Dysregulated PKA Activity Leads to Defective Neural Crest Differentiation and Schwann Cell Tumorigenesis

Dissertation

Presented in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in the Graduate School of The Ohio State University

By
Georgette Nicole Jones, B.S.
The Molecular, Cellular, and Developmental Biology Graduate Program

The Ohio State University
2010

Dissertation Committee:
Lawrence S. Kirschner, MD, PhD; Advisor
Denis Guttridge, PhD
Sissy Jhiang, PhD
Gustavo Leone, PhD
ABSTRACT

Proper regulation of cAMP-dependent protein kinase (PKA) is essential to normal cell proliferation, but PKA dysregulation leads to tumor formation in a range of endocrine tissues. Resulting from mutations in the main PKA regulatory subunit, PRKAR1A, Carney complex is characterized by tumors of various endocrine glands, as well as bone and cartilage tumors, Schwann cell tumors, and skin discoloration. Since PKA dysregulation affects several neural crest-derived tissues, we sought to examine the role of PKA in the neural crest.

The cranial neural crest (CNC) undergoes complex molecular and morphological changes during embryogenesis in order to form the vertebrate skull, and nearly 75% of all birth defects result from defective craniofacial development. The molecular events leading to CNC differentiation have been extensively studied; however, the role of PKA during craniofacial development has only been described in palate formation. Selective inactivation of Prkar1a in the CNC results in perinatal lethality caused by dysmorphic craniofacial bone and cartilage development and subsequent asphyxiation. Aberrant differentiation of Prkar1a-null CNC mesenchymal cells resulted in anomalous intramembranous ossification and cartilage dysplasia. These observations provide new evidence for the importance of PKA regulation in craniofacial
development, which may be beneficial to understanding and treating craniofacial birth defects.

In addition to the facial structures, proper PKA regulation is required in Schwann cells. Tissue-specific ablation of Prkar1a in a subset of neural crest precursor cells caused schwannomas with high frequency. Previously, signaling events leading to Schwann cell tumor initiation have been characterized in the context of Neurofibromatosis (NF). At the molecular level, Prkar1a-null tumors revealed loss of both NF proteins, despite the fact that transcript levels were increased, implying post-transcriptional regulation. Furthermore, the small G-proteins Ras, Rac1, and RhoA are all regulated by NF signaling. In Prkar1a-null tumors, all three molecules showed modest increases in total protein, but only Rac1 was activated. These data suggest that dysregulated PKA activation causes Schwann cell tumorigenesis via pathways that overlap but are distinct from those described in NF tumorigenesis.

Genetic interaction studies aimed at identifying the role of NF protein loss in Prkar1a-null tumors revealed that loss of Nf2, specifically, is rate-limiting for PKA Schwann cell tumorigenesis. Because Rac1 was highly activated in Prkar1a-null schwannomas, the p21-activated kinases (Paks) were evaluated for their activity. Pak6 was significantly over-expressed in the schwannomas at both the transcript and protein levels, however its activity was not different from normal Schwann cells. Despite no change in transcript levels, Pak2 protein expression was upregulated, and it was highly activated in the tumors.
Downstream targets of Pak were also up-regulated in Prkar1a-null tumors suggesting *in vivo* activation of Pak signaling. Altogether these results suggest that PKA suppression of Nf2 causes Schwannomas via Rac1-Pak signaling.

The results from this work reveal that proper PKA regulation is required in the developing neural crest, as well as in the adult neural crest-derived tissues. Not only do these studies highlight novel roles for PKA in both differentiation and proliferation, they further emphasize the importance of precise PKA regulation from embryogenesis to adulthood.
DEDICATION

Dedicated to Mom and Dad.
ACKNOWLEDGEMENTS

First, and foremost, I would like to acknowledge and thank my PI, Dr. Larry Kirschner, for his mentorship, expertise, generosity, and friendship throughout my graduate tenure. I sincerely appreciate all that you have done for me, and I fear that my words will not appropriately express my gratitude. With your help, I feel more confident that I can succeed as a young scientist. You helped me to refine my writing skills by offering me constructive criticism; you challenged me to be more creative with my experimental designs; you taught me never to underestimate the straight-forwardness of an experiment; you infused me with an enthusiasm for science, despite the obstacles that are ever-present; and you instilled in me the independence I need to progress in the research field. For these reasons, and many more, I hold an enormous debt of gratitude to you, and I will forever remember my time in your lab with fond memories.

To all the past and present members of the Kirschner lab, I offer my sincerest appreciation for all of your help and friendship over the past six years: Zhirong Yin, Parmeet Manchanda, Kiran Nadella, Emilia Mahoney, Daphne Pringle, Mei Zhang, William Towns, and Ben Glasner.
I would like to specially acknowledge and thank my graduate committee members for their thoughtful criticism and advice with my thesis project: Dr. Sissy Jhiang, Dr. Donna Kusewitt, Dr. Denis Guttridge, and Dr. Gustavo Leone. My appreciation also extends to Dr. David Bisaro, the director of MCDB, for his unending patience and willingness to help me and other graduate students in this program.

The Children’s Tumor Foundation (CTF) deserves my utmost gratitude as a Young Investigator Awardee. I thank Mr. John Risner, the president of CTF; Dr. Kim Hunter-Schaedle, chief scientific officer; and Ms. Min Wong, research program director for their extreme generosity and service to me. They also deserve special recognition for their hard work and devotion to the CTF mission of “Ending Neurofibromatosis Through Research.”

For their help and expertise, I gratefully acknowledge the following people who contributed to the studies presented in this thesis: Dr. Nancy Ratner and Jon Williams for sharing their expertise with mouse Schwann cell culture; Dr. Sung Ok Yoon and Chhavy Tep for their help with the G-protein assays and for generously donating rat Schwann cells for some of my experiments; Dr. Krista La Perle, Alan Fletchner, Dr. Anat Stemmer-Rachamimov, Dr. Ramiro Toribio, Brandy Marlow, and Lisa Rawahneh for their expertise with pathology and histology; Dr. Kimerly Powell, Michelle Carlton, Dr. Petra Schmalbrock, and Gina Mihai for their expertise with imaging and radiology; Dr. Michael Weinstein for his thoughtful perspectives; and Dr. Ian
Tonks and Dr. Graham Kay for their generous donation of the cre mice used in the studies presented here.

Finally, I would like to thank those friends and family members who have supported me during my time as a graduate student. Mom and Dad, you have more than inspired me to reach to my farthest potential. You have been supremely supportive to me emotionally and financially, and for that I cannot thank you enough. Dad, your health struggles have taught me never to give up on anything, and you will forever inspire me to work my hardest in the fight against cancer. Mom, your friendship and support are what keeps me going. Without your guidance, I am certain I would not be as strong-willed or successful. To my brothers, Shane and Brett, and their wives Tonya and Jessica, I sincerely thank you for your encouragement and advice over the years. And finally, to my closest friends Tim Butterfield, Bridget Ashcraft, Janell Ellenbecker, Adam Smith, Jen Robb, Tim Yulish, Cheryl Bolinger, Nael Alami, Mike Carathers, and many others, I thank you from the bottom of my heart for being the most wonderful, silly, generous, supportive, and understanding friends anyone could ask for.
VITA

January 21, 1981.............................................Born in Rochester, Minnesota

August 1995 to June 1999.........................Rochester Lourdes High School

August 1999 to May 2003............................B.S. Biology, University of Wisconsin – La Crosse

September 2003 to present.......................Graduate Research Associate,

                                 The Ohio State University

January 2005 to March 2005.......................Graduate Teaching Associate,

                                 The Ohio State University

June 2006 to May 2008...............................Fellowship, Young Investigator Award, Children’s Tumor Foundation
PUBLICATIONS


FIELDS OF STUDY

Major Field: Molecular, Cellular, and Developmental Biology
# TABLE OF CONTENTS

ABSTRACT .................................................................................................................. ii

DEDICATION ............................................................................................................... v

ACKNOWLEDGEMENTS ......................................................................................... vi

VITA .............................................................................................................................. ix

LIST OF TABLES ....................................................................................................... xiii

LIST OF FIGURES ...................................................................................................... xiv

LIST OF ABBREVIATIONS ......................................................................................... xvii

**CHAPTER 1: INTRODUCTION** ............................................................................... 1

1.1 THE cAMP-DEPENDENT PROTEIN KINASE (PKA) .............................. 1
    The PKA Signaling Pathway ................................................................. 1
    The PKA Holoenzyme ...................................................................... 3
    PKA Knock-Out Mouse Models ......................................................... 7

1.2 THE CARNEY COMPLEX ........................................................................... 13
    General Pathology ............................................................................. 13
    Genetics of Carney Complex ............................................................ 16
    Mouse Models of Carney Complex .................................................... 17

1.3 THE NEURAL CREST .................................................................................. 21
    Cranial Neural Crest ......................................................................... 21
    Neurocristopathies ........................................................................... 24
    PKA in Palate Development ............................................................... 26

1.4 THE NEUROFIBROMATOSIS (NF) SYNDROMES ..................................... 28
    General Pathology ............................................................................. 28
    Genetics of NF .................................................................................. 35
    Mouse Models of NF ......................................................................... 38
    PKA Interactions with the NF Proteins ............................................. 43
LIST OF TABLES

2.1: Expected and Observed Frequencies from Genetic Interaction Studies with

  TEC1KO ........................................................................................................ 63

3.1: Primer Sets Used for Real-Time PCR Analysis ................................. 94

4.1: Primer Sets Used for Real-Time PCR Analysis ................................. 114
LIST OF FIGURES

CHAPTER 1

1.1: Schematic of PKA Signaling ................................................................. 2
1.2: Carney Complex General Pathology ................................................... 15
1.3: Prkar1a<sup>+/−</sup> Mouse Model of PKA Tumorigenesis ......................... 19
1.4: Features of the Neurofibromatoses ...................................................... 32

CHAPTER 2

2.1: Gross Phenotype of TEC1KO Embryos ................................................. 51
2.2: Histological Progression of PNS Defects Found in TEC1KO Embryos ... 53
2.3: LacZ Staining of Transverse Sections Through the Nasal Septum ....... 54
2.4: MicroCT Scans of E17.5 Embryos ...................................................... 56
2.5: Alcian Blue Staining of the PNS .......................................................... 58
2.6: Histology of the E17.5 Palate and Craniofacial Bones ....................... 60
2.7: Phospho-CREB Immunohistochemistry ............................................. 62

CHAPTER 3

3.1: Tumor Incidence and Anatomic Localization of Tumors in TEC3KO Mice ................................................................................................. 75
3.2: MRI Analysis of TEC3KO Tumors ................................................................. 76
3.3: Cre Expression in TEC3KO Embryos .......................................................... 77
3.4: TEC3KO Schwannomas Exhibit Enhanced Staining for CyclinD1 but Show Heterogeneous Cre Activity ............................................................... 78
3.5: Decreased Expression of the NF Proteins in TEC3KO Schwannomas ... 80
3.6: Down-Regulation of Nf1 and Nf2 Occurs at the Post-Transcriptional Level ................................................................. 82
3.7: Loss of Akt and ERK Pathway Activation in TEC3KO Schwannomas .... 83
3.8: Signaling Analysis in TEC3KO Tumors ...................................................... 85
3.9: Small G-Proteins are Up-Regulated at the mRNA Level in TEC3KO Tumors ................................................................................. 86

CHAPTER 4
4.1: Tumor Onset Rate is Altered in TEC3KO by Conventional Mutations ... 101
4.2: Relative mRNA Expression of the Six Pak Isoforms in TEC3KO Schwannomas . ................................................................................. 103
4.3: Protein Expression Analysis Revealed Over Expression of Pak2 and Pak6 ......................................................................................... 104
4.4: Pak2 is Significantly Activated in TEC3KO Schwannomas .............. 106
4.5: Pak Signaling is Activated in vivo in TEC3KO Tumors ...................... 107
CHAPTER 5

5.1: Proposed Model for PKA-Mediated CNC Development .................................. 116

5.2: Schematic for Schwann Cell Tumorigenesis Involving PKA Signaling .. 120
LIST OF ABBREVIATIONS

AID ........................................................................................................... Autoinhibitory domain
AKAP ........................................................................................................... A-kinase anchoring protein
αMHC ........................................................................................................... Alpha myosin heavy chain
ATP ........................................................................................................... Adenosine triphosphate
BA ........................................................................................................... Branchial arch
BMP ........................................................................................................... Bone morphogenetic protein
Cα ........................................................................................................... PRKACα/PrkaCα
CALS ........................................................................................................... Café au lait spots
cAMP ........................................................................................................... Cyclic-AMP
Cβ ........................................................................................................... PRKACβ/PrkaCβ
Cγ ........................................................................................................... PRKACγ
CKO ........................................................................................................... Cardiac knock-out
CNC ........................................................................................................... Cranial neural crest
CRE ........................................................................................................... CREB response element
CREB ........................................................................................................... cAMP response element binding protein
CSRD ........................................................................................................... Cysteine-serine-rich domain
CTD ........................................................................................................... C-terminal domain
DDAH .............................................................. N\textsuperscript{G},N\textsuperscript{G}-dimethylarginine dimethylaminohydrolase
E .................................................. Embryonic day (post-conception)
ECM ........................................................... Extra-cellular matrix
EMT .......................................................... Epithelial to mesenchymal transition
ERM ........................................................ Ezrin/Radixin/Moesin family of proteins
FERM .................................................. Four-point-one (4.1), Ezrin, Radixin, Moesin
FNP .......................................................... Frontonasal process
GAP .......................................................... GTPase activating protein
Gαs ............................................................. Stimulatory G-protein
GDI .......................................................... GTP-dissociation inhibitor
GEM .................................................. Genetically modified mouse
GH .......................................................... Growth hormone
GPCR .................................................. G-protein coupled receptor
GTP .......................................................... Guanosine triphosphate
HE .......................................................... Hematoxylin and eosin
Hox .......................................................... Homeobox genes
Ihh .......................................................... Indian hedgehog
IP .......................................................... Immunoprecipitation
KO .......................................................... Knock-out
LimK .................................................. Lim Kinase
LOH .......................................................... Loss of heterozygosity
LP .......................................................... Lamina propria
LTD .......................................................... Long-term depression
LTP .............................................................. Long-term potentiation
MEFs ......................................................... Mouse embryonic fibroblasts
MPNST ...................................................... Malignant peripheral nerve sheath tumor
MSC .......................................................... Mesenchymal stem cells
NF1 ............................................................ Neurofibromatosis type I
NF2 ............................................................ Neurofibromatosis type II
NP ........................................................... Nf1\textsuperscript{+/−};p53\textsuperscript{+/−}
OE ........................................................... Olfactory epithelium
Pak ............................................................ p21 activated kinase
Pax ......................................................... Paired box transcription factors
PDB .......................................................... Paxillin binding domain
PDE .......................................................... Phosphodiesterase
pitKO ...................................................... Pituitary knock-out
PKA .......................................................... Protein kinase A
PMS ....................................................... Psammomatous melanotic schwannoma
PNS ........................................................ Primordial nasal septum
PNST ....................................................... Peripheral nerve sheath tumor
PPNAD ................................. Primary pigmented nodular adrenocortical disease
PRL .......................................................... Prolactin
r .............................................................. Rhombomeres
rGHRHR .............................. Rat growth hormone releasing hormone receptor
SC .......................................................... Schwann cells
Shh ................................................................................................................. Sonic hedgehog

TEC1 .............................................................................................. Tyrosinase expressing cre 1

TEC1KO .................................................................................................. TEC1;Prkar1a^{loxP/loxP}

TEC3 .............................................................................................. Tyrosinase expressing cre 3

TEC3KO .................................................................................................. TEC3;Prkar1a^{loxP/loxP}

TSH .................................................................................................... Thyroid stimulating hormone

WT ............................................................................................................ Wild-type
CHAPTER 1: INTRODUCTION

1.1 THE cAMP-DEPENDENT PROTEIN KINASE

The PKA Signaling Pathway

In the inactive state, the cyclic-AMP-dependent protein kinase, also known as Protein Kinase A (PKA), is a heterotetramer composed of a homodimer of two regulatory subunits each bound to a catalytic subunit. Activation of PKA starts when extracellular hormonal ligands bind to and stimulate G-protein coupled receptors (GPCR) which are large proteins consisting of seven transmembrane domains that can bind the heterotrimeric G-proteins (Figure 1.1). Upon ligand binding to the GPCR, a conformational change occurs in the receptor that allows the stimulatory G-protein (Gαs) to bind GTP and dissociate from both the GPCR and the other two G-protein subunits. Now active, Gαs goes on to activate membrane-bound adenylyl cyclase which facilitates the reaction whereby ATP is converted to cyclic-AMP (cAMP). As the levels of cAMP rise in the cytosol, these small molecules cooperatively bind to the PKA regulatory subunits and cause a conformational change that releases the catalytic subunits. Once freed, the active catalytic subunits can either remain in the cytosol or translocate to the nucleus where they can phosphorylate a range of downstream targets.
PKA is a serine/threonine kinase that can either activate or repress its targets via phosphorylation. There have been hundreds of proteins characterized as targets for PKA phosphorylation, and perhaps the most well-known target is the cAMP response element binding protein (CREB) transcription factor (1-4). Upon phosphorylation by PKA, CREB becomes activated and binds to regions of DNA known as CREB response elements (CRE) to activate gene expression. Because CRE is found in the promoter region of a large number of genes, PKA activation can lead to widespread
increases in gene transcription. Moreover, combined with hundreds of phosphorylation targets, it becomes clear that PKA signaling is very complex and that many cellular functions may be involved.

In the 1960s, when PKA was first characterized, cAMP signaling was described as a key feature of glucose metabolism. Studies since then have shown that cAMP and PKA signaling are involved in a number of cellular processes, including cell growth and proliferation, differentiation, and migration (5-7). Interestingly, the specific function for PKA signaling seems to be cell type specific, and characterizing the specific role it plays in different cell types has proven to be complex. Studies aimed at identifying unique characteristics about each subunit have helped to identify the role for PKA in various tissues.

The PKA Holoenzyme

In humans, there are four regulatory subunit isoforms, and three catalytic isoforms. The regulatory subunits can interact with any of the catalytic subunits, however the frequency of the various interactions may depend on tissue specific expression of each protein. Both human and mouse studies have aided in the characterization of each subunit, and further elucidated the role for specific subunits in a tissue-specific manner.
The Regulatory Subunits

There are four regulatory subunits for PKA which are divided into two groups, type I and type II. Each subunit contains an inhibitory domain that binds to the active site on the catalytic subunit, thereby inhibiting its activity. Type II isoforms contain a phosphorylation site in the inhibitory domain, whereas type I subunits do not; and this may result in altered binding affinity for the catalytic subunits (8, 9). Additionally, type II isoforms are known to bind A-kinase anchoring proteins (AKAP) which anchor the subunits to the cellular and subcellular membranes (Figure 1.1; (10, 11)). In contrast, type I regulatory subunits are typically localized to the cytosol.

In humans, the four regulatory genes PRKAR1A and PRKAR1B, and PRKAR2A and PRKAR2B encode the type I and type II subunits, respectively. These genes are evolutionarily conserved in mouse, and correspondingly encode the proteins Prkar1a, -r1b, -r2a, and -r2b. Studies in mice have revealed that Prkar1a and Prkar2a are both ubiquitously expressed, however the general expression levels of Prkar2a are lower than that of Prkar1a and it appears that Prkar2a function is most important in skeletal muscle and sperm (12-14). Most of the remaining tissues rely on Prkar1a for proper regulation of the catalytic subunits. In general, the b isoforms (Prkar1b and Prkar2b) are expressed in the neural tissues. Both Prkar1b and Prkar2b are expressed in the brain, albeit with differing patterns within the brain; but Prkar2b is also highly expressed in brown and white adipose tissue (8, 15, 16).
An interesting aspect of Prkar1a is that it has been shown to compensate for the other regulatory subunits if they have been mutated. Although Prkar2a was shown to play an important role in sperm function, Prkar1a was able to ameliorate the effects of Prkar2a mutation so that sperm function was not altered (13). Additionally, it was shown that in some cases Prkar1a can bind to AKAPs to compensate for loss of Prkar2a and maintain proper subcellular localization of PKA (12). Finally, Prkar1a was also able to compensate for mutations in Prkar1b and Prkar2b via increased protein stability (17). These findings revealed a back-up mechanism by which the cell can still maintain proper PKA regulation to avoid disruption of the many cellular functions involved with this signaling pathway.

The Catalytic Subunits

At the genetic level, humans have three PKAC subunit genes, designated as \textit{PRKACα} (\(C\alpha\)), \textit{PRKACβ} (\(C\beta\)), and \textit{PRKACγ} (\(C\gamma\)). Functional studies have not identified significant functional differences among the three isoforms, although there is tissue specificity of expression. By contrast, there are only two isoforms of PKA catalytic subunits found in mice – \(C\alpha\) and \(C\beta\) (encoded by \textit{Prkaca} and \textit{Prkacb}). Of these two isoforms, there also exists splice variants that have similarities to those found in humans.

The \(C\alpha\) subunit consists of two splice variants defined as \(C\alpha1\) and \(C\alpha2\), also referred to as \(C\alpha-s\) (18-20). Although the \(C\alpha1\) isoform is expressed
ubiquitously in mice, the expression of Ca2 is restricted to male germ cells, and it was shown that Ca2 is the only catalytic subunit of PKA expressed in mature sperm (21). Cβ, on the other hand, has three splice variants – Cβ1, Cβ2, and Cβ3 (19, 20, 22). Cβ1 is ubiquitously expressed, albeit at lower levels than Ca1, however, Cβ2 and Cβ3 expressions are limited to the brain. More specifically, Cβ2 expression is highest in the limbic regions of the brain, whereas Cβ3 is more diffusely expressed throughout various brain regions at low levels (22). While the Cβ isoforms comprise the major PKAC component in the brain, Ca1 is considered to be the predominant source of PKA catalytic activity in non-neuronal tissues (19, 20).

Both catalytic isoforms exhibit alternative splicing events at the first exon, resulting in amino-terminal differences between transcript variants. Interestingly, the Ca1 and Cβ1 isoforms exhibit around 91% homology, and they are both myristoylated at the N-terminus (19-23). Conversely, Ca2, Cβ2, and Cβ3 are all non-myristoylated due to splicing of alternative first exons that provide novel translation start sites and result in truncated amino termini (21, 22). It is thought that N-myristoylation of Ca1 and Cβ1 is not important for membrane tethering, as is commonly assumed with this type of co-translational modification; rather it is important for protein structural integrity and thermal resistance (23). However, the lack of myristoylation in the truncated isoforms suggests that this modification is not essential for protein stability.
PKA Knock-Out Mouse Models

Much work has been accomplished with targeted disruption of the various regulatory and catalytic subunits of PKA in mouse. These studies illustrated the importance for each subunit in embryonic development and adult mice, and further elucidated the function of each isoform in a range of tissues.

Regulatory Subunit Knock-out Mice

Complete loss of any of the regulatory subunits resulted in viable offspring, with the exception of Prkar1a−/− mice that exhibited early embryonic lethality before E10.5 (24, 25). The Prkar1a-null embryos displayed significant deficits in growth between E7.5 and E10.5, particularly in the trunk region, and the ultimate cause of death was due to failed heart tube formation (24). Although the total PKA activity in these embryos was decreased by about 40%, the basal activity was significantly increased compared to wild-type embryos (24). Similarly, Prkar1a−/− mouse embryonic fibroblasts exhibited significantly increased basal PKA activity (26). These findings further support that, as mentioned above, Prkar1a can compensate for the loss of the other regulatory subunits, but it cannot be compensated for. In addition, concomitant mutations in Ca (Prkar1a−/−;Ca−/−) resulted in decreased basal PKA activation and partial rescue of the embryonic phenotype, although they were still lethal at later stages of gestation (24).
Despite global expression of Prkar2a, complete loss of the gene resulted in healthy, normal-looking mice (12). Prkar1a compensation was observed in these mice, although there was a slight decrease in basal PKA activation (12). Prkar2a binding to AKAPs is essential in skeletal muscle to potentiate calcium signals during contraction. In response to Prkar2a mutation, there was increased binding of Prkar1a to AKAPs in order to maintain subcellular localization, even though Prkar1a has a lower binding affinity to the anchoring proteins (12). Since Prkar2a has been shown to play a role in sperm motility, loss of the protein was expected to result in altered fertility of male mice. Again, Prkar1a was able to compensate for the loss of Prkar2a, although the subcellular localization of the holoenzyme switched from being predominantly in the flagellum to instead being in the cytoplasm (13). Sperm motility was essentially unchanged, however, suggesting that proper PKA regulation (versus subcellular localization) was most important for sperm function.

PKA dysregulation has been shown to affect hippocampal long-term potentiation (LTP) which is involved in learning and memory (27). In another process known as long-term depression (LTD), sustained low-frequency stimulation results in desensitization of hippocampal synapses. Because of the brain specific localization of the Prkar1b, LTD and LTP were investigated in Prkar1b$^{-/-}$ mice. Prkar1b-null mice exhibit deficits in LTD, LTP, and depotentiation of stimuli along the Shaffer collateral-CA1 pathway that were not compensated for by Prkar1a (28). Whole brain homogenates did not, however,
show any change in total or basal PKA activation. This discrepancy may be explained by the loss of AKAP binding to Prkar1b, and compensation from Prkar1a may have mis-localized the holoenzyme (28). Therefore, both proper regulation of PKA and proper localization of the enzyme appear to be essential during learning and memory.

Prkar2b expression is highest in the brain, and more specifically in the striatum which contains dopamine-responsive neurons responsible for motor behaviour (29). PKA activity is normally high in the striatum, but Prkar2b−/− mice exhibited dramatically reduced PKA activation (29). This was attributed to inefficient compensation by Prkar1a which lead to proteolysis of the catalytic subunits. Prkar2b-null mice showed deficits in experience-dependent locomotor behaviors and were sensitized to amphetamine. Moreover, Prkar2b is also highly expressed in brown and white adipose tissue, and Prkar2b-null mice were lean compared to normal littermates (16). Compensation for this loss resulted in a switch from the type II holoenzyme to type I, which was more sensitive to cAMP signaling and caused induction of uncoupling proteins involved in metabolism. This chronic increase in metabolism lead to increased lipolysis and resistance to obesity (16, 30).

Catalytic Subunit Knock-out Mice

Skalhegg et al. (31) described a mouse in which both the Cα1 and Cα2 subunits were effectively knocked out by replacing exons 6–8 with a neomycin
resistance cassette. This targeted deletion of the Ca subunits led to perinatal lethality or delayed growth in mice. Because Ca1 is not only ubiquitously expressed, but also predominantly activated in non-neuronal tissues, the effect on homozygous null (Prkaca−/−) animals was widespread. Additionally, due to the specificity of Ca2 to the testis, there were also dramatic defects seen in sperm function in these animals.

The most notable defect seen in Prkaca−/− mice was their delayed growth. Most mutant animals died perinatally, however, ~27% survived to adulthood. Of this small percentage of mice, there was a marked decrease in overall body size at three months of age, such that mutant mice weighed only about 65% as much as their wild-type (WT) littermates (31). More recently, it has been shown that the epithelial growth factor receptor protein levels are significantly decreased in Prkaca−/− mice, which highlight a possible mechanism by which loss of PKA activation potential results in an impaired ability for cells to convey growth signals (32).

In contrast to Prkaca−/− mice, the conventional knockout (KO) mice for Cβ1 (Prkacb[β1]−/−) are viable, fertile, and phenotypically indistinguishable from their WT littermates (33). The only differences between normal mice and those lacking Cβ1 were observed in the hippocampus. Cβ1-null hippocampal slices could be stimulated acutely, but they were unable to potentiate electrostimuli along the Schaffer Collateral-CA1 pathway to the same extent as normal hippocampal slices in vitro (33). Additionally, Cβ1 null synapses were not
subject to LTD (33). Given the nature of these defects, and the tissue affected by Cβ1 mutation, a role for PKA in contextual learning becomes more apparent.

Additional evidence for the role of PKA in learning and memory is found in mice harboring mutations that ablate all three splice variants of Cβ (Prkacb<sup>−/−</sup>). Prkacb<sup>−/−</sup> mice carry a targeted deletion of exon 2, which is shared among splice variants, and results in phenotypically normal mice (34). Although the basal activity for PKA was significantly decreased in the amygdala, hippocampus, and cortex of Prkacb<sup>−/−</sup> mice, loss of Cβ resulted in a compensatory increase in Cα expression, causing the activation potential for PKA in the brain to remain largely unaffected (34). Activation of PKA in both the amygdala and the hippocampus is known to play a role in learning and memory formation. Despite compensatory up-regulation of Cα, Prkacb<sup>−/−</sup> mice were less affected by cued fear conditioning, although these observations appear to be strain specific (34). Nevertheless, given that Prkacb<sup>[β1]−/−</sup> mice have impaired hippocampal synapse potentiation and Prkacb<sup>−/−</sup> animals display learning defects despite compensation by Cα, it is apparent that the activation of the specific isoforms of PKA catalytic subunits is essential for proper molecular signaling during learning and memory.

To further address the role of PKA activation per total in the mouse, complete KO mice for both Cα subunits and Cβ1 were generated (35). Complete KO of the catalytic subunits (Prkaca<sup>−/−</sup>;Prkacb<sup>[β1]−/−</sup>) resulted in early embryonic lethality; however, when one allele of either Cα (including both splice
variants) or Cβ1 remained unaffected (Prkaca<sup>+/−</sup>;Prkacb[β1]<sup>−/−</sup> or Prkaca<sup>−/−</sup>;Prkacb[β1]<sup>+/−</sup>), there were significant defects in neural tube development leading to spina bifida and/or exencephaly. Although Prkaca<sup>+/−</sup>;Prkacb[β1]<sup>−/−</sup> animals survived birth, all of them exhibited severe spina bifida resulting from failure of vertebral arch fusion at the dorsal midline between the forelimbs and hindlimbs. No Prkaca<sup>−/−</sup>;Prkacb[β1]<sup>+/−</sup> animals survived gestation, and all of them exhibited severe neural tube defects leading to spina bifida. A small percentage (25%) of these animals had additional cranial defects that led to exencephaly, although this phenotype was not observed in Prkaca<sup>−/−</sup>;Prkacb[β1]<sup>+/−</sup> pups (35). Intriguingly, both Prkaca<sup>−/−</sup>;Prkacb[β1]<sup>+/−</sup> and Prkaca<sup>−/−</sup>;Prkacb[β1]<sup>+/−</sup> embryos were histologically indistinguishable when looking at neural tube defects in early development. Also, both genotypes displayed morphological defects in dorsal root ganglia organization, with increased apoptosis and regression by embryonic day 12.5. Altogether, these observations point to an essential role for proper PKA activation that cannot be compensated regardless of the subunit affected. Furthermore, molecular analysis of these models suggests that during neural tube development, the role of PKA in suppressing Sonic hedgehog signaling is paramount for cells to undergo patterning and differentiation into the proper neural precursors that make up the adult spinal column (35).
1.2 THE CARNEY COMPLEX

General Pathology

The Carney complex (OMIM #160980) was first described by Dr. J. Aidan Carney in 1985 as the complex of spotty skin pigmentation, myxoma, endocrine overactivity, and schwannoma (36). This autosomal dominant multiple endocrine neoplasia syndrome is very rare, and affects a wide range of endocrine and non-endocrine tissues.

One of the features of Carney complex is spotty skin pigmentation, known as lentigines, that commonly presents prior to any other manifestation of the disease (Figure 1.2A). These small freckle-like lesions are typically found on the lips, eyelids, and ears; although they can also be found in mucosal regions such as the mouth and vagina (37). Other skin lesions associated with this syndrome include blue nevi and cutaneous myxomas.

Myxomas are benign tumors involving connective tissue that are often times found in the heart. Cardiac myxoma occurs in approximately half of all Carney complex patients and they are the leading cause of death for patients because of cardiac infarcts, emboli, or surgical complications (Figure 1.2C; (38)). Additionally myxomas of the bone (osteochondromyxoma), breast, oropharynx, and the female genital tract have been described, but they are less common (38).

The most common endocrine manifestation of this syndrome is primary pigmented nodular adrenocortical disease (PPNAD; Figure 1.2D; (38)). PPNAD
is a benign disease that is characterized by increased cortisol secretion and the presence of numerous brown-colored adrenal nodules. Although this disease is generally associated with Cushing syndrome, clinical studies revealed that PPNAD in conjunction with cardiac and other myxomas was indicative of Carney complex (39). In addition to adrenal nodules, Carney complex patients often develop thyroid nodules which are typically characterized as follicular adenomas histologically (40). Less commonly, pituitary adenomas and acromegaly have been described. Although most patients exhibit increased growth hormone and prolactin secretion, fewer than 20% of them develop tumors or acromegaly (38, 41, 42). Finally, the incidence of large-cell calcifying Sertoli cell tumors is increased, with nearly one third of male patients affected (38); and approximately half of female patients may develop ovarian cysts or tumors (43).
Figure 1.2: Carney Complex General Pathology. (A) Spotty skin pigmentation (lentigines) are a common skin lesion in patients. (B) Surgical image of psammomatous melanotic schwannoma (black arrow) adjacent to the spinal cord (yellow arrow). (C) Ultrasound showing cardiac myxoma (arrow). (D) Histological section of the adrenal gland from a Carney Complex patient. Note the presence of numerous cortical nodules (arrows).

Although the main cause of death for Carney complex patients is usually due to cardiac myxoma, another common cause is from metastatic conversion of psammomatous melanotic schwannoma (PMS; Figure 1.2B). PMS is a rare form of schwannoma that, as its name implies, is highly pigmented with melanin and is histologically characterized by the presence of psammoma bodies. Despite the rarity of this tumor, approximately half of all patients who are diagnosed with PMS are also diagnosed with Carney complex (44). In most cases, PMS is a benign dumb-bell shaped tumor that may occur in the
peripheral nerves of the gastrointestinal tract, retroperitoneum, and lower spinal regions, including the pelvis (38, 44). Often times these tumors are difficult to resect surgically due to their location; and they can be a significant source of morbidity for affected patients. In addition, PMS tumors may become malignant; and as a result they are the cause of death for approximately 15% of Carney complex patients (38).

**Genetics of Carney Complex**

Initial linkage analysis of the Carney complex gene identified two candidate loci: 2p16 and 17q22-24 (45, 46). To date, no gene has been identified on the second chromosome that is causative for Carney complex. However, in 2000, Kirschner et al. identified mutations in the gene PRKAR1A that lead to the disease in nearly half of the patients tested (47, 48). Most mutations involved base pair substitutions that created an early termination codon, and subsequently lead to transcript degradation via nonsense-mediated mRNA decay (48). As a result of these mutations, PRKAR1A protein was not produced, and dysregulated PKA activation was implicated as the cause of the disease.

Studies since the discovery of PRKAR1A as the Carney complex gene have implicated the gene encoding phosphodiesterase 11A (PDE11A) in adrenocortical hyperplasia (49). PDE proteins function to break phosphodiester bonds that form the cyclic nucleotides such as cAMP. This in turn decreases
the amount of free cAMP available to bind the PKA regulatory subunits, and therefore acts as an inhibitor of cAMP/PKA signaling. Patients with PDE11A mutations were clinically diagnosed with Cushing syndrome, and did not share any other clinical or genetic features of Carney complex (49). Nonetheless, given the genetic overlap and clinical presentation similar to, but distinct from, PPNAD; it becomes clear that PKA signaling in the adrenal gland is essential for normal function.

Mouse Models of Carney Complex

As stated above, Prkar1a−/− mice die in utero before E10.5; however, Prkar1a heterozygotes (Prkar1a+/−) survive to adulthood and serve as a genetic model for Carney complex (25). Moreover, tissue specific ablation studies for Prkar1a further elucidated the need for proper PKA signaling in the pituitary and embryonic heart.

Prkar1a Conventional Heterozygotes

Targeted deletion of the second exon of Prkar1a, which contains the start codon, resulted in complete loss of protein expression (25). Prkar1a−/− mice died early in embryogenesis, but Prkar1a+/− mice survived and exhibited tumor formation in a range of cAMP-responsive tissues. The most common tumors found in these animals were in the tail and were pathologically similar to osteochondromyxoma, a feature of Carney complex (Figure 1.3A). Nearly half
of Prkar1a<sup>+/−</sup> mice exhibit these bony lesions by 8 months of age, and by 1 year over 80% of these mice were affected (25). Although the tail tumors were most frequently observed, they typically did not lead to premature mortality in these mice. Instead, most mutant mice required early euthanasia due to rapid progression of schwannomas found on the head, limbs, and rump (Figure 1.3B,C; (25)). By one year of age, approximately one third of Prkar1a<sup>+/−</sup> mice developed these neoplasms which were similar to, but distinct from, the schwannomas found in Carney complex patients. In contrast to PMS, these tumors were not pigmented and did not undergo metastasis; nonetheless, they consisted mainly of Schwann cells and were clinically characterized as schwannomas with divergent differentiation (25).

Another, less common, phenotype observed in the Prkar1a<sup>+/−</sup> mice was thyroid neoplasia, which occurred in approximately 11% of the animals (Figure 1.3D). Histologically, the masses were characterized as either thyroid adenoma or carcinoma, and the generation of this mouse represented the first conventional genetic model of spontaneous thyroid cancer (25).
Figure 1.3: *Prkar1a*± Mouse Model of PKA Tumorigenesis. (A) *Prkar1a* heterozygotes develop tail tumors (arrows) with high frequency by 1 year of age. (B,C) Non-pigmented schwannomas with divergent differentiation were observed in the rump (B) and head (C, arrows) in addition to other areas in approximately 33% of these animals. (D) Less frequently, thyroid adenomas or carcinomas were observed (arrow). Figure adapted from images in Kirschner, *et al.* (2005) *Cancer Research* 65(11): 4506-4514.

**Tissue Specific Knock-out of *Prkar1a***

Conditional *Prkar1a* knock-out mice were generated by adding *loxP* sites flanking the second exon of the gene. In combination with Cre expression, this sufficiently ablated *Prkar1a* protein expression in specific tissues (25).

Using the α-Myosin heavy chain (αMHC) promoter as a Cre driver, selective knock-out of *Prkar1a* was achieved in the embryonic heart in an attempt to model cardiac myxoma (50). These cardiac knock-out (CKO) mice died *in utero* before E12.5 due to specific defects associated with the ventricular wall. Excess PKA signaling in the embryonic heart lead to loss of
cardiac-specific transcription factor expression, and subsequently resulted in decreased transcription of genes required for cardiac development. Reduced cardiomyocyte proliferation, thinning of the myocardium, ventricular dilation, and sarcomere disorganization all contributed to the inability for the CKO embryos to efficiently circulate blood; and therefore caused growth retardation and lethality (50).

Even though the CKO mice died embryonically, all was not lost in the effort to model cardiac myxoma. Despite decreased proliferation in the CKO heart, about half of the animals displayed focal areas of myxomatous lesions adjacent to the left atrium; which is the same site as nearly three quarters of human cardiac myxomas (50). The cardiomyocytes in these lesions underwent myxoid degeneration and developed a gland-like appearance which is indicative of myxoma and a diagnostic characteristic for human tumors. Owing to the fact that Prkar1a+/− mice do not exhibit myxomagenesis, the CKO mice serve as the first model for cardiac myxoma that emulates early stages of the human condition seen in Carney complex.

Finally, to assess the role of PKA signaling specifically in the pituitary, the rat growth hormone releasing hormone receptor (rGHRHR) promoter was used to drive cre expression in the Pit1 lineage of anterior pituitary cells (51). Pit1 is a transcription factor that is expressed in a subset of pituitary cells that secrete growth hormone (GH), prolactin (PRL), and thyroid stimulating hormone (TSH). The rGHRHR-Cre mice were mated with Prkar1a-conditional animals to
generate pituitary-specific knock-out mice (pitKO, (52)). Pituitary tumors were found in about 50% of pitKO mice at 18 months of age, compared to less than 20% in control animals. Prolactinomas occur somewhat frequently in older genetically normal mice, so the observation of these tumors between groups was negligible and accounted for the majority of tumors seen in controls. However, nearly 30% of the tumors found in pitKO mice were characterized as non-prolactinomas and in most cases they highly expressed the hormones GH, PRL, and TSH (52). These mice also exhibited significantly increased levels of GH in serum samples, even in the absence of a defined tumor. Overall, the data from this mouse model illustrates the importance for proper PKA regulation in the pituitary for both regulated cell growth and homeostasis.

1.3 THE NEURAL CREST

Cranial Neural Crest

The cranial neural crest (CNC) is responsible for the development of most of the head structures, including the bones and cartilage, connective tissues, smooth muscle, and skin of the face (53). CNC cells originate and are inducted from the ectodermal-neuroectodermal ridges of the perfusion cephalic neural folds by E8 (54-56). At this stage, neural crest cells undergo epithelial-mesenchymal transition (EMT), which triggers CNC induction and the expression of a number of factors that mediate CNC migration (see below). By
E9, the CNC has already migrated to the frontonasal process (FNP) and branchial arches (BA) 1 and 2. The FNP is later divided into the medial nasal process and the lateral nasal processes which eventually make up the nose and cheeks, respectively. BA1 gives rise to both the mandibular and maxillary processes, and part of the external ear; and BA2 will contribute to the hyoid bone, styloid process and parts of the middle and external ear. To accomplish the development of these structures, the FNP and BA undergoes differentiation into bone, cartilage, smooth muscle, and glial fates.

**Markers of CNC Migration**

Some of the earliest genes expressed in the CNC are the zinc finger transcription factors Snail and Slug, which are important for induction and migration of the crest cells (57). Premigratory CNC cells express Snail in addition to the cell adhesion molecules E-cadherin and N-cadherin. During EMT, Snail represses E-cadherin and upregulates Slug expression to induce CNC migration (57-59). Furthermore, N-cadherin is also repressed after induction to promote cell migration (60, 61). Slug, and the Sox proteins (namely Sox8, 9, and 10), are key to promoting crest formation by influencing EMT and therefore CNC induction (62-64). During migration, CNC cells express a number of factors that facilitate the migratory capacities of the cells. One such marker is the receptor ErbB4, which binds to neuregulin in order to drive “pathfinding” of the cells to their proper endpoint locations (65). Another marker
of CNC migration is N-cofilin which breaks down actin filaments and accelerates actin treadmilling to facilitate movement of the cell (66). Some studies have also implicated the extracellular matrix (ECM) as an important player during CNC migration. Factors including a number of collagens, fibronectin, hyaluronic acid, fibrillin, and others are deposited by surrounding cells into the ECM to aid in the pathfinding and cessation of CNC migration (reviewed in (53, 67)).

Markers of CNC Differentiation

The most well-known markers of patterning and differentiation are the Homeobox (Hox) genes. During cranial development, the primitive hindbrain is made up of rhombomeres (r) that contain Hox-expressing CNC cells which eventually migrate to the branchial arches. BA1 is populated by r1 and r2, but it does not express any Hox genes. Nonetheless, BA2 is populated by r3 and r4 CNC cells that robustly express Hoxa2 (68, 69). Similarly to the Hox genes, the Dlx transcription factors are also expressed in a pattern that corresponds to the development of specific skeletal structures. For example, Dlx1 and Dlx2 are expressed mostly in the CNC of the proximal BAs to promote chondrocranial and dermatocranial development of the hyoid and styloid processes, the middle and inner ear, and the dermis. Likewise, Dlx5 is expressed distally in the olfactory and otic placodes as well as the CNC of the mandibular process of BA1 to support chondrogenesis and osteogenesis in these tissues (67). Some
other markers of bone differentiation in the CNC include the Bone Morphogenetic Proteins (BMPs). BMPs are expressed within both the CNC (BMP2 and 7) and peripheral cells of the mesoderm and ectoderm (BMP4 and 5) to induce bone and cartilage formation (67). Also expressed in both CNC and peripheral tissues are the *Paired box* (*Pax*) transcription factors. In the CNC, *Pax3* is important for the development of Schwann cells and melanocytes, while *Pax6* plays a central role in the development of bones, cartilage, and glial cells of FNP and BA1 derived tissues (70-72). While most *Pax* genes are expressed in the CNC, *Pax1*, 2, and 5 are also expressed outside of the CNC where they all play important roles in patterning and differentiation during craniofacial development (67). Finally, Sonic Hedgehog (Shh) and Indian hedgehog (Ihh) are both expressed outside the CNC where they function to organize the mesodermal midline and promote cartilage and bone formation, respectively (73, 74).

**Neurocristopathies**

In 1974, Dr. Robert P. Bolande first coined the term “neurocristopathies” to define human syndromes that involve dysmorphogenesis and/or tumorigenesis of neural crest cells or neural crest-derived tissues (75). These diseases can be further categorized based on the properties of the neural crest that are affected, such as migration, proliferation, and differentiation. For example, tumor syndromes such as Neurofibromatosis can result from
complications with proliferation and/or differentiation of neural crest-derived tissues; whereas birth defects like mandibulofacial dysostosis and cleft palate result more from defects in neural crest migration.

In addition to the CNC-derived tissues, including bone, cartilage, smooth muscle, glia, and melanoblasts; the trunk and cardiac neural crest gives rise to the adrenal medulla, pigment cells, neurons and glia of the peripheral nervous system, and connective tissue that separates the major heart vessels (76, 77). With so many tissues dependent upon proper neural crest development, genetic mutations that affect any aspect of this may have widespread pathological effects.

One very common neurocristopathy is Neurofibromatosis (described in more detail below), which results from defects in proliferation of neural crest-derived tissues. Neurofibromatosis is a tumor predisposition syndrome that affects several neural crest-derived tissues such as the adrenal medulla, melanocytes, and glia. Other tumorigenic syndromes include the multiple endocrine neoplasia (MEN) syndromes, Sipple syndrome, and multiple mucosal neuroma syndrome (reviewed in (78)). Given that Carney complex also affects several neural crest derivatives, it might also be considered a neurocristopathy. Specific tumors associated with these syndromes, and other neurocristopathies not listed here, include pheochromocytoma, a tumor of the chromaffin adrenal medullary cells; neuroblastoma, a common childhood cancer of the peripheral nervous system; and medullary thyroid cancer (78).
Defects in neural crest migration are known to cause mandibulofacial dysostosis (also known as Treacher Collins syndrome), Hirschprung disease, and cleft palate. Treacher Collins syndrome affects approximately 1 in 50,000 individuals and is characterized by dysmorphogenesis of the craniofacial bones resulting in microtia, micrognathia, and cleft palate (79-81). Hirschprung disease results from a lack of ganglia in the bowel, and is present in approximately 1 in 5,000 live births (82). In addition to other genes, RET is commonly mutated in patients with Hirschprung disease; and given that RET mutations also cause thyroid carcinoma and possibly neuroblastoma, the importance of this gene in the neural crest becomes apparent (83-85). Finally, observed in 1:500 to 1:1000 live births, cleft lip and palate are among the most common birth defects seen in humans (86). Typically these malformations result from defective neural crest cell migration, and PKA signaling has been shown to play an important role in palatogenesis.

**PKA in Palate Development**

PKA regulation and CREB phosphorylation have been shown to play an important role during palate formation, but a function for this signaling pathway has not otherwise been described in the CNC (87-89). The PKA regulatory subunits are differentially expressed in the palate, which may elucidate a complex mechanism by which PKA regulation occurs during palatogenesis (87). *Prkar1a* and *Ca* mRNA was constitutively expressed throughout palate
development, but mRNA for the *Prkar2a* and *Prkar2b* subunits was only elevated during early stages of palatogenesis (E12, (87)). After palatal shelf fusion (E13-E14), the expression of the type II regulatory subunit mRNAs steadily decreased (87). Likewise, the type II regulatory subunits were the predominant source for PKA regulation in early palatogenesis, while the type I regulatory subunits (particularly Prkar1a) were more active after palate fusion (90, 91). In addition to the global expression patterns of the subunits in the developing palate, subcellular localization of the regulatory subunits was investigated. Prkar1a was generally localized to the nuclear fractions of palate epithelial cells, whereas both of the type II regulatory subunits were generally localized to the cytosol (87, 91). Interestingly, AKAP95 mutations may impart susceptibility to cleft palate, although the role of type II PKA regulatory subunits in this process has not been evaluated (92). Taken together, these studies revealed a complex mechanism whereby palate development may rely on the localization of specific isozymes of PKA to properly regulate cell proliferation, migration, and differentiation.

Because the levels of CREB protein expression remain constant throughout palate development, most studies have focused on the phosphorylation status and localization of this transcription factor (88, 89). Just prior to palatal shelf fusion (on E12), there is a spike in intracellular levels of cAMP and therefore PKA activation, and this is accompanied by an increase in CREB phosphorylation (89, 90, 93, 94). Immediately following fusion, on E13-
14, the level of CREB phosphorylation declined, which may be attributed to increased phosphatase expression (88). Interestingly, TGF-β signaling was shown to also induce CREB phosphorylation in a PKA-independent manner (95). Murine embryonic palate mesenchyme cells, treated with TGF-β showed time- and dose-dependent increases in CREB phosphorylation, however PKA, calcium/calmodulin kinase II (caMKII), and Erk1/2 activation were all not involved in this mechanism (95). This novel finding suggests cross-talk between the PKA and TGF-β signaling pathways during palatogenesis, and further adds to the complexity by which gene regulation occurs in this developing tissue.

1.4 THE NEUROFIBROMATOSIS (NF) SYNDROMES

General Pathology

The Neurofibromatosis (NF) syndromes are generally characterized by the formation of multiple peripheral nerve sheath tumors (PNST), and there are two types of NF, which are genetically and pathologically distinct. Familial schwannomatosis (OMIM #162091) is also characterized by PNST formation, however less is known about it genetically and it will not be discussed further here. Interestingly, the NF syndromes, familial schwannomatosis, and Carney complex are the only inherited diseases in humans that predispose patients to the development of PNSTs.
**Neurofibromatosis Type I (NF1)**

NF1 (OMIM +162200), also known as von Recklinghausen disease, is the most commonly inherited tumor syndrome in humans, and it is diagnosed in approximately 1 of every 3000 individuals (96, 97). As mentioned above, this disease is considered a neurocristopathy because it affects several neural crest-derived tissues, including melanocytes, Schwann cells, and the adrenal medulla.

The most common tumors found in NF1 patients are neurofibromas, of which three forms exist: cutaneous (dermal), subcutaneous, and plexiform. These benign PNSTs consist mainly of Schwann cells, fibroblasts, perineurial cells, and mast cells; and they can arise virtually anywhere along the length of peripheral nerves (96-98). Nearly 100% of NF1 patients develop dermal neurofibromas which occur on nerve endings in the skin (Figure 1.4A; (99)). Because patients can develop hundreds of these growths in their lifetime, they are a significant source of morbidity and mental distress for many patients (99, 100). Subcutaneous neurofibromas are similar to the dermal neoplasms in that they arise from the Schwann cells of a single nerve, however they originate along peripheral nerves beneath the skin (96, 98, 99, 101). Plexiform neurofibromas involve multiple nerves, and are found in about 25-30% of NF1 patients (102-104). Because of the involvement of multiple nerves, vessels, and surrounding tissue, plexiform neurofibromas are very difficult to resect. Unlike dermal and subcutaneous neurofibromas, the plexiform tumors have been
reported to transform into malignant peripheral nerve sheath tumors (MPNST) in about 2-5% of patients, and the lifetime risk of this malignancy may be up to 10% (105, 106). MPNSTs can metastasize to a wide range of tissues including bone, brain, lung, liver, and retroperitoneum; and are generally associated with a poor prognosis (105). Tumor size, location, age of onset, family history, and degree of surgical intervention are all factors that contribute to the overall prognosis for NF1 patients affected by MPNST, with an average 2-year survival rate of about 50% (107).

Other neural crest-derived tissues affected in NF1 patients are the skin (melanocytes) and adrenal medulla. Café-au-lait spots (CALS) are large, benign, pigmented regions of skin that occur in about 95-99% of NF1 patients (Figure 1.4B; (108, 109)). Although these lesions are not specific to NF1, the presence of multiple CALS greater than 5 mm in diameter (in children) or greater than 15 mm in diameter (in adults) is characteristic of the disease (98, 109). CALS can be found anywhere on the body, with the exception of the palms of the hands, soles of the feet, and scalp; and exposure to sunlight can deepen their pigmentation (96). Skin fold freckling in areas such as the groin, under the breast, and in the fold of the eyelid are also common (110). The locations for these freckles are atypical in that they are not sun-exposed regions of the body, and they are specifically associated with NF1 (110). In addition to skin pigmentation, NF1 patients are prone to pheochromocytoma which is a typically benign tumor of the chromaffin cells of the adrenal medulla.
Pheochromocytoma is also not specific to NF1 as it is found in patients with multiple endocrine neoplasia type 2 (MEN2), von Hippel-Lindau syndrome, familial paraganglioma syndrome, and rarely in the normal population (111). There is an increased risk of pheochromocytoma in NF1 patients, however less than 5% of patients actually develop the disease (112). Additionally, malignant transformation can occur in these tumors, although only 11% of NF1 patients with pheochromocytoma exhibited this change (113).
Figure 1.4: Features of the Neurofibromatoses. (A-C) Specific features of NF1. (A) Dermal neurofibromas, note the large number of tumors in such a small area of the body. (B) CALS present on the rear torso. (C) Lisch nodules (arrows) which are distinct from natural eye pigmentation. (D-F) Specific features of NF2. (D) MRI image depicting bilateral vestibular schwannomas (arrows). Note the extension of tumor growth into the intra-auditory canals (arrow heads). (E) MRI image of the cervical spine depicting spinal menigioma (arrows) with elongations along the axis of the spine (arrow heads), and probable intramedullary ependymoma (black arrow head). (F) Forearm of a NF2 patient showing multiple subcutaneous schwannomas. These images adapted from figures in references (99, 110, 114).

Additional diagnostic criteria for NF1 include the presence of pigmented lesions on the iris known as Lisch nodules (Figure 1.4C). These lesions are found in about half of all NF1 patients by the age of 30, and nearly 100% of patients will develop Lisch nodules by the time they are 60 years old (115). Finally, learning disabilities, osseous lesions, optic gliomas, myeloid disorders, and astrocytomas are also all diagnostic features of NF1 (reviewed in (98, 115)).
Neurofibromatosis Type II (NF2)

NF2 (OMIM #101000) is less common than NF1, occurring in about 1 in every 40,000 individuals, and the pathology of the disease is distinct from NF1. Neurofibromas, multiple CALS, Lisch nodules, and osseous lesions are very rarely seen in NF2 patients. Instead, bilateral vestibular schwannomas are the hallmark feature of NF2, occurring in about 90-95% of patients (116-118). These benign Schwann cell tumors develop along the vestibular portion of cranial nerve VIII (also known as the vestibulocochlear nerve) which transmits nerve impulses from the inner ear to the brain, and their size and location often presents a challenge for surgical intervention (Figure 1.4D). As a result, about 30% of children and 60% of adults with NF2 exhibit hearing loss (119-121). Non-vestibular schwannomas of the remaining cranial nerves are observed as well, with cranial nerve III and V tumors occurring in approximately 15% and 72% of patients, respectively (122).

Meningiomas are somewhat common in the general population, but NF2 patients are particularly prone to their development. About 50-60% of patients develop intracranial meningiomas, and about 20% may have meningiomas in the spinal column (Figure 1.4E; (116, 123)). In contrast to sporadic cases, multiple meningiomas may be present in NF2 patients, and the proliferative potential of the tumor cells is significantly higher in the NF2 tumors (124).

Intramedullary spinal tumors are also a key feature of NF2, occurring in 20-50% of patients (114, 125). Ependymomas account for approximately 75%
of all intramedullary tumors (Figure 1.4E), and astrocytomas and gliomas also occur somewhat frequently (114, 126, 127). Less commonly, intramedullary schwannomas have been observed (126). In contrast, multiple schwannomas (apart from those found in the cranial nerves) account for nearly 90% of all extramedullary spinal tumors, and are often localized to the spinal nerve root (125).

As a direct result of nerve compression by peripheral and spinal nerve tumors, patients often develop peripheral neuropathies such as distal pain, numbness, and paralysis (114). In some cases, these symptoms may lead to the discovery of PNST in a patient that was otherwise undiagnosed. Interestingly, many patients may experience peripheral neuropathy in the absence of a frank tumor. This may be attributed to a number of factors including multiple microscopic schwannomas along the length of a single nerve, Schwann cell hyperplasia causing nerve constriction, or defective myelination by the Schwann cells (116, 128). Additional features of NF2 include ocular opacities (60-80%), retinal hamartomas (10-20%), cataracts (10-25% in patients younger than 50), skin plaques (40-50%), and singular (not multiple) CALS (30-50%; reviewed in (114)). Multiple dermal and subcutaneous neoplasms are observed in approximately 60-70% of NF2 patients (Figure 1.4F), although they are typically not neurofibromas, as is seen in NF1 patients (116, 123). Instead, they are characterized as schwannomas and the average
number of growths per patient is 7, compared to hundreds of dermal neurofibromas that can occur in a single NF1 patient (116, 123).

**Genetics of NF**

**NF1: A GAP in the Sequence**

In 1990, the causative gene for NF1 was identified on chromosome 17q11.2 as a large, 270 kb gene encoding a 13 kb transcript (129-131). The resulting gene product (Neurofibromin) showed sequence similarities to the yeast IRA1 and IRA2 proteins which function as GTPase activating proteins (GAP) for the small GTPase signaling molecule, Ras (132, 133). Further studies revealed that the GAP related domain of Neurofibromin indeed interacted with Ras, from both human and yeast, to downregulate its activity (134, 135). Ras is a well known oncogene that, when bound to GTP, works to transmit extracellular hormone or growth factor signaling cascades throughout the cell, thereby stimulating growth or proliferation. Activating Ras mutations have been identified in approximately 20% of all human cancers (136). GAP proteins facilitate the hydrolysis of GTP to GDP, and in turn deactivate Ras signaling. In NF1, the loss of this GAP protein leads to constitutively activated Ras, and therefore dysregulated cell proliferation in the peripheral nerve sheath cells (137).

Analysis of the mutation spectrum in NF1 patients revealed that most mutations involved splice site alterations. Ars *et al.* reported that up to half of
the patients tested showed mutations disrupting consensus splice sites, leading to either exon skipping or cryptic splice sites (138). In the same study, 44 types of mutations were identified, and 32% percent of them pertained to splicing. Additionally, frameshift mutations accounted for 41% of the described genetic changes, although they were not as frequently detected in patients as the splicing mutations (138). Alternative splicing defects were also recently reported to predict disease severity, which varies from person to person. Specific mutations related to splicing may explain up to 93.5% of the variability seen in human patients with NF1 (139). Other mutations described in NF1 include nonsense and missense mutations which, in addition to the splice site and frame shift mutations, may cause truncation of the transcript and subsequent nonsense-mediated mRNA decay (138-141).

**NF2: Merlin Casts a Spell**

The NF2 gene was mapped to chromosome 22q12, and later identified as a 4.5 kb transcript encoding the protein Schwannomin (so named for its role in schwannoma development), which has sequence homology to the Ezrin/Radixin/Moesin (ERM) family of cytoskeletal proteins (142-144). Trofatter et al. later renamed the protein Merlin (Moesin-Ezrin-Radixin-like protein) to more appropriately signify its relation to the ERM proteins (144). This family of cytoskeletal proteins functions mainly to tether actin filaments to the cytoplasmic membrane and thereby regulate cell polarity, motility, adhesion,
endo- and exocytosis, and signal transduction (reviewed in (145)). Although Merlin shares many similarities with the ERM family proteins, both at the genetic and functional levels, it is distinct in that it is the only ERM family member that has been described as a tumor suppressor. Via interactions with CD44 at the plasma membrane, it has been shown that Merlin plays an essential role in contact inhibition of cell growth (146). Additionally, reports have shown that Rac1 and RhoA directly, and Ras indirectly, are inactivated by Merlin; which illustrates the mechanisms by which Merlin exerts its tumor suppressive function (147-149).

Several studies have shown that, similarly to NF1, the specific type of mutation observed in NF2 may well predict the severity of clinical presentation in a patient (150-155). In general, those mutations which preserved the integrity of the 3’ (C-terminal) end of the gene (protein) resulted in milder disease (153). The majority of mutations (approximately 65%) involved nonsense and frameshift mutations that ultimately lead to truncated protein production, and a more severe phenotype (150, 152, 153). Interestingly, patients with these types of mutations encountered a higher rate of intramedullary tumors than those patients with splice-site or missense mutations or in-frame deletions (125). About 25% of NF2 somatic mutations involve splicing, and the phenotypic severity depends on the specific exons affected. Those patients with altered splicing affecting exons 1-7 had a significantly higher susceptibility to meningiomas than those whose mutations affected exons 8-13 (151, 153, 156-
In general, very few splicing mutations affect exons 14-17. The remaining 10% of mutations are missense mutations that generally are associated with milder presentations (152).

**Mouse Models of NF**

**NF1 Mouse Models**

The mouse *Nf1* gene shares over 98% sequence homology to the human *NF1* gene, and *Nf1*−/− conventional knock-out mice die embryonically before E14.5 most likely due to heart malformations and exencephaly (160-164). Although it was interesting to note the importance of *Nf1* in embryonic development, these knock-out mice were not considered adequate models for NF1 tumorigenesis. When considering the hallmarks of the human NF1 phenotype (neurofibromas, CALS, and Lisch nodules), the *Nf1*+/− mice also failed to serve as an accurate model (161, 162). All was not lost, however, in the effort to model certain aspects of the disease. Three quarters of *Nf1*+/− mice developed lung adenocarcinoma, lymphoma, lymphoid leukemia, myeloid leukemia, and/or neurofibrosarcoma (75%; (162)). Although these lesions are not chiefly associated with NF1, there have been reports of patients with neurofibrosarcomas and myeloid disorders (115, 165). In closer relation to NF1 patients, 30% of the heterozygous mice developed pheochromocytoma; representing a unique mouse model for this type of tumor (162).
Since the development of the conventional knock-out mice, several labs have joined in the effort to understand NF1-related tumorigenesis using a variety of approaches including alternative targeted knock-out alleles, chimeric mice, tissue-specific knock-out, and secondary gene mutations. The *Nf1* mutant mice described above harbored mutant alleles affecting exon 31, which is a region often mutated in human subjects (161, 162). Targeted mutations affecting exon 23, also commonly affected in human patients, resulted in learning defects but did not predispose the mice to tumorigenesis (166). Chimeric mice were used to avoid the lethal effects of conventional loss of *Nf1*, yet still retain “hits” to both copies of the gene in somatic cells. Mice that exhibited a moderate amount of *Nf1*<sup>−/−</sup> chimerism (based on degree of coat color variability) developed plexiform neurofibromas in the dorsal root ganglia or peripheral nerves of the limbs with 100% penetrance (167). Moreover, the number of these lesions per animal was between 10-100, showing striking similarly to the multiplicity of tumorigenesis in humans.

To assess the role of *Nf1* in specific tissues, conditional knock-out mice were generated using the *loxP/cre* approach (*Nf1<sup>loxP/loxP</sup>; (168)). Loss of *Nf1* in neuronal cells, with the use of *Synapsin-l cre*, resulted in reduced cerebral cortex thickness, growth retardation, and astrogliosis marked by significant increases in astrocyte number throughout the brain and specifically in the cerebral cortex (168). Unfortunately, these mice never developed astrocytomas, optic gliomas, or neurofibromas despite the obvious neural and glial impacts. In
a different approach, $Nf1^{loxP/-}$ mice that express $Krox20$-cre (Schwann cell-specific cre) generated plexiform neurofibromas with 100% penetrance by 1 year of age (169). These tumors were molecularly and histologically similar to human plexiform neurofibromas. Of note, loss of heterozygosity (LOH) often occurs early in tumorigenesis in human patients. The $Krox-20;Nf1^{loxP/-}$ model closely mimics the genetics of the human disease because it is a conventional heterozygote that in essence exhibits LOH in the Schwann cells of the peripheral nerves. $Krox-20;Nf1^{loxP/loxP}$ and $Nf1^{+/-}$ mice were not subject to peripheral nerve tumors, a finding that further elucidates the role of LOH and the microenvironment during tumorigenesis (169).

Finally, mutations in the tumor suppressor p53 have been associated with MPNST, thus mouse models with both $Nf1$ and $p53$ mutations were developed (167, 170-173). Because these two genes are on the same chromosome in mice (chromosome 11), and because LOH often times results in deletion of large chromosomal regions, both cis and trans mutations were generated (167, 173). $Nf1^{+/-};p53^{+/-}$ (NP) trans mice developed tumors similarly to the conventional mice with heterozygous mutations for either gene, albeit sooner and at greater frequency (167). On the other hand, NP cis mice developed MPNST with high penetrance, they survived for only 5 months on average, and LOH for both genes was consistently seen (167, 173). Interestingly, the NP cis mice also develop astrocytoma and glioblastoma, and the severity of tumor development was strain specific; which may serve as an
informative model for phenotypic variations observed in human patients (172).

Although several of *Nf1* mouse models summarized here do recapitulate neurofibroma or MPNST development, unfortunately no mouse models currently exist that adequately model the formation of dermal neurofibromas.

**NF2 Mouse Models**

Similarly to the NF1 mouse models, a number of approaches have been utilized to accurately model NF2 tumorigenesis in the murine system. *Nf2*−/− conventional knock-out mice died embryonically before E7.5 due to failure of extra-embryonic ectoderm formation (174). As with the *Nf1*−/− animals, it is interesting to note the importance of *Nf2* during development, but these particular animals did not provide insight into the tumorigenic potential of *Nf2* mutations. Conventional heterozygotes for *Nf2* survived and developed tumors such as osteosarcoma, fibrosarcoma, and hepatocellular carcinoma; although they did not mimic the human condition (175). Interestingly, metastatic lesions were highly prevalent in the *Nf2*+/− mice compared to control animals, suggesting a novel role for Merlin in metastasis (175). *Nf2* is also located on chromosome 11 in mice, so concomitant mutations in *p53* (as in the *Nf1* models) were generated in *cis* and *trans* with *Nf2*. Surprisingly, *Nf2*+/−;*p53*+/− *cis* and *trans* mice both developed osteosarcomas and fibrosarcomas more rapidly and more frequently than *Nf2*+/− alone, and they exhibited metastatic potential as well (175). Unfortunately, none of the above mice developed schwannoma which is
the key feature of NF2. Thus, alternative genetic studies were employed to better understand the role of Nf2 in schwannomagenesis.

In keeping with the relevance to human disease, mutations in the Nf2 gene were generated that mimic specific mutations commonly found in patients. One such mutation was a C-terminal deletion after base 314 of the cDNA (Nf2\(^{\Delta Cter}\)), and another involved deletion of bases 39-121 (representing exons 2-3) without frameshift (Nf2\(^{\Delta 39-121}\); (176)). Transgenic mice were developed expressing either of these mutant forms of Nf2 under the control of the rat P0 promoter (Schwann cell specific; (176)). The premise behind this approach was that these mutant genes may act as a dominant negative when overexpressed in the Schwann cells of otherwise normal mice. Although the P0;Nf2\(^{\Delta Cter}\) mice did not develop tumors beyond the normal spectrum for older wild-type mice, one third of the P0;Nf2\(^{\Delta 39-121}\) mice did develop tumors originating from the Schwann cells of peripheral nerves in addition to Schwann cell hyperplasia (176). This therefore suggests that indeed the loss of exons 2 and 3 in Nf2 acts as a dominant negative allele in Schwann cells, and gives insight into the mechanism by which NF2 tumorigenesis may occur in humans.

To address the role of Nf2 specifically in the Schwann cells, a conditional allele of Nf2 was generated that flanked exon 2 with loxP sites (177). In conjunction with cre expressed under the control of the P0 promoter, these P0;Nf2\(^{loxP/loxP}\) mice were highly susceptible to tumor formation (177). More specifically, they developed malignant schwannomas (4-35%), Schwann cell
hyperplasia (75-100%), cataracts (14-59%), and cerebral calcifications (~30%); all documented features of NF2. The addition of a p53 heterozygous mutation in this model lead to a significantly increased potential for MPNST development (33-77%) and invasion, and lead to a significantly shorter lifespan (2-5 months) compared to the $P0;Nf2^{loxP/loxP}$ alone (10-24 months; (177, 178)).

While some of the mouse models presented here did exhibit a number of features similar to the human NF2 phenotype, unfortunately none of the models exhibited the formation of meningiomas or ependymomas. Since these tumors occur quite frequently in human NF2 patients, the development of $Nf2$ models for these lesions would be highly beneficial to discover mechanisms by which they are formed.

**PKA Interactions with the NF Proteins**

Because Carney complex and the NF syndromes are among the only inherited diseases known to cause PNST formation, it is important to understand what the causative genes may have in common with each other on the molecular level. Several studies have shown that PKA can directly interact with and phosphorylate Neurofibromin (Nf1) and Merlin (Nf2) in vitro, however the exact function or downstream mechanism for these interactions and how that pertains to tumor development is as yet undetermined (179-182).

At least six putative PKA phosphorylation sites exist on Nf1, three of which are in the cysteine-serine-rich domain (CSRD) and the other three are
near or in the C-terminal domain (CTD; (180)). The specific function for both the CSRD and the CTD of Nf1 have not been fully defined yet, but due to the high rate of mutation in these areas that result in loss of protein production, it was shown that these regions may serve as autoregulatory regions (183). Binding of $N^G,N^G$-dimethylarginine dimethylaminohydrolase (DDAH) to the CTD increases the accessibility of the CSRD to PKA for phosphorylation (182). It was suggested that this mechanism underlies the intersection of Ras and cAMP signaling pathways (182). DDAH has been characterized previously as an enzyme that metabolizes inhibitors of nitric oxide synthetases, but a role for this particular function in relation to Nf1 is not understood. Likewise, the GAP function of Nf1 has not been examined after PKA phosphorylation, so the specific regulatory effects PKA exerts are not fully known.

Additional studies have shown overlap between Nf1 and PKA signaling. For example, loss of Nf1 in mouse Schwann cells resulted in a concomitant increase in cAMP levels, suggesting that Nf1 may serve to antagonize the production of cAMP in the cell (184). In contrast, mutations in the Drosophila $nf1$ gene resulted in decreased adenylyl cyclase activity, and caused an overall reduction in body size (185). Interestingly, the $nf1$-null defect was rescued by activation of PKA (186). Altogether these data implicate cell-type and species-specific functions for PKA and Nf1 signaling overlap, but the role for these interactions in tumorigenesis remains to be seen.
Little is known about the precise functionality of PKA phosphorylation of Nf2, although it likely plays an inhibitory (growth permissive) role. Previously the p21-activated kinase (Pak) proteins were shown to phosphorylate Nf2 at serine 518, thereby inducing a conformational change that inactivated the protein (187-189). PKA was also reported to phosphorylate at serine 518 independently of Pak, and this lead to heterodimerization of Nf2 and its family member Ezrin (179). Two separate studies have shown that both Nf2 and Ezrin may serve as AKAPs by binding to the Prkar1b and Prkar2a subunits of PKA respectively (190, 191). It appeared that the tumor suppressive function of Nf2 occurred when it served as an AKAP, but upon phosphorylation by PKA there was a switch whereby Nf2 dissociated from Prkar1b and dimerized with Ezrin to assume a growth permissive function (179, 191). The cross-talk between Nf2 and PKA appears to play an important role in the propagation of cell growth signals, however the exact downstream mechanisms affected by these interactions has not been uncovered.

A second PKA phosphorylation site exists on Nf2 at serine 10, which lies in the actin binding region of the protein (181). This is a unique site in that PAK has not been shown to phosphorylate at that residue. Phosphorylation at serine 10 does not affect the ability for serine 518 to be phosphorylated, and therefore it also does not inhibit dimerization of Nf2 and Ezrin (181). Serine 10 phosphorylation appeared to involve actin cytoskeleton alterations and it therefore affected cell morphology and migration. In particular, phosphorylation
of serine 10 resulted in stabilized actin filaments, which lead to increased filopodia formation and increased migration compared to cells expressing a non-phosphorylatable isoform of Nf2 (181). It is clear from these observations, as well as those seen with serine 518 phosphorylation, that PKA is a key regulator of Nf2 function; however more work is needed to fully understand how these interactions confer tumorigenicity in Schwann cells.
CHAPTER 2

NEURAL CRESC SPECIFIC LOSS OF \textit{Prkar1a} CAUSES PERINATAL LETHALITY RESULTING FROM DEFECTS IN INTRAMEMBRANOUS OSSIFICATION

2.1 INTRODUCTION

One of the most important and dynamic cell types involved in embryonic development is the neural crest. These cells are responsible for the proper development of a large number of adult structures ranging from the enteric ganglia to the bones of the face. As described in chapter 1.3, the CNC cells originate in the ectodermal-neuroectodermal ridges of the cephalic neural folds and subsequently undergo epithelial to mesenchymal transition (54, 56). This triggers the expression of soluble factors that mediate CNC migration to the FNP and BA1 and BA2. As the FNP develops, it gives rise to the nose and cheeks, as well as to the bony structures of the sinus cavity. The BAs gives rise to the mandibular and maxillary processes, the hyoid bone, styloid process, and the middle and external ear. Precisely regulated differentiation of the CNC ultimately leads to the orchestrated development of all of the craniofacial structures including bone, cartilage, smooth muscle, and glia.
Precisely regulated PKA signaling is required in the embryo in order for normal development of neural and mesodermal structures. When only one allele of either catalytic subunit is functional (e.g. Ca\(^{+/−}\);Cβ\(^{−/−}\) or Ca\(^{−/−}\);Cβ\(^{+/−}\)), causing dramatic decreases in total PKA activity, severe developmental defects arise, including spina bifida and exencephaly (reviewed in (192)). On the other hand, when PKA is abnormally activated, such as in the case of Prkar1a\(^{−/−}\) embryos, aberrant primitive streak migration occurs causing defects in tissues derived from the mesenchyme, such as the BA, heart tube, somites, and portions of the head (24). As a result of these problems, Prkar1a\(^{−/−}\) mice exhibit embryonic lethality before E9.5 (24, 25). This phenotype was improved by loss of one or both alleles of Ca, but viable Prkar1a\(^{−/−}\) mice have never been recovered (24).

Nearly three quarters of all birth defects described in humans result from problems with craniofacial development (193). One very common birth defect is cleft palate, which occurs in 1:500 to 1:1000 live births (86). PKA activation and regulation has been previously described to play an important role in CREB phosphorylation during palate formation, however its function in relation to the CNC has not been elucidated (89). Although CREB is responsive to PKA activation, it can also be phosphorylated by other kinases in response to growth signals (194). For example, studies have shown that cAMP signaling triggered by TGF-β can induce CREB phosphorylation independent of PKA during
palatogenesis, which suggests cross-talk between the PKA and TGF-β signaling pathways (95).

Our interest in the role of Prkar1a in the neural crest also stems from studies of the Carney complex. These patients exhibit manifestations in neural crest-derived tissues, including melanocyte hyperplasia (lentiginosis) and Schwann cell tumorigenesis (195). In $Prkar1a^{+/−}$ mice, Schwann cell tumors are observed in 33% of animals and knock-out of $Prkar1a$ in a subset of facial neural crest cells caused increased schwannoma predisposition (25). These observations give further support to the importance for $Prkar1a$ in the neural crest.

In light of these previous findings, we sought to develop a neural crest-specific knock out mouse for $Prkar1a$ to better understand the role of PKA regulation during the development of neural tissue. In this chapter, we report that ablation of $Prkar1a$ from post-migratory neural crest cells results in perinatal lethality due to asphyxiations caused by severe malformations of the craniofacial bones. Abnormalities associated with aberrant differentiation of CNC-derived mesenchymal cells in craniofacial tissues were consistently seen, and we observed clear evidence of defects in intramembranous ossification of the developing bones of the face and skull. Furthermore, we show that the excess PKA activity was due to the Cα subunit, as genetic reduction of this isoform rescued the mutant phenotype. These data indicate a novel role for
proper PKA regulation during embryonic development in the CNC, which may be beneficial to the understanding and treatment of craniofacial birth defects.

2.2 RESULTS

**Loss of Prkar1a in the Neural Crest Causes Perinatal Lethality**

The *Tyrosinase Expressing Cre 1 (TEC1)* murine line was designed to express cre under the *Tyrosinase* promoter and enhancer, which is expressed specifically in the neural crest cells by E10.5 (196). *TEC1* mice were mated with mice harboring the *Prkar1a* conditional allele (*Prkar1a*\^loxP/loxP; (25)) to generate a homozygous neural crest-specific knock-out, *TEC1;Prkar1a*\^loxP/loxP.

Genotyping of 21 day old pups revealed that *TEC1;Prkar1a*\^loxP/loxP (hereafter referred to as *TEC1KO*) mice were not observed at expected Mendelian frequencies (Table 2.1). Thus, it was surmised that *TEC1KO* embryos died in utero, and time course experiments were conducted to determine the stage of embryonic demise. No significant morphological differences were noted between wild-type and *TEC1KO* littermates until E17.5 when mutants exhibited obvious swelling and rounding of the head (Figure 2.1A,C). Mutant embryos appeared generally larger than their wild-type littermates, and body weight comparisons revealed that *TEC1KO* embryos were significantly heavier at E17.5 than normal embryos (*p* = 0.0036; Figure 2.1B). Late-stage *TEC1KO* embryos displayed normal embryonic movements,
and surprisingly survived through birth; however, immediately following birth, they were unable to breathe. Within 1-2 hours post-delivery, all TEC1KO pups exhibited cyanosis and severe abdominal distension, indicating ingestion of air while attempting to breathe (Figure 2.1D), and they ultimately succumbed to asphyxiation.

Figure 2.1: Gross Phenotype of TEC1KO Embryos. (A) E14.5 TEC1KO embryos (right) are indistinguishable from wild-type littermates (left). Although the mutant appears slightly smaller than the wild-type in this figure, this was not a consistent finding. (B) E17.5 TEC1KO litters weighed significantly more than wild-type litters (p=0.0036), and (C) the mutants (right) exhibited increased swelling and rounding of the frontonasal (black arrow) and maxillary bones (white arrow) compared to wild-type embryos of the same age (left). (D) Within 1-2 hours post-delivery, TEC1KO neonates showed signs of asphyxiation, such as facial cyanosis. Abdominal distention (arrow) results from swallowing air during attempts to breathe. * p ≤ 0.01

TEC1KO embryos were analyzed for malformations in the craniofacial region, specifically focusing on the sinus cavity. Histological analysis of normal and TEC1KO embryos at E12.5 showed no obvious differences, but at E14.5
hematoxylin and eosin (HE) staining revealed mild deviation of the primordial nasal septum (PNS) in TEC1KO embryos (Figure 2.2A,B). Some thickening of the lamina propria (LP) that separates the PNS from the olfactory epithelium occurred at this stage as well, which was attributed to edema (Figure 2.2F, bar/arrow). The severity of deviation in the PNS and swelling in the LP increased with age to E17.5 (Figure 2.2C,D and G,H). In addition, the PNS was significantly thicker than that of age-matched controls (Figure 2.2I-L, bars; p<0.0001). As mice are only able to breathe through their nasal passages, it is likely that obstruction of the airway caused by excessive swelling of the LP and septal deviation was the cause of death for these animals.
Figure 2.2: Histological Progression of PNS Defects Found in TEC1KO Embryos. (A-D) 4x magnified views of transverse sections of embryonic sinuses. For orientation, the brain is to the right in each panel, and the nasal openings are to the left. (A) Wild-type E14.5 embryo. (B) E14.5 TEC1KO sinus showing slight deviation (arrow) of the primordial nasal septum (PNS). (C) Wild-type E17.5 embryo. (D) E17.5 TEC1KO embryo. Note that septal deviation is exacerbated at this stage (arrow). (E-H) 10x magnified views of the boxed areas from panels A-D, respectively. (E) Wild-type E14.5 embryo. Note the thickness of the lamina propria (LP, bar). (F) E14.5 TEC1KO sinus showing edema (arrow) and thickening (bar) of the LP. (G) Wild-type E17.5 embryo, the bar indicates thickness of the LP. (H) Mutant E17.5 sinus showing increased edema (arrow) and thickening (bar) of the LP. (I-L) 40x views of the PNS from panels E-H, respectively. The black bars represent thickness of the septum, which was significantly increased in mutants by E17.5 (L, p<0.0001). Abbreviation: OE, olfactory epithelium.

Histological sections of E14.5 and E17.5 embryos that carried a copy of the Rosa26$^{LacZ}$ cre reporter (197) were stained for X-gal in order to confirm cre expression in the affected tissues of TEC1KO embryos. Staining was observed throughout the PNS and LP, but not in the olfactory epithelium (Figure 2.3). These results directly paralleled the HE staining that showed severe alterations in the PNS and LP of TEC1KO embryos, whereas the olfactory epithelium was unaffected.
LacZ staining of E14.5 (left panels) and E17.5 (right panels) in the primordial nasal septum (PNS) and the lamina propria (LP), indicating cre expression in those tissues. The olfactory epithelium (OE) was devoid of staining, confirming morphologic observations in mutants exhibiting nasal septal defects, whereas the olfactory epithelium is essentially normal.

**MicroCT Imaging of TEC1KO Mice Reveals Both Qualitative and Quantitative Craniofacial Bone Defects**

MicroCT scans were performed in order to more completely visualize the malformations of the craniofacial bones in TEC1KO embryos. Total body scans showed no difference in the appendicular or portions of the axial skeletons of E17.5 mutant embryos, consistent with the fact that those bones are not neural crest derivatives. However, there were remarkable malformations in the neural crest-derived bones of the face, such that the premaxilla and nasal bones showed increased radio-density in the mutants (Figure 2.4C, white arrows).
This equated to a statistically significant increase in premaxilla volume (p≤0.01), but the density of the two bones was essentially unchanged (Figure 2.4, bottom). In addition, the mandibles of TEC1KO embryos had variably radiolucent areas (Figure 2.4C, dashed arrow), and they were significantly larger and less dense than those of the wild-type controls (p≤0.01; Figure 2.4, bottom).

Perhaps most striking was the apparent loss of palate bones and the zygomatic arch in TEC1KO animals (Figure 2.4D, dashed oval/arrow). In this analysis, it was not clear if the bone was truly absent or rather not calcified enough to be visualized by microCT scanning. Surprisingly, there was no statistically significant difference in volume between TEC1KO and wild-type palates, although this may be due to variability in the wild-type samples. The small amount of bone that was present in the TEC1KO palate was much less dense than that of wild-type (p≤0.01).
Figure 2.4: MicroCT Scans of E17.5 Embryos. (A) Sagittal view of wild-type skull. (B) Axial view of wild-type palate. The dashed oval signifies the region of the palate. (C) Sagittal view of TEC1KO skull. Note the increased opacity of the premaxilla (Pm) and nasal (N) bones (solid white arrows). These bones, in addition to the frontal (F), parietal (P), mandible (M, dashed arrow) and maxilla (Mx) are irregularly shaped with variably radiolucent areas. (D) Axial view through TEC1KO palate. Note the absence of a zygomatic arch (Z, arrow) and central palate bones (dashed oval). (Bottom) Volumetric (left) and densitometric (right) measurements for individual craniofacial bones. Volume is expressed as mm$^3$, and density is expressed in Hounsfield units (HU). Note that Palate/Maxilla measurements included all of the bones found in panels B and D with the exception of the premaxilla. Also, the parietal, occipital, and other small bones of the face and neck were collectively measured and designated as Extras in the graphs. * p ≤ 0.05, ** p ≤ 0.01.
TEC1KO Embryos Exhibit Defects in CNC Mesenchymal Cell Differentiation and Intramembranous Ossification

Because proper proliferation, apoptosis, migration, and differentiation of the neural crest are all critical to normal CNC development, each of these mechanisms was investigated in TEC1KO CNC tissues to evaluate the role of Prkar1a in those cells. Results from BrdU incorporation assays and cleaved caspase-3 immunolabeling experiments showed no significant differences between mutant and wild-type embryos, indicating that the proliferative and apoptotic indices were unaffected (data not shown). Additionally, tracking of the neural crest cells revealed no changes in CNC migration between wild-type and TEC1KO embryos. This observation may be partly explained by the fact that the majority of the neural crest has already migrated prior to E10.5 when cre expression initiates.

To determine if proper differentiation of the CNC-derived tissues was achieved in TEC1KO, histological sections from E14.5 and E17.5 embryos were stained with Alcian blue to evaluate the morphology of the cartilage in the PNS. Although no significant changes in the cartilage were observed at E14.5, the staining revealed distinct differences between TEC1KO and wild-type embryos at E17.5 (Figure 2.5). The mutant cartilage consisted of disorganized chondrocyte lacunae with elongated, crescent-shaped nuclei (Figure 2.5, black arrows) and variable amounts of glycosaminoglycan matrix formation (Figure 2.5, white arrows). Although the septae were thicker, there was no difference in
the number of chondrocytes present in TEC1KO PNS compared to wild-type. This result is consistent with our observations that there were no alterations in proliferation or apoptosis in that tissue. Based on these observations, we propose that the increased thickness of the mutant cartilage was due to disorganization of the glycosaminoglycan matrix and chondrocyte dysplasia; which may have promoted pliability and therefore deviation of the PNS.

**Figure 2.5: Alcian Blue Staining of the PNS.** Wild-type (left) and TEC1KO (right) sections through the PNS were stained with Alcian blue for the presence of cartilage. Note that wild-type chondrocytes contain rounded nuclei and are uniformly aligned, whereas the disorganized mutant chondrocyte nuclei are typically elongated and crescent-shaped (black arrows). In addition, the amount of glycosaminoglycan matrix (white arrows) is highly variable in the TEC1KO PNS which likely contributed to the overall flexibility of the tissue causing severe deviation.

In addition to cartilage, we also examined the palate and craniofacial bones histologically to identify possible differentiation defects present in those tissues. Although cleft palate was anticipated based on the microCT scans, HE staining of coronal sections revealed no sign of cleft palate in the mutants (Figure 2.6A,B). Despite this, there were still obvious abnormalities in the
secondary palate, including edema, apoptotic cells, and mitotic figures (Figure 2.6J, inset). Additionally, while the wild-type mice exhibited bone formation encircling the nasopharynx, this bone was only present lateral to the airway in TEC1KO animals (Figure 2.6E,F). As a result, the nasopharynx lacked the necessary support to maintain an open airway, which may have further contributed to the inability of these animals to draw air into their lungs.

Besides the palatal defects, atypical cartilaginous structures flanked the palate region in the mutants (Figure 2.6B,F), and excess cartilage extended into the region of the frontal bone, which typically is devoid of cartilage at this stage of development (Figure 2.6C,D). The mutant frontal bone had thicker trabeculation (Figure 2.6H, bar), and there was a dramatic increase in fibrous connective tissue deposition in both the frontal bone and the mandible (Figure 2.6H,L). This phenotypic change was accompanied by marked osteolysis, as evidenced by the presence of numerous osteoclasts (Figure 2.6L, arrows). Combined, these features contributed to the increased volume of the bones, and directly paralleled the CT densitometry data which showed decreased radiodensity in the mandible and palate.
Figure 2.6: Histology of the E17.5 Palate and Craniofacial Bones. (A,B) 4x views of coronal sections through the embryonic palate. (A) Wild-type embryo showing completely fused secondary palate (SP). (B) TEC1KO palate. Note that the secondary palate is fused, however it is dramatically thicker than normal. Also note that the nasopharynx (NP) is collapsed due to loss of bony support. (C,D) 10x views of the frontal bone. Note the presence of cartilage in the mutant (D, arrows) that is not found in the wild-type (C). (E,F) The boxed areas in panels A and B, respectively, are shown at 10x magnification. Note the erroneous cartilage flanking the mutant (F). Also, note that the bone found in wild-type (E) encircling the nasopharynx is found only lateral to the palate in TEC1KO. (G,H) 40x magnification of the areas boxed in panels C and D, respectively. Bone trabeculation (bars) was thicker and disorganized in mutant (H) compared to normal (G), and there was increased deposition of fibrous connective tissue (arrows). (I,J) 40x view of the boxed areas in panels E and F, respectively. (I) Wild-type bone adjacent to the nasopharynx. (J) TEC1KO secondary palate. Note the absence of bone and the presence of apoptotic cells (inset, white arrowhead) and mitotic figures (inset, black arrowhead). (K) 40x view of wild-type mandible. (L) 40x view of TEC1KO mandible. Note the increased fibrous connective tissue and numerous osteoclasts (arrows) indicating osteolysis. Abbreviations: B, bone; Br, brain; C, cartilage; E, eye; T, tongue.

Taken together, these unusual observations indicated that the loss of Prkar1a had a dramatic effect on CNC cell differentiation, causing interference with intramembranous ossification of the craniofacial bones. Specifically, we observed that CNC-derived mesenchymal cells that normally would differentiate
directly into osteoblast precursors produced a mix of cartilage and disordered bone, whereas other cells produced ectopic cartilage only (Figure 2.6).

**Ablation of Prkar1a is Associated with Increased PKA Signaling**

In order to understand signaling changes associated with loss of Prkar1a, we studied signaling events involved in CNC differentiation. In our previous tissue-specific knockouts of Prkar1a, we observed enhanced PKA activity (26, 50). In order to confirm PKA activity in the CNC, we performed immunostaining for the phosphorylated (activated) form of CREB and found increased pCREB staining in the cartilage of the FNP and in the abnormal CNC-derived bone (Figure 2.7). Knowing that TFG-β signaling could also account for increased CREB phosphorylation, we stained for phospho-Smad 1/5/8, which is also a marker of BMP signaling required for bone formation. There was no difference in staining between wild-type and TEC1KO sections, indicating that TGF-β and BMP signaling was not altered in these animals (data now shown). Additionally, previous reports have shown the importance of PTHrP downregulation of the Runx2 transcription factor during bone development, which is dependent upon PKA activation (198, 199). Staining for PTHrP and Runx2 also revealed no differences between wild-type and mutant embryos (data not shown). Finally, staining for Sox9, a marker for cartilage differentiation, was also not changed between wild-type and TEC1KO (data not shown). Taken as a whole, these data indicate that loss of Prkar1a causes dysregulated PKA activation leading
to enhanced CREB phosphorylation. This subsequently interferes with normal CNC differentiation via a mechanism independent of other known signaling pathways involved in craniofacial development.

Figure 2.7: Phospho-CREB Immunohistochemistry. (Top left) 40x view of the wild-type nasal septum (PNS). Staining was observed in the lamina propria (LP) but not in the PNS. (Top right) 40x magnification of TEC1KO PNS. Increased nuclear pCREB staining was observed throughout the LP and the PNS (arrows). Additionally, pCREB staining was elevated in the fibrous connective tissue of the TEC1KO mandible (bottom right, arrows) compared to wild-type (bottom left).

Proper Regulation of PrkaCα is Crucial for Normal Craniofacial Development.

In order to define which catalytic isoform of PKA is most important for craniofacial development, TEC1;Prkar1αloxP/+ mice were mated to Ca+/−;Prkar1αloxP/loxP and Cβ+/−;Prkar1αloxP/loxP animals to generate TEC1KO pups.
carrying a mutation in either the Cα or Cβ subunit. The Ca crosses yielded no significant deviation from expected Mendelian frequencies (p=0.1489), indicating that a 50% reduction in Cα was sufficient to rescue the TEC1KO phenotype (Table 2.1). Ca+/−;TEC1KO animals survived to adulthood, were fertile, and they generally appeared to have a normal phenotype. Conversely, the Cβ crosses did not yield any rescue of the mutant phenotype (p<0.0001), suggesting that the bulk of PKA signaling in the CNC comes from Cα. Based on these experiments, it was concluded that Prkar1a specifically regulates Cα activity after E12.5 in the primordial craniofacial structures derived from the CNC in order to allow proper differentiation of the cartilaginous and bony tissues that make up the face.

Table 2.1: Expected and Observed Frequencies from Genetic Interaction Studies with TEC1KO.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Observed</th>
<th>Expected</th>
<th>n</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEC1KO</td>
<td>0</td>
<td>22.50</td>
<td>90</td>
<td>≤ 0.0001</td>
</tr>
<tr>
<td>Ca+/−;TEC1KO</td>
<td>10</td>
<td>15.60</td>
<td>74</td>
<td>0.1489</td>
</tr>
<tr>
<td>Cβ+/−;TEC1KO</td>
<td>0</td>
<td>9.88</td>
<td>74</td>
<td>≤ 0.0001</td>
</tr>
</tbody>
</table>

2.3 DISCUSSION

The TEC1KO phenotype is complex, and it involves defects associated with several tissue types. The most detrimental deformations were found in the nasal region where edema and severe deviation of the PNS prevented these
animals from breathing upon birth. Dysplastic chondrocytes, paired with
disorganization of the glycosaminoglycan matrix contributed to an increased
thickness of the PNS, and loss of structural integrity led to dramatic curvature of
the tissue. Loss of skull shape caused by defects in CNC-derived bone likely
also contributed to this phenotype by removing a rigid support structure for the
developing nasal septum. Additionally, increased swelling in the LP and
deficient nasopharyngeal support further constricted the airway in these
animals, ultimately resulting in asphyxiation.

At the developmental level, there are two mechanisms by which bone
can be formed: intramembranous ossification and endochondral ossification.
The former mechanism is utilized during the formation of the majority of the flat
bones of the skull and face, and this same process appears to play a role in the
healing of bone fractures (200). During intramembranous ossification of the
skull, mesenchymal cells of the CNC differentiate into osteoprogenitor cells
which further differentiate directly into osteoblasts (reviewed in (201)). Little is
known about the molecular basis of intramembranous ossification, and PKA
signaling has not been previously reported to play a role. In TEC1KO mice, we
observed that dysregulated PKA activation in the neural crest was sufficient to
cause impaired intramembranous ossification. As a result, in some areas bone
was inappropriately resorbed, whereas in other areas the trabecular bone was
thicker and dysmorphic. Additionally, Prkar1a−/− CNC cells gave rise to ectopic
cartilage in locations such as the frontal bone and near the palate. Our
observations suggest that a portion of Prkar1a<sup>−/−</sup> mesenchymal cells that would normally undergo intramembranous ossification are instead triggered to differentiate into cartilage. Those cells that are able to initiate intramembranous ossification, however, do so inefficiently and are unable to produce normal bone. An alternative possibility is that defective cross-talk between those cells undergoing intramembranous ossification and those in the process of endochondral ossification may in part be responsible for those islands of cartilage.

In the areas where bone is present, such as the mandible and frontal bones, there was a marked increase in osteolysis. There are at least two possibilities to explain this observation: either the bone never formed properly so it was being resorbed, or bone development occurred prematurely and remodeling started prenatally. Examination of E14.5 bone in the mutants revealed mild changes in bone development; however mature bone was not present, consistent with an unaffected timing of mineralization. This evidence supports the likelihood that there was improper bone formation and substantiates the hypothesis that PKA plays a role in the early events that lead up to CNC mesenchymal cell differentiation into osteoprogenitor cells during intramembranous ossification.

This study showcases a range of defects associated with the inability of CNC cells to undergo proper differentiation in the process of intramembranous ossification. As our lab has previously reported, Prkar1a<sup>−/−</sup> mice exhibit an
increased risk of bone tumors in the tail vertebrae (25). This location suggested to us that the tumors might initiate at the site of local trauma. As intramembranous ossification is part of the healing process for bone fractures (200), we now believe that these tumors may reflect the same defect observed in intramembranous ossification in the TEC1KO model. Specifically, Prkar1a+/− tail tumors exhibited increased fibrous connective tissue, similar to that observed in TEC1KO frontal and mandibular bones (25, 202). Additionally, although Prkar1a+/− tail tumors were of osteoblastic nature, there was significant turnover of the bone marked by decreased mineralization of the tissue and increased osteoclasts (202). In the TEC1KO mandibles, the presence of osteoclasts suggested increased osteolysis as well. Similar bone tumors have also been reported in Carney complex patients, primarily in the sinus region (203). Notably, these lesions were often congenital abnormalities, and given their location in the sinus region, there may be similarities to the TEC1KO model. However, the bone defects observed in TEC1KO mice are not necessarily associated with tumorigenesis. Taken as a whole, the previous studies, combined with the observations in TEC1KO embryos, suggest that proper PKA regulation is essential for both normal bone development as well as homeostasis of adult bone.

Here, we show new data suggesting a novel role for PKA in CNC differentiation, but PKA is already known to play an important role in palate development. Loss of PKA activity can cause cleft palate, and in TEC1KO
embryos dysregulated activation of the enzyme actually increased palate thickness. CREB phosphorylation was increased in these and other CNC-derived tissues in the TEC1KO sections, independent of TGF-β or BMP signaling alterations. In addition, given that there were no changes in PTHrP and Runx2 staining, we postulate that the TEC1KO phenotype is indeed PKA dependent, and does not involve alterations in other known pathways involved in craniofacial development.

Recently it was shown that epigenetic control of the transcription factors Otx2 and Lhx1 by histone deacetylase 8 (Hdac8) plays an important role in neural crest cell specification of the craniofacial bones (204). Conventional Hdac8 knock out mice, as well as neural crest-specific Hdac8 knock-outs, exhibited ossification defects of the frontal and interparietal bones that led to brain trauma and perinatal lethality. Although the pathology described in the Hdac8 knock out mice was distinct from that of TEC1KO animals, the previous study may provide clues for future studies aimed at characterizing the molecular basis for PKA-related ossification defects.

We demonstrate here that a fine balance of activation and repression of Ca exists in order for craniofacial features to develop normally. It is still unclear, however, whether the individual TEC1KO defects occur independently or if they result from problems in a coordinated aspect of development that has not been previously understood. Future work in this model may identify mechanisms by which tissues previously thought to differentiate independently, may in fact rely
more heavily upon surrounding tissues for proper differentiation and maturation. Additionally, this model could serve as a useful tool for better understanding the molecular basis of craniofacial bone development, which may in turn be beneficial for treatment strategies for craniofacial birth defects found in humans.

2.4 MATERIALS AND METHODS

**Mouse Experiments**

The generation of Prkar1a<sup>loxP/loxP</sup> and TEC1 mice has been previously described (25, 196). TEC1 and Prkar1a<sup>loxP/loxP</sup> mice were mated to produce the neural crest specific knockout mouse, TEC1;Prkar1a<sup>loxP/loxP</sup> (TEC1KO). Embryonic experiments were carried out via timed matings and observation of vaginal plugs. 6 litters (11 TEC1KO embryos, 35 wild-type embryos) were weighed at E17.5, and the results were analyzed using a two sample T-test for statistical relevance.

The Ca<sup>+/−</sup> and Cβ<sup>+/−</sup> mice have been described previously (31, 33) and were obtained from the Mutant Mouse Regional Resource Centers (www.mmrrc.org). TEC1;Prkar1a<sup>loxP/+</sup> mice were mated with Ca<sup>+/−</sup>;Prkar1a<sup>loxP/loxP</sup> and Cβ<sup>+/−</sup>;Prkar1a<sup>loxP/loxP</sup> animals to generate Ca<sup>+/−</sup>;TEC1;Prkar1a<sup>loxP/loxP</sup> (Ca<sup>+/−</sup>-TEC1KO) and Cβ<sup>+/−</sup>;TEC1;Prkar1a<sup>loxP/loxP</sup> (Cβ<sup>+/−</sup>-TEC1KO) animals.
Histology and Immunohistochemistry

All embryos were fixed in 10% formalin, processed with ethanol and xylene, and embedded in paraffin wax. Sections were taken using a microtome and mounted on glass slides for further staining. HE staining was performed using a Leica autostainer (Leica Microsystems Inc., Bannockburn, IL). Samples were stained with hematoxylin (Thermo Scientific, Waltham, MA) for 8 min, eosin (Sigma Aldrich, St. Louis, MO) for 30 sec, dehydrated in ethanol and xylenes, and coverslipped. Alcian blue staining was performed manually in 1% Alcian blue (Sigma Aldrich) prepared in 0.1 N HCl for 30 min. Nuclei were counterstained using Nuclear Fast Red (Sigma) for 5 min before dehydration and coverslipping.

For immunostaining, histological sections were deparaffinized and boiled in antigen retrieval solution (Vector Labs, Burlingame, CA) for 20 min. The slides were blocked in 5% goat serum and incubated in primary antibody (phospho-CREB, Cell Signaling Technology, Danvers, MA) diluted in block solution. Secondary biotinylated antibodies were used (Vector Labs), and color development was achieved using the ABC Elite kit (Vector Labs) and DAB chromogen (Dako, Carpinteria, CA).

To accomplish LacZ staining, primary embryonic tissues were harvested, embedded in Tissue-Tek O.C.T Compound (Sakura, Torrance, CA), and frozen to -80°C. Histological sections were obtained using a cryostat and fixed with 0.2% glutaraldehyde. Staining was achieved using 1 mg/ml X-gal (Sigma Aldrich, St.
Louis, MO) at 37°C overnight. Slides were subsequently dehydrated and mounted with coverslips.

**MicroCT Scans**

MicroCT images were obtained on an Inveon microCT *in vivo* scanner (Siemens USA, Malvern PA) for 4 wild-type and 4 TEC1KO E17.5 embryos. Full body scans were done using 2 projections per degree, a 650 msec exposure and 0.5 mm of Al filtration. These settings were selected to maximize soft tissue contrast without sacrificing the scan to data artifacts. A settle time of 500 msec was used to keep the sample and acquisition steady at maximum magnification. Initial resolution acquisitions were 10.1 µm, however this was decreased to 20.2 µm to reduce noise in the scan. Densitometric and volumetric measurements of individual craniofacial bones were obtained using Inveon Research Workplace software (Siemens), and the results were compared using a standard two sample T-test to check for statistical relevance.
CHAPTER 3

TISSUE-SPECIFIC ABLATION OF Prkar1a CAUSES SCHWANNOMAS BY SUPPRESSING NF PROTEIN PRODUCTION

3.1 INTRODUCTION

There are four human diseases associated with the formation of Schwann cell tumors. These lesions are the hallmarks of the Neurofibromatosis (NF) syndromes, NF1 and NF2, as well as familial schwannomatosis and Carney complex. NF1 is caused by mutations in the NF1 tumor suppressor (encoding Neurofibromin) and patients may clinically manifest Schwann cell neoplasia as neurofibromas or as malignant peripheral nerve sheath tumors (MPNSTs). NF2 is also caused by mutations in a tumor suppressor gene (NF2, which encodes the protein Merlin). Patients with NF2 develop multiple benign schwannomas, including both the pathognomonic vestibular schwannomas as well as non-vestibular schwannomas. Familial schwannomatosis, which has recently been suggested to be caused by mutations in the SMARCB1 gene (205), appears only to involve benign, non-vestibular schwannomas.

Schwannomas are also a component of Carney complex, as they are observed in approximately 14% of patients (36, 206). More specifically, these
tumors have been designated histopathologically as psammomatous melanotic schwannomas (PMS) for their histopathologic appearance and high degree of pigmentation. Because of their location in and around the spinal column, these tumors are a significant cause of morbidity and mortality for CNC patients (38). At the genetic level, CNC is caused by inactivating mutations in PRKAR1A, and patient tumors exhibit enhanced PKA activity (47). In a Prkar1a<sup>+/−</sup> mouse model, schwannomas were observed in approximately 33% of animals, and facial neural crest specific knock-out of the Prkar1a gene recapitulated Schwann cell tumorigenesis (25).

Mouse models of NF1 and NF2 have also been generated by creating the appropriate knockout alleles. In contrast to the observations in the Prkar1a<sup>+/−</sup> model, neither Nf1<sup>+/−</sup> nor Nf2<sup>+/−</sup> mice develop Schwann cell tumors (207). However, tissue-specific knock-out of these genes does recapitulate neoplasia in Schwann cells and other tissues (169, 208-210).

Because the genetics of NF have been well described, this information has been used to study signaling pathways that may contribute to Schwann cell tumorigenesis. Neurofibromin contains a GAP domain, which promotes the return of Ras to its inactive GDP-bound state. When Neurofibromin is lost, Ras signaling is upregulated, thereby causing activation of downstream effectors such as ERK and Akt (211-215). In contrast, Merlin is a member of the ERM family of proteins that links the cytoskeleton to membrane signaling complexes. Although Merlin does not have a GAP motif, nor does it directly interact with
Ras, it can interfere with the complex of ERM family proteins that couples Ras signaling to cytoskeletal changes that occur during cell division (148). As a result, alterations in ERK and Akt signaling may also occur (148, 216). Loss of Merlin can also cause activation of the small G-proteins Rac and Rho and their downstream effectors (149). These pathways are also activated indirectly by loss of Neurofibromin in tumors via the downstream effectors of Ras signaling, including the PI3K pathway (217).

In contrast to studies showing the effects of mutations in the NF genes, activation of the PKA pathway does not have a well established role in Schwann cell tumorigenesis, despite the fact that both humans and mice with PRKAR1A/Prkar1a mutations develop these neoplasms. In this study, we sought to characterize, in detail, the Schwann cell tumors arising in our tissue-specific knock-out model of Prkar1a, and to study the effects of this genetic manipulation on the function of Neurofibromin and Merlin. We report that ablation of Prkar1a in Schwann cells leads to post-transcriptional loss of both the Nf1 and Nf2 gene products. Despite these observations, this unique model of Schwann cell tumorigenesis occurs in the absence of Ras (and therefore ERK or Akt) pathway activation. However, expression of the small G-proteins Rac and Rho is increased in the tumors, and Rac activity is significantly elevated. Collectively, these data indicate that PKA dysregulation triggered by loss of Prkar1a causes Schwann cell tumorigenesis via pathways that overlap, but are distinct from, those that cause NF1 and NF2.
3.2 RESULTS

Characterization of TEC3;Prkar1a\textsuperscript{loxP/loxP} (TEC3KO) Mice

The TEC3 line was developed to express cre under the control of the enhancer-less Tyrosinase promoter, which enables transgene expression in a limited subset of facial neural crest derivatives (196). This cre line was crossed with mice carrying a conditional null allele of Prkar1a (25) to generate tissue specific knock-out mice (TEC3;Prkar1a\textsuperscript{loxP/loxP}, henceforth called TEC3KO animals). These mice were born at expected Mendelian frequencies (data not shown), but developed uni- or bilateral tumors on the face (Figure 3.1A, B) within a few months. Observation of a cohort of 35 animals revealed that the penetrance of the tumor phenotype was approximately 50% by 18 weeks of age, and nearly 80% by 40 weeks (Figure 3.1C). Although most mice manifested the phenotype early in life, tumors could develop as late as one year.
Figure 3.1 Tumor Incidence and Anatomic Localization of Tumors in TEC3KO Mice. (A, B) A typical schwannoma is shown in a TEC3KO mouse. Note the gelatinous consistency of the tumor and its localization to the facial nerve lateral to the orbit. (C) Kaplan-Meier plot of tumor onset in a cohort of TEC3KO animals (n = 35). Animals were monitored for 40 weeks of life, and tumor onset was considered when a tumor reached 0.5 cm in diameter.

To identify the tissue of origin for the schwannomas, TEC3KO and control mice were studied by magnetic resonance imaging (MRI) at a time before palpable tumor development (Figure 3.2). Coronal and axial scanning revealed small masses on the side of the face and lateral to the orbit in TEC3KO animals. Although they frequently caused extrusion of the orbit when larger (data not shown), these studies did not indicate an origin from the optic nerve. Based on these data and careful, anatomic observation, we determined that the tumors originated from Schwann cells originating in the trigeminal ganglion and supporting the 5th cranial nerve, also referred to as the trigeminal nerve. Because the TEC3 transgene was not initially reported to be expressed
in this location, we re-analyzed expression of the cre by crossing it to mice carrying the $\textit{Rosa26}^{\text{lacZ}}$ reporter allele (197). This analysis revealed robust expression of cre in Schwann cells of the trigeminal ganglion (Figure 3.3), further supporting our observations that $\text{TEC3KO}$ tumors arise from progeny of these cells.

Figure 3.2: MRI Analysis of $\text{TEC3KO}$ Tumors. Mice were subjected to monthly MRI scans starting at 1 month of age and continuing for 5 months. Images shown are from 4-month-old mice. Note the presence of small tumor growth in the $\text{TEC3KO}$ mouse (arrows).
Figure 3.3: Cre Expression in TEC3KO Embryos. (A–D) Histologic sections of lacZ-stained WT (A and C) or TEC3KO (B and D) embryos are shown at low (A and B) and high (C and D) power. Note the localization of staining to the trigeminal ganglion (Tg). (E) Whole-mount embryo staining of TEC3KO animals localizes cre activity to the lateral aspect of the face. BA1 indicates branchial arch 1; E, eye; FNP, frontonasal process; HB, hind-brain; SCG, superior cervical ganglion.

Histopathology of TEC3KO Tumors.

We have previously described the TEC3KO tumors as schwannomas, although detailed histopathologic characterization was not performed (25). To better understand the biology of these lesions, they were analyzed according to the recently published guidelines described for the Genetically Engineered Mouse (GEM) classification of PNSTs (97). TEC3KO tumors were very myxoid.
(Figure 3.1A, B, and Figure 3.4), and consisted mainly of spindle cells with many mitotic figures and elongated nuclei (Figure 3.4A, B). There were areas of marked cellularity and cellular atypia, and in some cases inflammatory cells were present.

![Image of cells and staining](image)

**Figure 3.4: TEC3KO Schwannomas Exhibit Enhanced Staining for Cyclin D1 but Show Heterogeneous Cre Activity.** (A and B) Hematoxylin and eosin staining of a TEC3KO tumor, showing schwannoma characteristics and heterogeneity. (B) Example of mitotic figures within the tumor (arrows). (C) Immunohistochemistry for cyclin D1 from a typical TEC3KO schwannoma shows high-level staining in most cells, although areas with low staining are also noted. (D) LacZ enzymatic staining of frozen tumor sections reveals heterogeneous activity of cre, with both cre-positive (blue) and cre-negative (non-blue) cells present in the tissue. Nuclei are visualized with nuclear fast red.

According to the diagnostic criteria for PNSTs, the TEC3KO tumors were classified as GEM schwannoma, grades II and III (97). This classification signified that, similar to the schwannomas in Carney complex patients, these tumors may behave as malignant schwannomas. Additionally, nearby tissue was impacted such that the bones of the skull underwent remodeling to
accommodate larger tumors. While there was no actual invasion of the skull cavity, and the mice showed no signs of neurological problems; the tumors would often obstruct the eye causing visual impairment. Interestingly, there have been no signs of metastases with these schwannomas, even as they grew to nearly 1.5 cm in diameter (data not shown).

A previous report from our lab showed that Cyclin D1 was upregulated in Prkar1a-null mouse embryonic fibroblasts (MEFs) (26). In line with these data, TEC3KO tumors revealed heavy staining for this marker by immunohistochemistry, even in areas where cell proliferation was not obvious (Figure 3.4C). The heterogeneity of these tumors was further observed by LacZ staining of frozen tissue sections taken from TEC3KO animals carrying the Rosa26\textsuperscript{lacz} allele (197). Analysis of this staining revealed that both cre-positive and cre-negative cells were present in the tumors, further confirming their heterogeneity (Figure 3.4D).

**Effects of Prkar1a Ablation on the NF Proteins in TEC3KO Schwannomas**

As described above, mutations in NF1 or NF2 cause human schwannomas, and tissue specific knock-out of these genes in mice can also cause tumorigenesis (169, 208-210). In order to determine the effects of Prkar1a knock-out on the NF proteins, the expression pattern of each of these gene products was tested in TEC3KO schwannomas. Immunofluorescence experiments confirmed the loss of Prkar1a within the tumors (Figure 3.5, top),
and interestingly, demonstrated loss of both Nf1 and Nf2 proteins as well (Figure 3.5, middle and bottom). These data were further confirmed by Western blotting of tumor lysates, which revealed almost complete lack of both Nf1 and Nf2 when compared to wild-type murine Schwann cells (Figure 3.6A). Additionally, similar loss of Nf2 was observed in primary TEC3KO tumor cultures (data not shown).

![Figure 3.5: Decreased Expression of the NF Proteins in TEC3KO Schwannomas.](image)

Frozen tissue sections from the same tumor were stained by immunofluorescence for Prkar1a, neurofibromin, and merlin proteins. Tumor stroma (S) stained positively and was therefore used as a control for staining. The dashed line marks the interface between stromal and tumor tissues. As expected, Prkar1a was not present in the tumor tissue (T), but surprisingly both neurofibromin and merlin were absent as well.
In order to determine if Prkar1a ablation caused down regulation of the NF genes at the transcriptional level, we performed quantitative real-time PCR on tumor samples (Figure 3.6B). As a control, primary murine embryonic Schwann cells were obtained from the dorsal root ganglia of E12.5 embryos (218). Because these cells are slow growing and have a short lifespan, all cells obtained from one litter of wild-type animals (n=6) were pooled and used for analysis. As expected, Prkar1a levels were significantly down-regulated in the tumors compared to WT Schwann cells. Residual Prkar1a message is likely detected due to heterogeneity of the tumors, as well as the presence of non-Schwann cells (e.g., endothelial cells and immune cells). Intriguingly, transcript levels for both Nf1 and Nf2 were elevated at least 10-fold (corresponding to a ΔΔCt value of 3.32) in TEC3KO schwannomas compared to wild-type Schwann cells. To rule out somatic mutation as a mechanism by which the protein can be down-regulated in the presence of normal levels of mRNA, we sequenced the Nf2 cDNA from 3 independent TEC3KO tumors and found no evidence of transcript alterations. These data suggest that there may be post-transcriptional regulation of NF gene expression after Prkar1a mutation, although the mechanism of these alterations is not yet known.
Protein lysates of WT Schwann cells and six different TEC3KO schwannomas were analyzed for Nf1 and Nf2 proteins. Note the marked down-regulation of both proteins in comparison to the actin control. (B) Real-time PCR analysis of mRNA for Prkar1a, Nf1, and Nf2 from WT Schwann cells and TEC3KO tumors. Results are shown as averaged expression among all six tumors compared with WT Schwann cells. ΔΔCt was calculated compared with a Gapdh standard, and error bars represent the relative standard deviation.

Signaling Pathways in TEC3KO Schwannomas

Previous work has shown that both Neurofibromin and Merlin can inhibit proliferation by down-regulating Ras signaling and therefore its downstream effectors, Akt and ERK (135, 148, 219). To examine the extent of activation of these pathways in the TEC3KO tumors, we analyzed tumors for total and activated (phosphorylated) Akt and ERK. Surprisingly, immunohistochemistry staining (Figure 3.7A) revealed a complete lack of activation of either of these pathways, which was confirmed by western blotting of tumor lysates (Figure 82...
Quantitative analysis of the western blot data revealed that there were significant decreases in total Akt (p<0.0001), phospho-Akt (p=0.0022) and total ERK (p=0.0072) in tumors. Phospho-ERK expression was essentially immeasurable in the tumors, making statistical analysis impractical. Furthermore, even though the levels of Ras expression appeared to be elevated in the TEC3KO tumors, the active (GTP-bound) form of the protein was significantly decreased compared to levels in normal rat Schwann cells (p=0.0096, Figure 3.8A,B).

Figure 3.7: Loss of Akt and ERK Pathway Activation in TEC3KO Schwannomas. (A) Paraffin-embedded sections of TEC3KO tumors were probed for the phosphorylated (activated) and unphosphorylated (total) forms of Akt and ERK. Stromal tissue stained positively for each protein and was therefore used as a control for staining. (B) Western blot analysis of the same lysates as in Figure 3.6A for p-Akt, Akt, p-ERK, and ERK. Actin is shown as a loading control. Note that ERK was probed from the same blot as in Figure 3A, and thus, the actin control lanes are the same. Quantitation of band intensities was performed, and tumor samples were compared with Schwann cells. Values shown below the blots represent the relative band intensity compared with the Schwann cell sample (set to 1.0) after normalization to actin.
Additional molecules thought to be important downstream effectors of the NF proteins include the small G-proteins Rac, Cdc42, and Rho. Although Neurofibromin may affect these signaling molecules as downstream targets from Ras, Merlin has been shown to play a direct role in the inhibition of Rac/Cdc42, Rho, and PAK1 signaling (148, 188, 189, 220). In TEC3KO tumors, Rac1 expression was not only significantly increased (p=0.0439), but so was its activity (p=0.0098) compared to normal rat Schwann cells (Figure 3.8A,B). Rho expression was increased overall in the tumors (p=0.0092), however its activation was highly variable, failing to produce a significant difference between levels of Rho-GTP in normal Schwann cells compared to the panel of 18 tumors taken as a whole (p=0.1789; Figure 3.8A,B, and data not shown). Cdc42, which is highly homologous to Rac1, was observed in wild-type Schwann cells, and essentially unchanged in the tumors (data not shown). Finally, real-time PCR was performed to check the transcript levels of each G-protein (Ras, Rac1, and RhoA); and similarly to the protein expression, mRNA expression was elevated for each gene (Figure 3.9).
Figure 3.8: Signaling Analysis in TEC3KO Tumors. (A) Representative blots from small G-protein activity assays performed on 18 TEC3KO tumors. Samples were probed for the proteins indicated at left, and actin was used as a loading control. (B) Quantification of results from panel (A). Note the up-regulation of total forms of each protein; however, significant increases in activity levels were seen only with Rac1. *P < .05, **P < .01.
Figure 3.9: Small G Proteins are Up-regulated at the mRNA Level in TEC3KO Tumors. Real-time PCR analysis of mRNA for the indicated small G-proteins was performed for WT Schwann cells and TEC3KO tumors. Results are shown as tumor expression compared to WT Schwann cells, and ΔΔCt was calculated compared to a Gapdh standard.

3.3 DISCUSSION

As in many biochemical processes, the study of inherited syndromes has contributed substantially to our knowledge of functional interactions. For Schwann cell tumorigenesis, the major syndromes that are involved are NF1, NF2, schwannomatosis, and the Carney complex, all multiple neoplasia syndromes associated with Schwann cell tumors.

At the biochemical level, it was previously reported that there are interactions between PKA and the NF proteins, as PKA is known to phosphorylate Neurofibromin, although the functional effects of this reversible modification are unclear (180, 182). Additionally, loss of Neurofibromin has
been shown to upregulate cAMP levels, which would presumably increase PKA activity (184). PKA has also been shown to phosphorylate Merlin at Serine-518, the same site which is phosphorylated by p21-activated kinase (Pak) (179). At the functional level, this phosphorylation reduces the ability of Merlin to suppress cell growth, potentially via heterodimerization with Ezrin. Moreover, Merlin has been shown to function as an AKAP by binding directly to the Prkar1b subunit, although no binding of Prkar1a was observed (191).

In TEC3KO schwannomas, the most striking observation was the marked down regulation of both Neurofibromin and Merlin in the tumors, shown both by immunofluorescence (Figure 3.5) and by western blotting (Figure 3.6A). Furthermore, this alteration occurs at the post-transcriptional level, as mRNA levels of both genes were significantly elevated when compared to normal Schwann cells (Figure 3.6B). Moreover, at least in the case of Nf2, there were no somatic mutations found in the gene that would account for its down regulation. These findings suggest that PKA may play a role in regulating the stability of the NF proteins. Such a role for PKA has previously been described, as phosphorylation by PKA has been shown to promote the degradation of proteins such as GRIP1 and Matrin 3 by triggering ubiquitin-dependent proteolysis (221, 222). The converse can also occur, as PKA causes cellular redistribution of RhoA, and via phosphorylation, reduces its degradation rate (223). We have observed the same phenomenon in Prkar1a knock-out MEFs, in which an enhanced stability of the cell cycle progression marker cyclin D1.
was observed (26). These observations are all consistent with the recently proposed role of Prkar1a in modulating autophagy and the mTOR pathway (224). Further studies are required to elucidate the role of autophagy or proteasomal-mediated degradation in the present observations.

From prior studies of the function of the NF genes, it has been proposed that there are three major downstream pathways that contribute to the Schwann cell tumorigenesis phenotype. Ablation of Neurofibromin causes activation of the Ras, ERK, and Akt pathways; whereas loss of Merlin causes increases in small G-protein (Rac/Cdc42/Rho) signaling (149, 225). This latter pathway is also activated by mutations in Nf1, as Rac and Rho may be activated by pathways downstream of Ras (226, 227).

Because we observed striking downregulation of both Neurofibromin and Merlin in the TEC3KO model, we expected to find increases in activation of each of the 3 pathways (ERK, Akt, and small G proteins). Surprisingly, Ras activity was significantly decreased in TEC3KO tumors compared to wild-type Schwann cells, and furthermore, there was a significant decrease in the activation of both Akt and ERK in the tumors. These results mimic earlier findings from our lab in Prkar1a knock-out MEFs, which also showed that immortalization occurred independently of ERK or Akt activation (26). Interestingly, previous studies on Neurofibromin have indicated that Akt signaling, particularly via mTOR, is most important for tumor growth (211, 212), whereas loss of Merlin appears to upregulate signaling primarily through ERK.
Data from the TEC3KO model indicates that there may be an alternative pathway, mediated by PKA activation, that can effectively promote Schwann cell hyperplasia as well. In addition, we observed a marked upregulation of Rac activity, whereas Rho was not consistently activated. Rac and Rho are small G-proteins of the Rho family, and although they have different functions they are both shown to be important for normal Schwann cell function. Rac appears to be involved in membrane ruffling and establishment of Schwann cell-neuron interactions during myelin formation (225, 228). On the other hand, Rho, which is activated by integrin signaling, is required for stress fiber formation and cell motility (229). Nonetheless, despite their differences in function, both proteins are known to be dysregulated in Schwann cell neoplasias (149).

There is good evidence that PKA can specifically affect the activity of Rac and Rho, although clearly those effects are cell type and condition specific (230). The interaction between PKA and Rho is better defined, such that PKA directly phosphorylates Rho at S188 and negatively regulates its activity (231). The mechanism of this regulation entails PKA phosphorylation leading to enhanced binding of Rho to the cytosolic Rho GTP-dissociation inhibitor (Rho-GDI) protein, which sequesters Rho from its active location at the cell membrane. However, relocation from the membrane is not solely associated with enhanced GTPase activity. It appears that PKA phosphorylation can also cause dissociation between the total GTP-loaded form of Rho and its intracellular activity (231-233).
Unlike Rho, Rac lacks a direct PKA phosphorylation site, although enhanced PKA activity increases GTP-loaded Rac and appears to also enhance its activity. The mechanism by which this occurs has yet to be elucidated, but it has been proposed that alterations in Rac-GTP exchange factors may be involved (234).

For both Rho and Rac, PKA activation has generally been associated with alterations in protein activity without changes in protein level. The exception to this is the decreased degradation of Rho, where GDI binding S188 phospho-Rho enhances its stability. Thus, although we observe significantly increased expression levels of both Rho and Rac in our tumors, the effect on signaling through these pathways is not so straightforward at the present time.

In summary, studies to date of pathways leading to schwannoma and PNST formation have revolved around Nf1 and Nf2 signaling through Ras, PI3K, Akt and ERK, as well as the downstream effectors Rac, Cdc42 and Rho. Although PKA is known to be important for Schwann cell growth, its role in this process has not yet been elucidated. In this chapter, we present data indicating that PKA may have multiple roles in promoting Schwann cell tumorigenesis. First, it appears to modulate the stability of the NF proteins, such that dysregulation of PKA leads to post-transcriptional loss of these proteins during tumorigenesis. Secondly, PKA appears to signal, either directly or indirectly, to the same downstream effectors activated by mutations in the NF genes. However, unlike activation of Schwann cell tumorigenesis by NF mutations,
PKA appears to promote tumorigenesis by a mechanism that excludes activation of Ras, ERK, and Akt. Thus, PKA promotes Schwann cell tumors by mechanisms that overlap, but are distinct from those seen in NF-associated tumorigenesis.

3.4 MATERIALS AND METHODS

Mouse Experiments

All mice were maintained in a sterile environment under 12-hour light/dark cycles. All animal experiments were carried out in accordance with the highest standards of animal care under an IACUC-approved protocol. The generation of \( Prkar1a^{loxp/loxp} \) and TEC3 mice has been previously described (25, 196). For this study, \( TEC3KO \ (TEC3;Prkar1a^{loxp/loxp}) \) animals were monitored weekly for tumor onset, which was defined as the age at which a tumor of 0.5 cm linear dimension (as measured by calipers) was first detected. Mice with bilateral tumors were designated as having tumor onset at the time the first tumor reached 0.5 cm.

Immunohistochemistry

Tissue samples were fixed overnight in 10% formalin, processed, and embedded in paraffin. Immunohistochemistry was performed on 8 \( \mu m \) sections after antigen retrieval (Vector Labs, Burlingame, CA) with the following
antibodies: phospho-Akt, Akt, phospho-ERK, ERK, and cyclin-D1 (Cell Signaling Technology, Danvers, MA). Antigens were incubated with the appropriate secondary antibodies and color was developed by adding DAB chromogen reagent (Vector Labs) to each section for 1-2 min before counterstaining with hematoxylin, dehydration, and coverslipping. Samples were analyzed on an Olympus BX50 microscope, and images were captured using Spot Basic v4.1 software. LacZ staining of frozen tissues was performed as described (51), and visualized as above.

**Immunofluorescence**

Freshly dissected tissue samples were frozen in Tissue Tek® Optimal Cutting Temperature Compound (Sakura Finetek U.S.A. Inc., Torrence, CA). 8 μm sections were fixed using cold acetone and permeabilized in 0.1% sodium citrate with 0.1% Triton-X100 detergent (Sigma, St. Louis, MO). The following primary antibodies were prepared as per the manufacturers recommendations in 3% BSA in PBS: Neurofibromin (sc-67), Merlin (sc-331; Santa Cruz Biotechnology, Santa Cruz, CA); Prkar1a (#610610; BD Biosciences, San Jose, CA). Slides were incubated with primary antibodies at room temperature for 45 min, washed, and incubated with Alexfluor-488 conjugated secondary antibodies (Invitrogen, Carlsbad, CA) for 25 min in the dark. Samples were then washed, mounted with DAPI mounting medium (Vector Labs), and visualized using a Zeiss Axioskop 40 microscope and Axiovision software.
Western Blot Analysis

Primary Schwann cells were isolated and maintained as previously described (218). Wild-type cells were harvested for protein using Mammalian Protein Extraction Reagent (M-PER) with protease inhibitors (Pierce Biotechnology, Rockford, IL). Primary tumor samples were dissected from TEC3KO animals and homogenized in protein lysis buffer containing 20 mM HEPES buffer, 20 mM NaCl, 5 mM EDTA, 5 mM EGTA, 0.5% Triton-X 100, 1 mM DTT, and protease inhibitors. Samples were resolved by SDS-PAGE prior to transfer onto nitrocellulose membranes (Pall Corporation, East Hills, NY). Antibodies were obtained from the following sources, and used according to the manufacturers recommendations: Nf1, Nf2 (Santa Cruz Biotechnology, Santa Cruz, CA); Actin (20-33; Sigma, St. Louis, MO); phospho-ERK (Thr202/Tyr204; #9101), ERK (#9102), phospho-Akt (Ser473; #9271), and Akt (#9272), (Cell Signaling Technology, Danvers, MA). Blots were developed using Western Lightning Chemi-luminescence reagents (PerkinElmer, Waltham, MA), exposed to Blue Lite Autorad film (ISC BioExpress, Kaysville, UT), and images were captured using the GeneLine imaging system (Spectronics Corporation, Westbury, NY). Quantitation of blots was determined by first normalizing all the samples to the actin loading control using Genetools imaging software (Spectronics Corporation, Westbury, NY). Band intensities of the tumor samples were expressed as percentages in comparison to the control Schwann
cells (set to 1.0 arbitrary units). Statistics were calculated using two-tailed T test to generate p-values.

**Real-Time PCR**

Primary murine Schwann cells and tumor samples (n=6) were harvested, and mRNA was prepared as described (26). Wild-type Schwann cells were collected from one litter of embryos (n=6) pooled together to comprise the control sample. cDNA was prepared using the iScript cDNA Synthesis kit (BioRad, Hercules, CA) and analyzed by quantitative real-time PCR using iQ SybrGreen Supermix (BioRad). Each sample was run in triplicate, and the values for all six tumors were averaged and compared to that of the wild-type Schwann cells. The primer sets used for this study can be found in Table 3.1.

| Table 3.1: Primer Sets Used for Real-Time PCR Analysis. |
|-----------------|-----------------|-----------------|
| Gene            | Forward (5' - 3') | Reverse (5' - 3') |
| Prkar1a         | AGATCGTGGTGCAAGGAGAG | CGGTCAAACCTTACGCA CTTC |
| Nf1             | CAGGTGACCCCATGTAT | TCCGTATCCATTTGTC |
| Nf2             | GGGATTCTTAGCAGAGG | ATCCACTCGAGCAGCAT |
| Rac1            | TGCAGACCTCCAGAGTT | CAAAGCTAGTGCTAGGTC |
| H-Ras           | GAAAGAGGCGGAGGAG | ACTGAGAGGGGTGGAGACT |
| K-Ras           | TGTGCTCGGCAAGATCT | TCAAAGCTAGTGCTAGG |
| N-Ras           | GTTGTGCTCGGCAAGG | AGCTGAGGCTGTGCTGTT |
| RhoA            | AGCGTCATAGGCTATA | CTGGTCAGACAGGTTGACA |
| GAPDH           | GCAAATTCAACGGCACAGTT | GTTCACACCCATCAAAACATG |
glycerol, 20 mM Tris (pH 8.0), 137 mM NaCl, 1 mM MgCl2, 0.5 mM EDTA, and 10 mM Sodium pyrophosphate. The lysis buffer also contained the following protease inhibitors: 1 ug/ml Aprontinin, 1 ug/ml Leupeptin, 100 ug/ml PMSF, 1 mM Sodium vanadate, and 5 mM Sodium Fluoride. For each sample, 0.5 mg (Ras and Rac) and 1 mg (Rho) of protein was kept for 1 hr at +4°C, rocking, with agarose beads conjugated to the substrate for the appropriate activated G-proteins: Raf1-RBD (Ras), Pak1-PBD (Rac), and Rhotekin-RBD (Rho) (Upstate, Charlottesville, VA). Following incubation, all samples were washed three times with lysis buffer, resuspended in Laemmli buffer, and run on 12% SDS-PAGE for Western blot using the following primary antibodies: anti-Ras (clone RAS10; #05-516), anti-Rac1 (clone 23A8; #05-389; Upstate), and RhoA (26C4; sc-418; Santa Cruz Biotechnology, Santa Cruz, CA). Separate Western blots were performed to test for the total amount of each G-protein, using Actin (Sigma) as a loading control.
CHAPTER 4

PKA-MEDIATED SCHWANNOGENESIS IS ASSOCIATED WITH ACTIVATION OF SPECIFIC ISOFORMS OF p21-ACTIVATED KINASES

4.1 INTRODUCTION

The p21-activated kinases (Paks) are serine/threonine kinases that were first described in 1994 as targets of activated Rac1/Cdc42 (235). There are two groups of Pak proteins: Group I which consists of Paks 1-3, and Group II that consists of Paks 4-6. Although the Group I Paks share 93% homology with each other, they are only 54% homologous to the Group II Paks – the main difference being an autoinhibitory domain (AID) that exists only in Group I (236). Group II Paks are 60-75% homologous to each other (236). In the inactive state, Group I Paks occur as homodimers where the AID of each molecule binds to and inhibits the catalytic domain of the other kinase (237). Since Group II Paks do not have an AID, their basal activity is generally higher than Group I Paks. Binding of GTP-bound Rac1/Cdc42 (p21) to Group I Paks causes dissociation of the homodimer and subsequent autophosphorylation. Now active, Pak goes on to interact with and phosphorylate its downstream targets. Group II PAKs are not subject to enhanced activation by Rac1/Cdc42 binding; however
changes in subcellular localization upon p21 binding may play a role in downstream signaling from these kinases (238, 239).

Approximately 40 targets have been described for Pak phosphorylation (reviewed in (240)), and Pak was originally reported to play a role in cytoskeletal dynamics and cell motility (241, 242). Additional studies have implicated the Pak proteins in cell growth and transformation (243-245), and their over expression and activation occurs in a number of human cancers including breast, colon, ovarian, bladder, pancreatic, and kidney (reviewed in (240, 246)). Pak activation is also reported in tumors associated with the Neurofibromatosis syndromes. Due to activated Ras in NF1 tumors, downstream MAP kinase and PI3 kinase signaling leads to the activation of Pak1 (245). It is known that Nf2 phosphorylation by Pak results in a conformational change that inactivates the protein and allows for cell growth. However, non-phosphorylated (active) Nf2 can also inhibit Pak1 by competing for Rac1 binding (220). As a result, when Nf2 is lost, Pak1 activation increases. Pak1, -2, and -6 have all been reported to phosphorylate Nf2 at serine 518 in vitro, however given that Pak2 and Pak6 are not highly expressed in the Schwann cells, it was thought that their interaction with Nf2 may not be relevant during schwannoma development (187-189).

In the previous chapter, we showed that loss of Prkar1a in the facial subset of neural crest-derived Schwann cells (TEC3KO) caused schwannoma development in mice with nearly 80% penetrance by 10 months of age.
Consequently, both Nf1 and Nf2 proteins were post-transcriptionally down regulated, despite increased transcript expression. Also, all three small G-proteins (Ras, Rac1, and RhoA) were over expressed at the protein level, but Rac1 was the only one that was highly activated. In light of these data, we sought to define the relevance of NF protein loss during dysregulated PKA schwannoma development, and define whether Pak signaling downstream of activated Rac1 occurs in this model.

Concomitant conventional Nf1 mutations in the TEC3KO mouse had no effect on the onset of tumorigenesis. In contrast, there was a trend towards increased tumorigenicity when conventional Nf2 mutations were introduced. Similarly to the TEC1KO mice, conventional mutations in Cα completely rescued the TEC3KO tumor phenotype. Cβ mutations also allowed partial rescue, suggesting that both PKA catalytic subunits may be involved in PKA-mediated Schwann cell tumorigenesis. Moreover, Pak expression and activity analysis revealed over expression of Pak6 at the protein level, but Pak2 exhibited significant activation. These results indicate that the loss of Nf2 may be a key step in PKA-related schwannomagenesis, and that the downstream effects are likely mediated through Pak signaling pathway activation.
4.2 RESULTS

**Nf2 Down Regulation may be a key step in TEC3KO Tumorigenesis**

Little is known about the functional relevance underlying the interactions between PKA and the NF proteins. In this study, we aimed to better define this mechanism with reference to tumorigenicity *in vivo*. As described in chapter 3.2, *TEC3KO* mice exhibit tumor onset beginning at approximately 2.5 months of age, and nearly 80% of all *TEC3KO* mice developed schwannomas by 10 months of age (Figure 3.1B). Since loss of both Nf1 and Nf2 was observed in *TEC3KO* schwannomas, germline mutations in either gene were introduced into the *TEC3KO* mice to assess which gene is most important for tumor initiation. Owing to the two-hit hypothesis for tumorigenesis, these additional heterozygous mutations might, in theory, accelerate tumor onset in the already tumor-prone animals.

*Nf1<sup>+/−</sup>;TEC3KO* mice (n=27) were phenotypically similar to their *TEC3KO* counterparts, and they showed a similar pattern of tumor onset rate as compared to the *TEC3KO* mice alone (n=85; Figure 4.1). *Nf2<sup>+/−</sup>;TEC3KO* mice (n=17) were also phenotypically analogous to *TEC3KO* animals, however there was a trend towards more rapid tumor onset; although it did not reach statistical significance with the limited number of animals studied (p=0.1 to 0.15; Figure 4.1). Tumor onset occurred no earlier than approximately 2 to 2.5 months for any of the three groups of mice. 50% tumor onset was achieved for both *Nf1<sup>+/−</sup>;TEC3KO* and *TEC3KO* mice by 5 months of age (20 weeks), however this
timing was reduced to only 4 months (16 weeks) for the \textit{Nf2}^{+-};\textit{TEC3KO} animals. By 10 months of age, 75-80\% of both \textit{Nf1}^{+-};\textit{TEC3KO} and \textit{TEC3KO} succumbed to tumor formation. In contrast, approximately 90\% of the \textit{Nf2}^{+-};\textit{TEC3KO} mice had tumors by this time. Although there were no differences in onset rate between the \textit{Nf1}^{+-};\textit{TEC3KO} and \textit{TEC3KO} groups, there was a slight growth rate advantage associated with the \textit{Nf1}^{+-};\textit{TEC3KO} tumors; however this was not statistically significant (data not shown). Similarly, the growth rate of \textit{Nf2}^{+-};\textit{TEC3KO} tumors was slightly increased (data not shown), which was also not statistically significant. The lack of significance of these findings may be due to the small number of mice involved in the study, and a larger study might demonstrate findings that reached statistical cutoffs. Nevertheless, these data provide clues to the molecular mechanism by which loss of the NF protein products drives Schwann cell tumorigenesis. Because \textit{Nf1} mutations do not alter tumor initiation, but \textit{Nf2} mutations slightly do, it suggests that \textit{Nf2} repression may lie downstream from PKA signaling during schwannoma development.
Figure 4.1: Tumor Onset Rate is Altered in TEC3KO by Conventional Mutations. Germline mutations in either Nf1 or Nf2 were introduced into the TEC3KO mice to assess the genetic interactions that lead to tumorigenesis. Likewise, germline mutations in Cα or Cβ were introduced to determine which catalytic subunit confers tumorigenicity when dysregulated. All animals were monitored weekly for the presence of tumors up to 10 months of age. Nf2\(^{+/--;}\)TEC3KO mice (n=17) showed enhanced tumorigenicity compared to TEC3KO alone (n=85), whereas Nf1 mutations (Nf1\(^{+/--;}\)TEC3KO; n=27) did not alter tumor initiation. Both Cα\(^{+/--;}\)TEC3KO (n=15) and Cβ\(^{+/--;}\)TEC3KO (n=17) mice were rescued from tumor development, however the Cβ mutations had only a partial rescuing effect.

Using the same approach as with the TEC1KO embryos described in chapter 2, germline mutations in either Cα or Cβ were introduced into the TEC3KO mice to determine which catalytic subunit causes tumor development when dysregulated. Both Cα\(^{+/--;}\)TEC3KO (n=15) and Cβ\(^{+/--;}\)TEC3KO (n=17) animals were resistant to tumor initiation, but the Cα mutations resulted in complete suppression of the tumor phenotype (p≤0.001; Figure 4.1). Mutations in Cβ were associated with a partial rescue since about 10-15% of them still developed schwannomas (p=0.006). These data support our hypothesis that dysregulated PKA activity alone is sufficient for Schwann cell tumorigenesis.
Given that Cα is ubiquitously expressed and Cβ is generally localized to neural tissues, it is likely that both subunits are expressed in the Schwann cells and play a role in the total PKA activity in this cell type.

**Pak Transcripts and Proteins are Over Expressed in TEC3KO**

Because Rac1 was highly activated in TEC3KO schwannomas (Figure 3.8), we sought to analyze its downstream effector molecules, the Paks, for their expression in the tumors. Real-time PCR was performed on 9 TEC3KO tumors and rat Schwann cells (SC) to detect the expression of each Pak isoform. Comparative analysis of the two sample groups revealed no change in expression for Pak1 and Pak2, or Pak5 (Figure 4.2). On the other hand, Pak3, Pak4, and Pak6 were significantly over expressed in tumors compared to SC (Figure 4.2). Pak6 showed the greatest fold increase of any Pak isoform (p=0.0001), which was interesting considering previous findings that Pak6 can phosphorylate Nf2 but is not normally expressed in the SC (189).
Figure 4.2: Relative mRNA Expression of the Six Pak Isoforms in TEC3KO Schwannomas.

Real-time PCR data showing TEC3KO mRNA expression of the Pak isoforms after normalization to rat SC. Error bars represent standard deviation. Note that Pak3 and Pak6 are significantly over expressed, and Pak4 expression is also significantly increased, albeit to a lesser degree. *p≤0.05, **p≤0.01.

Western blotting was performed to verify the real-time PCR data for each Pak isoform. Analysis of up to 14 TEC3KO tumors revealed no change in Pak1 or Pak5 protein expression, which was consistent with the real-time PCR data (Figure 4.3). Although Pak2 mRNA was not increased, its protein expression was slightly elevated (p=0.098). This change is not statistically significant, however, so these data are in keeping with the real-time data to suggest no change in total Pak2 expression. In contrast to the real-time data, there was no Pak3 protein in either rat SC or TEC3KO schwannomas, and Pak4 expression was decreased in tumors, despite increased transcript levels for both genes. The most striking observation in this study was the dramatic increase in Pak6
Rat SC exhibited very little Pak6 expression, which is consistent with previous reports, but the TEC3KO schwannomas showed a highly significant overexpression of the protein (p≤0.0001). This observation was in keeping with the real-time data which showed Pak6 transcript expression was the most highly over-expressed of any Pak isoform compared to normal rat SC. It also suggested that Pak6 may be involved in PKA-mediated SC tumorigenesis.

![Figure 4.3: Protein Expression Analysis Revealed Over Expression of Pak2 and Pak6.](image)

(A) Western blots showing representative data for 14 TEC3KO tumors that were tested for expression of the Pak isoforms. HEK293T cells transfected with expression vectors for Pak3 and Pak5 were used as a positive control for those isoforms since no expression was detected in rat SC or tumors. MEFs were used as an alternative positive control. β-actin was probed as a loading control. (B) Quantitation of all western blot data analyzed for Pak expression. Since Pak3 and Pak5 were not expressed in rat SC or tumors, they were not quantitated. Note that Pak2 is overexpressed (p=0.098), and Pak6 is significantly overexpressed. **p≤0.0001.
**TEC3KO Tumors Exhibit Pak Activation**

In addition to the expression analysis for each Pak isoform, it was important to understand the activation status of each of these proteins in *TEC3KO* schwannomas. To that end, immunoprecipitation (IP) was performed to pull down each Pak isoform from 6 tumors and rat SC. Subsequent kinase assays were done to assess the activity of each protein. Because Pak3 and Pak5 were not expressed in either rat SC or *TEC3KO* schwannomas, their activity was not evaluated in this study.

*TEC3KO* tumors had elevated kinase activity for each isoform tested, however Pak2 was the only isoform that was significantly more activated in the tumors (p≤0.0001; Figure 4.4). Pak1 activity was slightly higher in tumors compared to rat SC, but there was no statistical difference between the two groups. There was also a marked increase in Pak4 activation, however it fell just below the 95% confidence interval for statistical relevance (p=0.0586). Interestingly, despite the high over expression of Pak6 at both the transcript and protein levels, its activity in the tumors was only marginally increased from normal rat SC (Figure 4.4). This may be explained by the fact that Group II Paks are not increasingly activated by Rac1/Cdc42 interactions, however it is surprising that the basal activity level was not higher than the other isoforms tested, as was previously described (238-240). Altogether these data suggest that Pak2 over expression and activation are increased in *TEC3KO* tumors,
which further supports the proposed mechanism that PKA dysregulation causes Rac1, and subsequently Pak, activation to drive Schwann cell tumorigenesis.

![Graph showing Pak2 activity](image)

**Figure 4.4: Pak2 is Significantly Activated in TEC3KO Schwannomas.** Results from IP-kinase assays represented as counts per minute of $^{32}$P. These data represent Pak activity in 6 TEC3KO tumors compared to rat SC. Pak3 and Pak5 were not tested for their activity since there was no protein detected by western blot for either rat SC or TEC3KO schwannomas. Note that Pak2 activity is highest, whereas Pak6 is only slightly more active than in normal SC. Pak4 activity also increased although it was just below statistical significance ($p=0.0586$). Error bars represent standard deviation. **$p\leq0.0001$.**

To test the activation status of the Pak proteins *in vivo*, western blots were performed on TEC3KO schwannomas to look for expression of activated downstream targets of Pak. Lim kinase (LimK) is a direct target for Pak phosphorylation, and it requires this post-translational modification for activation. When phosphorylated in the kinase domain, activated LimK proceeds to phosphorylate its own targets, including Cofilin which regulates actin dynamics.
LimK was overexpressed in TEC3KO schwannomas, however the phosphorylated (active) form of the protein was not specifically tested (Figure 4.5). Total and phospho-Cofilin levels were slightly increased in tumors compared to rat SC, possibly suggesting activation downstream of Pak signaling.

**Figure 4.5: Pak Signaling is Activated in vivo in TEC3KO Tumors.** Western blot results showing increased LimK expression as well as its target Cofilin. LimK, total-Cofilin, and phospho-Cofilin levels were increased in TEC3KO schwannomas compared to rat SC (rSC) which suggests activation of Pak signaling. Paxillin expression decreased in tumors, which may have implications regarding Pak interactions with Nf2. MEFs were used as an alternate control.

As stated above, Nf2 is a direct target for Pak phosphorylation. For this to occur, Paxillin is required to bind one of the Paxillin binding domains (PDB) on Nf2 (247). This induces translocation of Nf2 and Paxillin to the plasma membrane where activated Pak can come in contact with Nf2 and negatively regulate its activity. Furthermore, Pak activation by Cdc42, not Rac1, seems to drive Nf2 phosphorylation (247). To indirectly assess whether Pak was capable
of phosphorylating Nf2 in the TEC3KO tumors, we probed for Paxillin by western blot. Paxillin expression was decreased in the tumors compared to normal rat SC, suggesting that Pak phosphorylation of Nf2 could not take place (Figure 4.5). Moreover, there was no change in Cdc42 expression between tumors and normal SC (data not shown). Since both PKA and Pak can phosphorylate and inhibit Nf2, it might be possible that the loss of Nf2 in TEC3KO tumors was a result of Pak activation, rather than direct interactions with PKA. These data suggest that although Pak signaling is activated in TEC3KO tumors, it does may not function to inhibit Nf2 in this model. Therefore, loss of Nf2 protein (as described in chapter 3) may likely be a direct result of phosphorylation by PKA, not Pak.

4.3 DISCUSSION

In the previous chapter, we showed that both Nf1 and Nf2 proteins are down regulated during PKA dysregulation in Schwann cells. At the time, it was not clear what the molecular relevance of this loss was, and what it meant in terms of tumor initiation. Based on the findings from the genetic interaction studies where additional germline mutations in the NF genes were introduced into the TEC3KO mice, it is possible that the loss of Nf2 specifically increases tumorigenicity in this model. The fact that the tumors were histopathologically characterized as schwannomas is in keeping with these findings since NF1
mutations in humans very rarely result in schwannoma development. The loss of Nf2 protein in TEC3KO mice may therefore be a rate-limiting step in schwannoma development when PKA is dysregulated, although further experimentation is necessary to confirm this hypothesis. Furthermore, the precise mechanism by which Nf2 is lost in this model remains to be understood.

Given that the mRNA transcript levels for Nf2 are elevated in TEC3KO tumors, but the protein is lost (chapter 3), it suggests that post-transcriptional modifications may lead to Nf2 degradation. The Pak proteins are known to phosphorylate and inactivate Nf2, however there is no data to suggest that this leads to degradation of the protein. Furthermore, it is not known what effects PKA phosphorylation has on Nf2. Both PKA and Pak can phosphorylate Nf2 at serine 518, which induces a conformational change in the protein thereby causing inactivation, but not degradation. Interestingly, one study revealed that mutations in the FERM domain of Nf2 resulted in subsequent ubiquitination and proteasomal degradation (248). The FERM domain (short for Four-point-one (4.1), Ezrin, Radixin, Moesin) is found at the N-terminus of many cytoskeletal proteins, and it facilitates membrane localization (249). Since a second PKA phosphorylation site, serine 10, is found in the FERM domain of Nf2, it is possible that this may have detrimental effects on protein stability. Further experimentation to understand the role of serine 10 phosphorylation on Nf2 stability is required to better define the precise mechanism by which the protein is lost.
Based on protein expression and activity, it is likely that Pak1, Pak3, and Pak5 do not play a large role in TEC3KO tumorigenesis. On the other hand, Pak2, Pak4, and Pak6 all exhibited alterations in expression or activation that suggest they may either individually or collectively contribute to tumor formation. Previous reports have shown that both Pak2 and Pak6 can phosphorylate Nf2, but Pak4 was never tested for this interaction (187-189). Nonetheless, since Paxillin was down regulated in TEC3KO tumors, and Cdc42 expression was not increased, it is likely that Pak phosphorylation of Nf2 is not required for schwannoma development in this model. Pak activation may therefore confer growth signals in an Nf2-independent manner.

Constitutive activation of Group II Paks has been shown to cause cellular transformation, whereas Group I Paks typically stimulate cell motility (236). Based on these findings, it is possible that Pak4 and Pak6 activation in TEC3KO tumors may work in concert to drive tumorigenesis in the Schwann cells. The slightly increased activation of Pak4, in spite of decreased protein expression, suggests that it may be important for translating growth signals; however the precise role for this kinase in PKA-Nf2 schwannomagenesis is not clear. Pak6 was the only isoform that exhibited significant over expression at both the transcript and protein levels, but this kinase was not significantly activated in TEC3KO tumors. NetPhosK 1.0 analysis of the Pak6 amino acid sequence revealed at least two strong PKA phosphorylation sites, suggesting that PKA may play a role in stabilization or activation of the kinase (data not
shown). The Pak6 autophosphorylation site at serine 561 may also be phosphorylated by PKA, which could cause increased activation. This does not likely occur in the TEC3KO mice, however, considering there is no increased activation of Pak6. A second, stronger, PKA phosphorylation site is located at serine 113, which could possibly cause increased stability of the protein; thereby resulting in significant over expression. Additionally, since phospho-CREB is known to have hundreds of targets, it is possible that activation of this transcription factor could cause increased transcription of the Pak6 gene; which would explain why the mRNA was so highly over-expressed as well. Examination of the Pak6 promoter region would be required to determine if this is the case, though.

Pak2 was the only isoform to show significantly increased kinase activity during PKA dysregulation. Furthermore, given the role for Group I Paks in cell motility, the increased LimK and phospho-Cofilin expression suggest that it may also be activated in vivo in TEC3KO tumors. Previously, our lab reported that PKA phosphorylation of LimK enhanced Pak activation of LimK, resulting in increased migration of Prkar1a-null MEFs (7). As mentioned in chapter 3, the TEC3KO tumors showed no signs of invasion or metastases, so the relevance for increased LimK activity in these tumors is not likely linked to cell motility. Several reports have shown that Pak2 may play a role in apoptosis via caspase-3 and BAD interactions, although its effects appear to be cell-type and condition specific (250-255). Indeed, further experiments are required to identify
whether Pak2 activation can suppress apoptosis in the TEC3KO schwannoma model.

Taken together, the results presented in this chapter revealed that PKA dysregulation causes schwannomagenesis in a process possibly mediated by loss of Nf2 protein. The downstream effects of loss of Prkar1a resulted in increased Rac1, and therefore Pak, activation. More specifically, Pak2, Pak4, and Pak6 may function independently or in concert to convey growth signals leading to cell transformation, although the specific downstream effector molecules are not yet identified.

4.4 MATERIALS AND METHODS

Mouse Experiments

All mice were maintained in a sterile environment under 12-hour light/dark cycles in accordance with the highest standards of animal care under an IACUC-approved protocol. The generation of Nf1+/− and Nf2+/− mice has been previously described (161, 162, 175). Ca+/−;Prkar1a<sup>loxP/loxP</sup>, Cβ+/−;Prkar1a<sup>loxP/loxP</sup>, Nf1+/−;Prkar1a<sup>loxP/loxP</sup>, or Nf2+/−;Prkar1a<sup>loxP/loxP</sup> mice were mated with TEC3KO animals to generate Ca+/−;TEC3KO (n=15), Cβ+/−;TEC3KO (n=17), Nf1+/−;TEC3KO (n=27), and Nf2+/−;TEC3KO (n=17) progeny. A total of 85 TEC3KO animals were studied as controls, which included the animals monitored in chapter 3. For this study, all animals were monitored weekly for tumor onset,
which was defined as the age at which a tumor of 0.5 cm linear dimension (as measured by calipers) was first detected. Mice with bilateral tumors were designated as having tumor onset at the time the first tumor reached 0.5 cm.

**Western Blotting**

Immunoblotting was performed as described in chapter 3.4 using the following antibodies diluted to in 5% milk in TBST per the manufacturer's recommendation: Pak1, Pak2, Pak3, Pak4, LimK, Cofilin, phospho-Cofilin, and Paxillin (Cell Signaling Technology, Danvers, MA); Pak5 and Pak6 (Santa Cruz Biotechnology, Santa Cruz, CA); and Actin (Sigma, St. Louis, MO).

**Real-Time PCR Analysis**

Primary rat SC and TEC3KO tumor samples (n=9) were harvested, and mRNA was prepared as described (26). cDNA was prepared using the iScript cDNA Synthesis kit (BioRad, Hercules, CA) and analyzed by quantitative real-time PCR using iQ SybrGreen Supermix (BioRad). Each sample was run in triplicate, and the ∆CT values for all nine tumors were averaged and compared to that of the wild-type SC to generate the ∆∆CT values expressed in Figure 4.2 where the ∆∆CT=0 for rat SC. The primer sets used for this study can be found in Table 4.1.
Table 4.1: Primer Sets Used for Real-Time PCR Analysis.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (5' - 3')</th>
<th>Reverse (5' - 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pak1</td>
<td>GAACACCTCATTTGATTTGGAAAAACC</td>
<td>ACCATTTCACTAGAATAGCACAGG</td>
</tr>
<tr>
<td>Pak2</td>
<td>TTGTCTTTTCTCTGGCTTCTCTCTCTCTCT</td>
<td>AGTGAATAAGGTTGATTCTCTCTCTCTCT</td>
</tr>
<tr>
<td>Pak3</td>
<td>CCAAAATAAAGAAGATATCCACCTTTTG</td>
<td>CTAGTGCTGTGTAACCTCGTCTCTCTCTCT</td>
</tr>
<tr>
<td>Pak4</td>
<td>TACTGGAACCTCGTTGATTTGATTTGAT</td>
<td>CACACACACACACACACACACACACACAC</td>
</tr>
<tr>
<td>Pak5</td>
<td>GACATCGTACAAACTCCGTTACGAT</td>
<td>TCATTGCAAATACATTACACTGTCC</td>
</tr>
<tr>
<td>Pak6</td>
<td>CAGAATCAATAGCAGACATGTTTGTG</td>
<td>CACACGTTCCTTTTTACAGAAAGAT</td>
</tr>
<tr>
<td>GAPDH</td>
<td>GCAAATTCAACGGCACAGTCAAG</td>
<td>GTTCACACCCATCACAACATGG</td>
</tr>
</tbody>
</table>

**IP-Kinase Assays**

200 µg of protein from rat Schwann cells, sciatic nerves (positive control), and 6 TEC3KO tumors was used to IP each Pak isoform for subsequent kinase activity assays. The IP (lysis) buffer used for these experiments was the same as that described in chapter 3.4 for the G-protein activity assays. All samples were pre-cleared with 1.0 µg rabbit IgG prior to IP, and the antibodies used for Pak western blots were the same used for these IPs. Protein A/G beads (Santa Cruz Biotechnology, Santa Cruz, CA) were used to pull down the IgG-Pak complexes which were subsequently resuspended in and equal volume of 0.5 mg/ml myelin basic protein (Sigma, St. Louis, MO). Kinase assays were performed by adding 3x hot mix containing 50 mM Hepes (pH 7.5), 10 mM MgCl₂, 150 µM ATP, and 10 uCi/reaction γ⁻³²P ATP. Samples were incubated at 30°C for 30 min and then 10 ul of each reaction was spotted on P81 paper. Counts per minute were read on a scintillation counter, and all samples were normalized to background readings from negative controls. Each sample was tested in duplicate for kinase activity to control for variability.
CHAPTER 5: CONCLUDING REMARKS

PKA signaling in and of itself is very complex, and paired with the complexities associated with neural crest development, the process becomes even more compounded. It is clear from the studies presented here that PKA plays a pivotal role in both differentiation and proliferation of neural crest-derived tissues of the embryo and adult. When dysregulated, excess PKA signaling in the CNC causes impaired differentiation of the craniofacial tissues, and Schwann cells are prone to tumorigenic growth. Given that genetic knockdown of the catalytic subunits in the TEC1KO and TEC3KO mice was sufficient to rescue the mutant phenotypes, we can surmise that precise on/off regulation of PKA in the neural crest is imperative for normal development and function of the adult neural crest-derived tissues.

The TEC1KO model serves as a good tool for delineating the effects of constitutively active PKA signaling in a specific cellular compartment that includes the bone and cartilage precursor cells. The mesenchymal stem cells (MSC) derived from the CNC have the capacity to differentiate into either bone or cartilage, or a number of other tissues such as connective tissue or muscle (Figure 5.1). The effects of PKA dysregulation in the CNC had the biggest impact on the ability for MSCs to properly form bone and cartilage. Nearly
complete resorption of the frontal bone and mandible, and loss of defined bone encircling the nasopharynx suggested that the MSCs were incapable of differentiating into normal bone. Furthermore, erroneous cartilage deposition implied that there was a switch in signaling within the MSC that favored cartilage differentiation (Figure 5.1). The cartilage that was formed, however, was not normal either considering that the glycosaminoglycan matrix was disrupted and there was evidence of dysplastic chondrocyte growth. The other supportive tissues, such as connective tissue or muscle, appeared to develop normally, however there was evidence of edema in areas such as the LP that suggested improper functioning of those cells. As a result of impaired differentiation, the craniofacial tissues formed improperly and constricted the airway of TEC1KO neonates, causing asphyxiation.

**Figure 5.1: Proposed Model for PKA-Mediated CNC Development.** Mesenchymal stem cells (MSC) arising from the CNC are subject to differentiation into either bone or cartilage, or other cell types including connective tissue or muscle. When PKA is dysregulated, such as in TEC1KO embryos, the MSCs are driven towards cartilage differentiation moreso than bone. The fate of other cell types is less clear based on the studies presented here, but it appears as though those tissues may develop normally.

On a molecular level, it is difficult to identify the precise mechanism by which MSC differentiation goes awry in this model. The findings so far have
been largely descriptive, but future work could be done to better define the molecular interactions that lead up to the observed phenotype. For example, cartilage and bone isolated by laser capture microdissection from TEC1KO and wild-type embryos could be used to gather information from microarray experiments. Comparing gene expression between the normal and mutant tissues could yield a wealth of information regarding which genes specific for bone or cartilage differentiation are up or down regulated at the mRNA level. Alternatively, MSCs deficient for Prkar1a could be stimulated to differentiate in vitro into either cartilage or bone fates. This approach is currently underway in the lab to define whether knock-down of Prkar1a in MSCs undergoing bone differentiation can drive cartilage formation instead.

Since Prkar1a+/− mice are prone to osteochondromyxoma of the tail vertebrae, which are not neural crest-derived, and there are clear deficits in bone and cartilage development in TEC1KO; it may be important to investigate the role of PKA signaling in skeletal development per total in the mouse. Bone-specific cre mice, such as Osteocalcin cre (256), or cartilage-specific cre mice, like the Type II Collagen a1 (Col2a1) cre (257), could be mated with Prkar1a conditional mice to generate animals with bone and cartilage specific PKA dysregulation. Given the results from the TEC1KO model, it might be predicted that loss of Prkar1a in the bones would predispose the animals to osteolysis and loss of bone density. PKA dysregulation in the bone precursors may result in similar differentiation defects as seen in the TEC1KO bones such where
chondrocyte differentiation is favored. Moreover, it may also be possible that bone-specific loss of Prkar1a may lead to increased susceptibility to osteochondromyxoma development. In contrast, loss of Prkar1a in the cartilage may result in dysplastic growth and possibly loss of rigidity in those tissues. It would be interesting to see if these animals could survive embryogenesis, and if they do it would be interesting to observe whether any cartilage defects present challenges in the adult derived structures during endochondral ossification.

In addition to the TEC1KO model, TEC3KO animals serve as a unique model for neural crest dependence upon PKA regulation with relation to Schwann cell tumorigenesis. Since Carney complex and the Neurofibromatosis syndromes are among the only inherited diseases known to cause Schwann cell tumors, it was exceedingly important to identify the role of PKA in this process. Our data supports previous findings that PKA can interact with and inhibit both Nf1 and Nf2, and we were able to show that this may lead to enhanced degradation of Nf2 specifically during schwannomagenesis.

In normal Schwann cells, the PKA holoenzyme oscillates between the on (active) and off (inactive) forms depending upon extracellular signals. In the active state, the catalytic subunits can freely interact with Nf2 to phosphorylate and inhibit the protein. This releases growth suppression, which allows for Rac1 and Pak mediated cell proliferation (Figure 5.2A). When extracellular hormone signaling ceases, the PKA heterotetramer forms to inhibit catalytic activity. Nf2
can therefore resume its tumor suppressor activity to inhibit proliferation of the cell and maintain normal growth. In TEC3KO Schwann cells, Prkar1a is knocked out leading to constitutive activation of the catalytic subunits. This results in increased PKA-Nf2 interactions that may lead to Nf2 degradation, and therefore loss of growth suppression (Figure 5.2B). Either directly or indirectly, Rac1 is highly activated by PKA dysregulation and subsequently Pak2 is then activated as well. Although Pak2 is known to phosphorylate and inhibit Nf2, this interaction does not likely occur in this model; which could be due to lack of Nf2 protein availability if it is being degraded. The combination of growth signals from both PKA and Pak2, as well as the loss of growth inhibition by Nf2, ultimately leads to schwannoma development. Additionally, since Pak6 was so highly over expressed in TEC3KO schwannomas, it may be possible that PKA can interact with and stabilize Pak6 via phosphorylation. Despite no changes in activation, increased stabilization could potentially play a role in Pak6-mediated cell transformation; but further experimentation is required before this conclusion can be made with any certainty.
Figure 5.2: Schematic for Schwann Cell Tumorigenesis Involving Dysregulated PKA Signaling. (A) In the normal Schwann cells, PKA activity oscillates between the on and off states. In the on (activated) state, the regulatory (R) subunits are bound to cAMP, and the catalytic (C) subunits are freed. PKA can then phosphorylate and inhibit Nf2 from suppressing growth. Likewise, Rac1 is no longer inhibited, which may signal for Pak activation that would also permit cell growth. In the off (inactive) state the R subunits are bound to the C subunits, therefore Nf2 is active as a tumor suppressor to inhibit growth of the cell. (B) When PKA activity is dysregulated by the loss of Prkar1a, there is constitutive inactivation of Nf2; likely due to degradation. This causes significant activation of both Rac1 and Pak2 and leads to uncontrolled cell growth. Since Pak6 was highly overexpressed, and it may play a role in cellular transformation, it is possible that PKA activation may lead to Pak6 stabilization and therefore allow for tumorigenic growth.

There is a lot of overlap in signaling between the TEC3KO model of PKA tumorigenesis and that of the NF syndromes. Not surprisingly, small G-protein signaling appears to play an important role in propagating growth signals in both models, and TEC3KO schwannomas seem to arise from Rac1 activation specifically. Future work with the TEC3KO model will involve selective down regulation of Rac1 in order to rescue the phenotype. Rac1 conditional knock-out mice are currently being mated with TEC3KO animals to generate TEC3KO;Rac1^{loxP/loxP} progeny. The premise behind this mating strategy is that if these animals are resistant to schwannoma development then PKA signaling
through Rac1 (and presumably Pak2) is the predominant mechanism by which these tumors are formed. Likewise, mice harboring constitutively activated Rac1 under the control of the *TEC3* promoter are being made to support the hypothesis that activated Rac1 alone is sufficient for schwannoma development. According to this hypothesis, these animals should develop tumors similar to those seen in *TEC3KO*.

With reference to the impact of PKA activation on Nf2, further experimentation is ongoing to determine if phosphorylation of serine 10 causes degradation of the protein. Site directed mutants for serine 10 and serine 518 (or both) were generated to make both non-phosphorylatable and phosphomimetic forms of Nf2. These constructs will be transfected into rat schwannoma cells or immortalized mouse Schwann cells that lack Nf2 to evaluate the stability of the resulting proteins. If serine 10 phosphorylation is indeed capable of causing Nf2 degradation, the half-life of the protein would be compromised, and the total expression would be decreased. Another method to test this hypothesis would be to treat normal Schwann cells with cAMP analogs, such as 8-bromo-cAMP, and then determine whether the half-life of Nf2 decreases.

Additionally, further experiments could be performed to analyze the role of Pak2 and Pak6 in PKA-mediated tumorigenesis. Amino acid sequence analysis using NetPhosK 1.0 shows that PKA may phosphorylate both Pak2 and Pak6 (data not shown), however the data more strongly suggests that Pak6 is a true target of PKA. Co-IP experiments would help to determine if PKA
can indeed interact with either Pak protein, and further in vitro kinase assays using Pak2 or Pak6 as a substrate would show whether or not PKA can phosphorylate them. Moreover, protein half-life experiments would show whether PKA phosphorylation of Pak6 can indeed stabilize the protein. If this is the case, it may be that sustained basal activation of Pak6, versus increased stimulation, may play a pivotal role in schwannoma development in this model. Finally, microarrays or microRNA arrays could be performed on TEC3KO schwannoma cells compared to normal Schwann cells to identify changes in gene expression downstream of Pak and/or PKA activation that have not been previously observed.

The results presented in this thesis showcase two unique models for PKA dysregulated development and tumorigenesis. Future work with these systems may provide more clues to the complex nature of PKA signaling, and hopefully define novel targets for treatment strategies aimed at ameliorating phenotypes associated with craniofacial birth defects, Neurofibromatosis, or other neurocristopathies.
REFERENCES


Bernards A, Snijders AJ, Hannigan GE, Murthy AE, & Gusella JF (1993) Mouse neurofibromatosis type 1 cDNA sequence reveals high degree of


136


