UNCOVERING MOLECULAR PROPERTIES OF NEURAL CREST CELLS

Thesis by

Meghan Adams

In Partial Fulfillment of the Requirements

for the Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California

2009

(Defended May 26, 2009)

ACKNOWLEDGEMENTS

I thank my mentor, Marianne Bronner-Fraser, for her endless and tireless support. Her boundless enthusiasm for experimentation, her ability to edit well and quickly, and her skill at teaching are legend, but she also took the time to make sure that I became the best scientist and teacher I could, even when I doubted myself the most. Thank you for being an inspiration. I thank the members of my committee, Kai Zinn, Ellen Rothenberg, and Scott Fraser for their discussions and insights into many aspects of my work as well as life as a scientist. Thank you for your guidance and excellent advice, without which both this thesis and I would have suffered a great deal. Since I had the somewhat unique experience of working with one of my committee members before starting graduate school (and then worked just next door during my thesis), I would also like to draw special attention to Scott Fraser and the members of his lab who have taught me way more than I ever thought I would know about confocal microscopes and provided many a happy hour(s) of long discussion.

To Members of the Bronner-Fraser Lab: To Laura, for encouraging me to work on a great project, being a great officemate and answering all my questions over the years; To Tatjana, for teaching me countless scientific techniques, making me a better scientist, and for being a critic when I needed one, and being a good friend always; To Meyer, who shared a love of audiobooks and great writers, and is a font of scientific knowledge. To my fellow graduate students over the years: Tanya, Dan, Sujata, Martín, Jane K, Celia, Paola, Houman and the next generations…keep fighting the good fight, and be good to yourselves. Current and former officemates: to Sara, Tanya, Sonja, Sujata (again!), Laura (also again!) and Katherine—thank you for putting up with me and taking the peanut butter cups away from me when I started eating too many, and always being there when I needed you for advice on a figure, method, or life. To everyone (the vast legions over the years) for all for the tea times, dancing, waterfights, and discussions (both rigorously scientific and, well, not)—you have made my life much more entertaining and I wouldn't have missed it for anything. I would also like to thank Mary, Gary, Martha, Matt, David, Connie, Sam, Joanne, and all of the other people who let us do what we do and make sure the place doesn't fall down around us, and for providing extra pairs of hands, late night conversations, and good music.

To the teacher who inspired me the most: Paul Darman, and his wonderful family. Thank you for reminding me how much I love science and for all the loud bangs! To friends outside the lab: Jordan, Amber, Mark, Devin, Adria, and the pottery and knitting groups who have tolerated my presence and encouraged my good habits.

I dedicate this thesis to my parents, Mary Susan and Gregory Carl Adams: the inspiration and support for so many of my life's adventures. Without you, I would not have been born—but even more importantly, I would not have lived well.

Last, but never least, to Julien—my best friend, partner, and travel buddy in the adventures of the last several years. Your unwavering support and good sense of humor are an inspiration, and there is no one with whom I would rather argue or laugh. All my love, always. To Julien's family, the Muffats and Tapponniers: thank you for sharing your wonderfully warm Christmases, New Years, and family vacations with me, for always including me as a member of your family, and for not laughing too much at my French.

ABSTRACT

The neural crest is a transient population of cells that arises at the border between the neural and non-neural ectoderm. These cells are induced, undergo an epithelial-to-mesenchymal transition, and then migrate along stereotypical pathways to form a wide array of derivatives. While these cells have long been studied, much about these cells and their interactions is still not understood. In order to better define these cells, we performed a screen for genes involved in neural crest cell development based on an *in vitro* culture system that produces neural crest cells. This highly successful screen resulted in a large number of candidates to examine, and we performed in situ hybridization to define the mRNA expression of 112 these genes. Moreover, we performed QPCR on several transcription factors that resulted from this screen to determine the level at which they were upregulated in our *in vitro* culture system. We also present loss-of-function analyses of two different genes that were discovered in our screen for neural crest effectors. These genes, Adh5 and Ccar1, are both functionally relevant in neural crest cells and the loss of either one through morpholino knockdown significantly decreases the mRNA of *Sox10* on the injected side. Furthermore, we also show that Adh5 morpholino knockdown also results in a reduction of *Snail2* and *FoxD3* mRNA. Taken as a whole, this body of work represents the discovery of many new genes involved neural crest cell development, and the demonstration that at least two of these genes are functionally important for neural crest cells.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

KEYWORDS

Chicken embryo

Premigratory neural crest

Migratory neural crest

Screen

Electroporations

Morpolino

Cranial neural crest

Trunk neural crest

Adh5

Ccar1

Transcription factor

QPCR

Chapter I: Introduction

Portions of this chapter are published as: The role of neural crest cells in the endocrine system. Adams MS and Bronner-Fraser M. Endocrine Pathology. 2009 Apr 18. Other portions were written in collaboration with Katherine Fishwick, Herve A, and M. Angela Nieto to be published as: Epithelial-to-Mesenchymal Transitions: Importance of changing cells' state in development and disease. Acloque H, Adams MS, Fishwick K, Bronner-Fraser M and Nieto MA; Journal of Clinical Investigations. [In Press].

1.1: Neural crest cells

The neural crest (NC) is a pluripotent cell population that arises at the junction of the neural tube and the dorsal ectoderm (Figure 1). The neural crest has been widely studied and been the subject of pivotal advances in areas of developmental biology such as inductive interactions and long-range migration. More recently, studies of neural crest cells have also proven useful in areas such as cancer research, stem cell research (including regenerative medicine), and understanding the evolution of structures as well as gene networks (Zito, Richiusa et al. 2008; Sieber-Blum, Schnell et al. 2006; Song, Lee et al. 2008).

NC cells are first specified at the end of gastrulation at the border between neural and non-neural tissues (Basch, Bronner-Fraser et al. 2006). After neurulation, NC precursors within the neural tube undergo an epithelial to mesenchymal transition and subsequently migrate away from the neuroepithelium in a rostral to caudal progression. These highly migratory cells subsequently form many divergent derivatives including neurons and glia of the sensory, sympathetic, and enteric systems, melanocytes, and the bones, cartilage, and connective tissues of the face (Figure 1) (Anderson 1993; Unsicker 1993; Baker and Bronner-Fraser 1997; Le Dourarin and Kalcheim 1999; Hall 1999)**.** In addition, they contribute to endocrine cells like the chromaffin cells of the adrenal medulla, and the C-cells (parafollicular cells) of the thyroid gland (Le Douarin and Teillet 1974; Le Douarin, Fontaine et al. 1974; Pearse 1966; Bussolati and Pearse 1967; Polak, Pearse et al. 1974). The advent

of neural crest cells is linked to the development of the "new head" (and, as some argue, the "new neck") and the evolution of predatory behaviors in vertebrates (Gans and Northcutt 1983; Kuratani 2008). The gene regulatory network underlying NC formation is predicted to have evolved over 500 million years ago during the early Cambrian period leading up to the inception of the vertebrate lineage (Meulemans and Bronner-Fraser 2005; Sauka-Spengler and Bronner-Fraser 2006; Sauka-Spengler, Meulemans et al. 2007; Sauka-Spengler and Bronner-Fraser 2008).

Neural crest cells can be divided into several subpopulations based on their site of origin and the locations to which they migrate. These groups are the cranial, trunk, cardiac, and vagal neural crest cells. The cranial neural crest cells are those that emerge from the presumptive brain region to form many of the craniofacial cartilages and bones as well as nerves and connective tissues. The skeletogenic potential of the cranial neural crest has been widely explored and documented throughout the Vertebrata subphylum. Though it was for some time assumed that trunk neural crest cells did not form bone or cartilage elements, they have since been shown to contribute in a limited way to to these derivatives in a few extant and extinct animals (Clark, Bender et al. 2001; Smith, Murray et al. 1994; Sanchez-Martin, Rodriguez-Garcia et al. 2002; Cebra-Thomas, Betters et al. 2007; Smith, Murray et al. 1994; Cano, Perez-Moreno et al. 2000; Freitas, Zhang et al. 2006; Graveson, Smith et al. 1997; Lumsden 1988). These examples, however, are the exception rather than the rule as the trunk neural crest cells of many model organisms have been shown to not have the ability to give rise to cartilage or bone (Smith and Hall 1990; Noden 1991;

Couly, Coltey et al. 1992; Couly, Coltey et al. 1993). The vagal neural crest cells, originating in the neck region, colonize the intestine to form the ganglia of the enteric nervous system (ENS), which controls intestinal peristalsis. Defects in innervation can lead to megacolon disorder, also known as Hirschsprung's disease (Heanue and Pachnis 2007).The cardiac neural crest, a subpopulation of the vagal neural crest, is a relatively recent discovery and has been shown to contribute to the musculoconnective tissue of the primary blood vessels as well as the septum that divides the outflow tract from the heart into the aorta and pulmonary artery (Le Lievre and Le Douarin 1975; Jiang, Choudhary et al. 2002). In quail, clonal analysis of derivatives has shown that the cardiac neural crest cells can give rise to melanocytes, smooth muscle, chondrocytes, connective tissue, and sensory neurons (Ito and Sieber-Blum 1991).

1.2: Historical aspects

Beginning in the time of Aristotle (about 350 B.C.), we have records of studies based on the chicken embryo. Long domesticated, the chicken and its eggs have been not only a valuable food source, but also a source of material for scientific inquiry. When using chicken embryos as a model organism, there are not only opportunities to perform classical embryological studies involving grafting, excision or implantation, but one can also place beads soaked in myriad solutions, inject cloned constructs or manufactured oligonucleotides and elecroporate them into cells, or even use viral transfection techniques. Further studies are made possible by the elucidation of the

chicken genome, and on the genomes of other organisms as they can be used for comparative inquiries. Our lab and others use chicken embryos of different ages to study neural crest cells.

Neural crest cells were first described in 1868 by Wilhelm His in chicken embryos when he named a band of cells between the dorsal ectoderm and the neural tube the "*Zwichenstrang*" or "intermediate cord" (His 1868; Hörstadius 1950). This initial appellation gave way to the more descriptive and familiar term "neural crest" in a 1879 paper by Arthur Milnes Marshall (Hall 1999). Initially, neural crest cells were thought to simply be the precursors of the cranial and spinal ganglia and neurons. This first impression was soon questioned and in 1888 Kastschenko suggested that cranial mesenchyme was derived from neural crest cells based on his observations in shark embryos. In the 1890s, Julia Platt was the first person to demonstrate that the neural crest cells were the origin of the mesenchyme of the visceral arch and further demonstrated that they were also the origin of the cartilage of the visceral skeleton and the dentine of the teeth (in the mud puppy, *Necturus*) (Hall 1999). A quarter of a century passed before Landacre (1921) confirmed a neural crest origin for visceral cartilages in *Ambystoma jeffersonianum,* and the ramifications of this work continue to be important in the community (Landacre 1921). Most of the original studies of neural crest cells were performed using amphibian embryos, but many laboratories subsequently moved into chicken embryos and mammals such as the mouse in order to exploit the many advantages offered by these embryos. In the 1960s, Chibon and Weston used tritiated thymidine to label cells in amphibians and avians (respectively)

in order to determine the fates of the cells they were examining (Chibon 1967; Weston 1963). This technique, though transient, opened up these organisms to new questions that were more thoroughly answered by the more stable quail-chicken chimeras pioneered by Nicole Le Douarin. Among others, these techniques allowed for the construction of fate maps beginning in the 1970s—work that continues to the present day. There are discrepancies between different fate maps, both because of the organisms in which they were performed as well as the techniques used. It will be critical to resolve these differences in order to understand the behavior of NC cells in humans, where such lineage analyses are not possible (Gross and Hanken 2008).

More interspecific grafts were used in an attempt to understand the nature of the induction of the neural crest cells. In the late 1970s and the early 1980s, Rollhäuser-ter Horst performed a series of experiments using grafts between different species of urodeles that suggested the necessity of both neural and non-neural ectoderm in the induction of neural crest cells (Rollhauser-ter Horst 1979; Rollhauser-ter Horst 1980). Moury and Jacobson went on to use pigmented and albino axolotl embryos to demonstrate that boundaries created between neural plate and epidermal tissues would give rise to both neural folds and neural crest cells (Moury and Jacobson 1989; Moury and Jacobson 1990). Selleck and Bronner-Fraser created quail-chicken chimeras in which the neural plate of quail were cultured with chicken non-neural ectoderm *in vivo*; these gave rise to migratory HNK-1 positive neural crest cells (Selleck and Bronner-Fraser 1995). These experiments, while informative, used *in vivo* techniques in which one could not separate the effects of neural plate-epidermal

interactions from the presence and influence of the underlying mesoderm. Therefore, *in vitro* culture techniques were used to determine what tissues could induce neural crest cell formation. To this end, further experiments were performed to show that combining neural and non-neural ectoderm induces *Slug*, a gene seen in neural crest cells (Dickinson, Selleck et al. 1995; Gammill and Bronner-Fraser 2002; Adams, Gammill et al. 2008) or demonstrates the formation of melanocytes and catecholaminergic neurons (Dickinson, Selleck et al. 1995). Mancilla and Mayor also demonstrated the upregulation of *Slug* in co-cultured neural plate and epidermis from *Xenopus laevis* embryos (Mancilla and Mayor 1996). Furthermore, the neural crest cells were shown to come from both of the cultured tissues (Selleck and Bronner-Fraser 1995; Mancilla and Mayor 1996). This co-culturing scheme was then used to perform a successful subtractive screen to identify members of the genetic cascade that is involved in the induction and migration of neural crest cells (Gammill and Bronner-Fraser 2002; Adams, Gammill et al. 2008).

The use of vital dyes *in vivo* combined with imaging has also been useful for examining contributions of neural crest populations (one example: (Bronner-Fraser and Fraser 1988)). As the use of vital dye has some limitations, electroporations of avian embryos *in ovo* and in various types of culture dishes were developed in order to study the effect of over-expression and knock-down of genes in a spatially and temporally controlled manner (Funahashi, Okafuji et al. 1999; Nakamura, Watanabe et al. 2000; Katahira and Nakamura 2003; Sauka-Spengler and Barembaum 2008). The use of constructs such as morpholinos and RNAi has been invaluable for

dissection of genetic interactions in organisms that are less amenable to classical genetic manipulations. These can be used to investigate the effect of knocking down genes that are involved in vital processes like gastrulation as well as later processes like the migration of neural crest cells (in which genetic null embryos would not survive past gastrulation and thus the gene's effect could not be examined at the later stage) (Sauka-Spengler and Barembaum 2008; Katahira and Nakamura 2003). The advances in imaging techniques have also allowed striking advances in this area, especially because better signal collection and the use of less laser power, have allowed for less damaging imaging techniques and the possibility of longer observation times in live tissue imaging.

Some antibodies have served as useful neural crest markers. For example, the HNK-1 (CD57) antibody identifies a cell surface sulfoglucuronyl glycolipid and is used to label avian premigratory and early migratory neural crest cells as well as neural crest cells in some other species (some reptiles and mammals). It is not exclusive to the crest populations (as it also stains cerebellar and motor neurons as well as some leukocytes) and is not useful as a label in amphibians ; Erickson, Loring et al. 1989; Chou, Schachner et al. 2002). Despite these limitations, it is a good marker for neural crest cells in avian embryos (Tucker and Erickson 1984; Rickmann, Fawcett et al. 1985; Bronner-Fraser 1986; Erickson, Loring et al. 1989; Sadaghiani and Vielkind 1990; Jeffery, Strickler et al. 2004) and has been shown to also stain turtle neural crest cells (Hou 1999).

1.3: The EMT process in NCC with distinct involved factors

General information

The neural crest represents a definitive example of a cell type that undergoes a classical epithelial to mesenchymal transformation (EMT) in which epithelial cells in the dorsal neural tube are transformed into a migratory mesenchymal phenotype (Newgreen and Gibbins 1982; Nichols 1987). Neural crest cells form at the border of the neural plate and non-neural ectoderm (Selleck and Bronner-Fraser 1995; Le Dourarin and Kalcheim 1999) as a result of signals emanating from both of these tissues. This phenomenon gives rise to the 'neural plate border specifier' signals expressed by both of these populations (Knecht and Bronner-Fraser 2002; Meulemans and Bronner-Fraser 2004). Cells at the border form the dorsal neural folds and further signaling refines a sub-population of these into pre-migratory neural crest cells that express 'neural crest specifier' genes such as *Sox-10*, *Snail,* and *FoxD3* (Meulemans and Bronner-Fraser 2004; Sauka-Spengler and Bronner-Fraser 2006). These cells then undergo a crucial epithelial-to-mesenchymal transition, escape the neural folds and migrate to distinct regions of the developing embryo prior to re-aggregation and further differentiation.

Epithelial and mesenchymal cells make up the majority of the body plan in metazoan animals. These cell types differ both morphologically and functionally. Epithelial cells display a characteristic apico-basal polarity and are adherent cells that are

attached to their neighbors by intercellular adhesion complexes, thereby forming coherent layers. Mesenchymal cells lack this polarity and intercellular junctions, and have the ability to move throughout the extracellular matrix as individual cells (Thiery and Sleeman 2006). However, the epithelial- and mesenchymal-cell phenotypes are not irreversible, and during embryonic development, cells can convert between the epithelial and mesenchymal states. Using the chick primitive streak (a structure that forms early in avian, reptile, and mammal development and that is one of the first signs of gastrulation) as a model, Elizabeth Hay was the first to propose that epithelial cells can undergo dramatic phenotypic changes that reflect their "transformation" to mesenchymal cells (Hay 1968). The term “transition” is now preferred, since this "transformation" is reversible and mesenchymal cells can revert to epithelial cells via MET (mesenchymal to epithelial transition). This EMT process is essential for germ layer formation and cell migration in the early vertebrate embryos (Hay 1968).

The sequential events in EMT have been defined as individual steps by Levayer & Lecuit in 2008:

> “1) specification of a group of cells destined to undergo EMT
>
> 2) loss of intercellular adhesion mediated by cadherins at adherens junctions
>
> 3) loss of polarity markers
>
> 4) cytoskeletal reorganization to actively drive cell delamination
>
> 5) degradation of the basement membrane.

These steps do not necessarily occur consecutively and are not all necessarily present in a given example of EMT" (Levayer and Lecuit 2008). It is worth noting that, although many signaling pathways and molecules have been shown to be consistently involved in neural crest formation and migration, as yet there have been no individual or combinatorial signals that have been shown to function identically in these processes (Sauka-Spengler and Bronner-Fraser 2008). In addition, large amounts of the information that are gathered on the EMT process derive from work with cell lines and work on different forms of cancer (since this is a crucial process that cancer cells need to undergo to become migratory, and therefore metastatic). Despite this heterogeneity, some studies pinpoint the action of certain molecules and pathways in the EMT process undertaken by neural crest cells as they migrate out of the neural tube.

Regulation of the Cadherin proteins during EMT

Snail family genes bind to targets sites on DNA via conserved E-box sequences within both their own promoter (Sakai, Suzuki et al. 2006) and the promoters of their targets such as E-Cadherin (Cano, Perez-Moreno et al. 2000), claudin-3,-4 and -7, and occludin (Ikenouchi, Matsuda et al. 2003). This interaction appears dependent on these elements and at least in some cases, the presence of the N-terminal SNAG domain within the Snail genes (Ikenouchi, Matsuda et al. 2003). Further transcription factors have been found which bind these target sites but do not belong to the Snail superfamily; these are SIP-1 and SIP-2 (Smad Interacting Proteins -1 and -2, respectively). SIP-1 (also known as ZEB-2, ZFHX1B, or SMADIP1) is necessary for

EMT in neural crest cells in the mouse as crest cells in homozygous null embryos are unable to exit the neural tube, and vagal neural crest (forming at the level of somites 1–7) does not form at all, suggesting a particular requirement of this subtype of crest for SIP-1 (Van de Putte, Maruhashi et al. 2003).

Neural crest cells delaminate from the neural folds or dorsal neural tube shortly before or after its closure. A crucial step in this is the transition from polarized epithelial cells tightly linked together to individual mesenchymal cells; therefore initiation of neural crest migration relies upon a decrease in cell-cell adhesion. For any given cell, cadherin expression is a critical determinant of adhesion to neighboring cells or to the extracellular matrix (ECM). Cadherins are transmembrane proteins which mediate attachments to neighboring cells through homophillic binding to cadherins expressed on neighboring cells. This homophilic interaction initiates signal transduction from the ECM to the cytoskeleton within the cell (reviewed in (Gumbiner 2005). Ectodermal cells express epithelial cadherins such as E-Cadherin (also known as L-CAM in chick), a product of the Cdh1 gene which is initially expressed through the entire ectoderm and is subsequently downregulated in cells undergoing EMT (Edelman, Gallin et al. 1983). Loss of E-Cadherin can occur through several mechanisms; the most common appears to be transcriptional repression through transcription factors such as *Snail*, *Snail2,* or *SIP-1* (Batlle, Sancho et al. 2000; Cano, Perez-Moreno et al. 2000; Comijn, Berx et al. 2001; Perez-Moreno, Locascio et al. 2001; Bolos, Peinado et al. 2003). However, E-Cad levels may also be reduced by ubiquitination and lysosomal targeting of the protein

(Palacios, Tushir et al. 2005); hypermethylation of the E-Cad promoter (Graff, Herman et al. 1995); methylation of the promoter (Machado, Oliveira et al. 2001) or mutations in the E-Cad gene in regions regulating its adhesive properties, reviewed in (Hajra and Fearon 2002). These latter mechanisms have been uncovered in transformed cell lines and it would be interesting to investigate their occurrence during EMT in normal embryonic development.

E-Cadherin is not the only cadherin that must be repressed in order for EMT to occur. In the chick, both E-cadherin and Cadherin 6B (Cad6B) are expressed within the neural folds (Nakagawa and Takeichi 1995; Taneyhill, Coles et al. 2007), but only Cad6B is known to be a direct target of *Snail2,* which binds E-box elements within the Cad6B promoter to act as a transcriptional repressor (Taneyhill, Coles et al. 2007). This was the first work to describe a direct target of *Snail2* during neural crest EMT within the developing embryo. Furthermore, Cadherin 6B was shown to regulate the timing of EMT and neural crest cell emigration from the neural tube (Coles, Taneyhill et al. 2007). Another type I cadherin, N-cadherin (N-Cad, also known as A-CAM) is also present in the neural plate but downregulated during EMT in vivo. Interestingly, the downregulation of N-Cad can stimulate premature neural crest cell migration (Newgreen and Gooday 1985). Expression of N-Cad returns in specific lineages such as the dorsal root ganglia as cells reaggregate (Duband, Volberg et al. 1988; Akitaya and Bronner-Fraser 1992; Hatta and Takeichi 1986).

As neural crest cells exit the neural tube, cadherins such as Cad-7 are expressed on migratory cells in place of E-Cad and Cad6B (Nakagawa and Takeichi 1995; Nakagawa and Takeichi 1998). A further type II cadherin, Cad11 has also been described on migrating neural crest cells in *Xenopus* (Hadeball, Borchers et al. 1998) and mouse (Hoffmann and Balling 1995; Kimura, Matsunami et al. 1995).

Signaling cascades mediating crest cell EMT

Wnts have been shown to regulate several important stages in neural crest cells such as induction, the proliferation of NC precursors, the regulation of neural crest cell delamination through modulation of cell division, and the regulation of their migration (Garcia-Castro, Marcelle et al. 2002). Wnt signaling cooperates with other signal cascades such as the BMP signaling pathway during the specification and differentiation process of neural crest cells (LaBonne and Bronner-Fraser 1998).

The protolithic member of the TGFB superfamily was named a “transforming growth factor” due to its ability to “transform” the phenotype of cells grown in soft agar (de Larco and Todaro 1978). This name is quite appropriate for our purposes in that several family members are associated with the epithelial to mesenchymal transformation. There is a great deal of difficulty in interpreting the effect of TGFb family members and signaling pathways on the EMT, not least because three TGFb isoforms have been documented in mammals and four reported in the chicken (Ahmed and Nawshad 2007). In addition to being involved in EMT (Boyer, Ayerinskas et al. 1999), the TGFb family of peptide growth factors regulates many

different biological functions such as inhibiting cell growth, inducing cell proliferation and inducing ECM synthesis (Massague 1990). Antisense oligonucleotides to TGFb2 mRNA were shown to inhibit EMT in chicken by Potts et al. in 1991 and other labs have gone on to demonstrate that functional antibodies to TGFB type II and type III receptors can inhibit EMT and the migration of the mesenchymal cells after transformation (Brown, Boyer et al. 1996; Boyer, Ayerinskas et al. 1999; Brown, Boyer et al. 1999). Additionally, Potts et al. also showed that TGFb3 is required for EMT in the chicken (Potts, Dagle et al. 1991). Further studies have gone on to show that although exogenous TGFB1, 2, or 3 can promote EMT, these factors are not sufficient and require the presence of additional ECM components to be effective (Nakajima, Krug et al. 1994).

Pez/PTPN14 is a tyrosine phosphatase that is thought to regulate cell adhesion through tyrosine phosphorylation of adherens junction proteins (Muller, Choidas et al. 1999; Wadham, Gamble et al. 2003). Overexpression of Pez/PTPN14 in epithelial MDCK cells is sufficient to cause TGF-beta mediated EMT, and knockdown in zebrafish resulted in defective pigmentation and craniofacial deformities (Wyatt, Wadham et al. 2007).

The signaling of the bone morphogenetic protein (BMP) family (also members of the TGFb superfamily) has been shown to regulate the EMT in which neural crest cells delaminate from the neural tube (Sela-Donenfeld and Kalcheim 1999; Sela-Donenfeld and Kalcheim 2000). Individual family members are still being examined

using new techniques to determine their specific effects throughout neural crest development in different species (Raible 2006; Correia, Costa et al. 2007). *Noggin*, a BMP inhibitor, prevents neural crest cells from delaminating until it is downregulated by signals from the somite. Another regulator of BMP signaling in neural crest is Cv-2, a vertebrate homolog of *Drosophila* crossveinless, which regulates BMP signaling and the onset of migration in trunk but not cranial neural crest cells (Coles, Christiansen et al. 2004). Additionally, BMP signaling has been shown to upregulate the transcription of *wnt1* while inhibiting *wnt1* results in lower expression levels of BMP regulated genes, strongly suggesting that these two signaling pathways are interconnected during the neural crest EMT (Burstyn-Cohen, Stanleigh et al. 2004). In chick embryos, BMP4 is expressed in neural folds and has the ability to transform competent neural plate cells into migratory neural crest cells. (Selleck, Garcia-Castro et al. 1998; Sela-Donenfeld and Kalcheim 1999). In the avian neural crest, BMP4 signaling activates *c-myb*, which activates transcription of *msx-1* and *Snail2*, both genes required for the formation of neural crest (Liem, Tremml et al. 1995; Sela-Donenfeld and Kalcheim 1999; Karafiat, Dvorakova et al. 2005). Notch signaling is also necessary for cranial neural crest to form (Endo, Osumi et al. 2002; Endo, Osumi et al. 2003).

Role of the extracellular matrix during crest cell EMT

Further to changes in cellular adhesion, there is a breakdown in the basement membrane that surrounds the neural tube from which cranial neural crest cells migrate. (Erickson and Perris 1993). This disruption in the basement membrane

occurs immediately before or after the onset of cranial neural crest cell migration in both mice and avians (Duband and Thiery 1987; Nichols 1981). In the trunk region, no basal lamina (and therefore, no basement membrane) exists at the neural fold before neural crest cell migration begins, which removes this step in neural crest cell development (Martins-Green and Erickson 1987).

MMP-2, or matrix metalloprotease- 2 (MMP-2, 72-kD type IV collagenase or gelatinase A), is a protein important for degrading a variety of extracellular matrix (ECM) molecules including collagen type I, IV, V, VII, X, XI, elastin, gelatins, laminin, aggrecan, and vitronectin (Alexander and Werb 1992; Parsons, Watson et al. 1997). It is expressed during EMT in several cell types including the neural crest, and is rapidly downregulated as the crest cells migrate away. MMP inhibitors, including BB-94 and TIMP-2 (tissue inhibitor of metalloprotease-2), prevent EMT in vitro and in vivo during neural crest formation. Additionally, in the Patch mouse mutant (which has lost the alpha subunit of the platelet derived growth factor receptor), craniofacial mesenchyme does not disperse properly and Robbins et al showed that MMP-2 activity is depressed (Robbins, McGuire et al. 1999). Specific morpholino knockdown of MMP-2 expression in the dorsal neural tube perturbs the neural crest cell EMT (Duong and Erickson 2004).

The ECM is not a passive structure allowing the movement of neural crest cells but rather provides an active signaling input into cell migration from the neural tube. It is also important to consider that the ECM is not a homogenous entity and different

basement membranes may differentially regulate cell movement. Studies point to several components of the ECM being of primary importance in NC cell migration and as such are defined as 'permissive' i.e. supporting and allowing cell movement (Perris and Perissinotto 2000): namely fibronectin, laminins, proteoglycans and the movement inhibitory aggrecan proteoglycan. In addition, the complement of integrin molecules expressed by the migrating cells that recognize these ECM components also appears to be necessary for the correct regulation of migration. Genes regulating EMT may also contribute to determining the surrounding ECM; In epithelial cells overexpressing *Snail*, *Snail2* or a further transcriptional repressor of E-Cadherin, *E-47*, all have upregulated *collagen1a2* and *integrin-a5* gene expression and downregulated expression of *keratin-19* and *integrin*-b*6*; these cells also have upregulated expression of an ECM modifying protein, TIMP-1 (Moreno-Bueno, Cubillo et al. 2006). Much work has been done to understand the many interactions between ECM and E-Cadherin and adherens junctions in cancerous cells. Just as work on neural crest may inform our understanding of metastasis, the reverse is also true (e.g. (Giehl and Menke 2008)).

1.4: Neural crest stem cell plasticity and pluriopotency

The first direct evidence of neural crest cell multipotency came in 1980, when Sieber-Blum and Cohen took individual trunk neural crest cells from quail and cultured them. They found that these cells gave rise to one of three types of clones: unpigmented (which solely consisted of adrenergic neurons), pigmented (which

solely consisted of melanocytes), and mixed clones (containing both types of cells) (Sieber-Blum and Cohen 1980). When cultured quail neural crest cell colonies were reintroduced back into embryos, these cells were able to contribute to neural crest derivatives such as the sympathetic ganglia, adrenal gland, and aortic plexus as well as various populations of neurons. Furthermore, when single neural crest cells were injected into host embryos, they proved able to give rise to many daughter cells of different neural crest cell lineages (Bronner-Fraser, Sieber-Blum et al. 1980).

These results were later confirmed *in vivo* without the inherent complications of transplantation when Bronner-Fraser and Fraser used lysinated rhodamine dextran (LRD) to label individual dorsal neural tube cells *in vivo*. Descendants of these individual cells were not only found in the neural tube, but also went on to form such diverse neural crest derivatives as sensory neurons, pigment cells, glia, and adrenomedullary cells (Bronner-Fraser and Fraser 1988). This was the first evidence for the pluripotency of neural crest cells *in vivo* and later work went on to definitively confirm the pluripotency of both premigratory and early migrating neural crest cells (Bronner-Fraser and Fraser 1989). Pluripotent neural crest cells have been found in avians (Sieber-Blum and Cohen 1980; Bronner-Fraser and Fraser 1988; Bronner-Fraser and Fraser 1989; Sieber-Blum 1989; Ito and Sieber-Blum 1991) as well as in mammals (Stemple and Anderson 1992; Morrison, White et al. 1999), demonstrating the conservation of this concept, at least among these groups.

Although avian, murine, and human NCSCs have been reported, it is important to note that these cells are vastly in the minority and that not all migratory neural crest cells show the same degree of commitment—approximately 80% of some populations of neural crest cells demonstrated some level of pluripotency, while the remaining 20% gave rise to only one type of cell (Baroffio, Dupin et al. 1988; Baroffio, Dupin et al. 1991; Le Douarin, Creuzet et al. 2004). Since it was not possible to "challenge" these cells to see if they could give rise to more derivatives under other circumstances, their full range of developmental potential could not be assessed.

In the cardiac neural crest cells, Ito and Sieber-Blum showed that a small percentage of the cells can give rise to all the described derivatives, that more can give rise to restricted progeny (melanocytes and smooth muscle) and that still others are committed early to becoming only melanocytes (Ito and Sieber-Blum 1991). Mice have also been shown to have similar unipotent and multipotent cardiac neural crest cells, with some of the multipotent cells displaying the property of self-renewal (Youn, Feng et al. 2003). Additionally, in mice an enriched population of NCSCs has been obtained through p75 sorting (Stemple and Anderson 1992). In the quail, *in vitro* analysis of neural crest cells grown on fibroblast feeder cells with endothelin 3-supplemented media have shown the existence and rarity (approximately 1% of the trunk and 3% of cranial clones) of NCSCs that can generate many different expected cell types (Trentin, Glavieux-Pardanaud et al. 2004). Human NCSCs have been derived from human embryonic stems cells (HESCs) at the neural rosette stage and can be propagated and differentiated *in vitro* to give rise to many neural crest cell

derivatives, such as Schwann cells, smooth muscle cells, peripheral neurons, osteogenic cells, and chondrogenic cells. Furthermore, transplantation of the human NCSCs into embryonic chickens and adult mice have demonstrated the viability, migratory and differentiation capacities of these cells (Lee, Kim et al. 2007).

While the pluripotency of these cells had been demonstrated in short-term analysis, it remained to discover how long these cells retain stem cell properties in the embryo. It is now apparent that some cells capable of forming a broad range of neural crest derivatives appear to persist into adulthood. In 2004, pluripotent NCSCs were identified in the adult mouse hair follicle and termed the epidermal NCSCs (eNCSCs or EPI-NCSCs). Clonal analysis from bulge explants yielded neurons, smooth muscle cells, Schwann cells and melanocytes while serial cloning revealed the self-renewing capacity of these cells (Sieber-Blum, Grim et al. 2004). Further work has revealed the existence of NCSCs in bone marrow, the dorsal root ganglia, and the whisker pad of adult mice (Nagoshi, Shibata et al. 2008). Hu et al have also provided data describing genes that are differentially expressed in the EPI-NCSCs and have generated a 19 gene “molecular signature” to help identify and isolate these cells (Hu, Zhang et al. 2006). NCSCs are already being utilized with some success in attempts to repair disorders such as the lesioned spinal cord (Sieber-Blum, Schnell et al. 2006; Song, Lee et al. 2008).

1.5: Neural crest cells and neuroendocrine tumors

The established neural crest derivatives give rise to pheochromocytomas and extra-adrenal paragangliomas, neuroblastomas and medullary thyroid carcinomas. The tumors can be sporadic or can occur as components of hereditary syndromes with variable penetrance [71]. Although controversies concerning embryological origin appear to have mostly been resolved, questions persist concerning the pathobiology of each tumor type. The answers to those questions are likely to come from greater understanding of neural crest cells and the pathways involved in their formation, migration and differentiation.

Sympathoadrenal tumors

Salient questions pertaining to sympathoadrenal neural crest cells include why pheochromocytomas in the adrenal gland are often adrenergic while extra-adrenal paragangliomas are almost always noradrenergic. The idea that the transcription factor Egr-1 is a simple master switch for the adrenergic phenotype (Wong 2003) has not held up in gene expression profiling studies of adrenergic and noradrenergic tumors (Huynh, Pacak et al. 2006). Likewise, the idea that adrenal glucocorticoids are the major adrenergic determinant has not held up in light of mouse studies showing that sympathoadrenal progenitors are already heterogeneous when they reach the adrenal cortical primordium (Unsicker, Huber et al. 2005). Other questions that remain unanswered include those that concern the increased malignancy of extra-adrenal paragangliomas as compared to their intra-adrenal counterparts, and those

concerning the developmental basis for the varying manifestations of different hereditary syndromes that include pheochromocytomas/paragangliomas.

Pheochromocytomas and extra-adrenal paragangliomas are both types of tumors of the autonomic nervous system found in neural crest-derived endocrine cells or organs (paraganglia). In 2004, the WHO (World Health Organization) formalized the current definition of pheochromocytomas as tumors that "arise in the adrenal medulla and are derived from chromaffin cells of neural crest origin" and that they are "intra-adrenal sympathetic paraganglioma[s]". Those tumors that "arise from chromaffin cells in sympathoadrenal and parasympathetic paraganglia" are termed "extra-adrenal paragangliomas". (DeLellis 2004). Pheochromocytomas and extra-adrenal paragangliomas show differences including a lower malignancy rate in pheochromocytomas (roughly four fold higher at ~20% in extra-adrenal paragangliomas associated with the sympathetic nervous system)(Linnoila, Keiser et al. 1990), a tendency to be associated with certain genetic disorders like multiple endocrine neoplasia type 2 (MEN2) (Bryant, Farmer et al. 2003), von Hippel-Lindau (VHL) disease, or familial paraganglioma syndrome and neurofibromatosis type 1 (NF1) (Maher and Eng 2002) (Eisenhofer, Bornstein et al. 2004), and are more often adrenergic in nature (whereas extra-adrenal paragangliomas are more often than not noradrenergic) (Lloyd, Sisson et al. 1986). Despite their differences, these tumors have similar morphologies, neuroendocrine profiles, and some genetic predispositions (Tischler, Kimura et al. 2006).

The von Hippel-Lindau and neurofibromatosis 1 genes are named for the diseases that mutations in them can cause. All of the VHL mutations decrease its ability to suppress the function of JunB, which normally acts as an antagonist to cJun (Lee, Nakamura et al. 2005). The disease is divided into two types (type I and type II) based on the susceptibility to pheocychromotomas or paragangliomas (Tischler 2008). Those patients who do develop pheochromocytomas may have examples that show some unusual characteristics like a thick vascular capsule, intermixed small vessels, myxoid (mucoid: resembling mucous) and hyalinized (glasslike or transparent) stroma, small cells with clear or amphophilic cytoplasm, and a lack of hyaline globules (Koch, Mauro et al. 2002), although the cause of these atypical features is not currently understood. These pheochromocytomas are usually noradrenergic and therefore phenylethanolamine N-methyltransferase negative (Tischler 2008; Eisenhofer, Huynh et al. 2004). It is possible to test for mutations in the VHL gene, which leads to easier identification of the genetic underpinning for people who suffer from this disease. The NF1 gene, on the other hand, is quite large and does not have the “hot spots”, or areas that are frequently mutated and might allow for genetic testing. However, some mouse *NF1* heterozygous knockouts display incidence of pheochromocytomas at an increased frequency, providing a gene-specific pheochromocytoma model for study (Jacks, Shih et al. 1994; Tischler, Shih et al. 1995). Furthermore, one study of these mice has shown that almost 20% of the genes expressed in these pheochromocytomas are also expressed in early neural development (Powers, Evinger et al. 2007), which makes the results of studies working to elucidate these early genes even more relevant. Interestingly, development

of the tumors depends on the genetic background of the mice, possibly mirroring the variable penetrance of hereditary human tumor syndromes. In mice, the influence of genetic background may be due to strain-dependent differences in tissue specific expression of Nf1 and other tumor suppressors (Reilly and Van Dyke 2008; Couto and Cardiff 2008). Contributions of the tumor microenvironment might also be critical for some types of tumors to develop (Reilly and Van Dyke 2008). Interestingly, some mutations of murine *Nf1* have been to most types of tumors seen in neurofibromatosis (Joseph, Mosher et al. 2008). Kaelin and co-workers have proposed the attractive hypothesis that different genetic mutations that lead to pheochromocytoma/paraganglioma do so by promoting survival of progenitors that would otherwise undergo programmed death (Lee, Nakamura et al. 2005) (Nakamura and Kaelin 2006). The actions of the various tumor-susceptibility genes at different times in development would help to account for the genotype-phenotype correlations.

C-cell neoplasms

The thyroid gland is formed from two different embryonic lineages: the thyroxine-producing follicular cells (of endodermal origin) (Hilfer 1968) and the parafollicular calcitonin-producing C-cells (Pearse and Carvalheira 1967; Le Lievre and Le Douarin 1975; Fontaine 1979). According to current understanding, these two lineages are derived from the thyroid primordium, which arises from an outpocketing of the floor of the pharynx, and the ultimobranchial bodies (UBBs), which arise from caudal lateral outpocketings of the fourth pharyngeal pouches (Biddinger and Ray 1993;

Manley and Capecchi 1998; Di Lauro and De Felice 2001). The UBB are populated by neural crest-derived C-cell precursors and subsequently incorporated into the right and left thyroid lobes (Pearse and Carvalheira 1967; Moseley, Matthews et al. 1968; Pearse and Polak 1971; Le Lievre and Le Douarin 1975; Williams, Toyn et al. 1989). The C-cells, which comprise only 0.1% (by mass) of the epithelial portion of the human thyroid gland (Biddinger and Ray 1993), are the major producers of the hormone calcitonin.

Medullary thyroid carcinoma (MTC) is a cancer first described at the beginning of the 20th century as "malignant goiter with amyloid" and definitively defined in 1959 as "solid non-follicular carcinoma with co-existing amyloid", although the amyloid deposition is not a necessary feature (Hazard, Hawk et al. 1959; Gimm and Dralle 1999). It arises from the parafollicular C-cells (calcitonin cells) and accounts for between 5–10% of thyroid carcinomas (Williams 1966; Al-Rawi and Wheeler 2006). As this tumor is derived from C-cells, those seeking identification and treatment for MTC are provided at least one invaluable immunohistochemical and biochemical marker, calcitonin. Additionally, TTF1 is reported to be strongly expressed in MTCs, while paragangliomas do not show expression (Agoff, Lamps et al. 2000).

Medullary thyroid carcinoma is a good example of the sort of disorder that makes it so critical for scientists to more thoroughly understand the genetic network that functions during the normal and neoplastic states of cells and organs. While the current treatment modality for this and many other pathologies is surgical resection of

tissues, it is more than possible that understanding the disease would allow new treatments to be attempted in order to augment chances of patient survival and hopefully to someday obviate the need for drastic surgical intervention and, in the cases of other cancers, the use of agents damaging to healthy tissues.

1.6: Known human craniofacial defects

Syndromes with known genotypic basis

Craniofacial defects are one of the most common birth defects and affect roughly 10% of live births; generally somewhere between 1:500 and 1:1000 babies. Commonly, this may manifest as a cleft lip or palate or defects in dentition. There are three kinds of cleft palate: incomplete, complete and bilateral. These can occur with or without a cleft lip, and children who are born with this condition cannot create suction and will have trouble trying to suckle and get enough milk to thrive. Invariably, the cells that are affected in these difficulties are the neural crest cells. Craniofacial defects can also include serious malformations of the skull including missing or drastically reduced bones. This is critically important, as the frontal bones are necessary to protect the brain. Since these disorders are so numerous and widespread, there are many distinct named syndromes as well as several different gene mutations available for study.

Apert syndrome is an autosomal dominant mutation named for the French neurologist who first described it in 1906. A brachysphenocephalic type of acrocephaly

characterizes the skull malformations seen in patients in addition to a highly arched and sometimes cleft palate, a hypoplastic midface, and defects in the growth of the orbital bones. The underlying mutation was discovered to be fibroblast growth factor receptor type 2 (FGFR2) in 1995 by the group of Andrew Wilkie (Wilkie, Slaney et al. 1995). Similar defects in cartilage and bone development are seen in the $FGFR2^{+/S252W}$ mouse model (Wang, Xiao et al. 2005). Mutations in FGFR2 and FGFR3 have also been implicated in Crouzon syndrome (acanthosis nigricans is also a feature apparent with the FGFR3 mutation). Crouzon craniofacial dysostosis is an autosomal dominant disorder with craniosynostosis, hypoplastic orbital and facial bones and highly arched palate (Reardon, Winter et al. 1994; Glaser, Jiang et al. 2000). Eswarakumar et al. generated an animal model of Crouzon syndrome in 2006 using a dominant mutation in the mesenchymal splice form of Fgfr2 (Eswarakumar, Ozcan et al. 2006). One further syndrome associated with mutations in FGFR2 is Pfeiffer syndrome (Lajeunie, Ma et al. 1995; Rutland, Pulleyn et al. 1995; Schell, Hehr et al. 1995) and Muenke reported in 1996 that this was the mutation in the family originally described by Pfeiffer in 1964 (Pfeiffer 1964). Mutations in FGFR1 have also been found in familial Pfeiffer syndrome (Muenke, Schell et al. 1994). There are three types of Pfeiffer syndrome, with types 2 and 3 being more severe. Patients with Pfeiffer syndrome display craniostenosis and craniofacial dysplasia, including midfacial hypoplasia (Muenke, Schell et al. 1994; Lajeunie, Ma et al. 1995; Rutland, Pulleyn et al. 1995; Schell, Hehr et al. 1995).

Human TFAP2A mutants have branchiooculofacial syndrome (BOFS) with bilateral branchial cleft sinuses, congenital strabismus, obstructed nasolacrimal ducts, broad nasal bridge, protruding upper lip, and carp mouth. Sometimes there are colomba, micropthalmia, auricular pits, lip pits, highly arched palate, and dental anomalies (Lee, Root et al. 1982; Hall, deLorimier et al. 1983; Fujimoto, Lipson et al. 1987; Milunsky, Maher et al. 2008). AP2 and AP2-alpha knockout mice have severe craniofacial defects such as severe dysmorphogenesis of the face, skull, sensory organs and cranial ganglia (Schorle, Meier et al. 1996; Zhang, Hagopian-Donaldson et al. 1996). Another TFAP family member, TFAP2B, has a role in facial development. TFAP2B mutations result in Char syndrome, in which typical facial features include a flat midface, wide-set eyes, a flat nasal bridge and a nasal tip that is both flat and broad, thickened and everted ("duck-bill") lips and a triangular mouth (Char 1978; Satoda, Zhao et al. 2000). Attempts to study the neural crest cell defects in mouse models of TFAP2B have been unsuccessful as the knockout mice die soon after birth due to polycystic kidney disease (Moser, Pscherer et al. 1997).

Those who suffer from Pierre Robin syndrome display craniofacial defects like high, narrow palates, fused mandibles, and micrognathia (Murray, Oram et al. 2007). It has been associated with deletion 2q32.3-q33.2 (Houdayer, Portnoi et al. 2001). In mice, a neural-crest specific deletion of snail1 in a snail2 knockout background has been found to resemble this syndrome, and while the defects are thought to result from problems in neural crest cells, they are not found to occur in the initial delamination or migration stages of their development (Murray, Oram et al. 2007). Knockouts of

Snail2 are also found in Waardenburg-Shah syndrome type 2 and non-Kit related piebaldism (heterozygous) (Waardenburg 1951; Read and Newton 1997; Sanchez-Martin, Rodriguez-Garcia et al. 2002; Spritz, Chiang et al. 2003). Waardenburg syndrome type 2 has also been associated with MITF in some families. Two other Waardenburg-Shah syndromes, types 1 and 3, have mutations in the Pax3 gene (Waardenburg 1951; Read and Newton 1997; Spritz, Chiang et al. 2003). Waardenburg syndrome has a number of common features including pigment defects, hearing impairments, and cranial dysmorphisms like synophrys, telecanthus, broad and high nasal roots, and lower lacrimal dystopia (Pardono, van Bever et al. 2003).

DiGeorge syndrome (DGS, also sometimes known as the DGA or DiGeorge anomaly) results in many cases from a deletion in the DGCR, or DiGeorge syndrome chromosome region, on chromosome 22 (Strong 1968; Wilson, Cross et al. 1991). Several genes including zinc finger transcription factor ZNF74 are known to be in this region (Aubry, Demczuk et al. 1993). The phenotype seen in patients includes cleft lip and is the result of cervical neural crest cell migration into the pharyngeal arches and pouches. Based on its presence among the genes in the DGCR, a transcription factor of the T-box family, Tbx1, was investigated by Jerome and Papaioannou in 2001. Mice homozygous null for this gene showed many mutations including abnormal facial structures (known as conotruncal anomaly face) and cleft palate (Jerome and Papaioannou 2001; Baldini 2002; Yagi, Furutani et al. 2003). Subsequent analysis of patients with this syndrome showed that mutations in Tbx1 could result in this syndrome (Yagi, Furutani et al. 2003). A mouse knockout of

HoxA3 also produces a recessive phenocopy of DGS, but mutations in the human gene have not as yet been associated with this syndrome (Chisaka and Capecchi 1991).

TGFBR1 mutations have been described in LDS (Loeys-Dietz syndrome) type 1A and 2A along with non-FBN1-related Marfan syndrome while TGFBR2 mutations are associated with LDS type 1B and 2B along with non-FBN1-related Marfan syndrome. LDS types 1 and 2 are clinically indistinguishable based on symptoms, which are characterized by a triptych of arterial tortuosity and aneurysms, hypertelorism, and bifid uvula or cleft palate. LDS type 1 has craniofacial involvement such as craniosynostosis or cleft palate, while type 2 has no history of these traits (Loeys, Chen et al. 2005; Loeys, Schwarze et al. 2006). Marfan syndrome shows striking clinical variability but often displays a highly arched palate with crowded teeth (McKusick, Traisman et al. 1972; Pyeritz, Murphy et al. 1979). TGFBR2 conditional mouse mutants made with a Wnt-cre line show severe skull defects, missing frontal and severely retarded parietal bones, cleft palate, and reduced mandible and maxilla bones (Sponseller, Hobbs et al. 1995; Ito, Yeo et al. 2003; Loeys, Chen et al. 2005). TGFBR2 has been shown to be a direct target of EWS-FLI1 (Hahm, Cho et al. 1999), a translocation known to be commonly involved with another neurocristopathy called Ewing's sarcoma (Giovannini, Biegel et al. 1994). Paris-Trousseau type Thrombocytopenia (TCPT) is in many cases the result of a homozygous deletion of Fli1, one of the two genes that form this translocation, in humans. In addition to thrombocytopenia, the characteristics of TCPT include mental retardation and facial

dysmorphism among other characteristic neural crest defects (Favier, Jondeau et al. 2003).

Mutations in the human Treacle gene result in Treacher Collins syndrome (TCS; also called Treacher Collins-Franceschetti or TCOF), a disorder presenting with severe neural crest abnormalities, including micrognathia, microtia, and other ear deformities, hypoplastic zygomatic arches and macrostomia as well as hearing loss and cleft palate (Dixon 1996). Mouse mutants haploinsufficient for *Tcof1* have similar craniofacial defects including reduced head size, frontonasal dysplasia, poorly formed nasal passages, and cleft palate resulting from the failure of palatal shelves to fuse. Studies on these mice have shown that the gene product is required for ribosome biogenesis necessary for NC cell proliferation (Dixon, Jones et al. 2006; Dixon, Trainor et al. 2007). Treacle mutations or loss of mRNA and accompanying craniofacial defects have also been seen in additional animals such as Resus monkeys (Macaca mulatta) and dogs (Haworth, Islam et al. 2001; Shows, Ward et al. 2006). Nager acrofacial dystosis, another syndrome, resembles TCS in that it often displays with a high nasal bridge, micrognathia, external ear defects, cleft palate, cleft lip, and an absent soft palate. This syndrome in distinguishable in that it always presents with limb defects (McDonald and Gorski 1993). Saethre-Chotzen syndrome also displays limb deformities, but results from a mutation in the human gene Twist. The symptoms of Saethre-Chotzen syndrome also include craniosyntosis and dysmorphic facial features including facial asymmetry (el Ghouzzi, Le Merrer et al. 1997; Howard, Paznekas et al. 1997). In mice, the knockout is embryonic lethal and

displays branchial arch malformations (Chen and Behringer 1995). Therefore, a conditional mouse mutant was created in which the typical craniofacial defects can be seen (Chen, Akinwunmi et al. 2007).

Human FoxD3 deficiencies present as autosomal dominant vitiligo and as a regulator of melanoblast differentiation [323]. Zebrafish FoxD3 mutant *mother superior* has a protruding lower jaw, loss of cartilage in the hyoid and posterior branchial arches, as well as pigment defects and a defective peripheral nervous system (Montero-Balaguer, Lang et al. 2006). Complete FoxD3 mouse knockout embryos fail early in embryogenesis, around the time of implantation. Tissue specific knockout of FoxD3 in the neural crest cells results in perinatal death with catastrophic loss of craniofacial structures and other neural crest derivatives (Teng, Mundell et al. 2008).

SIP1 (Zeb2, ZFHX1B, SMADIP1) mutations result in Mowat-Wilson syndrome, a disorder including Hirschprung symptoms as well as microcephaly, mental retardation, hypertelorism, short stature and submucosal cleft palate (Mowat, Croaker et al. 1998; Wakamatsu, Yamada et al. 2001; Zweier, Albrecht et al. 2002; Ishihara, Yamada et al. 2004). In the mouse, it has been shown that the similar defects that are seen in mutants of this gene result from an early arrest in cranial neural crest cell migration (Van de Putte, Francis et al. 2007).

Bardet-Biedl syndrome (BBS) patient have subtly flattened and hypoplastic midfacial bones resulting from aberrant NCC migration. Twelve genes have been discovered to

have involvement in this syndrome and are known as BBS1–12. Both zebrafish and mouse mutant models have been examined and show similar defects to the human condition (Tobin, Di Franco et al. 2008).

Sox 9 mutation in humans results in campomelic dysplasia, a mutation that includes craniofacial defects such as a cleft palate, micrognathia, and a flat face (Houston, Opitz et al. 1983; Wagner, Wirth et al. 1994; Kwok, Weller et al. 1995). The zebrafish mutant of Sox9a is the *jellyfish* mutant and lacks cartilaginous elements of the neurocranium and branchial arches (Yan, Miller et al. 2002).

Syndromes with no known genotypic basis:

Hemifacial Microsomia (HFM), also known as Goldenhar syndrome or oculoauriculovertebral dysplasia (OAVS), is a defect involving first and second branchial arch derivatives (Nijhawan, Morad et al. 2002). It has been hypothesized that maternal diabetes can interfere with neural crest cell migration as this syndrome has increased prevalence among the children of diabetic mothers (Wang, Martinez-Frias et al. 2002). Thus far, there have been no genotype-phenotype correlations among human patients with this syndrome, though different genes have been investigated (Kelberman, Tyson et al. 2001; Splendore, Passos-Bueno et al. 2002). A transgenic mouse line with the HFM phenotype has been isolated and described by Naora et al. They designated the locus of the autosomal dominant insertion HFM and mapped to mouse chromosome 10 (Naora, Kimura et al. 1994). Another syndrome

with no known genetic origin, Hallerman-Streiff syndrome, displays natal teeth and other dental abnormalities as well as a characteristic “bird like” face and dyscephalia with a small mouth and a highly arched palate (Nicholson and Menon 1995).

1.6: Goal of this thesis

This brief survery demonstrates the importance of the neural crest derivatives in adult organisms, and the fascinating properties of the developing neural crest as a model to study induction, migration, and determination of cellular fate. My thesis project specifically sought to expand the genes known to be involved in the induction and migration of neural crest cells, in order to refine our current understanding and illuminate mechanisms of normal development, while pointing out candidate protagonists in various human disorders. To do this, we took a two-pronged approach: to screen for involved molecules *in vitro* and then to test their functional relevance in neural crest cells *in vivo*. We have discussed the importance of this cell type as well as some of the normal and pathological situations it is involved in.

In Chapter 2, we first show the results of the screen performed to identify new genes involved in neural crest induction and migration. This screen takes advantage of a culturing technique in which cDNA derived from individually cultured pieces of non-neural ectoderm and intermediate neural plate are subtracted from cDNA expressed by co-cultures. This technique allows for the enrichment of gene transcripts that are whose expression levels are modulated concurrent with neural crest induction and

development. Subsequently we discuss examinations of these genes by in situ hybridization as well as selected transcription factors by QPCR. Performing these experiments has allowed us to increase the number of genes likely involved in neural crest development by more than 100, and also to further define the *in vitro* culture assay system used in our screen. These results will allow us to more fully define the current neural crest genetic network.

In Chapter 3, we examine a selected subset of the genes uncovered by the screen discussed in Chapter 2 for their involvement in neural crest cell development. To do this we study the effects of perturbations of these gene products through the use of morpholino oligos and overexpression vectors, performing classic loss-of-function and gain-of-function analysis of these genes.

We have demonstrated here that Ccar1 mRNA is present in the both the dorsal neural folds and in the migratory neural crest cells in early chicken embryos (Figures 1 and 2). It presence in migrating neural crest cells was then further confirmed by overlay with HNK-1 on a section of a Ccar1 in situ. We also quantitated the expression level of Ccar1 in non-neural ectoderm and intermediate neural plate isolates and conjugates as described above. Ccar1 showed a significant difference between these two populations with an average conjugate value of 0.777, standard deviation 0.104 and an average non-conjugate value of 0.464, standard deviation 0.149. The student t-test value is significant, with $p=0.0405$ (Chapter 3, Figure 2). Upon perturbation using morpholino oligos, we discovered that loss of the Ccar1 protein causes a decrease in

Sox10 mRNA on the injected side, and that there is an increase in death in the dorsal neural folds of these embryos (Chapter 3, Figure 3).

The transcript of the second gene that we examined, Adh5, is also present in the dorsal neural folds and the migrating neural crest, and in early chicken embryos does not appear to be present at significant levels in other tissues. When injected with a morpholino against the 5'UTR of the Adh5 gene, chicken embryos show a marked reduction in the mRNA (by in situ hybridization) of three transcription factors known as neural crest specification: *Sox10, FoxD3,* and *Snail2*. These results are statistically significant, at $p<0.0001$, $p=0.0086$, and $p=0.0065$, respectively.

Thus, we have been able to not only increase the numbers of genes known to be involved in neural crest cell development, but we have also been able to confirm the functional relevancy of two of these genes.

Table I. Disorders relating to neural crest genes/EMT

Summary of genes that cause neural crest defects when mutated. Increasing numbers of studies are probing the ways in which many genes involved in EMT have a dual nature with importance in both early embryonic development and cancer. Mutations within these genes can lead to uncontrolled EMTs within the developing or adult organism resulting in tumor development (Ansieau, Bastid et al. 2008) and metastasis. This can cause spreading of the cancer to multiple sites within the body, which may lead to decreased chances of survival. The expression of several genes involved in EMT, such as Snail1 and Snail2, has been reported in human tumors (Peinado, Olmeda et al. 2007).

Since the neural crest population is highly proliferative, migratory, and multipotent, it is not surprising to see the catastrophic effects of neural crest cell derived tumors, such as neuroblastomas and medulloblastomas. As neural crest cells contribute to a wide range of cell types, mutations in genes that specify the neural crest or its sublineages may lead to serious or even lethal consequences. Null mutations often lead to failure to survive past early developmental stages. Accordingly, conditional knockouts in mice have been employed to elucidate the role of these genes in later development. Occasionally, gene knockouts have mild effects that may be due to gene redundancy or plasticity within the neural crest population.

AP2 (TFAP2A) (human)	Branchiooculofacial syndrome (BOFS)	Low birth weight, retarded postnatal growth, bilateral branchial cleft sinuses, congenital strabismus, obstructed nasolacrimal ducts, broad nasal bridge, protruding upper lip, and carp mouth. Occasionally seen are coloboma, microphthalmia, auricular pits, lip pits, highly arched palate, dental anomalies, and subcutaneous cysts of the scalp	(Lee, Root et al. 1982)
AP2 (mouse)	AP2-/- AP2 alpha -/-	Die perinatally with cranioabdominoschisis and severe dysmorphogenesis of the face, skull, sensory organs, and cranial ganglia; Neural tube defects followed by craniofacial and body wall abnormalities	(Schorle, Meier et al. 1996) (Zhang, Hagopian-Donaldson et al. 1996)
TFAP2B	Char syndrome	Flat midface, wide-set eyes, flat nasal bridge, broad and flat nasal tip, thickened and everted "duck-bill" lips, triangular mouth	(Lee, Root et al. 1982)
FGFR1 (human)	Familial Pfeiffer syndrome	Craniostenosis and craniofacial dysplasia, including midfacial hypoplasia	[257-261]
FGFR2 (human)	Apert syndrome Crouzon syndrome Pfeiffer syndrome	Autosomal dominant; Characterized by brachiosphenocephalic skull defects; highly arched, sometimes cleft palate; hypoplastic midface; orbital bone defects. Autosomal dominant; craniosynostosis; hypoplastic orbital and facial bones; highly arched palate	[252] [254, 255] [257-260]
FGFR2 (mouse)	$FGFR2^{+/S252W}$ Dominant mutation in mesenchymal splice form	Similar to human Apert syndrome with defects in cartilage and bone development Similar to human Crouzon syndrome	[253] [256]
FGFR3	Crouzon syndrome	Crouzon syndrome with acanthosis nigricans	[254, 255]

Fli1 (human)	Paris-Trousseau type Thrombocytopenia (TCPT): Homozygous deletion of Fli1.	Mental retardation, facial dysmorphism, clinodactyly, pyloric stenosis, and thrombocytopenia	(Favier, Jondeau et al. 2003)
FoxD3 (human)		Autosomal dominant vitiligo; regulator of melanoblast differentiation	[323]
FoxD3 (mouse)		Embryonic lethality at E6.5, required for maintenance of epiblast cells and expansion	[324]
FoxD3 (zebrafish)	*mother superior* mutation	Depletion of crest derivatives, leading to a protruding lower jaw, loss of cartilage in hyoid and posterior branchial arches, pigmentation defects particularly in the trunk iridophores and reduced number of neural precursors resulting in defective peripheral nervous system.	(Montero-Balaguer, Lang et al. 2006)
Mi200 family		Regulates EMT/MET by targeting ZEB1 and ZEB2, which control E-cadherin expression	(Park, Gaur et al. 2008)
Pax3 (human)	Waardenburg-Shah syndrome 1 and 3 Tumors	Dystopia canthorum and type 3 also includes contractures or hypoplasia of upper-limb joints and muscles Rhabdomyosarcoma, tumors of NCC origin including melanoma and neuroblastoma	(Waardenburg 1951)
SIP1 (Zeb2, ZFHX1B, SMADIP1) (human)	Mowat-Wilson syndrome	Hirschprung associated with microcephaly, mental retardation, hypertelorism, submucous cleft palate, short stature	(Wakamatsu, Yamada et al. 2001)
SIP1 (Zeb2, ZFHX1B, SMADIP1) (mouse)		Early arrest in cranial neural crest migration	(Van de Putte, Francis et al. 2007)
Snail1/Snail2 (mouse)	Double knockout resembles Pierre Robin Sequence	Micrognathia, fused mandible, enlarged parietal foramen in skull vault; defects occur in later development (not in initial delamination and migration)	(Murray, Oram et al. 2007)
Snail2 (human)	Waardenburg-Shah syndrome 2 Piebaldism (non-Kit related)	Defects in melanocytes, also seen with Mitf mutation Congenital white forelock, scattered normal pigmented and hyperpigmented macules, and a triangular shaped depigmented patch on forehead. Caused by a heterozygous deletion of SNAI2 gene.	(Waardenburg 1951) (Sanchez-Martin, Rodriguez-Garcia et al. 2002)

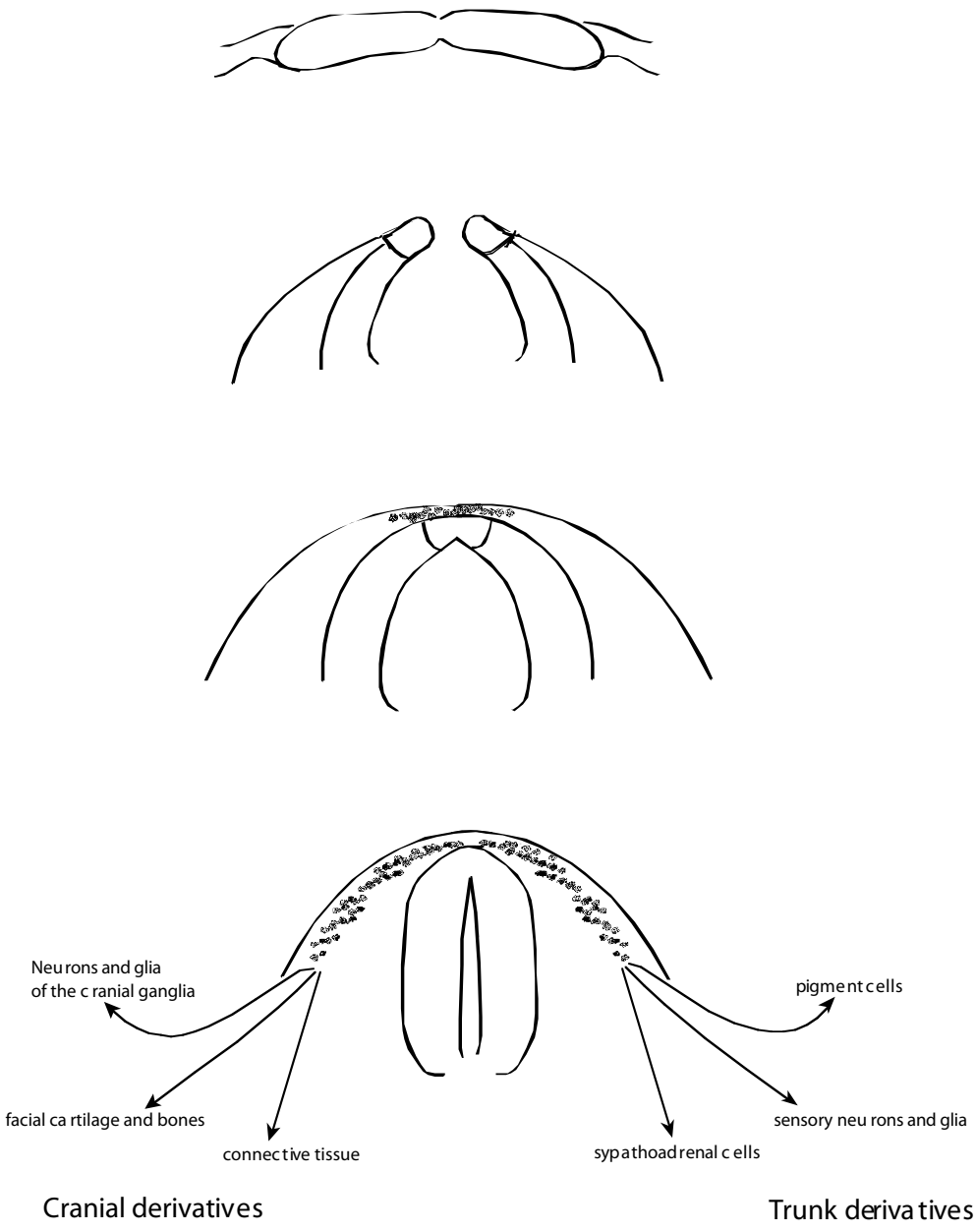
Snail2 (chicken)		Cooperates with ets-1 to recruit cells and initiate EMT	(Theveneau, Duband et al. 2007)
Sox8 (mouse)		No effect as a single mutation but increases the severity of *Sox10* heterozygote; may be jointly required with *Sox10* for maintenance of vagal neural crest cells.	(Maka, Stolt et al. 2005)
Sox9 (human)	Campomelic dysplasia	Congenital skeletal malformation syndrome, includes cleft palate, low set ears, loss of the 12th pair of ribs, abnormal pelvic bones, small chest and hip dislocations; may also include absence of the olfactory bulbs, renal and/or cardiac defects and XY sex reversal with genital malformations. Also is frequently associated with conductive and sensorineural hearing loss.	(Wagner, Wirth et al. 1994; Kwok, Weller et al. 1995)
Sox9 conditional (mouse)	*Sox9$^{flox/flox}$;Foxg1^{Cre}* Cre activity driven in the prospective otic ectoderm	Sox9 controls adhesive properties of placode cells and regulates *Epha4* expression; otic placodes are normally specified but invagination is impaired.	(Barrionuevo, Naumann et al. 2008)
Sox9a (zebrafish)	*Jellyfish* mutation	Lack cartilage elements of the neocranium, branchial arches and pectoral girdle; cartilage morphogenesis also disrupted.	(Yan, Miller et al. 2002)
Sox10 haploinsufficiency (human)	Hirschsprung's disease (HSCR) Waardenburg-Shah syndrome 4	Failure of enteric ganglia to populate the distal colon. Deafness/pigmentation defect in addition to HSCR; also seen in EDN3 and EDNRB mutants.	(Badner, Sieber et al. 1990; Herbarth, Pingault et al. 1998; Pingault, Bondurand et al. 1998) (Herbarth, Pingault et al. 1998)

Sox10-/- (mouse, *Xenopus*, zebrafish)	Colorless (D. rerio)	Loss of melanocytes, autonomic and enteric neurons and peripheral glia. Crest cells form but fail to differentiate as these lineages and die, can result in embryonic lethality.	(Southard-Smith, Kos et al. 1998; Britsch, Goerich et al. 2001; Dutton, Pauliny et al. 2001; Paratore, Goerich et al. 2001; Honore, Aybar et al. 2003)
Tbx1 (human)	Di George syndrome	Cardiac outflow tract anomalies, absence or hypoplasia of the thymus and parathyroid glands, nasal voice (often associated with cleft palate or submucosal cleft palate), and facial dysmorphism, known as conotruncal anomaly face.	(Yagi, Furutani et al. 2003)
TGFBR1 (human)	LDS1A LDS2A Non-FBN1 related Marfan syndrome	LDS1 and 2 are clinically indistinguishable autosomal dominant aortic aneurysm syndromes characterized by the triad of arterial tortuosity and aneurysms, hypertelorism, and bifid uvula or cleft palate; Type 1 category has craniofacial involvement consisting of cleft palate, craniosynostosis, or hypertelorism was observed. Patients assigned to the type 2 category had no evidence of these traits, but some had an bifid uvula. Marfan syndrome shows striking pleiotropism and clinical variability. The cardinal features occur in 3 systems: skeletal, ocular, and cardiovascular; a highly arched palate with crowding of the teeth are frequent skeletal features	(Loeys, Chen et al. 2005; Loeys, Schwarze et al. 2006) (McKusick, Traisman et al. 1972; Pyeritz, Murphy et al. 1979; Sponseller, Hobbs et al. 1995)
TGFBR2 (human)	Loeys-Dietz syndrome (LDS) type 1B and 2B Non-FBN1 related Marfan syndrome		

TGFBR2 (mouse)	TGFBR2 fl/fl; Wnt1-cre	Severe skull defects, missing frontal and severely retarded parietal bone, cleft palate, reduced mandible and maxilla; skull size is 25% smaller than littermates	(Ito, Yeo et al. 2003; Loeys, Chen et al. 2005)
Treacle (human)	Treacher-Collins syndrome	Antimongoloid slant of the eyes, coloboma of the lid, micrognathia, microtia and other ear deformities, hypoplastic zygomatic arches and macrostomia. Conductive hearing loss and cleft palate often apparent	(Dixon 1996)
Treacle (mouse)	*Tcof*+/- haploinsufficient embryos generated	Reduced head size, frontonasal dysplasia, failure of palatal shelves to fuse resulting in cleft palate, poorly formed nasal passages, die within 24h of birth from respiratory arrest due to cranioskeletal malformations. Required for ribosome biogenesis necessary for crest cell proliferation.	(Dixon, Jones et al. 2006)
Treacle (Macaca mulatta)	Resus Tcof1 homolog is 93.8% identical in terms of protein	Infant macaque discovered displaying Treacher-Collins phenotype. No mutation found in TCOF1 coding or splice sites, but 87% reduction of spleen TCOF1 mRNA	(Shows, Ward et al. 2006)
Treacle (dog)		Genetic analysis showed mapping to this region of mutation leading to brachycephaly in domestic dog breeds	(Haworth, Islam et al. 2001)
Twist (human)	Saethre-Chotzen syndrome	Craniosyntosis, limb deformities and dysmorphic facial features including facial asymmetry.	(el Ghouzzi, Le Merrer et al. 1997; Howard, Paznekas et al. 1997)
Twist (mouse) Twist conditional (mouse)	*Twist1-/-*	Embryonic lethal at E11.5, open cranial neural tube, retarded forelimb bud development and branchial arch malformations. Heterozygotes have craniofacial defects and polydactyly.	(Chen and Behringer 1995) (Chen, Akinwunmi et al. 2007)

Figure I. Schematic of Neural Crest Development

Representation of the early embryo (A) as well as the subsequent steps of neural crest induction (B), early migration (C), and later migration with some delineated derivatives (D). Figure adapted from Knecht et al 2002.



Adams, M. S., L. S. Gammill, et al. (2008). "Discovery of transcription factors and other candidate regulators of neural crest development." Dev Dyn **237**(4): 1021-33.

Agoff, S. N., L. W. Lamps, et al. (2000). "Thyroid transcription factor-1 is expressed in extrapulmonary small cell carcinomas but not in other extrapulmonary neuroendocrine tumors." Mod Pathol **13**(3): 238-42.

Ahmed, S. and A. Nawshad (2007). "Complexity in interpretation of embryonic epithelial-mesenchymal transition in response to transforming growth factor-beta signaling." Cells Tissues Organs **185**(1-3): 131-45.

Akitaya, T. and M. Bronner-Fraser (1992). "Expression of cell adhesion molecules during initiation and cessation of neural crest cell migration." Dev Dyn **194**(1): 12-20.

Al-Rawi, M. and M. H. Wheeler (2006). "Medullary thyroid carcinoma--update and present management controversies." Ann R Coll Surg Engl **88**(5): 433-8.

Alexander, C. M. and Z. Werb (1992). "Targeted disruption of the tissue inhibitor of metalloproteinases gene increases the invasive behavior of primitive mesenchymal cells derived from embryonic stem cells in vitro." J Cell Biol **118**(3): 727-39.

Anderson, D. J. (1993). "Molecular control of cell fate in the neural crest: the sympathoadrenal lineage." Annu Rev Neurosci **16**: 129-58.

Ansieau, S., J. Bastid, et al. (2008). "Induction of EMT by twist proteins as a collateral effect of tumor-promoting inactivation of premature senescence." Cancer Cell **14**(1): 79-89.

Aubry, M., S. Demczuk, et al. (1993). "Isolation of a zinc finger gene consistently deleted in DiGeorge syndrome." Hum Mol Genet **2**(10): 1583-7.

Badner, J. A., W. K. Sieber, et al. (1990). "A genetic study of Hirschsprung disease." Am J Hum Genet **46**(3): 568-80.

Baker, C. V. and M. Bronner-Fraser (1997). "The origins of the neural crest. Part I: embryonic induction." Mech Dev **69**(1-2): 3-11.

Baldini, A. (2002). "DiGeorge syndrome: the use of model organisms to dissect complex genetics." Hum Mol Genet **11**(20): 2363-9.

Baroffio, A., E. Dupin, et al. (1988). "Clone-forming ability and differentiation potential of migratory neural crest cells." Proc Natl Acad Sci U S A **85**(14): 5325-9.

Baroffio, A., E. Dupin, et al. (1991). "Common precursors for neural and mesectodermal derivatives in the cephalic neural crest." Development **112**(1): 301-5.

Barrionuevo, F., A. Naumann, et al. (2008). "Sox9 is required for invagination of the otic placode in mice." Dev Biol **317**(1): 213-24.

Basch, M. L., M. Bronner-Fraser, et al. (2006). "Specification of the neural crest occurs during gastrulation and requires Pax7." Nature **441**(7090): 218-22.

Batlle, E., E. Sancho, et al. (2000). "The transcription factor snail is a repressor of E-cadherin gene expression in epithelial tumour cells." Nat Cell Biol **2**(2): 84-9.

Biddinger, P. W. and M. Ray (1993). "Distribution of C cells in the normal and diseased thyroid gland." Pathol Annu **28 Pt 1**: 205-29.

Bolos, V., H. Peinado, et al. (2003). "The transcription factor Slug represses E-cadherin expression and induces epithelial to mesenchymal transitions: a comparison with Snail and E47 repressors." J Cell Sci **116**(Pt 3): 499-511.

Boyer, A. S., Ayerinskas, II, et al. (1999). "TGFbeta2 and TGFbeta3 have separate and sequential activities during epithelial-mesenchymal cell transformation in the embryonic heart." Dev Biol **208**(2): 530-45.

Britsch, S., D. E. Goerich, et al. (2001). "The transcription factor Sox10 is a key regulator of peripheral glial development." Genes Dev **15**(1): 66-78.

Bronner-Fraser, M. (1986). "Analysis of the early stages of trunk neural crest migration in avian embryos using monoclonal antibody HNK-1." Dev Biol **115**(1): 44-55.

Bronner-Fraser, M. and S. Fraser (1989). "Developmental potential of avian trunk neural crest cells in situ." Neuron **3**(6): 755-66.

Bronner-Fraser, M. and S. E. Fraser (1988). "Cell lineage analysis reveals multipotency of some avian neural crest cells." Nature **335**(6186): 161-4.

Bronner-Fraser, M., M. Sieber-Blum, et al. (1980). "Clonal analysis of the avian neural crest: migration and maturation of mixed neural crest clones injected into host chicken embryos." J Comp Neurol **193**(2): 423-34.

Brown, C. B., A. S. Boyer, et al. (1996). "Antibodies to the Type II TGFbeta receptor block cell activation and migration during atrioventricular cushion transformation in the heart." Dev Biol **174**(2): 248-57.

Brown, C. B., A. S. Boyer, et al. (1999). "Requirement of type III TGF-beta receptor for endocardial cell transformation in the heart." Science **283**(5410): 2080-2.

Bryant, J., J. Farmer, et al. (2003). "Pheochromocytoma: the expanding genetic differential diagnosis." J Natl Cancer Inst **95**(16): 1196-204.

Burstyn-Cohen, T., J. Stanleigh, et al. (2004). "Canonical Wnt activity regulates trunk neural crest delamination linking BMP/noggin signaling with G1/S transition." Development **131**(21): 5327-39.

Bussolati, G. and A. G. Pearse (1967). "Immunofluorescent localization of calcitonin in the 'C' cells of pig and dog thyroid." J Endocrinol **37**(2): 205-9.

Cano, A., M. A. Perez-Moreno, et al. (2000). "The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression." Nat Cell Biol **2**(2): 76-83.

Cebra-Thomas, J. A., E. Betters, et al. (2007). "Evidence that a late-emerging population of trunk neural crest cells forms the plastron bones in the turtle Trachemys scripta." Evol Dev **9**(3): 267-77.

Char, F. (1978). "Peculiar facies with short philtrum, duck-bill lips, ptosis and low-set ears--a new syndrome?" Birth Defects Orig Artic Ser **14**(6B): 303-5.

Chen, Y. T., P. O. Akinwunmi, et al. (2007). "Generation of a Twist1 conditional null allele in the mouse." Genesis **45**(9): 588-92.

Chen, Z. F. and R. R. Behringer (1995). "twist is required in head mesenchyme for cranial neural tube morphogenesis." Genes Dev **9**(6): 686-99.

Chibon, P. (1967). "[Nuclear labelling by tritiated thymidine of neural crest derivatives in the amphibian Urodele Pleurodeles waltlii Michah]." J Embryol Exp Morphol **18**(3): 343-58.

Chisaka, O. and M. R. Capecchi (1991). "Regionally restricted developmental defects resulting from targeted disruption of the mouse homeobox gene hox-1.5." Nature **350**(6318): 473-9.

Chou, D. K., M. Schachner, et al. (2002). "HNK-1 sulfotransferase null mice express glucuronyl glycoconjugates and show normal cerebellar granule neuron migration in vivo and in vitro." J Neurochem **82**(5): 1239-51.

Clark, K., G. Bender, et al. (2001). "Evidence for the neural crest origin of turtle plastron bones." Genesis **31**(3): 111-7.

Coles, E., J. Christiansen, et al. (2004). "A vertebrate crossveinless 2 homologue modulates BMP activity and neural crest cell migration." Development **131**(21): 5309-17.

Coles, E. G., L. A. Taneyhill, et al. (2007). "A critical role for Cadherin6B in regulating avian neural crest emigration." Dev Biol **312**(2): 533-44.

Comijn, J., G. Berx, et al. (2001). "The two-handed E box binding zinc finger protein SIP1 downregulates E-cadherin and induces invasion." Mol Cell **7**(6): 1267-78.

Correia, A. C., M. Costa, et al. (2007). "Bmp2 is required for migration but not for induction of neural crest cells in the mouse." Dev Dyn **236**(9): 2493-501.

Couly, G. F., P. M. Coltey, et al. (1992). "The developmental fate of the cephalic mesoderm in quail-chick chimeras." Development **114**(1): 1-15.

Couly, G. F., P. M. Coltey, et al. (1993). "The triple origin of skull in higher vertebrates: a study in quail-chick chimeras." Development **117**(2): 409-29.

Couto, S. S. and R. D. Cardiff (2008). "The genomic revolution and endocrine pathology." Endocr Pathol **19**(3): 139-47.

de Larco, J. E. and G. J. Todaro (1978). "Growth factors from murine sarcoma virus-transformed cells." Proc Natl Acad Sci U S A **75**(8): 4001-5.

DeLellis, R., Lloyd, RV, Heitz, PU, Eng, C. , Ed. (2004). Tumours of Endocrine Organs. Pathology and Genetics. Lyon, World Health Organization IARC Press.

Di Lauro, R. and M. De Felice (2001). Thyroid gland: anatomy and development. Philadelphia, Saunders.

Dickinson, M. E., M. A. Selleck, et al. (1995). "Dorsalization of the neural tube by the non-neural ectoderm." Development **121**(7): 2099-106.

Dixon, J., N. C. Jones, et al. (2006). "Tcof1/Treacle is required for neural crest cell formation and proliferation deficiencies that cause craniofacial abnormalities." Proc Natl Acad Sci U S A **103**(36): 13403-8.

Dixon, J., P. Trainor, et al. (2007). "Treacher Collins syndrome." Orthod Craniofac Res **10**(2): 88-95.

Dixon, M. J. (1996). "Treacher Collins syndrome." Hum Mol Genet **5 Spec No**: 1391-6.

Duband, J. L. and J. P. Thiery (1987). "Distribution of laminin and collagens during avian neural crest development." Development **101**(3): 461-78.

Duband, J. L., T. Volberg, et al. (1988). "Spatial and temporal distribution of the adherens-junction-associated adhesion molecule A-CAM during avian embryogenesis." Development **103**(2): 325-44.

Duong, T. D. and C. A. Erickson (2004). "MMP-2 plays an essential role in producing epithelial-mesenchymal transformations in the avian embryo." Dev Dyn **229**(1): 42-53.

Dutton, K. A., A. Pauliny, et al. (2001). "Zebrafish colourless encodes sox10 and specifies non-ectomesenchymal neural crest fates." Development **128**(21): 4113-25.

Edelman, G. M., W. J. Gallin, et al. (1983). "Early epochal maps of two different cell adhesion molecules." Proc Natl Acad Sci U S A **80**(14): 4384-8.

Eisenhofer, G., S. R. Bornstein, et al. (2004). "Malignant pheochromocytoma: current status and initiatives for future progress." Endocr Relat Cancer **11**(3): 423-36.

Eisenhofer, G., T. T. Huynh, et al. (2004). "Distinct gene expression profiles in norepinephrine- and epinephrine-producing hereditary and sporadic pheochromocytomas: activation of hypoxia-driven angiogenic pathways in von Hippel-Lindau syndrome." Endocr Relat Cancer **11**(4): 897-911.

el Ghouzzi, V., M. Le Merrer, et al. (1997). "Mutations of the TWIST gene in the Saethre-Chotzen syndrome." Nat Genet **15**(1): 42-6.

Endo, Y., N. Osumi, et al. (2002). "Bimodal functions of Notch-mediated signaling are involved in neural crest formation during avian ectoderm development." Development **129**(4): 863-73.

Endo, Y., N. Osumi, et al. (2003). "Deltex/Dtx mediates NOTCH signaling in regulation of Bmp4 expression in cranial neural crest formation during avian development." Dev Growth Differ **45**(3): 241-8.

Erickson, C. A., J. F. Loring, et al. (1989). "Migratory pathways of HNK-1-immunoreactive neural crest cells in the rat embryo." Dev Biol **134**(1): 112-8.

Erickson, C. A. and R. Perris (1993). "The role of cell-cell and cell-matrix interactions in the morphogenesis of the neural crest." Dev Biol **159**(1): 60-74.

Eswarakumar, V. P., F. Ozcan, et al. (2006). "Attenuation of signaling pathways stimulated by pathologically activated FGF-receptor 2 mutants prevents craniosynostosis." Proc Natl Acad Sci U S A **103**(49): 18603-8.

Favier, R., K. Jondeau, et al. (2003). "Paris-Trousseau syndrome : clinical, hematological, molecular data of ten new cases." Thromb Haemost **90**(5): 893-7.

Fontaine, J. (1979). "Multistep migration of calcitonin cell precursors during ontogeny of the mouse pharynx." Gen Comp Endocrinol **37**(1): 81-92.

Freitas, R., G. Zhang, et al. (2006). "Developmental origin of shark electrosensory organs." Evol Dev **8**(1): 74-80.

Fujimoto, A., M. Lipson, et al. (1987). "New autosomal dominant branchio-oculo-facial syndrome." Am J Med Genet **27**(4): 943-51.

Funahashi, J., T. Okafuji, et al. (1999). "Role of Pax-5 in the regulation of a mid-hindbrain organizer's activity." Dev Growth Differ **41**(1): 59-72.

Gammill, L. S. and M. Bronner-Fraser (2002). "Genomic analysis of neural crest induction." Development **129**(24): 5731-41.

Gans, C. and R. G. Northcutt (1983). "Neural Crest and the Origin of Vertebrates: A New Head." Science **220**(4594): 268-273.

Garcia-Castro, M. I., C. Marcelle, et al. (2002). "Ectodermal Wnt function as a neural crest inducer." Science **297**(5582): 848-51.

Giehl, K. and A. Menke (2008). "Microenvironmental regulation of E-cadherin-mediated adherens junctions." Front Biosci **13**: 3975-85.

Gimm, O. and H. Dralle (1999). "C-cell cancer--prevention and treatment." Langenbecks Arch Surg **384**(1): 16-23.

Giovannini, M., J. A. Biegel, et al. (1994). "EWS-erg and EWS-Fli1 fusion transcripts in Ewing's sarcoma and primitive neuroectodermal tumors with variant translocations." J Clin Invest **94**(2): 489-96.

Glaser, R. L., W. Jiang, et al. (2000). "Paternal origin of FGFR2 mutations in sporadic cases of Crouzon syndrome and Pfeiffer syndrome." Am J Hum Genet **66**(3): 768-77.

Graff, J. R., J. G. Herman, et al. (1995). "E-cadherin expression is silenced by DNA hypermethylation in human breast and prostate carcinomas." Cancer Res **55**(22): 5195-9.

Graveson, A. C., M. M. Smith, et al. (1997). "Neural crest potential for tooth development in a urodele amphibian: developmental and evolutionary significance." Dev Biol **188**(1): 34-42.

Gross, J. B. and J. Hanken (2008). "Review of fate-mapping studies of osteogenic cranial neural crest in vertebrates." Dev Biol **317**(2): 389-400.

Gumbiner, B. M. (2005). "Regulation of cadherin-mediated adhesion in morphogenesis." Nat Rev Mol Cell Biol **6**(8): 622-34.

Hadeball, B., A. Borchers, et al. (1998). "Xenopus cadherin-11 (Xcadherin-11) expression requires the Wg/Wnt signal." Mech Dev **72**(1-2): 101-13.

Hahm, K. B., K. Cho, et al. (1999). "Repression of the gene encoding the TGF-beta type II receptor is a major target of the EWS-FLI1 oncoprotein." Nat Genet **23**(2): 222-7.

Hajra, K. M. and E. R. Fearon (2002). "Cadherin and catenin alterations in human cancer." Genes Chromosomes Cancer **34**(3): 255-68.

Hall, B. D., A. deLorimier, et al. (1983). "Brief clinical report: a new syndrome of hemangiomatous branchial clefts, lip pseudoclefts, and unusual facial appearance." Am J Med Genet **14**(1): 135-8.

Hall, B. K. (1999). The neural crest in development and evolution. New York, Springer-Verlag.

Haworth, K. E., I. Islam, et al. (2001). "Canine TCOF1; cloning, chromosome assignment and genetic analysis in dogs with different head types." Mamm Genome **12**(8): 622-9.

Hay, E. D. (1968). Organization and fine structure of epithelium and mesenchyme in the developing chick embryo. Epithelial–Mesenchymal Interactions. R. a. B. Fleischmajer, R.E. . Baltimore, MD, USA, Williams & Wilkins Co**:** 31–55.

Hazard, J. B., W. A. Hawk, et al. (1959). "Medullary (solid) carcinoma of the thyroid; a clinicopathologic entity." J Clin Endocrinol Metab **19**(1): 152-61.

Heanue, T. A. and V. Pachnis (2007). "Enteric nervous system development and Hirschsprung's disease: advances in genetic and stem cell studies." Nat Rev Neurosci **8**(6): 466-79.

Herbarth, B., V. Pingault, et al. (1998). "Mutation of the Sry-related Sox10 gene in Dominant megacolon, a mouse model for human Hirschsprung disease." Proc Natl Acad Sci U S A **95**(9): 5161-5.

Hilfer, S. R. (1968). Cellular interactions in the genesis and maintenance of thyroid characteristics. Baltimore, Williams and Wilkins Co.

His, W. (1868). ""Die erste Entwicklung des Hühnchens im Ei: Untersuchungen über die erste Anlage des Wirbelthierleibes"."

Hoffmann, I. and R. Balling (1995). "Cloning and expression analysis of a novel mesodermally expressed cadherin." Dev Biol **169**(1): 337-46.

Honore, S. M., M. J. Aybar, et al. (2003). "Sox10 is required for the early development of the prospective neural crest in Xenopus embryos." Dev Biol **260**(1): 79-96.

Hörstadius, S. (1950). The Neural Crest: its Properties and Derivatives in the Light of Experimental Research. London, Oxford University Press.

Hou, L. (1999). "Effects of local tissue environment on the differentiation of neural crest cells in turtle, with special reference to understanding the spatial distribution of pigment cells." Pigment Cell Res **12**(2): 81-8.

Houdayer, C., M. F. Portnoi, et al. (2001). "Pierre Robin sequence and interstitial deletion 2q32.3-q33.2." Am J Med Genet **102**(3): 219-26.

Houston, C. S., J. M. Opitz, et al. (1983). "The campomelic syndrome: review, report of 17 cases, and follow-up on the currently 17-year-old boy first reported by Maroteaux et al in 1971." Am J Med Genet **15**(1): 3-28.

Howard, T. D., W. A. Paznekas, et al. (1997). "Mutations in TWIST, a basic helix-loop-helix transcription factor, in Saethre-Chotzen syndrome." Nat Genet **15**(1): 36-41.

Hu, Y. F., Z. J. Zhang, et al. (2006). "An epidermal neural crest stem cell (EPI-NCSC) molecular signature." Stem Cells **24**(12): 2692-702.

Huynh, T. T., K. Pacak, et al. (2006). "Transcriptional regulation of phenylethanolamine N-methyltransferase in pheochromocytomas from patients with von Hippel-Lindau syndrome and multiple endocrine neoplasia type 2." Ann N Y Acad Sci **1073**: 241-52.

Ikenouchi, J., M. Matsuda, et al. (2003). "Regulation of tight junctions during the epithelium-mesenchyme transition: direct repression of the gene expression of claudins/occludin by Snail." J Cell Sci **116**(Pt 10): 1959-67.

Ishihara, N., K. Yamada, et al. (2004). "Clinical and molecular analysis of Mowat-Wilson syndrome associated with ZFHX1B mutations and deletions at 2q22-q24.1." J Med Genet **41**(5): 387-93.

Ito, K. and M. Sieber-Blum (1991). "In vitro clonal analysis of quail cardiac neural crest development." Dev Biol **148**(1): 95-106.

Ito, Y., J. Y. Yeo, et al. (2003). "Conditional inactivation of Tgfbr2 in cranial neural crest causes cleft palate and calvaria defects." Development **130**(21): 5269-80.

Jacks, T., T. S. Shih, et al. (1994). "Tumour predisposition in mice heterozygous for a targeted mutation in Nf1." Nat Genet **7**(3): 353-61.

Jeffery, W. R., A. G. Strickler, et al. (2004). "Migratory neural crest-like cells form body pigmentation in a urochordate embryo." Nature **431**(7009): 696-9.

Jerome, L. A. and V. E. Papaioannou (2001). "DiGeorge syndrome phenotype in mice mutant for the T-box gene, Tbx1." Nat Genet **27**(3): 286-91.

Jiang, X., B. Choudhary, et al. (2002). "Normal fate and altered function of the cardiac neural crest cell lineage in retinoic acid receptor mutant embryos." Mech Dev **117**(1-2): 115-22.

Joseph, N. M., J. T. Mosher, et al. (2008). "The loss of Nf1 transiently promotes self-renewal but not tumorigenesis by neural crest stem cells." Cancer Cell **13**(2): 129-40.

Karafiat, V., M. Dvorakova, et al. (2005). "Transcription factor c-Myb is involved in the regulation of the epithelial-mesenchymal transition in the avian neural crest." Cell Mol Life Sci **62**(21): 2516-25.

Katahira, T. and H. Nakamura (2003). "Gene silencing in chick embryos with a vector-based small interfering RNA system." Dev Growth Differ **45**(4): 361-7.

Kelberman, D., J. Tyson, et al. (2001). "Hemifacial microsomia: progress in understanding the genetic basis of a complex malformation syndrome." Hum Genet **109**(6): 638-45.

Kimura, Y., H. Matsunami, et al. (1995). "Cadherin-11 expressed in association with mesenchymal morphogenesis in the head, somite, and limb bud of early mouse embryos." Dev Biol **169**(1): 347-58.

Knecht, A. K. and M. Bronner-Fraser (2002). "Induction of the neural crest: a multigene process." Nat Rev Genet **3**(6): 453-61.

Koch, C. A., D. Mauro, et al. (2002). "Pheochromocytoma in von hippel-lindau disease: distinct histopathologic phenotype compared to pheochromocytoma in multiple endocrine neoplasia type 2." Endocr Pathol **13**(1): 17-27.

Kuratani, S. (2008). "Evolutionary developmental studies of cyclostomes and the origin of the vertebrate neck." Dev Growth Differ **50 Suppl 1**: S189-94.

Kwok, C., P. A. Weller, et al. (1995). "Mutations in SOX9, the gene responsible for Campomelic dysplasia and autosomal sex reversal." Am J Hum Genet **57**(5): 1028-36.

LaBonne, C. and M. Bronner-Fraser (1998). "Neural crest induction in Xenopus: evidence for a two-signal model." Development **125**(13): 2403-14.

Lajeunie, E., H. W. Ma, et al. (1995). "FGFR2 mutations in Pfeiffer syndrome." Nat Genet **9**(2): 108.

Landacre, F. L. (1921). "The fate of the neural crest in the head of urodeles." Journal of Comparative Neurology(33): 1-44.

Le Douarin, N., J. Fontaine, et al. (1974). "New studies on the neural crest origin of the avian ultimobranchial glandular cells--interspecific combinations and cytochemical characterization of C cells based on the uptake of biogenic amine precursors." Histochemistry **38**(4): 297-305.

Le Douarin, N. M., S. Creuzet, et al. (2004). "Neural crest cell plasticity and its limits." Development **131**(19): 4637-50.

Le Douarin, N. M. and M. A. Teillet (1974). "Experimental analysis of the migration and differentiation of neuroblasts of the autonomic nervous system and of

neurectodermal mesenchymal derivatives, using a biological cell marking technique." Dev Biol **41**(1): 162-84.

Le Dourarin, N. M. and C. Kalcheim (1999). The Neural Crest. Cambridge, UK, Cambridge University Press.

Le Lievre, C. S. and N. M. Le Douarin (1975). "Mesenchymal derivatives of the neural crest: analysis of chimaeric quail and chick embryos." J Embryol Exp Morphol **34**(1): 125-54.

Lee, G., H. Kim, et al. (2007). "Isolation and directed differentiation of neural crest stem cells derived from human embryonic stem cells." Nat Biotechnol **25**(12): 1468-75.

Lee, S., E. Nakamura, et al. (2005). "Neuronal apoptosis linked to EglN3 prolyl hydroxylase and familial pheochromocytoma genes: developmental culling and cancer." Cancer Cell **8**(2): 155-67.

Lee, W. K., A. W. Root, et al. (1982). "Bilateral branchial cleft sinuses associated with intrauterine and postnatal growth retardation, premature aging, and unusual facial appearance: a new syndrome with dominant transmission." Am J Med Genet **11**(3): 345-52.

Levayer, R. and T. Lecuit (2008). "Breaking down EMT." Nat Cell Biol **10**(7): 757-9.

Liem, K. F., Jr., G. Tremml, et al. (1995). "Dorsal differentiation of neural plate cells induced by BMP-mediated signals from epidermal ectoderm." Cell **82**(6): 969-79.

Linnoila, R. I., H. R. Keiser, et al. (1990). "Histopathology of benign versus malignant sympathoadrenal paragangliomas: clinicopathologic study of 120 cases including unusual histologic features." Hum Pathol **21**(11): 1168-80.

Lloyd, R. V., J. C. Sisson, et al. (1986). "Immunohistochemical localization of epinephrine, norepinephrine, catecholamine-synthesizing enzymes, and chromogranin in neuroendocrine cells and tumors." Am J Pathol **125**(1): 45-54.

Loeys, B. L., J. Chen, et al. (2005). "A syndrome of altered cardiovascular, craniofacial, neurocognitive and skeletal development caused by mutations in TGFBR1 or TGFBR2." Nat Genet **37**(3): 275-81.

Loeys, B. L., U. Schwarze, et al. (2006). "Aneurysm syndromes caused by mutations in the TGF-beta receptor." N Engl J Med **355**(8): 788-98.

Lumsden, A. G. (1988). "Spatial organization of the epithelium and the role of neural crest cells in the initiation of the mammalian tooth germ." Development **103 Suppl**: 155-69.

Machado, J. C., C. Oliveira, et al. (2001). "E-cadherin gene (CDH1) promoter methylation as the second hit in sporadic diffuse gastric carcinoma." Oncogene **20**(12): 1525-8.

Maher, E. R. and C. Eng (2002). "The pressure rises: update on the genetics of phaeochromocytoma." Hum Mol Genet **11**(20): 2347-54.

Maka, M., C. C. Stolt, et al. (2005). "Identification of Sox8 as a modifier gene in a mouse model of Hirschsprung disease reveals underlying molecular defect." Dev Biol **277**(1): 155-69.

Mancilla, A. and R. Mayor (1996). "Neural crest formation in Xenopus laevis: mechanisms of Xslug induction." Dev Biol **177**(2): 580-9.

Manley, N. R. and M. R. Capecchi (1998). "Hox group 3 paralogs regulate the development and migration of the thymus, thyroid, and parathyroid glands." Dev Biol **195**(1): 1-15.

Martins-Green, M. and C. A. Erickson (1987). "Basal lamina is not a barrier to neural crest cell emigration: documentation by TEM and by immunofluorescent and immunogold labelling." Development **101**(3): 517-33.

Massague, J. (1990). "The transforming growth factor-beta family." Annu Rev Cell Biol **6**: 597-641.

McDonald, M. T. and J. L. Gorski (1993). "Nager acrofacial dysostosis." J Med Genet **30**(9): 779-82.

McKusick, V. A., H. S. Traisman, et al. (1972). "More speculation on Marfan syndrome." J Pediatr **80**(3): 530-1.

Meulemans, D. and M. Bronner-Fraser (2004). "Gene-regulatory interactions in neural crest evolution and development." Dev Cell **7**(3): 291-9.

Meulemans, D. and M. Bronner-Fraser (2005). "Central role of gene cooption in neural crest evolution." J Exp Zoolog B Mol Dev Evol **304**(4): 298-303.

Milunsky, J. M., T. A. Maher, et al. (2008). "TFAP2A mutations result in branchio-oculo-facial syndrome." Am J Hum Genet **82**(5): 1171-7.

Montero-Balaguer, M., M. R. Lang, et al. (2006). "The mother superior mutation ablates foxd3 activity in neural crest progenitor cells and depletes neural crest derivatives in zebrafish." Dev Dyn **235**(12): 3199-212.

Moreno-Bueno, G., E. Cubillo, et al. (2006). "Genetic profiling of epithelial cells expressing E-cadherin repressors reveals a distinct role for Snail, Slug, and E47 factors in epithelial-mesenchymal transition." Cancer Res **66**(19): 9543-56.

Morrison, S. J., P. M. White, et al. (1999). "Prospective identification, isolation by flow cytometry, and in vivo self-renewal of multipotent mammalian neural crest stem cells." Cell **96**(5): 737-49.

Moseley, J. M., E. W. Matthews, et al. (1968). "The ultimobranchial origin of calcitonin." Lancet **1**(7534): 108-10.

Moser, M., A. Pscherer, et al. (1997). "Enhanced apoptotic cell death of renal epithelial cells in mice lacking transcription factor AP-2beta." Genes Dev **11**(15): 1938-48.

Moury, J. D. and A. G. Jacobson (1989). "Neural fold formation at newly created boundaries between neural plate and epidermis in the axolotl." Dev Biol **133**(1): 44-57.

Moury, J. D. and A. G. Jacobson (1990). "The origins of neural crest cells in the axolotl." Dev Biol **141**(2): 243-53.

Mowat, D. R., G. D. Croaker, et al. (1998). "Hirschsprung disease, microcephaly, mental retardation, and characteristic facial features: delineation of a new syndrome and identification of a locus at chromosome 2q22-q23." J Med Genet **35**(8): 617-23.

Muenke, M., U. Schell, et al. (1994). "A common mutation in the fibroblast growth factor receptor 1 gene in Pfeiffer syndrome." Nat Genet **8**(3): 269-74.

Muller, T., A. Choidas, et al. (1999). "Phosphorylation and free pool of beta-catenin are regulated by tyrosine kinases and tyrosine phosphatases during epithelial cell migration." J Biol Chem **274**(15): 10173-83.

Murray, S. A., K. F. Oram, et al. (2007). "Multiple functions of Snail family genes during palate development in mice." Development **134**(9): 1789-97.

Nagoshi, N., S. Shibata, et al. (2008). "Ontogeny and multipotency of neural crest-derived stem cells in mouse bone marrow, dorsal root ganglia, and whisker pad." Cell Stem Cell **2**(4): 392-403.

Nakagawa, S. and M. Takeichi (1995). "Neural crest cell-cell adhesion controlled by sequential and subpopulation-specific expression of novel cadherins." Development **121**(5): 1321-32.

Nakagawa, S. and M. Takeichi (1998). "Neural crest emigration from the neural tube depends on regulated cadherin expression." Development **125**(15): 2963-71.

Nakajima, Y., E. L. Krug, et al. (1994). "Myocardial regulation of transforming growth factor-beta expression by outflow tract endothelium in the early embryonic chick heart." Dev Biol **165**(2): 615-26.

Nakamura, E. and W. G. Kaelin, Jr. (2006). "Recent insights into the molecular pathogenesis of pheochromocytoma and paraganglioma." Endocr Pathol **17**(2): 97-106.

Nakamura, H., Y. Watanabe, et al. (2000). "Misexpression of genes in brain vesicles by in ovo electroporation." Dev Growth Differ **42**(3): 199-201.

Naora, H., M. Kimura, et al. (1994). "Transgenic mouse model of hemifacial microsomia: cloning and characterization of insertional mutation region on chromosome 10." Genomics **23**(3): 515-9.

Newgreen, D. and I. Gibbins (1982). "Factors controlling the time of onset of the migration of neural crest cells in the fowl embryo." Cell Tissue Res **224**(1): 145-60.

Newgreen, D. F. and D. Gooday (1985). "Control of the onset of migration of neural crest cells in avian embryos. Role of Ca++-dependent cell adhesions." Cell Tissue Res **239**(2): 329-36.

Nichols, D. H. (1981). "Neural crest formation in the head of the mouse embryo as observed using a new histological technique." J Embryol Exp Morphol **64**: 105-20.

Nichols, D. H. (1987). "Ultrastructure of neural crest formation in the midbrain/rostral hindbrain and preotic hindbrain regions of the mouse embryo." Am J Anat **179**(2): 143-54.

Nicholson, A. D. and S. Menon (1995). "Hallerman-Streiff syndrome." J Postgrad Med **41**(1): 22-3.

Nijhawan, N., Y. Morad, et al. (2002). "Caruncle abnormalities in the oculo-auriculo-vertebral spectrum." Am J Med Genet **113**(4): 320-5.

Noden, D. M. (1991). "Cell movements and control of patterned tissue assembly during craniofacial development." J Craniofac Genet Dev Biol **11**(4): 192-213.

Palacios, F., J. S. Tushir, et al. (2005). "Lysosomal targeting of E-cadherin: a unique mechanism for the down-regulation of cell-cell adhesion during epithelial to mesenchymal transitions." Mol Cell Biol **25**(1): 389-402.

Paratore, C., D. E. Goerich, et al. (2001). "Survival and glial fate acquisition of neural crest cells are regulated by an interplay between the transcription factor Sox10 and extrinsic combinatorial signaling." Development **128**(20): 3949-61.

Pardono, E., Y. van Bever, et al. (2003). "Waardenburg syndrome: clinical differentiation between types I and II." Am J Med Genet A **117A**(3): 223-35.

Park, S. M., A. B. Gaur, et al. (2008). "The miR-200 family determines the epithelial phenotype of cancer cells by targeting the E-cadherin repressors ZEB1 and ZEB2." Genes Dev **22**(7): 894-907.

Parsons, S. L., S. A. Watson, et al. (1997). "Matrix metalloproteinases." Br J Surg **84**(2): 160-6.

Pearse, A. G. (1966). "The cytochemistry of the thyroid C cells and their relationship to calcitonin." Proc R Soc Lond B Biol Sci **164**(996): 478-87.

Pearse, A. G. and A. F. Carvalheira (1967). "Cytochemical evidence for an ultimobranchial origin of rodent thyroid C cells." Nature **214**(5091): 929-30.

Pearse, A. G. and J. M. Polak (1971). "Cytochemical evidence for the neural crest origin of mammalian ultimobranchial C cells." Histochemie **27**(2): 96-102.

Peinado, H., D. Olmeda, et al. (2007). "Snail, Zeb and bHLH factors in tumour progression: an alliance against the epithelial phenotype?" Nat Rev Cancer **7**(6): 415-28.

Perez-Moreno, M. A., A. Locascio, et al. (2001). "A new role for E12/E47 in the repression of E-cadherin expression and epithelial-mesenchymal transitions." J Biol Chem **276**(29): 27424-31.

Perris, R. and D. Perissinotto (2000). "Role of the extracellular matrix during neural crest cell migration." Mech Dev **95**(1-2): 3-21.

Pfeiffer, R. A. (1964). "[Dominant Hereditary Acrocephalosyndactylia.]." Z Kinderheilkd **90**: 301-20.

Pingault, V., N. Bondurand, et al. (1998). "SOX10 mutations in patients with Waardenburg-Hirschsprung disease." Nat Genet **18**(2): 171-3.

Polak, J. M., A. G. Pearse, et al. (1974). "Immunocytochemical confirmation of the neural crest origin of avian calcitonin-producing cells." Histochemistry **40**(3): 209-14.

Potts, J. D., J. M. Dagle, et al. (1991). "Epithelial-mesenchymal transformation of embryonic cardiac endothelial cells is inhibited by a modified antisense oligodeoxynucleotide to transforming growth factor beta 3." Proc Natl Acad Sci U S A **88**(4): 1516-20.

Powers, J. F., M. J. Evinger, et al. (2007). "Pheochromocytomas in Nf1 knockout mice express a neural progenitor gene expression profile." Neuroscience **147**(4): 928-37.

Pyeritz, R. E., E. A. Murphy, et al. (1979). "Clinical variability in the Marfan syndrome(s)." Birth Defects Orig Artic Ser **15**(5B): 155-78.

Raible, D. W. (2006). "Development of the neural crest: achieving specificity in regulatory pathways." Curr Opin Cell Biol **18**(6): 698-703.

Read, A. P. and V. E. Newton (1997). "Waardenburg syndrome." J Med Genet **34**(8): 656-65.

Reardon, W., R. M. Winter, et al. (1994). "Mutations in the fibroblast growth factor receptor 2 gene cause Crouzon syndrome." Nat Genet **8**(1): 98-103.

Reilly, K. M. and T. Van Dyke (2008). "It takes a (dysfunctional) village to raise a tumor." Cell **135**(3): 408-10.

Rickmann, M., J. W. Fawcett, et al. (1985). "The migration of neural crest cells and the growth of motor axons through the rostral half of the chick somite." J Embryol Exp Morphol **90**: 437-55.

Robbins, J. R., P. G. McGuire, et al. (1999). "Diminished matrix metalloproteinase 2 (MMP-2) in ectomesenchyme-derived tissues of the Patch mutant mouse: regulation of MMP-2 by PDGF and effects on mesenchymal cell migration." Dev Biol **212**(2): 255-63.

Rollhauser-ter Horst, J. (1979). "Artificial neural crest formation in amphibia." Anat Embryol (Berl) **157**(1): 113-20.

Rollhauser-ter Horst, J. (1980). "Neural crest replaced by gastrula ectoderm in amphibia. Effect on neurulation, CNS, gills and limbs." Anat Embryol (Berl) **160**(2): 203-11.

Rutland, P., L. J. Pulleyn, et al. (1995). "Identical mutations in the FGFR2 gene cause both Pfeiffer and Crouzon syndrome phenotypes." Nat Genet **9**(2): 173-6.

Sadaghiani, B. and J. R. Vielkind (1990). "Distribution and migration pathways of HNK-1-immunoreactive neural crest cells in teleost fish embryos." Development **110**(1): 197-209.

Sakai, D., T. Suzuki, et al. (2006). "Cooperative action of Sox9, Snail2 and PKA signaling in early neural crest development." Development **133**(7): 1323-33.

Sanchez-Martin, M., A. Rodriguez-Garcia, et al. (2002). "SLUG (SNAI2) deletions in patients with Waardenburg disease." Hum Mol Genet **11**(25): 3231-6.

Satoda, M., F. Zhao, et al. (2000). "Mutations in TFAP2B cause Char syndrome, a familial form of patent ductus arteriosus." Nat Genet **25**(1): 42-6.

Sauka-Spengler, T. and M. Barembaum (2008). "Gain- and loss-of-function approaches in the chick embryo." Methods Cell Biol **87**: 237-56.

Sauka-Spengler, T. and M. Bronner-Fraser (2006). "Development and evolution of the migratory neural crest: a gene regulatory perspective." Curr Opin Genet Dev **16**(4): 360-6.

Sauka-Spengler, T. and M. Bronner-Fraser (2008). "A gene regulatory network orchestrates neural crest formation." Nat Rev Mol Cell Biol **9**(7): 557-68.

Sauka-Spengler, T. and M. Bronner-Fraser (2008). "Insights from a sea lamprey into the evolution of neural crest gene regulatory network." Biol Bull **214**(3): 303-14.

Sauka-Spengler, T., D. Meulemans, et al. (2007). "Ancient evolutionary origin of the neural crest gene regulatory network." Dev Cell **13**(3): 405-20.

Schell, U., A. Hehr, et al. (1995). "Mutations in FGFR1 and FGFR2 cause familial and sporadic Pfeiffer syndrome." Hum Mol Genet **4**(3): 323-8.

Schorle, H., P. Meier, et al. (1996). "Transcription factor AP-2 essential for cranial closure and craniofacial development." Nature **381**(6579): 235-8.

Sela-Donenfeld, D. and C. Kalcheim (1999). "Regulation of the onset of neural crest migration by coordinated activity of BMP4 and Noggin in the dorsal neural tube." Development **126**(21): 4749-62.

Sela-Donenfeld, D. and C. Kalcheim (2000). "Inhibition of noggin expression in the dorsal neural tube by somitogenesis: a mechanism for coordinating the timing of neural crest emigration." Development **127**(22): 4845-54.

Selleck, M. A. and M. Bronner-Fraser (1995). "Origins of the avian neural crest: the role of neural plate-epidermal interactions." Development **121**(2): 525-38.

Selleck, M. A., M. I. Garcia-Castro, et al. (1998). "Effects of Shh and Noggin on neural crest formation demonstrate that BMP is required in the neural tube but not ectoderm." Development **125**(24): 4919-30.

Shows, K. H., C. Ward, et al. (2006). "Reduced TCOF1 mRNA level in a rhesus macaque with Treacher Collins-like syndrome: further evidence for haploinsufficiency of treacle as the cause of disease." Mamm Genome **17**(2): 168-77.

Sieber-Blum, M. (1989). "Commitment of neural crest cells to the sensory neuron lineage." Science **243**(4898): 1608-11.

Sieber-Blum, M. and A. M. Cohen (1980). "Clonal analysis of quail neural crest cells: they are pluripotent and differentiate in vitro in the absence of noncrest cells." Dev Biol **80**(1): 96-106.

Sieber-Blum, M., M. Grim, et al. (2004). "Pluripotent neural crest stem cells in the adult hair follicle." Dev Dyn **231**(2): 258-69.

Sieber-Blum, M., L. Schnell, et al. (2006). "Characterization of epidermal neural crest stem cell (EPI-NCSC) grafts in the lesioned spinal cord." Mol Cell Neurosci **32**(1-2): 67-81.

Smith, M. M. and B. K. Hall (1990). "Development and evolutionary origins of vertebrate skeletogenic and odontogenic tissues." Biol Rev Camb Philos Soc **65**(3): 277-373.

Smith, S. H., R. G. Murray, et al. (1994). "The surface structure of Leptotrichia buccalis." Can J Microbiol **40**(2): 90-8.

Song, Y. S., H. J. Lee, et al. (2008). "Human neural crest stem cells transplanted in rat penile corpus cavernosum to repair erectile dysfunction." BJU Int **102**(2): 220-4; discussion 224.

Southard-Smith, E. M., L. Kos, et al. (1998). "Sox10 mutation disrupts neural crest development in Dom Hirschsprung mouse model." Nat Genet **18**(1): 60-4.

Splendore, A., M. R. Passos-Bueno, et al. (2002). "TCOF1 mutations excluded from a role in other first and second branchial arch-related disorders." Am J Med Genet **111**(3): 324-7.

Sponseller, P. D., W. Hobbs, et al. (1995). "The thoracolumbar spine in Marfan syndrome." J Bone Joint Surg Am **77**(6): 867-76.

Spritz, R. A., P. W. Chiang, et al. (2003). "Human and mouse disorders of pigmentation." Curr Opin Genet Dev **13**(3): 284-9.

Stemple, D. L. and D. J. Anderson (1992). "Isolation of a stem cell for neurons and glia from the mammalian neural crest." Cell **71**(6): 973-85.

Strong, W. B. (1968). "Familial syndrome of right-sided aortic arch, mental deficiency, and facial dysmorphism." J Pediatr **73**(6): 882-8.

Taneyhill, L. A., E. G. Coles, et al. (2007). "Snail2 directly represses cadherin6B during epithelial-to-mesenchymal transitions of the neural crest." Development **134**(8): 1481-90.

Teng, L., N. A. Mundell, et al. (2008). "Requirement for Foxd3 in the maintenance of neural crest progenitors." Development **135**(9): 1615-24.

Theveneau, E., J. L. Duband, et al. (2007). "Ets-1 confers cranial features on neural crest delamination." PLoS ONE **2**(11): e1142.

Thiery, J. P. and J. P. Sleeman (2006). "Complex networks orchestrate epithelial-mesenchymal transitions." Nat Rev Mol Cell Biol **7**(2): 131-42.

Tischler, A. S. (2008). "Pheochromocytoma and extra-adrenal paraganglioma: updates." Arch Pathol Lab Med **132**(8): 1272-84.

Tischler, A. S., N. Kimura, et al. (2006). "Pathology of pheochromocytoma and extra-adrenal paraganglioma." Ann N Y Acad Sci **1073**: 557-70.

Tischler, A. S., T. S. Shih, et al. (1995). "Characterization of Pheochromocytomas in a Mouse Strain with a Targeted Disruptive Mutation of the Neurofibromatosis Gene Nf1." Endocr Pathol **6**(4): 323-335.

Tobin, J. L., M. Di Franco, et al. (2008). "Inhibition of neural crest migration underlies craniofacial dysmorphology and Hirschsprung's disease in Bardet-Biedl syndrome." Proc Natl Acad Sci U S A **105**(18): 6714-9.

Trentin, A., C. Glavieux-Pardanaud, et al. (2004). "Self-renewal capacity is a widespread property of various types of neural crest precursor cells." Proc Natl Acad Sci U S A **101**(13): 4495-500.

Tucker, R. P. and C. A. Erickson (1984). "Morphology and behavior of quail neural crest cells in artificial three-dimensional extracellular matrices." Dev Biol **104**(2): 390-405.

Unsicker, K. (1993). "The chromaffin cell: paradigm in cell, developmental and growth factor biology." J Anat **183 (Pt 2)**: 207-21.

Unsicker, K., K. Huber, et al. (2005). "The chromaffin cell and its development." Neurochem Res **30**(6-7): 921-5.

Van de Putte, T., A. Francis, et al. (2007). "Neural crest-specific removal of Zfhx1b in mouse leads to a wide range of neurocristopathies reminiscent of Mowat-Wilson syndrome." Hum Mol Genet **16**(12): 1423-36.

Van de Putte, T., M. Maruhashi, et al. (2003). "Mice lacking ZFHX1B, the gene that codes for Smad-interacting protein-1, reveal a role for multiple neural crest cell defects in the etiology of Hirschsprung disease-mental retardation syndrome." Am J Hum Genet **72**(2): 465-70.

Waardenburg, P. J. (1951). "A new syndrome combining developmental anomalies of the eyelids, eyebrows and nose root with pigmentary defects of the iris and head hair and with congenital deafness." Am J Hum Genet **3**(3): 195-253.

Wadham, C., J. R. Gamble, et al. (2003). "The protein tyrosine phosphatase Pez is a major phosphatase of adherens junctions and dephosphorylates beta-catenin." Mol Biol Cell **14**(6): 2520-9.

Wagner, T., J. Wirth, et al. (1994). "Autosomal sex reversal and campomelic dysplasia are caused by mutations in and around the SRY-related gene SOX9." Cell **79**(6): 1111-20.

Wakamatsu, N., Y. Yamada, et al. (2001). "Mutations in SIP1, encoding Smad interacting protein-1, cause a form of Hirschsprung disease." Nat Genet **27**(4): 369-70.

Wang, R., M. L. Martinez-Frias, et al. (2002). "Infants of diabetic mothers are at increased risk for the oculo-auriculo-vertebral sequence: A case-based and case-control approach." J Pediatr **141**(5): 611-7.

Wang, Y., R. Xiao, et al. (2005). "Abnormalities in cartilage and bone development in the Apert syndrome FGFR2(+/S252W) mouse." Development **132**(15): 3537-48.

Weston, J. A. (1963). "A radioautographic analysis of the migration and localization of trunk neural crest cells in the chick." Dev Biol **6**: 279-310.

Wilkie, A. O., S. F. Slaney, et al. (1995). "Apert syndrome results from localized mutations of FGFR2 and is allelic with Crouzon syndrome." Nat Genet **9**(2): 165-72.

Williams, E. D. (1966). "Diarrhoea and thyroid carcinoma." Proc R Soc Med **59**(7): 602-3.

Williams, E. D. (1966). "Histogenesis of medullary carcinoma of the thyroid." J Clin Pathol **19**(2): 114-8.

Williams, E. D., C. E. Toyn, et al. (1989). "The ultimobranchial gland and congenital thyroid abnormalities in man." J Pathol **159**(2): 135-41.

Wilson, D. I., I. E. Cross, et al. (1991). "DiGeorge syndrome with isolated aortic coarctation and isolated ventricular septal defect in three sibs with a 22q11 deletion of maternal origin." Br Heart J **66**(4): 308-12.

Wong, D. L. (2003). "Why is the adrenal adrenergic?" Endocr Pathol **14**(1): 25-36.

Wyatt, L., C. Wadham, et al. (2007). "The protein tyrosine phosphatase Pez regulates TGFbeta, epithelial-mesenchymal transition, and organ development." J Cell Biol **178**(7): 1223-35.

Yagi, H., Y. Furutani, et al. (2003). "Role of TBX1 in human del22q11.2 syndrome." Lancet **362**(9393): 1366-73.

Yan, Y. L., C. T. Miller, et al. (2002). "A zebrafish sox9 gene required for cartilage morphogenesis." Development **129**(21): 5065-79.

Youn, Y. H., J. Feng, et al. (2003). "Neural crest stem cell and cardiac endothelium defects in the TrkC null mouse." Mol Cell Neurosci **24**(1): 160-70.

Zhang, J., S. Hagopian-Donaldson, et al. (1996). "Neural tube, skeletal and body wall defects in mice lacking transcription factor AP-2." Nature **381**(6579): 238-41.

Zito, G., P. Richiusa, et al. (2008). "In vitro identification and characterization of CD133(pos) cancer stem-like cells in anaplastic thyroid carcinoma cell lines." PLoS ONE **3**(10): e3544.

Zweier, C., B. Albrecht, et al. (2002). ""Mowat-Wilson" syndrome with and without Hirschsprung disease is a distinct, recognizable multiple congenital anomalies-mental retardation syndrome caused by mutations in the zinc finger homeo box 1B gene." Am J Med Genet **108**(3): 177-81.

Chapter II: Discovery of transcription factors and other candidate regulators of neural crest development

This chapter has been published as Discovery of transcription factors and other candidate regulators of neural crest development. Adams MS, Gammill LS, Bronner-Fraser M. Dev Dyn. 2008 Apr; 237(4):1021–33.

2.1: Abstract

Neural crest cells migrate long distances and form divergent derivatives in vertebrate embryos. Despite previous efforts to identify genes upregulated in neural crest populations, transcription factors have proved to be elusive due to relatively low expression levels and often transient expression. We screened newly induced neural crest cells for early target genes with the aim of identifying transcriptional regulators and other developmentally important genes. This yielded numerous candidate regulators, including fourteen transcription factors, many of which were not previously associated with neural crest development. Quantitative real-time PCR confirmed upregulation of several transcription factors in newly induced neural crest populations *in vitro*. In a secondary screen by in situ hybridization, we verified the expression of >100 genes in the neural crest. We note that several of the transcription factors and other genes from the screen are expressed in other migratory cell populations and have been implicated in diverse forms of cancer.

2.2: Introduction

The neural crest is a cell population that forms at the border between the neural and non-neural ectoderm of vertebrate embryos. As the neural folds elevate, cells induced to become neural crest cells become incorporated into the dorsal neural tube, and subsequently undergo an epithelial to mesenchymal transition and depart from the future central nervous system. These multipotent precursors then migrate along prescribed and stereotypic pathways to defined and diverse sites in the periphery. Upon completion of migration, they differentiate into a wide variety of derivatives including melanocytes, peripheral neurons and glia, adrenomedullary cells, and much of the bone and cartilage of the face (Knecht and Bronner-Fraser 2002; Le Douarin, Brito et al. 2007). As a population, the neural crest is of widespread interest because of its plasticity, mobility, and contribution to developmental disorders and cancers.

Previous work in a number of species, including mice, chick, frogs and zebrafish, has implicated a set of transcriptional regulators in conferring neural crest identity. This set of genes, termed neural crest specifier genes (Meulemans and Bronner-Fraser 2004), include *Sox9*, *Sox10*, *FoxD3*, *Snail2/Slug*, *c-Myc* and *AP2* (Spokony, Aoki et al. 2002; Honore, Aybar et al. 2003; Sasai, Mizuseki et al. 2001; Aoki, Saint-Germain et al. 2003; Luo, Lee et al. 2003; Bellmeyer, Krase et al. 2003). These transcription factors are expressed in premigratory and/or early migrating neural crest cells and thought to be involved in induction and survival of the premigratory population, their epithelial to mesenchymal transition from the neural tube, migratory

ability, and cell lineage decisions (Anderson 1994; Cheung, Chaboissier et al. 2005). However, it seems unlikely that these complicated processes would be controlled by the relatively few transcription factors identified to date in newly formed neural crest cells.

In a genomic analysis previously performed in our lab, a large number of genes were identified as upregulated in premigratory and migratory neural crest (Gammill and Bronner-Fraser 2002). However, this yielded only two transcription factors that had not been previously associated with the neural crest. Because transcription factors are essential for understanding proximal portions of the gene cascades that regulate neural crest migration and differentiation, it is critical to identify the complete repertoire of genes in order to build testable gene regulatory networks (Davidson 2006; Ben-Tabou de-Leon and Davidson 2007). Data from various organisms have led to the formulation of putative gene regulatory networks in different organisms (Mayor, Morgan et al. 1995; Mayor and Aybar 2001; Meulemans and Bronner-Fraser 2004; Steventon, Carmona-Fontaine et al. 2005). Further work in identifying the function of transcription factors and downstream genes will help refine and expand upon these networks. In turn, this work may give new insight into related gene circuits that may be operative in other migratory and metastatic cell types.

To identify putative neural crest transcription factors, we completed a subtractive screen for newly induced neural crest cells using a macroarrayed cDNA library from

early chick embryos. This allowed identification of over 200 genes, many of which were not previously associated with neural crest development. As a secondary screen, we performed in situ hybridization to verify expression of 101 genes in premigratory and/or migrating neural crest cells. Quantitative PCR (QPCR) was used to confirm the level of upregulation of several transcription factors in newly induced neural crest cells. The results reveal many previously unknown transcriptional regulators in the neural crest and suggest interesting commonalities between neural crest cells, various metastatic cancers and other migratory cell types.

2.3: Materials and methods

Embryos and Explants

Fertile chicken eggs were incubated from 24 hours to three days to obtain embryos from HH stage 4 to HH stage 24. Embryos to be used for in situ hybridization were fixed overnight in 4% paraformaldehyde (PFA) at 4°C. They were then washed 4 x 5 minutes in PTW and dehydrated in a MeOH/PTW series at RT before being stored at –20°C in 100% MeOH for more than 3 days. Explants were dissected from embryos in Ca2+/Mg2+-free Tyrodes saline with .05% trypsin (Sigma). Conjugates were assembled and allowed to recover as previously described before embedding in collagen gels and culturing for 24 hours (Dickinson, Selleck et al. 1995; Gammill and Bronner-Fraser 2002).

RNA preparation and QPCR

RNA was prepared from tissues in collagen gels using Stratagene's RNA isolation kit. Collagen gels were transferred to an Eppendorf microcentrifuge tube with forceps and triturated in lysis buffer.

Quantitative polymerase chain reaction (QPCR) was performed using the 96 well plate ABI 7000 QPCR machine in a TaqMan assay (Applied Biosciences) with Sybrgreen Itaq Supermix with ROX (BioRad), 300 nM of each primer, and 200-500 ng of cDNA in a 25 μL reaction volume. Gene specific primers were designed using the Primer Express program (Applied Biosystems) and synthesized by IDT.

During the exponential phase of the QPCR reaction a threshold and baseline was set according to the protocols of Applied Biosystems. The threshold cycle (C_T) is defined as the cycle number at which the fluorescence generated by a reaction meets this threshold and is critical in determining the amount of the amplicon produced in the RT-QPCR reaction. The results for different samples were then interpolated on a line created by running standard curves for each primer set and then normalized against the results for a housekeeping gene, which is assumed to be a measure of the amount of cDNA used in each reaction. These calculations were performed according to the standard curve assay method, which is detailed in the Applied

Biosystems protocols. Each reaction plate was run with five point standard curves (three replicates each) for the gene of interest (GOI) and the chosen housekeeping gene, 18s. Each plate also held three non-template controls (in which no amplification was seen) for each set of primers. Standards are always run on the same plate as the unknown samples in order to ensure the validity of C_T measurements.

QPCR primer sequences were as follows:

slug forward 5’-GGGCTGCTACCGTCTCCTCTA-3’

slug reverse 5’-GCTGACCCTTCCCAAAGATG-3’

gcnf forward 5'-TTCCCCCAAGATGTCATTGAC-3'

gcnf reverse 5'-TGATGGCTGCAATGCAGAAG-3'

elk-3 forward 5'-CAGCCCCACCTTTGGTTCTT-3'

elk-3 reverse 5'-TGAGGGCAATGGAACCGATA-3'

zf469 forward 5'-CCAGCTCCAAAGAAGCAACAA-3'

zf469 reverse 5'-TGCCGAGAAGAGTCCAAAGTG-3'

srebp2 forward 5'-GATCACAGGCTGGCTTCTCC-3'

srebp2 reverse 5'-GGCTTCCTGGCTCTGAATCA-3'

gtfIIe forward 5'-CCAAGTATGTCGTGCGTGGT-3'

gtfIIe reverse 5'-CCAGAGCCAAAGCGTGTTC-3'

In Situ Hybridization

Antisense, digoxygenin labeled RNA probes were prepared from clones categorized as being upregulated by the macroarray screen. The probes were hydrolyzed and in situs were performed as described (Xu 1992). Embryos were visually examined at different angles to aid in scoring and photographed on a Zeiss Axioskop2 Plus. Selected embryos were then coarsely sectioned using a scalpel or finely sectioned using a cryostat (Bright) and mounted or immunostained.

Immunostaining

Wholemount in situs were post-fixed in 4% PFA (paraformaldehyde) + 0.02% glutaraldehyde and washed in PTW 4x15 minutes. They were nutated at 4°C overnight in 15% sucrose, kept for 8 hours at 37°C in 7.5% gelatin/15% sucrose and between 8 and 20 hours in 20% gelatin. The embryos were then embedded, frozen and cryosectioned at approximately 25 μM. Sections were briefly dried onto slides and degelatinized for 20 minutes at 42°C in PBS. They were then placed in PBS (phosphate buffered saline) + 0.1% Tween 20 + 5% goat serum block for hours at room temperature before an overnight 4°C incubation with 1:20 anti-HNK-1 antibody (American Type Culture Collection hybridoma). Sections were then washed several times in PBS + 0.1% Tween over several hours, blocked for 3 hours at RT with PTW (phosphate-buffered saline [PBS], pH 7.5, 0.1% Tween 20) and 5% goat serum, and

incubated with 1:400 anti-IgM RRX goat anti mouse (Jackson ImmunoResearch) in PTW at 4°C overnight. Slides were then washed again in PTW several times, rinsed twice in dH_20 and then coverslipped using PermaFluor (Immunotech).

2.4: Results and discussion

In order to identify novel neural crest regulators, we performed a genome-level screen for genes upregulated in newly induced neural crest cells. Neural crest induction was recapitulated in vitro by culturing intermediate neural plate and non-neural ectoderm in apposition (Dickinson, Selleck et al. 1995; Selleck and Bronner-Fraser 1996; Gammill and Bronner-Fraser 2002). cDNA enriched for neural crest genes was generated by subtracting intermediate neural plate and non-neural ectoderm cultured in isolation from tissue cultured as conjugates, and a macroarrayed early chick cDNA library screened as previously described (Gammill and Bronner-Fraser 2002).

In this paper, we now describe the completion of the neural crest screen. We previously reported the early results from this screen and the identification of only three transcription factors, including *snail-2* (*slug*) (Gammill and Bronner-Fraser 2002). We and others went on to demonstrate the functional importance of candidate neural crest regulators from the screen, validating its relevance for neural crest

development (Gammill, Gonzalez et al. 2006; Coles, Gammill et al. 2006; Gessert, Maurus et al. 2007; Taneyhill, Coles et al. 2007; Eroglu, Wang et al. 2006; Suzuki, Sakai et al. 2006) (the unknown from Gammill and Bronner-Fraser, 2002 was ultimately identified as cadherin-6b). In characterizing additional clones, we were particularly interested in identifying new transcription factors expressed in the neural crest. We reasoned that because transcription factors are generally the least abundant transcripts, they would be represented with the fewest clones in the arrayed library and would exhibit the weakest hybridization signals since they would have the lowest copy number in the hybridized cDNA. It seemed highly probable, therefore, that by picking all upregulated clones, not just the most obvious, that we should increase the number of transcription factors in our collection. As we previously performed only a sampling of upregulated clones (Gammill and Bronner-Fraser 2002), here we comprehensively analyzed all genes with a two-fold or greater hybridization signal from the subtracted probe compared to the unsubtracted probe. It is important to appreciate that this differential hybridization signal is not a direct measure of gene expression levels, rather it reflects the efficiency of subtraction for that gene, which is determined by a variety of factors including differential expression levels, sequence polymorphism, and non-specific loss. Clones meeting this two-fold criterion were picked, sequenced, and compared to the Genbank non-redundant database and the GO ontology database. This resulted in the identification of 223 new candidate neural crest genes that fell into logical functional categories.

To independently confirm the specificity of our results, in situ hybridization analysis was performed at multiple stages of neural crest development on 112 of the identified genes from the most functionally interesting categories: chromatin, cytoskeletal, ECM, mitochondria, mitosis/cell cycle, nucleocytoplasmic export, protein production and degradation, receptors or downstream signaling molecules, rho pathway genes, RNA binding proteins, secreted signals or signal production, transcription coupled processes, transcription factors, transporters, and unknown genes (Figures 1–6). This provided the additional advantage of revealing dynamic aspects of gene expression patterns over the course of neural crest development. In this way, we confirmed that 101 of 112 genes were strongly expressed in premigratory neural crest cells in the neural folds and/or in migrating neural crest relative to neighboring tissues. These clones were kept as verified candidate neural crest regulators (Table 1).

Transcription factors:

By completing the screen, we identified several additional transcription factors expressed in the neural crest. These include: *Ccar1*, *CTCF*, *Elk-3*, *GCNF*, *GTFIIE*, *ILF2*, *ILF3*, *PhD*, *SRF*, *SREBP2*, *Similar to Tcf20/AR1/SPBP*, *WHSC1L1*, *ZFP3*, and *ZFP469*. All of the genes were expressed in premigratory neural crest cells within the neural folds and/or migrating neural crest cells moving through the periphery.

We verified the upregulation of a subset of transcription factors by quantitative polymerase chain reaction (QPCR). Intermediate neural plate and non-neural

ectoderm were dissected and cultured as isolates or as conjugates in exactly the same manner as the subtracted probe was generated. We then quantitated the expression level of known (Snail2/Slug) and novel (identified in our screen) transcription factors in isolates and conjugates. The QPCR results showed that Snail2/Slug levels in the conjugated tissues consistently were upregulated by 5-7 fold (5.998 $\pm$ 0.642 s.d. in conjugates compared with 0.775 $\pm$ 0.059 s.d. in isolates, p=0.00015 by Student's t test for our samples) (Fig.2). Most of the transcription factors identified in our screen were upregulated approximately two-fold in the conjugates by QPCR (Figs. 2-6, Table 2). These levels were reproducible across replicates and experiments. This minimal induction likely reflects a variety of factors. Some genes may be expressed at low levels in isolates, and were upregulated relative to background levels in conjugates. Other genes may not have reached maximal expression levels at the endpoint of the assay, which represented premigratory crest (Dickinson, Selleck et al. 1995), and so the difference by QPCR likely appeared less dramatic than by in situ (for example in the case of *Elk-3*, Fig.3). Moreover, in all cases the conjugates contained neural and ectodermal cells in addition to newly induced neural crest cells, which will have diluted the neural crest signal. In sum, these results confirm that the transcription factors identified in the screen were upregulated in conjugates, and illustrate the advantages of the spatial and temporal information available when screening clones by in situ hybridization.

Two of the transcription factors, Elk-3 (Fig.3) and Serum Response Factor (SRF), were expressed in migrating neural crest cells but absent from the premigratory neural

crest. *Elk-3* (also known as *Sap-2*, *ERP* and *Net*) is a member of the ternary complex factor (TCF) subfamily of the Ets transcription factors. With the exception of migrating neural crest cells and some cranial mesenchyme, *Elk-3* mRNA was not observed in other tissues at the developmental stages examined. Mice with a homozygous mutation resulting in the deletion of the DNA binding domain in Elk-3 have defects in both vasculature and wound healing (Ayadi, Zheng et al. 2001). Elk-3 has further been shown to act as a repressor of PAI-1 to allow cellular migration (Buchwalter, Gross et al. 2005). The pattern of *SRF* is similar to that of *Elk-3*. SRF regulates the expression of cytoskeletal genes and is required for neuronal migration in the mouse forebrain (Alberti, Krause et al. 2005). When examined closely, SRF deficient murine ES cells display impaired migration, adhesion and cell spreading (Schratt, Philippar et al. 2002), consistent with an important role in cell movement.

The majority of transcription factors identified in our screen were expressed in both premigratory and migratory neural crest cells. Several appear to be transcriptional activators (*TCF20*, *GTFIIH* and *SREBP2*), or of unknown function (*ZFP3*, *ZFP469*). *TCF20* (*AR1*, *SPBP*) is a nuclear factor that can enhance the transcriptional activity of various transcription factors, such as *c-Jun*, *Ets*, *Sp1*, and *Pax6*, which suggests that TCF20 is a transcriptional activator. It probably acts as a coactivator for structurally and functionally disparate transcription factors binding to target sequences in promoters or enhancers (Rekdal, Sjottem et al. 2000). *Germ cell nuclear factor* (*GCNF*, *NR6A1*) (Fig.4) is a nuclear orphan receptor that was first described in the mouse testis. GCNF is expressed in the neural plate and the neural crest of *Xenopus*

midneurula embryos, with later expression in the central nervous system and branchial arches (Joos, David et al. 1996). When GCNF is depleted from the *Xenopus* embryo using a morpholino, there is improper formation of the neural tube as well as defective differentiation in the head (Barreto, Reintsch et al. 2003). GTFIIE (Fig.5) is the alpha subunit (56kDa) of general transcription factor IIE is a part of a tetramer that is required to recruit TFIIH and in the opening of an RNA polymerase II promoter (Holstege, van der Vliet et al. 1996). *SREBP2* (Fig.6) is a member of a family of basic-helix-loop-helix-leucine zipper (bHLH-ZIP) transcription factors that recognize sterol regulatory element 1 (SRE-1) (Hua, Yokoyama et al. 1993). Little is known about PhD finger protein 12 except that it can bind DNA and is shown by GO gene ontology to have transcription factor activity. Similarly, *ZFP3* and *ZFP469* are zinc-finger transcription factors about which not much is known.

Intriguingly, many of the transcription factors identified in our screen also are abnormally regulated in cancer cells. This is not surprising since many of the characteristics of a neural crest cell – motility, the ability to undergo an epithelial to mesenchymal transition, the ability to break through the basal lamina and form many derivatives – are also features of cancer cells. Genes found in both neural crest and associated with tumorigenesis include *Ccar1*, *CTCF*, *ILF2* and *WHSC1L1*. For example, *Cell Cycle and Apoptosis Regulatory Protein-1* (*Ccar1* or *Carp1*) was first identified by a yeast two hybrid screen and found to interact with DED caspases (McDonald and El-Deiry 2004). It mediates apoptosis signaling by CD437, a retinoid that causes cell cycle arrest and apoptosis in a number of cancer cells including HBC

(human breast carcinoma) by utilizing an undefined retinoic acid receptor/retinoid X receptor-independent mechanism. Reduced levels of Ccar1 result in inhibition of apoptosis by CD437 or adriamycin, whereas increased expression of Ccar1 causes elevated levels of cyclin-dependent kinase inhibitor p21WAF1/CIP1 and apoptosis (Rishi, Zhang et al. 2006). CTCF is an evolutionarily conserved zinc finger containing transcription factor and insulator binding protein that was originally identified and characterized as a transcriptional repressor of the c-Myc oncogene. It has been shown to regulate transcriptional activation as well as repression, and in addition is involved in cell-cycle regulation, apoptosis and cell differentiation (Pugacheva, Kwon et al. 2006; Ladomery and Dellaire 2002). In Xenopus, CTCF was shown to have strong expression in the neural tube and developing brain as well as later expression in the branchial arches (Burke, Hollemann et al. 2002). Mutations of *CTCF* have been identified in Wilm's tumors, as well as in breast and prostate cancers (Pugacheva, Kwon et al. 2006) (Ladomery and Dellaire 2002). ILF2 (NF45) associates with ILF3 (NF90) in the nucleus to regulate IL-2 gene transcription (Zhao, Shi et al. 2005) (Reichman, Muniz et al. 2002). Both of these proteins are widely expressed in normal tissues and are seen at higher levels in the brain (Zhao, Shi et al. 2005) (Buaas, Lee et al. 1999). ILF2 is increased in lymphoma, leukemia, and hepatocellular carcinoma cell lines while ILF3 expression is increased in nasopharyngeal carcinomas, suggestive of a role in cell proliferation and the cell cycle (Zhao, Shi et al. 2005). *WHSC1L1* (Wolf-Hirschhorn syndrome candidate 1 like 1) is another gene that is frequently rearranged in cancers, including breast cancer. *WHSC1L1* is closely related to *WHSC1*, a disorder in which patients display a

craniofacial defect known as 'Greek helmet face' (Bergemann, Cole et al. 2005). It had previously been suggested that this gene may be involved in embryonic development as well as cancers (Stec, van Ommen et al. 2001), and our identification of the gene in the neural crest is consistent with a possible role in craniofacial development.

Other genes implicated in metastasis:

In addition to the transcription factors we identified, several genes in other categories that are likely to be downstream targets also have been associated with various types of cancer. Of these *Lactate dehydrogenase A*, *Septin 9*, and *Cathepsin B* were observed in premigratory but not in migrating neural crest cells. LDHA (lactate dehydrogenase A) was originally shown to be required for cell transformation by c-Myc (Hirota, Katsumata et al. 1990; Lewis, Prescott et al. 2000). Additionally, injection of cells co-expressing both LDHA and Rcl are able to form tumors in nude mice in a similar manner to c-Myc expressing cells, suggesting that these genes are downstream of c-Myc (although neither Rcl or LDHA overexpression alone induces tumorigenesis) (Lewis, Prescott et al. 2000). Similarly, *Septin 9* (*Sept9*, *Sint1*, *Septin D1*, *MSF*, *MLL septin-like fusion*) was originally described as a fusion partner gene of MLL in acute myeloid leukemia and maps to a region commonly affected in breast and ovarian cancers. The septin gene is a member of a family of nucleotide-binding proteins first discovered in yeast due to their role in the cell division cycle. Consistent

with our findings, Sept9 expression has been reported in murine neural crest cells (Sorensen, Warming et al. 2002). *Cathepsin B* is a lysosomal cysteine proteinase involved in the effectiveness of invasive and metastatic cancers (Szpaderska and Frankfater 2001) and cells appear to have differential abilities to migrate invasively based on cathepsin B expression both in vitro and in vivo (Szpaderska and Frankfater 2001; Lakka, Gondi et al. 2004). Direct injections into tumor-containing animals of plasmids containing cathepsin B inhibited gliomal tumor growth and regressed pre-established tumor cells (Lakka, Gondi et al. 2004).

Our screen revealed that the *Neurofibromatosis 2* (*NF2*) gene, implicated in tumor production, is upregulated by neural crest induction. NF2 was previously shown to be expressed throughout development in the neural tube as well as in migrating neural crest cells and their derivatives (Akhmametyeva, Mihaylova et al. 2006). The *NF2* gene encodes the protein merlin, which is a member of the ezrin, radaxin, and moesin (ERM) family of membrane-cytoskeleton associated proteins. It is thought to have roles in cell adhesion, motility, and proliferation during development. Mutations in *NF2* result in a predisposition to schwannomas, tumors of a neural crest origin. Familial schwannomas and meningiomas contain mutations that result in truncations of *NF2*. *NF2* homozygous mutants are lethal at an early stage. However, conditional mutants targeted to Schwann and neural crest cells recapitulate the types of tumors seen in humans (Giovannini, Robanus-Maandag et al. 2000).

Two different type IV collagens, col4a5 and col4a6, were expressed by migratory neural crest and also have been implicated in malignant cell types. There are six collagen IV molecules that are arranged in a paired head-to-head fashion on three different chromosomes. *Col4a5* and *Col4a6* are the pair located on chromosome X and are found in patients with Alport syndrome associated with diffuse leiomyomatosis.

Other genes found in our screen, including PGK (Fig.1), EWS, and HEPH, have transcripts that are expressed in both the premigratory and migratory neural crest cells and associated with various types of cancers. Phosphoglycerate kinase (PGK) is a glycolytic enzyme that catalyzes the reversible conversion of 1,3-diphosphoglycerate to 3-phosphoglycerate. It also is secreted by mouse fibrosarcoma tumor cells, and may participate in angiogenesis (Lay, Jiang et al. 2000). EWS is a member of the TET family (TLS/FUS, EWS, TAFII68) that occurs in a translocation breakpoint associated with Ewing's sarcoma, a primitive neuroectodermal tumor. The translocation generates a fusion with FLI-1, a member of the ETS family of transcription factors, and the fusion can function as a transcription factor (Ladomery and Dellaire 2002). HEPH is involved in iron transport and shows altered expression in human colorectal carcinogenesis, resulting in increased intracellular iron that may induce proliferation and repress cell adhesion (Brookes, Hughes et al. 2006). Wound healing assays have long been used to look for cell migration defects. We found several genes in addition to those already described above as associated with wound healing and cell migration.

Genes involved in wound healing and cell migration:

Col1a2 and *Von Willebrand factor type A and cache domain containing 1* genes were expressed in the premigratory but not the migratory neural crest. *Col1a2* is a major collagen subunit that plays a role in neural crest cell migration and may regulate cellular differentiation (He, Feng et al. 2005). In mouse, it is expressed in the head folds at e8.5-9.0. In adult tissues, it is upregulated in response to wounding, indicating that it may be involved in cell migration (Ponticos, Abraham et al. 2004). Col1a2 also seems to mediate the adhesion and migration of germ cells during spermatogenesis in the mouse (He, Feng et al. 2005). *Von Willebrand factor type A and cache domain containing 1* (*VWCD1*) is likely to be a voltage gated calcium channel or an ECM protein; genes containing similar domains participate in cell adhesion, migration, homing, pattern formation, and signal transduction (Colombatti, Bonaldo et al. 1993).

Paxillin, *periplakin*, and *G-protein coupled receptor GPR144* are genes associated with wound healing and cell migration. They were distributed in the migrating neural crest but absent from premigratory neural crest. Paxillin expression was previously described in migrating mouse neural crest cells and derivatives such as dorsal root and trigeminal ganglia, and branchial arches. Loss of paxillin led to a decrease in focal adhesions and lamellipodia (Hagel, George et al. 2002). In Drosophila, overexpression of paxillin results in a “blistered wing” phenotype considered

characteristic of changes in cell adhesion. *Paxillin* also plays a role in the developing Drosophila eye (Chen, Turano et al. 2005). Plakins are proteins that make contact with intermediate filaments (Aho, McLean et al. 1998). Periplakin, a member of this family, localizes to the plasma membrane and is believed to play a major role in cell-cell adhesion (Nishimori, Tomonaga et al. 2006). Plakins were shown by Aho et al. to be well expressed in the brain (Aho, McLean et al. 1998). G-protein coupled receptor GPR144 is a membrane bound adhesion protein with a GPS domain in the N-terminal region as well as a pentraxin domain (Bjarnadottir, Fredriksson et al. 2004). Its developmental role is as yet unexplored.

Other genes previously implicated in wound healing and migration include *TPM-γ*, *TRIO*, *MACF/ACF7*, *nonerythroid α-spectrin*, *PTK9L*. Their mRNA transcripts were present in premigratory and migratory neural crest cells. Tropomyosins such as TPM-γ are components of the microfilament cytoskeleton that bind to the helical groove of the actin filament. Several tropomyosin isoforms have been shown to regulate cell contractility, morphology, migration and the organization of the cytoskeleton (Bryce, Schevzov et al. 2003). TRIO is a guanine nucleotide exchange factor (GEF) that functions in the activation of Rho GTPases such as RhoA, Rac1, and Cdc42 (Medley, Buchbinder et al. 2003). It is essential for embryonic development and for the development of neural tissue in mouse (O'Brien, Seipel et al. 2000). Furthermore, the TRIO-like unc-73 gene is essential for cell migration and axon guidance in *C. elegans* (Steven, Kubiseski et al. 1998). MACF/ACF7 is from the family of cytoskeletal

linkers and is the vertebrate homolog of Drosophila *Shortstop/Kakapo*. It binds both to microtubules through its GAR and GSR-repeat domains and to actin (Leung, Liem et al. 2001). In MACF-null cells, microtubules are destabilized and microtubule tracking along actin cables is perturbed (Kodama, Karakesisoglou et al. 2003). It was previously shown to be expressed in the spinal cord and brain of the developing mouse as well as neural crest derivatives such as the dorsal root ganglia (Leung, Sun et al. 1999). Our data confirm that MACF1 is also expressed in the premigratory and migratory neural crest of chickens. Spectrins, including nonerythroid α-spectrin, are members of the cytoskeletal proteins that cross-link actin filaments or link actin to the cell membrane (Djinovic-Carugo, Gautel et al. 2002). Anti-spectrin antibodies lead to the collapse of the intermediate filament network in cultured cells. Furthermore, depletion of alpha-spectrin in Drosophila results in the loss of cell-cell contact (Lee, Coyne et al. 1993). Very little is known about PTK9L (protein tyrosine kinase 9-like or A6RP), but preliminary immunolocalization studies have previously indicated that it might be involved in focal adhesions (Rohwer, Kittstein et al. 1999).

Genes associated with human craniofacial and other birth abnormalities:

Because it contributes to such a diversity of structures, the neural crest has been implicated in many birth abnormalities, including those that involve craniofacial malformations, intestinal innervation defects, and malformations of the cardiac system (Chai and Maxson 2006; Iwashita, Kruger et al. 2003) (Wurdak, Ittner et al. 2005). Several of the identified genes from this screen have been associated with

birth abnormalities that are reminiscent of alterations in the normal course of neural crest cell development.

Nectins are molecules that are involved in adhesion, migration and polarization. We observed nectin-1 expression in migrating neural crest cells, but not in the dorsal neural tube, suggesting a possible role in cell movement and morphogenesis. Consistent with this, human mutations in *nectin-1* result in various cleft lip/palate and ectodermal dysplasia disorders. Nectin functions as a cell adhesion molecule and are highly concentrated at adherens junctions. They signal through alpha-catenin, and are associated with F-actin bundles (Nakanishi and Takai 2004; Takai and Nakanishi 2003). Trans-interactions of nectin induce the activation of Cdc42 and Rac small G proteins. The nectin-induced activation of Cdc42 increases the number of both filopodia and cell-cell contact sites while the activation of Rac induces formation of lamellipodia (Nakanishi and Takai 2004). They also appear to form micro-clusters at sites of cell-cell contact (Takai, Irie et al. 2003).

GARS-AIRS-GART, and *WBSCR20A* (present in the region critical for the Williams-Beuren syndrome) have transcripts in both premigratory and migratory neural crest. Human *GARS-AIRS-GART* encodes two different proteins that are differentially expressed during human brain development but down-regulated after birth. They continue to be expressed postnatally in the cerebellum of patients with Down syndrome and have been suggested to contribute to this syndrome (Brodsky, Barnes

et al. 1997). Williams-Beuren syndrome displays many features consistent with neural crest pathology, including congenital heart and vascular disease, dental abnormalities, dysmorphic facial features, and mental retardation. One of the genes that are present in the region critical for this syndrome is *WBSCR20A* (*Nol1*, *p120*), a 120 kDA protein that was found to be expressed in the human fetal brain by RT-PCR (Merla, Ucla et al. 2002).

Cell cycle genes:

Neural crest cells actively proliferate as they delaminate and migrate. Thus, it is not surprising that numerous genes identified in our screen were associated with the cell cycle including *Lamin B2* (Figure 1), *Cyclin B2*, *Cyclin B3*, *FMS Interacting Protein*, *Mcm2* and *Mcm5*. All of these were expressed in both premigratory and migratory neural crest cells in early development. Lamin proteins are filaments that form a mesh that lines the inner side of the nuclear envelope (Lehner, Stick et al. 1987). *Lamin B2* is an essential gene that is associated with spindles in mitosis and deficit results in spindle defects (Harborth, Elbashir et al. 2001; Tsai, Wang et al. 2006). Cyclin B2 has been shown to be essential for the control of the cell cycle at the G2/M transition and, in embryonic heart cells undergoing EMT, appears to be regulated by RhoA (Tavares, Mercado-Pimentel et al. 2006). Cyclin B3 is localized to the nucleus and is degraded during anaphase entry. Its prolonged expression interferes with progression through G1 and entry into S-phase (Nguyen, Manova et al. 2002). Human Mcm2 and Mcm5 proteins form a complex that localizes to the nucleus. Blocking Mcm2 inhibits

DNA synthesis, indicating that these proteins may play a role in DNA replication and regulation of the cell cycle (Tsuruga, Yabuta et al. 1997). Finally, FMIP (FMS Interacting Protein) has been shown to control differentiation of granulocytes and macrophages (Tamura, Mancini et al. 1999).

Neural crest cells are induced at the border of the neural plate and the non-neural ectoderm, migrate through stereotyped pathways, and finally form such disparate cell types as melanocytes and cartilage. Identifying the different gene cascades that are active in these cells is critical to a greater understanding of neural crest cells as well as the many disorders in which they are implicated. With the results from this screen, we expand the known genes, including transcription factors, involved in neural crest development. These data provide new players to be included in existing gene transduction networks and further our understanding of the development of neural crest cells.

2.5: Acknowledgements

We would like to thank Tatjana Sauka-Spengler and Lisa Taneyhill for technical guidance and the Bronner-Fraser lab for helpful discussions. This work was supported by grants from the NIH (DE015309 to L. S. G. and NS36585 to M. B. F.) as well as the Betty and Gordon Moore Fellowship (M. S. A.).

Figure 1:

Neural crest gene expression: In situ hybridization was performed to confirm the expression of genes seen to be upregulated in macroarray screen. Selected genes from different categories are shown in dorsal view with anterior at the top. (A) chromatin: *safB1*; (B) cytoskeletal: *nestin*; (C) ECM: *lamin B2*; (D) mitochondria: *hexokinase 2*; (E) mitosis/cell cycle: *TD-60*; (F) Nucleocytoplamic export: *importin 9*; (G) protein production/degradation: *makorin*; (H) receptors/downstream signaling: *MHC-B*; (I) rho pathway: *kinectin*; (J) RNA binding proteins: *HNRPM*; (K) secreted signals/signal production: *PEBP*; (L) transport: *OSBP2*; (M) miscellaneous: *PGK*; (N) unknown: *chEST872f20*

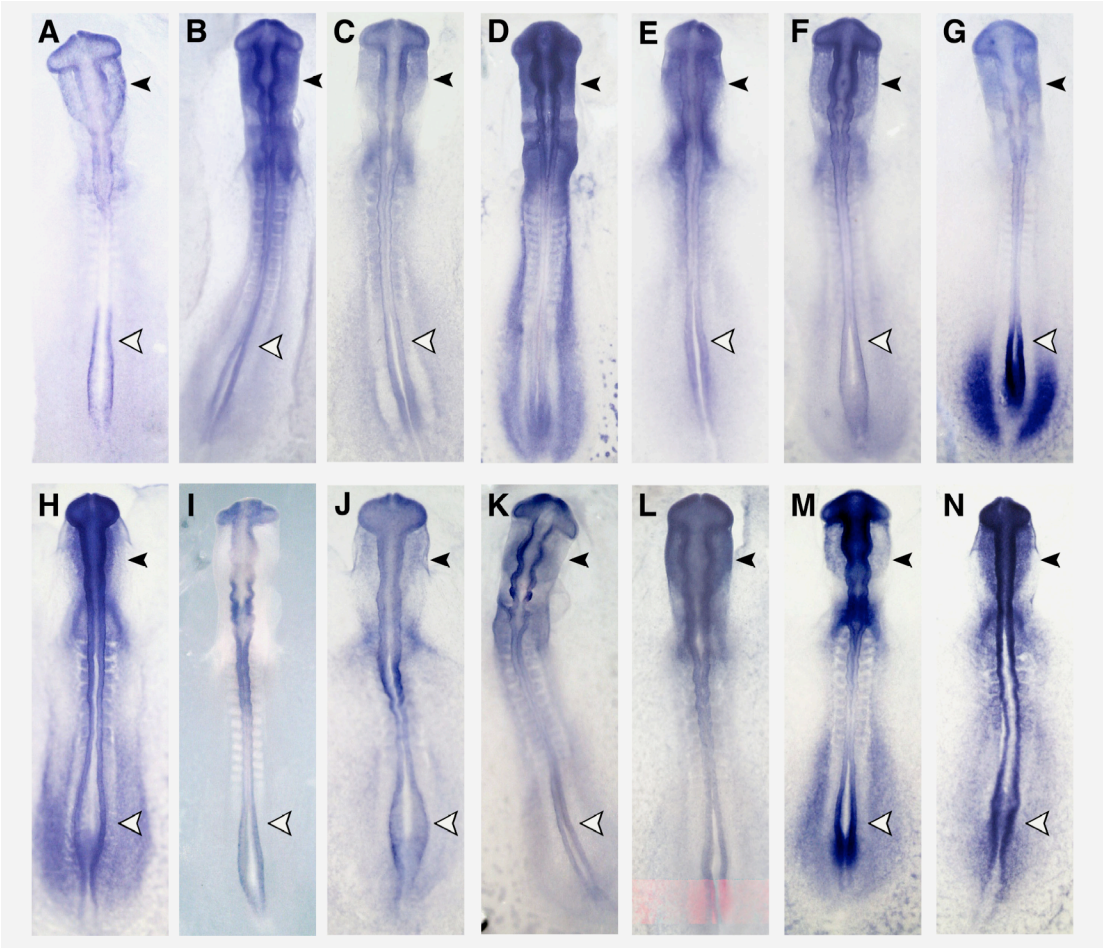


Figure 2:

Neural crest expression of and QPCR results for Snail 2 (slug). (A–D) Snail2 expression is seen specifically in premigratory (dorsal neural tube) and migratory neural crest. Shown are in situ hybridizations of 10 somite (A) and 13 somite (B–D) embryos in whole mount (A and B) and sections of whole mount stained embryos (C and D). Immunofluorescence for HNK-1 is in red (D). Arrowheads mark premigratatory neural crest, arrows mark migratory neural crest. (E) Results of conjugate QPCR showing a very highly significant difference between control and conjugate tissues ($p = 0.00015$)

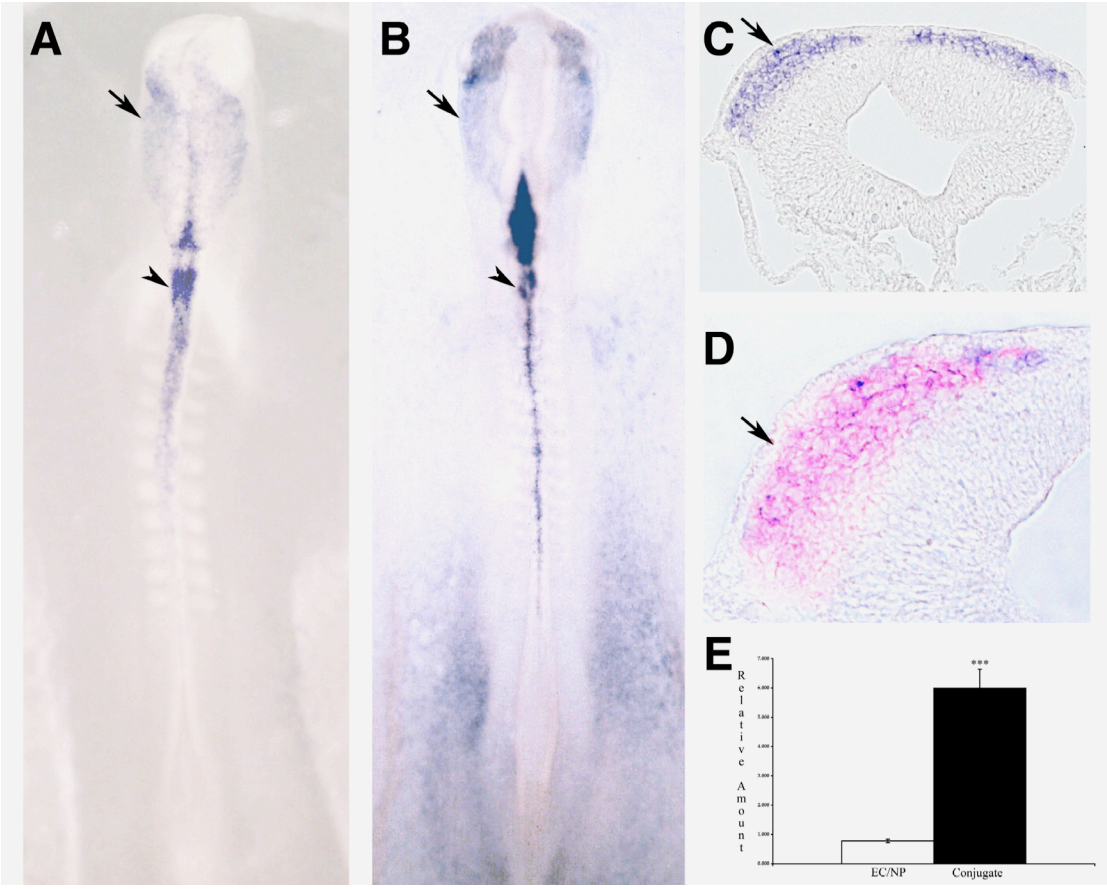


Figure 3:

Neural crest expression of and QPCR results for Elk-3. (A–D) Elk-3 expression is seen specifically in premigratory (dorsal neural tube) and migratory neural crest. Shown are in situ hybridizations of 10 somite (A) and 16 somite (B–D) embryos in whole mount (A and B) and sections of whole mount stained embryos (C and D). Immunofluorescence for HNK-1 is in red (D). Arrowheads mark premigratatory neural crest, arrows mark migratory neural crest. (E) QPCR results showing a highly significant difference between control and conjugated tissues ($p = 0.005$)

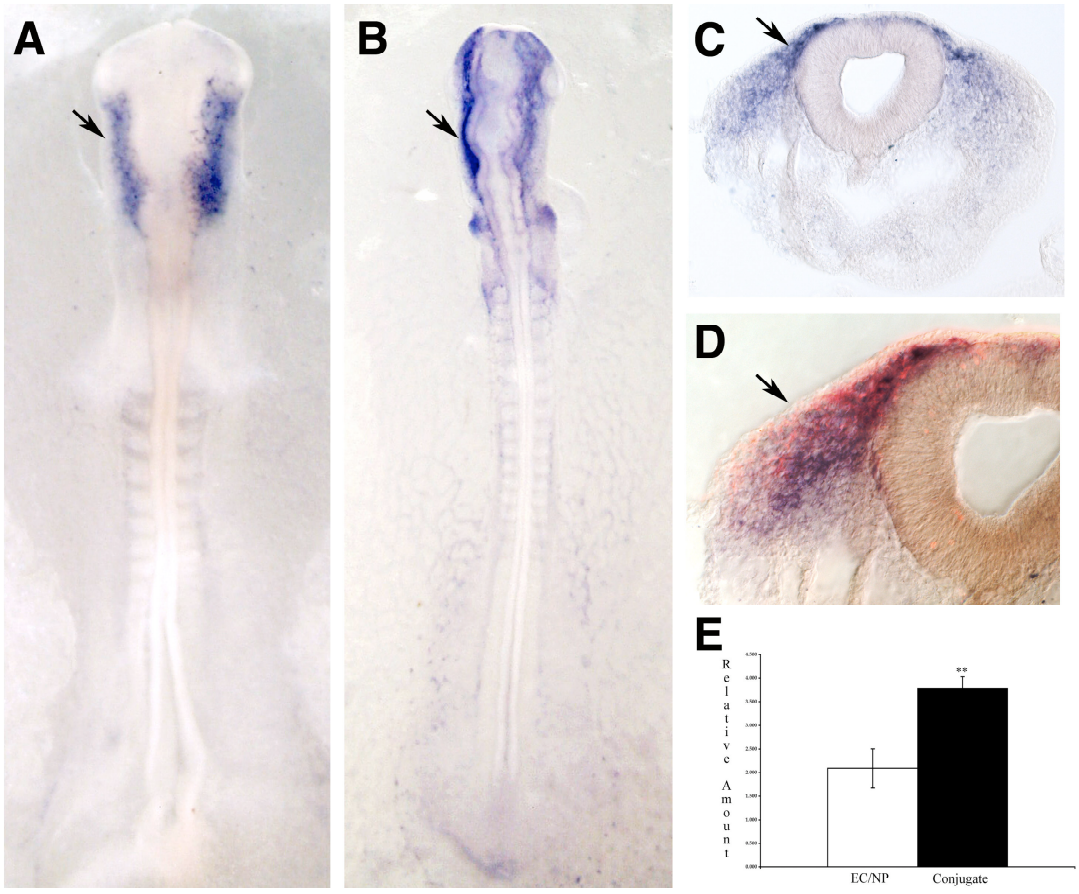


Figure 4:

Neural crest expression of and QPCR results for GCNF. (A–D) GCNF expression is seen specifically in premigratory (dorsal neural tube) and migratory neural crest. Shown are in situ hybridizations of 12 somite (A) and 15 somite (B–D) embryos in whole mount (A and B) and sections of whole mount stained embryos (C and D). Immunofluorescence for HNK-1 is in red (D). Arrowheads mark premigratatory neural crest, arrows mark migratory neural crest. (E) Results of QPCR showing a significant difference between control and conjugated tissues ($p = 0.0384$)

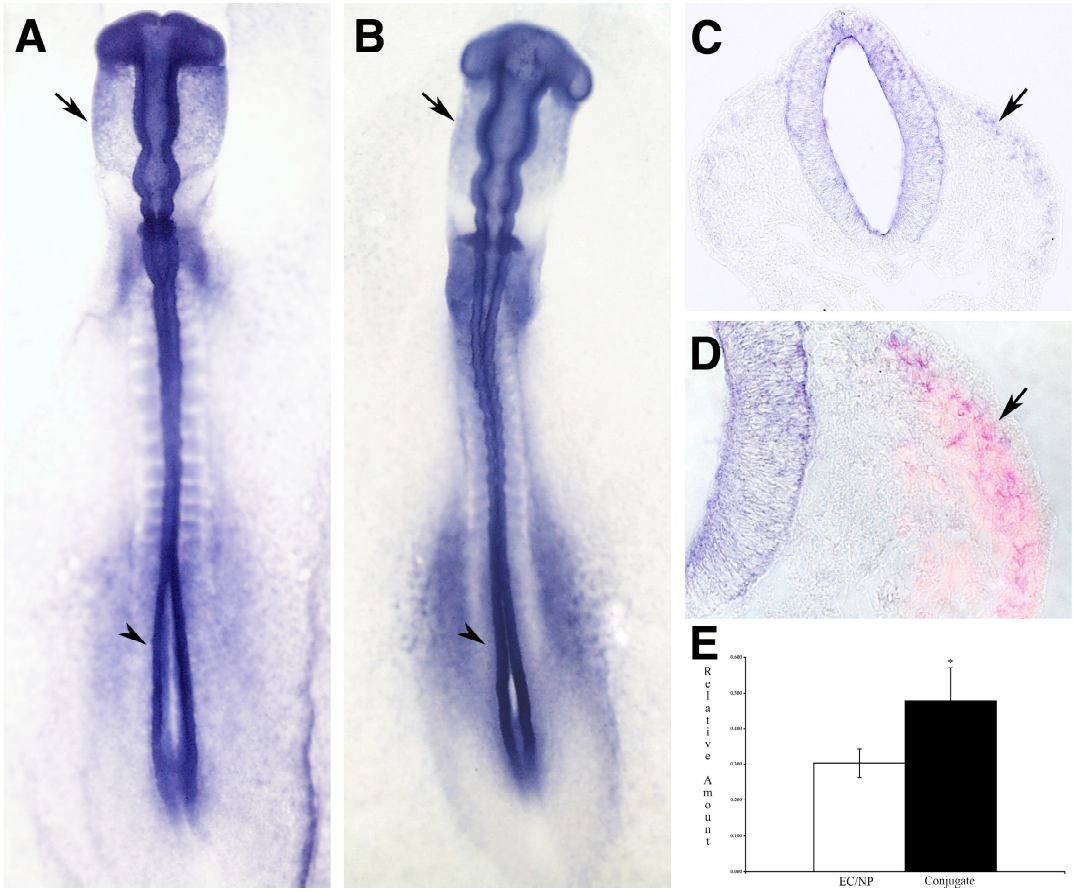


Figure 5:

Neural crest expression of and QPCR results for GTF2E. (A–D) GTF2E expression is seen specifically in premigratory (dorsal neural tube) and migratory neural crest. Shown are in situ hybridizations of 10 somite (A) and 12 somite (B–D) embryos in whole mount (A and B) and sections of whole mount stained embryos (C and D). Immunofluorescence for HNK-1 is in red (D). Arrowheads mark premigratatory neural crest, arrows mark migratory neural crest. (E) Results of QPCR showing a significant difference between control and conjugated tissues ($p = 0.041$)

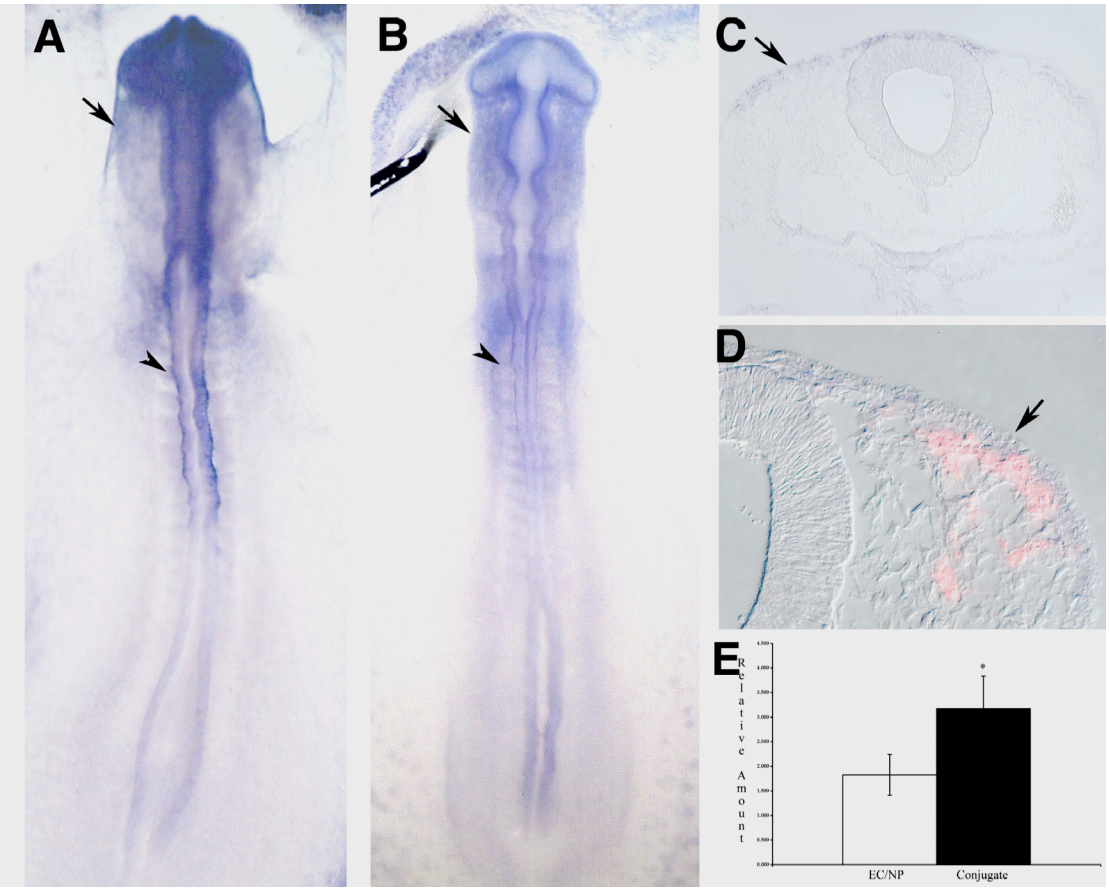


Figure 6:

Neural crest expression of and QPCR results for SREBP2. (A–D) SREBP2 expression is seen specifically in premigratory (dorsal neural tube) and migratory neural crest. Shown are in situ hybridizations of 9 somite (A) and 18 somite (B–D) embryos in whole mount (A and B) and sections of whole mount stained embryos (C and D). Immunofluorescence for HNK-1 is in red (D). Arrowheads mark premigratatory neural crest, arrows mark migratory neural crest. (E) Results of QPCR showing a significant difference between control and conjugated tissues ($p = 0.030$)

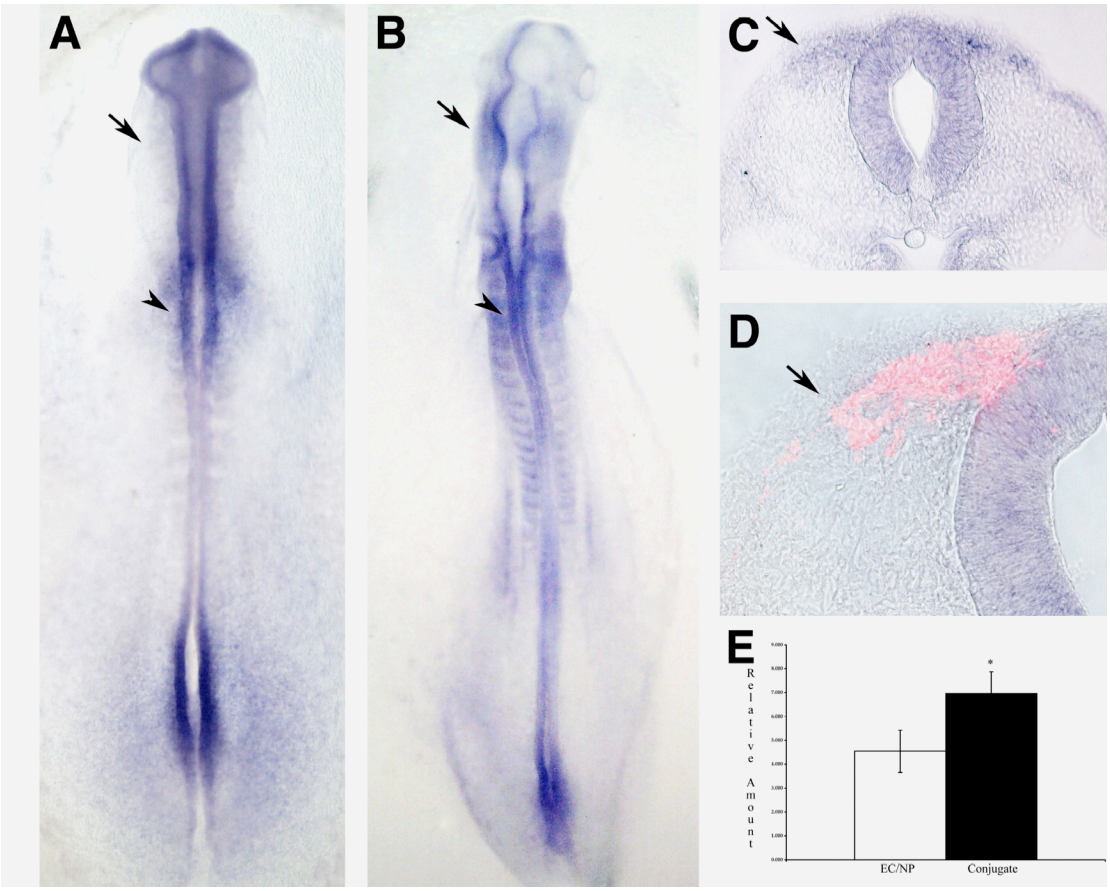


Table 1 Summary of functional categories and genes identified.

Clones were organized into categories based upon functions assigned by sequence homology. Degree of homology is indicated by colored spots (● = BLAST bit score > 200; ● = bit score 80–200; ● = bit score 50–80; ● = bit score 40–50; ● = bit score < 40). In situ hybridization patterns in 4–16 somite embryos were scored for expression in neural folds (premigratory neural crest; pNC) and cranial migratory neural crest (mNC) under the assumption that the *in vitro* induced neural crest examined in the subtraction corresponds to a timepoint between these stages of neural crest development. General specificity of expression (SP) was also scored (-, expression in many tissues at varying levels; +/-, expression broad, but discrete patterns obvious; +, expression limited to only a few tissues; ++, expression highly specific).

	A. Chromatin	**pNC**	**mNC**	**SP**	
●	H3,3B	+	+	-	replacement histone; restricted to cells of meiotic prophase
●	HP1BP74	+	+	+/-	nucleosome assembly; DNA binding
●	SAFB1	+	+	+/-	may act in attaching base of chromatin loops to nuclear matrix
	B. Cytoskeleton				
●	□lpha E-catenin	+	+	+/-	major binding partner of beta-catenin
●	CAP-23	-	+	+/-	BASP1; regulates cell cortex actin dynamics
●	MACF1	+	+	+/-	may stabilize actin where microtubules and microfilaments meet
●	NFM	+	+	+/-	neurofilament triplet M; intermediate filament family
●	Nectin1	+	+/-	+/-	part of adherens junctions; may interact with cadherin-catenin
●	Nestin	+	+	+/-	NES; intermediate filament protein
●	Merlin	-	+	+/-	gene responsible for neurofibromatosis-2
●	Nonerythroid □-spectrin	+	+	+/-	SPTAN1; filamentous cytoskeletal protein
●	Paxillin	-	+	+/-	important for labile adhesions required for rapid cell migration
●	Periplakin	-	+	+/-	thought to contact the intermediate filaments of keratinocytes
●	PTK9L	+	+	+/-	protein contains an actin-binding site and an ATP-binding site
●	TPM-□	+	+	+/-	may alter actin filament organization, cell size, and shape
	C. ECM				
●	COL1A2	+	-	+/-	fibrillar forming collagen; collagen □2 precursor
●	COL4□5	-	+	+/-	may be involved in Alport syndrome: deafness and renal problems
●	COL4□6	-	+	+/-	involved in basement membrane formation, Alport syndr variant
●	LMNB2	+	+	+/-	Lamin B2: intermediate filament protein
	D. Mitochondria				
●	BID	+	+	-	death agonist; member of BCL-2 family of cell death regulators
●	Hexokinase 2	-	+	+/-	catalyzes first committed step of glycolysis
●	HADHB	+	+	-	part of multienzyme complex; mitochondrial fatty acid oxidation
●	LDHA	+	-	+/-	promoter contains essential binding sites for HIF-1;
●	Similar to BLCAP	+	+	+/-	seen in mitochondria; downregulated during bladder cancer
●	Similar to NADH dehydrogenase	+	-	-	first enzyme in the mitochondrial electron transfer chain
	E. Mitosis/cell cycle				
●	CycB2	+	+	+/-	essential for the control of the cell cycle at the G2/M transition

CycB3	+	+	+/-	nuclear sublocalization, belongs to cyclin family AB
MCM2	+	+	+/-	may play an important role in 2 crucial steps of the cell cycle
MCM5	+	+	+/-	forms complex with MCM2
Septin 9	-	+	+/-	cell division control protein
SEC14-like 1	+	-	+/-	may be involved in E-cadherin-mediated adhesion and signaling
TD-60	+	+	+/-	RCC1 family; binds nucleotide-free form of Rac1 preferentially
F. Nucleocytoplasmic export				
DEAD box polypeptide 23	+	+	+/-	RNA helicase, nuclear pore protein, mRNA export
Importin 9	+	+	+/-	can mediate nuclear import of core histones into cell nuclei
G. Protein production/degradation				
Cathespin B	-	+	-	intracellular degradation and turnover of proteins
CBS	-	+	+/-	enzyme deficient in classical homocystinuria
GARS-AIRS-GART	+	+	+/-	catalyzes 2nd, 3rd and 5th steps of de novo purine biosynthesis
Glutathione S-transferase CL2	+	+	-	conjugates reduced glutathione to hydrophobic electrophiles
Makorin 1	+	+	+/-	E3 ubiquitin ligase, promotes proteasomal degradation
PSMD3	-	+	-	proteasome 26S subunit, non-ATPase; subunit 3
Roxan	+	+	-	contains TPR, LD, and zf motifs, forms complex with eIF4G
H. Receptors/downstream signaling				
Anamorsin (Ciapin)	+	+	+/-	critical in hematopoiesis, med. of cytokine-induced antiapoptosis
Axl-related RTK (Rek)	+	-	+/-	Axl/Tyro3 family with a potential role in neural cell development
MHC B complex protein	+	+	+/-	non-classical MHC protein, encodes G-protein like molecule
FMIP	+	+	-	serum/glucocorticoid related kinase 2
GPR144	+	-	+/-	G protein coupled receptor; integral part of membrane
MHC Bcomplex protein	+	+	+/-	non-classical MHC protein, encodes G-protein like molecule
Ptprf	+	+	-	protein tyrosine phosphatase LAR: interacts with TRIO
SPINT1	+	-	+	member of Kunitz family of serine protease inhibitors
I. Rho pathway				
KTN1	+	-	+	mediator of RhoG and RhoA activity
Trio	+	+	+/-	Interacts with PTPRF; displays two Rho-GEF domains
J. RNA binding proteins				
Akap1	+	+/-	+/-	bifunctional helical element: for ER and mitochondrion targeting
ANKRD17 isoform a	+	-	+/-	Studies suggest that this protein is involved in liver development.
G3BP1	+	+	+/-	element of the Ras signal transduction pathway
EWS	+	+	+/-	mutated in Ewing sarcoma: cancer thought to have NCC origin
HnrpA2/B1	+	+	+/-	involved with pre-mrna processing
Hnrp M	+	+	+/-	pre-mRNA-binding proteins in vivo
Lark	+	+	+/-	has RT zinc finger; regulates protein production; RNA binding
NONO	+	-	-	mammalian homolog of nonAdiss; nuclear RNA binding protein
WBSCR20A	+	+	+/-	p120; Proliferation-associated nucleolar protein
SH3DBP	+	+	+/-	binds Ras-GTPase-activating protein; associates with SH3 domain
K. Secreted signals/signal production				
Collapsin-2	+	-	+	Sema3D
CFR	+	+	+/-	p70; ER and golgi localization
PEBP	+	+	+/-	RAF kinase inhibitor protein; regulates Raf/MEK/ERK module
Vav2	+	+	+/-	oncogene; activates c-fos serum response element and CD69
L. Transcription Coupled Processes				
Rel assoc pp40	+	+	+/-	functionally related to I kappa B (inhibitor of the NF-kappa B)
M. Transcription Factors				
Ccar1	+	+	+/-	DNA binding transcriptional regulator; conserved SAP domain
CTCF	+	+	+/-	CCCTC-binding factor (zinc finger protein); represses c-myc

Elk3	-	+	+	ETS domain transcription factor family; TCF subfamily
GTFIIE	+	+	+/-	part of a multiprotein complex near the transcription start site
GCNF	+	+	+/-	essential transcription factor in vertebrate embryogenesis
ILF2	+	+	+/-	binds NFAT and stimulates its ability to enhance gene expression
ILF3	+	+	-	subunit of nuclear factor of activated T cells, dimerizes with ILF2
PHD	+	+	+/-	has PHD-finger domain; folds into interleaved type of Zn-finger
SRF	-	+	+	Serum Response Factor: binds specific sequences as a homo-dimer
SREBP2	+	+	+/-	bHLH protein that stimulates transcription by binding a SRE
Sim to TCF20	+	+	+/-	stromylesin 1 PDGF-responsive element-binding protein
WHSC1L1	+	+	+/-	Wolf-Hirschhorn syndrome candidate 1-like 1; multiple ZF repeats
Sim to Zinc finger protein 3	+	+	-	binds and regulates the J and/or S elements in MHC II promoter
ZFP469	+	+	+/-	may be involved in txnl regulation; potential nuclear localization
N. Transporters				
ATP1A1	+	+	+/-	plays a very important role in nerve cell membranes
HEPH, variant 2	+	+	-	Cu-containing iron oxidase; iron export from intestinal enterocytes
OSBP2	+	+	+/-	may mediate oxysterol cytotoxicity in tissues
RANBP3	+	+	-	promote export complexes of the CRM1/NES/RanGTP type
VWCD1	+	-	+	Ca^{++}-channel protein; important in excitation-contraction coupling
O. Miscellaneous				
Adat1	-	+	+/-	participate in the pre-mRNA editing of nuclear transcripts
Adh5	+	+	+/-	class III : glutathione-dependent formaldehyde dehydrogenase
Aldehyde dehydrogenase 9a1	+	+/-	-	catalyzes dehydrogenation of γ-aminobutyraldehyde to GABA
sim to autoantigen NGP-1	-	+	+/-	gtpase that associates with pre-60s ribosomal subunits in nucleolus
GTBP/mutS	+	+	+/-	forms heterodimer with hMSH2; binds G/T mismatches in dsDNA
Heparin binding protein RI-HB	+	-	-	RA-induced heparin-binding protein; essential for embryogenesis
JIF-1	+	+	-	interacts with presinillin1 to suppress function of c-Jun homodimers
PGK	+	+	+/-	catalyses 1,3-diphosphoglycerate is to 3-phosphoglycerate
RNH1	+	-	+/-	blocks function of ribonucleases
Taldo1	+	+	-	key enzyme of the pentose phosphate pathway
Tumor protein D52-like 2	-	+	+/-	involved in cell proliferation, Ca2+-mediated signal transduction
P. Unknown/EST/no homology				
finished cDNA ChEST648l12	+	+	+/-	similar to Homo sapiens K+ channel tetramerization protein
finished cDNA ChEST872f20	+	+	-	
Gallus sim to hypoth LOC419534	+	+	+/-	
Gallus sim to FLJ00068 protein	+	-	+	
Hypothetical protein MGC15523	+	-	-	

Table 2 QPCR Results

	Slug	Elk-3	GCNF	GTF2E	SREBP2
Isolates Avg	0.775	2.086	0.303	1.826	4.539
Isolates StDev	0.059	0.416	0.040	0.413	0.886
Conjugates Avg	5.998	3.786	0.479	3.171	6.960
Conjugates StdDev	0.642	0.243	0.092	0.664	0.909
P-value	0.00015	0.005	0.0384	0.041	0.030

Aho S, McLean WH, Li K, Uitto J. 1998. cDNA cloning, mRNA expression, and chromosomal mapping of human and mouse periplakin genes. Genomics 48:242-247.

Akhmametyeva EM, Mihaylova MM, Luo H, Kharzai S, Welling DB, Chang LS. 2006. Regulation of the Neurofibromatosis 2 gene promoter expression during embryonic development. Dev Dyn 235:2771-2785.

Alberti S, Krause SM, Kretz O, Philippar U, Lemberger T, Casanova E, Wiebel FF, Schwarz H, Frotscher M, Schutz G, Nordheim A. 2005. Neuronal migration in the murine rostral migratory stream requires serum response factor. Proc Natl Acad Sci U S A 102:6148-6153.

Anderson DJ. 1994. Stem cells and transcription factors in the development of the mammalian neural crest. Faseb J 8:707-713.

Aoki Y, Saint-Germain N, Gyda M, Magner-Fink E, Lee YH, Credidio C, Saint-Jeannet JP. 2003. Sox10 regulates the development of neural crest-derived melanocytes in Xenopus. Dev Biol 259:19-33.

Ayadi A, Zheng H, Sobieszczuk P, Buchwalter G, Moerman P, Alitalo K, Wasylyk B. 2001. Net-targeted mutant mice develop a vascular phenotype and up-regulate egr-1. Embo J 20:5139-5152.

Barreto G, Reintsch W, Kaufmann C, Dreyer C. 2003. The function of Xenopus germ cell nuclear factor (xGCNF) in morphogenetic movements during neurulation. Dev Biol 257:329-342.

Bellmeyer A, Krase J, Lindgren J, LaBonne C. 2003. The protooncogene c-myc is an essential regulator of neural crest formation in xenopus. Dev Cell 4:827-839.

Ben-Tabou de-Leon S, Davidson EH. 2007. Gene regulation: gene control network in development. Annu Rev Biophys Biomol Struct 36:191.

Bergemann AD, Cole F, Hirschhorn K. 2005. The etiology of Wolf-Hirschhorn syndrome. Trends Genet 21:188-195.

Bjarnadottir TK, Fredriksson R, Hoglund PJ, Gloriam DE, Lagerstrom MC, Schioth HB. 2004. The human and mouse repertoire of the adhesion family of G-protein-coupled receptors. Genomics 84:23-33.

Brodsky G, Barnes T, Bleskan J, Becker L, Cox M, Patterson D. 1997. The human GARS-AIRS-GART gene encodes two proteins which are differentially expressed during human brain development and temporally overexpressed in cerebellum of individuals with Down syndrome. Hum Mol Genet 6:2043-2050.

Brookes MJ, Hughes S, Turner FE, Reynolds G, Sharma N, Ismail T, Berx G, McKie AT, Hotchin N, Anderson GJ, Iqbal T, Tselepis C. 2006. Modulation of iron transport proteins in human colorectal carcinogenesis. Gut 55:1449-1460.

Bryce NS, Schevzov G, Ferguson V, Percival JM, Lin JJ, Matsumura F, Bamburg JR, Jeffrey PL, Hardeman EC, Gunning P, Weinberger RP. 2003. Specification of actin filament function and molecular composition by tropomyosin isoforms. Mol Biol Cell 14:1002-1016.

Buaas FW, Lee K, Edelhoff S, Disteche C, Braun RE. 1999. Cloning and characterization of the mouse interleukin enhancer binding factor 3 (Ilf3) homolog in a screen for RNA binding proteins. Mamm Genome 10:451-456.

Buchwalter G, Gross C, Wasylyk B. 2005. The ternary complex factor Net regulates cell migration through inhibition of PAI-1 expression. Mol Cell Biol 25:10853-10862.

Burke LJ, Hollemann T, Pieler T, Renkawitz R. 2002. Molecular cloning and expression of the chromatin insulator protein CTCF in Xenopus laevis. Mech Dev 113:95-98.

Chai Y, Maxson RE, Jr. 2006. Recent advances in craniofacial morphogenesis. Dev Dyn 235:2353-2375.

Chen GC, Turano B, Ruest PJ, Hagel M, Settleman J, Thomas SM. 2005. Regulation of Rho and Rac signaling to the actin cytoskeleton by paxillin during Drosophila development. Mol Cell Biol 25:979-987.

Cheung M, Chaboissier MC, Mynett A, Hirst E, Schedl A, Briscoe J. 2005. The transcriptional control of trunk neural crest induction, survival, and delamination. Dev Cell 8:179-192.

Coles EG, Gammill LS, Miner JH, Bronner-Fraser M. 2006. Abnormalities in neural crest cell migration in laminin alpha5 mutant mice. Dev Biol 289:218-228.

Colombatti A, Bonaldo P, Doliana R. 1993. Type A modules: interacting domains found in several non-fibrillar collagens and in other extracellular matrix proteins. Matrix 13:297-306.

Davidson EH. 2006. The regulatory genome : gene regulatory networks in development and evolution. Amsterdam ; Boston: Elsevier/Academic Press. xi, 289 pp.

Dickinson ME, Selleck MA, McMahon AP, Bronner-Fraser M. 1995. Dorsalization of the neural tube by the non-neural ectoderm. Development 121:2099-2106.

Djinovic-Carugo K, Gautel M, Ylanne J, Young P. 2002. The spectrin repeat: a structural platform for cytoskeletal protein assemblies. FEBS Lett 513:119-123.

Eroglu B, Wang G, Tu N, Sun X, Mivechi NF. 2006. Critical role of Brg1 member of the SWI/SNF chromatin remodeling complex during neurogenesis and neural crest induction in zebrafish. Dev Dyn 235:2722-2735.

Gammill LS, Bronner-Fraser M. 2002. Genomic analysis of neural crest induction. Development 129:5731-5741.

Gammill LS, Gonzalez C, Gu C, Bronner-Fraser M. 2006. Guidance of trunk neural crest migration requires neuropilin 2/semaphorin 3F signaling. Development 133:99-106.

Gessert S, Maurus D, Rossner A, Kuhl M. 2007. Pescadillo is required for Xenopus laevis eye development and neural crest migration. Dev Biol 310:99-112.

Giovannini M, Robanus-Maandag E, van der Valk M, Niwa-Kawakita M, Abramowski V, Goutebroze L, Woodruff JM, Berns A, Thomas G. 2000. Conditional biallelic Nf2 mutation in the mouse promotes manifestations of human neurofibromatosis type 2. Genes Dev 14:1617-1630.

Hagel M, George EL, Kim A, Tamimi R, Opitz SL, Turner CE, Imamoto A, Thomas SM. 2002. The adaptor protein paxillin is essential for normal development in the mouse and is a critical transducer of fibronectin signaling. Mol Cell Biol 22:901-915.

Harborth J, Elbashir SM, Bechert K, Tuschl T, Weber K. 2001. Identification of essential genes in cultured mammalian cells using small interfering RNAs. J Cell Sci 114:4557-4565.

He Z, Feng L, Zhang X, Geng Y, Parodi DA, Suarez-Quian C, Dym M. 2005. Expression of Col1a1, Col1a2 and procollagen I in germ cells of immature and adult mouse testis. Reproduction 130:333-341.

Hirota Y, Katsumata A, Takeya T. 1990. Nucleotide and deduced amino acid sequences of chicken lactate dehydrogenase-A. Nucleic Acids Res 18:6432.

Holstege FC, van der Vliet PC, Timmers HT. 1996. Opening of an RNA polymerase II promoter occurs in two distinct steps and requires the basal transcription factors IIE and IIH. Embo J 15:1666-1677.

Honore SM, Aybar MJ, Mayor R. 2003. Sox10 is required for the early development of the prospective neural crest in Xenopus embryos. Dev Biol 260:79-96.

Hua X, Yokoyama C, Wu J, Briggs MR, Brown MS, Goldstein JL, Wang X. 1993. SREBP-2, a second basic-helix-loop-helix-leucine zipper protein that stimulates transcription by binding to a sterol regulatory element. Proc Natl Acad Sci U S A 90:11603-11607.

Iwashita T, Kruger GM, Pardal R, Kiel MJ, Morrison SJ. 2003. Hirschsprung disease is linked to defects in neural crest stem cell function. Science 301:972-976.

Joos TO, David R, Dreyer C. 1996. xGCNF, a nuclear orphan receptor is expressed during neurulation in Xenopus laevis. Mech Dev 60:45-57.

Knecht AK, Bronner-Fraser M. 2002. Induction of the neural crest: a multigene process. Nat Rev Genet 3:453-461.

Kodama A, Karakesisoglou I, Wong E, Vaezi A, Fuchs E. 2003. ACF7: an essential integrator of microtubule dynamics. Cell 115:343-354.

Ladomery M, Dellaire G. 2002. Multifunctional zinc finger proteins in development and disease. Ann Hum Genet 66:331-342.

Lakka SS, Gondi CS, Yanamandra N, Olivero WC, Dinh DH, Gujrati M, Rao JS. 2004. Inhibition of cathepsin B and MMP-9 gene expression in glioblastoma cell line via RNA interference reduces tumor cell invasion, tumor growth and angiogenesis. Oncogene 23:4681-4689.

Lay AJ, Jiang XM, Kisker O, Flynn E, Underwood A, Condron R, Hogg PJ. 2000. Phosphoglycerate kinase acts in tumour angiogenesis as a disulphide reductase. Nature 408:869-873.

Le Douarin NM, Brito JM, Creuzet S. 2007. Role of the neural crest in face and brain development. Brain Res Rev 55:237-247.

Lee JK, Coyne RS, Dubreuil RR, Goldstein LS, Branton D. 1993. Cell shape and interaction defects in alpha-spectrin mutants of Drosophila melanogaster. J Cell Biol 123:1797-1809.

Lehner CF, Stick R, Eppenberger HM, Nigg EA. 1987. Differential expression of nuclear lamin proteins during chicken development. J Cell Biol 105:577-587.

Leung CL, Liem RK, Parry DA, Green KJ. 2001. The plakin family. J Cell Sci 114:3409-3410.

Leung CL, Sun D, Zheng M, Knowles DR, Liem RK. 1999. Microtubule actin cross-linking factor (MACF): a hybrid of dystonin and dystrophin that can interact with the actin and microtubule cytoskeletons. J Cell Biol 147:1275-1286.

Lewis BC, Prescott JE, Campbell SE, Shim H, Orlowski RZ, Dang CV. 2000. Tumor induction by the c-Myc target genes rcl and lactate dehydrogenase A. Cancer Res 60:6178-6183.

Luo T, Lee YH, Saint-Jeannet JP, Sargent TD. 2003. Induction of neural crest in Xenopus by transcription factor AP2alpha. Proc Natl Acad Sci U S A 100:532-537.

Mayor R, Aybar MJ. 2001. Induction and development of neural crest in Xenopus laevis. Cell Tissue Res 305:203-209.

Mayor R, Morgan R, Sargent MG. 1995. Induction of the prospective neural crest of Xenopus. Development 121:767-777.

McDonald ER, 3rd, El-Deiry WS. 2004. Suppression of caspase-8- and -10-associated RING proteins results in sensitization to death ligands and inhibition of tumor cell growth. Proc Natl Acad Sci U S A 101:6170-6175.

Medley QG, Buchbinder EG, Tachibana K, Ngo H, Serra-Pages C, Streuli M. 2003. Signaling between focal adhesion kinase and trio. J Biol Chem 278:13265-13270.

Merla G, Ucla C, Guipponi M, Reymond A. 2002. Identification of additional transcripts in the Williams-Beuren syndrome critical region. Hum Genet 110:429-438.

Meulemans D, Bronner-Fraser M. 2004. Gene-regulatory interactions in neural crest evolution and development. Dev Cell 7:291-299.

Nakanishi H, Takai Y. 2004. Roles of nectins in cell adhesion, migration and polarization. Biol Chem 385:885-892.

Nguyen TB, Manova K, Capodieci P, Lindon C, Bottega S, Wang XY, Refik-Rogers J, Pines J, Wolgemuth DJ, Koff A. 2002. Characterization and expression of mammalian cyclin b3, a prepachytene meiotic cyclin. J Biol Chem 277:41960-41969.

Nishimori T, Tomonaga T, Matsushita K, Oh-Ishi M, Kodera Y, Maeda T, Nomura F, Matsubara H, Shimada H, Ochiai T. 2006. Proteomic analysis of primary esophageal squamous cell carcinoma reveals downregulation of a cell adhesion protein, periplakin. Proteomics 6:1011-1018.

O'Brien SP, Seipel K, Medley QG, Bronson R, Segal R, Streuli M. 2000. Skeletal muscle deformity and neuronal disorder in Trio exchange factor-deficient mouse embryos. Proc Natl Acad Sci U S A 97:12074-12078.

Ponticos M, Abraham D, Alexakis C, Lu QL, Black C, Partridge T, Bou-Gharios G. 2004. Col1a2 enhancer regulates collagen activity during development and in adult tissue repair. Matrix Biol 22:619-628.

Pugacheva EM, Kwon YW, Hukriede NA, Pack S, Flanagan PT, Ahn JC, Park JA, Choi KS, Kim KW, Loukinov D, Dawid IB, Lobanenkov VV. 2006. Cloning and characterization of zebrafish CTCF: Developmental expression patterns, regulation of the promoter region, and evolutionary aspects of gene organization. Gene 375:26-36.

Reichman TW, Muniz LC, Mathews MB. 2002. The RNA binding protein nuclear factor 90 functions as both a positive and negative regulator of gene expression in mammalian cells. Mol Cell Biol 22:343-356.

Rekdal C, Sjottem E, Johansen T. 2000. The nuclear factor SPBP contains different functional domains and stimulates the activity of various transcriptional activators. J Biol Chem 275:40288-40300.

Rishi AK, Zhang L, Yu Y, Jiang Y, Nautiyal J, Wali A, Fontana JA, Levi E, Majumdar AP. 2006. Cell cycle- and apoptosis-regulatory protein-1 is involved in apoptosis signaling by epidermal growth factor receptor. J Biol Chem 281:13188-13198.

Rohwer A, Kittstein W, Marks F, Gschwendt M. 1999. Cloning, expression and characterization of an A6-related protein. Eur J Biochem 263:518-525.

Sasai N, Mizuseki K, Sasai Y. 2001. Requirement of FoxD3-class signaling for neural crest determination in Xenopus. Development 128:2525-2536.

Schratt G, Philippar U, Berger J, Schwarz H, Heidenreich O, Nordheim A. 2002. Serum response factor is crucial for actin cytoskeletal organization and focal adhesion assembly in embryonic stem cells. J Cell Biol 156:737-750.

Selleck MA, Bronner-Fraser M. 1996. The genesis of avian neural crest cells: a classic embryonic induction. Proc Natl Acad Sci U S A 93:9352-9357.

Sorensen AB, Warming S, Fuchtbauer EM, Pedersen FS. 2002. Alternative splicing, expression, and gene structure of the septin-like putative proto-oncogene Sint1. Gene 285:79-89.

Spokony RF, Aoki Y, Saint-Germain N, Magner-Fink E, Saint-Jeannet JP. 2002. The transcription factor Sox9 is required for cranial neural crest development in Xenopus. Development 129:421-432.

Stec I, van Ommen GJ, den Dunnen JT. 2001. WHSC1L1, on human chromosome 8p11.2, closely resembles WHSC1 and maps to a duplicated region shared with 4p16.3. Genomics 76:5-8.

Steven R, Kubiseski TJ, Zheng H, Kulkarni S, Mancillas J, Ruiz Morales A, Hogue CW, Pawson T, Culotti J. 1998. UNC-73 activates the Rac GTPase and is required for cell and growth cone migrations in C. elegans. Cell 92:785-795.

Steventon B, Carmona-Fontaine C, Mayor R. 2005. Genetic network during neural crest induction: from cell specification to cell survival. Semin Cell Dev Biol 16:647-654.

Suzuki T, Sakai D, Osumi N, Wada H, Wakamatsu Y. 2006. Sox genes regulate type 2 collagen expression in avian neural crest cells. Dev Growth Differ 48:477-486.

Szpaderska AM, Frankfater A. 2001. An intracellular form of cathepsin B contributes to invasiveness in cancer. Cancer Res 61:3493-3500.

Takai Y, Irie K, Shimizu K, Sakisaka T, Ikeda W. 2003. Nectins and nectin-like molecules: roles in cell adhesion, migration, and polarization. Cancer Sci 94:655-667.

Takai Y, Nakanishi H. 2003. Nectin and afadin: novel organizers of intercellular junctions. J Cell Sci 116:17-27.

Tamura T, Mancini A, Joos H, Koch A, Hakim C, Dumanski J, Weidner KM, Niemann H. 1999. FMIP, a novel Fms-interacting protein, affects granulocyte/macrophage differentiation. Oncogene 18:6488-6495.

Taneyhill LA, Coles EG, Bronner-Fraser M. 2007. Snail2 directly represses cadherin6B during epithelial-to-mesenchymal transitions of the neural crest. Development 134:1481-1490.

Tavares AL, Mercado-Pimentel ME, Runyan RB, Kitten GT. 2006. TGFbeta-mediated RhoA expression is necessary for epithelial-mesenchymal transition in the embryonic chick heart. Dev Dyn 235:1589-1598.

Tsai MY, Wang S, Heidinger JM, Shumaker DK, Adam SA, Goldman RD, Zheng Y. 2006. A mitotic lamin B matrix induced by RanGTP required for spindle assembly. Science 311:1887-1893.

Tsuruga H, Yabuta N, Hashizume K, Ikeda M, Endo Y, Nojima H. 1997. Expression, nuclear localization and interactions of human MCM/P1 proteins. Biochem Biophys Res Commun 236:118-125.

Wurdak H, Ittner LM, Lang KS, Leveen P, Suter U, Fischer JA, Karlsson S, Born W, Sommer L. 2005. Inactivation of TGFbeta signaling in neural crest stem cells leads to multiple defects reminiscent of DiGeorge syndrome. Genes Dev 19:530-535.

Xu QaW, D.G., editor. 1992. In situ hybridization: A practical approach. Oxford University Press.

Zhao G, Shi L, Qiu D, Hu H, Kao PN. 2005. NF45/ILF2 tissue expression, promoter analysis, and interleukin-2 transactivating function. Exp Cell Res 305:312-323.

Chapter III: Functional perturbations of candidate regulators of neural crest development

This chapter presents the analysis of two different genes discovered in the screen for genes with functional relevance in neural crest described in Chapter 2. M.S.A.'s contribution to the work on Ccar1 encompasses the spatiotemporal analysis of the mRNA pattern of Ccar1, a portion of the morpholino injections of Ccar1, and the analysis of those injected embryos by in situ for Sox10. M.S.A. also performed the entirety of the work described herein on Adh5.

3.1: Abstract

Here we present the results of our studies on two different candidates that have resulted from our screen for genes important in neural crest cell development. We have characterized the expression patterns of these genes and examined their functional importance using perturbation analysis via morpholino-mediated knock-down. The levels of Ccar1 in neural crest cells induced in vitro were analyzed by quantitative PCR.

3.2: Introduction

To date, a dozen or so genes have been functionally analyzed and found to be important members of a neural crest gene regulatory network involved in specification and formation of this important cell type. However, many more genes are likely to be involved in this network. With this in mind, we successfully performed a screen (described in Chapter 2) to increase the number of genes that might be functionally investigated.

To begin functional characterization of the genes discovered in our screen (seen in Chapter 2), we chose two of the genes that previous literature suggested might be particularly relevant to the neural crest. Ccar1 is a transcription factor that was found not only in the screen described in this thesis, but also emerged in a concurrent screen performed to identify vertebrate orthologues of genes involved in long-range migration of the hermaphrodite specific neuron in *Caenorhabditis elegans*. The second gene, Adh5,

is an enzyme of unknown function, but is related to enzymes involved in retinoic acid signaling.

Ccar1:

Cell cycle and apoptosis-regulatory protein-1 (CCAR1 or CARP1) is a perinuclear protein first identified by Rishi et al. as a regulator of apoptotic signaling (Rishi, Zhang et al. 2003). The Ccar1 sequence has been identified in many different genomes including nematodes, honey bees, frogs, avians, mice, rats, dogs, chimpanzees, and humans and there is a great deal of sequence similarity and highly conserved domains, indicating its importance across the evolutionary scale (Zhang, Levi et al. 2007; Rishi, Zhang et al. 2006; Anantharaman and Aravind 2008). The human Ccar-1 gene is located on the long arm of chromosome 10 and the mRNA is approximately 3.5 kb long, encoding a 1146 aa protein with a mass of 130 kDa (Zhang, Levi et al. 2007; Rishi, Zhang et al. 2003; Kim, Yang et al. 2008). The protein includes a 35 amino acid SAP domain, which is common in chromatin-associated proteins (Aravind and Koonin 2000; Kim, Yang et al. 2008).

Ccar1 has been traditionally thought to be a strictly apoptotic molecule in malignant cell types such as breast or prostate cancers as well as leukemias (Zhang, Levi et al. 2007). Reduced levels of Ccar1 in these types of malignant cells result in a decrease in apoptosis signaling by CD437 ([3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid), the chemotherapeutic (anthracycline toxin) adriamycin, etoposide, and EGFR inhibitor(s) (Rishi, Zhang et al. 2006). CD437 is an apoptotic retinoid from a family that is known to cause growth arrest and stimulate apoptosis in malignant cell types such as breast cancer and leukemia (among others) through a retinoic acid receptor/retinoid X receptor-

independent mechanism (Shao, Dawson et al. 1995; Hsu, Rishi et al. 1997; Liang, Fontana et al. 1999). Ccar1 had been further shown to bind to p53 and to perform a critical role in transcriptional activation by p53 (Kim, Yang et al. 2008).

Although in the literature, Ccar1 is mostly considered an inducer of apoptosis, it also has been shown to act as a coactivator for several other classes of transcription factors as it is important in the recruitment of the Mediator to their target genes. As an example, it was convincingly demonstrated that Ccar1 also has a role in estrogen-induced cell proliferation (Kim, Yang et al. 2008).

ADH5:

ADH family members are known to catalyze the conversion of alcohol to acetaldehyde as well as the metabolism of retinoids, steroids and 5-hydroxyfatty acids (Beisswenger, Holmquist et al. 1985; Pares and Vallee 1981; Wagner, Pares et al. 1984; Kaiser, Holmquist et al. 1988; Giri, Linnoila et al. 1989; Hempel and Pietruszko 1979; Iborra, Renau-Piqueras et al. 1992). There are four defined classes of ADH genes; Class I and II and IV have a high affinity for alcohol and have functions catalyzing ethanol metabolism (Iborra, Renau-Piqueras et al. 1992). Adh5 is a member of the class III (X) alcohol dehydrogenase family and is the only isozyme shown to be present in the human, simian, equine, bovine, canine, and rat brain, where it is likely to be present in the nucleus (Beisswenger, Holmquist et al. 1985; Iborra, Renau-Piqueras et al. 1992). Furthermore, this class does not have a large capacity for the oxidation of ethanol or methanol as many of the other alcohol dehydrogenase genes do, but it efficiently oxidizes long chain

alcohols and ω-hydroxyfatty acids (Beisswenger, Holmquist et al. 1985; Pares and Vallee 1981; Wagner, Pares et al. 1984; Kaiser, Holmquist et al. 1988; Giri, Linnoila et al. 1989). For this reason, Adh5 is also known as FALDH or FDH (NAD^+/glutathione-dependent formaldehyde dehydrogenase) (Uotila and Koivusalo 1974; Iborra, Renau-Piqueras et al. 1992). This enzyme functions as a homodimer and has a zinc at the active site that is essential (Wagner, Pares et al. 1984).

The human ADH5 gene was first mapped to chromosome 4, and this was further refined to chromosome 4q21-q25 (or 4q21-q25) where seven genes are clustered (Meera Khan, Wijnen et al. 1984) (Smith 1986). The corresponding murine gene has been mapped to chromosome 3, along with the other mouse ADH genes. The human Adh5 gene contains 9 exons and 8 introns, and the region 5' of ADH5 is extremely G and C rich and contains neither a TATA box nor a CAAT box but does contain a multitude of known binding sites. Several of these binding sites are for proteins important for neural crest development such as Sp1 (specificity protein 1) and AP2 as well as those that are known to be involved in pathologies of neural crest origin, like NF-1 (Hur and Edenberg 1992). Sp1 is a sequence specific Krüppel-like C2H2-type zinc finger superfamily member and has been shown to activate many viral and cellular genes as well as being important for the transactivation of the RET promoter within a known neural crest-derived cell line (Suske 1999). FBI-1, a POZ domain transcription factor, is a potent inhibitor of ADH5 and represses transcription by interfering with the DNA binding domain of Sp1 (Lee, Suh et al. 2002).

Chi-like alcohol dehydrogenases are highly conserved through mammalian evolution and have also been seen in very basal organisms such as *Escheria coli* (60% amino acid sequence identity) (Sharma, Fox et al. 1989; Giri, Krug et al. 1989; Edenberg, Brown et al. 1991; Hur and Edenberg 1992; Gutheil, Holmquist et al. 1992). The only family member seen in invertebrates is a glutathione-dependent formaldehyde dehydrogenase, most closely related to Class III family members (and sometimes referred to as ADH3) and specifically homologous to Adh5 (Koivusalo, Baumann et al. 1989; Canestro, Albalat et al. 2002; Gonzalez-Duarte and Albalat 2005). Studies of ADH genes in fish have lead to the discovery of an enzyme that has the characteristics of a class I ADH gene, but has more structural similarity to a member of class III. This suggests that a class III Adh gene was the evolutionary precursor of the ADH family (Kaiser, Fernandez et al. 1993).

3.3: Materials and methods

Embryos and Explants

Fertile chicken eggs were incubated from 24 hours to three days to obtain embryos from HH stage 4 to HH stage 24. Embryos to be used for in situ hybridization were fixed overnight in 4% paraformaldehyde (PFA) at 4°C. They were then washed 4 x 5 minutes in PTW and dehydrated in a MeOH/PTW series at RT before being stored at –20°C in 100% MeOH for more than 3 days. Explants were dissected from embryos in Ca2+/Mg2+-free Tyrodes saline with .05% trypsin (Sigma). Conjugates were assembled and allowed to recover as previously described before embedding in collagen gels and

culturing for 24 hours (Dickinson, Selleck et al. 1995; Gammill and Bronner-Fraser 2002)

RNA preparation and QPCR

RNA was prepared from tissues in collagen gels using Stratagene's RNA isolation kit. Collagen gels were transferred to an Eppendorf microcentrifuge tube with forceps and triturated in lysis buffer.

Quantitative polymerase chain reaction (QPCR) was performed using the 96 well plate ABI 7000 QPCR machine in a TaqMan assay (Applied Biosciences) with Sybrgreen Itaq Supermix with ROX (BioRad), 300 nM of each primer, and 200-500 ng of cDNA in a 25 μL reaction volume. Gene specific primers were designed using the Primer Express program (Applied Biosystems) and synthesized by IDT. During the exponential phase of the QPCR reaction a threshold and baseline was set according to the protocols of Applied Biosystems as described in Chapter 2.

QPCR primer sequences were as follows:

Snail2 forward 5'-GGGCTGCTACCGTCTCCTCTA-3'

Snail2 reverse 5'-GCTGACCCTTCCCAAAGATG-3'

ccar1 forward 5'-TCGACCAGCTGCTGCTTGTA-3'

ccar1 reverse 5'-CCGCTCAGAGTGGGAAACC-3'

Cloning

Partial chick cDNA fragments of Ccar1 and ADH5 were identified in a 8-12 somite stage macroarrayed chick cDNA library. Primers were designed to allow the cloning of these genes into pCIG-GFP and pCIG-RFP from Topo-TA the vector after High-Fidelity PCR amplification.

Adh5 has been identified as NM_001031152.1 and the sequence of the gene is
ATGGCCAGCGGGGTTATTAAATGCAAGGCTGCTGTTGCCTGGGAGGCAGGTA
AACCTCTCTCTATTGAGGAGGTGGAAGTCGCTCCGCCAAAAGCTCATGAAGT
TCGCATCAAGATAGTTGCCACTGCTCTCTGTCACACTGATGCCTATACTCTGA
GTGGTGCTGATCCCGAGGGATGTTTCCCTGTGATTCTGGGTCATGAAGGAGC
AGGAATTGTAGAAAGTGTTGGGGAAGGAGTAACAAAAGTAAAGCCAGGGGA
CACCGTGATCCCTCTGTACATCCCCCAGTGTGGCGAGTGCAAGTACTGCAAG
AATCCTAAAACTAATCTGTGCCAAAAGATAAGAGTTACTCAAGGGAAAGGAC
TCATGCCTGATGGTACGATCAGATTCACCTGCAAAGGAAAGCAGATTTACCA
CTTCATGGGGACTAGCACCTTCTCGGAGTACACAGTGGTGGCCGATATCTCA
GTAGCTAAGATAGATCCTGCAGCACCTTTCGATAAAGTGTGCCTGCTGGGTTG
TGGCGTCTCTACAGGTTATGGGGCTGCTGTTAACACTGCTAAGGTGGAACCTG
GCTCCACGTGTGCAGTCTTTGGTTTAGGAGGAGTTGGGCTGGCTACTGTTATG
GGCTGTAAAGCGGCAGGAGCATCCCGGATCATTGGCATTGACATAAACAAGA
ATACCTATGCCAAAGCCAAGGAGTTTGGAGCTGCTGAGTGCATTAGCCCTCA
AGACTTTGAGAAGCCCATACAGGAGGTGCTGGTTGAAATGACTGATGGTGGT
GTAGACTACTCATTTGAGTGTATTGGTAACGTTGGAGTCATGAGGGCTGCCTT
GGAAGCCTGCCACAAAGGCTGGGGAGTCAGTGTGATAGTTGGAGTAGCTGCT
GCTGGTCAGGAGATCTCAACGCGGCCGTTCCAGCTAGTAACCGGTCGGACAT
GGAAAGGGACTGCGTTTGGAGGCTGGAAGAGCGTAGACAGTGTACCAAAGC
TGGTGAATGACTACATGGCCAAAAAGATCAAAGTAGATGAGTTTGTGACTCA
TACCCTGCCTTTTGACAAAATTAATGAGGCTTTTGATCTCCTGCATAAAGGAA
AGAGCATTCGAACAGTTCTAAAGTTTTAG

The Adh5 amino acid sequence is:
MASGVIKCKAAVAWEAGKPLSIEEVEVAPPKAHEVRIKIVATALCHTDAYTLSG

ADPEGCFPVILGHEGAGIVESVGEGVTKVKPGDTVIPLYIPQCGECKYCKNPKTN
LCQKIRVTQGKGLMPDGTIRFTCKGKQIYHFMGTSTFSEYTVVADISVAKIDPAA
PFDKVCLLGCGVSTGYGAAVNTAKVEPGSTCAVFGLGGVGLATVMGCKAAGA
SRIIGIDINKNTYAKAKEFGAAECISPQDFEKPIQEVLVEMTDGGVDYSFECIGNV
GVMRAALEACHKGWGVSVIVGVAAAGQEISTRPFQLVTGRTWKGTAFGGWKS
VDSVPKLVNDYMAKKIKVDEFVTHTLPFDKINEAFDLLHKGKSIRTVLKF

Ccar1 has been identified as XM_421573 and the sequence of the gene is
ATGGCTCAGTTCGGAGGACAGAAGAACCCTCCGTGGGCCACTCAGTTTACAG
CCACCGCCGTGTCCCAGCCAGCTGCTCTTGGTGTGCAGCAGCCGTCGCTGCTT
GGAGCCTCTCCTACAATATACACCCAGCAGACGGCGCTGGCCGCGGCGGGCC
TGACTACGCAAACACCAACCAACTACCAGCTAACTCAGACGGCCGCTTTGCA
GCAGCAGGCGGCAGCTGCAGCAGCTGCCTTACAGCAGCAATATTCACAGCCT
CAGCAGACTCTCTATAGTGTACAGCAACAGTTGCAGCAACCTCAGCAGACCA
TTTTAACTCAGCCAGCTGTTGCACTACCTACCAGTCTTAGCCTATCTACTCCTC
AGCCAGCAGCCCAGATAACTGTTTCTTACCCAGCACCCCGCTCAAGTCAGCA
GCAAACACAACCACAAAAGCAGCGCGTCTTCACTGGGGTCGTTACTAAACTG
CATGATACGTTCGGTTTTGTGGATGAAGATGTCTTTTTTCAACTTAGTGCTGTT
AAAGGGAAGACACCTCAAGTGGGCGACAGAGTTTTAGTAGAAGCTACTTACA
ATCCTAATATGCCGTTCAAATGGAATGCACAAAGGATCCAGACGCTTCCAAA
TCAGAACCAGACACAGGCTCAACCGTTACTCAAGACGCCTCCGGCAGTTCTT
CAGCCCATTGCGCAGCAGACGGCGTTTGGTGTTCAGGCACAGCCTCAGCCAC
AGTCCTTGCTGCAGGCACAGATATCGGCAGCTTCAATCACACCTCTGCTTCAG
ACACAGCCTCAGCCATTGCTGCAGCAGCCACAGCAAAAAGGTGGTTTGTTAC
AGCCCCCTGTCCGTCTAGTTTCACAGCCTCAACCAGCTCGAAGATTGGACCCA
CCATCCAGATTTTCTGGGAGGAATGACAGAGGAGGAGATCCTATGCCAAACC
GAAAAGATGACAGGAGCCGTGAGAGAGATCGGGAGAGACGTCGATCTCGGG
AAAGATCACCCCAGAGAAAACGCTCCAGAGAGAGATCGCCTCGACGAGAGA
GGGAGAGGTCGCCTCGAAGACCGCGACGTGTTGTTCCTCGTTACACCGTTCA
GTTTTCTAAGTTTTCATTGGACTGCCGCAGCTGTGATATGATGGAGCTGAGGA
GGCGTTACCAGAACTTGTATATCCCAAGTGATTTCTTCGATGCTCAGTTTACA
TGGGTGGATGCTTTCCCAATGTCTAGACCATTTCAACTGGGAAACTACTGTAA
CTTCTATGTAATGCATAGGGAAGTAGACCCTATAGATAAAAATGCTGCTGTC
CTCGATCCACCTGATGCTGATCATCTGTACAGTGCTAAGGTGATGTTGATGGC
TAGCCCTAGTATGGAAGACCTCTATCACAAGTCCTGTGCTCTGGCTGAAGATC
CCCAAGAACTTCGTGATGGATTTCAGCATCCTGCTAGACTTGTAAAGTTTTTA
GTGGGTATGAAAGGCAAAGATGAAGCTATGGCTATTGGAGGACACTGGTCCC
CATCACTAGATGGACCTGATCCGGAGAAGGACCCTTCGGTGCTGATAAAGAC
GGCTATTCGTTGTTGCAAGGCTCTTACAGGGATTGACTTAAGTGTGTGCACAC
AATGGTACCGTTTTGCAGAGATTCGCTACCATCGCCCTGAGGAGACGCACAA
GGGGCGTACAGTTCCAGCTCATGTGGAGACAGTGGTTTTATTTTTCCCGGATG
TTTGGCATTGCCTTCCCACCCGCTCAGAGTGGGAAACCCTCTCCCGAGGATAC
AAGCAGCAGCTGGTCGAGAAGCTTCAGGGTGAACGCAAGGAGGCTGATGGA
GAACAGGATGAAGAGGAAAAGGATGATGGGGAAGCAAAAGAGATCTCTACA
CCTACACACTGGTCTAAACTGGATCCGAAAACAATGAAGGTAAATGACCTTC

GCAAAGAATTAGAAAGTCGAACTCTTAGCTCTAAAGGACTGAAATCTCAGTT
GATAGCTCGACTGACAAAGCAACTGAAAGTAGAGGAGCAAAAAGAAGAGCA
AAAGGAGCTAGAGAAGTCTGAGAAAGAAGAGGAAGAGGAGGAGGATAGGA
AATCTGAAGATGACAAAGAGGAAGAGGAAAGAAAGCGTCAAGAAGAAATG
GAACGTCAGCGACGGGAGAGACGGTACATCTTGCCTGATGAACCAGCGATCA
TTGTGCATCCTAACTGGGCAGCCAAGAGCGGGAAGTTTGACTGTAGCATAAT
GTCACTCAGTGTTCTTCTGGACTATAGGTTGGAGGATAATAAAGAACATTCCT
TTGAGGTTTCATTGTTTGCAGAACTCTTCAATGAAATGCTTCAGAGGGATTTT
GGTGTCAGAATTTATAAAGCACTGATTTCCCTCCCAGAGAGGGAGGACAAAA
AAGATAAAAAAAGCAAAAAAGATGAACGAAAAGAAAAAAAGGAAGAAAAA
GAAGAAGAAAATGATGAACCAAAACCGAAGAGGAGAAAATCTGGAGATGAT
AAAGAAAAAAAAGAAGATAAAGATGAAAAGAAGAGGGAAGAGAAAAGGAA
AGATGATTCCAAAGATGAAGAAGAAACTGAAGATGACAATAATCAAGAAGA
ATATGATCCAATGGAGGCGGAAGAAGCTGAAGATGAAGATGAAGAATGCAG
ACCACTCGCAACTCCCATTGAAGTCAGTAGGAGATACCGGGAAGAAGAAGA
AATGAATAAACGAGAGGACAGAAGAGAGGGAAATAAGCATTGTAAAGAGAG
GTCATCTAAAGATAAAGAGAAAGACAAGACACAGATGGTAACTGTTAATAG
GGATCTTCTGATGGCTTTCGTTTATTTTGACCAAAGTCACTGTGGATATCTTCT
GGAGAAGGACATGGAGGAGATACTGTATACTCTTGGACTACACCTGTCTCGT
GCTCAGGTCAAGAAGCTACTTAACAAAGTAGTACTTAGAGAATCCTGCTTCT
ACAGAAGACTAACAGATACTTCTAAAGATGAGGAAAACCAAGAAGAATCAG
AAGAACTACAAGAAGACATGTTAGGAAACAGATTGCTGTTGCCATCACCTAC
AGTAAAGCAAGAATCAAAAGCTGTAGAAGAAAATGTTGGCCTTATTGTATAC
AACGGAGCTATGGTAGATGTCGGGAGCCTTTTACAGAAGCTGGAGAAGAGTG
AAAGAGTGCGGGCAGAGATAGAACAAAAGCTACAGCTCCTTGAAGAAAAAA
CAGATGAGGATGAAAAGACCATACTGCAGCTGGAAAATTCTAACAAAAGTCT
GTCTGCGGAGCTCAGAGATGTCAAAAAGGACCTTAGTCAGCTGCAAGAGAAC
TTGAAGATCTCAGATGAGAAAAATTTGCAATTTGAGAGTCAGCTGAATAAGA
CAATCAAAAATTTAGCTACTGTTATGGATGAAATGCAGAGTGTGCTTAAGCA
GGATATTGTGAAAAGTGAAGATAAAGATCAGAAATCCAAAGAAAATGGAGC
AAGCGTATGA

The Ccar1 amino acid sequence is:

MAQFGGQKNPPWATQFTATAVSQPAALGVQQPSLLGASPTIYTQQTALAAAGL
TTQTPTNYQLTQTAALQQQAAAAAAALQQQYSQPQQTLYSVQQQLQQPQQTIL
TQPAVALPTSLSLSTPQPAAQITVSYPAPRSSQQQTQPQKQRVFTGVVTKLHDTF
GFVDEDVFFQLSAVKGKTPQVGDRVLVEATYNPNMPFKWNAQRIQTLPNQNQT
QAQPLLKTPPAVLQPIAQQTAFGVQAQPQPQSLLQAQISAASITPLLQTQPQPLLQ
QPQQKGGLLQPPVRLVSQPQPARRLDPPSRFSGRNDRGGDPMPNRKDDRSRERD
RERRRSRERSPQRKRSRERSPRRERERSPRRPRRVVPRYTVQFSKFSLDCRSCDM
MELRRRYQNLYIPSDFFDAQFTWVDAFPMSRPFQLGNYCNFYVMHREVDPIDKN

AAVLDPPDADHLYSAKVMLMASPSMEDLYHKSCALAEDPQELRDGFQHPARLV
KFLVGMKGKDEAMAIGGHWSPSLDGPDPEKDPSVLIKTAIRCCKALTGIDLSVCT
QWYRFAEIRYHRPEETHKGRTVPAHVETVVLFFPDVWHCLPTRSEWETLSRGYK
QQLVEKLQGERKEADGEQDEEEKDDGEAKEISTPTHWSKLDPKTMKVNDLRKE
LESRTLSSKGLKSQLIARLTKQLKVEEQKEEQKELEKSEKEEEEEEDRKSEDDKEE
EERKRQEEMERQRRERRYILPDEPAIIVHPNWAAKSGKFDCSIMSLSVLLDYRLE
DNKEHSFEVSLFAELFNEMLQRDFGVRIYKALISLPEREDKKDKKSKKDERKEKK
EEKEEENDEPKPKRRKSGDDKEKKEDKDEKKREEKRKDDSKDEEETEDDNNQE
EYDPMEAEEAEDEDEECRPLATPIEVSRRYREEEEMNKREDRREGNKHCKERSS
KDKEKDKTQMVTVNRDLLMAFVYFDQSHCGYLLEKDMEEILYTLGLHLSRAQV
KKLLNKVVLRESCFYRRLTDTSKDEENQEESEELQEDMLGNRLLLPSPTVKQESK
AVEENVGLIVYNGAMVDVGSLLQKLEKSERVRAEIEQKLQLLEEKTDEDEKTIL
QLENSNKSLSAELRDVKKDLSQLQENLKISDEKNLQFESQLNKTIKNLATVMDE
MQSVLKQDIVKSEDKDQKSKENGASV

Morpholino oligo design:

Morpholinos were designed against the above sequences as shown below:

The Adh5 morpholino is designed against the 5’-UTR region of the gene shown above. The oligo was designed and manufactured by GeneTools in both a lissaminated and a FITC-labeled form.

ADH5 UTR morpholino: 5'- TGTCGTTCCCGGACGGAGAAGAAAG -3'

The Ccar1 morpholino is designed against the start site of the gene shown above. The oligo was designed and manufactured by GeneTools in both a lissaminated and a FITC-labeled form.

Ccar1 start site morpholino: 5'- CTTCTGTCCTCCGAACTGAGCCATG-3'

Electroporation

Chicken embryos were collected on filter paper rings and electroporated *ex ovo* using the a vertical stage 4 electroporation apparatus. They were electroporated at 7 volts 5 pulses, 50 msec on and 100 msec off. They were then cultured in a 37°C incubator for between 12 and 36 hours in dishes on a minimal layer of thin albumin.

In Situ Hybridization

Antisense, digoxygenin labeled RNA probes were prepared from clones categorized as being upregulated by the macroarray screen. The probes were hydrolyzed and in situs were performed as described (Xu 1992). Embryos were visually examined at different angles to aid in scoring and photographed on a Zeiss Axioskop2 Plus. Selected embryos were then coarsely sectioned using a scalpel or finely sectioned using a cryostat (Bright) and mounted or immunostained.

Sections

Wholemount in situs were post-fixed in 4% PFA (paraformaldehyde) + 0.02% glutaraldehyde and washed in PTW 4x15 minutes. They were nutated at 4°C overnight in 15% sucrose, kept for 8 hours at 37°C in 7.5% gelatin/15% sucrose and between 8 and 20 hours in 20% gelatin. The embryos were then embedded, frozen and cryosectioned at approximately 25 μM. Sections were briefly dried onto slides and degelatinized for 20 minutes at 42°C in PBS.

Immunostaining

Sections were performed as described above. They were then placed in PBS (phosphate buffered saline) + 0.1% Tween 20 + 5% goat serum block for hours at room temperature before an overnight 4°C incubation with 1:20 anti-HNK-1 antibody (American Type Culture Collection hybridoma). Sections were then washed several times in PBS + 0.1% Tween over several hours, blocked for 3 hours at RT with PTW (phosphate-buffered saline [PBS], pH 7.5, 0.1% Tween 20) and 5% goat serum, and incubated with 1:400 anti-IgM RRX goat anti mouse (Jackson ImmunoResearch) in PTW at 4°C overnight. Slides were then washed again in PTW several times, rinsed twice in dH_20 and then coverslipped using PermaFluor (Immunotech).

3.4: Results

Ccar1

Ccar1 mRNA is present in the both the dorsal neural folds and in the migratory neural crest cells (Figures 1 and 2). This is further confirmed by overlay with HNK-1 on a section of a Ccar1 in situ. In addition, up-regulation of the gene in neural plate/ectoderm conjugates was confirmed by QPCR. To this end, intermediate neural plate and non-neural ectoderm were dissected and cultured as isolates or as conjugates in exactly the same manner as the subtracted probe was generated in order to perform our screen for

genes important in neural crest cells (described in Chapter 2). We then quantitated the expression level of Snail2 and Ccar1 in isolates and conjugates. Ccar1 showed asignificant difference between these two populations with an average value in conjugates of 0.777, standard deviation 0.104 and an average value in control tissue of 0.464, standard deviation 0.149. The student t-test value is significant, with p=0.0405. In this same set of samples, the values of Snail2 were an average conjugate value of 5.995, standard deviation 0.637 and an average non-conjugate value of 0.748, standard deviation 0.241 (results for Snail2 reported previously in another paper) (Adams, Gammill et al. 2008). The student t-test value for the Snail2 results is also significant, with p=0.0002. Since the culture system is designed as a subtractive scheme in which the difference between the cultures should reflect the additional cell type induced therein, namely the neural crest, it makes sense that transcripts of Ccar-1 would be increased in this population.

Next, we examined the effects of loss-of-function of Ccar-1 using translation-blocking morpholino antisense oligonucleotides. Using Sox10 as a neural crest marker, in situ hybridization revealed that Ccar11 morpholino injected embryos showed a significant reduction of Sox10 mRNA on the injected side as compared with the contralateral side. Out of 26 electroporated embryos, 13 showed no difference, 10 showed a slight deficit, and 3 showed a severe deficit of Sox10 transcripts. In contrast, control morpholino electroporated embryos under identical conditions at the same time, showed no deficits on the injected side (20/22 embryos) or very mild deficits (2/22 embryos). Contingency testing on this data set using Prism software gave a Chi-square value of 9.551 with a p

value of 0.0084, a statistically significant difference.

Our examination of these embryos for the root cause of the loss of *Sox10* mRNA on the morpholinated side of the embryo has led us to investigate the relative levels of cell death seen there, as this would very well explain the loss of *Sox10* expressing cells. Our preliminary data indicates that these embryos have a significant cell death increase in the dorsal neural folds of the injected side of the embryo when compared to the control side of the embryo and with embryos injected with the control morpholino (Figure 3).

Adh5

In situ hybridization in very early embryonic stages has not previously been reported for Adh5 mRNA, nor has the expression pattern of this gene been previously published in chicken embryos. While previous reports have indicated that Adh5 mRNA is present in a ubiquitous manner during development, the reports examined embryos older than those studied here, and thus are not reflective of early neural crest development. We therefore performed a survey of expression during early development, confirming the presence of Adh5 mRNA in both the dorsal neural folds and the early migrating neural crest cells (Figure 4).

Functional analysis was carried out by perturbing the translation of Adh5 using a morpholino with a FITC or lissamine tag (along with control morpholinos carrying the same fluorescent tag). Sox10 in situ hybridization performed on Adh5 morpholino injected embryos showed a significant reduction of Sox10 mRNA on the injected side

(Figure 5: A and A'). Out of 17 electroporated embryos, 4 showed no difference, 7 showed a slight deficit, and 6 showed a severe deficit of Sox10 mRNA on the injected side of the embryo. Control morpholino electroporated embryos from the same batch showed only slight deficits in 2/22 emryos on the injected side and none with severe deficits (Figure 5: B and B'). Contingency testing on this data set using Prism software gave a Chi-square value of 19.12 with a p value of <0.0001, a statistically significant difference.

When Snail2 was examined by in situ hybridization on Adh5 morpholino injected embryos, there was a significant reduction of Snail2 mRNA on the injected side. Out of 13 electroporated embryos, 5 showed no difference, 5 showed a slight deficit, and 3 showed a severe deficit of Snail2 mRNA on the injected side of the embryo (Figure 5: E and E'). This result is validated through comparison to embryos electroporated with a control morpholino at the same time, which showed slight deficits on the injected side in only two out of 17 embryos and no embryos exhibiting severe deficits (Figure 5: F and F'). Contingency testing on this data set using Prism software gave a Chi-square value of 10.06 with a p value of 0.0065, a statistically significant difference.

Finally, embryos injected with the Adh5 morpholino were examined for FoxD3 expression by in situ hybridization. This also showed a significant reduction of FoxD3 mRNA on the injected side. Out of 12 electroporated embryos, 5 showed no difference, 4 showed a slight deficit, and 3 showed a severe deficit of FoxD3 mRNA on the injected side of the embryo (Figure 5: C and C'). In control morpholino electroporated embryos

performed concurrently, slight deficits on the injected side were observed in only one out of 16 embryos, with none exhibiting severe deficits (Figure 5: D and D'). Contingency testing on this data set using Prism software gave a Chi-square value of 9.421 with a p value of 0.009, a statistically significant difference.

3.5: Discussion

Here we have demonstrated the functional relevance of two of the genes we discovered in our screen for novel neural crest effectors. When we began to study induced populations of neural crest cells, we used an *in vitro* method that has been used for many years and across several organisms. This culture method, in which non-neural ectoderm and intermediate neural plate are co-cultured, produces cells that are considered to be neural crest as they are migratory, and HNK-1 positive. As we discovered the involvement of several other transcription factors in neural crest cell development, we further reaffirmed the upregulation in the co-cultured tissues of several transcription factors, including Ccar1, by QPCR. These results not only confirm the relative levels of upregulation seen in the cultures, giving us an idea of the sensitivity of our screen, but also further validates this culture method as one that produces neural crest cells that approach those seen *in vivo*.

The genes that we chose to examine further, Ccar1 and Adh5, have expression patterns that confirm a spatiotemporal mRNA presence that would support their involvement in

the developing neural crest (despite earlier reports that indicated the ubiquitous presence of Adh5 in the developing embryo). We have further examined both Ccar1 and Adh5 for functional relevance in the neural crest cells through morpholino knockdown. We have found that the loss of Ccar1 or Adh5 causes a loss of the mRNA expression of a neural crest specifier gene, *Sox10,* as compared to the contralateral (uninjected) side of the embryo or embryos injected with a control morpholino. Furthermore, the loss of Adh5 through morpholino-mediated knockdown results in the loss of the mRNA expression of two other neural crest specifier genes, *Snail2* and *FoxD3*, on the injected side as compared to the aforementioned controls. Upon closer examination through Tunel staining, we have preliminary data that suggests that the loss of *Sox10* mRNA in Ccar1-morpholino injected sites of the embryo is most likely due to cell death.

Although our understanding of the root causes of the changes observed in these morpholinated embryos is not yet complete, we know that the described differences in these embryos are statistically significant, indicating the functional relevance of these genes. With continued experimentation, we should be able to firmly seat both of these genes and their effects in our understanding of the way in which neural crest cells develop, and in the genetic network controlling neural crest cells.

Figure 1: Ccar1 expression

Ccar1 mRNA is present in stage 4 and 5 embryos in the neural plate border, in the dorsal neural folds of stage 7–9 embryos, and in the dorsal neural folds and migrating neural crest of older embryos.

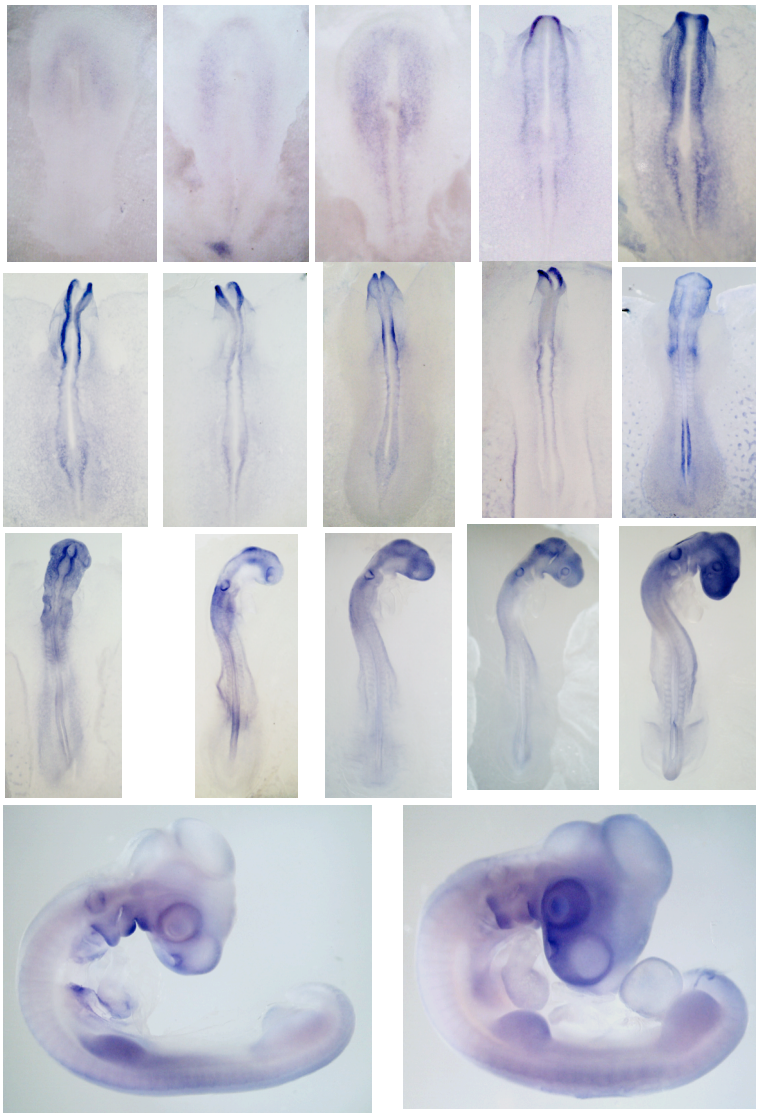


Figure 2: Ccar1 in situs with HNK1 overlay and QPCR of *in vitro* cultures

Ccar1 in situs of st 10 (A) and stage 12 (B) embryos are shown along with a section through the older embryo (C). A closeup of HNK-1 immunostaining of the same section shown in (C) is seen in (D). (E) QPCR in vitro conjugates as compared to individually cultured tissue pieces: an average conjugate value of 0.777, standard deviation 0.104 and an average non-conjugate value of 0.464, standard deviation 0.149. The student t-test value is significant, with p = 0.0405.

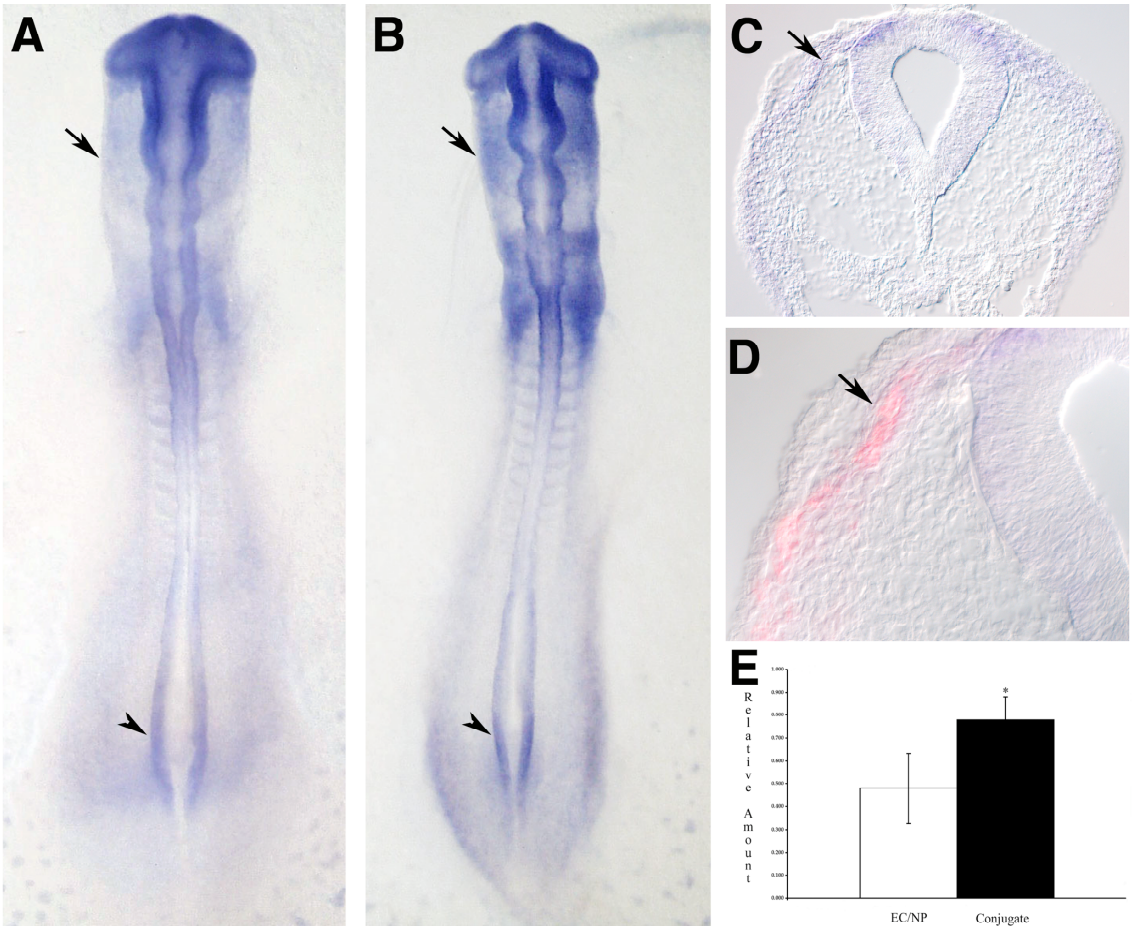


Figure 3: Morpholino knockdown of Ccar1, Sox10 in situ, and Tunel staining

(A) Sox10 in situs of embryos injected with Ccar1 morpholino (a and c) while the distribution of the lissaminated morpholino is shown in the adjoining photograph (b and d). (B) Tunel staining showing the difference between cell death in sections from Carp-1 (a–c) and (d–f) control morpholino injected embryos. Adjoining photos show DAPI staining and the morpholino distribution.

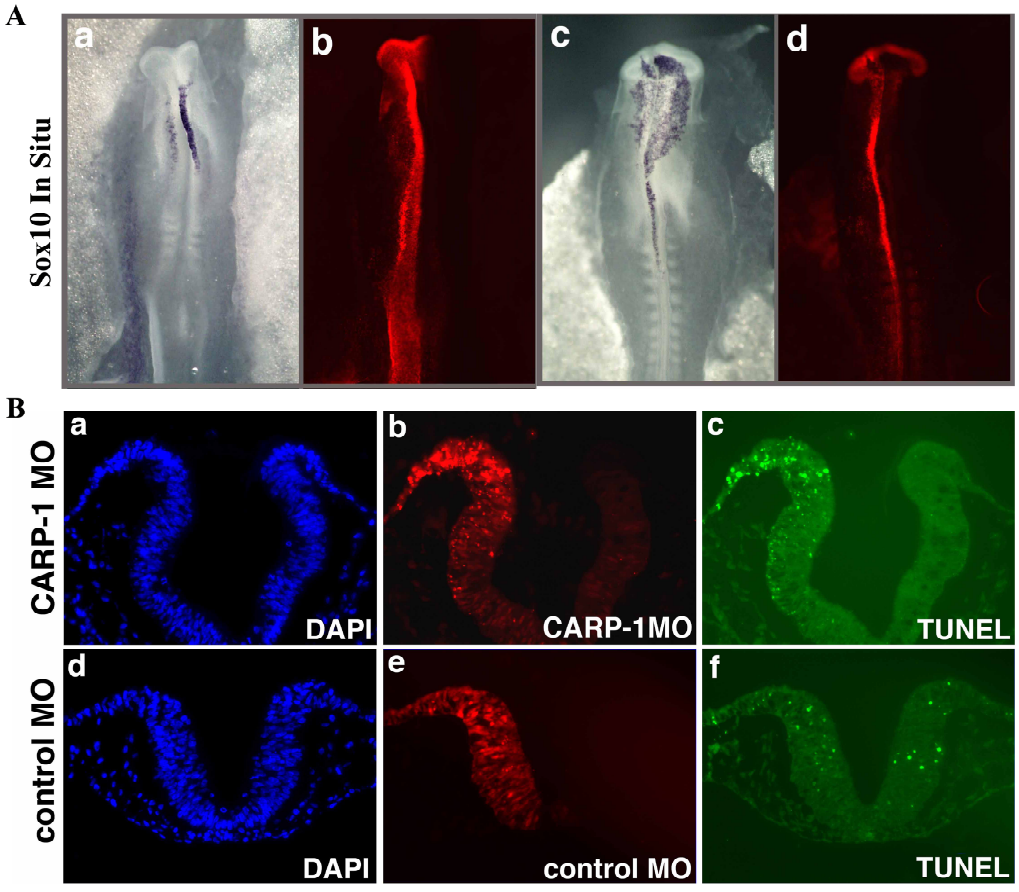


Figure 4: In situs of ADH5

In situ hybridizations of Adh5 mRNA reveal its specific expression in the dorsal neural folds and the migrating neural crest cells. Below are embryos representing stages 8–15 of chicken development.

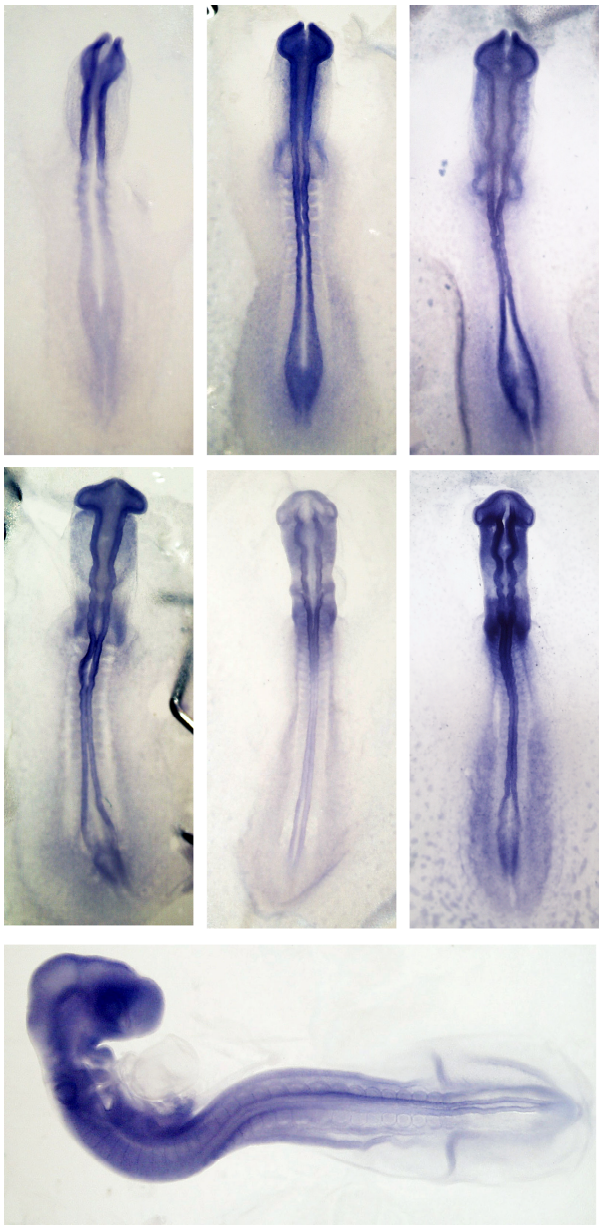
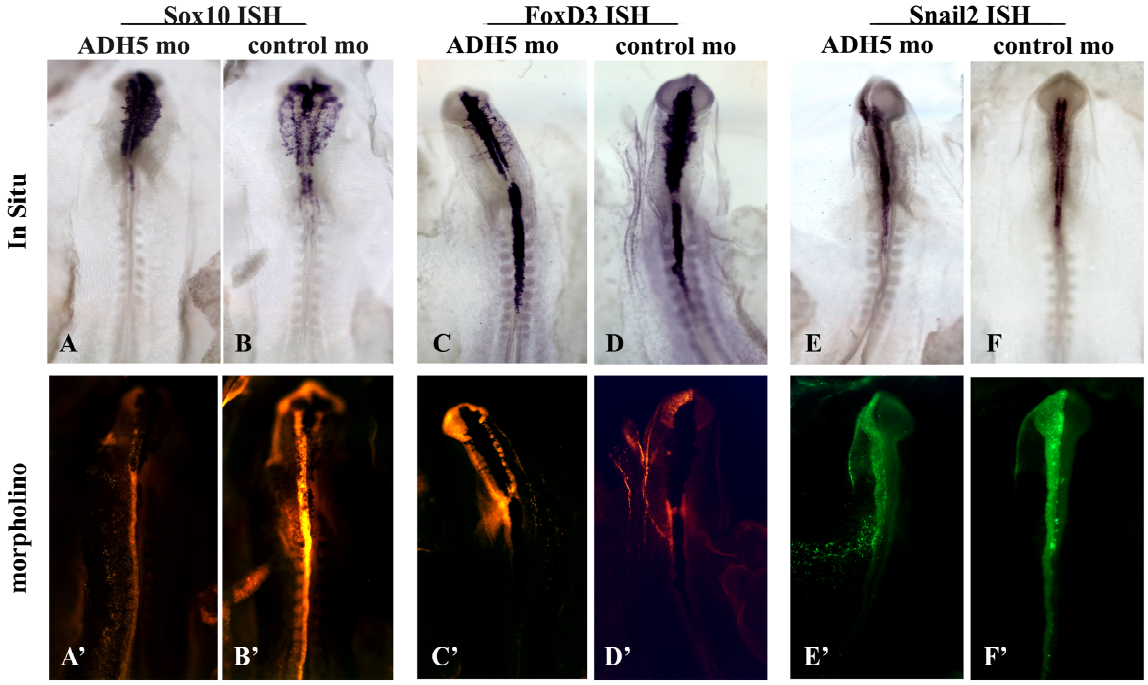


Figure 5: Morpholino knockdown of ADH5, Sox10, slug, and foxd3 in situ

Significant differences were seen in embryos injected with ADH5 morpholino and control morpholino by Sox10, FoxD3, and Snail2 in situ hybridizations.



Adams, M. S., L. S. Gammill, et al. (2008). "Discovery of transcription factors and other candidate regulators of neural crest development." Dev Dyn **237**(4): 1021-33.

Anantharaman, V. and L. Aravind (2008). "Analysis of DBC1 and its homologs suggests a potential mechanism for regulation of sirtuin domain deacetylases by NAD metabolites." Cell Cycle **7**(10): 1467-72.

Aravind, L. and E. V. Koonin (2000). "SAP - a putative DNA-binding motif involved in chromosomal organization." Trends Biochem Sci **25**(3): 112-4.

Beisswenger, T. B., B. Holmquist, et al. (1985). "chi-ADH is the sole alcohol dehydrogenase isozyme of mammalian brains: implications and inferences." Proc Natl Acad Sci U S A **82**(24): 8369-73.

Canestro, C., R. Albalat, et al. (2002). "Ascidian and amphioxus Adh genes correlate functional and molecular features of the ADH family expansion during vertebrate evolution." J Mol Evol **54**(1): 81-9.

Dickinson, M. E., M. A. Selleck, et al. (1995). "Dorsalization of the neural tube by the non-neural ectoderm." Development **121**(7): 2099-106.

Edenberg, H. J., C. J. Brown, et al. (1991). "Alcohol dehydrogenase gene expression and cloning of the mouse chi-like ADH." Adv Exp Med Biol **284**: 253-62.

Gammill, L. S. and M. Bronner-Fraser (2002). "Genomic analysis of neural crest induction." Development **129**(24): 5731-41.

Giri, P. R., J. F. Krug, et al. (1989). "Cloning and comparative mapping of a human class III (chi) alcohol dehydrogenase cDNA." Biochem Biophys Res Commun **164**(1): 453-60.

Giri, P. R., M. Linnoila, et al. (1989). "Distribution and possible metabolic role of class III alcohol dehydrogenase in the human brain." Brain Res **481**(1): 131-41.

Gonzalez-Duarte, R. and R. Albalat (2005). "Merging protein, gene and genomic data: the evolution of the MDR-ADH family." Heredity **95**(3): 184-97.

Gutheil, W. G., B. Holmquist, et al. (1992). "Purification, characterization, and partial sequence of the glutathione-dependent formaldehyde dehydrogenase from Escherichia coli: a class III alcohol dehydrogenase." Biochemistry **31**(2): 475-81.

Hempel, J. D. and R. Pietruszko (1979). "Human stomach alcohol dehydrogenase: isoenzyme composition and catalytic properties." Alcohol Clin Exp Res **3**(2): 95-8.

Hsu, C. A., A. K. Rishi, et al. (1997). "Retinoid induced apoptosis in leukemia cells through a retinoic acid nuclear receptor-independent pathway." Blood **89**(12): 4470-9.

Hur, M. W. and H. J. Edenberg (1992). "Cloning and characterization of the ADH5 gene encoding human alcohol dehydrogenase 5, formaldehyde dehydrogenase." Gene **121**(2): 305-11.

Iborra, F. J., J. Renau-Piqueras, et al. (1992). "Immunocytochemical and biochemical demonstration of formaldhyde dehydrogenase (class III alcohol dehydrogenase) in the nucleus." J Histochem Cytochem **40**(12): 1865-78.

Kaiser, R., M. R. Fernandez, et al. (1993). "Origin of the human alcohol dehydrogenase system: implications from the structure and properties of the octopus protein." Proc Natl Acad Sci U S A **90**(23): 11222-6.

Kaiser, R., B. Holmquist, et al. (1988). "Class III human liver alcohol dehydrogenase: a novel structural type equidistantly related to the class I and class II enzymes." Biochemistry **27**(4): 1132-40.

Kim, J. H., C. K. Yang, et al. (2008). "CCAR1, a key regulator of mediator complex recruitment to nuclear receptor transcription complexes." Mol Cell **31**(4): 510-9.

Koivusalo, M., M. Baumann, et al. (1989). "Evidence for the identity of glutathione-dependent formaldehyde dehydrogenase and class III alcohol dehydrogenase." FEBS Lett **257**(1): 105-9.

Lee, D. K., D. Suh, et al. (2002). "POZ domain transcription factor, FBI-1, represses transcription of ADH5/FDH by interacting with the zinc finger and interfering with DNA binding activity of Sp1." J Biol Chem **277**(30): 26761-8.

Liang, J. Y., J. A. Fontana, et al. (1999). "Synthetic retinoid CD437 induces S-phase arrest and apoptosis in human prostate cancer cells LNCaP and PC-3." Prostate **38**(3): 228-36.

Meera Khan, P., L. M. Wijnen, et al. (1984). "Human formaldehyde dehydrogenase (FDH) and its assignment to chromosome 4." Cytogenet Cell Genet **38**(2): 112-5.

Pares, X. and B. L. Vallee (1981). "New human liver alcohol dehydrogenase forms with unique kinetic characteristics." Biochem Biophys Res Commun **98**(1): 122-30.

Rishi, A. K., L. Zhang, et al. (2003). "Identification and characterization of a cell cycle and apoptosis regulatory protein-1 as a novel mediator of apoptosis signaling by retinoid CD437." J Biol Chem **278**(35): 33422-35.

Rishi, A. K., L. Zhang, et al. (2006). "Cell cycle- and apoptosis-regulatory protein-1 is involved in apoptosis signaling by epidermal growth factor receptor." J Biol Chem **281**(19): 13188-98.

Shao, Z. M., M. I. Dawson, et al. (1995). "p53 independent G0/G1 arrest and apoptosis induced by a novel retinoid in human breast cancer cells." Oncogene **11**(3): 493-504.

Sharma, C. P., E. A. Fox, et al. (1989). "cDNA sequence of human class III alcohol dehydrogenase." Biochem Biophys Res Commun **164**(2): 631-7.

Smith, M. (1986). "Genetics of human alcohol and aldehyde dehydrogenases." Adv Hum Genet **15**: 249-90.

Suske, G. (1999). "The Sp-family of transcription factors." Gene **238**(2): 291-300.

Uotila, L. and M. Koivusalo (1974). "Formaldehyde dehydrogenase from human liver. Purification, properties, and evidence for the formation of glutathione thiol esters by the enzyme." J Biol Chem **249**(23): 7653-63.

Wagner, F. W., X. Pares, et al. (1984). "Physical and enzymatic properties of a class III isozyme of human liver alcohol dehydrogenase: chi-ADH." Biochemistry **23**(10): 2193-9.

Xu, Q. a. W., D.G., Ed. (1992). In situ hybridization: A practical approach, Oxford University Press.

Zhang, L., E. Levi, et al. (2007). "Transactivator of transcription-tagged cell cycle and apoptosis regulatory protein-1 peptides suppress the growth of human breast cancer cells in vitro and in vivo." Mol Cancer Ther **6**(5): 1661-72.

Chapter IV: Summary and future directions

The importance and relevance of studying the neural crest cells

Arising concurrently with the vertebrate lineage, neural crest cells cells were largely responsible for the development of the "new head" and "new neck" of these animals as well as the predatory behaviors that follow from these morphological changes (Gans and Northcutt 1983) (Kuratani 2008). Indeed, much of the diversity immediately visible not only in humans, but in such animals as Darwin's finches (where beak shape and size determines the niches they may occupy) is due to the control of neural crest cells that form the bones and cartilage of the face, as well as other determinants such as melanocytes. Crest cells, which form at the border between the neural and non-neural ectoderm, are unique in that they are able to migrate great distances along stereotypical paths before giving rise to derivatives that are far flung and incredibly diverse (see Chapter 1 for a more comprehensive overview) (Knecht and Bronner-Fraser 2002; Le Douarin, Brito et al. 2007). As we study the neural crest cells, each piece of data allows us to see a bit more into the intricate levels of control that allow this population to achieve its tasks in the developing embryo, and later during processes such as types of wound healing in adults. In fact, our understanding is thus not restricted to the developmental potential of these cells, but also informs our knowledge of other self-renewing populations such as somatic stem cells and cancers. In all these cases, the potential for translational approaches is great, as the genes that control the neural crest may ultimately represent therapeutic targets.

Pathologies that involve the neural crest are astounding, due to their rates of proliferation, their ability to metastasize, and their resiliency against different treatment schemes. While a great deal has been learned about the neural crest and related pathologies, many questions remain. These will continue to inspire researchers and clinicians to study the formation and differentiation of these complex cells and tissues. As new results come from genomic screens and evolutionary analyses of neural crest cells, medical researchers will benefit from new markers and the discovery of new genetic pathways. Analysis of these genes as well as NCSCs may lead to better understandings of why certain cancers, including those of the endocrine system, are so persistent, and subsequently lead to new approaches in diagnosis and treatment.

Identification of new genes involved in the neural crest

This thesis project was designed to discover new genes important in the induction and migration of neural crest cells, and to validate a selected subset of those genes through functional perturbation. Previous studies have laid the groundwork for understanding the control of neural crest cells (Mayor, Morgan et al. 1995; Mayor and Aybar 2001) (Meulemans and Bronner-Fraser 2004) (Steventon, Carmona-Fontaine et al. 2005). One network module is composed of transcriptional regulators seen in early migrating and/or premigratory neural crest. This grouping includes *Sox9*, *Sox10*, *FoxD3*, *Snail2/Slug*, *c-Myc,* and *AP2* and has been termed the "neural crest specifier genes" (Meulemans and Bronner-Fraser 2004) (Spokony, Aoki et al. 2002) (Honore, Aybar et al. 2003) (Sasai, Mizuseki et al. 2001) (Aoki, Saint-Germain et al. 2003) (Luo, Lee et al. 2003)

(Bellmeyer, Krase et al. 2003). For all of the excellent work that has been done, however, we are still far from a complete picture of the neural crest cell and the genetic control that it is under.

Thus, we performed a screen based on a subtraction scheme in which an *in vitro* culture system known to give rise to crest was compared to its component parts. The subtracted and unsubtracted pools of cDNA were sequentially hybridized against a macroarrayed library generated from 8–12 somite stage chicken embryos, and the selected clones were catalogued. In the initial analysis of this work, a large number of genes with possible importance in premigratory and migratory neural crest were identified. (Gammill and Bronner-Fraser 2002). Despite this surge of new candidates to analyze, this initial analysis only revealed two transcription factors previously unassociated with the neural crest. Since transcription factors are generally upstream of other factors present in these networks, and as these upstream genes are critically important when attempting to build testable gene regulatory networks, we set about trying to unmask more of them (Davidson 2006) (Ben-Tabou de-Leon and Davidson 2007). Since transcription factors are generally upstream of other factors present in these networks, and are thus generally not as abundant at the transcriptional level, we reasoned that they would be represented by fewer library clones and weaker hybridization signals in the screen. While this differential hybridization signal is certainly not a direct measure of gene expression levels but rather reflects the efficiency of subtraction for that gene. Nonetheless, we decided to revisit the screen results using different selection criteria to see if we might discover additional new genes, and amongst them, more transcriptional regulators. As initially

only a sampling of upregulated clones was examined, we opted to comprehensively analyze all clones with a two-fold or greater hybridization signal from the subtracted probe as compared to the unsubtracted probe (Gammill and Bronner-Fraser 2002).

Therefore, we examined a total of 553 clones for a total of 223 genes with possible implications in the development of neural crest cells. The results of sequencing analysis allowed us to blast search for gene identity against the newly released chicken genome. The identification of clones by blast permitted us to search for background information about these genes, which directed our focus to those genes and gene classes we found to be particularly interesting. We then performed in situ hybridization on chicken embryos of different ages to examine the spatiotemporal pattern of 112 different genes' mRNA including all identified transcription factors and several unknown genes (summarized in Table 1, Chapter 2). Our screen was highly successful as more than 90% of the *in situ* patterns revealed expression in the premigratory and/or migratory neural crest cells. Remarkably, our attempt to identify new transcription factors with expression in the neural crest was also successful. Indeed, 14 transcription factors (*Ccar1*, *CTCF*, *Elk-3*, *GCNF*, *GTFIIE*, *ILF2*, *ILF3*, *PhD*, *SRF*, *SREBP2*, *Similar to Tcf20/AR1/SPBP*, *WHSC1L1*, *ZFP3*, and *ZFP469)* were all expressed in premigratory neural crest cells within the neural folds and/or migrating neural crest cells moving through the periphery.

In order to understand the sensitivity of our screen more precisely, we further characterized the upregulation of several of the transcription factors that arose from this screen and in so doing we also aided in the definition of the *in vitro* culture system used

as its basis. All of the transcription factors that were analyzed by QPCR showed significant upregulation in the non-neural ectoderm and intermediate neural plate conjugates when compared to those tissues cultured separately. Snail2/Slug levels in the conjugated tissues were used as a positive control and were consistently upregulated by 5–7 fold (Chapter 2, Figure 2 and Table 2). The other transcription factors identified in our screen were upregulated approximately two-fold in the conjugates by QPCR (statistically significant and reproducible across replicates and experiments) (Chapter 2, Figs. 2-6, Table 2 and Chapter 3, Figure 2).

Determining relevance of two newly identified genes through functional perturbation

As one of our goals with this thesis was to understand more about the impact of the identified genes on neural crest cell development, we performed loss-of-function analyses of two of these genes using morpholino oligos. To that end, we performed in situ hybridizations for a panel of three different neural crest specifiers, *Sox10*, *Snail2* (*slug*), and *FoxD3,* on morpholinated embryos.

Ccar1 (Carp-1) is a transcription factor that piqued our interest when we analyzed it by in situ and by QPCR. It was concurrently identified by another screen in our lab that was attempting to identify genes important in neural crest cells through analysis of genes

known to be important in hermaphrodite specific neuron (HSN) migration in *Caenorhabditis elegans*. Therefore, Ccar1 was a good candidate for functional perturbation. When we injected morpholinos designed against the start site of Ccar1, we were able to see significant differences between the injected and non-injected sides of the embryo through examination of *Sox10* expression by in situ hybridization. Additional analysis through Tunel staining revealed that this effect is likely due to an increase in cell death on the injected side. We were initially surprised to see this increase in cell death, as Ccar1 is mostly considered to be an inducer of apoptosis, and its loss might be considered to be anti-apoptotic. However, it has recently been discovered that Ccar1 has also been shown to act as a coactivator for several other classes of transcription factors as it is important in the recruitment of the Mediator complex to their target genes. As an example, it was convincingly demonstrated that Ccar1 has a role in estrogen-induced cell proliferation (Kim, Yang et al. 2008). With the complexity of the processes occurring in the embryo and the amount of regulation between different genes, it is impossible to predict exactly what the results might be from an experimental perturbation. It is exactly for this reason that functional experiments are interesting as they can truly reveal what it happening in the embryo itself.

We also investigated the functional importance of Adh5 (alcohol dehydrogenase 5, formaldehyde dehydrogenase) in neural crest cells of the developing chicken. This gene is highly evolutionarily conserved and previously reported to be the only member of its family present in the brain. While it has also been reported to be expressed widely in the embryo, we find that in the early stages of chicken development the mRNA is expressed

quite specifically in the dorsal neural folds and the migrating neural crest. When we injected morpholinos designed against the 5'UTR region of Adh5, we were able to see significant differences between the injected and non-injected sides of the embryos through examination of *Sox10*, *FoxD3*, and *Snail2* (Slug) expression by in situ hybridization. We look forward to examining these embryos for the root cause of these changes, and discovering what partners of Adh5 might be involved in this phenotype.

Further directions

Future work on this project would necessarily include trying to form a deeper understanding of the mechanism by which the knockdown of Adh5 affects the expression of certain neural crest genes (Sox10, FoxD3, and Snail2) on the injected side. First, we would like to examine the pattern of HNK-1 in these embryos through the use of an antibody. This will give us a better indication as to whether the mRNA of the markers is missing, or if there is a loss of neural crest cells on the injected side. If there are fewer neural crest cells on the injected side, additional analysis though Tunel and phosphohistone H3 staining will reveal whether the decrease is due to cell death or to a decreased amount of proliferation (or combination of the two).

Rescue experiment would also be important; we will test whether overexpression of a construct containing the gene of interest (Adh5 or Ccar1) is sufficient to promote expression recovery of the neural crest specifiers. This will allow us to say that the knockdown and subsequent phenotypic recovery is entirely based on the absence or

presence of the gene in question. We will further make use of this construct in overexpression experiments to determine the effect of adding more of the gene products in areas where they are expressed endogenously as well as ectopically.

In the long term, it will also be important to assess the other genes known to have neural crest cell involvement for their functional effects, and to be able to insert them into the neural crest gene network. It will be exciting to see the genes discussed in our screen (Chapter 2) continue to follow this path and to see their relevance determined. Finally, this future expansion of the neural crest gene network will hopefully have far reaching consequences in terms of understanding NCSC development, their use for therapeutic regeneration, and an even greater ability to devise treatments for pathologies involving their derivatives.

Adams, M. S., L. S. Gammill, et al. (2008). "Discovery of transcription factors and other candidate regulators of neural crest development." Dev Dyn **237**(4): 1021-33.

Agoff, S. N., L. W. Lamps, et al. (2000). "Thyroid transcription factor-1 is expressed in extrapulmonary small cell carcinomas but not in other extrapulmonary neuroendocrine tumors." Mod Pathol **13**(3): 238-42.

Ahmed, S. and A. Nawshad (2007). "Complexity in interpretation of embryonic epithelial-mesenchymal transition in response to transforming growth factor-beta signaling." Cells Tissues Organs **185**(1-3): 131-45.

Aho, S., W. H. McLean, et al. (1998). "cDNA cloning, mRNA expression, and chromosomal mapping of human and mouse periplakin genes." Genomics **48**(2): 242-7.

Akhmametyeva, E. M., M. M. Mihaylova, et al. (2006). "Regulation of the Neurofibromatosis 2 gene promoter expression during embryonic development." Dev Dyn **235**(10): 2771-2785.

Akitaya, T. and M. Bronner-Fraser (1992). "Expression of cell adhesion molecules during initiation and cessation of neural crest cell migration." Dev Dyn **194**(1): 12-20.

Al-Rawi, M. and M. H. Wheeler (2006). "Medullary thyroid carcinoma--update and present management controversies." Ann R Coll Surg Engl **88**(5): 433-8.

Alberti, S., S. M. Krause, et al. (2005). "Neuronal migration in the murine rostral migratory stream requires serum response factor." Proc Natl Acad Sci U S A **102**(17): 6148-53.

Alexander, C. M. and Z. Werb (1992). "Targeted disruption of the tissue inhibitor of metalloproteinases gene increases the invasive behavior of primitive mesenchymal cells derived from embryonic stem cells in vitro." J Cell Biol **118**(3): 727-39.

Anantharaman, V. and L. Aravind (2008). "Analysis of DBC1 and its homologs suggests a potential mechanism for regulation of sirtuin domain deacetylases by NAD metabolites." Cell Cycle **7**(10): 1467-72.

Anderson, D. J. (1993). "Molecular control of cell fate in the neural crest: the sympathoadrenal lineage." Annu Rev Neurosci **16**: 129-58.

Anderson, D. J. (1994). "Stem cells and transcription factors in the development of the mammalian neural crest." Faseb J **8**(10): 707-13.

Ansieau, S., J. Bastid, et al. (2008). "Induction of EMT by twist proteins as a collateral effect of tumor-promoting inactivation of premature senescence." Cancer Cell **14**(1): 79-89.

Aoki, Y., N. Saint-Germain, et al. (2003). "Sox10 regulates the development of neural crest-derived melanocytes in Xenopus." Dev Biol **259**(1): 19-33.

Aravind, L. and E. V. Koonin (2000). "SAP - a putative DNA-binding motif involved in chromosomal organization." Trends Biochem Sci **25**(3): 112-4.

Aubry, M., S. Demczuk, et al. (1993). "Isolation of a zinc finger gene consistently deleted in DiGeorge syndrome." Hum Mol Genet **2**(10): 1583-7.

Ayadi, A., H. Zheng, et al. (2001). "Net-targeted mutant mice develop a vascular phenotype and up-regulate egr-1." Embo J **20**(18): 5139-52.

Badner, J. A., W. K. Sieber, et al. (1990). "A genetic study of Hirschsprung disease." Am J Hum Genet **46**(3): 568-80.

Baker, C. V. and M. Bronner-Fraser (1997). "The origins of the neural crest. Part I: embryonic induction." Mech Dev **69**(1-2): 3-11.

Baldini, A. (2002). "DiGeorge syndrome: the use of model organisms to dissect complex genetics." Hum Mol Genet **11**(20): 2363-9.

Baroffio, A., E. Dupin, et al. (1988). "Clone-forming ability and differentiation potential of migratory neural crest cells." Proc Natl Acad Sci U S A **85**(14): 5325-9.

Baroffio, A., E. Dupin, et al. (1991). "Common precursors for neural and mesectodermal derivatives in the cephalic neural crest." Development **112**(1): 301-5.

Barreto, G., W. Reintsch, et al. (2003). "The function of Xenopus germ cell nuclear factor (xGCNF) in morphogenetic movements during neurulation." Dev Biol **257**(2): 329-42.

Barrionuevo, F., A. Naumann, et al. (2008). "Sox9 is required for invagination of the otic placode in mice." Dev Biol **317**(1): 213-24.

Basch, M. L., M. Bronner-Fraser, et al. (2006). "Specification of the neural crest occurs during gastrulation and requires Pax7." Nature **441**(7090): 218-22.

Batlle, E., E. Sancho, et al. (2000). "The transcription factor snail is a repressor of E-cadherin gene expression in epithelial tumour cells." Nat Cell Biol **2**(2): 84-9.

Beisswenger, T. B., B. Holmquist, et al. (1985). "chi-ADH is the sole alcohol dehydrogenase isozyme of mammalian brains: implications and inferences." Proc Natl Acad Sci U S A **82**(24): 8369-73.

Bellmeyer, A., J. Krase, et al. (2003). "The protooncogene c-myc is an essential regulator of neural crest formation in xenopus." Dev Cell **4**(6): 827-39.

Ben-Tabou de-Leon, S. and E. H. Davidson (2007). "Gene regulation: gene control network in development." Annu Rev Biophys Biomol Struct **36**: 191.

Bergemann, A. D., F. Cole, et al. (2005). "The etiology of Wolf-Hirschhorn syndrome." Trends Genet **21**(3): 188-95.

Biddinger, P. W. and M. Ray (1993). "Distribution of C cells in the normal and diseased thyroid gland." Pathol Annu **28 Pt 1**: 205-29.

Bjarnadottir, T. K., R. Fredriksson, et al. (2004). "The human and mouse repertoire of the adhesion family of G-protein-coupled receptors." Genomics **84**(1): 23-33.

Bolos, V., H. Peinado, et al. (2003). "The transcription factor Slug represses E-cadherin expression and induces epithelial to mesenchymal transitions: a comparison with Snail and E47 repressors." J Cell Sci **116**(Pt 3): 499-511.

Boyer, A. S., Ayerinskas, II, et al. (1999). "TGFbeta2 and TGFbeta3 have separate and sequential activities during epithelial-mesenchymal cell transformation in the embryonic heart." Dev Biol **208**(2): 530-45.

Britsch, S., D. E. Goerich, et al. (2001). "The transcription factor Sox10 is a key regulator of peripheral glial development." Genes Dev **15**(1): 66-78.

Brodsky, G., T. Barnes, et al. (1997). "The human GARS-AIRS-GART gene encodes two proteins which are differentially expressed during human brain development and temporally overexpressed in cerebellum of individuals with Down syndrome." Hum Mol Genet **6**(12): 2043-50.

Bronner-Fraser, M. (1986). "Analysis of the early stages of trunk neural crest migration in avian embryos using monoclonal antibody HNK-1." Dev Biol **115**(1): 44-55.

Bronner-Fraser, M. and S. Fraser (1989). "Developmental potential of avian trunk neural crest cells in situ." Neuron **3**(6): 755-66.

Bronner-Fraser, M. and S. E. Fraser (1988). "Cell lineage analysis reveals multipotency of some avian neural crest cells." Nature **335**(6186): 161-4.

Bronner-Fraser, M., M. Sieber-Blum, et al. (1980). "Clonal analysis of the avian neural crest: migration and maturation of mixed neural crest clones injected into host chicken embryos." J Comp Neurol **193**(2): 423-34.

Brookes, M. J., S. Hughes, et al. (2006). "Modulation of iron transport proteins in human colorectal carcinogenesis." Gut **55**(10): 1449-60.

Brown, C. B., A. S. Boyer, et al. (1996). "Antibodies to the Type II TGFbeta receptor block cell activation and migration during atrioventricular cushion transformation in the heart." Dev Biol **174**(2): 248-57.

Brown, C. B., A. S. Boyer, et al. (1999). "Requirement of type III TGF-beta receptor for endocardial cell transformation in the heart." Science **283**(5410): 2080-2.

Bryant, J., J. Farmer, et al. (2003). "Pheochromocytoma: the expanding genetic differential diagnosis." J Natl Cancer Inst **95**(16): 1196-204.

Bryce, N. S., G. Schevzov, et al. (2003). "Specification of actin filament function and molecular composition by tropomyosin isoforms." Mol Biol Cell **14**(3): 1002-16.

Buaas, F. W., K. Lee, et al. (1999). "Cloning and characterization of the mouse interleukin enhancer binding factor 3 (Ilf3) homolog in a screen for RNA binding proteins." Mamm Genome **10**(5): 451-6.

Buchwalter, G., C. Gross, et al. (2005). "The ternary complex factor Net regulates cell migration through inhibition of PAI-1 expression." Mol Cell Biol **25**(24): 10853-62.

Burke, L. J., T. Hollemann, et al. (2002). "Molecular cloning and expression of the chromatin insulator protein CTCF in Xenopus laevis." Mech Dev **113**(1): 95-8.

Burstyn-Cohen, T., J. Stanleigh, et al. (2004). "Canonical Wnt activity regulates trunk neural crest delamination linking BMP/noggin signaling with G1/S transition." Development **131**(21): 5327-39.

Bussolati, G. and A. G. Pearse (1967). "Immunofluorescent localization of calcitonin in the 'C' cells of pig and dog thyroid." J Endocrinol **37**(2): 205-9.

Canestro, C., R. Albalat, et al. (2002). "Ascidian and amphioxus Adh genes correlate functional and molecular features of the ADH family expansion during vertebrate evolution." J Mol Evol **54**(1): 81-9.

Cano, A., M. A. Perez-Moreno, et al. (2000). "The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression." Nat Cell Biol **2**(2): 76-83.

Cebra-Thomas, J. A., E. Betters, et al. (2007). "Evidence that a late-emerging population of trunk neural crest cells forms the plastron bones in the turtle Trachemys scripta." Evol Dev **9**(3): 267-77.

Chai, Y. and R. E. Maxson, Jr. (2006). "Recent advances in craniofacial morphogenesis." Dev Dyn **235**(9): 2353-75.

Char, F. (1978). "Peculiar facies with short philtrum, duck-bill lips, ptosis and low-set ears--a new syndrome?" Birth Defects Orig Artic Ser **14**(6B): 303-5.

Chen, G. C., B. Turano, et al. (2005). "Regulation of Rho and Rac signaling to the actin cytoskeleton by paxillin during Drosophila development." Mol Cell Biol **25**(3): 979-87.

Chen, Y. T., P. O. Akinwunmi, et al. (2007). "Generation of a Twist1 conditional null allele in the mouse." Genesis **45**(9): 588-92.

Chen, Z. F. and R. R. Behringer (1995). "twist is required in head mesenchyme for cranial neural tube morphogenesis." Genes Dev **9**(6): 686-99.

Cheung, M., M. C. Chaboissier, et al. (2005). "The transcriptional control of trunk neural crest induction, survival, and delamination." Dev Cell **8**(2): 179-92.

Chibon, P. (1967). "[Nuclear labelling by tritiated thymidine of neural crest derivatives in the amphibian Urodele Pleurodeles waltlii Michah]." J Embryol Exp Morphol **18**(3): 343-58.

Chisaka, O. and M. R. Capecchi (1991). "Regionally restricted developmental defects resulting from targeted disruption of the mouse homeobox gene hox-1.5." Nature **350**(6318): 473-9.

Chou, D. K., M. Schachner, et al. (2002). "HNK-1 sulfotransferase null mice express glucuronyl glycoconjugates and show normal cerebellar granule neuron migration in vivo and in vitro." J Neurochem **82**(5): 1239-51.

Clark, K., G. Bender, et al. (2001). "Evidence for the neural crest origin of turtle plastron bones." Genesis **31**(3): 111-7.

Coles, E., J. Christiansen, et al. (2004). "A vertebrate crossveinless 2 homologue modulates BMP activity and neural crest cell migration." Development **131**(21): 5309-17.

Coles, E. G., L. S. Gammill, et al. (2006). "Abnormalities in neural crest cell migration in laminin alpha5 mutant mice." Dev Biol **289**(1): 218-28.

Coles, E. G., L. A. Taneyhill, et al. (2007). "A critical role for Cadherin6B in regulating avian neural crest emigration." Dev Biol **312**(2): 533-44.

Colombatti, A., P. Bonaldo, et al. (1993). "Type A modules: interacting domains found in several non-fibrillar collagens and in other extracellular matrix proteins." Matrix **13**(4): 297-306.

Comijn, J., G. Berx, et al. (2001). "The two-handed E box binding zinc finger protein SIP1 downregulates E-cadherin and induces invasion." Mol Cell **7**(6): 1267-78.

Correia, A. C., M. Costa, et al. (2007). "Bmp2 is required for migration but not for induction of neural crest cells in the mouse." Dev Dyn **236**(9): 2493-501.

Couly, G. F., P. M. Coltey, et al. (1992). "The developmental fate of the cephalic mesoderm in quail-chick chimeras." Development **114**(1): 1-15.

Couly, G. F., P. M. Coltey, et al. (1993). "The triple origin of skull in higher vertebrates: a study in quail-chick chimeras." Development **117**(2): 409-29.

Couto, S. S. and R. D. Cardiff (2008). "The genomic revolution and endocrine pathology." Endocr Pathol **19**(3): 139-47.

Davidson, E. H. (2006). The regulatory genome : gene regulatory networks in development and evolution. Amsterdam ; Boston, Elsevier/Academic Press.

de Larco, J. E. and G. J. Todaro (1978). "Growth factors from murine sarcoma virus-transformed cells." Proc Natl Acad Sci U S A **75**(8): 4001-5.

DeLellis, R., Lloyd, RV, Heitz, PU, Eng, C. , Ed. (2004). Tumours of Endocrine Organs. Pathology and Genetics. Lyon, World Health Organization IARC Press.

Di Lauro, R. and M. De Felice (2001). Thyroid gland: anatomy and development. Philadelphia, Saunders.

Dickinson, M. E., M. A. Selleck, et al. (1995). "Dorsalization of the neural tube by the non-neural ectoderm." Development **121**(7): 2099-106.

Dixon, J., N. C. Jones, et al. (2006). "Tcof1/Treacle is required for neural crest cell formation and proliferation deficiencies that cause craniofacial abnormalities." Proc Natl Acad Sci U S A **103**(36): 13403-8.

Dixon, J., P. Trainor, et al. (2007). "Treacher Collins syndrome." Orthod Craniofac Res **10**(2): 88-95.

Dixon, M. J. (1996). "Treacher Collins syndrome." Hum Mol Genet **5 Spec No**: 1391-6.

Djinovic-Carugo, K., M. Gautel, et al. (2002). "The spectrin repeat: a structural platform for cytoskeletal protein assemblies." FEBS Lett **513**(1): 119-23.

Duband, J. L. and J. P. Thiery (1987). "Distribution of laminin and collagens during avian neural crest development." Development **101**(3): 461-78.

Duband, J. L., T. Volberg, et al. (1988). "Spatial and temporal distribution of the adherens-junction-associated adhesion molecule A-CAM during avian embryogenesis." Development **103**(2): 325-44.

Duong, T. D. and C. A. Erickson (2004). "MMP-2 plays an essential role in producing epithelial-mesenchymal transformations in the avian embryo." Dev Dyn **229**(1): 42-53.

Dutton, K. A., A. Pauliny, et al. (2001). "Zebrafish colourless encodes sox10 and specifies non-ectomesenchymal neural crest fates." Development **128**(21): 4113-25.

Edelman, G. M., W. J. Gallin, et al. (1983). "Early epochal maps of two different cell adhesion molecules." Proc Natl Acad Sci U S A **80**(14): 4384-8.

Edenberg, H. J., C. J. Brown, et al. (1991). "Alcohol dehydrogenase gene expression and cloning of the mouse chi-like ADH." Adv Exp Med Biol **284**: 253-62.

Eisenhofer, G., S. R. Bornstein, et al. (2004). "Malignant pheochromocytoma: current status and initiatives for future progress." Endocr Relat Cancer **11**(3): 423-36.

Eisenhofer, G., T. T. Huynh, et al. (2004). "Distinct gene expression profiles in norepinephrine- and epinephrine-producing hereditary and sporadic pheochromocytomas: activation of hypoxia-driven angiogenic pathways in von Hippel-Lindau syndrome." Endocr Relat Cancer **11**(4): 897-911.

el Ghouzzi, V., M. Le Merrer, et al. (1997). "Mutations of the TWIST gene in the Saethre-Chotzen syndrome." Nat Genet **15**(1): 42-6.

Endo, Y., N. Osumi, et al. (2002). "Bimodal functions of Notch-mediated signaling are involved in neural crest formation during avian ectoderm development." Development **129**(4): 863-73.

Endo, Y., N. Osumi, et al. (2003). "Deltex/Dtx mediates NOTCH signaling in regulation of Bmp4 expression in cranial neural crest formation during avian development." Dev Growth Differ **45**(3): 241-8.

Erickson, C. A., J. F. Loring, et al. (1989). "Migratory pathways of HNK-1-immunoreactive neural crest cells in the rat embryo." Dev Biol **134**(1): 112-8.

Erickson, C. A. and R. Perris (1993). "The role of cell-cell and cell-matrix interactions in the morphogenesis of the neural crest." Dev Biol **159**(1): 60-74.

Eroglu, B., G. Wang, et al. (2006). "Critical role of Brg1 member of the SWI/SNF chromatin remodeling complex during neurogenesis and neural crest induction in zebrafish." Dev Dyn **235**(10): 2722-35.

Eswarakumar, V. P., F. Ozcan, et al. (2006). "Attenuation of signaling pathways stimulated by pathologically activated FGF-receptor 2 mutants prevents craniosynostosis." Proc Natl Acad Sci U S A **103**(49): 18603-8.

Favier, R., K. Jondeau, et al. (2003). "Paris-Trousseau syndrome : clinical, hematological, molecular data of ten new cases." Thromb Haemost **90**(5): 893-7.

Fontaine, J. (1979). "Multistep migration of calcitonin cell precursors during ontogeny of the mouse pharynx." Gen Comp Endocrinol **37**(1): 81-92.

Freitas, R., G. Zhang, et al. (2006). "Developmental origin of shark electrosensory organs." Evol Dev **8**(1): 74-80.

Fujimoto, A., M. Lipson, et al. (1987). "New autosomal dominant branchio-oculo-facial syndrome." Am J Med Genet **27**(4): 943-51.

Funahashi, J., T. Okafuji, et al. (1999). "Role of Pax-5 in the regulation of a mid-hindbrain organizer's activity." Dev Growth Differ **41**(1): 59-72.

Gammill, L. S. and M. Bronner-Fraser (2002). "Genomic analysis of neural crest induction." Development **129**(24): 5731-41.

Gammill, L. S., C. Gonzalez, et al. (2006). "Guidance of trunk neural crest migration requires neuropilin 2/semaphorin 3F signaling." Development **133**(1): 99-106.

Gans, C. and R. G. Northcutt (1983). "Neural Crest and the Origin of Vertebrates: A New Head." Science **220**(4594): 268-273.

Garcia-Castro, M. I., C. Marcelle, et al. (2002). "Ectodermal Wnt function as a neural crest inducer." Science **297**(5582): 848-51.

Gessert, S., D. Maurus, et al. (2007). "Pescadillo is required for Xenopus laevis eye development and neural crest migration." Dev Biol **310**(1): 99-112.

Giehl, K. and A. Menke (2008). "Microenvironmental regulation of E-cadherin-mediated adherens junctions." Front Biosci **13**: 3975-85.

Gimm, O. and H. Dralle (1999). "C-cell cancer--prevention and treatment." Langenbecks Arch Surg **384**(1): 16-23.

Giovannini, M., J. A. Biegel, et al. (1994). "EWS-erg and EWS-Fli1 fusion transcripts in Ewing's sarcoma and primitive neuroectodermal tumors with variant translocations." J Clin Invest **94**(2): 489-96.

Giovannini, M., E. Robanus-Maandag, et al. (2000). "Conditional biallelic Nf2 mutation in the mouse promotes manifestations of human neurofibromatosis type 2." Genes Dev **14**(13): 1617-30.

Giri, P. R., J. F. Krug, et al. (1989). "Cloning and comparative mapping of a human class III (chi) alcohol dehydrogenase cDNA." Biochem Biophys Res Commun **164**(1): 453-60.

Giri, P. R., M. Linnoila, et al. (1989). "Distribution and possible metabolic role of class III alcohol dehydrogenase in the human brain." Brain Res **481**(1): 131-41.

Glaser, R. L., W. Jiang, et al. (2000). "Paternal origin of FGFR2 mutations in sporadic cases of Crouzon syndrome and Pfeiffer syndrome." Am J Hum Genet **66**(3): 768-77.

Gonzalez-Duarte, R. and R. Albalat (2005). "Merging protein, gene and genomic data: the evolution of the MDR-ADH family." Heredity **95**(3): 184-97.

Graff, J. R., J. G. Herman, et al. (1995). "E-cadherin expression is silenced by DNA hypermethylation in human breast and prostate carcinomas." Cancer Res **55**(22): 5195-9.

Graveson, A. C., M. M. Smith, et al. (1997). "Neural crest potential for tooth development in a urodele amphibian: developmental and evolutionary significance." Dev Biol **188**(1): 34-42.

Gross, J. B. and J. Hanken (2008). "Review of fate-mapping studies of osteogenic cranial neural crest in vertebrates." Dev Biol **317**(2): 389-400.

Gumbiner, B. M. (2005). "Regulation of cadherin-mediated adhesion in morphogenesis." Nat Rev Mol Cell Biol **6**(8): 622-34.

Gutheil, W. G., B. Holmquist, et al. (1992). "Purification, characterization, and partial sequence of the glutathione-dependent formaldehyde dehydrogenase from Escherichia coli: a class III alcohol dehydrogenase." Biochemistry **31**(2): 475-81.

Hadeball, B., A. Borchers, et al. (1998). "Xenopus cadherin-11 (Xcadherin-11) expression requires the Wg/Wnt signal." Mech Dev **72**(1-2): 101-13.

Hagel, M., E. L. George, et al. (2002). "The adaptor protein paxillin is essential for normal development in the mouse and is a critical transducer of fibronectin signaling." Mol Cell Biol **22**(3): 901-15.

Hahm, K. B., K. Cho, et al. (1999). "Repression of the gene encoding the TGF-beta type II receptor is a major target of the EWS-FLI1 oncoprotein." Nat Genet **23**(2): 222-7.

Hajra, K. M. and E. R. Fearon (2002). "Cadherin and catenin alterations in human cancer." Genes Chromosomes Cancer **34**(3): 255-68.

Hall, B. D., A. deLorimier, et al. (1983). "Brief clinical report: a new syndrome of hemangiomatous branchial clefts, lip pseudoclefts, and unusual facial appearance." Am J Med Genet **14**(1): 135-8.

Hall, B. K. (1999). The neural crest in development and evolution. New York, Springer-Verlag.

Harborth, J., S. M. Elbashir, et al. (2001). "Identification of essential genes in cultured mammalian cells using small interfering RNAs." J Cell Sci **114**(Pt 24): 4557-65.

Hatta, K. and M. Takeichi (1986). "Expression of N-cadherin adhesion molecules associated with early morphogenetic events in chick development." Nature **320**(6061): 447-9.

Haworth, K. E., I. Islam, et al. (2001). "Canine TCOF1; cloning, chromosome assignment and genetic analysis in dogs with different head types." Mamm Genome **12**(8): 622-9.

Hay, E. D. (1968). Organization and fine structure of epithelium and mesenchyme in the developing chick embryo. Epithelial–Mesenchymal Interactions. R. a. B. Fleischmajer, R.E. . Baltimore, MD, USA, Williams & Wilkins Co**:** 31–55.

Hazard, J. B., W. A. Hawk, et al. (1959). "Medullary (solid) carcinoma of the thyroid; a clinicopathologic entity." J Clin Endocrinol Metab **19**(1): 152-61.

He, Z., L. Feng, et al. (2005). "Expression of Col1a1, Col1a2 and procollagen I in germ cells of immature and adult mouse testis." Reproduction **130**(3): 333-41.

Heanue, T. A. and V. Pachnis (2007). "Enteric nervous system development and Hirschsprung's disease: advances in genetic and stem cell studies." Nat Rev Neurosci **8**(6): 466-79.

Hempel, J. D. and R. Pietruszko (1979). "Human stomach alcohol dehydrogenase: isoenzyme composition and catalytic properties." Alcohol Clin Exp Res **3**(2): 95-8.

Herbarth, B., V. Pingault, et al. (1998). "Mutation of the Sry-related Sox10 gene in Dominant megacolon, a mouse model for human Hirschsprung disease." Proc Natl Acad Sci U S A **95**(9): 5161-5.

Hilfer, S. R. (1968). Cellular interactions in the genesis and maintenance of thyroid characteristics. Baltimore, Williams and Wilkins Co.

Hirota, Y., A. Katsumata, et al. (1990). "Nucleotide and deduced amino acid sequences of chicken lactate dehydrogenase-A." Nucleic Acids Res **18**(21): 6432.

His, W. (1868). ""Die erste Entwicklung des Hühnchens im Ei: Untersuchungen über die erste Anlage des Wirbelthierleibes"."

Hoffmann, I. and R. Balling (1995). "Cloning and expression analysis of a novel mesodermally expressed cadherin." Dev Biol **169**(1): 337-46.

Holstege, F. C., P. C. van der Vliet, et al. (1996). "Opening of an RNA polymerase II promoter occurs in two distinct steps and requires the basal transcription factors IIE and IIH." Embo J **15**(7): 1666-77.

Honore, S. M., M. J. Aybar, et al. (2003). "Sox10 is required for the early development of the prospective neural crest in Xenopus embryos." Dev Biol **260**(1): 79-96.

Hörstadius, S. (1950). The Neural Crest: its Properties and Derivatives in the Light of Experimental Research. London, Oxford University Press.

Hou, L. (1999). "Effects of local tissue environment on the differentiation of neural crest cells in turtle, with special reference to understanding the spatial distribution of pigment cells." Pigment Cell Res **12**(2): 81-8.

Houdayer, C., M. F. Portnoi, et al. (2001). "Pierre Robin sequence and interstitial deletion 2q32.3-q33.2." Am J Med Genet **102**(3): 219-26.

Houston, C. S., J. M. Opitz, et al. (1983). "The campomelic syndrome: review, report of 17 cases, and follow-up on the currently 17-year-old boy first reported by Maroteaux et al in 1971." Am J Med Genet **15**(1): 3-28.

Howard, T. D., W. A. Paznekas, et al. (1997). "Mutations in TWIST, a basic helix-loop-helix transcription factor, in Saethre-Chotzen syndrome." Nat Genet **15**(1): 36-41.

Hsu, C. A., A. K. Rishi, et al. (1997). "Retinoid induced apoptosis in leukemia cells through a retinoic acid nuclear receptor-independent pathway." Blood **89**(12): 4470-9.

Hu, Y. F., Z. J. Zhang, et al. (2006). "An epidermal neural crest stem cell (EPI-NCSC) molecular signature." Stem Cells **24**(12): 2692-702.

Hua, X., C. Yokoyama, et al. (1993). "SREBP-2, a second basic-helix-loop-helix-leucine zipper protein that stimulates transcription by binding to a sterol regulatory element." Proc Natl Acad Sci U S A **90**(24): 11603-7.

Hur, M. W. and H. J. Edenberg (1992). "Cloning and characterization of the ADH5 gene encoding human alcohol dehydrogenase 5, formaldehyde dehydrogenase." Gene **121**(2): 305-11.

Huynh, T. T., K. Pacak, et al. (2006). "Transcriptional regulation of phenylethanolamine N-methyltransferase in pheochromocytomas from patients with von Hippel-Lindau syndrome and multiple endocrine neoplasia type 2." Ann N Y Acad Sci **1073**: 241-52.

Iborra, F. J., J. Renau-Piqueras, et al. (1992). "Immunocytochemical and biochemical demonstration of formaldhyde dehydrogenase (class III alcohol dehydrogenase) in the nucleus." J Histochem Cytochem **40**(12): 1865-78.

Ikenouchi, J., M. Matsuda, et al. (2003). "Regulation of tight junctions during the epithelium-mesenchyme transition: direct repression of the gene expression of claudins/occludin by Snail." J Cell Sci **116**(Pt 10): 1959-67.

Ishihara, N., K. Yamada, et al. (2004). "Clinical and molecular analysis of Mowat-Wilson syndrome associated with ZFHX1B mutations and deletions at 2q22-q24.1." J Med Genet **41**(5): 387-93.

Ito, K. and M. Sieber-Blum (1991). "In vitro clonal analysis of quail cardiac neural crest development." Dev Biol **148**(1): 95-106.

Ito, Y., J. Y. Yeo, et al. (2003). "Conditional inactivation of Tgfbr2 in cranial neural crest causes cleft palate and calvaria defects." Development **130**(21): 5269-80.

Iwashita, T., G. M. Kruger, et al. (2003). "Hirschsprung disease is linked to defects in neural crest stem cell function." Science **301**(5635): 972-6.

Jacks, T., T. S. Shih, et al. (1994). "Tumour predisposition in mice heterozygous for a targeted mutation in Nf1." Nat Genet **7**(3): 353-61.

Jeffery, W. R., A. G. Strickler, et al. (2004). "Migratory neural crest-like cells form body pigmentation in a urochordate embryo." Nature **431**(7009): 696-9.

Jerome, L. A. and V. E. Papaioannou (2001). "DiGeorge syndrome phenotype in mice mutant for the T-box gene, Tbx1." Nat Genet **27**(3): 286-91.

Jiang, X., B. Choudhary, et al. (2002). "Normal fate and altered function of the cardiac neural crest cell lineage in retinoic acid receptor mutant embryos." Mech Dev **117**(1-2): 115-22.

Joos, T. O., R. David, et al. (1996). "xGCNF, a nuclear orphan receptor is expressed during neurulation in Xenopus laevis." Mech Dev **60**(1): 45-57.

Joseph, N. M., J. T. Mosher, et al. (2008). "The loss of Nf1 transiently promotes self-renewal but not tumorigenesis by neural crest stem cells." Cancer Cell **13**(2): 129-40.

Kaiser, R., M. R. Fernandez, et al. (1993). "Origin of the human alcohol dehydrogenase system: implications from the structure and properties of the octopus protein." Proc Natl Acad Sci U S A **90**(23): 11222-6.

Kaiser, R., B. Holmquist, et al. (1988). "Class III human liver alcohol dehydrogenase: a novel structural type equidistantly related to the class I and class II enzymes." Biochemistry **27**(4): 1132-40.

Karafiat, V., M. Dvorakova, et al. (2005). "Transcription factor c-Myb is involved in the regulation of the epithelial-mesenchymal transition in the avian neural crest." Cell Mol Life Sci **62**(21): 2516-25.

Katahira, T. and H. Nakamura (2003). "Gene silencing in chick embryos with a vector-based small interfering RNA system." Dev Growth Differ **45**(4): 361-7.

Kelberman, D., J. Tyson, et al. (2001). "Hemifacial microsomia: progress in understanding the genetic basis of a complex malformation syndrome." Hum Genet **109**(6): 638-45.

Kim, J. H., C. K. Yang, et al. (2008). "CCAR1, a key regulator of mediator complex recruitment to nuclear receptor transcription complexes." Mol Cell **31**(4): 510-9.

Kimura, Y., H. Matsunami, et al. (1995). "Cadherin-11 expressed in association with mesenchymal morphogenesis in the head, somite, and limb bud of early mouse embryos." Dev Biol **169**(1): 347-58.

Knecht, A. K. and M. Bronner-Fraser (2002). "Induction of the neural crest: a multigene process." Nat Rev Genet **3**(6): 453-61.

Koch, C. A., D. Mauro, et al. (2002). "Pheochromocytoma in von hippel-lindau disease: distinct histopathologic phenotype compared to pheochromocytoma in multiple endocrine neoplasia type 2." Endocr Pathol **13**(1): 17-27.

Kodama, A., I. Karakesisoglou, et al. (2003). "ACF7: an essential integrator of microtubule dynamics." Cell **115**(3): 343-54.

Koivusalo, M., M. Baumann, et al. (1989). "Evidence for the identity of glutathione-dependent formaldehyde dehydrogenase and class III alcohol dehydrogenase." FEBS Lett **257**(1): 105-9.

Kuratani, S. (2008). "Evolutionary developmental studies of cyclostomes and the origin of the vertebrate neck." Dev Growth Differ **50 Suppl 1**: S189-94.

Kwok, C., P. A. Weller, et al. (1995). "Mutations in SOX9, the gene responsible for Campomelic dysplasia and autosomal sex reversal." Am J Hum Genet **57**(5): 1028-36.

LaBonne, C. and M. Bronner-Fraser (1998). "Neural crest induction in Xenopus: evidence for a two-signal model." Development **125**(13): 2403-14.

Ladomery, M. and G. Dellaire (2002). "Multifunctional zinc finger proteins in development and disease." Ann Hum Genet **66**(Pt 5-6): 331-42.

Lajeunie, E., H. W. Ma, et al. (1995). "FGFR2 mutations in Pfeiffer syndrome." Nat Genet **9**(2): 108.

Lakka, S. S., C. S. Gondi, et al. (2004). "Inhibition of cathepsin B and MMP-9 gene expression in glioblastoma cell line via RNA interference reduces tumor cell invasion, tumor growth and angiogenesis." Oncogene **23**(27): 4681-9.

Landacre, F. L. (1921). "The fate of the neural crest in the head of urodeles." Journal of Comparative Neurology(33): 1-44.

Lay, A. J., X. M. Jiang, et al. (2000). "Phosphoglycerate kinase acts in tumour angiogenesis as a disulphide reductase." Nature **408**(6814): 869-73.

Le Douarin, N., J. Fontaine, et al. (1974). "New studies on the neural crest origin of the avian ultimobranchial glandular cells--interspecific combinations and cytochemical characterization of C cells based on the uptake of biogenic amine precursors." Histochemistry **38**(4): 297-305.

Le Douarin, N. M., J. M. Brito, et al. (2007). "Role of the neural crest in face and brain development." Brain Res Rev **55**(2): 237-47.

Le Douarin, N. M., S. Creuzet, et al. (2004). "Neural crest cell plasticity and its limits." Development **131**(19): 4637-50.

Le Douarin, N. M. and M. A. Teillet (1974). "Experimental analysis of the migration and differentiation of neuroblasts of the autonomic nervous system and of neurectodermal mesenchymal derivatives, using a biological cell marking technique." Dev Biol **41**(1): 162-84.

Le Dourarin, N. M. and C. Kalcheim (1999). The Neural Crest. Cambridge, UK, Cambridge University Press.

Le Lievre, C. S. and N. M. Le Douarin (1975). "Mesenchymal derivatives of the neural crest: analysis of chimaeric quail and chick embryos." J Embryol Exp Morphol **34**(1): 125-54.

Lee, D. K., D. Suh, et al. (2002). "POZ domain transcription factor, FBI-1, represses transcription of ADH5/FDH by interacting with the zinc finger and interfering with DNA binding activity of Sp1." J Biol Chem **277**(30): 26761-8.

Lee, G., H. Kim, et al. (2007). "Isolation and directed differentiation of neural crest stem cells derived from human embryonic stem cells." Nat Biotechnol **25**(12): 1468-75.

Lee, J. K., R. S. Coyne, et al. (1993). "Cell shape and interaction defects in alpha-spectrin mutants of Drosophila melanogaster." J Cell Biol **123**(6 Pt 2): 1797-809.

Lee, S., E. Nakamura, et al. (2005). "Neuronal apoptosis linked to EglN3 prolyl hydroxylase and familial pheochromocytoma genes: developmental culling and cancer." Cancer Cell **8**(2): 155-67.

Lee, W. K., A. W. Root, et al. (1982). "Bilateral branchial cleft sinuses associated with intrauterine and postnatal growth retardation, premature aging, and unusual facial appearance: a new syndrome with dominant transmission." Am J Med Genet **11**(3): 345-52.

Lehner, C. F., R. Stick, et al. (1987). "Differential expression of nuclear lamin proteins during chicken development." J Cell Biol **105**(1): 577-87.

Leung, C. L., R. K. Liem, et al. (2001). "The plakin family." J Cell Sci **114**(Pt 19): 3409-10.

Leung, C. L., D. Sun, et al. (1999). "Microtubule actin cross-linking factor (MACF): a hybrid of dystonin and dystrophin that can interact with the actin and microtubule cytoskeletons." J Cell Biol **147**(6): 1275-86.

Levayer, R. and T. Lecuit (2008). "Breaking down EMT." Nat Cell Biol **10**(7): 757-9.

Lewis, B. C., J. E. Prescott, et al. (2000). "Tumor induction by the c-Myc target genes rcl and lactate dehydrogenase A." Cancer Res **60**(21): 6178-83.

Liang, J. Y., J. A. Fontana, et al. (1999). "Synthetic retinoid CD437 induces S-phase arrest and apoptosis in human prostate cancer cells LNCaP and PC-3." Prostate **38**(3): 228-36.

Liem, K. F., Jr., G. Tremml, et al. (1995). "Dorsal differentiation of neural plate cells induced by BMP-mediated signals from epidermal ectoderm." Cell **82**(6): 969-79.

Linnoila, R. I., H. R. Keiser, et al. (1990). "Histopathology of benign versus malignant sympathoadrenal paragangliomas: clinicopathologic study of 120 cases including unusual histologic features." Hum Pathol **21**(11): 1168-80.

Lloyd, R. V., J. C. Sisson, et al. (1986). "Immunohistochemical localization of epinephrine, norepinephrine, catecholamine-synthesizing enzymes, and chromogranin in neuroendocrine cells and tumors." Am J Pathol **125**(1): 45-54.

Loeys, B. L., J. Chen, et al. (2005). "A syndrome of altered cardiovascular, craniofacial, neurocognitive and skeletal development caused by mutations in TGFBR1 or TGFBR2." Nat Genet **37**(3): 275-81.

Loeys, B. L., U. Schwarze, et al. (2006). "Aneurysm syndromes caused by mutations in the TGF-beta receptor." N Engl J Med **355**(8): 788-98.

Lumsden, A. G. (1988). "Spatial organization of the epithelium and the role of neural crest cells in the initiation of the mammalian tooth germ." Development **103 Suppl**: 155-69.

Luo, T., Y. H. Lee, et al. (2003). "Induction of neural crest in Xenopus by transcription factor AP2alpha." Proc Natl Acad Sci U S A **100**(2): 532-7.

Machado, J. C., C. Oliveira, et al. (2001). "E-cadherin gene (CDH1) promoter methylation as the second hit in sporadic diffuse gastric carcinoma." Oncogene **20**(12): 1525-8.

Maher, E. R. and C. Eng (2002). "The pressure rises: update on the genetics of phaeochromocytoma." Hum Mol Genet **11**(20): 2347-54.

Maka, M., C. C. Stolt, et al. (2005). "Identification of Sox8 as a modifier gene in a mouse model of Hirschsprung disease reveals underlying molecular defect." Dev Biol **277**(1): 155-69.

Mancilla, A. and R. Mayor (1996). "Neural crest formation in Xenopus laevis: mechanisms of Xslug induction." Dev Biol **177**(2): 580-9.

Manley, N. R. and M. R. Capecchi (1998). "Hox group 3 paralogs regulate the development and migration of the thymus, thyroid, and parathyroid glands." Dev Biol **195**(1): 1-15.

Martins-Green, M. and C. A. Erickson (1987). "Basal lamina is not a barrier to neural crest cell emigration: documentation by TEM and by immunofluorescent and immunogold labelling." Development **101**(3): 517-33.

Massague, J. (1990). "The transforming growth factor-beta family." Annu Rev Cell Biol **6**: 597-641.

Mayor, R. and M. J. Aybar (2001). "Induction and development of neural crest in Xenopus laevis." Cell Tissue Res **305**(2): 203-9.

Mayor, R., R. Morgan, et al. (1995). "Induction of the prospective neural crest of Xenopus." Development **121**(3): 767-77.

McDonald, E. R., 3rd and W. S. El-Deiry (2004). "Suppression of caspase-8- and -10-associated RING proteins results in sensitization to death ligands and inhibition of tumor cell growth." Proc Natl Acad Sci U S A **101**(16): 6170-5.

McDonald, M. T. and J. L. Gorski (1993). "Nager acrofacial dysostosis." J Med Genet **30**(9): 779-82.

McKusick, V. A., H. S. Traisman, et al. (1972). "More speculation on Marfan syndrome." J Pediatr **80**(3): 530-1.

Medley, Q. G., E. G. Buchbinder, et al. (2003). "Signaling between focal adhesion kinase and trio." J Biol Chem **278**(15): 13265-70.
Meera Khan, P., L. M. Wijnen, et al. (1984). "Human formaldehyde dehydrogenase (FDH) and its assignment to chromosome 4." Cytogenet Cell Genet **38**(2): 112-5.
Merla, G., C. Ucla, et al. (2002). "Identification of additional transcripts in the Williams-Beuren syndrome critical region." Hum Genet **110**(5): 429-38.
Meulemans, D. and M. Bronner-Fraser (2004). "Gene-regulatory interactions in neural crest evolution and development." Dev Cell **7**(3): 291-9.
Meulemans, D. and M. Bronner-Fraser (2005). "Central role of gene cooption in neural crest evolution." J Exp Zoolog B Mol Dev Evol **304**(4): 298-303.
Milunsky, J. M., T. A. Maher, et al. (2008). "TFAP2A mutations result in branchio-oculo-facial syndrome." Am J Hum Genet **82**(5): 1171-7.
Montero-Balaguer, M., M. R. Lang, et al. (2006). "The mother superior mutation ablates foxd3 activity in neural crest progenitor cells and depletes neural crest derivatives in zebrafish." Dev Dyn **235**(12): 3199-212.
Moreno-Bueno, G., E. Cubillo, et al. (2006). "Genetic profiling of epithelial cells expressing E-cadherin repressors reveals a distinct role for Snail, Slug, and E47 factors in epithelial-mesenchymal transition." Cancer Res **66**(19): 9543-56.
Morrison, S. J., P. M. White, et al. (1999). "Prospective identification, isolation by flow cytometry, and in vivo self-renewal of multipotent mammalian neural crest stem cells." Cell **96**(5): 737-49.
Moseley, J. M., E. W. Matthews, et al. (1968). "The ultimobranchial origin of calcitonin." Lancet **1**(7534): 108-10.
Moser, M., A. Pscherer, et al. (1997). "Enhanced apoptotic cell death of renal epithelial cells in mice lacking transcription factor AP-2beta." Genes Dev **11**(15): 1938-48.
Moury, J. D. and A. G. Jacobson (1989). "Neural fold formation at newly created boundaries between neural plate and epidermis in the axolotl." Dev Biol **133**(1): 44-57.
Moury, J. D. and A. G. Jacobson (1990). "The origins of neural crest cells in the axolotl." Dev Biol **141**(2): 243-53.
Mowat, D. R., G. D. Croaker, et al. (1998). "Hirschsprung disease, microcephaly, mental retardation, and characteristic facial features: delineation of a new syndrome and identification of a locus at chromosome 2q22-q23." J Med Genet **35**(8): 617-23.
Muenke, M., U. Schell, et al. (1994). "A common mutation in the fibroblast growth factor receptor 1 gene in Pfeiffer syndrome." Nat Genet **8**(3): 269-74.
Muller, T., A. Choidas, et al. (1999). "Phosphorylation and free pool of beta-catenin are regulated by tyrosine kinases and tyrosine phosphatases during epithelial cell migration." J Biol Chem **274**(15): 10173-83.
Murray, S. A., K. F. Oram, et al. (2007). "Multiple functions of Snail family genes during palate development in mice." Development **134**(9): 1789-97.
Nagoshi, N., S. Shibata, et al. (2008). "Ontogeny and multipotency of neural crest-derived stem cells in mouse bone marrow, dorsal root ganglia, and whisker pad." Cell Stem Cell **2**(4): 392-403.

Nakagawa, S. and M. Takeichi (1995). "Neural crest cell-cell adhesion controlled by sequential and subpopulation-specific expression of novel cadherins." Development **121**(5): 1321-32.

Nakagawa, S. and M. Takeichi (1998). "Neural crest emigration from the neural tube depends on regulated cadherin expression." Development **125**(15): 2963-71.

Nakajima, Y., E. L. Krug, et al. (1994). "Myocardial regulation of transforming growth factor-beta expression by outflow tract endothelium in the early embryonic chick heart." Dev Biol **165**(2): 615-26.

Nakamura, E. and W. G. Kaelin, Jr. (2006). "Recent insights into the molecular pathogenesis of pheochromocytoma and paraganglioma." Endocr Pathol **17**(2): 97-106.

Nakamura, H., Y. Watanabe, et al. (2000). "Misexpression of genes in brain vesicles by in ovo electroporation." Dev Growth Differ **42**(3): 199-201.

Nakanishi, H. and Y. Takai (2004). "Roles of nectins in cell adhesion, migration and polarization." Biol Chem **385**(10): 885-92.

Naora, H., M. Kimura, et al. (1994). "Transgenic mouse model of hemifacial microsomia: cloning and characterization of insertional mutation region on chromosome 10." Genomics **23**(3): 515-9.

Newgreen, D. and I. Gibbins (1982). "Factors controlling the time of onset of the migration of neural crest cells in the fowl embryo." Cell Tissue Res **224**(1): 145-60.

Newgreen, D. F. and D. Gooday (1985). "Control of the onset of migration of neural crest cells in avian embryos. Role of Ca++-dependent cell adhesions." Cell Tissue Res **239**(2): 329-36.

Nguyen, T. B., K. Manova, et al. (2002). "Characterization and expression of mammalian cyclin b3, a prepachytene meiotic cyclin." J Biol Chem **277**(44): 41960-9.

Nichols, D. H. (1981). "Neural crest formation in the head of the mouse embryo as observed using a new histological technique." J Embryol Exp Morphol **64**: 105-20.

Nichols, D. H. (1987). "Ultrastructure of neural crest formation in the midbrain/rostral hindbrain and preotic hindbrain regions of the mouse embryo." Am J Anat **179**(2): 143-54.

Nicholson, A. D. and S. Menon (1995). "Hallerman-Streiff syndrome." J Postgrad Med **41**(1): 22-3.

Nijhawan, N., Y. Morad, et al. (2002). "Caruncle abnormalities in the oculo-auriculo-vertebral spectrum." Am J Med Genet **113**(4): 320-5.

Nishimori, T., T. Tomonaga, et al. (2006). "Proteomic analysis of primary esophageal squamous cell carcinoma reveals downregulation of a cell adhesion protein, periplakin." Proteomics **6**(3): 1011-8.

Noden, D. M. (1991). "Cell movements and control of patterned tissue assembly during craniofacial development." J Craniofac Genet Dev Biol **11**(4): 192-213.

O'Brien, S. P., K. Seipel, et al. (2000). "Skeletal muscle deformity and neuronal disorder in Trio exchange factor-deficient mouse embryos." Proc Natl Acad Sci U S A **97**(22): 12074-8.

Palacios, F., J. S. Tushir, et al. (2005). "Lysosomal targeting of E-cadherin: a unique mechanism for the down-regulation of cell-cell adhesion during epithelial to mesenchymal transitions." Mol Cell Biol **25**(1): 389-402.

Paratore, C., D. E. Goerich, et al. (2001). "Survival and glial fate acquisition of neural crest cells are regulated by an interplay between the transcription factor Sox10 and extrinsic combinatorial signaling." Development **128**(20): 3949-61.

Pardono, E., Y. van Bever, et al. (2003). "Waardenburg syndrome: clinical differentiation between types I and II." Am J Med Genet A **117A**(3): 223-35.

Pares, X. and B. L. Vallee (1981). "New human liver alcohol dehydrogenase forms with unique kinetic characteristics." Biochem Biophys Res Commun **98**(1): 122-30.

Park, S. M., A. B. Gaur, et al. (2008). "The miR-200 family determines the epithelial phenotype of cancer cells by targeting the E-cadherin repressors ZEB1 and ZEB2." Genes Dev **22**(7): 894-907.

Parsons, S. L., S. A. Watson, et al. (1997). "Matrix metalloproteinases." Br J Surg **84**(2): 160-6.

Pearse, A. G. (1966). "The cytochemistry of the thyroid C cells and their relationship to calcitonin." Proc R Soc Lond B Biol Sci **164**(996): 478-87.

Pearse, A. G. and A. F. Carvalheira (1967). "Cytochemical evidence for an ultimobranchial origin of rodent thyroid C cells." Nature **214**(5091): 929-30.

Pearse, A. G. and J. M. Polak (1971). "Cytochemical evidence for the neural crest origin of mammalian ultimobranchial C cells." Histochemie **27**(2): 96-102.

Peinado, H., D. Olmeda, et al. (2007). "Snail, Zeb and bHLH factors in tumour progression: an alliance against the epithelial phenotype?" Nat Rev Cancer **7**(6): 415-28.

Perez-Moreno, M. A., A. Locascio, et al. (2001). "A new role for E12/E47 in the repression of E-cadherin expression and epithelial-mesenchymal transitions." J Biol Chem **276**(29): 27424-31.

Perris, R. and D. Perissinotto (2000). "Role of the extracellular matrix during neural crest cell migration." Mech Dev **95**(1-2): 3-21.

Pfeiffer, R. A. (1964). "[Dominant Hereditary Acrocephalosyndactylia.]." Z Kinderheilkd **90**: 301-20.

Pingault, V., N. Bondurand, et al. (1998). "SOX10 mutations in patients with Waardenburg-Hirschsprung disease." Nat Genet **18**(2): 171-3.

Polak, J. M., A. G. Pearse, et al. (1974). "Immunocytochemical confirmation of the neural crest origin of avian calcitonin-producing cells." Histochemistry **40**(3): 209-14.

Ponticos, M., D. Abraham, et al. (2004). "Col1a2 enhancer regulates collagen activity during development and in adult tissue repair." Matrix Biol **22**(8): 619-28.

Potts, J. D., J. M. Dagle, et al. (1991). "Epithelial-mesenchymal transformation of embryonic cardiac endothelial cells is inhibited by a modified antisense oligodeoxynucleotide to transforming growth factor beta 3." Proc Natl Acad Sci U S A **88**(4): 1516-20.

Powers, J. F., M. J. Evinger, et al. (2007). "Pheochromocytomas in Nf1 knockout mice express a neural progenitor gene expression profile." Neuroscience **147**(4): 928-37.

Pugacheva, E. M., Y. W. Kwon, et al. (2006). "Cloning and characterization of zebrafish CTCF: Developmental expression patterns, regulation of the promoter region, and evolutionary aspects of gene organization." Gene **375**: 26-36.

Pyeritz, R. E., E. A. Murphy, et al. (1979). "Clinical variability in the Marfan syndrome(s)." Birth Defects Orig Artic Ser **15**(5B): 155-78.

Raible, D. W. (2006). "Development of the neural crest: achieving specificity in regulatory pathways." Curr Opin Cell Biol **18**(6): 698-703.

Read, A. P. and V. E. Newton (1997). "Waardenburg syndrome." J Med Genet **34**(8): 656-65.

Reardon, W., R. M. Winter, et al. (1994). "Mutations in the fibroblast growth factor receptor 2 gene cause Crouzon syndrome." Nat Genet **8**(1): 98-103.

Reichman, T. W., L. C. Muniz, et al. (2002). "The RNA binding protein nuclear factor 90 functions as both a positive and negative regulator of gene expression in mammalian cells." Mol Cell Biol **22**(1): 343-56.

Reilly, K. M. and T. Van Dyke (2008). "It takes a (dysfunctional) village to raise a tumor." Cell **135**(3): 408-10.

Rekdal, C., E. Sjottem, et al. (2000). "The nuclear factor SPBP contains different functional domains and stimulates the activity of various transcriptional activators." J Biol Chem **275**(51): 40288-300.

Rickmann, M., J. W. Fawcett, et al. (1985). "The migration of neural crest cells and the growth of motor axons through the rostral half of the chick somite." J Embryol Exp Morphol **90**: 437-55.

Rishi, A. K., L. Zhang, et al. (2003). "Identification and characterization of a cell cycle and apoptosis regulatory protein-1 as a novel mediator of apoptosis signaling by retinoid CD437." J Biol Chem **278**(35): 33422-35.

Rishi, A. K., L. Zhang, et al. (2006). "Cell cycle- and apoptosis-regulatory protein-1 is involved in apoptosis signaling by epidermal growth factor receptor." J Biol Chem **281**(19): 13188-98.

Robbins, J. R., P. G. McGuire, et al. (1999). "Diminished matrix metalloproteinase 2 (MMP-2) in ectomesenchyme-derived tissues of the Patch mutant mouse: regulation of MMP-2 by PDGF and effects on mesenchymal cell migration." Dev Biol **212**(2): 255-63.

Rohwer, A., W. Kittstein, et al. (1999). "Cloning, expression and characterization of an A6-related protein." Eur J Biochem **263**(2): 518-25.

Rollhauser-ter Horst, J. (1979). "Artificial neural crest formation in amphibia." Anat Embryol (Berl) **157**(1): 113-20.

Rollhauser-ter Horst, J. (1980). "Neural crest replaced by gastrula ectoderm in amphibia. Effect on neurulation, CNS, gills and limbs." Anat Embryol (Berl) **160**(2): 203-11.

Rutland, P., L. J. Pulleyn, et al. (1995). "Identical mutations in the FGFR2 gene cause both Pfeiffer and Crouzon syndrome phenotypes." Nat Genet **9**(2): 173-6.

Sadaghiani, B. and J. R. Vielkind (1990). "Distribution and migration pathways of HNK-1-immunoreactive neural crest cells in teleost fish embryos." Development **110**(1): 197-209.

Sakai, D., T. Suzuki, et al. (2006). "Cooperative action of Sox9, Snail2 and PKA signaling in early neural crest development." Development **133**(7): 1323-33.
Sanchez-Martin, M., A. Rodriguez-Garcia, et al. (2002). "SLUG (SNAI2) deletions in patients with Waardenburg disease." Hum Mol Genet **11**(25): 3231-6.
Sasai, N., K. Mizuseki, et al. (2001). "Requirement of FoxD3-class signaling for neural crest determination in Xenopus." Development **128**(13): 2525-36.
Satoda, M., F. Zhao, et al. (2000). "Mutations in TFAP2B cause Char syndrome, a familial form of patent ductus arteriosus." Nat Genet **25**(1): 42-6.
Sauka-Spengler, T. and M. Barembaum (2008). "Gain- and loss-of-function approaches in the chick embryo." Methods Cell Biol **87**: 237-56.
Sauka-Spengler, T. and M. Bronner-Fraser (2006). "Development and evolution of the migratory neural crest: a gene regulatory perspective." Curr Opin Genet Dev **16**(4): 360-6.
Sauka-Spengler, T. and M. Bronner-Fraser (2008). "A gene regulatory network orchestrates neural crest formation." Nat Rev Mol Cell Biol **9**(7): 557-68.
Sauka-Spengler, T. and M. Bronner-Fraser (2008). "Insights from a sea lamprey into the evolution of neural crest gene regulatory network." Biol Bull **214**(3): 303-14.
Sauka-Spengler, T., D. Meulemans, et al. (2007). "Ancient evolutionary origin of the neural crest gene regulatory network." Dev Cell **13**(3): 405-20.
Schell, U., A. Hehr, et al. (1995). "Mutations in FGFR1 and FGFR2 cause familial and sporadic Pfeiffer syndrome." Hum Mol Genet **4**(3): 323-8.
Schorle, H., P. Meier, et al. (1996). "Transcription factor AP-2 essential for cranial closure and craniofacial development." Nature **381**(6579): 235-8.
Schratt, G., U. Philippar, et al. (2002). "Serum response factor is crucial for actin cytoskeletal organization and focal adhesion assembly in embryonic stem cells." J Cell Biol **156**(4): 737-50.
Sela-Donenfeld, D. and C. Kalcheim (1999). "Regulation of the onset of neural crest migration by coordinated activity of BMP4 and Noggin in the dorsal neural tube." Development **126**(21): 4749-62.
Sela-Donenfeld, D. and C. Kalcheim (2000). "Inhibition of noggin expression in the dorsal neural tube by somitogenesis: a mechanism for coordinating the timing of neural crest emigration." Development **127**(22): 4845-54.
Selleck, M. A. and M. Bronner-Fraser (1995). "Origins of the avian neural crest: the role of neural plate-epidermal interactions." Development **121**(2): 525-38.
Selleck, M. A. and M. Bronner-Fraser (1996). "The genesis of avian neural crest cells: a classic embryonic induction." Proc Natl Acad Sci U S A **93**(18): 9352-7.
Selleck, M. A., M. I. Garcia-Castro, et al. (1998). "Effects of Shh and Noggin on neural crest formation demonstrate that BMP is required in the neural tube but not ectoderm." Development **125**(24): 4919-30.
Shao, Z. M., M. I. Dawson, et al. (1995). "p53 independent G0/G1 arrest and apoptosis induced by a novel retinoid in human breast cancer cells." Oncogene **11**(3): 493-504.
Sharma, C. P., E. A. Fox, et al. (1989). "cDNA sequence of human class III alcohol dehydrogenase." Biochem Biophys Res Commun **164**(2): 631-7.

Shows, K. H., C. Ward, et al. (2006). "Reduced TCOF1 mRNA level in a rhesus macaque with Treacher Collins-like syndrome: further evidence for haploinsufficiency of treacle as the cause of disease." Mamm Genome **17**(2): 168-77.

Sieber-Blum, M. (1989). "Commitment of neural crest cells to the sensory neuron lineage." Science **243**(4898): 1608-11.

Sieber-Blum, M. and A. M. Cohen (1980). "Clonal analysis of quail neural crest cells: they are pluripotent and differentiate in vitro in the absence of noncrest cells." Dev Biol **80**(1): 96-106.

Sieber-Blum, M., M. Grim, et al. (2004). "Pluripotent neural crest stem cells in the adult hair follicle." Dev Dyn **231**(2): 258-69.

Sieber-Blum, M., L. Schnell, et al. (2006). "Characterization of epidermal neural crest stem cell (EPI-NCSC) grafts in the lesioned spinal cord." Mol Cell Neurosci **32**(1-2): 67-81.

Smith, M. (1986). "Genetics of human alcohol and aldehyde dehydrogenases." Adv Hum Genet **15**: 249-90.

Smith, M. M. and B. K. Hall (1990). "Development and evolutionary origins of vertebrate skeletogenic and odontogenic tissues." Biol Rev Camb Philos Soc **65**(3): 277-373.

Smith, S. H., R. G. Murray, et al. (1994). "The surface structure of Leptotrichia buccalis." Can J Microbiol **40**(2): 90-8.

Song, Y. S., H. J. Lee, et al. (2008). "Human neural crest stem cells transplanted in rat penile corpus cavernosum to repair erectile dysfunction." BJU Int **102**(2): 220-4; discussion 224.

Sorensen, A. B., S. Warming, et al. (2002). "Alternative splicing, expression, and gene structure of the septin-like putative proto-oncogene Sint1." Gene **285**(1-2): 79-89.

Southard-Smith, E. M., L. Kos, et al. (1998). "Sox10 mutation disrupts neural crest development in Dom Hirschsprung mouse model." Nat Genet **18**(1): 60-4.

Splendore, A., M. R. Passos-Bueno, et al. (2002). "TCOF1 mutations excluded from a role in other first and second branchial arch-related disorders." Am J Med Genet **111**(3): 324-7.

Spokony, R. F., Y. Aoki, et al. (2002). "The transcription factor Sox9 is required for cranial neural crest development in Xenopus." Development **129**(2): 421-32.

Sponseller, P. D., W. Hobbs, et al. (1995). "The thoracolumbar spine in Marfan syndrome." J Bone Joint Surg Am **77**(6): 867-76.

Spritz, R. A., P. W. Chiang, et al. (2003). "Human and mouse disorders of pigmentation." Curr Opin Genet Dev **13**(3): 284-9.

Stec, I., G. J. van Ommen, et al. (2001). "WHSC1L1, on human chromosome 8p11.2, closely resembles WHSC1 and maps to a duplicated region shared with 4p16.3." Genomics **76**(1-3): 5-8.

Stemple, D. L. and D. J. Anderson (1992). "Isolation of a stem cell for neurons and glia from the mammalian neural crest." Cell **71**(6): 973-85.

Steven, R., T. J. Kubiseski, et al. (1998). "UNC-73 activates the Rac GTPase and is required for cell and growth cone migrations in C. elegans." Cell **92**(6): 785-95.

Steventon, B., C. Carmona-Fontaine, et al. (2005). "Genetic network during neural crest induction: from cell specification to cell survival." Semin Cell Dev Biol **16**(6): 647-54.

Strong, W. B. (1968). "Familial syndrome of right-sided aortic arch, mental deficiency, and facial dysmorphism." J Pediatr **73**(6): 882-8.

Suske, G. (1999). "The Sp-family of transcription factors." Gene **238**(2): 291-300.

Suzuki, T., D. Sakai, et al. (2006). "Sox genes regulate type 2 collagen expression in avian neural crest cells." Dev Growth Differ **48**(8): 477-86.

Szpaderska, A. M. and A. Frankfater (2001). "An intracellular form of cathepsin B contributes to invasiveness in cancer." Cancer Res **61**(8): 3493-500.

Takai, Y., K. Irie, et al. (2003). "Nectins and nectin-like molecules: roles in cell adhesion, migration, and polarization." Cancer Sci **94**(8): 655-67.

Takai, Y. and H. Nakanishi (2003). "Nectin and afadin: novel organizers of intercellular junctions." J Cell Sci **116**(Pt 1): 17-27.

Tamura, T., A. Mancini, et al. (1999). "FMIP, a novel Fms-interacting protein, affects granulocyte/macrophage differentiation." Oncogene **18**(47): 6488-95.

Taneyhill, L. A., E. G. Coles, et al. (2007). "Snail2 directly represses cadherin6B during epithelial-to-mesenchymal transitions of the neural crest." Development **134**(8): 1481-90.

Tavares, A. L., M. E. Mercado-Pimentel, et al. (2006). "TGFbeta-mediated RhoA expression is necessary for epithelial-mesenchymal transition in the embryonic chick heart." Dev Dyn **235**(6): 1589-98.

Teng, L., N. A. Mundell, et al. (2008). "Requirement for Foxd3 in the maintenance of neural crest progenitors." Development **135**(9): 1615-24.

Theveneau, E., J. L. Duband, et al. (2007). "Ets-1 confers cranial features on neural crest delamination." PLoS ONE **2**(11): e1142.

Thiery, J. P. and J. P. Sleeman (2006). "Complex networks orchestrate epithelial-mesenchymal transitions." Nat Rev Mol Cell Biol **7**(2): 131-42.

Tischler, A. S. (2008). "Pheochromocytoma and extra-adrenal paraganglioma: updates." Arch Pathol Lab Med **132**(8): 1272-84.

Tischler, A. S., N. Kimura, et al. (2006). "Pathology of pheochromocytoma and extra-adrenal paraganglioma." Ann N Y Acad Sci **1073**: 557-70.

Tischler, A. S., T. S. Shih, et al. (1995). "Characterization of Pheochromocytomas in a Mouse Strain with a Targeted Disruptive Mutation of the Neurofibromatosis Gene Nf1." Endocr Pathol **6**(4): 323-335.

Tobin, J. L., M. Di Franco, et al. (2008). "Inhibition of neural crest migration underlies craniofacial dysmorphology and Hirschsprung's disease in Bardet-Biedl syndrome." Proc Natl Acad Sci U S A **105**(18): 6714-9.

Trentin, A., C. Glavieux-Pardanaud, et al. (2004). "Self-renewal capacity is a widespread property of various types of neural crest precursor cells." Proc Natl Acad Sci U S A **101**(13): 4495-500.

Tsai, M. Y., S. Wang, et al. (2006). "A mitotic lamin B matrix induced by RanGTP required for spindle assembly." Science **311**(5769): 1887-93.

Tsuruga, H., N. Yabuta, et al. (1997). "Expression, nuclear localization and interactions of human MCM/P1 proteins." Biochem Biophys Res Commun **236**(1): 118-25.

Tucker, R. P. and C. A. Erickson (1984). "Morphology and behavior of quail neural crest cells in artificial three-dimensional extracellular matrices." Dev Biol **104**(2): 390-405.

Unsicker, K. (1993). "The chromaffin cell: paradigm in cell, developmental and growth factor biology." J Anat **183 (Pt 2)**: 207-21.

Unsicker, K., K. Huber, et al. (2005). "The chromaffin cell and its development." Neurochem Res **30**(6-7): 921-5.

Uotila, L. and M. Koivusalo (1974). "Formaldehyde dehydrogenase from human liver. Purification, properties, and evidence for the formation of glutathione thiol esters by the enzyme." J Biol Chem **249**(23): 7653-63.

Van de Putte, T., A. Francis, et al. (2007). "Neural crest-specific removal of Zfhx1b in mouse leads to a wide range of neurocristopathies reminiscent of Mowat-Wilson syndrome." Hum Mol Genet **16**(12): 1423-36.

Van de Putte, T., M. Maruhashi, et al. (2003). "Mice lacking ZFHX1B, the gene that codes for Smad-interacting protein-1, reveal a role for multiple neural crest cell defects in the etiology of Hirschsprung disease-mental retardation syndrome." Am J Hum Genet **72**(2): 465-70.

Waardenburg, P. J. (1951). "A new syndrome combining developmental anomalies of the eyelids, eyebrows and nose root with pigmentary defects of the iris and head hair and with congenital deafness." Am J Hum Genet **3**(3): 195-253.

Wadham, C., J. R. Gamble, et al. (2003). "The protein tyrosine phosphatase Pez is a major phosphatase of adherens junctions and dephosphorylates beta-catenin." Mol Biol Cell **14**(6): 2520-9.

Wagner, F. W., X. Pares, et al. (1984). "Physical and enzymatic properties of a class III isozyme of human liver alcohol dehydrogenase: chi-ADH." Biochemistry **23**(10): 2193-9.

Wagner, T., J. Wirth, et al. (1994). "Autosomal sex reversal and campomelic dysplasia are caused by mutations in and around the SRY-related gene SOX9." Cell **79**(6): 1111-20.

Wakamatsu, N., Y. Yamada, et al. (2001). "Mutations in SIP1, encoding Smad interacting protein-1, cause a form of Hirschsprung disease." Nat Genet **27**(4): 369-70.

Wang, R., M. L. Martinez-Frias, et al. (2002). "Infants of diabetic mothers are at increased risk for the oculo-auriculo-vertebral sequence: A case-based and case-control approach." J Pediatr **141**(5): 611-7.

Wang, Y., R. Xiao, et al. (2005). "Abnormalities in cartilage and bone development in the Apert syndrome FGFR2(+/S252W) mouse." Development **132**(15): 3537-48.

Weston, J. A. (1963). "A radioautographic analysis of the migration and localization of trunk neural crest cells in the chick." Dev Biol **6**: 279-310.

Wilkie, A. O., S. F. Slaney, et al. (1995). "Apert syndrome results from localized mutations of FGFR2 and is allelic with Crouzon syndrome." Nat Genet **9**(2): 165-72.

Williams, E. D. (1966). "Diarrhoea and thyroid carcinoma." Proc R Soc Med **59**(7): 602-3.

Williams, E. D. (1966). "Histogenesis of medullary carcinoma of the thyroid." J Clin Pathol **19**(2): 114-8.

Williams, E. D., C. E. Toyn, et al. (1989). "The ultimobranchial gland and congenital thyroid abnormalities in man." J Pathol **159**(2): 135-41.

Wilson, D. I., I. E. Cross, et al. (1991). "DiGeorge syndrome with isolated aortic coarctation and isolated ventricular septal defect in three sibs with a 22q11 deletion of maternal origin." Br Heart J **66**(4): 308-12.

Wong, D. L. (2003). "Why is the adrenal adrenergic?" Endocr Pathol **14**(1): 25-36.

Wurdak, H., L. M. Ittner, et al. (2005). "Inactivation of TGFbeta signaling in neural crest stem cells leads to multiple defects reminiscent of DiGeorge syndrome." Genes Dev **19**(5): 530-5.

Wyatt, L., C. Wadham, et al. (2007). "The protein tyrosine phosphatase Pez regulates TGFbeta, epithelial-mesenchymal transition, and organ development." J Cell Biol **178**(7): 1223-35.

Xu, Q. a. W., D.G., Ed. (1992). In situ hybridization: A practical approach, Oxford University Press.

Yagi, H., Y. Furutani, et al. (2003). "Role of TBX1 in human del22q11.2 syndrome." Lancet **362**(9393): 1366-73.

Yan, Y. L., C. T. Miller, et al. (2002). "A zebrafish sox9 gene required for cartilage morphogenesis." Development **129**(21): 5065-79.

Youn, Y. H., J. Feng, et al. (2003). "Neural crest stem cell and cardiac endothelium defects in the TrkC null mouse." Mol Cell Neurosci **24**(1): 160-70.

Zhang, J., S. Hagopian-Donaldson, et al. (1996). "Neural tube, skeletal and body wall defects in mice lacking transcription factor AP-2." Nature **381**(6579): 238-41.

Zhang, L., E. Levi, et al. (2007). "Transactivator of transcription-tagged cell cycle and apoptosis regulatory protein-1 peptides suppress the growth of human breast cancer cells in vitro and in vivo." Mol Cancer Ther **6**(5): 1661-72.

Zhao, G., L. Shi, et al. (2005). "NF45/ILF2 tissue expression, promoter analysis, and interleukin-2 transactivating function." Exp Cell Res **305**(2): 312-23.

Zito, G., P. Richiusa, et al. (2008). "In vitro identification and characterization of CD133(pos) cancer stem-like cells in anaplastic thyroid carcinoma cell lines." PLoS ONE **3**(10): e3544.

Zweier, C., B. Albrecht, et al. (2002). ""Mowat-Wilson" syndrome with and without Hirschsprung disease is a distinct, recognizable multiple congenital anomalies-mental retardation syndrome caused by mutations in the zinc finger homeo box 1B gene." Am J Med Genet **108**(3): 177-81.