Beyond cell Adhesion: Exploring the Role of Cadherin-11 Extracellular Processing by ADAM Metalloproteases in Cranial Neural Crest Migration

Catherine D. McCusker

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BEYOND CELL ADHESION: EXPLORING THE ROLE OF CADHERIN-11
EXTRACELLULAR PROCESSING BY ADAM METALLOPROTEASES IN
CRANIAL NEURAL CREST MIGRATION.

A Dissertation Presented

by

CATHERINE D. MCCUSKER

Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
of the requirements for the degree of

DOCTOR OF PHILOSOPHY

February 2010

Program of Molecular and Cellular Biology
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DEDICATION

I dedicate this dissertation to my loved ones. These people have supported me throughout the triumphs and tragedies of my life – they had unwavering faith in me along this journey. It is hard for me to express how much each of these people mean to me, and the profound effect these relationships have had on my life. This dissertation is dedicated to my mother Patricia McCusker, my father Richard McCusker, my “little” brother James McCusker, my “big” brother Michael McCusker, and the rest of my extended family especially my grandparents, Margaret and Francis McCusker, and Ruth and Roger Koltz. I also dedicate this dissertation to my “adopted” family members, my loving and supportive fiancé Julian Sosnik, and my life-long friend Colleen Greene. I love you all!
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I have many people to thank for help along this journey. I would like to start with thanking members of the Alfandari research group. I recognize Dominique and Helene as the main contributors to my becoming a developmental biologist. You have been extremely supportive, and have always pushed me to become a better scientist. My experience with Dominique, Helene, Russ, Erin, and Wei has been amazing, and it has been a great honor to be part of this team. I would also like to acknowledge my thesis committee; Rolf Karlstrom, Rachel Fink, and Patricia Wadsworth. I appreciate all of your encouragement and effort over the last four years. I acknowledge my brother James McCusker as being my first collaborator in the engineering field –thank you for all of your hard work on developing a quantitative method to analyze cell directionality (Chapter 4). To my friends inside and outside the lab –thank you. You have all brought me a tremendous amount of happiness and inspiration. I also want to acknowledge the MCB program, and the VASCI department. Thank you for providing an enriching and supportive environment in which to grow.
ABSTRACT

BEYOND CELL ADHESION: EXPLORING THE ROLE OF CADHERIN-11 EXTRACELLULAR PROCESSING BY ADAM METALLOPROTEASES IN CRANIAL NEURAL CREST MIGRATION.

February 2010

CATHERINE D. MCCUSKER, B.S., BRIDGEWATER STATE COLLEGE
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Directed by: Professor Dominique Alfandari

The migration of the cranial neural crest is an essential part of cranio-facial development in every vertebrate embryo. The cranial neural crest (CNC) is a transient population of cells that forms the lateral border of the anterior neural plate. In the tailbud stage *Xenopus* embryo, the neural crest cells delaminate from the neural tube, and undergo a large-scale migration from the dorsal to ventral region of the embryo. The CNC travels along distinct pathways, and populates specific regions of the embryos face. Once the CNC ceases migrating, it differentiates into a variety of tissues that are essential for cranio-facial structure and function. Some of these tissues include bones, muscle, cartilage, and ganglia. The CNC receives a concert of signals from neighboring tissues during and after CNC migration as well as signals transmitted among CNC cells, which act together to determine the fate of each CNC cell. Therefore, the proper migration of the CNC is an essential part of cranio-facial development.

What molecules are important for the process of CNC migration? As one might imagine, a milieu of different molecules and interactions are essential for this complicated embryological process to occur. The work presented in this dissertation will focus on the role of a cell adhesion molecule that is important for *Xenopus* CNC migration. Typically, the amount of cell adhesion decreases within tissues undergoing migration. This behavior is essential to allow fluidity within the tissue as it moves. However, cell adhesions are fundamental for cell migration to occur because the moving cells need a platform on which to mechanically propel themselves. These interactions can occur between the migrating cell and extracellular matrix molecules (ECM), or can happen between cells.

The cranial neural crest utilizes both cell-ECM and cell-cell interactions during the process of migration. The amount of cell adhesion mediated by either of these mechanisms will depend on where the cell is located within the CNC. Cells located at the periphery of the CNC tissue, which is surrounded by a matrix of ECM, will have more cell-ECM interactions. Cells located deeper in the CNC tissue, where there is little ECM, will rely more on cell-cell interactions. The work presented in this thesis focuses on a
cell-cell adhesion molecule that is part of the cadherin superfamily of molecules. With this in mind, these studies should be descriptive of the environment within the CNC, and to a less degree the environment between the CNC and the surrounding tissues.

The work presented in this dissertation will focus on cadherin-11, which is a classical cadherin that is specifically expressed in the cranial neural crest during its migration. How does cadherin-11 function in the CNC during this process? The work presented here suggests that the main role of cadherin-11 in the CNC is to perform as a cell adhesion molecule. However, too much cell adhesion is inhibitory to migration. In this respect, many of the studies described in this work indicate that cadherin-11 mediated cell adhesion is tightly regulated during CNC migration. Here I show that cadherin-11 is extracellularly processed by ADAM metalloproteases, ADAM9 and ADAM13, which removes the adhesive domain of cadherin-11. This extracellular cleavage event occurs throughout CNC migration, and is likely the main mechanism that regulates cadherin-11 mediated cell adhesion. Cleavage of cadherin-11 by ADAMs does not seem to affect its ability to interact with cytoplasmic binding partners, β-catenin and p120-catenin. This observation supports the idea that the “purpose” of cadherin-11 cleavage is to regulate cell adhesion, and not to induce (cell autonomous) signaling events.

Additionally, the secreted extracellular domain of cadherin-11 (EC1-3) retains biological activity. This fragment can bind to a number of cell surface molecules in tissue culture including full-length cadherin-11 and specific members of the ADAM family. This observation suggests that EC1-3 may interact with full-length cadherin-11 molecules in vivo, and inhibit cadherin-11 mediated cell adhesion during CNC migration. EC1-3 can rescue CNC migration in embryos that overexpress cadherin-11, further supporting this hypothesis. Many of the above observations have been published in my first-author paper entitled “Extracellular processing of cadherin-11 by ADAM metalloproteases is essential for Xenopus cranial neural crest migration” published in the journal Molecular Biology of the Cell in 2009.

Some of the unpublished work in this dissertation further focuses on how EC1-3 effects CNC migration in an ex vivo environment. During these studies, the observation was made that overexpression of EC1-3 in a cranial neural crest explant produces abnormal directional movement. In these experiments, it appeared as though certain regions of the CNC explant were “attracting” other regions of the explant. The preliminary studies described in chapter IV are aimed at answering the question; does EC1-3 attract migrating CNC cells? Here, we generated a Matlab program in order to effectively quantify the amount of directional movement of CNC explants presented with a source of EC1-3. In addition to quantifying cell directionality, this program can also decipher between cells moving with random or directed motion, and measure the velocity of cell migration within certain coordinates. Therefore, this program should be useful other ex vivo studies that require the observation of these features.

To conclude, the work presented in this dissertation suggests that the role of cadherin-11 during cranial neural crest migration is predominately based on the adhesive function. In order for CNC migration to proceed, the amount of cadherin-11 mediated cell-cell adhesion is tightly regulated throughout this process. These cell-cell interactions are likely important for “sheet” and “branch” migration where CNC cells maintain a lot of cell-cell cohesion.
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CHAPTER I

INTRODUCTION

Abstract

The Cranial Neural Crest (CNC) is a morphogenic population of cells that undergo a large-scale migration to populate different regions in the face of the developing embryo. These cells will differentiate into many types of tissue, such as bones, muscle, cartilage, and ganglia, and are essential for proper craniofacial development. The work presented in this thesis will focus on specific molecular interactions that occur in the CNC during migration. One group of molecules that have proved to be very important for CNC migration, as well as many other embryological processes, is the cadherin superfamily of cell adhesion molecules. Indeed, the embryo utilizes a subset of cadherin molecules during different phases of CNC development. The work presented in this thesis will primarily focus on the molecule cadherin-11, which is expressed in the CNC throughout migration.

This chapter will introduce the reader to the ADAM family of metalloproteases. This family is responsible for the processing of many different substrates that are involved in the major signaling pathways utilized during embryogenesis. This thesis will focus on a subset of these ADAMs that are expressed in the CNC during migration. The expression and activity of some of these ADAMs have already been tied to CNC development and migration. This thesis will present results showing that members of the ADAM family can process cadherin-11 during CNC migration, and this cleavage event is essential for this morphogenic process. This is the first example of an ADAM/cadherin...
interaction playing a role during embryogenesis. This thesis will also explore the biological activity of the resulting C-terminal and N-terminal cadherin-11 cleavage fragments, and how these molecules may also promote CNC migration.

Section I: General background of cranial neural crest development

Almost 150 years ago Wilhelm His, a Swiss embryologist, discovered a population of cells located between the neural tube and epidermal ectoderm of the chick embryo. This group of cells would later be coined as the neural crest. Since the discovery, embryologists have found the neural crest to be a pluripotent population of cells that arises in all vertebrates and is essential for the development of many embryological tissues.

The NC is initially induced at the border of the neural plate between the neural and the non-neural ectoderm through the cross talk of three major pathways, the BMP, FGF, and Wnt signaling pathways (Figure 1.1 and reviewed in LaBonne 1998). As neurulation continues, the expression of NC specifiers, most of which are transcription factors such as members of the Pax, Snail, Hox, and Zic families, are thought to play a role in NC development by potentiating the NC identity (reviewed in Sauka-Spengler 2006). However, because not all cells within this specified region become NC, a particular cell is not considered a NC precursor until it delaminates from the neural tube (NT) and begins to migrate ventrally (Figure 1.1B.3).

In the embryo’s trunk, the neural crest cells start their migratory path between the somites and the neural tube and overlying epidermal ectoderm. This region of the NC is
known as the trunk neural crest (TNC) (Figure 1.1B.3). Upon reaching their destination, the TCN cells differentiate into a number of different tissue types such as neurons, glia, and pigment cells (Table 1). The anterior (or rostral) region of the NC is known as the cranial neural crest (CNC), which displays a number of distinguishing characteristics from the other NC cells. The CNC migrates along specific pathways in the embryo head, known as the mandibular, hyoid, and branchial arches, to populate different regions of the embryos face (Figure 1.1B.3 and Figure 1.2). Once the CNC cells cease migrating they differentiate into a number of connective and sensory tissues including bone, muscle, cartilage, and ganglia (Table 1). While specific craniofacial structures will vary from one species of animal to another, generally the most anterior CNC cells (such as the mandibular and part of the hyoid arches) will take part in the development of the frontonasal skeleton. The more posterior CNC, including those that populate the branchial arches, form the cartilage and bone of the inner ear, the jaw, and the neck. The work presented in this thesis will focus on the migration of the cranial neural crest population of the neural crest (reviewed in Santagati 2003).

It is thought that as NC cells migrate they receive signaling cues from the cells surrounding the pathway that will help specify what type of tissue that NC cell will differentiate into. This differentiation process is further supported by signals emitted by the tissues surrounding the NC cell when it ceases migration (reviewed in García-Castro 1999). The migration of the CNC is absolutely essential for proper craniofacial development as well as the development of a number of tissues and organs throughout the embryo. The work presented in this thesis will look at some of the molecular interactions
that occur in the CNC during migration to further understand this complicated embryological process.

**Section II: Introduction to cadherin molecules utilized in NC development and migration.**

**General Cadherin background**

The cadherin superfamily is comprised of Ca\(^{++}\) dependent transmembrane cell-adhesion molecules. This superfamily is divided into five major subgroups, the classical cadherins, desmosomal cadherins, atypical cadherins, protocadherins, and cadherin-like molecules (Figure 1.3A and reviewed in Hulpiau 2009). While many of these subgroups play important roles during different embryological processes in vertebrates, for brevity sake I will focus on classical cadherin molecules. Members of this subgroup were the first family members to be discovered, and because of this they are known as the “classical” cadherins. The general domain organization of classical cadherins is depicted in figure 1.3A. Classical cadherins are further divided into two groups: type I and type II molecules. This differentiation is primarily based on the genetic characteristic of having fewer introns in type I cadherins, as well as a slight variation within their “adhesive sequence” located in the first extracellular (EC1) domain (figure 1.3) (Nollet, 2000). Type I cadherins such as E-cadherin, N-cadherin, and M-cadherin have an HAV adhesive sequence; while type II cadherins such as cadherin-11, cadherin-6, and cadherin-7 have a QAV sequence in this region. While classical cadherins have been designated as type I or type II proteins, this division does not imply any functional or behavioral differences among the molecules. For example “mesenchymal” cadherins, which are thought to
mediate the “looser” adhesive bonds in mesenchymal or migratory cells, are comprised of both type I and type II molecules.

Generally, cadherin molecules mediate cell-cell adhesion by forming homophilic interactions with other cadherins of the same type on the surface of a neighboring cell (Figure 1.3B). Many classical cadherins have been shown to form oligomeric zipper-like clusters at the interface of neighboring cells, which increase the strength of their adhesive bonds (Derycke 2004). Cadherin mediated cell adhesion is regulated in 4 major ways; 1) at the transcriptional level, such as E-cadherin down regulation by the Slug transcription factor during and epidermal to mesenchymal transition (EMT), 2) by internalizing the molecules into endosomes, which can either be degraded or recycled to a different region of the cell, such as the leading edge of a migrating cell, 3) through the activity of intracellular binding partners, such as β-catenin, which needs to bind to cadherins cytoplasmic tail in order to have a rigid extracellular domain capable of forming adhesive bonds, and 4) through proteolytic processing of the extracellular region of a cadherin molecule, which removes it’s adhesive sequence. Along with the important adhesive function, the extracellular domain of some cadherins can also bind to and activate cell surface receptors, such as EGF receptors, and promote downstream signaling. This topic will be explored further in Chapter V.

Cadherins can also mediate a variety of cellular responses through their cytoplasmic domain. This region binds to a number of molecules that perform an array of cellular tasks. Perhaps the best-characterized cytoplasmic binding partner of most classical cadherins is the Wnt signaling molecule β-catenin (Figure 1.3) (Kawaguchi 1999). β-catenin interacts with α-catenin, linking cadherin to the cytoskeleton (Oyama
This interaction is extremely important in linking the intercellular structures of the cell with the cell membrane, and to other cells, to maintain tissue integrity. The cadherin/β-catenin complex also performs another function. That is, it sequesters a pool of β-catenin at the cell membrane, protecting it from GSK3 mediated degradation (Sadot 1994). Upon dramatic cellular changes, β-catenin can be released from the cytoplasmic tail of cadherin, and can then promote downstream signaling by interacting with T-cell factor and lymphoid enhancer factor (TCF/LEF) transcription factors (Maretzky 2005; Ozawa 1998; Behrens 1996).

The juxta-membrane region in the cytoplasmic tail of classical cadherins can interact with another catenin, p120-catenin (Yanagisawa 2006). P120-catenin is also functionally connected to the cytoskeleton, but not in the manner of β-catenin. Instead, p120-catenin is involved with the regulation of the GTPases that control the cytoskeletal dynamics important for cellular movement (Noren 2000). More specifically, p120 regulates the activity of the Rho family exchange factor Vav2, which activates Rho-dependent actin polymerization that produces protrusions in the plasma membrane during cell migration (Noren 2000). Also unlike β-catenin, p120-catenin can mediate this signaling while still bound to the cytoplasmic tail of cadherin. In fact, some studies suggest that p120-catenin must be bound to cadherin in order to relay pro-migratory signaling (Yanagisawa 2006).

Though there are some exceptions, β-catenin and p120-catenin are considered the loyal cytoplasmic binding partners of classical cadherin molecules. As one may expect, there are other cytoplasmic binding partners that appear to be somewhat specific for individual family members. For brevity sake I will only cite one such example, the
interaction between cadherin-11’s cytoplasmic tail and the GEF-Trio signaling molecule. Trio is a small Guanine nucleotide Exchange Factor (GEF) that specifically interacts with the cytoplasmic domain of cadherin-11, and not to that of other classical cadherins (Backer 2007). Trio activates GTPases Rac1, RhoG/CDC42, and RhoA/ROK, which promotes cytoskeletal dynamics (Bateman 2001). This interaction between cadherin-11 and Trio was recently shown to be important for CNC migration in *Xenopus* embryos (Kashef 2009).

**Classical cadherins in neural crest development.**

The adhesive function and signaling capabilities of classical cadherins are highly utilized throughout embryogenesis. While there are a number of structural and functional similarities shared among classical cadherins, it is the subtle differences between these molecules that make them so useful in specific developmental processes. One excellent example of this characteristic is the involvement of cadherins during neural crest development (Akitaya 1992; Kimura 1995; Nakagawa 1995; Inoue 1997; Hadeball 1998; Vallin 1998; Borchers 2001; Coles 2007).

A general picture of the role of classical cadherins during neural crest migration can be put together using what researchers have learned about these molecules in the NC of *G.gallus, M.musculus, X.laevis* embryos. The neural crest expresses E-cadherin and N-cadherin during induction. As the induction process completes, E-cadherin expression decreases, and the expression of new cadherins, namely cadherin-6 (or cadherin-6B), cadherin-7, and cadherin-11, commences. Just prior to the onset of neural crest migration N-cadherin and cadherin-6 proteins are downregulated (Akitaya 1992; Nakagawa 1995.
Here, N-cadherin is extracellularly processed by a metalloprotease (Shoval 2007). Following this extracellular cleavage event N-cadherin is further processed, releasing a soluble C-terminal-fragment (CTF) (as well as β-catenin), into the cytosol. These molecules then translocate to the nucleus and promote the expression of genes, such as cyclin-D1, essential for the delamination of the neural crest from the surrounding tissue (Shoval 2007).

Once neural crest cells begin to migrate they express cadherin-7 and cadherin-11 (Kimura 1995; Nakagawa 1995; Hadeball 1998; Vallin 1998). These classical cadherins are expressed throughout neural crest migration, and overexpression of these cell adhesion molecules blocks this process (Nakagawa 1995; Dufour 1999, Borchers 2001; Coles 2007; Shoval 2007). However, the results described in Chapter II, and results recently published in (Kashef 2009), will show that depletion of at least cadherin-11 also blocks neural crest migration \textit{in vivo}. This observation suggests that the amount of cadherin-11, and possibly cadherin-7, expressed at the cell surface needs to be tightly regulated in order for NC cell migration to progress.

How might cadherin-11 and cadherin-7 surface levels be regulated throughout migration? Since both molecules are expressed throughout migration, it is possible that they are not regulated transcriptionally. This hypothesis is supported by the fact that the RNA of both of these molecules is consistently expressed during this process. Some work has suggested that the turnover rate of at least cadherin-7 is rapid when compared to the turnover rate of N-cadherin (Dufour 1999). Faster turnover rates could be mediated by two major processes; 1) internalization and degradation of the molecules by endosomes, and 2) proteolytic processing of the cadherin at the cell surface. Studies conducted in
tissue culture have shown that both cadherin-7 and cadherin-11 can be extracellularly processed, suggesting that this event may also occur in vivo (Kawaguchi 1999; Pishvaian 1999; Kawano 2002).

Section III: Introduction to the ADAM family, and their activity in the embryo

General ADAM Background

A Disintegrin And Metalloproteases (ADAM) comprise a family of zinc metalloproteases that process extracellular ligands involved in cell signaling, cell-cell and cell-extracellular matrix adhesion, and cell migration (Wolfsberg 1995). Members of the ADAM family can process a variety of substrates including growth factor ligands such as EGF and TNFα (Gschwind 2003; Yan 2002), signaling molecules such as Notch from the Notch/Delta pathway (Tortorella 1999), chemokines and cytokines (Schulte 2007), ECM molecules (Alfandari 2001; Rosendahl 1997; Millichip 1998), and cell adhesion molecules such as cadherins (Shoval 2007; McCusker 2009), making them important players during embryogenesis.

ADAM proteolytic activity can result in the activation or suppression of signaling cascades. For example ADAM10 cleaves Heparin-Binding EGF (HB-EGF) to activate EGF receptors and downstream signaling (Yan 2002). On the other hand, ADAM10 processing of Ephrin ligand, ephrin-A5, promotes the repulsion between two cells and terminates the signal between the ephrin ligand and receptor (Janes 2005).

Additionally, many ADAMs, such as ADAM10 and ADAM17, are functionally redundant since they can process some of the same substrates (Jarriault 2005, Horiuchi
The general domain organization of ADAM metalloproteases is depicted in figure 1.4. All ADAMs contain a metalloprotease domain, but only about half of these ADAMs are proteolytically active, suggesting that ADAM function can also be attributed to their non-proteolytic domains. However, for the purposes of this thesis, I will focus solely on the metalloprotease function of the ADAM molecules.

A single point mutation in the catalytic domain of protease-active ADAMs can render them proteolytically inactive, and creates a dominant negative (DN) form of the molecule probably by sequestering substrate from the wild-type protein (Figure 1.4). Loss-of-function approaches through the use of DN forms of ADAMs, knock down through the use of morpholino oligonucleotides, or ADAM inhibition through the use of small molecule inhibitors, along with gain-of-function approaches have allowed us to shed light on how specific members of the ADAM family function during frog embryogenesis.

**ADAM family members are important for CNC development.**

Of the ADAM family, multiple members of the meltrin subgroup (known as mesenchymal ADAMs) are enriched in the CNC during frog development (Figure 2.4 from Chapter II). Through loss of function approaches, our lab and others have resolved that ADAM9, ADAM13, and ADAM19 expression are important for CNC development (Alfandari 2001; Neuner 2008), and may also play a role in CNC induction by cleaving ephrin-B ligands (DeSimone communication in (Alfandari 2009)). ADAM19 also appears to play a role in CNC induction, as morpholino knock down of this molecule decreases the expression of CNC markers (Neuner 2008), and inhibits CNC migration
(Figure 1.5). By a targeted knock down approach, we have found that depletion of ADAM9, ADAM13, and ADAM19 individually can inhibit CNC migration in *Xenopus* embryos (Appendix V). This phenomenon is more pronounced by depleting two or more of these ADAMs in the same tissue. Single knock out of any of these ADAMs in the mouse model, except for ADAM13 which has not been cloned in any other vertebrate models, does not result in CNC abnormalities, though ADAM19 knockouts do have problems with heart development (Weskamp 2004; Kurohara 2004; Horiuchi 2006). Yet, it is clear that these molecules retain functional redundancy, and it is likely that multiple meltrins will need to be knocked out in the mouse before seeing a clear CNC phenotype. Some of the results discussed in the following chapters will elucidate how meltrins can compensate for each other’s function in the CNC.

There are other, non-meltrin ADAMs, namely ADAM10 and ADAM11, also expressed in the CNC during induction and migration. ADAM10 is one of the most studied ADAM family members because of the essential role in the notch/delta pathway (Pan 1997; Hartmann 2002). ADAM10 also processes a number of other ligands, such as epidermal growth factor (EGF), and cadherin family members N-cadherin, E-cadherin, and the protocadherin PCDHγ (Blobel 2005; Maretzky 2005; Reiss 2005). ADAM10 processing of N-cadherin and E-cadherin promotes an epithelial to mesenchymal transition (EMT) in tissue culture (Reiss 2005; Maretzky 2005).

ADAM11 is also expressed in the CNC during development (Cai 1998). Since this ADAM is not proteolytically active, the role of ADAM11 in the CNC may be to act as an integrin ligand. This hypothesis is supported by the fact that ADAM22 (also proteolytically inactive and closely related to ADAM11), and to a lesser extent
ADAM11, have been shown to inhibit cell proliferation in gliomas and brain specimens through their disintegrin domains via an integrin-dependent mechanism (D'Abaco 2006).

Section IV: Functional ties between ADAMs and Cadherins

As has already been touched upon in the previous sections, there have now been numerous examples of ADAMs interacting with cadherin molecules. Here, these previous examples will be compiled with others to help the reader appreciate the growing relationship between these two families.

Ham and colleagues performed the first study suggesting that an ADAM molecule was interacting with a cadherin molecule in 2002. In this work, the authors showed that ADAM15 and VE-cadherin co-localize to adherens junctions (AJ). They also showed that VE-localization to the AJ was a prerequisite to ADAM15 localization, and that an increase or decrease in VE-cadherin expression resulted in the corresponding alteration in ADAM15 expression (Ham 2002). Note that at the conception of this thesis work, this was the only published link between ADAMs and cadherins. Recently, ADAM15 was shown to cleave E-cadherin in breast cancer cell lines (Najy 2008). The soluble extracellular fragments of E-cadherin can then bind to epidermal growth factor receptors, namely ErbB receptor family members HER2 and HER3, and activate ErbB downstream signaling (Najy 2008).

The ADAM with the most established interactions with cadherin family members is ADAM10. As was described in the previous sections, ADAM10 can process both N-cadherin and E-cadherin (Reiss 2005; Uemura 2006; Shoval 2007; Maretzky 2005).
ADAM10 cleavage of both of these cadherins was shown to regulate cell-cell adhesion, as well as promote EMT and cell migration (Reiss 2005; Uemura 2006; Maretzky 2005). ADAM10 can also process γ-Protocadherin C3 and VE-cadherin, which plays a role in the regulation of cell adhesion and cell transmigration mediated by these molecules (Reiss 2005; Schulz 2009).

Adding to the list, ADAM9 along with ADAM15 and ADAM17 all appear to be involved in the processing of Desmoglein 2, a desmosomal cadherin, resulting in a decrease in cell adhesion in a cancer cell line (Klessner 2009). ADAM9 expression has also been tied to E-cadherin shedding in cancer cell lines (Hirao 2005). Additionally, the results presented in Chapter II will reveal that ADAM9 and ADAM13 can process cadherin-11 in tissue culture and in vivo (McCusker 2009). Research conducted over the last five years by our lab and others has shown that ADAM processing of cadherin family members is not an anomaly, but rather an integral part of cadherin regulation and function.

Section V: History of the work presented in this dissertation.

While this thesis focuses on meltrin processing of cadherin-11 in the CNC, the early scope of this project was broad. The goal at the onset of this project was to identify an extracellular ligand that was cleaved by an ADAM, specifically ADAM13, which could potentially play a role in CNC development. Within the published literature, candidate substrates were chosen based on their expression in the CNC and their potential to promote an activity that is important for CNC development such as the promotion of
EMT or involvement in cell migration. Additional relevance was placed on candidates whose activity was regulated through an extracellular processing event, and whose overexpression had been shown to inhibit CNC migration. Using these criteria our principle substrate candidates were insulin growth factor binding protein 5 (IGFBP-5), Src homology 2 domain containing protein tyrosine phosphatase substrate-1 (SHPS-1), ephrin ligands B1 and B3, and cadherin-11.

Pursuit of these substrates initiated with the cloning of the molecule if not yet cloned in *Xenopus*. Biochemical assays were then performed in tissue culture to analyze the ability for ADAM/candidate binding and shedding capability. Additionally, *in vivo* assays were conducted to determine whether ADAM13 co-overexpression rescued the inhibition of CNC migration caused by the overexpression of the candidate. The results from these initial studies that are not presented in this thesis are included in Appendix I. With this approach, cadherin-11 stood out from the rest of the candidates as a likely target for ADAM13 processing in the CNC during migration. At this time, there were no cadherin-11 specific antibodies that could be used to study the endogenous protein in *Xenopus*, thus one of our first objectives when we pursued this molecule was to generate a monoclonal antibody directed against the cytoplasmic tail of cadherin-11. The antibody was targeted to this region because it contained more variation than the extracellular region of classical cadherins, and would minimize the potential of cross reactivity. The generation and screening of this monoclonal antibody is described in Appendix II. Production of the cadherin-11 monoclonal antibody, 1B4, was exceptionally useful in the resolution of cadherin-11 as a target of meltrin family members during CNC migration.
Chapter II describes results showing that both ADAM9 and ADAM13 can cleave the extracellular domain of cadherin-11. In vivo, both of these ADAMs can rescue the CNC phenotype that occurs with cadherin-11 over-expression. Also discussed are results exploring how extracellular cleavage of cadherin-11 affects the interaction with known cytoplasmic binding partners, β-catenin and p120-catenin. The chapter is concluded with studies looking at the effect cadherin-11 depletion has on CNC migration in vivo and ex vivo.

Chapter III will focus on the biological activity of the cadherin-11 extracellular cleavage fragment (EC1-3). Here, it is shown that EC1-3 can bind to a number of cell surface molecules in tissue culture. In addition, I describe results demonstrating that EC1-3 can rescue the CNC phenotype that occurs with cadherin-11 over expression and with ADAM depletion. And lastly, I show some preliminary experiments looking at EC1-3’s affect on cadherin-11 mediated cell sorting, and cadherin-11 turnover rate.

In the final “results chapter”, Chapter IV, I will explain results suggesting that EC1-3 can affect cell migration of CNC cells ex vivo. The results in this chapter are highly quantitative. This section will describe results elucidating how CNC cells move when placed next to a source of cells secreting EC1-3. This chapter ends with some preliminary experiments conducted to shed light on which receptor EC1-3 may be acting through to promote CNC cell migration.

In order to thoroughly discuss both the biochemical and embryological implications of the studies conducted in this thesis project, the discussion of this thesis is divided into two chapters. The first chapter, Chapter V, focuses on the signaling capabilities of classical cadherin cleavage fragments. The second chapter, Chapter VI,
will focus more on the role of cadherin/ADAM interactions during CNC migration, and potentially other developmental processes.
Figure 1.1: Illustration of neural crest induction. (A) Dorsal view of a *Xenopus* embryo during early neurulation. The neural crest (purple) is induced at the border between the neural (blue) and non-neural (red) ectoderm. While all of the ectoderm initially expresses BMP, a gradient of BMP signaling is formed by the expression of BMP antagonists Chordin and Noggin from the notochord. These molecules, namely Chordin and Noggin inhibit BMP signaling in the neural plate. The border of the neural plate is less inhibited by the signals from the notochord because it is further away, and as a result has an intermediate level of BMP signaling. BMP signaling in the epidermal ectoderm is high.  

1. **Neural crest induction in the neurula stage embryo.** The neural crest is induced at the border between the neural and non-neural ectoderm, where there are intermediate levels of BMP signaling. This specific amount of BMP signal, along with the FGF and Wnt signals secreted by the underlying mesoderm, and the Wnt signal secreted by the epidermal ectoderm induces the neural crest.  

2. **Neural crest cells form at the apex of the neural folds.** The neural crest cells are internalized when the neural folds fuse.  

3. **Neural crest delamination and migration in the trunk and cranial regions.** BMP and non-canonical Wnt signaling have both been shown to be important for NC delamination, though the exact source of these signals still need to be resolved. Left side: trunk neural crest cells delaminate from the neural tube and migrate between the neural tube and the somites, and the somite and overlying epidermis. Right side: cranial neural crest cells form in the anterior region of the embryo, and migrate between the epidermis and underlying mesoderm in the head. *This figure has not been published, but was inspired by (Sauka-Spengler and Bronner-Frasier 2008).*
Figure 1.2: Detailed illustration of CNC migration in *Xenopus* embryo: The CNC migrates in a ventral direction along three major pathways, the Mandibular (M), the Hyoid (H), and Branchial (B) arches. The Mandibular arch migrates around (and a few cells migrate over) the optic vesicle (1). The mandibular branches will fuse at the ventral region below the optic vesicle (3) and eventually condense to form Meckel’s cartilage that will become the upper jaw. The CNC cells from the hyoid arch will take part in the otic vesicle (2) that will form the tympanic membrane, as well as the ceratohyal (4) that will form the lower jaw, and the anterior pharyngeal pouches (5) that will take part in the formation of “gill” cartilage and the pharyngeal cartilages. The branchial arches also migrate to populate the pharyngeal pouches (5). This figure is adapted from Mayor et al., 1999.
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<th>Cranial Crest</th>
<th>Trunk Crest</th>
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<td>and parafollicular cells of thyroid</td>
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<td><strong>Skeleton</strong></td>
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<td>Face and skull bones, and visceral cartilages</td>
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Table 1: **Main derivatives of neural crest cells** (Adapted from Santagati 2002)
Figure 1.3: Illustration of the cadherin super-family: (A) The cadherin super-family is divided into five major subgroups. The classical cadherins consist of type I and II cadherins, all of which contain five extracellular (EC) domains in their extracellular region, a single pass through the plasma membrane, and a cytoplasmic domain that interacts with p120-catenin and β-catenin. Classical cadherins have an adhesive sequence within the EC1 domain (HAV for type I, or QAV for type II) to perform the homophilic interactions described in (B). Desmosomal cadherins also have 5 extracellular domains and a single pass through the plasma membrane, and their cytoplasmic domain interacts with plakoglobin and desmoplakin. Atypical cadherins contain 5 extracellular domains, are anchored to the plasma membrane via a single pass or GPI anchor, and contain little or no cytoplasmic domain. Protocadherins can contain up to seven EC domains, a single pass through the membrane, and a cytoplasmic domain that can interact with kinases to promote intracellular signaling events. Cadherin-like (or related) proteins, including the Flamingo and FAT subgroup of cadherins, can have a large number of EC domains, several passes through the plasma membrane, and a cytoplasmic tail. (B) Classical cadherins (as well as a few other cadherin subfamilies) can form homophilic interactions with the extracellular domain of other cadherin molecules on the surface of neighboring cells. Classical cadherins can also form heterotypic interactions, but these are less common (and potentially less stable). The cytoplasmic binding partners of classical cadherins are also known to regulate the stability of classical cadherins. A common marker for an “active” cadherin that is forming cell-cell adhesions is co-localization with β-catenin molecule at the plasma membrane. This figure was adapted from (Taneyhill, 2008).
Figure 1.4: Domain organization of a typical ADAM molecule. The extracellular region of ADAM proteins generally contains five domains. The pro-domain covers the metalloprotease (MP) domain and renders it inactive. The pro domain is cleaved off by Furins within the endoplasmic reticulum (Loechel 1999), before the active ADAM is expressed at the cell surface. The metalloprotease domain (MP) is stabilized by zinc ions, and chelation of these ions by molecules such as EDTA or other protease inhibitors renders the ADAM inactive. A glutamic acid (E) to alanine (A) substitution at residue #341 within the MP domain produces a proteolytic inactive form of the ADAM that may also act as a “dominant negative” form by sequestering substrate from the wild type molecule. The disintegrin (DI) domain is known to interact with integrins, and support integrin mediated cell adhesion (Takahashi 2001; Bridges 2002; Eto 2002). The DI domain is thought to act together with the cystine-rich (CR) domain to bind to substrate. The EGF domain contains EGF repeats. The function of this domain has yet to be resolved, and not all ADAMs contain this region. Most ADAM family members also contain a single pass through the plasma membrane (TM), and a cytoplasmic domain (Cyto) that may play a role in intracellular signaling. This figure has not been published.
**Figure 1.5: Inhibition of ADAM activity can block CNC migration.** Overexpression of a dominant negative E/A mutant of ADAM13 in the CNC inhibits migration. Image is an in situ hybridization for CNC marker xTwist, showing the E/A expressing embryos have inhibited migration when compared to the positive control. *(Images are from Alfantari 2001)*
CHAPTER II

CADHERIN-11 IS PROCESSED BY ADAM METALLOPROTEASES IN VIVO DURING CRANIAL NEURAL CRESCENT MIGRATION

Abstract

The studies described in this chapter will focus on the role of cadherin-11 during the process of CNC migration. Here we will show that similar to N-cadherin, cadherin-11 is processed by ADAM metalloproteases to promote the migration of these cells. Since full-length cadherin-11 has a number of cytoplasmic binding partners, some of the studies described in this chapter will examine how cadherin-11 cleavage may affect these interactions. These studies indicate that cleavage of cadherin-11 has a different biochemical outcome than what has been previously shown for N-cadherin. The implications of these distinct biochemical behaviors, and their specific developmental roles in neural crest development will be discussed in chapter VI.

Section I: Introduction

The CNC expresses both N-cadherin and cadherin-11 prior to migration. Coinciding with NC delamination from the neural tube, N-cadherin is extracellularly processed and removed from the NC cell surfaces. This event sets off a signaling cascade, which appears to be the final step in the EMT of the NC. As this transition occurs the NC begins to migrate. Cadherin-11 expression remains in the CNC throughout this process.

What is the role of cadherin-11 during CNC migration? In order to speculate about this activity, let us first reflect on how classical cadherins generally operate during
cell migration. Besides the signaling potential of these molecules, cadherins use their cell-cell adhesive properties in combination with their structural function as the cell migrates through other cells. Here, a cadherin will form a homophilic bond with another cadherin molecule (of the same kind) on the surface of a neighboring cell. The cadherin-cadherin bond persists until it reaches the trailing edge of the cell where it is broken by either mechanical constraint on the bond, or by an extracellular processing event on one of the cadherin molecules. In the migrating cell, the cadherin can then be internalized and targeted for degradation, or “treadmilled” to the leading edge of the cell where it is recycled for use (Figure 2.1 and reviewed in Jones 2006). Additionally, classical cadherins interact with β-catenin, which interacts with α-catenin and anchors the complex to the actin cytoskeleton. This structural link likely helps in the modification of the cell body as the cadherin molecule travels from the leading edge to the trailing edge of the cell.

How may this system work in the context of the embryo during CNC migration? In this instance CNC cells are presented with various environments depending on their orientation in the CNC (reviewed in Newgreen 1993). Surrounding the CNC is a complicated network of extracellular matrix molecules. This ECM separates the CNC from the surrounding tissues, and is secreted, processed, and rearranged by CNC cells as they travel on it (reviewed in Macdonald 2001). Migration in this environment is highly dependent on the use of integrins, which mediate the cell to ECM interactions. However, the environment is different within the CNC. Here, there is far less ECM, and much more cell-cell contact. It is within this environment that cadherin-11 probably plays a role in CNC migration. If this is true, cadherin-11 is likely to be more important for the first
phase of migration, where the CNC migrates as a sheet, and in branch migration where CNC cells maintain contact with other CNC cells (phases of CNC migration discussed in Alfandari 2003). However it is less likely that cadherin-11 is important during single CNC cell migration, where the cell is either migrating through ECM, or through cells that do not express cadherin-11.

The work presented in this chapter will look at how cadherin-11 cell surface levels are regulated during CNC migration. Here we show that cadherin-11 is processed during CNC migration by ADAM family members, and that this cleavage event is important for this process. It is possible that cadherin-11 cleavage helps keep the intensity of cell-cell adhesion in check during migration, and may also play a role in the breaking of cadherin-cadherin bonds at the trailing edge of cells within the CNC. Additionally, this chapter will show that cleavage of cadherin-11 does not affect the interaction with cytoplasmic binding partners p120-cateinin or β-catenin. The maintenance of the cadherin-11/β-cateinin interaction suggests that the structural function of cadherin-11 is retained after the “adhesive domain” is removed.

Much of this research has been published in my first author paper entitled “Extracellular cleavage of cadherin-11 by ADAM metalloproteases is essential for Xenopus cranial neural crest migration” in the journal Molecular Biology of the Cell (McCusker 2009). The studies in this chapter that have not yet been published are depicted in figures C of 2.5, C of 2.7, 2.12, B of 2.13, 2.15, 2.16, and 2.17. The experiments performed by other members of the Alfandari lab are depicted in figures 2.9, A from 2.10, and 2.11.
Section II: Endogenous cadherin-11 is cleaved in the head of the Xenopus embryos.

Since a secreted form of cadherin-11 had been observed in tissue culture, we predicted that migrating CNC cells could regulate cadherin-11 surface levels by an extracellular cleavage event (Kawaguchi 1999). To investigate this possibility, we produced a monoclonal antibody directed against the cytoplasmic domain of cadherin-11 and studied the expression and changes in molecular weight of cadherin-11 during CNC migration in Xenopus laevis embryos. Using this cadherin-11 antibody, we have shown that cadherin-11 is predominantly expressed in the embryo head between stages 17 and 26 (Figure 2.1).

The western blot analysis in Figure 2.3A depicts the expression of endogenous cadherin-11 at the beginning (stage 19) and during CNC migration (stages 21 and 23). In these embryos, the amount of total cadherin-11 increases as the CNC migrates. Furthermore, we can detect the presence of a cadherin-11 cleavage product of approximately 75-80 kDa that also increases during migration. This cleavage product corresponds in size to the cytoplasmic, transmembrane, and a portion of the extracellular domain, and retains at least one glycosylation site since it can be purified on concanavalin A-beads (ConA). Using the primary amino acid sequence and the putative N-glycosylation sites, we estimate the cleavage site to be between the EC3 and EC4 domain of the cadherin-11 protein (Figure 2.3B). The timing and sizes of the cadherin-11 fragments suggest that the homophilic binding site in the first cadherin domain (EC1) is removed during CNC migration thus decreasing cell-cell interactions.
Section III: Who is cleaving cadherin-11 \textit{in vivo}?

**ADAM9 and ADAM13 cleave cadherin-11 in Cos-7 cells**

The next objective was to find which protease is responsible for extracellularly processing cadherin-11 during CNC migration. Since ADAM10 was previously shown to cleave members of the cadherin superfamily, we first asked whether an ADAM could also process cadherin-11. There are a number of ADAM metalloproteases expressed in the CNC during migration (Figure 2.4). ADAM9, ADAM13, and ADAM19 are the most similar by domain organization and sequence homology, and are all members of the meltrin subfamily of ADAMs. ADAM11 is also expressed in the CNC, but is proteolytically inactive and serves as a negative control in our studies. ADAM10 is one of the best-characterized ADAMs, and can process a number of ligands involved in different signaling pathways, such as Notch, EGFs, E-cadherin and N-cadherin (described in Chapter I).

In order to determine whether these ADAMs may cleave cadherin-11 \textit{in vivo}, protein cleavage was assayed in Cos-7 cells overexpressing cadherin-11 with each of the above listed ADAMs. Western blot analysis on the cell extract revealed the presence of the 80 kDa cadherin-11 fragment in ADAM9 and ADAM13 cotransfected cells, but not with their proteolytic inactive E/A mutants (Figure 2.5). This fragment was not present in the ADAM10, ADAM11 and ADAM19 cotransfections (Figure 2.5).
Cadherin-11 binds to ADAM family members in tissue culture and *in vivo*

To further investigate whether meltrins are directly interacting with cadherin-11 we tested the ability of cadherin-11 and ADAM13 to co-precipitate. Indeed, ADAM13 co-precipitates with cadherin-11 in extracts from transfected Cos-7 cells (Figure 2.6A), as well as from embryos overexpressing these two proteins (Figure 2.6B). Interestingly, while overexpressed cadherin-11 binds to both the pro and mature forms of ADAM13 (2.5B lanes 2 and 3), endogenous cadherin-11 only co-precipitates the overexpressed mature ADAM13 (2.5B lane 4), suggesting that cadherin-11 preferentially binds with this form in embryos. Additionally, other ADAM family members, namely ADAM9 and ADAM10, can co-precipitate with cadherin-11 from tissue culture extract. These results, and others, are displayed in Appendix III.

Cadherin-11 can also co-precipitate with ADAM13 from tissue culture extract, although it is much more difficult to detect (Figure 2.6C). There are multiple reasons why this co-precipitation is subtler than the reverse co-immunoprecipitation results. It is possible that the antibody used to pull-down ADAM13 interferes with the ability to interact with cadherin-11. Additionally, the antibodies used when detecting ADAM13 that pulls down with cadherin-11 may be much more sensitive than the antibody used to detect cadherin-11 in the reverse co-immunoprecipitation. Regardless, cadherin-11 does clearly co-precipitate with ADAM13.

To determine when the interaction between endogenous ADAM13 and cadherin-11 occurs during early development we performed another co-immunoprecipitation experiment using wildtype embryos at four different stages of development (Figure 2.7).
We used blastula (Stage 7) embryos as negative control since neither ADAM13 nor cadherin-11 is expressed at that stage. We also used gastrula stage embryos (St 10.5) because both proteins are expressed but the CNC has not yet been induced. Finally we used neurula stage embryos (Stage 19) where the CNC have just begun migration and tailbud stage (Stage 23) when the CNC migration is nearly completed. While this experiment was performed by extracting whole embryos, we have shown that the majority of the cadherin-11 is expressed in the head of the embryo (Figure 2.2), and previous work has shown that cadherin-11 expression is highly localized to the CNC within the head region (Hadeball 1998). The results show that ADAM13 co-precipitates with cadherin-11 during the migration of CNC cells, but not at blastula or gastrula stages. In addition, only the mature form of endogenous ADAM13 (M) is bound to endogenous cadherin-11 (Figure 2.7, top panel), while both the pro and mature forms of ADAM13 are clearly detectable in the embryo extract (Figure 2.7, bottom panel). As expected, the levels of the 80 kDa cadherin-11 cleavage fragment increase as the CNC is migrating (Figure 2.7, middle panel). We find that cadherin-11 cleavage is also occurring at gastrula stage, while no detectable level of ADAM13 is associated, suggesting that another ADAM, possibly ADAM9, may also cleave cadherin-11 during gastrulation in vivo.

Section IV: Extracellular processing of cadherin-11 is important for CNC migration.

**Meltrins can rescue CNC migration in embryos overexpressing cadherin-11**

Previous work has shown that overexpression of cadherin-11 results in the inhibition of CNC migration in *Xenopus* embryos (Borchers 2001). Our results indicate
that cadherin-11 levels are regulated by proteolytic cleavage during CNC migration. Our hypothesis is that endogenous ADAM13 cannot regain an endogenous level of cell-cell adhesion when cadherin-11 is overexpressed. To investigate this possibility we tested whether the overexpression of ADAM13 could rescue CNC migration in embryos overexpressing cadherin-11. In 2-cell stage embryos one blastomere was injected with synthetic mRNA for cadherin-11 alone, or in combination with ADAM13 (Figure 2.8A and B). Synthetic mRNA for β-galactosidase was also included to identify the injected side of the embryos. The non-injected sides of these embryos serve as a stage-match control for embryo development and were used in each case to quantify the extent of normal migration. At stage 25 embryos were fixed and processed for whole mount in situ hybridization using a mix of RNA probes for Sox10 and Twist to label CNC. Sox10 was used in combination with Twist because previous work had shown that Twist could be down regulated in CNC overexpressing cadherin-11 (Borchers 2001). Our results confirm that overexpression of cadherin-11 severely disrupted the CNC migration on the injected side (Figure 2.8A). In contrast, expression of both cadherin-11 and ADAM13 was able to rescue CNC migration in a large fraction of the injected embryos (Figure 2.8A and B).

We hypothesized that cadherin-11 overexpression blocks CNC migration because it overwhelms the activity of the endogenous meltrin proteases, and this causes an increase in the amount of cell-cell adhesion that inhibits CNC cell movement. With this in mind, we suspected that ADAM13 co-overexpression rescues CNC migration because it processes the excess cadherin-11 resulting in a decrease in cell-cell adhesion. In line with this hypothesis, we see a concomitant increase in the cleavage of overexpressed
cadherin-11 when the expression of mature overexpressed ADAM13 increases (Figure 2.8C).

Since ADAM9 and ADAM19 are also expressed in CNC during migration, we analyzed their ability to rescue the migration of CNC cells overexpressing cadherin-11. We performed a targeted injection at the 16-cell stage with synthetic mRNA for RFP with cadherin-11 and either ADAM9, 13, 19, or 13-E/A, and analyzed the in vivo migration of the fluorescent CNC cells (Figure 2.9). In this instance, ADAM9 rescued CNC migration at a similar rate as ADAM13. However, co-expression of ADAM19 and ADAM13-E/A failed to rescue the migration of cells overexpressing cadherin-11. Note that ADAM9 and ADAM13, but not ADAM19, cleaves cadherin-11 in Cos cells (Figure 2.5). In combination with the cleavage experiments performed in vitro, these rescue studies indicate select members of the meltrin family process that cadherin-11 in vivo.

**Inhibition of cadherin-11 cleavage also inhibits CNC migration in vivo**

We have previously shown using a dominant negative approach that ADAM13 is critical for CNC migration in vivo (Alfandari 2001). To resolve the importance of cadherin-11 cleavage by ADAMs during CNC migration, we further investigated the effect of blocking ADAM function on this process. We first used a hydroxamate-based inhibitor Marimastat that inhibits a wide range of metalloprotease function including ADAMs (Orth 2004). Cos-7 cells transfected with ADAM13 and cadherin-11 were treated with various concentrations of Marimastat. Western blot analysis shows that Marimastat inhibits ADAM13 cleavage of cadherin-11 in a dose dependant manner (Figure 2.10A).
We then examined the effect Marimastat treatment has on CNC migration *in vivo* by injecting the inhibitor in the pathway of the migrating cells. At stage 22 the CNC cells of the embryo injected with the carrier solution containing 10% DMSO (10 nl) migrated in the hyoid, branchial, and mandibular segments (Figure 2.10B). In contrast, injection of the inhibitor blocked CNC migration *in vivo* in a similar but more robust fashion as the ADAM13 DN (Figure 2.10B) (Alfandari 2001). These results suggest that at least one metalloprotease inhibited by Marimastat, possibly ADAM13, is essential for releasing cadherin mediated cell-cell adhesion during CNC migration.

In order to further investigate this hypothesis, we knocked down individual ADAM metalloproteases via morpholino injection (Figure 2.11A). The embryos used in this study were injected with morpholino oligonucleotides (MO) to ADAM9, ADAM13 and ADAM19 and then raised to tailbud stage (Stage 24) before the analysis. The total proteins were then extracted and the glycoproteins purified by affinity to ConA. Western blot using antibodies to each ADAM, cadherin-11, PACSIN2, and the β1-integrin subunit were performed. The results show that MOs directed against ADAM9, ADAM13, and ADAM19 decreased the translation of their corresponding proteins. Western blot analysis also revealed that the level of un-cleaved cadherin-11 at 120 kDa is increased by about twofold in embryos with each of the ADAM MO, suggesting that ADAM9, 13 and 19 may all participate in the cleavage of cadherin-11 *in vivo*. As a control we tested cadherin-11 mRNA level using real-time quantitative PCR and found no increase in expression of the gene (Figure 2.12), confirming that the increase in cadherin-11 protein level is due to “stabilization” of the protein and not increased gene expression. In support of this hypothesis, injection of an ADAM9, ADAM13, and ADAM19 MO cocktail
significantly decreases the amount of cleaved cadherin-11 at stage 21 (Figure 2.11B). Additionally, injection of the MO cocktail (3MO) also blocks CNC migration in vivo (Figure 2.11C). The effect of the ADAM19 MO on cadherin-11 protein can be explained by the observation that reduction of ADAM19 protein also reduce the ADAM13 protein by over 60% (Fig.2.10A and unpublished information).

To further test whether cleavage of cadherin-11 was essential for CNC migration we attempted to make a non-cleavable form of cadherin-11 to see whether it was capable of rescuing cadherin-11 depleted CNC. If cadherin-11 cleavage were essential for migration, we would expect that a non-cleavable mutant could not rescue this phenotype. However, making a non-cleavable form of cadherin-11 has proven to be very challenging. Further description of these attempts is provided in Appendix IV.

Section V: Cleavage of cadherin-11 does not affect the ability of the cytoplasmic tail to bind β-catenin or p120-catenin.

Cleaved cadherin-11 maintains its interaction with β-catenin

Cadherin-11, like many other classical cadherin proteins, can bind to β-catenin via the cytoplasmic domain. Cleavage of N-cadherin by ADAM10 decreases the ability to bind β-catenin, increasing the cytoplasmic pools, and resulting in the stimulation of Wnt downstream markers c-myc, cyclin-D1, and c-jun in tissue culture (Reiss 2005). Furthermore, overexpression of cadherin-11 in Xenopus decreased the expression of Twist, a CNC marker that is also downstream of canonical Wnt signaling. This effect is caused by cadherin-11 sequestering β-catenin at the cell surface, since co-overexpression of β-catenin rescues Twist expression (Borchers 2001).
In light of these findings, we considered two possible ways ADAM13 processing of cadherin-11 could affect β-catenin. Either cleavage of cadherin-11 destabilizes the interaction with β-catenin, increasing the cytoplasmic pool and possibly promoting nuclear signaling, or ADAM13 cleavage of cadherin-11 only affects the adhesive properties and not the ability to bind β-catenin.

In order to test how cadherin-11 cleavage effects the interaction with β-catenin we overexpressed cadherin-11 in embryos either alone or with ADAM13. Cadherin-11 was then immunoprecipitated and the association with β-catenin was tested by western blot (Figure 2.13A). Interestingly, the level of associated β-catenin was not detectably affected by the co-expression of ADAM13 suggesting that the 80 kDa fragment is still capable of binding to β-catenin, and that cadherin-11 cleavage does not directly affect the cytoplasmic pool of β-catenin.

In parallel with this binding assay, we sought to see whether the amount of β-catenin interacting with endogenous cadherin-11 increased in ADAM knockdown embryos. Here embryos were injected with morpholinos directed against ADAM9, ADAM13, and ADAM19, and were extracted at stage 19 to perform immunoprecipitation for cadherin-11. Western blot analysis probing for β-catenin shows that there is no detectable difference in the amount of β-catenin that co-precipitates with cadherin-11 in wt or morpholino injected embryos (2.13B).

However, the amount of cadherin-11 cleavage fragment is only a fraction of the total amount of cadherin-11 expressed in cells or in embryos. Therefore it is possible that cleavage of cadherin-11 does release β-catenin from the cytoplasmic domain, yet the amount of β-catenin released is so minimal the difference cannot be detected. To resolve
this issue we performed additional co-immunoprecipitation in Cos-7 cells that had been transfected with wt or an extracellularly truncated form of cadherin-11, which was made to mimic the c-terminal fragment after proteolysis (ΔEC1-3). Cross co-precipitations were performed to determine whether ΔEC1-3 can associate with β-catenin, and *vice versa*. Indeed ΔEC1-3 and β-catenin do co-precipitate with each other; further indicating that cleaved cadherin-11 maintains the interaction with β-catenin (2.13C).

To further test whether cadherin-11 cleavage affects the interaction with β-catenin real-time quantitative PCR analysis was performed, analyzing the expression of canonical Wnt target genes xTwist, cyclin-D1, and c-myc. This study was done on cDNA from stage 21 embryos injected with the same mRNA combination as described in figure 2.13A. As a positive control embryos were incubated in a LiCl solution that dorsalizes embryos by inhibiting GSK3, the kinase responsible for initiating the degradation process of β-catenin. UV irradiation of embryos is known to ventralize *Xenopus* embryos, and serves as a negative control. We found that co-expression of ADAM13 with cadherin-11 did not stimulate the expression of any of the markers (Figure 2.14).

Since canonical Wnt signaling can also stimulate cell proliferation, we tested whether phosphorylated histone-H3 levels were stimulated in mRNA-injected embryos. No change in p-HH3 levels was detected by western blot analysis on embryo extracts or by whole mount immunostaining (data not shown). In combination with the studies described above, these results strongly indicate that cadherin-11 cleavage does not inhibit the interaction with β-catenin.
Cadherin-11 cleavage does not prohibit its interaction with p120-catenin

P120-catenin is another cytoplasmic signaling molecule that also interacts with the cytoplasmic domain of cadherin-11 (Yanagisawa 2006) P120 activity is involved in the dynamic cytoskeletal rearrangements that occur during cell migration (Noren 2000). Additionally, p120 expression is strongly expressed in the CNC, and *Xenopus* embryos knocked-down for this protein have less craniofacial cartilage and a smaller craniofacial skeleton (Ciesiolka 2004).

Due to the involvement of p120 in cell migration, the formation of facial structures, and ability to bind cadherin-11, we hypothesized that cadherin-11 cleavage may affect the interaction with p120-catenin and promote signaling through this molecule. In a similar manner to the previously described studies with β-catenin and cadherin-11, binding experiments between cadherin-11 and p120-catenin were carried out. First, extract from cells transfected with cadherin-11, p120-mt, and ADAM9 or ADAM13 were immunoprecipitated for cadherin-11. In this instance, the amount of p120 that pulls down with cadherin-11 is not detectably different in the presence of ADAM9 or ADAM13 (Figure 2.15A). Additionally, a co-immunoprecipitation experiment was performed on cells transfected with the extracellularly truncated form of cadherin-11 and p120-catenin. Here, the truncated form of cadherin-11 does coimmunoprecipitate with p120-catenin, indicating that cadherin-11 cleavage does not prevent this interaction (Figure 2.15B).
Section VI: Knock down of cadherin-11 blocks CNC migration *in vivo*, but not *ex vivo*.

Others and we have shown that cadherin-11 plays an important role in the migration of the CNC. Overexpression of this adhesion molecule blocks CNC migration *in vivo* (Borchers 2001 and Figure 2.8A) and *ex vivo* (Figure 2.17B). Depletion of cadherin-11, through the use of morpholinos, also blocks CNC migration *in vivo* (Figure 2.16 and Kashef 2009). These phenotypes indicate that a defined level of cadherin-11 is expressed on the surface of migrating CNC cells, and if the balance is shifted in either direction CNC migration is inhibited.

However, we have found that cadherin-11 depleted CNCs do migrate when dissected from the embryo and placed on a 2-dimensional substrate coated with fibronectin (Figure 2.17C). This result is contradictory to what has been published recently by Kashef and colleagues, who showed that CNC explants depleted of cadherin-11 do not migrate *ex vivo*. However CNC explants are extremely sensitive *ex vivo*, and the discrepancies between my study and that published in Kashef 2009 may be attributed to slight differences in how this assay was performed. For example my studies were performed by observing CNC migration on Fn-coated plastic dishes, while Kashef performed their analysis on Fn-coated glass slides. While this seems like a minor detail, our lab has observed that CNC explants exhibit more robust migration on plastic than on glass.

It is difficult to understand why the CNC requires such tight regulation of cadherin-11 levels *in vivo*, but less so in an *ex vivo* environment. I hypothesize that cadherin-11 depleted CNCs are capable of migrating, but perhaps *in vivo* they cannot
overcome the mechanical constraints imposed by the surrounding tissues. For example, a substantial amount of extracellular matrix completely surrounds the CNC \textit{in vivo}. This matrix supports integrin mediated cell migration, but is also processed and rearranged by the CNC cells as they migrate upon it. In contrast, our experimental setting supplies the CNC tissue with a single ECM protein, fibronectin, on only one side of the explant. In this \textit{ex vivo} situation, fibronectin simply acts a substrate in which to migrate upon instead of a complicated 3-dimensional structure to rearrange and eventually migrate through. Additionally, CNC cells migrating as a single cell through head mesenchyme need to migrate throughout tissues that maintain cell-cell interactions. An \textit{ex vivo} environment does not have these obstacles. It is probable that cadherin-11 depletion does compromise CNC migration. Therefore, these cells fail to migrate \textit{in vivo} when they are presented with the difficult task of migrating through a network of cell-ECM and cell-cell interactions. However, CNC cells knocked down for cadherin-11 can, and do, migrate in the simplified \textit{ex vivo} environment.

Section VII: Chapter II Discussion

Cadherin-11 is a cell-cell adhesion molecule that is expressed in the CNC throughout migration. We hypothesized that the amount of cadherin-11 expressed at the cell surface is tightly regulated during CNC migration to control the amount of cell-cell adhesion.

In this chapter we show that endogenous cadherin-11 is extracellularly processed \textit{in vivo} during CNC migration. Based on the size of the cleavage fragment on SDS-
PAGE, we predict that cleavage occurs between the EC3 and EC4 domains in the extracellular region of cadherin-11. We pursued ADAM family members as candidates for this activity because of the established interactions between the ADAM and cadherin families in addition to the expression of multiple ADAM family members in the CNC during migration. Protein cleavage assays run in tissue culture showed that both ADAM9 and ADAM13, two closely related ADAMs were capable of producing the 80 kDa cadherin-11 cleavage fragment. On the other hand, ADAM10, which had been previously shown to process both N-cadherin and E-cadherin does not process cadherin-11. Interestingly ADAM19, another ADAM family member that is closely related to both ADAM9 and ADAM13, does not cleave cadherin-11 in tissue culture. These results imply that not all closely related ADAMs can process the same substrates. This is an important distinction because it suggests that individual ADAM family members process specific substrates during embryogenesis.

In order to determine whether ADAMs are directly interacting with cadherin-11, a number of binding experiments were performed. These studies showed that ADAM13 co-precipitates with cadherin-11 when overexpressed in tissue culture and in embryos. Interestingly, only the mature form of ADAM13 co-precipitated with endogenous cadherin-11. This suggests that cadherin-11 may have a higher binding affinity for the mature form of this ADAM. Alternately, the interaction between endogenous cadherin-11 and ADAM13 may be regulated by their cellular localization. It is possible that cadherin-11 normally binds to ADAM13 when it is expressed at the plasma membrane as an active protease. The likely reason why both pro and mature forms of ADAM13 co-precipitate with cadherin-11 when these molecules are overexpressed is because much of the
overexpressed cadherin-11 localizes to the ER (data not shown) where it could interact with the pro-form of ADAM13.

A portion of the studies described in this chapter focused on resolving the importance of cadherin-11 cleavage in CNC migration. These studies indicate that the extracellular processing of cadherin-11 is important for CNC migration. First, overexpression of the ADAM9 and ADAM13 metalloproteases can rescue CNC migration in embryos overexpressing cadherin-11. Biochemical analysis of the embryos overexpressing cadherin-11 and ADAM13 showed an increase in cadherin-11 cleavage that corresponded with and increase in the expression of mature ADAM13, suggesting that this cleavage event was at least partially responsible for the rescue of these embryos. In support of this hypothesis, co-overexpression of the proteolytic inactive form of ADAM13 does not rescue CNC migration, indicating that the proteolytic activity of ADAM13 is essential for the rescue. In addition to these studies, we showed that inhibition of cadherin-11 cleavage through the use of a small molecule inhibitor or by the injection of morpholino oligonucleotides directed against ADAMs also inhibits CNC migration.

I propose that cadherin-11 cleavage promotes CNC migration by multiple mechanisms. First, processing of cadherin-11 likely regulates the level of cell-cell adhesion mediated by this molecule by removing the adhesive sequence. In addition, cadherin-11 cleavage also produces an extracellular cleavage fragment, which may also promote migration by acting as a competitive inhibitor for cadherin-11 binding, as well as potentially binding to a cell-surface receptor that transmits a pro-migratory signal. The
following two chapters will explore the potential biological activity of cadherin-11 extracellular cleavage fragments.

The possibility that extracellular processing affects the interaction between cadherin-11 and cytoplasmic binding partners was also explored. These studies show that the cytoplasmic tail of cleaved cadherin-11 maintains its interaction with both β-catenin and p120-catenin. The implications of this observation will be discussed in Chapter VI. We have not investigated whether cadherin-11 cleavage affects the ability to interact with cytoplasmic binding partner Trio.

The last section of this chapter provided an interesting twist on the role of cadherin-11 in CNC migration. Overexpression of cadherin-11 clearly inhibits CNC migration in vivo and ex vivo. On the other hand, depletion of cadherin-11 inhibits CNC migration only in the embryo. So, despite the potential for the cytoplasmic and extracellular regions to relay a pro-migratory signal, these signals are not absolutely essential for motility. This was a surprising result because it had been recently shown by our collaborators that the cytoplasmic tail of cadherin-11 promotes CNC migration by interacting with the GEF-Trio (Kashef 2009). Yet, it appears that the regulation of the intensity of cell-cell adhesion generated by cadherin-11 trumps the effect these pro-migratory signals may have in CNC migration. This observation also supports the hypothesis that cadherin-11 function is not essential for single cell migration throughout the embryo head since head mesenchyme cells do not express cadherin-11.
Section VIII: Materials and Methods

Eggs and Embryos

Eggs were obtained from *Xenopus laevis*, fertilized, and cultured as described previously (Alfandari 1997). Embryos were staged according to (Nieuwkoop and Faber 1967). UV irradiation and LiCl treatments were performed as described (Pickard 2004). For *ex vivo* migration studies, CNC explants were dissected at stage 15-17, and healed before placing in a fibronectin coated 96-well culture dish. For *in vivo* migration assays, synthetic mRNA (including mRNA encoding RFP-mt) was targeted into a CNC precursor cell (B1.1) at the 16-cell stage.

Cell Culture

Cos cells were cultured in RPMI media complemented with Pen/Strep, L-Glut, Sodium Pyruvate and FBS (10U/ml, 2 mM, 0.11 mg/ml, 10%; Hyclone, South Logan, UT, USA). Transfections were performed using Fugene 6 reagent (Roche, Basel, Switzerland) following the manufacture’s instructions.

DNA Constructs

The cloning of *Xenopus* ADAM9, 10, 13 and the E/A mutants have been previously described (Alfandari 2001; Smith 2002; Cai 1998). ADAM19 was cloned by homologous PCR using sequences from mouse, chick and *Xenopus* tropicalis. 5’ and 3’ ends were obtained by RACE PCR using the generacer kit (Invitrogen). All full-length ADAM were cloned into the pCS2 vector for expression. The ADAM9-E/A construct was produced using the Quick Change Mutagenesis Kit (Stratagene, La Jolla, CA, USA).
full-length cadherin-11 in pcDNA3 was a gift from Dr. Dorris Wedlich and was re-cloned into pCS2. The EC1-3 construct was made by introducing a myc- tag and stop codon between the EC3 and EC4 sequences of cadherin-11. The ΔEC1-3 construct was made by deletion using all around PCR with PFU. All constructs were sequenced and tested for expression using the appropriate antibodies in both Cos-7 cells and embryos. The p120-mt construct is in pCS2 and was a generous gift from Frans van Roy’s Lab.

**Morpholino oligonucleotides**

Morpholino oligonucleotides were directed against the 5’ UTR of ADAM9, ADAM13, and ADAM19 diluted in water at 5 mg/ml (Gene Tools LLC, Philomath, OR, USA). 10 ng of MO was injected into each embryo at the 1-cell stage, or 1 ng was injected at the 16-cell stage. The Morpholino sequences directed against the ADAM9, 13, and 19 are listed in Table 1. The morpholino oligonucleotides directed against cadherin-11 spans across the start codon and binds to the sequence 5’-acaactacaaaATGaagaaagac-3’.

The cadherin-11 Mo was a generous gift from D. Wedlich’s Lab, and was the same one used in (Kashef 2009).

xCadherin-11 Antibody Production and Screening.

Appendix II is dedicated to describing the production and screening of the cadherin-11 1B4 monoclonal antibody. In brief, a His-tagged fusion protein (pET 30 Vector, Novagen, San Diego, CA, USA) encoding 157 C-terminal amino acids of the cytoplasmic domain of cadherin-11 was purified using standard methods. 100-300 mg of the fusion protein was combined with Freund’s adjuvant and injected intraperitoneally into Balb/c
mice. Hybridoma fusion protocol was performed using standard methods (Harlow 1988). Hybridomas were screened by ELISA, Western Blot, Immunofluorescence, and Immunoprecipitation to test immunoreactivity to endogenous cadherin-11 and minimal cross reactivity to N- and C-cadherin. The monoclonal antibody (mAb) 1B4 showed a very low affinity for overexpressed N-cadherin and no detectable affinity for C-cadherin.

**Antibodies**

Rabbit 6615F affinity purified polyclonal antibody (pAb) to ADAM13 is used at a 0.1 mg/ml concentration in western blot (Alfandari 1997). The rabbit β-catenin pAb (abcam, Cambridge, MA, USA) was used at a 1:2000 dilution. Rabbit anti ADAM9 was described earlier (Cai 1998). Rabbit anti ADAM19 was produced against a fusion protein to the ADAM19 cytoplasmic domain and affinity purified prior to use. As loading controls antibody to the b1 integrin subunit (mAb 8C8) and PACSIN2 (mAb 3D8) were used (Gawantka, 1994, Cousin 2000). To perform western blot after immunoprecipitation, we biotinylated mAb 1B4 while bound to the antigen using NHS-LC Biotin (Pierce). 9E10 mAb and a-mouse-FITC (1:200) were used to detect EC1-3-mt.

**Microinjection Experiments**

Transcription reactions and injections were performed as previously described in (Cousin 2000). An injection volume of 5 nl was determined by capillary calibration of the injection needle.
**Whole mount in situ hybridization**

Whole mount in situ hybridization was performed as previously described (Harland 1991). Diogoxigenin-rUTP-labeled transcripts were synthesized *in vitro* from *Xenopus* Sox10 and Twist plasmids. Synthetic mRNA encoding β-galactosidase was also included in the microinjections of embryos that were analyzed *via* in situ hybridization. The x-gal reaction was performed as in (Smith 1991) to indicate the site of injection. Embryos that were expressing β-galactosidase in the posterior region were excluded from our statistical analysis. Images were recorded using a Nikon D50 camera on a Nikon SMZ1500 dissecting scope.

**Protein extraction and analysis**

For direct western blot analysis of transfected Cos-7 cells, each well of a 6-well plate was extracted with 200 μL of reducing Laemmli buffer, and 10% of the extract was applied to a SDS-PAGE gel. Immunoprecipitation were carried out exactly as described in (Alfandari 1995) using Protein-G beads (Roche) and 10 μg of mAb-1B4. Western blot protocol was followed as previously reported (Cousin 2000). Embryo extraction and analysis was performed similar to above but 1X MBS was used instead of 1X TBS in the extraction buffer and washes. 20 μl of extraction buffer was used per embryo. Total embryo number for each experiment is noted in the figure legends. Glycoproteins were purified from total protein extract using concanavalin-A agarose beads (Vector) as previously described (Alfandari 1997).
Quantitative PCR Analysis

RNA from stage 21 embryos was purified using guanidine isothiocyanate as described in (Alfandari 1995). Reverse transcription reactions were performed as in (Alfandari 1997). Sequences for xActin, Sox8, xTwist, cyclin-Dl, and c-myc probes are listed in the Table 2. Q-PCR reactions and data generation were performed using SYBR Green Premix Ex Taq (Takara, Kyoto, Japan) and the LightCycler system 1.5 (Roche). The 2 \(^{-\Delta\Delta CT}\) method was used for target quantification (Livak 2001), where Actin was used to normalize for total cDNA quantities.
**Figure 2.1: Cadherin molecules are recycled to the leading edge during cell migration.** Cadherins expressed on the surface of the migrating cell and the supporting cells form adhesive bonds (homophilic interactions). 1. At the trailing edge of the cell, cadherin molecules are endocytosed into the cell. These endosomes are either sent for degradation (2), or are shuttled to the leading edge (3) where the cadherin is recycled to the surface of the cell. The recycled cadherin can then perform homophilic interactions at the leading edge. *This figure was drawn for dissertation and has not been published.*
**Figure 2.2: Cadherin-11 is predominantly expressed in the embryo head during CNC migration.** 10 embryos were dissected into three parts, head (top panel), dorsal (second panel), and ventral (third panel) at 4 different embryological stages. Stage 17 corresponds with CNC induction, stage 21 is during early CNC migration, and stage 23 and 26 are two later stages of CNC migration. Tissues were extracted, glycoproteins purified using ConA agarose and western analysis for cadherin-11 and β-catenin (loading control) was performed. 10 whole embryos were also extracted and purified on ConA (bottom panel) to compare the amount of cadherin-11 in each tissue to the total amount of cadherin-11 at that embryological stage. *This figure was a supplementary figure in* (McCusker 2009).
Figure 2.3: Cadherin-11 is cleaved *in vivo* during cranial neural crest migration. (A) Wild type embryos were extracted at stage 19, stage 21, and stage 23 representative of the different phases of CNC migration and purified on ConA agarose (20 embryos/lane). Western blot analysis for Cad-11 shows an increase in full-length protein (120 kDa.) as migration proceeds, as well as the appearance of one 80 kDa cleavage product. (B) Schematic representation of full-length Cad-11. The EC1 domain contains a QAV homophilic binding motif consistent with type II Cadherins (Hadeball, 1998). The cleavage site (*) is determined by calculating the relative molecular mass of the C-terminal fragment taking into account the N-glycosylation sites. The cytoplasmic region of Cad-11 can bind to β-catenin (Kawaguchi, 1999). *This figure was published in (McCusker 2009).*
Figure 2.4: Multiple members of the ADAM family are expressed in the CNC during migration. (left) Schematic representation of the general RNA expression patterns of ADAM family members, ADAM9, ADAM10, ADAM11, ADAM13, and ADAM19. ADAM9 and ADAM10 are expressed in the CNC during migration, but are also expressed in many other anterior tissues. ADAM11, ADAM13, and ADAM19 all have expression patterns that are more restricted to the CNC. ADAM11 is not proteolytically active, but all other ADAMs listed here are active. (right) The general domain organization of each ADAM shows that all ADAMs listed, with the exception of ADAM10 that does not have an EGF repeat domain, have a Pro domain (Pro), a metalloprotease domain (MP), a disintegrin (DI), a cysteine rich (CR), an EGF repeat (EGF), transmembrane (T), and a cytoplasmic region (cyto). The catalytic sequence within the metalloprotease domain for each ADAM is labeled with an arrow. Mutation of the glutamic acid amino acid (E) to an alanine (A) produces a proteolytic inactive form of the ADAM. This figure has not been published—it was made for this dissertation. References for ADAM expression in Xenopus (the inspiration for this figure) are included in the text of this chapter.
Figure 2.5: ADAMs in the meltrin family can induce cadherin-11 cleavage. (A) Transfected Cos-7 cells over-expressing cadherin-11 with different ADAMs were extracted and cadherin-11 processing was visualized by western blot analysis. An 80 kDa cadherin-11 cleavage fragment is present in cells co-transfected with ADAM9 (lane 1) and ADAM13 (lane 5), but not with their proteolytic-inactive E/A mutants (lanes 2 and 6). Co-transfection of cadherin-11 with ADAM10, ADAM11, or ADAM19 does not stimulate Cad-11 cleavage. (B) Quantification of the % cadherin-11 cleavage, calculated by densitometric analysis, of the study described in A (n=9). (C) A shedding experiment with cadherin-11 in the presence of ADAM19 (lane 5) in tissue culture. While ADAM9 (lane 4), ADAM13 (lane 2), and ADAM19 (lane 5) are all meltrin family members; only ADAM9 and ADAM13 can cleave cadherin-11 in tissue culture. This figure was published as a supplementary figure in (McCusker 2009).
**Figure 2.6: ADAM13 binds to cadherin-11 in vitro and in vivo.** (A) Cos-7 overexpressing cadherin-11 alone (lane 1), with ADAM13 (lane 2), with ADAM13 E/A (lane 3), or ADAM13 alone (lane 4) were extracted and immunoprecipitated for cadherin-11. Western blot analysis for ADAM13 show that both pro (120 kDa.) and mature (100 kDa.) forms of ADAM13 coimmunoprecipitate with cadherin-11 (lanes 2 and 3). ADAM13 is not detected in IP from Cos-7 cells that have not been co-transfected with cadherin-11 (lane 4). (B) A similar binding experiment was performed on Stage 19 embryos overexpressing cadherin-11 alone (lane 1), cadherin-11 and ADAM13 (lane 2), cadherin-11 and ADAM13 E/A (lane 3), ADAM13 alone (lane 4), or nothing (lane 5) (10 embryos/lane). As was shown in Cos-7, both pro (120 kDa.) and mature (100 kDa.) forms of ADAM13 coimmunoprecipitate with overexpressed cadherin-11 (top panel, lanes 2 and 3). Only the mature form of overexpressed ADAM13 pulls down with endogenous cadherin-11 (lane 4). This membrane was stripped and re-probed for cadherin-11 (lower panel). The amount of full-length (120 kDa.), cleavage fragment 1 (80 kDa.), and cleavage fragment 2 (30 kDa.) all increase when cadherin-11 is overexpressed in embryos (lanes 1-3 vs. lanes 4-5). (C) Co-precipitation of cadherin-11 with ADAM13 from Cos-7 extract. Cos cells were transfected with ADAM13, ADAM13E/A, ADAM13 + cadherin-11, ADAM13E/A + cadherin-11, cadherin-11, or non-transfected, immunoprecipitated for ADAM13 (with 15F antibody), and western blot analysis was performed detecting cadherin-11. Light bands for full-length cadherin-11 are detected with ADAM13 WT (lane 3) and E/ mutant (lane 4), but not for cadherin-11 transfected alone (lane 5). *Part A and B are supplementary figures in (McCusker 2009). Part C has not been published.*
Figure 2.7: Binding of endogenous ADAM13 and Cadherin-11 occurs during CNC migration, and corresponds with Cadherin-11 cleavage. Wild-type embryos (50 embryos/stage) were extracted at stages 7, 10.5, 19, and 23. Embryo extracts were immunoprecipitated for Cad-11 and detected by Western blot for ADAM13 (A) and Cad-11 (B). (C) Glycoproteins from five embryos were purified using Con-A agarose, separated by SDS PAGE, and blotted using ADAM13 antibodies. Both pro- (P) and mature (M) forms are detected. This figure was published in (McCusker 2009).
Figure 2.8: ADAM13 rescues CNC migration in cells overexpressing cadherin-11. (A) In situ hybridization was performed using a combination of CNC markers xTwist and Sox10. Embryos were injected into 1 blastomere at the 2-cell stage with synthetic mRNA for either Cad-11 alone (top left), or in combination with ADAM13 (bottom left). The site of injection was determined by co-injecting mRNA for β-galactosidase. The right panels correspond to the non-injected side of each embryo. Disruption of CNC migration was determined by comparing the distance migrated on the injected side (left panels) vs. the non-injected side (right panels) of the same embryo. (B) Quantification of 3 independent rescue experiments. N=30 for GFP injected embryos, n=79 for Cad-11 injected embryos, and n=80 for Cad-11 and ADAM13 injected embryos. (C) Shedding assay on overexpressed cadherin-11 and ADAM13 in embryos. Here, embryos were injected at the 2-cell stage with mRNA encoding each of these proteins, and were extracted at later stages of development. Note that the expression curve of overexpressed proteins does not match their endogenous protein expression profile. At the earliest time point, the (120 kDa.) pro-form of ADAM13 is predominantly expressed. Correspondingly, there is very little cadherin-11 cleavage fragment at this stage. At later stages, stage 15 and stage 19, the mature (100 kDa) form of ADAM13 is predominantly expressed. Here, the amount of cadherin-11 cleavage increases dramatically. At the final time-point very little cadherin-11 and ADAM13 are over expressed, probably due to the degradation of the synthetic mRNA injected into these embryos. Figures A and B were published in (McCusker 2009); figure C is unpublished.
Figure 2.9: ADAM9 and ADAM13 can both rescue CNC migration in embryos overexpressing cadherin-11. (A) Visualization of CNC cell migration in vivo using RFP as a lineage tracer. One dorsal animal cell at the eight-cell stage was injected with mRNA encoding RFP and cadherin-11 to inhibit CNC migration. Synthetic RNA (0.25 ng) encoding ADAM9, ADAM13, ADAM19, and ADAM13-E/A were each co-injected with cadherin-11 to determine their ability to rescue migration. (B) Histograms representing the percentage of embryos in which the RFP-labeled cells migrated. Significance was determined by student’s t-test p < 0.05. The number of embryos analyzed for each injection set is as follows: RFP = 51, cadherin-11 = 61, cadherin-11 + ADAM9 = 67, cadherin-11 + ADAM13 = 59, cadherin-11 + ADAM13-E/A = 70, and cadherin-11 + ADAM19 = 56. This figure was published in (McCusker 2009).
Figure 2.10: The ADAM inhibitor, Marimastat, inhibits cadherin-11 cleavage, and blocks CNC migration in vivo. (A) Cos-7 cells over-expressing cadherin-11 and ADAM13 were treated with 0 μM, 1 μM, or 10 μM of Marimastat. cadherin-11 cleavage was determined by western blot analysis of Cad-11 (top panel). ADAM13 levels were also detected by western blot (lower panel). (M): mature-form ADAM13 (P): Pro-form of ADAM13. (B) Lateral view of tailbud stage embryos treated by whole mount in situ hybridization using slug to label neural crest cells. Embryos at stage 17 were injected under the epidermis with 10 nl of 10% DMSO (left) or the same amount of 1 mM Marimastat in 10% DMSO (right). At tailbud stage the CNC in control embryos have migrated in the Hyoid, Branchial, and Mandibular segments (100%, n=24). In contrast, 87.5% of the embryos injected with the Marimastat inhibitor have severe inhibition of CNC migration (n=24). The experiment described in (A) was performed by D. Alfandari. The experiment described in (B) was performed by H. Cousin. This figure was published in (McCusker 2009).
Figure 2.11: Morpholino knock down of ADAMs decreases cadherin-11 cleavage, and inhibits CNC migration in vivo. (A) Western blot analysis detecting ADAM and cadherin-11 expression in control non-injected embryos (NI) or injected with morpholinos directed against ADAM9 (MO9), ADAM13 (MO13), or ADAM19 (MO19). Each lane represents the glycoproteins from 5 embryos equivalent. PACSIN2 and the β1 integrin protein levels are unaffected by MO injection. In contrast, the un-cleaved cadherin-11 protein level is increased twofold with each MO. (B) ADAM9, 13, and 19 protein expression was knocked down using a cocktail of all three specific MO. Embryos were extracted at stage 15 (pre-migration) or at stage 21 (mid-migration), and were immunoprecipitated for cadherin-11. Cadherin-11 was then detected by western blot (20 embryos/lane). At stage 21, the cadherin-11 cleavage fragments is reduced in embryos injected with the 3MO. (C) In vivo migration analysis of embryos injected at the 16-cell stage with mRNA encoding GFP alone (0.5 ng/ injection), or combined with 1 ng of the 3MO cocktail (0.33 ng of each MO/ injection). The CNC in GFP mRNA injected embryos migrated in 21 out of 21 embryos. The CNC in GFP mRNA combined with 3MOs migrated in only 8 out of 33 embryos (24%). The experiment described in (A) was performed by D. Alfandari. This figure was published in (McCusker 2009).
<table>
<thead>
<tr>
<th>Target</th>
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<tr>
<td>xADAM13</td>
<td>GTCCCAGCCGACCCCTCCGACCCCAT</td>
</tr>
<tr>
<td>xADAM9</td>
<td>GGTGTCCTCCTCATCTACATCCACTG</td>
</tr>
<tr>
<td>xADAM19</td>
<td>GAGTCCTGTAGCTCCTCCATCCGA</td>
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Table 2: Sequences of morpholino oligonucleotides directed against ADAMs.
Figure 2.12 ADAM Mo does not increase cadherin-11 transcription. Embryos injected with control Morpholino or antisense to ADAM9, ADAM13, ADAM19 and a combination of ADAM13 and ADAM19 were grown until stage 22. Following mRNA purification and reverse transcription the cDNA were analyzed using primers specific to alpha tubulin (control) and cadherin-11. The relative expression calculated using the $2^{-\Delta\Delta CT}$ method and represented as a Log2 (fold change) is presented. The relative expression of cadherin-11 mRNA appears to decrease following each MO injection when compared to the control MO. Results are from 3 independent experiments. This shows that the increase of uncleaved cadherin-11 protein level is not due to increased transcription. This experiment was performed by D.Alfandari This figure was published in (McCusker 2009).
Figure 2.13: Cadherin-11 cleavage does not inhibit the ability to bind to β-catenin. (A and B) Co-immunoprecipitation experiments of endogenous β-catenin with cadherin-11. (A) Embryos were injected with synthetic mRNA (1 ng each mRNA/injection) for cadherin-11 (lane 1), cadherin-11 and ADAM13 (lane 2), cadherin-11 and ADAM13-E/A (lane 3), no mRNA (lane 4), ADAM13 (lane 5), or ADAM13-E/A (lane 6). Extracted embryos (10 embryos/lane) were immunoprecipitated for cadherin-11 and bound β-catenin was detected by western blot analysis. (B) Embryos were injected at the 1-cell stage with the following morpholinos; Control (C) lane 1, ADAM13 (13) lane 2, ADAM19 (19) lane 3, ADAM13 and ADAM19 (13/19) lane 4, ADAM9 + ADAM13 + ADAM19 (3MO) lane 5, and non-injected in lane 6. 20 Embryos per set were extracted at stage 19, and immunoprecipitated for cadherin-11 with the 1B4 antibody. Western blot analysis for β-catenin in immunoprecipitated sample (top panel) and whole embryo extract (lower panel) was performed. (C) (top) Co-precipitation of endogenous β-catenin with overexpressed full-length cadherin-11 or an extracellular truncated form (ΔEC1-3) cadherin-11 designed to mimic the C-terminal portion of cleaved cadherin-11. The truncated form of cadherin-11 is still capable of binding to β-catenin. (bottom) Co-immunoprecipitation of cadherin-11 with β-catenin. Figure A and the top panel of Figure C were published in (McCusker 2009); figure B and the lower panel of C have not yet been published.
Figure 2.14: Cleavage of cadherin-11 does not activate transcriptional markers downstream of β-catenin signaling. Q-PCR analysis on cDNA made from embryos injected with mRNA encoding cadherin-11, cadherin-11 + ADAM13 (A13), cadherin-11 +A13E/A, A13, GFP, or had been UV irradiated (Ventralized), or treated with 0.1M LiCl. (Dorsalized) xTwist, Cyclin-D1, c-myc, and Sox8 expression levels were normalized to Actin. Transcript quantities for each injection set are presented relative to GFP injected embryos (considered 100% expression). ADAM13 does not rescue expression of Twist, Cyclin-D1, c-myc, and Sox8 that are down regulated by cadherin-11 overexpression (Compare Cad-11 to Cad-11+A13). In fact by itself, ADAM13 appears to reduce Twist expression and Cad-11+A13 has lower Twist expression level than cadherin-11 alone. The rescue of Twist expression would be expected if ADAM13 induced the cleavage of cadherin-11 and release from β-catenin. The released β-catenin could translocate to the nucleus and activate gene transcription. This figure was published in (McCusker 2009).
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<th>Probe</th>
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<tr>
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</tr>
<tr>
<td>Cyclin D1 anti-sense</td>
<td>TCTGATGAAAGCGTTGTGTGCTGC</td>
</tr>
<tr>
<td>c-myc sense</td>
<td>ACTGAAACGACAGCATTTCACAGC</td>
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<tr>
<td>c-myc anti-sense</td>
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<tr>
<td>xTwist sense</td>
<td>GCCATGTCAGGAGCGTCAGAGGA</td>
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<tr>
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<td>GATTGGCGAACCTACACGGGTCCC</td>
</tr>
<tr>
<td>Sox8 sense</td>
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</tr>
<tr>
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</tr>
<tr>
<td>Actin anti-sense</td>
<td>CAAAGTCAAGAGCAACATAGCAG</td>
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</table>

Table 3: Q-PCR probe sequences
Figure 2.15: Cleavage of cadherin-11 does not detectably interfere with the ability to bind p120-catenin. (A) (top) Western blot analysis for p120 catenin that co-immunoprecipitates with cadherin-11 in transfected Cos cells. All cells were transfected with p120-catenin alone, or in the presence of cadherin-11 alone or with ADAM9 or ADAM13. (bottom) Western blot analysis detecting p120-catenin from cell extract. (B) (top) Co-precipitation of cadherin-11 (lane 1) and an extracellularly truncated form (ΔEC) (lane 2) with p120-catenin. (bottom) Western blot analysis detecting cadherin-11 and ΔEC in cell extract. These figures have not yet been published.
**Figure 2.16: Knockdown of cadherin-11 by morpholinos inhibits CNC migration in vivo.** In vivo migration assay of embryos injected at the 16-cell stage with RFP alone (left) or with morpholinos directed against cadherin-11 (right). Representative images were captured of stage 25 embryos. 100% of RFP embryos had migrated (n = 33), 22% of C11-Mo embryos had migrated (n = 23). Similar results were published in Kashef 2009. *This figure has not been published.*
Figure 2.17: Overexpression but not knockdown of cadherin-11 inhibits CNC migration ex vivo. (A-C) 7.5-hour time laps analysis of CNC explants removed from stage 17 embryos and placed on a 2-dimensional fibronectin substrate. Images shown are every half-hour from T=0 to 7.5 hours. (A) WT explant migration (representative of 4/5 movies, where one movie showed slightly inhibited migration). (B) Migration of explant overexpressing cadherin-11 (representative of 7/9 movies, where 2 movies had generally normal migration). (C) Migration of explant knocked down for cadherin-11 (representative of 9/10 movies, where one explant had inhibited migration). Each of these movies are included in the Supplementary Movie files as Movie 1 – Movie 3. These figures have not yet been published.
CHAPTER III

THE EXTRACELLULAR CLEAVAGE FRAGMENT OF CADHERIN-11 RETAINS BIOLOGICAL ACTIVITY IN VIVO

Abstract

The studies presented in the previous chapter generates an interesting question; does cadherin-11 cleavage promote CNC migration by simply clearing away the excess protein, or do the resulting cleavage fragments also play a role in cell migration? This question was partially explored in Chapter II where some studies focused on how extracellular cleavage may affect the interaction between cadherin-11 and cytoplasmic binding partners. In this chapter, we will further investigate this issue by determining whether the extracellular cleavage fragment of cadherin-11 (EC1-3) retains biological activity in vivo. It is our premise that EC1-3 acts as a competitive inhibitor for cadherin-11 binding, and that this activity can promote cell migration by further decreasing cell-cell adhesion. While many of the studies described here agree with this hypothesis, others also suggest that the EC1-3 story may be more complicated than this.

Section I: Introduction

How does cadherin-11 function during CNC migration? It is likely that the role of cadherin-11 within the CNC is dependent on the capability to act as a cell-cell adhesion molecule. However, it is possible that the shed extracellular fragment of cadherin-11 (called EC1-3) may also play a role during CNC migration since this fragment retains the cadherin “adhesive sequence”. With this in mind, it was speculated that EC1-3 binds to
full-length cadherin-11 on the surface of the CNC cells, and inhibits the ability to bind to other full-length cadherin-11 molecules (Figure 3.0). Here, EC1-3 could act as an inhibitor for cell-cell adhesion within the CNC since cadherin-11 is expressed only in this region of the tailbud stage embryo. EC1-3 may help maintain low levels of cell adhesion, which could be important for tissue “fluidity” during CNC migration. Some of the studies presented in this chapter support the hypothesis that EC1-3 inhibits cadherin-11 mediated cell-cell adhesion in vivo.

Additionally, we hypothesized that the interaction between cadherin-11 and EC1-3 affects other properties of cadherin-11, such as turnover rates or ability to support selective adhesion. In the first case, increasing cadherin-11 turnover within the CNC could also help regulate the intensity of cell-cell adhesion. On the other hand, the effect of EC1-3 on cadherin-11 mediated cell sorting may have a different role in the CNC. Here, it was speculated that cadherin-11-cadherin-11 interactions could also take part in segregating the CNC from the surrounding tissue at the onset of migration. Since cadherin-11 is only expressed in the CNC, the ability to perform selective adhesion may help delineate the boundary between the CNC and the surrounding tissue.

Some of the experiments described in this chapter have been published in the paper entitled “Extracellular cleavage of cadherin-11 by ADAM metalloproteases is essential for Xenopus cranial neural crest migration” in the journal Molecular Biology of the Cell (McCusker 2009). These experiments include those depicted in figures A and B from 3.1, 3.2, 3.6, 3.7, and 3.8. The other experiments in this chapter have not been published. I have personally performed all of the experiments described in this chapter.
Section II: The extracellular domain of cadherin-11 can bind to a number of cell surface molecules.

Thus far we have provided evidence that ADAM cleavage of cadherin-11 produces an 80 kDa fragment that remains in the plasma membrane. Consequently, it is likely that the extracellular fragment containing the homophilic binding site is released from the cell surface, and may interfere with cadherin-11 function in cell adhesion. To test this hypothesis, we made a construct designed to mimic the cadherin-11 extracellular cleavage fragment (EC1-3). In order to investigate whether the fragment can bind to full-length cadherin-11, we applied the media from EC1-3 transfected cells onto live Cos-7 cells expressing either cadherin-11 or a cadherin-11 mutant missing the homophilic binding site (ΔEC1-3). Immunofluorescence detected the EC1-3 fragment only in wells transfected with cadherin-11 but not the ΔEC1-3 construct, suggesting that the extracellular cleavage fragment can bind to full-length cadherin-11 (Figure 3.1A and B).

We also found that EC1-3 binds to tissue culture cells transfected with select ADAM family members. ADAM13 and ADAM19 transfected cells were capable of binding to EC1-3 (Figure 3.1F and G). On the other hand, ADAM9, ADAM10, and ADAM11 were not able to bind EC1-3 (Figure 3.1C-E). It was surprising to see that EC1-3 binds to cells expressing ADAM19, since this ADAM did not cleave cadherin-11 in tissue culture (Figure 2.5C). It was also intriguing to see that ADAM9 expressing cells did not bind to EC1-3, since ADAM9 does cleave cadherin-11 in tissue culture (Figure 2.5A). Additionally, ADAM9 co-precipitates with full-length cadherin-11 (Figure A3.3 from Appendix III). These results show that the EC1-3 binds to ADAM13 and 19 and suggest that ADAM9 may bind to a different region of cadherin-11.
EC1-3 rescues CNC migration in embryos overexpressing cadherin-11

Since EC1-3 can bind to cells expressing full-length cadherin-11 we predicted that this fragment might also help promote CNC cell migration in embryos overexpressing this molecule. To further explore this hypothesis we overexpressed both of these proteins with GFP to follow CNC migration in vivo. While overexpression of cadherin-11 alone expectedly blocks CNC migration, co-expression of EC1-3 rescues this phenotype (Figure 3.2A). This result suggests that the cleavage fragment may compete with full-length cadherin-11 molecules for cell-cell adhesion, and that CNC cells require a defined ratio of cleaved to uncleaved cadherin-11 for migration to proceed.

EC1-3 colocalizes with groups of CNC cells overexpressing cadherin-11, but not with “rescued” CNC cells in vivo

We speculated that EC1-3 binds to cells overexpressing cadherin-11 in vivo, and this interaction promotes cell migration by decreasing the intensity of cell-cell adhesion. Thus, we performed co-localization studies of cryosectioned “rescued” cadherin-11 embryos to see whether EC1-3 colocalized with cadherin-11 in vivo. Here, an embryo was considered “rescued” if cadherin-11 overexpressing cells, visualized by RFP expression, migrated within the CNC branches. Embryos were injected at the 32-cell stage in the a2 cell with EC1-3-mt mRNA, and the b2 cell with cadherin-11 and RFP mRNA. At stage 27, embryos that exhibited rescued CNC migration were sorted for sectioning. Embryo heads were cryosectioned transversally, and EC1-3-mt was detected.
by immunofluorescence using the myc 9E10 monoclonal antibody (FITC secondary). The image in Figure 3.3 shows a section with a cluster of cells overexpressing cadherin-11 located dorsally in the embryo. Closer inspection of the cluster of these cells (Figure 3.4) shows that EC1-3 does colocalize (yellow color) to cells located at the periphery of the non-migrating cadherin-11 cell cluster. Ventrally, a group of cadherin-11 overexpressing cells can also be seen in the head mesenchyme (marked with an arrow and *). These cells are “rescued” CNC cells that have probably migrated along the mandibular or hyoid arches of the CNC. Surprisingly, EC1-3 does not localize to these cadherin-11 expressing cells. Figure 3.5 depicts another example of EC1-3 not binding to rescued CNC cells in vivo.

Note also that EC1-3 does seem to bind to a number of structures inside the embryos head (cement gland, optic vesicle, deep head mesenchyme). While we do not yet know what molecules EC1-3 is interacting with in these structures, it is interesting to see how extensively this secreted molecule can penetrate through the head tissues.

Section IV: EC1-3 can rescue embryos knocked down for ADAM expression.

To test if the EC1-3 fragment could promote migration in CNC cells with reduced levels of ADAM protein, we chose two complementary approaches (Figure 3.6 and 3.7). The first one consists of injecting the MO at the one cell stage, producing ADAM depleted embryos, and then injecting either a lineage tracer alone or with the EC1-3 fragment at the 16-cell stage (Figure 3.6). The cell targeted at the 16-cell stage is defined as D1-2 and contributes to a large fraction of the CNC cell population (Moody 1987). In
that case we can compare using *in situ* hybridization the position of CNC cells that express the lineage tracer to the ones that do not. The second approach is to inject the MO with the lineage tracer at the 16-cell stage in D1-2 and follow the position of the injected cells in live embryos thus directly assessing the capacity of the injected cells to migrate (Figure 3.7).

The first approach shows that the EC1-3 cadherin-11 fragment can rescue CNC positioning in embryos with reduced level of ADAM13 and ADAM19 with the same efficiency as the injection of an ADAM13 mRNA lacking the MO target sequence (R13, Figure 3.6C). Using the second approach we find that combination of morpholino oligonucleotides to ADAM9, 13 and 19 can all decrease CNC migration *in vivo* (Figure 3.7). The most efficient inhibition was found using all three MOs (66% inhibition). All MO combinations containing ADAM13 MO were rescued by the expression of EC1-3 (Figure 3.7C). However, this molecule had no affect on CNC inhibited by the ADAM9 and ADAM19 MO combination. These results suggest that meltrin ADAMs may all participate in CNC migration or may compensate for each other *in vivo*. Because the cadherin-11 extracellular domain could only rescue migration in embryos that had decreased ADAM13 (MO13+9, MO13+19 or all 3MO), but not in embryos lacking both ADAM9 and 19, it is likely that ADAM13 is the principal ADAM responsible for cadherin-11 cleavage during CNC migration in *Xenopus*.

The studies described in figures 3.2, 3.6, and 3.7 show that EC1-3 can rescue CNC migration when co-expressed in cells that are either overexpressing full-length cadherin-11, or have knocked-down ADAM expression through the use of morpholinos. To determine whether this rescue is cell-autonomous or not, we created embryos whose
CNC was mosaic with ADAM depleted cells (3MO) and cells expressing the EC1-3 by performing dual injections into the a2 and b2 (CNC precursor) cells at the 32-cell stage (Figure 3.8A). In this instance, EC1-3 expression can still rescue migration in a separate population of CNC cells with knocked down ADAM expression (Figure 3.8B-C). Surprisingly, RFP expressed in the cells with knocked-down ADAM expression (to visualize CNC migration in vivo) remained stable throughout the later stages of CNC cell differentiation. This unexpected feature made it possible to analyze whether the rescued ADAM depleted cells took part in cranio-facial structures (Figure 3.8D-E). Indeed, embryos that were mosaic for EC1-3 and ADAM depleted cells had more ADAM depleted cells in the developing facial cartilages and muscles than embryos not expressing EC1-3 (Figure 3.8E). These results show that EC1-3 can rescue migration non-cell autonomously, and suggest that rescued ADAM depleted cells retain the molecular characteristics that are essential for differentiation into cranio-facial structures.

Section V: EC1-3 does not rescue migration in CNC cells knocked down for cadherin-11.

In Chapter II we showed that knockdown of cadherin-11 inhibits CNC migration in vivo. In this chapter, we have shown that the extracellular cleavage fragment of cadherin-11 retains biological activity in vivo. In an attempt to see whether EC1-3 activity in vivo requires the expression of the full-length molecule, we expressed the EC1-3 domain in CNC depleted of cadherin-11 (Figure 3.9). This study was conducted similar to the one described in the previous section, using cadherin-11 specific morpholinos. Here, EC1-3 does not rescue CNC migration in cells depleted of cadherin-
11. This result could mean that EC1-3 requires the full-length cadherin-11 molecule to promote CNC migration, as would be expected for a competitive inhibitor. However, it is important to note that the cytoplasmic tail of cadherin-11 interacts with a number of potential signaling molecules. Therefore, loss of cadherin-11 may affect a number of pathways, which could also have an inhibitory affect on CNC migration. It is possible that these multiple sources of inhibition are too much to overcome, even if EC1-3 does promote CNC cell migration via a cadherin-11 independent mechanism.

Section VI: EC1-3 does not seem to affect full-length cadherin-11 turnover, or cadherin-11 mediated cell sorting.

**EC1-3 does not seem to affect cadherin-11 turnover in tissue culture cells**

Dufour and colleagues had previously conducted a comparative analysis of two cadherin molecules expressed in the chick neural crest, N-cadherin and cadherin-7 (Dufour 1999). Again, N-cadherin is cleared from the neural crest at the onset of migration, and cadherin-7 is expressed in the neural crest throughout migration. They found that cells overexpressing N-cadherin tended to cluster together when grafted into a host embryo, while those expressing cadherin-7 would spread out into the tissue. They also showed that cells expressing N-cadherin migrated with a slower velocity than those expressing cadherin-7. Interestingly, they found that cadherin-7 had a greater turnover rate than N-cadherin. The authors suggested that the difference in the behavior of cells overexpressing each of these cadherins is in part due to divergent turnover rates.
The study from Dufour and colleagues inspired us to question whether EC1-3 may promote CNC migration by increasing the turnover rate of cadherin-11. To answer this question a shedding study was performed in Cos-7 cells overexpressing cadherin-11 in the presence of media from cells transfected with EC1-3-mt or GFP-mt. Figure 3.10A shows that EC1-3 is secreted into the media from these cells. EC1-3 media was incubated on cadherin-11 transfected cells from 0 to 4 hours, and cells were washed and extracted for analysis. The western blot in figure 3.10B shows no significant decrease in the amount of full length cadherin-11 expressed by these cells when incubated in the presence of EC1-3. However, this experiment does not rule out the possibility that the cadherin-11 expressing cells are producing new cadherin-11 protein when the surface levels decrease. Therefore, this experiment should be conducted in the presence of cycloheximide to block protein translation and rule out this possibility. Alternatively, cell surface labeling experiments could be used to only analyze the surface pool of cadherin-11.

To further explore the possibility that EC1-3 promotes cadherin-11 turnover, we conducted turnover studies on xtc cells expressing a cadherin-11-GFP fusion protein. Performing time-lapse microscopy on these cells in the presence of EC1-3 media or GFP media, we concluded that EC1-3 did not detectably decrease the amount of cadherin-11-GFP located at the cell surface (Movie 4: included in supplementary movie files). This observation, combined with the one described in figure 3.10, suggests that EC1-3 does not have a dramatic effect on cadherin-11 surface levels.
EC1-3 does not seem to affect cadherin-11 mediated cell sorting.

In an attempt to further understand how EC1-3 promotes CNC migration in cells overexpressing cadherin-11, we questioned whether EC1-3 could affect the ability of cadherin-11 to promote selective adhesion. Selective adhesion is a result of cadherins that preferentially form homophilic bonds with other like cadherin molecules. When two populations of cells expressing different cadherin molecules are mixed together, the cells expressing the same cadherin will stick together, and the initially heterogeneous population of cells will segregate into homogenous clusters. We know that EC1-3 binds to full-length cadherin-11, and thus could inhibit the ability to form an adhesive bond with other full-length molecules. Therefore, if EC1-3 inhibits the ability of cadherin-11 to perform homophilic interactions, it could decrease the amount of cell segregation seen by mixing animal cap cells expressing cadherin-11 with non-overexpressing animal cap cells (Figure 3.11). However this phenomenon does not occur. Instead, cells expressing cadherin-11 will segregate from wt cells despite EC1-3 co-expression (Figure 3.11). Yet, we can’t rule out that EC1-3 could have an effect on selective adhesion promoted by cells expressing endogenous levels of cadherin-11.

Section VII : Chapter III Discussion

While cadherins are mostly thought of as cell adhesion molecules, it is becoming clear that they are capable of performing other functions. In this chapter, we show that the extracellular cleavage fragment of cadherin-11 maintains biological activity in vivo. Here, EC1-3 can rescue CNC migration in embryos that have an excess of cadherin-11
whether it is achieved by overexpression or by the depletion of ADAMs (Figures 3.2 and 3.6-3.8). These results suggest that EC1-3 can bind to cadherin-11 and act as an inhibitor for homophilic binding with other full-length molecules. The fact that EC1-3 can bind to tissue culture cells expressing the full-length cadherin-11 but not to an extracellularly truncated form supports this hypothesis (Figure 3.1). Additionally, EC1-3 cannot rescue CNC migration in embryos with knocked down cadherin-11 expression. This suggests that EC1-3 requires the full-length cadherin-11 molecule to promote CNC migration. Alternately, given that the cytoplasmic tail of cadherin-11 can bind to multiple signaling molecules, it is also possible that the absence of the cadherin-11 signaling may inhibit CNC migration in ways that cannot be overcome by an EC1-3 mediated pro-migratory signal.

So, it is conceivable that cadherin-11 cleavage promotes CNC migration by tightly regulating the cell-surface levels of full-length cadherin-11, and through the generation of an extracellular cleavage fragment that decreases cell-cell adhesion. Yet, there are a number of details that can be observed within the experiments described in this chapter that suggest the story does not end here. The fact that EC1-3 can bind to select members of the ADAM family suggest that it may also be involved in the regulation of proteolytic activity mediated by these molecules (Figure 3.1). Additionally, while EC1-3 binds to tissue culture cells overexpressing cadherin-11 and colocalizes with clusters of CNC cells overexpressing cadherin-11 in vivo, EC1-3 does not colocalize to “rescued” cadherin-11 overexpressing CNC cells in vivo.

I propose two hypotheses to explain the observation that EC1-3 does not colocalize with “rescued” CNC cells (Figure 3.12). First, it is possible that EC1-3 binds
to full-length cadherin-11 on the leading-edge of the CNC. This interaction decreases the amount of cell-cell adhesion between the edge cells and their neighbors, and allows them to migrate away from the group. Once they have started migrating, they are no longer surrounded by cells expressing cadherin-11, and utilize other adhesion molecules (such as integrins) to move ventrally. CNC cells knocked down for cadherin-11 can migrate normally ex vivo (Figure 2.17C), as shown in chapter II, which suggests that cadherin-11 is not required for migration on ECM.

An alternative hypothesis is that EC1-3 promotes a pro-migratory signal in rescued CNC cells. In this instance, CNC cells overexpressing cadherin-11 could bind to EC1-3, which activates downstream signaling events that stimulate migration. Once the cells break away from the non-migrating cluster of cells and migrate ventrally, EC1-3 is internalized with the surface receptor. CNC cells that do not migrate cannot overcome the dramatic increase in cell adhesion that occurs when overexpressing cadherin-11, and probably loose their identity as CNC, and adopt a neural cell fate (Borchers 2001). Exploration of the possibility that EC1-3 promotes a pro-migratory signal is described in Chapter IV.

The possibility that cadherin-11 cleavage may promote signaling was initially speculated after an experiment described in Chapter II (Figure 2.8). This study was designed to see whether the interaction between cadherin-11 and ADAM13 has a physiological function during CNC migration. Here, we showed that co-overexpression of ADAM13 rescues CNC migration in embryos overexpressing cadherin-11. Surprisingly, we found that a considerable portion of the rescued crests (~20% of total embryos) had actually migrated further on the injected side of the embryo (Figure 2.8B).
This phenotype may simply be the result of earlier delamination from the neural tube. Alternately, this phenotype may be caused by cadherin-11 cleavage producing a pro-migratory signal, which increases in intensity when more of the molecule is cleaved (such as when cadherin-11 and ADAM13 are overexpressed).

The results described in the next chapter will reveal the possibility that the extracellular cleavage fragment of cadherin-11 may be acting as a signaling molecule, which aids in CNC cell migration.

Section VIII: Materials and methods:
(The following are additional materials and methods not listed in Chapter II)

Tissue culture binding study
To harvest EC1-3 media: Cos-7 cells were transfected with 1 µg of EC1-3-mt DNA in 1 well of a six well plate. 24 hours post-transfection, the media was removed from the transfected well and replaced with 2 ml of warmed (37 °C) S6 media (serum and protein free media) for 6 hours. S6 media from EC1-3 transfected wells was removed and spun for 5 min. at 1000 G to pellet any floating cells. The supernatant from this centrifugation was used to replace the media on cells transfected (36 hours prior) with cadherin-11, ΔEC1-3-cad-11, ADAM9, ADAM10, ADAM13, or ADAM19, which had been pre-washed with 1x PBS. EC1-3/S6 media was incubated on test-wells for one hour in the incubator, washed with 1x PBS, and fixed with 3.7% formaldehyde in PBS. The immunofluorescence procedure was performed using 9E10 mAb and α-mouse-FITC (1:200) to detect EC1-3-mt. Photographs were taken using a Zeiss Axiovert 200M
inverted microscope (Thornwood, NY) equipped with a Hamamatsu Orca camera (Bridgewater, NJ).

**DNA Constructs**

The PAPC construct is in pCS2 and was a generous gift from B. Gumbiner. The EC1-3 construct was made by introducing a myc- tag and stop codon between the EC3 and EC4 sequences of cadherin-11. The ΔEC1-3 construct was made by deletion using all around PCR with Pyrococcus furiosus DNA polymerase. All constructs were sequenced and tested for expression using the appropriate antibodies in both Cos-7 cells and embryos.

**In vivo migration assay**

Embryos were injected in the D1.2 cell at the 16-cell, or in the a2 and b2 cells of a 32-cell embryo using RFP and GFP as lineage markers. See figure legends for details on injections. An embryo was scored as “migration positive” if it had fluorescent cells traveling along the hyoid and/or branchial arches. Embryos in which the CNC had only migrated along the mandibular archway were not counted as positive because this archway is permissive (Alfandari 2001). Images were recorded using a Nikon D50 camera on a Nikon SMZ1500 dissecting scope (Melville, NY).

**In vivo EC1-3/cadherin-11 colocalization study**

The embryos that were cryosectioned were first injected at the 32-cell stage in the a2 and b2 cells. The a2 cell was injected with mRNA encoding RFP and cadherin-11, the b2 cell was injected with mRNA encoding EC1-3-mt. “Rescued” embryos were sorted by the
ability of RFP expressing cells to migrate, prior to fixing in MEMFA for 1 hour at room temperature. Cryosectioning procedure was performed as in (Neuner 2008). EC1-3-mt was detected in tissue sections by performing immunofluorescence using mAb 9E10 for the myc epitope tag, and a FITC-conjugated secondary antibody. DAPI was used to detect nuclei, which helped highlight the structures within the embryo head.

**Dissociation and reaggregation assay**

This assay was modified from (Rangarajan 2006). Briefly, embryos were injected with RFP alone, or with cadherin-11 or PAPC; or were injected with GFP alone, or with EC1-3-mt. At blastula stage, animal caps were removed from embryos, and incubated in calcium and magnesium-free media for 2 hours. During this time the pigmented epidermal layer was carefully removed and discarded, and the underlying animal cap cells were allowed to dissociate. Dissociated animal cap cells were then mixed in a 5 to 3 ratio of GFP to RFP expressing cells, and were placed in a BSA coated well of a 96-well v-bottom plate with 1 x MBS. Microspheres were allowed to form overnight at 18 to 20°C. Images were recorded using a Nikon D50 camera on a Nikon SMZ1500 dissecting scope (Melville, NY)
Figure 3.0: Possible biological activities of the cleavage fragment of cadherin-11. Full-length cadherin forms homophilic interactions with other cadherin-11 molecules on the surface of neighboring cells. We hypothesized that EC1-3, the extracellular cleavage fragment of cadherin-11, may maintain biological activity. 1. EC1-3 contains the adhesive sequence of cadherin-11. Therefore, EC1-3 may interact with full-length cadherin-11 through this adhesive domain, preventing a homophilic interaction with another full-length cadherin-11. 2. EC1-3 could also interact with a cell-surface receptor and promote downstream signaling events.
Figure 3.1: The extracellular cleavage fragment (EC1-3) of cadherin-11 binds to Cos-7 cells expressing cadherin-11, ADAM13, and ADAM19. EC1-3 binding experiment performed in cell culture. Conditioned media from EC1-3-mt transfected cells was incubated with live cells transfected with full-length cadherin-11, an extracellular truncated form (ΔEC1-3), ADAM9, ADAM10, ADAM11, ADAM13, and ADAM19. After 20 min, the cells were washed, fixed and processed for immunofluorescence using mAb 9E10 (myc). The green fluorescence represent EC1-3-mt bound to cells, while DAPI was used to stain all cell nuclei. Part A and B of this figure were published in (McCusker 2009); parts C through G have not yet been published.
Figure 3.2: EC1-3 rescues migration in embryos overexpressing cadherin-11. (A) Lateral view of embryos (St 26) that were injected in one CNC precursor cell at the 16-cell stage with synthetic mRNA for; GFP alone (GFP), GFP and cadherin-11 (Cadherin-11), or GFP plus cadherin-11 and EC1-3. 0.5 ng of GFP mRNA, 1 ng of cadherin-11 mRNA, and 1 ng of EC1-3 mRNA was used. The extent of CNC migration was determined by tracking GFP fluorescence, and results from 3 independent experiments are plotted in the histogram (B). Bars represent the percentage of embryos in which CNC migration was observed. The total number of embryos counted was: GFP alone (n=40), GFP+Cad-11 (n=75), or GFP+Cad-11+EC1-3 (n=82). The asterisk represents statistical significance at P<0.05. This figure was published in (McCusker 2009).
Figure 3.3: Cryosection of embryo overexpressing cadherin-11 cut transversally through the head. A) Diagram of a 32-cell *Xenopus* embryo. According to fate map studies performed by Moody *et al* (1977), the a2 and b2 blastomeres are major contributors to the neural crest. In my experiments I inject the two blastomeres separately in order to produce a mosaic CNC population. B) Representative section to show large scale morphology of sections (upcoming figures are at higher magnification). Images were taken with a 5x objective. Embryos were injected at the 32-cell stage with mRNA encoding RFP and cadherin-11 (in a2 cell), and EC1-3mt (in b2 cell). Embryos sectioned had rescued CNC migration in cells overexpressing cadherin-11 (sorted for phenotype as whole embryos). Immunofluorescence for EC1-3mt (Green channel, 9E10 for myc with FITC secondary antibody) was performed. Full-length cadherin-11 overexpressing cells were identified by RFP fluorescence (red channel). All cells were labeled with DAPI stain for nuclei (blue channel). Head structures are outlined in white. *Part A of this figure was taken from xenbase.com and is based on the studies performed by (Moody 1997). Part B of this figure has not been published.*
Figure 3.4: EC1-3 colocalizes to the border population of cells overexpressing cadherin-11. Fluorescence microscopy was performed as described in Figure 3.3. Image was taken with a 10x objective. Yellow color indicates co-localization of EC1-3 with cells expressing RFP (overexpressing cadherin-11) (white arrow). Arrow with * marks “rescued” CNC cells migrating in the head mesenchyme. These cells do not co-localize with EC1-3. This figure has not been published.
Figure 3.5: EC1-3 does not co-localize to “rescued” CNC cells. Fluorescence microscopy was performed as described in figure 3.3. Image was taken with a 10x objective. White arrows mark “rescued” migrating CNC cells that overexpress cadherin-11. EC1-3 (green) does not co-localize to these cells. This figure has not been published.
Figure 3.6: The cadherin-11 extracellular cleavage fragment rescues CNC migration in embryos with reduced ADAM13 expression. (A) Schematic representation of the experimental method. Embryos were injected at the one cell stage with MO13 and MO19 (5 ng each) and then again at the 16 cell stage, with a lineage tracer and mRNA encoding the various constructs, in D1.2 to target CNC. In this case we are testing the ability of the mRNA to rescue CNC migration. (B) In situ hybridization using Twist and Sox10 to label the CNC. The left panels represent the control side where the MO inhibited migration. The right panels represent the experimental side injected either with β-Gal or β-Gal and the EC1-3 mRNA. The black lines represent the extent of migration of the most posterior segment. Following injection of the EC1-3 migration is rescued. (C) Quantification of 3 individual experiments described above (2MO is MO13+19). The total number of embryos for each injection set was n=77 (2MO + β-gal), n= 96 (2MO + β-gal + EC1-3), n= 82 (2MO + β-gal + R13), n=66 (2MO + β-gal + C11), and n= 91 (non-injected + β-gal + EC1-3). This figure was published in (McCusker 2009).
Figure 3.7: EC1-3 rescues migration in CNC depleted of melrin proteins. (A) Embryos were injected at the 16-cell stage in D1-2 with the MO (0.33 ng each) and GFP as a lineage tracer to test their ability to prevent CNC migration. (B) Lateral views of representative embryos at tailbud stage. Migration was determined by the presence of GFP labeled cells in the CNC pathways as evident in the GFP control. (C) Histogram representing the percentage of migration in embryos injected with the various MO (0.33 ng of each A9, A13, and A19) with (black bars) or without (grey bars) the EC1-3 mRNA. This figure was published in (McCusker 2009).
Figure 3.8: The cadherin-11 extracellular fragment (EC1-3) can act non-cell-autonomously. (A) Schematic representation of the experimental design. The EC1-3 mRNA was coinjected with GFP mRNA at the 32-cell stage in the a2 cell. The 3MO cocktail (0.5 ng ADAM9, 13 and 19) was injected with RFP mRNA in the b2 cell of the same embryo (all mRNA were at 0.25 ng). Embryos were grown to stage 26 before imaging the GFP and RFP fluorescence (B). The percentage of embryos with migrating CNC cells expressing RFP was then counted and is presented in C. Asterisks indicate statistical significance as determined by Student's t test (p < 0.05). (D) Late stage (stages 45–47) analysis of RFP localization in differentiated facial structures in the dual injected embryos from above. Embryos were scored for having strong, little, or trace to no expression in the developing facial cartilage. (E) Histogram representing scoring data from late stage embryo analysis. This figure was published in (McCusker 2009).
Figure 3.9: The EC1-3 does not rescue CNC migration in embryos knocked down for cadherin-11. The EC1-3 mRNA was co-injected with GFP mRNA at the 32-cell stage in the a2 cell. The cadherin-11 morpholino was injected with RFP mRNA in the b2 cell of the same embryo. Embryos were grown to stage 26 before imaging the GFP and RFP fluorescence. (A) Representative fluorescent images of stage 26 embryos. Cadherin-11 depletion inhibits CNC migration (top right), which is not rescued by EC1-3 (bottom). Note that the EC1-3 cells (green channel) migrate normally in the cadherin-11 Mo embryos. (B) Graphic representation of migration assay depicted in (A). The number of embryos scored for each injection set is as follows; GFP/RFP = 33, GFP+EC1-3/RFP+C11-Mo = 38, GFP/RFP+C11-Mo = 38. This figure has not yet been published.
Figure 3.10: EC1-3 does not detectably alter the turnover of cadherin-11 in tissue culture cells. (A) Western blot analysis of media taken from cells transfected with vector (lane 1), EC1-3-mt (lane 2), or GFP-mt (lane 3). Membrane was probed for EC1-3-mt with mAb 9E10. (B) (top) Western blot analysis for cadherin-11 on transfected 293T cells incubated with EC1-3-mt media (lanes 2-7), or GFP-mt media (lanes 8-13), lane 1 is the non-transfected control. Treatment media was incubated on wells for 0, 15, 30, and 60 minutes, 2 hours, and 4 hours before cells were washed and extracted. (bottom) Western analysis for GAPDH as a loading control. *This figure has not yet been published.*
Figure 3.11: EC1-3 does not affect the ability of cadherin-11 to promote cell segregation. Embryos were injected in all 4 blastomeres at the 4-cell stage with mRNA for RFP alone or with cadherin-11 or PAPC (top image of each pair), or GFP alone or with EC1-3 (bottom image of each pair). Animal caps were dissected at stage 10, were dissociated in calcium and magnesium free media (removing the pigmented layer), and mixed together so that 3 RFP expressing animal caps were combined with 5 GFP expressing animal caps. Microspheres formed over a 24-hour period. Microspheres above are representative images of 4 microspheres per group. 100% of each group showed the phenotypes as shown in pictures. A) Representative microsphere combining RFP and GFP expressing A.C. cells. B) Representative images from microsphere expressing RFP and cadherin-11 a.c. cells mixed with GFP a.c. cells. C) Image of a microsphere made my mixing RFP and PAPC a/c/ cells with GFP expressing cells. D-E) Microspheres with the same combination of a.c. cells described in A-C, except GFP a.c’s are also expressing EC1-3. This figure has not yet been published.
Figure 3.12: Models for EC1-3 “rescue” activity *in vivo*: Overexpression of cadherin-11 (red cells) inhibits CNC migration, and the presence of EC1-3 rescues this phenotype. “Rescued” CNC cells overexpress cadherin-11 (blue lines) and migrate along the CNC arches A) EC1-3 (green lines) might inhibit cell adhesion by competing with full-length cadherin-11 molecules. This activity promotes CNC cell migration by decreasing cell-cell adhesion. Once the cell overexpressing cadherin-11 breaks away from the cluster of CNC cells, its migration is no longer inhibited by cell-cell adhesion. Rescued cells migrate on ECM and through head mesenchymal cells. B) EC1-3 might activate a promigratory signal that enables the border CNC cells to break away from the CNC cluster. It is possible that EC1-3 interacts with a cell surface receptor (orange lines) to initiate this signaling pathway.
CHAPTER IV

CNC CELLS MIGRATE TOWARD A SOURCE SECRETING THE EXTRACELLULAR CLEAVAGE FRAGMENT OF CADHERIN-11.

Abstract

The aim of the studies presented in this chapter is to further resolve the mechanism of EC1-3 biological activity. Our initial hypothesis was that EC1-3 promotes CNC migration by interacting with full-length molecules and inhibiting cell-cell adhesion. The results presented in the previous chapter suggest that this is one function of EC1-3, yet closer observation of these results suggests that EC1-3 may also be involved in cell-signaling events that promote CNC migration. In this chapter we describe results that support the idea that EC1-3 can act as a signaling molecule. The possible developmental function and potential mechanism of this activity will be discussed at the end of the chapter.

Section I: Introduction

How does cadherin-11 function during CNC migration? The previously described results have shown that cadherin-11 is extracellularly processed by ADAM metalloproteases, and this cleavage event is important for the progression of migration. I have also shown that extracellular cleavage does not detectably affect the interaction with cadherin-11 and cytoplasmic binding partners. The results described in Chapter III revealed that the extracellular cleavage fragment of cadherin-11 retains biological activity and appears to act as an inhibitor for cell adhesion in vivo. These
pieces of evidence suggest that the primary function of cadherin-11 in CNC migration is based on the adhesive properties. This is in contrast to the role of N-cadherin in the neural crest, which clearly plays a signaling role during NC delamination from the neural tube. In this instance, extracellular processing of N-cadherin by metalloproteases releases the cytoplasmic domain and binding partners, which promote downstream signaling (Shoval 2007). This is not to say that the adhesive properties of N-cadherin are not an important part of N-cadherin function during NC development. But it does indicate that classical cadherins that contain distinct biochemical behaviors are expressed in the NC during different stages of migration.

In order to further explore how cadherin-11 mediated cell adhesion is regulated during CNC migration, we studied how EC1-3 affects CNC cell behavior in an ex vivo environment. Initially, these experiments were designed to explore whether EC1-3 is acting as an inhibitor for cell adhesion. Here, EC1-3 was overexpressed in a CNC explant, and migration on a 2-dimensional substrate was recorded. If EC1-3 inhibits cell-cell adhesion, it might increase the amount of single cell migration from the explant because it would promote cell de-adhesion from the surrounding CNC tissue. As will be described in the results section of this chapter, instead of observing a dramatic increase in the amount of single cell migration in these explants, we found that they performed abnormal directional migration patterns. Since EC1-3 seemed to affect the directional cell movements of CNC cells, I decided to further explore this behavior. I placed a wild-type CNC explant next to a source of cells secreting EC1-3, and hypothesized that this source may attract migrating CNC cells. While the result of this experiment does show that CNC cells migrate more toward a source secreting
EC1-3 than a non-injected source, much more work needs to be done to determine how and why this is occurring.

The figures presented in this chapter are preliminary studies observing how CNC migration is affected by the presence of relatively large amounts of EC1-3. These results have not yet been published because of the highly exploratory and preliminary state of this work. I have personally performed all of the experiments in this chapter. The quantification method used in the analysis of the movies though this chapter was created by D. Alfandari, J. McCusker, and myself and is described in Appendix VI. The data displayed in figures 4.3, 4.4, 4.5, 4.6, and B of 4.7 was generated by using a Matlab program written by J. McCusker.

**Section II: Overexpression of EC1-3 in a CNC explant produces abnormal cell migration.**

Generally, cadherin molecules perform their adhesive function by forming homophilic interactions with other cadherins on the surface of neighboring cells. Therefore, we suspected that the extracellular cleavage fragment of cadherin-11 might act as a competitive inhibitor for cadherin-11 binding, further decreasing the amount of cell adhesion mediated through these molecules. To investigate this hypothesis we overexpressed the EC1-3 domains of cadherin-11, which mimics the cadherin-11 cleavage fragment, in cranial neural crest cells to see whether these explants dissociated faster than WT explants. We then performed time-lapse photography of the migration of CNC cells removed from the embryo and placed on a 2-dimensional FN substrate (Figure 4.1).
While EC1-3 expressing explants did not exhibit an increase in single cell dissociation from the explant, we did observe some unusual migration patterns (Figure 4.1B). Similar to WT explants, EC1-3 explants spread on the FN substrate and underwent branch migration and some single cell migration. However, unlike WT explants that continue branch and single cell migration in an outward direction (Figure 4.1A), EC1-3 branch migration reversed directionality so that it was moving toward the center of the explant (Figure 4.1B). Movies of other EC1-3 expressing explants exhibit similar migration behavior where branches of cells retract onto the explant or sometimes break away and rotate in a pinwheel-like manner (examples in supplementary movie files 6 and 7). Because of these migration patterns, we speculated that EC1-3 might be acting as an attractant to migrating CNC cells.

Section III: Single source movies show that cells secreting EC1-3 attract CNC cells.

To further investigate the possibility that CNC cells are attracted to cells secreting the extracellular cleavage fragment of cadherin-11 we used RNA injected animal cap (AC) cells as our source and performed time lapse video microscopy. Animal cap cells were selected because it is considered a naive tissue from the early embryo, and these cells efficiently secrete EC1-3 into the culture media (Figure 4.2A).

Using the ImageJ open sourced software, we traced the cell trajectories from 4-5 cells from each quadrant, 16-20 cells per movie, to see whether they migrated toward a non-injected source (Figure 4.2B) or a source of EC1-3 (Figure 4.2C). Due to the abundance of data, we divided the trajectories into two groups; 1) cells moving with a
random path, or “random cell movement” (RCM) and 2) cells moving with a more direct path, or “directed cell movement” (DCM). Definitions of the terms used throughout this chapter are located in Appendix VI. Our method for quantifying cell migration toward a target is also described in Appendix VI.

Analysis of the directionality of the cell trajectories from these movies showed that a large population of cells from a CNC explant move toward a source secreting EC1-3 (Figure 4.3B). Additionally, most of the CNC cells that migrate toward EC1-3 have “directed cell movement” (Figure 4.3B). This increase in DCM toward the EC1-3 source is not caused by an increase in the number of cells with DCM. This is demonstrated by further analysis of the cell trajectory populations that show the presence of EC1-3 does not greatly affect the amount of random or directed cell migration compared to the negative control (Figure 4.4).

A small population of CNC cells also moves toward the negative control source, suggesting that animal cap cells may endogenously secrete molecules that can influence the directionality of migration (Figure 4.3A). Since significantly more CNC cells move toward an EC1-3 source we hypothesized that EC1-3 may affect CNC cell directionality by one of the following mechanisms: 1) EC1-3 may be acting as a cell attractant to CNC cells, or 2) EC1-3 may be acting as a general stimulant of cell migration. If EC1-3 promotes a pro-migratory signal, it could appear to attract CNC cells because cell migration would be stimulated as they near the source (see figure 4.9 from discussion).
Section IV: CNC cells are most responsive to an EC1-3 source within a 450 µm radius *ex vivo*.

Further analysis of the cell trajectories showed that cells close to the EC1-3 source have an increased prevalence of directionality toward this target. This observation was made by plotting the “difference angle” of each trajectory against the distance from the source. This plot indicates that cells with “directional migration” toward the EC1-3 source (i.e. having a angle difference of 30° or less) are closer to that source (Figure 4.5B). This plot also indicates that cells must be within a range of 0 µm to 450 µm distance from the EC1-3 source in order to be affected. In the *Xenopus* embryo, the CNC travels a distance of between 200 and 300 µm during migration. Relatively speaking, the effective distance for a cell to “sense” EC1-3 *ex vivo* is well within the range that one would expect in an embryo. This is not to say that 450 um is the physiologically effective distance of EC1-3. Since protein diffusion is far easier through a liquid medium than through tissue, this “effective” distance should be much shorter in the embryo. The idea of the figure described in this section was simply to show that the effect of EC1-3 on CNC cell migration was concentration dependent, and that cells exposed to larger doses of this molecule were more receptive to it.

Section V: Exposure to EC1-3 increases CNC cell movement.

We have counted in each frame the number of cells present and their distance to the control or experimental “source” of EC1-3. The results presented in Figure 4.6A
suggest that more cells travel close to a source expressing EC1-3. This observation is consistent with a model in which EC1-3 “attracts” CNC cells.

To determine whether the CNC cells increased in speed when presented with increasing concentrations of EC1-3, we plotted the average instantaneous speed of CNC cells within distance ranges (i.e. 0-100 μm, 100 -200 μm, and so on) (Figure 4.6B). As can be seen in the histogram, cells closer to the EC1-3 source increase in velocity (6 μm/min compared to 2 μm/min at further distances). Additionally, CNC cells exposed to EC1-3 have a higher average velocity than the control for each distance interval except for the distance superior at 700 μm. Taken together these data represent a total number of 22000 trajectories with an average velocity of 2.37 μm/min for the cells exposed to the EC1-3 and 1.54 μm/min for the control. It is interesting to note that cells that are moving toward the negative control source also have an increase in velocity when approaching the source, but this is significantly less than the one observed with EC1-3.

It is striking that no cells were found within 100 μm of the control source. However, the lack of CNC cells within 100 um of the negative control source may be attributed to our method of quantification. In these assays, the “source” is identified as a single point in the center of the actual source. However, the actual size of the source ranges between 150 and 250 um in diameter. Upon reaching, and often invading, the source, the trajectory of a cell was no longer tracked due to difficulty in visualizing the cell as it moved throughout the animal cap tissue. Therefore, many cells may not have been identified as reaching the source (i.e. within 100 um of the sources center) even though they actually did reach the source. In order to alleviate this issue, we would need
to adjust our quantification method to incorporate an “area” reference instead of a “point reference” for the sources.

Section VI: Bait and choose assay reveals that CNC cells migrate toward both NI and EC1-3 sources when placed together.

In order to further analyze EC1-3’s ability to act as a cell attractant or a stimulator of cell migration we designed an experiment to analyze cell directionality in the presence of both the negative control and EC1-3 sources. We named this assay the “bait and choose” assay because the CNC explant was “baited” with two potential sources, and could preferentially migrate toward one of these (Figure 4.7A). In these assays, one source was injected with EC1-3 mRNA, and the other with GFP mRNA to identify it as the negative control. Similar to the single source movies, cells were tracked in the bait and choose assay, and were divided by “random” or “directed” cell movement. In these movies, the angle difference for each trajectory was determined for both sources.

Our analysis shows that CNC “baited” with both EC1-3 and negative control sources will migrate toward both sources (Figure 4.7B). We hypothesize that the source cells endogenously express molecules, such as FGFs, that could also act as a directional cue for the CNC cells while they migrate. Indeed, animal cap cells do express molecules such as FGF-2, FGF-8, and PDGFα, which have all been shown to act as cell attractants (Kubota 2002; Nagel 2004).

The fact that CNC cells do not preferentially migrate toward the EC1-3 source in the bait and choose assay suggests that EC1-3 may not act as a cell attractant. However, this confronts us with an interesting paradox: CNC cells will migrate toward the negative
control source if a source secreting EC1-3 is also present. It is possible that a source secreting EC1-3 does attract CNC cells, but the close proximity of the EC1-3 source to the negative control source combined with the abundance of directional cues secreted by both sources, inhibits the ability of CNC cells to “sense” EC1-3. On the other hand, EC1-3 may be acting as a stimulant to cell migration, which promotes cell movement to the vicinity of both sources. At this closer distance both sources could attract CNC cells, since both sources could secrete endogenous directional cues. At this point, we need to conduct further studies to truly resolve whether EC1-3 is acting through these possible mechanisms.

**Section VII: Discussion and future directions**

The initial aim of the studies conducted in this chapter was to further test our hypothesis that EC1-3 promotes CNC migration by acting as a competitive inhibitor for cadherin-11 mediated cell-cell adhesion. While the results in this chapter do not refute this possibility, we unveiled an unexpected affect on CNC cell migration. Here we show that CNC cells migrate toward a source of EC1-3. However, more studies need to be conducted to understand the mechanism of this activity. We propose that EC1-3 may either be acting as a cell attractant, or as a general stimulator of cell migration (Figure 4.8). These two possibilities will be discussed below.
Is EC1-3 a CNC cell attractant?

To start, what are the cell mechanisms influenced by cellular attractants? One possibility is to act as a “directional cue” for a migrating cell. However, another potential function of a cell attractant is to cluster cells that are receptive to this signal. Not only do these two functions promote unique physiological effects, they also imply drastically different spatial parameters. Cell attraction as a directional cue can occur from short to long-range distances, whereas cell attraction to cluster cells must occur over very short distances.

There is evidence that both functions of cell attraction are utilized by the CNC during migration. For almost ten years it has been known that mouse CNC cells were receptive to a long-range attraction signal (Kubota 2002). This signal is thought to aid in the directional migration of CNC cells toward the ventral side of the embryo. However, the molecules responsible for this signal have not been uncovered, though some investigators believe it may be an FGF-mediated signal since they are expressed in specific regions within the mandibular mesenchyme and ventral epithelium. If EC1-3 behaves as a cell attractant it probably does not act as a cell directional cue since the cells it would be “directing” (i.e. CNC cells) secrete it.

Conceptually, it is possible that EC1-3 could work as a short-range “clustering attractant”. Such a signal could be used to keep CNC cells in a group as they undergo branch migration. To my knowledge, there have been no published examples of this type of attractant. Yet, it is plausible that a “clustering attractant” could be used by a group of migrating cells, such as the CNC, to keep this group of cells together without increasing the amount of cell adhesion (Figure 4.8).
It is also possible that the ability of EC1-3 to act as an attractant within the CNC plays a role in the directional migration of these cells. Roberto Mayor has hypothesized that a combination of a short-range signal with “contact inhibition of locomotion” between CNC promotes the directional movement of the CNC (revealed in R. Mayor’s seminar at the 68th National SDB Meeting). Contact inhibition of locomotion is a phenomenon originally observed in fibroblast tissue culture by (Abercrombie 1954). Abercrombie observed that when the leading edge of two migrating cells meet, they become “paralyzed” and then retract. Following retraction, new leading edges are established on the opposite side of the cells, and they travel in this new direction.

Recently, contact inhibition of locomotion has been observed in vivo by Carmona-Fontaine of Dr. Mayor’s research team (Carmona-Fontaine 2008). Carmona-Fontaine reported that this phenomenon occurs among migrating zebrafish CNC cells in situ, and found that contact inhibition of locomotion was essential for migration in the ventral direction. They also found that non-canonical Wnt signaling was essential for this process. In his talk, Dr. Mayor described a mathematical model that incorporates contact inhibition of locomotion during directional migration of the CNC. According to his model, the effect of contact inhibition of locomotion on directional migration of the CNC was not enough to account for directional migration of the CNC over the time frame in which it occurs. He found that the mathematical model works if he incorporated a short-range attractant signal within the CNC (this model has not yet been published). With this in mind, it is possible that EC1-3 acts as a CNC cell co-attractant, which together with contact inhibition promotes directional migration of the CNC (Figure 4.8).
Does EC1-3 stimulate cell migration?

Another possible explanation for EC1-3 affect on CNC cell migration is that EC1-3 promotes a pro-migratory signal (Figures 4.8 and 4.9). Interaction with a cell surface receptor might be essential for the ability of EC1-3 to either promote cell attraction or to transmit a pro-migratory signal. However, in this instance EC1-3 would act as a general stimulator of cell migration. In vivo, this activity may potentiate CNC cell migration as the cells travel ventrally (Figure 4.8). Ex vivo, EC1-3 could appear to attract CNC cells because it is presented to them in an artificial gradient (Figure 4.9). So, cells that are closer to a source secreting EC1-3 would generally move more than cells that are further away, which is what we observe in our ‘single source’ analysis. However, if EC1-3 performs as a general stimulant to cell migration, we would expect CNC migration toward this source to consist of both random and directed cell movement. Our observations show that this is not the case in that most of the migration toward an EC1-3 source consists of “directed” cell movement (DCM).

Does EC1-3 interact with a cell surface receptor?

Whether EC1-3 promotes CNC migration through a pro-migratory signal or by acting as a cell attractant it will likely need to promote this cell behavior by interacting with a cell surface receptor. Thus, one focus of our current studies is identify which receptor for EC1-3. Our first candidate has been the FGF Receptor 1 (FGFR1). This receptor is an attractive candidate for multiple reasons. First, FGFR1 activity was shown to be important for CNC induction and it is expressed in the Xenopus CNC throughout migration (Monsoro-Burq 2003; Trokovic 2003; Golub 2000). FGFR1 was also shown to
mediate the attraction of mouse CNC cells toward sources secreting FGFs \((ex \ vivo)\) (Kubota 2002). Additionally, full-length cadherin-11 can bind to FGFR1 and promote downstream signaling (Boscher 2008). This interaction occurs through the extracellular domain of cadherin-11 suggesting that EC1-3 may behave in the same manner.

Studies pursuing this receptor as a mediator of EC1-3 signaling are already underway, and are described in Appendix VII. To briefly summarize, we are using a dominant negative form of FGFR1 as well as a FGFR1 small molecule inhibitor to see whether inhibition of FGFR1 signaling attenuates the effects promoted by EC1-3.

However, there are other likely receptors that EC1-3 may interact with to promote signaling events. One such receptor is ErbB3, which is specifically expressed in the \textit{Xenopus} CNC during migration and may be important for this process since knockdown of this receptor inhibits the formation of head structures (Nie 2006). Additionally, the extracellular cleavage fragment of E-cadherin was recently shown to interact with ErbB receptors in tissue culture and promote downstream signaling that resulted in cell migration and proliferation (Najy 2008).

\textbf{Does EC1-3, or a molecule EC1-3 stimulates in the AC cells, promote this affect on CNC migration?}

We have not overlooked the possibility that EC1-3 may be stimulating the expression of another molecule in the animal cap cells that promotes CNC cell migration toward that source. One experiment that we propose to elucidate this possibility is to use an “inert” source to secrete purified EC1-3 in ‘single source’ studies. In this case, we will alleviate the presence of any signaling molecules that the animal cap source may secrete endogenously, or in response to EC1-3. If we find that a source secreting the EC1-3 molecule alone does not ‘attract’ migrating CNC cells, we are still left with the exciting
possibility that EC1-3 promotes the expression of signaling molecules from both CNC (Figure 4.1) and animal cap (Figure 4.2) cells.

Section VIII: Materials and Methods

(not yet described in previous chapters)

Antibodies

Anti-ACTIVE ® MAPK pAb was purchased from Promega (Madison, WI, USA), and was used for western blot analysis using the manufactures directions. Phospho-Akt (Thr308) Antibody was purchased from Cell Signaling (Danvers, Ma, USA), and was used for western blot analysis following the manufactures instructions. As a loading control mouse GAPDH monoclonal antibody from Millipore (Saint-Charles, MO, USA) was used following manufactures instructions.

Embryo and embryo tissue care

Eggs were obtained from Xenopus laevis, cultured and fertilized as described previously (Alfandari 1995). Embryos were staged according to Nieukoop and Faber (1967). For our studies, animal cap tissue was dissected from the embryo at stage 10, and healed in 1 x MBS with 50 µg/ml of gentamycin. CNC explants were dissected at stage 15-17, and healed Danilchik’s media before using in ex vivo assays.
**Ex vivo migration assays**

Time lapse of *ex vivo* migration was performed similar to (Alfandari 1997) using 10 ug/ml of bovine fibronectin (FN, Sigma, St. Lois, MO, USA). Animal cap tissue was dissected from stage 10 embryos, being careful to remove the pigmented layer. The animal cap tissue healed for 4 hours before being placed into a well of a 96-well culture dish, and being incubated at 18°C while CNC explants were dissected. In the bait and choose assay, animal cap tissue was aligned to an external grid so that the two animal caps would be roughly the same distance apart (roughly 400-600 µM) among the wells. CNC explants were dissected from stage 15 to 17 embryos, and healed in DC media for 30 minutes. Explants were placed into well of a 96-well plate, and were oriented to an external grid so that the closest edge was between 200-300 µm away from the animal cap tissue. When the FGFR1 inhibitor SU5402 was used, the inhibitor was mixed with the media before the animal cap tissue and the explants were added to the wells. Animal caps and explants were incubated for 30 minutes to 1 hour at 18°C before starting time-lapse photography. Before time laps, fluorescence images were taken to identify the negative control animal cap (GFP-mt). Images were obtained using a Zeiss Axiovert 200M microscope with a Hamamatsu Orca Ag Camera and Openlab software (Improvision, Lexington, MA, USA). Cell trajectory data was obtained by using the ImageJ (http://rsb.info.nih.gov/ij/index.html) open sourced software, using the Manual Tracking plugin (written by Fabrice Cordelires, Institut Curie, Orsay, France). Data analysis and quantification was performed using Matlab software (address) (see description below for quantification).
CNC cell migration quantification method

The directionality of the cell trajectories was quantified using the commercially available software package Matlab (Natick, Ma, USA). For this, the individual trajectories from the tracked cell data were analyzed independently. Each cell trajectory was simplified to a vector passing through both the initial and final cell locations, which we describe as the actual trajectory vector. We then calculate the ideal trajectory vector as the vector passing through both the initial cell and target locations. The absolute angle between the actual and ideal trajectory vectors are used to describe the directionality of the migrating cell. This analysis is repeated for all trajectories and all data sets and the trajectory angles are collected and represented through a directional histogram using the Matlab rose command. (see also Appendix VI).
Figure 4.1: Time-lapse photography of *ex vivo* migration reveals CNC explants secreting EC1-3 performing ‘retractile’ cell migration. A) Time-lapse images of a representative wildtype CNC explant migrating on a 2-dimensional substrate. B) Time-lapse images of the *ex vivo* migration of a representative CNC explant overexpressing EC1-3. A and B) Images represent the net migration occurring over 30 minutes for a total of 5.5 hours. The initial image of T=0 explant is overlayed on each following time-point. Blue arrows trace the migration of a CNC branch. Wildtype CNC branch extends in an outward direction (A), while EC1-3 CNC branch initially extends, and then retracts after 2.5 hours from T=0 of movie (B). *This figure has not been published. Movies are included in supplementary movies: Movie 5 and 6.*
Figure 4.2: CNC cells migrate toward a source of cells secreting EC1-3. A) Western blot analysis detecting EC1-3-mt secreted into the culture media. Lane 1; media from cells transfected with empty vector, Lane 2; media from cells transfected with EC1-3-mt. B and C) Example cell trajectories from CNC explants migrating for 3 hours on a 2-D fibronectin substrate. B) Trajectories from an explant placed next to a negative control source. C) Trajectories from an explant placed next to a source secreting EC1-3. This figure has not been published. Movies are included in supplementary movies: Movie8 and Movie 9.
Figure 4.3: *Ex vivo* analysis of CNC cell directionality toward a source secreting EC1-3. A and B) Rose diagrams of the angles from the trajectories of CNC explants placed next to a negative control source (A) or a source secreting EC1-3 (B). Trajectory data from 10 movies each was combined, and separated into all trajectories (left panels), random trajectories (middle panels), and directed trajectories (right panels). *This figure has not been published.*
Figure 4.4: Close proximity to EC1-3 source does not increase prevalence of cells with “directed cell migration”. A and B) Plots of distance vs. ratio (total displacement/trajectory) of cell trajectories from CNC explants exposed to non-injected source (A) or EC1-3 source (B). “Distance” refers to the distance a single cell was located from the source at T = 0. The “ratio” refers to the difference between the lengths of “cell displacement”/“actual cell path”. Cells with a ratio of 0.5 or greater are cells moving with “directed cell migration”. Cells below this threshold are considered to be moving “randomly”. Cells with “close proximity” (i.e. 450 µm or less) to the negative control or EC1-3 sources exhibit both random and directed cell migration. This figure has not yet been published.
Figure 4.5: CNC cells that migrate toward a source secreting EC1-3 are within 450µm. A and B) Angles from the data described in figure 4.3 were plotted against the distance of the cell from the source at the initial time point. A) Angle vs. distance plot from CNC cells next to negative control sources, or B) EC1-3 sources. The horizontal line on each graph indicates the threshold for cells moving toward the target, where cells below this line (30°) are considered to be moving toward the source. *This figure has not been published.*
Figure 4.6: CNC cells move with a greater velocity in the presence of a source secreting EC1-3. A) The distribution of trajectory points from all of the time-lapse movies. The distance of each point from every trajectory to the source was plotted for EC1-3 movies (black bars), and negative control (NI = non-injected) (grey bars). B) Each trajectory from the time-lapse movies was divided into mini-trajectories (2 min length). The velocity of each mini-trajectory was determined, and plotted against the distance the mini-trajectory was to the source. Black bars (EC1-3), grey bars (Non-injected) C) 1. A representative single trajectory plot from a CNC cell migrating for 110 minutes. 2. The trajectory plot from “1” was split into 11 mini-trajectories, representing the distance and direction the cell moved over 10 minutes. Blue arrows represent the actual cell trajectories, green arrow represent the “ideal” cell trajectory to move toward the EC1-3 source. *This figure has not been published.*
Figure 4.7: Bait and choose assay show that CNC cells can migrate toward both sources. A) A pictorial description of bait and choose assay design. Source cells are made by injecting the animal hemisphere of the early embryo with mRNA encoding GFP-mt or EC1-3-mt, and dissecting out the animal cap at stage 10. Healed animal caps (one from each injection set), were placed on a 2-D substrate, roughly 300–400 μm apart. The CNC was then dissected from a stage 15-17 embryo and placed 250 – 400 μm away from animal cap sources. Time-lapse photography filmed the migration of the CNC cells over a 4-hour period. B) Rose diagrams of angles from cell trajectories in the bait and choose assay. Top panels represent cell directionality toward the GFP-mt source, lower panels represent cell directionality toward the EC1-3 source. Data was obtained from the trajectories of 19 movies. This figure has not been published.
A

How does EC1-3 promote CNC migration?

1. EC1-3 decreases cell adhesion by inhibiting cad-11-cad-11 interactions.
2. EC1-3 stimulates cell migration
3a. EC1-3 attracts migratory CNC cells
3b. EC1-3 works together with “contact inhibition of locomotion” to promote directional CNC migration

EC1-3 clusters CNC cells without increasing cell-cell adhesion

B

Contact Inhibition of Locomotion

- move in opposite direction
- Net directional movement

Contact Inhibition of locomotion & Cell attraction

- Cell attraction increases directional movement
- Net directional movement
**Figure 4.8: Potential mechanisms for EC1-3 promotion of CNC migration *in vivo.***

A) I hypothesize that EC1-3 promotes CNC migration by the following mechanisms: (1) EC1-3 inhibits cadherin-11 homophilic binding and decreases cell-cell adhesion in the CNC. Decreasing cell-cell adhesion helps maintain “fluidity” within the CNC (2) EC1-3 stimulates a promigratory signal. This signal potentiates cell movement during CNC migration. (3) EC1-3 attracts CNC cells. EC1-3 attraction of CNC cells might promote CNC migration by the following mechanisms; (3a) EC1-3 attraction acts together with contact inhibition of locomotion (described in B) to promote directional migration of the CNC, or (3b) EC1-3 acts as a CNC “clustering” agent, which keeps the CNC cells together without increasing cell-cell adhesion. This may be particularly important in a morphogenic cell type, such as the CNC, where an increase in cell adhesion inhibits migration. B) (left) Contact inhibition of locomotion occurs when two migrating cells collide, and results in the redirection of cell migration in the opposite orientation. As a consequence the cells move on a trajectory perpendicular to the original direction of motion, and displacement in the ventral direction is small. (right) Contact inhibition of locomotion combined with cell attraction results in subtler redirection where co-attraction prevents cell migration in the opposite direction. Instead, both cells migrate in the same direction, which results in a larger cell displacement in the ventral direction. *This figure has not been published.*
Figure 4.9: Diagram of models describing why CNC cells migrate toward EC1-3 *ex vivo*. A) Model for CNC cell migration toward EC1-3 source *ex vivo*, if EC1-3 stimulates cell migration. Concentric rings (orange) around the EC1-3 source represent a gradient of EC1-3 molecules surrounding the source. If EC1-3 stimulates migration, CNC cells that are closer to the source exhibit more migration because they are exposed to a larger quantity of EC1-3 molecules. This results in more CNC migration close to the EC1-3 source, which gives the impression of directional migration toward that source. B) Model for CNC cell migration toward an EC1-3 source *ex vivo* if EC1-3 is a cell attractant. A non-polarized CNC cell expresses receptors (purple) that can bind to EC1-3. A gradient of EC1-3 (concentric orange rings) is presented to the cell, predominantly activating EC1-3 receptors on one side of the cell. Orange circles represent EC1-3 molecules. Polarized activation of EC1-3 receptors provides positional information and activates a downstream cascade that promotes cell migration toward the EC1-3 source. *This figure has not been published*
CHAPTER V

DISCUSSION I: EXPLORING THE SIGNALING CAPABILITIES OF
CLASSICAL CADHERIN MOLECULES

Forward

The research presented in this thesis focuses on the developmental role of molecular interactions that occur between cadherin-11 and ADAM family members during CNC migration. Thus, the logic and design of these experiments were drawn from both biochemical and embryological perspectives. In order to thoroughly discuss the implications of the results from these experiments, I have divided the discussion into two chapters. This chapter will focus on the role of classical cadherin cleavage fragments to influence specific cellular behaviors. The text of this chapter was recently published as a mini-review in the journal Communicative and Integrative Biology (see Appendix X). The following chapter, Chapter VI, will discuss the role of classical cadherins in the context of the embryo.

Section I: Introduction

Classical cadherins are a group of Ca\(^{++}\) dependent transmembrane cell adhesion molecules, mostly known for their ability to perform homophilic interactions with like-cadherin molecules on the surface of neighboring cells. Over the past decade, many studies have also established cadherins as key players of intracellular signaling events by modifying the activity of Rho GTPases, members of the Wnt signaling pathway, and receptor tyrosine kinases. Given the utility of these molecules, it is not surprising that
they play multiple roles during different embryological and adult processes. Yet, these activities have been primarily tied to their full-length molecules. And, while the activity of full-length molecules is undoubtedly an essential part of how cadherins perform in vivo, it is becoming increasingly evident that the proteolytic fragments of these molecules may also play a role. This is an exciting development because proteolysis of cadherins was previously thought to be a simple clearing-mechanism meant to regulate the levels of cadherin molecules on the cell-surface.

Here, I will further discuss our recent findings, showing that both N-terminal and C-terminal fragments of cadherin-11 retain biological activity in Xenopus embryos. I will also review the current literature demonstrating that both the extracellular and intracellular fragments of other classical cadherins are capable of activating certain signaling events tied to epithelial to mesenchymal transitions (EMTs), cell survival, cell proliferation, and cell migration.

Section II: Proteolysis of classical cadherins

Complete processing of a full-length cadherin molecule involves multiple proteases. It is generally accepted that the extracellular region is processed first, and that multiple metalloproteases containing a disintegrin domain (ADAM), matrix metalloproteases (MMPs), and other transmembrane proteases can perform this event (Davies 2001; Hermant 2003; Hunter 2001; Ito 1999; Maretzky 2005, McCusker 2009; Najy 2008; Steinhusen 2001). This initial cleavage results in the shedding of the extracellular N-terminal fragment (NTF), and the generation of a first C-terminal
fragment (CTF1) that contains the transmembrane and cytoplasmic domains of the molecule. In many cases CTF1 is further processed by the presenilin-1 (PS-1) complex in the juxta-membrane region, releasing the cytoplasmic domain (CTF2) along with any associated proteins (Ferber 2008; Marambaud 2003; Uemura 2006). Among the classical cadherins, there are multiple examples showing that the NTF, CTF1, and CTF2 can each perform diverse biological activities.

Section III: Biological activities of shed cadherin fragments

In our recent publication, we showed that cadherin-11 cleavage by ADAM metalloproteases (ADAM9, and 13) was essential for cranial neural crest (CNC) migration in vivo (McCusker 2009). One likely purpose of this processing is to control the overall cell-adhesion levels mediated by the full-length cadherin-11 molecules. Yet, I have also discovered that the NTF itself can promote migration in vivo. In Xenopus embryos, CNC migration is inhibited when full-length cadherin-11 is overexpressed or when ADAM9, 13, and 19 expression are knocked down. This phenotype can be rescued by overexpressing the cadherin-11 NTF. We suspect that the cadherin-11 NTF acts as an antagonist of homophilic interactions since we have shown that it binds to full-length cadherin-11 in cell culture (McCusker 2009). Interestingly, I have also found that the NTF can bind to select members of the ADAM family (unpublished observations), regardless of their ability to process full-length cadherin-11. Future work will determine whether this interaction affects the function of these ADAMs and plays a role in the embryo.
The NTFs of both N-cadherin and E-cadherin have also been shown to have biological activities. Endogenous N-cadherin NTF has been detected \textit{in vivo} in the extracellular matrix (ECM) surrounding denervated muscle fibers, as well as in embryonic retinal tissue (Cifuentes-Diaz 1994; Paradies 1993). N-cadherin NTF that is associated with the ECM may perform an important biological function in cell adhesion and neurite outgrowth, as immobilized NTF promotes both of these activities in cell culture (Utton 2001; Paradies 1993). N-cadherin NTF can also associate with and activate FGF receptors, resulting in the activation of PI3K and Akt, and decreasing the levels of apoptosis (Lyon 2008). Likewise, E-cadherin NTF was shown to bind to, and activate the human ErbB receptors, Her2 and Her3, leading to the activation of downstream signaling that results in cell migration and cell proliferation in cell culture (Najy 2008). Therefore, the NTFs of these classical cadherins can affect cell adhesion properties, as well as bind to and activate cell-surface receptors.

\textbf{Section IV: Signaling through the CTFs}

The cadherin fragment signaling capabilities do not end with the extracellular region. The CTFs of classical cadherins can also influence intracellular signaling events by interacting with molecules involved in multiple pathways. For example, the N-cadherin CTF2 associates with CREB binding protein (CBP), targets it for degradation, and inhibits CREB-mediated transcription in cell culture (Marambaud 2003). Marambaud and colleagues have speculated that this activity may be important for neuronal growth and survival. In addition, Ferber and colleagues have shown that the E-cadherins CTF2
can activate the expression of Wnt-related genes that are involved in cell proliferation and differentiation in cell culture (Ferber 2008). In this instance, E-cadherins CTF2 binds to p120 catenin, translocates to the nucleus, and can activate transcription of Wnt target genes by blocking the repressor Kaiso (Ferber 2008).

The CTFs of N-cadherin, E-cadherin, and cadherin-11 all associate with the Wnt signaling molecule, β-catenin. N-cadherin and E-cadherin CTF2 both interact with β-catenin, protect it from degradation, and relocate it to the nucleus to promote gene transcription (Maretzky 2005; Marambaud 2002; Reiss 2005; Sadot 1998; Uemura 2006). As a result, the CTF2 of N-cadherin and E-cadherin can both effect the transcription of a number of downstream targets such as cyclin D1, c-myc, and c-jun, and can enhance cell behaviors such as cell proliferation and cell migration in cell culture and in vivo (Uemura 2006; Shoval 2007; Reiss 2005; Maretzky 2005). Yet, it is not entirely resolved whether CTF2 alone or the CTF2/β-catenin complex is responsible for all of these processes.

On the other hand, the interaction between cadherin-11 and β-catenin appears to deviate somewhat from what has been established with the other classical cadherins. While we can detect a small cadherin-11 CFT2-sized fragment in embryos, we have not yet established whether it complexes with β-catenin (McCusker 2009). However, I have shown that the cadherin-11 CTF1 maintains the interaction with β-catenin, and does not stimulate the activation of the transcriptional targets described above (McCusker 2009). In the case of CNC migration we suspect that is an important detail, since β-catenin signaling can block CNC migration if activated exogenously during this process (de Melker 2004).
Section V: Teasing apart the role of full-length vs. cadherin fragment affect on signaling

One of the future objectives of our laboratory is to determine whether endogenous cadherin fragments have a physiological function. As we pursue this goal we are challenged with a major difficulty: how do we decipher between the activities of a full-length cadherin molecule and that of the cleavage fragment?

For example, in order to see whether a cleavage fragment elicits a certain phenotype others and we often utilize recombinant technology to express peptides meant to mimic the NTFs and CTFs of a cadherin. In this instance, it is difficult to determine whether phenotypes observed with overexpressed fragments are specifically caused by their “fragment” properties, or their ability to mimic the full-length molecule. It is probable that the structure of endogenous and recombinant “fragments” varies significantly from the uncleaved peptides, making them molecularly distinguishable. However, these peptides have been shown to interact with many of the same binding partners of the full-length molecule, suggesting that the full-length and cleaved peptides share significant similarities. In addition, it also unclear if the physiological level of a cleavage fragment is enough to promote the same cell behaviors observed when overexpressing recombinant cleavage fragments. So, while the use of recombinant proteins is a useful and essential step to help realize the signaling potential of these fragments, we cannot be sure that the endogenous fragments are performing in the same manner.

The use of chemical inhibitors to block protease activity is another tool that we have used to help determine whether the cleavage of a cadherin is important for a
physiological process. The advantage of using these inhibitors is that cadherin cleavage can be blocked temporally. However, it is unlikely that they will help us resolve whether signaling events promoted by the endogenous fragments are important for a specific cellular behavior. One reason for this is that we could not be sure that a phenotype we observed was a result of the inability to generate a cleavage fragment and signaling events, or the inability to process (remove) the full-length molecule. But more importantly, these inhibitors block multiple proteases, and any phenotype we observe could easily be attributed to any of their proteolytic targets.

Perhaps the main source of the experimental difficulties described above is due to the fact that cadherins have important cellular functions before they are processed. Therefore, we must be careful not to interfere with the activity of the full-length cadherin while studying possible signaling activities of the cleavage fragments. One possibility is to generate antibodies that recognize a cryptic site revealed on the cleavage fragment upon processing. This would allow us to see exactly where and when the fragments are generated during a particular biological process. It is also possible that one such antibody could block the “function” of NTFs and would be extremely useful in deciphering a role for these molecules in vivo. Alternately, the generation of small, “function blocking” peptides that are expressed under inducible promoters would be a useful tool for understanding the roles of endogenous CTFs. These peptides would make it possible to temporally block CTF activity in vivo.

Another tool that could further decipher the role of cadherin cleavage would be to generate a mutant in which the cleavage site has been replaced by a sequence cleaved by an exogenous protease. This mutant could be expressed in embryos lacking the wild type
cadherin and perform all the functions of the full-length protein but would be unable to be processed by the natural protease. In return, the exogenous protease could be provided at a defined time to observe the change in cell behavior. The two main technical difficulties of such an experiment is 1) to find a sequence that would not be cleaved by the natural protease which is always difficult with ADAM and 2) to identify an exogenous protease whose expression would not adversely affect embryo development.

To conclude, it is clear that we will need to come up with creative solutions as we determine the physiological relevance of cadherin cleavage fragments. Yet, as new tools and technologies emerge, we will be able to resolve these issues.
Figure 5.1: The extracellular (NTF) and intracellular (CTF) cleavage fragments of classical cadherins retain biological activities. (A) N-cadherin NTF and CTF can interact with the FGFR, β-catenin, and CREB signaling molecules. These interactions can lead to transcriptional activation or inhibition, and can promote cell migration. (B) E-cadherin cleavage fragments can interact with EGF receptors, β-catenin, and p120 catenin signaling molecules. These fragments can protect cells from apoptosis, support cell migration, and promote the transcription of a number of genes. (C) Cadherin-11 cleavage fragments can interact with full-length cadherin-11, ADAM13, ADAM19, and β-catenin, and promote cell migration in vivo. This figure was published in Communicative and integrative Biology (McCusker 2009)
CHAPTER VI

EXPLORING THE CADHERIN-11/ADAM INTERACTION IN THE EMBRYO

Forward

In the previous chapter I focused on the possible biological roles of cadherin-11 cleavage fragments from a cell biological perspective. This chapter will concentrate on the role of cadherin-11 cleavage by ADAMs in the context of the embryo. I will begin by discussing the various adhesive environments an individual CNC cell can encounter along its journey. Here, I seek to give the reader a global perspective of how the CNC utilizes both cell-cell and cell-ECM interactions during migration. In this chapter I will also highlight distinguishing characteristics among the classical cadherins that are expressed in the neural crest, focusing on their role as both cell adhesion molecules and molecules that have the potential to promote signaling events. Additionally, I will discuss how specific ADAM family members mediate the function of these cadherins expressed in the NC. I will speculate whether ADAM regulation of cadherin molecules is restricted to migratory cells, or may also play a role in other non-migratory embryological processes. This chapter closes with a summary of the main results from my thesis work.

Section I: Introduction

Cells use a variety of cell-adhesion molecules during the process of migration. Cell adhesion molecules supply a link with the surroundings of a migrating cell, which serves as a platform in which to propel the cell body. CNC cells use cadherins, which
mediate cell-cell interactions, and integrins, which mediate cell-ECM interactions. At the onset of CNC migration, individual CNC cells are presented with various environments. The CNC is surrounded by a meshwork of ECM molecules, which separates the CNC from the overlying epidermis and underlying mesoderm. As a result, cells at the periphery of the CNC have a lot of contact with the ECM. On the other hand, there is little ECM within the CNC, and these cells have far more cell-cell interaction. Although a detailed study of the localization of both cadherin and integrin bonds in the CNC has not been performed, it seems intuitive that the “outside” layer of CNC would have more integrin-ECM interactions than the inside of the CNC, which would have more cadherin-cadherin interactions. This is not to say that specific regions of the CNC express more or less of these types of cell adhesion molecules. I think that it is likely that individual CNC cells express both integrins and cadherins in preparation for the adhesive environment it may encounter.

An individual CNC cell is also presented with a variety of environments throughout migration (Figure 6.0). At the onset of migration, the CNC migrates as a cohesive sheet: this phase is called “sheet migration”. As described above, there are generally two types of CNC adhesion-environments at this stage, those with high contact with the ECM, and those with more cell-cell contact. During sheet migration, the cells at the leading edge of the CNC process and rearrange the ECM molecules that we hypothesize to help “clear the pathway” for the following CNC cells (discussed in Alfandari 2004). As migration progresses the CNC travels along distinct archways: this phase is called “branch migration”. During this stage the surface to volume ratio of the CNC increases, and an individual CNC cell is more likely to be exposed to the “outside”
of the CNC. While ECM molecules are still located in the outside environment, the presence of the ECM is not as strong as at the onset of migration. Here, it is likely that CNC cells are also interacting with cells from the head mesenchyme. During the later stages of CNC migration, individual CNC cells can break away from the arches, and invade the head mesenchyme. Therefore, CNC cells must also use cell-cell adhesions as they increasingly interact with the cells in the head mesenchyme. Cadherins or other cell-cell adhesion molecules, such as Immunoglobulin-like adhesion molecules or selectins, may mediate these cell-cell bonds.

Although the work presented in this dissertation has focused on the role a specific cadherin family member in the CNC, it is clear that this molecule must act in concert with other types of cell adhesion molecules during the process of CNC migration. The following sections will further discuss the role of cadherin molecules during CNC migration. Additionally, I will discuss how ADAMs regulate cadherins during CNC migration: as well as how ADAM regulation of cadherins may play a role in other developmental processes.

Section II: The differential roles of classical cadherin molecules in neural crest migration.

One focus of this work was to further understand the role of cadherins during neural crest migration. Among the frog, chick and mouse model systems a number of classical cadherins have shown to be expressed during the early and late stages of migration. Both N-cadherin and cadherin-6B (cadherin-6 in mouse) are expressed during NC induction, and are down-regulated shortly after the onset of migration (Akitaya 1992;
Nakagawa 1995; Inoue 1997). On the other hand, cadherin-11 and cadherin-7 expression is upregulated throughout NC migration (Kimura 1995; Nakagawa 1995; Hadeball 1998; Nakagawa 1998; Vallin 1998; Nollet 2000). The following text will discuss what is known about each of these cadherins in NC development separating them into two groups; 1) cadherins expressed during early NC migration (Part I), and 2) cadherins expressed throughout migration (Part II) (Figure 6.1).

**Initiation of NC migration: The role of N-cadherin and cadherin-6B in EMT and delamination of the NC.**

As induction draws to a close, and the NC cells prepare for migration, subtle changes in the NC begin to happen. Pre-migratory neural crest cells express a number of molecules that are considered as mesenchymal markers, such as Snail, Slug, Twist, N-cadherin and cadherin-11. Yet, these cells maintain adherens junctions and a general cell polarity, which is not characteristic of mesenchymal or migratory cell types. Just prior to migration a switch occurs which initiates the epithelial to mesenchymal transition (or epithelial to mesenchymal-like transition (if referring to *Xenopus* CNC), resulting in the loss of adherens junctions, loss of cell polarity, and the delamination of NC cells from the surrounding tissue.

**N-cadherin**

Part of this EMT switch occurs when BMP signaling induces the extracellular processing of N-cadherin by metalloproteases, possibly ADAM10 (Shoval 2007). The C-terminal fragment (CTF) containing the cytoplasmic tail of N-cadherin is then further processed in the membrane by the gamma-secretase, releasing the cytoplasmic tail and
associated proteins (such as β-catenin). This second cleavage fragment (CTF2) translocates to the cell nucleus, and induces the expression of genes, such as cyclin-D1 and β-catenin, which are important for the G1/S transition, completion of the EMT, and the onset of cell migration (Shoval 2007; Burstyn-Cohen 2002; Akimoto 1999).

NC cells synchronously migrate from the neural tube in the S-phase of the cell cycle. The transition from the G1-phase to the S-phase in the NC cells is an important transition during the EMT, as inhibition of the G1/S transition blocks delamination and migration (Burstyn-Cohen 2002). Both cyclin-D1 and β-catenin, two molecules upregulated by the CTF2 of N-cadherin, promote the transition from G1-phase to S-phase in the NC (Akimoto 1999; Burstyn 2004).

**Cadherin-6B**

Along with N-cadherin, cadherin-6B is also expressed during NC induction through the onset of NC delamination, when it is quickly downregulated. The mechanism for cadherin-6B protein downregulation in the crest has yet to be resolved, and no studies have been published on the potential for cadherin-6B to mediate signaling events in a similar manner to N-cadherin. However, cadherin-6B regulation in the NC does vary from that of N-cadherin. N-cadherin protein is downregulated by metalloproteases during NC delamination, yet N-cadherin RNA is strongly present throughout NC migration (Simonneau 1992). On the other hand, cadherin-6B protein and transcripts are both downregulated at the beginning of NC migration. Here, cadherin-6B transcription is directly repressed by the Snail2 transcription factor (Taneyhill 2007).
The function of cadherin-6B in the NC still remains to be determined. One hypothesis is that cadherin-6B expression in the NC helps delineate the boundary between the NC and the neighboring neural tissue (since both tissues express N-cadherin) (Nakagawa 1998). It has also been suggested that cadherin-6B retains the NC from migrating until it completes induction, since targeted depletion of cadherin-6B results in premature delamination and migration from the neural tube (Coles 2007).

Neural crest migration: How do cadherin molecules 7 and 11, promote this process?

A common feature of EMT or EMLT is a process called cadherin switching. During this process cadherins expressed in epithelial cells, such as E-cadherin, are downregulated and replaced with mesenchymal cadherins like cadherin-11. It is generally thought that these mesenchymal cadherins maintain “looser” interactions, which are more conducive to mesenchymal and migrating cells. However, recent work has shown that the variation in dissociation constants among cadherin molecules does not have a profound effect on the strength of cadherin-cadherin binding (Patel 2006). Moreover, overexpression of any classical cadherin in the NC or in migrating tissue culture cells will inhibit migration (Nakagawa 1995; Nakagawa 1998; Dufour 1999; Borchers 2001; Coles 2007; Shoval 2007). This suggests that too much of a mesenchymal cadherin will increase the amount of cell adhesion and inhibit migration regardless if they form “looser” cell-cell contacts than their epidermal counterparts.

How then do mesenchymal cadherins promote cell migration? This is an intriguing question because the classical cadherins share many similar characteristics such as their general domain organization, intracellular binding partners, and the ability
to perform homophilic interactions to promote cell-cell adhesion. The results from our lab and others suggest that, at least in the case of the migrating neural crest, the amount of cell adhesion mediated by mesenchymal cadherins such as cadherin-11 is strongly influenced by regulatory proteins.

**Cadherin-11**

Cadherin-11 is expressed during both phases of CNC migration, the initial sheet phase where the CNC migrates as a cohesive tissue, and the second phase where CNC cells migrate in the archways and as single cells. Dorris Wedlich’s lab and I have both shown that overexpression and depletion of cadherin-11 inhibits CNC migration *in vivo*, suggesting that a defined balance of this molecule is required during migration. I have shown that cadherin-11 is continuously regulated by meltrins ADAM9 and ADAM13 *via* an extracellular cleavage event during CNC migration in *Xenopus*. This cleavage event promotes CNC migration by multiple mechanisms. First, it regulates the amount of full-length cadherin-11 at the cell surface, keeping in check the strength of cell-cell adhesion mediated by this molecule during migration. Secondly, it produces an extracellular cleavage fragment that exhibits pro-migratory activity.

The pro-migratory activity of the extracellular cleavage fragment of cadherin-11 (EC1-3) appears to be transmitted in multiple ways. Since EC1-3 retains the cadherin-11 adhesive sequence, it probably acts as a competitive inhibitor for cadherin-11 binding. My results show that EC1-3 can bind to cells expressing FL-cadherin-11, and can rescue migration in CNC cells overexpressing cadherin-11, which support this hypothesis. However, the results described in Chapter IV suggest that EC1-3 may play a more active
role in CNC migration, possibly by transmitting a cell-attraction or pro-migratory signal through the activation of a cell surface receptor.

The molecular interactions that occur with the cytoplasmic tail of cadherin-11 were also recently shown to be important for CNC migration (Kashef 2009). Here, the intracellular domain of cadherin-11 interacts with the GEF Trio, the activity of which stimulates GTPases such as Rac1, RhoG and RhoA. These molecules support the cytoskeletal dynamics required for the cell movements during CNC migration. It would be interesting to resolve whether extracellular processing of cadherin-11 by ADAMs affects the ability to support cytoskeletal rearrangements.

**Cadherin-7**

Similar to cadherin-11, cadherin-7 is expressed in the NC throughout migration. Unlike cadherin-11, which is expressed in both cranial and trunk neural crest populations, cadherin-7 is only expressed in a subset of the NC population (Nakagawa 1995). Far less work has been done on elucidating the function of cadherin-7 in NC migration. Some work has shown that cadherin-7 has a faster turnover rate in migrating NC cells when compared to N-cadherin (Dufour 1999). This turnover is likely due to proteolysis as shown in cell culture experiments, but the enzyme responsible for this proteolysis remains to be identified (Kawano 2002). It will be interesting to resolve whether cadherin-7 acts in a similar or different manner to cadherin-11 during NC migration with respect to the ability to promote cell-autonomous (GEF-Trio) and non cell-autonomous (EC1-3) stimulation of cell migration.
Section III: Specific ADAMs regulate cadherin function during different phases of neural crest development.

**Metalloprotease cleavage of N-cadherin versus meltrin cleavage of cadherin-11 in the neural crest.**

Although metalloprotease cleavage of N-cadherin was shown to play an important role in trunk neural crest delamination in chick, there are fundamental differences with the ADAM13 cleavage of cadherin-11 in the *Xenopus* CNC (Shoval 2007). First, as described previously, cleavage of cadherin-11 occurs continuously during migration, whereas cleavage of N-cadherin is part of the global down-regulation of this protein required at the onset of migration (Akitaya 1992; Shoval 2007). Second, in the avian neural crest, cleavage of N-cadherin releases the cytoplasmic domain and β-catenin that relocalize to the nucleus to activate the transcription of pro-migratory genes such as cyclin-D1 (Shoval 2007). Conversely, we have shown that cleavage of cadherin-11 does not affect the interaction with β-catenin.

Although β-catenin signaling appears to be important for the initial delamination of the neural crest, it seems to play a different role during neural crest cell migration (de Melker 2004; Shoval 2007). Some signaling through β-catenin is important for the expression of neural crest markers, such as Twist, during migration (Borchers 2001). However, conditional knock out of β-catenin in mouse embryos showed that β-catenin is not required for neural crest cell migration (Brault 2001). In fact, exogenous stimulation of β-catenin via LiCl treatment will stop the migration of avian neural crest *ex vivo* (de Melker 2004). It is possible that cadherin-11 helps to control the “intensity” of β-catenin signaling by sequestering a pool of this molecule at the cell membrane during CNC migration. On the other hand, it has been suggested that signaling through β-catenin is
involved in the differentiation of the neural crest cells once they reach their target locations (Hari 2002; Paratore 2002). Because neural crest differentiation occurs mostly after migration ceases, the interaction with cadherin-11 and β-catenin at the cell membrane may also play a role in the maintenance of an undifferentiated state while the cells are still moving.

**Which meltrin cleaves cadherin-11 during CNC migration?**

Meltrin family members ADAM9, 13, and 19 are all expressed in CNC cells during migration. Because all three of these proteins are active proteases, they could all potentially cleave cadherin-11 during this process. However, our studies *in vitro* reveal that ADAM9 and 13 can cleave cadherin-11, whereas ADAM19 cannot. Additionally, both ADAM9 and 13, but not ADAM19, are capable of rescuing the migration of CNC expressing an excess of cadherin-11 *in vivo*. Yet, resolving which meltrin predominantly cleaves cadherin-11 during CNC migration was complicated by the ability of the ADAMs to compensate for each other’s function.

We have shown further evidence of the compensation among the meltrins *in vivo* by detecting an increase in ADAM9 expression when either ADAM13 or 19 expression is knocked-down *via* morpholino oligonucleotide injection. To prevent the effects of compensation in our experiments, we used combinations of MOs to knock down at least two meltrins at one time. We have shown that injection of a combination of any two ADAM MOs blocks CNC migration in about half of the embryos screened. This phenotype can be rescued by the expression of the cadherin-11 extracellular domain when ADAM13 MO is included in the injection. However, the cadherin-11 extracellular
domain does not rescue embryos with double knockdown of ADAM9 and ADAM19. This distinction suggests that ADAM13 is the main enzyme responsible for cadherin-11 cleavage during CNC migration in *Xenopus*.

On the other hand, the above observation does not explain why depletion of either ADAM9 or ADAM19 can also block CNC migration. In this regard, we observed a decrease in ADAM13 expression in both ADAM9 and ADAM19 depleted embryos, suggesting that there is cross-talk among these meltrins. Thus the CNC migration phenotype, and the increase in uncleaved cadherin-11 levels in ADAM9 and 19 knocked down embryos could be at least partially attributed to this secondary effect on the ADAM13 protein level. Additionally, loss of either ADAM9 or ADAM19 may affect CNC induction. For example, conditional knockout of ADAM19 in the mouse neural crest does not prevent migration, but interferes with the specification of cardiac neural crest cells and the proper morphogenesis of the heart (Komatsu 2007). We have also observed that ADAM19 depletion interferes with CNC specification in *Xenopus*, (Neuner 2008), and this could contribute to the partial inhibition of CNC migration observed here.

We propose that ADAM13 is responsible for the cleavage of cadherin-11 during CNC migration and that other meltrins, such as ADAM9 can compensate for this function when ADAM13 protein expression decreases. These results also suggest that another protein cleaved by the meltrins may be important in the specification and/or migration of the CNC.
Section IV: Cadherin-11 and ADAMs outside the CNC.

Does ADAM regulation of cadherin-11 play a role in non-migratory embryological processes?

Eye development

Cadherin-11 and meltrins are considered to be mesenchymal proteins because they are often expressed in this tissue type. And while the expression of these molecules is restricted to mesenchymal cell types, not all of these are migratory cells like the CNC. Do meltrins interact with cadherin-11 in these other mesenchymal tissues in a similar manner to what we see with the CNC? There are a number of non-migratory tissues that cadherin-11 and meltrins are expressed in during embryogenesis, one example being the cells surrounding the optic vesicle. Meltrin family member ADAM13 is expressed in the mesenchymal cell layer in between the optic vesicle and the overlying epidermal layer in Xenopus embryos (Alfandari 1997). Cadherin-11 is also expressed in this same cell layer in mouse embryos (Kimura 1995). Cadherin-11 expression was found in the general optic vesicle region in Xenopus embryos, but a clear expression analysis of this region has yet to be performed in frogs (Hadeball 1998; Vallin 1998). By western blot analysis, I can detect both cadherin-11 and ADAM13 in eye extract from Xenopus embryos. (Appendix VIII)

Using a morpholino knock-down approach, I have found that cadherin-11 expression may be important for the development of other regions in the eye, most noticeably the pigment epithelium surrounding the optic cup. I have found that knock down of cadherin-11 in the optic vesicle does not alter the development of this tissue at
early stages, but appears to disrupt the differentiation of eye tissues at later stages (Appendix VIII). The differentiation of this eye tissue requires the presence of the overlying epidermis (Arresta 2005). It is possible that the interaction between the cadherin-11 and ADAM13 molecules expressed in the mesenchymal cell layer of the developing eye is involved in the development and differentiation of these cells. In support of the hypothesis that these molecules interact within the eye tissues, I have been able to co-immunoprecipitate small amounts of endogenous ADAM13 with cadherin-11 from embryo eye extract. (Appendix VII).

Further work is required to see whether depletion of ADAM13 in the mesenchymal cells overlying the optic vesicle phenocopies the eye defect seen with cadherin-11 knockdown. Additionally, it will be interesting to see whether ADAM13 interaction with cadherin-11 in the eye acts in the same, or different manner as the ADAM13/cadherin-11 interaction in the CNC. I suspect that the interaction between these proteins in the eye may produce a different biochemical result, since I have not yet seen the clear presence of a cadherin-11 cleavage fragment from eye extract. (Appendix VIII)

It will also be informative to determine whether ADAM13 and cadherin-11 are both expressed in the mesenchymal layer of the developing eye, or in neighboring tissue layers. If cadherin-11 expression in the *Xenopus* eye is different from cadherin-11 expression in the mouse, and it is truly expressed in the optic vesicle, these two molecules would be expressed in adjacent tissues. In this case, it is possible that ADAM13/cadherin-11 mediate a different cellular response whether they are interacting in the same tissue (cis) or neighboring tissues (trans).
Limb development

Another process that cadherin-11 is involved in is the condensation of the limb mesenchyme, which is important for cartilage formation in the developing limb. Although the expression of ADAM13 has not yet been detected in the frog limb bud, a number of other ADAMs have been detected in other model organisms. For example, ADAM12 (α-meltrin), ADAM10, and ADAMTS1 have all been detected in the limb mesenchyme of the chick or mouse models (Lewis 2004; Jin 2007; Thai 2002). Thus far, no studies have focused on the possible interaction between cadherin-11 and ADAM family members in the developing limb. However, cadherin-11 activity during tissue condensation does seem to rely more on the adhesive function (and not the ability to promote signaling). With this in mind, it would be interesting to determine whether cadherin/ADAM interactions occur predominantly in developmental processes that require regulation of cadherin mediated signaling, and not with the processes that mostly utilize their adhesive function. Further understanding of cadherin-11 function in the developing limb will provide an excellent platform in which to answer these sorts of questions.

Summary and concluding remarks.

One goal of the research presented in this dissertation was to further understand the differential roles of classical cadherin molecules in neural crest migration. Most of the previously published literature focused on the role of the classical cadherins, namely N-cadherin and cadherin-6B, which seem to play a role solely on the EMT and delamination
events that initiate NC migration. While the expression of other cadherins (cadherin-11 and cadherin-7) occurs throughout NC migration and appears to be important for this process, very little was known about how these molecules actually promote NC cell migration.

My work has shown that cadherin-11 is continuously processed throughout CNC migration, and that members of the meltrin subfamily of ADAM metalloproteases are responsible for this event. I have also shown that cleavage of cadherin-11 is essential for CNC migration, as inhibition of endogenous cadherin-11 processing also blocks CNC migration. While cleavage of full-length cadherin-11 must play a role in regulating the intensity of cell-cell adhesion mediated by this molecule during CNC migration, it also appears to promote CNC migration through the biological activity of the resulting cleavage fragments.

I have pursued the potential activity of both the cytoplasmic (C-terminal) and extracellular (N-terminal) cleavage fragments in the context of the cell and the embryo. I have found that unlike N-cadherin processing by metalloproteases, cadherin-11 processing by meltrins does not disrupt the ability to bind with cytoplasmic partner β-catenin. This divergence in the behavior among these cadherins may be extremely important for NC migration since β-catenin signaling is important for the progression of EMT, but sustained signaling through this molecule will prevent NC migration at later stages.

Much of my thesis project has focused on the biological activity of the extracellular cleavage fragment (EC1-3) of cadherin-11. Here I have shown that EC1-3 can rescue CNC migration in embryos that have an over-abundance of cadherin-11
mediated cell adhesion. While this activity suggests that EC1-3 functions by acting as a competitive inhibitor for full-length cadherin-11 binding, some of my experiments also indicate that EC1-3 may be promoting cell migration through the activation of an unknown signaling pathway. In this instance, I have found that CNC cells will migrate toward a source secreting EC1-3 \textit{ex vivo}. However, I have yet to resolve whether EC1-3 is acting as a cell attractant or as a stimulant of cell migration.

To conclude, the work presented in this thesis has furthered our understanding of how classical cadherins play a role in CNC cell migration. It also highlights some subtle, but significant, differences among the specific cadherin molecules that are expressed during different stages of NC migration. Lastly, these results support the hypothesis that specific ADAM molecules mediate the function of cadherins during many embryological processes. Future work will show whether ADAM/cadherin interactions are a general feature of cadherin function, or happen predominantly in mesenchymal and migratory cell types.
Figure 6.0: Cranial neural crest cells are presented with various adhesive environments during migration. On the left, the CNC is highlighted on stage 20, 23, and 26 Xenopus embryos. The red lines indicate where the frontal section (illustrated on the left) is made. On the right, illustrations of hypothetical frontal sections. Yellow indicates epidermis; grey indicates the ECM; green indicates the CNC; red indicates the mesoderm (or the head mesenchyme at later stages). The first phase of CNC migration starts in the stage 19-20 Xenopus embryo, and is known as “sheet migration”. As the name implies, the CNC migrates as a cohesive sheet in the ventral direction. At this stage CNC cells are presented with two main adhesive environments, the ECM (grey mesh) or other CNC cells (green). The “outside” layer of CNC cells (dark green) interacts with the ECM surrounding the CNC. These cells should use their integrins to interact with this substrate. The “inside CNC cells (light green), are mostly in contact with other CNC cells. Therefore, these cells should maintain mostly cell-cell adhesions through the use of cadherins, or other cell-cell adhesion molecules. The second phase of CNC migration is known as branch migration. At this stage the CNC travels along distinct pathways in the head. Here, more CNC cells are exposed the environment outside of the CNC. Some of these “outside” cells must interact with ECM molecules. However, since the ECM is processed throughout CNC migration, “outside” CNC cells may also come into contact with cells of the head mesenchyme. During the late stages of CNC migration, individual cells can break away from the CNC arches and invade the head mesenchyme. At this stage, fewer CNC cells have contact with other CNC cells, and more CNC cells are exposed to trace amounts of ECM or to the head mesenchyme. CNC migration on, or through, the head mesenchyme must also rely on cell-cell adhesion molecules. This figure was drawn for this dissertation and has not been published.
Figure 6.1: Differential expression of cadherin molecules during neural crest development. 1. In the neurula stage embryo, E-cadherin (grey) is expressed throughout the ectoderm, and N-cadherin (pink) is expressed in the neural ectoderm. 2. In the late neurula, cadherin-6 (or 6B) is expressed with N-cadherin at the lateral border between the neural plate and the non-neural ectoderm (red). This region will become the neural crest, which will also become the neural folds. 3. The neural folds move toward each other, and fuse along the midline. 4. Upon neural fold fusion, the NC is internalized, and will start to undergo migration in the ventral direction. At this stage N-cadherin and cadherin-6 are removed from the surface of NC cells. The NC “replaces” these cadherins with cadherin-11 (brown) and cadherin-7 (orange) (in a NC subpopulation). This figure was drawn for this dissertation and has not been published.
APPENDIX I
CANDIDATE SUBSTRATES FOR ADAM13 ACTIVITY

At the start point of my thesis work I was presented with the following goal: find a substrate that ADAM13 processes to promote *Xenopus* CNC migration. In collaboration with my mentor D. Alfandari, we chose a number of candidates by searching through the literature. Our initial criterion for a potential substrate was as follows: a candidate should be expressed in the CNC during development; and perturbation of the candidate activity through over or underexpression alters CNC migration. Additional emphasis was put on substrates that play a role in migration and could be processed by a metalloprotease. With these criteria, we pursued Insulin Growth Factor Binding Proteins (IGFBPs) 3 and 5, ephrin ligands B1 and B3, SH2 domain-containing protein tyrosine phosphatase substrate 1 (SHPS-1) (also Sirp-α), and cadherin-11. Further description of each of these candidates, as well as the work I performed on each one before completely pursuing cadherin-11, will be described in the following text.\(^1\) With the exception of IGFBP3 and SHPS-1, all of these molecules had been previously cloned in *Xenopus*. Therefore, it was necessary to clone IGFBP3 and SHPS-1 to pursue them as potential ADAM13 substrates.

IGFBPs 3 and 5

IGFBPs are secreted proteins that interact with Insulin Growth Factors (IGFs) with high affinity. They can either inhibit or potentiate IGF signaling capacity through IGF receptors depending on the IGFBP/IGF interaction. The general domain organization of IGFBPs is depicted in figure A1.1A. The N-terminal domain is responsible for interacting with IGFs, and the C-terminal binds with heparin and has homology with thyroglobulin type I. The central region of IGFBPs is not highly conserved among these proteins, and it is within this region that IGFBPs can be processed by metalloproteases (Cwyfan Hughes 1992). Cleavage of an IGFBP decreases affinity for IGFs and invariably inhibits the affect on IGF signaling (Cwyfan Hughes 1992). IGFBP5 had been previously cloned in *Xenopus*, and IGFBP mRNA was shown to locate to the anterior and dorsal regions in the tailbud embryo (Figure A1.1B). Overexpression of IGFBP5 resulted in an enlargement of head structures via an IGFR mediated mechanism (Pera 2001). IGFBP3 has not been cloned in *Xenopus*, but was shown to localize to migrating CsNC cells in the zebrafish embryo (Li 2005). While the

\(^1\) All of these studies were performed within the first six months of my graduate work with D. Alfandari. After this point my work focused on cadherin-11 as the primary ADAM13 candidate.
role of IGFBP signaling in CNC migration has yet to be studied, we hypothesized that ADAM13 cleavage of these molecules may help regulate the intensity of their signal during this process.

Figure A1.1: General information about IGFBPs. A) Generic domain organization of IGFBPs (taken from Pera 2001). The N-terminal domain (red) interacts with IGFs (consensus sequence indicated). The C-terminal region of IGFBPs has sequence homology with Thyrogobulin type I repeats (blue) and has been shown to interact with heparin. The central region of IGFBP (black line) is processed by metalloproteases. B) Localization of IGFBP5 mRNA detected by in situ hybridization in Xenopus embryos (Pera 2001). IGFBP5 is expressed in a variety of dorsal an anterior structures (such as the somites and the otic vesicle) including regions of the CNC.

IGFBP3 was cloned through PCR amplification using degenerate primers on cDNA libraries from stage 14 and stage 20 Xenopus embryos. Upon the second round of PCR, bands of approximately 600 base pairs (correct size based on location of primers in zebrafish sequence) were amplified (Figure A1.2A). This product was cloned into the TopoTA vector, transformed into XL1-Blue bacteria, and the DNA purified from the resulting colonies was tested for incorporation of the insert by digestion reaction (Figure A1.2B). Colonies positive for the insert were sent out for sequencing. Analysis of the resulting DNA sequences showed high homology with IGFBP5. The two colonies that showed the least DNA sequence homology with IGFBP5, had significant amino acid homology with zebrafish IGFBP3. These two colonies, xBP3.2 and xBP3.7, were grown into 50 ml cultures and their DNA was purified by Simaprep midiprep kit. The resulting DNA was further tested by digestion using SpeI, which cuts a unique site in IGFBP3. Both colonies were positive by this test (Figure A1.2C). This is the extent of the work I did on this molecule before I shifted my focus completely to cadherin-11.
Figure A1.2: Cloning of IGFBP3. A) Detection of amplified DNA bands separated on agarose gel with EtBr from PCR reactions using different combinations of degenerate IGFBP3 primers. The 600 base pair band present in reactions 1-5 is the predicted size of the amplified sequence. The band from colony #2 was cloned into topoTA. B) Detection of fragments dropped in restriction digests of colonies transfected with topo-TA product. All positive colonies were sent out for sequencing. C) Detection of restriction digest from 2 colonies from (B) that were likely to be IGFBP3. Digest was performed to target a unique sequence in IGFBP3. The DNA from both clones was positive for cleavage (lanes 3 and 5).

D. Alfandari had previously cloned Xenopus IGFBP5 by PCR into the CS2 vector adding a Flag tag to the C-terminus. Figure A1.3 depicts the IGFBP5-flag expression from the colonies transformed with this DNA. I attempted to perform co-precipitation study with ADAM13, yet no definitive results were obtained using this construct (data not shown). I also performed preliminary shedding experiments on IGFBP5, but they were not properly controlled (data not shown). My work on this molecule ended with these experiments.

Figure A1.3: Detection of IGFBP5 protein. Western blot analysis detecting the flag-tag on IGFBP5 from extracted cos cells that had been transfected with DNA from bacterial cultures transformed with the IGFBP5 construct.

It should be noted that while my work on both IGFBP3 and IGFBP5 subsided as I studied cadherin-11, I have not definitively shown that these molecules are or are not processed by ADAM13. Therefore, they remain as candidates for this activity.

Ephrin ligands B1 and B3

Ephrin ligands B1 and B3 are membrane-anchored ligands for the Eph receptor tyrosine kinases. The interaction between Eph receptors and their ligands perform forward (Eph mediated) and reverse (ephrin mediated) signaling that can result in
attractive or repulsive behavior in migrating cells (depending on the Eph/ephrin complex). A schematic of ephrin/Eph interactions is depicted in figure A1.4A. ADAM10 cleavage of Eph-A3 ligand, ephrin-A2, in trans was shown to promote the repulsive signal mediated by these molecules (Janes 2005). Both ephrin B1 and B3 ligands are expressed in the *Xenopus* CNC during migration (Helbling 1999) (Figure A1.4B). EphrinB1 loss of function experiments in mouse exhibit cranio-facial defects such as cleft palate (Compagni 2003). We hypothesized that ADAM13 cleavage of ephrin ligands could promote CNC migration by attenuating signaling mediated by Eph/ephrin interactions.

![Diagram of ephrin/Eph interactions](image)

**Figure A1.4:** General information about ephrin-B1 and ephrin-B3: A) Schematic of ephrin/Eph interactions (figure from (Pasquale 2005)). Ephrin B molecules have a single pass through the plasma membrane. The extracellular domain of ephrin-B molecules has both low and high affinity binding sites for Eph receptors. B) Localization of ephrin-B1 and ephrin-B3 mRNA detected by in situ hybridization in *Xenopus* embryos (figure from (Helbling 1998). Both ephrin-B molecules localize to the CNC.

Constructs containing the *Xenopus* ephrin B1 and the ephrin B3 sequences were made by D. Alfandari using PCR and cloned into the PCS2-SS-MT plasmid. This plasmid contains 6 myc epitope immediately after the signal sequence from ADAM13 and a multiple cloning site after the myc tag. Co-transfection of ephrin B1 or B3 with ADAM13 did not produce detectable cleavage fragments by western blot analysis. However, co-overexpression of A13-E/A seems to increase the amount of detectable ephrin B1 and B3 when compared to co-expression with ADAM13 (Figure A1.5). Additional shedding experiments performed by detecting secreted ephrin B1 or B3 in media from ADAM13 or ADAM13-E/A co-transfected cos cells (data not shown). These experiments did not show a detectable increase in the amount of shed ligand. Though,
this may be due to the presence of multiple ADAMs in Cos-7 cells that can digest both Ephrins.

Cos transfection

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WB: 9E10 for myc-tagged ephrins

WB: 15F for A13

**Figure A1.5: Shedding of ephrin B1 and B3 in the presence of ADAM13.** (Top panels) Western blot analysis was performed on cos extract detecting ephrin-B1 and B3 co-transfected with ADAM13 (left) or ADAM13-E/A (right). While multiple bands are detected when B1 and B3 are co-transfected with ADAM13, these bands are also present in the ADAM13-E/A cotransfections. (Bottom panels) Western blot analysis detecting ADAM13 was performed as a control for expression.

It is possible that ADAM13 cleavage of these ligands may occur in trans, and would therefore require the expression of the proper Eph receptor. Therefore, these shedding studies should be repeated in the presence of Eph receptors.

**SHPS-1 (Sirp-α)**

SHPS-1 is a single-pass glycoprotein expressed at the plasma membrane, and is involved in the promotion of pro-migratory signals mediated by Src homology 2 domains of the protein-tyrosine phosphatases (SHP-2). A schematic of SHPS-1 is depicted in figure A1.6. SHPS-1 also interacts with the cell surface molecule CD47, which inhibits cell migration through cell-cell adhesion (Motegi 2003). SHPS-1 is cleaved by metalloproteases, which disrupts the interaction with CD47, and results in the alleviation of inhibition on cell migration. While SHPS-1 has not been cloned in *Xenopus*, SHPS-1 binding partner SHP-2 has, and the mRNA is localized to the CNC during migration (Qian 2008). We hypothesized that ADAM13 cleavage of SHPS-1 may promote CNC migration by inhibiting the interaction with CD47.
Figure A1.6: Domain organization of SHPS-1. (Schematic from Ohnishi et al.) The extracellular region (ECR) of SHPS-1 interacts with SHP-2. SHPS-1 is processed in the juxta-membrane region by metalloproteases (cleavage sequence is labeled). SHPS-1 makes a single pass through the plasma membrane, and has a cytoplasmic domain.

Since SHPS-1 had yet to be cloned in *Xenopus*, much of my work on this molecule focused on performing this task. Cloning of SHPS-1 was carried out using degenerate primers based on the homology between human, mouse, and chicken sequences. Degenerate PCR was performed on a cDNA library from stage 20 *Xenopus* embryos. Following the second round of PCR, a ~300 bp band was detected, and cloned into TopoTA vector. Transfection of XL1-blue cells with this construct resulted in multiple colonies containing the insert (Figure A1.7).

**Figure A1.7: Cloning of Xenopus SHPS-1.** Detection of bands dropped from digestion reaction performed on topoTA colonies. Size of SHPS-1 band should be approximately 350 base pairs long. Positive colonies were sent out for sequencing.

We were also gifted a construct containing the mouse form of SHPS-1 in the pTracer vector and was cloned into the CS2 vector. My initial biochemical studies on this molecule as a candidate for ADAM processing were performed on the mouse construct. Here, I showed that mSHPS-1 selectively binds to ADAM13 and not to ADAM10 (Figure A1.8A). Additionally, H. Cousin from our research group showed that overexpression of mSHPS-1 in *Xenopus* has an inhibitory effect on CNC migration (data not shown). However, my multiple attempts at visualizing SHPS-1 shedding in the presence of ADAM13 were negative (Figure A1.8B). It is possible that SHPS-1 is processed by ADAM13 but I fail to detect it *via* technical error. However the fact that I
see an increase in the presence of SHPS-1 when co-transfected with ADAM13, suggests that ADAM13 interaction with this molecule may be independent of proteolytic activity (Figure A1.8 C). My pursuit of SHPS-1 as a potential substrate for ADAM13 ended with these studies.

**Figure A1.8: SHPS-1 interaction with ADAM13.** A) Western blot analysis detecting SHPS-1 (labeled Sirp) co-precipitated with ADAM13 (top) in immunoprecipitated cos extract. Cos cells were transfected with mSHPS-1 or myc-tagged DC10, ADAM10, or ADAM13. This membrane was reprobed for ADAM13 using the 15F antibody to control for ADAM13 precipitation. Western blots of ConA purified Cos-7 extract were probed for SHPS-1 or myc-tag, to control for transfection efficiency and expression of the constructs. B) Western blot analysis detecting SHPS-1 (top panel) in cos cells co-transfected with ADAM13 or ADAM13-E/A (bottom panel). No cleavage fragments of SHPS-1 were detected in these assays. C) Western blot analysis of extract from cos cells transfected with SHPS-1 and ADAM10 or an ADAM13 mutants. More SHPS-1 is detected when co-transfected with either ADAM13 or ADAM13-E/A.
Cadherin-11

A thorough description of the logic behind cadherin-11 as a candidate substrate has been described in Chapter 1 of this thesis, and for brevity sake, will not be repeated here. We speculated that ADAM13 cleavage of cadherin-11 might promote CNC migration by regulating cell-cell adhesion.

Constructs for *Xenopus* cadherin-11 were provided by our collaborator D. Wedlich, and were cloned into the CS2 vector for our studies. Cadherin-11 cleavage fragments were detected by western blot analysis on extract from cos cells co-transfected with ADAM13 and ADAM9 (Figure A1.9B) using serum from mice that had been immunized with a cadherin-11 fusion protein.

Gamma-secretase is a protease that localizes to cellular membranes, and processes a number of cell-surface molecules (including cadherins) within their transmembrane domain. Incubation of cells co-transfected with cadherin-11 and ADAM13 with a gamma-secretase inhibitor increases the presence of the cadherin-11 cleavage fragment, suggesting that gamma-secretase further processes the c-terminal fragment of cadherin-11 after ADAM processing (Figure A1.9C). In preliminary experiments I also found that co-overexpression of ADAM13 with cadherin-11 could rescue CNC migration *in vivo* (complete results depicted in Chapter II). Combined, these studies presented cadherin-11 as the most promising candidate for ADAM13 processing in the CNC, and my project shifted the focus solely on this molecule. In order to study endogenous cadherin-11, we developed a monoclonal antibody directed against this molecule. The screening of our most highly utilized cadherin-11 monoclonal AB, 1B4, is described in Appendix II.
Figure A1.9: Cadherin-11 is processed in the presence of ADAM9 and ADAM13. A) Western blot detection of cadherin-11 from non-transfected or transfected cos-7 cells using cadherin-11 immunized mouse serum. B) Western blot analysis detecting cadherin-11 (using mouse serum) from cos-7 cells co-transfected with cadherin-11 and ADAM9, ADAM9E/A, ADAM10, ADAM11, ADAM13, or ADAM13E/A. An 80 kDa cadherin-11 cleavage fragment is detected when cadherin-11 is cotransfected with ADAM9 or ADAM13. C) Western blot analysis of shedding experiment with cadherin-11 and ADAM13 in the presence of gamma-secretase inhibitor. Inhibitor was incubated on transfected cos for 0, 6 or 24 hours. 6-hour incubation with the inhibitor increased the amount of cadherin-11 cleavage products in ADAM13 transfected cells.
APPENDIX II

GENERATION OF A MONOCLONAL CADHERIN-11 AB

Due to the positive results obtained with cadherin-11 as a potential substrate to ADAM13, we decided to generate a monoclonal antibody in order to study the endogenous protein in Xenopus. There was no cadherin-11 ABs that could detect frog cadherin-11 at the time. We designed the antibody to immunoreact with the cytoplasmic tail of cadherin-11 because this region of classical cadherins has more variation than the extracellular domain, and we could minimize the potential for cross-reactivity among cadherins. To do so, we expressed and purified a fusion protein containing the Xenopus cadherin-11 cytoplasmic domain (named ΔEC-cad-11), which was used in a series of immunizations in mice. Blood samples were obtained after each immunization to test for reactivity against xcadherin-11. Some of the results for cadherin-11 shown in Appendix I were performed using this serum.

A fusion was performed with splenocytes from an immunoreactive mouse and SP20 cells using the protocol described in “Antibodies: A Laboratory Manual” by Ed Harlow and David Lane. The resulting hybridoma colonies were first screened by ELISA for reactivity with purified ΔEC-cad-11. Of 192 colonies initially tested, 11 colonies had an OD value of 0.3 or higher (strongly reactive) and 14 had an OD value between 0.25 and 0.3. The 1B4 colony (OD 2.7) that was eventually sub-cloned and used in many of my experiments was one of three colonies having an OD value of greater than 1.0. All of the above “hits” were then screened by western blot analysis on extract from cos-7 cells that had been transfected with full-length cadherin-11 (Figure A2.1). Blood serum from the immunoreactive mouse was used as a positive control. Media from the 1B4 colony was strongly positive by western blot analysis.

The 1B4 colony was further tested for immunoreactivity against endogenous cadherin-11 (Figure A2.2). Specificity of 1B4 was challenged by western blot analysis on extract from cos cells transfected with N-cadherin or C-cadherin, two classical cadherin molecules (Figure A2.3 and A2.4). 1B4 was shown to have slight reactivity toward N-cadherin when strongly expressed (Figure A2.4). 1B4 was also tested for the ability to immunoprecipitate cadherin-11 (Figure 2.6). We found that 1B4 does immunoprecipitate cadherin-11. However, we found that 1B4 is not capable of detecting cadherin-11 by immunofluorescence in transfected cos-7 cells (data not shown). The 1B4 colony was subcloned into monoclonal colonies. The colony 1B4-C7 is the monoclonal antibody used throughout the experiments described in this thesis.
Figure A2.1: Western blot screen of hybridoma media on cadherin-11 transfected cos extract. A “curtain gel” was run on cadherin-11 transfected cos extract. Each “lane” was incubated with media from a hybridoma colony ELISA “hit”. Blood serum from the immunized mouse was used as a positive control. Lane 4 shows the reactivity of 1B4 by western blot analysis. This colony was the strongest hit of all the hybridomas.

Figure A2.2: 1B4 clone detects overexpressed and endogenous cadherin-11. A) Western blot analysis on extract from stage 6 and stage 22 Xenopus embryos using 1B4 (polyclonal). Xenopus embryos do not express cadherin-11 at stage 6, but do express it strongly by stage 22. 1B4 is immunoreactive with a 120 kDa and ~50 kDa proteins at stage 22. B) Western blot analysis on extract from transfected Cos-7 cells. Lane 1 is non-transfected, lane 2 is transfected with cadherin-11. 1B4 detects the 120 kDa overexpressed cadherin-11 protein.
Figure A2.3: **1B4 is specifically reactive to cadherin-11 and not to N-cadherin or C-cadherin.** Western blot analysis using the 1B4 polyclonal antibody on Cos-7 cells transfected with N-cadherin, C-cadherin, and cadherin-11. Samples were run in duplicate. 1B4 strongly interacts with cadherin-11 when compared to N-cadherin or C-cadherin by western blot.

Figure A2.4 **1B4 has slight reactivity toward N-cadherin.** (top panel) Western blot analysis using 1B4 polyclonal AB on extract from cos-7 cells transfected with N-cadherin (lane 1) or cadherin-11 (lane 2), or non-transfected (lane 3). 1B4 can detect the 140 kDa N-cadherin band. (bottom panel) Western blot analysis detecting the myc-tag on N-cadherin shows that N-cadherin expression is very high in this transfection.
**Figure A2.5: 1B4 immunoprecipitates cadherin-11 from Cos-7 extract.** Western blot analysis using biotinylated-1B4 antibody to detect cadherin-11 from transfected cos-7 extract immunoprecipitated with 1B4. The biotinylated-1B4 allowed us to detect cadherin-11 in 1B4-precipitated samples because we could use streptavidin-HRP to detect the immunoprecipitated protein. This alleviated the strong detection of Ig bands that would have been present using a mouse secondary antibody. The ability of 1B4 to precipitate cadherin-11 has been further confirmed using a rabbit polyclonal antibody directed against xcadherin-11 by western blot analysis (data not shown).
APPENDIX III

ATTEMPTED GENERATION OF A NON-CLEAVABLE CADHERIN-11 MUTANT

We have been challenged with creating a non-cleavable form of cadherin-11. First, we do not know exactly where the cadherin-11 molecule is being cleaved by ADAM13, so we do not know the structure or sequence of this location. By approximation based on size, our results suggest that the cleavage is within the linker-region between EC3 and EC4, which is comprised of 12 amino acids. We have aligned this region with the cleavage sites of all known substrates of ADAM13 but no sequence similarity is present. However, it is important to note that there is no known consensus sequence for ADAM proteolysis. The cleavage-site structure and distance from the membrane appear to be the most important factors for this interaction.

We designed a “non-cleavable” mutant based on our general idea of the cleavage site. We removed a region of the linker between the EC3 and EC4 domains, and replaced it with a myc-tag (10 amino acids). A shedding experiment was performed Cos cells transfected with the cadherin-11 mutant and ADAM13 to see whether the mutant was resistant to proteolysis. Unexpectedly, the cadherin-11 mutant had substantially more cleavage fragment present than wild-type cadherin (Figure 3.1). This unexpected outcome is probably caused by a structural alteration within the cleavage region of cadherin-11, which makes it more vulnerable to proteolysis. So, in order to decrease the possibility of any large-scale structural changes additional attempts to make a non-cleavable mutant were done through the generation of point mutations within the linker region. However, these mutants were all also capable of being cleaved. While these attempts of making a non-cleavable form of cadherin-11 were not fruitful, we feel that the assays performed with the small molecule ADAM inhibitor, as well as with the ADAM morpholinos, strongly suggest that cadherin-11 cleavage is essential for CNC migration.
Figure 3.1: Generation of a non-cleavable form of cadherin-11 was unsuccessful. In order to generate a non-cleavable mutant of cadherin-11 a sequence within the EC3-EC4 linker, the general region of cadherin-11 cleavage, was removed and replaced with a myc-tag. A shedding experiment in transfected Cos cells was performed to analyze the levels of Cad-11-myc cleavage compared to WT cadherin-11 in the presence of ADAM13. Cad-11-myc had a very pronounced band (lane 5) representing the cleavage fragment of cadherin-11 when compared to the WT molecule (lane 2). Control western analysis was performed on ADAM13, and PACSIN2 as a loading control.
APPENDIX IV

CADHERIN-11/ADAM INTERACTIONS

Part I: Cadherin-11 ADAM interactions

Table A4.1: Chart of interactions found between ADAM family members and cadherin-11 in tissue culture and in vivo studies. Column 1, name of ADAM family member; Column 2, ability of ADAM to cleave cadherin-11 in tissue culture; Column 3, ability of ADAM to co-precipitate with cadherin-11. P = pro-form, M = mature form, 50 kDa, and 30 kDa represent the different forms of the ADAM that can co-precipitate. Noted in parenthesis is the tissue type used for immunoprecipitation (cos or embryo). Column 4, ability of EC1-3 region of cadherin-11 to bind to cos cells expressing each ADAM.
Part II: Interaction of cadherin-11 and ADAM13/ADAM10 chimeras.

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B

Figure A4.2: ADAM13/ADAM10 Chimeras co-precipitate with cadherin-11. A) Domain organization of ADAM13/ADAM10 chimeras. These chimeras were used to determine which region of ADAM13 is required for binding to cadherin-11. B) Western blot analysis was used to detect which forms of ADAM13/ADAM10 co-precipitate with cadherin-11 when overexpressed in tissue culture. ADAM13, ADAM10, 1013DC, 1013DC-E/A, 1013, 1310 constructs all contained a myc-tag which was used for detection with the 9E10 antibody. “Cont” lane refers to the negative, (non-transfected) control. Note that only the mature form of ADAM13 can be detected by western blot analysis. Since ADAM10 also co-precipitates with cadherin-11, it is not surprising that the ADAM13/ADAM10 chimeras also co-precipitate.
Figure A4.3: EC1-3 does not bind to ADAM13/ADAM10 chimeras or ADAM10 expressing tissue culture cells. This figure shows a binding experiment performed in tissue culture cells transfected with RFP and ADAM13, 1013, 1310, 1013DC, and ADAM10. RFP DNA was included to control for transfection efficiency. The binding experiment was performed as described in the materials and methods of chapter 3. EC1-3 was detected with 9E10 primary antibody, and anti-mouse-FITC secondary (Green fluorescence). DAPI (blue) was used to detect nuclei. Red fluorescence detects RFP.

This data shows that while the full-length chimeras and ADAM10 co-precipitate with cadherin-11, EC1-3 of cadherin-11 does not bind. This could suggest that cadherin-11 possesses multiple domains that can interact with ADAMs. ADAM13 can, at the very least, bind to the extracellular domain of cadherin-11. On the other hand, ADAM10 and the chimeras cannot bind to this region. Alternately, it is possible that very little of ADAM10 or the chimeras are expressed at the cell surface, and cannot interact with EC1-3 suspended in the tissue culture media. This hypothesis is supported by the western blot shown in Figure A3.1 where only the Pro-form of these molecules co-precipitate with cadherin-11. Only the mature forms of ADAMs are expressed at the cell surface.
Figure A4.4: ADAM9 co-precipitates with cadherin-11. Western blot analysis detecting ADAM9 and ADAM9-E/A proteins co-precipitated with cadherin-11 from transfected cos extract. To control for background bands, lane 1 was transfected with cadherin-11 alone. Both the pro and mature forms of ADAM9 co-precipitate with cadherin-11.
APPENDIX V

THE EFFECT OF SINGLE ADAM KNOCK DOWN ON CNC MIGRATION

Figure A5.1: Knockdown of meltrin family members ADAM9, ADAM13, ADAM19 through the use of morpholino oligonucleotides inhibits CNC migration. Each of these ADAMs are expressed in the CNC (see more detail in chapter II). (A) Images are a lateral view of stage 26 embryos that had been co-injected into the D1-2 cell at the 16-cell stage with mRNA encoding GFP and morpholinos directed against ADAM9, 13, or 19 (1 ng Mo per injection). (B) Histogram illustrating how ADAM knockdown can inhibit CNC migration. Bars represent the percent of embryos that had CNC migration. In all injection sets 1 ng of total Mo was used, to minimize the potential for toxicity. Injection of morpholinos against a single ADAM was most inhibitory when directed against ADAM13 (46%), and least inhibitory when directed against ADAM19 (67%). Coinjection of Mo directed against ADAM13 with either ADAM9 or ADAM19 Mo, substantially decreased the amount of migrating CNCs compared to A9 or A19 alone. Knockdown of all three meltrins had an additive effect on inhibiting migration (33% migrated). Results are a combination of three individual experiments. Error bars are the standard deviation among each group. N-values for each injection group are as follows (GFP = 47; A9 Mo = 56; A13 Mo = 69; A19 Mo = 67; A9+A13 Mo = 76; A9 + A19 Mo = 66; A13 + A19 Mo = 69; A9 + A13 + A19 Mo (3Mo) = 87)
APPENDIX VI

DESCRIPTION OF THE METHODOLOGY USED TO QUANTIFY CNC CELL EXPLANT CELL MIGRATION AND DIRECTIONAL MOVEMENT TOWARD A SOURCE.

Section I: Term definitions

Actual cell path: (ACP) The path of a migrating cell over a certain time frame. This path represents the x,y coordinates of the cell’s location at each time point.

Angle difference: The angle that measures the distance between the Actual cell trajectory and the Ideal cell trajectory.

Cell displacement: (CD) The vector representing the total directional displacement of a cell during a specific time frame. This vector is obtained by connecting the first and last points of the actual cell path.

Cell directionality: The overall direction of a cell’s movement. This movement does not refer to cell movement toward a specific target. (see Directional migration)

Directed cell movement: (DCM) A term describing the movement of a cell who’s Actual cell path length is close to the length of cell displacement. A cell with DCM must have a CD/ACP ratio of 0.5 or greater.

Directional migration: Migration of a cell moving toward a specific target. Cells with both DCM and RCM can have directional migration. A cell is considered to have directional migration if the angle difference is 30 degrees or less.

Ideal cell trajectory: (ICT) A vector representing the optimal trajectory a cell would take migrating directly toward a target (in these studies a negative control or EC1-3 source).

Random cell movement: (RCM) A term describing the movement of a cell who’s Actual cell path length is much greater than the Cell displacement. A cell with RCM has a CD/ACP ratio of less than 0.5.
Section II: Description of quantification method to determine cell directionality trends in CNC explants.

A) Trajectories from migrating CNC explant cells were separated into two groups, cells with a Random cell movement (RCM), and those with Directed cell movement (DCM). Here, the cell displacement (CD) (red lines) was compared to the actual cell path (ACP) (blue lines) between two time points (T1, T2). The ratio between these two distances was taken, and cells with a ratio of less than 0.5 were considered to have RCM, while cells with a ratio of 0.5 or greater move with DCM.

B) Determination of directional migration toward a source of animal cap cells. The CD (black lines) is the vector connecting the position of the cell at the first and last time-points of the ACP. The ideal cell trajectory (ICT) (red line for cell 1, blue line for cell 2), is generated by connecting the first time-point of the cell to the center of the source. The angle between the CD and the ICT was taken for each cell, and is termed the “angle difference”. Cells with an angle difference of 30° or less are considered to be moving toward the source (ex. Cell 2). This range of accepted angles for directional movement was designed to correct for the fact that the source is not a single point, but rather a larger target of roughly 100 µm in diameter. Using this method we can easily distinguish Cell 1, which moves on a trajectory away from the source, and Cell 2 that moves on a path toward the source.

Figure A6.0: Development of a quantitative method to determine CNC cell directionality toward a source secreting Ec1-3.
APPENDIX VII

IS EC1-3 AFFECT ON CNC MIGRATION MEDIATED THROUGH FGFR1?

The objective of the following studies was to determine whether the pro-migratory affect EC1-3 has on CNC migration is mediated through the FGF Receptor-1 (FGFR1). There are multiple pieces of evidence that support the hypothesis that EC1-3, or at the very least cadherin-11, may interact with this receptor during CNC development. First, FGFR1 signaling does play an active role in CNC induction and is expressed during migration of these cells. (Monsoro-Burq 2003). FGFR1 signaling also stimulates cell migration. It has been shown to mediate chemotaxis of mouse CNC cells toward a target secreting FGFs (Kubota 2000). Recently, full-length cadherin-11 was shown to bind to FGFR1 and activate downstream signaling (Boscher 2008). This interaction may be mediated through the HAV “adhesive sequence” that is present in the extracellular domain of both FGFR1 and classical cadherin molecules (Byers 1992). In addition, the extracellular fragment of N-cadherin was shown to bind to and activate FGF receptors, resulting in the activation of the PI3K and Akt pathways (Lyon 2008). I performed all of the experiments in this chapter. None of the figures in this appendix have been published.

Section I: Results

To pursue the possibility that EC1-3 was acting through the FGFR1 receptor, we decided to perform a “bait and choose” assay in the presence of a dominant negative form of FGFR1 (R1-Fc). The R1-Fc mutant is the extracellular domain of FGFR1, which is secreted into the media, and blocks FGFR1 signaling by sequestering ligand (Marics 2002). However, before these studies were conducted it was important to first show that the CNC is properly induced when R1-Fc is expressed since Harland et al showed that inhibition of FGFR1 via the expression of a transmembrane “dominant negative” mutant form can block induction of the CNC. To make sure that any effect we observed in the bait and choose assay with R1-Fc was not a result of defects in CNC induction, we performed in situ hybridization for xSlug, a CNC marker that was shown to be downregulated when FGFR1 activity is blocked (Monsoro-Burq 2003). Importantly, the amount of mutant expressed by these cells is not enough to block the induction of xSlug in the CNC in vivo. (Figure A7.1A) To further show that R1-Fc CNC cells were induced properly, we also performed an in vivo migration assay of CNC cells expressing R1-Fc along with RFP. In this assay R1-Fc CNC cells migrated just as efficiently as WT CNC cells (Figure A7.1A). Additionally, cranial cartilage development occurs normally in these embryos (Figure A4.1.1B). However, R1-Fc does decrease FGFR1 signaling, since larger doses of this mutant blocks the expression of xBra (a gene downstream of FGFR1) in the blastopore lip, as well as cause severe gastrulation defects shown to occur in (Marics 2002). (Figure 7.2)

Knowing that R1-FC does not inhibit CNC induction, it was safe to use in a bait and choose assay and trust that any affect of cell migration was caused by inhibition of FGFR1-signaling and not caused by an earlier developmental defect. “Bait and choose” assays were performed in the presence of the dominant negative form of the FGF
receptor, R1-Fc. We have shown that this mutant is efficiently secreted into the media by cells expressing it (Figure A7.3A). Our results show that R1-Fc inhibits CNC cell attraction toward both sources in the bait and choose assay (Figure A7.3B). We have also attempted this assay with an FGFR1 small molecule inhibitor (data not shown), yet so far we have been challenged with finding the proper concentration that does not completely inhibit single cell migration. Regardless, the above result suggests that R1-Fc inhibits the pro-migratory signal of EC1-3. However, further analysis of CNC migration in the presence of R1-Fc will need to be conducted in single source movies to determine whether R1-Fc inhibits the phenomenon we observe in cell migration when placed next to a source secreting EC1-3.

**EC1-3 does not detectably stimulate MAPK or Akt signaling in embryos.**

Use of the dominant negative FGFR1, inhibits the ability of CNC cells to migrate toward both sources in the bait and choose assay. This inhibition may be partially caused by sequestering FGF molecules secreted by the sources and blocking their ability to support a directional cue to the migrating CNC cells. Additionally, EC1-3 could stimulate cell migration by acting through FGFR1, which is also inhibited by R1-Fc. We suspect that EC1-3 may act in a similar manner to full-length cadherin-11, which has been shown to bind to and activate the FGFR1 receptor in tissue culture (Boscher 2008). It is possible that EC1-3 stimulates CNC cell migration through FGFR1 since both of these molecules are expressed in the CNC during this process.

In order to analyze the possibility that FGFR signaling is stimulated in the presence of EC1-3 we overexpressed these molecules in embryos or in tissue culture and performed biochemical analysis on markers downstream of FGFR. First we performed western blot analysis probing for phosphorylated MAPK and phosphorylated Akt (Figure A7.4). These molecules are downstream of FGFR1, and had been previously shown to be phosphorylated upon the activation of FGFR1 by full-length cadherin-11 (Boscher 2008). Our preliminary results show that EC1-3 does not detectably stimulate the phosphorylation of these proteins above background. However, co-expression of cadherin-11 with FGFR1 also does not stimulate signaling in these experiments. It should be noted that the effect Boscher and colleagues reported on MAPK and Akt stimulation by cadherin-11 interacting with FGFR1 was subtle. It is possible with future repeats of this assay we will refine our technique and be able to detect these changes. Alternately, it is also possible that we will not be able to detect alterations in MAPK and Akt activity because FGFR1 signaling (via FGFs) is already saturated in our assays.

**Cadherin-11, but not EC1-3 may interact with FGFR1 to promote the formation of cartilage.**

The expression of full-length cadherin-11, and the activation of FGFR1 have been separately shown to promote the formation of cartilage (Kii et al, Petiot et al). We hypothesized that EC1-3 activation of FGFR1 may also promote the formation of cartilage. In order to pursue this possibility, we made animal cap “sandwiches” from embryos expressing either EC1-3 or cadherin-11 with animal caps expressing FGFR1 alone or with FGFR1-Fc and stained them with alcian blue (Figure A7.5). Alcian blue binds to glycoproteins in the ECM and at the cell membrane, and is used as a cartilage stain because it has strong interactions with proteins in this tissue (though which proteins
it interacts with are unknown). In this assay, we found that EC1-3 alone or with FGFR1 did not increase alcian blue intensity when compared to the negative control. However, cadherin-11 and FGFR1 expression does increase the intensity of alcian blue staining, while cadherin-11 alone has only background staining. The increase in staining that was seen in cadherin-11 and FGFR1 animal caps is inhibited by the coexpression of the dominant negative FGFR1-Fc.

While the above result suggests that cadherin-11 and FGFR1 may interact to promote the differentiation of a specific CNC tissue type, more experiments need to be conducted to support this hypothesis. One experiment that we would like to conduct is similar to the one described above. However, instead of using alcian blue stain as a marker for cartilage formation we would perform this experiment using immunofluorescence to detect Collagen II expression in animal cap cells expressing cadherin-11 and the FGFR1 receptor. Collagen II is an extracellular matrix molecule that is expressed in early cartilage formation and has been shown to be induced with FGFR1 signaling (Wang 2001). We would also like to perform western blot analysis, and potentially quantitative-PCR, to determine whether collagen II and other proteins expressed in cartilage is increased in embryos that are expressing cadherin-11 and FGFR1.

Section II: Discussion

Here, we have shown that blocking FGFR signaling through the expression of a dominant negative mutant, attenuates the effects we see when incubating the CNC explant with an EC1-3 source. These studies suggest that EC1-3, or a molecule it induces, signals through the FGF receptor. It is possible that EC1-3 is directly binding to FGFR1 to promote cell migration, since full-length cadherin-11 was shown to bind to and activate this receptor in tissue culture (Boscher 2008).

Alternately, EC1-3 may bind to and activate different receptors that could then promote the expression of a molecule, such as a FGF, that stimulates FGFR1. Indeed, the extracellular cleavage fragment of other classical cadherins has been shown to directly interact with a diversity of cell surface receptors. CEC1-5, the extracellular domain of C-cadherin, binds to full-length C-cadherin. This interaction disrupts C-cadherins cytoplasmic interaction with aPKC, altering aPKCs phosphorylation state and signaling capabilities (Seifert 2009). We have shown that the extracellular cleavage fragment of cadherin-11 binds to a number of cell surface molecules such as full-length cadherin-11, ADAM13, and ADAM19 (Chapter III). Additionally, the extracellular cleavage fragment of E-cadherin binds and activates the ErbB receptors, Her2 and Her3, resulting in the stimulation of cell migration and cell proliferation (Najy 2008). However it is clear that we will need to conduct more studies to resolve whether EC1-3 does stimulate FGFR1 signaling.

One interesting observation that was also made in the studies describe in this Appendix was that full-length cadherin-11, when co-expressed with FGFR1, may promote the formation of cartilage. This phenomenon does not occur when EC1-3 is co-expressed with FGFR1. This could suggest that only full-length cadherin can promote signaling through FGFR1. Alternately, cartilage formation may require both cadherin-11 mediated FGFR1 signaling in addition to tissue condensation promoted by full-length...
cadherin-11 homophilic bonds. Some of these questions will be answered as we determine whether EC1-3 can stimulate FGFR1 signaling.

**Figure A7.1** R1-Fc does not inhibit CNC induction, migration, or differentiation in vivo. A) *In vivo* analysis of CNC induction and migration in embryos expressing R1-Fc (right panels). Top panels show representative images of in situ hybridization for Slug in stage 20 embryos. Embryos were injected at the 8-cell stage into CNC precursor cell, with mRNA for β-galactosidase alone (left), or with R1-Fc (right). The side of injection is marked with white arrow. Lower panels represent *in vivo* assay for CNC cell migration where embryos were injected at the 16-cell stage with mRNA encoding RFP alone (left), or with (0.25 ng) R1-Fc (right). CNC branches are marked with white arrows. B) Late stage analysis of cartilage formation in embryos expressing R1-Fc (lower panels). Embryos were injected on one side of the embryo at the 8-cell stage, and fixed at stage 45 for alcian blue staining.
Figure A7.2: Injection of FGFR1-Fc does inhibit signaling downstream of FGFR1. This assay is a repeat of the one published by Marics et al, and was performed solely for the purpose of ensuring that this molecule does inhibit FGFR1 signaling. A) and B) Embryos were injected at the 1-cell stage with 1 ng of FGFR1-Fc mRNA and 0.5 ng of β-gal mRNA (or β-gal mRNA alone). Some embryos were then fixed at gastrula stage (11.5), for β-gal reaction to detect site of injection, followed by in situ hybridization for xBrachyury. Other embryos were allowed to develop until stage 24 before scoring developmental defects. A) Images of stage 25 embryos from β-gal (left) or β-gal and FGFR1-Fc (right) injected embryos. 0 of 7 positive control embryos exhibited gastrulation defects, 5 out of 8 FGFR1-Fc injected embryos exhibited gastrulation defects. B) Representative images of xBra in situ hybridization of injected embryos. 90% of the β-gal embryos (left) exhibited normal xBra expression on the injected side (N = 40), while only 50% of the FGFR1-FC embryos (right) exhibited normal xBra expression on the injected side (N = 41). The results in A) and B) repeat those shown in Marics et al 2002
Figure A7.3: Expression of a dominant negative FGFR1 receptor prevents CNC cell attraction toward sources. A) Western blot analysis detecting the expression of R1-Fc in the culture media of cells transfected with empty vector (lane 1), or R1-Fc (lane 2). B) Rose diagrams from bait and choose assay of CNC explants expressing R1-Fc. Top panels represent directionality toward the GFP-mt source. Lower panels represent cell directionality toward EC1-3 source. Rose diagrams combine the data from 10 movies.
Figure A7.4 EC1-3 does not detectably stimulate MAPK phosphorylation in embryos or Akt phosphorylation in tissue culture. A) Western blot analysis detecting phosphorylated MAPK, cadherin-11, the myc-tag on EC1-3, GAPDH as a loading control, and PACSIN-2 as another loading control. Embryos were injected at the 1 cell stage with mRNA (0.5 ng each) encoding cadherin-11, EC1-3, FGFR1 (R1), or the dominant negative R1 (combinations for each injection set is marked with a “+” on the lane). B) Western blot analysis detecting phosphorylated Akt of transfected cos cells. Cells transfected with FGFR1 were cotransfected with cadherin-11 and incubated with cos or DN-R1 media, or FGFR1 cells were incubated with EC1-3 media alone or with DN-R1 media. GAPDH western serves as a loading control. In both A) and B) the presence of cadherin-11 serves as a positive control, and Fc-R1 (DN-R1) serves as a negative control. However, coexpression of cadherin-11 with FGFR1 did not appear to stimulate downstream signaling through MAPK or Akt activation as had been previously reported by Bosher et al.
Figure A7.5 Cadherin-11 and FGFR1 but not EC1-3 and FGFR1 may promote the formation of cartilage. A) Alcian blue staining of “animal cap sandwiches” made by sandwiching an RFP expressing animal cap with a GFP expressing animal cap. RFP animal caps were injected at the 4-cell stage in each balstomere with RFP alone, with cadherin-11, or with EC1-3 (0.25 ng each RNA/injection). GFP expressing animal caps were made by injecting GFP mRNA alone, with FGFR1, or with FGFR1 and FGFR1-Fc. Sandwiches were incubated until whole embryo controls were at stage 30, and control images (B) were taken to control for protein expression in each animal cap, before fixing and staining with alcian blue.
APPENDIX VIII

CADHERIN-11 AND ADAM13 IN THE DEVELOPING EYE

Figure A8.1: ADAM13 and Cadherin-11 colocalize to the mesenchymal layer separating the optic vesicle from the overlying epidermis. (A) Illustration of a transverse section through the anterior region of a Xenopus embryo. The optic vesicle (OV) is located lateral to the neural tube (NT) and the notochord (N) and is separated from these structures by the head mesenchyme. The mesenchyme surrounding the optic vesicle is referred to as the periocular mesenchyme, which mostly consists of CNC cells and is highly migratory. The lateral region of the OV is covered with an epidermis, which consists of a mesenchymal layer and an epidermal layer. (B) In frogs ADAM13 localizes to the epidermal layer covering the OV (Alfandari 1997). ADAM13 expression appears to locate to the mesenchymal layer under the epidermal layer. (C) Cadherin-11 expression in the optic vesicle region has also been studied in mice. In this model, cadherin-11 is also located to the mesenchymal layer covering the OV. In frogs, cadherin-11 expression has
also been seen in the OV region (Vallin 1998; Hadeball 1998), though a detailed analysis of the expression in this region of frogs has yet to be performed.

**Figure A8.2: Cadherin-11 and ADAM13 can be detected in Xenopus OV extract.** (A) Western blot analysis probing for cadherin-11 (top panel) or ADAM13 (bottom panel) from OV extract of stage 26 (lane 1) or stage 29 (lane 2) embryos (20 eyes per lane). Optic vesicles dissections contained the overlying epidermal layers, but not the periocular mesenchyme. Cadherin-11 expression is consistently expressed in the OV of both stage 26 and 29 embryos (detected with 1B4 antibody). Thus far, I have not been able to detect the 80 kDa cleavage fragment of cadherin-11 from OV extract. ADAM13 expression can also be detected (15F antibody) at both stages, with slightly less protein detected at the later stage. (B) Cadherin-11 expressed in the OV binds to ADAM13 expressed in the OV. Western blot analysis detecting cadherin-11 (top panel) or ADAM13 (bottom panel), from OV (lane 1) or whole embryo (lane 2) extract immunoprecipitated for cadherin-11. (10 OV or whole embryos per ip). While cadherin-11 expression is too faint to be detected in the IP of OV extract (top panel, lane 1), ADAM13 faintly but detectably coimmunoprecipitates (lower panel, lane 1). Surprisingly, it appears that the pro form of ADAM13 predominantly co-immunoprecipitations with cadherin-11 from the OV, while both forms of ADAM13 co-immunoprecipitation from embryo extract (lower panel, lane 2).
Figure A8.3: Cadherin-11 knockdown inhibits eye development. External view shows the developing eye from a stage 30 *Xenopus* embryo that had been injected at an early stage (16-cell) with morpholinos targeting cadherin-11 and mRNA for RFP protein. OV area is highlighted in white. While early development of the OV is apparently normal (unpublished observations) in the cadherin-11 Mo embryos, the later stages of differentiation are affected. OV cells knocked down for cadherin-11, located by RFP florescence, have inhibited formation of the dark pigment-layer surrounding the optic cup of the developing eye.
APPENDIX IX

LIST OF SUPPLEMENTARY MOVIE FILES

From Chapter II:

Movie 1: Migration of wild type CNC explant *ex vivo*. This is a 7.5-hour time laps analysis of CNC explants removed from stage 17 embryos and placed on a 2-dimensional fibronectin substrate. Images shown are every half-hour from T=0 to 7.5 hours. This movie is of a WT explant (representative of 4/5 movies, where one movie showed slightly inhibited migration).

Movie 2: *Ex vivo* migration of CNC explant overexpressing cadherin-11. This is a 7.5-hour time laps analysis of CNC explants removed from stage 17 embryos and placed on a 2-dimensional fibronectin substrate. Images shown are every half-hour from T=0 to 7.5 hours. This movie is of the migration of an explant overexpressing cadherin-11 (representative of 7/9 movies, where 2 movies had generally normal migration).

Movie 3: *Ex vivo* migration of CNC explant depleted of cadherin-11. This is a 7.5-hour time laps analysis of CNC explants removed from stage 17 embryos and placed on a 2-dimensional fibronectin substrate. Images shown are every half-hour from T=0 to 7.5 hours. This movie is of the migration of an explant morpholino knocked-down for cadherin-11 (representative of 9/10 movies, where one explant had inhibited migration).

From Chapter III:

Movie 4: Cadherin-11-GFP expressing Xtc cell treated with EC1-3. This is a 40-minute time-lapse movie of cadherin-11-GFP fluorescence in a transfected Xtc cell. Images were taken every 30 seconds using a 40x objective. Flash indicates when EC1-3 media was added to the tissue culture media. EC1-3 was added 25 minutes from movie start.

From Chapter IV:

Movie 5: *Ex vivo* migration of a WT explant. This is a 6-hour time-lapse analysis of a wild type CNC explant removed from stage 17 embryos and placed on a 2-dimensional fibronectin substrate.

Movie 6: *Ex vivo* migration of an explant overexpressing EC1-3 (exhibiting retractile behavior). This is a 6-hour time-lapse analysis of a CNC explant overexpressing EC1-3 and removed from stage 17 embryos and placed on a 2-dimensional fibronectin substrate.
Movie 7: *Ex vivo* migration of an explant overexpressing EC1-3 (exhibiting “pinwheel” migration behavior). This is a 6-hour time-lapse analysis of a CNC explant overexpressing EC1-3 and removed from stage 17 embryos and placed on a 2-dimensional fibronectin substrate.

Movie 8: *Ex vivo* migration of a CNC explant placed next to a negative control source. This is a 3-hour time-lapse analysis of a wild type CNC explant and removed from stage 17 embryos and placed next to a negative control source on a 2-dimensional fibronectin substrate.

Movie 9: *Ex vivo* migration of a CNC explant placed next to an EC1-3 source. This is a 3-hour time-lapse analysis of a wild type CNC explant and removed from stage 17 embryos and placed next to a source secreting EC1-3 on a 2-dimensional fibronectin substrate.
APPENDIX X
REPRINT 1

Mini-Review

Life after proteolysis
Exploring the signaling capabilities of classical cadherin cleavage fragments

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Abbreviations: ADAM, MME; NTE, CTIF, PS-1, CNG, ECM, FGF, P3K, ErbB, Her2 and Her3, CREB, CBP

Key words: N-cadherin, E-cadherin, cadherin-11, ADAM, proteases, proteolysis, signaling, cell migration

Classical cadherins are a group of Ca++ dependent transmembrane cell adhesion molecules, mostly known for their ability to perform homophilic interactions with like-cadherin molecules on the surface of neighboring cells. Over the past decade, many studies have also established cadherins as key players of intracellular signaling events by modifying the activity of Rho GTPases, members of the Wnt signaling pathway, and receptor tyrosine kinases. Given the utility of these molecules, it is not surprising that they play multiple roles during different embryological and adult processes. Yet, these activities have been primarily tied to their full-length molecules. And, while the activity of full-length molecules is undoubtedly an essential part of how cadherins perform in vivo, it is becoming increasingly evident that the proteolytic fragments of these molecules may also play a role. This is an exciting development because proteolysis of cadherins was previously thought to be a simple clearing-mechanism meant to regulate the levels of cadherin molecules on the cell-surface.

Here, we will further discuss our recent findings by McCusker and colleagues, showing that both N-terminal and C-terminal fragments of cadherin-11 retain biological activity in Xenopus embryos. We will also review the current literature demonstrating that both the extracellular and intracellular fragments of other classical cadherins are capable of activating certain signaling events tied to Epithelial to Mesenchymal Transitions (EMTs), cell survival, cell proliferation and cell migration.

Proteolysis of Classical Cadherins

Complete processing of a full-length cadherin molecule involves multiple proteases. It is generally accepted that the extracellular region is processed first, and that multiple metalloproteases containing a disintegrin domain (ADAM), Matrix Metalloproteases (MMPs), and other transmembrane proteases can perform this event.1,4 This initial cleavage results in the shedding of the extracellular N-terminal fragment (NTF), and the generation of a first C-terminal fragment (CTF1) that contains the transmembrane and cytoplasmic domains of the molecule. In many cases CTF1 is further processed by the Psenelin-1 (PS-1) complex in the juxta-membrane region, releasing the cytoplasmic domain (CTF2) along with any associated protein.6,7,8 Among the classical cadherins, there are multiple examples showing that the NTE, CTIF1 and CTF2 can each perform diverse biological activities (Fig. 1).

Biological Activities of Shed Cadherin Fragments

In the recent publication by McCusker and colleagues, we showed that cadherin-11 cleavage by ADAM metalloproteases (ADAM9, and 13) was essential for Cranial Neural Crest (CNC) migration in vivo.6 One likely purpose of this processing is to control the overall cell-adhesion levels mediated by the full-length cadherin-11 molecules. Yet, we have also discovered that the NTF itself can promote migration in vivo. In Xenopus embryos, CNC migration is inhibited when full-length cadherin-11 is overexpressed or when ADAM9, 13 and 19 expression are knocked down. This phenotype can be rescued by overexpressing the cadherin-11 NTE. We suspect that the cadherin-11 NTF acts as an antagonist of homophilic interactions since we have shown that it binds to full-length cadherin-11 in cell culture.6 Interestingly, we have also found that the NTF can bind to select members of the ADAM family (McCusker and Alfantadi, unpublished observations), regardless of their ability to process full-length cadherin-11. Future work will determine if this interaction affects the function of these ADAMs and if this interaction plays a physiological role in the embryo.

The NTFs of both N-cadherin and E-cadherin have also been shown to have biological activities. Endogenous N-cadherin NTF has been detected in vivo in the extracellular matrix (ECM) surrounding differentiated muscle fibers, as well as in embryonic intestinal tissue.13,14 N-cadherin NTF that is associated with the ECM may perform an important biological function in cell adhesion and neurite outgrowth, as immobilized NTF promotes both of these activities in cell culture.14,15 N-cadherin NTF can also associate with and activate FGF receptors, resulting in the activation of PI3K and Akt, and decreasing the levels of apoptosis.16 Likewise, E-cadherin NTF was shown to bind to, and activate the human ErbB receptors, Her2 and Her3, leading to the activation of downstream signaling that results...
in cell migration and cell proliferation in cell culture. Therefore, the NTIs of these classical cadherins can affect cell adhesion properties, as well as bind to and activate cell-surface receptors.

**Signaling Through the CTFs**

The cadherin fragment signaling capabilities do not end with the extracellular region. The CTFs of classical cadherins can also influence intracellular signaling events by interacting with molecules involved in multiple pathways. For example, the N-cadherin CTF2 associates with CREB Binding Protein (CBP), targets it for degradation, and prevents its ability to promote CREB-mediated transcription in cell culture. Mamand and colleagues have speculated that this activity may be important for neuronal growth and survival. In addition, Ferber and colleagues have shown that the E-cadherin CTF2 can activate the expression of Wnt-related genes that are involved in cell proliferation and differentiation in cell culture. In this instance, E-cadherin CTF2 binds to p120 catenin, translocates to the nucleus, and can activate transcription of Wnt target genes by blocking the repressor Kaiso.

The CTFs of N-cadherin, E-cadherin and cadherin-11 all associate with the Wnt signaling molecule, β-catenin. N-cadherin and E-cadherin CTF2 both interact with β-catenin, prevent its degradation and relocate it to the nucleus to promote gene transcription. As a result, the CTF2 of N-cadherin and E-cadherin can both affect the transcription of a number of downstream targets such as cyclin D1, c-myc and c-jun, and can enhance cell behaviors such as cell proliferation and cell migration in cell culture and in vivo.

On the other hand, cadherin-11’s interaction with β-catenin appears to deviate somewhat from what has been established with the other classical cadherins. While we can detect a small cadherin-11 CTF2-sized fragment in embryos, we have not yet established if it complexes with β-catenin. However, we have shown that the cadherin-11 CTF1 maintains its interaction with β-catenin, and does not stimulate the activation of the transcriptional targets described above. In the case of CNC migration we suspect that is an important detail, since β-catenin signaling can block CNC migration if activated exogenously during this process.

**Teasing Apart the Role of Full-Length vs. Cadherin Fragment**

One of the future objectives of our laboratory is to determine if endogenous cadherin fragments have a physiological function. As we pursue this goal we are challenged with a major difficulty: how do we decipher between the activities of a full-length cadherin molecule and that of its cleavage fragment?
For example, in order to see if a cleavage fragment elicits a certain phenotype or not, we often utilize transgenic technology to express peptides meant to mimic the NTFs and CTFs of a cadherin. In this instance, it is difficult to determine if phenotypes observed with overexpressed fragments are specifically caused by their “fragment” properties, or their ability to mimic the full-length molecule. It is probable that the structure of endogenous and recombinant “fragments” varies significantly from the uncropped peptides, making them molecularly distinguishable. However, these peptides have been shown to interact with many of the same binding partners of the full-length molecule, suggesting that the full-length and cleaved peptides share significant similarities. In addition, it also unclear if the physiological level of a cleavage fragment is enough to promote the same cell behaviors observed when overexpressing recombinant cleavage fragments. So, while the use of recombinant proteins is a useful and essential tool to help realize the signaling potential of these fragments, we cannot be sure that the endogenous fragments are performing in the same manner.

The use of chemical inhibitors to block protease activity is another tool that we have used to help determine if the cleavage of a cadherin is important for a physiological process. The advantage of using these inhibitors is that cadherin cleavage can be blocked temporarily. However, it is unlikely that they will help us resolve if signaling events promoted by the endogenous fragments are important for a specific cellular behavior. One reason for this is that we could not be sure that a phenotype we observed was a result of the inability to generate a cleavage fragment and its subsequent signaling events, or the inability to process (remove) the full-length molecule. But more importantly, these inhibitors block multiple proteases, and any phenotype we observe could easily be attributed to any of their proteolytic targets.

Perhaps the main source of the experimental difficulties described above is due to the fact that cadherins have important cellular functions before they are processed. Therefore, we must be careful not to interfere with the activity of the full-length cadherin while studying possible signaling activities of its cleavage fragments. One possibility is to generate antibodies that recognize a cryptic site revealed on the cleavage fragment upon processing. This would allow us to see exactly where and when the fragments are generated during a particular biological process. It is also possible that one such antibody could block the “function” of NTFs and would be extremely useful in deciphering a role for these molecules in vivo. Alternately, the generation of small, “function blocking” peptides that are expressed under inducible promoters would be a useful tool for understanding the roles of endogenous CTFs. These peptides would make it possible to temporally block CTF activity in vivo.

Another tool that could further decipher the role of cleaved cadherin would be to generate a mutant in which the cleavage site has been replaced by a sequence cleaved by an exogenous protease. This mutant could be expressed in embryos lacking the wild type cadherin and perform all the functions of the full-length protein but would be unable to be processed by its natural protease. In return, the exogenous protease could be provided at a defined time to observe the change in cell behavior. The two main technical difficulties of such an experiment is (1) to find a sequence that would not be cleaved by the natural protease which is always difficult with ADAM and (2) to identify an exogenous protease whose expression would not adversely affect embryonic development.

To conclude, it is clear that we will need to come up with creative solutions as we determine the physiological relevance of cadherin cleavage fragments. Yet, as new tools and technologies emerge, we will be able to resolve these issues.

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References
APPENDIX XI

REPRINT 2

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Extracellular Cleavage of Cadherin-11 by ADAM Metalloproteases Is Essential for Xenopus Cranial Neural Crest Cell Migration

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Cell adhesion molecules such as cadherins alternate their expression throughout cranial neural crest (CNC) development, yet our understanding of the role of these molecules during CNC migration remains incomplete. The "mesenchymal" cadherin-11 is expressed in the CNC during migration yet prevents migration when overexpressed in the embryo, suggesting that a defined level of cadherin-11–mediated cell adhesion is required for migration. Here we show that members of the meltrin subfamily of ADAM metalloproteases cleave the extracellular domain of cadherin-11 during CNC migration. We show that a fragment corresponding to the putative shed form of cadherin-11 retains biological activity by promoting CNC migration in vivo, in a non-cell-autonomous manner. Additionally, cleavage of cadherin-11 does not affect binding to β-catenin and downstream signaling events. We propose that ADAM cleavage of cadherin-11 promotes migration by modifying its ability to support cell-cell adhesion while maintaining the membrane-bound pool of β-catenin associated with the cadherin-11 cytoplasmic domain.

INTRODUCTION

The neural crest is a transient population of cells present in all vertebrate embryos. Induced at the border between the neural and nonneural ectoderm, these cells migrate from the dorsal part of the embryo to more ventral locations where they participate in the formation of muscle, cartilage, melanocytes, and ganglia of the peripheral nervous system (PNS; Dupin et al., 2006; Knight and Schilling, 2006; Sandell and Trainor, 2006; Sauka-Spengler and Brenner-Frazer, 2006; Harris and Erickson, 2007). Neural crest cells are separated in two distinct populations depending on their position on the anterior/posterior axis. The most anterior are called cranial neural crest (CNC), responsible for the facial structures, whereas the posterior are the trunk neural crest mostly contributing to the PNS and the melanocytes.

Neural crest cell migration requires tight control over cell adhesion molecules such as integrins and cadherins. To date, there have been four different Cadherin molecules implicated in neural crest migration among the mouse, chick, and Xenopus models (Akita and Bronner-Frazer, 1992; Kimura et al., 1995; Nakagawa and Takeichi, 1995; Inoue et al., 1997; Hadoball et al., 1998; Vallin et al., 1998; Borchers et al., 2001; Coles et al., 2007). These four molecules can be divided into two groups in relation to their expression during migration. The first group consisting of N-cadherin and cadherin-6 (also Cad-6A) are both expressed at the beginning of migration, and then their mRNA and protein expression is quickly downregulated (Akita and Bronner-Frazer, 1992; Nakagawa and Takeichi, 1995). The second group comprising cadherin-7 and -11 is continually expressed throughout neural crest cell migration (Kimura et al., 1995; Nakagawa and Takeichi, 1995; Hadeball et al., 1998; Vallin et al., 1998). Not surprisingly, overexpression of any of these four cell adhesion molecules in at least one of the above model organisms blocks neural crest migration (Nakagawa and Takeichi, 1995, 1998; Dufour et al., 1999; Borchers et al., 2001; Coles et al., 2007; Shoval et al., 2007). However, it is likely that there must be unique properties among these cadherins that make one group more conducive to cell migration than the other.

To further understand the role of cadherins in the neural crest, we have examined the regulation of cadherin-11 during CNC migration in Xenopus laevis. In the Xenopus embryo, N-cadherin is replaced by cadherin-11 expression during CNC migration. We suspected that a protease regulates cadherin-11 levels during CNC migration as an extracellular cleavage product of cadherin-11 had been previously detected in tissue culture cells (Kawaguchi et al., 1999). Among the proteases expressed in the embryo, a member of the ADAM metalloprotease family was a likely candidate for the regulation of cadherin-11 during this process. ADAMs and VE-cadherin have previously been shown to interact in various experimental systems. For example, ADAM15 and VE-cadherin colocalize to adherens junctions and increasing the expression of VE-cadherin results in a corresponding increase in ADAM15 (Ham et al., 2002). Additionally, ADAM10 activity can modify cell adhesion via the cleavage of both N- and E-cadherin (Mietzke et al., 2005). ADAM10 was also found to play a role in the global down-regulation of N-cadherin at the onset of trunk neural crest migration in chick embryos (Shoval et al., 2007). Yet, although ADAM10 is expressed dorsally in Xenopus, it is not enriched in the CNC. On the other hand, another ADAM, ADAM3, is specifically expressed in the Xenopus CNC during migration. Moreover, the proteolytic activity of ADAM13 was previously shown to play a vital role in the

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migration of this tissue (Alfaradhi et al., 2001). Our findings show that cadherin-11 is cleared during Xenopus CNC migration, and that ADAMs from the melanin subfamilies are responsible for this event. We propose that cadherin-11 cleavage occurs when complexed with that of other cadherins in the neural crest and provides further insight into the differential roles of cadherins during morphogenesis.

MATERIALS AND METHODS

Eggs and Embryos

Eggs were obtained from X. laevis, fertilized, and cultured as described previously (Alfaradhi et al., 1997). Embryos were staged according to Nieuwkoop and Faber (1967). UV irradiation and LCI mutagens were performed as described (Pickard and Demjanovski, 2000).

Cell Culture

CaCl2 was cultured in RPMI media complemented with Pen/Strep, 1-glut, sodium pyruvate, and F11/B1 [0.2 ml, 2 mM, 0.01 mg/ml, 10%. Hydram. South Logan, UT]. Transfections were performed using Fugene 6 reagent (Roche, Basel, Switzerland) following the manufacturer’s instructions.

DNA Constructs

The cloning of Xenopus ADAM 10, 13, and 19 and the E. A mutants have been previously described (Ca et al., 1998; Alfaradhi et al., 2001; Smith et al., 2003). Monomeric red fluorescent protein (mRFP) in CS2 was a generous gift from Dr. Jim Smith (Ganson Institute, Cambridge, United Kingdom). ADAM19 was cloned by homologous PCR using sequences from mouse, chick, and Xenopus tropins, 5’ and 3’ ends were obtained by RACE PCR using the gennester kit (Invitrogen, Carlsbad, CA). All full-length ADAM were cloned into the pCSE vector for expression. The ADAM/E/A construct was produced using the QuikChange Mutagenesis Kit (Stratagene, La Jolla, CA). The Xenopus full-length cadherin-11 in pCSE was a gift from Dr. Doris Welsch (University of Ulm, Germany) and was isolated into PCR. The E/C3 construct was made by introducing a micro-tag and stop codons between the E2 and C4 sequences of cadherin-11. The Z2/C3 construct was made by deleting all around PCR with Pyrocosus various DNA polymerase. All constructs were sequenced and tested for expression using the appropriate antibodies in both Ca/N cells and embryos.

Morpholino Oligonucleotides

Morpholino oligonucleotides were directed against the 5’ untranslated region of ADAM10, 13, and 19 eluted in water at 3 mg/ml (Gene Tools, Philomath, OR). Ten nanograms of morpholino (MC) was injected into each embryo at the one-cell stage or 1 ng was injected at the 36-cell stage. The MC sequences directed against the ADAM, 13, and 19 are listed in Table 1.

Cadherin-11 Antibody Production and Screening

A His-tagged fusion protein (PET 30 vector; Novagen, San Diego, CA) encoding 137 C-terminal amino acids of the cytoplasmic domain of cadherin-11 was purified using standard methods. Fusion protein, 100–300 μg, was combined with Freund’s adjuvant and injected intramuscularly into BALB/c mice. Hybridoma fusion protocol was performed using standard methods (Harlow and Lane, 1988). Hybridomas were screened by ELISA, Western blot, immunofluorescence, and immunoprecipitation to test immunoreactivity to endogenous cadherin-11 and neutralizing cross-reactivity to N- and C-cadherin. The mAb 8H8 showed a very low affinity for overexpressed N-cadherin and no detectable affinity for C-cadherin. The same fusion protein was used to immunize rabbits from which specific immunoglobulins were purified by affinity on the antigen according to Alfaradhi et al. (1997).

Antibodies

Rabbit polyclonal antibodies directed against ADAM13 (Cayman Chemical Co., Ann Arbor, MI) were used at a 1:30,000 dilution. Rabbit polyclonal antibodies directed against ADAM13 (Cayman Chemical Co., Ann Arbor, MI) were used at a 1:30,000 dilution.

Table 1. MO sequences directed against ADAM13, 13, and 19

<table>
<thead>
<tr>
<th>Target</th>
<th>Morpholino sequence</th>
</tr>
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<tr>
<td>xADAM13</td>
<td>GCCTCAACCAACCTCCTC</td>
</tr>
<tr>
<td>xADAM13</td>
<td>GCCCTGCCCTTCACTAC</td>
</tr>
<tr>
<td>xADAM19</td>
<td>CAGTTCACACAGTCTT</td>
</tr>
</tbody>
</table>

Table 2. Sequences for xActin, Sox8, xTwist, cyclin-D, and c-myc probes

<table>
<thead>
<tr>
<th>Probe</th>
<th>Sequence 5’</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclin D1 sense</td>
<td>ATCCACATGACGGCA</td>
</tr>
<tr>
<td>c-myc sense</td>
<td>GCCTCAACACCTCCTC</td>
</tr>
<tr>
<td>Sox8 sense</td>
<td>CAGTCAGAGCAAGGT</td>
</tr>
<tr>
<td>xTwist sense</td>
<td>GGTTGCCAGCACTA</td>
</tr>
<tr>
<td>Actin sense</td>
<td>AACACGCAAATAATGC</td>
</tr>
</tbody>
</table>

Rabbit anti-ADAM19 was produced earlier (Ca et al., 1998). Rabbit anti- ADAM13 was produced against a fusion protein to the ADAM19 cytoplasmic domain and affinity-purified before use. For loading controls, antibody to the β-lactamase subunit mAb (OCS) and FACS21 mAb (308) were used (Gaskin et al., 1994; Coussin et al., 2003). To perform Western blot after immunoprecipitation, we biotinylated mAb-1B4 whole bound to the antibody using NHS-LC Biotin (Pierce, Rockford, IL). 0.2 M NaOH and amion-TEM (125) were used to detect EC1 and/or via immunofluorescence. Photographs were taken using a Zeiss Axioscope 2041 inverted microscope (Thornwood, NY) equipped with a Hamamatsu Orca camera (Bridgeview, IL).

Microinjections

Transcription reactions and injections were performed as previously described (Desbrow et al., 2000). The injection volume was determined by capillary calibration of the injection needle. We injected 5 nl at the one- to eight-cell stages and 2.5–10 nl at 32–64-cell stages.

Whole Mount In Situ Hybridization

Whole mount in situ hybridization was performed as previously described (Holland, 1991). Digoxigenin-labeled transcripts were synthesized in vitro from Xenopus Xsox8 and Twist plasmids. Synthetic mRNA encoding x-galactosidase was also included in the mixture of embryos that were analyzed via in situ hybridization. The xgalt reaction was performed as in Smith and Holland (1991) to indicate the site of injection. Embryos that were expressing β-galactosidase in the posterior region were excluded from our statistical analysis. Images were recorded using a Nikon D90 camera on a Nikon SMZ1500 dissecting scope (Melville, NY).

Protein Extraction and Analysis

For direct Western blot analysis of transfected Ca/N cells, each well of a six-well plate was extracted with 250 μl of reducing Laemmli buffer, and 10% of the extract was applied to a SDS-PAGE gel. Immunoprecipitation were carried out exactly as described in Alfaradhi et al., 1998 using protein G beads (Roche, Indianapolis, IN) and 10 μg of mAb-D4. Western blot protocol was followed as previously reported (Ca et al., 2001). Embryonic extraction analysis was performed similar to above but 1:5/modified Barch’s saline (MBS) was used instead of 1:10. TRIS in the extraction buffer and washes. Extraction buffer, 2 μl was used per embryo. Total embryo number for each experiment is noted in the figure legends. Glycoproteins were purified from total protein extract using concanavalin-A agarose beads (Vector Laboratories, Burlington, CA) as previously described (Alfaradhi et al., 1997).

Quantitative PCR Analysis

DNA from stage-11 embryos was purified using guanidine thiocyanate as described in Alfaradhi et al., 1998. Reverse transcription reactions were performed as in Alfaradhi et al., 1998. Sequences for xActin, Sox8, xTwist, cyclin-D, and c-myc probes are listed in the Table 2. Quantitative PCR (QPCR) reactions and data generation were performed using CIRB Green Premix En Taq (Takara, Kyoto, Japan) and the LightCycler system 13 (Roche). The 2-ΔΔCt method was used for target quantification (Livak and Schmittgen, 2001), where actin was used to normalize for total DNA quantity.

RESULTS

Endogenous Cadherin-11 Is Cleaved In Vivo during CNC Migration

Because a shed form of cadherin-11 had been observed in tissue culture, we predicted that migrating CNC cells could
regulate cadherin-11 surface levels by an extracellular cleavage event (Kawaguchi et al., 1999). To investigate this possibility, we produced a mAb directed against the cytoplasmic domain of cadherin-11 and studied its expression and changes in molecular weight during CNC migration in X. laevis embryos. The Western blot analysis in Figure 1A depicts the expression of endogenous cadherin-11 at the beginning (stage 19) and during CNC migration (stages 21 and 23). In these embryos, the amount of total cadherin-11 increases as the crest progresses through migration. Furthermore, we can detect the presence of a cadherin-11 cleavage product of ~75-80 kDa, which also increases during migration. This cleavage product corresponds in size to the cytoplasmic and transmembrane domains, as well as a portion of the extracellular domain, and retains at least one glycosylation site because it can be purified on concanavalin A beads. Using the primary amino acid sequence and the putative N-glycosylation sites, we estimate the cleavage site to be between the ECD and EC4 domain of the cadherin-11 protein (Figure 1B). The timing and sizes of the cadherin-11 fragments suggest that the homophilic binding site in the first Cadherin domain (EC1) is preserved during CNC migration, thus decreasing cell-cell interactions.

Cadherin-11 Can Be Cleaved In Vitro by ADAM9 and 13

Our next objective was to find which protease is responsible for cleaving Cadherin-11 during CNC migration. Because ADAM10 was previously shown to cleave members of the cadherin superfamily, we first investigated if cadherin-11 could also be processed by an ADAM. Cos-7 cells were transfected with cadherin-11 and various ADAM constructs. We selected ADAMs that had been previously shown to be expressed in an overlapping pattern with cadherin-11, namely ADAM9, 10, 15, and 19. Western blot analysis on the cell extract revealed the presence of the 80-kDa cadherin-11 fragments in the ADAM9 and 13 cotransfected cells, but not with their proteolytically inactive E/A mutants (Supplemental Figure S1). This fragment was not present in the ADAM10 and 19 cotransfections.

Binding of Endogenous ADAM13 and Cadherin-11 Occurs During CNC Migration and Corresponds to Cadherin-11 Cleavage

As described above, both ADAM9 and 13 were shown to cleave cadherin-11 in tissue culture. However, we pursued ADAM13 as the protease most likely responsible for cadherin-11 cleavage because of its highly specific expression in the CNC and its previously established role in CNC migration (Allandari et al., 2001). To further investigate if ADAM13 is directly interacting with cadherin-11, we tested the ability of the two proteins to coprecipitate. Indeed, ADAM13 coprecipitates with cadherin-11 in extracts from transfected Cos cells, as well as from embryos overexpressing these two proteins (Supplemental Figure S2). Interestingly, although overexpressed cadherin-11 binds to both the pro and mature forms of ADAM13, endogenous cadherin-11 only communoprecipitates the overexpressed mature ADAM13, suggesting that cadherin-11 preferentially binds with this form in embryos (Supplemental Figure S2).

To determine when the interaction between endogenous ADAM13 and cadherin-11 occurs during early development, we performed another communoprecipitation experiment using noninjected embryos at four different stages of development (Figure 2). We used blastula (stage 7) embryos as negative control because neither ADAM13 nor cadherin-11 is expressed at that stage. We also used gastrula stage embryos (stage 10.5) because both proteins are expressed but the CNC has not yet been induced. Finally we used neurula stage embryos (stage 19), where the CNC have just begun migration and tailbud stage (stage 23), when the CNC migration is nearly completed. The results show that ADAM13 coprecipitates with cadherin-11 during the migration of CNC cells, but not at blastula or gastrula stages. In addition, only the mature form of endogenous ADAM13 (M) is bound to endogenous cadherin-11 (Figure 2A). At stage 22 a 20-kDa band also coprecipitated with cadherin-11. A similar size band was previously described for ADAM13 (Allandari et al., 1997) and could correspond to the protein lacking both the pro and metalloproteinase domain. Surprisingly this band is not significantly enriched when ADAM13 is purified by affinity to concanavalin-A (Figure 2C). As expected, the levels of the 80-kDa cadherin-11 cleavage fragment increase as the CNC is migrating (Figure 2B). We find that cadherin-11 cleavage is also occurring at gastrula stage, whereas no detectable level of ADAM13 is associated, suggesting that another ADAM, possibly ADAM9, may also cleave cadherin-11 during gastrulation in vivo. Although the mRNA for cadherin-11 is also expressed in the somites, Western blotting experiments suggest that during CNC migration the vast majority of the protein is restricted to CNC.
in the head of the embryo, and not in the trunk where the somites are (Supplemental Figure S3).

Overexpression of ADAM9 or 13 but Not ADAM19 Rescues Migration of CNC Cells Blocked by the Overexpression of Cadherin-11

Previous work has shown that overexpression of cadherin-11 results in the inhibition of CNC migration in Xenopus embryos (Horner et al., 2001). Our results indicate that the cadherin-11 level is regulated by proteolytic cleavage during CNC migration. Our hypothesis is that overexpression of ADAM19 rescues CNC migration by extracellular expression of cadherin-11. Embryos were injected into one blastomere at the two-cell stage with synthetic mRNA for cadherin-11 alone, or in combination with ADAM13 (Figure 3B). Synthetic mRNA for β-galactosidase was also included to identify the injected side of the embryos. The noninjected sides of these embryos serve as a stage-match control for embryo development and were used in each case to quantify the extent of migration. At stage 25 embryos were fixed and processed for whole mount in situ hybridization using a mix of RNA probes for Sox10 and Twist to label CNC. Sox10 was used in combination with Twist because previous work had shown that Twist could be down-regulated in CNC overexpressing cadherin-11 (Borchers et al., 2001). Our results confirm that overexpression of cadherin-11 severely disrupts the CNC migration on the injected side (Figure 3A). In contrast, expression of both cadherin-11 and ADAM13 was able to rescue CNC migration in a large fraction of the injected embryos (Figure 3, A and B; p < 0.05).

Because ADAM9 but not ADAM19 can cleave cadherin-11 in vitro, we analyzed their ability to rescue migration of CNC cells overexpressing cadherin-11 by targeted injection at the 16-cell stage. The cell targeted at the 16-cell stage is defined as D2-2 and contributes to a large fraction of the CNC cell population (Mould, 1987). To follow the ability of CNC cells to migrate, the various mRNA probes were injected with mRNAs for GFP (Figure 3C). ADAM13 was used in this assay as a positive control and the ADAM13/E/A mutant as a negative control. These experiments showed that ADAM9 can rescue migration with the same efficiency as ADAM13, whereas ADAM19 or the ADAM13/E/A do not (Figure 3D). This experiment shows that the ability of ADAM to rescue CNC migration blocked by an excess of cadherin-11 depends on the presence of the active proteolytic site and is specific of a subset of ADAM metalloproteases.

Inhibition of ADAM Activity Blocks Cadherin-11 Cleavage In Vivo

We have previously shown using a dominant negative approach that ADAM13 is critical for CNC migration in vivo (Alfandari et al., 2001). To resolve the importance of cadherin-11 cleavage by ADAMs during CNC migration, we further investigated the effect of blocking ADAM function on this process. We first used a hexamethylene-based inhibitor of ADAMs that inhibits a wide range of metalloprotease function including ADAM10 (Orth et al., 2004). C5-7 cells transfected with ADAM13 and cadherin-11 were treated with various concentrations of this inhibitor. Western blot analysis shows that treatment with ADAM13 inhibits ADAM13 cleavage of cadherin-11 in a dose-dependent manner (Figure 4A).

We then investigated the effect of treatment with CCC on CNC migration in vivo by injecting the inhibitor in the pathway of the migrating cells. At stage 22 the CCC treatment has no effect on CNC migration of the embryo injected with the carrier solution containing 10% DMSO (10 nl) migrated in the hyoid, branchial, and mandibular segments (Figure 4B). In contrast, injection of the inhibitor blocked CNC migration in vivo in a similar but more robust manner as the ADAM13-injected embryos (Figure 4B; Alfandari et al., 2001). These results suggest that at least one metalloprotease inhibited by naringin, possibly ADAM13, is essential for releasing cadherin-mediated cell-cell adhesion during CNC migration.

To further investigate this hypothesis, we knocked down individual ADAM metalloproteases via morpholino injection (Figure 4C). The embryos used in this study were injected with MOs against ADAM9, 13, and 19 and then were raised to tailbud stage (stage 24) for the analysis. The total proteins were extracted and the glycopolypeptide purified by affinity to concanavalin-A. Western blot using antibodies to each ADAM, cadherin-11, PACSIN2, and the β3-integrin subunit were performed. The results show that MOs directed against ADAM9, 13, and 19 decreased the translation of their corresponding proteins. Western blot analysis also revealed that the level of uncleaved cadherin-11 at 120 kDa is increased by about two-fold in embryos with each of the ADAM MO, suggesting that ADAM9, 13, and 19 may all participate directly or indirectly, in the cleavage of cadherin-11 in vivo. As a control we tested the cadherin-11 mRNA level using real-time qPCR and found no increase in expression of the gene (Supplemental Figure S4), confirming that the increase in cadherin-11 protein level is due to "stabilization" of the protein and not increased gene expression. In support of this hypothesis, injection of an ADAM9, 13, and 19 MO cocktail significantly decreases the amount of cleaved cadherin-11 at stage 21 (Figure 4D). Additionally, injection of the MO cock- tail (MO) also blocks CNC migration in vivo (Figure 4E).

The Extracellular Cleavage Fragment Binds to Full-Length Cadherin-11 Molecules and Promotes CNC Cell Migration

Thus far we have provided evidence that ADAM cleavage of cadherin-11 produces a 80kDa fragment that remains in the plasma membrane. Consequently, it is likely that the extracellular fragments containing the homophilic binding site are released by the shedding events and may interfere with cadherin-11 function in cell adhesion. To determine if the extracellular fragment may participate in the migration, we made a construct designed to mimic the cadherin-11 extra- cellular cleavage fragment (EC1-3). To test whether the fragment can bind to full-length cadherin-11, we applied the media from EC1-3-transfected cells onto live C5-7 cells overexpressing either cadherin-11 or a cadherin-11 mutant missing the homophilic binding site (ΔEC1-3). Immunofluorescence detected the EC1-3 fragment only on cadherin-11 transfected cells, suggesting that the extracellular cleavage fragment can bind to full-length cadherin-11 (Figure 5A). Because EC1-3 can bind to cells expressing full-length cadherin-11, we predicted that this fragment might also help promote CNC cell migration. To further explore this hypothesis we expressed both of these proteins with green fluorescent protein (GFP) to follow CNC migration in vivo. Although overexpression of cadherin-11 alone blocks CNC migration, overexpression of EC1-3 rescues this phenotype (Figure 5B; p < 0.05). This result suggests that the cleavage fragment may compete with full-length cadherin-11 molecules for cell-cell adhesion and that CNC cells require a defined ratio of cleaved to uncleaved cadherin-11 for migration.
Figure 3. ADAM9 and 13 rescue CNC migration in cells overexpressing cadherin-11. (A) In situ hybridization was performed using a combination of CNC markers sTwist and Sex10. Embryos were injected into one blastomere at the two-cell stage with synthetic mRNA for either Cad-11 alone (top left) or in combination with ADAM13 (bottom left). The site of injection was determined by coinjecting mRNA for β-galactosidase. The right panels correspond to the noninjected side of each embryo. Disruption of CNC migration was determined by comparing the distance migrated on the injected side (left panels) versus the noninjected side (right panels) of the same embryo. (B) Quantification of three independent rescue experiments. *p < 0.05 for GFP-injected embryos, **p < 0.01 for Cad-11-injected embryos, and ***p < 0.001 for Cad-11+ and ADAM13-injected embryos. (C) Visualization of CNC cell migration in vivo using RFP as a lineage tracer. One donor animal cell at the eight-cell stage was injected with mRNA encoding RFP and cadherin-11 to inhibit CNC migration. Synthetic RNA (0.25 ng) encoding ADAM9, ADAM13, ADAM9, and ADAM13/E/A were each coinjected with cadherin-11 to determine their ability to rescue migration. p < 0.05 Histograms representing the percentage of embryos in which the RFP-labeled cells migrated. Significance was determined by students t test (p < 0.05). The number of embryo analyzed was as follows: RFP = 51, Cad-11 = 61, Cad-11+ADAM9 = 67, Cad-11+ADAM13 = 59, Cad-11+ADAM13/E/A = 78, and Cad-11+ADAM19 = 56.
Figure 4. Reduction of ADAM function decreases cadherin-11 cleavage and CNC migration. (A) Cos-7 cells overexpressing Cad-11 and ADAM33 were treated with 0 μM, 1 μM, or 10 μM of mammary Cad-11 cleavage was determined by Western blot analysis of Cad-11 (top panel). ADAM33 levels were also detected by Western blot (bottom panel). M: mature form ADAM33; P: proform of ADAM33. (B) Lateral view of tailbud stage embryos treated with whole mount in situ hybridization using slg as a control neural crest cells. Embryos at stage 17 were injected with the epidermis with 10 nl of 1% DMSO (left) or 0.1 mM mammary in 10% DMSO (right). At tailbud stage the CNC in control embryos have migrated in the hyoid, brachial, and mandibular segments (100%, n = 24). In contrast, 5% of the embryos injected with the mammary inhibitor have severe inhibition of CNC migration (n = 24). (C) Western blot analysis detecting ADAM and Cad-11 expression in control uninjected embryos (NI) or injected with morpholinos directed against ADAM9 (MO9), ADAM3 (MO33), or ADAM9 (MO9). Each lane represents the glycoproteins from five embryos equivalent. PAGSN2 and the β1 integrin protein levels are unaffected by MO injection. In contrast, the unedited cadherin-11 protein level is increased twofold with each MO (D) ADAM9, 13, and 19 protein expression was knocked down using a cocktail of all three specific MO3. Embryos were extracted at stage 15 (premigration) or at stage 21(mid-migration), and were immunoprecipitated for Cad-11. Cad-11 was then detected by Western blot (20 embryos/lane). At stage 21, the cadherin-11 cleavage fragments are reduced in embryos injected with the 3MO. (E) In vivo migration analysis of embryos injected at the 1-cell stage with mRNA encoding GFP alone, or Mxi3 and GFP alone (0.5 ng/injection) or combined with 1 ng of the 3MO cocktail (0.25 ng of each MO injection). The CNC in GFP mRNA injected embryos migrated in 21 of 21 embryos. The CNC in GFP mRNA combined with 3MOs migrated in only eight of 33 embryos (24%).

**EC1-3 Rescues CNC Migration in Embryos with Reduced ADAM33 Expression**

To test if the EC1-3 fragment could promote migration in CNC cells with reduced level of ADAM protein, we chose two complementary approaches (Figures 6 and 7). The first consists of injecting the MO at the one-cell stage, to produce embryos with reduced level of ADAMs and then injecting either a lineage tracer alone or with the EC1-3 fragment at the 16-cell stage D1-2 (Figure 6). In that case we can compare using in situ hybridization the position of CNC cells that express the lineage tracer to the ones that do not. The second approach is to inject the MO with the lineage tracer at the 16-cell stage in D1-2 and follow the position of the injected cells in live embryos, thus directly assessing the capacity of the injected cells to migrate (Figure 7).

The first approach shows that the EC1-3 cadherin-11 fragment can rescue CNC positioning in embryos with reduced level of ADAM13 and 19 with the same efficiency as the injection of an ADAM13 mRNA lacking the MO target se-
Figure 6. The cadherin-11 extracellular cleavage fragment rescues CNC migration in embryos with reduced ADAM13 expression. (A) Schematic representation of the experimental method. Embryos were injected at the one-cell stage with MO13 and MO19 (5 ng each) and then again at the 16-cell stage, with a lineage tracer and mRNA encoding the various constructs, in D1.2 to target CNC. In this case we are testing the ability of the mRNA to rescue CNC migration. (B) In situ hybridization using Twist and Sox30 to label the CNC. The left panels represent the control side where migration was inhibited by the MO. The right panels represent the experimental side injected either with β-gal or the EC1-3 mRNA. The black lines represent the extent of migration of the most posterior segment. After injection of the EC1-3 mRNA is rescued. (C) Quantification of three individual experiments described above (2MO is MO13 + MO19). The total number of embryos for each injection set was n = 77 (2MO + β-gal), n = 76 (2MO + EC1-3), n = 82 (2MO + β-gal + A19), n = 66 (2MO + β-gal + C11), and n = 91 (control + β-gal + EC1-3).

The most efficient inhibition was found using all three MOs (66% inhibition). CNC migration in all MO combinations containing the ADAM13 MO was rescued by the expression of the EC1-3 domain of cadherin-11. However, this fragment had no effect on CNC migration, injected by the ADAM9 and 19 MO combination. These results suggest that both ADAMs may all participate in CNC migration or may compensate for each other in vivo. Because the cadherin-11 extracellular domain could only rescue migration in embryos that had decreased ADAM13 (MO13 + 5 MO13 + 19 or all 3MO), but not in embryos lacking both ADAM9 and 19, it is likely that ADAM13 is the principal ADAM responsible for cadherin-11 cleavage during CNC migration.

EC1-3 Promotes Migration in a Non-Cell-Autonomous Manner

The studies described in Figures 5, 6, and 7 show that EC1-3 can rescue CNC migration when coexpressed in cells that are either overexpressing full-length cadherin-11 or have knocked down ADAM expression through the use of MOs. To determine if this rescue is cell-autonomous or not, we injected embryos into the 32-cell stage with the EC1-3 and GFP mRNA in the a2 cell, while we injected the ADAM MO cocktail with RFP mRNA in b2. Both a2 and b2 contribute to the CNC. The result shows that expression of the EC1-3 domain rescued CNC migration of RFP expressing cells lacking ADAM proteins, whereas GFP alone did not, demonstrating that the cadherin-11 extracellular domain can act in a non-cell-autonomous manner.

Surprisingly, the RFP we expressed in the cells with knocked-down ADAM expression (to visualize CNC migration) remained stable throughout the later stages of CNC cell differentiation (stages 45-47). This unexpected feature made it possible to analyze if the rescued ADAM/KD cells also differentiated into craniofacial structures (Figure 8, D and E). Indeed, embryos that were mosaic for EC1-3 and ADAM knockdown (KD) cells had a tendency to have more ADAM KD cells in the developing facial cartilages and muscles than those embryos not expressing EC1-3 (Figure 8E). These results show that EC1-3 can rescue migration noncell autonomously and suggest that ADAM KD cells rescued by EC1-3 retain the ability to differentiate into craniofacial structures.

Cadherin-11 Cleavage Does Not Affect Canonical Wnt Signaling In Vivo

Cadherin-11, like many other cadherin proteins, can bind to β-catenin via its cytoplasmic domain. Cleavage of N-cadherin by ADAM10 decreases its ability to bind β-catenin, increasing the cytoplasmic pools, and resulting in the stimulation of Wnt downstream markers c-myc, cyclin-D1, and c-Jun in tissue culture (Reiss et al., 2005). Furthermore, overexpression of cadherin-11 in Xenopus decreased the expression of Twist, a CNC marker that is also downstream of canonical Wnt signaling. This effect is caused by cadherin-
Figure 8. The cadherin-11 extracellular fragment (EC1-3) is not cell-autonomous. (A) Schematic representation of the experimental design. The EC1-3 mRNA was co-injected with GFP mRNA at the 32-cell stage in the a2 cell. Cell membranes F were injected with RFP mRNA in the b2 cell of the same embryo (all mRNAs were at 0.25 ng). Embryos were grown to stage 26 before imaging. GFP and RFP fluorescence. (B) The percentage of embryos with migrating CNC cells expressing RFP was then counted and is presented in C. Asterisks indicate statistical significance as determined by Student's t-test (p < 0.05). (D) Late stage (stages 45–47) analysis of RFP localization in differentiated facial structures in the dual-injected embryos from above. Embryos were scored for having strong, little, or trace to no expression in the developing facial cartilage. (E) Histogram representing scoring data from late stage embryo analysis.

11–sequestering β-catenin at the cell surface, because coexpression of β-catenin rescues Twist expression (Bonhers et al., 2001). In light of these findings, we considered two possible ways ADAM processing of cadherin-11 could affect β-catenin. Either cleavage of cadherin-11 destabilizes its interaction with β-catenin, increasing the cytoplasmic pool and possibly promoting nuclear signaling, or ADAM cleavage of cadherin-11 only affects its adhesive properties and not its ability to bind β-catenin. To test how cadherin-11 cleavage affects its interaction with β-catenin, we overexpressed cadherin-11 in embryos either alone or with ADAM13. Cadherin-11 was then immunoprecipitated and the association with β-catenin was tested by Western blot (Supplemental Figure S1A). Here, the level of associated β-catenin was not affected by the coexpression of ADAM13, suggesting that the 80-kDa fragment is still capable of binding to β-catenin. This was further confirmed by coimmunoprecipitation experiments using a truncated form of cadherin-11 lacking the EC1-3 domains expressed in Cos cells (Supplemental Figure S1B). This truncated form associated with the endogenous β-catenin with efficiency similar to that of the wild-type cadherin-11. Finally, we performed real-time qPCR analysis on the expression of canonical Wnt target genes XTwist, cyclin-D1, and c-myc using cDNA from

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stage-21 embryos injected with the same mRNA combination as described above (Supplemental Figure SSC). Coexpression of ADAM13 with cadherin-11 did not stimulate the expression of any of the markers. Cumulatively, these results indicate that cadherin-11 cleavage by ADAM13 does not affect Wnt signaling through β-catenin.

**DISCUSSION**

**The Differential Role of Cadherins in the Neural Crest**

One objective of the studies described in this manuscript was to further understand the role of cadherins during neural crest migration. Among the different model organisms, both cadherin-11 and -7 are expressed in neural crest cells throughout migration (Kimura et al., 1995; Nakagawa and Takeichi, 1995; Hadeball et al., 1998; Nakagawa and Takeichi, 1998; Vallen et al., 1998). N-Cadherin and cadherin-6b are also expressed in neural crest cells but are down-regulated shortly after the onset of migration (Akiyama and Bronner-Fraser, 1996; Nakagawa and Takeichi, 1995; Inoue et al., 1997). Although N-cadherin and cadherin-6b are expressed at the beginning of migration, it is speculated that they may have an inhibitory function in this process (Coles et al., 2007; Shoval et al., 2007). This hypothesis is supported by the observation that down-regulating cadherin-6b in the chick neural crest results in premature migration of these cells (Coles et al., 2007).

One intriguing question is how these cadherins may either promote (Cad-7 and -11) or prevent (N-Cad and Cad-6b) cell migration since they all share similar domain organization, intracellular binding partners, and the ability to support cell–cell adhesion. Of course each cadherin family member has unique adhesive properties such as homophilic binding tendencies and exclusive dissociation constants that may make one more useful to migrating cells than the other (Bayas et al., 2006; Patel et al., 2006). However, we suspect that their adhesive properties are more strongly influenced by regulatory proteins during the process of migration. In line with this premise we have shown that cadherin-11 is continuously regulated by ADAM13 via an extracellular cleavage event during CNC migration in Xenopus. Similarly, cadherin-7 was shown to have a rapid turnover rate in migrating neural crest cells when compared with N-cadherin (Dautour et al., 1999). This turnover is likely due to proteolysis as shown in cell culture experiments, but the enzyme responsible for this proteolysis remains to be identified (Kotsos et al., 2002). Thus, the pairing of selected ADAM metalloprotease with cadherins may provide them with unique properties such as promoting cellular migration.

**Preadipogenic Function of the Cleaved Extracellular Domain**

We have also discovered that the cleavage of cadherin-11 produces a fragment that has preadipogenic activity in vivo. This extracellular fragment can rescue CNC migration when there is an overabundance of full-length cadherin-11, either via overexpression of cadherin-11 mRNA or by blocking the cleavage of endogenous cadherin-11 through MoAbs directed against ADAMs (Figures 5–7). This cleavage fragment can also rescue the migration of ADAM KD CNC cells via a non-cell-autonomous mechanism (Figure 8). Our results show that the fragment can bind to full-length cadherin-11 molecules (Figure 5), suggesting that in vivo it could act as a competitor and prevent cadherin-11-mediated interactions among cells (Figure 9). Some invasive cancers may use a similar mechanism to promote cell migration via the expression of an alternatively spliced cadherin-11 product. This variant encodes a secreted form of cadherin-11 and has been
found in aggressive cancer cell lines (Psheva et al., 1999; Feltes et al., 2002). Although competition with cadherin-11 is a likely hypothesis another possibility is that the cleaved cadherin-11 extracellular fragments may bind to an unrelated protein acting as a receptor similar to what has been shown for the L1 adhesion molecule (Figure 9). In the case of L1, the cleaved domain binds to the αvβ5 integrins and stimulate haptotactic migration (Mechtler et al., 2001).

Although the extracellular cleavage fragment of other cadherin molecules has also been shown to retain biological activity, it appears that no generalizations about their function are possible. For example, although the extracellular fragment of E-cadherin was shown to decrease cell–cell adhesion in vitro, the extracellular fragment of N-cadherin promotes mesenchymal cell–cell adhesions in chick embryos (Parades and Grunwald, 1993; Nee et al., 2001). Thus, the expression of specific cadherins along with the generation of their cleavage fragments seems to play an active role in mediating a specific cellular response, such as cell migration.

Which Metlins Cleaves Cadherin-11 during CNC Migration?

Meltin family members ADAM9, 13, and 19 are all expressed in CNC cells during migration. Because all three of these proteins are active proteases, they could all potentially cleave cadherin-11 during this process. However, our studies in vitro reveal that ADAM9 and 13 can cleave cadherin-11, whereas ADAM19 cannot (Supplemental Figure S1). Additionally, both ADAM9 and 13, but not ADAM19, are capable of rescuing the migration of CNC expressing an excess of cadherin-11 in vivo (Figure 3, C and D). Yet, resolving which meltin predominantly cleaves cadherin-11 during CNC migration was complicated by the ability of the ADAMs to compensate for each other’s function. We have shown further evidence of the compensation among the meltins in vivo by detecting an increase in ADAM9 expression when either ADAM13 or 19 expression is knocked down via MO injection (Figure 4C). To prevent the effects of compensation in our experiments, we used combinations of MOs to knock down at least two meltins at one time. We have shown that injection of a combination of any two ADAM MOs blocks CNC migration in about half of the embryos screened (Figure 7). This phenotype can be rescued by the expression of the cadherin-11 extracellular domain when ADAM13 MO is included in the injection. However, the cadherin-11 extracellular domain does not rescue embryos with double MO knockdown of ADAM9 and ADAM19. This distinction suggests that ADAM13 is the main enzyme responsible for cadherin-11 cleavage during CNC migration.

On the other hand, the above observation does not explain why MOs for ADAM9 and 19 can also block CNC migration. In this regard, we observed a decrease in ADAM13 expression in both ADAM9 and ADAM19 MO injected embryos (Figure 4C), suggesting that there is cross-talk among these meltins. Thus the CNC migration phenotype (Figure 7), and the increase in uncleaved cadherin-11 levels (Figure 4C) in ADAM9 and 19 knocked down embryos could be at least partially attributed to this secondary effect on the ADAM13 protein level. Additionally, loss of either ADAM9 or 19 may affect CNC induction. For example, conditional knockout of ADAM9 in the mouse neural crest does not prevent migration, but interferes with the specification of cardiac neural crest cells and the proper morphogenesis of the heart (Komatsu et al., 2007). We have also observed that ADAM19 KD interferes with CNC specification in Xenopus, (Neuner and Allandari, unpublished results), and this could contribute to the partial inhibition of CNC migration observed here.

We propose that ADAM3 is responsible for the cleavage of cadherin-11 during CNC migration and that other meltins, such as ADAM9, can compensate for this function when ADAM3 protein expression decreases. Our results also suggest that another protein cleaved by the meltins may be important in the specification and/or migration of the CNC.

ADAM10 Cleavage of N-Cadherin versus ADAM13 Cleavage of Cadherin-11 in the Neural Crest

Although ADAM10 cleavage of N-cadherin was shown to play an important role in trunk neural crest delamination in chick, there are fundamental differences with the ADAM3 cleavage of cadherin-11 in the Xenopus CNC (Shoval et al., 2007). First, as described previously, cleavage of cadherin-11 occurs continuously during migration, whereas cleavage of N-cadherin in part of the global down-regulation of this protein required at the onset of migration (Akitaya and Bronner-Fraser, 1992; Shoval et al., 2007). Second, in the avian neural crest, cleavage of N-cadherin releases β-catenin that relocates to the nucleus to activate the transcription of primordial gene such as cyclin-D1 (Shoval et al., 2007). Cyclin-D1 in addition to its role in controlling cell division also controls cell motility by inhibiting ROCK (Rho-associated protein kinase) signaling and TSP-1 expression (Li et al., 2006). Conversely, we have shown that cleavage of cadherin-11 does not affect its interaction with β-catenin.

Although β-catenin signaling appears to be important for the initial delamination of the neural crest, it seems to play a different role during neural crest cell migration (de Melker et al., 2004; Shoval et al., 2007). Some signaling through β-catenin is important for the expression of neural crest markers, such as Twist, during migration (Borchers et al., 2001). However, conditional knock out of β-catenin in mouse embryos showed that β-catenin signaling is not required for neural crest cell migration (Braut et al., 2001). In fact, exogenous stimulation of β-catenin via L1C treatment will stop the migration of avian neural crest ex vivo (de Melker et al., 2004). It is possible that cadherin-11 helps to control the “intensity” of β-catenin signaling by sequestering a pool of this molecule at the cell membrane during CNC migration. On the other hand, it has been suggested that signaling through β-catenin is involved in the differentiation of neural crest cells once they reach their target locations (Hart et al., 2002; Farah et al., 2002). Because neural crest differentiation occurs mostly after migration ceases, cadherin-11’s interaction with β-catenin at the cell membrane may also play a role in the maintenance of an undifferentiated state while the cells are still moving.

In summary, we propose that the differential role of cadherins in the neural crest is in part mediated by ADAM cleavage. Here we show that ADAM regulates cadherin-11 throughout CNC migration. The continuous cleavage of cadherin-11 could promote migration by removing its adhesive domain, and by producing an extracellular fragment that retains biological activity. The decrease in cell adhesion could be important to increase the fluidity of the CNC tissue as well as promote the dispersion into single cells during the second phase of CNC migration (Figure 9).
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