

ANALYSIS OF THE EXPRESSION AND FUNCTION OF CHICKEN
PROTOCOLADHERIN 1 IN NEURAL CREST CELL MIGRATION AND PERIPHERAL
NERVOUS SYSTEM FORMATION

by

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ABSTRACT

The necessary steps of development from a single cell to a multi-celled functional organism are complex. Many molecules have been identified and their roles characterized in this process. One interesting population of cells includes the highly migratory neural crest cells (NCCs) unique to the vertebrate embryo and existing transiently during early embryonic development. The NCCs migrate along specific pathways at specific time-points, stop at target locations, differentiate and give rise to a variety of cell types and tissues. Trunk NCCs must choose between two different migratory pathways: the ventral route, giving rise to neurons and glia of the dorsal root ganglia (DRG), sympathetic ganglia (SG), Schwann cells of the ventral root (VR); or the dorsolateral pathway, giving rise to melanocytes. Although many aspects of neural crest migration have been elucidated, cessation of migration and subsequent differentiation at target structures is not clearly defined. One family of molecules involved in various steps of NCC migration is the cell-cell adhesion molecules, the cadherins.

To investigate the involvement of cadherins in NCC migration and differentiation during development using the avian model system, a combination of experiments and techniques including a library screen, in situ hybridization, in ovo electroporation, immunohistochemical and immunofluorescence staining as well as live time-lapse confocal imaging were performed. Results from these experiments produced the discovery and isolation of a novel molecule in the family of cadherin adhesion molecules, chicken protocadherin-1 (cPcdh1). Expression analysis showed cPcdh1 expressed in migrating NCCs, the DRG, SG and Schwann cells along the VR. A distinct expression pattern showed cPcdh1 along the periphery of the DRG, where crest cells are in an undifferentiated and mitotically active state. Further testing with deletion constructs and siRNA demonstrated when cPcdh1 function is inhibited, a greater percentage of cells migrate to the SG and VR at the expense of the DRG. Time-lapse confocal imaging showed cPcdh1 cells having an elongated cell shape with contact primarily being formed with neighboring cells along the periphery and longer cell-cell contact than observed in the control. Collectively, the results provide evidence for cPcdh1 involvement in NCC migration arrest and DRG formation.

CHAPTER 1

INTRODUCTION

The process of change or progression from a simple to a more complex state can be associated with development and is clearly evident in the development of a living system. Developmental biology is an area of continuous inquiry and discovery with the concept of how development occurs being of universal interest. One group of cells that has a significant influence in early embryonic development are the neural crest cells.

Background of Neural Crest Cells

Unique to the vertebrate embryo is the neural crest, a distinct structure existing only transiently in the early embryo and considered to represent decisive issues of developmental biology. The neural crest was first described in chick embryos by Wilhelm His in 1868 as “the cord in between” (or “Zwischenstrang”), a strip of cells lying between the dorsal ectoderm and the developing neural plate, giving rise to spinal and cranial ganglia (Horstadius, 1950). The neural crest cell is a migratory, multipotent stem cell population arising in the central nervous system (CNS). These cells have to generate various differentiated cell types in the correct number and correct sequence during neural development in order for embryonic development to occur and to develop normally (Sommer and Rao, 2002). Their importance lies in the ability of neural crest cells to migrate extensively to diverse locations, cease migration at specific sites and to generate a wide variety of differentiated cell types. These include neurons and glia of the peripheral nervous system (sensory, sympathetic and parasympathetic ganglia, Schwann

cells), melanocytes, neuroendocrine cells of the adrenal medulla, craniofacial cartilage, bones, tendons, connective and adipose tissues. In addition, there is a small population of neural crest cells that remains within the dorsal neural tube and produces the proprioceptive sensory neurons of the mesencephalic trigeminal nucleus (Narayanan and Narayanan, 1978). Abnormal development of the neural crest and its derivatives can have severe consequences for many different organ systems. These pathologies are known as neurocristopathies (Bolande, 1997). Some of the most common birth defects traced to defects in neural crest cells include cleft palate, spina bifida, Hirschsprung disease, DiGeorge syndrome (craniofacial and heart defects), Waardenburg-Shah syndrome (hypopigmentation and aganglionic megacolon) and frontonasal dysplasia (multiple craniofacial defects) (Wilkie and Morriss-Kay, 2001; Goodman, 2003; Van de Putte et al., 2003; Huang and Saint-Jennet, 2004). Neural crest cells can be grouped into four main yet overlapping domains: cranial (cephalic); vagal and sacral; cardiac; and trunk neural crest cells. Cranial neural crest cells migrate dorsolaterally to give rise to most of the skeleton and cartilage of the skull and face, cranial neurons, glia and connective tissues of the face. The vagal and sacral neural crest cells generate the parasympathetic or enteric ganglia of the gut. The cardiac neural crest can generate the endothelium of the aortic arch arteries and the septum between the aorta and the pulmonary artery. The trunk neural crest cells contribute to melanocytes, sensory and sympathetic neurons, Schwann cells and adrenomedullary cells.

The generation of neural crest cells occurs during the process of neurulation. In the avian embryo, the ectodermal cells along the dorsal midline thicken to form the

neural plate. The cells that will form the neural crest lie along each side of the neural plate. An indentation forms along the midline as the neural plate invaginates and the edges elevate to form the neural folds. The folds fuse in the dorsal midline creating the formation of the neural tube, separated from the overlying ectoderm. Initially the neural crest cells lie between the neural tube and the ectoderm and eventually detach and migrate away. The anterior end of the neural tube differentiates into the hindbrain, midbrain and forebrain to form the adult brain with the remainder of the neural tube forming the spinal cord (Matthews, 2001).

Neural crest cells form distinct sets of derivatives depending on where they originate from on the anteroposterior axis. Only cranial neural crest for example, give rise to craniofacial cartilage while trunk neural crest give rise to sensory neurons and glia. Conversely, in avian embryos, heterotrophic transplantations of neural crest sections from different axial levels have shown premigratory neural crest cells are highly plastic. For example, neural crest cells derived from ectopically grafted tissue migrated into characteristic positions for their new location on the neuraxis and consequently adopted the appropriate fate for the new position (LeDouarin and Kalcheim, 1999; Trainor and Krumlauf, 2001).

Neural crest migration can be divided into three stages: induction/emigration from the neural tube; migration along defined pathways; and differentiation/cessation.

Induction/Emigration From the Neural Tube

At the end of gastrulation, ectodermal cells thicken to form the neural plate during the process of neurulation. As the dorsal neural tube closes, the neural crest forms at the

point of fusion of the neural tube margins. The neural crest has been considered the most dorsal derivative of the neural tube. The neural crest cells are part of the neuroepithelium before migration, originating from the lateral ridges (neural folds) of the closing neuroepithelium. To leave the neuroepithelium, neural crest cells must lose their epithelial characteristics and take on the properties of migratory mesenchymal cells.

Induction from the neural tube has been well studied and various genes, proteins and co-factors have been indicated to be involved. According to the widely accepted neural default model, the default fate of neural ectoderm is to form neural tissue (Knecht and Bronner-Fraser, 2002). Interactions between non-neural ectoderm, neural plate and the underlying mesoderm have been shown to specify the fate of the neural crest precursors. Cells have been induced to become neural crest cells by epidermis and neural plate contact (Dickinson et al., 1995). Members of the bone morphogenic proteins (BMPs), Wnt and fibroblast growth factor (FGF) families have been implicated in several steps of neural crest induction (Basch ML et al., 2004). BMPs are signaling molecules of the transforming growth factor- β (TGF- β) family and are expressed in the neural folds, dorsal neural tube and ectoderm. BMPs (specifically BMP-4 and BMP-7) have been suggested to function as a neural crest inducer due to their ability to induce the formation of neural crest cells from neural plate explants *in vitro* (Liem et al., 1995). BMP signaling within the dorsal neural tube is also important in the dorsoventral patterning of spinal cord-derived neurons and eliciting the delamination of neural crest progenitors from the neural epithelium (Sela-Donenfeld and Kalcheim, 1999). Wnts are secreted glycoproteins that interact with the frizzled transmembrane receptors. Activation of the canonical Wnt

signaling pathway inhibits glycogen synthase-3 β (GSK-3 β) resulting in stabilization and nuclear translocation of β -catenin, making β -catenin available for binding the TCF/LEF family of transcription factors and ultimately inducing targeted gene expression (Wodarz and Nusse, 1998). Wnts have shown to induce neural crest cell markers in ectoderm explants in conjunction with BMP inhibitors and at least one Wnt family member, Wnt6, has shown to be produced in the epidermis at the correct time and place to strongly implicate an involvement of Wnt6 as a neural crest cell inducer (LaBonne and Bronner-Fraser, 1998; Garcia-Castro et al., 2002). Neural crest cell formation therefore, relies in part on BMP and Wnt signaling.

Recent studies demonstrate neural crest induction may be underway earlier, during gastrulation well before the neural plate appears and initiated independently of mesodermal and neural tissue (Basch et al., 2006). A specific region of the chick epiblast (stage 3-4) has been shown to generate neural crest cells when explanted under non-inducing conditions. This region expresses the transcription factor Pax7 and when Pax7 translation was blocked, the expression of neural crest markers Slug, Sox9, Sox10 and HNK-1 were inhibited, establishing Pax7 as an early marker for neural crest formation (Basch et al., 2006).

Following induction, neural crest cells delaminate from the dorsal neural tube, undergo an epithelial-to-mesenchymal transition (EMT) aiding in the start of their migration to their target destinations, lose their contact with other neuroepithelial cells and invade the surrounding extracellular matrix filled spaces (Tucker 2004). For cells to leave the neural tube, certain proteins such as RhoB (a member of the Rho family of

GTP-binding proteins) and Slug (a member of the Snail family of zinc finger transcription factors activated by the binding of β -catenin/Tcf/Lef) must be present as well as extracellular signals such as BMPs and Wnts (as reviewed by Steventon et al., 2005). The EMT is accompanied by the onset of Slug expression, which is maintained during neural crest cell migration (Nieto et al., 1994). The expression of Slug also causes the dissociation of the junctions that anchor connecting filaments between neighboring cells in the epithelia (Savagner et al., 1997), thus allowing cells to delaminate from the neural tube. RhoB is expressed in the premigratory and early migrating neural crest cells and when inhibited, will prevent emigration but not differentiation of neural crest cells (Liu and Jessell, 1998).

Two cell adhesion molecules are expressed by the neural tube, N-CAM and N-cadherin while the epidermal ectoderm expresses another cell adhesion molecule E-cadherin up to the neural plate boundary. The expression of these molecules is lost from the presumptive neural crest cells as they appear in the neural folds but another adhesion molecule, Cad-6B is expressed in the neural folds. Cad-6B becomes downregulated at the onset of migration while another adhesion molecule, Cadherin-7 is upregulated and expression is maintained during ventral and dorsolateral migration (Nakagawa and Takeichi, 1995; Nakagawa and Takeichi, 1998). In addition to changes in cell-cell adhesion, a breakdown occurs in the basement membrane surrounding the neural tube in areas from which the neural crest cells migrate (Erickson and Perris, 1993). Recent studies in the mouse have shown the non-muscle-specific gene, n-cofilin, as a critical regulator for cytoskeletal changes accompanying neural crest cell delamination and

migration (Gurniak et al., 2005) and apparently essential for migration of neural crest cells in the paraxial mesoderm. This was demonstrated in mutant n-cofilin mouse embryos by Gurniak et al., (2005) showing the main defect observed was an impaired delamination and migration of neural crest cells, affecting the development of neural crest derived tissues.

Around the time of neural tube closure and as the neural crest cells start their migration into the periphery, neural crest cells progressively adopt specific fates as a result of both intrinsic and extrinsic influences. The neural crest forms according to a rostrocaudal gradient along the body axis and releases cells that follow definite migration routes at precise times of development to finally reach target embryonic sites where they settle and differentiate. How these diverse neural crest derived phenotypes are specified early and maintained later in the differentiated tissues is beginning to be understood. However, less understood are the molecules that initiate the segregation of different cell types and influences the process of differentiation into specific cell fates.

Neural Crest Cell Migration

The following discussion on migration will mainly focus on trunk-derived neural crest cells due to the focus of this thesis. Trunk neural crest cells begin to emigrate from the neural tube at stage 12, which corresponds to somite level 8-13 (cervical level of the trunk). The detachment from the neural tube progresses in an anterior-to-posterior wave and emigration from the neural tube ceases at approximately stage 18 at the cervical level (Serbedzija et al., 1989). At the trunk level in birds and mammals, neural crest cells have a choice of two primary migratory pathways: dorsolaterally between the somite and the

ectoderm (epidermis), or ventrally through the rostral half of each somatic sclerotome between the somites and the neural tube (Krull, 2001).

The first pathway taken by the early-migratory crest cells is the ventral route, initially between the somites while they remain epithelial and then into the rostral (anterior) part of the somite-derived sclerotome (Rickmann et al., 1985; Bronner-Fraser, 1986; Loring and Erickson, 1987; Serbedzija et al., 1990). The cells taking this path normally give rise to the components of the peripheral nervous system (Wakamatsu et al., 2000) such as the neurons and glial cells of the sensory and sympathetic ganglia (SG), the glial cells of the ventral root (VR), the adrenal medulla (somite level 18-24 only) and a subpopulation of the enteric crest (sacral level only). Specifically, the crest cells that remain in the anterior half of the sclerotome aggregate to form the DRG, becoming primary sensory neurons and associated glia. The cells that penetrate further ventrally adopt the SG and adrenal medullary phenotypes. This selective migration results in the formation of segmentally arranged streams of migrating neural crest cells. The segmental pattern of neural crest migration is believed responsible for the metameric organization of the ganglia of the peripheral nervous system (Kuan et al., 2004; LeDouarin and Kalcheim, 1999). This segmented arrangement of the ganglia relative to the somites, which form the vertebrae, is essential for the proper wiring of the ganglia and peripheral nerves to targets in the periphery.

Later migrating trunk neural crest cells take the second pathway, the dorsolateral route, beginning approximately 24 hours after embarking on the ventral path (Erickson and Goins 1995). Late neural crest cells proliferate in the migration staging area located

between the neural tube, somites and ectoderm for 24 hours, then follow the dorsolateral pathway (Pla et al., 2005). Neural crest cells were found to populate their derivatives in a ventral-to-dorsal order, with the latest emigrating cells migrating exclusively along the dorsolateral pathway (Serbedzija et al., 1989).

The neural crest cells first invade the intersomitic space possibly due to the reason that it is a large space providing a path of least resistance and it may be the only pathway immediately available because the dermatome/myotome has yet to begin to develop. The pathway is filled with fibronectin which neural crest cells adhere strongly to and migrate on *in vitro* (Newgreen and Thiery, 1980; Erickson and Turley, 1983; Tucker and Erickson, 1984). The preferential entry into the anterior rather than the posterior half-sclerotome by trunk cells taking the ventral migration pathway appears to be imposed by the somites with the anterior sclerotome being permissive and the posterior sclerotome being repulsive for neural crest migration (Bronner-Fraser and Stern, 1991; Kalcheim and Teillet, 1989). This was originally thought to be due to repulsion of the neural crest cells from the posterior sclerotome cells rather than attraction by the anterior cells (Keynes et al., 1996). For example, previous reports suggested Eph/ephrin signaling might pattern trunk neural crest migration (Krull et al., 1997; Wang and Anderson, 1997). An inhibitory mechanism involving Eph/ephrin interactions was shown to confer a rostrocaudal pattern to trunk neural crest cells, preventing neural crest entry into the caudal portion of each somite resulting in segmental migration in the trunk area (Krull et al., 1997) although when using *Eph* and ephrin mutant mice, researchers found the mice

failed to show any defects in trunk neural crest migration (Adams et al., 2001; Davy et al., 2004; Orioli et al., 1996; Wang et al., 1998).

Other inhibitory molecules identified along neural crest migratory pathways include the extracellular molecule F-spondin and a member of the semaphorin family, collapsin, indicating these molecules contribute to repelling and thus restricting neural crest cells and their migration (Debby-Brafman et al., 1999; Eickholt et al., 1999). The receptor neuropilin 1 and its ligand semaphorin 3A have also been suggested to play a role in trunk cell migration (Eickholt et al., 1999), but were not expressed at the right time (reviewed by Kuan et al., 2004) and were not required in the mouse for appropriate trunk neural crest migration (Kawasaki et al., 2002). The receptor neuropilin 2 (*Npn2*) was found expressed on neural crest cells while its repulsive ligand semaphorin 3F (*Sema3f*) was restricted to the posterior sclerotome. In addition, in *Npn2* and *Sema3f* mutant mice, the neural crest cells migrated as a uniform sheet, losing their segmental pattern of migration. The *Npn2*/*Sema3f* signaling was shown required for segmental neural crest migration but not for somite patterning and segmental migration was found not to be essential for the formation of the DRG (Gammill et al., 2005). The integrin receptors expressed by the neural crest cells themselves may also be involved in guiding neural crest cell migration. Neural crest cells express several integrins during migration in vivo, thus providing receptors for extracellular matrix molecules such as laminin, fibronectin and tenascin that signals cells to alter their motility. There is evidence for the presence of attractive, positive molecular interactions, which may also influence neural crest migration through the rostral half of each somite (Koblar et al., 2000; Krull, 2001).

A variety of molecular systems have been identified in the local environment of the crest that may direct neural crest cell migration. For example, the extracellular matrix contains motility-promoting permissive molecules as well as repulsive or motility-inhibiting molecules and both are considered to have a primary role in crest migration. Many of these macromolecules have been shown to be required for migration and are already present in the correct place prior to crest migration (Newgreen and Thiery, 1980; Duband and Thiery, 1987; Krotoski et al., 1986). For example, laminin was found to be widespread along the migration pathway of neural crest cells and shown to be responsible for directing neural crest cell migration between the dermatome/myotome and the sclerotome in the anterior half of the somite (Loring and Erickson, 1987). Other molecules considered permissive for crest migration include fibronectin and collagen types I, IV and VI. The molecules considered non-permissive for crest migration include a number of chondroitin sulphate proteoglycans, (collagen type IX and versican) and the tenascins (Perris, 1997). Both cranial and trunk neural crest cells have been shown to extend lamellipodia with filopodia extensions in many directions to interact with environmental cues as they migrate (Kasemeier-Kulesa et al., 2005; Teddy and Kulesa, 2004). Molecules known to be repulsive for growing axons (i.e., members of the collapsin/semaphorin and ephrin families) have also been implicated in crest guidance by repelling migrating cells from certain areas. Early migratory and condensing trunk neural crest cells have been found to express the Robo receptors, Robo1 and Robo2, which bind to the Slit ligand. Trunk neural crest cells will avoid cells or membranes expressing Slit2. For example, Slit2 expression was shown to be at the entrance of the gut, which the vagal

neural crest cells but not trunk neural crest cells invade (De Bellard et al., 2003) as well as expressed in the dermamyotome, confining the early migrating cells to the ventral pathway (Jia et al., 2005). One model even suggests early neural crest development involves a 'sequential activation of migratory potential, with a signal to migrate activating this potential in a subset of premigratory neural crest cells' (Gammill and Bronner-Fraser, 2002; Gammill et al., 2005).

Although it has been generally accepted that neural crest cells are pluripotent when they emigrate from the neural tube and differentiate according to molecular cues in the environment into which they migrate (Erickson and Reedy, 1998; LeDouarin and Kalcheim, 1999), a portion of the neural crest cell population that initially emerge from the neural tube (and at intervals thereafter) have been shown to consist of fate-restricted precursors that give rise to a single cell type, raising the possibility that trunk neural crest lineages may be established largely before or at the time they leave the neural tube (Henion and Weston, 1997). DiI-labeling studies (Serbedzija et al., 1989; Kitamura et al., 1992) have shown neural crest cells destined for the dorsolateral path leave the neural tube beginning at stage 18 at the wing bud level and marker analysis has demonstrated these late-migrating trunk neural crest cells are already specified as melanoblasts before they enter the dorsolateral path (Kitamura et al., 1992; Reedy et al., 1998). In addition, due to the temporal lineage segregation, different signaling pathways have been suggested to be responsible for their specification (Reedy et al., 1998; Henion and Weston, 1997; Jin et al., 2001). For example, studies have shown that high Wnt signaling specifies melanocytes at the expense of the neuronal and glial lineages while BMP

signaling is correlated with the migration of neuroblasts and glioblasts (Dorsky et al., 1998; Jin et al., 2001). In addition, the non-canonical Wnt signaling (also known as the planar cell polarity or Wnt-Ca(2+) pathway) has been shown in *Xenopus* to control migration of neural crest cells while the canonical Wnt signaling pathway is required for neural crest induction (De Calisto et al., 2005). Various theories exist on the multipotency of neural crest cells when they leave the neural tube. Early tests to determine if premigratory neural crest cells were predetermined to a particular fate used the chick-quail chimeric system. Quail trunk crest cells when transplanted anteriorly into the chick embryo, gave rise to the cholinergic neurons of the gut, which are normally derived from the more anterior crest cells. Likewise, when the quail anterior crest cells were transplanted to the trunk region in chick, they gave rise to adrenergic neurons and SG, the normal derivatives of trunk cells (LeDouarin et al., 1975). This was taken to imply the environment influences the fate of neural crest cells and they acquire information necessary to differentiate during their migration. As mentioned previously, in the trunk of avian and mammalian embryos, ventrally migrating crest cells give rise to DRG, SG and Schwann cells along the spinal nerve and includes both glia and neurons. Additionally, because neural crest progenitors continue to divide as they migrate along the migratory pathways until they coalesce at their final destinations, the fate of some cells may be dependent on intrinsic characteristics and/or the environment it finds itself (Anderson, 1997). *In vivo* cell tracing methods by retrovirus-mediated gene transfer or dye injection of a single cell has shown a single progenitor can produce multiple types of neurons, glia and or both neurons and glia (Frank and Sanes, 1991; Bronner-Fraser and

Fraser, 1989), demonstrating multipotentiality in the progenitors that give rise to the DRG.

Neural Crest Cell Differentiation/Cessation

Specific stop signals and mechanisms regulating the cessation of migration at target sites such as the DRG are less characterized. Research has shown at the end of migration, cessation of movement has been shown to correlate with restoration of N-cadherin binding accompanied by changes in matrix adhesion and integrin molecules (Thiery et al., 1982; Duband and Thiery, 1987; Duband et al., 1988; Bronner-Fraser et al., 1992).

Once neural crest cells have left the neural tube, they encounter cues in their migration pathway and at their final destination which influence their proliferation and survival as described previously. Along the dorsoventral migration pathway, recent evidence shows trunk neural crest cells to migrate in chain-like formations and to be highly motile with extensive interaction among neighboring cells and the environment (Kasemeier-Kulesa et al., 2005). Tests to determine if premigratory crest cells are committed to a particular fate showed in general, crest cells acquire instructions to differentiate during their migration as well as when they arrive at their final destinations. For example, when trunk cells, which normally give rise to cells of the sympathetic nervous system were transplanted to the vagal region in quail embryos, they gave rise to the neurons of the gut. The more anterior crest cells from the vagal region, which normally give rise to neurons that innervate the gut, were transplanted into the trunk region of quail embryos, the transplanted vagal crest cells migrated along the trunk

pathways and differentiated into neurons of the sympathetic nervous system (LeDouarin et al., 1982). This has been taken to indicate that neural crest cells show elasticity in responding to local environmental cues. Migrating neural crest cells appear to respond to various unique factors resulting in commitment to a particular fate and limiting their potential. As the pluripotent neural crest cells migrate along particular routes, they segregate into several more specialized progenitors, losing their pluripotency most likely as a result of different factors. For example, glial growth factor (GGF) can drive neural crest cells towards a glial fate (Anderson, 1997). GGF is a member of the epidermal growth factor/TGF- α family of ligands. The GGF receptor is expressed in all neural crest cells while only cells that become neural in the forming peripheral ganglia express GGF2. When new cells enter the ganglion, they are exposed to the GGF2 signal and adopt a glial fate, thus the cells are sensitive to GGF once they arrive at their destinations (Jessen and Mirsky, 1994, 2005; Shah et al., 1994).

Recently, the chemokine stromal cell-derived factor-1 (SDF1) was shown to attract sensory neuron progenitors. Migrating neural crest cells as well as neural crest cells in the DRG were shown to express the chemokine receptor CXCR4 while the ligand SDF1 was found expressed along the neural crest cell pathway to the DRG. CXCR4 mutant mice had small and malformed DRGs, indicating an affect on the migration and cessation of neural crest cells by a failure to stop and form a normal DRG. *In vitro*, SDF1 acted as a chemoattractant for neural crest cells (Belmandani et al., 2005). Neural crest cells expressing CXCR4 generally stained for Brn3a, a transcription factor for DRG neurons, whereas only a small number of CXCR4 positive cells expressed the

transcription factor *Phox2b*, indicating the catecholaminergic phenotype of sympathetic neurons. The chemokine SDF1 appears to function in segregating the progenitors of the DRG from the SG.

The first cells to leave the neural tube in the trunk region of the chick embryo are the SG precursors (Serbedzija et al. 1989). These cells migrate beyond the DRG to sites adjacent to the dorsal aorta where the primary SG chain will form. BMP-4 produced by the aorta itself has been shown to promote the differentiation of the crest cells into sympathetic neurons (Shah et al., 1996; Reissman et al., 1996). Here they begin to express the transcriptional regulator *Mash1*. *Mash1* is a bHLH transcription factor acting as a determinant of the neuronal state. Downstream targets regulated by *Mash1* include the homeobox gene *Phox2a* (Lo et al., 1998). Previous experiments have shown *Phox2a* is necessary but not sufficient to direct neuronal fate (Lo et al., 1998) and *Phox2b* function appears to be required for differentiation of the entire autonomic neuron population (Pattyn et al., 1999). A recent study involving time-lapse confocal imaging has shown trunk neural crest cells tend to migrate in chain-like arrays through the rostral somite from the forming dorsal root ganglia to the presumptive sympathetic ganglia. At the dorsal aorta, the cells spread in the anteroposterior direction and mix with neighboring neural crest cell populations instead of coalescing to form a separate ganglion (Kasemeier-Kulesa et al., 2005).

The DRG are organized as bilateral metameric units along the spinal cord. Each DRG forms in the rostral part of the somite and is populated by neural crest cells migrating longitudinally and laterally from the part of the neural tube facing the same

somite and the preceding somite (Teillet et al., 1987). Neural crest cells that form the DRG consist of various subtypes of sensory neurons along with satellite (a glial subtype that ensheaths neuronal somata) cells. Although fate-restricted precursor cells have been detected within the crest cells that migrate to the DRG, it is generally accepted that cell fate is progressively acquired by interaction with the local environment (Le Douarin and Kalcheim, 1999). For the sensory ganglia, it is unclear how the environment contributes to fate restriction within the neurogenic crest cells and whether interactions between the neural crest cells themselves might be involved. Studies of chick embryo DRG have shown that Notch signaling is involved in the neuron/glial cell decision. When forming the DRG, Notch-1 is expressed preferentially by cells in the outer periphery of the ganglion whereas the expression of Delta-1 and early neuronal markers such as Islet is localized to the core region. In quail DRG cell cultures, Notch activation prevented neuronal differentiation and kept cells in an undifferentiated, actively proliferating state although they could eventually form glia (Wakamatsu et al., 2000). Two proteins shown required for the formation of the DRG are the basic-helix-loop-helix transcription factors Neurogenin 1 and Neurogenin 2. Ma et al., demonstrated null mutations in both of these genes in mice resulted in a failure to form DRGs (Ma et al., 1999). An intact β -catenin signaling pathway has been shown to be required for the formation of the DRG as well (Hari et al., 2002). The migrating neural crest cells that eventually form the DRG and SG have been shown to reorganize as they form the DRG and SG as well as while in route to their target destinations. For example, in early stages of development, cells will reorient

ventrally or dorsally from their original locations and contribute to another ganglion type such as DRG versus SG (Kasemeier-Kulesa et al., 2005).

The sensory neurons in the DRG can be divided into two major neuronal subtypes based on their birthdate and size. Birthdate studies in the chick embryo demonstrated the major neuronal production in the DRG occurs between E4.5 and E6.5 for the ventrolateral population and between E4.5 and E7.5 for the dorsomedial neurons (McMillan-Carr and Simpson, 1978).

The neuron size can be further divided into the large ventrolateral neurons and the secondly, the small dorsomedial neurons (Pannese, 1974; McMillan-Carr and Simpson, 1978; Hamburger et al., 1981). In mammalian DRG, equivalent neuron populations were classified as the large “light” and the small “dark” populations (Lawson et al., 1974). The small diameter neurons send central afferents to laminae I and II of the dorsal horn, transmitting nociceptive (pain) and thermoceptive (heat) sensations. The large diameter neurons transmit mechanoreceptive (pressure, muscle contraction) and proprioceptive (body movement and position) stimuli (Koerber and Mendell 1992; Snider and Wright, 1996). DRG neurons in the chicken embryo have a spatial organization with the proprioceptive neurons located in the ventral-lateral third of the ganglia and the cutaneous neurons more broadly distributed. When sensory neurons differentiate in the mature DRG, they extend two axonal processes growing in opposite directions from cell bodies to reach discrete central and peripheral targets.

Some researchers believe melanocytes are specified in the migration staging area lateral to the neural tube, prior to their migration and they preferentially choose the

dorsolateral pathway in avian embryos (Wehrle-Haller and Weston, 1995). Specific localized factors are required by crest-derived subpopulations for survival and differentiation. Inappropriately located cells deprived of these factors are eliminated by apoptosis as a ‘proof-reading mechanism’ (Erickson and Goins 1995; Wakamatsu et al, 1998).

The migration of the melanocyte progenitors along the dorsolateral pathway has shown to be regulated by the ligand-receptor system stem cell factor (SCF, also known as Steel factor) and the receptor tyrosine kinase c-kit. Melanocyte precursors express c-kit as they leave the neural tube while SCF is expressed by cells of the dermatome and ectoderm before the onset of neural crest cell migration between these tissues. In mice lacking a functional SCF, the progenitors appear alongside the neural tube but fail to migrate. A secreted form of SCF is needed to promote cell migration along the dorsolateral path while a membrane-bound form is required for the cell’s survival (Wehrle-Haller and Weston, 1995). One transcription factor important for melanocyte differentiation and/or survival is *mitf*. *c-kit* is known to be positively regulated by *mitf* thus linking melanocyte specification with SCF dependence (Tsujimura et al., 1996; Wakamatsu et al., 1998).

Endothelin receptors (ETRs) are G protein-coupled receptors with seven transmembrane domains (for review see Pla and Larue, 2003). In the trunk, one ETR subtype, endothelin receptor B (*ETRB*), is expressed in all neural crest cells before and after they delaminate from the neural tube and is expressed on the crest cells that migrate ventrally and on their neural derivatives (Hou et al., 2004). The neural crest cells that

migrate on the dorsolateral pathway down-regulate the *ETRB* gene and express the other *ETRB* subtype, *ETRB2*. *ETRB2* is not present in the ventrally migrating neural crest cells but is activated in neural crest cells located in the migration staging area just prior to the initiation of the dorsolateral migration and is expressed throughout melanocyte differentiation (Lecoin et al., 1998).

Endothelin 3 (ET3) is the ligand of ETRB and ETRB2 and is produced in the gut mesenchyme and ectoderm (Nataf et al., 1998). Previous research has shown ET3 promotes the differentiation of neural crest cells into melanocytes, stimulates ETRB2 expression and increases the survival and proliferation rates of committed melanocyte precursors in trunk neural crest cell cultures (Reid et al., 1996). In birds, ET3 is thought to exert a paracrine action in the skin on neural crest cells through ETRB2. ET3 has been shown to have an effect on the proliferation of already differentiated melanocytes undergoing a transdifferentiation process with some cells losing their melanocytic properties and exhibiting glial markers (Dupin et al., 2000). ETRB has been found to be required for the migration of melanoblasts along the dorsolateral pathway with the major function associated with terminal migration (Shin et al., 1999; Lee et al., 2003).

Complicating the issue of cell fate is the idea it may be plausible for one factor to have multiple roles, perhaps both in migration as well as differentiation. For example, N-myc was shown by Wakamatsu et al., (1997) to have both an effect on migration and neuronal differentiation of trunk neural crest cells (Wakamatsu et al., 1997). N-myc protein expression in neural crest cells was found uniformly expressed in migrating cells followed by limited expression to those cells undergoing neuronal differentiation. A high

expression of exogenous *N-myc* gene induced early ventral migration of neural crest cells and promoted neuronal differentiation although N-myc expression itself was not an absolute requirement for neuronal differentiation in the neural crest (Wakamatsu et al., 1997; Sawai et al., 1993).

Members of the neuregulin family and their receptors (ErbB) have been implicated in neural crest cell fate decisions. Neuregulins are EGF-related proteins, some isoforms of which are expressed as a transmembrane precursor while others are secreted. Neuregulins are expressed in motor neurons and the neurons of differentiating ganglia *in vivo* and could instruct uncommitted neural crest cells to become glia rather than neurons (Ho et al., 1995; Britsch et al., 1998). In fact, Neuregulin-1 has been shown to promote glial differentiation, proliferation and survival (Dong et al., 1995; Leimeroth et al., 2002; Shah et al., 1994). These environmental factors however do not appear to be sufficient in ganglionic locations where both neurons and satellite glia differentiate whereas NOTCH-mediated lateral inhibition has been shown to be involved in this process (as discussed previously). Delta-1, a Notch ligand is presented on the surface of neurons and activates Notch signaling in neighboring cells to prevent their neuronal differentiation (Wakamatsu et al., 2000). Only a few transcriptional factors to date have been shown to regulate glial differentiation in the PNS. One example is *Sox10*, which is expressed in early migrating crest cells with restricted expression to glial cells (Bondurand et al., 1998; Cheng et al., 2000). *Sox10* has been shown to be required for both survival of crest-derived cells and their glial differentiation as well as for the generation of Schwann cell precursors from the neural crest (Jessen and Mirsky, 2005; Paratore et al., 2001). Expression of *P0* and

Seraf (Schwann cell-specific EGF-like repeat autocrine factor) are considered the earliest markers for Schwann cell differentiation of crest cells. *P0* encodes a cell surface protein involved in myelination and is a marker for glial cells in the PNS (Bhattacharyya et al., 1991). The expression of the gene *Seraf* indicates an early segregation of Schwann cell precursors and can affect the distribution of Schwann cells when introduced to chicken embryos during neural crest cell migration (Wakamatsu et al., 2004). As the previous examples demonstrate, multiple factors have been implicated in the fate of neural crest cells. How these factors are involved in the fate decisions of neural crest cells as they migrate as well as when they reach their target areas and begin to differentiate remains to be fully explained.

CHAPTER 2

CADHERINS AND PROTOCADHERINS

As evident in the previous details regarding neural crest cell induction, migration, cessation of migration and differentiation, many factors play multiple roles. A particular class of cell surface proteins, the cell adhesion molecules (CAMs) mediate adhesion between cells and to the extracellular matrix as well as the migration of cells in many embryonic tissues. CAMs are necessary to instruct cells to remain at particular sites, to associate specifically with neighboring cells or to migrate directionally. CAMs are classified into one of four different protein families: the immunoglobulin-like, the integrin, the cadherin or the selectin family. One family of CAMs shown to be involved in multiple neural crest processes and implicated in cell-cell recognition and interactions, cell sorting, cell transformations and histogenesis is the cadherins (Nollet et al., 2000; Yagi and Takeichi, 2000).

Cadherins

The cadherin superfamily comprises a large and diverse number of transmembrane proteins with five to thirty-four extracellular cadherin domains that mediate Ca^{2+} dependent binding, typically in a homophilic manner. The classical cadherins (E-cadherin, P-cadherin and N-cadherin) are single-span transmembrane proteins in general with five extracellular cadherin domains and are located primarily within adherens junctions (Yap et al, 1997; Angst 2001). The adhesion activities of

classical cadherins relies on two common features: the conserved Trp2 in the first cadherin-repeat domain (EC1) necessary for homophilic binding; and the conserved catenin-binding motifs in the cytoplasmic domain necessary for signaling and linking to the actin cytoskeleton (Gumbiner, 2005). Cadherins link to the cytoskeleton by binding the conserved cytoplasmic tail to β -catenin, which in turns binds to α -catenin. α -catenin links the cadherin- β -catenin complex to the actin cytoskeleton via actin-binding proteins such as α -actinin and profilin (Neuhoff, et al., 2005). It has been reported that α -catenin also recruits formin-1 to nucleate actin polymerization at the adherens junctions (Kobielak and Fuchs, 2004; Kobielak et al., 2004). Cadherin function was initially thought to be restricted to the non-migrating epithelial tissues (Kemler, 1992). By the mid-to-late 1990's discovery of additional cadherins (cadherin-11, cadherin-6 and cadherin-7) indicated a role for cadherins in neural crest cell migration and differentiation (Tanihara et al., 1994; Hoffman and Balling, 1995; Nakagawa and Takeichi, 1995; Nakagawa and Takeichi 1998; Inoue et al., 1997; Vallin et al., 1998).

Protocadherins

Protocadherins represent the largest subgroup of the cadherin superfamily. Protocadherins are transmembrane glycoproteins that contain six or more conserved cadherin-repeats in their extracellular domains (Yagi and Takeichi, 2000; Frank and Kemler, 2002). Protocadherin genes typically include a large exon that encodes most of the extracellular domain, a region of the transmembrane and part of the cytoplasmic domain. The extracellular cadherin domains of protocadherins have characteristics of

calcium-dependent adhesion molecules but do not have the Trp2 residue in the extracellular domain. Protocadherin cytoplasmic domains do not have the catenin-binding motifs and thus have no similarity to the cytoplasmic domains of classical cadherins (Obata et al., 1995; Kohmura et al., 1998; Wu and Maniatis, 1999; Nollet et al., 2000). Protocadherins have been divided into several subfamilies often based on the alignment of their cytoplasmic domains, the first two extracellular cadherin repeats and/or structural differences between genes (Nollet et al., 2000; Wolverton and Lalande, 2001; Redies et al., 2005). The majority of protocadherins are divided into three subgroups, α -, β - and γ -protocadherins which are encoded by three gene clusters on chromosome 5q31 in the human genome. Recently, a novel subgroup of protocadherin has been identified named δ -protocadherin (Redies et al., 2005). Other subgroups include the flamingo-like or CELSR cadherins and the Fat-and Dachshous-related protocadherins. Analysis using either human or murine sequences based on the cytoplasmic domains or the first two extracellular cadherin repeats have shown; the α -, β - and γ -protocadherins each have a specific genomic organization; the δ -protocadherins contain two previously identified amino acid motifs in their cytoplasmic domains; the CELSR protocadherin subgroup contains seven-pass transmembrane domains; the Fat-like protocadherins have significantly more than seven extracellular domains; the two Dachshous protocadherins 1 and 2, each with 27 extracellular calcium-binding cadherin repeats being distinct from each other and from the Fat-like protocadherin family (Redies et al., 2005).

Cadherins and protocadherins have been shown to be involved in cell adhesion in development and specifically in the developing nervous system. For example, during

neural tube formation in the early embryo, *Xenopus* NF-protocadherin (NFPC) whose expression was found within the deep, sensorial layer of the ectoderm and in a group of cells in the neural folds, was shown to mediate cell adhesion during differentiation of the epidermis (Bradley et al., 1998). Another protocadherin, paraxial protocadherin is expressed in the trunk mesoderm in *Xenopus*, zebrafish and mouse (Kim et al., 1998; Yamamoto et al., 1998; Yamamoto et al., 2000). Specifically in *Xenopus*, paraxial protocadherin has been shown to mediate cell sorting of blastomeres and has an essential role in paraxial mesodermal convergence and extension movements during gastrulation. Paraxial protocadherin is also involved in the establishment of somite boundaries during early *Xenopus* development (Kim et al., 1998; Kim et al., 2000). Another protocadherin, axial protocadherins is expressed in the axial mesoderm notochord in *Xenopus* and functions as a mediator of prenotochord cell sorting (Kuroda et al., 2002). Paraxial protocadherin together with axial protocadherin cooperate to sort notochord from somite and divide the gastrulating mesoderm into the paraxial and axial domains. Recently paraxial protocadherin has also been shown to mediate cell sorting and tissue morphogenesis by downregulating C-cadherin activity (Chen and Gumbiner, 2006).

A majority of the protocadherins discovered to date appear to have roles in the central nervous system. For example, in humans, expression of protocadherin 2 has been found to appear relatively early in development of the fetal central nervous system and is involved in induction, fasciculation and extension of axons (Omi and Kitagawa, 2004). Recently, a protocadherin PCNS (Protocadherin in Neural Crest and Somites) was discovered to have a dual function in the migration of cranial neural crest cells and

somitogenesis in *Xenopus* (Rangarajan et al., 2006). Researchers showed with a gain-of-function analysis by ectopic overexpression of PCNS in whole embryos, a failure of 96% of the embryos in completing gastrulation. To investigate if the gastrulation effects may be due to abnormally high levels of cell adhesion, an ectodermal cell dissociation and reaggregation assay was used. Results indicated that PCNS did not impart specific cell-cell adhesion and authors speculated PCNS overexpression interfered with one or more signaling pathways. For a loss-of-function analysis, antisense morpholinos were used to inhibit translation of mRNA. Results showed PCNS was required for the migration of cranial neural crest cells in *Xenopus* by a strong inhibition of migration from the dorsal neural tube into the branchial arches. This was confirmed with cultured explants of cranial neural crest tissue. The additional role of *PCNS* function in the segmentation of the somites was demonstrated with the injection of the antisense morpholinos followed by probing with somite-specific markers *MyoD* and *Thylacine-1*.

Research using the avian system has also pointed to role of protocadherins in the developing central nervous system. Two α -protocadherin family members in chick have been found in multiple regions of the chick central nervous system including the cerebellum, olfactory bulb, forebrain and in the autonomic nervous system.

Immunohistochemistry has shown these two α -protocadherin proteins in nicotinic cholinergic pathways including the parasympathetic and somatic motor system. During development (as well as in the adult), localization appeared in presynaptic terminals at perisynaptic sites but absent from the postsynaptic side of nicotinic synapses on both neurons and muscles. A continuing synaptic function, presumably involving heterophilic

interactions with other kinds of molecules was suggested by the researchers (Blank et al., 2004). Another protocadherin OL-protocadherin, was initially found in the developing mouse brain and later in the chick visual system at E11 to E19 with localized protein expression along axon fibers in peripheral motor nerves of the E3.5 to E6.5 chicken nervous system (Hirano et al., 1999; Muller et al., 2004; Nakao et al., 2005). Since OL-protocadherin failed to interact with other protocadherins or classical cadherins with similar amino acid sequences in their extracellular domains in transfected cells, OL-protocadherin was suggested to function as a homophilic adhesion molecule (Hirano et al., 1999). The strong expression of OL-protocadherin in axon fibers together with the inability of OL-protocadherin to localize at cell junctions indicates OL-protocadherins may function in axon growth or guidance (Nakao et al., 2005). In 2003, Capehart and Kern identified a protocadherin (protocadherin γ A-like) expressed during chick development. Current research has shown members of the protocadherin- γ family expressed in the chick central nervous system, specifically in the fore-, mid- and hindbrain at stages 23 and 25 as well as in the spinal cord and optic lobe at stages 27 and 43 respectively (Cronin and Capehart, 2007). By using double-label in situ on mice from E14 through birth, protocadherin- γ expression was found throughout the central and peripheral nervous system with highest levels in the brain, spinal cord and DRG (Wang, et al., 2002). Further experiments showed protocadherin- γ present at, but not strictly confined to, some synapses. Using mutant mice with the entire protocadherin- γ locus deleted, researchers found multiple characteristics of neurogenesis including neuronal migration, axon outgrowth and synapse formation proceeded normally although at late

stages of embryogenesis, apoptosis of many spinal interneurons occurred. The mutants at these later stages showed a lack of coordinated movement and neonatal lethality indicating the protocadherin- γ genes appear to have a role in neuronal maturation or survival rather than patterning (Wang, et al., 2002). Later experiments were aimed at circumventing apoptosis and neurodegeneration in protocadherin- γ mutant mice.

Researchers used analysis of mutants lacking both the protocadherin- γ proteins and the proapoptotic protein Bax along with generating a hypomorphic allele of protocadherin- γ with minimal apoptosis. Results showed a preservation of spinal interneurons but with a severely decreased number of spinal cord synapses, neurological defects and a compromised synaptic function in hypomorph cultured neurons. These results were taken as evidence for a direct role of protocadherin- γ s in synaptic development (Weiner et al., 2005). Due to the discoveries of protocadherin- γ A5 also being expressed in the dorsal horn and DRG (Capehart and Kern, 2003) and the more recently of protocadherin- γ transcripts in stage 27 chick embryos in the dorsal horn and floorplate regions of the developing spinal cord as well as in the DRG (Cronin and Capehart, 2007) provides evidence of protocadherins in the peripheral nervous system. This also indicates a possible role for one or more members of the protocadherin- γ family in interactions between interneurons and the DRG while peripheral sensory connections with the central nervous system are being established (Cronin and Capehart, 2007).

When cells undergo an EMT, an obvious phenotypic change is the loss of cell-cell contact. One protein whose expression and function is altered is E-cadherin. E-cadherin binds to another E-cadherin in an adjacent cell in a homophilic interaction and when the

expression is downregulated, there is a concurrent decrease in cell adhesion. The EMT is accompanied by the onset of the transcription factor Slug whose expression is maintained during the neural crest cell migration period (Nieto et al., 1994). Slug has been shown to repress E-cadherin expression and induce EMT, thus allowing neural crest cells to migrate (Cano et al., 2000; Bolos et al., 2003). In addition, the cell adhesion molecules N-CAM and N-cadherin are expressed in neural tube cells. The presumptive neural crest cells lose the expression of these adhesion molecules as they appear in the neural folds. Another cadherin, cadherin 6B, becomes expressed at this stage, but also becomes downregulated at the commencement of migration (Nakagawa and Takeichi 1995). Once cells cease migrating, coalesce and differentiate in the DRG, cells re-express N-CAM and N-cadherin while another population of cells in the dorsal and ventral roots express cadherin-7 (Hatta et al., 1987; Nakagawa and Takeichi 1995).

Although the involvement of cadherins in the beginning steps of neural crest cell migration is well established, cadherin-mediated adhesive activity in neural crest cell migration and differentiation is less obvious due to a lack of conclusive experiments. Previous research has shown overexpression of cadherin-11 blocked neural crest cell emigration from the neural tube in *Xenopus* while ectopic expression of a dominant-negative cadherin-11, which lacked the extracellular domain, resulted in premature emigration (Borchers et al., 2001). When N-cadherin or cadherin-7 was overexpressed in the chick embryo, neural crest cell migration was inhibited with an aggregation of neural crest cells in the dorsal neural tube and an associated decrease of crest cells in the DRG (Nakagawa and Takeichi, 1998). The evident regulation of cadherins necessary for the

departure of neural crest cells from the neural tube leaves open the question of what roles cadherins and protocadherins play in neural crest migration and especially with regards to differentiation is currently unknown. Another major unresolved question in developmental biology is when the fates of individual neural crest cells become different from one another. For example, neural crest cells migrating along the ventral pathway choose to stop adjacent to the neural tube and populate the DRG or to continue more ventrally to the dorsal aorta to populate the SG or continue still further to form the enteric nervous system. Unclear is whether the appearance of differentiated cells in precise locations is the result of the differential localization of developmentally distinct precursors or whether factors in these locations instruct developmentally ambiguous precursors to adopt specific fates. Yet to be completely resolved is what determines the choices for neural crest migration and cessation as well as what stop signals exist. Although the involvement of cell adhesion molecules in early events of development such as neural tube formation is well established, the cell-cell interactions during migration and differentiation at target areas in the peripheral nervous system is less understood. We postulate that members of the protocadherin family are likely involved in this process. Therefore to identify and analyze the role of protocadherins in neural crest migration in the chick embryo I initiated a screen for novel cadherin members expressed in the developing peripheral nervous system resulting in the isolation of chicken protocadherin-1 (cPcdh1).

CHAPTER 3

MATERIALS AND METHODS

Library Screening

A chick E4.5 DRG bacteriophage library (gift of Dr. Frances Lefcort) was screened for the identification and isolation of novel chicken protocadherins expressed in neural crest cells. PCR primers specific for conserved regions in the extracellular domains of both cadherins and protocadherins (Bradley et al., 1998) were used to generate a radiolabeled DNA probe for screening. Several positive clones were initially isolated and after three rounds of screening a novel protocadherin cDNA clone (sequenced by Iowa State Sequencing Facility) was identified and subsequently named cPcdh1 (GenBank accession number: AY676328). The entire reading frame was determined to be 3150 nucleotides in length. Basic Local Alignment Search Tool (BLAST) showed highest homology with *Xenopus* axial protocadherin (82% identical) and human protocadherin 1 (81%). The NCBI conserved domain search identified 6 cadherin extracellular repeat domains.

Whole Mount In Situ Hybridization

Fertilized white leghorn chicken eggs (CBT Farms, Chestertown, MD, USA; or Charles River Laboratories, Inc., Wilmington, MA, USA) were placed on a rocking shelf and incubated at 37°C (Kuhl, Flemington, NJ, USA). The embryos were staged according to Hamilton and Hamburger (HH) (Hamburger and Hamilton, 1951). Stage 5 (19 hours)

up through stage 34 (E8) were dissected in 1x phosphate buffered saline (PBS), pH 7.4 and fixed in 4% paraformaldehyde (PFA)/1x PBS for approximately 2 to 4 hours at 4°C. The embryos were rinsed twice with 1x PBS, dehydrated through a series of methanol washes (25%, 50%, 75% and 100% in 1xPBS containing 0.15% Tween-20; PBST) and stored in 100% methanol at -20°C until time of in situ hybridization.

For probe preparation, a restriction enzyme digest of cPcdh1 was performed using Not 1 (Promega, Madison WI) for the sense probe and Sal 1 (Promega, Madison WI) for the antisense probe. To verify linearization, the digests were run on a 1% agarose gel containing ethidium bromide for band visualization. The correct digests were subject to phenol/chloroform extraction and a sodium acetate/ethanol precipitation. Following precipitation, digoxigenin-labeled cRNA antisense and sense probes were generated by in vitro transcription using DIG RNA labeling mix (Roche Diagnostics, Indianapolis, Indiana, USA) with either a T3 enzyme for the sense probe or T7 enzyme for the antisense probe. After a sodium acetate and ethanol precipitation, the pellets were resuspended in a freshly prepared sodium bicarbonate/sodium carbonate solution and hydrolyzed for approximately 35 minutes at 60°C for a 1.5 kb probe to generate 300 nucleotide fragments. Following hydrolysis, the probe was subject to a final sodium acetate and ethanol precipitation and the pellet was resuspended in DEPC treated water. The probe concentration was determined by UV spectrophotometry, then diluted with prehybridization solution to a final stock concentration of 0.1 µg/ul, aliquoted and stored at -80°C until use.

The protocol described previously by Nieto et al., 1996 and Wilkinson and Nieto, 1993 was followed for the whole mount in situ hybridization. Briefly, the embryos stored in 100% methanol were prepared for in situ hybridization by performing the methanol washes in reverse with a final rinse in 1xPBS. The embryos were incubated 1 hour in 6% hydrogen peroxide. According to their stage of development, the embryos were next proteinase K treated (10 µg/ml) from 10 minutes (young embryos, stage 5) through 35 minutes (E8 embryos). This step was later removed for those embryos used for immunohistochemistry and siRNA injections. Following proteinase K treatment, embryos were washed with glycine (2 mg/ml) in 1x PBST briefly and refixed with 0.2% glutaraldehyde/4% PFA/1x PBST for 20 minutes. After washing briefly with 1xPBST, the embryos were incubated with prehybridization buffer for 1 hour at 70°C. Following prehybridization, the embryos were hybridized with 1 µg/ml digoxigenin-labeled RNA probe and incubated overnight at 70°C. The following day, embryos were washed with 3 different solutions. The embryos were washed first in solution 1 (50% formamide, 5x SSC pH 4.5, 10% SDS, 15% H₂O) 3 times for 30 minutes at 70°C. Next the embryos were washed with a 1:1 ratio of solution 1 and solution 2 (.5M NaCl, .01M Tris pH 7.5, 0.25% Tween-20, 88.75% H₂O) 10 minutes at 70°C. This was followed by 3 washes for 5 minutes in solution 2 at room temperature. Embryos were RNASE A treated (100 ug/ml) in solution 2 for 20 minutes at 37°C, This was followed by a 5 minute wash at room temperature in solution 2. Embryos were then washed with solution 3 (50% formamide, 2x SSC pH 4.5, 40% H₂O) for 5 minutes at room temperature followed by 3 washes for 30 minutes at 60°C. Next embryos were preblocked with 10% heat inactivated goat

serum/1x PBST for 1 hour at room temperature. The embryos were next incubated with 1ul anti-digoxigenin alkaline phosphatase antibody (Roche Diagnostics, Indianapolis, Indiana, USA) in 4 mL of 1% heat inactivated goat serum/1x PBST at 4°C overnight with gentle rocking. The following day, embryos were washed a minimum of 4 times with 1x Tris buffered saline solution (TBS) containing 0.05% Tween-20 (TBST) and levamisole (2mM/ml). Each wash consisted of 1 hour of gentle rocking at room temperature. Next, embryos were washed with a solution containing 100 mM NaCl, 100 mM Tris-HCL pH 9.5, 50 mM, MgCl₂ 0.1% Tween-20, H₂O and levamisole (2mM/ml) for 10 minutes at room temperature. After the third and final wash, embryos were developed in 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT; Sigma-Aldrich, St. Louis, MO) at room temperature in the dark until the purple color developed to the desired extent. Typically embryos showed color development by 40 minutes and were allowed to further develop up to 3 hours. Once satisfied with color development, embryos were washed with 1x PBST at room temperature for 10 to 30 minutes. Embryos were then post fixed with 1x PBS containing 3.7% formaldehyde and incubated overnight at 4°C. The following day embryos were washed twice in 1x PBS. Embryos were prepared for embedding and cryostat sectioning by taking the embryos through a series of washes (5%, 15%, 30%) in optimal cutting temperature (OCT) compound (Tissue-Tek) in 1x PBST at 4°C until embryos sank or in the case of the 30% wash, allowed to wash overnight at 4°C. The final washes consisted of a 1:1 wash of 30% OCT with 100% OCT for 2 hours followed by a wash in 100% OCT for 2 hours both at 4°C. The embryos were individually embedded in 100% OCT in peel-away sectioning molds and allowed to

freeze in a dry ice and ethanol slurry before storing in -80°C until time of cryostat sectioning. Embryos that were to be vibratome sectioned were embedded in 5% agarose/8% sucrose/1x PBS in a peel-away tapered mold. Cryostat sections were serially sectioned typically at 30 μ m and vibratome sections at 100 μ m for in situ stained embryos. The glass slides were coverslipped after pipetting a 1:1 solution of glycerol and 1x PBST onto the glass slides containing the sections and the edges were sealed with fingernail polish.

Whole mount images were acquired using Axiovision 3.1 software (Zeiss) with an AxioCam HRC digital video camera (Zeiss). Images of the in situ hybridization and immunochemistry sections were captured by 2 different methods. Initially, sections were photographed with a Kodak Elite Chrome 100 (fine grain) color slide film and a 35-mm camera (Nikon FX-35DX) attached to a Nikon Microphot-FXA fluorescence scope via a phototube assembly. A Nikon Super Coolscan 4000 slide scanner was used with Nikon Scan software to acquire digital images of the color slides. Section images were later captured using a Zeiss digital camera adapter along with the Axiovision 3.1 software.

Cloning

To clone cPcdh1FL, the PCR oligonucleotide forward primer 821-1 (5'-TGATCAGAGGAGGCGATGCAGAC-3') designed to introduce a Bcl I site and the reverse primer 821-2 (5'-GTTGTCGTTGATATCGAGCAC-3') designed to introduce an EcoR V site were created for the 5' end of cPcdh1 (Sigma Genosys, The Woodlands, TX). For the 3' end of cPCDH1, PCR forward primer 821-3 (5'-AGCTCTTCCAGATCTCTCCCC-3'), reverse primers 821-4 (5'-

GGCGCCACTCCCACACCTCGGTC-3') designed with a Nar I site and 821-5 (5'-ATCGATACTCCCACACCTCGGTC-3') designed with a Cla I site were created (Sigma Genosys, The Woodlands, TX). The PCR primers 821-1 and 821-2 were cloned into pBluescript and cut out with Sac II and EcoR V and ligated to cPcdh1 cut with EcoR V and Sac II. The PCR primers 821-3 and 821-4 were cloned into the pGEM T-vector (Promega, Madison, WI) and cut out with Bgl II and Sac II and ligated to the cPcdh1 ligated with PCR primers 821-1 and 821-2 described above in pBluescript. This plasmid containing PCR primers 821-1, 821-2, 821-3 and 821-4 with their respective cloning sites in pBluescript was cut with Bcl I and Nar I and cloned into the CS2mt vector cut with BamH I and Cla I.

To generate the deletion construct cPcdhΔE, which lacks the extracellular domain, PCR primers 821-4 and 53-1 (5'-GGATCCCTGGTTCACCTTCTATGTC-3') were subcloned into the pGEM-T vector, cut with BamH I and Nar I and cloned into the CS2mt vector cut with BamH I and Cla I. In order to introduce the signal sequence into the deletion construct cPcdhΔE, NFAE cloned into CS2mt containing the signal sequence from *Xenopus* N-cadherin (Bradley et al., 1998) was cut out with BamH I and cloned into the CS2MT vector containing the 821-4 and 53-1 PCR products described above.

To generate the deletion construct cPcdhΔC lacking the cytoplasmic domain a new PCR reverse primer 821-CT (5'-ATCGATGTCGGCAGTAGCGCAC-3') was designed with a Cla I site and was used with the forward primer 821-3 to subclone into the CS2MT vector digested with BamH I and Cla I and next cloned into the cPcdh1 in CS2MT (described above) cut with Hind III and Bgl I.

To generate the deletion construct cPcdh1 Δ TM encoding a secreted protein lacking the transmembrane and cytoplasmic domains, cPcdh1 in CS2MT was cut with Sac I, which cut out the transmembrane and cytoplasmic domains. This was religated on itself to give cPch1 Δ TM in CS2MT.

All constructs were subcloned into the pMiwII vector under the control of the chicken β -actin promoter (Araki and Nakamura, 1999) by cutting with Hind III and Xba I or Xho I.

For live time-lapse imaging, cPcdh1 in CS2MT was cut with Hind III and Xba I and subcloned into the pBluescript vector. This product was cloned into the pMES2 vector, a modified version of pMES (gift of C. Krull) cut with Cla I and Xba I. The pMES vector contains the chicken β -actin promoter to drive expression of the gene and an internal ribosome entry site (IRES) for green fluorescent protein (GFP) expression. All cloning was checked on agarose gels and confirmed with sequencing from Iowa State Sequencing Facility.

In Ovo Microinjection and Electroporation

Fertilized White Leghorn chicken eggs (CBT Farms, Chestertown, MD, USA; or Charles River Laboratories, Inc., Wilmington, MA, USA) were placed on a rocking shelf and incubated at 37°C (Kuhl, Flemington, NJ, USA). The night prior to electroporation, the eggs were placed on their sides to allow the embryo to move to the top of the egg. Eggs were removed from the incubator, lightly sprayed with 70% ethanol and 3 ml of albumin was withdrawn from the end of each egg with a 10 ml syringe. The eggs were

opened first, then staged according to Hamburger and Hamilton (HH) staging (Hamburger and Hamilton, 1951) and opened by cutting a small circle on the topside of the egg. Each DNA construct to be injected and electroporated was prepared just prior to use at a concentration of 0.20 ug/ul with the addition of 1% fast green dye (Fast Green FCF, Sigma, St.Louis, MO, USA) at 8% of total volume to aid in visualization of the construct *in ovo*. *In ovo* microinjection and electroporation of the various plasmids were performed as previously described (Muramatsu et al., 1997; Swartz et al., 2000).

Embryos at HH st.10 were initially injected with one of the following constructs pCDH1(FL), pCDH1ΔE, pCDH1ΔC and pCDH1ΔTM all tagged with a C-terminal myc epitope tag. As a control embryos were injected with either CS2MT or a vector encoding the myc epitope cloned into pMiWII (pMiWII-MT). The DNA construct was loaded into a borosilicate glass capillary pulled needle (World Precision Instruments) and microinjected into the lumen of the neural tube, filling the neural tube from the hindbrain area (mesencephalon and rhombencephalon) down thru the posterior neuropore. Gold-plated electrodes (Harvard Apparatus, model 512) with a 4 mm gap were placed on either side of the neural tube and a square wave electroporator (BTX Electro Square Porator ECM 830, Genetronics, Inc) was used to apply 5 pulses each with a 23 volt current for a pulse length of 50 msec. After electroporation, a few drops of Hanks Balanced Salt Solution (Gibco, Grand Island, NY, USA) was placed on top of the embryo, the egg was resealed with adhesive tape and placed back into the 37°C incubator. Eggs were allowed to develop until HH st. 20-23, approximately 50 hours from the time of electroporation.

Embryos were dissected in 1x PBS, fixed in 4% paraformaldehyde for 2 hours at 4°C, rinsed in 1xPBS and processed for whole mount immunostaining.

Whole Mount Immunostaining

Whole mount immunostaining was performed with the same basic procedure used for slide immunohistochemistry. Briefly, the embryos were dissected and fixed in 4% paraformaldehyde/1xPBS for 2 hours at 4°C. Embryos were either stained using immunochemical methods (HRP and DAB substrate), or by immunofluorescence. For immunochemical staining embryos were rinsed with 1x PBST and endogenous peroxidases were quenched with hydrogen peroxide for 1 hour at room temperature when using horseradish peroxidase (HRP) conjugated secondary antibodies. Embryos were rinsed again with 1x PBST and then blocked for 1 hour at room temperature with 20% heat inactivate goat serum/1x PBST. For immunofluorescence staining, embryos were rinsed with 1x PBST and then blocked for 1 hour at room temperature with normal goat serum (NGS; 0.15M NaCl, 0.03M Tris pH 7.5, 1% w/v glycine, 10% inactivated goat serum, 0.4% Triton X-100). Embryos were incubated 1-3 days at 4°C with primary anti-myc (9E10; rabbit or mouse). Following primary antibody incubation, immunochemical staining embryos were rinsed with 1x PBST, blocked with 20% heat incubated goat serum in 1x PBST for 1 hour at room temperature followed by incubation overnight at 4°C with secondary antibody (FAB fragment peroxidase conjugated goat anti-mouse IgG & IgM. The next day embryos were rinsed with 1x PBST and developed with 3,3'-diaminobenzidine (DAB) staining. Embryos were developed in a 1.5 ml DAB (0.5 mg/ml

DAB in 1x PBST) for 20 minutes at room temperature, followed by the addition of H_2O_2 and allowed to stain (usually 5-20 minutes) until desired darkness. Embryos were then washed twice in 1x PBS and postfixed in 3.7% formaldehyde/1x PBS overnight at 4°C. Embryos were washed in 1x PBS and prepared for vibratome sectioning as described previously. Following primary antibody incubation, embryos for immunofluorescence staining were rinsed 1x TBS, blocked with NGS for 1 hour at room temperature, followed by incubation with secondary antibody goat anti-mouse/rabbit cy3 overnight at 4°C. Following secondary incubation, embryos were rinsed with 1x TBS and checked for fluorescence. Embryos were postfixed and prepared for either vibratome or cryostat sectioning as previously described.

BrdU Injections

At stage 23, a few hours prior to dissection, the embryos were injected with BrdU (1 mg/ml) and allowed to develop approximately 4 additional hours in the incubator. The embryos were then dissected and fixed as previously described for pCDH1 in situ hybridization with the following changes. After washing in 1x PBST, embryos were incubated in 2N HCL for 1 hour at room temperature. Embryos were blocked in 3% heat inactivated goat serum/1x PBST for 1 hour at room temperature and then washed in Roche incubation buffer (Roche Diagnostics, Indianapolis, IN) twice for 10 minutes. Embryos were incubated 2 days at 4°C in mouse BrdU antibody diluted 1:60 in incubation buffer. Following incubation, embryos were washed 4x for 1 hour in 1x PBST

at room temperature. Embryos were blocked in 20% heat inactivated goat serum/1x PBST for 1 hour followed by incubation in goat anti-mouse HRP secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) diluted 1:300 in 20% goat serum/1x PBST overnight at 4°. The following day, embryos were washed 5 times in 1xPBST for 1 hour at room temperature. The embryos were developed following the DAB staining protocol as described previously. After staining, embryos were fixed and processed either for vibratome or cryostat sectioning as previously explained.

BrdU staining was also performed on cryostat sections with a similar protocol. The slides were rehydrated with 1x TBS for 20 minutes at room temperature. Next, slides were incubated in 2N HCL for 45 minutes at 37°C. Slides were washed 3 times for 5 minutes with 1x TBS and incubated with 0.1M Borax (sodium borate, Sigma, St. Louis, MO) for 10 minutes at room temperature. Slides were washed 5 times for 1 minute in 1x TBS at room temperature followed by block in NGS for 1 hour at room temperature. Slides were placed in a humidified chamber and incubated in primary antibody (rat Brdu) at 1:1000 diluted in NGS overnight at 4°C. The following day, slides were washed 5 times with NGS for 10 minutes at room temperature. Slides were incubated in the dark for 1 hour at room temperature with a 1:1000 dilution of the secondary antibody (goat anti-rat cy3). Slides were washed 5 times for 10 minutes in 1x TBS. Prolong Antifade (Molecular Probes, Eugene, OR USA) was applied, slides were coverslipped and viewed on a Nikon Microphot-FXA fluorescence scope.

Morpholino Injections

For the morpholino experiment, a 25-basepair oligomer, complementary to the 821 ATG start site was identified by Genetools, LLC as a good candidate for making an antisense morpholino and was made and purchased from Genetools (5'-TGATCCAGCGGCGTCTGCATCGCCT-3'). This morpholino was modified to include a 3' carboxyfluorescein tag, to begin 6 bases upstream from the start and to have a 64% GC content, more specifically, a 28% guanine content. Electroporations were performed as previously described with a few changes including no fast green dye, a pulse of 23 volts, 5 pulses for 100 msec and in dark conditions. The experiments with morpholinos also included a fluorescein tagged control morpholino oligomer designed and provided by Genetools, LLC. The control is a 25-mer morpholino based on the sequence of the mutated β -globin of human thalassemia patients and should not have a specific effect in chicken cells.

siRNA

The design of the siRNA involved the use of the Whitehead website (<http://jura.wi.mit.edu/siRNAext/>) which offered suggestions for RNAi design. Once potential candidates were chosen, the sequences were BLASTED in the database to assure they did not overlap with other chicken genes and thus block other protein expression. A similar procedure was used with the Dharmacon website (Dharmacon.com). Two different sense strand sequences were chosen for siRNA injections and electroporations (siRNA1: ACACACCACUUGACAUAGAUU; siRNA2:

CCAACUCGCUGAAGGUACA UU), which each provided as a duplex bound to the antisense strand. The control siRNA used was Dharmacon siCONTROL non-targeting siRNA #1. The siRNAs were diluted in RNase-free 1xPBS to a stock concentration of 2 ug/ul and stored at -20°C.

The siRNA in ovo injections and electroporations were performed as previously described for the other constructs with a few modifications. The siRNAs were co-injected with the pMes2 vector (for identification by GFP fluorescence) or with the LacZ/CS2 vector (for β -gal staining) added to a final concentration of 0.2 ug/ul. A 1:10 dilution (1%) of DEPC-treated fast green dye was added and injections and electroporations were performed under as RNase-free conditions as possible. Embryos were allowed to develop as previously described. Dissections were performed with DEPC-treated 1xPBS and the embryos were processed either for cryostat sectioning and immunohistochemistry or stained for β -gal and processed for in situ hybridization followed by cryostat sectioning.

Immunohistochemistry

Immunochemistry was performed on embryo sections from the in situ hybridization as well as embryos electroporated with the various constructs prior to coverslipping. The procedure is basically the same for either cryostat or vibratome sections. The sections were blocked with 10% NGS in TBS, pH 7.5 for 1 hour at room temperature. The sections were incubated overnight at 4°C with various primary antibodies diluted in NGS (for detailed information see Table 1: antibody spreadsheet). The slides were rinsed 4 times in NGS or 1x TBS at room temperature and incubated for

1 hour in the dark at room temperature with the appropriate secondary antibody (as shown in Table 1). The slides were washed 5 times for 10 minutes in 1x TBS. Prolong Antifade or PVA/DABCO was applied and the slides were coverslipped.

Xenopus microinjection

cPcdh1 RNA was prepared for *Xenopus* microinjection by first cutting 10 ug of DNA from the cPcdh1 Δ C, cPcdh1 Δ E, and cPcdh1(FL) constructs, cloned into CS2MT, with the restriction enzyme Not 1 (Promega, Madison, WI) and verified by checking on a 1% agarose gel. Transcription was carried out with a combination of 5x transcription buffer (Promega, Madison, WI) DTT, stock nucleotides (A, C and U), nucleotide G diluted 1:10 in DEPC water, RNasin, GPPG (5'-cap), DEPC treated water, SP6 and the specific DNA construct under Rnase free conditions. After incubation at 37°C for 2 hours, DNase was added to the microcentrifuge tube and allowed to incubate at 37°C for an additional 30 minutes. Following incubation, DEPC treated water and phenol/chloroform was added, mixed by vortexing and centrifuged at 14,000 rpm for 5 minutes. The supernatant was removed into a new microcentrifuge tube and ammonium acetate and 100% ethanol was added and the DNA was precipitated overnight at -20°C. The following day the tube was centrifuged at full speed (14,000 rpm) and the supernatant was removed. The pellet was resuspended in 25 ul of DEPC treated water and a small amount was removed and checked on a RNA gel. A final precipitation with sodium acetate and ethanol was performed overnight at -20°C. The following day the tube was centrifuged at full speed and the supernatant was removed and the pellet

Primary Antibody	Identification	Dilution	Source	Secondary Antibody	Dilution	Source
anti-myc (mouse)	monoclonal to myc tag	1:20	Developmental Studies Hybridoma Bank (9E 10)	goat anti-mouse cy3	1:250	
				FAB fragment peroxidase conjugated goat anti-mouse IgG & IgM (H+L)		
				goat anti-mouse alexafluor 488 IgM	1:2000	Molecular Probes
anti-myc (rabbit)		1:250		goat anti-rabbit cy3	1:250	
anti-HNK (mouse)	neural crest marker	1:100		goat anti-mouse alexafluor 488 IgM	1:2000	Molecular Probes
				goat anti-mouse cy2	1:200	
anti-BEN (mouse)	cell adhesion molecule; expressed on axons; labels proliferating cells in the neural tube and somites	1:10		goat anti-mouse cy3	1:250	
				goat anti-mouse cy2	1:200	
anti-phosphohistone H3 (rabbit)	mitosis marker; proliferating cells in metaphase	1:200	Upstate	goat anti-rabbit cy3	1:250	
				goat anti-rabbit alexafluor 488 IgM	1:1500	Molecular Probes
anti-Tuj1 (mouse)	neuron marker	1:1500	Berkeley Antibody Company	goat anti-mouse Tritc	1:300	
				goat anti-mouse cy3	1:250	
anti-TrkA (rabbit)	marker for nociceptive sensory afferents; peripheral nervous system	1:8200		goat anti-rabbit cy3	1:250	
anti-neurofilament (rabbit)		1:250		goat anti-rabbit alexafluor 488 IgM	1:1500	Molecular Probes
anti-Isl 1 (mouse)	sensory and motor neurons; an early marker for neuronal differentiation of DRG cells	1:10	Developmental Studies Hybridoma Bank	goat anti-mouse alexafluor 488 IgM	1:2000	Molecular Probes
anti-TrkC	marker for proprioceptive sensory afferents					
DAPI	binds to natural double-stranded DNA			DAPI	1:3000	
anti-FITC (rabbit)		1:500		goat anti-rabbit cy3	1:250	

Table 1. Antibody spreadsheet showing primary and secondary antibodies used for staining including dilutions used.

resuspended in 25 μ l DEPC treated water. UV spectrophotometry was used to check the ratio and concentration of the RNA. Embryos were obtained from hormone-induced egg-laying adult *X. laevis* females artificially fertilized using standard methods (Sive et al., 2000). Microinjection of *Xenopus* eggs was performed as previously described (Heggen and Bradley, 2003). The RNA was diluted to approximately 0.200 μ g/ μ l with DEPC treated water and the dilution was used with or without *LacZ* mRNA. Injection settings were set to a 200-300 duration and approximately 20 psi of pressure. Glass capillary needles were loaded with 2 μ l of RNA with RNase free gel loading tips. The tip of the needle was broken in order to yield a drop size of 0.5mm diameter when viewed under the microscope. The *Xenopus* eggs were injected at the appropriate stage of development (2, 4 or 8 cell stage). Eggs were kept at 14°C in 0.5x MMR plus 1% Ficoll while injecting to slow down cell division. After injection, dead embryos were removed, the eggs were placed in 0.5x MMR plus 1% Ficoll and allowed to remain at room temperature for 15-30 minutes. Any new dead embryos were removed, the solution changed to 0.1x MMR plus 1% Ficoll and eggs were placed in 19°C incubator overnight.

The next day the embryos were checked, dead embryos were removed and the remaining embryos were devitellined. Embryos were fixed at stage 15 in fresh MEMFA (0.1 M MOPS, pH 7.4), 2 mM EGTA, 1 mM MgSO₄, 3.7% formaldehyde) for 1-2 hours at room temperature. After fixing, embryos were washed in NaPO₄ pH 6.3 for 5 minutes at room temperature. The embryos coinjected with *LacZ* were stained for β -galactosidase activity with X-Gal as previously described (Bradley et al., 1998). The embryos not injected with *LacZ* were washed in 1x PBST, blocked with 20% goat serum for 1 hour

and primary antibody (anti-myc mouse from Developmental Hybridoma Studies Bank; 9E 10) was applied (1:20) diluted in 20% goat serum in 1x PBST for 1-2 days at 4°C. Following primary antibody incubation, the embryos were washed in 1x PBST for a few hours at room temperature with several solution changes. Embryos were blocked with 20% goat serum/1x PBST at room temperature for 1 hour. Embryos were incubated in secondary antibody (anti-mouse cy3; 1:250) for 1-2 days at 4°C. After secondary antibody incubation, embryos were washed 4-5 hours with 1x PBST with several changes. Embryos were developed using DAB as previously described. Embryos were rinsed in 1x PBS and photographed using a Zeiss Stemi SV 11 Apo camera and microscope.

Xenopus Cell Mixing and Aggregation Assay

To test for adhesion and cell sorting, cell dispersal as well as dissociation and reaggregation assays with cPcdh1-MT, cPcdh1ΔE-MT, cPcdh1ΔC-MT, cPcdh1ΔTM-MT and control GFP mRNAs were injected into *Xenopus* embryos as described in Kim et al., 1998 and Chen and Gumbiner, 2006. Control cells have been shown to intersperse with surrounding unlabeled cells (Chen and Gumbiner, 2006). Analysis of the dissociation and reaggregation studies involved determining if blastomeres dissociated from embryos injected with either cPcdh1-MT or one of the deletion constructs sorted distinctly apart from blastomeres obtained from uninjected embryos, which is considered as an indication of sorting abilities (blastomeres from control injected embryos do not sort out from uninjected blastomeres).

The cell mixing assay was performed as previously described (Rashid et al., 2006). Briefly, *Xenopus* embryos were injected into a single dorsal blastomere at the 16-cell stage, fixed and stained at stage 15 with the anti-myc antibody (9E10 mentioned above) followed by goat anti-mouse cy3 for immunofluorescence. To quantify cell mixing, whole-mount embryos were viewed on a Nikon M2Bio fluorescent dissecting scope and the fluorescent cells not in contact with any other labeled cell were counted. An average was obtained for 10-30 embryos for each construct injected. The t-test for independent groups was used to determine the statistical difference between conditions (VassarStats.com).

Cell Counting and Statistics

Counts of fluorescent cells in cryostat sections were performed using a Nikon Microphot-FXA fluorescence scope. Individual embryos for each construct were cryostat sectioned onto slides, which were individually counted based on the number of fluorescent cells in each target area (DRG, VR, SG, melanocyte, or other). Percentages of cells counted per area per construct were calculated. Total cell counts per embryo of less than 100 were not used for future calculations. The mean and standard error were calculated using VassarStats (VassarStats.com) and Statdisk version 10.4.0 (Statdisk.com). Using an independent sample weighted one-way analysis of variance (Anova) was performed to test the equality of the means (VassarStats.com).

Confocal Live Time-Lapse Imaging

To analyze the migration of pCDH1 by live confocal imaging, the pCDH1 construct was subcloned into the vector pMES2 as described above. Embryos were electroporated with the cPCDH1 in pMES2 at stage 9-11 as previously described. The embryos were allowed to develop to stages 14 through 20 and examined for strong expression of fluorescence. A transverse slice was prepared per current protocol established by Dr. Lynn George (Lefcort lab, personal communication). In this case, a GFP-positively expressing embryo was dissected and examined for strong expression through the spinal cord. The embryo was then embedded in 5% Hanks/agarose medium and vibratome sectioned at approximately 190-200 μm . The sections were examined for GFP expression along with tissue integrity. The best section was transferred to a culture plate and onto a Millipore filter soaked in Neural Basal Media media (Sigma, St. Louis, MO) containing B-57. A round coverslip was placed on top of the well in the culture plate containing the section. The plate was sealed with parafilm prior to imaging. The section was imaged in a custom-made incubator as previously described (Kasemeier-Kulesa et al., 2005) using a laser scanning Olympus confocal microscope. To locate the Z-level with the desired expression of cells, the section was scanned using the Fluoview version 5.0 software. A starting Z-scan was taken over approximately 10 μm every 1-2 μm with a Kalman of 2. The software program was next set to image typically over a 16-hour time span, taking a scan every fifteen minutes. Aperature, PMT, gain and laser settings varied depending on the fluorescence of individual construct sections. Temperature was maintained between 35-38°C. Analysis consisted of counting the number of contacts

particular neural crest cells made with each other and the length of time the contact was maintained. This was performed for the most promising videos of each construct imaged by two independent individuals on three separate occasions.

CHAPTER 4

RESULTS

To identify protocadherins involved in formation of the peripheral nervous system, specifically neural crest cell migration and differentiation, PCR of the chick E4.5 DRG library was performed using primers specific for the extracellular domain of protocadherins. The subsequent sequencing of the PCR products revealed a novel protocadherin, which was then used to isolate a full-length cDNA. Upon conducting a Basic Local Alignment Search Tool (BLAST) comparison of the predicted protein sequence, results showed this cDNA to have the highest homology with *Xenopus* axial protocadherin (82%) and human protocadherin 1 (81%) (Figure 1). Therefore this novel protocadherin was named chick protocadherin 1 (cPcdh1, AY676328). The NCBI conserved domain search identified 6 cadherin extracellular repeat domains, a transmembrane domain and a novel cytoplasmic domain. Recent phylogenetic analysis based on conserved motifs in the cytoplasmic domains shows cPcdh1 to be a member of the δ -protocadherin subfamily, specifically within the $\delta 2$ -subgroup which includes protocadherin-8,-10,-17,-18 and -19 (Redies et al., 2005; Vanhalst et al., 2005).

To analyze the expression pattern of cPcdh1 in chick embryos, we first performed an in situ hybridization. Results from whole-mount in situ hybridization from stage 5 (Hamburger and Hamilton, 1951) through stage 34 (E8) of development showed cPcdh1 expression at the early stages primarily in the notochord (Figure 2A and data not shown).

1	MOTPLDQRS	-----	GSRHGAQRPHPTPL	KHRMRTLTSL	ALWFLCQLPALACGTR	Chick PC821										
1	MDSGAGGRRCPEAALLIL	-----	GPPRMEHLRHSP	GGGQRLLLPSML	LALLLL-LAPSPGHATR	human PC1										
1	MK-----	-----	RTATAC	FLWLFF	QLPIILGSSITK	Xenopus Axial PC										
52	VVYKVP	EEQPPN	TLIGSLASDYGF	PDVGHLYKLEVGAPYLRVDGK	TGDIYTTET	SIUREG	Chick PC821									
68	VVYKVP	EEQPPN	TLIGSLASDYGF	PDVGHLYKLEVGAPYLRVDGK	TGDIYTTET	SIUREG	human PC1									
25	VAIRVP	EEQPPN	TLIGSLASDYGF	PDVGHLYKLEVGAPYLRVDGK	TGDIYTTET	SIUREG	Xenopus Axial PC									
112	LRFCQHLL	PGGPCYLE	FEFVSTDL	MONNSPRLL	LEGQIEVLD	INDNTPNFASPVLT	LTTPF	Chick PC821								
120	LRFCQHLL	PGGPCYLE	FEFVSTDL	MONNSPRLL	LEGQIEVLD	INDNTPNFASPVLT	LTTPF	human PC1								
85	LRFCQHLL	PGGPCYLE	FEFVSTDL	MONNSPRLL	LEGQIEVLD	INDNTPNFASPVLT	LTTPF	Xenopus Axial PC								
172	NTNIGTL	FSIPRAMDRD	SGPNHGVASYEL	LAGPEAQEL	FGLQVAED	QEEKQ	PQLIVMGNLD	Chick PC821								
180	NTNIGSLFP	IPLASDRD	AGPNHGVASYEL	LAGPEAQEL	FGLQVAED	QEEKQ	PQLIVMGNLD	human PC1								
143	NTMVGMLFP	IPLASDRD	AGPNHGVASYEL	LAGPEAQEL	FGLQVAED	QEEKQ	PQLIVMGNLD	Xenopus Axial PC								
232	REQWDSY	DLTIKVQDGG	NPPRASSALL	RLTITIV	DMNDNAPK	FEKALYEAE	LSSENSPV	IGHSV	Chick PC821							
240	REQWDSY	DLTIKVQDGG	NPPRASSALL	RLTITIV	DMNDNAPK	FEKALYEAE	LSSENSPV	IGHSV	human PC1							
283	REQWDSY	DLTIKVQDGG	NPPRASSALL	RLTITIV	DMNDNAPK	FEKALYEAE	LSSENSPV	IGHSV	Xenopus Axial PC							
292	IQVKAND	SDQGANAE	IDYSFHQAS	DMVRRLL	RLDRNTGL	ITVQGGP	IDREDVSILKFS	SVMA	Chick PC821							
300	IQVKAND	SDQGANAE	IDYSFHQAS	DMVRRLL	RLDRNTGL	ITVQGGP	IDREDVSILKFS	SVMA	human PC1							
263	IQVKAND	SDQGANAE	IDYSFHQAS	DMVRRLL	RLDRNTGL	ITVQGGP	IDREDVSILKFS	SVMA	Xenopus Axial PC							
352	KDKGANPK	SARTQVVV	TIKOMNDNAPS	IEIRGIGLV	THQDGMAN	ISEDV	VPVETAV	LVQV	Chick PC821							
360	KDKGANPK	SARTQVVV	TIKOMNDNAPS	IEIRGIGLV	THQDGMAN	ISEDV	VPVETAV	LVQV	human PC1							
323	KDKGANPK	SARTQVVV	TIKOMNDNAPS	IEIRGIGLV	THQDGMAN	ISEDV	VPVETAV	LVQV	Xenopus Axial PC							
412	SDRDEGEN	AVVTCV	VAGDV	PFQLRQ	ASETGS	DSKKKY	FLQTTT	PLDY	ESVK	DYTTIE	LVAV	Chick PC821				
420	SDRDEGEN	AVVTCV	VAGDV	PFQLRQ	ASETGS	DSKKKY	FLQTTT	PLDY	ESVK	DYTTIE	LVAV	human PC1				
383	SDRDEGEN	AVVTCV	VAGDV	PFQLRQ	ASETGS	DSKKKY	FLQTTT	PLDY	ESVK	DYTTIE	LVAV	Xenopus Axial PC				
472	DSGNPPL	LSSTNS	LKVQV	VDVNDN	APVFS	QSFT	EVAF	PENN	EPND	LVMEV	SASD	ADSG	SNA	Chick PC821		
480	DSGNPPL	LSSTNS	LKVQV	VDVNDN	APVFS	QSFT	EVAF	PENN	EPND	LVMEV	SASD	ADSG	SNA	human PC1		
443	DSGNPPL	LSSTNS	LKVQV	VDVNDN	APVFS	QSFT	EVAF	PENN	EPND	LVMEV	SASD	ADSG	SNA	Xenopus Axial PC		
532	KLVYSL	VTDPSS	KDSFT	IDPDS	GETRYK	KAVLD	REQRE	RYEF	LVVA	ADKGS	PSLKG	TAS	VA	Chick PC821		
540	ELVYSL	LEPEPA	AKGLFT	ISPET	GETQ	VKTS	LDREQ	RESY	ELKV	YAADR	GS	PSLQGT	ATL	VA	Chick PC821	
583	QLHYS	ILPDP	SARGV	FSIN	PDSSQ	QIRV	NMYLD	REQRE	HVDH	YAVD	KG	IPSLKG	TAS	VA	Xenopus Axial PC	
592	INVMDR	NDNDPK	FMLSG	YNF	SVMEN	MPRLSP	VGMV	TVID	ADK	GENA	RIQL	SVEQ	DNDG	DFV	Chick PC821	
600	VNVLDC	NDNDPK	FMLSG	YNF	SVMEN	MPRLSP	VGMV	TVID	ADK	GENA	RIQL	SVEQ	DNDG	DFV	human PC1	
563	ITVLD	NDNDPK	FMLSG	YNF	SVMEN	MPRLSP	VGMV	TVID	ADK	GENA	RIQL	SVEQ	DNDG	DFV	Xenopus Axial PC	
652	IQNGTGT	ILSSIS	FDREQ	QSTYT	FR	LKAV	DGGD	PPR	SAY	VGVT	INVLD	ENDN	APYIT	AP	Chick PC821	
660	IQNGTGT	ILSSIS	FDREQ	QSTYT	FR	LKAV	DGGD	PPR	SAY	VGVT	INVLD	ENDN	APYIT	AP	human PC1	
623	IQNGTGT	ILSSIS	FDREQ	QSTYT	FR	LKAV	DGGD	PPR	SAY	VGVT	INVLD	ENDN	APYIT	AP	Xenopus Axial PC	
712	NATYKH	LHPHT	SPGQ	QVSK	VKAED	IDTGV	NAAEL	YISIT	GGNP	FELF	QIS	PSQ	SGD	IT	LEKE	Chick PC821
720	NTSHKL	LTPTQ	RLGET	VSQAA	EDF	SGVNA	ELIYS	IAGGN	PYGL	FQIG	SHSG	AIT	LEKE	Chick PC821		
683	NTSHKL	LTPTQ	RLGET	VSQAA	EDF	SGVNA	ELIYS	IAGGN	PYGL	FQIG	SHSG	AIT	LEKE	human PC1		
772	ILRKHH	GLHRL	LVVR	VNDK	GKPS	SRHGT	ALVHF	YVNET	LANR	YLLD	TLVGH	SLDT	PLD	DTA	Chick PC821	
780	ILRKHH	GLHRL	LVVR	VNDK	GKPS	SRHGT	ALVHF	YVNET	LANR	YLLD	TLVGH	SLDT	PLD	DTA	human PC1	
743	ILRKHH	GLHRL	LVVR	VNDK	GKPS	SRHGT	ALVHF	YVNET	LANR	YLLD	TLVGH	SLDT	PLD	DTA	Xenopus Axial PC	
832	GDPEYER	SKQR	SNIL	FGV	IAG	VAVT	LVIV	LVVL	VRYC	RQRE	AKSG	YQAG	KKET	KD	LYAP	Chick PC821
840	GDPEYER	SKQR	SNIL	FGV	IAG	VAVT	LVIV	LVVL	VRYC	RQRE	AKSG	YQAG	KKET	KD	LYAP	human PC1
883	GDPEYER	SKQR	SNIL	FGV	IAG	VAVT	LVIV	LVVL	VRYC	RQRE	AKSG	YQAG	KKET	KD	LYAP	Xenopus Axial PC
892	KDAKSGK	-SKNKV	KKSK	SPKPP	KTE	DEE	ETGL	QKSL	KENL	MSD	SVGS	SPRI	HLPL	NY	Chick PC821	
900	KDAKSGK	-SKNKV	KKSK	SPKPP	KTE	DEE	ETGL	QKSL	KENL	MSD	SVGS	SPRI	HLPL	NY	human PC1	
863	KDAKSGK	-SKNKV	KKSK	SPKPP	KTE	DEE	ETGL	QKSL	KENL	MSD	SVGS	SPRI	HLPL	NY	Xenopus Axial PC	
951	PGSPDL	GRHYR	SN	SPLP	STQL	QPS	SPS	ASKKH	QVVD	LPANT	FEVGT	GDN	NSTG	SED	YS	Chick PC821
960	PGSPDL	GRHYR	SN	SPLP	STQL	QPS	SPS	ASKKH	QVVD	LPANT	FEVGT	GDN	NSTG	SED	YS	human PC1
922	PGSPDL	GRHYR	SN	SPLP	STQL	QPS	SPS	ASKKH	QVVD	LPANT	FEVGT	GDN	NSTG	SED	YS	Xenopus Axial PC
1811	YVYRTN	PKYPS	KQV	GGP	FLST	PLP	HPY	HGA	I-WTE	VVE	Chick PC821					
1820	YVYRTN	PKYPS	KQV	GGP	FLST	PLP	HPY	HGA	I-WTE	VVE	human PC1					
982	YVYRTN	PKYPS	KQV	GGP	FLST	PLP	HPY	HGA	I-WTE	VVE	Xenopus Axial PC					

Figure 1. Sequence homology results comparing the predicted protein sequence with BLAST. Results show the cDNA from the library screen to have the highest homology with *Xenopus* axial protocadherin (82%) and human protocadherin 1 (81%). Red underline shows transmembrane section; Black underline shows cytoplasmic domain; BLAST: Basic Local Alignment Search Tool.

Starting at stage 15 cPcdh1 is detected in the newly developing DRG and by stage 20 the DRG are strongly stained (Figure 2B and 2C). Sections through a stage 24 embryo reveal cPcdh1 expression is highly localized to the lateral margins of the DRG, with expression reduced at the center (Figure 2G). cPcdh1 is also found in Schwann cells along the VR, in the SG and in the dermamyotome (DM). As a negative control, embryos hybridized with a sense cPcdh1 probe showed no specific stain (Figure 2E and 2H). To localize more precisely cPcdh1 protein, a rabbit antiserum was generated to the cPcdh1 extracellular domain. The antiserum recognizes cPcdh1 protein by immunoblotting (data not shown) and immunolocalization in embryos reveals cPcdh1 protein is localized to migrating neural crest cells, DRG, VR, SG and DM, confirming the in situ hybridization results (Figure 2I-J). Specifically, by stage 20 cPcdh1 protein is primarily found along the dorsolateral margins of the DRG. In conclusion, results from the whole mount in situ hybridization and immunolocalization demonstrated cPcdh1 is expressed in most trunk neural crest cell derivatives with distinct localization to the periphery of the DRG. In contrast, cPcdh1 is not found in premigratory neural crest cells or in neural crest cells that migrate along the dorsal pathway to become melanocytes. These results suggest cPcdh1 is found only in neural crest cells along the ventral migratory pathway.

The localized expression of cPcdh1 in the DRG is suggestive of a role in localizing a subset of cells at the margins. Within the DRG, the cells at the margin are mitotically active and undifferentiated, while cells in the center are differentiating into neurons and glial cells (Wakamatsu et al. 2000). To determine whether cPcdh1 co-localizes with the mitotically active cells at the margins of the DRG, stage 24 chick

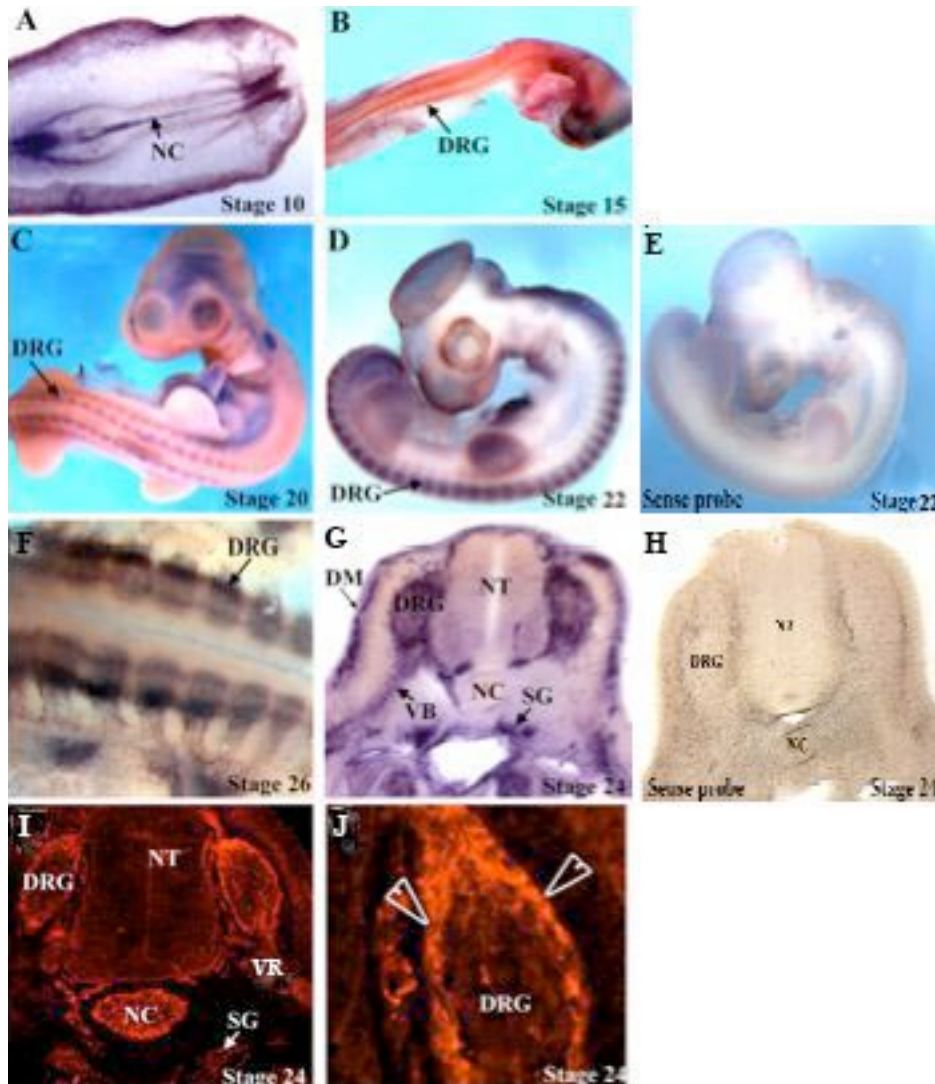


Figure 2. Results of in situ hybridization and antibody staining showing cPcdh1 staining through various stages of chick embryo development. (A) staining of notochord at stage 10; (B,C,F) specific and strong staining of DRG beginning at stage 15 and continuing through stage 26; (E,H) Sense staining of whole mount and transverse section at stage 24; (G) Transverse vibratome section of a stage 24 embryo showing staining in floor plate of NT, NC, DM, DRG, SG, and Schwann cells along the VR; (I-J) rabbit antiserum generated to the cPcdh1 extracellular domain recognizes cPcdh1 protein by immunolocalization in embryos showing cPcdh1 protein is localized to migrating neural crest cells, DRG, VR, SG and DM, confirming the in situ hybridization results. Note specific staining along periphery of DRG shown by arrowheads in (H). DRG: dorsal root ganglia, NT: neural tube, NC: notochord, DM: dermatome, SG: sympathetic ganglia, VR: ventral root.

embryos were subjected to in situ hybridization for cPcdh1, followed by immuno-labeling with an antibody against HNK, a pan neural crest marker, or phosphohistone H3, a marker of cells undergoing mitosis. As shown in Figure 3A, the HNK antibody labels all DRG cells, including the region that expresses cPcdh1. However, anti-phosphohistone H3 only labels a subset of cells in the DRG, mainly at the margins, and these cells are also positive for cPcdh1 expression (Figure 3B). Because phosphohistone H3 only labels a small subset of mitotically active cells in the DRG, BrdU labeling was also performed. Results confirm cPcdh1 is expressed in mitotically active cells in the DRG as shown in Figure 3C. In contrast, a comparison of cPcdh1 expression to that of Tuj1 or Islet 1/2, two markers of differentiated sensory neurons, shows cPcdh1 expression is highest in regions in which Tuj1 or Islet-1 are not expressed. Collectively, these results suggest cPcdh1 labels neural crest cells within the DRG that are mitotically active and undifferentiated and is downregulated as cells migrate to the center of the DRG and differentiate.

Because the results of the expression profile indicated cPcdh1 is expressed in the DRG, VR and SG, we hypothesized cPcdh1 may function to halt neural crest migration at these peripheral nervous system structures and/or function as a cell adhesion molecule. In particular, cPcdh1 may be involved in halting neural crest cells at the DRG and holding the cells at the periphery in a mitotically active undifferentiated state. To test this hypothesis, functional analysis studies were performed in vivo in *Xenopus* and chick embryos. To ectopically express cPcdh1 in embryos, cPcdh1 was cloned into vectors suitable for injection and ovo electroporation. This was followed by the creation of three

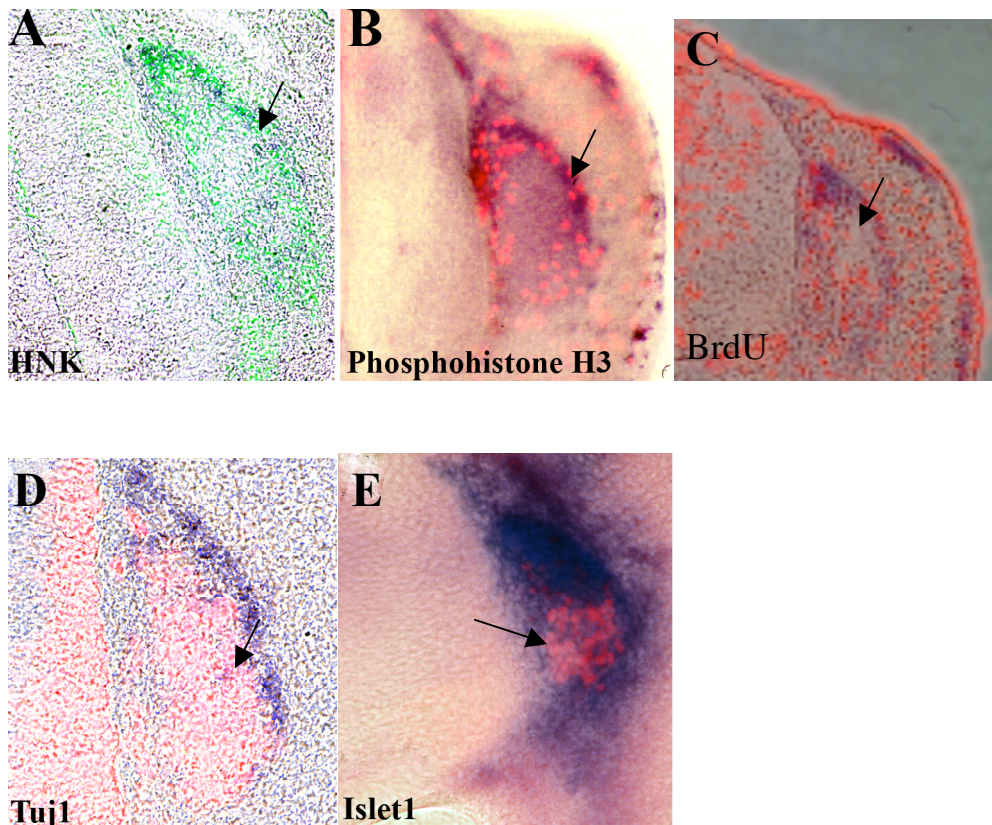


Figure 3. Stage 24 in situ hybridization with cPcdh1 followed by immunolabeling of cell markers indicating co-localization of cPcdh1 with mitotically active and undifferentiated cells in the DRG (A-C) and non-localization with differentiated cell types (D, E). Results show within the DRG cPcdh1 labels mitotically active and undifferentiated neural crest cells and is downregulated as cells migrate to the center of the DRG and differentiate. In all figures, the in situ stain is the dark blue/purple color. (A) HNK stain: green color, (B) Phosphohistone H3: bright red color, (C) BrdU stain: bright red color, (D) Tuj1 stain: red/pink color, (E) Islet-1 stain: bright red color, DRG: dorsal root ganglia, arrows indicate DRG.

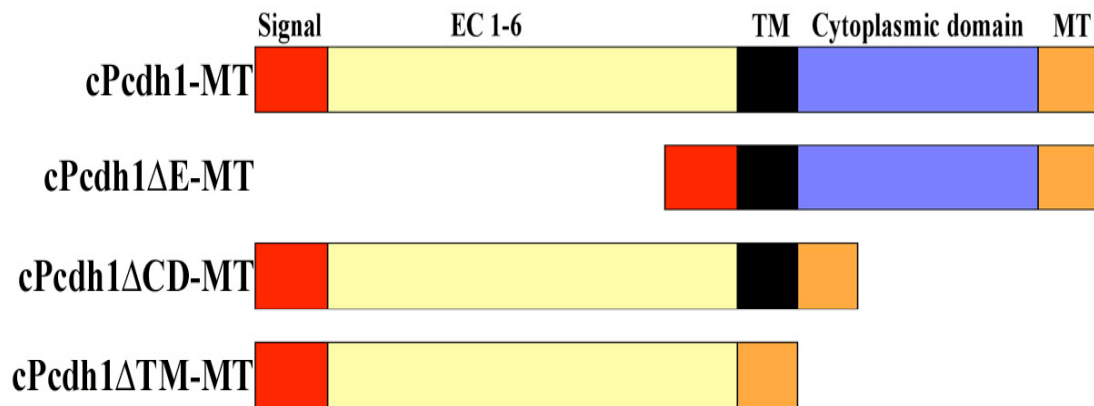


Figure 4. Schematic of cloning constructs used for in ovo electroporation to test function of cPcdh1.

cPcdh1-MT: full-length cPcdh1 tagged with a C-terminal myc tag.

cPcdh1ΔE-MT: mutant construct lacking the extracellular domain.

cPcdh1ΔCD-MT: construct lacking the entire cytoplasmic domain replaced by a C-terminal myc tag.

cPcdh1ΔTM-MT: construct encoding a secreted protein lacking both the transmembrane and cytoplasmic domains.

TM: transmembrane domain, MT: myc-tag, EC: extracellular repeats.

deletion constructs for injections and ovo electroporation in order to disrupt cPcdh1 function. The constructs used are depicted in Figure 4 and consisted of a full-length cPcdh1 tagged with a C-terminal myc tag (cPcdh1-MT) and the three mutant constructs: one lacking the extracellular domain (cPcdh1 Δ E-MT); a construct lacking the entire cytoplasmic domain, replaced by a C-terminal myc tag (cPcdh1 Δ CD-MT); and a construct encoding a secreted protein lacking both the transmembrane and cytoplasmic domains (cPcdh1 Δ TM-MT). The cPcdh1 Δ E-MT deletion construct design was based on previous research showing cadherin constructs typically functioned as dominant-negatives when missing the extracellular domain (Kintner 1992; Bradley et al., 1998; Borchers et al., 2001). The cPcdh1 Δ CD-MT construct was designed based on research demonstrating a gain-of-function effect when axial protocadherin in *Xenopus* lacked the cytoplasmic domain (Kuroda et al., 2002). In *Xenopus*, an axial protocadherin construct lacking both the transmembrane and cytoplasmic domain functioned as a secreted dominant-negative form (Kuroda et al., 2002), thus the rationale for the design of cPcdh1 Δ TM-MT. The constructs were subcloned into the pMiWII vector, which utilizes a chicken β -actin promoter to drive expression of the cPcdh1 construct.

The Ability of cPcdh1 to Mediate Cell Adhesion

Preliminary tests for cell adhesion involved microinjection of *Xenopus* embryos with cPcdh1-MT, cPcdh1 Δ E-MT and cPcdh1 Δ CD-MT constructs along with a control of the CS2-MT vector only encoding mRNA. The results from these initial experiments showed all constructs were properly expressed and targeted to the cell membrane. Only

the full-length construct (cPcdh1-MT) exhibited strong cell-cell adhesion, which was demonstrated by a reduced cell mixing at the borders of the clone of expressing cells (data not shown). These preliminary results prompted a more thorough investigation with a greater number of embryos and injection of all constructs; cPcdh1-MT, cPcdh1 Δ E-MT, cPcdh1 Δ C-MT, cPcdh1 Δ TM-MT and control GFP mRNAs into *Xenopus* embryos as described in Kim et al., 1998 and Chen and Gumbiner, 2006. In cell mixing studies, if cells from cPcdh1-MT or one of the deletion constructs formed tight patches and maintained sharp boundaries with unlabeled neighbor cells, this is interpreted as having cell sorting activity. In other words, a lack of single-labeled cells not in contact with other labeled cells but instead, in contact with many labeled cells, is recognized as an ability to adhere or be cohesive. Results from the *Xenopus* cell mixing assay demonstrate cPcdh1-MT had a statistically significant (by paired t-test) reduced number of isolated cells not in contact with other cells as compared to the deletion constructs and the control CS2-MT (Figure 5). These results support the hypothesis cPcdh1 may be functioning as a cell-adhesion molecule.

Role of cPcdh1 in Targeting Neural Crest Cells to the Peripheral Nervous System

Based on the in situ results from the chick embryos showing cPcdh1 expression in peripheral tissue areas, cPcdh1 may also function to target neural crest cells to these areas. Although the constructs worked in the *Xenopus* system, we needed to establish the constructs would express in chick embryos and in the peripheral nervous system areas. Therefore, chick embryos were subjected to in ovo electroporation at stage 10, allowed to

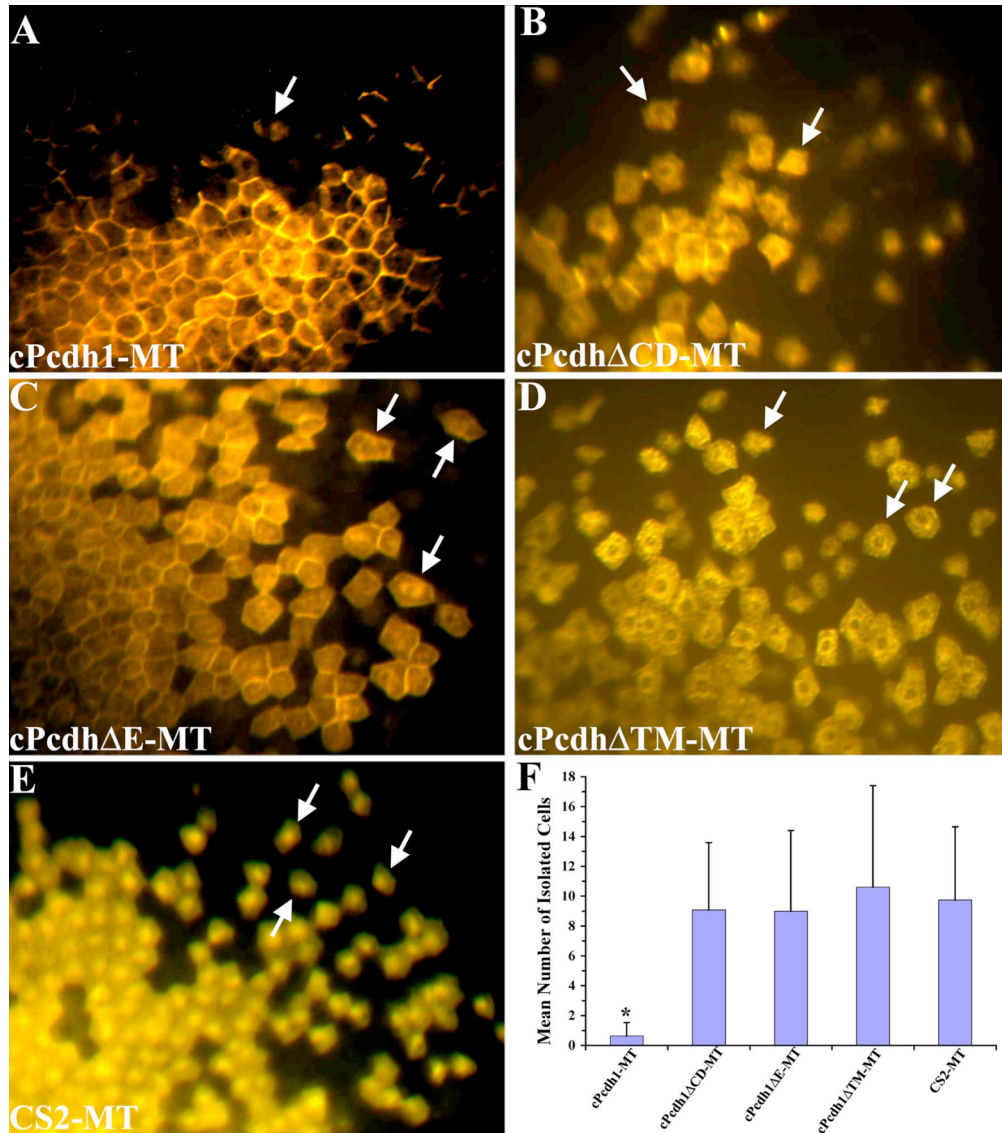


Figure 5. Cell mixing assay in *Xenopus*.

Xenopus embryos were injected with RNA encoding the constructs and immunostained for the myc tag. Individual cells not in contact with another cell were counted. cPcdh1-MT showed a minimal amount of isolated cells as compared to the deletion constructs and the control CS2-MT indicating cPcdh1 may function to promote or maintain cell adhesion. Arrows indicate isolated cells. Statistical analysis was performed using paired t-test. Asterisk indicates statistical significance.

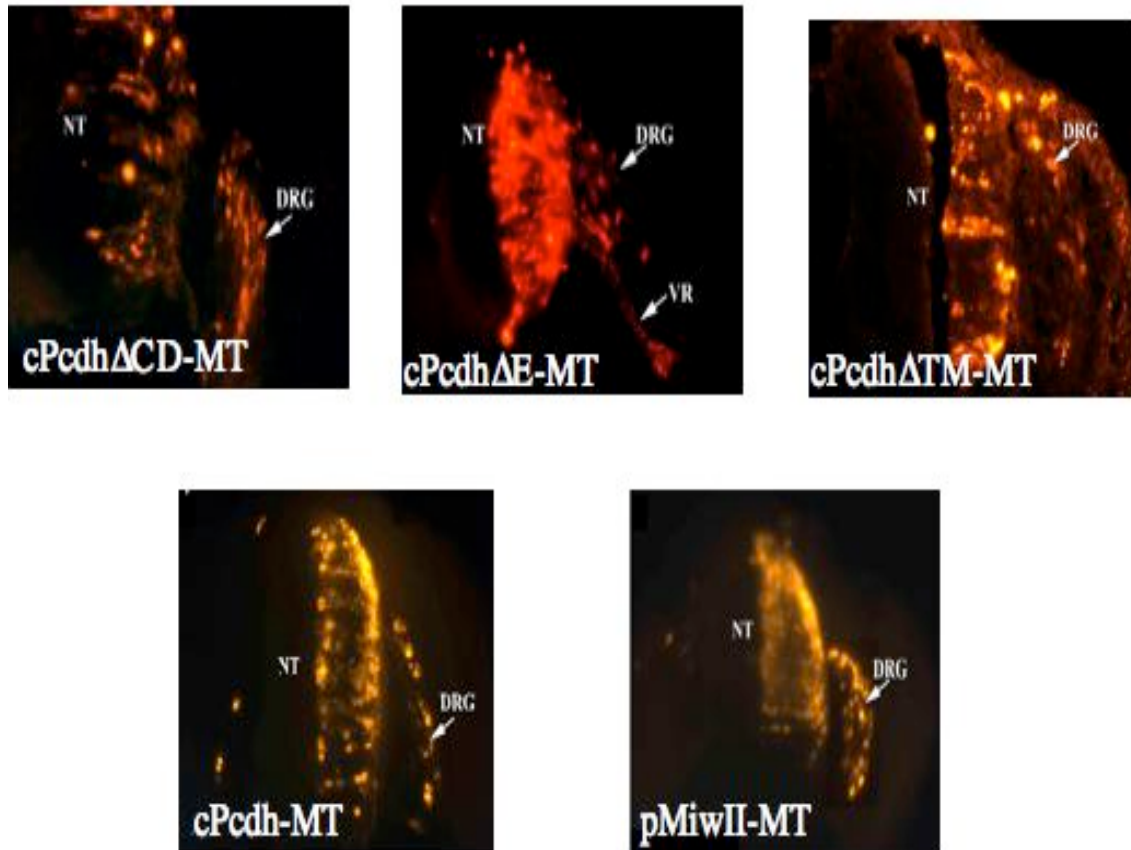


Figure 6. Examples of successful in ovo injection and electroporation of each construct in a chick embryo. Pictures are transverse sections of stage 20-22 embryos. cPcdh1-MT: full-length cPcdh1 tagged with a C-terminal myc tag, cPcdh1ΔE-MT: mutant construct lacking the extracellular domain, cPcdh1ΔCD-MT: construct lacking the entire cytoplasmic domain replaced by a C-terminal myc tag, cPcdh1ΔTM-MT

develop until stage 20-23, then fixed, cryo-sectioned and immunostained for the myc-epitope. As shown in Figure 6, all four constructs and control were successfully electroporated and expressed in the chick neural tube and neural crest cells.

In order to determine if cPcdh1 is involved in targeting neural crest cells to specific peripheral nervous system areas, a fate map analysis was undertaken in the DRG. Embryos electroporated with the four constructs (cPcdh1-MT, cPcdh1 Δ E-MT, cPcdh1 Δ TM-MT and cPcdh1 Δ CD-MT) as well as the control, vector only construct (pMiWII-MT) were used. Fate map analysis involves the counting of myc-positive stained cells at the peripheral nervous system target locations for individual embryos of each construct. Using immunostained and cryo-sectioned embryos, individual neural crest cells ectopically expressing the myc tag were counted in target areas including the DRG, VR, SG, melanocytes as well as 'other' which included cells that were not clearly located in the previously mentioned areas. The number of cells counted for each area was recorded as a percentage of the total number of labeled trunk neural crest cells for each embryo. The mean percent and standard error was calculated for each construct per area. Equality of the means was tested with one-way ANOVA and Tukey test. The calculation results are presented in Table 2. As shown in Figure 7, the results indicate over-expression of full-length cPcdh1-MT is not significantly different from the control pMiWII showing cPcdh1-MT did not alter the distribution of cells in the target areas (DRG: 72.48% vs. 78.17%, VR: 17.8% vs. 11.32%, SG: 5.62% vs 7.07%, melanocyte: 3.87% vs.3.00% respectively, p-value<0.01). In contrast, the deletion constructs show a marked difference from cPcdh1-MT and control pMiWII-MT in the distribution of cells

Construct	DRG-mean	VR-mean	SG-mean	Melano-mean	Other-mean	Islet	Islet n	NF	NF n	H3
MiWII (n=12)	72.48	17.8	5.62	3.87	0.21	36.15	n=6		74.75 n=4	5.11
cPcdh1-MT(n=11)	78.17	11.32	7.07	3	0.43	14.04	n=6		46.72 n=3	13.93
cPcdhE (n=8)	52.49	23.37	18.97	3.15	2.01	46.71	n=2		68.73 n=1	7.04
cPcdhTM(n=14)	56.27	23.41	17.57	2.53	0.17	50.89	n=9		84.56 n=4	2.63
cPcdhCD(n=11)	63.91	23.31	12.05	2.03	0.38	59.55	n=3		66.26 n=4	15.84
siControl (n=7)	73.15	18.41	4.26	4.09	0.09	28.89	n=5			
siRNA 1 (n=5)	54.45	25.27	17.68	2.68	0.12	33.5	n=5			
si RNA 3 (n=3)	56.81	28.89	12.96	1.16	0.17	44.81	n=3			
pMES2 (n=6)	51.92	35.52	9.75	2.74	0.07					
Ncad (n=5)	69.49	19.98	6.79	3.68	0.05					
CBR (n=5)	29.02	62.84	6.07	2.02	0.04					
	DRG-std.err	VR-std.error	SG-std.error	Melano-std.er	Other-std.err	Islet-std.err		NF-std.err		H3-std.err
MiwII	1.47	0.82	1.34	0.99	0.07	6.26		2.78		0.74
cPcdh1-MT	2.72	1.76	1.67	0.47	0.15	2.87		13.78		0.28
cPcdhE	4.91	1.16	3.82	0.83	1.61	0.56				
cPcdhTM	3.06	1.04	2.64	0.38	0.07	4.23		2.28		0.39
cPcdhCD	4.73	6.32	1.98	0.45	0.16	3.85		4.28		3.96
siControl	2.86	3.01	0.81	0.99	0.09	3.84				
siRNA 1	4.83	4.19	2.41	0.77	0.07	5.69				
siRNA 3	6.5	3.98	3.26	0.62	0.15	4.21				
N-Cad	6.53	4.45	2.6	2.7	0.03					
CBR	10	9.92	1.97	0.33	0.02					
	DRG-std.dev	VR-std.dev	SG-std.dev	Melano-std.de	Other-std.dev	Islet-std.dev		NF-std.dev		H3-std.dev
MiwII	5.08	4.63	3.42	2.85	0.25	15.32		5.57		1.48
cPcdh1-MT	9.02	5.84	5.54	1.57	0.5	7.03		23.87		0.49
cPcdhE	13.9	3.29	10.81	2.35	4.56	0.65				
cPcdhTM	11.46	3.87	9.86	1.41	0.25	12.7		4.57		0.77
cPcdhCD	15.7	20.96	6.57	1.48	0.54	6.66		8.56		6.86
siControl	7.58	7.96	2.14	2.63	0.23	8.6				
siRNA 1	10.8	9.38	5.38	1.71	0.15	12.73				
siRNA 3	11.26	6.89	5.64	1.07	0.27	7.28				
N-Cad	14.6	9.95	5.82	6.03	0.07					
CBR	22.36	22.19	4.4	0.73	0.04					

Table 2. Table showing mean percent, standard error, standard deviation and number of embryos used for calculations (n) for each construct. DRG: dorsal root ganglia; VR:ventral root; SG: sympathetic ganglia; Melano: melanocytes; NF: neurofilament; H3: phosphohistone H3

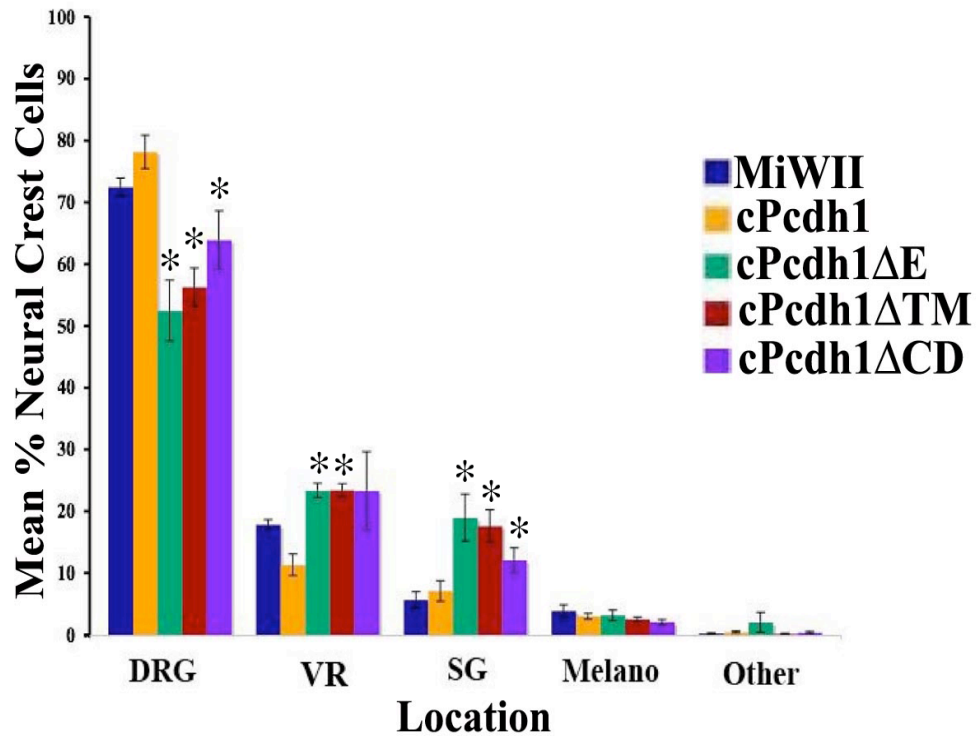


Figure 7. Graph of results from fate map analysis at specific peripheral nervous system areas. Embryos were subject to in ovo electroporation at stage 10 with the different constructs as well as control, followed by immunostaining, cryo-sectioning and fate map analysis at stage 20. Percentages are presented as mean percent of neural crest cells for the total number of embryos counted per target area. One-way Anova and Tukey tests were used to determine statistical significance at $p < 0.05$. The over-expression of cPcdh1-MT showed no significant distribution difference of cells in target areas from that of the control (MiWII-MT). The deletion constructs did show a marked difference from cPcdh1-MT with fewer cells targeting the DRG and more cells targeting the other peripheral nervous system areas such as the VR, SG with the melanocytes showing the least amount of change. Melanocytes and Other were not significantly changed. Additional data is presented in Table 2. DRG: dorsal root ganglia, VB: ventral branch, SG: sympathetic ganglia, Melano: melanocytes, Other: stained cells that were not clearly in one of the specific target areas, MiWII: MiWII-MT. Asterisks indicate statistical significance as compared to cPcdh1.

in specific areas. Specifically, in the DRG, all three deletion constructs showed a marked decrease in the number of cells halting at the DRG as compared to cPcdh1-MT and the control pMiWII-MT (cPcdh1 Δ E: 52.49%; cPcdh1 Δ TM: 56.27%; cPcdh1 Δ CD: 63.91 vs. cPcdh1-MT: 78.17%; pMiWII-MT: 72.48%). Concomitant with the decrease in localization to the DRG, the deletion constructs resulted in a marked increase in neural crest cells that localize to more ventral locations. For example, the cPcdh1 Δ E-MT, cPcdh1 Δ TM-MT and cPcdh1 Δ CD-MT constructs resulted in 18.97%, 17.57% and 12.05% of neural crest cells respectively, localizing to the SG as compared to 7.07% of neural crest cells value for cPcdh1-MT. In the VR, all three constructs resulted in a greater percentage of neural crest cells than either control or cPcdh1-MT. In conclusion, these results demonstrate cPcdh1 functions in halting neural crest cells at the DRG and when this function is disrupted, as shown by the deletion constructs, cells can now aggregate at target areas more ventral to the DRG, such as the VR and SG, at the expense of the DRG.

Because these initial experiments rely on the ability of the deletion constructs to inhibit endogenous cPcdh1 function, they could also function to inhibit other molecules, especially related adhesion molecules such as other protocadherins that may also be expressed in neural crest cells. To verify the deletion constructs inhibit only cPcdh1, we next chose to disrupt cPcdh1 expression at the mRNA level. Initially a morpholino construct was chosen for microinjection to interfere with the translation of cPcdh1. Morpholinos had previously been used successfully in chick embryo, specifically in the trunk-level of the neural tube (Kos et al., 2003). A morpholino from Genetools LLC was

purchased and injected. Preliminary results indicated a potential reduction in cells halting at the DRG (data not shown) but the results were inconsistent and the technique itself had a few limitations. Problems with morpholinos include nonspecific side effects, uneven uptake of morpholinos by cells as well as variable penetration and phenotype (Heasman, 2002; Kos et al., 2003; Rao et al., 2004).

As morpholino injections proved problematic, we turned to siRNA to confirm our initial findings that cPcdh1 is required for neural crest cell migration and possibly to detain cells at the DRG periphery. The two siRNA oligos from Dharmacon targeting different areas of the cPcdh1 mRNA were used in order to disrupt the expression of cPcdh1 at the mRNA level. The two siRNAs were electroporated into stage 10 chick embryos, with the addition of a GFP-encoding vector (pMES) to allow for visualization, similar to the previous experiments. In addition, a non-targeting siControl oligo provided from Dharmacon was electroporated as a negative control. The siRNA-injected chick embryos were subjected to fate map analysis as described previously. The results are nearly identical to what we initially found where the deletion constructs disrupted the function of cPcdh1, producing a decrease in cells arresting at the DRG. As shown in Figure 8, the siControl had basically the same effect as full-length cPcdh1-MT and the control vector pMiWII-MT in the DRG, VR, SG, and melanocytes. The two cPcdh1-specific siRNA oligos (siRNA 1 and siRNA 3) resulted in a decrease of cells in the DRG with a corresponding significant increase in neural crest cells that now localize in the VR and SG.

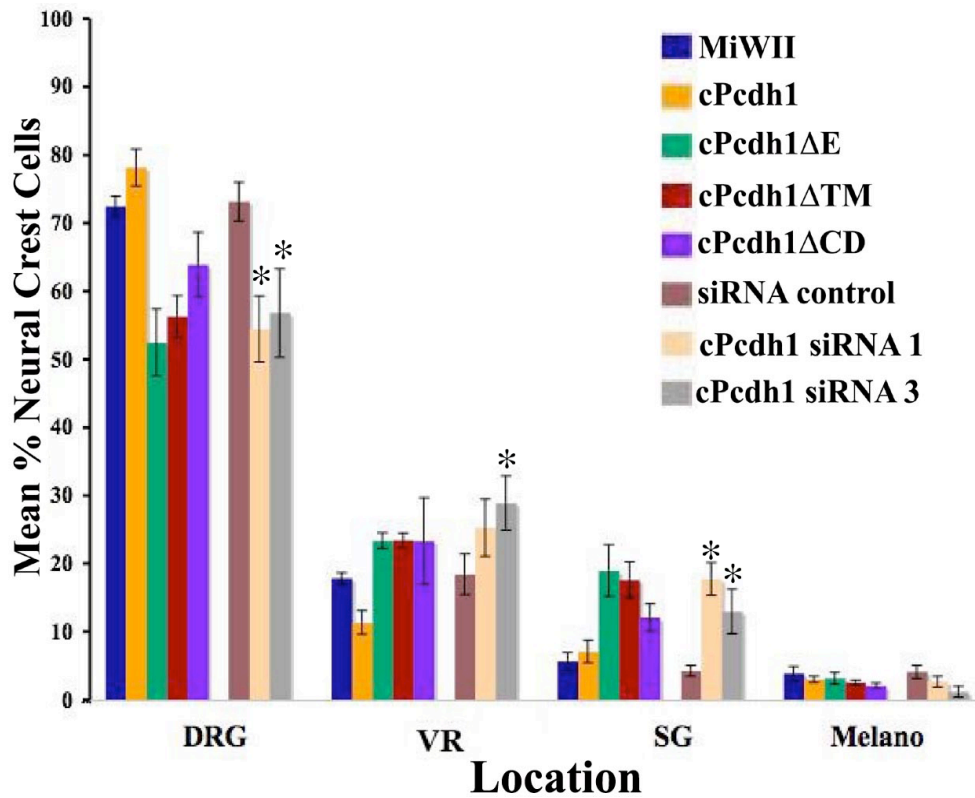


Figure 8. Graph of results from fate map analysis including siRNA oligos at specific peripheral nervous system target areas. Embryos were subjected to in ovo electroporation at stage 10, allowed to develop until stage 20, immunostained and cryo-sectioned for fate map analysis. Percentages are presented as mean percent of neural crest cells for the total number of embryos counted per target area. One-way Anova and Tukey tests were used to determine statistical significance at $p < 0.05$. Results for the three deletion constructs are explained in the text as well as in Figure 7. Results for siRNA oligos confirm deletion construct results, showing when cPcdh1 function is disrupted, an increase in the number of cells is found in the VR and SG at the expense of the DRG. Additional data is presented in Table 2. The Other category is not shown in this figure due to negligible results comparable to Figure 7. DRG: dorsal root ganglia, VB: ventral branch, SG: sympathetic ganglia, Melano: melanocytes, MiWII: MiWII-MT. Asterisks indicate statistical significance from siRNA control.

To confirm that the cPcdh1-specific siRNA oligos did indeed inhibit the expression of cPcdh1, embryos were injected with the siRNA constructs along with a LacZ expression vector (to localize electroporated cells) and then subjected to in situ hybridization for cPcdh1 and cryo-sectioned. The in situ results indicated cPcdh1 mRNA levels are reduced when cPcdh1-specific siRNA oligos are electroporated into neural crest cells (Figure 9). Specifically, within the developing DRG, staining for cPcdh1 mRNA is reduced as compared to embryos electroporated with the control siRNA. Furthermore, the localization of stained neural crest cells corroborate the previous experiments; when cPcdh1 function is disrupted, the accumulation of cells at the periphery of the DRG appears to dissipate with a more widespread distribution of cells throughout the DRG as shown in Figure 9. These results further confirm cPcdh1 may be functioning to arrest neural crest cells at the DRG and disruption by either deletion constructs or at the mRNA level with siRNAs results in an increase of cells migrating past the DRG and arresting more ventrally at the expense of the DRG.

cPcdh1 is Required for Proper Localization of Cells Within the DRG

As the in situ hybridization and immunolocalization results demonstrated strong cPcdh1 staining in the dorsolateral margins of the DRG and due to the fate mapping results showing cPcdh1 functions to localize neural crest cells to the DRG, we next asked if cPcdh1 was functioning to localize a subpopulation of neural crest cells within the marginal zone of the DRG. In order to test this hypothesis, embryos were microinjected and electroporated with control pMiwII-MT, cPcdh1-MT, cPcdh1 Δ TM-MT,

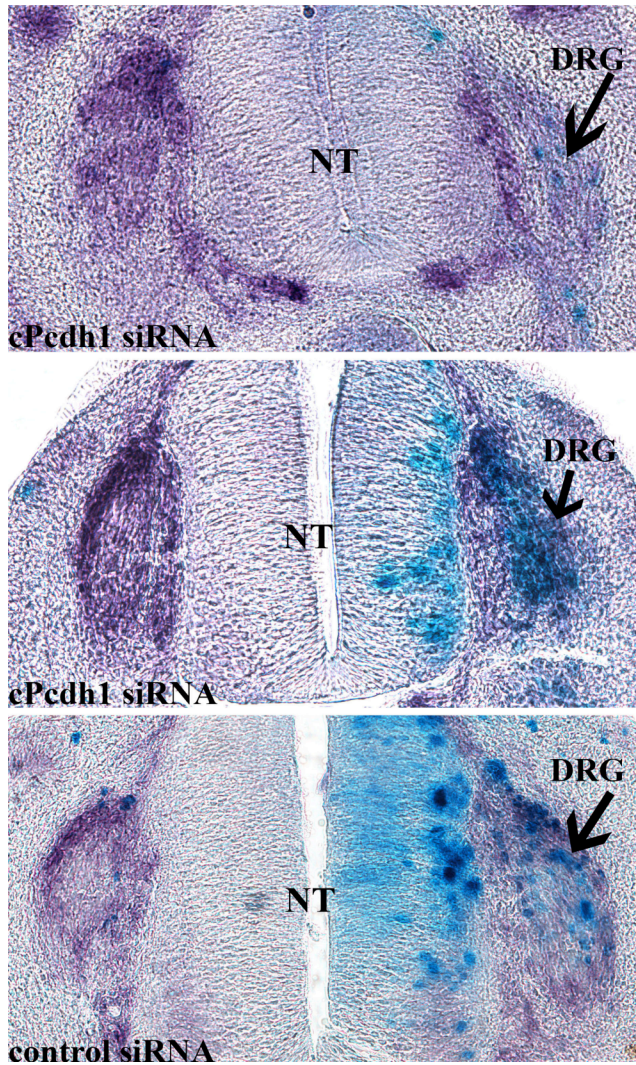


Figure 9. Electroporation of siRNA 1 and siControl with the LacZ expression vector for visualization and localization of cells followed by in situ hybridization for cPcdh1. Disruption of cPcdh1 by siRNA shows loss of cells on the margins of the DRG. Blue cells: LacZ expression vector, Purple stain: in situ hybridization, NT: neural tube, DRG: dorsal root ganglia.

cPcdh1 Δ CD-MT, siControl, siRNA 1 and siRNA 3 constructs and analyzed for the locations of ectopically expressing neural crest cells within the DRG. After harvesting embryos at stage 20, immunofluorescence using Islet-1 (a label for sensory and motor neurons), neurofilament (a neuron-specific marker) and phosphohistone H3 (a marker of cells undergoing mitosis) was performed with fate map and statistical analysis, to identify where, within the DRG, the ectopical expressed cells localized. As shown in Table 2 and Figure 10, overexpression of cPcdh1-MT resulted in a significant decrease in the number of myc+/Islet+ cells within the DRG as compared to the control pMiWII-MT. Conversely, the deletion constructs showed a marked increase in the number of myc+/Islet+ labeled cells over the control embryos as shown in Figure 11 (statistical analysis by paired t-test; p-value<0.01). Furthermore, cPcdh1-MT showed a marked increase in the number of cells double-stained with the phosphohistone H3 marker as compared to the control and cPcdh1 Δ TM-MT (13.93% vs. 5.11% and 2.63% respectively; paired t-test p-value<0.01) as shown in Figure 12. Collectively, these results confirm our hypothesis that cPcdh1 has a role in maintaining cells at the periphery of the DRG in an undifferentiated yet mitotically active state. Further, when this function is disrupted, an increase in ectopically expressing cells is found in the interior of the DRG, where they co-label for Islet-1, indicating that not only is cell-adhesion affected but also cell fate. These observations suggest a possible role for cPcdh1 in localizing newly arrived neural crest cells to the margins of the DRG, where they undergo mitosis and remain undifferentiated.

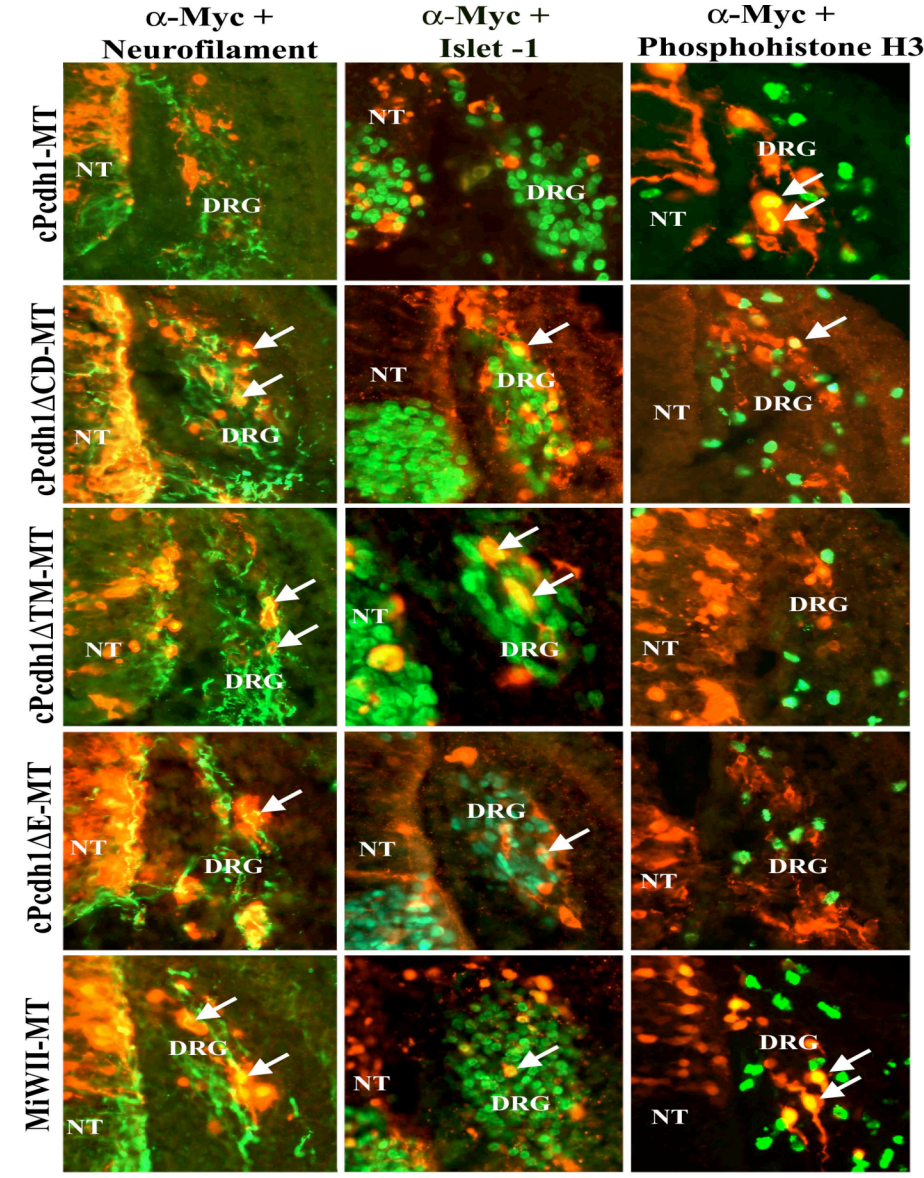


Figure 10. Immunofluorescent staining of cryosectioned embryos showing disruption of cPcdh1-MT alters the location and differentiation of neural crest cells in the DRG. Stage 10 chick embryos were electroporated with the different constructs, cryosectioned and stained for the myc-epitope tag (red fluorescence) and neurofilament, Islet-1 or phosphohistone H3 (green fluorescence). Cells ectopically expressing cPcdh1 exhibit a decrease in neurofilament and Islet-1 staining with a corresponding increase in phosphohistone H3 staining. Conversely, cells that ectopically express the deletion constructs cPcdh1 Δ E-MT, cPcdh1 Δ TM-MT or cPcdh1 Δ CD colocalize with neurofilament and Islet-1 but are less likely to be phosphohistone H3 positive. The MiWII-MT vector expressing only the myc-epitope tag was electroporated and stained as a control.

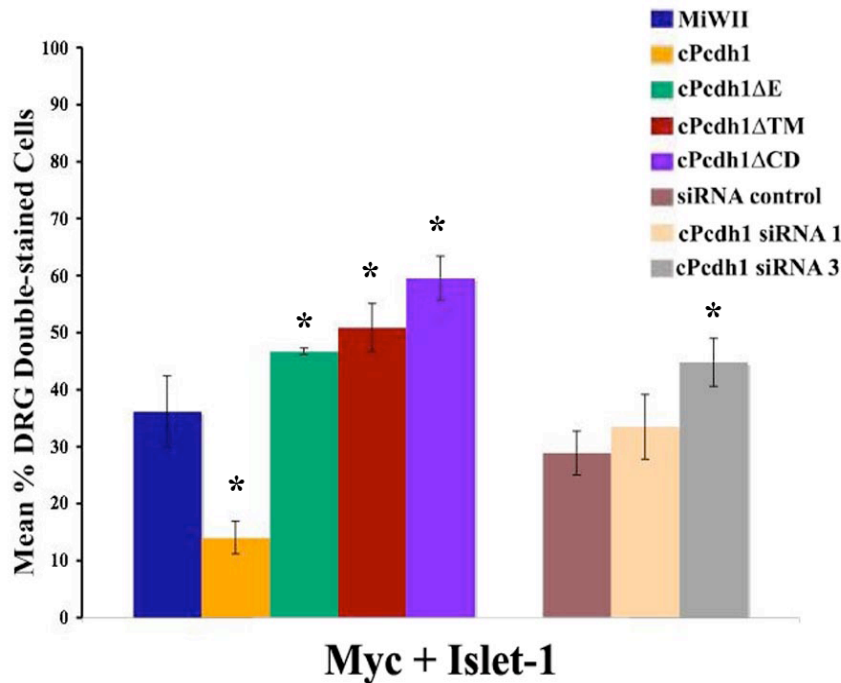


Figure 11. Fate map analysis showing disruption of cPcdh1 function results in an increase in the number of neural crest cells that differentiate into sensory neurons. Embryos were subject to in ovo electroporation at stage 10 with the different constructs as well as control, followed by immunostaining with Islet-1, cryo-sectioning and fate map analysis. Percentages are presented as mean percent of neural crest cells for the total number of embryos counted per construct for the DRG. Ectopic over-expression of cPcdh1-MT resulted in fewer total DRG Islet-1 positive cells as compared to the control. Deletion constructs or siRNA 3 oligo conversely show an increase in cells that are positive for Islet-1 in the DRG (paired t-test; $p < 0.01$; siControl and siRNA 1 were not statistically different; $p > 0.05$). Additional data presented in Table 2. E: cPcdh1ΔE-MT, TM: cPcdh1ΔTM-MT, CD: cPcdh1ΔCD-MT. Asterisks indicate statistically significant difference from the control MiWII for the deletion constructs, or from the control siRNA for the cPcdh1-specific siRNAs.

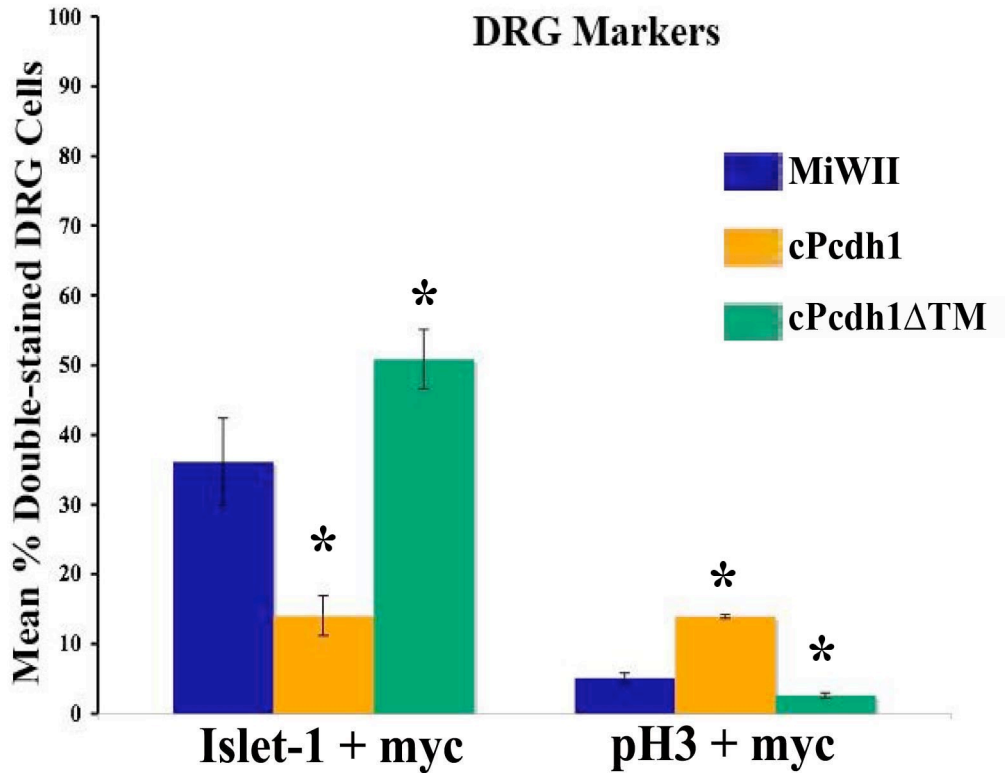


Figure 12. Fate map analysis showing disruption of cPcdh1 function results in an increase of neural crest cells in the DRG that differentiate into sensory neurons and a decrease in mitotically active cells.

Embryos were subject to in ovo electroporation at stage 10 with the different constructs as well as control, followed by immunostaining with Islet-1 or phosphohistone H3 (to detect cells undergoing mitosis), cryosectioning and fate map analysis. Percentages are presented as mean percent of neural crest cells for the total number of embryos counted per construct for the DRG. Ectopic over-expression of cPcdh1-MT resulted in fewer total DRG-Islet-1 positive cells (as shown in Figure 9) and an increase in the number of DRG-phosphohistone H3 labeled cells as compared to the control. In contrast, embryos electroporated with the deletion construct cPcdh1ΔTM-MT showed an increase in total DRG-Islet-1 positive cells and a decrease of DRG-phosphohistone H3 labeled cells as compared to control and cPcdh1-MT (paired t-test; $p < 0.05$). Additional data in Table 2. H3: phosphohistone H3; MiWII: MiWII-MT; TM: cPcdh1ΔTM-MT. Asterisks indicate statistical significance between cPcdh1 and cPcdh1ΔTM-MT.

To further confirm our results showing a possible function for cPcdh1 in localizing neural crest cells to the mitotically active DRG margins, time-lapse confocal live imaging was conducted using cPcdh1 subcloned into pMES2, a GFP expressing vector (cPcdh1-p2). Because our earlier results using siRNA 1 and siRNA 3 confirmed their ability to work in inhibiting cPcdh1 expression (similar to what was observed with the deletion constructs), the siRNA oligos were also imaged. Because the siRNA oligos were not fluorescently labeled, they were co-injected with GFP. The vector pMES2 was used as a negative control. Initially embryos were used at stage 24-25 but because we wanted to capture the neural crest cells early in their migratory route in hopes of showing a ‘decision’ or influence on the cells to localize on the perimeter of the DRG, earlier stages (stage 17-20) were used for most of the imaging experiments.

The control vector (pMES2) showed more movement in all directions and cell shape changes. As shown in Figure 13, the cell shapes appeared more oval and fuller with filopodia in many directions. The neural crest cells ectopically expressing the control vector showed an average of two to three contacts with another cell, either by cell-cell or cell-filament connections (Figure 17 and Figure 19A). On average, at least one contact was maintained for a minimum of 50 minutes but not often longer than 70 minutes. There did not appear to be any preference by the cells for the outside margin. More interior movement was noted with the interior cells showing numerous filament-like extensions in all directions. In approximately 75% of the images viewed, cells were observed to separate and move to the margins of the DRG from the interior and change shape often,

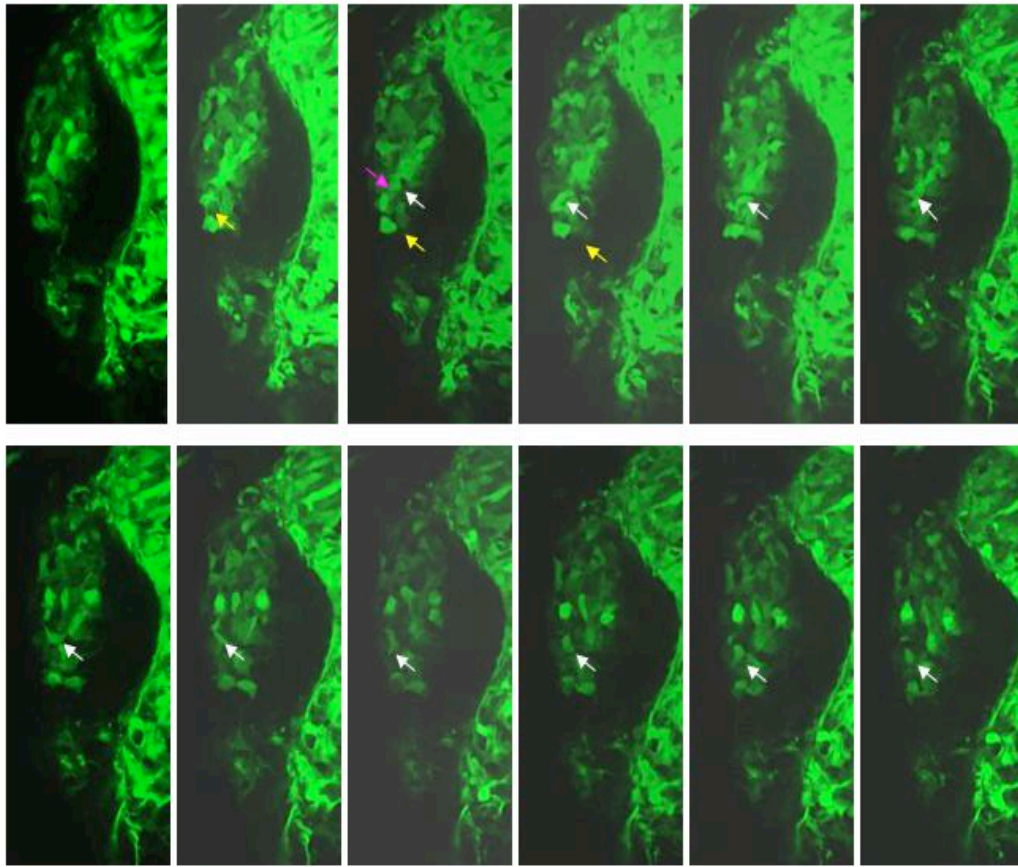


Figure 13. Serial views (left-to-right and top-to-bottom) of pMES2 (stage 20-21) time-lapse confocal imaging

Cells are found throughout DRG, not being localized to any particular area. Overall cell shape appears “roundish” or oval.

White arrow indicates a cell changing shape and moving from the interior to the exterior.

The final shape adopted allows the cell to fit into its’ new location.

Yellow arrow shows cell with at least two filopodia extensions in contact with another cell. Contact is broken and another contact with another cell is made within 10 minutes.

Magenta arrow shows new filament-like contact of cell after breaking one filament contact.

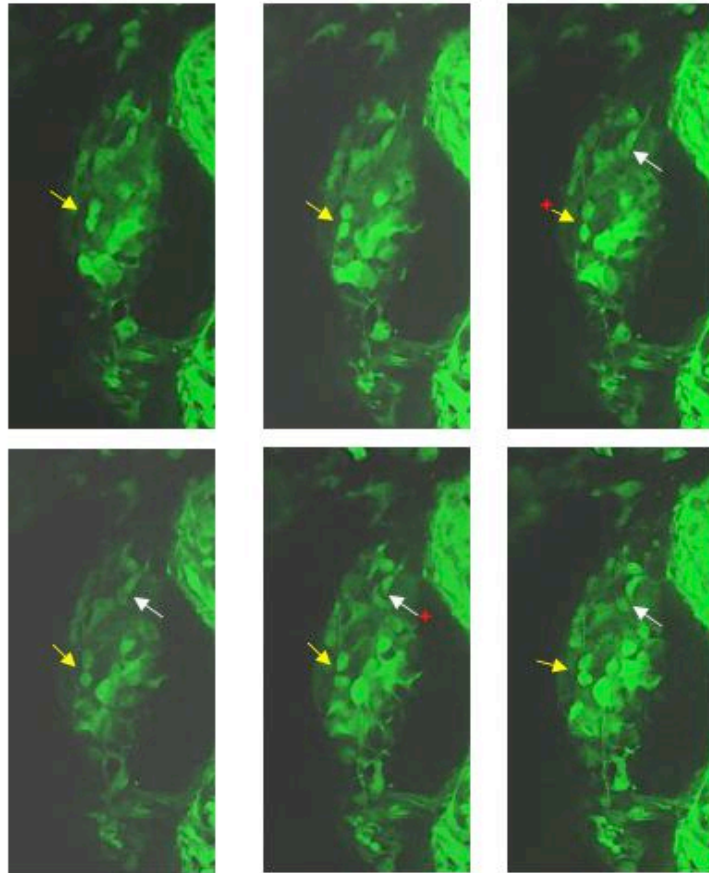


Figure 14. Serial confocal live imaging views of pMES2 control vector stage 20-21. Images are from left-to-right and top-to-bottom in consecutive order. Yellow arrows indicate cell-cell contact being interrupted and re-established after 30 minutes. Top white arrows represent a separation with a cell shape change to a more oval shape. The red asterisk represents time-point when cell-contact is broken.

possibly to “fit” in their new location (see Figure 13 and 14). Cells were noticed to even move back into the neural tube at the top of the DRG in some pMES2 constructs (data not shown). Different cell shapes appeared to be made more frequently when the cells were trying to make contact with another cell. When contact with other cells was broken, contact with another cell was not re-established for 10 to 30 minutes (as shown in Figure 13 and 14) with 20 minutes being the average time of separation. As cells were attempting to make contact with another cell, they would often extend or elongate and once contact was made, the cell would then “roll-up” to their more typical oval shape (see Figure 19A as example of typical pMES2 roundish cell shape).

In general, cPcdh1-p2 cells maintained cell-cell contact longer, up to 2 or 3 hours, as compared to the control pMES2 cells. Although on average, the cell-cell contact was less for cPcdh1-GFP than pMES2, cPcdh1-GFP cells appeared to always have at least one cell-cell contact (Figure 17) and if one contact would break, the other contact would remain. The contacts appeared more dorsal-to-ventral along the periphery and to have a much more elongated cell shape overall as shown in Figure 15, 16 and 19B. Although a cell may first be observed as round-shaped, the cell would become more elongated and remain in that form over time. Contact between cells appeared directed towards each other on the periphery of the DRG rather than directed to interior cells. Although the interior DRG cells moved to the same extent as in pMES2, they did not tend to move as often to the periphery of the DRG as was observed in pMES2 images. Cells appeared to attempt to make more contacts, “reaching” or “talking” to their nearest neighbor, mainly on the periphery (Figure 15 and 16). The peripheral cells appeared more

elongated at least along the top two-thirds of the DRG periphery (Figure 19B). In approximately 50% of images studied, there were more extensions between perimeter cells from dorsal-to-ventral than observed in pMES2 (Figure 19B), although interior cells in both constructs often showed the same amount of extensions, roughly 4 to 5 extensions per cell. Because the cells appeared to stay on the periphery once they moved to their specific location along the outside of the DRG (Figure 16), the cells could possibly be “talking” with their neighbor but yet not often changing positions.

Preliminary imaging results show the siRNA (1 or 3) construct with cell shapes overall being more elongated than round but with a “jagged” appearance. The elongated cells were observed along the same axis in the direction of rostral-caudal rather than along the sides as in cPcdh1-p2. Numerous extensions/filopodia were observed, often with a “frayed” appearance (Figure 18 and Figure 19C).

In summary, the novel chick protocadherin discovered in this work is expressed in neural crest derivatives found in the peripheral nervous system. Specifically in the DRG, cPcdh1 is found predominantly expressed along the dorsal-lateral periphery, an area associated with mitotically active and undifferentiated cells. When the function of cPcdh1 is disrupted either with deletion constructs or by cPcdh1-specific siRNA oligos, fewer neural crest cells arrest in the DRG with a concurrent increase of cells migrating to the VR and SG. Fate map analysis demonstrates when cPcdh1 is over-expressed, an increase of cells co-localize with mitotically active cells along the perimeter of the DRG and when cPcdh1 function is disrupted, there is an increase in the number of neural crest cells that have differentiated into sensory neurons. Confocal time-lapse live imaging has shown

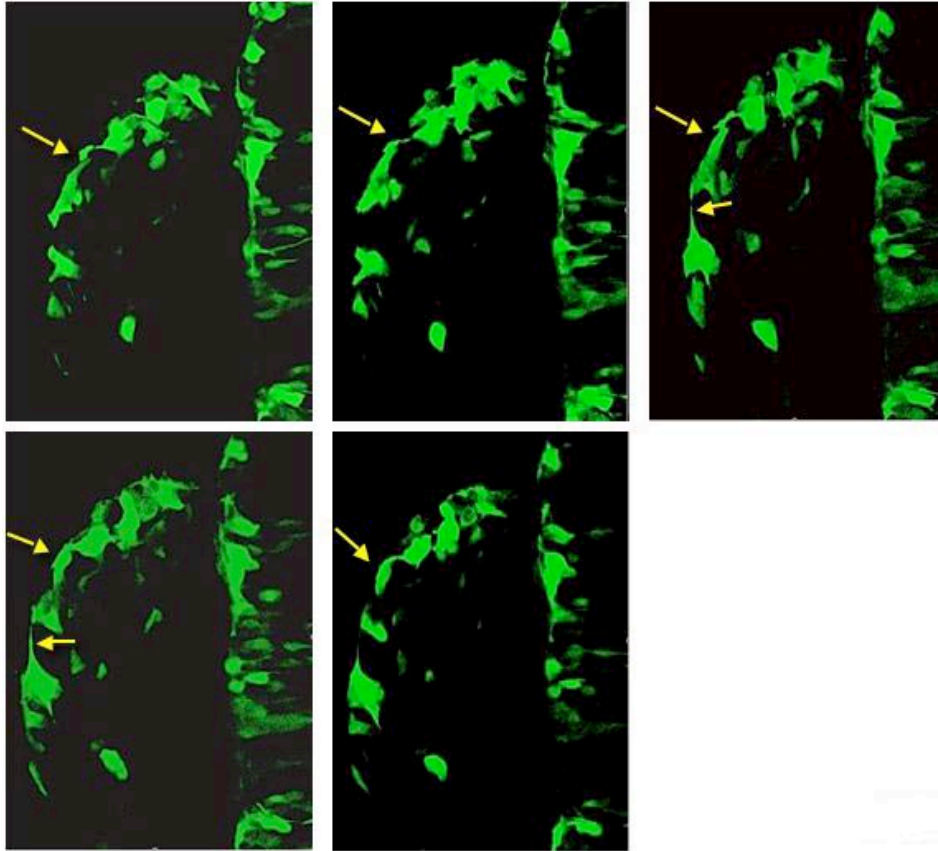


Figure 15. Confocal time-lapse live imaging of cPcdh1-p2 stage 19 (transverse section) Right-to-left and top-to-bottom are consecutive views. Yellow arrows indicate typical elongated shape of cells; extensions toward neighboring cell along DRG periphery.

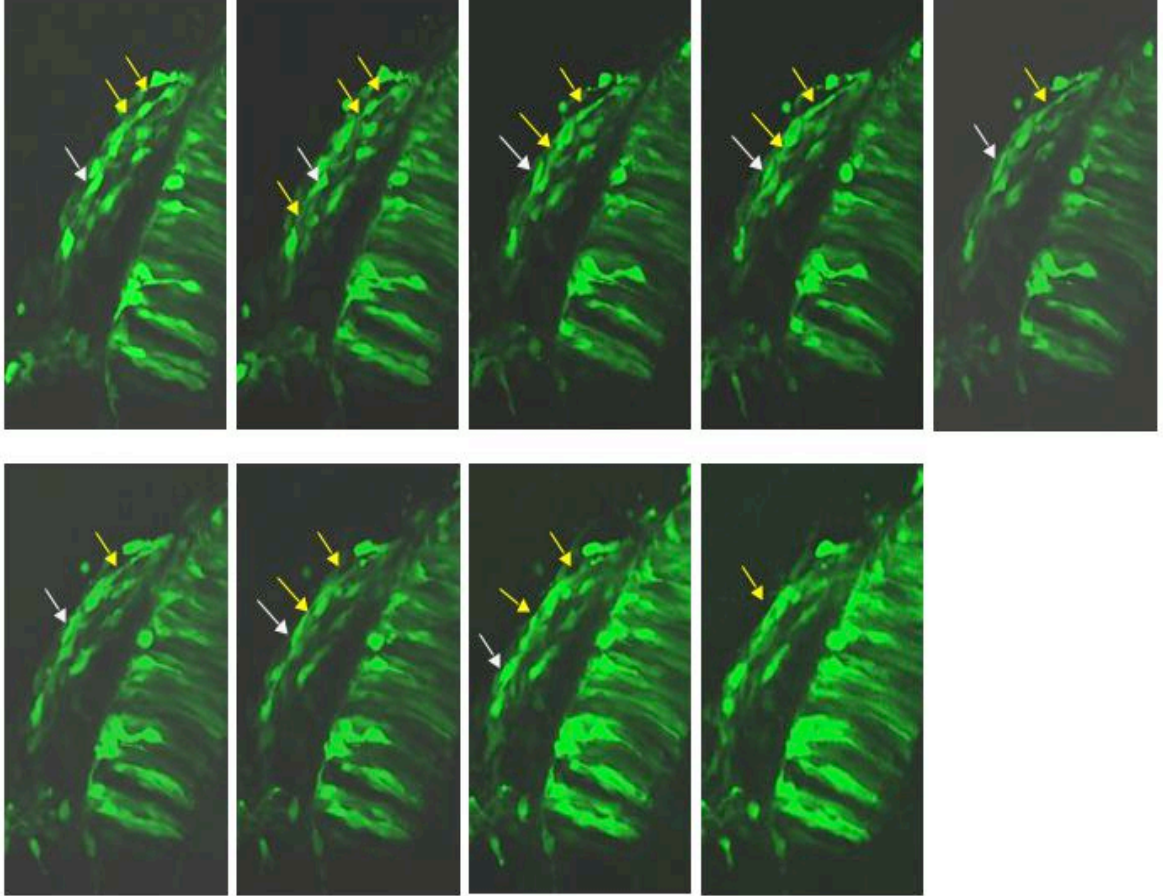


Figure 16. Serial time-points from confocal live imaging of cPcdh1-p2 stage 18 (transverse section).

Frames are from left-to-right and top-to-bottom in consecutive order.

Yellow arrows show cells exhibiting extensions along DRG periphery.

White arrows indicate typical elongated shape of cells and cells maintaining their position along DRG periphery.

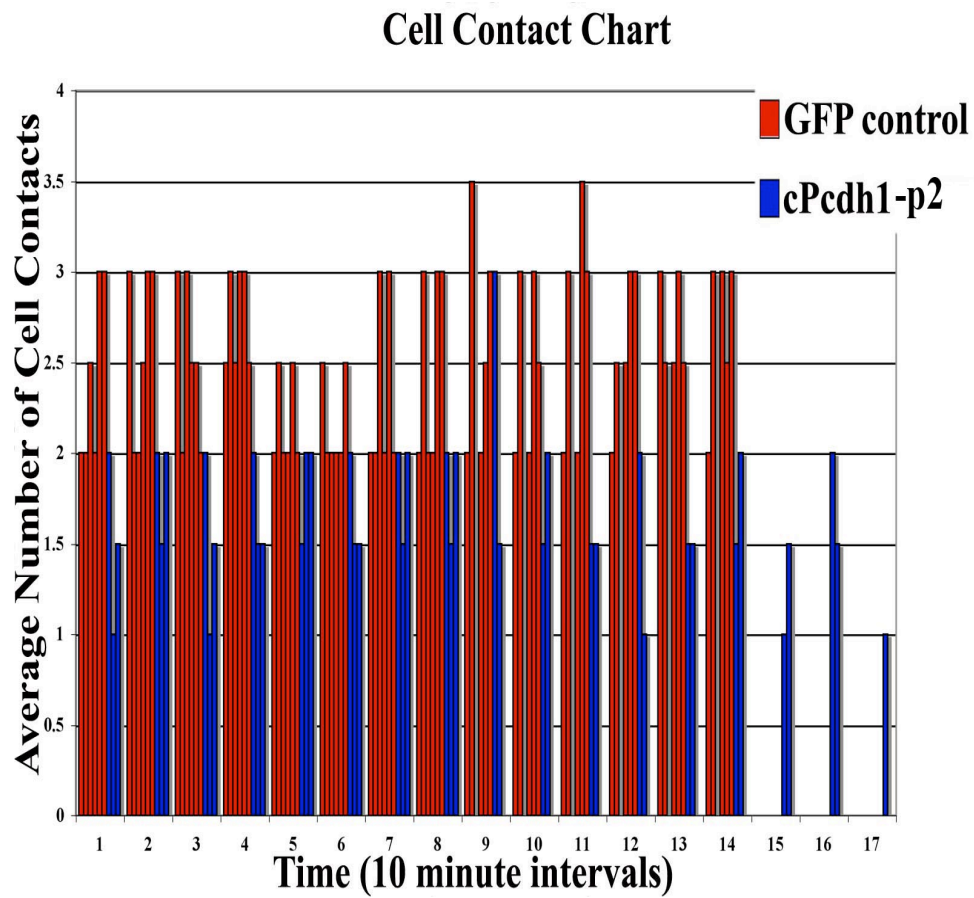


Figure 17. Graph of live-imaging results showing average number of contacts of individual cells for control vector pMES2 and cPcdh1-p2 over time.

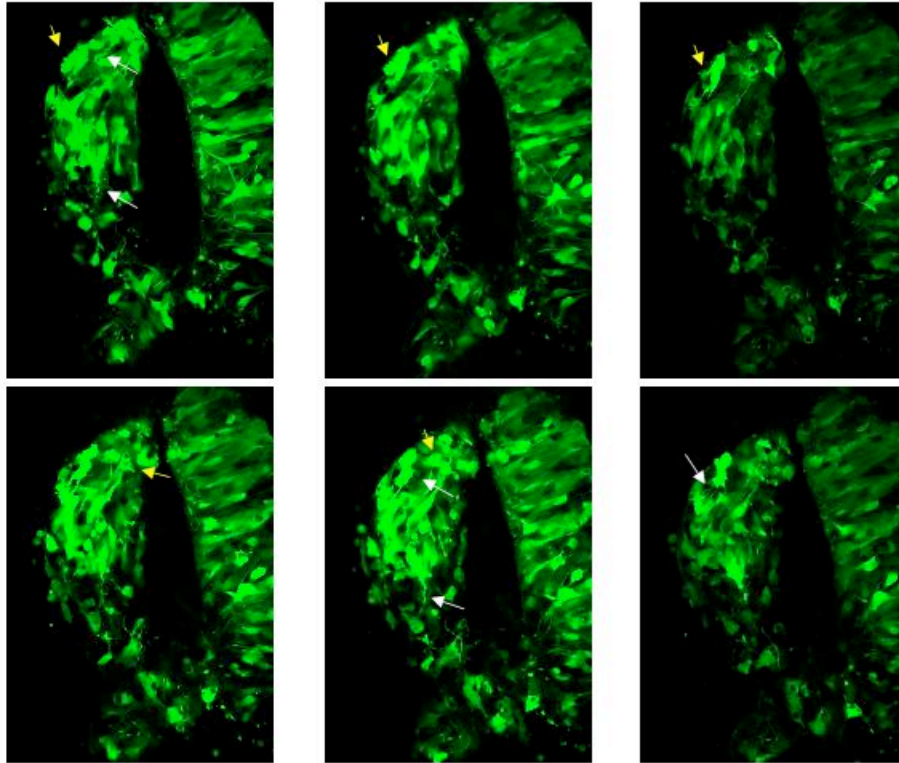


Figure 18. Serial view of time-lapse confocal imaging of siRNA 1 stage 20 (transverse section).

Top row: consecutive 30-minute time-point views from left-to-right; Bottom row: consecutive 30-minute time-point views from left-to-right approximately 30-minutes later.

Yellow arrows indicate “jagged-edged” cell shape.

White arrows indicate numerous filopodia, often with a “jagged” extension appearance.

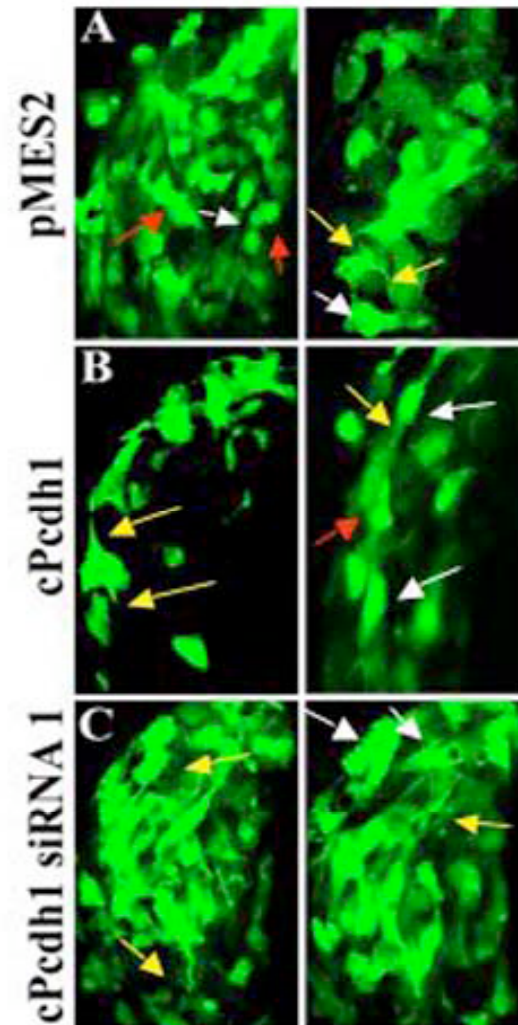


Figure 19. Magnification of DRG confocal live imaging results. (A) Control vector showing “roundish” shape of cells and extensions in a variety of directions besides dorsal-to-ventral. (B) cPcdh1 expressing cells showing dorsal-to-ventral filopodia extensions between cells and elongated cell shape along DRG periphery. (C) cPcdh1-specific siRNA1 expressing cells showing “jagged” cell appearance, multiple and often short extensions in various directions of filopodia. White arrows indicate cell shape; Yellow arrows show filopodia extensions; Red arrows show a typical cell contact used for counting and the cell contact chart shown in Figure 17.

cPcdh1 cells to be elongated mainly along the DRG periphery where the cells also appear to reach to their neighboring cells along the periphery. Although cPcdh1 cells make and break cell contacts, the time of separation is brief, approximately 10 minutes and a cell will remain in contact with at least one other cell for long periods of time, up to 3 hours or longer. In conclusion, when cPcdh1 expression comes on, cells tend to stop in the DRG indicating cPcdh1 may normally function to both target a sub-population of migrating neural crest cells to the DRG and retain the newly arrived cells at the periphery where the cells remain in an undifferentiated and mitotically active state prior to differentiating into sensory neurons.

CHAPTER 5

DISCUSSION AND CONCLUSION

In avians, neural crest cells are generated at the crest of the closing neural folds and migration of the neural crest begins after the neural tube closes. The capacity to generate a wide variety of cell types and tissues has made neural crest cells an important biological system to study cell migration, tissue morphogenesis and gene expression. Chick embryos are used as a model to study developmental biology and in particular, neural crest development for a number of reasons: the accessibility of the embryo makes the chick amenable to surgical manipulation, cell marking techniques, cell culture and transgenesis, in ovo injection and electroporation and retrovirally mediated gene transfer, the low-cost of use, the relatively short development period and the well-characterized development stages from pregastrulation throughout almost all developmental stages.

The developmental stages referred to in this study are based on Hamburger and Hamilton staging (Hamburger and Hamilton, 1951). Neural crest cells begin to emigrate from the neural tube at stage 12 which corresponds to somite level 8-13 (cervical level of the trunk). The detachment from the neural tube progresses in an anterior-to-posterior wave and emigration from the neural tube ceases at approximately stage 18 at the cervical level (Serbedzija et al., 1989).

In the chick, DRG develop from migrating neural crest cells that coalesce laterally to the neural tube beginning at E2.75-3 (Teillet et al., 1987; Lallier and Bronner-Fraser 1988). E4.5-5 in the avian embryo is the peak of neurogenesis in the immature DRG,

followed by target innervation and programmed cell death of postmitotic neurons between E5 and E12 with a peak period between E7 and E9 (Carr and Simpson, 1978). At E4.5, 30% of the cells are mitotically active progenitor cells with the remaining 70% of the cells being nascent postmitotic neurons (Goldstein et al., 1995; Rifkin et al., 2000; Wakamatsu et al., 2000). Gliogenesis is dominant after E6 and DRG neurogenesis is complete by E8.5, with the majority of neurons expressing their mature phenotypes (Carr and Simpson, 1978; Oakley et al., 1997).

The goal of this work was to discover protocadherins involved in the developing nervous system, specifically in relation to neural crest cell migration, cessation of migration and differentiation in the peripheral nervous system. Previous research has shown cadherins and protocadherins to be involved in many aspects of development from the early embryo and adhesion in the neural folds to later events such as forming synapses. Cadherins such as E- and N-cadherin are found involved in neural crest cell migration but to date, little research has focused on protocadherins role in trunk neural crest cells, the cessation of their migration and the subsequent differentiation to form the various peripheral nervous system trunk derivatives. The results from this research work show the discovery and isolation of a new protocadherin, cPcdh1. We examined the identity of cPcdh1 expressing cells, first asking if they are really neural crest-derived. Second, we asked how these cells find their way to the DRG, VR and SG and what determines where they stop and why. Results showed cPcdh1 to be involved in mediating trunk neural crest cell migration, functioning as a cell adhesion molecule with possible involvement in the

differentiation and fate choices of cells in the developing peripheral nervous system, specifically with regards to the DRG.

cPcdh1 belongs in the recently identified δ -protocadherin subfamily group consisting of approximately nine other protocadherins besides cPcdh1. The δ -protocadherin subfamily is further divided into two subgroups, $\delta 1$ - and $\delta 2$ -protocadherins. $\delta 1$ -protocadherins consist of the human protocadherins 1(axial protocadherin in *Xenopus*), 7 (NF-protocadherin in *Xenopus*), 9 and 11, the only known protocadherins with seven extracellular cadherin domains (Redies et al., 2005; Nollet et al., 2000; Hirano et al., 2003; Vanhalst et al., 2005; Yoshida and Sugano 1999). This group contains the conserved CM1 and CM2 amino acid motifs in their cytoplasmic domains (Wolverton and Lalande 2001) as well as a highly conserved RRVTF sequence in the CM3 domain necessary for the binding of protein phosphatase-1 α to $\delta 1$ -protocadherins. The $\delta 2$ -protocadherin subgroup, consisting of protocadherins 8 (arcadlin in rat; paraxial protocadherin in *Xenopus*), 10 (OL-protocadherin in mouse and chick), 17 and 18, contains six extracellular domains and the CM1 and CM2 but not the CM3 motif. Based on sequence similarity (82% with axial protocadherin and 81% with human protocadherin 1) we initially placed cPcdh1 in the δ -protocadherin subfamily. However, because cPcdh1 has six extracellular cadherin domains and does not contain the CM3 motif, cPcdh1 may belong to the $\delta 2$ -protocadherin subgroup.

cPcdh1 and Neural Crest Cell Migration

Our results show cPcdh1 expressed in migratory neural crest cells and most of the peripheral nervous system tissues but not expressed in premigratory neural crest cells in the neural tube or in those cells migrating along the dorsal pathway. These results imply cPcdh1 is involved in the cell-cell adhesion among the ventrally migrating neural crest cells.

Based on the in situ hybridization results from this study, cPcdh1 is not expressed in the premigratory cells. There was no obvious change in DRG morphological size at the time points we examined (st.20-23) when cPcdh1 was over-expressed or when deletion constructs were used indicating a lack of action on neural crest precursor cells and their potential to differentiate during normal development. Results from the staining with the pan-neural crest cell marker HNK show not all HNK-positive crest cells express cPcdh1. The HNK results combined with the in situ hybridization results showing extensive and specific cPcdh1 staining on the margins of the DRG indicate some of the ectopically expressing cPcdh1 neural crest cells are likely to be pre-destined to stop in the DRG. When cPcdh1 is inhibited with either deletion constructs or cPcdh1 mRNA-specific siRNA oligos, some ectopically expressing cells still stop in the DRG, indicating that cPcdh1 may only be involved in a certain sub-population of neural crest cells.

Slug has been shown to regulate neural crest cell formation and EMT (Nieto et al., 1994; LaBonne and Bronner-Fraser, 2000; del Barrio and Nieto, 2002). For example, the onset of *Slug* expression allows for the dissociation of junctions between neighboring cells and downregulates N-cadherin expression, which promotes the delamination of neural crest cells from the neural tube. Future immunohistochemistry experiments could

include using the neural crest marker *Slug* as well as other adhesion molecules such as N-cadherin or even cadherin 7 with cPcdh. cPcdh1 expression should not be expressed in *Slug*-expressing neural crest cells prior to delamination but cpcdh1 expression could be turned on by *Slug* after delamination. In addition, if cPcdh1 expression becomes upregulated at later stages when other adhesion molecules are either up or down-regulated, this could help to understand if cPcdh1 expression affects the expression of other genes.

Neuregulin and its receptors are implicated in the ventral migration of neural crest cells. Targeted deletion of neuregulin, *erbB2* or *erbB3* genes result in incomplete development of the primary chain of SG (Britsch et al., 1998). Neural crest cells leave the neural tube but fail to migrate ventrally toward the dorsal aorta and contribute to the formation of the SG and instead, their migration is stopped in the vicinity of the DRG. What we observe when cPcdh1 function is inhibited with the deletion constructs or cPcdh1-specific siRNA oligos is similar: neural crest cells do not stop to form the DRG but instead continue more ventrally and is arrested near the SG, thus cPcdh1 may function in the same pathway as neuregulin with a role in the ventral migration of neural crest cells.

β -catenin molecules have been found primarily in association with N-cadherin in the region of the intercellular contacts in most migrating neural crest cells. Only early migrating cells situated in proximity with the dorsal side of the neural tube showed detectable β -catenin in their nuclei, indicating β -catenin may be recruited for signaling in neural crest cells only transiently at the onset of migration and that sustained β -catenin signals are not necessary for the progression of migration (de Melker et al., 2004).

Potentially, cPcdh1 could function only transiently to signal neural crest cells to stop and coalesce at the DRG.

cPcdh1 and Sensory Neurogenesis

Mature DRG contain approximately twenty types of post-mitotic neurons that differ in their sensory properties, innervation targets, morphologies and biochemistry (Scott, 1992). Immature ganglia contain a less well-defined population of cells consisting primarily of nascent, undifferentiated neurons and mitotically active progenitor cells that can generate neurons and/or glial cells. Our studies of cPcdh1 expression can help in better understanding these processes.

The time line of sensory neuron differentiation by marker analysis shows Hu, Delta and trkC to be among the first sensory neuron specific genes expressed, followed by neurofilament and then Islet-1. Research has shown in stage 23 chick embryos, that both motor neurons and DRG cells express Islet-1 after leaving the cell cycle while SG cells express Islet-1 while still dividing (Avivi and Goldstein, 1999). As our results demonstrated, the most evident results were with Islet-1, indicating a potential overlap of cPcdh1 expression as it turns off and earlier sensory neuron markers as they turn on. Besides labeling differentiated neurons in the central and peripheral nervous system, Hu is also expressed in a subpopulation of crest-derived cells early in development prior to differentiation. For example, Hu labels some neurogenic neural crest cells in a population of cells that are considered nonneuronal (those cells lacking neuron-specific antibodies such as neurofilament) but have a neurogenic ability (Vogel et al., 1993). Likewise, previous research has shown DRG younger than E7 to contain nonneuronal cells

immunoreactive to Hu whereas DRG older than E7 lack neurogenic ability and thus did not show Hu⁺ cells (Marusich and Weston, 1992). To further support the timeline of cPcdh1 expression, staining for Hu protein before and after E7 to indicate post-mitotic differentiating neurons and the decline in neurogenic ability in the DRG could be useful.

The forkhead family transcription factor, Foxs1, has recently been shown to be an early sensory neuronal marker with expression turned on in the sensory neuron precursors of the trunk (Montelius et al., 2007). Migrating neural crest cells expressing Foxs1 were found at the anterior dorsal somitic lip at stage 18. These cells showed limited proliferation and migrated to form a cluster in the ventral region of the coalescing ganglion and were surrounded by Foxs1(-)/Sox10(+) migrating neural crest cells maintaining a high proliferation rate. Expression of Sox 10 is known to be present in undifferentiated neural crest cells (Hong and Saint-Jeannet 2005) and not be expressed with Foxs1 (Montelius et al., 2007). Beginning with expression of neurogenin1 and Brn3a, sensory neurogenesis of the Foxs1(-)/Sox10(+) precursors occurred within the condensed DRG. The authors suggest a “sequential emergence of neuronal precursors of the sensory nervous system with different molecular characteristics, starting during migration and continuing well after DRG condensation.” Because cPcdh1 was expressed in the peripheral nervous system tissues such as the DRG and SG at the time they coalesce as well as with strong expression along the DRG periphery, cPcdh1 could be a part of the “sequential emergence of neuronal precursors”. The function of cPcdh1 in this process may be the maintenance of cells at the DRG periphery or perhaps acting alone or

with other molecules to downregulate proliferation which would allow cells such as Foxs1(-)/Sox10(+) to migrate to the ventral region of the DRG.

Early work using dissociated chick DRG cells *in vitro* at varying developmental stages (E5-E14) quantified the appearance of differentiated cell types in the DRG. The proportion of glial cells increased throughout the E5-E14 time period while the proportion of neurons increased until E7-E8 but then decreased due to the glial proliferation at that stage. Analysis of the proportions of neurons, glial cells and fibroblast-like cells also showed a large population of undifferentiated cells present at E5 and E6 although not at later stages of development (Rohrer et al., 1985). The presence of the undifferentiated cells correlates with the period of neuron birth in DRG: at E6.5 a majority of neuroblasts in the ventrolateral region and approximately 70% of the mediodorsal neurons have withdrawn from the mitotic cycle (Carr and Simpson, 1978). The undifferentiated population of cells contained neuronal progenitor cells that differentiated into neurons within 1 day in culture. Researchers observed the morphological differentiation of neuronal precursor cells preceding the differentiation of neuron-like membrane properties (electrically excitable) *in vitro* using chick and quail migratory and pre-migratory neural crest cells as well as in dissociated chick DRG cells (Ziller et al., 1983; Rohrer et al., 1985). Evaluation of our results in conjunction with a potential function of cPcdh1 indicates cells may be retained in the DRG periphery until time for differentiation. This could be due to the local environment being non-permissive for differentiation because other cellular events have not taken place yet. For example, one difference among sensory neurons in the developing chick DRG is a separation into

two spatially distinct subpopulations. The ventrolateral region in the DRG contain neurons with large somata and a light staining cytoplasm. The dorsomedial region contains smaller neurons with a dense cytoplasm (Hamburger and Levi-Montalcini, 1949). Older DRGS give rise to Schwann cells (ensheath axons) but not to satellite cells (ensheath neuronal somata) when transplanted into younger embryos (Schweitzer et al., 1983). Although these cell types arose from a common ganglionic environment, evidence has shown the local environmental cues such as contact with axons versus cell bodies to influence the choice between a Schwann cell or satellite cell (Frank and Sanes, 1991). Lineage-tracing tests would be interesting to perform in order to determine the fate of cPcdh1 cells at a later stage after E4-4.5/stage 23 (stage 31-34 when the ventrolateral and dorsomedial boundary is distinct). Sensory neurons, Schwann cells, satellite cells and glia could be determined by either in situ hybridization with a cPcdh1 probe or in ovo injection of cPcdh1 (co-injected with lacZ as with the siRNA/in situ method we used) followed by either immunohistochemistry or immunofluorescence with specific antibodies and fate-map analysis. In addition, analysis of both ventrolateral and dorsomedial neurons as well as large and small neurons which innervate distinct targets would be informative in understanding the possible affect on cell fate by cPcdh1 or if cPcdh1 plays a part in any later events. When sensory neurons differentiate, two axonal processes are extended in different directions to reach the peripheral and central target fields. Because the innervation of the targets is done with extreme precision, the possibility exists that sensory neurons become specified before innervation (LeDouarin and Kalcheim, 1999). Possibly cPcdh1 has a later role in development, specifically

during gliogenesis. Because the majority of gliogenesis in the DRG occurs after stage 27 (E5) in the avian embryo this possibility was not tested at this time but would be of interest for future studies, which could determine if cPcdh1 may function later during glial differentiation and/or may show that cPcdh1 cells later become satellite glia.

Influence by the environment does not completely answer the question whether some cells may be pre-determined prior to emigration from the tube or if the entire population of cells responds in the same way to the same environmental cues. How do cells choose between two or more fates when exposed to a similar environment? Do the local signals at the homing sites dictate the cell type that develops or are cells lineage-restricted such that progressive cell divisions specify the fate of daughter cells? Possibly the choice is truly random and the environmental factors only influence the probability of a choice rather than actively directing cell fate. Intrinsic characteristics such as clocks or temporal changes in the homing site itself could affect cell fate. Because most neurons in the DRG are born before most glia (Carr and Simpson, 1978) and the progenitors are multipotential, an early withdrawal from the mitotic cycle may predispose a cell to become a neuron and a later withdrawal would promote a cell to become glia. Exogenous N-myc expression was shown to act on the population of neural crest cells still not totally committed to a non-neuronal lineage, direct them to ganglion-forming areas of an embryo and then promote on site neuronal differentiation. Additionally, N-myc deficient mouse embryos showed a significant reduction in the number of mature neurons of the DRG and SG (Wakamatsu et al., 1997). Due to our ectopic overexpression of cPcdh1 and results with the sensory neuron marker Islet-1 indicating a limited overlap of cPcdh1 expression

as Islet-1 is turned on, cPcdh1 may be functioning in the neuron/glia fate decision of a cell promoting the decision to be glia by holding the cells in a mitotically active yet undifferentiated state at the DRG periphery to ensure a later withdrawal from the cell cycle. Because there may be distinct classes of mitotically active progenitor cells residing in the immature DRG that give rise to distinct sensory neurons and glia, cPcdh1 may be functioning in cooperation to maintain the progenitor cells in this state until ready for differentiation. Intercellular signaling interactions between nascent (emerging) post-mitotic neurons and progenitor cells could be taking place and cPcdh1 may be playing a role in this signaling.

cPcdh1 and Cell Fate/Transcription Factors

Neurogenin and NeuroD3 are considered as determination genes for neuronal fate in the vertebrate nervous system (Ma et al., 1996). Studies of trunk DRG in mice have shown neurogenin2 required for the development of the early-generated trkC⁺ and trkB⁺ neurons while neurogenin1 is required for the later-generated small-diameter nociceptive trkA⁺ neurons as well as a subset of trkC⁺ and trkB⁺ neurons (Ma et al., 1999). In the chick, large-diameter trkC⁺ (and some trkB⁺) neurons are located in the ventrolateral region of the DRG and the small-diameter trkA⁺ neurons located in the dorsomedial region (Carr and Simpson, 1978; Lefcort et al., 1996; Oakley et al., 1995). In addition, birthdate and neuronal death studies show birth, differentiation and death of the large neurons precedes the small-diameter neurons (Carr and Simpson, 1978). Differential expression of markers has also been noted for the two major neuronal subgroups. For example, in avian DRG, substance P immunoreactivity is limited to the small-diameter

dorsomedial DRG neurons and first appears at E5 (Fontaine-Perus et al., 1985).

Neurofilament protein markers can characterize the large-diameter ventrolateral neurons. Neurofilament is strongly expressed in sensory neurons in the early migrating cells aggregating in the ventrolateral area of the DRG; less strongly expressed in late maturing cells and no neurofilament expression is found in satellite cells (Price, 1985; Lawson et al., 1993). Similar to our studies with Islet-1, staining for substance P in cPcdh1 (and deletion constructs) injected avian embryos developed until E5 and later, as the DRG matures and neurons differentiate, could provide information on whether cPcdh1 has a role only in one of the two major subsets of neuron populations. If a difference in the number of substance P⁺ cells was found and no change in neurofilament⁺ cells, this could suggest cPcdh1 plays a role in the later-forming smaller-diameter DRG neurons. Alternatively, no change in substance P but a change in neurofilament⁺ cells would be suggestive cPcdh1 participates in the early-forming large-diameter DRG neurons. Our early preliminary results with the neurofilament marker at E4 have shown a decrease in myc⁺/neurofilament⁺ cells in the DRG in cPcdh1 injected embryos, as compared to the control pMiWII and all three deletion constructs. In situ at later stages such as E6 and E8/8.5 showed cPcdh1 expression more evenly distributed throughout the DRG (as well as staining along the periphery of hind and forelimb forming digits) with less strong staining along the periphery. These results indicate cPcdh1 is not associating as closely with the undifferentiated cells along the periphery but instead is downregulated and likely to be expressed in the later maturing cells such as the glia cells associated with the ventrolateral region of the DRG. Further work could support this hypothesis (or the

converse) by staining for *trkC* and *neurogenin2* to indicate if these cells could be ventrolateral precursors. Because most of *neurogenin1* expression occurs after ganglion condensation while *neurogenin2* is expressed in early neural crest cell migration, the expression of *neurogenin2* has been suggested to be required for the ventrolateral precursors specified for a sensory fate (final destination of large-diameter cells are within the deeper laminae of the dorsal horn of the spinal cord, sending mechanoreceptive and proprioceptive stimuli; Koerber and Mendell 1992; Snider and Wright, 1996) shortly after they emigrate from the neural tube (Ma et al., 1999). In addition, by stage 32 (E7.5/8) *trkC* expression is primarily in the ventrolateral region of the DRG, correlating with a time of differentiation and neurogenesis in this region, while the time period of programmed cell death for the large diameter ventrolateral neurons is well advanced (Carr and Simpson, 1978; Hamburger et al., 1981; Lefcort et al., 1996). Further analysis of *cPcdh1* function at this time period could determine if *cPcdh1* is specified for this subtype of sensory neurons.

The monoclonal antibody 7B3 recognizes an avian transitin-like protein expressed by neural crest cells in early development. 7B3 initially recognizes neural crest cells as they emerge from the neural tube, is apparent in neural crest cells on the dorsomedial but not lateral pathway and becomes progressively restricted to a smaller subpopulation of non-neuronal cells at later stages of development. Using both quail trunk neural crest cell cultures and in vivo examination showed 7B3 expression along the VR in Schwann cells and in presumptive satellite glial cells that surround DRG neurons but absent in the neurons themselves (Henion et al., 2000). Due to the ability of 7B3 to recognize trunk-

derived glial cells early in development and the progressive restricted expression during development, this marker could be used to indicate if cPcdh1 may be involved in glial cell fate-restriction of trunk neural crest cells.

Analysis of neural crest cells with different markers can provide useful information regarding differentiation of neural crest cells specifically as it pertains to different stages of development but does not answer the question as to the state of commitment of these cells. In order to test the commitment of an individual cell, cells known to have a particular fate during development need to be identified and then exposed to a different environment. Regardless of the new environment, a committed cell will retain its original fate (Vogel et al., 1993). For example, cPcdh1 cells could be cultured to various stages and then exposed to different environments such as with or without the presence of neurotrophins, growth factors etc to determine how this may affect the fate of cPcdh1 cells.

cPcdh1 and Cell Sorting/Cell Adhesion

A possible function of cPcdh1 is in cell sorting as demonstrated in both the *Xenopus* cell-mixing and aggregation assays as well as in fate map analysis results. The cell-mixing and aggregation assays showed cPcdh1-MT had more cells in contact with other labeled cells as compared to the control or the deletion constructs, which did not suppress cell mixing. Besides indicating a role of cPcdh1 in adhesion, this also points to a function in cell sorting when combined with the fate map analysis results demonstrating the inhibition of cPcdh1 altered the distribution of cells between the DRG and SG. Previous work has shown when a rostral somite is grafted into an embryo, the DRG

increases in size but at the expense of the SG (Goldstein and Kalcheim, 1991).

Additionally, recent confocal imaging has shown reorientation back to the DRG of a sub-population of neural crest cells that have migrated to the SG (Kasemeier-Kulesa et al., 2005). Because the SG develops just prior to the DRG (Serbedzija et al., 1989), cPcdh1 may function to sort the SG cells from the DRG cells by aiding in the arrest of migrating neural crest cells at the DRG. To examine the possible function of cPcdh1 with regards to SG and DRG cells, inhibition of cPcdh1 after the SG has typically formed (stage 18) would prove informative (we injected at stage 10, prior to neural crest migration). If more cells were still found in the SG at the expense of the DRG, this would imply cPcdh1 was also functioning in the sorting of those cells that already migrated to the SG and would normally migrate back to the DRG.

In mouse, paraxial protocadherin was shown differentially expressed at the border between somites (Rhee et al., 2004). The expression of mouse paraxial protocadherin on the posterior side of the somite border has been proposed to promote the organization of cells along that border (Rhee et al., 2004). When looking at results of cPcdh1 in situ hybridization staining, a correlation could be drawn to paraxial protocadherin. The expression of cPcdh1 on the periphery of the DRG could be proposed to promote organization of those cells along the DRG border. The disruption of cPcdh1 could result in a disruption in the organization of the cells at the border, which is primarily what we have discovered in our studies. This disruption includes the lack of localization at the periphery of the DRG and an increased cell migration to other PNS locations at the expense of cells migrating to the DRG.

Classical cadherins binds to the same cadherin type in an adjacent cell in a homophilic interaction. The intracellular domain of classical cadherins is linked to the actin cytoskeleton through tandem binding to β - and α -catenin. Lateral clustering of cadherin molecules at the plasma membrane adds adhesive strength for cytoskeleton stabilization of the adherens junctions involved in keeping adjacent epithelial cells together (Trojanovsky 1999; Vleminckx and Kemler, 1999). Epigenetic, transcriptional and posttranslational mechanisms can regulate cadherin levels at the plasma membrane. Methylation of the E-cadherin gene, observed in a number of human cancers (Conacci-Sorrell et al., 2002) is one example of epigenetic regulation. A posttranslational mechanism involved in regulation of cell-cell adhesion is tyrosine phosphorylation. The dissociation of the adherens junctions in order to decrease cell-cell adhesion has been shown to be a result of tyrosine phosphorylation of cadherins or catenins (Ayalon and Geiger, 1997; Ozawa and Kemler, 1998; Roura et al., 1999). Another mechanism proposed to regulate E-cadherin expression in EMTs is transcriptional repression. Snail (Batlle et al., 2000; Cano et al., 2000), Slug (Bolos et al., 2003) E12/E47 (Perez-Moreno et al., 2001) transcription factors among others have been found to be upregulated during EMT and can repress E-cadherin transcription. Thus the possibility of cPcdh1 expression being regulated by other factors can not be ruled out. Potentially these type of mechanisms could work with cPcdh1 or to downregulate cPcdh1 in order to affect adhesion and allow cells to move interior to differentiate.

A further example of interactions among adhesion molecules is demonstrated with protocadherins. For example, the protocadherin- γ family was shown expressed in the

chick central nervous system, specifically in the fore-, mid- and hindbrain at stages 23 and 25 as well as in the spinal cord and optic lobe at stages 27 and 43 respectively (Cronin and Capehart, 2007). Transcripts of protocadherin- γ members were found in stage 27 chick embryos in the dorsal horn and floorplate regions of the developing spinal cord as well as in the DRG (Cronin and Capehart, 2007). Due to protocadherin- γ A5 also being expressed in the dorsal horn and DRG, a possible role for one or more members of the protocadherin- γ family in interactions between interneurons and the DRG while peripheral sensory connections with the CNS are being established has been suggested (Capehart and Kern, 2003; Cronin and Capehart, 2007). Further support of protocadherins working together was shown with the analysis of the expression of the protocadherin- γ family in the optic lobe of stage 43 chick embryos. Protocadherin- γ was found expressed in multiple laminae of the stratum griseum and fibrosa superficiale and within the thalamic projection neurons of the stratum griseum centrale (Cronin and Capehart, 2007). Protocadherin- γ A5, protocadherin- α and OL-protocadherin are expressed in a pattern similar to protocadherin- γ in the chick optic lobe at later stages implying multiple protocadherin families for neuronal organization and interconnection in the chick visual system are involved in essential adhesive interactions and may be working cooperatively with other cell adhesion molecules in neuronal differentiation and the establishment of neural networks in the developing brain (Capehart and Kern, 2003; Blank et al., 2004; Muller et al., 2004; Cronin and Capehart, 2007). Another member of the protocadherin family, Arcadlin, a member of the δ 2-protocadherin subfamily, has shown homophilic adhesion activity. Arcadlin is a synaptic protein that binds to itself in a

Ca^{2+} -dependent manner (Yamagata et al., 1999). Some δ -protocaderins family members have shown homophilic over heterophilic adhesive interactions and are able to mediate cell sorting. For example, axial protocadherin and paraxial protocadherin are involved in prenotochord and paraxial mesoderm sorting during *Xenopus* gastrulation (Kuroda et al., 2002; Kim et al., 1998). Additionally, in cell dispersal and reaggregation assays in *Xenopus*, cells that maintain sharp boundaries with unlabeled neighbor cells are considered as having cell sorting abilities as described by Kim et al., (1998) and Chen and Gumbiner (2006). In our tests for adhesion and cell sorting using cPcdh1-MT, cPcdh1 Δ E-MT, cPcdh1 Δ C-MT, cPcdh1 Δ TM-MT and control GFP mRNAs injected into *Xenopus* embryos, cells from cPcdh1-MT formed tight patches, further confirming cell sorting abilities of protocadherins and specifically cPcdh1. The consensus throughout these results show an emerging picture of protocadherins having adhesion properties with the potential to interact with each other or other adhesion family members. This broadens the function of cPcdh1 to potentially include working with other cell adhesion molecules.

One method of which cPcdh1 could be acting as an adhesion molecule is through the extracellular domain. Previous findings have found clustering effects of the transmembrane or extracellular E-cadherin domain could be sufficient to mediate cell adhesion (Ozawa and Kemler, 1998; Huber et al., 1999). Support for this theory is evident when evaluating our results with cPcdh1 Δ E-MT where the extracellular domain is deleted, showing a decrease in cells halting at the DRG and subsequently migrating and coalescing at other peripheral target areas.

Besides the potential effects due to the extracellular domain, the cytoplasmic domain also could prove to be important in adhesion. For example, the cytoplasmic domain of α -protocadherins does not show any similarity with classical cadherins but has been suggested to be a factor in anchoring and signal transduction. The noncatalytic domain of tyrosine Fyn was used in a yeast two-hybrid screen to isolate α -protocadherins and thus is thought to be an intracellular binding partner of α -protocadherins (Kohmura et. al., 1998). More recently, using transfected cells, two cloned α -protocadherins from the chick ciliary ganglion were shown at the cell surface with an accumulation at cell-cell contacts. The authors demonstrated expression at the surface was independent of the cytoplasmic domain and participation by the α -protocadherins in homophilic interactions. Importantly, the cytoplasmic domain was found to enhance the interaction as well as to link the α -protocadherins to the cytoskeleton (Triana-Baltzar and Blank, 2005). A similar combination of experiments could be pursued with cPcdh1. These include tagging the N- and C-terminal of cPcdh1 with different tags such as GFP and FLAG. Transfection of dorsal root ganglion cells from cell culture could be used for analysis of cell-cell contacts. Based on results from our *Xenopus* cell mixing experiments, cell-cell contact by cPcdh1 will be found. The next question would be two-fold; can a detection be made as to if the C-or N-terminal is involved in any cell-cell contact and do any of the currently made deletion constructs affect this contact? Again, based on the results of the *Xenopus* injections, a significantly greater number of single-labeled cells not in contact with other single-labeled cells were observed when the control or the deletion constructs were injected as compared to when cPcdh1 was injected, indicating a loss of adhesiveness.

Based on the statistical numbers from counting the single-labeled cells, all three deletion constructs showed no statistical significant differences among each other, therefore if a particular domain is more capable to target/stabilize cPcdh1 at cell-cell contacts remains unanswered. The results do indicate both the extracellular and cytoplasmic domains of cPcdh1 are required for cell adhesion. Further experiments to test the possibility of homophilic interactions as well as the importance of the extracellular versus the intracellular domain could include co-immunoprecipitation and pull-down assays using both transfected cell cultures and homogenized chick embryo tissue with the C- and N-terminal tagged constructs and the deletion constructs with the goal of determining what domain may be more significant in cell-cell contact which was not completely obvious from our cell-fate mapping analysis. In addition, using the cPcdh1 Δ E-MT deletion construct for pull-down assays would help to determine what binds the cytoplasmic domain. Future studies examining structure could also prove insightful as to the function of cPcdh1. This could include first searching the protein databases and finding consensus domains among other proteins and their related functions.

cPcdh1 and Signaling

As mentioned earlier, studies of chick embryo DRG have shown that Notch signaling is involved in the neuron/glia cell decision (Wakamatsu et al., 2000). When forming the DRG, Notch-1 is expressed preferentially by cells in the outer periphery of the ganglion whereas the expression of Delta-1 and early neuronal markers such as Islet is localized to the core region. In quail DRG cell cultures, Notch activation prevented neuronal differentiation and kept cells in an undifferentiated, actively proliferating state

although they could eventually form glia. From the results of our work showing a preferential expression pattern of cPcdh1 on the DRG dorsolateral margin and the increase of Islet+ cells when cPcdh1 is disrupted by deletion constructs or siRNA oligos, cPcdh1 may possibly be a part of the Notch signaling pathway as a downstream target.

β -catenin molecules have been found primarily in association with N-cadherin in the region of the intercellular contacts in most migrating neural crest cells. β -catenin was shown to be involved in cell-cell contacts in migrating cells, recruited for signaling in neural crest cells only transiently at the onset of migration. Additionally sustained β -catenin signals were not necessary for the progression of migration (de Melker et al., 2004). In addition, an intact β -catenin signaling pathway has been shown to be required for the formation of the DRG (Hari et al., 2002). For example, DRGs and melanocytes were found to be absent in mice with a deletion of β -catenin in their neural crest progenitors (Hari et al., 2002). In contrast, when a constitutively active form of β -catenin in neural crest cells was overexpressed in transgenic mice, an increase in sensory neural cells at the expense of other neural crest derivatives resulted (Lee et al., 2004). In *Xenopus*, neural crest induction was shown to require the canonical Wnt signaling pathway through β -catenin while the non-canonical Wnt pathway operating separately from β -catenin is required for neural crest cell migration and the formation of sensory ganglia (Veeman et al., 2003; Bejsovec 2005; De Calisto et al., 2005).

Initially paraxial protocadherin was shown to be involved in convergence and extension movements of paraxial mesoderm and in the establishment of somite boundaries in *Xenopus* (Kim et al., 1998; 2000). Studies in *Xenopus* with a dominant-

negative form of paraxial protocadherin (one lacking the cytoplasmic domain, similar to cPcdh1 Δ C) indicated the intracellular domain was not only required for but had an inhibitory effect on cell adhesion (if the cytoplasmic region was intact, sorting activity was found to decrease) (Kim et al., 1998). Although this was initially taken as support for the proposed function of paraxial protocadherin as an adhesion molecule to mediate cell sorting events (Kim, et al., 1998), more recent evidence suggests paraxial protocadherin does not act as a true homophilic cell-cell adhesion molecule. Instead, paraxial protocadherin has been suggested to mediate activin-induced down-regulation of C-cadherin adhesion activity (Chen et al., 2006). Further studies have found paraxial protocadherin to interact with the *Xenopus* Frizzled-7 (Xfz7) receptor and activate RhoA and JNK (two intracellular targets of the planar cell polarity pathway signaling cascade) via the non-cononical Wnt pathway in regulating tissue separation and convergent extension movements (Medina et al., 2004; Unterseher et al., 2004). The Xfz7 receptor has been shown to regulate tissue separation through modulation of protein kinase C α (PKC α) activity (Winklbauer et al., 2001). This result allows us to consider in more detail that cPcdh1 could also have a signaling function. Recently, ankyrin repeats domain protein 5 (xANR5), a novel FGF target gene product that regulates cell-protrusion formation and tissue separation was shown to interact with paraxial protocadherin in both a physical and functional manner to regulate early morphogenesis. This research shows xANR5 to act through Rho in regulating gastrulation. Moreover, xANR5 appears to be a cytoplasmic partner of paraxial protocadherin (Chung et al., 2007). Although protocadherins have been shown to function using cytoplasmic co-factors (Yoshida et al.,

1999; Heggem and Bradley 2003; Redies et al., 2005), there has yet to be any indication that δ -protocadherins interact with β -catenin. However, when reviewing evidence from the previous studies mentioned, specifically paraxial protocadherin's interaction with Xfz7 and C-cadherin, our results from fate map analysis demonstrating a change in neural crest sorting when cPcdh1 is disturbed could be an indirect cause from a classical cadherin cell-adhesion or Wnt signaling.

Many cadherins have been shown to be involved in the development of the nervous system. For example, N-cadherin and cadherin-6B are both expressed in the neural folds and premigratory crest cells and both are downregulated as the neural crest begins to migrate. N-cadherin is a classical cadherin thought to function as a cell-cell adhesion molecule in neural crest cells. N-cadherin is re-expressed on cells migrating to the DRG. Overexpression of N-cadherin inhibits neural crest cell migration resulting in neural crest cells aggregating in the dorsal neural tube and a decrease of neural crest cells in the DRG (Nakagawa and Takeichi 1998). Obviously N-cadherin activity is regulated during neural crest cell migration events. Because cPcdh1 is expressed in migrating neural crest cells and when they coalesce at the DRG, is it possible that cPcdh1 and N-cadherin could cooperate in neural crest migration, cessation and differentiation of crest cells at the DRG? This could be tested with *Xenopus* injections or in a similar method to what was used by Gumbiner and Chen (2006) with the use of CHO cells which do not express endogenous cadherins and have been used previously for studying classical cadherins' adhesive properties (Brieher et al., 1996; Chappuis-Flament et al., 2001; Niessen and Gumbiner, 2002). If we can obtain a purified cPcdh1 protein, then we

could also test the ability to adhere to a substrate of cPcdh1. In cell aggregation assays, determine the number of cells that aggregate as compared to control and a cell line expressing N-cadherin. If no cPcdh1 aggregation occurs, this indicates a lack of mediated homophilic cell adhesion by cPcdh1 in CHO cells. If they do aggregate, then the opposite would be true and cPcdh1 may be functioning by homophilic cell adhesion. Adhesion assays could also be performed as further confirmation of the aggregation results.

Jin et al., have shown in the avian embryo Wnt and BMP signaling to have antagonistic functions in the trunk neural crest. Wnt signaling stimulated melanogenesis in cells grown in culture while BMPs stimulated neuronal and glial cell differentiation while repressing melanogenesis (Jin et al., 2001). The authors postulate BMP and Wnt signaling are working cooperatively in segregation of the neural crest lineage and further speculate this is due to opposing gradients of Wnt and BMP. Is there a gradient of cPcdh1 expression that may affect a signaling pathway such as Wnt? Possibly cPcdh1 may be antagonistic or binds and inhibits Wnt3a so cells can become neurons and glial cells rather than melanocytes. Although the fate mapping results showed melanocyte differentiation was not affected when cPcdh1 function was inhibited by the deletion constructs or siRNA, this does not completely rule out cPcdh1 could be involved with Wnts or BMPs. Based on DiI-labelling and because melanocyte precursors do not express the HNK-1 epitope, presumptive pigment precursor cells were shown to first appear at stage 21 on the dorsolateral pathway (Serbedijida et al., 1989). The most active migration of melanoblasts occurs before the sixth day of incubation with massive seeding of the epidermis by melanoblasts occurring at the end of E5 and during E6 and melanocyte

differentiation occurring at E9 in chick (LeDouarin and Kalcheim, 1999). The majority of our analysis in this work was performed on stage 20-23 chick embryos and as such, may not be as accurate when evaluating melanocytes. To address this potential problem, further fate-map analysis should be conducted at stages 22-29.

Examination of the *Xenopus* cell sorting results show: cPcdh1 Δ TM-MT expressing cells are caught in the endoplasmic reticulum; cPcdh1 Δ E-MT expressing cells are on the plasma membrane; and cPcdh1 Δ CD-MT expressing cells remain between cells or endocytosed when no cell-cell contact is present. Additionally, the filopodia observed in the cPcdh1-specific siRNA expressing cells from the live imaging experiments show short and multiple extensions, sometimes with a branched or jagged appearance. Because external signals such as growth factors can activate filopodia, membrane ruffles and stress fibers, the possibility exists of cPcdh1 involvement in this process. For example, the binding of growth factors to receptor tyrosine kinases activate intracellular signaling pathways directed by Ras-like molecules such as Rac, Rho and Cdc42 belonging to the GTPase family (Hall, A., 1998). RhoA regulates focal adhesion formation and stress fibers (Ridley and Hall, 1992; Nobes and Hall, 1995; Chrzanowska-Wodnicka and Burridge, 1996) while Cdc42 plays a role upstream of Rho in filopodia formation and migration (Price et al., 1998; Schoenwaelder and Burridge, 1999). Focal complexes form at the ends of filopodia and once formed, the actin-rich lamellipodia propel spreading. Lamellipodia are a collection of actin filaments that polymerize in a branching or dendritic pattern in a Rac-dependent manner upstream of Rho (Hall, A., 1998). Based on our preliminary results from live-imaging using cPcdh1-specific siRNA showing

filopodia with short and numerous extensions, cPcdh1 may be upstream of Cdc42 in the Cdc42, Rac, Rho pathway.

Inhibition of cPcdh1

In order to give us a better understanding of the function of cPcdh1, we chose to study the effects when cPcdh1 is inhibited. We chose to construct deletion constructs as described in the materials and methods section and schematic shown in Figure 4.

The cPcdh1 Δ E-MT deletion construct design was based on previous research showing cadherin constructs typically functioned as dominant-negatives when missing the extracellular domain (Kintner 1992; Bradley et al., 1998; Borchers et al., 2001). The cPcdh1 Δ CD-MT construct was designed based on research demonstrating a gain-of-function effect when axial protocadherin in *Xenopus* lacked the cytoplasmic domain (Kuroda et al., 2002). In addition, a *Xenopus* Xcad-11 construct lacking the cytoplasm domain (complete loss of the β -catenin binding site) also acted as a gain-of-function to inhibit migration of cranial neural crest cells (Borchers et al., 2005). In *Xenopus*, an axial protocadherin construct lacking both the transmembrane and cytoplasmic domain functioned as a secreted dominant-negative form that interfered with sorting activity of cells (Kuroda et al., 2002), thus the rationale for the design of cPcdh1 Δ TM-MT.

All three constructs altered the distribution of neural crest cells as shown in Figure 7. This suggests that each encodes dominant-negative proteins that can inhibit endogenous cPcdh1 function. Each deletion construct could also be inhibiting cPcdh1 function by different methods. For example, the deletion construct cPcdh1 Δ TM is a secreted

molecule and may inhibit function by binding to the extracellular domain of other protocadherins. The other deletion constructs may function in an opposite manner by binding independently to other co-factors either extracellularly (as in the case of cPcdh1 Δ CD) or intracellularly (as with cPcdh1 Δ E). Whether other endogenous proteins were being affected by the use of these constructs and because it was unclear as to if one of the deletion constructs was a better candidate for a true dominant-negative, we attempted to inhibit endogenous cPcdh1 by alternative methods.

The first of these was the use of morpholinos. Although morpholinos have been successfully employed as a method to study loss-of-function events and used at the trunk level of avian embryos (Kos et al., 2003), our initial attempts proved problematic. As Corey and Abrams mention, antisense technology rarely, if ever, duplicates complete 'loss-of-function' mutations and have noted results can be marginal with the observed phenotypes being due to a wide variety of unexpected non-antisense effects. The non-antisense effects can be caused by unintended interactions with proteins and binding to non-target nucleic acid sequences (Corey and Abrams, 2001). Factors that lead to uneven uptake include the amount of morpholino injected into the lumen of the neural tube, the dilution of morpholinos as they spread from the site of injection, and the positioning of the electrodes (Kos et al., 2003). In addition, microinjections and in ovo electroporations were found to work best if performed in the absence of light (i.e. 'dark-room' conditions). During this time, the use of siRNA was becoming more established as a method to study loss-of-function, therefore we chose to pursue this method.

SiRNA targets mRNA causing degradation of mRNA and results in gene silencing/knockdown. The use of cPcdh1-specific siRNA oligos proved helpful in confirming our deletion construct results. When using siRNA oligos from Dharmacon, typically a number of oligos are suggested and are ranked according to how well they match certain criteria shown to be effective for siRNA silencing. The criteria include: a G/C content that ranged between 30-50% (criterion I); low internal stability of the siRNA at the 5' antisense end as measured by the number of A/U basepairs with three or more A/U basepairs as a minimum (criterion II); lack of stable internal repeats as estimated by the predicted melting temperatures where high melting temperatures favor internal hairpin structures (criterion III); an A at position 19 of the sense strand (criterion IV); an A at position 3 of the sense strand (criterion V); the presence of a U in position 10 of the sense strand (criterion VI); the absence of a G or C at position 19 of the sense strand (criterion VII); and the absence of a G at position 13 of the sense strand (criterion VIII) (Reynolds et al., 2004). The criteria are extremely informative and useful when choosing siRNA sequences, but they are not absolutes. When choosing the siRNA oligos for our experiments, we attempted to choose the oligos that were closest to the criteria listed above. Although our results demonstrate the two siRNA oligos chosen (siRNA1 and siRNA3) were effective in reducing cPcdh1 mRNA levels as well as the number of siRNA ectopically expressing cells that stop at the DRG, a different siRNA oligo candidate may have provided more evident results. This also explains why the two siRNA oligos used for our experiments did not yield exactly the same results. Overall, the

siRNA results indicate cPcdh1 is necessary for holding neural crest cells at the DRG periphery and confirm the deletion constructs acted as dominant-negatives.

cPcdh1 and Live Imaging

The initial experiments in this research are valuable and meaningful in elucidating the function and role(s) of cPcdh1 but are static. *In vivo* imaging can provide additional information as well as providing confirmation to the results and conclusions from our earlier work. Time-lapse imaging of live neural crest cells as they migrate through their environment could help to identify the cellular and molecular interactions involved (both known and unknown) and possible sorting events. To this end, our hypothesis based on our initial results that cPcdh1 is functioning as an adhesion type molecule to hold undifferentiated neural crest cells at the periphery of the DRG and a better determination if sorting is taking place with cPcdh1 can be further tested.

Limitations of the transverse slice preparation technique include what would be considered typical when working with live tissue. Over time there is a loss of tissue morphology due to a number of reasons. A sliced tissue is exposed to the external environment, including light and lasers, which could affect both the tissue and subsequently the cells we are attempting to image. In addition, although media is provided, it is not the same nutrition provided by the egg itself. The technique could be considered somewhat invasive in that it involves dissection, embedding in warm agarose, sectioning and handling of the slice, all resulting in some damage to the tissue.

Although not static, the time-lapse imaging does capture cells at various determined time points (every 10 minutes for this study) and thus could miss some cell

movement. In addition, due to equipment limitations, a time-lapse imaging over the Z-scan range preferred (approximately 10 μm section scan with a scan taken every 0.5 μm through the Z-level) was not possible and the original idea of actually capturing cPcdh1 cells moving from the periphery to the interior of the DRG was not possible. The counting of contacts and contact time was subjective and limited by lack of 3D view. To compensate for subjective interpretations, two people performed the counts independently on three separate occasions and results averaged.

To aid in understanding different aspects of the function and role(s) of cPcdh1, future live imaging analysis should include measurements of the distance cPcdh1, pMES2 and siRNA expressing cells migrate. Neural crest cells have been shown to be inherently migratory (Erickson et al., 1980; Erickson, 1985; Keller and Spieth, 1984; Tucker and Erickson, 1984). In culture, neural crest cells move rather quickly away from explanted neural tubes at rates in the range of 20-60 $\mu\text{m/hr}$ (Newgreen et al., 1979; Davis, 1980; Erickson and Turley, 1983; Rovasio et al., 1983; Tucker and Erickson, 1984). The rate of *in vitro* migration of crest cells that have dispersed through the somite and along the surface of the dermatome/myotome has been estimated at 20-30 $\mu\text{m/hr}$ (Loring and Erickson, 1987). The fastest movement of neural crest cells *in vitro* was reported to occur on planar substrata coated with fibronectin, serum, or chick-fibroblast-derived extracellular matrix. When cells are suspended in a collagen matrix, lower rates have been reported and are dependent on the density of the matrix or collagen concentration (Davis, 1980; Tucker and Erickson, 1984). The rate and distance estimated for travel by a cell does not always take into account that the pathway(s) may be slightly curved and the

cells being followed are often assumed to be moving in a horizontal flat plane, which is not the real-life case. Movement measurements in previous studies possibly were often based only on selected cells that may have unobstructed movement or more or less contact inhibition with other cells. The path length in previous studies was assumed for calculation purposes to not change significantly over the time of imaging. As growth of an embryo takes place and development continues, embryonic changes occur such as an increase in size of the DRG and the turning on/off of other signals. As many of these limitations as possible should be addressed when calculating distance measurements. When attempting to determine the rate of migration, cPcdh1 slice explants should be imaged when placed on the different surfaces mentioned above. This could then be used to compare to the above reported rates and give a better understanding of any differences or similarities that these cells may be experiencing. If cPcdh1 expressing cells showed a difference in rate or distance of migration, the possibility exists that pre-destined neural crest cells may experience a different rate of migration. This could help to explain how a population of cells may experience different environments at different times of development, which could affect cell fate or indicate potential intrinsic characteristics of selected cells.

In particular, live imaging of cPcdh1 Δ CD-MT could be informative regarding intrinsic characteristics of cPcdh1 as well as useful when evaluating the effects caused by this deletion construct. Besides neural tube closure defects seen in n-cofilin mutant mouse embryos, the embryos also showed a heavily deformed neural tube similar to what has been observed in knockout mice of the Delta1-Notch pathway (Gurniak et al., 2005;

Oka et al., 1995). This neural tube deformation is generally interpreted as a secondary consequence of hyperproliferation although for n-cofilin mutants the opposite was true with a lack of proliferation. For cPcdh1 Δ CD-MT embryos, although a deformed neural tube was occasionally observed there was no apparent change in proliferation. The high levels of n-cofilin expressed in neural crest cells and the lack of migration *in vitro* in mutant explants suggest the differences observed in the embryos are largely due to a cell autonomous migration defect (Gurniak et al., 2005). With cPcdh1 and the deletion constructs there was not a lack of migration as determined by fate analysis, but rather a disruption of the normal migration patterns and cessation of migration at the DRG with an increase of migration to other areas such as the SG at the expense of the DRG. Because of this contrast between n-cofilin and cPcdh1 constructs, the migration defect could be due to a cell non-autonomous effect rather than a cell autonomous effect. Thus the surrounding environment and other factors/molecules could be working in concert with cPcdh1 to arrest neural crest cell migration at the DRG and/or to hold cells at the periphery of the DRG.

The DRG are organized as bilateral metameric units along the spinal cord. Each DRG forms in the rostral part of the somite and is populated by neural crest cells migrating longitudinally and laterally from the part of the neural tube facing the same somite and the preceding somite. Neural crest cells derived from the rostral or caudal part of the neural tube remain segregated along the rostrocaudal level of each DRG in a way that corresponds to their origin along the neuraxis (Teillet et al., 1987). Because this segregation pattern remains throughout development of the ganglia, it has been suggested

at the end of the migration process, the progenitor cells that have migrated close to each other because they arose from the same segmental level, may tend to associate with their neighbors due to cell adhesion mechanisms that become active at gangliogenesis (Le Douarin and Kalcheim, 1999). Using time-lapse confocal live imaging of cPcdh1 could provide evidence for this theory although the technique and stage of imaging may have to be altered.

Examination of the shape and extensions of the imaged cells show similarities to growth cones. Growth cones send out long filopodia and can retract them quickly or branch out in response to the environment. High resolution imaging in chick have shown both short and long filopodia in neural crest cells, suggesting filopodia are involved in the direction a neural crest cell chooses to take (Teddy and Kulesa, 2004). This is similar to what was observed in our live imaging experiments; filopodia of different lengths and in different directions were observed as if “sensing” the environment. The cells expressing cPcdh1-GFP showed extensions directed towards their nearest neighbor on the periphery perhaps in an attempt to interact or communicate to provide information pertaining to the local environment or target. Previous research has shown cells on the perimeter of the incipient DRG exhibiting greater movement than cells within the central core with the cells on the perimeter actively extending and retracting filopodia into the surrounding environment. In addition, Teillet et al., (1987) described repositioning of neural crest cells along the rostral-caudal axis of the neural tube during the formation of the DRG. In streams of migrating neural crest cells, shape changes appear dependent on the position of the cell in the stream. For example, at the front of the stream, neural crest cells have

many lamellipodial and filopodial extensions in nearly all directions while at the middle of the stream, neural crest cells have a bipolar shape (Teddy and Kulesa, 2004). Our live imaging results also showed changes in cell shapes, which differed when cells ectopically expressed cPcdh1 versus the control.

Further experiments could be directed to establishing in more detail the fate of cPcdh1 by imaging at a later stage such as stage 31 when the neurons have separated into dorsomedial and dorsolateral regions. Various sections from the same embryo could be prepared with one used for imaging, and others cultured for different amounts of time and then stained for the presence of trkA,B, or C, substance P or NF. The section used for live imaging could also be stained for these markers after a limited period of imaging and compared with the cultured slices. The aim would be to try to capture the division of neurons in the DRG in progress and to evaluate any changes that may occur with cPcdh1 expression during this time (i.e. a change in location of expression or a decrease in expression overall as if cPcdh1 was being downregulated as the sensory neurons mature).

CHAPTER 6

Conclusion

Collectively, the results confirm our hypothesis cPcdh1 has a role in maintaining cells in the dorsolateral margins of the DRG in an undifferentiated yet mitotically active state. Further, when this function is disrupted, an increase in ectopically expressing cells is found in the interior of the DRG, where they co-label for Ist-1, indicating that not only is cell-adhesion affected but also cell fate. This finding is further supported by a closer examination of the in situ hybridization (Figure 2G), immunolocalization with rabbit antibody (Figure 2I and 2J) and in ovo electroporation experiments showing the location of neural crest cells over-expressing full-length cPcdh1 in the DRG is consistently along the perimeter (cPcdh1-MT in Figure 5). Re-evaluation of ectopic expression of cPcdh1 Δ E, cPcdh1 Δ C or cPcdh1 Δ TM shows a breakdown in this defined separation between the perimeter and the interior of the DRG with neural crest cells now located throughout the DRG (cPcdh1 Δ CD-MT, cPcdh1 Δ E-MT and cPcdh1 Δ TM-MT in Figure 5). Further analysis using time-lapse confocal imaging has shown ectopically expressing cPcdh1 cells to be mainly along the DRG periphery and appear elongated in shape. These cells also appear to reach to their neighboring cells along the periphery with “dorsal-to-ventral” extensions. As observed with cells from other constructs, cPcdh1 cells make and break cell contacts but the time of separation is brief, approximately 10 minutes. In addition, a cPcdh1 expressing cell will remain in contact with at least one other cell for extended periods of time, up to 3 hours or longer. When cPcdh1 expression comes on,

cells tend to stop in the DRG indicating cPcdh1 may normally function to both target a sub-population of migrating neural crest cells to the DRG and retain the newly arrived cells at the periphery where the cells remain in an undifferentiated and mitotically active state prior to differentiating into sensory neurons. In conclusion, the results from this study show cPcdh1 is expressed in the trunk peripheral nervous system, is required for proper neural crest cell migration, functions in protocadherin-mediated cell adhesion, is required for mediating migration arrest and localizing neural crest cells to the dorsolateral margin of the DRG and is required for the proper formation of the DRG.

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