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Interactions between Endothelin Receptor B and Transcription Factors Sox10 and Pax3 in the Melanocyte Lineage

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INTERACTIONS BETWEEN ENDOTHELIN RECEPTOR B AND TRANSCRIPTION FACTORS SOX10 AND PAX3 IN THE MELANOCYTE LINEAGE

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To: Dean Kenneth Furton
College of Arts and Sciences

This dissertation, written by Marcia K. Lowenstein, and entitled Interactions between Endothelin receptor b and Transcription Factors Sox10 and Pax3 in the Melanocyte Lineage, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this dissertation and recommend that it be approved.

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The dissertation of Marcia K. Lowenstein is approved.

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Dean George Walker
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Florida International University, 2009

DEDICATION

To my husband, Henry, whose love and support gave me the strength to pursue my dreams,

To my children, Max, Ray, Elan and Aaron, whose love and smiling faces helped carry me through and always re-energized me,

And to my parents, Esther and Jack Kravec and Ralph and Bronia Lowenstein, whose love and confidence encouraged me to reach for the stars.

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ABSTRACT OF THE DISSERTATION

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Genetic interactions that underlie developmental processes such as cell differentiation and pattern formation are complex and difficult to elucidate. Neural Crest (NC) cells and their derivatives offer an optimal system in which to probe for these complex interactions as they acquire different cell fates and constitute a variety of structures. The transcription factors Sox10 and Pax3 as well as the transmembrane receptor Endothelin receptor b (Ednrb) are temporally and spatially co-expressed early in NC cells and mutations in these genes lead to similar hypopigmentation phenotypes due to a reduced number of NC-derived melanocyte precursors, the melanoblasts. The goal of this study was to establish whether Sox10 and Ednrb or Pax3 and Ednrb interact to promote normal murine melanocyte development. Crosses of Sox10 or Pax3 with Ednrb heterozygous mutants showed that the double heterozygous hypopigmentation phenotype was significantly more pronounced than

phenotypes of single heterozygotes, implying that a synergistic interaction exists between Sox10 and Ednrb and Pax3 and Ednrb.

This interaction was further explored by the attempt to rescue the Sox10 and Pax3 hypopigmentation phenotypes by the transgenic addition of Ednrb to melanoblasts. Pigmentation was completely restored in the Sox10 and partially restored in the Pax3 mutant mice. The comparison of the number of melanoblasts in transgenic and non-transgenic Sox10 mutant embryos showed that the transgenic rescue occurred as early as E11.5, a critical time for melanoblast population expansion. Cell survival assays indicated that the rescue was not due to an effect of the transgene on melanoblast survival.

A novel phenotype arose when studying the interaction between Ednrb and Pax3. Newborns appeared normal but by 3.5 weeks of age, the affected pups were smaller than normal littermates and developed a dome-shaped head; some also developed thoracic kyphosis. Affected pups were dead by 4 weeks of age: 80% were $Pax3^{Sp/+}$ and 75% were female. When compared to normal littermates, affected mice had brains with enlarged 4^{th} ventricles and more glia while skeletal staining showed kyphosis, wider rib cages and pelvic differences. An epistatic interaction resulting from the mixing of genetic backgrounds that is exacerbated in the presence of Pax3 heterozygosity is suspected.

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LIST OF ABBREVIATIONS AND ACRONYMS

BCIP = 5-Bromo-4-chloro-3-indolyl-phosphate
bp = base pair
BS = belly spot
C = centigrade
cDNA = complimentary DNA
DNA = deoxyribose nucleic acid
Dct = DOPAchrome tautomerase
Dct-Ednrb = transgene that delivers Ednrb to melanoblasts
der = dermis
DMSO = dimethyl sulfoxide
dNTP = deoxynucleotides
DePC = diethylpyrocarbonate
E = embryonic day
Edn3 = Endothelin3
Ednrb = Endothelin receptor b
EMT = epithelial mesenchymal transition
ep = epidermis
ET-B = avian Endothelin receptor b
EDTA = ethylene diaminetetra acetic acid
EtOH = ethanol
F1 = filial 1 = generation 1
F2 = filial 2 = generation 2
F3 = filial 3 = generation 3
g = gram
GFAP = Glial Fibrillary Acidic Protein
H_2O_2 = hydrogen perioxide
hDβH = dopamine-β-hydroxylaze
hf = hair follicle
hr = hour
LB = Luria Broth
LiCl = lithium chloride
Kitl = *Kit* ligand
mM = millimolar
M = molar
Mb = melanoblast
MeOH = methanol
min = minutes
Mitf = Microphthalmia-associated transcription factor
ml = milliliter
MSA = Migration Staging Area
n = sample size
N = generation produced by backcrossing a progeny with a parent

NaCl = Sodium Chloride
NBT = 4-Nitro blue tetrazolium chloride
NC = neural crest
NF = Neurofilament
NIH = National Institutes of Health
NS = not significant
NTMT = Sodium-Tris-Magnesium-Tween-20
P1 = Postnatal day 1
PBS = Phosphate Buffered Saline
PBT = PBS + 0.1% Tween-20
PCR = polymerase chain reaction
PF = paraformaldehyde
PNS = peripheral nervous system
RT = room temperature
S = seconds
SDS = Sodium Dodecyl Sulfate
SSC = Sodium chloride, Sodium citrate, Citric acid
Taq = thermostable polymerase named after *Thermus aquaticus*
TAE = Tris Acetate EDTA
TBE = Tris Borate EDTA
TBS = Tris buffered saline
TBST = TBS + 0.1% Tween-20
TE = Tris EDTA
Tg = transgene
Trp-1 = Tyrosinase protein related-1
Trp-2 = Tyrosinase protein related-2; Dct
µg = microgram
µl = microliter
µM = micromolar
WS = Waardenburg syndrome
WT = wildtype

Chapter I.

INTRODUCTION

I. INTRODUCTION

From fertilization to birth, cells undergo a series of changes that results in the transformation of nonspecific cells into a complex living organism that has all the necessary structures in the correct location time and time again. The transformation of cells that look identical into specialized cells with specific functions is the developmental process known as differentiation. As cells differentiate they must also receive signals that dictate the structure into which they will be incorporated; for instance keratinocytes, melanocytes, and fibroblasts need spatial and temporal orientation to become functional skin. These questions of differentiation and pattern formation can be elucidated by studying the genetic networks that control these processes. In vertebrates, the neural crest has served as a good model to study these questions.

1. Neural Crest

The neural crest (NC) is an ideal cell type in which to study cell fate and specification. Unique to vertebrates, NC cells arise in the developing neural tube and migrate to the far reaches of the developing organism, all the while differentiating in accordance to environmental signals they encounter along the way. Arising from the epithelium and undergoing mesenchymal transition (Duband et al., 1995), NC cells emigrate from the dorsal aspect of the neural tube. Depending on their migratory path these multipotent progenitor cells differentiate into a variety of cells (Figure 1), including melanocytes, Schwann cells, satellite cells, enteric glia, neurons and facial cartilage and bones (Anderson, 1989; LeDouarin and Kalcheim, 1999). The diversity of structures

arising from the NC has long intrigued developmental biologists; the central question being, how does a group of cells that appears homogeneous give rise to such diverse structures within the organism (Dupin et al., 2001; Gammill and Bronner-Fraser, 2003; Shah et al., 1994)?

Development of the NC by protochordate ancestors is considered a major evolutionary event in vertebrates and thus appears to be conserved in species ranging from zebrafish to humans (Gammill and Bronner-Fraser, 2003; Gans and Northcutt, 1983), enabling us to study NC cells and their gene networks across species. Specifically the generation of NC cells begins as the ectoderm separates into the neural ectoderm (also known as the neural plate which gives rise to the central nervous system) and the non-neural ectoderm (which will give rise to the epidermis); the border between these two areas will give rise to NC cells (Gammill and Bronner-Fraser, 2003). As neurulation proceeds, a subset of these border cells will begin their emigration (Selleck and Bronner-Fraser, 1995; Serbedzija et al., 1994) and subsequently populate the entire length of the embryo. This process is exhaustive and once NC cells emerge and migrate, this border known as the neural crest ceases to exist.

Early NC markers include *Snail* (for mice and fish; Slug for birds) (Locascio et al., 2002), *Foxd3* (Kos et al., 2001) and Group E Sox family members *Sox9* (Mori-Akiyama et al., 2003) and *Sox10* (Southard-Smith et al., 1998). *Snail/Slug* have been shown in cultured cells to be important for the epithelial-mesenchymal transition (Cano et al., 2000) and *in vivo* for the proper specification (Aybar et al., 2003; LaBonne and Bronner-Fraser, 2000) and

migration (LaBonne and Bronner-Fraser, 2000; Nieto et al., 1994) of NC progenitor cells. *Foxd3* is essential for proper differentiation of NC cells and appears to act in concert with *Snail/Slug* on downstream targets necessary for neural crest determination (Sasai et al., 2001).

Neural crest cells respond to both cell autonomous and external signals based on spatiotemporal and developmental sensitivities (Le Douarin et al., 2004; Paratore et al., 2002b; Shah and Anderson, 1997). Often referred to as neural crest stem cells (NCSCs), many lineages appear to maintain a stem-like quality throughout life (Dupin et al., 2000; Kim et al., 2003). Stem cells are defined as those cells that remain undifferentiated and multipotent throughout their lives, and generate progeny that can either remain multipotent or have a bias towards terminal differentiation depending on external cues. While embryonic stem cells remain totipotent, many other types of stem cells are partially committed, probably due to the niche they inhabit; in turn, NCSCs give rise only to neural crest derivatives (Le Douarin et al., 2004; Morrison et al., 1997; Sommer, 2001).

In addition to the effects of intrinsic and extrinsic signals, terminal differentiation into specific tissues and structures also depends on the axial level from which the NC cells originate (Figure 2). Cranial NC cells migrate into the branchial arches and the face to form bones and cartilage of the face and neck, cranial nerves and some pigment cells. The vagal and sacral NC cells migrate from different regions in the embryo but both are instrumental in innervating the gastrointestinal tract of vertebrates. The cardiac NC contributes

to, among other things, the musculoconnective tissue of large arteries of the heart and the septum that permits separation of the aorta and pulmonary artery (Le Douarin, 1982; Le Lievre and Le Douarin, 1975). Avian trunk NC cells migrate in two waves, the first wave migrating ventromedially and ultimately forming the sympathetic nervous system, glia of the peripheral nervous system (PNS) and the dorsal root ganglia while the second wave migrates dorsolaterally and is comprised of melanocyte precursors known as melanoblasts (Henion and Weston, 1997; Le Douarin and Teillet, 1974). Murine trunk NC cells also migrate in a temporally specified manner but vary from avian NC migration in that murine NC cells are seen in the dorsolateral pathway throughout the migratory period and it is those cells that will travel via the ventromedial and ventrolateral pathways that are temporally different (Serbedzija et al., 1994; Serbedzija et al., 1990). As with the avian NC, all NC cells that migrate via the dorsolateral pathway will become melanocytes while cells migrating via ventromedial and ventrolateral pathways will become glia of the PNS and the dorsal root ganglia as well as several other derivatives (LeDouarin and Kalcheim, 1999). The spatial-temporal pattern of NC cell migration may be indicative of some level of predetermination of cell lineage or may help restrict developmental potential as a consequence of timing and migratory path.

2. Melanocyte Development

The journey from NC cell to a fully mature pigmented melanocyte begins with the induction of the neural tube and the emergence of the NC. Cell lineage

experiments show that at approximately E8.5, the first wave of murine NC cells begin their emigration through the dorsolateral and ventromedial pathways; migration down the ventrolateral pathway begins shortly afterwards (Serbedzija et al., 1994). By E11.5, melanoblasts have populated all regions of the murine embryo (Figure 3); as the embryo continues to develop, the melanoblasts cross the basement membrane that divides the dermis from the epidermis and most murine melanoblasts will subsequently take up residence in the hair follicle by E16.5 while the others remain in contact with the basement membrane on the epidermal side (Mayer, 1973). Once in the hair follicle, some melanoblasts will settle into a stem cell niche and remain there undifferentiated until the proper signaling pathway stimulates differentiation (Ohlstein et al., 2004). Other cells will continue differentiating and will begin the process of producing melanin shortly after birth. In addition to populating the hair follicle and the epidermis, melanocytes also provide pigment cells to the stria vascularis of the inner ear, the choroid of the eye, the Harderian gland and the atrioventricular valves of the heart (Brito and Kos, 2008; LeDouarin and Kalcheim, 1999).

3. Pigmentation Mutants

Mouse pigmentation mutants have been a source of fascination and interest to people over the ages. In fact, ancient Chinese documents reference mouse pigmentation mutants that include albino and yellow mice. In the 1700s, mice were popular pets in Europe, China and Japan due to the ease of care and their many variations of coat color. Once Mendel's laws of inheritance were rediscovered in the early 1900's, it was quickly recognized that mice provided a

situation similar to that of the peas – that genetics could be studied using coat color variation in mice. In this case however the model organism is a mammal and shares 95% genome homology with humans. In addition, the mouse genome has been extensively characterized and is easily manipulated thereby making the mouse an excellent candidate in which to study genetic interactions and disease (Jackson Laboratory, 2009; National Human Genome Research Institute, 2002).

Given that the NC is so important that it has been deemed the 4th germ layer, melanocyte development – or the lack of – has afforded the opportunity to study the dynamics and specifics of NC specification and differentiation using pigmentation models to track NC cell development. Spontaneous pigmentation mutants such as *Piebald lethal*, *Dom* and *Splotch* have proven to be valuable models for understanding gene regulatory networks involved in the development of NC cells.

The *piebald lethal* mutation (*Ednrb*$^{S\text{-}l}$) spontaneously arose at Jackson Laboratory in April of 1959 in a second generation cross between a C3H/HeJ female mouse with a head blaze and a bellyspot and a wildtype C57BL/6J male. Almost immediately, it was recognized that this mutation was a recessive allele as most pups were born black with white belly spots while several in each litter were born white with black eyes and a black spot or two around the ears and occasionally a black spot at the base of the tail. In addition to the difference in coat color, the white pups typically died within the neonatal period of a deficiency of myenteric ganglion cells (a decrease in the innervation of the

lower intestinal tract). The mutant named *lethal spotting* also exhibited a hypopigmentation phenotype, although less severe than *piebald lethal*, and the same gastrointestinal lethality (Lane, 1966). Of interest is that the pigment cells (melanocytes) and the enteric neurons, which are responsible for proper innervation of the gastrointestinal tract, are both derived from NC cells (Le Douarin and Kalcheim, 1999).

The gene affected in *piebald lethal* mice encodes *Endothelin receptor b (Ednrb*), a G-coupled seven transmembrane receptor (Hosoda et al., 1994), whose human homolog, *EDNRB*, is mutated in approximately 5% of cases of Hirschsprung disease (Chakravarti, 1996; Puffenberger et al., 1994). The *Ednrb* mutation has also been identified at the *spotting lethal* locus in the rat (Ceccherini et al., 1995; Gariepy et al., 1996; Shin et al., 1997) and in horses with the lethal white foal syndrome (Santschi et al., 1998).

The murine *lethal spotting* locus ($Edn3^{ls}$) encodes the *Ednrb* ligand, *Endothelin 3* (*Edn3*) (Baynash et al., 1994). Endothelin 3 is a member of a group of three related peptides, Edn1, Edn2 and Edn3, each encoded by a separate gene. The peptides are produced as inactive preproendothelins that undergo successive proteolytic cleavages to generate the 22 amino acid active endothelins.

The developmental expression patterns of *Ednrb* and *Edn3* have been analyzed in avian and murine embryos. These studies showed that *Edn3* is expressed in the environment through which NC cells migrate while *Ednrb* is expressed by the NC cells themselves (Hou et al., 2004; Nataf et al., 1998;

Nataf et al., 1996). Avian cells giving rise to melanocytes, however, express not Ednrb but a closely related receptor, *Ednrb2* (Lecoin et al., 1998; Stone et al., 1997).

In mice homozygous for *Ednrb*, there is a drastic reduction in the number of melanocyte precursors from the time they can be detected by the expression of the DOPAchrome tautomerase marker (*Dct*, also referred to as tyrosinase related protein-2, *Trp2*). *DOPAchrome tautomerase* is first detected at E10.5 as melanocyte precursors have already undertaken the dorsolateral pathway. This suggested that the expression of functional *Ednrb* is important for the proper migration of melanocytes before or just before E10.5 (Pavan and Tilghman, 1994). *Ednrb* was then expressed at different stages of embryogenesis under the control of the tetracycline inducible system and confirmed that Ednrb expression is required from E10.5-12.5 (Shin et al., 1999). Broad expression of *Ednrb* is seen throughout the developing embryo; the Ednrb/Edn3 signaling pathway appears to be critically important at E10.5-12.5 for the migration of melanoblasts and enteric neuron precursors (Lee et al., 2003; Shin et al., 1999).

In vitro studies in the quail showed that *Edn3* markedly increases the proliferation of pluripotent NC cells and eventually stimulates dramatic increases in the number of melanocytes (Lahav et al., 1996; Stone et al., 1997). In primary mouse NC cultures, *Edn3* also increases the number of melanocyte precursors (Reid et al., 1996). These observations are consistent with the genetic data of *Ednrb* mutations and the previous demonstration that

melanocyte precursors in vitro have binding sites for radio labeled *Edn1*, which binds to *Ednrb* (as well as to a second murine *Endothelin* receptor – *EdnrA*) (Reid et al., 1996).

In an attempt to rescue the mutant phenotype (spotting and enteric aganglionosis) of the *lethal spotting* rat in which functional expression of *Ednrb* is abolished, a transgenic rat expressing *Ednrb* under the control of the human dopamine-β-hydroxylase (hDβH) promoter was created (Gariepy et al., 1998). Rescue of the enteric aganglionosis phenotype was obtained in the progeny resulting from the cross of the *spotting lethal* rat and the hDβH-*Ednrb* transgenic rat. However, the pigmentation phenotype was not rescued. This partial rescue can be attributed to the fact that in the rat, the hDβH promoter directs expression only to a subset of central nervous system and PNS precursor cells, not to melanocytes or their precursors. Expression is driven in vagal NC cells (precursors of enteric neurons) from the stage at which they begin to colonize the foregut but not during their migratory steps (Kapur et al., 1991). The hDβH-*Ednrb* transgene's ability to prevent enteric aganglionosis shows that the critical time for the action of *Ednrb* during the development of enteric neurons occurs after the divergence from the melanocytic lineage and after they have reached the gut.

The *Dominant megacolon* (*Sox10*^{Dom}) mutant also arose spontaneously in a hybrid stock carrying bouncy (B6C3Fe *a*/*a-bc*) at N12 in 1979. Heterozygous animals are characterized by variable white spotting and aganglionic megacolon. Homozygous animals are embryonic lethal and die around E13.5

depending on the genetic background. These phenotypes result from the synthesis of a C-terminus truncated Sox10 (Sry-like high mobility box 10) protein due to a point mutation that introduces a premature termination codon in the *Sox10* gene (Kuhlbrodt et al.,1998; Southard-Smith et al., 1998). *Sox10* belongs to a class of transcription factors of the high mobility group (HMG) showing HMG domain homology to the testis-determining factor SRY. It has been proposed that the truncated *Sox10* allele may exert its phenotypic effect by acting in a dominant negative manner (Potterf et al., 2000).

Sox10 has been implicated in regulation of cell survival, proliferation and cell specification (Schepers et al., 2002; Wegner, 1999). The heterozygous mutation in humans results in neurocristopathies, the best known being Waardenburg syndrome type II or type IV (Bondurand et al., 2007; Herbarth et al., 1998; Paratore et al., 2002a; Pusch et al., 1998). A mouse mutant was created in which *LacZ* was inserted into the *Sox10* locus. The mutant displays the same phenotype as Sox^{Dom} (Figure 5); apart from being important in the development of melanocytes and enteric ganglia, *Sox10* is also critical for the proper differentiation of Schwann cells (Britsch et al., 2001) In addition, it has been reported that *Sox10* deficient mice lack an adrenal medulla as a result of inappropriate specification of NC cells at the onset of migration and subsequent cell death (Reiprich et al., 2008).

Sox proteins are found in all tissues and cells that have been analyzed thus far, and not surprisingly, the individual Sox proteins have very precise species-specific patterns and locations (Carney et al., 2006; Hou et al., 2006; Wegner,

1999). An additional feature of Sox proteins is that, depending on the cellular context, they partner exclusively with other proteins in order to activate downstream targets, as in the case of *Sox10* and the transcription factor *Pax3* for downstream activation of *Mitf*, a master melanocyte regulatory transcription factor (Bondurand et al., 2000; Kuhlbrodt et al., 1998). In the case of *Sox10* and *Pax3*, activation of *Mitf* varies from 500-fold with *Sox10* to 10-fold with *Pax3* to >1500-fold with *Sox10* and *Pax3* (Bondurand et al., 2000).

Cell survival, proliferation and specification are the first roles played by *Sox10* in the melanocyte lineage (Lee et al., 2000; Mollaaghababa and Pavan, 2003). Neural crest cells initially appear normal in the absence of *Sox10* and even begin migration; it is shortly after, in the post-migratory NC population that abnormalities begin: cells become apoptotic and homozygous embryos start dying shortly afterward (Kapur, 1999; Southard-Smith et al., 1998). During specification of melanocytes, *Sox10* has been shown to activate *Mitf*, and together they activate *DOPAchrome tautomerase* (*Dct*). *Dct* functions in the biosynthetic pathway of eumelanin (black pigment), initiating melanogenesis (Ludwig et al., 2004; Tsukamoto et al., 1992).

Splotch (*Pax3*^{sp}) is another spontaneous mouse mutant that displays a spotting phenotype. Splotch is a point mutation within intron 3 of the paired homeobox 3 (*Pax3*) gene that partners with *Sox10*. The mutation interferes with normal splicing of intron 3 and results in 4 aberrantly spliced mRNAs with exon 4 deleted. The *Pax3*^{Sp} mutation also disrupts the homodimerization of the protein, a function associated with the octapeptide-encoding central segment of

the gene (Epstein et al., 1993) that partners with *Sox10*. *Pax3* is a member of the paired class homeodomain family of transcription factors and first appears in the murine embryo at E8.5 precisely at the dorsal part of the neural tube just before closure (Goulding et al., 1991). It is subsequently found in the developing brain, the dermomyotome of the developing somites, in the limb buds and some craniofacial structures. Mice heterozygous for *Pax3* mutations display belly spotting, with occasional dorsal spotting on the back or tail. *Pax3*, like *Sox10*, is essential for life and homozygous mutants fail to develop many structures determined by the NC and are embryonic lethal by E13.5. Homozygous embryos display limb abnormalities, exencephaly, heart defects, open neural tube (spina bifida) and abnormal tail morphology. In humans, heterozygous loss of function *PAX3* mutations cause Waardenburg Syndrome I and III (WSI,III). WSI phenotypes include abnormal placement of the inner canthus of the eye resulting in a wide nasal bridge formation, skin hypopigmentation, heterochromia iridis and deafness. WSIII phenotypes include skeletal abnormalities and cardiopulmonary defects together with the phenotypes observed in WSI.

Pax3 is crucial for survival of melanocyte progenitors until they reach the migrating staging area (MSA; in chick) where they stay for 24 hours before continuing their migration (Hornyak et al., 2001). Required for the expansion of the progenitor population, one of the most interesting of *Pax3*'s functions is its promotion of melanocyte development while simultaneously inhibiting terminal differentiation. This is accomplished by *Pax3* activating *Mitf* while competing

with *Mitf* for binding sites on the *Dct* promoter (Corry and Underhill, 2005; Hornyak et al., 2001; Lang et al., 2005).

4. Genes Governing Murine Melanocyte Development

The identification of the genes mutated in $Ednrb^{S-l}$, $Sox10^{Dom}$, $Pax3^{Sp}$ as well as other spontaneous pigmentation mutants including *Dominant white* (Kit^{w-v}), *Slaty* (Dct^{slt}), *Silver* ($Pmel17^{si}$), Belted ($Adamts20^{bt-2J}$) coupled to genetics and developmental biology studies on insertional and targeted mutants has allowed for the delineation of the molecular sequence of events involved in the process of skin melanocyte development (Figure 6). Pre-migratory NC cells express transcription factors such as *Snail/Slug*, *Sox10* and *Pax3* that do not impose a particular fate until certain signaling molecules change the patterns of gene expression (Bondurand et al., 2000; Locascio et al., 2002). It appears that the members of the *Wnt* family, *Wnt1* and *Wnt3*, are required for these cells to commit to the melanocytic lineage (Bronner-Fraser, 2004; Dunn et al., 2000; Hari et al., 2002). The *Wnt* pathway transcriptionally regulates the expression of *Mitf* by the activation of *β-catenin* and *Lef1* (Takeda et al., 2000). At this point, those NC cells that left the neural tube and took the dorsolateral pathway have undergone their first commitment step to becoming a melanoblast. The *Kitl/Kit* pathway is required for the proliferation and survival of these cells by activating Mitf at the protein level (Nishikawa et al., 1991; Opdecamp et al., 1997; Yoshida et al., 1996). During this first commitment step, *Sox10* and *Mitf* cooperatively activate the expression of *dopachrome tautomerase* (*Dct*) (Jiao et al., 2004; Ludwig et al., 2004; Potterf et al., 2000).

Dct is a melanogenic enzyme and it is not clear why it is expressed at this early stage since melanin is only produced at the end of the process of differentiation, about six days later. Another specification marker expressed at this time is the melanosomal protein *Pmel17* (Baxter and Pavan, 2003). *Kitl/Kit* signaling is no longer required, and the *Edn3/Ednrb* pathway takes over controlling the expansion of the committed melanoblast population and, most likely, allowing these cells to migrate further and reach the prospective skin (Shin et al., 1999; Yoshida et al., 1996). *Edn3/Ednrb* signaling regulates the expression of *Mitf* both at the transcriptional and translational level, guaranteeing melanoblast survival and regulation of the melanogenic proteins Dct, Trp1, and Pmel17 (Sato-Jin et al., 2008). While traveling along the dermis, extracellular matrix proteins and metaloproteases such as *Adamts20*, are not only required for their proper migration but also for their survival (Silver et al., 2008). As melanoblasts cross the dermal/epidermal junction they undergo final differentiation by expressing tyrosinase, the rate limiting enzyme for melanin production. While *Edn3/Ednrb* signaling is not necessary for those melanoblasts that have reached the epidermis, it is still capable of regulating the expression of *tyrosinase* both transcriptionally and translationally (Sato-Jin et al., 2008). In the epidermis, *Kitl/Kit* signaling becomes critical one more time allowing for the survival of fully committed melanocytes (Yoshida et al., 1996). Those melanocytes that reach the hair follicles remain, and some de-differentiate to become the melanocyte stem cells that generate new transition

cells at each hair cycle stage. No differentiated melanocytes remain in the epidermis.

5. Genetic Backgrounds and Modifiers

Epistatic relationships between genes is a concept that dates back more than half a century (Haldane, 1941). More recently, definitions describing this concept have emerged with the majority opinion converging on the explanation that a modifier is a gene that alters the expression of another gene, typically at another loci (Futuyma, 1998; Suzuki et al., 2004). As the complexities of genetic interactions come into focus, there is more and more interest from both scientists and medical clinicians to investigate modifier genes: from the standpoint of understanding underlying genetic networks to being able to treat both monogenic and multigenic diseases that present with varying levels of expressivity and penetrance in human patients. An example of a disease state in which gene expression and penetrance of severity can vary between families but also within families is Waardenburg Syndrome (WS). Waardenburg syndrome is a neurocristopathy (disease of the NC) in which one of several melanogenic genes are responsible for the majority of the cases in which the genetics have been elucidated. Mutations in the genes *SOX10*, *PAX3*, *MITF*, *SNAI2*, *EDN3* and *EDNRB* have been identified in variant forms of WS and associated with particular signs and symptoms which can include white forelock, cochlear deafness, craniofacial anomalies, aganglionosis and skeletal deformities of the upper limbs (Bondurand et al., 2007; Puffenberger et al., 1994; Sanchez-Martin et al., 2002; Tassabehji et al., 1994; Tassabehji et al.,

1992). Fortuitously, mice carrying similar mutations of the aforementioned genes are good models for the study of WS in humans (Tachibana et al., 2003).

Studying modifier genes in WS or other diseases turns out to be complicated and results are difficult to interpret due to the multitude of unknown factors that may potentially affect any aspect of gene expression. Not only is the type of mutation important, but expressivity and penetrance may also be influenced by the position of the mutation (i.e., which exon carries the mutation) or whether the mutation is in a cis or trans position. In addition, mutations of the same gene may have pleiotropic effects on phenotypic expression (Genin et al., 2008; Goldfarb et al., 1992; Gouya et al., 2006).

One study that looked at epidermal growth factor receptor (*Egfr*^{tm1Mag}) during embryonic development identified that the timing of the interaction was dictated by the background in which the mouse was bred and that these background strains significantly influenced the timing of homozygous lethality (Strunk et al., 2004). Depending on the background, the critical time for epistatic influence on the known genetic mutation varied in distinct windows of time: from E4.5-9.5, E10.5-13.5 and E14.5-18.5. Over 15 different in-bred background strains were analyzed but of particular interest to the current study were that embryos on a C57BL/6J background died in mid-gestation while those embryos on a C3H background died in late gestation. Strunk and colleagues (2004) concluded that modifiers were present in all backgrounds yet function varied according to varying temporal programs. And while the *Egfr* homozygous condition was consistently embryonic lethal in all background strains, the study provided a

view into the temporal window of epistatic connections and that what may appear as stochastic in nature is not.

In another study, a mutation that interferes with cardiac formation actually proceeded to different stages of development depending on the background strain of the mouse (Astrof et al., 2007). Mice with the *FN*-null mutation (absence of fibronectin) die very early during embryonic development; embryos on the 129S4 background die of cardia bifida while those embryos on a C57BL/6J background develop a central looped heart and die at a later embryonic age. To identify novel gene candidates for modifiers of cardiac development, F2 intercrosses between 129S4 and C57BL/6J strains were used for genetic mapping and haplotype analysis. These analyses revealed a 1Mbp interval on mouse chromosome 4 that contains a modifier of cardiogenesis in *FN*-null mice. Microarray analysis on wildtype, heterozygous and homozygous *FN* embryos identified 21 candidate genes, 5 of which had differential expression between the two background strains.

Neural crest development appears to be influenced by modifiers in background strains as well. As previously discussed, NC progenitors destined for the enteric nervous system are modulated by the interaction between *Sox10* and *Ednrb*; their mutations also appear to be modulated by modifiers present in the C57BL/6J and C3H backgrounds. The C57BL/6J background aggravated the aganglionosis phenotype and decreased hypopigmentation while the C3H background produced the opposite effects. In fact, the effects on aganglionosis penetrance were so significant that only 15% of pups on the C57BL/6J

background survived compared to 78% of pups on the C3H background (Cantrell et al., 2004).

Another set of intrasubspecific crosses of *Ednrb*^{s} mice (Mayer *Ednrb*^{s} /*Ednrb*^{s} and C3HeB/FeJ *Ednrb*^{s}/*Ednrb*^{s}) has identified *mast cell growth factor* (*MGF*, now known as *Kitl*) as a modifier for the patterning of hypopigmentation displayed by *Ednrb* mutants (Rhim et al., 2000) This study suggested that alterations of *Kitl* expression in C3H mice may be responsible for the appearance of white forelock hypopigmentation phenotype characteristic of Waardenburg syndrome. Recent studies employing crosses between *Ednrb* and *Kit* mutant as well as the transgenic addition of *Edn3* to *Kit* mutants have corroborated the existence of a synergistic interaction between the *endothelin* and *Kitl* and signaling pathways in melanocyte development (Aoki et al., 2005; Garcia et al., 2008)

A mutagenesis screen to expose modifiers responsible for the difference in severity in Sox10 heterozygous WS identified 3 modifiers of *Sox10*, one of which is on the *GLI-Kruppel family member 3* (*Gli3*; a component of the hedgehog signaling pathway) which exacerbates the pigmentation defect of *Sox10* heterozygosity (Matera et al., 2008). Finally, a detailed analysis of the expression of various melanogenic genes and melanocyte viability on different in-bred background strains revealed different numbers of melanoblasts among the 8 parts of the embryo according to background strain (Loftus et al., 2009).

6. Research questions

Investigations into whether *Ednrb*/*Edn3*, *Sox10* and *Pax3* interact genetically sprung from their spatiotemporal patterns of expression in early embryogenesis. Based on these patterns of co-expression and similarity of phenotypes in mutants, it is likely that *Sox10* and/or *Pax3* interact with *Ednrb* and possibly regulate its expression. Interactions between *Sox10* and *Ednrb* have been shown to modulate the development of enteric neurons (Cantrell et al., 2004; Zhu et al., 2004), and an *Ednrb* enhancer that is activated specifically in enteric neuron precursors has been identified that contains multiple binding sites for *Sox10* (Zhu et al., 2004). Recently, in human melanocytes, *Sox10* has been shown to directly regulate *Ednrb* at the transcriptional level through two different promoters (Yokoyama et al., 2006). Given that *Sox10* and *Pax3* transactivate downstream targets in melanocyte development and that both transcription factors are essential for survival and/or proliferation of melanocyte progenitors, my research questions directly addressed whether or not these transcription factors interact with *Ednrb* in the melanocyte lineage.

Question #1: Does *Sox10* interact with *Ednrb* at the genetic level?

The analysis of the phenotype of *Ednrb*/*Sox10* compound heterozygote mouse mutants will allow us to establish whether *Sox10* interacts with *Ednrb* in an additive, diminishing or synergistic manner. Crosses between $Ednrb^{S\text{-}l/+}$ and $Sox10^{LacZ/+}$ mice over 3 generations were examined and hypopigmentation of resulting single mutants were compared to the hypopigmentation of double

mutants. An aggravated phenotype beyond the additive expectation will imply a genetic interaction.

Question #2: Does *Pax3* interact with *Ednrb* at the genetic level?

The analysis of the phenotype of *Ednrb*/*Pax3* compound heterozygote mouse mutants allowed us to establish whether *Pax3* interacts with *Ednrb* in an additive, diminishing or synergistic manner. Crosses between $Ednrb^{S\text{-}l/+}$ and $Pax3^{Sp/+}$ mice over 3 generations were examined and hypopigmentation of resulting single mutants compared to the hypopigmentation of double mutants. An aggravated phenotype beyond the additive expectation will imply a genetic interaction.

Question #3: Can the transgenic delivery of *Ednrb* to *Sox10* haploinsufficient melanocyte progenitor cells rescue the hypopigmentation phenotype of *Sox10* mutant mice?

$Sox10^{LacZ/+}$ mice were crossed with transgenic mice that express *Ednrb* under the control of the *Dct* promoter (Ittah, Ph.D. dissertation, Florida International Univ., 2006) and any differences in pigmentation between the *Sox10* heterozygote and the *Sox10* heterozygote with the transgene were evaluated. A normal coat color will imply that the additional *Ednrb* supplied early on in development was sufficient to rescue the emerging melanoblasts and restore coat color.

Question #4: Can the transgenic delivery of *Ednrb* to *Pax3* haploinsufficient melanocyte progenitor cells rescue the hypopigmentation phenotype?

$Pax3^{Sp/+}$ mice were crossed with transgenic Dct-*Ednrb* mice to evaluate any differences in pigmentation between the *Sox10* heterozygote and the *Sox10*

heterozygote with the transgene. A normal coat color will imply that the additional *Ednrb* supplied early on in development was sufficient to rescue the emerging melanoblasts and restore coat color.

Question #5: How early can the transgenic delivery of *Ednrb* to the *Sox10* heterozygote mutant rescue its spotting phenotype?

Sox10$^{LacZ/+}$ mice were crossed with the transgenic Dct-*Ednrb* mice. Thus, *Sox10* heterozygous mice carrying the transgene expressed additional levels of *Ednrb* in their melanocyte precursors. Embryos from the cross *Sox10*$^{LacZ/+}$ and Dct-*Ednrb* were harvested at E11.5 and analyzed via in situ hybridization using the *Pmel17* riboprobe. If a qualitative difference emerges in the number of melanocyte precursors between *Sox10* heterozygotes with and without the transgene, it will imply that the transgene is acting prior to E11.5 and is involved in the expansion of the melanocyte precursor population.

Question #6: Does the transgenic delivery of *Ednrb* rescue melanoblasts in the *Sox10* heterozygote from an untimely death by apoptosis?

Sox10$^{LacZ/+}$ mice were crossed with the transgenic Dct-*Ednrb* mice. Thus, *Sox10* heterozygous mice carrying the transgene expressed additional levels of *Ednrb* in their melanocyte precursors. Embryos from the cross *Sox10*$^{LacZ/+}$ and Dct-*Ednrb* were harvested at E11.5 and E12.5 and analyzed using *LacZ* and LysoTracker Red double staining to elucidate whether or not the melanoblasts are dying at either E11.5 or E12.5. An increase in the number of double stained cells traveling along the dorsolateral pathway will imply that there is a decrease in the number of melanoblasts that make it to their final destination, resulting in hypopigmentation.

Question #7: What are the characteristics of the novel phenotype that arose from the *Ednrb*$^{S\text{-}l/+}$ cross with *Pax3*$^{Sp/+}$?

A novel phenotype arose from crossing *Ednrb*$^{S\text{-}l/+}$ with *Pax3*$^{Sp/+}$. The most obvious and striking characteristics were the development of hydrocephaly during the 3rd and 4th weeks of life, 100% lethality and skeletal deformity. Gross brain morphology was assessed with the aid of antibody staining to determine if an increase in astrocytes (as a response to stress) was visible. In addition, skeletal staining to describe skeletal differences between normal and mutant littermates was attempted.

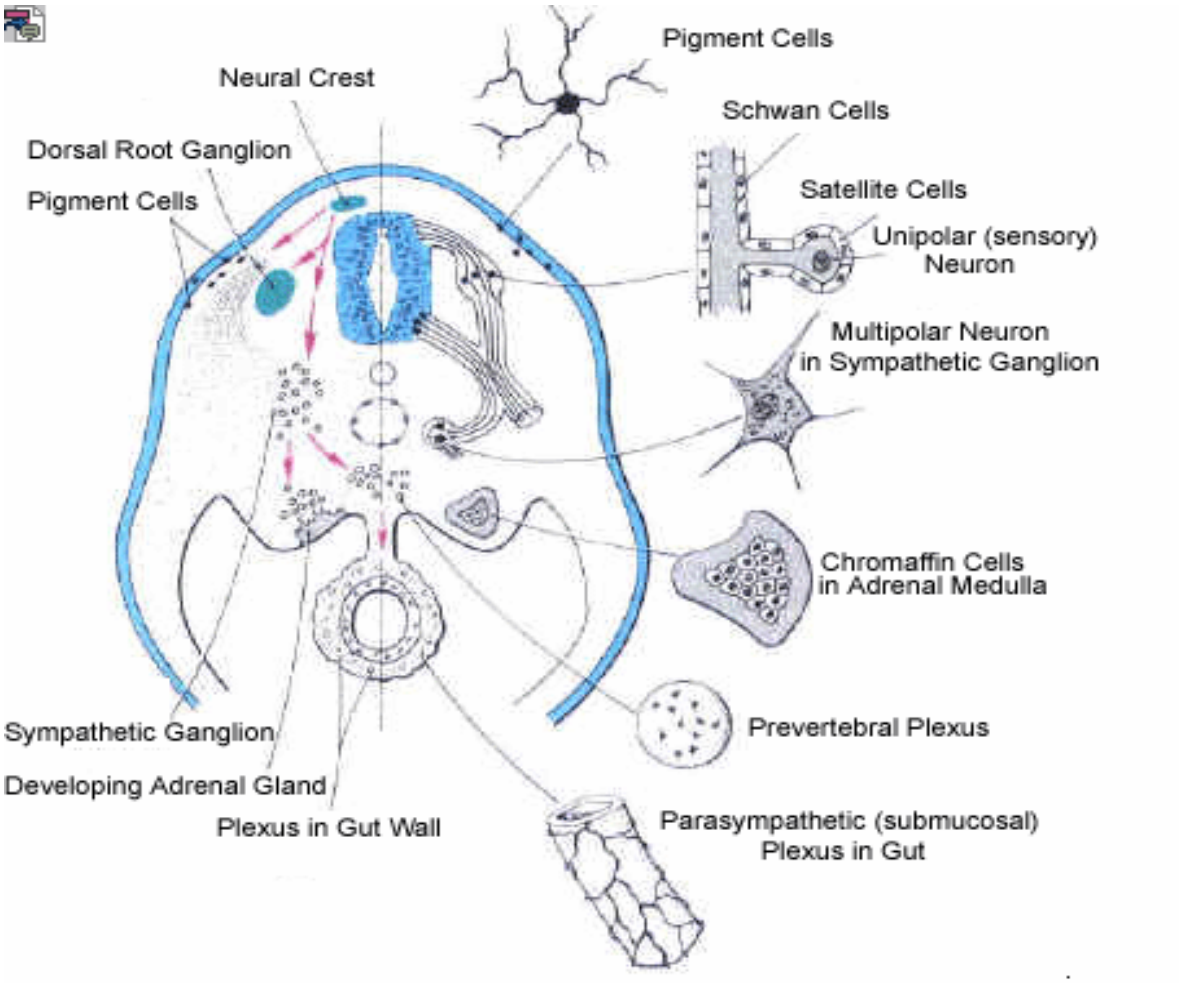

Figure 1.1 Derivatives of neural crest cells (Alberts et al., 2002).

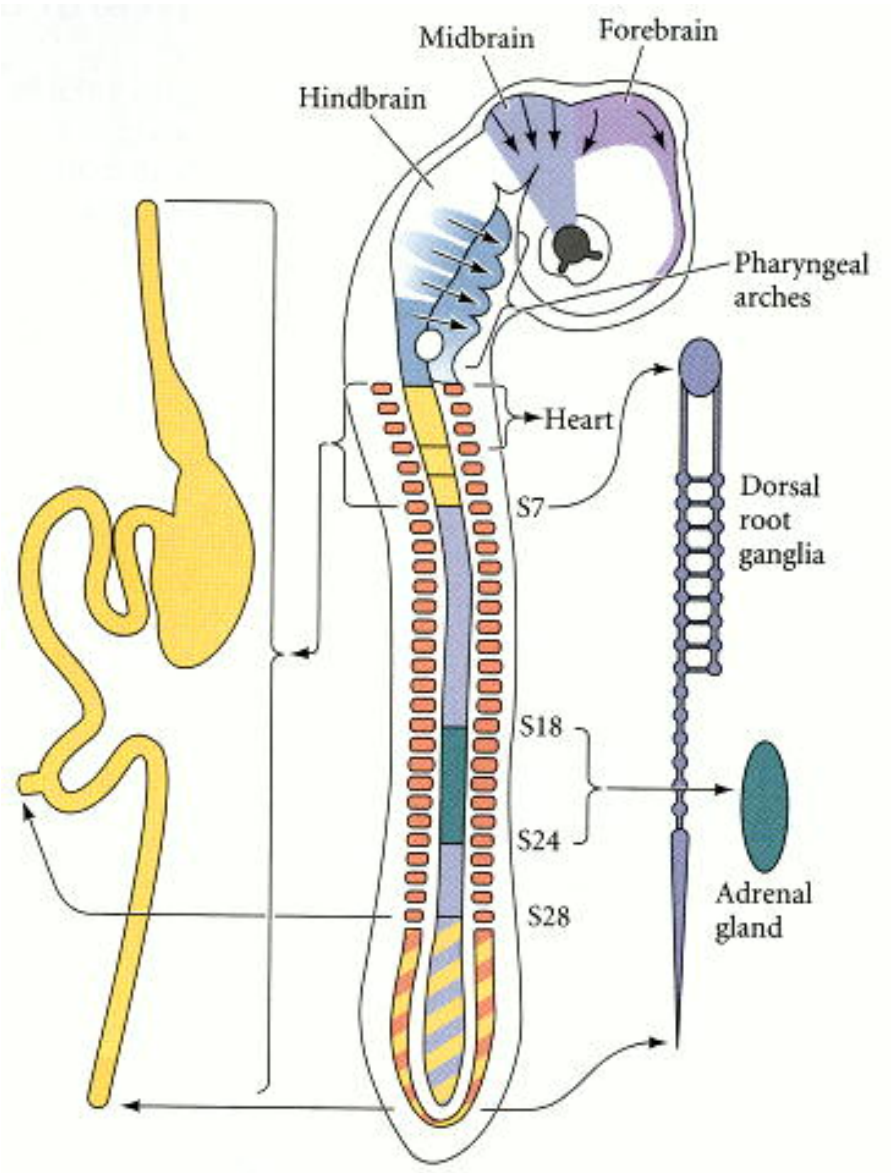

Figure 1.2 Regions of the neural crest. Regions include the cranial NC, vagal NC (near somites 1-7), cardiac NC (somites 1-3), trunk NC (about somite 6 to the tail) and the sacral NC posterior to somite 28 (Gilbert, 2003).

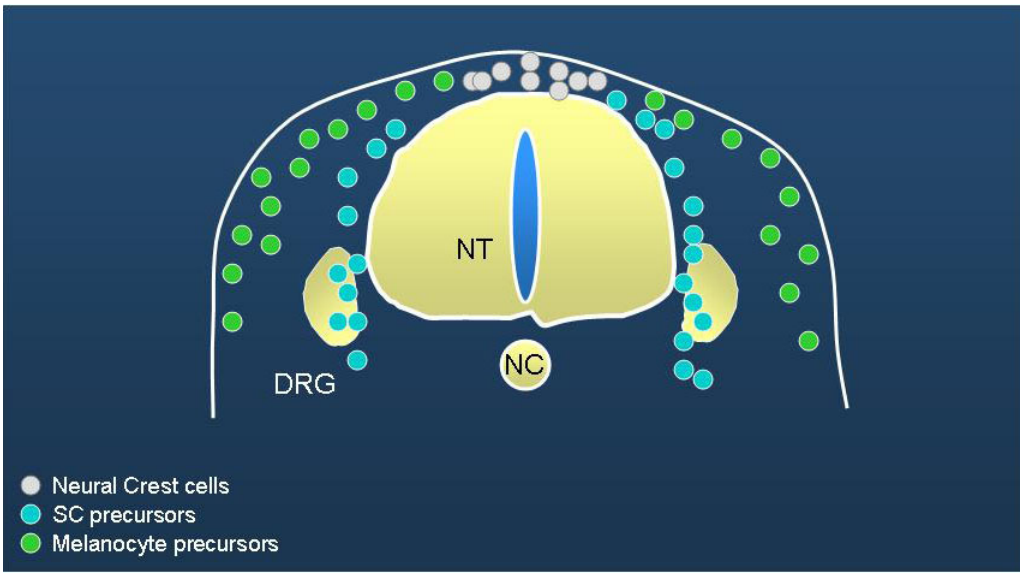

Figure 1.3 Neural crest cell migration. Neural crest cells delaminate from the dorsal aspect of the neural tube. As migration progresses, Neural crest cells traveling via the ventromedial pathway begin to take on the characteristics of Schwann cell precursors (among other phenotypes). Neural crest cells that migrate via the dorsolateral pathway exhibit melanocyte precursor traits. This cartoon depicts the murine neural tube at E11.5. NT = neural tube; NC = Notochord; DRG = dorsal root ganglia.

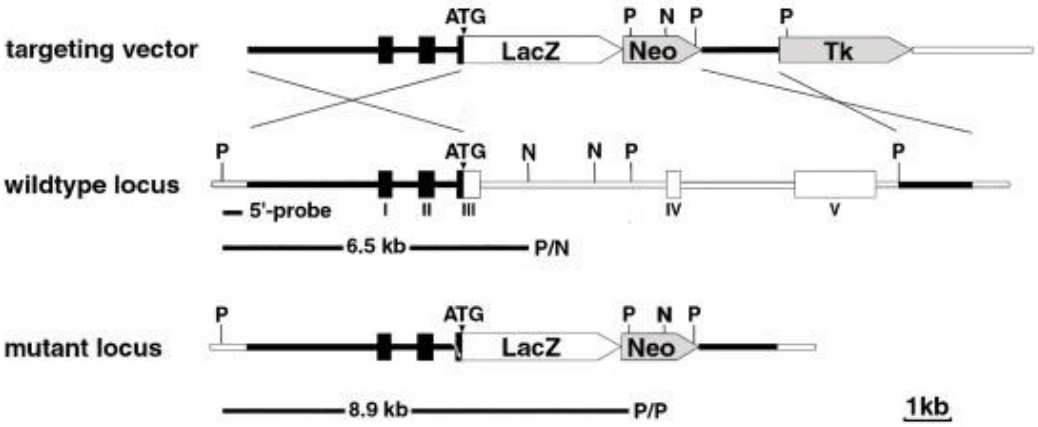

Figure 1.4 *Sox10* mutant locus. Schematic representation of targeted deletion of *Sox10* locus and insertion of the reporter gene *LacZ*. The targeting vector (top) via homologous recombination disrupted the *Sox10* locus (middle) inserting the *LacZ* gene (bottom) in its place (Britsch et al., 2001). Noncoding exons are represented by black boxes while coding exons are represented by white boxes. P = restriction site *Spe*I; N = restriction site *Nco*I.

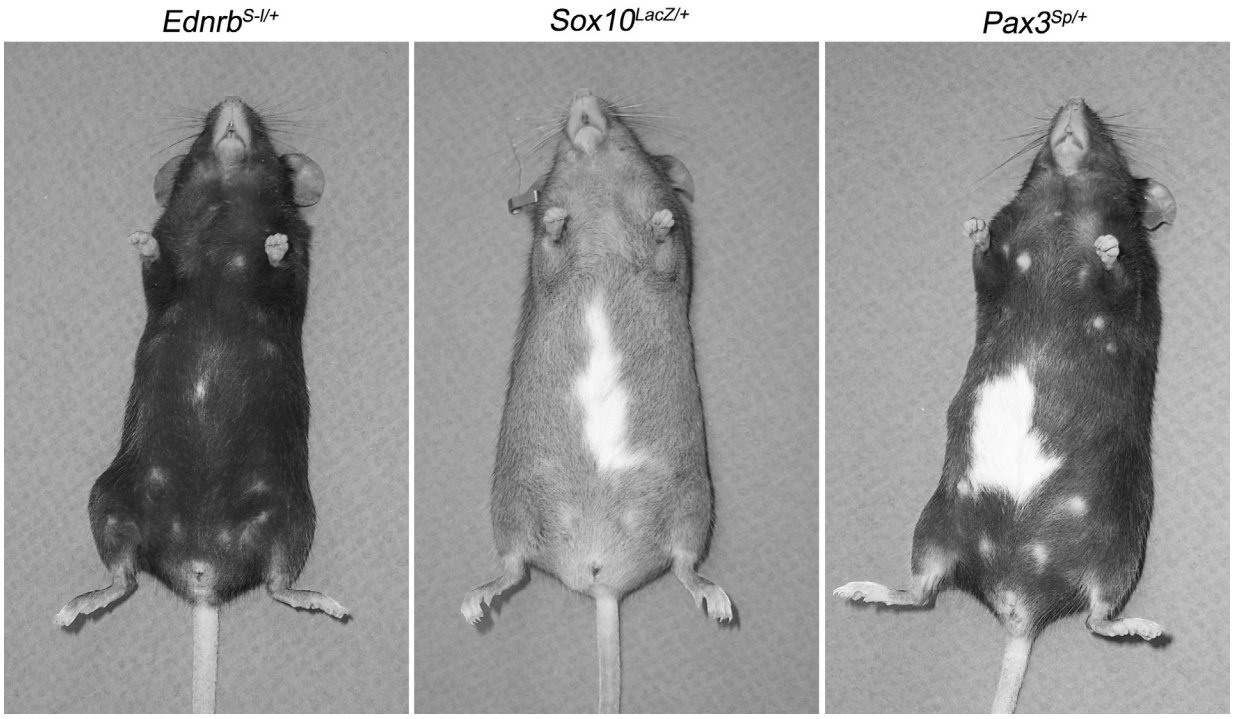

Figure 1.5 Pigmentation mutants. Typical hypopigmentation phenotypes of mutant mice used in studies. The *Ednrb*$^{S-l/+}$ hypopigmentation is quite small while the *Sox10*$^{LacZ/+}$ hypopigmentation (when present) and *Pax3*$^{Sp/+}$ hypopigmentation (always present) are larger. Pigmentation defects in all three genotypes are confined to the ventral aspect of the animals.

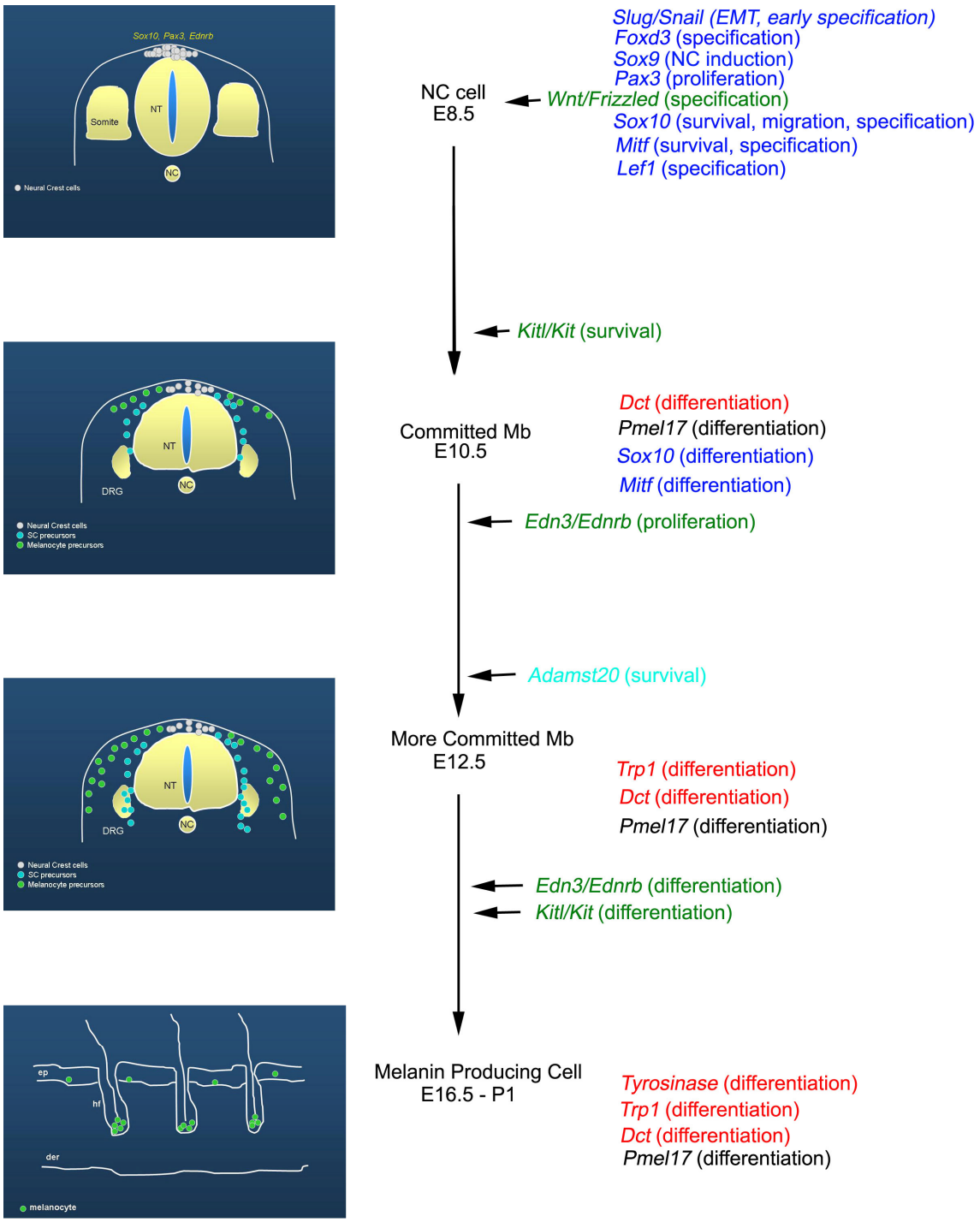

Figure 1.6 Genes governing murine melanocyte development. A timeline of melanogenic genes are detailed from the emergence of premigratory melanoblasts (E8.5) through the differentiation of melanin producing cells (E16.5). Transcription factors present as the neural tube closes at E8.5 are essential for EMT, NC induction, specification of NC cells, survival and migration. In addition, the *Wnt/Frizzed* signaling pathway is critically important for proper cellular specification at this time. As NC cells emerge, *Kitl/Kit*

becomes important for the survival of melanoblasts and at E10.5, *Dct*, *Pmel17*, *Sox10* and *Mitf* are all important differentiation factors. Between E10.5-12.5, the *Edn3/Ednrb* signaling pathway is necessary for proliferation and towards the end of this time period *Adamst20* is important for survival. Other necessary differentiation factors that either turn on or remain activated in the next phase of development (E12.5-16.5) are *Trp1*, *Dct* and *Pmel17*, along with the signaling pathways Edn3/Ednrb and *Kitl/Kit*. At E16.5, tyrosinase is activated and along with *Trp1*, *Dct* and *Pmel17* the maturing melanocyte becomes a pigment producing cell. Color key: transcription factors are in blue, signaling pathways are green, enzymes in red, melanosomal protein in black, extracellular matrix component in turquoise. EMT = epithelial mesenchymal transition, ep = epidermis, hf= hair follicle, der = dermis, P1 = postnatal day 1, *Kitl* = *Kit ligand*, Dct = DOPAchrome tautomerase, *Edn3* = *Endothelin 3*, *Ednrb* = *Endothelin receptor b*, *Trp1* = *Tyrosinase related protein 1*, Mb = Melanoblast.

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Chapter II.

INTERACTION BETWEEN THE TRANSCRIPTION FACTORS *Sox10* AND *Pax3* WITH *Endothelin receptor b* IN THE MELANOCYTE LINEAGE

II. INTERACTION BETWEEN THE TRANSCRIPTION FACTORS *SOX10* AND *PAX3* WITH *ENDOTHELIN RECEPTOR B* IN THE MELANOCYTE LINEAGE

1. Abstract

Genetic interactions, the cornerstone of cell fate and specification, are often difficult to elucidate due to the complex nature of cell development. Neural crest (NC) cells arise in the developing neural tube and migrate throughout the organism, all the while differentiating in accordance to environmental signals they encounter along the way. The diverse array of NC derivatives, which includes melanocytes, provides an intriguing system in which to explore the complex interactions necessary for cell differentiation. *Sox10*, *Pax3* and *Endothelin receptor b* are temporally and spatially co-expressed early in NC cells and mutations in these genes lead to similar hypopigmentation phenotypes due to a reduced number of melanocyte precursors. **The goal of this study is to establish whether *Sox10* and *Ednrb* and/or *Pax3* and *Ednrb* interact to promote normal murine melanocyte development.** Breeding experiments crossing heterozygous mutants of *Sox10* and *Ednrb* or *Pax3* and *Ednrb* were set to determine if animals with mutations in both genes exhibited an aggravated phenotype. Hypopigmentation was measured and analyzed using Canvas 9, Matlab and Box-Cox transformations. Double heterozygous mutants display a phenotype that is significantly more pronounced than phenotypes of single heterozygous animals implying that a synergistic interaction exists between *Sox10* and *Ednrb* and *Pax3* and *Ednrb*.

2. Introduction

In the mouse, emigration of NC cells from the neural tube begins around embryonic day (E)8.5. Melanocyte precursors are few in number early in development, despite the later need for numerous cells to populate the entire skin (Mintz, 1967; Schaible, 1969). Mutations that decrease the number of melanocyte progenitors early in embryogenesis cause significant alterations of phenotype, resulting in areas of hypopigmentation. In fact, depending on the timing and severity of the insult, the effects on hypopigmentation will vary since melanocyte precursors proliferate along their migratory path. Melanocyte progenitors emerge according to their axial level and the embryo is populated from both the rostral and caudal ends with rostral melanoblasts emerging and migrating slightly ahead of caudal melanoblasts; after melanoblasts have populated the entire length of the embryo via the dorsolateral pathway they turn inward to travel towards ventral aspects of the embryo (LeDouarin and Kalcheim, 1999; Pavan and Tilghman, 1994). For this reason, many hypopigmentation phenotypes present with ventral white areas, and depending on the severity of the effect, dorsal spots and belts are seen. In the most extreme cases, the animal is completely white.

The spatial and temporal specification, migration, proliferation and differentiation of cells depend on the genetic networks controlling developmental processes and phenotypes. It is this highly coordinated process that generates cell diversity and proper morphology within each individual regardless of species, and it is also what motivates developmental biologists

and geneticists to elucidate the transient stages between conception and senescence.

Sox10 and *Pax3* and *Ednrb* are present as the neural tube closes and NC cells begin their emigration (Baynash et al., 1994; Goulding et al., 1991; Wegner, 1999) and all appear to help orchestrate the NC cell journey towards final determination. *Sox10* is involved in the regulation of cell survival, proliferation and cell specification (Mollaaghababa and Pavan, 2003; Schepers et al., 2002) while *Pax3* is crucial for the survival of melanocyte progenitors until they reach the migratory staging area (MSA) and then later for proper migration (Hornyak et al., 2001). *Ednrb* is not instructive in the initial specification of melanocyte progenitors but is essential for survival, migration, proliferation and later indirectly activating the downstream targets of melanin production (Hou et al., 2004b; Lee et al., 2003).

Investigations into whether *Ednrb*, *Sox10* and/or *Pax3* interact genetically sprung from their spatiotemporal patterns of expression in early embryogenesis and that mutations of these genes can cause variations of Waardenburg syndrome (Read and Newton, 1997; Tachibana, 1999). Waardenburg syndrome (WS) was originally clinically described as four different types, all displaying pigmentary defects and some extent of hearing loss; the presence of additional signs and symptoms determined further classification (Read and Newton, 1997). Waardenburg syndrome Type I (WS I [MIM 193500]) were the original cases described by the Dutch ophthalmologist and geneticist Petrus J. Waardenburg back in 1947 in a meeting of the Dutch Ophthalmological Society

(Waardenburg, 1951). The additional features included dystopia canithorum and broad nasal root. Type III WS (WS III [MIM 148820]) is a more extreme version of Type I and most patients with either type I or III are heterozygous for *Pax3* (Read and Newton, 1997; Tassabehji et al., 1992). Type II WS (WS II [MIM 193510]) patients are profoundly deaf and have pigmentation defects; mutations in *Mitf* are seen in 15% of the cases and there have been a couple of cases with mutations in *SNAI2* (Read and Newton, 1997; Tassabehji et al., 1994), leaving approximately 85% of the cases from unknown mutations. Type IV WS (WS IV [MIM 277580]) has the additional feature of Hirschprung's disease, the failure of the distal colon to be innervated by NC derived enteric neurons which results in loss of peristalsis and inability to defecate (Shah et al., 1981). Some Type IV patients have been diagnosed with mutations in *Sox10*, *Ednrb* and/or *Edn3* (McCallion and Chakravarti, 2001; Paratore et al., 2002; Puffenberger et al., 1994).

Given the pattern of co-expression, similarity of phenotypes and the presence of mutations in *Sox10*, *Pax3* and *Ednrb* in the various forms of WS, investigations into possible genetic interactions have begun. In fact, a synergistic interaction between *Sox10* and *Pax3* has been identified in the transcriptional regulation of *Mitf* (Bondurand et al., 2000) and *Edn3* regulates *Mitf* in an *Ednrb*-dependent manner (Sato-Jin et al., 2008) in the melanocyte lineage. Typically, *Sox10* mutations have been identified as nonsense or frameshift mutations (Bondurand et al., 1999; Pingault et al., 1998; Touraine et al., 2000) but recently long deletions at the *Sox10* locus has been found to be

the cause of deleterious effects, including the novel finding that *Sox10* is mutated in some cases of Type II WS (Antonellis et al., 2006; Bondurand et al., 2007).

Taken together, I wanted to elucidate whether or not synergistic genetic interactions exist among *Sox10*, *Pax3* and *Ednrb* in the development of the melanocyte lineage and generation of a normal coat color pattern. In order to do so, specified matings were set to evaluate whether or not the phenotype of double heterozygous animals (*Sox10* $^{LacZ/+}$::*Ednrb*$^{S\text{-}l/+}$ or *Pax3*$^{Sp/+}$::*Ednrb*$^{S\text{-}l/+}$) was significantly exacerbated when compared to the phenotype of the single heterozygous animals. Areas of hypopigmentation were quantified and statistical analyses performed.

3. Materials and methods

3.1 Animals and Genotyping

Heterozygous *Ednrb*$^{s\text{-}l}$ on SSL/Le background and *Pax3*^{Sp} on C57BL/6J background were originally obtained from Jackson Laboratory and maintained in the FIU Animal Care Facility. Heterozygous *Ednrb*$^{s\text{-}l}$ mice display small to medium areas of ventral hypopigmentation while homozygous animals are almost completely white, with small areas of pigmentation around the pinna and base of the tail. Tail biopsies were used as sources of genomic DNA for PCR genotyping (Appendix I.A). To detect the *Ednrb*$^{s\text{-}l}$ allele, the tightly linked *D14Mit7* microsatelite polimorphic marker (Metallinos et al., 1994) was used to distinguish between *Ednrb*$^{s\text{-}l}$ and C57B/6J mice using conditions previously described (Pavan and Tilghman, 1994; and Appendix I.B). *Pax3*^{Sp} animals

carry a point mutation within intron 3 of the paired homeobox 3 (*Pax3*) gene on mouse Chromosome 1. The mutation interferes with normal splicing of intron 3 and leads to at least 4 aberrantly spliced mRNAs with exon 4 deleted. *Pax3*^{Sp} heterozygotes consistently display ventral hypopigmentation; mice homozygous for this mutation are embryonic lethal by E13.5. To genotype *Pax3*^{Sp} mice, the intron 3/exon 4 boundary of *Pax3* was amplified with primers: P3in3F (5'-GAGAGGGTTGAGTACGTTAGCTGG-3') and P3ex4 (5'-CTCGCTCACTCAGGATGCC-3'). Products were visualized on a 15% polyacrylamide gel resulting in a single band around 230bp for a wildtype and a heteroduplex band at this same site for the heterozygote (Appendix I.B).The PCRs were performed for 30 cycles under the following conditions: 94°C for 30 s, 58°C for 30 s, 72°C for 30 s.

The generation of *Sox10*^{tm1WegLacZ} (from here on noted as *Sox10*^{LacZ}) mice was described by Britsch et. al. (2001). Briefly, *Sox10* was mutated using a targeting vector that deleted the coding sequence and inserted *LacZ* into the allele. We have maintained these mice by backcrossing and keeping them on their original mixed C3H background. While many *Sox10*$^{LacZ/+}$ mice have ventral hypopigmentation, it is not a consistent phenotype thereby requiring genotyping. Tail biopsies were used as sources of genomic DNA for PCR genotyping using the following primers: 5' Sox10 (5'-CAGGTGGGCGTTGGGCTC-3'); 3' Sox10 (5'-CAGAGCTTGCCTAGTGTCTT-3'); and 3' LacZ (5'-TAAAAATGCGCTCAGGTCAA-3'). The PCRs were performed for 32 cycles under the following conditions: 94ºC for 30 s, 55ºC for

30 s, and 72ºC for 30 s. Products were visualized on a 2% agarose gel resulting in a 500bp band for wildtype and a 600bp for the mutated allele (Appendix I.B). Animals homozygous for *Sox10* are embryonic lethal, typically by E13.5.

All animals used in this study were housed in the Animal Care Facility at Florida International University (Miami, FL). Water and murine chow were fed *ad libitum* and light/dark was cycled every 12 hours. All animal work was performed according to institutional guidelines established by NIH (Guide for the Care and Use of Laboratory Animals, 2009; IACUC protocol # 08-001).

3.2 Crosses and Phenotype Analysis

Sox10$^{LacZ/+}$ mice were crossed with *Ednrb*$^{s-l/+}$ mice to produce offspring that were either heterozygous for *Sox10*, *Ednrb* or double heterozygous for *Sox10* and *Ednrb*. At 2 months of age hypopigmentation was measured and the area calculated using the Canvas 9 program; double heterozygous animals were euthanized at this time. First generation single heterozygous animals were crossed and hypopigmentation of F_2 offspring was evaluated at 2 months of age as described. As with F_1, F_2 double heterozygous animals were euthanized and single heterozygous animals were crossed to produce a third generation of animals. Again, at 2 months of age hypopigmentation was measured and all F_3 animals were euthanized.

Pax3$^{Sp/+}$ mice were crossed with *Ednrb*$^{s-l/+}$ mice as described above, also for three generations, to produce progeny that were either *Pax3*$^{Sp/+}$, *Ednrb*$^{s-l/+}$ or *Pax3*$^{Sp/+}$ ::*Ednrb*$^{s-l/+}$.

3.3 Statistical Analysis

In order to evaluate the data, hypopigmentation areas of the single heterozygous animals were added and then compared to the hypopigmentation of animals manifesting both mutations. These data were then analyzed using a one-tailed t-test, the Wilcoxon rank sum test and Box-Cox square root transformations. These statistical tests are non-parametric, rely on independent samples and do not assume normality of data. In addition, individual data points were plotted on a scatter plot using Matlab (The MathWorks, Inc.).

4. Results

4.1 Genetic Interaction between *Sox10* and *Ednrb*

To elucidate whether or not genetic interactions exist between *Sox10* and *Ednrb* in the melanocyte lineage, I analyzed the sum of hypopigmentation of mice carrying heterozygous alleles in *Sox10* and *Ednrb* and compared it to the hypopigmentation of mice that were heterozygous for both genes (Figure 1). Representative pictures of the three generations of mice imply some interaction between *Sox10* and *Ednrb* in generations 2 and 3. $Ednrb^{s\text{-}l/+}$ mice typically have proper pigmentation or a very small area of ventral hypopigmentation, whereas null mutant ($Ednrb^{s\text{-}l/s\text{-}l}$) mice are completely white with the occasional dark spot near the pinna and/or at the base of the tail. $Sox10^{LacZ/+}$ mice have ventral hypopigmentation approximately three-quarters of the time ($Sox10^{LacZ/LacZ}$ mice are embryonic lethal). A scatter plot of hypopigmentation areas of individual animals (Figure 2) indicates that a genetic interaction becomes apparent in generations 2 and 3 since the areas of ventral hypopigmentation look larger in

the double heterozygous mice than the areas of hypopigmentation in single heterozygous animals.

Hypopigmentation was measured by photographing mice at two months of age and using the Canvas 9 program to outline and calculate the area. While areas of hypopigmentation varied, the individual values were used in non-parametric assays that did not assume normality of data. Three statistical tests were used to analyze the data: Box-Cox square root transformations, Wilcoxon rank sum test and the one-tailed t-test, each of which took into account unequal size of the groups and variability and standard error of the samples. All analyses produced similar results (Tables 1 and 2).

In generation one (n=71), surprisingly, hypopigmentation of *Sox10*$^{LacZ/+}$ was larger than hypopigmentation seen in *Ednrb*$^{s\text{-}l/+}$ or *Sox10*$^{LacZ/+}$*::Ednrb*$^{sl/+}$ mice. However, in generations 2 (n=52) and 3 (n=27) the hypopigmentation of *Sox10*$^{LacZ/+}$*::Ednrb*$^{s\text{-}l/+}$ mice was significantly larger (means of 404 and 298; p values ranging from .005-.012, and .005-.026, respectively for F_2 and F_3) than the sum of the single heterozygous animals (means of 66.5 and 37.7, respectively for F_2 and F_3; Table 3) implying that background exerts an influence on pigmentation (Cantrell et al., 2004) and that as the C3H and C57BL/6J backgrounds blend the nature of the interaction between *Sox10*$^{LacZ/+}$ and

Ednrb$^{s\text{-}l/+}$ becomes apparent.

4.2 Genetic Interaction between *Pax3* and *Ednrb*

As with the cross mentioned above, *Pax3*^{Sp} and *Ednrb*$^{s-l}$ heterozygous mice were crossed to reveal whether or not a genetic interaction exists between these melanocytic genes (Hornyak et al., 2001; Pavan and Tilghman, 1994). All animals were genotyped and progeny crossed as described above, and at 2 months of age, hypopigmentation was photographed and measured using the Canvas 9 program. Representative pictures (Figure 3) of the three generations of mice imply some interaction between *Pax3* and *Ednrb*. As mentioned above, *Ednrb*$^{s-l/+}$ mice exhibit either proper pigmentation or a very small area of ventral hypopigmentation while the *Ednrb*$^{s-l/s-l}$ mice are completely white with the occasional dark spot near the pinna and/or at the base of the tail. *Pax3*$^{Sp/+}$ mice, on the hand, consistently have ventral hypopigmentation and *Pax3*$^{Sp/Sp}$ mice are embryonic lethal by E13.5.

A scatter plot of the hypopigmentation areas of individual animals (Figure 4) indicates that a genetic interaction becomes apparent in generations 2 and 3. The three statistical analyses (Tables 4 and 5), Box-Cox square root transformations, Wilcoxon rank sum test and the one-tailed t-test, all indicate that a significant difference exists between the area of hypopigmentation of the double heterozygous mutants in generations 2 and 3 (means of 477.8 and 340.0; p values ranging from .010-.079 and .0004-.021, respectively for F_2 and F_3) when compared to the sum of the areas of hypopigmentation of the single heterozygous mutants (means of 12.5 and 84.0, respectively for F_2 and F_3;

Table 6) even when considering the unequal sample sizes of the groups and the natural variability between the animals.

5. Discussion

Manipulating pigmentation phenotypes has long been a model system in which to elucidate genetic crosstalk and influences in health and disease. In the case of Waardenburg Syndrome, four out of the five genes responsible for variants of this disease are also involved in the development of melanocytes: *Sox10*, *Pax3*, *Ednrb* and *Mitf*. Investigating genetic interactions in the context of biological systems has the tremendous potential to increase our understanding of cellular development and the etiology of disease. One way to do this is by creating compound heterozygotes in animal models to investigate *in vivo* genetic interactions. Recent examples that have employed this approach include studies investigating candidate genes for autism spectrum disorder and cardiac development.

PTEN (PI3K pathway) and *SLC6A4* (serotonin pathway) are considered autism candidate genes (Bartlett et al., 2005; Herman et al., 2007). Page and colleagues (2009) examined the question of whether these two genes, both candidates for a complicated disease and whose pathways intersect (Ji et al., 2006) , interact by creating double heterozygous animals and evaluating their phenotype. In a completely different system, the effect of using compound heterozygous mice was employed to study genetic interactions between *Gata4/6* and *Tbx5* in cardiac development. *Gata4* and *Gata6* belong to a (zinc finger) transcription family, are expressed in a variety of cells and are important

in cardiovascular development (Molkentin, 2000; Pu et al., 2004); *Tbx5* is a transcription factor whose haploinsufficiency causes a variety of cardiac defects (Bruneau, 2008). Based on earlier experiments indicating a possible interaction between *Gata4* and *Tbx5*, heterozygous *Gata4* or *Gata6* mice were crossed with *Tbx5* heterozygous mice to generate compound heterozygous $Gata4^{+/-}$ ::$Tbx5^{+/-}$ or $Gata6^{+/-}$ ::$Tbx5^{+/-}$ embryos and pups in order to determine whether or not synergistic interactions occur between these genes (Maitra et al., 2009). Both of these studies helped elucidate interactions between genes of interest.

In this study, I have taken mice that were heterozygous for either of the transcription factors *Sox10* or *Pax3* and crossed them with mice harboring the heterozygous mutation of *Ednrb*. Progeny of the $Sox10^{LacZ/+}$ and $Ednrb^{s-l/+}$ cross, alongside progeny of the $Pax3^{Sp/+}$ and $Ednrb^{s-l/+}$ cross, were evaluated over the course of three generations to see if there was a genetic interaction between either transcription factor and *Ednrb*.

The $Sox10^{LacZ/+}$ and $Ednrb^{s-l/+}$ crossed over three generations produced second and third generation double heterozygous progeny in which the area of hypopigmentation was significantly larger than the sum of the area of hypopigmentation of single heterozygous animals from the same generation. A previous study using an *Ednrb* hypomorph ($Ednrb^{s}$) crossed with a spontaneously occurring *Sox10* mutation ($Sox10^{Dom/+}$) or the knock-in $Sox10^{LacZ/+}$ revealed that the pigmentation phenotype, in addition to the hearing and aganglionosis phenotypes, seen in *Ednrb* and *Sox10* haploinsufficient mice were aggravated in the double heterozygous animals (Stanchina et al., 2006).

In addition, pups born with a mutation in both *Ednrb* and *Sox10*$^{Dom/+}$ had increased mortality regardless of the *Ednrb* allele used in the cross (Cantrell et al., 2004; Stanchina et al., 2006). The *Ednrb* hypomorph (*Ednrb*^{s}) used in these studies retains about 25% of functional receptor activity; even without losing all the functional capacity of the mutated allele, a genetic interaction was seen between *Ednrb* and *Sox10*$^{Dom/+}$. This study used the *Ednrb*$^{s-l}$ allele which has essentially no receptor activity.

However, data from two other studies suggested no interaction between *Sox10* and *Ednrb* in the melanocyte lineage (Cantrell et al., 2004; Hakami et al., 2006). The differences in the results found in these studies could be attributed to the allelic variants of the mouse mutants that were used. Cantrell et al (2004) and Hakami et al (2006) used the *Sox10* variant *Dom*. *Sox10*^{Dom} mice lack the transactivation domain (Herbarth et al., 1998; Southard-Smith et al., 1998) of the gene; *Sox10*^{LacZ} mice (used by Stanchina et al and in this study) have *LacZ* knocked into the gene at the initiation codon (Britsch et al., 2001). Cantrell et al. (2004) used *Ednrb*^{tm1Ywa} in which 4.2kb is replaced by a neomycin cassette in exon 3 rendering the gene null (Hosoda et al., 1994) while Hakami et al. (2006) and Stanchina et al. (2006) used a *Ednrb*^{s} hypomorph where a 5.5kb retrotransposon was placed in intron 1 rendering the gene 25% functional (Roix et al., 2001). Cantrell et al. (2004) and Hakami et al. (2006) also used *Ednrb*$^{s-l}$ in which all coding exons are deleted and there is no functional transcript available (Hosoda et al., 1994). This study consistently used *Sox10*^{LacZ} and *Ednrb*$^{s-l}$ mice to assess whether an interaction between

these genes exists; neither heterozygous animal had residual functional effects from the mutated allele.

Another difference between these studies that may have influenced the varying outcomes is the background of the mouse strains used. Although the mice originally came from the same sources (e.g. Jackson Labs; Dr. Wegner, Universitat Erlangen), they had typically resided in the animal facilities of the respective labs for some time. Some mice were outcrossed to switch the background (Cantrell et al., 2004; Hakami et al., 2006) while other mice were maintained in their mixed background for a number of years (Stanchina et al., 2006). The *Ednrb*$^{s-l}$ mice used in this study were originally switched over from an LLE background to a C57BL/6J background where they have been maintained for years. The *Sox10*^{LacZ} mice were originally on a C3H mixed background and have been backcrossed since their arrival. Colony size and number of years being inbred or outcrossed could increase, or decrease, the expression of background modifiers and genetic heterogeneity which in turn might affect gene expression and penetrance (Cantrell et al., 2004; Genin et al., 2008; Strunk et al., 2004). Biological complexities of studying genetic interactions *in vivo* imply that a myriad of genes, allelic variants of those genes, modifiers and/or environmental influences can impact gene expression underscoring the need to perform experiments over generations and in varying backgrounds to truly elucidate genetic interactions. The C57BL/6J background is known to diminish pigmentation phenotypes (Cantrell et al., 2004), which is possibly why the hypopigmentation of generation 1 double heterozygous

animals was not significantly different than the hypopigmentation of the single heterozygous animals. As the single heterozygous animals from each subsequent generation were crossed, the backgrounds continued to mix to create a more homogeneous (mixture of C57BL/6J + C3H) background allowing the phenotype from the genetic interaction to become apparent.

Given that *Ednrb* and *Sox10* are spatially and temporally co-expressed as neural crest cells emigrate from the dorsal aspect of the neural tube and melanocyte and enteric neuron precursors migrate via the dorso-lateral and ventro-medial pathways, respectively (Le Douarin and Dupin, 2003), one might suspect that the expression of one might be dependent on the expression of the other. In fact, interactions between *Sox10* and *Ednrb* have been shown to modulate the development of enteric neurons (Cantrell et al., 2004; Zhu et al., 2004), and an *Ednrb* enhancer that is activated specifically in enteric neuron precursors has been identified that contains multiple binding sites for *Sox10* (Zhu et al., 2004). Recently, in human melanocytes, *Sox10* has been shown to directly regulate *Ednrb* at the transcriptional level through two different promoters (Yokoyama et al., 2006).

In situ hybridization has shown that *Sox10* $^{Dom/Dom}$ embryos still express *Ednrb* and that, at least for neural crest cells explants, the converse is true as well, that *Ednrb* null mutant cells express *Sox10* (Hakami et al., 2006). However, the expression of either gene in the mutant of the other occurs in a significantly reduced manner. Of interest is that in these mutants certain structures (e.g., the PNS in *Sox10* homozygous mice) or cells (e.g.,

melanoblasts in *Ednrb* homozygous mice) begin to deteriorate or are missing. Structures that are deteriorating or missing prevent the distinction between the lack of expression of the gene of interest due to regulation of the mutated gene or simply because the tissues and structures where the gene is expressed are not there *Ednrb* and *Sox10* may interact in a functional hierarchical manner at a specified time during development to promote melanoblast differentiation. For example, while *Ednrb* is not required for initial melanoblast specification, it is required during E10.5-E12.5 for proper melanocyte development (Lee et al., 2003; Shin et al., 1999). This is the same critical window in which *Sox10* expression is needed for melanoblasts to proceed normally in development (Britsch et al., 2001).

Sox proteins are abundant in tissues, each of them present in a variety of tissues and transcriptionally active at different times throughout the vertebrate lifecycle (Wegner, 1999). Besides being important architectural modifiers, Sox proteins cooperatively bind with other proteins to ensure specificity of their action (Kuhlbrodt et al., 1998; Schlierf et al., 2002). *Sox10* undoubtedly needs a partner to activate (Kamachi et al., 2000) and become fully functional in melanoblast development and, since *Sox10* and *Pax3* work coordinately to transcriptionally activate the melanocytic downstream target *Mitf*, could they possibly each work to activate *Ednrb* as well?

Pax3$^{Sp/+}$ mice have a phenotype similar to *Ednrb*$^{s\text{-}l/+}$ and *Sox10*$^{LacZ/+}$ (ventral hypopigmentation), but unlike the other mutants *Pax3*$^{Sp/+}$ is a fully penetrant phenotype, apparent in all heterozygous mice. *Pax3* is present in the dorsal

aspect of the neural tube as NC cells begin their emigration and while the prospective melanocytes are in the MSA prior to their emigration. Acting as a nodal point in melanocyte stem cell differentiation, *Pax3* in conjunction with *Sox10* coordinately regulates *Mitf* (Bondurand et al., 2000; Potterf et al., 2000) and subsequently acts to repress this activation as well (Lang et al., 2005). In NC derived enteric neurons, Sox10 and Pax3 synergistically interact to regulate c-RET (Lang et al., 2000) Intriguingly, a *Sox10* transgene put under the control of the *Pax3* promoter rescues *Sox10* deficient melanocytes (Hou et al., 2004a). Given the seemingly related roles that *Sox10* and *Pax3* play in melanogenesis, the question of whether *Pax3* and *Ednrb* interact needed to be addressed.

Heterozygous mutants of *Pax3* and *Ednrb* were crossed to evaluate the hypopigmentation of single and double heterozygous animals for 3 generations. The results of these crosses indicate that there is indeed a genetic interaction between *Pax3* and *Ednrb* in the melanocyte lineage. As with the *Sox10* experiment the progeny of $Pax3^{Sp/+}$ crossed with $Ednrb^{s\text{-}l/+}$ showed that the hypopigmentation of the double mutants in generations 2 and 3 was significantly larger than the sum of the hypopigmentation of the single mutants.

Haploinsufficiency of *Sox10* or *Pax3* in combination with haploinsufficiency of *Ednrb* caused an aggravated hypopigmentation phenotype in second and third generation progeny. First generation results may have been obscured by initial mixing of varying genetic backgrounds (C3H, C57BL/6J and LLE) which may have altered the expressivity and penetrance of the pigmentation

phenotype. Taken together, these results suggest a genetic interaction between *Sox10* and *Ednrb* as well as *Pax3* and *Ednrb*.

The question that naturally follows is whether the hypopigmentation phenotype of these mutants could be rescued by the targeted addition of the complimentary gene. For instance, could the *Sox10*/*Pax3* phenotype be rescued by additional *Ednrb* or could the *Ednrb* phenotype be rescued by additional *Sox10*/*Pax3*? In light of previous research showing that *Ednrb* contains multiple binding sites for *Sox10* in enteric neurons (Zhu et al., 2004) and in human melanocytes (Yokoyama et al., 2006), I will attempt to answer this question by delivering additional *Ednrb* to $Sox10^{s\text{-}l/+}$ and $Pax3^{Sp/+}$ mice.

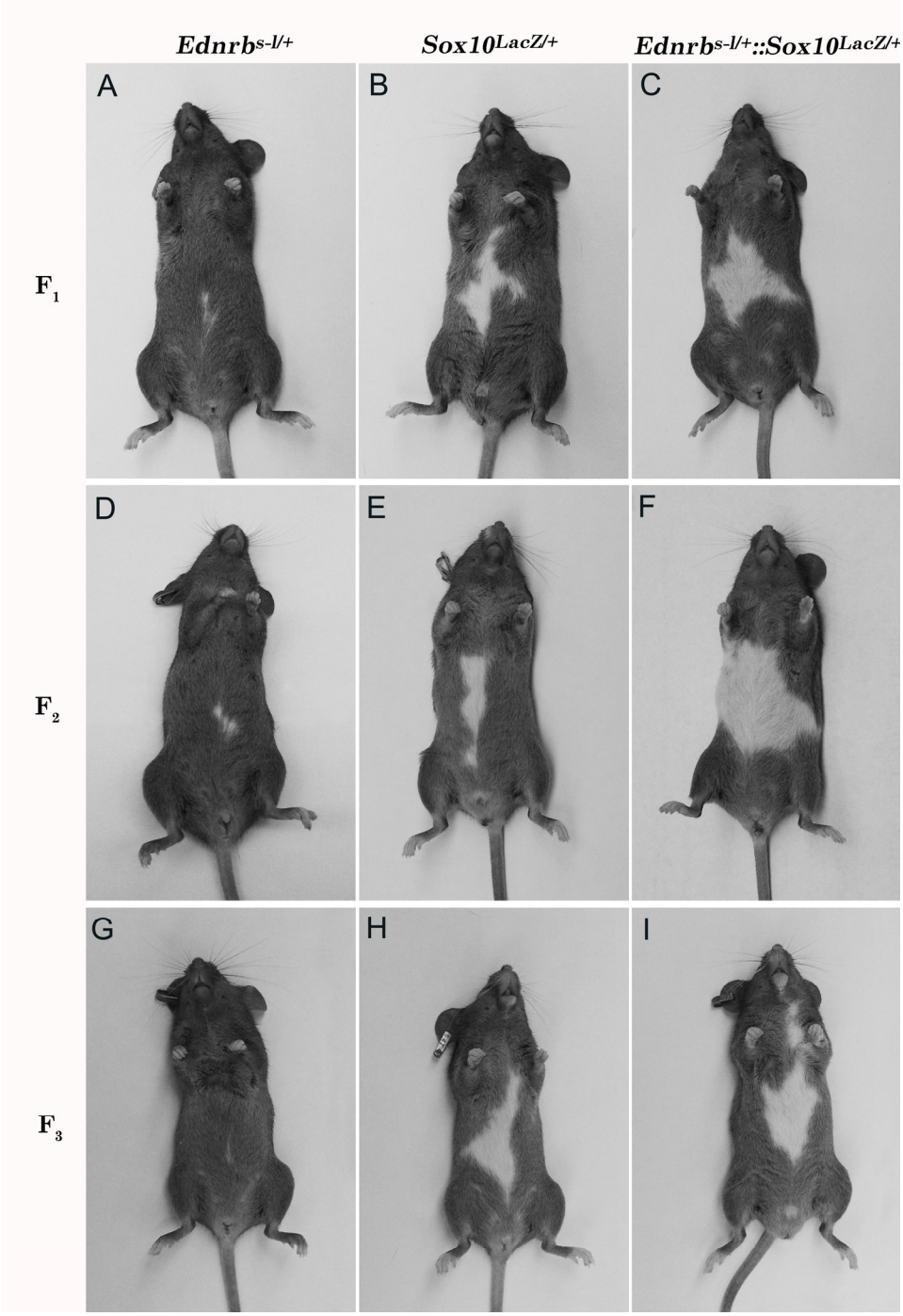

Figure 2.1 *Sox10* and *Ednrb* double heterozygosity results in an aggravated hypopigmentation phenotype. Representative pictures of hypopigmentation for each genotype show ventral hypopigmentation of double heterozygous mutants for generation 1 was not different than the hypopigmentation of single mutants. In generations 2 and 3, double heterozygous mice had larger areas of hypopigmentation than did the *Sox10* heterozygous and the *Ednrb* heterozygous animals (F_1 = 1st generation, F_2 = 2nd generation, F_3 = 3rd generation).

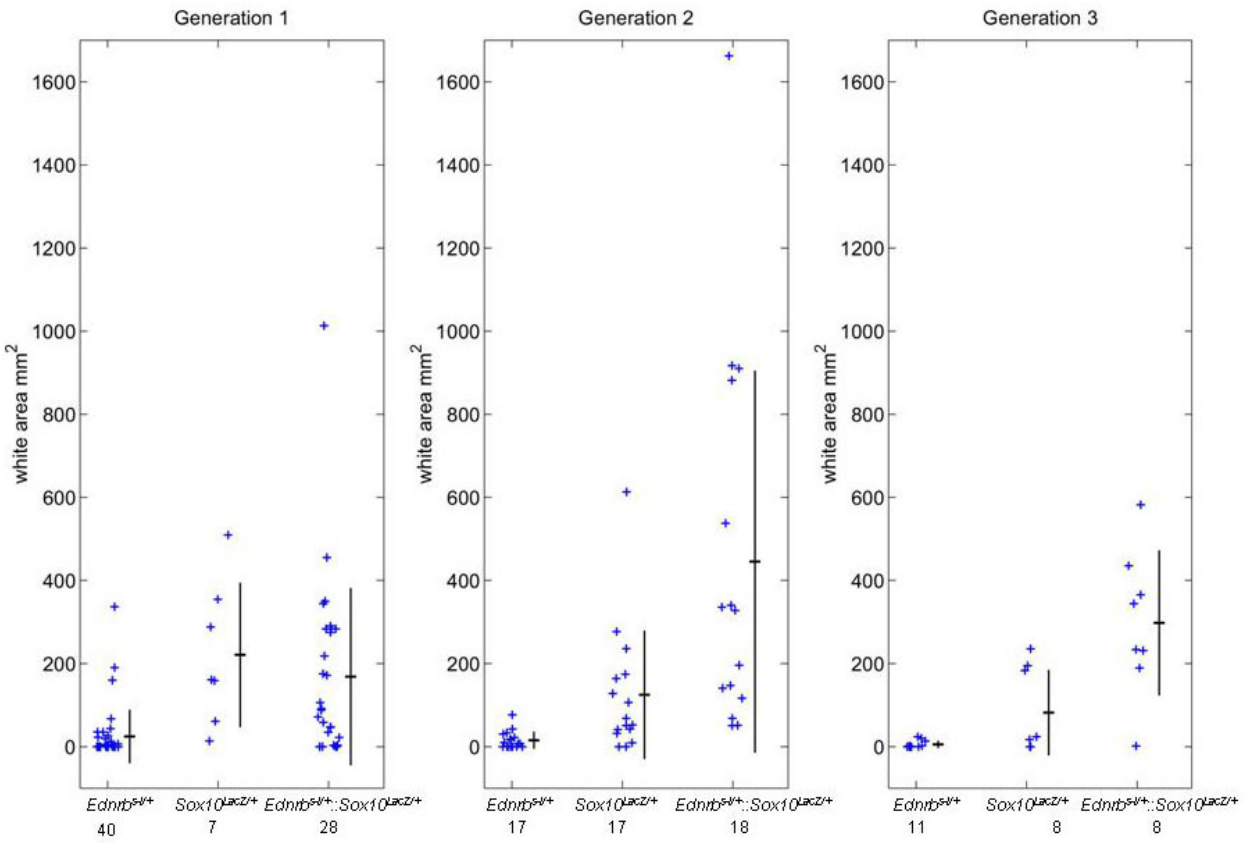

Figure 2.2 Double heterozygous progeny of generations 2 and 3 exhibit larger areas of hypopigmentation than single heterozygous animals. Numerical scatter plots of individual progeny of *Sox10*$^{LacZ/+}$ crossed with *Ednrb*$^{s-l/+}$ over three generations, including means and standard deviations. The numbers below the genotype represent the number of animals in that group; white area in mm^2 denotes area of hypopigmentation.

	Box-Cox square root transformation	Wilcoxon rank sum test	One-tailed t-test
F1	NS	NS	NS
F2	p = .012	p = .005	p = .010
F3	p = .026	p = .014	p = .005

Table 2.1 *Sox10* and *Ednrb* double heterozygosity results in an aggravated phenotype. Various analyses were employed given that samples were uneven in size. Statistical analyses robustly indicate that in generations 2 and 3 a statistically significant difference exists between the sum of hypopigmentation of single heterozygous animals (*Sox10*$^{lacZ/+}$ and *Ednrb*$^{s\text{-}l/+}$) and the hypopigmentation of double heterozygous animals (*Sox10*$^{lacZ/+}$::*Ednrb*$^{s\text{-}l/+}$; F_1 = 1st generation, F_2 = 2nd generation, F_3 = 3rd generation).

Generation	Genotype	Number of animals	Mean area of hypopigmentation	Significance
F1	*Ednrb*$^{s-l/+}$	40	25	NS
	Sox10$^{LacZ/+}$	7	221	
	Ednrb$^{s-l/+}$:: *Sox10*$^{LacZ/+}$	28	168	
F2	*Ednrb*$^{s-l/+}$	17	15	p=.005
	Sox10$^{LacZ/+}$	17	118	
	Ednrb$^{s-l/+}$:: *Sox10*$^{LacZ/+}$	18	404	
F3	*Ednrb*$^{s-l/+}$	11	6	p=.014
	Sox10$^{LacZ/+}$	8	82	
	Ednrb$^{s-l/+}$:: *Sox10*$^{LacZ/+}$	8	298	

Table 2.2 Wilcoxon rank sum test of hypopigmentation in *Sox10* and *Ednrb* single heterozygotes versus double heterozygosity. In F2 and F3 progeny, hypopig-mentation is significantly larger in *Ednrb*$^{s-l/+}$::Sox10$^{LacZ/+}$ mice than in mice with either gene mutated alone (NS = not significant, F_1 = 1st generation, F_2 = 2nd generation, F_3 = 3rd generation).

Sox10$^{LacZ/+}$ x *Ednrb*$^{S-l/+}$	Average of Hypopigmentation of Single Heterozygous	Average of Hypopigmentation of Double Heterozygous
F_1	53.6	168.5
F_2	66.5	404.0
F_3	37.7	298.0

Table 2.3 Sums of averages. The sum of hypopigmentation averages for single heterozygous animals (*Sox10*$^{LacZ/+}$ x *Ednrb*$^{S-l/+}$) compared to the hypopig-mentation average for double heterozygous siblings (*Sox10*$^{LacZ/+}$::*Ednrb*$^{S-l/+}$). Levels of significance are listed by statistical measure used in Table 1. (F_1 = 1st generation, F_2 = 2nd generation, F_3 = 3rd generation).

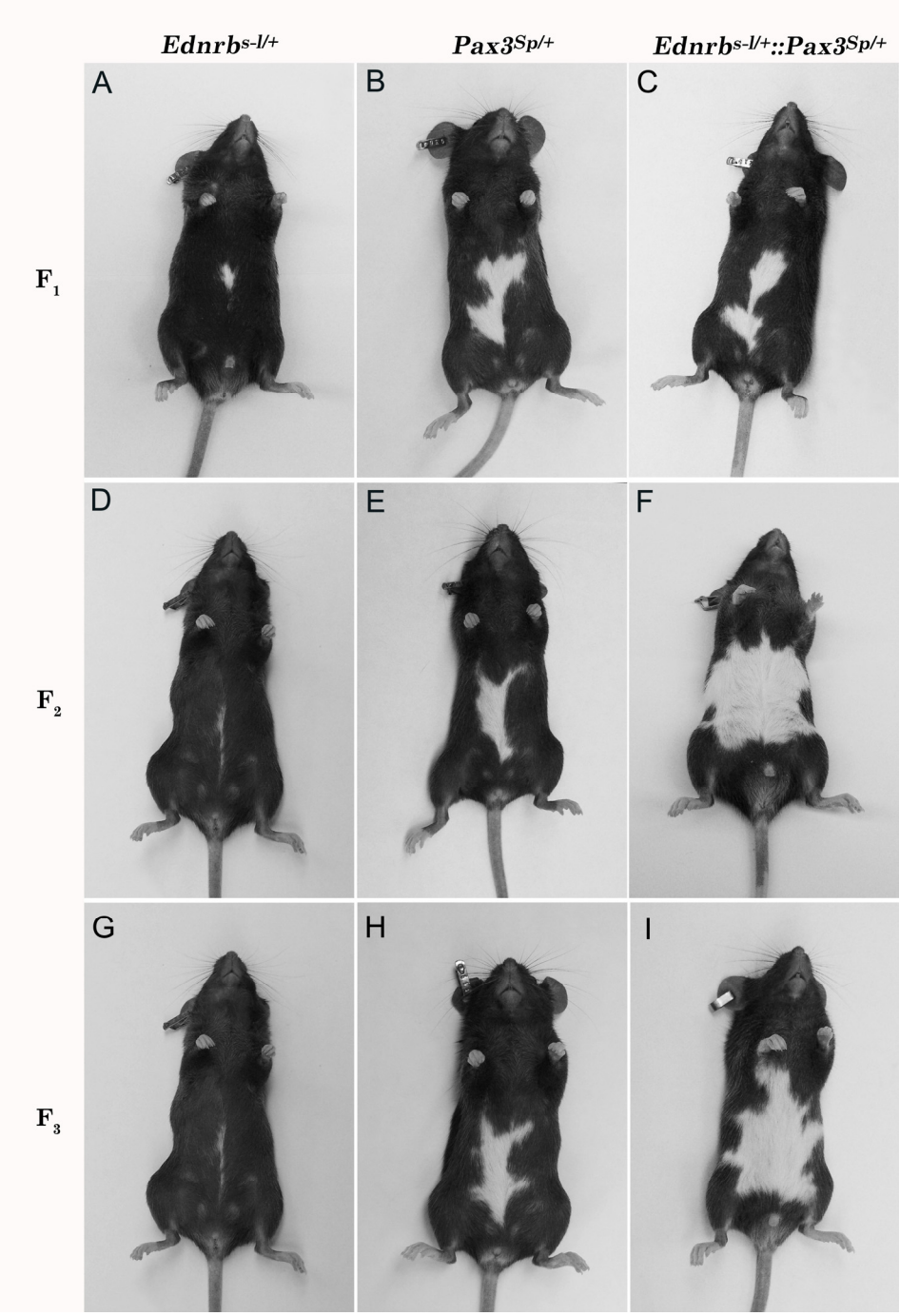

Figure 2.3 *Pax3* and *Ednrb* double heterozygosity results in an aggravated hypopigmentation phenotype. Representative pictures of hypopigmentation for each genotype show ventral hypopigmentation of double heterozygous mutants for generation 1 was not different than the hypopigmentation of single mutants. In generations 2 and 3, double heterozygous mice had much larger areas of hypopigmentation than did the *Pax3* heterozygous and the *Ednrb* heterozygous animals (F_1 = 1st generation, F_2 = 2nd generation, F_3 = 3rd generation).

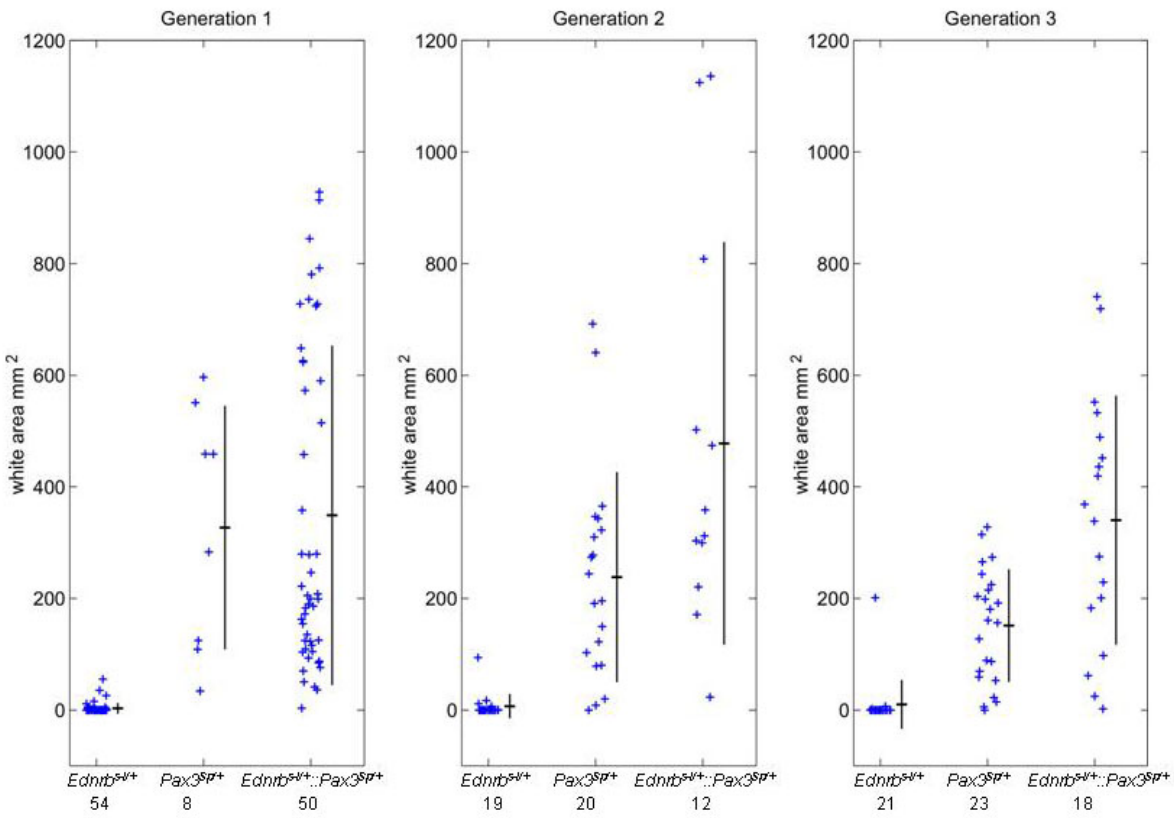

Figure 2.4 Double heterozygous progeny of generations 2 and 3 exhibit larger areas of hypopigmentation than single heterozygous animals. Numerical scatter plots of individual progeny of *Pax3*$^{Sp/+}$ crossed with *Ednrb*$^{s-l/+}$ over three generations, including means and standard deviations. The numbers below the genotype represent the number of animals in that group.

	Box-Cox square root transformation	Wilcoxon rank sum test	One-tailed t-test
F1	NS	NS	NS
F2	p = .079	p = .037	p = .010
F3	p = .021	p = .004	p = .0004

Table 2.4 *Pax3* and *Ednrb* double heterozygosity results in an aggravated phenotype. Various analyses were employed given that samples were uneven in size. Statistical analyses robustly indicate that in generations 2 and 3 a statistically significant difference exists between the sum of hypopigmentation of single heterozygous animals (*Pax3*$^{Sp/+}$ and *Ednrb*$^{s-l/+}$) and the hypopig-mentation of double heterozygous animals (*Pax3*$^{Sp/+}$::*Ednrb*$^{s-l/+}$; F_1 = 1st generation, F_2 = 2nd generation, F_3 = 3rd generation).

Generation	Genotype	Number of animals	Mean area of hypopigmentation	Significance
F1	$Ednrb^{+/-}$	54	3	NS
	$Pax3^{+/-}$	8	327	
	$Pax3^{+/-}$::$Ednrb^{+/-}$	50	349	
F2	$Ednrb^{+/-}$	19	7	p=.037
	$Pax3^{+/-}$	20	238	
	$Pax3^{+/-}$::$Ednrb^{+/-}$	12	478	
F3	$Ednrb^{+/-}$	21	10	p=.004
	$Pax3^{+/-}$	23	152	
	$Pax3^{+/-}$::$Ednrb^{+/-}$	18	340	

Table 2.5 Wilcoxon rank sum test of hypopigmentation in *Pax3* and *Ednrb* single heterozygous versus double heterozygosity. In F2 and F3 progeny, hypopig-mentation is significantly larger in $Ednrb^{s-l/+}$::$Pax3^{Sp/+}$ mice than in mice with either gene mutated alone (NS = not significant, F_1 = 1st generation, F_2 = 2nd generation, F_3 = 3rd generation).

$Pax3^{LacZ/+}$ x $Ednrb^{S-l/+}$	Average of Hypopigmentation of Single Heterozygous	Average of Hypopigmentation of Double Heterozygous
F_1	45.0	349.1
F_2	125.5	477.8
F_3	84.0	340.0

Table 2.6 Sums of Averages. The sum of hypopigmentation averages for single heterozygous animals ($Pax3^{LacZ/+}$ x $Ednrb^{S-l/+}$) compared to the hypopig-mentation average for double heterozygous siblings ($Pax3^{LacZ/+}$::$Ednrb^{S-l/+}$). Levels of significance are listed by statistical measure used in Table 1. (F_1 = 1st generation, F_2 = 2nd generation, F_3 = 3rd generation).

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Chapter III.

TRANSGENIC EXPRESSION OF *Endothelin receptor b* RESUCES THE HYPOPIGMENTATION PHENOTYPE OF *Sox10* AND *Pax3* HETEROZYGOUS MICE

III. TRANSGENIC EXPRESSION OF *ENDOTHELIN RECEPTOR B* RESCUES THE HYPOPIGMENTATION PHENOTYPE OF *SOX10* AND *PAX3* HETEROZYGOUS MICE

1. Abstract

Given the synergistic effect of *Sox10* and *Ednrb* as well as *Pax3* and *Ednrb* on the hypopigmentation phenotype of mice carrying mutations in these genes, I investigated the effects of a transgenic addition of *Ednrb* to melanocyte precursors. Using a transgene that uses the *Dopachrome tautomerase* (*Dct*) promoter to selectively deliver *Ednrb* to melanoblasts, I first elucidated the effect of delivering additional *Ednrb* to the melanocyte precursors in $Sox10^{LacZ}$ and $Pax3^{Sp}$ mutant mice. Coat color was completely restored in the $Sox10^{LacZ/+}$::Dct-*Ednrb* mice and partially restored in the $Pax3^{Sp/+}$::Dct-*Ednrb* mice. Then, I compared melanoblast development in *Sox10* mutant mice with and without this Dct-*Ednrb* transgene by harvesting embryos at E11.5 and E12.5, a critical time in melanoblast expansion, and comparing the amount of melanoblasts between transgenic and non-transgenic embryos. The transgenic addition of *Ednrb* rescued melanocyte precursors in *Sox10* heterozygous animals but not in *Sox10* homozygous animals. Cell survival assays indicate that the *rescue is not due to an effect of the transgene on melanoblast survival*. In conclusion, my data demonstrates that both *Sox10* and *Pax3* interact with *Ednrb* in the development of the melanocyte lineage. Transgenic expression of additional *Ednrb* may compensate for the detrimental effects of *Sox10* haploinsufficiency in this lineage, and the reduced number of melanocyte precursors in *Sox10* mutants is not due to increased apoptosis.

2. Introduction

The results described in Chapter 2 established a synergistic genetic interaction between the transcription factors *Sox10* and *Pax3* and the G-coupled receptor *Ednrb*. By crossing *Sox10* or *Pax3* heterozygotes with *Ednrb* heterozygotes, a clear aggravation of the pigmentation phenotype was observed. To further explore these genetic interactions, I will use a transgenic mouse that puts the delivery of *Ednrb* under the control of the Dct promoter to attempt the rescue of the hypopigmentation phenotype of the *Sox10* and *Pax3* mutants.

The first transgenic mouse was created in mid-1980's (Palmiter and Brinster, 1985) and opened the way to examine genes and their mutations at the organismal level. Transgenically, genes can be manipulated to cause loss of function, gain of function, the addition of a new gene or exploration of activation and/or deactivation of genes during critical times of gene expression. This ability to modulate gene expression *in vivo* enables elucidation of the cascade of genetic interactions and eventually helps us determine whether these interactions are direct, indirect or working along parallel pathways. Untangling of genetic interactions is often difficult due to the complex, non-linear influences that genes and other modulating factors have on each other's expression. For example, the C57BL/6J background diminishes the penetrance of the $Ednrb^{S\text{-}l/+}$ hypopigmentation phenotype while augmenting the aganglionosis of $Sox10^{Dom/+}$ mice when compared to other background strains (Cantrell et al., 2004).

Gene expression in higher eukaryotes is predominantly regulated by transcriptional control; and it is this highly coordinated, tightly controlled activation and deactivation of genes that dictates cellular differentiation. A direct genetic interaction implies that one gene binds directly with another gene, as in the case of *Sox10* and *Mitf* where *Sox10* binds to the proximal region of the *Mitf* promoter activating it in melanoblasts (Bondurand et al., 2000; Hornyak et al., 2001; Potterf et al., 2000). An example of an indirect genetic interaction is that of *Sox5* and *Sox10*: *Sox5* binds and recruits *CtBP2* and *HDAC1* as transcriptional co-repressors that bind to the *Sox10* regulatory region thereby decreasing *Sox10* activity (Stolt et al., 2008). In some cases, mutations in multiple genes can cause a disease without ever interacting with each other as in the case of Hirschsprung's disease whereby mutations in *Ret* (Edery et al., 1994) or mutations in *Ednrb* (Puffenberger et al., 1994) result in similar disease phenotypes. In addition to these possibilities, enhancer regions can have binding sites for multiple transcription factors. This complexity adds to the difficulty of deciphering specific gene functions, sometimes necessitating more than one approach in order to elucidate whether or not an interaction exists.

In one study *Sox10* was shown to bind directly to an *Ednrb* enhancer in migrating murine enteric neuroblasts (Zhu et al., 2004). In fact, *Sox10* had 3 binding sites on the *Ednrb* enhancer and depending on the binding site(s) that were affected and the extent of the mutation, the penetrance and variability of the phenotype appeared to be affected. The pigmentation phenotype was not rescued suggesting that *Sox10* regulation of *Ednrb* through this particular

enhancer might function in enteric neurons and not in melanoblasts. However in another study, the expression of *EDNRB* in human melanocytes appeared directly dependent on *Sox10* (Yokoyama et al., 2006). In fact in the presence of *Sox10* siRNA, EDNRB protein was reduced by 46% and the presence or absence of *Sox10* affected *EDNRB* promoter activity. Three putative *SOX10* binding sites on the *EDNRB* promoter were evaluated and all three dramatically influenced *EDNRB* expression. Another transcription factor, *Sp1*, was found to work in conjunction with *Sox10* to upregulate *EDNRB* even more than *SOX10* alone and may contribute to specificity of *SOX10* activation in the melanocyte lineage.

Using a transgene is another way in which to evaluate whether or not genetic interactions exist: typically a transgene adds to the genome but can also replace a gene, either entirely or partially, essentially creating a knock-out or knock-in mutant depending on the resulting gene expression. The Dct-*Ednrb* transgene used here delivers additional *Ednrb* to the *Dct* expressing melanoblasts of the murine embryo with expression at E11.5 confirmed (Ittah, 2005). *Dct* has been previously shown to start being expressed during E11.5 – E12.5 (Mackenzie et al., 1997; Potterf et al., 2001; Steel et al., 1992) and is a well know melanoblast marker (Hornyak et al., 2001; Zhao and Overbeek, 1999). In fact, the *Dct* promoter has been used before to successfully deliver specific genes to melanoblasts (Hakami et al., 2006; Zhao and Overbeek, 1999) and also to track migrating melanoblasts (Silver et al., 2008).

Recently, a *Sox10* transgene was put under the control of the Dct promoter to see if additional *Sox10* in an *Ednrb* mutant mouse could rescue the hypopigmentation phenotype (Hakami et al., 2006). While, the Dct-*Sox10* transgene was able to adequately rescue *Sox10* heterozygosity (implying that the transgene correctly targeted *Sox10* positive cells), the Dct-*Sox10* transgene was unable to rescue the pigmentation phenotype of $Ednrb^{S\text{-}l/+}$ mice. In this study I will attempt to rescue the $Sox10^{LacZ/+}$ hypopigmentation phenotype via the Dct-*Ednrb* transgene. If the transgenic addition of *Ednrb* can rescue the hypopigmentation of *Sox10* heterozygosity, these results taken together with those results from Hakami et al. (2006), will imply that *Sox10* directly regulates *Ednrb* expression during the critical window of melanoblast expansion.

3. Materials and Methods

3.1 Animals and Genotyping

$Sox10^{LacZ}$, $Pax3^{Sp}$ and $Ednrb^{s\text{-}l}$ animals were maintained and genotyped as described in Chapter 2. $Sox10^{LacZ/LacZ}$ are embryonic lethal at approximately E13.5; since embryos were harvested at E11.5 and E12.5 developmental changes in the *Sox10* homozygous condition were observed (Appendix II.C). Staged embryos were dated 0.5d at noon the day of vaginal plug. Embryos were harvested by euthanizing the pregnant dam via cervical dislocation and dissecting embryos through a transverse abdominal incision. Embryos were immediately placed in PBS (the pH of which was determined by the subsequent procedure, see Appendices II.D and II.H) and yolk sacs were saved for DNA extraction (Appendix II.B). Those destined for double staining with LysoTracker

Red and LacZ, were immediately started on that protocol (Appendix II.H). Embryos for in situ hybridization were dehydrated through a methanol (MeOH; Fisher Scientific) series (25%:75% MeOH:PBS; 50%:50%; 75%:25% MeOH:PBS for 5min each) and stored in 100% MeOH at -20ºC.

Dct-*Ednrb* transgenic animals were generated in our laboratory (Avner Ittah, FIU dissertation 2005). The full length *Ednrb* cDNA (1958bp, genbank accession # U32329**)** obtained from pmET-B (Hosoda et al., 1994) was inserted into the vector pmDct that contains 3431 bp of the mouse *Dct* promoter and the BGH poly adenylation signal (Hornyak et al., 2000). Dct-*Ednrb* transgenic mice were generated by microinjection of the transgene into FVB F_1 zygotes. The studies presented here were carried out with a line that initially had 150 copies of the transgene. This line was crossed for over 10 generations with C57BL/6J mice from our colony. Mice were genotyped using 1μM of each primer Dct-*Ednrb*f (5'-ACAAGGAAGACTGGCGAGAA-3'); and, Dct-*Ednrb*r (5'-TCCTCCCCCTTGCTGTCCTGC-3'; Invitrogen), 1X buffer, 2mM MgCl, 1 unit Taq (Bioline), 5% DMSO (Fisher Scientific), 2.5mM of each dNTP (Invitrogen) and 1μl DNA. The amplified DNA was visualized on a 1% agarose gel resulting in a 2217bp band (Appendix I).The PCRs were performed for 35 cycles under the following conditions: 94°C for 30 s, 57°C for 30 s, and 72°C for 2 min.

All animals used in this study were housed in the Animal Care Facility at Florida International University (Miami, FL). Water and murine chow were fed *ad libitum* and light/dark was cycled every 12 hours. All animal work was

performed according to institutional guidelines established by NIH (Guide for the Care and Use of Laboratory Animals, 2009; IACUC protocol # 08-001).

3.2 Crosses and Phenotype Analysis

Sox10$^{LacZ/+}$ mice were crossed with Dct-*Ednrb* mice to produce offspring that were *Sox10*$^{LacZ/+}$::Dct-*Ednrb*. These transgenic *Sox10*$^{LacZ/+}$ mice were examined for the presence of pigmentation defects over the course of 4 generations. *Pax3*$^{Sp/+}$ mice were also crossed with Dct-*Ednrb* mice to produce offspring that were *Pax3*$^{Sp/+}$::Dct-*Ednrb* in order to evaluate potential changes in pigmentation defects for 1 generation.

Sox10$^{LacZ/+}$::Dct-*Ednrb* mice were crossed to each other to produce E11.5 and E12.5 embryos that were *Sox10*$^{LacZ/LacZ}$::Dct-*Ednrb* and *Sox10*$^{LacZ/+}$::Dct-*Ednrb*. *Sox10*$^{LacZ/LacZ}$ (with or without the transgene) often times disintegrated during dissection or during the procedures of in situ hybridization or Lysotracker Red/*LacZ* but all embryos, regardless of presentation or staining, were genotyped.

3.3 In Situ Hybridization

In situ hybridization (ISH) was performed on E11.5 embryos using the *Pmel17* riboprobe. *Pmel17* is a highly sensitive and early melanoblast marker with expression starting at E9.5 (Baxter and Pavan, 2003). The full length cDNA (1935bp, genbank accession number M77348) was inserted into DH5α *Escherichia coli* and this plasmid was purified and linearized prior to *Pmel17* riboprobe synthesis (Appendix II.E). *Pmel17* structure and homology to other melanocytic markers has been previously described by Kwon et al. (1991).

On day 1 of ISH, embryos were rehydrated via a MeOH series using PBT* (PBS** plus 0.1% Tween-20; Fisher Scientific) 5min for each of the following steps: 75%:25% MeOH:PBT; 50%50%; 25%:75% MeOH:PBT and two washes in 100% PBT. Embryos were then bleached in 6% H_2O_2 (Fisher Scientific) in PBT for 1hr at RT and then permeabilized using 10µg/ml proteinase K (Promega) in PBT (15min for E11.5 and 18min for E12.5). To neutralize this reaction, 2mg/ml glycine (Fisher Scientific) in PBT was added for 10min at RT and then washed twice with PBT. Embryos were postfixed with 4% paraformaldehyde (Fisher Scientific), 0.2% gluteraldehyde (Sigma) in PBT for 20min. Pre-hybridization was accomplished by adding 1ml hybridization solution (50% formamide, 5xSSC pH4.5 1% SDS, Fisher Scientific; 50µg/ml yeast tRNA, Sigma; 50µg/ml heparin, Sigma) and incubated in a 70ºC water bath for 1hr. The pre-hybridization solution was removed and fresh hybridization solution containing 2µl *Pmel17* riboprobe per ml of hybridization solution added and left overnight in the 70ºC water bath.

Day 2 consisted of embryos being briefly rinsed in solution 1 (50% formamide, 5xSSC and 1%SDS; Fisher Scientific) and then washed 3 times more with solution1 (30min each time in a 70ºC water bath). Washing was continued, 3 more times, using solution 2 (50% formamide and 2xSSC; Fisher Scientific) and placed in a 65ºC water bath for 30min each time, followed by washing with TBS plus 0.1% Tween-20 (TBST) 3 times for 5min each time. Blocking of embryos in 10% goat serum (Fisher Scientific) in TBST for 2hrs, rocking at RT, during which time the preabsorb anti-digoxigenin antibody was

prepared by adding a pinch of embryo powder to microcentrifuge tubes containing 0.5ml TBST (1 microcentrifuge tube/embryo) and placed in 70ºC water bath for 30min. Microcentrifuge tubes were removed from water bath, vortexed and cooled prior to the addition of 5µl of 100% goat serum and 1µl anti-digoxigenin antibody (Roche) and rocked at 4ºC for 1hr. After centrifuging for 5min, the supernatant was collected and 1.5ml of 1% goat serum in TBST per microcentrifuge tube was added. When embryos were ready, the blocking solution was removed, the antibody solution added and embryos were left rocking overnight at 4ºC.

To remove the antibody, on day 3 embryos were briefly rinsed in TBST and then washed 4 times in TBST for 1hr each time at RT. Levamisole (2mM; Sigma) was added to a 5th TBST wash and left overnight, rocking at 4ºC. Finally, day 4 was the detection of the antibody in which the embryos were washed in NTMT (100mM NaCl, 100mM Tris pH9.5, 50mM MgCl2, 0.1% Tween-20; Fisher Scientific; 2mM levamisole; Sigma) 3 times for 10min each time. While protecting from light, 45µl 4-Nitro blue tetrazolium chloride (NBT; Roche) and 35µl 5-Bromo-4-chloro-3-indolyl-phosphate (BCIP; Roche) were added to 10ml NTMT and added to each embryo. Still protected from light, embryos were monitored every 30min to control the level of antibody development (Wilkinson 1998).

Cells labeled with the *Pmel17* probe that were located in the torso were counted from a picture taken of the area at 3.2x magnification. The angle of lines drawn to define the area intersected the posterior aspect of the forelimb

and the anterior aspect of the hindlimb. Lines drawn to define the area, lighting and magnification were kept constant for all pictures taken.

3.4 LysoTracker Red and *LacZ* Staining

Embryos were dissected and rinsed in cold 0.05M PBS (pH7.2) and placed in 5μM LysoTracker Red DND-99 (Invitrogen), protected from light, at 37ºC for 30min. Continuing to protect the embryos from light, they were washed twice in PBS, 2% paraformaldehyde (PF; Fisher Scientific) for 1hr at room temperature (RT), washed twice more with PBS and then left overnight at 30ºC in *LacZ* solution (5μM ferricyannide, 5μM ferrocyannide, 2mM $MgCl_2$, 0.01% sodium deoxycholate (Fisher Scientific), 0.02% nonidate p-40 (Roche) and 1mg/ml X-gal (Invitrogen). Rinsed twice in PBS to stop reaction and a 10% sucrose (Fisher Scientific) solution was added to embryos for overnight incubation at 4ºC, rocking gently. Finally, embryos were placed in 20% sucrose solution for 4-6hrs and embedded in Tissue-Tek Tissue Freezing Medium (Sakura). Embryos were stored at -80ºC.

Transverse cryosections of 10μm were taken of embryos from the brachial nerve to the sciatic nerve. Every third section was kept from E11.5 embryos while every 4^{th} section was kept from E12.5 embryos. Of these sections, 30 equally spaced sections (e.g. every other section) were selected for counting. Cells that were blue (*LacZ*+), elongated and appeared to be traveling along the dorsolateral pathway were counted and checked for double labeling with Lysotracker Red.

4. Results

4.1 Dct-*Ednrb* Transgene Rescues the *Sox10*$^{LacZ/+}$ and Partially Rescues the *Pax3*$^{Sp/+}$ Hypopigmentation Phenotypes

The transgenic delivery of *Ednrb* to the melanoblasts of *Sox10*$^{LacZ/+}$ revealed that the hypopigmentation caused by *Sox10* heterozygosity can be rescued by additional *Ednrb* (Figure 1). In fact, while 47% (n=83) of *Sox10*$^{LacZ/+}$ mice did not exhibit ventral hypopigmentation (53% did; n= 95); 100% (n=33) of the *Sox10*$^{LacZ/+}$ carrying the transgene had fully colored coats (Table 1).

In contrast, the *Pax3*$^{Sp/+}$ hypopigmentation was only partially rescued by the transgene. The hypopigmentation defect of *Pax3*$^{Sp/+}$ is completely penetrant; all Pax3 heterozygous mice have ventral hypopigmentation. *Pax3*$^{Sp/+}$::Dct-*Ednrb* mice exhibited no hypopigmentation 24% (n=5) of the time and a partial rescue (considerably smaller area of hypopigmentation) 10% (n=2) of the time. The other 66% of the animals displayed typical *Pax3* heterozygous hypopigmentation (Figure 2, Table 2).

4.2 Transgenic Embryos Show Increased Numbers of Melanoblasts

In situ hybridization using *Pmel17* as a marker shows an increased number of melanoblasts in the transgenic embryos when compared to the non-transgenic embryos of the same genotype (Table 3). Easily apparent in E11.5 embryos, counts taken of trunk melanoblasts supports this observation. Comparison of *Sox10*$^{LacZ/+}$ embryos to *Sox10*$^{LacZ/+}$::Dct-*Ednrb* embryos results in a significant increase in trunk melanoblasts (p=0.04). Unexpectedly, an even more significant increase in trunk melanoblasts (p=0.01) was seen when

comparing wildtype (*Sox10*$^{+/+}$) embryos to transgenic wildtype (*Sox10*$^{+/+}$::Dct-*Ednrb*) embryos. Three embryos were counted for each genotype (Figure 3).

4.3 Increased Numbers of Melanoblasts in Transgenic Embryos Cannot Be Accounted for by an Effect on their Survival

Double staining with LysoTracker Red and *LacZ* indicate that the increased numbers of melanoblasts observed in transgenic embryos is not due to a putative role of the transgene in preventing the apoptosis of melanoblasts in *Sox10*$^{LacZ/+}$ embryos at E11.5 and E12.5. *LacZ*+ cells traveling along the dorsolateral pathway that were morphologically identified as melanoblasts were counted and then checked to see if they were double labeled with LysoTracker Red (Tables 4 and 5). While cell death was seen throughout the embryo in a characteristic pattern, there were very few *LacZ*+ cells that also stained positive for LysoTracker Red (figures 4 and 5). In fact, in E11.5 embryos it was very rare that a cell was double stained for *LacZ* and LysoTracker Red (Table 4). 11.5 day old homozygous *Sox10* embryos rarely had a *LacZ*+ cell while the transgenic *Sox10* homozygous embryo did have a few (between 4 and 16) LacZ+ cells but there was no significant difference (p=0.26). *Sox10* heterozygous embryos had *LacZ*+ 38-67 cells but the transgenic *Sox10* heterozygous embryos had significantly more *LacZ*+ cells (135-180 cells; p=0.003).

Rapid increase in the amount of these *LacZ*+ (presumptive melanocytic) cells was seen when comparing numbers obtained from E11.5 (Table 4) and E12.5 (Table 5) embryos. Interestingly, not only do the numbers increase tremendously in the *Sox10*$^{LacZ/+}$ embryos from E11.5 to E12.5 (with or without

the transgene), but the number of cells increased somewhat in the homozygous embryos as well. In the E12.5 *Sox10*$^{LacZ/+}$ embryos, the number of *LacZ*+ cells increased significantly in the transgenic heterozygous embryos (p=0.012) when compared to the heterozygous embryos without the transgene; while in the homozygous embryos there was no significant difference in the number of *LacZ*+ cells between *Sox10*$^{LacZ/LacZ}$ and *Sox10*$^{LacZ/LacZ}$::Dct-*Ednrb*. There were several more double stained cells (0-20) at this age in the heterozygous embryos but this seemed to be relative to the total number of *LacZ*+ cells observed. *Sox10* homozygous mutants were difficult to obtain at E12.5 as they often disintegrated during dissection or during the staining process.

5. Discussion

In vivo analysis of coat color and embryonic melanoblasts indicated that the *Sox10* hypopigmentation phenotype was consistently rescued by the transgenic addition of *Ednrb*; in the case of *Pax3* hypopigmentation phenotype the additional *Ednrb* conferred a partial rescue on coat color. *Sox10*, which is colocalized with *Ednrb*, may be required as premigratory melanoblasts emerge to ensure melanoblast survival and differentiation (Potterf et al., 2001). *Dct*, a well known melanocyte marker (Hornyak et al., 2001; Zhao and Overbeek, 1999), is partially regulated by *Sox10* (Jiao et al., 2004; Ludwig et al., 2004; Potterf et al., 2000) so that in *Sox10* heterozygous mice the level of *Dct* expression is less than it would normally be in melanoblasts of wildtype mice. *Sox10* is known to activate *Mitf*, a major melanocyte-specific transcriptional regulator and *Mitf* in turn regulates *Dct* expression (Bondurand et al., 2000;

Yokoyama et al., 2006). In the case of *Sox10* heterozygosity, *Mitf* activation is diminished as is *Dct* expression with *Dct* expression being affected by decreased levels of both *Sox10* and *Mitf*. In $Sox10^{Dom/+}$ embryos, *Dct* expression is greatly reduced but melanoblasts still populate the embryo in a temporally and spatially correct manner with a dramatic increase in *Dct* expression at E13.5 and normal melanoblast population by E14.5 (Potterf et al., 2001). Melanoblasts in $Sox10^{LacZ/+}$ embryos also appear to migrate in a temporally and spatially correct manner at E11.5 and E12.5. *Sox10* haploinsufficiency must provide sufficient *Ednrb* activation so that melanoblasts migrate normally and populate most of the embryo, except for the ventral aspect. Trunk melanoblasts migrate at a later time than do other melanoblast populations (Serbedzija et al., 1990; Yoshida et al., 1996) and therefore may have a later requirement for *Ednrb*. Since the critical window for *Ednrb* expression in melanoblasts lasts through E12.5, the use of a *Dct* promoter, whose expression begins at E11.5, appears to act early enough to compensate for the decreased amount of *Sox10* activity and rescue the melanoblasts.

While a Dct-*Sox10* transgene could not rescue the hypopigmentation phenotype of $Ednrb^{S-l/+}$ (Hakami et al., 2006), the Dct-*Ednrb* transgene consistently rescued the hypopigmentation phenotype of the $Sox10^{LacZ/+}$ mice. Dct-*Sox10* adds more *Sox10* to a system with limited *Ednrb*. It may be that once the limited amount of functional *Ednrb* has been fully activated by *Sox10* the addition of more *Sox10* does nothing because there are no more binding sites available on *Ednrb*. Conversely, the transgenic addition of *Ednrb* to a

Sox10$^{LacZ/+}$ does what *Sox10* would have normally done – provide sufficient levels of *Ednrb* signaling to promote proper melanoblast dispersal and thereby rescues the hypopigmentation phenotype of the *Sox10* heterozygous mutant.

Pax3$^{Sp/+}$ mutants, which exhibit a completely penetrant ventral hypopigmentation phenotype, experienced a partial rescue with the Dct-*Ednrb* transgene. Present by E8.5 (Goulding et al., 1991), *Pax3* is important in the initiation of the melanogenic pathway while simultaneously acting in the prevention of terminal differentiation (Lang et al., 2005). Similar to *Sox10*, *Pax3* activates *Mitf* (which then activates *Dct*) but unlike *Sox10*, *Pax3* competes with *Mitf* for binding sites on *Dct* (Fuchs et al., 2004; Potterf et al., 2000; Yasumoto et al., 2002). *Dct* expression is normal in *Pax3*$^{Sp/+}$ embryos at E11.5 indicating that *Dct* expression is not affected by *Pax3* haploinsufficiency (Potterf et al., 2001). Prior to *Mitf* activation of *Dct*, *Mitf* is present in the dorsal aspect of the neural tube and is important in the survival of premigratory melanoblasts shortly before they reach the MSA and for a short while after they start migrating (Hornyak et al., 2001). It is possible therefore, that *Pax3* may exert influence over the expression of *Mitf* at this early stage (E8.5-E10.0) as well and that the hypopigmentation phenotype seen in *Pax3* heterozygous mice is at least partially due to this early insult on *Mitf*. If melanoblasts are more severely affected by decreased levels of *Pax3* at the premigratory phase rather than later once *Dct* is expressed, then the transgenic expression of *Ednrb* may be too late to rescue coat color in the *Pax3* mutant.

In order to elucidate the timing of the rescue in *Sox10*$^{LacZ/+}$ embryos, in situ hybridization with *Pmel17* was performed on E11.5 as this is the first time melanoblasts are migrating throughout the length of the embryo. *Pmel17* hybridizes to a larger number of murine melanoblasts than does *Dct* and at an earlier age (E10.5 vs. E11.5), as presumptive melanoblasts are starting to emerge (Baxter and Pavan, 2003). Initially identified in human and murine DNA (Kwon et al., 1991), *Pmel17* is now used in a variety of animals including horses (Brunberg et al., 2006), dogs (Clark et al., 2006) and chickens (Kerje et al., 2004) to study pigmentation genetics. Sensitivity of *Pmel17* to identify melanoblasts may be due to the fact that it is the only melanosomal component that has membrane bound and soluble forms (Valencia et al., 2006), possibly making detection easier.

The *Pmel17* promoter is not regulated by *Sox10* (Loftus et al., 2009) implying that expression of *Pmel17* is not influenced by *Sox10* haploinsufficiency. In addition to its early expression, being *Sox10*-independent makes *Pmel17* an excellent candidate for identifying whether or not the transgenic addition of *Ednrb* into a *Sox10* heterozygote increases the number of melanoblasts. By E11.5, significant differences in the number of melanoblasts were seen between *Sox10*$^{LacZ/+}$ and *Sox10*$^{LacZ/+}$::Dct-*Ednrb* embryos and interestingly, between wildtype and wildtype transgenic embryos. It appears that increasing the amount of *Ednrb* in the melanocyte lineage allows for greater expansion of migrating precursors.

Since the *LacZ* reporter gene has been knocked into the *Sox10* locus, migrating melanoblasts (and other *Sox10* positive cells) can be easily identified. Double staining of embryos was employed to explore whether the melanoblast density difference between transgenic and non-transgenic embryos was due to increased apoptosis in the non-transgenic embryos. As this was not the case, the increased numbers of melanoblasts in transgenic embryos may be due to a variety of reasons. The Dct-*Ednrb* transgene may have compensated for the lack of *Sox10* activation by saturating the system with *Ednrb* thereby increasing any survival component that *Ednrb* bestows on melanoblasts as they emerge from the MSA. Since *Ednrb* appears to be required for the migration of emerging melanoblasts (Shin et al., 1999), the additional *Ednrb* may have compensated for the haploinsufficient *Sox10* activation of *Ednrb* and may ensure proper melanoblast migration. And finally, given that *Edn3*, the preferred ligand for *Ednrb*, is a known proliferative agent for this lineage (Lahav et al., 1996) perhaps the increase in functional receptor allowed for an increase in *Edn3* proliferative action resulting in an even larger number of melanoblasts at E11.5 and E12.5. Taken together, these results support the direct interaction of *Sox10* and *Ednrb* because it is the *Sox10* activation of *Ednrb* which enables the receptor to promote survival, migration and subsequently the proliferation of emerging melanoblasts needed to rescue the pigmentation phenotype.

While *Sox10* and *Pax3* work together to transactivate *Mitf* in the melanocyte lineage, it appears that the relationship of *Pax3* to *Ednrb* is more complex than that of *Sox10* and *Ednrb*. As shown earlier, mice harboring both $Pax3^{Sp/+}$ and

Ednrb$^{S\text{-}l/+}$ mutations exhibited aggravated pigmentation phenotypes (Chapter 2), yet there is only a small partial rescue of the coat color with the transgenic addition of *Ednrb* in the *Pax3*$^{Sp/+}$ mutant. Perhaps an interaction between *Pax3* and *Ednrb* does exist but in a much more indirect fashion with one or more intermediate transcription factors or signaling pathways in the genetic cascade. Or perhaps the insult of *Pax3* haploinsufficiency on neural crest specification is too great for *Ednrb* to rescue. And finally, it is possible that the *Dct* promoter used to deliver the *Ednrb* becomes active too late to completely rescue melanoblasts from their *Pax3* deficiency. Any of these possibilities would explain the increased hypopigmentation of the double mutant and the lack of rescue using the Dct transgene.

In conclusion, the complete rescue of hypopigmentation in the *Sox10* heterozygote supports an interaction between *Sox10* and *Ednrb* in the melanocyte lineage. Still unclear is the mode of rescue although there may be a putative survival effect of the transgene by E11.5. Future experiments should include looking earlier in development to evaluate whether or not cell death is occurring prior to E11.5 in *Sox10* heterozygous mice. Proliferation assays (such as labeling with P-Histone3) from the time that premigratory melanoblasts emerge from the MSA until E12.5 when melanoblasts have populated the entire length of the embryo may help illuminate the mode of rescue as well. In regards to the *Pax3* heterozygous condition, the possible interaction between *Pax3* and *Ednrb* remains elusive. Evaluating possible effects of *Pax3* haploinsufficiency on neural crest specification and on *Mitf* expression prior to and during the time

premigratory melanoblasts begin their journey may help elucidate any interaction, albeit indirect, between *Pax3* and *Ednrb* in the melanocyte lineage.

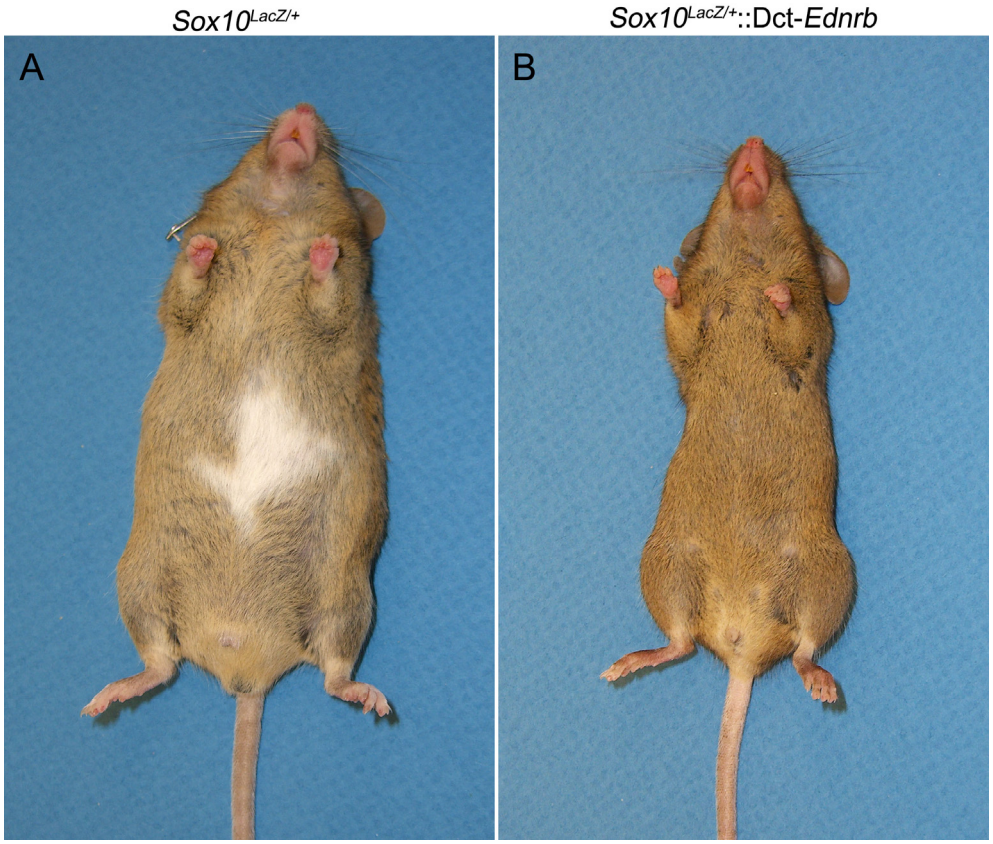


Figure 3.1 Transgenic rescue of the *Sox10*$^{LacZ/+}$ pigmentation phenotype. (A)Typical hypopigmentation of *Sox10*$^{LacZ/+}$ mice. (B)The targeted addition of *Ednrb* to the melanocyte precursors consistently rescued the hypopigmentation phenotype of *Sox10*$^{LacZ/+}$ mice.

	BS	No BS
Sox10$^{LacZ/+}$ (n=188)	53% (n=95)	47% (n=83)
Sox10$^{LacZ/+}$ w/Dct::*Ednrb* (n=33)	0%	100% (n=33)

Table 3.1 Complete rescue of *Sox10*$^{LacZ/+}$ pigmentation phenotype by Dct-*Ednrb* transgene. Targeted addition of *Ednrb* to melanoblasts rescues the pigmentation phenotype in *Sox10* heterozygous mice 100% of the time. Homozygous *Sox10* embryos did not have rescued melanoblasts and were still embryonic lethal, possibly due to the more serious developmental anomalies incurred by the near complete absence of *Sox10*. BS = Belly Spot.

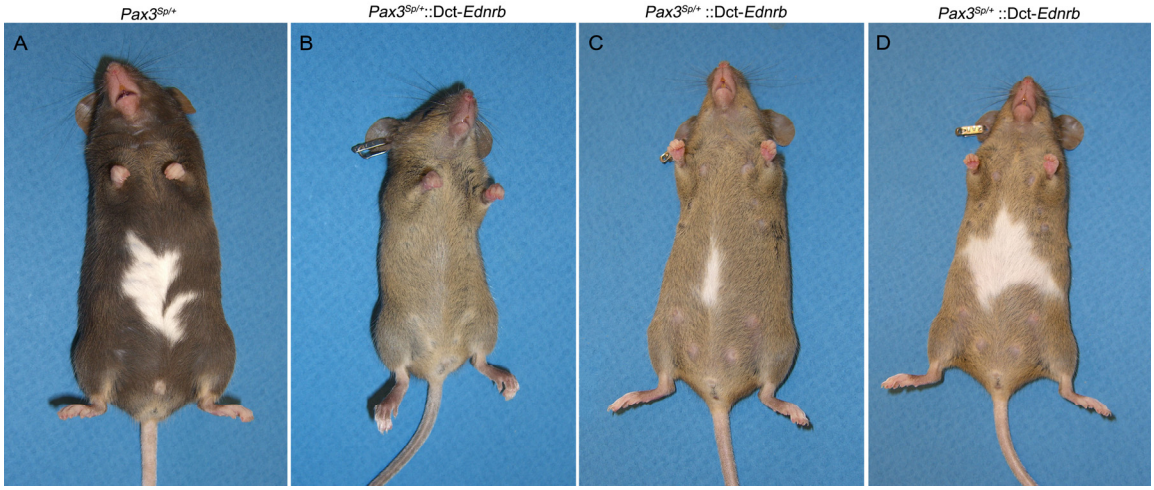


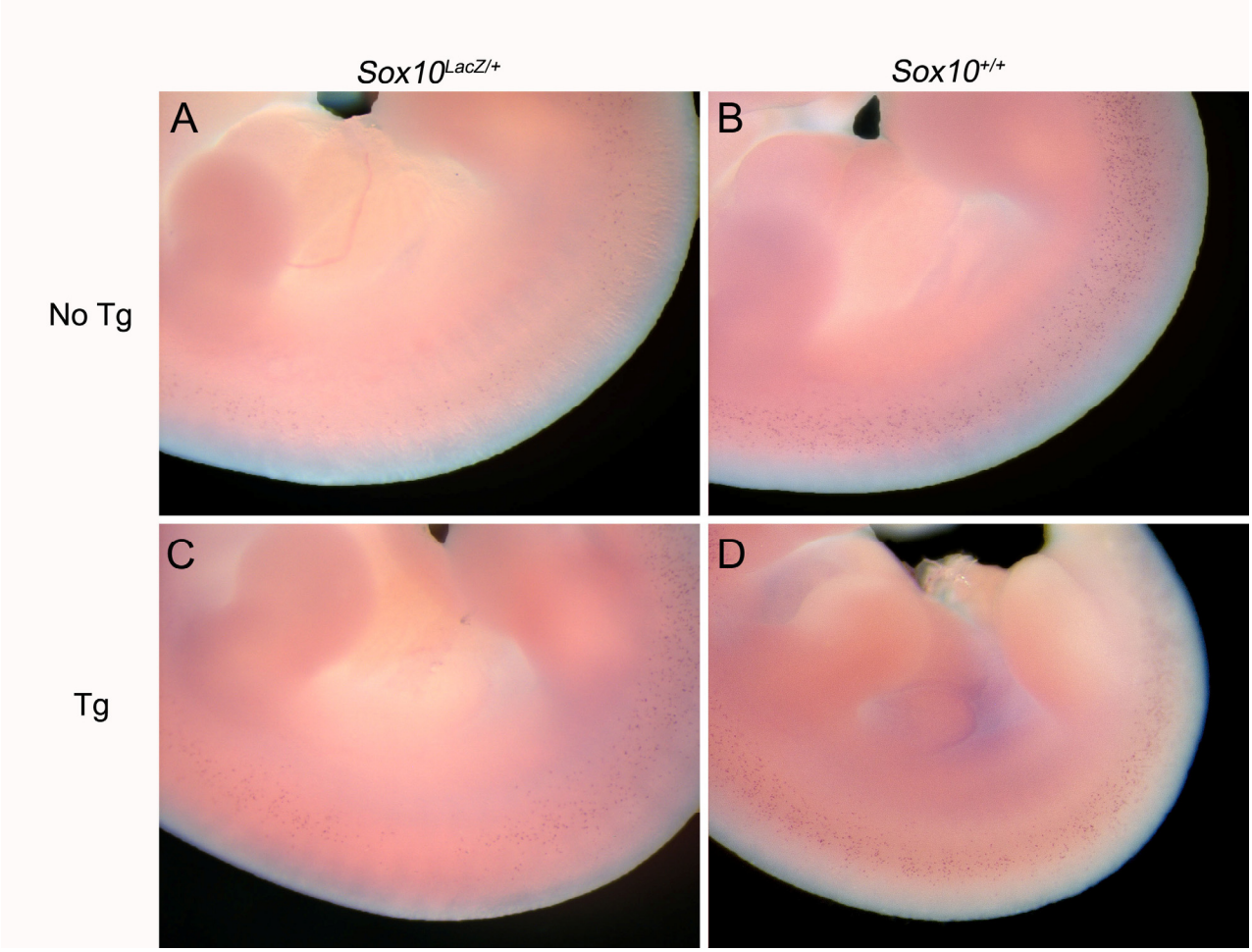
Figure 3.2 Dct-*Ednrb* transgene partially rescues the *Pax3*$^{Sp/+}$ pigmentation phenotype. (A) Mice who did inherit the transgene have the characteristic *Pax3*$^{Sp/+}$ pigmentation; transgenic delivery of *Ednrb* (B) completely rescues the *Pax3*$^{Sp/+}$ pigmentation phenotype 24% of the time and (C) partially rescues 10% of the time. (D) The transgene was not able to rescue 66% of the time.

	BS	Reduced BS	No BS
Pax3$^{Sp/+}$ (n=120)	100% (n=120)	0%	0%
Pax3$^{Sp/+}$ w/Dct::*Ednrb* (n=21)	66% (n=14)	10% (n=2)	24% (n=5)

Table 3.2 Transgenic expression of *Ednrb* partially rescues the *Pax3*$^{Sp/+}$ phenotype. Targeted addition of *Ednrb* to melanoblasts rescues the pigmentation phenotype in *Pax3* heterozygous mice nearly a quarter of the time and partially rescues 10% of the time. The transgene was not able to rescue melanoblasts in homozygous embryos, possibly due to the more serious developmental anomalies caused by the absence of *Pax3*, such as the lack of structures and tissues necessary to support life. BS = Belly Spot.

	Sox10$^{LacZ/+}$	*Sox10*$^{+/+}$
No Tg	161 229 108	325 445 385
Tg	279 255 305	623 613 531

Table 3.3 *Pmel17* labeled trunk melanoblasts. Cell counts for each of the embryos in which trunk melanoblasts were counted show an increase in the number of melanoblasts in the transgenic counterpart for each genotype: *Sox10*$^{LacZ/+}$ vs. *Sox10*$^{LacZ/+}$::Dct-*Ednrb* and *Sox10*$^{+/+}$ vs. *Sox10*$^{+/+}$::Dct-*Ednrb*. Tg = Transgene.


Sox10LacZ/+
Sox10+/+
No Tg
Tg
A
B
C
D

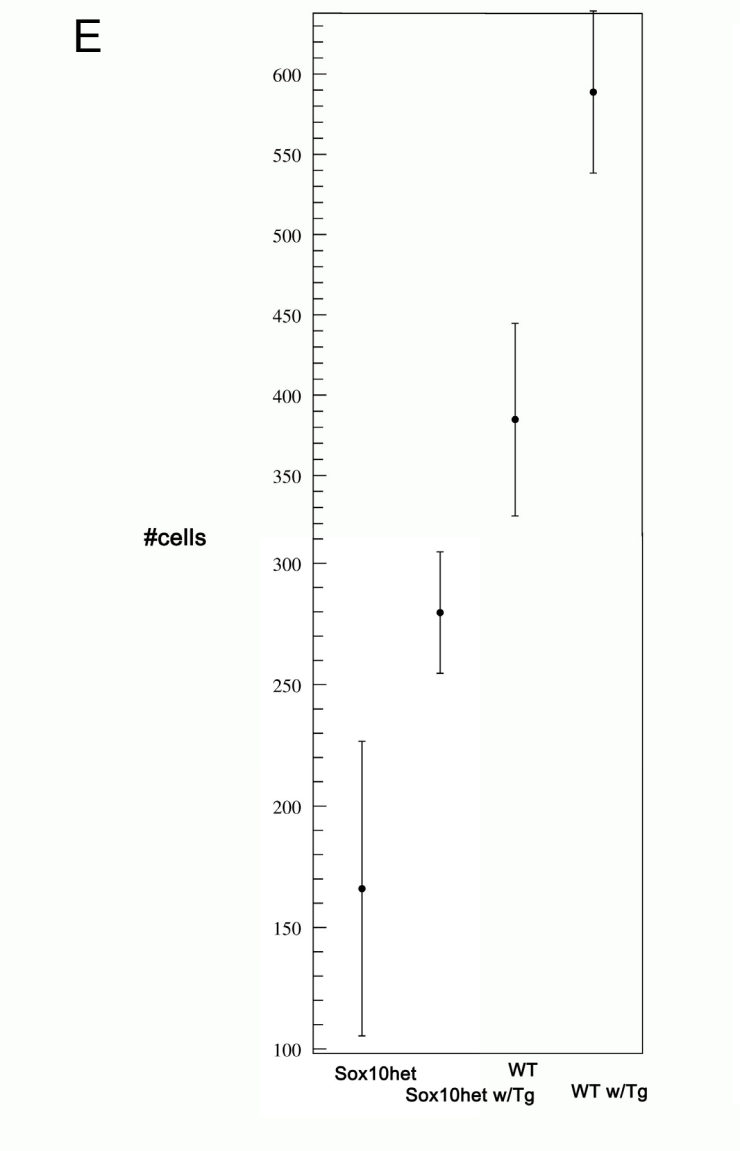


Figure 3.3 In situ hybridization with *Pmel17* riboprobe shows increased numbers of truck melanoblasts in transgenic embryos at E11.5. Non-transgenic embryos of both (A) *Sox10*$^{LacZ/+}$ and (B) wildtype embryos have fewer melanoblasts than their (C, D) transgenic counterparts. (E) *Pmel17* labeled cell count shows transgene increases number of melanoblasts in *Sox10*$^{LacZ/+}$ and in wildtype embryos. In each condition, the presence of Dct-*Ednrb* significantly increased the number of melanoblasts. *Sox10*$^{LacZ/+}$ vs. Transgenic *Sox10*$^{LacZ/+}$, p=0.04; wildtype vs. transgenic wildtype p=0.01. Three embryos were counted for each condition. No Tg = No Transgene; Tg = Transgene.

	Sox10$^{LacZ/LacZ}$	*Sox10*$^{LacZ/+}$
No Tg	1*/0** 0 0	67/0 38/0 38/0
Tg	4/0 16/1 0	143/0 180/0 135/0

Table 3.4 Transgenic rescue of *Sox10* haploinsufficient cells is not due to an effect on melanoblast survival at E11.5. *LacZ* and LysoTracker Red double stained *Sox10* positive cells indicate that apoptosis is not the cause of decreased melanoblasts in E11.5 embryos. It is a rare cell in E11.5 embryos that is stained by *LacZ* and also stained by Lysotracker Red, even though ample cell death is seen throughout the embryo. *Number of *LacZ*+ cells; **Number of LysoTracker Red+ cells. No Tg = No Transgene; Tg = Transgene.

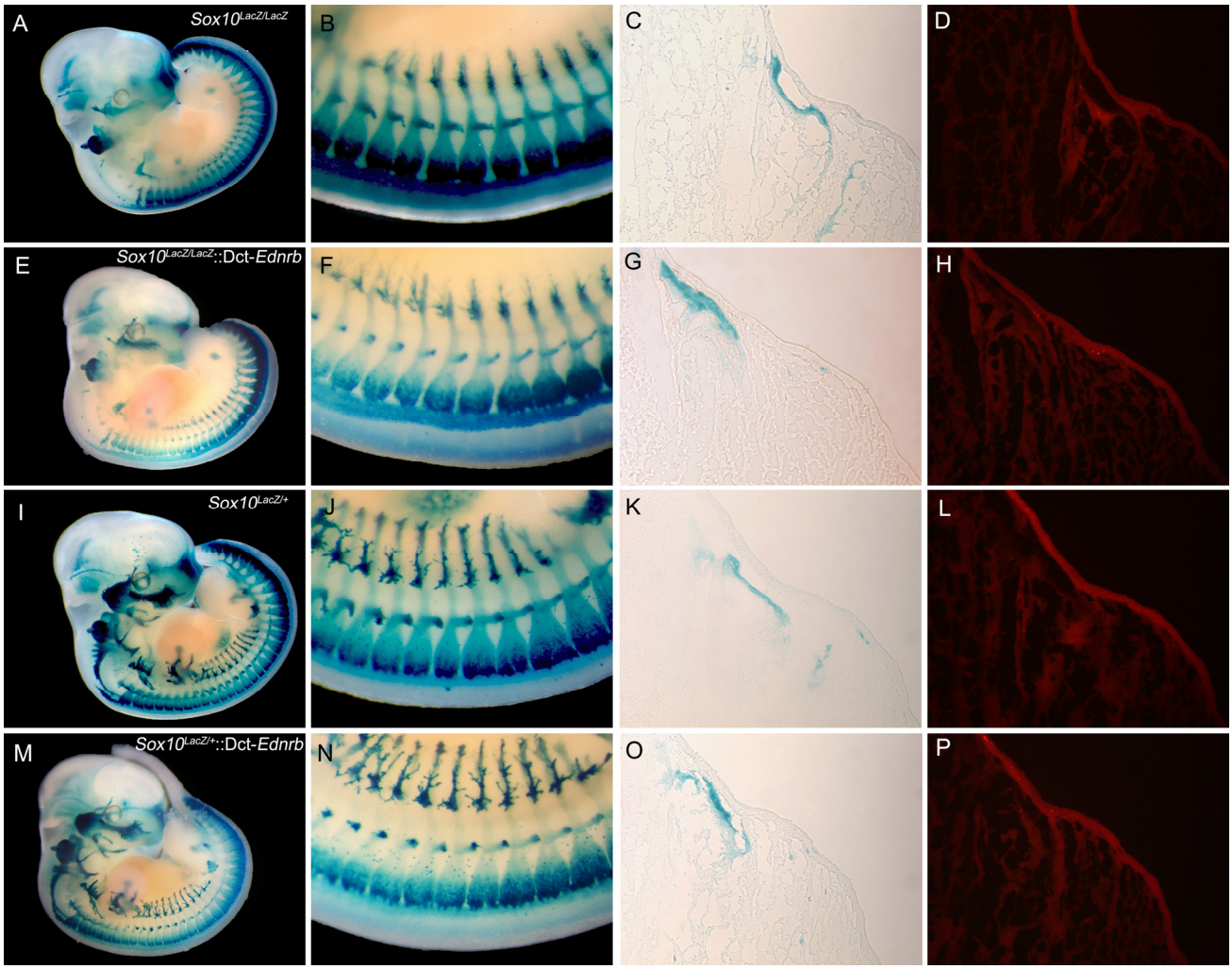


Figure 3.4 *LacZ* staining of E11.5 *Sox10* embryos shows transgenic rescue of melanoblasts in *Sox10* heterozygotes. (A,B) *Sox10* homozygous embryos appear void of melanoblasts in the truck region, (E,F) regardless of transgenic expression of Ednrb. (C,D,G,H) In reality, homozygous embryos with or without the transgene have very few if any melanoblasts, none of which stained positive for LysoTracker Red. Melanoblasts are seen populating the trunk region in (I,J) $Sox10^{LacZ/+}$ embryos; (M,N) *Sox10* heterozygous embryos exhibit a large increase in the number of melanoblasts in transgenic animals. (K,L,O,P) Again, none of the melanoblasts were double labeled with LysoTracker Red implying that the difference in melanoblast numbers is not due to increased cell death at E11.5.

	Sox10$^{LacZ/LacZ}$	*Sox10*$^{LacZ/+}$
No Tg	7/0 19/6 12/0	166/4 207/0 148/0
Tg	17/0	987/10 622/6 1216/20

Table 3.5 Transgenic rescue of *Sox10* haploinsufficient cells is not due to an effect on melanoblast survival at E12.5. *LacZ* and LysoTracker Red double stained *Sox10* positive cells indicate that apoptosis is not the cause of decreased melanoblasts in E12.5 embryos. Very few cells in E12.5 embryos that are stained by *LacZ* are also stained by Lysotracker Red, even though ample cell death is seen throughout the embryo. *Number of *LacZ*+ cells; **Number of LysoTracker Red+ cells. No Tg = No Transgene; Tg = Transgene.

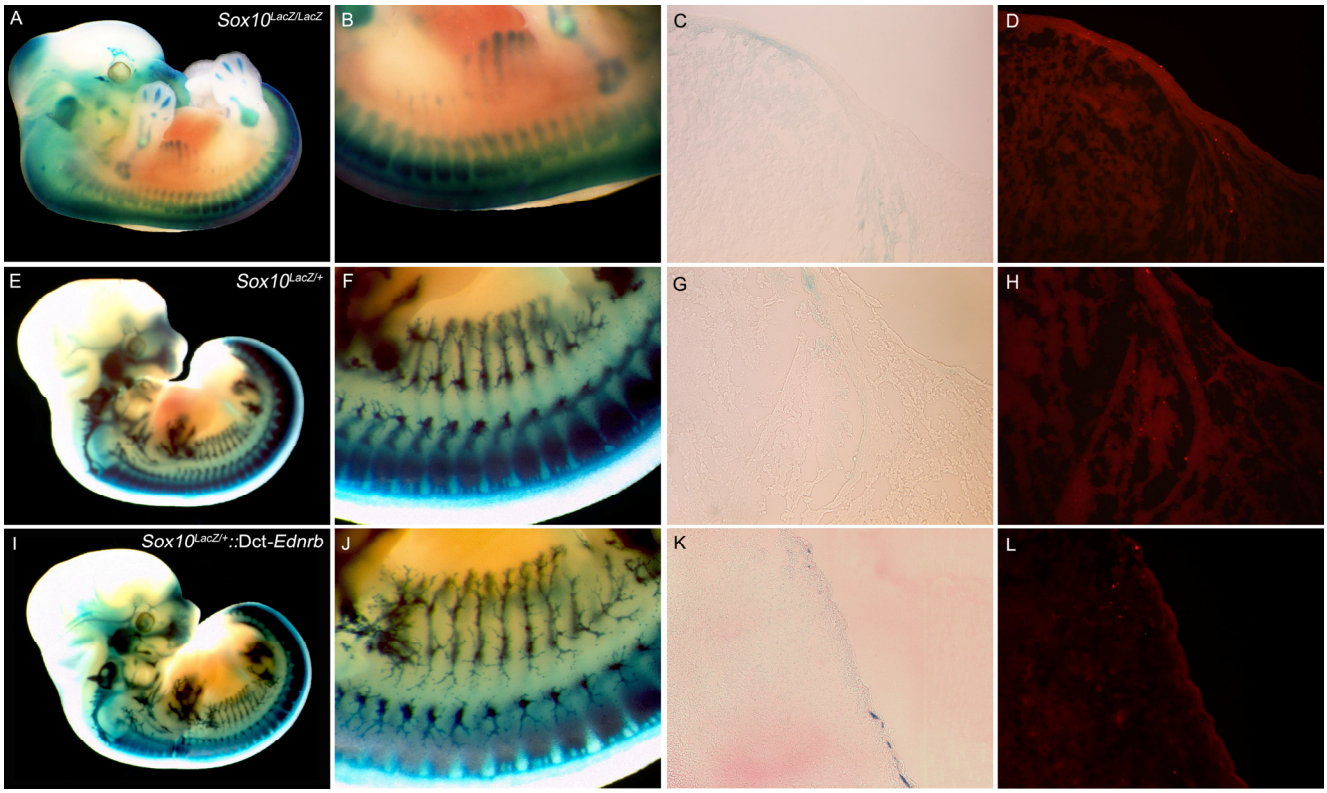


Figure 3.5 *LacZ* staining of E12.5 *Sox10* embryos shows transgenic rescue of melanoblasts in *Sox10* heterozygotes. (A,B) Homozygous *Sox10* embryos have relatively few melanoblasts that can be seen in (C) cryosections. (E,F) *Sox10* heterozygous embryos had visible melanoblasts that were also better visualized in (G) cryosections. (I,J,K) Transgenic *Sox10* heterozygous embryos had significantly more melanoblasts than non-transgenic heterozygous embryos. (D,H,L) No significant cell death was seen in any of the embryos regardless of genotype.

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Chapter IV.

CROSS BETWEEN *Ednrb*$^{S-l}$ AND *Pax3*^{Sp} RESULTS IN A NOVEL PHENOTYPE: HYDRCEPHALUS AND INTERRMITTENT SKELETAL DEFORMITIES

IV. CROSS BETWEEN *Ednrb*$^{S-l}$ AND *Pax3*^{Sp} RESULTS IN A NOVEL PHENOTYYPE: HYDROCEPHALUS AND INTERMITTENT SKELETAL DEFORMITIES

1. Abstract

A novel phenotype arose when studying the potential interaction between *Ednrb* and *Pax3*. All pups were born normal in appearance and behavior but by 3 to 3.5 weeks of age, the affected pups were smaller than their normal littermates and developed a dome-shaped head; some also developed distinct thoracic kyphosis. All affected pups were dead by 4 weeks of age except one which survived until the 8th week of life. **The goal of this study was to describe the basic characteristics seen in affected pups**. Seventy-five percent of affected pups were female although there was no gender bias based on genotype or generation. Of the affected animals, 80% were heterozygous for *Pax3*. Upon histologic examination, brains of affected mice had an enlarged 4th ventricle and increased glial processes when compared to brains of normal littermates. Skeletal staining showed kyphosis and rounded wide rib cages in those animals which developed skeletal abnormalities. An epistatic interaction resulting from the mixing of genetic backgrounds that is potentially exacerbated in the presence of *Pax3* heterozygosity is suspected.

2. Introduction

Endothelin receptor b and *Pax3* are involved in early NC development and mutations in either one result in similar phenotypes, such as a decrease in melanocytes or the presence of variant forms of Waardenburg's Syndrome (WS) (2001; Read and Newton, 1997; Tassabehji et al., 1994). *Pax3*

heterozygous mice have a completely penetrant ventral hypopigmentation phenotype; *Ednrb* heterozygous mice often have hypopigmentation while the homozygous mutants are almost completely white (Hornyak et al., 2001; Pavan and Tilghman, 1994). As detailed in chapter one (Figure 1), *Pax3* and the *Ednrb/Edn3* signaling pathway are both involved in the proliferation of melanocyte progenitors.

Melanocyte numbers are dramatically reduced in mice that are *Ednrb* homozygotes (Hosoda et al., 1994; Hou et al., 2004). In addition to the reduction of skin melanocytes, these animals are often deaf due to the absence of melanocytes in the stria vascularis of the inner ear (Matsushima et al., 2002) and typically die in the neonatal period due to aganglionosis which arises as a consequence of improper innervation of the gut by enteric neuronal precursors (Lee et al., 2003; Shin et al., 1999). *Endothelin receptor b* has been suggested to be an essential migration factor for the emerging neural crest cells from E10.5-12.5 (Shin et al., 1999). Expressed by a variety of cells along the migratory path of the increasingly committed NC progenitor cells, *Ednrb* must be functional in a cell autonomous fashion for melanoblasts to eventually become pigmented and for correct spatial distribution of enteric neurons to occur (Druckenbrod et al., 2008; Hou et al., 2004). Neither of these NC derivatives can successfully reach terminal differentiation in the absence of intrinsic *Ednrb*.

Pax3 is a critically important transcription factor during early embryonic development with a temporally and spatially restricted expression pattern. *Pax3*

expression begins in the mouse embryo at E8.5 and is essential for patterning of the vertebrate nervous system, melanocyte development and skeletal muscle formation (Bajard et al., 2006; Goulding et al., 1991; Hornyak et al., 2001). While *Pax3* is known to be a necessary proliferative factor for emerging melanoblasts, it also transactivates (along with *Sox10*) the major melanocyte regulatory transcription factor *Mitf* (Bondurand et al., 2000; Hornyak et al., 2001; Potterf et al., 2000). One of the most interesting features of *Pax3* is that as it activates *Mitf* it also serves as an inhibitor to *Dct*, a downstream target of *Mitf*, thereby promoting the melanogenic cascade while at the same time preventing terminal differentiation (Lang et al., 2005).

Development is the complex interaction of genes selectively being activated and deactivated in a spatially and temporally restricted fashion in order to dictate cell fate and specification. While *Ednrb* and *Pax3* have several distinct roles in embryonic development, in the melanocyte lineage they are expressed at similar times and in similar locations, and indeed mutants have similar pigmentation and disease phenotypes. This was the original impetus for examining whether or not a genetic interaction existed between *Ednrb* and *Pax3* in the melanocyte lineage. In the previous study detailed in chapter 2, I crossed $Ednrb^{S\text{-}l/+}$ mice with $Pax3^{Sp/+}$ mice to see if double heterozygosity would affect the hypopigmentation phenotype. The hypopigmentation was significantly aggravated in double heterozygous progeny in generations two and three. While collecting data for this first study, a novel phenotype arose. These mice were born looking normal but by 3.5-4 weeks of age appeared to

have large dome-shaped heads, were hunched over and smaller than their normal looking littermates. All affected animals died shortly after the appearance of the domed head.

In this study, I wanted to examine the changes occurring both in the head and skeletal structure of these animals. My aim was to provide a descriptive account, in morphologic terms, of the alterations leading up to the certain death of these animals. None of the affected animals survived.

3. Materials and Methods

3.1 Animals and Genotyping

Heterozygous *Ednrb*$^{s-l}$ on SSL/Le background and *Pax3*^{Sp} on C57BL/6J background were originally obtained from Jackson Laboratory and maintained in the FIU Animal Care Facility. Heterozygous *Ednrb*$^{s-l}$ mice display small to medium areas of ventral hypopigmentation while homozygous animals are almost completely white, with small areas of pigmentation around the pinna and base of the tail. Tail biopsies were used as sources of genomic DNA for PCR genotyping (Appendix I.A). To detect the *Ednrb*$^{s-l}$ allele, the tightly linked *D14Mit7* microsatelite polimorphic marker (Metallinos et al., 1994) was used to distinguish between *Ednrb*$^{s-l}$ and C57B/6J mice using conditions previously described (Pavan and Tilghman, 1994; and Appendix I.B). *Pax3*^{Sp} animals carry a point mutation within intron 3 of the paired homeobox 3 (*Pax3*) gene on mouse Chromosome 1. The mutation interferes with normal splicing of intron 3 and leads to at least 4 aberrantly spliced mRNAs with exon 4 deleted. *Pax3*^{Sp} heterozygotes consistently display ventral hypopigmentation; mice homozygous

for this mutation are embryonic lethal by E13.5. To genotype *Pax3*^{Sp} mice, the intron 3/exon 4 boundary of *Pax3* was amplified with primers: P3in3F (5'-GAGAGGGTTGAGTACGTTAGCTGG-3') and P3ex4 (5'-CTCGCTCACTCAGGATGCC-3'). Products were visualized on a 15% polyacrylamide gel resulting in a single band around 230bp for a wildtype and a heteroduplex band at this same site for the heterozygote (Appendix I.B).The PCRs were performed for 30 cycles under the following conditions: 94°C for 30 s, 58°C for 30 s, 72°C for 30 s.

All animals used in this study were housed in the Animal Care Facility at Florida International University (Miami, FL). Water and murine chow were fed *ad libitum* and light/dark was cycled every 12 hours. All animal work was performed according to institutional guidelines established by NIH.

3.2 Crosses and Phenotype Analysis

Crossing of *Ednrb*$^{S\text{-}l/+}$ mice with *Pax3*$^{Sp/+}$ mice resulted in mice of all possible genotypes: *Ednrb*$^{S\text{-}l/+}$, *Pax3*$^{Sp/+}$, double heterozygous mutants (*Ednrb*$^{S\text{-}l/+}$ ::*Pax3*$^{Sp/+}$) and wildtype for these mutations. While genotyping of all animals was done, only characterization of progeny that exhibited the overt phenotype in tandem with one normal littermate was undertaken. These animals were euthanized shortly before the natural death of the affected sibling, typically at 3.5-4 weeks of age and either used for skeletal analysis or for brain antibody staining. These animals were progeny from the first experiment (Chapter 2).

3.3 Skeletal Staining

Animals were euthanized once hydrocephaly was visible and death was imminent, typically between 3.5 and 4 weeks of age. They were immediately skinned, eviscerated and placed in a 95ºC water bath for 90 seconds to loosen fat and muscle, which was then peeled off the bones. The skeletal specimens were fixed overnight in 4% paraformaldehyde (Fisher Scientific) at 4ºC, briefly rinsed with deionized water and placed in acetone (Fisher Scientific) overnight. They were then washed 3 times with deionized water, covered with Alcian Blue (Acros) solution and placed in total darkness overnight. The next day, specimens were washed 3 times with 70% ethanol (Florida Distillers, Co.) changing the solution every 2.5 hours and left overnight in a 1% KOH (Acros) solution. KOH was removed and Alizarin Red (Ricca Chemical Co.) solution added to cover specimens and left again in complete darkness overnight. Specimens were then placed in a 20% glycerol (Fisher Scientific) in 1%KOH for 2 days and then stored in a 1:1 glycerol:ethanol solution. Adapted from Nagy et al. (2003).

3.4 Brain Dissection

Animals used for brain studies were also euthanized once hydrocephaly was visible and death was imminent, typically at 3.5 to 4 weeks of age. Animals were anesthetized and perfused initially with 0.1M PBS, pH 7.4 (Fisher Scientific) and immediately followed with 4% paraformaldehyde. Animals were decapitated, the head skinned and the skull chipped away using rongeurs. Brains were rinsed in PBS, measured and put in 4% paraformaldehyde, gently

rocking at 4ºC overnight. The following sequence was followed over subsequent days and solutions were changed when the brains had sunk to the bottom of the 50ml tube: 10% sucrose; 20% sucrose; 30% sucrose and finally a 1:1 solution of 60% sucrose:Tissue-Tek Freezing Medium (Sakura). Brains were then embedded in pure Tissue-Tek Freezing Medium and stored at -80ºC. Later, transverse cryosections of 10µm were taken of the frozen brains and used for antibody staining.

3.5 Antibody Staining

Selected slides were placed on a slide warmer for 2 hours at 37ºC. Slides were washed 3 times in PBS pH7.4 for 15 minutes each time. A blocking solution of 10% goat serum (Fisher Scientific) in PBT (PBS plus 0.1% Tween-20; Fisher Scientific) was added in two intervals, once for 15 minutes and then for 1.25 hours. The primary antibody in 5% goat serum in PBT was added to slides and left in a humidity chamber overnight at 4ºC: 200µl of Glial fibrillary acidic protein (GFAP; Sigma) at 1:50. In the morning, the primary antibody were neutralized using PBS and the secondary antibody (Alexa Fluor 488; Invitrogen) was diluted 1:100 in 5% goat serum in PBT and left on slides for 2 hours. Slides were washed with PBS and mounted.

4. Results

4.1 Realization of Novel Phenotype

As data was being collected to examine whether or not an interaction exists between *Ednrb* and *Pax3* in the melanocytic lineage (previously described in Chapter 2), a number of animals were born that exhibited the same

developmental anomalies with complete lethality. All pups appeared normal at birth; at approximately 3 weeks of age a number of the progeny appeared smaller than their littermates and their heads became unusually dome shaped. The heads of the affected pups seemed to grow larger as the week went on, especially in the frontal areas. By 4 weeks of age, all pups were dead except for one which lived to approximately 8 weeks of age. Some of the affected pups also appeared to develop kyphotic curvatures of the thoracic spine (Figure 1).

4.2 Sample Size and Demographics

A total of 23 affected pups were among the 428 animals born from the *Ednrb*$^{S\text{-}l/+}$ x *Pax3*$^{Sp/+}$ cross signifying that 5.4% of all progeny born in the study developed this lethal phenotype. Of these 23, 3 died prior to the realization that a trend was emerging and because genotyping is done at 4 weeks of age, no genetic data is available on these 3 pups other that they were born in the second generation. The remaining 20 pups were genotyped and examined for brain and skeletal abnormalities.

Fifteen of the 20 affected pups (75%) evaluated were female. Although the total was skewed toward female gender, the distribution among genotypes does not seem to be favored by one gender or the other, at least within this small sample. Males and females appear in all genotype categories: 8 females and 2 males were *Pax3*$^{Sp/+}$, 4 females and 2 males were *Ednrb*$^{S\text{-}l/+}$ ::*Pax3*$^{Sp/+}$ and 3 females and 1 male were wildtype at these two loci (Table 1). Initially there appeared to be generational bias (favoring generations 2 and 3) but one mutant pup was a first generation progeny. Generation 2 produced 11 mutant

pups and generation 3, 8 mutant pups. Twenty-two of the 23 mutant animals died between 3.5 and 4 weeks of age; one mutant mouse, who was not a runt, did not develop hydrocephaly until the 7th week of life and was dead by the 8th week. Of interest, several of the affected mice had microphthalmia.

4.3 Skeletal Abnormalities Seen in Mutant Mice

All affected mice developed hydrocephaly and were smaller than their littermates; the only affected mouse that was not smaller than his littermates was the one mouse that didn't develop hydrocephaly until the 7th week of life and survived until the 8th week. Upon examination of the affected progeny, several of them had a curvature of the spine equivalent to thoracic kyphosis, their rib cages appeared wider and their organs were tucked further up into their rib cages than their unaffected littermates (Figure 2). Note the difference in head shape between the normal (Figure 2A) and the affected (Figure 2B) littermates. The abnormal shape of the head is seen again in the affected (Figure 3A) versus the normal (Figure 3B) littermates. The dorsal view seen in the bottom of figure 3 exemplifies the difference in size seen between affected (C) and non-affected (D) mice. The difference in the density and development of the pelvic area is striking.

4.4 Differences in Brain Morphology

The brains of the affected mice were always larger than the brains of their normal littermates in all dimensions measured: length, width and depth (Table 2). Often times the skulls of affected mice were soft and the meninges thick. During dissections, a clear liquid commonly leaked out of brains of affected

mice and often times during the fixation process these brains would deflate somewhat. Brains of affected mice often had blood in the skull cavity, the cortical area of the olfactory nucleus and between the two hemispheres. During cryosectioning along the coronal plane, blood was often seen in the outer cortical layer and would diminish as sectioning progressed towards the caudal aspect of the brain.

The most striking difference between the brains of the affected and unaffected mice was the size of the ventricles. In particular, the 4^{th} ventricle was typically huge when comparing the affected brain to the normal brain. An increase in glial cells and processes was clear when comparing the brains of the affected mice to the brains of the normal mice (Figure 4).

5. Discussion

Expansion of the cranial cavity from a normal sloping forehead to a domed-shaped head during the neonatal period suggested the development of hydrocephaly, and upon further evaluation, morphological studies supported this initial hypothesis. Approximately 5% of the progeny born from an *Ednrb*$^{S\text{-}l/+}$ and *Pax3*$^{Sp/+}$ cross developed this abnormal phenotype which resulted in 100% lethality. Presence of this novel phenotype was more prevalent in second and third generation progeny but one pup born in the first generation developed hydrocephaly.

Hydrocephaly literally means water (“hydro”) on the brain (“cephaly”); the water being cerebrospinal fluid (CSF). Cerebrospinal fluid bathes the brain and spinal column and then is reabsorbed back into the bloodstream all the while

serving as a protective cushion, and nutrient supplier and waste removal system for the brain. A blockage of any type can trap CSF within the confines of the ventricular system causing pressure on sensitive brain tissue, subsequent swelling and tissue damage (Rekate, 2009). Reactive gliosis, the generation of astroglia, is seen as a measure of stress and tissue damage in the central nervous system (Aoyama et al., 2006; Miller and McAllister, 2007). It has been estimated that 1 in 500 children are affected with hydrocephaly (National Institute of Neurological Disorders and Stroke, 2009) and a quick search on PubMed reveals a plethora of research activity (over 22,000 articles). Yet little is known about the mechanisms of development or truly adequate treatment of hydrocephaly.

Hydrocephaly appears to be a complicated, multigenetic disorder (Takahashi et al., 2008; Town et al., 2008; Zador et al., 2007) often with the co-concurrence of polycystic kidney disease (Liebau et al., 2007; Town et al., 2008). *Stumpy*, a recently characterized highly conserved gene required for mammalian ciliogenesis, results in complete penetrance of hydrocephalus and polycystic kidney disease in mice with the homozygous null condition (Town et al., 2008). Interestingly, several observable development trends seen in my mice are similar to theirs in that both our mutant pups were born with normally shaped heads which became domed shaped during the neonatal period and these pups were smaller than normal littermates yet had larger brains. Two of their control pups (<1%) manifested the condition which they concluded may represent spontaneous development of hydrocephaly in C57BL/6J mice. The

“wildtype” animals in my population that manifested hydrocephaly may have an allele of a yet unidentified gene that has low causative influence on the development of hydrocephalus. Perhaps in the presence of *Pax3* haploinsufficiency, this unidentified gene is modified and there is a dramatic increase in the expressivity and penetrance of this defect (80% of affected pups were *Pax3* heterozygotes compared to 20% with two normal *Pax3* alleles). In addition, it is possible that a mutation in *Pax3* exacerbates the expression of the phenotype in the presence of a homozygous-null modifier. In other words, background strains mixed as generations of progeny were born could increase incidence of this unidentified allele occurring in the homozygous-null state and perhaps it is under these circumstances that a *Pax3* mutation modifies its expression. This would explain the preponderance of affected mice in generations 2 and 3 while there was only one affected mouse in generation one. It would be interesting to investigate whether or not these affected mice had abnormal renal morphology, and whether or not their expression of *Stumpy* is normal.

The skeletal abnormalities seen in some of the affected animals insinuate that the genetic network underlying this phenotype may be dependent on the allelic variations present at the locus. In the case of *piebald*, the varying lengths of deletions of the *piebald* gene affected gene expression and dictated whether the mice displayed hydrocephaly, skeletal defects, cartilage malformations of the head or a combination of these defects (O'Brien et al., 1996). The possibility

remains open that the presentation of allelic variance will manifest in similar but varying phenotypes in our mutant mice as well.

The complexity of elucidating genetic networks is magnified further by the possibility of subunits of a modifier gene having substantially different effects on gene expression. In the case of the *Reelin* signaling pathway, the *Pafah1b* Alpha subunits have profoundly different effects on brain development (Assadi et al., 2008). Appropriate *Pafah1b1* association with genes in the *Reelin* pathway is necessary for proper layering of the brain and defects in this interaction results in layering defects and hydrocephaly. Mutations in *Pafah1b3* aggravate the layering defects while mutations in *Pafah1b2* strongly suppress the development of hydrocephaly in mutant mice destined to develop this condition.

Background strains also have tremendous impact on gene penetrance and expressivity. To establish whether mice of five commonly used inbred background strains were born with similar ventricular size and volume capacity, Hino and colleagues (2009) examined ventricles in newborn mice on MRL/Mp, C57BL/6, C3H/He, DBA/2 and BALB/c background strains. The MRL/Mp strain had the largest ventricles and the lipid droplets lining the ventricles were fewer in number and smaller than those present in other backgrounds. The authors concluded that the initial increased volume of CSF and the decreased number of lipid molecules (which supply nutrients) may increase the risk of developing hydrocephaly. Other studies have also shown that the C57BL/6J background decreases the incidence of hydrocephaly in mice (Takahashi et al., 2008;

Tapanes-Castillo et al., 2009) just as it decreases the hypopigmentation phenotype. However, while severity of hypopigmentation decreases on a C57BL/6J background, the incidence and severity of aganglionosis increases (Cantrell et al., 2004) implying that background modifiers can exacerbate or diminish gene expression. It is possible that the incidence of hydrocephaly in my population is only 5.4% due to the effect of C57BL/6J background modifiers and that an interaction between *Ednrb* and *Pax3*, or a modifier of one or both of these, is minimized because of the background strain. The *Ednrb* mice were originally on an LLE background, and although there have been several years of generations on a C57BL/6J background, it is possible that a modifier of *Pax3* (although dilute) exists in the *Ednrb* background. And as in the case of *Stumpy*, perhaps until that modifier had the opportunity to exist in a homozygous state in the presence of a *Pax3* mutation it went along relatively undetected.

Much work needs to be done in order to characterize the etiology of the hydrocephaly and skeletal deformities seen here. Determining candidate genes, single polymorphisms, types of mutations involved and microarray analysis may begin to illuminate the genetic interaction underlying the development of hydrocephaly in this particular population.

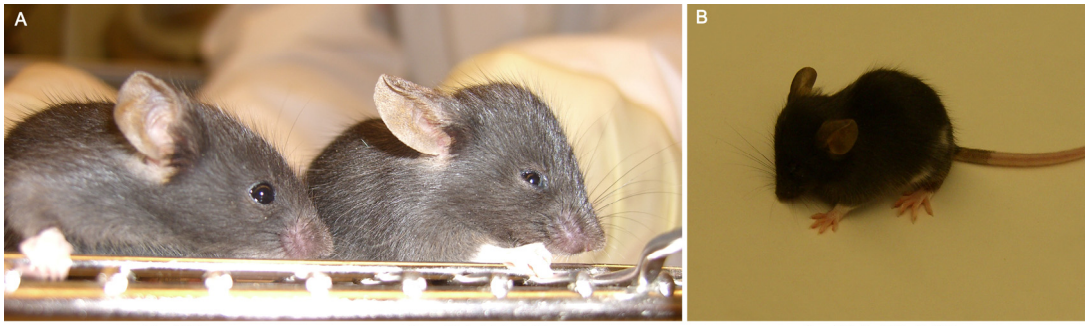

Figure 4.1 Normal and affected littermates. (A) Littermates at 4 weeks minus a day, the mouse on the left appears normal while the mouse on the right is developing a domed-shaped head and eyes appear smaller than its sibling's eyes. (B) is the same affected mouse from panel (A). The body shape is more hunched and rounder than those of its littermate. Both animals are female.

	Pax3$^{Sp/+}$	*Pax3*$^{Sp/+}$::*Ednrb*$^{S-l/+}$	Wildtype
F_1		♀	
F_2*	♀♀♀♀ ♂	♀♀ ♂	♀♀ ♂
F_3	♀♀♀♀ ♂	♀ ♂	♀
Totals:	8♀ 2♂	4♀ 2♂	3♀ 1♂

*Three animals died prior to genotyping or recognition of novel phenotype.

Table 4.1 Distribution of affected progeny according to generation, genotype and gender. Animals are classified by generation according to their genotype and gender. Of the 20 animals genotyped, 10 were *Pax3*$^{Sp/+}$, 4 were *Pax3*$^{Sp/+}$::*Ednrb*$^{S-l/+}$ and 4 were wildtype for both these genes. While no gender pattern emerged via genotype or generation there were a predominance of females (15 females out of 20 animals). Three animals (F_2) died prior to genotyping or recognition of novel phenotype. F_2 = 2nd generation.

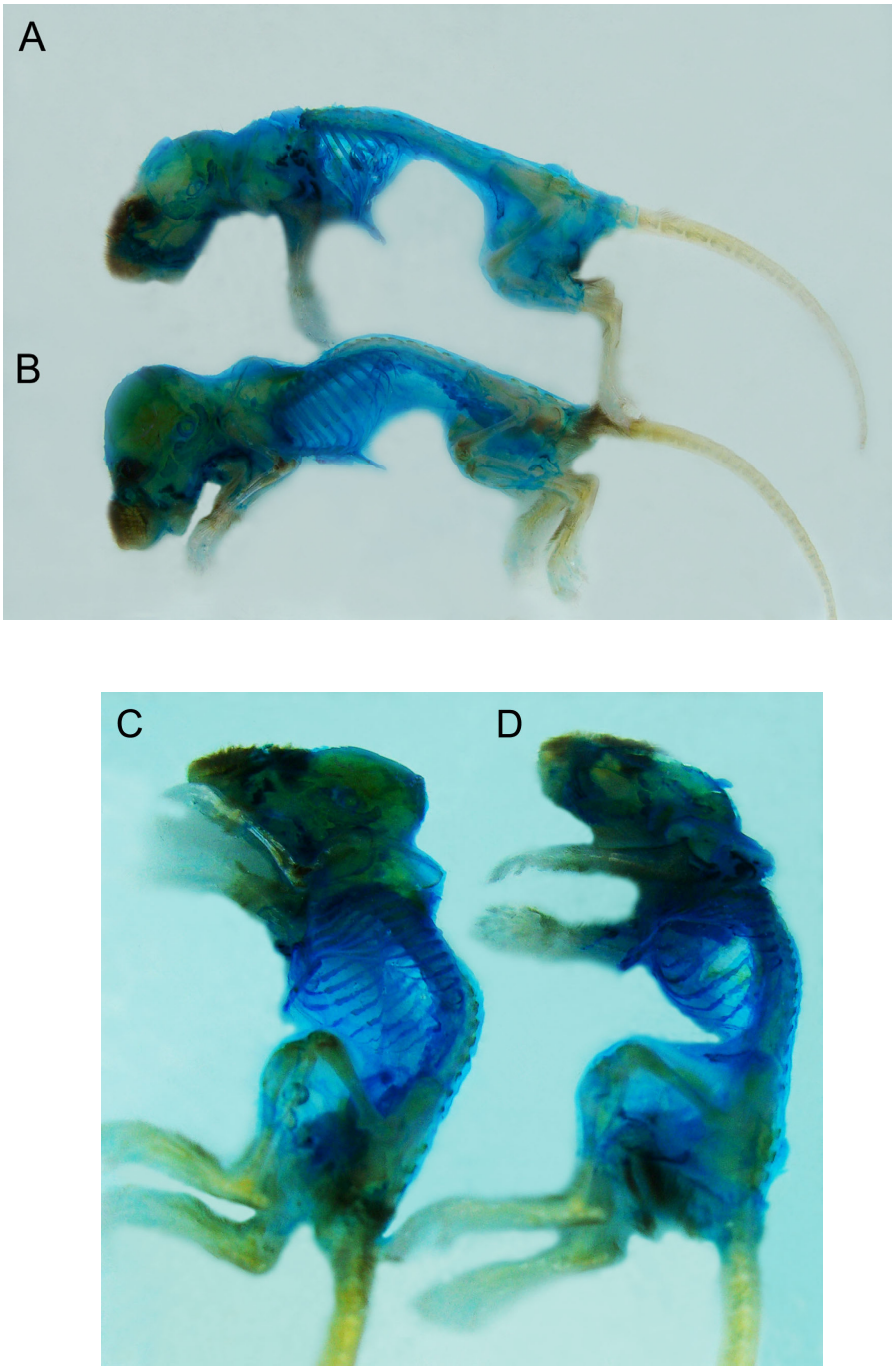

Figure 4.2 Skeletal staining showing curvature of the spine, rib cage expansion and hydrocephalic head. (A) Normal curvature of the mouse spine in the normal mouse as compared to (B) kyphotic curve of the thoracic part of the spine seen in the affected mouse. (C) Rib cage had normal number of ribs but has rounded and wider when compared to (D) the unaffected littermate. Both specimens are female, age 4 weeks old. These mice were depicted in Figure 1; the affected mouse is *Pax3*$^{Sp/+}$::*Ednrb*$^{S\text{-}l/+}$ and the normal littermate is *Pax3*$^{Sp/+}$.

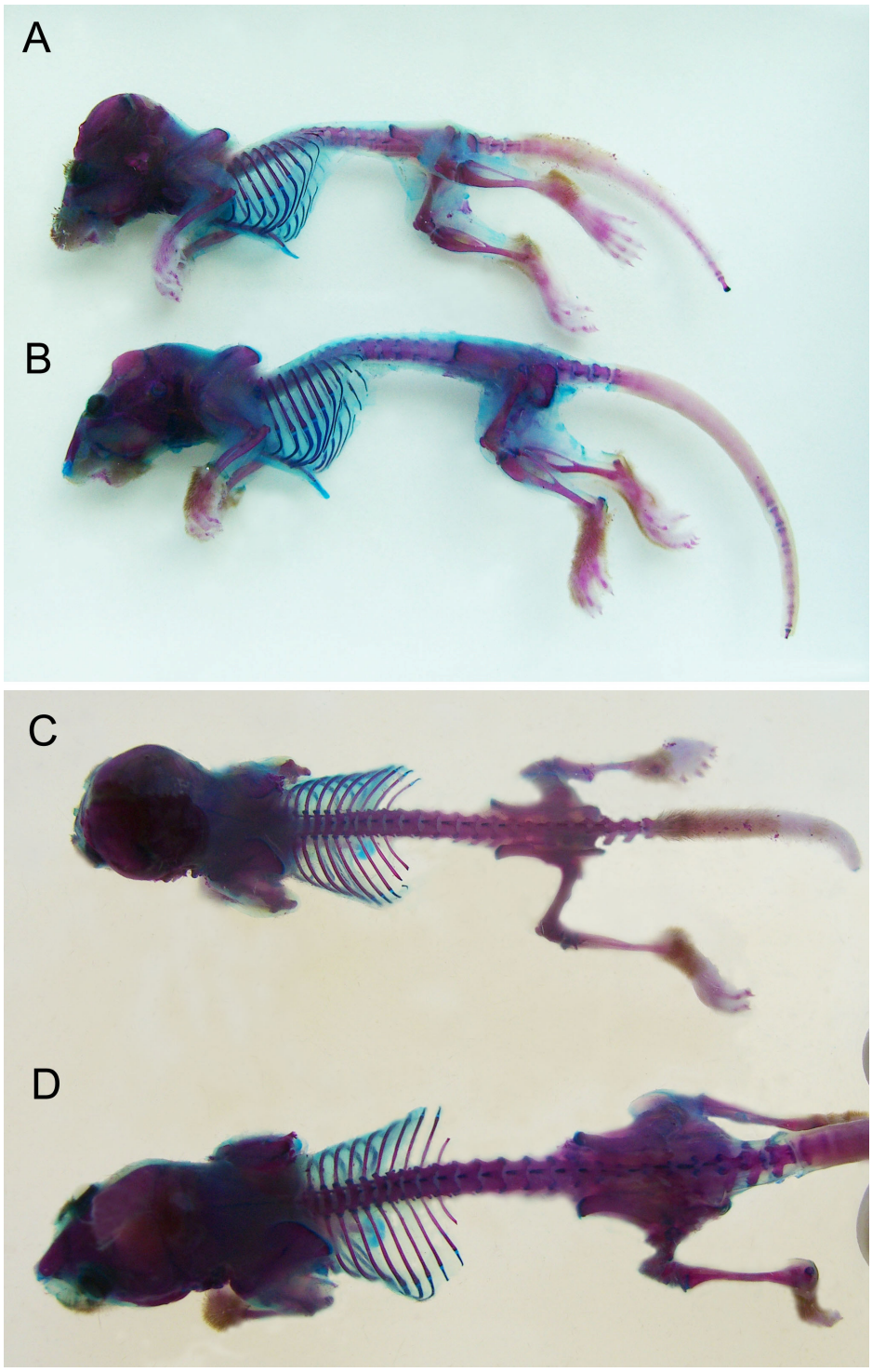

Figure 4.3 Skull and pelvic differences. (A) Affected mouse exhibits misshapen head secondary to hydrocephaly as compared to (B) unaffected littermate. (C) Affected mouse is much smaller in overall size and also has considerably less bone in the pelvic region as compared to (D) its littermate. Specimens are female, 4 weeks old and Pax3 heterozygotes.

	Normal Brain	Mutant Brain
Length	15cm	17.1cm
Width	10cm	11.5cm
Depth	5cm	6.5cm

Table 4.2 Dimensions of two brains. The brain from the affected mouse is larger than the brain of its unaffected littermate in all dimensions. All affected and unaffected brains followed this pattern; this example was chosen as it was an average example of the difference in one pair of littermate brains. Brains come from female littermates who were 3.5 weeks old and both were Pax3 heterozygotes.

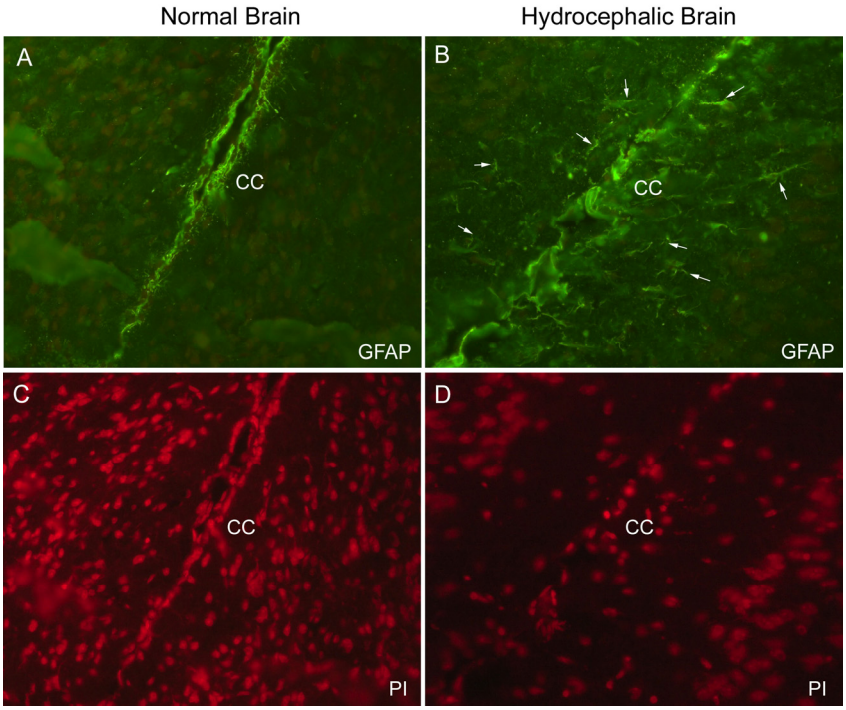

Figure 4.4 Brain morphology differences. Antibody staining of 4-week old female murine brains with GFAP shows an increase in glia in the (B) affected brain when compared to (A) a normal brain. In addition, there are fewer cells in (D) the brain tissue of the hydrocephalic brain than in (C) the normal brain. CC = corpus callosum, Magnification: 40X; arrows indicate glia.

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Chapter V.

CONCLUSIONS, FUTURE DIRECTIONS AND IMPLICATIONS

V. Conclusions, Future Directions and Implications

1. Conclusions and Future Directions

Genetic interactions provide a complicated network of signals that modulate penetrance and expressivity of gene expression. To elucidate some of the most basic mechanisms of development is to unravel the process of multipotent progenitor cells determining cell fate and subsequently differentiating into highly specialized cells. Neural crest cells emerge as a population of multipotent cells that generate a diverse array of cells and tissues, among them the pigment cells. Melanogenic genes have been described and major melanogenic transcription factors identified but the study of the genetic interactions between the genes that modulate the differentiation of these cells is underway. To that regard, I have determined that interactions between *Sox10* and *Ednrb*, and *Pax3* and *Ednrb* do exist.

Mouse pigmentation phenotypes were used as they are an excellent system in which to elucidate the underpinnings of genetic interactions due to their high homology with the human genome and their short generation time. In chapter 2, I showed that progeny harboring double heterozygous mutations in $Sox10^{LacZ}$ and $Ednrb^{S\text{-}l}$ displayed significantly aggravated pigmentation phenotype in generations two and three of the crosses: in other words, animals who were double heterozygotes had areas of hypopigmentation significantly larger that the sum of the hypopigmentation of $Sox10^{LacZ/+}$ and $Ednrb^{S\text{-}l/+}$ areas. This suggests that rather than there being an additive effect of the mutations, a synergistic effect occurred indicating that *Sox10* and *Ednrb* do interact in the

melanocyte lineage. A similar outcome was seen in the *Pax3*$^{Sp/+}$ cross with *Ednrb*$^{S\text{-}l/+}$ in which double heterozygote progeny in generations two and three exhibited significantly larger areas of hypopigmentation than the progeny harboring only one of the two mutations.

Melanocytes are derived from the NC. Given that NC cells provide progenitor cells for diverse tissues and organ systems throughout the vertebrate body, it is of no surprise that when a mutation affects NC development early on other cells may be compromised as well. In the case of *Sox10* mutations, haploinsufficient mice can also develop aganglionosis or hearing phenotypes in addition to hypopigmentation. Other studies have shown an interaction between *Sox10* and *Ednrb* but with varying results. Stanchina and colleagues (2006) showed aggravated phenotypes (pigmentation and aganglionosis) when using an *Ednrb* hypomorph crossed with *Sox10*^{Dom} or *Sox10*^{LacZ} while two other studies showed no effect of double heterozygosity on pigmentation phenotype (Cantrell et al., 2004; Hakami et al., 2006). Differences between these results may lie in some yet unidentified background modifier that is present in higher levels in some of these in-bred strains than in other in-bred strains. If no genetic interaction existed between *Sox10* and *Ednrb* then all studies would have produced negative results; in addition, in human melanocytes two *Sox10* binding sites have been identified on *Ednrb* (Yokoyama et al., 2006). I do not believe that these mixed results are due to stochastic events but rather that gene expression and penetrance may be dependent on modifiers of which we are not yet aware. A clear analysis of

phenotypic expression and the background in which it is expressed will help elucidate the myriad of interactions that comprise the signals necessary for the process of cell fate determination and differentiation. It is this process of discovery that will enable us to unravel and understand how diseases vary in their onset and severity.

In chapter 3, I showed that when using a transgene to deliver additional *Ednrb* to the melanoblasts of *Sox10* deficient animals, the hypopigmentation phenotype was rescued in all progeny. *Sox10* heterozygous mice display a hypopigmented ventral spot approximately half of the time, yet when crossed with a transgenic mouse that was created to deliver additional *Ednrb* to melanocyte precursors there was a complete rescue of coat color. If *Sox10* activates the expression of *Ednrb* as I suspect it does, then *Sox10* heterozygous mice may have a larger belly spot than the *Ednrb* heterozygous mice due to the initial effect of *Sox10* haploinsufficiency on the premigratory and early migratory melanoblasts in addition to the insufficient activation of *Ednrb*. *Sox10* heterozygous mice survive with few, if any side effects, implying that the level of *Sox10* in these mice is still adequate to sustain overall cellular function. The delivery of additional *Ednrb* to melanoblasts that have survived *Sox10* haploinsufficiency appears to compensate for the lower level of *Ednrb* activation thereby rescuing the pigmentation phenotype. Interestingly, the transgenic addition of *Sox10* to *Ednrb* heterozygotes did not rescue the hypopigmentation phenotype (Hakami et al., 2006). The delivery of additional *Sox10* to a system with limited *Ednrb* provides no additional *Ednrb* to

compensate for heterozygosity. In other words, once the limited amount of functional *Ednrb* has been fully activated by *Sox10* the addition of more *Sox10* does nothing because there is no more *Ednrb* available to be activated by *Sox10*. Conversely, the addition of *Ednrb* to a *Sox10* limited system does what *Sox10* would have normally done – provide sufficient levels of *Ednrb* signaling to promote proper melanoblast dispersal and thereby rescues the hypopigmentation phenotype of the *Sox10* heterozygous mutant.

Pax3 and *Ednrb* have a more complicated relationship. *Pax3* heterozygous mice exhibit a completely penetrant hypopigmentation phenotype and the transgenic delivery of *Ednrb* produced a partial rescue. Double heterozygous animals also displayed a significantly larger area of hypopigmentation as discussed above and in chapter 2. However, the transgene rescued the hypopigmentation in only 24% of the progeny and in another 10% there was a partial rescue. The ability of the transgene to rescue the pigmentation phenotype may differ due to the temporal expression of *Sox10* and *Pax3*. *Sox10* and *Ednrb* are co-expressed temporally while *Pax3* expression is downregulated at the time when *Ednrb* is being upregulated. If this is the case, then *Ednrb* may be activated too late to rescue melanocyte progenitors in the $Pax3^{Sp/+}$. It is also possible that the insult of *Pax3* haploinsufficiency is more devastating to the premigratory melanoblast precursors and that the additional *Ednrb* is insufficient to rescue more of the cells.

In order to establish the timing of the rescue in the *Sox10* heterozygous mice, *Sox10*$^{LacZ/+}$ embryos with and without the transgene were evaluated at E11.5 (the age at which melanoblasts have first populated the entire length of the embryo). In situ hybridization using a *Pmel17* riboprobe showed that the transgenic expression of *Ednrb* rescued the *Sox10* deficient melanoblasts by E11.5. *Pmel17* was used as its expression is not regulated by *Sox10* and it is among the most sensitive of melanoblast markers (Loftus et al., 2009; Valencia et al., 2006). Once the rescue was established, the mechanism of this rescue came into question. Cell survival assays in E11.5 and E12.5 embryos indicate that the rescue is not due to the effect of the transgene on melanoblast survival. Proliferation and migration studies at E11.5 or earlier will help elucidate the answer to this question.

During the *Pax3*$^{Sp/+}$ and *Ednrb*$^{S\text{-}l/+}$ study, a novel phenotype in the progeny became apparent. Approximately 5% of pups born in this experiment developed hydrocephaly and died by 4 weeks of age. In addition to the hydrocephaly, some pups also exhibited skeletal abnormalities that included kyphosis of the thoracic region and smaller pelvic regions that appeared less ossified than the pelvic regions of their unaffected littermates. All affected pups except one were much smaller than their littermates. Interesting characteristics of this population include that 80% of all affected animals were *Pax3*$^{Sp/+}$ and 75% were female. All except one of the affected animals were born in the second or third generation of the cross.

All affected progeny developed severe hydrocephaly (which became apparent in the 4th week of life) from which they died. Brain morphology studies showed enlarged brains and bilaterally distended 4th ventricles in all animals. Many of the skulls of these animals were softer than those of their unaffected littermates. In addition, all affected animals were smaller than their normal littermates (except one) and skeletal studies confirmed that their skeletons were indeed smaller than usual. Also of interest was that some of the affected animals had kyphosis, a curvature of the spine in the thoracic region, and had a much smaller pelvis than normal littermates.

The founders used to create this *Pax3*$^{Sp/+}$::*Ednrb*$^{S\text{-}l/+}$ population originally came on different backgrounds and were backcrossed (independently) for several years prior to being used in this study. The *Pax3* mutant mice came on a C57BL/6J background and the *Ednrb* mutant mice were originally on an LLE background and were later transitioned to a C57BL/6J background. We have many different lines of mice on the C57BL/6J background and on other backgrounds as well, and we have created double and triple mutants of some of these lines and have never seen hydrocephaly develop in any of these progeny. In this study however, it was apparent early on that some unknown interaction was occurring inducing hydrocephalus and intermittently inducing skeletal deformities. This was the first time in the history of our lab that the *Pax3*$^{Sp/+}$ animals were crossed with *Ednrb*$^{S\text{-}l/+}$ animals and this tangential finding was intriguing. Eighty percent of the affected animals were *Pax3* heterozygotes while 20% were not. So the question remains as to what kind of

modifier lies in the *Ednrb* background that, when in the presence of *Pax3* haploinsufficiency, increases the incidence of hydrocephaly and skeletal deformities?

The complexity of illuminating genetic cascades and networks is a monumental task that will undoubtedly take years of research. DNA analyses to determine candidate genes, single polymorphisms and microarray analyses could be used to begin to identify potential genes that are being modified by a mutation in Pax3.

2. Implications

One of the mysteries of medical science is that individuals who harbor the same mutations can vary tremendously in the course of the disease and in how much they suffer. Gene expression and penetrance varies according to the presence or absence of other genes, or to background modifiers that were previously undetected. The four types of Waardenburg's syndrome bring to light some of the difficulties of studying genetic interactions: (1) similarity of signs and symptoms despite defects in different genes; and, (2) differences in severity and penetrance of the disease between families as well as within families. These complexities arise from the myriad of genes involved in every transaction that occurs along the developmental path. To make matters even more difficult, genes typically function in a nonlinear fashion and may be affecting expression of more than one gene at any given time.

It should be of no surprise that genetic networks contain a level of unprecedented complexity given the intricate and delicate balance of

developmental pathways, biochemical cycles, immunological responses and the many other highly sophisticated systems that integrate to become a living organism. Phenotypic expression is anchored in gene expression and it is a matter of time and patience until the underlying connections and mechanisms are uncovered. Elucidation of genetic interactions will help us understand how the mutated gene is modulated, with the intention of one day being able to provide gene therapy to individuals in need.

3. References:

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APPENDICES

APPENDIX I.

A. Isolation of DNA from Tail Biopsies

Animal is briefly anesthetized using a few drops of Isoflurane (Abbott). Working quickly, a numbered ear tag is placed in the right ear and the tip of the tail is clipped (0.5cm). The tail is briefly cauterized to prevent bleeding. Each tail is placed in 500µl of tail lysis buffer (100mM Tris-Cl pH 8.0, 5mM EDTA pH 8.0, 200mM NaCl, 0.2% SDS; Fisher Scientific) and 5µl of proteinase K (10mg/ml; Promega) and placed in a 55-60ºC water bath overnight.

Digested tails were cooled with 167µl 5M NaCl (Fisher Scientific) for 10 minutes. Samples were then centrifuged for 10min at 4ºC; supernatant transferred to clean microcentrifuge tubes and 1 volume of cold 100% EtOH (Florida Distillers Co.) added. Solution was shaken vigorously (DNA strands became visible) and centrifuged for 10min at 4ºC. Supernatant was poured off and the remaining pellet was dissolved in 1X TE (100mM Tris-Cl pH 8.0, 10mM EDTA pH 8.0; Fisher Scientific) (Miller et al., 1988).

B. Genotyping of Mouse Mutants

DNA extracted from tails was used to genotype mice. Individual PCR reactions (10µl total volume/reaction) were set up for each of the genes being tested.

PCR reaction for *Ednrb* consisted of 1µM of each primer (Ednrbf: 5'-AATGTATGGGCATGTGCGTG-3'; and Ednrbr: 5'-GAGATAGTCAACCAAAACAA-3'; Invitrogen), 1X buffer, 1.5mM MgCl, 1 unit of Taq (Promega), 2.5mM of each of the four deoxynucleotides (dNTPs; Invitrogen). Reactions were cycled 30 times at 94ºC for 30s, 58ºC for 30s and 72ºC for 30s. Amplified DNA was visualized on a 15% polyacrylamide gel made from 30% acrylamide/bis-acrylamide with 10% of 5X TBE, 1% ammonium persulfate (Fisher Scientific) and 0.1% TEMED (Sigma). All gels were run at 350volts for 3.5 hours at 4ºC. Ethidium bromide (Fisher Biotech) was used to visualize DNA.

To genotype $Pax3^{Sp}$ mice, the point mutation was amplified with primers: P3in3F (5'-GAGAGGGTTGAGTACGTTAGCTGG-3') and P3ex4 (5'-CTCGCTCACTCAGGATGCC-3'; Invitrogen). The reaction protocol and thermocycler program are identical to that of *Ednrb* (listed above). Products are visualized on a 15% polyacrylamide gel resulting in a single band around 230bp for a wildtype and a heteroduplex band at this same site for the heterozygote.

$Sox10^{LacZ/+}$ is a knock-in mutant and requires 3 primers: 5' Sox10 (5'-CAGGTGGGCGTTGGGCTC-3'); 3' Sox10 (5'-CAGAGCTTGCCTAGTGTCTT-3'); and 3' LacZ (5'-TAAAAATGCGCTCAGGTCAA-3'). In addition to the 1µM of

each primer (Invitrogen), 1X buffer, 2mM MgCl, 1 unit Taq (Promega), 5% DMSO (Fisher Scientific), 2.5mM of each dNTP (Invitrogen) and 1µl DNA. Thermocycler conditions were 94ºC for 30 s, 55ºC for 30 s, and 72ºC for 30 s for 32 cycles. Products were visualized on a 2% agarose (Fisher Scientific) gel resulting in a 500bp band for wildtype and a 600bp for the mutated allele. Animals homozygous for *Sox10* are embryonic lethal, typically by E13.5.

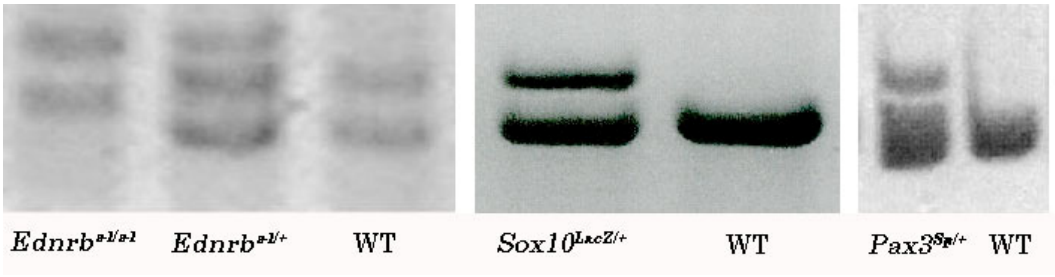

Figure A.1 Possible results of gel electrophoresis of mice tail DNA genotyping. *Ednrb* genotyping of tail DNA can result in either homozygous (*Ednrb*$^{s\text{-}l/s\text{-}l}$), heterozygous (*Ednrb*$^{s\text{-}l/+}$) or wildtype identification of animals. *Sox10* and *Pax3* genotyping of tail DNA results in either heterozygous (*Sox10*$^{LacZ/+}$ or *Pax3*$^{Sp/+}$) or wildtype animals. Both *Sox10* and *Pax3* null mutants are embryonic lethal by E13.5-14.5).

C. Buffers

Tris Acetate EDTA (TAE) (typically made as 50X solution)
40mM Tris-acetate
1mM EDTA
(24.2g Tris, 5.7ml Glacial Acetic acid, 10.0ml 0.5M EDTA pH 8.0, bring volume up to 100ml with dH_20 and autoclave.)

Tris Borate EDTA (TBE) (typically made as a 5X solution)
45mM Tris-borate
1mM EDTA
(54g Tris, 27.5g Boric acid, 20ml 0.5M EDTA pH8.0, bring volume up to 1 liter with dH_20 and autoclave.)

Tris EDTA (TE) (typically made as a 10X solution)
10mMTris, pH 7.5
1mM EDTA pH8.0
(10ml 1.0M Tris, 2ml 0.5M EDTA pH 8.0, bring volume up to 100ml with dH_20. Adjust pH to 8.0 and autoclave.)

APPENDIX II.

A. Staging and Harvesting of Embryos

Mice were mated overnight and staged embryos were dated as 0.5d noon the day the vaginal plug was observed. Embryos were harvested at E11.5 and E12.5 by euthanizing the pregnant dam via cervical dislocation and dissecting embryos through a transverse abdominal incision. Embryos were immediately placed in PBS (the pH of which was determined by the subsequent procedure, see Appendices II.D and II.H) and yolk sacs were saved for DNA extraction (Appendix II.B). Those destined for double staining with LysoTracker Red and LacZ, were immediately started on that protocol (Appendix II.I). Embryos for in situ hybridization were dehydrated through a methanol (MeOH; Fisher Scientific) series (25%:75% MeOH:PBS; 50%:50%; 75%:25% MeOH:PBS for 5min each) and stored in 100% MeOH at -20ºC.

B. Isolation of DNA from Tail Biopsies or Embryonic Yolk Sacs

Isolation of DNA from tail biopsies was achieved using the protocol listed in Appendix I.A. To genotype embryos, yolk sacs were harvested by taking the innermost of the 3 embryonic membranes surrounding the developing embryo. This membrane was placed in 250µl of yolk sac buffer (10mM Tris-Cl pH 8.0, 10mM EDTA pH 8.0, 100mM NaCl, 0.5% SDS; Fisher Scientific) plus 10µl of Proteinase K (10mg/ml; Promega) and placed in a 55-60ºC water bath overnight.

Digested yolk sacs were cooled with 200µl 5M NaCl (Fisher Scientific) for 10 minutes. Samples were then centrifuged for 10min at 4ºC; supernatant transferred to clean microcentrifuge tubes and 1 volume of cold 100% EtOH (Florida Distillers Co.) added. Solution was shaken vigorously (DNA strands became visible) and centrifuged for 10min at 4ºC. Supernatant was poured off and the remaining pellet was dissolved in 1X TE (100mM Tris-Cl pH 8.0, 10mM EDTA pH 8.0; Fisher Scientific) (adapted from Miller et al., 1988).

C. Genotyping of Mouse Mutants

Sox10, *Pax3* and *Ednrb* mutant animals were genotyped as described in the materials and methods section of Chapter 2 and Appendix I.B. *Sox10*$^{LacZ/LacZ}$ are embryonic lethal at approximately E13.5, hence *Sox10* embryos were harvested at E11.5 and E12.5 enabling the possibility of examining developmental changes in the *Sox10* homozygous condition. DNA amplification of embryos homozygous for *Sox10* generates a 600bp band while wildtype embryos generate a 500bp band; heterozygous embryos produce both bands.

Dct-*Ednrb* transgenic animals were generated in our laboratory (Avner Ittah, FIU dissertation 2005). The full length *Ednrb* cDNA (1958bp, genbank accession # U32329**)** obtained from pmET-B (Hosoda et al., 1994) was inserted into the vector pmDct that contains 3431 bp of the mouse *Dct* promoter and the BGH poly adenylation signal (Hornyak et al., 2000). Dct-*Ednrb* transgenic mice were generated by microinjection of the transgene into FVB F_1 zygotes. The studies presented here were carried out with a line that initially had 150 copies of the transgene. This line was crossed for over 10 generations with C57BL/6J mice from our colony. Mice were genotyped using 1µM of each primer Dct-*Ednrb*f (5'-ACAAGGAAGACTGGCGAGAA-3'); and, Dct-*Ednrb*r (5'-TCCTCCCCCTTGCTGTCCTGC-3'; Invitrogen), 1X buffer, 2mM MgCl, 1 unit Taq (Bioline), 5% DMSO (Fisher Scientific), 2.5mM of each dNTP (Invitrogen) and 1µl DNA. The amplified DNA was visualized on a 1% agarose gel resulting in a 2217bp band (Appendix I).The PCRs were performed for 35 cycles under the following conditions: 94°C for 30 s, 57°C for 30 s, and 72°C for 2 min.

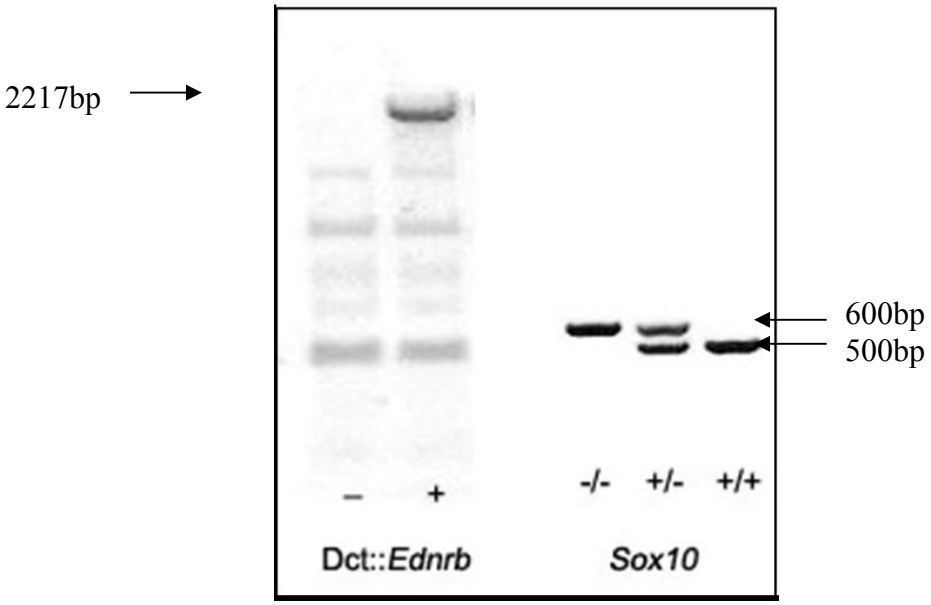

Figure A.2 Possible results of gel electrophoresis of mice tail or yolk sac DNA genotyping. Dct-*Ednrb* results either in a positive 2217bp band indicating presence of the transgene, or no band at this level indicating that the transgene was not transmitted. Other bands are non-specific. *Sox10* gel shows a 600bp band for *Sox10*$^{LacZ/LacZ}$ (-/-), 600bp and 500bp bands for *Sox10*$^{LacZ/+}$ (+/-) and a 500bp band for the wildtype (+/+).

D. In Situ Hybridization of Mouse Embryos

In situ hybridization took 4 days to complete. Day 1 (pretreatment and hybridization): Embryos were rehydrated via a MeOH series using PBT* (PBS** plus 0.1% Tween-20 Fisher Scientific) 5min for each of the following steps: 75%:25% MeOH:PBT; 50%50%; 25%:75% MeOH:PBT and two washes in

100% PBT. Embryos were then bleached in 6% H_2O_2 (Fisher Scientific) in PBT for 1hr at RT and then permeabilized using 10µg/ml proteinase K (Promega) in PBT (15min for E11.5 and 18min for E12.5). To neutralize this reaction, 2mg/ml glycine (Fisher Scientific) in PBT was added for 10min at RT and then washed twice with PBT. Embryos were postfixed with 4% paraformaldehyde (Fisher Scientific), 0.2% gluteraldehyde (Sigma) in PBT for 20min. Pre-hybridization was accomplished by adding 1ml hybridization solution (50% formamide, 5xSSC pH4.5 1% SDS, Fisher Scientific; 50µg/ml yeast tRNA, Sigma; 50µg/ml heparin, Sigma) and incubated in a 70ºC water bath for 1hr. The pre-hybridization solution was removed and fresh hybridization solution containing 2µl pMel17 riboprobe per ml of hybridization solution added and left overnight in the 70ºC water bath.

Day 2 (washing and antibody addition): Embryos briefly rinsed in solution 1 (50% formamide, 5xSSC and 1%SDS; Fisher Scientific) and then washed 3 times more with solution1 (30min each time in a 70ºC water bath). Washing was continued, 3 more times, using solution 2 (50% formamide and 2xSSC; Fisher Scientific) and placed in a 65ºC water bath for 30min each time, followed by washing with TBS plus 0.1% Tween-20 (TBST) 3 times for 5min each time. Blocking of embryos in 10% goat serum (Fisher Scientific) in TBST for 2hrs, rocking at RT, during which time the preabsorb anti-digoxigenin antibody was prepared by adding a pinch of embryo powder to microcentrifuge tubes containing 0.5ml TBST (1 microcentrifuge tube/embryo) and placed in 70ºC water bath for 30min. Microcentrifuge tubes were removed from water bath, vortexed and cooled prior to the addition of 5µl of 100% goat serum and 1µl anti-digoxigenin antibody (Roche) and rocked at 4ºC for 1hr. After centrifuging for 5min, the supernatant was collected and 1.5ml of 1% goat serum in TBST per microcentrifuge tube was added. When embryos were ready, the blocking solution was removed, the antibody solution added and embryos were left rocking overnight at 4ºC.

Day 3 (washing the antibody): Embryos were briefly rinsed in TBST and then washed 4 times in TBST for 1hr each time at RT. Levamisole (2mM; Sigma) was added to a 5th TBST wash and left overnight, rocking at 4ºC.

Day 4 (detection): Embryos were washed in NTMT (100mM NaCl, 100mM Tris pH9.5, 50mM MgCl2, 0.1% Tween-20; Fisher Scientific; 2mM levamisole; Sigma) 3 times for 10min each time. While protecting from light, 45µl 4-Nitro blue tetrazolium chloride (NBT; Roche) and 35µl 5-Bromo-4-chloro-3-indolyl-phosphate (BCIP; Roche) were added to 10ml NTMT and added to each embryo. Still protected from light, embryos were monitored every 30min to control the level of antibody development (Wilkinson 1998).

*All water used for solutions was Diethylpyrocarbonate (DePC; Sigma) treated water and therefore RNase-free.
***All PBS used in this protocol was 0.05M, pH7.4.

E. *Pmel17* Template and Riboprobe Preparation

Bacterial Transformation: 2.5µl of *Pmel17* plasmid was added to 50µl of DH5α competent *Escherichia coli* and incubated on ice for 30min, heat shocked for 20sec at 37ºC and then immediately placed in ice for 1min. 950µl Luria Broth (LB) was added and mixture was incubated for 1hr at 37ºC (30min into the hour 30µl Ampicillin was added). An LB plate containing Ampicillin was inoculated and left to incubate overnight at 37ºC.

Glycerol Stock of *Pmel17* Plasmid: Inoculated 5ml LB with isolated colonies from LB plate and incubated overnight at 37ºC shaking at 250rpm. 750µl of freshly grown transformed bacteria was added to 750µl sterile glycerol stock and stored at -80ºC.

DNA Isolation and Purification: 5ml LB was inoculated with glycerol stock of *Pmel17* plasmid (stored in -80ºC) and incubated overnight at 37ºC at 250rpm. Wizard Plus SV Mini Prep: DNA Purification System (Promega catalog #A1460) was used for the following steps. 2ml of fresh culture was centrifuged and pellet was resuspended in 250µl Resuspension Solution. To this, 250µl Cell Lysis Solution was added, inverted 4 times followed by the addition of10µl Alkaline Protease Solution and again inverted 4 times to mix gently, then incubated at room temperature for 5min. 350µl Neutralization Solution was added, centrifuged for 10min and the clear lysate was decanted into the spin column and centrifuged again for 1min. Flowthrough was discarded and 750µl Wash Solution added, centrifuged for 1min, 250µl Wash Solution, centrifuged for 2min and transferred to spin column to remove Wash Solution. Finally, 50µl of nuclease free H_2O was added and stored at -20ºC.

Template Preparation: 10µl of *Pmel17* plasmid was digested with 2µl of the restriction enzyme KPN1 (Promega), 2µl buffer J and 6µl UVH_2O at 37ºC for 2hrs. Linearized template was then cleaned via phenol-chloroform (Fisher Scientific) extraction and ethanol (Florida Distillers Co.) precipitation and resuspended in H_2O to a concentration of approximately 1µg/µl.

Riboprobe Synthesis: 5µl template was added to 4µl 5x transcription buffer (Roche), 2µl nucleotide tri-phosphate (NTP) labeling mix (Roche), 2µl T_3 polymerase (Promega), 1µl RNase inhibitor (Promega) and 6µl DePC H_2O and incubated at 37ºC for 2hrs. At this point, 2µl 0.2M EDTA, 2.5µl 4M LiCl and 75µl EtOH were added to precipitate riboprobe; pellet was then washed in 70% EtOH, resuspended in 22.5µl DePC H_2O, 2.5µl 4M LiCl and 75µl EtOH to precipitate again and a final wash in 70% EtOH. This pellet was dissolved in 20µl DePC H_2O and 80µl hybridization solution and stored at -20ºC.

F. Embryo Powder

12.5-14.5 day old embryos were homogenized in cold PBS pH7.4. Four volumes of acetone were added and mixture was incubated on ice for 30min, then centrifuged for 10min at 4ºC. Supernatant was discarded, the pellet washed again in cold acetone and spun down for another 10min. Supernatant was again discarded and pellet was macerated into a fine powder and air-dried. Embryo powder was stored at 4ºC.

G. Cell Counts of *Pmel17* labeled Melanoblasts

Cells labeled with *Pmel17* that were located in the torso from the fore limb to the hind limb were counted from a picture taken of the area at 3.2x magnification. The angle of lines drawn to define the area, lighting and magnification were kept constant for all pictures taken.

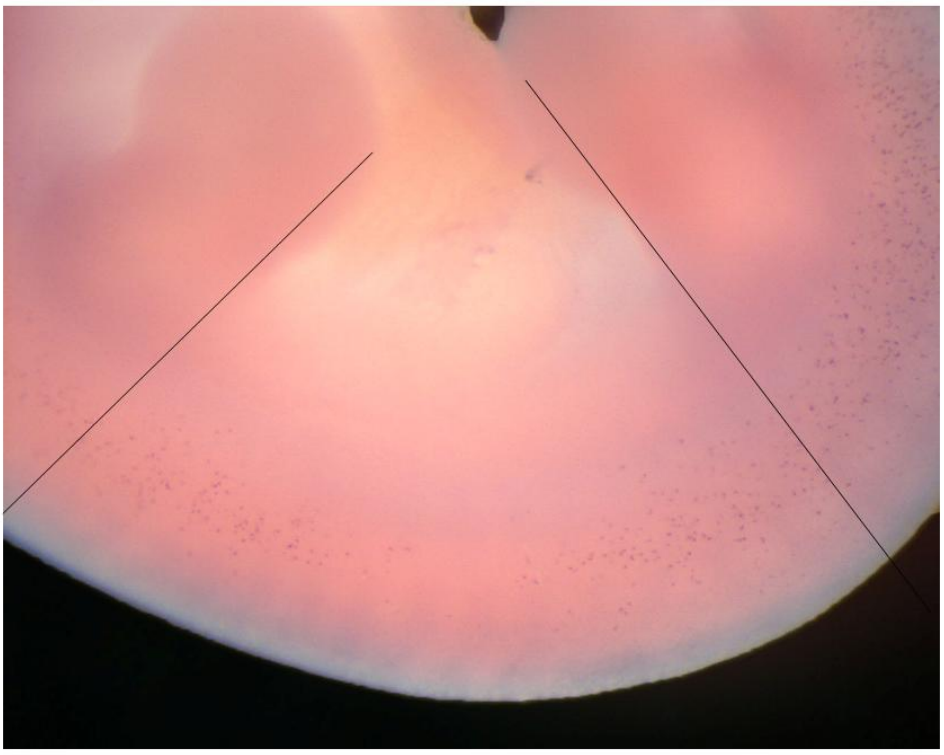

Figure A.3 Defining the area for cell counts of *Pmel17* positive cells. Lines were drawn that intersect the posterior most aspect of the forelimb and the anterior most aspect of the hindlimb. E11.5 Sox10 heterozygote.

H. Lysotracker Red and *LacZ* Double Staining of Mouse Embryos

Embryos were dissected and rinsed in cold 0.05M PBS* and placed in 5μM LysoTracker Red DND-99** (Invitrogen) at 37ºC for 30min. Embryos were washed twice in PBS, 2% paraformaldehyde (PF; Fisher Scientific) for 1hr at room temperature (RT), washed twice more with PBS and then left overnight at 30ºC in *LacZ* solution (5μM ferricyannide, 5μM ferrocyannide, 2mM $MgCl_2$, 0.01% sodium deoxycholate (Fisher Scientific), 0.02% nonidate p-40 (Roche) and 1mg/ml X-gal (Invitrogen). Rinsed twice in PBS to stop reaction and a 10% sucrose (Fisher Scientific) solution was added to embryos for overnight incubation at 4ºC, rocking gently. Finally, embryos were placed in 20% sucrose solution for 4-6hrs and embedded in Tissue-Tek Tissue Freezing Medium (Sakura). Embryos were stored at -80ºC.

*All PBS used in this protocol was pH7.2.
**From this point on, all embryos and solutions were protected from light at all times.

I. Counts of Lysotracker Red and *LacZ*+ Cells

Transverse cryosections of 10μm were taken of embryos from the brachial nerves to the sciatic nerve. Every third section was kept in E11.5 embryos while every 4^{th} section was kept in E12.5 embryos. Of these sections, 30 equally spaced sections (e.g. every other section) were selected for counting. Cells that were blue (*LacZ*+), elongated and appeared to be traveling along the dorsolateral pathway were counted and checked for double labeling with Lysotracker Red.

J. Buffers and Solutions

<u>DePC H_2O</u>
0.1%DePC in 1liter dH_2O mixed for a minimum of 2hrs on the stir plate in the hood with an open top; autoclaved and left venting in hood with loose cap.

<u>Glycerol Stock</u>
100% glycerol was added in equal volumes to dH_2O and autoclaved.

<u>Luria Broth</u> (done in hood)
1% Tryptone powder
0.5% Yeast Extract powder
1% NaCl
Brought up to 100ml with dH_2O, calibrated to pH 7.0, autoclaved, cooled and added 50μl of 100μg/ml Ampicillin. Stored at 4ºC.

Luria Broth Agar Plates (done in hood)
10g Tryptone powder
5g Yeast Extract
10g NaCl
15g Agar
Brought up to 1liter with dH_2O, autoclaved, cooled slightly to add 250µl of 100µg/ml Ampicillin and poured plates. Once set, stored at 4ºC.

Phosphate Buffered Saline (PBS) (typically made in a 10X solution)
For pH7.4: 11.2ml of 1M Potassium Phosphate Monobasic (KH_2PO4)
38.8ml of 1M Potassium Phosphate Dibasic (K_2HPO4)
Brought up to 100ml with dH_2O and autoclaved

For pH7.2: 16.8ml of 1M Potassium Phosphate Monobasic (KH_2PO4)
34.2ml of 1M Potassium Phosphate Dibasic (K_2HPO4)
Brought up to 100ml with dH_2O and autoclaved

Phosphate Buffer Saline with 0.1% Tween-20 (PBT; made fresh)
1µl of 100% Tween-20 added for each ml of PBS

Tris Buffered Saline with 0.1% Tween-20 (TBST; made fresh)
1µl of 100% Tween-20 added for each ml of TBS

SSC
20xSSC: 175.3gNaCl and 88.2g NaCitrate, volume brought up to 1liter; pH adjusted to 7.0.
10xSSC, pH4.5: 250ml 20xSSC + 30ml citric acid + 220ml DePC H_2O

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VITA

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1992	Intensive Course in Maternal Nutrition Department of Obstetrics and Gynecology University of Florida, Gainesville, FL
1988	Intensive Course in Pediatric Nutrition University of Iowa Children's Hospital Iowa City, Iowa
1984	Masters of Science in Nutrition and Dietetics Florida International University Miami, Florida.
1983	Bachelor of Science in Human Nutrition and Food Science University of Florida, Gainesville, Florida.

PRESENTATIONS:

March 2009. Interaction between the transcription factor *Sox10* and *Endothelin receptor B* in the melanocyte lineage. Poster presentation 10th Annual Biology Symposium, Florida International University, Miami FL.

December 2008. Interaction between the transcription factor *Sox10* and *Endothelin receptor B* in the melanocyte lineage. Poster presentation 48th Annual Meeting American Society for Cell Biology, San Francisco, California.

February 2008. Interaction between the transcription factor, *Sox10*, and *Endothelin receptor B* in the melanocyte lineage. Tenth Annual Florida International Biology Symposium, Kampong Gardens, Miami, Florida.

March 2007. Interactions Among *Endothelin Receptor B* and Transcription Factors, *Sox10* and *Pax3*, in Melanocyte and Glial Lineages. Biological Diversity: Cellular and Molecular Interactions. Biomedical and Comparative Immunology Symposium. Florida International University, Miami, Florida.

March 2006. Transgenic Expression of *Ednrb* Rescues the Spotting Phenotype of *Sox10* Mutant Mice. Annual Scholarly Forum, Graduate Student Association. Florida International University, Miami, Florida.

October 2005. Transgenic Expression of *Ednrb* Rescues the Spotting Phenotype of *Sox10* Mutant Mice. MBRS-Rise (Minority Biomedical Research Initiative for Scientific Enhancement) Mini-symposium, Florida Internation University, Miami, Florida.

September 2005. Transgenic Expression of *Ednrb* Rescues the Spotting Phenotype of *Sox10* Mutant Mice. Plenary speaker, Developmental Biology 1: Melanoblast/RPE Specification, Development, Survival and Apoptosis. International Pigment Cell Conference, Reston VA.

Fall 2004. The Effect of *Endothelin-3* Signaling on Early Schwann Cell Development. MBRS-Rise (Minority Biomedical Research Support Research Initiative for Scientific Enhancement) Mini-symposium, Florida International University, Miami, Florida.

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