Strong in vivo genetic interactions between transcription factors and target genes in melanocyte development have been observed with *Sox10* and *Mitf* (Potterf et al., 2000) and with *Mitf* and *Bcl2* (McGill et al., 2002), providing a very sensitive assay for interacting alleles that affect coat color. In fact, strong in vivo effects were observed on enteric ganglia in *Sox10, Ednrb* double heterozygous mutant mice ($Ednrb^{tm1Ywa/+}$, $Sox10^{Dom/+}$)(Cantrell et al., 2004). Therefore, if *Sox10* acted upstream of *Ednrb* in the melanocyte lineage, one would predict that

a significant lowering of both SOX10 and EDNRB levels in *Sox10*, *Ednrb* double heterozygous mice would result in noticeably increased hypopigmentation compared to heterozgotes for either gene. However, we did not observe such synergistic interaction between *Sox10* and *Ednrb*.

Our genetic hypothesis that SOX10 and EDNRB function independently in the melanocyte lineage suggests a stark and very interesting difference with the recent finding that SOX10 directly activates *Ednrb* during the development of enteric neural crest (Zhu et al., 2004) and the report of a strong genetic interaction between these genes in the enteric nervous system (Cantrell et al., 2004). Therefore, SOX10 regulation of a single gene (*Ednrb*) appears to be dependent on the cellular context. As co-activators are typically involved in SOX gene function, it is possible that the differing milieu of co-factors in enteric and melanocyte derived neural crest cells accounts for the differing transcriptional regulation.

MATERIALS AND METHODS

Mouse Strains

Dominant megacolon (*Sox10*$^{Dom/+}$) mice were obtained from the Jackson Laboratory and were maintained on a C57BL6/C3HeB/FeJLe-a/a background. Animals with a *LacZ* knock-in engineered at the *Sox10* locus (*Sox10*^{tm1Weg}, here referred to as *Sox10*$^{LacZ/+}$) were obtained on a mixed genetic background (Britsch et al., 2001) and were crossed to C57BL6/J mice before use in rescue assays. *Ednrb*$^{s-l/s}$ mice were obtained from the Jackson Laboratory and maintained on SSL/LeJ background. Mice with a *LacZ* knock-in engineered at the *Ednrb* locus (Lee et al., 2003) were maintained on a mixed C57BL6/C3H/HeJ genetic background.

Generation of *Tg(Dct-Sox10)* mice

For the *Tg(Dct-Sox10)* construct a 0.5 kbp BamHI fragment containing a spliceable intron (MP1) and Poly A sequences was inserted within the unique *BamHI* restriction site of a pBluescript II SK(+) vector (pBluescript II-MP1). Next, a *XhoI*/*EcoRV* fragment of the murine *Sox10* cDNA encompassing the full-length open reading frame (Potterf et al., 2000) was directionally cloned into the *XhoI* and *EcoRV* sites of the MCS within pBluescript II-MP1, with the 3' end of the cDNA fragment directly upstream of the MP1 and Poly A sequences (pBluescript II-mSox10-MP1). The *Sox10* cDNA fragment used carries 278bp of sequences upstream of the methionine initiation codon and 106bp of sequences downstream of the termination codon, along with a carry-over 45bp fragment from the MCS of pcDNA3.1-mSox10 at the 5' end. A 3.68kbp minimal *Dct* promoter fragment (Budd and Jackson, 1995) was obtained by *XhoI*/*NotI* double digestion of pMG-1 vector (gift of Dr. Glenn Merlino at the National Cancer Institute) and was subcloned into pBluescript II-mSox10-MP1, directly upstream of the *Sox10* cDNA fragment (pDctp-mSox10-Mp1). For this subcloning, the *NotI* end of the promoter piece and the end of a carry-over *SalI* site in pBluescript II-mSox10-MP1 were filled in for blunt-end ligation at these sites. For generation of transgenic mice, pDct-mSox10-MP1 was digested with *PvuI* and *NotI* and the linear fragment carrying the transgenic cassette was purified and injected into fertilized FVB/N eggs. Candidate founders were tested for presence of the transgene either by Southern or by PCR amplification using the following primers: 5' primer: agcagtatggctggagcact; 3' primer: tccagtcgtagccgctgagca, corresponding to sites within the *Dct* promoter and the *Sox10* cDNA respectively (Figure 3). Lines of transgenic mice were maintained by crossing to C57BL/6J mice.

Whole Mount RNA In-situ Hybridizations

Transgene expression was analyzed by whole mount RNA in-situ analysis of E10.5 – E12.5 embryos as described (Loftus et al., 2002). Reverse-transcribed digoxigenin-conjugated probe was made from a PCR-amplified product containing the entire *Sox10* ORF region, with the T7

polymerase binding site introduced at the 3' end. (Forward Primer: ccagggtgtttggtggtgagga; Reverse Primer (contains T7 binding site): gcgggtaatacgactcactatagggcagctcagtcagggcttggcct). The probes for analysis of endogenous *Dct* and *Ednrb* expression pattern were prepared as described (Kos et al., 1999; Southard-Smith et al., 1998).

Immunohistochemistry of Neural Tube Explant Cultures

Neural tubes were dissected from E9.5 embryos and explant cultures were prepared as previously described (Hou et al., 2004a). Two-day old explant cultures were fixed for 25 minutes at RT using 4% paraformaldehyde/1 X PBS (pH 7.5), followed by permeabilization for 5 minutes in 0.1% Triton X-100. For double indirect immunolabeling, the explants were incubated with monoclonal anti-β-galactosidase antibody (Promega) and purified anti-SOX10 antibody (Mollaaghababa and Pavan, 2003). The primary antibody signals were revealed using TRITC-coupled goat anti-rabbit (Fab)2 and FITC-coupled goat anti-mouse (Fab)2 (Molecular Probes). MITF expression in explant cultures was as described (Hou et al., 2004b).

Transgenic animal crosses

$Sox10^{LacZ/+}$ and *Tg(Dct-Sox10)/+* heterozygous mice were mated, and the offspring were scored for the presence of the belly spot phenotype. All progeny were genotyped by PCR for both the $Sox10^{LacZ}$ allele and the *Dct-Sox10* transgene. For the $Sox10^{LacZ}$ allele, a modification of the published procedure (Britsch et al., 2001) was used; the presence of *lacZ* sequence was tested using the Sox10-F and Sox10-LacZ primers, while concomitantly Sox10-F and Sox10-R primers were used in a second reaction to verify the presence of the wild type *Sox10* allele. For statistical analysis, the Fisher's exact test was performed to obtain the P value for the colleted data.

For crosses involving *Ednrb* mutants, *Tg(Dct-Sox10)/+* heterozygous mice from the CF1-10 line were mated to $Ednrb^{s/s-l}$ compound heterozygous animals. Recovered *Tg(Dct-Sox10)/+ ;* $Ednrb^{s/+}$ double heterozygotes were back-crossed to $Ednrb^{s/s-l}$ animals and the severity of hypopigmentation was scored in their progeny. In a second set, hypopigmentation was scored in progeny from mating of *Tg(Dct-Sox10)/+ ;* $Ednrb^{s-l/+}$ mice with $Ednrb^{s-l/s}$ animals. *Ednrb* genotypes were assigned according to coat pigmentation phenotypes. The severity of spotting was determined by visual examination of dorsal and ventral surfaces of the mice.

Analysis of Synergistic Interactions

$Ednrb^{s-l/s}$ mutants were outcrossed with B6 to expand the line and recover $Ednrb^{s-l/+}$ individuals. $Ednrb^{s-l/+}$ and $Sox10^{Dom/+}$ mice were crossed to generate $Ednrb^{s-l/+}$, $Sox10^{Dom/+}$ animals that were backcrossed to $Ednrb^{s-l/+}$ mice to obtain pups for analysis. Pups were genotyped for both the $Ednrb^{s-l}$ and the $Sox10^{Dom}$ alleles as described (Cantrell et al., 2004). In separate crosses, $Ednrb^{s-l/s-l}$ SSLe females were mated to $Sox10^{Dom/+}$ males and offspring were genotyped for the $Sox10^{Dom}$ allele and analyzed.

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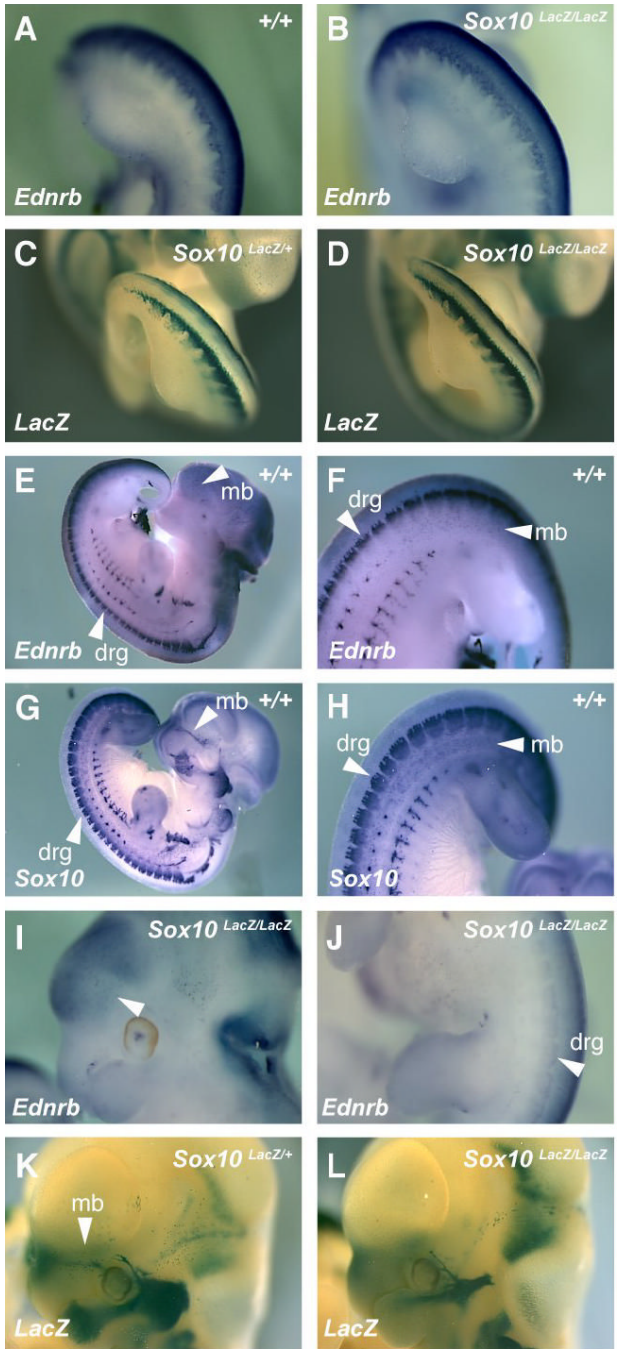

Figure 1.

Ednrb and *Sox10* have similar patterns of expression in neural crest cells at E10.5 and E11.5. Neural crest gene expression was examined by in situ hybridization for *Ednrb* (A, B, E, F, I and J), by in situ hybridization for *Sox10* (G and H), and by β-galactosidase expression for *Sox10* (C,D,K and L). Embryos were analyzed at E10.5 (A – D) and at E11.5 (E – L). Embryos were wild type (A, E–H), $Sox10^{LacZ}/+$ (C and K), or $Sox10^{LacZ/LacZ}$ (B, D, I, J and L). The expression of *Ednrb* and *Sox10* are similar in migratory neural crest at E10.5 (A versus C) and not significantly reduced in $Sox10^{LacZ/LacZ}$ embryos (A versus B). In E11.5 wild type embryos, similar expression patterns of *Ednrb* and *Sox10* are observed in the dorsal root ganglia (drg) and melanoblasts (mb) (E and F versus G and H). While *Ednrb* expression is vastly reduced

in E11.5 homozygous *Sox10*$^{LacZ/LacZ}$ embryos, punctate cells are still present dorsal to the eye (white arrow in I) and in the drg (white arrow in J). While *Sox10*-expressing cells are observed rostral to the eye at 11.5 in *Sox10*$^{LacZ/+}$ embryos (K), they are vastly reduced in *Sox10*$^{LacZ/LacZ}$ embryos (L). Genotypes of embryos are shown in upper right of each panel. For in situ hybridizations, probe used is shown in lower left of panel. Panels marked with LacZ show β-galactosidase stained embryos.

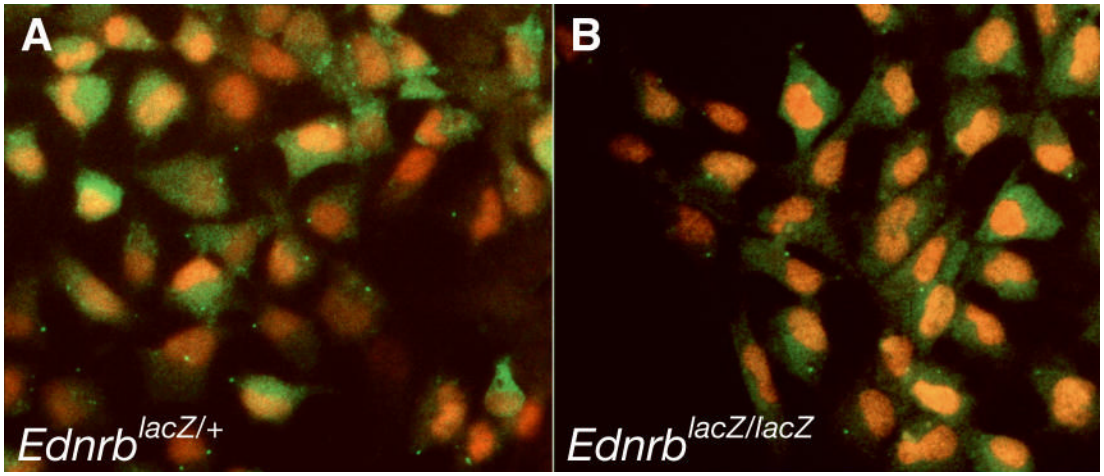

Figure 2.

Sox10 and *Ednrb* are co-expressed in cultured neural crest cells. Double staining of neural tube explant cultures with anti-SOX10 (red) and anti-β-galactosidase (green) antibodies. (A) Heterozygous $Ednrb^{LacZ/+}$ neural crest progenitors demonstrating co-expression of products from the *Sox10* and $Ednrb^{LacZ}$ genes. (B) Homozygous $Ednrb^{LacZ/LacZ}$ neural crest progenitors demonstrating continued expression of SOX10 in the absence of EDNRB.

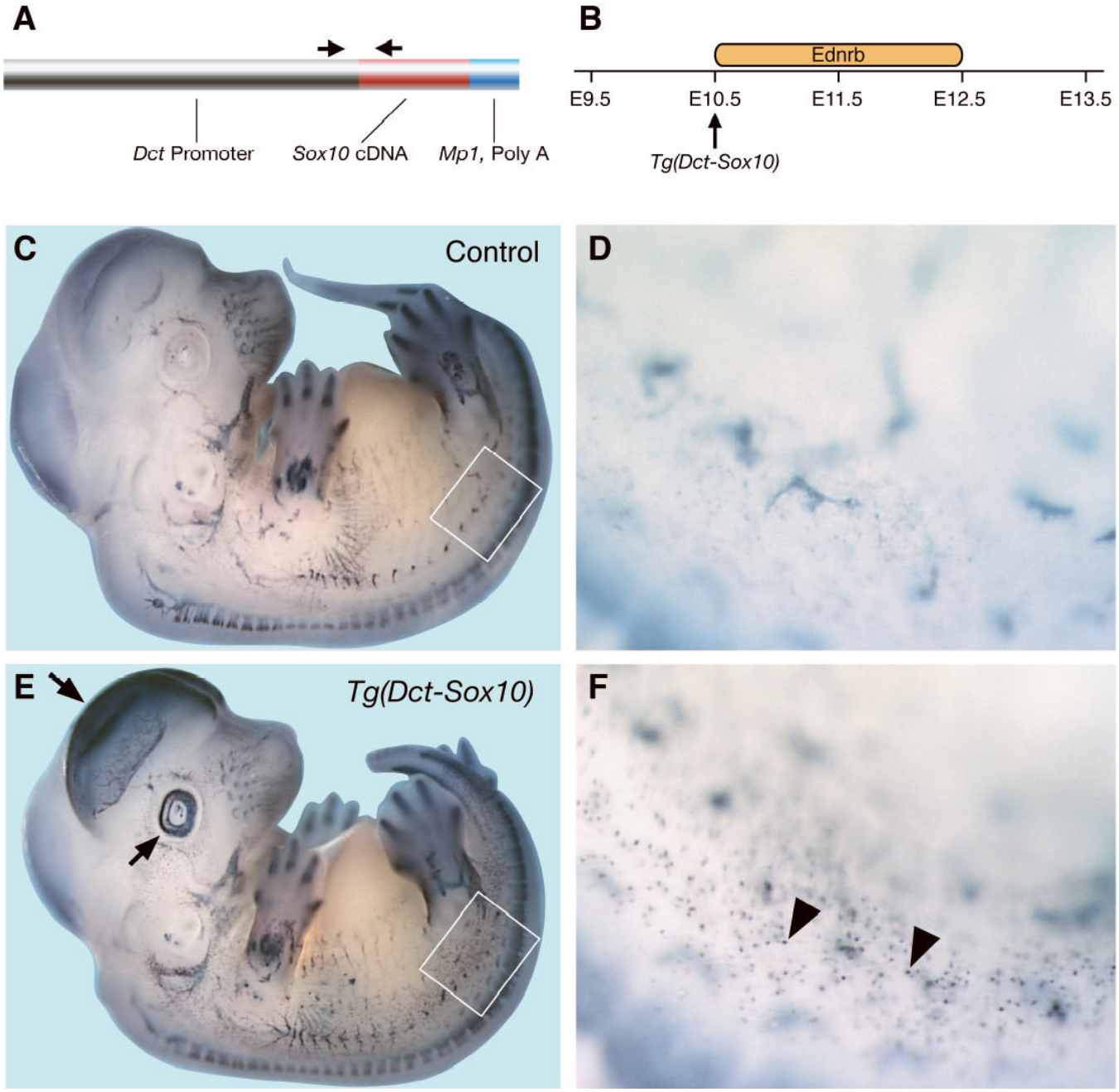

Figure 3.

The transgenic mouse line *Tg(Dct-Sox10)* overexpresses SOX10 in melanoblasts in the time frame critical for EDNRB function. (A) Schematic drawing of *Tg(Dct-Sox10)* construct. The arrows indicate locations of PCR primers used for genotyping transgenic animals. (B) Time-line for commencement of *Sox10* transgene expression during embryogenesis with respect to the time-window that is critical for *Ednrb* function. (C–F) Whole mount RNA in-situ hybridization for *Sox10* expression in non-transgenic and sibling transgenic embryos at the E11.5 stage: (C) Non-transgenic embryo (+/+); (D) Higher magnification of the area in panel C delineated by a white box; (E) Transgenic embryo (*Tg(Dct-Sox10)*/+); (F) Higher magnification of the area in panel E delineated by a white box. Arrows indicate transgene expression in the eyes and the telencephalon. Arrowheads show *Sox10* over-expression in

melanoblasts of transgenic embryos compared to non-transgenic siblings (compare panels D and F).

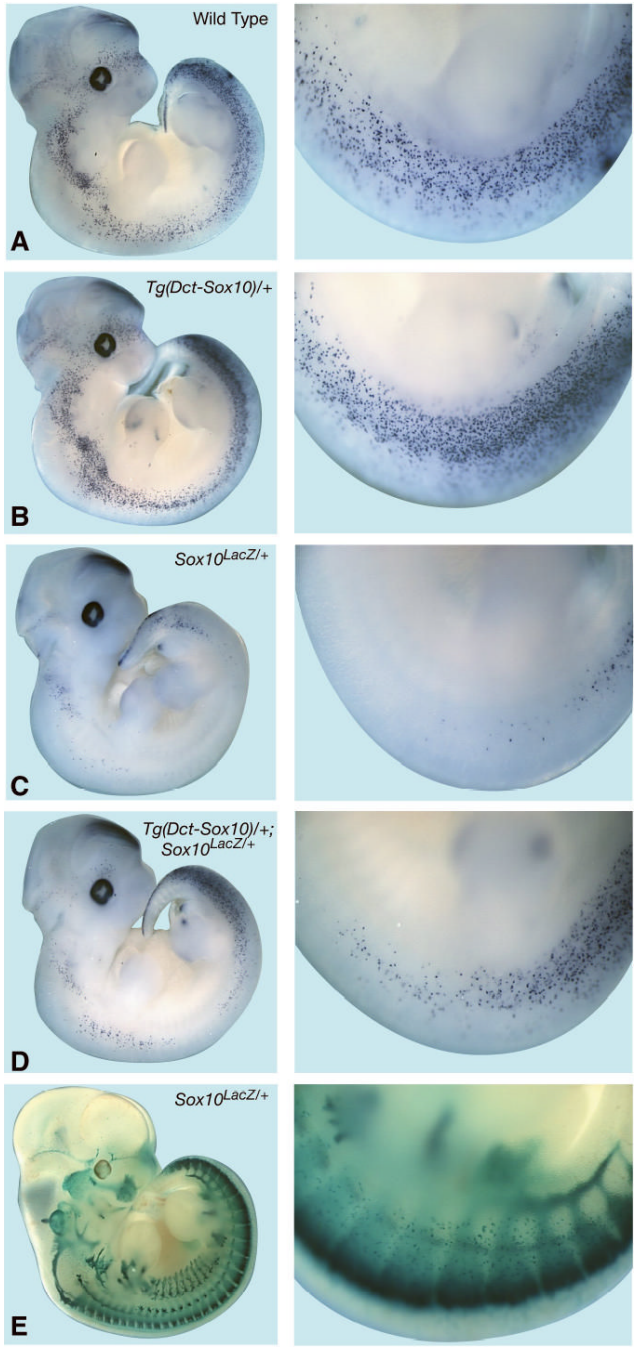

Figure 4.

SOX10 transgene rescues *Dct* expression in *Sox10*$^{LacZ/+}$ embryos. Whole mount RNA in-situ for *Dct* expression is shown for E11.5 littermate embryos of the following genotypes: (A) Wild type (non-trangenic); (B) *Tg(Dct-Sox10)/+*; (C) *Sox10*$^{LacZ/+}$; (D) *Tg(Dct-Sox10)/+ ; Sox10*$^{LacZ/+}$. (E) X-gal staining of E11.5 *Sox10*$^{LacZ/+}$ embryos to visualize the melanoblast population with reduced *Dct* expression.

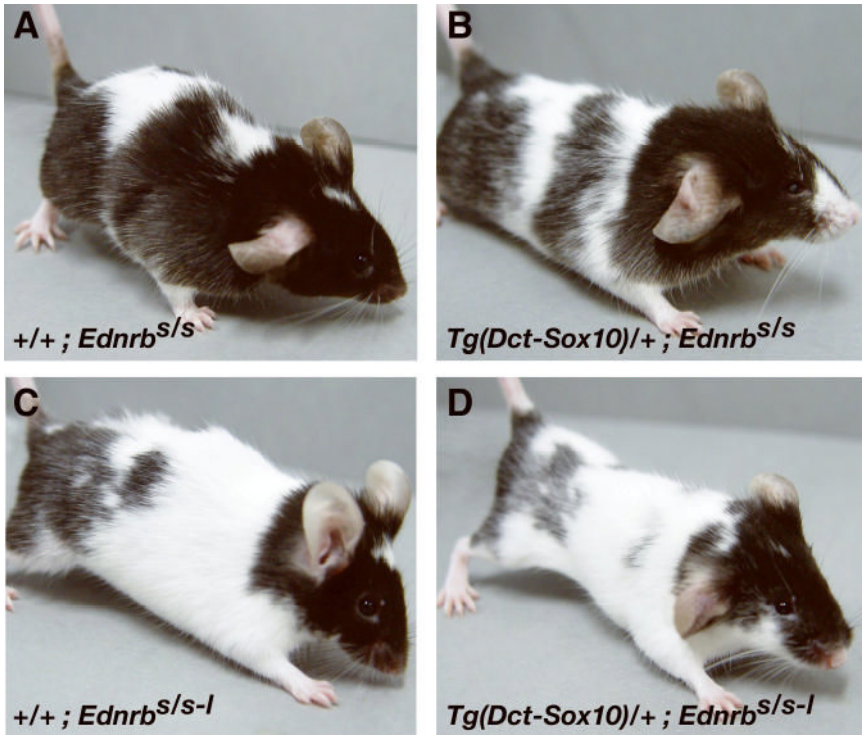

Figure 5.

Tg(Dct-Sox10) cannot rescue hypopgmentation in *Ednrb* mutant mice. Typical examples of the following genotypes are shown: (A) +/+; *Ednrb*$^{s/s}$; (B) *Tg(Dct-Sox10)*/+; *Ednrb*$^{s/s}$; (C) +/+; *Ednrb*$^{s/s–l}$; (D) *Tg(Dct-Sox10)*/+; *Ednrb*$^{s/s-l}$

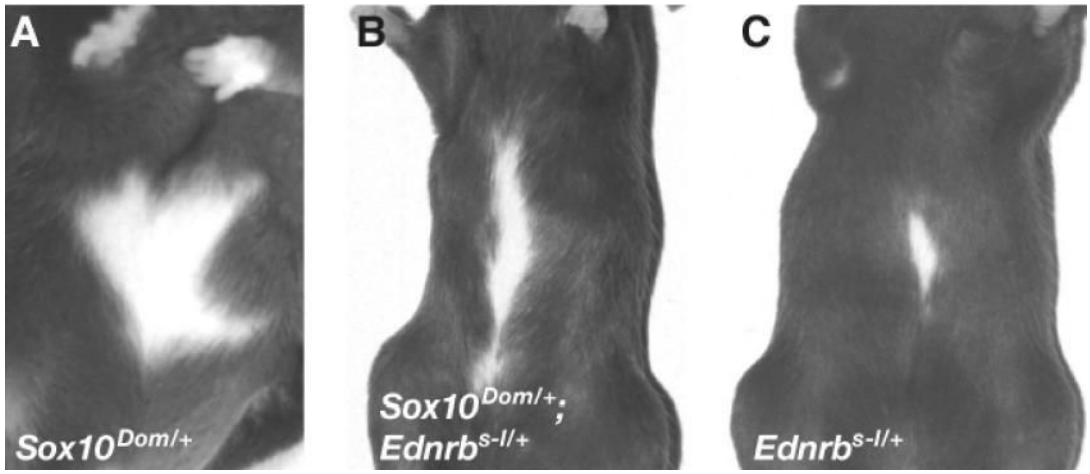

Figure 6.
Lack of synergistic hypopigmentation between *Sox10* and *Ednrb*. Ventral photographs of mice: (A) *Sox10*$^{Dom/+}$; (B) *Sox10*$^{Dom/+}$; *Ednrb*$^{s-l/+}$; (C) *Ednrb*$^{s-l/+}$

Table 1

Effects of varying SOX10 and EDNRB dosage on hypopigmentation

Genotype	No.	Severity of Hypopigmentation				
		I	II	III	IV	V
Tg(Dct-Sox10)/+$^{CF1-10}$	20	100%	0	0	0	0
Tg(Dct-Sox10)/+^{2272}	17	100%	0	0	0	0
Tg(Dct-Sox10)/+^{2245}	8	100%	0	0	0	0
Tg(Dct-Sox10)/+^{2274}	14	86%	14%	0	0	0
Sox10$^{LacZ/+}$	23	39%	61%	0	0	0
Sox10$^{LacZ/+}$; Tg $^{CF1-10}$	23	96%	4%	0	0	0
Ednrb$^{s/s}$	5	0	0	100%	0	0
Ednrb$^{s/s}$; Tg $^{CF1-10}$	6	0	0	100%	0	0
Ednrb$^{s/s-l}$	9	0	0	0	100%	0
Ednrb$^{s/s-l}$; Tg $^{CF1-10}$	6	0	0	0	100%	0
Ednrb$^{s-l/s-l}$	1	0	0	0	0	100%
Ednrb$^{s-l/s-l}$; Tg $^{CF1-10}$	2	0	0	0	0	100%
Ednrb$^{s/+}$ or *Ednrb*$^{s-l/+}$	16	44%	56%	0	0	0
Ednrb$^{s/+}$; Tg $^{CF1-10}$ or *Ednrb*$^{s-l/+}$;Tg $^{CF1-10}$	10	20%	80%	0	0	0
Ednrb$^{s-l/+}$	23	78%	22%	0	0	0
Ednrb$^{s-l/+}$; *Sox10*$^{Dom/+}$	37	16%	84%	0	0	0
Sox10$^{Dom/+}$	14	7%	93%	0	0	0

No.:Number of animals used for the analysis

I: No hypopigmentation on dorsal or ventral surfaces

II: Ventral hypopigmentation only

III: Hypopigmentation over approximately 25% of the body surface area.

IV: Hypopigmentation over approximately 50 – 70% of the body surface area.

V: Hypopigmentation over approximately 95% of the body surface area.