Xenopus ADAM13 and ADAM19 are Important for Proper Convergence and Extension of the Notochord

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XENOPUS ADAM13 AND ADAM19 ARE IMPORTANT FOR PROPER CONVERGENCE AND EXTENSION OF THE NOTOCHORD

A Dissertation Presented

By

RUSSELL D. NEUNER

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 2011

Molecular and Cellular Biology
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XENOPUS ADAM13 AND ADAM19 ARE IMPORTANT FOR PROPER CONVERGENCE AND EXTENSION OF THE NOTOCHORD

A Dissertation Presented

By

RUSSELL D. NEUNER

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DEDICATION

I dedicate this dissertation to everyone who has helped me over the years. To those who have helped shape me into the person that I am today. To everyone that has supported me through the triumphs and tragedies of my life. I cannot express how sincerely grateful I am to have each of you by my side. This dissertation is dedicated to my father David Neuner, my mother Janet Allen, my brother Scott Neuner, my sister Stephanie Luster and the rest of my extended family especially my loving grandparents, Fred and Doris Neuner. I also dedicate this dissertation to the special people in my life; Martha Espy, my loving and supportive fiancé Lindsay Grigg, the entire Grigg family and my life-long friends Eric Harmon and Luke Woolley.

For Everything – Thank You!
ACKNOWLEDGMENTS

There are a lot of people to acknowledge through my journey here at UMass. I would like to start by thanking everyone from the Alfandari research team. I recognize Dominique Alfandari and Helene Cousin for their dedication to my growth and development as a scientist. Your relentless support has instilled values that will continue to grow throughout the rest of my scientific career. For that, I sincerely thank you for everything. I would also like to thank Kate McCusker and Julian Sosnik for their support as previous lab mates and as friends. My experience with Mike Coyne, Erin Kerdavid, Wei Ding and Genevieve Abbruzzese has been great and it has been an honor to be part of this research team. I would like to acknowledge my thesis committee; Rolf Karlstrom, Rafael Fissore and Jesse Mager. I greatly appreciate your help, constructive criticism and enduring support over the years. To all of my friends outside of the lab, especially Ankit Vahia and Nick Griner – Thank you. You have brought a tremendous amount of happiness and laughter to my life. I would also like to acknowledge the Molecular and Cell Biology Program and the Veterinary and Animal Science Department for fostering an outstanding environment for my graduate studies here at UMass.
ABSTRACT

XENOPUS ADAM13 AND ADAM19 ARE IMPORTANT FOR PROPER CONVERGENCE AND EXTENSION OF THE NOTOCORD

FEBRUARY 2011

RUSSELL D. NEUNER, B.S., PENNSYLVANIA STATE UNIVERSITY
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Gastrulation is a fundamental process that reorganizes the primary germ layers to shape the internal and external features of an early embryo. Morphogenetic movements underlying this process can be classified into a variety of different types of cellular movements. I will focus on investigating in this thesis two types of cell movements in the dorsal mesoderm; mediolateral cell intercalation and convergence and extension. During gastrulation, mesoderm cells send protrusions to gain traction on neighboring cells and the surrounding extracellular matrix; a process called mediolateral cell intercalation. Mesoderm cells use this type of cell movement to converge and extend the dorsal mesoderm tissue during gastrulation; a process called convergence and extension. These morphogenetic movements are essential to form the early embryo and are important for later development.

There are a number of different proteins involved in regulating the morphogenetic movements during gastrulation. The Planar Cell Polarity Signaling Pathway helps establish individual cell polarity and is activated in dorsal mesoderm cells undergoing convergence and extension. In addition, dorsal mesoderm cells migrate by using integrin receptors and the surrounding extracellular matrix to correctly position the mesoderm in the embryo. I will focus my efforts on analyzing the function of ADAM proteins during Xenopus laevis gastrulation. The ADAM family of metalloproteases is important for a variety of biological processes. ADAM proteins function as ectodomain sheddases by cleaving membrane bound proteins involved in signal transduction, cell-cell adhesion,
and cell-extracellular matrix adhesion. I will focus on investigating the roles of two ADAM family members; ADAM13 and ADAM19 during gastrulation. Both ADAM13 and ADAM19 are expressed in the dorsal mesoderm during gastrulation. Throughout early embryonic development, ADAM13 is expressed in the somitic mesoderm and cranial neural crest cells. ADAM19 is expressed in dorsal, neural and mesodermal derived structures such as the neural tube, notochord, the somitic mesoderm, and cranial neural crest cells.

Since ADAM13 and ADAM19 are expressed in similar tissues, I investigated if both proteins functionally interacted. I show that a loss of ADAM13 protein in the embryo reduces the level of ADAM19 protein by 50%. In the opposite experiment, a loss of ADAM19 protein in the embryo reduces the level of ADAM13 protein by 50%. This suggests that both ADAM13 and ADAM19 are required to maintain proper protein levels in the embryo. This might be explained through their physical interaction in a cell. The ADAM19 Proform binds to the ADAM13 Proform in cultured cells. Through domain analysis, I show that ADAM19 binds specifically to the cysteine-rich domain of ADAM13. When co-overexpressed in a cell, the level of Mature ADAM13 (compared to the Proform) is reduced suggesting a complex form of regulation. I propose a few models that discuss how ADAM19 may function as a chaperone to stabilize and regulate the further processing of ADAM13 protein.

Some of the unpublished work discussed in this thesis focuses on the roles of ADAM13 and ADAM19 in the dorsal mesoderm during gastrulation. Specific emphasis is made on investigating the axial mesoderm during notochord formation. I show that ADAM19 affects gene expression important for the A-P polarity of the notochord while ADAM13 does not. The changes in gene expression can be partially rescued by the EGF ligand Neuregulin1β, a known substrate for ADAM19 in the mouse. ADAM13 and ADAM19 are important for convergence and extension movements of the axial mesoderm during gastrulation. Specifically, a loss of ADAM13 or ADAM19 causes a delay in mediolateral cell intercalation resulting in a significantly wider notochord compared to control embryos. These defects occur without affecting dishevelled intracellular localization or the activation of the PCP signaling pathway. However, a loss
of ADAM13 or ADAM19 reduces dorsal mesoderm cell spreading on a fibronectin substrate through $\alpha_5\beta_1$ integrin.

To conclude, the work presented in this thesis focuses on the similarities and differences of ADAM13 and ADAM19 in the early embryo. Although ADAM13 and ADAM19 are required for normal morphogenetic movements during gastrulation, my data suggests they have different functions. ADAM13 appears to function in regulating cell movements while ADAM19 appears to function in regulating cell signaling. I propose a few models that discuss how each ADAM metalloprotease may function in the dorsal mesoderm and contribute to convergence and extension movements during gastrulation.
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CHAPTER I

INTRODUCTION

Abstract

Gastrulation is a morphogenetic process that reorganizes the primary germ layers (ectoderm, mesoderm, and endoderm) to shape the internal and external features of an early embryo. The morphogenetic movements that drive this process of early development are mediolateral cell intercalation and convergence and extension in the dorsal mesoderm. As a result of these movements, the paraxial mesoderm and axial mesoderm develop during gastrulation to form the somites and notochord, respectively. This is important to properly lengthen the embryo along its anterior-posterior axis. There are a large number of molecules that regulate convergence and extension during gastrulation. I have focused my efforts on understanding how one protein family called ADAM metalloproteases contribute to these morphogenetic movements and function during gastrulation.

SECTION I: General Background of Gastrulation in Amphibians

Approximately 150 years ago, a German scientist named Ernst Haeckel observed and illustrated for the first time one of the earliest phases in Metazoan embryonic development called gastrulation. Haeckel proposed the term “gastraea” to describe an early embryo that has a structurally simple hollow diploblastic ovoid embryonic shape
(Haeckel, 1875). In the early 1920’s, the Amphibian embryo became a prominent model organism for experimental embryologists. This was largely due to the influence of another German scientist, Hans Spemann, and his discovery of the organizing center (Spemann and Mangold, 1924). The organizing center is a group of cells that express specific molecules important for patterning the dorsal side of the embryo. These experiments were performed by transplanting organizer cells into the ventral side of a host embryo. What they observed was the formation of a two headed, dual-axis tadpole. Spemann’s work had huge significance in the field of embryology by stimulating a search, which continues to this day, to investigate the identity of the organizer signals (Oelgeschlager et al., 2003). Concurrently, the work of Walter Vogt helped establish an initial understanding of fate mapping in the early embryo (Vogt, 1925). The work of these early pioneers paved the way for more modern scientists such as Pieter Nieuwkoop to investigate a more elaborate fate mapping in the early embryo (Nieuwkoop and Faber, 1967) and for Ray Keller to investigate in detail the morphogenetic movements during *Xenopus laevis* gastrulation (Keller, 1975). Today a lot of the same principles and methodology are still used extensively in the Xenopus gastrula. Gastrulation can be defined as a morphogenetic process that reorganizes the primary germ layers (ectoderm, mesoderm, and endoderm) to shape the internal and external features of an early embryo.

After fertilization, the embryo undergoes a series of cleavages that divide a large single cell into a hollow ball of a few thousand cells called a blastula. At this point, the process of gastrulation begins and is marked by the appearance of the dorsal blastopore lip. The dorsal lip itself is the initial structure that forms a circular opening on the vegetal pole of the embryo called the blastopore. The blastopore closes through a variety of
different cell movements during gastrulation (Figure 1.1). The four evolutionarily conserved movements include internalization, epiboly, convergence and extension (Solnica-Krezel, 2005). Internalization marks the beginning of gastrulation and in amphibians, creates the dorsal blastopore lip. The dorsal lip is composed of large “bottle” cells that apically constrict to initiate the involution of the prospective mesoderm (Hardin and Keller, 1988). The involuting mesoderm enters through the blastopore and migrates inside the embryo. This process is essential to correctly position the three germ layers for proper embryonic development. Epiboly movements spread tissue by increasing the surface area and decreasing thickness. The driving force behind epiboly occurs when cells interdigitate themselves between deep and superficial layers; a process known as radial intercalation (Keller, 1980).

The dorsal mesoderm undergoes dynamic movements to converge and extend the tissue along the anterior-posterior axis; a process called convergence and extension (Keller et al., 1985). Dorsal mesoderm cells undergo this type of movement by forming mediolateral polarized protrusions to gain traction on neighboring cells and the surrounding extracellular matrix (ECM). This cellular behavior is known as mediolateral cell intercalation. The dorsal mesoderm can be microdissected from a gastrulating embryo and cultured ex vivo in a ‘Keller Sandwich’ (Keller and Danilchik, 1988) and an ‘Open Face Explant’ (Wilson et al., 1989). When cultured ex vivo, the dorsal mesoderm retains the ability to undergo convergence and extension movements. On the ventral side of the gastrulating embryo, the lateral and ventral mesoderms undergo dynamic movements to converge the tissue without extension; a process called convergence and thickening (Keller and Danilchik, 1988). These two complementary morphogenetic
movements during gastrulation cause the blastopore to close asymmetrically towards the ventral side of the embryo (Figure 1.2) (Keller and Shook, 2008).

Once gastrulation is complete, the developing embryo enters the neurulation phase as the neural tube forms. The neural tube develops from the neuroectoderm on the dorsal side of the embryo. In addition to neural tube closure, the paraxial mesoderm forms the developing somites and the axial mesoderm continues to form the developing notochord during this phase. Mediolateral cell intercalation continues during neurulation in the paraxial mesoderm, axial mesoderm and the neural tissue. This drives the process of convergence and extension to properly lengthen the embryo along the anterior-posterior axis. These cellular movements are essential for the embryo to develop and survive to the tailbud stage.

At the molecular level, the major signaling pathways important for mesoderm induction are Fibroblast Growth Factor (FGF) signaling (Kimelman et al., 1988) and Transforming Growth Factor β (TGFβ) signaling (Thomsen et al., 1990). FGF and TGFβ are known as morphogens because they are signaling molecules that produce specific responses in a cell. How morphogens act on a cell depends on their concentration in the cellular microenvironment. The addition of either FGF or TGFβ to naïve animal cap cells isolated from the blastula stage will cause cells to develop into mesoderm tissue, such as muscle and notochord. FGF or TGFβ treated explants are not only induced to express mesoderm specific genes but can also undergo convergence and extension movements (Kimelman and Maas, 1992; Thomsen et al., 1990). There are a large number of molecules that regulate convergence and extension during gastrulation (Table 1 in (Solnica-Krezel, 2005)). The major signaling pathway that is critical for regulating
convergence and extension is the Non-canonical Wnt signaling pathway (or Planar Cell Polarity (PCP) Pathway) (Yin et al., 2008). The PCP signaling pathway is extensively studied in the fly and shown to be evolutionarily conserved in vertebrates. This signaling pathway is important for establishing individual cell polarity in the dorsal mesoderm (Tada and Kai, 2009). Cell polarity is established through the localization of specific PCP components to the medial and lateral edges of cells undergoing mediolateral cell intercalation (Jiang et al., 2005; Zallen and Wieschaus, 2004). A gain or loss of function to any of the signaling components in the PCP pathway results in convergence and extension defects (Roszko et al., 2009). An observed feature of these embryos is a shortened anterior-posterior body axis.

Convergence and extension movements also involve the regulation of cell adhesion molecules. During gastrulation, the involuting mesoderm enters the blastopore and migrates on the inner surface of the blastocoel roof. This migration event is highly dependent upon the extracellular matrix protein, fibronectin, which localizes along the blastocoel roof (Boucaut and Darribere, 1983; Lee et al., 1984). The interaction between fibronectin and its integrin receptor, α5β1, also contributes to convergence and extension movements (Davidson et al., 2006; Rozario et al., 2009). For example, depletion of fibronectin by an antisense morpholino randomizes mediolateral cell protrusions resulting in convergence and extension defects (Davidson et al., 2006). In addition, the disruption of fibronectin to α5β1 integrin by the microinjection of a function blocking antibody randomizes mediolateral cell protrusions resulting in convergence and extension defects (Davidson et al., 2006). Furthermore, it is suggested that the interaction between α5β1 integrin and fibronectin may cross talk with the PCP signaling pathway to regulate
convergence and extension movements (Marsden and DeSimone, 2001). In particular, disruption of the PCP components Vangl2 and Frizzled7 by dominant negative RNA injection impairs proper fibronectin matrix assembly during gastrulation (Goto et al., 2005).

Convergence and extension movements also involve the regulation of cell-cell adhesion proteins such as cadherins and protocadherins (Hammerschmidt and Wedlich, 2008). For example, the Xenopus paraxial protocadherin C (PAPC) regulates cell-cell adhesion by downregulating the adhesive activity of C-cadherin (Chen and Gumbiner, 2006). PAPC can also physically interact with Sprouty2, a known inhibitor of the PCP signaling pathway, to promote convergence and extension movements during gastrulation (Wang et al., 2008). Recent work in zebrafish has also analyzed the role of matrix metalloproteases in cell movements during gastrulation (Coyle et al., 2008). Membrane-type I matrix metalloprotease (MT1-MMP/MMP14) regulates mediolateral cell intercalation by affecting dorsal mesoderm cell polarity. Loss of MMP14 results in convergence and extension defects without affecting mesoderm patterning. Furthermore, MMP14 can degrade a fibronectin matrix in a gastrulating zebrafish embryo (Latimer and Jessen, 2009). This suggests that fibronectin remodeling via MMP14 may be important for regulating cell polarity and mediolateral cell intercalation in the zebrafish embryo.

Our lab studies another zinc metalloprotease protein family called ADAM metalloproteases. I will focus my efforts on analyzing the function of ADAM proteins during *Xenopus laevis* gastrulation.
SECTION II: Introduction to the ADAM Family of Metalloproteases

General ADAM background

Members of the ADAM family of zinc metalloproteases are best characterized for their enzymatic processing of other membrane bound proteins; a process called ectodomain shedding (Alfandari et al., 2009; Schlondorff and Blobel, 1999). Members of the ADAM family function in signal transduction through their cleavage of signaling molecules such as epidermal growth factor (EGF), tumor necrosis factor-α (TNF-α) and Notch (Dyczynska et al., 2007; Ohtsu et al., 2006; Pan and Rubin, 1997; Yan et al., 2002). ADAM members also function in cell-cell adhesion through their cleavage of cadherins (Maretzky et al., 2005; McCusker et al., 2009; Reiss et al., 2005; Uemura et al., 2006), and cell-extracellular matrix adhesion through their processing of ECM molecules (Alfandari et al., 2001; Millichip et al., 1998). ADAM metalloproteases exist in two main states; an enzymatically inactive state called the Proform and an enzymatically active state called the Mature form (Figure 1.3). The difference between the two forms is that a Prodomain is present in the N-terminus of the Proform. The Prodomain interacts with the metalloprotease active site rendering the ADAM catalytically inactive. The prodomain is cleaved by a Furin-like enzyme in the Golgi complex which releases the inhibition on the active site (Loechel et al., 1998). This allows the ADAM Mature form to become catalytically active (Figure 1.3).

There are more than 30 members in the ADAM family of metalloproteases and they can be divided into three major classes. The first class contains the principle
sheddases (ADAM10 and ADAM17) that are known to cleave the majority of the ADAM substrates. For example, ADAM10 cleaves heparin-binding EGF (HB-EGF) to activate the EGF signaling cascade and resultant gene expression (Yan et al., 2002). The second class is the mesenchymal ADAMs or the meltrin subfamily (ADAM9, 12, 13, 19, 33) that are known to be involved in skeletal muscle, bone and heart development. For example, ADAM19 knockout mice exhibit severe defects in heart morphogenesis leading to their perinatal death (Zhou et al., 2004). The third class is the non-proteolytic ADAMs (ADAM11, 22, 23) because they lack a conserved sequence within the active site of their metalloprotease domain rendering these members catalytically inactive. Members of this class have an important role in neural development. For example, ADAM22 forms a complex with the epilepsy-related ligand and its receptor in neurons to regulate synaptic transmissions in the brain (Fukata et al., 2006).

In addition to their main role as ectodomain sheddases, ADAM members can physically interact with integrin receptors in a cell via their disintegrin domains (Lu et al., 2007). This interaction affects cell-extracellular matrix adhesion (Huang et al., 2005) and cell migration (Chen et al., 2008). Recently, a potential function of the cytoplasmic domain of ADAM members has emerged. Numerous proline-rich and SH3 binding motifs contained within the cytoplasmic domain are found to interact with intracellular proteins such as the cytoplasmic domains of ADAM12, ADAM15 and ADAM19 with FISH (Abram et al., 2003) and the cytoplasmic domain of ADAM22 with 14-3-3β (Zhu et al., 2002). The cytoplasmic domain can function independently of the extracellular region for ADAM10 (Tousseyn et al., 2009) and ADAM13 (Cousin et al., submitted 2010). For ADAM10, it was proposed that a two-step processing event occurs. The first
cleavage by another ADAM member occurs within the cysteine-rich domain releasing the extracellular fragment of ADAM10. This triggers a second cleavage by γ-secretase at the plasma membrane for the intracellular release of the ADAM10 cytoplasmic domain. The released cytoplasmic domain translocates into the nucleus where it is believed to function (Tousseyn et al., 2009). For ADAM13, our lab has shown that γ-secretase also cleaves the cytoplasmic domain of ADAM13. The released cytoplasmic domain translocates into the nucleus to regulate gene transcription of approximately 2000 different genes. Furthermore, our lab is the first to show that the ADAM13 cytoplasmic domain functions in vivo and is essential for proper cranial neural crest cell migration in Xenopus laevis embryos (Cousin et al., submitted 2010).

ADAM Family Members Function in Xenopus Gastrulation

Members of the meltrin subfamily of ADAMs, specifically ADAM9, ADAM13 and ADAM19, are expressed during gastrulation (Alfandari et al., 1997; Cai et al., 1998; Neuner et al., 2009). ADAM12 and ADAM15 have not been cloned yet in Xenopus laevis but are expressed during gastrulation of a closely related phylogenetic species; Xenopus tropicalis (Wei et al., 2010). ADAM9 is ubiquitously expressed in all cells, while ADAM13 and ADAM19 are restricted to the dorsal mesoderm. Through a loss of function approach by microinjection of an antisense morpholino oligonucleotide, our lab has shown that single knock down of ADAM9 or ADAM19 does not affect blastopore closure. However, knock down of ADAM13 delays gastrulation by approximately two hours. This suggests that ADAM9 and ADAM19 do not function in regulating blastopore closure during gastrulation, while ADAM13 does. ADAM19 does however
affect early gene expression at the gastrula stage and will be discussed further in Chapter II of this thesis (Figure 2.4).

ADAM10 is also expressed during gastrulation; however a potential function has never been investigated (Pan and Rubin, 1997). ADAM10 is one of the most studied ADAM family members because of its essential role in the Notch/Delta signaling pathway (Pan and Rubin, 1997). The Notch/Delta signaling pathway has recently been shown to regulate morphogenetic movements as both activation or inhibition of this pathway delays blastopore closure and affects the segregation of the three germ layers (Revinski et al., 2010). Thus, it is possible that ADAM10 functions in segregating the three germ layers during gastrulation.

This thesis will focus on investigating the role of two ADAM family members, ADAM13 and ADAM19, during gastrulation. Specific emphasis will be on analyzing the morphogenetic movements that form the notochord. In final, I will attempt to understand whether ADAM13 and ADAM19 function independently of each other or whether they have overlapping functions during gastrulation.

SECTION III: Overview of the Work Presented in this Thesis

I began this project five years ago with the initial characterization of ADAM19. The goal at the onset of the project was to identify another meltrin subfamily member that could compensate for ADAM13 function, specifically in the cranial neural crest. Of the other meltrin subfamily members, ADAM19 had the highest sequence homology at the amino acid level to ADAM13 making it an ideal candidate. At the time there was
nothing known about the role of ADAM19 in *Xenopus laevis*. Therefore, the second goal of this project was broad and it was to characterize ADAM19 in the early embryo. This was critical in order to identify a biologically relevant substrate to ADAM19 proteolytic activity in the embryo. The initial experiments were to clone full length *Xenopus laevis* ADAM19 and determine its expression pattern. At the time there were no ADAM19 antibodies available that could be used to study the endogenous protein in Xenopus. Thus, one of the first major objectives was to generate a number of antibodies that could recognize different structural domains of the ADAM19 protein. I successfully made three antibodies to the ADAM19 protein and they are briefly described:

1. **2C5 E1**: Mouse monoclonal antibody directed against the cytoplasmic domain of ADAM19.
2. **C2 and C3**: Rabbit polyclonal antibody directed against both variants (C2 and C3) of the cytoplasmic domain of ADAM19.
3. **DC19**: Goat polyclonal antibody directed against the disintegrin-cysteine rich domains of ADAM19.

The cytoplasmic domain was selected because this domain amongst other ADAM family members is the most variable at the amino acid level. This would minimize the potential of cross reactivity with other ADAM family members. The disintegrin-cysteine rich domain was selected because this domain amongst other ADAM family members is the most conserved at the amino acid level and would increase our chances of ADAM19 detection. The generation and screening of all three antibodies is described in Appendix I.
Chapter II describes the results of the initial characterization of ADAM19 in the early embryo. I show that ADAM19 is expressed in dorsal, neural and mesodermal derived structures during early embryogenesis. Through a loss of function approach using an antisense morpholino oligonucleotide to ADAM19, I show that ADAM19 affects gene expression important for the anterior-posterior (A-P) polarity of the notochord during gastrulation. The changes in gene expression are accompanied by a decrease in phosphorylated AKT, a downstream target of the EGF signaling pathway. At the neurula stage, loss of ADAM19 affects neural crest induction by a reduction in Sox8 and Slug gene expression. At the tailbud stage, loss of ADAM19 affects muscle differentiation by a reduction in myosin light chain (MLC) gene expression and a reduction in 12/101 protein level. Lastly, I show that loss of ADAM19 affects somite organization and perturbs fibronectin localization at the intersomitic boundary.

Chapter III focuses on ADAM13 and ADAM19 protein regulation in cultured cells and in the embryo. I show that a loss of ADAM13 protein reduces the level of ADAM19 protein by 50%. In addition, a loss of ADAM19 protein reduces the level of ADAM13 protein by 50%. This suggests that both ADAM13 and ADAM19 are required to maintain a proper protein level in the embryo. I propose this outcome may be explained by the physical interaction of ADAM13 and ADAM19 in a cell. When co-overexpressed the ADAM19 Proform binds to the ADAM13 Proform in a cell. Through protein domain analysis, I show that ADAM19 binds specifically to the Cysteine-Rich Domain of ADAM13. When co-overexpressed in a cell, the level of Mature ADAM13 (compared to the Proform) is reduced suggesting a complex form of regulation. Lastly, I
describe a few models that could explain how ADAM19 regulates the level of ADAM13 in a cell.

Chapter IV describes ADAM13 and ADAM19 function in the dorsal mesoderm, with specific emphasis on the notochord. Through a loss of function approach using antisense morpholino oligonucleotides to ADAM13 or ADAM19, I show that ADAM19 affects gene expression important for the A-P polarity of the notochord while ADAM13 does not. The changes in gene expression can be partially rescued by the EGF ligand Neuregulin1β, a known substrate for ADAM19 in the mouse. I show that a loss of ADAM13 or ADAM19 affects convergence and extension movements during notochord formation. Specifically, a loss of ADAM13 or ADAM19 causes a delay in mediolateral cell intercalation resulting in significantly wider notochords compared to control embryos. This defect occurs without affecting dishevelled intracellular localization or the activation of the PCP signaling pathway. However, a loss of ADAM13 or ADAM19 reduces dorsal mesoderm cell spreading on a fibronectin substrate through α5β1 integrin.

Chapter V will explore the similarities and differences of ADAM13 and ADAM19 in the dorsal mesoderm during gastrulation. Although both ADAM13 and ADAM19 affect convergence and extension movements, I hypothesize their contribution may involve different mechanisms. ADAM19 mediated shedding of Neuregulin1β maintains Brachyury expression in the notochord. ADAM13 affects dorsal mesoderm cell adhesion to fibronectin through α5β1 integrin. In final, a few models will explain how ADAM13 and ADAM19 function in the dorsal mesoderm and contribute to convergence and extension movements.
Figure 1.1: The Different Types of Cell Movement during Gastrulation. (A) Diagrams the four main types of cell movements; Epiboly, Internalization, Convergence and Extension. The final tissue shape is compared to the original (dashed rectangle). (B) Radial Intercalation. Mesoderm cells from deep tissue layers interdigitate between deep and superficial cell layers to thin the overall tissue. (C) Directed Migration. Involuting mesoderm cells respond to an environmental signal via a net migration or displacement in a single direction. (D) Involution. Mesoderm cells of the dorsal blastopore lip turn in upon themselves to create this type of cell movement. (E) Invagination. This is the beginning step to involution where cells change shape in order to fold inward. (F) Ingression. Involuting mesoderm cells undergo an EMT and migrate along the blastocoel roof through this type of cell movement. (G) Mediolateral Cell Intercalation. Dorsal mesoderm cells become polarized in the mediolateral axis to gain traction in order to converge and extend a tissue. This figure was taken from (Solnica-Krezel, 2005).
Figure 1.2: Illustration of Gastrulation in the *Xenopus laevis* Embryo. (A) The vegetal view of the early Xenopus gastrula (left) compared to the early neurula (right). The morphogenetic movements during gastrulation to properly form the notochord (dark grey) and somitic mesoderm (light grey) along the anterior-posterior axis (A-P arrow) are shown. The overall directionality of blastopore closure is depicted (black arrows inside blastopore). (B) The process of convergence and extension in the dorsal mesoderm of an open face explant cultured *ex vivo*. This figure is modified from (Keller and Shook, 2008).
Figure 1.3: Schematic Diagram of an ADAM Family Member. The extracellular region of an ADAM protein contains five amino acid domains. The prodomain (Pro, grey) binds to the Metalloprotease domain through a cysteine switch mechanism rendering the enzyme inactive called the ADAM Proform (A). The prodomain is cleaved by a Furin-like enzyme which releases the inhibition allowing for the ADAM to become proteolytically active called the ADAM Mature form (B) (Loechel et al., 1998). The Metalloprotease domain (Met, yellow) contains the zinc active site (green dot). A glutamic acid mutation to an alanine (E/A) at residue #341 renders the ADAM Metalloprotease proteolytically inactive. This can act as a “dominant negative” form by competitively binding to substrates and sequestering them from the endogenous ADAM. The Disintegrin domain (Dis, red) interact with integrins, an important property for cell adhesion. Together with the Cysteine-rich domain (CR, blue) interacts directly with protein substrates. The EGF-like domain contains a number of EGF amino acid repeats, yet its function has not been determined. Most ADAM family members have a single pass transmembrane domain (TM, black) containing a Cytoplasmic domain (Cyto, orange). The Cytoplasmic domain contains proline-rich and SH3 binding motifs that function within intracellular signaling.
CHAPTER II

XENOPUS ADAM19 IS INVOLVED IN NEURAL, NEURAL CREST AND MUSCLE DEVELOPMENT

Most of the work presented in this chapter is published in the journal

Mechanism of Development (Neuner et al., 2009)

*Data generated by myself are depicted in Figures 2.2 (A-D); 2.3; 2.4; 2.5A; 2.6A; 2.7A; 2.8G; 2.8A,B; Table 2.1

*Data generated by other members of the Alfandari Lab are depicted in Figures 2.1; 2.2(E-I); 2.3B; 2.4B, C; 2.7B; 2.8(A-F, H); 2.9C

*The only unpublished figures are Figure 2.2(A, B) and 2.9(A, B).

Abstract

ADAM19 is a member of the meltrin subfamily of ADAM metalloproteases. In Xenopus, ADAM19 is present as a maternal transcript. Zygotic expression begins during gastrulation in the dorsal blastopore lip and increases through neurulation and tailbud formation. ADAM19 is expressed in dorsal, neural and mesodermal derived structures such as the neural tube, the notochord, and the somites. Using a Morpholino knock down approach, I show that a reduction of ADAM19 protein in gastrula stage embryos reduces Brachyury expression in the notochord concomitant with an increase in the dorsal markers, Goosecoid and Chordin. The changes in gene expression are accompanied by a decrease in phosphorylated AKT, a downstream target of the EGF signaling pathway.
The changes in gene expression occur while the blastopore closes at the same rate as the control embryos. During neurulation and tailbud formation, ADAM19 knock down reduces neural fates as determined by the expression of N-tubulin and NRPI but not Sox2. In the somitic mesoderm, the expression of MLC is also decreased while MyoD is not. ADAM19 knock down affects neural crest induction as determined by the reduction in Sox8 and Slug gene expression prior to cell migration. Neural crest induction is also decreased in embryos treated with an EGF receptor inhibitor suggesting that this pathway is necessary for neural crest cell induction. Using targeted knock down of ADAM19 I show that ADAM19 function is required in the neural crest and that the migration of the cranial neural crest is perturbed. In addition, I show that a reduction in ADAM19 protein affects somite organization, reduces 12-101 protein expression and perturbs fibronectin localization at the intersomitic boundary. There was nothing known about ADAM19 in Xenopus laevis development prior to our published work described in this Chapter.

SECTION I: Introduction

Post-translational modification of proteins is a critical step in most biological processes. Among these modifications, cleavage of cell surface proteins controls a diverse range of cellular processes such as cell signaling, cell adhesion and cell migration. Members of the ADAM family function in signal transduction through their cleavage of signaling molecules such as epidermal growth factor (EGF), tumor necrosis factor-α (TNF-α) and Notch (Dyczynska et al., 2007; Ohtsu et al., 2006; Pan and Rubin, 1997; Yan et al., 2002). ADAM members also function in cell-cell adhesion through
their cleavage of cadherins (Maretzky et al., 2005; McCusker et al., 2009; Reiss et al., 2005; Uemura et al., 2006), and cell-extracellular matrix adhesion through their processing of ECM molecules (Alfandari et al., 2001; Millichip et al., 1998).

ADAM19 is a member of the meltrin subfamily and is shown, in mouse, to cleave TNF-α, Kit1 Ligand, TRANCE, HB-EGF, and Neuregulin-1β (Chesneau et al., 2003; Horiuchi et al., 2005; Kawaguchi et al., 2007; Shirakabe et al., 2001). The knockout (KO) of ADAM19 causes defects in heart development, believed to be due to defective HB-EGF signaling (Kurohara et al., 2004; Zhou et al., 2004). Conditional KO of ADAM19 specifically in the cardiac neural crest cells reproduced the heart phenotype, suggesting that ADAM19 is critical for cardiac neural crest cell specification but not migration (Komatsu et al., 2007). In mouse, the major enzyme responsible for the cleavage of both HB-EGF and Neuregulin-1β appears to be ADAM17. In addition, mice lacking ADAM17 show very similar heart defects to ADAM19 KO mice (Sahin et al., 2004). Furthermore, a double knock out of both ADAM17 and ADAM19 results in a more severe phenotype, demonstrating a possible overlapping function of these ADAMs during embryonic development (Horiuchi et al., 2005). Fibroblasts derived from ADAM19 KO mice are able to process both Neuregulin-1β and HB-EGF, suggesting that the ADAM19 substrate responsible for the cardiac defects remains to be identified.

To test whether ADAM19 has a unique role during early embryonic development, I cloned the *Xenopus laevis* homologue and analyzed its expression pattern. ADAM19 is present at low levels as a maternal transcript with zygotic expression beginning during gastrulation and localizing to the dorsal blastopore lip. During later stages of embryonic development, ADAM19 is expressed in the dorsal mesoderm, neuroectoderm and neural
crest cells. Loss of function experiments using an antisense morpholino knock down approach reveal that ADAM19 is essential for the specification of the dorsal mesoderm, neuronal derivatives and cranial neural crest cells. The apparent lack of an overlapping function for ADAM19 in *Xenopus laevis* may help resolve the biologically relevant substrate for ADAM19 during early embryonic development.

**Results**

**SECTION II: ADAM19 Expression Pattern in *Xenopus laevis***

I cloned the *Xenopus laevis* homologue of ADAM19 using degenerate oligonucleotides to human, mouse, quail, and the genomic sequence of *Xenopus tropicalis*. The full-length sequence was generated by 5’ and 3’ RACE PCR and assembled from multiple PCR amplicons. The deduced amino acid sequence is 49% identical to human ADAM19 and 84% identical to *Xenopus tropicalis* ADAM19. I found two variants in the cytoplasmic domain at the amino acid level, one of which is most common to the *Xenopus tropicalis* sequence (Genbank accession # EU770696).

I investigated ADAM19 expression throughout development using real-time PCR. I used poly(A) purified messenger RNA to eliminate any trace of genomic DNA in our samples. ADAM19 is expressed as a maternal transcript with zygotic expression beginning from gastrulation through early tailbud formation (Figure 2.1). The expression level of ADAM19 at gastrulation is low but is confirmed by *in situ* hybridization where it appears restricted to the dorsal lip of the blastopore (Figure 2.2A).
Using whole mount *in situ* hybridization, I investigated the distribution of ADAM19 mRNA during gastrulation, neurulation and tailbud formation (Figure 2.2). To avoid background staining due to either antibody or substrate precipitation in the blastocoel cavity, I performed *in situ* hybridization on bisected gastrula embryos. At the late gastrula (stage 12), the mRNA encoding ADAM19 is localized to the dorsal lip of the blastopore where the dorsal mesoderm invaginates (Figure 2.2A). During early tailbud stages, ADAM19 is expressed in the dorsal region of the embryo including the neural tissue and the dorsal mesoderm. ADAM19 is detected in the neural tube, the notochord, and the cement gland (Figure 2.2B, C and F). In the mesoderm, ADAM19 is expressed both in the segmented (somite) and non-segmented (caudal) dorsal mesoderm (Figure 2.2G and H). When stained embryos are observed in PBS rather than Murray’s clear to visualize superficial staining, ADAM19 is detected in the segments of the cranial neural crest cells (CNC) (Figure 2.2I). The expression in the CNC cells was confirmed by Reverse Transcriptase PCR on explants dissected from stage 17 embryos (Figure 2.2I insert). At stage 26, ADAM19 expression is evident in the cement gland, the brain, the somites, the otic vesicle and the branchial arches (Figure 2.2D). At late tailbud (stage 36), ADAM19 is still expressed in the entire head, the branchial arches, the somites and the pronephros (Figure 2.2E). The staining intensity decreases in the somite and by contrast appears stronger in the neural tube.

To determine the role of ADAM19 in early development, I designed antisense morpholino oligonucleotides corresponding to the sequence near the ATG start codon for ADAM19 and common to all amplicons identified (Table 2.2). I also generated two ADAM19 polyclonal antibodies in order to study the ADAM19 protein (Appendix I).
One antibody was directed against the disintegrin-cysteine rich domains of ADAM19 and raised in the goat called DC19. The other antibody was directed against both variants of the cytoplasmic domain of ADAM19 and raised in rabbit called C2 and C3. All antibodies recognized the cloned full length ADAM19 protein in transfected Cos-7 extract and embryos injected with the ADAM19 mRNA (Figure 2.3C and data not shown). When the antibodies are used in combination by immunoprecipitation in embryos at stage 22, a 120 kilodalton (kDa) polypeptide and a 90 kDa polypeptide are recognized. Based on the predicted molecular weights, the 120 kDa polypeptide corresponds to the endogenous ADAM19 Proform and the 90 kDa polypeptide corresponds to the endogenous ADAM19 Mature form. In embryos injected with ADAM19 MO (MO19) both polypeptides recognized by the antibody are reduced by 90 to 100%, depending on the injection, when normalized to GAPDH (Figure 2.3A and B).

In addition to the endogenous protein, MO19 prevents translation of the ADAM19 mRNA while a morpholino to ADAM13 (MO13) does not (Figure 2.3C, lane 5 versus lane 6). Mutation of four nucleotides in the ADAM19 mRNA allows translation of the protein in injected embryos (Figure 2.3C, lane 4). While translation of ADAM19 mRNA is still affected by the MO19 (Figure 2.3C, lane 2 versus lane 4), the mRNA translates into an excess amount of ADAM19 protein compared to the endogenous protein level (Figure 2.3C, lane 4 versus lane 1). I believe that the four nucleotides changes (out of 26 total) may still allow partial binding of the morpholino to the mRNA when both are concentrated in a tube before the injection. This construct is named ADAM19 Rescue (A19R) and is used in all of the rescue experiments described in this thesis. Before performing knock down analysis in MO19 injected embryos, I tested by
microinjection for toxicity of 1, 5, 10, 25 and 50 nanogram (ng) dosages of MO19 per embryos. At the highest dose of 50 ng per embryos, I observed that early cleavage and gastrulation movements were unaffected. However, at the end of neurulation a significant percentage failed to close their neural tube and died (data not shown). While the 50 ng MO19 dose produced significant death, I did not see a more significant decrease in the level of ADAM19 protein compared to the 10 ng MO19 dose. For this reason I selected 10 ng as an optimal dosage to use in all subsequent experiments described in this thesis.

SECTION III: ADAM19 Affects Early Patterning during Gastrulation

ADAM19 is expressed in the dorsal mesoderm during gastrulation. The dorsal mesoderm undergoes dynamic cell movements to close the blastopore during gastrulation. To investigate the role of ADAM19 in blastopore closure, I measured the size of the blastopore during gastrulation in MO19 embryos compared to controls. Using the average diameter of the blastopore, I found that embryos injected with MO19 closed the blastopore at the same rate as embryos injected with a control morpholino (CMO) (Figure 2.4).

Although ADAM19 does not affect blastopore closure, I investigated the role of ADAM19 in early gene expression during gastrulation. I performed whole mount in situ hybridization for Brachyury (Xbra), Chordin, and Goosecoid expression to visualize the mesoderm in MO19 injected embryos at the late gastrula stage (Figure 2.5). Brachyury is a T-box transcription factor that is expressed in all mesoderm tissues during gastrulation.
(Cunliffe and Smith, 1992). Brachyury is critical for gastrulation movements and for establishing the anterior-posterior axis in the early embryo. Our results show that Xbra is expressed in the ventral and lateral mesoderm but is absent in the notochord of 63% of the embryos with reduced ADAM19 protein (n=30) (Figure 2.5A). To test whether the dorsal mesoderm was entirely missing, I assessed Chordin and Goosecoid expression in the notochord and cephalic mesenchyme, respectively. Goosecoid is a paired homeobox transcription factor that is expressed in anterior, dorsal mesoderm during gastrulation (Cho et al., 1991). As a homeobox gene, goosecoid is critical for the early patterning of anterior dorsal mesoderm in the embryo. Chordin is a secreted Bone Morphogenetic Protein (BMP) antagonist that is expressed in the axial mesoderm during gastrulation (Sasai et al., 1994). Chordin is a dorsalizing factor because it binds and inhibits proteins that induce ventral tissue such as BMP in the early embryo (Sasai et al., 1994). At the late gastrula stage, I found that most MO19 embryos (65% n=30) had a wider, more anterior shift in the localization of Chordin expression while 35% showed a similar staining to the controls. A more detailed analysis into Xbra and Chordin expression will be presented in Chapter IV of this thesis. Furthermore, 76% of the embryos injected with MO19 had the same level of Goosecoid staining while the remaining 24% (n=20) showed a wider expression. Another notochord marker (not-1) was not affected by the knock down of ADAM19 (data not shown). These results show that the dorsal mesoderm and the notochord are present in embryos with a reduced level of ADAM19 but that the patterning of the notochord may be altered.

Using quantitative real-time PCR, I tested the level of expression of these genes in embryos injected with a CMO, MO19 and a combination of MO19 and ADAM19 Rescue
mRNA (Figure 2.5B). The results confirm a small reduction of Xbra expression (16% decrease) possibly due to the absence of expression in the notochord. In contrast, I found that both Chordin and Goosecoid levels were increased by approximately two fold in embryos with reduced ADAM19 protein suggesting that there is an increase in specific dorsal mesoderm markers. These changes in gene expression were all rescued by the co-injection of ADAM19 Rescue mRNA.

Since the expression of Xbra in the notochord is absent, I performed a targeted injection at the 8-cell stage with β-galactosidase as a lineage tracer. The cells with a reduced level of ADAM19 were still able to incorporate into the notochord and the somites, suggesting that ADAM19 expression is not essential for the formation of these tissues (data not shown).

SECTION IV: ADAM19 Affects Neural Patterning and Neural Crest Induction

To investigate the role of ADAM19 in neural induction, I performed in situ hybridization using Sox2 as a general neural marker in gastrula stage embryos. The results show that MO19 does not prevent neural induction (Figure 2.5A). While the overall expression of Sox2 was not affected, careful analysis revealed some subtle changes. For example, Sox2 expression in control embryos is lighter in the neural plate directly above the notochord (future floor plate, Figure 2.5A, red arrowhead). This decrease of Sox2 at the midline is not visible in 30% of the embryos injected with MO19 (n=30), suggesting that a reduction in ADAM19 protein may affect axial tissue patterning across the mesoderm/neuroectoderm tissue boundary. However at that point, it is not
clear whether the affect on the neural plate is direct or an indirect result of defective signaling from the notochord.

To further investigate the role of ADAM19 in the neural tissue, I performed in situ hybridization using N-tubulin, Sox2, and NRP-1 at the tailbud stage (stage 24). Sox2 and NRP1 were used as general neural markers and N-tubulin as a marker of neuronal precursors. Embryos were injected in one blastomere of the two-cell stage with 10 ng of either CMO or MO19 with mRNA encoding β-galactosidase (Figure 2.7A). The expression of Sox2 was unaffected by a reduction in ADAM19 protein while both N-tubulin and NRP1 appear reduced on the injected side (Figure 2.7A). The reduction of N-tubulin expression was confirmed by quantitative real-time PCR at both stage 17 and stage 24 (Figure 2.7B). While the decrease was small (30%), it was highly reproducible (p-value<0.05) and was fully rescued by the injection of ADAM19 Rescue mRNA. This suggests that only a fraction of neurons expressing N-tubulin are affected by the ADAM19 knock down. In contrast to N-tubulin, ADAM19 knock down had no effect on Sox2 expression at both stage 17 or stage 24 confirming the in situ hybridization results.

ADAM19 is also expressed in the CNC cells. I investigated the role of ADAM19 in neural crest induction at the open neural plate stage (Stage 15) before CNC migration. I performed in situ hybridization and real time PCR for the expression of two early neural crest markers, Sox8 and Slug (Figure 2.6). Our results show that both Sox8 and Slug expression are reduced in the MO19 injected half of the embryos compared to the uninjected half serving as an internal control (Figure 2.6A). Real time PCR shows a reduction of approximately two fold for Sox8 (50% reduction) and Slug (40% reduction; Figure 2.6B). In contrast, the neural marker Sox2 increased at this stage. Since the
neural crest is induced at the border of the neural plate, it is likely that the cells that are not induced to become neural crest will remain neural and maintain Sox2 expression.

To investigate the role of ADAM19 in CNC cell migration, I performed targeted injection of the MO19 together with GFP mRNA at the 16-cell stage in a dorso-lateral animal blastomere. The dorso-lateral animal blastomere will give rise to the cranial neural crest. Cell migration was evaluated by observing the distribution of GFP in the different CNC segments (Figure 2.6C). Our results show that, while GFP is present in all segments of the CNC that migrate in control embryos, no migration was observed in 33% of the embryos injected with MO19. Together these results show that ADAM19 is required for neural crest cell induction and affects CNC cell migration. Moreover, our results show that there is a significant reduction of xTwist expression in the MO19 injected side (Figure 2.7A, arrowhead). To investigate if ADAM19 affects CNC specification or solely xTwist expression, I determined ADAM11 expression as a marker of CNC by quantitative PCR. While Twist is a widely used marker for CNC by in situ hybridization, it also labels other cells at the tailbud stage. On the other hand, ADAM11 is more restricted with expression in both the CNC and one line of dorsal neurons (Cai et al., 1998). Confirming the in situ hybridization, I observed a decrease of ADAM11 expression at both stage 17 (58%) and stage 24 (50%) (Figure 2.7B). This expression can be rescued with ADAM19 rescue mRNA.

To investigate whether the reduction of the neural and neural crest markers were due to the reduction of ADAM19 in these tissues or a secondary effect of the dorsal mesoderm patterning, I injected MO19 in both dorsal animal blastomeres at the 8-cell stage to target only ectodermal derivatives (Figure 2.7B, right graph). Only 1 ng of
MO19 was injected in each cell at the 8-cell stage to adjust for the reduction in cell size. The results show that a reduction of ADAM19 protein in the ectoderm is sufficient to reduce N-tubulin and ADAM11 in the same proportion as those observed when the MO19 is injected at the one cell stage. In contrast no change was observed in the somitic mesoderm marker Myosin Light Chain (MLC), confirming the proper targeting of our injection. These results suggest that ADAM19 is required in the neural ectoderm and neural crest cells to perform its function.

SECTION V: Role of ADAM19 in Somitic Cell Organization and Muscle Differentiation

The expression of ADAM19 increases at neurula and tailbud stages and is mainly concentrated in the dorsal mesoderm and neural tissue. To determine if ADAM19 plays a role in somite development, I performed in situ hybridization using MLC and MyoD at the tailbud stage (st.24) (Figure 2.7A). The results show that ADAM19 knock down dramatically reduces MLC expression in the somites. In contrast, MyoD, which is also expressed in the somites, did not appear to be reduced on the injected side. Furthermore, MyoD staining shows that the correct segmentation of the somites is achieved even in embryos with reduced ADAM19 protein. The reduction of MLC expression was confirmed by quantitative PCR at stage 17 (93% decrease) and at stage 24 (58% decrease) (Figure 2.7B). Expression of MLC was rescued by the co-injection of ADAM19 mRNA (Figure 2.7B). In fact, the same pattern of gene expression (reduction of MLC but no changes in MyoD) was also observed at stage 15 (data not shown). These
results suggest that a reduction in the ADAM19 protein level does not affect early induction and segmentation (MyoD) of the somitic mesoderm but may interfere with the differentiation of the muscle (MLC).

To further investigate the role of ADAM19 in somite formation, I used our ADAM19 antibody (C3) to detect the protein localization on sections of tailbud stage embryos (Figure 2.8). I found that ADAM19 localizes to the notochord, the intersomitic boundary and the ventral half of the neural tube (Figure 2.8A-C). In addition, I found staining within the branchial arches (Figure 2.8A). The distribution of the protein is consistent with the mRNA expression pattern (Figure 2.2) and provides new information about where the protein may be functioning in these tissues. I performed the same staining on embryos injected in one side with MO19. The most dramatic change is the complete absence of ADAM19 staining at the intersomitic junction (Figure 2.8D). As seen for the in situ hybridization with MyoD (Figure 2.7A), the segmentation of the somites is apparent in both sides of the embryos. While the segmentation is unaffected, the organization of the cells within each somite is abnormal in embryos with reduced ADAM19 protein. Furthermore, the nuclei on the control side are aligned at the center of each cell, however this alignment is disrupted on the side with reduced ADAM19 protein (Figure 2.8D). In addition to these somite defects, staining with the mAb 12-101, a marker of muscle differentiation, is decreased on the MO19 injected side (Figure 2.8E). This decrease was observed in the posterior somites but not in the most anterior somites suggesting a delay in 12-101 expression rather than a complete inhibition (data not shown). Moreover, the decrease of 12-101 expression was confirmed by western blot on embryos injected at the one cell stage with MO19 (Figure 2.8G).
To visualize the intersomitic boundary of embryos injected with MO19, I performed staining with antibodies to fibronectin (FN). The staining confirms the presence of somite boundaries in the injected side similar to the control side. Interestingly, I also found that some of the intersomitic boundaries lacked FN (Figure 2.8F). This was confirmed by whole mount immunostain where the entire embryo can be visualized at once (Figure 2.8H). Using this technique, I found that the fibronectin staining was present but slightly abnormal. The defect was seen only in the somites located between the “chevron” and the “straight” shaped somites (approximately 7th somite). Compared to this mild and restricted FN defect, the abnormal distribution of the nuclei affected a larger number of somites. Together, the results presented in Figure 2.7 and 2.8 show that a reduction of the ADAM19 protein affects both the organization and differentiation of somite cells into muscle but not the initial induction of the paraxial mesoderm or the segmentation.

SECTION VI: ADAM19 Affects EGF Signaling during Gastrulation

In mouse, ADAM19 can cleave members of the EGF family of ligands, including Neuregulin-1β (Chesneau et al., 2003; Horiuchi et al., 2005; Kawaguchi et al., 2007; Shirakabe et al., 2001). Upon cleavage the EGF ligands bind to the EGF receptor and activate a signaling cascade involving AKT/PKB (protein kinase B). To investigate if Xenopus ADAM19 plays a role in the EGF signaling pathway, I performed western blot analysis of late gastrula stage embryos injected with either CMO or MO19. Our results show that phosphorylated AKT (AKTp) is decreased significantly in embryos with
reduced ADAM19 protein (Figure 2.9). In contrast, neither phosphorylated MAPK (MAPKp) nor the cytoplasmic adaptor protein PACSIN2 was reduced. To investigate whether the EGF signaling pathway was involved in neural crest cell induction, I used a pharmacological inhibitor of the EGF receptor (AG1478, 100 µM). I treated embryos during gastrulation between stage 10 and stage 12 with AG1478 compared to a DMSO control treatment. In addition, I also used a broad-spectrum inhibitor of metalloproteases called Marimastat (10 µM) to inhibit most ADAM protease activity (Orth et al., 2004). Based on the blastopore size, neither inhibitor affected gastrulation movements (data not shown). However using real time PCR, I found that Marimastat and AG1478 significantly decreased Slug expression (Figure 2.9C). This result confirms the importance of the EGF signaling pathway and metalloprotease activity in neural crest cell induction. As a control, I performed western blot with the AKTp antibody and found a 50% reduction in embryos treated with the EGF receptor inhibitor confirming its activity in vivo. Similar to what was observed with MO19 (Figure 2.9B), I also found that Marimastat reduced the level of phosphorylated AKT (data not shown), suggesting that metalloprotease activity, possibly of ADAM19, is important for EGF signaling during gastrulation.

SECTION VII: Discussion

ADAM19 Expression and Function in the Early Embryo

This chapter describes the cloning and molecular analysis of the Xenopus laevis homologue of ADAM19. Maternal ADAM19 mRNA is present during the initial cleavage stages. Zygotic expression is first detected during gastrulation in the dorsal
mesoderm of the dorsal blastopore lip. During later development, ADAM19 mRNA is localized to dorsal, neural and mesodermal derived structures including the neural tube, the notochord, and the somites. Loss of function experiments using antisense morpholino oligonucleotides shows a reduction of Brachyury in the notochord of gastrula stage embryos and an increase in the dorsal mesoderm markers Goosecoid and Chordin. During neurulation and tailbud formation a reduction of ADAM19 protein results in the reduction of neuronal, neural crest and somitic markers. The segmentation of somites is not perturbed but the organization of cells within an individual somite is disrupted. Lastly, I present evidence that the EGF signaling pathway is affected by ADAM19 knock down.

ADAM19 mRNA is expressed during early mouse embryonic development in a pattern similar to the one described here for Xenopus. ADAM19 is present in dorsal tissues including the spinal cord, the craniofacial and dorsal root ganglia, and the peripheral nervous system. In the mouse, ADAM19 is also present in mesenchymal condensation of the tailbud and the heart (Kurisaki et al., 1998). ADAM19 KO mice die perinatally due to defects observed in heart formation (Kurohara et al., 2004; Zhou et al., 2004), confirming the importance of ADAM19 in heart morphogenesis. The heart defects were shown to be due to a lack of the ADAM19 protein in cardiac neural crest cells rather than in the endothelial cells (Komatsu et al., 2007). The cardiac neural crest cell migration was not affected by the lack of ADAM19; however the ability of the cells to participate in heart morphogenesis was perturbed. In Xenopus, a reduction of ADAM19 protein affects early gene expression in the gastrula, neurula and early tailbud stage. I have also observed a decrease in the expression of three cardiac specific genes
during heart formation (Appendix II), suggesting that ADAM19 is also involved in heart morphogenesis in amphibians. It is interesting to note that ADAM12 (meltrin-α) was not found during early embryogenesis in *Xenopus laevis*, while ADAM9 and ADAM13 are both expressed in overlapping patterns with ADAM19 (Alfandari et al., 1997; Cai et al., 1998). This suggests that ADAM9, ADAM13 and ADAM19 may have overlapping functions during *Xenopus laevis* gastrulation.

In addition to the prominent role of ADAM19 in cardiac morphogenesis, the ADAM19 KO mice also have minor defects found in other tissues. Most of the ADAM19 KO mice (~80%) die perinatally due to defects shown in heart development. However, approximately 20% survive to adulthood and appear healthy and fertile. If the adult ADAM19 KO mice become pregnant, they die late in pregnancy due to an inability to compensate for the increased circulatory demands (Zhou et al., 2004). ADAM19 is highly enriched in the placenta during pregnancy and believed to be involved in trophoblast invasion of rhesus monkeys, *Macaca mulatta*, (Wang et al., 2005) and in humans (Zhao et al., 2009). This strongly suggests that ADAM19 may function in the placenta during pregnancy. Another group has reported that some ADAM19 KO mice carrying a mixed genetic background of 129/Sv and C57BL/6 survive to adulthood but have defects in the peripheral nervous system (Kurohara et al., 2004). The differentiation of Schwann cells from promyelin to myelinating stages is drastically delayed in ADAM19 KO mice upon sciatic nerve injury. It is interesting to note, that this defect is due to a delay in the AKT-dependent upregulation of Krox20. These results further support the role of ADAM19 in the AKT signaling pathway.
Role of ADAM19 in Early Patterning of the Embryo

A reduction of ADAM19 in gastrula stage embryos results in a decrease of Brachyury expression in the notochord and an increase of the dorsal mesoderm markers, Goosecoid and Chordin. This result is consistent with the observation that the upregulation of Goosecoid can repress Brachyury expression (Boucher et al., 2000). Thus ADAM19, which is expressed in the dorsal mesoderm, may participate in restricting Goosecoid gene expression. On the other hand, ADAM19 may be important to maintain Brachyury expression in the notochord. At the moment, it is not clear whether Goosecoid overexpression is preventing Brachyury expression in the notochord or if these are independently affected by ADAM19. Further analysis into ADAM19 function on Brachyury expression will be investigated in Chapter IV of this thesis. Previous work has shown that high doses of TGF-β can induce Goosecoid and Chordin at the same time repress Brachyury expression (Latinkic and Smith, 1999; Latinkic et al., 1997). Thus ADAM19 knock down may increase TGF-β signaling in the early embryo. This suggests that ADAM19 function is to negatively regulate TGF-β signaling. In mouse, ADAM12 promotes TGF-β signaling by interacting with the TGF-β type II receptor to control its subcellular localization (Atfi et al., 2007). Therefore ADAM19 may also interact with the TGF-βII receptor but decrease rather than increase TGF-β signaling. In addition, the EGF-CFC/FLR1 protein was shown to modulate TGF-β signaling via nodal related proteins (Cheng et al., 2003; Dorey and Hill, 2006; Yabe et al., 2003). Cleavage of FLR-1 by ADAM19 may reduce TGF-β signaling at the end of gastrulation coinciding with the disappearance of FLR-1 mRNA expression. Thus there are several possible ways that
ADAM19 may control TGF-β signaling during gastrulation. Furthermore, the expression patterns of Brachyury and Chordin in the axial mesoderm, as been shown to control the anterior-posterior (A-P) polarity of the notochord. Chordin localizes to the anterior notochord while Brachyury localizes to the posterior notochord (Ninomiya et al., 2004). This suggests that embryos with reduced ADAM19 protein have notochords that are “anterior-ized” compared to control embryos. Further analysis into the A-P polarity of the notochord will be investigated in Chapter IV of this thesis.

Through various gene expression patterns for the notochord, paraxial mesoderm and neural ectoderm, I have found that the separation between the axial and paraxial tissues may be delayed or impaired in embryos with reduced ADAM19 protein. ADAM metalloproteases can cleave cell-cell adhesion molecules such as cadherins or protocadherins (Maretzky et al., 2005; McCusker et al., 2009; Reiss et al., 2005). The role of the protocadherins AXPC and PAPC in notochord and somite formation is elegantly demonstrated in Xenopus (Chen and Gumbiner, 2006; Kim et al., 2000; Kim et al., 1998; Kuroda et al., 2002; Unterseher et al., 2004; Wang et al., 2008) and it is possible that the activity of these proteins is regulated by proteolytic processing. Therefore, ADAM19 may be involved in separating the axial mesoderm from paraxial mesoderm by cleaving cell adhesion molecules such as protocadherins.

**Role of ADAM19 in EGF Signaling of the Embryo**

The EGF signaling pathway is the best characterized signaling pathway involving ADAM19 (Blobel, 2005). In mice, ADAM19 can cleave some members of the EGF
ligand family including Neuregulin1-β (NRG1β). In Xenopus, NRG1β is expressed as a maternal transcript with zygotic expression beginning during gastrulation through tailbud formation (Chung and Chung, 1999; Yang et al., 1999; Yang et al., 1998a). Purified recombinant NRG1β can induce naïve animal cap cells to become mesoderm and express Brachyury (Chung and Chung, 1999). Therefore, ADAM19 mediated cleavage of NRG1β in the dorsal mesoderm may be important for maintaining Brachyury expression in the notochord. NRG1β signals through the EGF receptors ErbB3/B4 tyrosine kinases to activate the MAPK and PI3K/AKT signaling pathways (Esper et al., 2006). Furthermore, the ErbB signaling pathway also contributes to convergence and extension movements during gastrulation (Nie and Chang, 2007b). However, a knock down of ErbB3 or ErbB4 did not inhibit Brachyury expression in the notochord, suggesting that ADAM19 function may act through a different signaling pathway (Nie and Chang, 2007a; Nie and Chang, 2007b). I have found that ADAM19 knock down decreases AKT<sub>p</sub>, a downstream component of the EGF signaling pathway. In addition, the EGF receptor inhibitor AG1478 affects neural crest gene expression similar to the ADAM19 knock down. These results suggest that ADAM19 is important for EGF receptor activation and that the EGF signaling pathway is important for neural crest cell induction.

**Role of ADAM19 in Neural, Neural Crest and Muscle Development**

During neurulation and tailbud formation, embryos with reduced ADAM19 protein show a reduction of N-tubulin and NRPI in the neural tissue and MLC in the somites. Interestingly, for both the dorsal mesoderm and neural tissue, the overall
induction is not affected as shown by the expression of Sox2 in the neural tube and MyoD in the dorsal mesoderm. This suggests that ADAM19 may function only as a modifier rather than a master regulator of cell fate. ADAM19 knock down reduces the expression of CNC cell markers xTwist, Sox8, Slug and ADAM11. *In situ* hybridization using xTwist reveals that at least some CNC maintain xTwist expression and are capable of migration. However, our lab has shown that CNC with a reduced level of ADAM19 do not migrate as efficiently as wild type cells *in vivo*. Together with the conditional ADAM19 KO mice in the cardiac neural crest, ADAM19 may be required for both neural crest cell induction and function. This could be due to either a direct affect on migration or the observed defect in neural crest induction. Additional experiments are required to test these two hypotheses. Once again, Neuregulin appears as an attractive candidate as a substrate for ADAM19 because Neuregulin can induce both neural and muscle specific markers in Xenopus animal cap assays (Chung and Chung, 1999). Moreover, the role of NRG1β in neuronal cell specification has been studied extensively (Britsch et al., 1998; Cameron et al., 2001).

In the paraxial mesoderm, a reduction of ADAM19 protein does not prevent muscle induction or somite segmentation. In addition, the fibronectin rich extracellular matrix is deposited at the intersomitic boundary. While the general organization of the somites does not depend on ADAM19, cell organization within each somite appears to be affected by a reduction of ADAM19 protein. Specifically, the morphotype suggests that cell rotation may be delayed or abnormal. Together with somite organization, at least two markers of muscle differentiation (MLC and 12-101) are reduced in ADAM19 knock down embryos. It is not clear at the moment whether the effects on cell organization and
muscle differentiation are linked. As for the early gastrulation defects, either a signaling role for ADAM19 or a structural role via cleavage of a cell adhesion molecule could account for the defects seen following ADAM19 knock down. Loss of function and complementation experiments using the putative substrates for ADAM19 will be necessary to determine which of the signaling pathways ADAM19 regulates. The Xenopus system may provide a unique way to dissect in vivo the role of ADAM19 in early embryonic development.

SECTION VIII: Materials and Methods

Embryo Culture
Xenopus laevis eggs were fertilized and the resultant embryos were de-jellied in 2% cysteine and cultured as previously described (Newport and Kirschner, 1982a; Newport and Kirschner, 1982b). Staging of embryos was according to (Nieuwkoop, 1967a; Nieuwkoop, 1967b).

Cloning of ADAM19
Degenerate oligonucleotide primers were designed from sequences conserved in human, mouse, quail, and Xenopus tropicalis. PCR was carried out with cDNA from stage 20, stage 36 and adult liver using S1 (amino acids LNIRIAL) and AS3 (amino acids CNNNKN) at an annealing temperature of 50°C. The corresponding PCR product was cloned into TOPO-TA (Invitrogen) and sequenced. Specific primers were designed and used in 5’ and 3’ RACE-PCR with the Generacer kit (Invitrogen) according to the
manufacturer’s instructions. Clones from at least 3 independent PCR reactions were sequenced to obtain the full length ADAM19.

**Quantitative PCR**

Total RNA was isolated from 5 embryos according to (Chomczynski and Sacchi, 1987) followed by LiCl precipitation and poly A purification using the Oligotex kit (Qiagen) according to the manufacturer instructions. The cDNA was reversed transcribed using the Quantum Qscript kit (Quanta Biosciences). All PCR reactions were carried out using SYBR green (Takara) on a MX3005P light cycler (Stratagene). All experiments were repeated at least 3 times on separate injection days and the real time PCR was also performed in triplicate on separate days to maximize variation due to PCR efficiency. The results were analyzed using the 2^{-ΔCT} method (Livak and Schmittgen, 2001) and are presented as the Log2 fold change. All data were normalized using alpha tubulin. The list of the real time PCR primers is shown in Table 2.1.

**Plasmid Constructs and mRNA Microinjection**

Full-length Xenopus ADAM19 was amplified by PCR using 2.5 units of *Pfu* DNA polymerase (Stratagene) and cloned into the pCS2+ vector (Turner and Weintraub, 1994). Rescue ADAM19 mRNA was generated using a sense oligonucleotide containing 4 nucleotide silent mutations at the MO binding site (5’ – CATGGAAGGTTGCAACAGGAGTC – 3’, mutation are in bold). Non-rescue ADAM19 mRNA corresponds to the original cDNA sequence. The pCS2-ADAM19 construct was linearized by NotI and transcribed by SP6 RNA polymerase. All mRNA was prepared for
microinjection as described in (Cousin et al., 2000). Transcripts were injected close to the animal pole region at the indicated dosages and embryo stages stated in the figure legends. For embryos where one blastomere at the two-cell stage was injected, the uninjected half of the embryo served as an internal control for each embryo.

**Antisense Morpholino experiments**

An antisense Morpholino (MO) was synthesized (Gene Tools Carvalis, OR) just upstream of the ATG start codon for ADAM19 (MO19). The sequence was selected to be complementary with all the 5’ cDNA clone obtained by RACE PCR and is also a perfect match to the published sequence for ADAM19 in *Xenopus tropicalis*. A control MO (CMO) was used to test for non-specific toxicity in the dose response experiments. The sequences of the morpholinos used in this dissertation are in Table 2.1. Serial dilutions of MO19 (50 ng to 1 ng) were injected in 5 nl volume into wild-type embryos to test for toxicity. A dosage of 10 ng of MO19 was selected and was shown to inhibit translation of 1 ng of the wild type ADAM19 mRNA in embryos while allowing translation of a messenger containing the 4 nt mismatch. When the MO where injected in a subset of cells to target specific tissues, β-galactosidase or GFP-myc RNA was injected to trace the lineage with reduced ADAM19 protein.

**Whole Mount β-galactosidase Detection and In Situ Hybridization**

Embryos were fixed in MEMFA (0.1 M MOPS pH 7.4, 2 mM EGTA pH 8, 1 mM MgSO4, and 3.7% formaldehyde) for 1hr at 20°C. They were washed in PBS and stained at 37°C in PBS solution containing 1 mg/ml X-gal, 20 mM K3Fe(CN)6, 20 mM
K₄Fe(CN)₆, and 2 mM MgCl₂. The embryos were dehydrated in methanol and stored at -20°C. Whole mount *in situ* hybridization was performed exactly as described previously using albino or wild-type embryos (Harland, 1991).

**Western Blot Analysis**

Embryo protein extraction and western blot analysis were performed exactly as described previously (Alfandari et al., 1997). Monoclonal antibody to PACSIN2 (3D8) and GAPDH (Millipore) was used as a loading control as described previously (Cousin et al., 2000). For quantification, blots were detected using chemiluminescence on a G-Box HR16 (Syngene) and analyzed using the Genetool analysis software. Alternatively for weaker signals, the blots were exposed on X-ray films for various time points and the films were scanned and analyzed by densitometry using the same software.

**Immunofluorescence and Whole Mount Immunostain**

Embryos were fixed using either MEMFA or DENTS (20% DMSO in 80% Methanol) and stored in methanol before sectioning. Embedding was performed by increasing doses of 15% fish skin gelatin (Sigma) and 15% sucrose in PBS. After a final impregnation overnight at 4°C, embryos were mounted in OCT (Tissue Teck). Cryosections (14 to 16 µm) were blocked in PBS containing 20% goat serum and 1% BSA (Sigma). Primary antibodies were used at 10 µg/ml and secondary antibodies (Jackson ImmunoResearch) were used at 5 µg/ml. Slides were mounted using Vectashield (Vector) and imaged using a Zeiss Axiovert 200M inverted microscope equipped with a Ludl XY-stage control and a Hamamatsu Orca camera. Images were taken using the ApoTome structured light
illumination system (Zeiss) and the AxioVision software (Zeiss). Whole mount immunostaining was performed as previously described (Dent et al., 1989).

**Time Lapse Imaging**

Embryos at late blastula stage were individually placed in holes made in an agarose coated plastic dish. Time lapse movies were made using an Axiovert inverted microscope (Zeiss) equipped with a Ludl XY-stage control and an Orca camera (Hamamatsu) and controlled by the OpenLab software (Improvision). Measurements were done using the same software.
Figure 2.1: ADAM19 expression. Quantitative real-time PCR expression of ADAM19 during early Xenopus development. Polyadenylated RNA from 2.5 embryos was used to make cDNA. PCR amplification was performed on a light cycler for 40 cycles. (A) The amplification product from both the control (-RT) and the experimental (+RT) were separated on a 2% agarose gel. (B) The relative abundance of the ADAM19 mRNA was measured by the $2^{(-ΔCT)}$ method (Livak and Schmittgen, 2001) using stage 10 as a value of 1. Values are not adjusted to a control gene but to identical number of embryos. The CT values for each reaction are given below the graph. No CT values were obtained for the -RT control of each stage. ADAM19 mRNA is present as a maternal transcript at stage 2. Zygotic transcription starts during gastrulation and increases during neurulation (Stage 16) continuing into early tailbud stage (Stage 21).
Figure 2.2: ADAM19 mRNA distribution. Whole mount in situ hybridization using a probe corresponding to Xenopus ADAM19. (A) Lateral view of a bisected gastrula with the dorsal side to the left and the animal pole at the top (ap). (B; G) Dorsal views of early tailbud stage embryos with the anterior at the top. (C; D; E; I) Lateral views of tailbud stage embryos with anterior to the left. (F) Ventral view of an early tailbud stage embryo. Photographs in A, F and I were taken with embryos in PBS to visualize superficial rather than internal staining. All other embryos were imaged in Murray’s clear. (A) ADAM19 is first expressed in the dorsal mesoderm of the gastrula, with the most intense signal in the dorsal blastopore lip (dbl). (B; F; G; H) At early tailbud stage, ADAM19 is found in the dorsal mesoderm including the notochord (n), somites (s), the cement gland (cg) and the neural tube (nt). (C; D; I) ADAM19 expression in these tissues persists during tailbud formation. In the anterior, ADAM19 is expressed in the brain (br), the otic (ot) and optic (o) vesicles as well as CNC segments and branchial arches (ba). (E) At stage 36, the expression in the pronephros (p) is also detected.
Figure 2.3: Morpholino oligonucleotide knock down of ADAM19. Western blot of protein extract from embryos injected with a control morpholino or a morpholino to either ADAM13 (MO13) or ADAM19 (MO19). The morpholinos (10 ng) were injected at the one cell-stage and embryos were extracted at stage 22. (A) Immunoprecipitation of ADAM19 using the goat DC19 antibody separated by SDS-PAGE and probed with a rabbit polyclonal antibody to the ADAM19 cytoplasmic domain (AP ADAM19 C3). A monoclonal antibody to GAPDH was used as a loading control. (B) The densitometric analysis of the blot depicted in A shows that the ADAM19 protein is reduced by approximately 100% by the MO19 when normalized to the GAPDH expression level. (C) The MO19 is capable of preventing translation of 1 ng of ADAM19 mRNA when co-injected in embryos (A19NR) but not a messenger with 4 mutations in the sequence corresponding to the MO19 (A19R). The ADAM13 morpholino does not affect ADAM19 translation.
Figure 2.4: ADAM19 is not required for blastopore closure. Time-lapse imaging of gastrula stage embryos injected with either a control MO (CMO) or MO19. Embryos were microinjected with 10 ng of MO at the one-cell stage and allowed to develop through the end of gastrulation. The blastopore size in micrometers was measured using the OpenLab software at three different time points during gastrulation. The values represent the average of three different experiments using 8 embryos each for the control and the experimental. Error bars correspond to standard deviation from the mean. Representative images are shown below the histogram.
Figure 2.5: Gene Expression analysis of ADAM19 knock down at the gastrula stage. (A) Embryos were microinjected with 10 ng of either the MO19 or a control MO at the one-cell stage and allowed to develop until gastrula stage (stage 12 and 13). Whole mount in situ hybridization were performed using the markers Brachyury, Sox2 (Xbra Sox2, stage 12), and Chordin and Goosecoid (Chd and Gsc, stage 13). For all embryos, the dorsal view is presented with the anterior up. The red arrowheads point to the notochord. The number of embryos and the frequency of the phenotype depicted in the photographs are indicated in the text. (B) Quantitative real-time PCR was performed on embryos injected with either MO19 alone or together with ADAM19 Rescue mRNA (MO19+A19R). The relative abundance of Chordin (Chd), Goosecoid (Gsc) and Brachyury (Xbra) gene expression was measured by the $2^{-\Delta\Delta CT}$ method using either embryos injected with the control MO or non-injected and normalized to alpha-tubulin. There was no effect of the control MO to gene expression. The asterisks represent statistical significance at $P<0.05$ using a student t test. There is a small decrease in Xbra (16%, not significant) and a robust increase in both Chd (187%) and Gsc (230%) which are rescued by the injection of the ADAM19 mRNA.
Figure 2.6: ADAM19 is critical for neural crest induction. (A) Whole mount *in situ* hybridization of neurula (stage 15) embryos using Slug and Sox8 to detect neural crest cells. The injected side is to the left. Embryos injected with MO19 show a decrease in expression of both neural crest markers (red arrowhead). (B) Real time PCR analysis at stage 15. The level of gene expression was measured by the $2^{-\Delta CT}$ method and normalized to alpha-tubulin. Results are presented as the Log2 of the fold change compared to non injected embryos. Variation of Sox2, Sox8, and Slug are all significant (p<0.01) in embryos injected with MO19 when compared to the control MO. (C) Lateral view of tailbud stage embryos (stage 24) visualizing GFP. Embryos were injected at the 16-cell stage in a dorso-lateral-animal blastomere to target neural crest cells. In the control, GFP is found in all CNC segments (white arrows). This is absent in 33% of the embryos injected with MO19.
Figure 2.7: Gene Expression analysis of ADAM19 knock down at the tailbud stage. (A) Whole-mount *in situ* hybridization of tailbud stage embryos (stage 22-24). Embryos were injected in one blastomere of the two-cell stage with 10 ng of CMO or MO19 with mRNA encoding beta-galactosidase (β-Gal) as a lineage tracer. The embryos were processed by whole mount *in situ* hybridization with the markers Myosin Light Chain (MLC), N-tubulin, NRP1, Sox2 and ADAM11. The injected side, as detected by β-Gal activity (light blue), is on the left side for all embryos. The percentages of embryos displaying these phenotypes are indicated and represent the average of 3 independent experiments. The black arrowheads point to the area of decreased gene expression. For Twist the embryo heads are presented as a side view and the injected side of each embryo is to the left. A decrease in xTwist expression is visible (arrowhead) in embryos injected with MO19. (B) Quantitative real-time PCR was performed on embryos injected at the one-cell stage with either the MO19 alone or with the ADAM19 rescue mRNA (MO19+A19R) and allowed to develop until neurula (stage 17) and tailbud stage (stage 24). On the right, the MO19 was injected in two dorsal-animal blastomeres at the 8-cell stage (MO19 Ectoderm) to target dorsal ectoderm but not mesoderm derivatives. All genes were compared to non-injected control embryos. The relative abundance of MLC, N-tubulin, Sox2 and ADAM11 gene expression was measured by the 2-ΔCT method and normalized to alpha-tubulin. Results are presented as the Log2 of the fold change and error bars correspond to the standard error. Asterisks represent a p<0.05 using a standard t test assuming unequal variance.
Figure 2.8: ADAM19 is essential for somite organization and muscle differentiation. Histological and molecular analysis of embryos with reduced ADAM19 protein. (A-F) Frozen sections of tailbud stage embryos. (A-D) Immunofluorescence using the rabbit polyclonal antibody to the ADAM19 cytoplasmic domain (AP C3). (A) Frontal section, anterior is to the left. The ADAM19 protein is localized to the intersomitic boundary (arrowhead), the notochord (n), the neural tube (nt) and in the branchial arches (ba). (B) Transverse section. ADAM19 is in red, the muscle marker 12-101 in green and the nuclei (DAPI) in blue. ADAM19 staining appears associated with the ventral basal side of the neural tube (arrowhead) and the notochord (n). (C) Magnification of the somite from a frontal section, ADAM19 is in red, the arrowhead points to the intersomitic boundary. (D-F) Frontal sections of tailbud stage embryos injected at the two-cell stage in one cell with 5 ng of MO19. The injected side is labeled (yellow dots) and was detected either by using the ADAM19 antibody C3 (D and E) or GFP-myc (F) that was co-injected with the MO19. The injection of MO19 completely abolished staining of the intersomitic boundary by the ADAM19 antibody. While the intersomitic boundary is still visible, the nuclei are not aligned on the injected side (white line). (E) ADAM19 is in green (left), 12-101 in red (center) and the merge presented with DAPI staining (right). There is a clear reduction of 12-101 staining on the injected side (MO19). (F) The MO19 was co-injected with GFP-myc to detect the cells that were targeted using the 9E10 mAb. Positive cells are visible in the right somites and some of the cells that contribute to the notochord (n). Fibronectin was detected using a rabbit polyclonal antibody (32F). Some of the FN staining is absent in part of the intersomitic boundary (arrowhead). (G) Western blot using 12-101 on total protein extract. Stage 7 extract is used as a negative control. At stage 22, MO19 injected embryos but none of the other injected embryos have less 12-101 protein. (H) Whole mount immunostain using mAb 4H2 to detect fibronectin. The injected side was detected prior to fixation using GFP fluorescence. The side injected with the MO19 shows an abnormal pattern of FN staining at some of the intersomitic boundaries (arrowhead).
Figure 2.9: ADAM19 may interfere with EGF signaling. (A) Western blot using an antibody to phosphorylated AKT, β-catenin, phosphorylated MAP kinase, and PACSIN2. Embryos were injected at the one-cell stage with either the control MO (CMO) or MO19 and developed to late gastrula (stage 12). Total protein 0.5 embryo equivalent were used for each blot. (B) Histogram representing the quantification of the western blot from 3 independent injection experiments. All measures were normalized to PACSIN2. Variations in pMAPK were not significant while the decrease in pAKT and β-catenin was significant (p<0.05). (C) Real time PCR analysis of Slug expression. Embryos were treated with 10 µM of Marimastat (Mar), 100 µM of AG1478 or DMSO (1/1000) as a control, between stage 10 and stage 12, based on the blastopore size (none of the treatment affected gastrulation timing in these conditions). Marimastat decreased Slug expression by an average of 34% while AG1478 decrease Slug by 60%. 
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**Table 2.1: Quantitative Real Time PCR Primer Sets used in Chapter II.**
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Table 2.2: Morpholino Sequences used in this Thesis
CHAPTER III

ADAM13 AND ADAM19 PROTEIN REGULATION

All of the work presented in this chapter is data completed by myself and is unpublished.

Abstract

This chapter focuses on ADAM13 and ADAM19 protein regulation in cultured cells and in the embryo. I show that a loss of ADAM13 protein reduces the level of ADAM19 protein by 50%. In addition, a loss of ADAM19 protein reduces the level of ADAM13 protein by 50%. This suggests that both ADAM13 and ADAM19 are required to maintain a proper protein level in the embryo. I propose this outcome may be explained by physical interactions of ADAM13 and ADAM19 in a cell. The ADAM19 Proform binds to the ADAM13 Proform in cultured cells. Through domain analysis, I show that ADAM19 binds specifically to the cysteine-rich domain of ADAM13. When co-overexpressed in a cell, the level of Mature ADAM13 (compared to the Proform) is reduced suggesting a complex form of regulation. Lastly, I propose a few models that could explain how ADAM19 regulates the level of ADAM13 in a cell.

SECTION I: Introduction

Cell surface membrane bound proteins undergo a number of post-translational modifications before reaching their final destination in a cell. The importance of these
modifications allows a cell to have a rapid response to changes within its microenvironment. Members of the ADAM family of metalloproteases are known to function as rapid responders to cellular events, such as inflammation (Murphy, 2009). ADAM proteins are synthesized in the endoplasmic reticulum where a number of glycosylations occur post-translationally. The proper folding of an ADAM protein, based on data for ADAM10 and ADAM17, involves the ER chaperone ERp57 in conjunction with calnexin and calreticulin (Jessop et al., 2007). Once folded, the ADAM protein is transported from the ER and through the Golgi network. Newly synthesized ADAM proteins emerge in an inactive state called a Proform. The ADAM Proform is kept catalytically inactive through a ‘cysteine switch’ mechanism where a single cysteine residue in their prodomain interacts with the Zinc atom located in the active site of their Metalloprotease domain (Van Wart and Birkedal-Hansen, 1990). This mechanism keeps the Prodomain in close contact with the Metalloprotease domain rendering the enzyme inactive. Further maturation occurs in the Trans Golgi compartment where a proprotein convertase, specifically Furin, cleaves the prodomain of an ADAM at the consensus recognition site, RxKR (Loechel et al., 1998). This processing event is shown for some ADAM members to be a key step toward ADAM “activation” into the active state called a Mature form (Figure 1.3). At this point, the Mature ADAM protein can be transported to the cell surface to carry out its function. This is the ‘classical’ post-translational modification route for an ADAM protein.

Not all ADAM proteins can function at the cell surface and depending on the cell type; many ADAM proteins are stored in a perinuclear compartment believed to be the ER-Golgi complex (Schlondorff et al., 2000). Furthermore, potential ADAM substrates
localize to various cellular compartments as well as travel a similar post-translational modifications route in a cell. It is determined that two ADAM members, ADAM17 and ADAM19, cleave the EGF ligand Neuregulinβ1 (NRGβ1) in a cell (Horiuchi et al., 2005). ADAM19 cleaves NRGβ1 in the Golgi complex while ADAM17 cleaves NRGβ1 at the cell surface (Yokozeki et al., 2007). These experiments were carried out by western blotting for ADAM17 and ADAM19 on subcellular fractionation extracts (Yokozeki et al., 2007). Additionally for ADAM19, experiments using a NRGβ1 construct that is N-terminally fused to GFP were analyzed under fluorescence correlation spectroscopy (FCS). FCS is used to detect fluctuations of fluorescent intensity in a confocally defined volume in living cells. Thus, the trafficking and subcellular localization of ADAM proteins and their substrates may provide spatial and temporal control of how and where an ADAM protein functions within a cell (Murphy, 2009).

The overexpression of an ADAM and a potential substrate protein in cell culture results in the ectodomain shedding of the substrate into the cell supernatant. This technique generated an initial list of potential biologically relevant substrates to ADAM family members (Table 1 in (Huovila et al., 2005)). In addition, multiple ADAM proteins can cleave the same substrate in a cell, showing that ADAM family members have overlapping functions (Schlondorff and Blobel, 1999). Studies done on knockout mice have provided some clues into ADAM protein function during embryogenesis. However, single knockout of many ADAM members (ADAM8, ADAM9, ADAM12, ADAM15) result in viable mice with no observable phenotype (Horiuchi et al., 2003; Kelly et al., 2005; Kurisaki et al., 2003; Weskamp et al., 2002). Even the double (ADAM 9/15−/−) and triple (ADAM 9/12/15−/−) knockout mice are viable with no
observable phenotype (Sahin et al., 2004). It is highly unlikely that these ADAM members do not function during embryogenesis because they have broad expression patterns in the embryo. Therefore, it is more likely that ADAM family members have overlapping functions during embryogenesis in vivo (Alfandari et al., 2009).

Since multiple ADAM members are capable of cleaving the same substrates, it is suggested that ADAM proteins may work as a team in a tissue specific manner (Hartmann et al., 2002). The idea that two ADAM family members can function together may be an integral part of ADAM metalloprotease regulation. For example, the first two ADAM family members discovered were ADAM1 and ADAM2, also known as Fertilin-α and Fertilin-β respectively. Both proteins form a heterodimer complex on the plasma membrane of sperm and are important for sperm migration into the oviduct and binding to the zona pellucida of the oocyte (Nishimura et al., 2007; Yamaguchi et al., 2009). In this chapter I will show that both ADAM13 and ADAM19 are required to maintain a proper protein level in the embryo. Furthermore, this regulation may occur at the protein level through their physical interaction in a cell.

**Results**

**SECTION II: ADAM13 and ADAM19 Regulation in the Embryo**

Xenopus ADAM13 and ADAM19 are members of the meltrin subfamily of ADAM metalloproteases (Alfandari et al., 1997; Neuner et al., 2009). The full length proteins share 41% identity at the amino acid level with the Disintegrin domain sharing the highest identity (64%) and the Cytoplasmic domain sharing the lowest identity (13%)
ADAM13 and ADAM19 have predicted N-glycosylation sites (NxS/T) located in their extracellular region (Gooley et al., 1991). N-glycosylation occurs in the ER and, based on data for ADAM10 and ADAMTS13, is important for further processing and stability of the full length protein (Escrevente et al., 2008; Zhou and Tsai, 2009). In addition, N-glycosylation of ADAM10 is important for enzymatic activity in vitro (Escrevente et al., 2008). A conserved Metalloprotease active site (HExGHxxGxxHD) is found in the Metalloprotease domain. The Disintegrin domain contains a “Disintegrin Loop” motif that, in its three-dimensional structure, is shown to fold into a β-turn believed to bind directly to integrin receptors (McLane et al., 1998; Norris et al., 2003). The Cysteine-rich domain is important for substrate selectivity and for ADAM13, is the site for autocatalysis or self-shedding (Gaultier et al., 2002). The cytoplasmic domain contains a number of SH3 binding motifs capable of protein-protein interactions. In addition, a number of Serine and Threonine phosphorylation sites are predicted throughout ADAM13 and ADAM19 cytoplasmic domains, as well as, a predicted Tyrosine phosphorylation site in the cytoplasmic domain of ADAM19 (Blom et al., 1999) (Figure 3.1). Phosphorylation of membrane bound proteins is a form of regulation that modifies their intrinsic properties or creates a docking site for interacting with other proteins (Deribe et al., 2010).

ADAM13 is expressed as a maternal transcript with zygotic expression beginning from early gastrulation through tailbud formation. During gastrulation, ADAM13 and ADAM19 are expressed in the dorsal mesoderm of the dorsal blastopore lip (Neuner et al., 2009; Wei et al., 2010). Throughout early embryonic development, ADAM13 has a more restricted expression pattern, compared to ADAM19, being localized to the somitic
mesoderm and cranial neural crest cells (Alfandari et al., 1997). Since ADAM13 and ADAM19 are expressed in similar tissues, I investigated whether ADAM13 and ADAM19 functionally interacted.

Antisense Morpholino oligonucleotides corresponding to the sequence near the ATG start codon for ADAM13 (MO13) and ADAM19 (MO19) were used to prevent protein translation (Table 2.1). Embryos injected with MO13 showed a clear absence of ADAM13 protein at the tailbud stage (st. 22) confirming the specificity of both our morpholino sequence and our antibodies (Figure 3.2). This result was shown by glycoproteins purified on Concanavalin-A agarose beads and by immunoprecipitation for ADAM13. Embryos injected with MO13 showed a 50% reduction in the level of ADAM19 at the tailbud stage (st. 22) (Figure 3.3). In the reverse experiment, embryos injected with MO19 showed a clear absence of ADAM19 protein at the tailbud stage confirming the specificity of both our morpholino sequence and our antibodies (Figure 3.3). Embryos injected with MO19 showed a 50% reduction in the level of ADAM13 at the tailbud stage (Figure 3.2). The results suggest that both ADAM13 and ADAM19 need to be present in the embryo in order to maintain a proper protein level.

I propose two hypotheses explaining why ADAM13 and ADAM19 are both required to maintain a proper protein level in the embryo. One hypothesis is that ADAM13 and ADAM19 regulate each other’s gene expression. Our lab has shown that the cytoplasmic domain of ADAM13 is cleaved off and translocates into the nucleus to regulate gene expression important for CNC cell migration (Cousin et al., 2010). In addition, the cytoplasmic domain of ADAM19 can also modulate gene expression and promote CNC cell migration (Cousin et al., 2010). A loss of ADAM19 affects the
expression of a number of genes involved in CNC induction and muscle differentiation (Neuner et al., 2009). Since the expression pattern of ADAM13 is restricted to these tissues and that ADAM11 expression is reduced in CNC cells; it is possible that ADAM19 regulates ADAM13 gene expression. Therefore, ADAM13 and ADAM19 could regulate gene expression through the function of their cytoplasmic domain in the nucleus. To investigate gene expression, I will extract RNA from embryos injected with MO13 or MO19 for real time PCR. In embryos lacking ADAM13, ADAM19 gene expression will be measured. In embryos lacking ADAM19, ADAM13 gene expression will be measured. Since both ADAM13 and ADAM19 are required in the embryo to maintain a proper protein level, my second hypothesis is that ADAM13 and ADAM19 act as chaperones for each other. This is important to regulate their protein stability in a cell. The second hypothesis will be investigated next in Section III of this chapter.

SECTION III: ADAM13 and ADAM19 Physical Interaction in Cultured Cells

To determine if ADAM13 and ADAM19 act as chaperones for protein stability, I transfected 293T cells with ADAM13, ADAM19, or in combination. Total protein extracts were used to determine ADAM13 and ADAM19 protein levels. When ADAM13 and ADAM19 are cotransfected together, the level of ADAM13 is significantly reduced compared to ADAM13 alone (Figure 3.4A, left middle gel lane 2 versus lane 4). However, there is no change in the level of ADAM19 (Figure 3.4A, left middle gel lane 3 versus lane 4). Although the overall level of ADAM13 is reduced, there is a stronger reduction in the Mature form (100kD). The ratio of Proform to Mature
form is approximately 2:1 when compared to ADAM13 alone with an approximate ratio of 1:1. This suggests that the transition from Proform to Mature form of ADAM13 may be regulated by ADAM19.

To determine if ADAM13 and ADAM19 physically interact, I transfected 293T cells with ADAM13, ADAM19, or in combination and total protein extracts were prepared for coimmunoprecipitation. Using an antibody to immunoprecipitate ADAM13 (6615F), a 120kD band was detected by the ADAM19 antibody (2C5 E1) in the cotransfected sample (Figure 3.4A, left). This band corresponds to the Proform of ADAM19. In the reverse experiment, using an antibody to immunoprecipitate ADAM19 (2C5 E1), a 120kD band was detected by the ADAM13 antibody (6615F) in the cotransfected sample (Figure 3.4A, left). This band corresponds to the Proform of ADAM13. The results suggest that both ADAM13 Proform and ADAM19 Proform physically interact when overexpressed in 293T cells.

The coimmunoprecipitation experiment was repeated using the frog cell line, XTC. This cell line endogenously expresses ADAM13 (Alfandari et al., 1997), but does not express ADAM19. I transfected XTC cells with GFP alone or ADAM19 alone and total protein extracts were prepared for coimmunoprecipitation. Using an antibody to immunoprecipitate ADAM13 (6615F), the Proform of ADAM19 (120kD) was detected by the ADAM19 antibody (2C5 E1) only in the ADAM19 transfected sample. In the reverse experiment, using an antibody to immunoprecipitate ADAM19 (2C5 E1), the Proform of ADAM13 and the Mature form of ADAM13 were detected by the ADAM13 antibody (6615F) only in the ADAM19 transfected sample. As a control, ADAM13 was immunoprecipitated from red fluorescent protein (RFP) expressing XTC cells to detect
endogenous ADAM13 in our experiment (Figure 3.4B). The results show that endogenous ADAM13 Proform and Mature form physically interact with overexpressed ADAM19 Proform in XTC cells.

To identify the domain of interaction between ADAM13 and ADAM19, I transfected 293T cells that overexpress different myc-tagged ADAM13 constructs. The Disintegrin Domain alone (D13), the Cysteine Rich Domain alone (CR13), or the Disintegrin-Cysteine Rich Domain (DC13) were cotransfected with full length ADAM19 and total protein extracts were prepared for coimmunoprecipitation. Using an antibody to immunoprecipitate the myc-tagged ADAM13 constructs (9E10), the Proform of ADAM19 was detected by the ADAM19 antibody (C3) (Figure 3.5, first gel). In the reverse experiment, using an antibody to immunoprecipitate ADAM19 (C3), both DC13 and CR13 were detected by the myc-tag antibody (9E10) (Figure 3.5, second gel). Interestingly, DC13 and CR13 strongly interacted with ADAM19 while D13 showed a weaker interaction with ADAM19 (Figure 3.5, second gel). Total cell extract was probed for the myc-tagged ADAM13 constructs and ADAM19 to ensure proper transfections. The results show that ADAM19 binds preferentially to the Cysteine Rich Domain of ADAM13.

Previous work has suggested that ADAM19 may function primarily within a cell at a perinuclear compartment with very little ADAM19 at the cell surface (Murphy, 2009; Yokozeki et al., 2007). To determine where in a cell Xenopus ADAM19 may function, I investigated the subcellular localization of ADAM19 by immunofluorescence. Transfected XTC cells that overexpress ADAM19 were fixed and processed for immunofluorescence. Using an antibody to ADAM19 (2C5 E1) and an antibody to the
ER-specific protein Calnexin, I observed strong colocalization of ADAM19 and Calnexin in the ER (Figure 3.6). Very little to no expression of ADAM19 was found at the plasma membrane in lamellipodia. Since the outer membrane of the nucleus is continuous with the ER, some punctuate expression of Calnexin was observed in the nucleus and this is consistent with previous observations (Gilchrist and Pierce, 1993; Wada et al., 1991). The results show that overexpressed ADAM19 localizes to the endoplasmic reticulum of XTC cells.

Since ADAM13 and ADAM19 physically interact in XTC cells, I investigated where in a cell ADAM13 and ADAM19 interact. Transfected XTC cells that overexpress ADAM19 were fixed and processed for immunofluorescence. Using an antibody to ADAM19 (2C5 E1) and an antibody to ADAM13 (6615F), I observed strong colocalization of overexpressed ADAM19 and endogenous ADAM13 (Figure 3.7). The colocalization occurs within a perinuclear compartment, possibly the endoplasmic reticulum. Endogenous ADAM13 was also observed at the cell surface in lamellipodia (white arrows) consistent with previous observations (Cousin et al., 2000). The results show that ADAM13 and ADAM19 colocalize to a perinuclear compartment of XTC cells.

SECTION IV: Discussion

ADAM Metalloprotease ER Retention

Protein subcellular localization is a major part of the post translational modification mechanism. The sorting process begins in the endoplasmic reticulum
immediately upon protein folding and is further refined in the Golgi complex. A protein can be retained in the endoplasmic reticulum if it contains a specific amino acid sequence known as an ER retention signal (Wieland et al., 1987). Arginine motifs such as ‘RR’ or RxR’ (where ‘x’ is any amino acid) are one class of ER retention signals that do not need to be exposed at the C-terminus of a membrane protein but rather incorporated into the intracellular domain (Michelsen et al., 2005; Zhou et al., 2002). For example, ER retention mediated by an RxR motif is shown for the NMDA receptor (Scott et al., 2001). Upon cell treatment with PMA, a known PKC activator, cell surface expression of the NMDA receptor increased. When two predicted PKC phosphorylation sites adjacent to the RxR motif were mutated to alanine, it completely abolished the affects of PMA. Thus no cell surface expression of the NMDA receptor was observed. Therefore, the receptors release from the endoplasmic reticulum is regulated by PKC phosphorylation of serine residues adjacent to the ER retention signal. This class of ER retention signals has also been found in the cytoplasmic domains of a number of ADAM family members (Table 3.2).

In addition to phosphorylation regulating ER exit, the recruitment of 14-3-3 proteins to the cytoplasmic tail of the $K_{\text{ATP}}$ channel $\alpha$ subunit, Kir6.2 is shown to be important for regulating ER retention (Nufer and Hauri, 2003; Yuan et al., 2003). Upon phosphorylation at a serine residue adjacent to the RxR motif, 14-3-3 is recruited and binds to the cytoplasmic tail of Kir6.2. This masks the ER retention signal allowing for anterograde transport through the secretory pathway and increases the level of Kir6.2 at the cell surface. Furthermore, the authors show that $\beta$-COPI directly competes with 14-3-3 binding at this motif to regulate retrograde transport back to the endoplasmic
The same mechanism for the regulation of a protein at the cell surface is reported for the MHCII chain, lip35 (Khalil et al., 2003), the HIV co-receptor GPR15 (Shikano et al., 2005), and the neuronal $\alpha 4$ acetylcholine receptor (O'Kelly et al., 2002).

Therefore, at least for some membrane bound proteins, their regulation to the cell surface involves both phosphorylation of residues close to the RxR motif and 14-3-3 interaction.

14-3-3 proteins are involved in similar biological functions as ADAM metalloproteases such as cell signaling (Radhakrishnan and Martinez, 2010; van Hemert et al., 2001), cell migration (Deakin et al., 2009; Hou et al., 2010; Lu et al., 2009), and cell-cycle regulation (Douglas et al., 2010; Kasahara et al., 2010). The consensus binding motif for 14-3-3 proteins as well as SH3 binding motifs are present in the cytoplasmic domain of a number of ADAM family members (Table 3.2). For example, 14-3-3 proteins bind to the cytoplasmic domain of ADAM22. This interaction masks the adjacent ER retention signal (RxR) to regulate the level of ADAM22 at the cell surface (Godde et al., 2006). In addition, $\beta$-COPI was identified as a binding partner to the cytoplasmic domain of ADAM19 in fetal brain tissue (Huang et al., 2002). For ADAM10 and ADAM12, it is hypothesized that adaptor proteins bind to their cytoplasmic domains to regulate their exit from the endoplasmic reticulum to the cell surface (Cao et al., 2002; Hougaard et al., 2000; Marcello et al., 2010). Therefore, it is believed that the regulation of an ADAM protein to the cell surface is regulated through the interaction of adaptor proteins such as 14-3-3 with the ADAM cytoplasmic domain. More importantly, this might contribute to the deregulation of an ADAM protein in disease such as cancer.
How Does ADAM19 Regulate ADAM13 in a Cell?

When overexpressed the Proforms of ADAM13 and ADAM19 physically interact in a cell. Both ADAM13 Proform and ADAM19 Proform can still physically interact, via coimmunoprecipitation, without the cytoplasmic domain of ADAM13 (ADAM13Δcyto) or their metalloprotease activity (ADAM13 E/A and ADAM19 E/A) (data not shown). Therefore, the physical interaction is independent of the ADAM13 cytoplasmic domain and the proteolytic activity of ADAM13 or ADAM19. ADAM19 binds to the Cysteine-rich Domain of ADAM13 within a perinuclear compartment, possibly the endoplasmic reticulum. Previous work has suggested that ADAM19 may function primarily within a cell at a perinuclear compartment with very little ADAM19 at the cell surface (Murphy, 2009; Yokozeki et al., 2007). Our observations show that overexpressed ADAM19 strongly localizes to the endoplasmic reticulum. The cytoplasmic domain of ADAM19 contains four ER retention signals (RxR) suggesting that ADAM19 may localize and function primarily in the endoplasmic reticulum. When ADAM13 and ADAM19 are co-overexpressed in a cell, the overall level of ADAM13 is reduced. Furthermore, there is a strong reduction specifically in the Mature form of ADAM13. This occurs without changing the protein level of ADAM19. Previously, our lab has shown that only the Mature form of ADAM13 is expressed at the cell surface (Alfandari et al., 1997). Therefore, the results suggest that ADAM19 may regulate the protein stability of ADAM13 and possibly, the level of Mature ADAM13 that localizes to the cell surface.
I propose that ADAM19 regulates ADAM13 through two different hypotheses. One hypothesis is that ADAM19 cleaves ADAM13 in its extracellular domain. This cleavage event would shed a soluble form of ADAM13 outside of the cell. When ADAM13 is detected in the embryo by western blot four molecular weight bands are observed; the Proform (120kD), the Mature form (100kD), soluble ADAM13 (50kD) and the cytoplasmic tail (17kD). The different forms of ADAM13 are deduced from their predicted molecular weights and are considered to be functional in the embryo. Previously our lab has shown that ADAM13 is autcatalytic by cleaving itself within the Cysteine-rich Domain (Gaultier et al., 2002). The shed fragment of ADAM13 (50kD) retains biological function outside of a cell. Soluble ADAM13 binds to the HepII domain of fibronectin and cleaves fibronectin in vitro (Gaultier et al., 2002). Additionally, an autocatalytic processing event is important in vitro for the proteolytic function of ADAMTS4 (Flannery et al., 2002), ADAM8 (Schlomann et al., 2002) and ADAM19 (Kang et al., 2002).

Therefore, the working hypothesis model is that ADAM19 cleaves ADAM13 into a soluble form to promote an interaction between soluble ADAM13 and fibronectin. This cleavage event would be important for remodeling the fibronectin matrix outside of a cell and more importantly, the cells ability to adhere and migrate on fibronectin (Figure 3.8). To investigate if ADAM19 cleaves ADAM13, I would detect soluble ADAM13 from the cell supernatant of co-transfected cultured cells compared to ADAM13 alone and ADAM13E/A alone. Since ADAM13 is autcatalytic, the ADAM13 alone sample is a positive control and the ADAM13E/A alone sample is a negative control to determine baseline levels of soluble ADAM13 shed into the supernatant.
It is possible that ADAM19 does not cleave ADAM13. Since ADAM19 binds specifically to the Cysteine-Rich Domain of ADAM13, this physical interaction could directly perturb ADAM13 cleavage. My second hypothesis is that ADAM19 binds to ADAM13 in a perinuclear compartment and functions to sequester ADAM13 from further processing. ADAM19 could function by preventing the autocatalysis of ADAM13 into soluble ADAM13 by masking the cleavage site in the Cysteine-Rich Domain (Figure 3.8). This would reduce soluble ADAM13 from being shed outside of the cell to interact with fibronectin. As a result, this would affect fibronectin matrix remodeling and the cells ability to adhere and migrate on fibronectin.

The above models only address the in vitro results on ADAM13 and ADAM19 co-overexpression. However in the embryo, a loss of ADAM13 protein reduces the level of ADAM19 protein by 50%. In addition, a loss of ADAM19 protein reduces the level of ADAM13 protein by 50%. This suggests that both ADAM13 and ADAM19 are required to maintain a proper protein level in the embryo. This conclusion was also seen previously for ADAM13 expression (Figure 4 in (McCusker et al., 2009)). However, the level of ADAM19 was not reduced because the conditions using a combination of the ADAM19 antibodies had not been determined yet (Figure 4 in (McCusker et al., 2009)). I propose an alternative hypothesis where ADAM13 and ADAM19 proteins function as chaperones to stabilize each other in a perinuclear compartment. This is important to regulate the level of each ADAM protein that translocates to the cell surface. Once at the cell surface, the ADAM protein carries out its proteolytic function by cleaving a potential substrate (Figure 3.8). For example, ADAM19 could regulate the level of ADAM13 protein that translocates to the cell surface of CNC cells. This would regulate cell-cell
adhesion by affecting the ability of ADAM13 to cleave the cell adhesion molecule Cadherin-11 (McCusker et al., 2009). To investigate if ADAM19 affects the level of ADAM13 at the cell surface in vivo, I would detect ADAM13 by western blot from cell surface biotinylated CNC cells (or dorsal mesoderm cells) from embryos that were injected with CMO or MO19. Embryos injected with MO13 would be used as a negative control. The ADAM13 Mature form will only be analyzed since it is the only form of ADAM13 present on the cell surface.

SECTION V: Materials and Methods used in Chapter III

Cell Culture
293T cells were cultured in RPMI media supplemented with 10U/ml Pen/Strep, 2mM L-Glutamine, 0.11mg/ml Sodium Pyruvate and 10% Fetal Bovine Serum (Hyclone). Transient transfections were performed using Fugene 6 reagents (Roche) following the manufacturer’s instructions. XTC cells were cultured in L15 media supplemented with 10U/ml Pen/Strep, 2mM L-Glutamine and 10% Fetal Bovine Serum (Hyclone). Transient transfections for XTC cells were performed using Fugene HD reagents (Roche) following the manufacturer’s instructions.

Immunofluorescence
After 48 hours of expression, XTC cells were trypsinized and seeded onto glass coverslips coated with 10µg/ml of Fibronectin for 12 hours. Cells were fixed for 20 minutes in 3.7% formaldehyde in MBS at room temperature, permeabilized in PBS 0.5% Triton X100 for 5 minutes at room temperature and blocked in 1% BSA in PSB 0.1%
Tween20 overnight at 4C. Antibody incubations were performed sequentially (one hour at room temperature for each antibody) in 1% BSA in PBS 0.1% Tween20. The primary antibodies used: ADAM13 (6615F) 1:200; ADAM19 (2C5 E1) 1:200; and Calnexin (Assay Designs, Stressgen) 1:200. DAPI was used at 1:1000 to stain the nuclei. All secondary antibodies were used at 1:500 and purchased from Jackson Labs.
Figure 3.1: Amino Acid Alignment of Xenopus ADAM13 to Xenopus ADAM19. The individual amino acid domains are labeled. The consensus N-glycosylation sites (NxS/T) are shown in red (Gooley et al., 1991), the consensus Furin cleavage site of the Prodomain is shown in blue, the Metalloprotease active site is shown in yellow, the Disintegrin Loop is shown in grey (McLane et al., 1998), the Transmembrane domain is boxed, the SH3 Binding Motif (RPxPxxP) is shown in green (Cicchetti et al., 1992), and the Predicted Serine/Threonine/Tyrosine Phosphorylation sites (NetPhos 2.0) are shown in pink (Blom et al., 1999).
Table 3.1: Amino Acid Percent Identity of Xenopus ADAM13 to Xenopus ADAM19. Each amino acid domain is aligned and the percent identity is shown.

<table>
<thead>
<tr>
<th>Domain</th>
<th>Identity (%)</th>
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<tr>
<td>Prodomain</td>
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<tr>
<td>Metalloprotease Domain</td>
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<td>Disintegrin Domain</td>
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<tr>
<td>Cytoplasmic Domain</td>
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Figure 3.2: ADAM13 Expression in ADAM19 Knockdown Embryos. Western blot of protein extract from embryos injected with a control morpholino (CMO) or a morpholino to either ADAM13 (MO13) or ADAM19 (MO19). The morpholinos (10 ng) were injected at the one cell-stage and embryos were extracted at stage 22. (A) Glycoproteins from 10 embryos were purified on Concanavalin A-agarose beads, separated by SDS-PAGE and probed with a rabbit polyclonal antibody to the ADAM13 cytoplasmic domain (6615F from (Alfandari et al., 1997)). (B) The densitometric analysis of the blot depicted shows that the ADAM13 protein is reduced by approximately 50% in MO19 embryos when normalized to Integrin β1 expression level (8C8 from (Gawancka et al., 1992)). (C) Immunoprecipitation of ADAM13 using the goat ADAM13 antibody from five embryos (St. 22), separated by SDS-PAGE and probed with a rabbit polyclonal antibody to the ADAM13 cytoplasmic domain (6615F). A monoclonal antibody to GAPDH was used as a loading control.
Figure 3.3: ADAM19 Expression in ADAM13 Knockdown Embryos. Western blot of protein extract from embryos injected with a control morpholino (CMO) or a morpholino to either ADAM13 (MO13) or ADAM19 (MO19). The morpholinos (10 ng) were injected at the one cell-stage and embryos were extracted at stage 22. Immunoprecipitation of ADAM19 using the goat DC19 antibody from 20 embryos (St. 22), separated by SDS-PAGE and probed with a rabbit polyclonal antibody to the ADAM19 cytoplasmic domain (C3). A monoclonal antibody to GAPDH was used as a loading control. The densitometric analysis of the blot depicted shows that the ADAM19 protein is reduced by approximately 50% in MO13 embryos when normalized to the GAPDH expression level.
Figure 3.4: ADAM13 and ADAM19 Physically Interact in Transfected 293T and XTC Cells. (A) Transfected 293T cells (left panel) that overexpress ADAM13 alone (A13+CS2), ADAM19 alone (A19+CS2) or ADAM13 and ADAM19 (A13+A19) were extracted for coimmunoprecipitation. A 120kD band was detected in the cotransfected (A13+A19) sample, corresponding to the Proform of ADAM13 (second blot) or the Proform of ADAM19 (top blot). Total cell extract was separated by SDS-PAGE and probed with an antibody to ADAM13 (6615F), ADAM19 (C3), and GAPDH as a loading control. Transfected XTC cells (right panel) that overexpress GFP-myc tagged (GFPmt) or ADAM19 were extracted for coimmunoprecipitation. XTC cells endogenously express ADAM13 (Alfandari et al., 1997). A 120kD band was detected for the Proform of ADAM19 (top blot) and ~120kD and 90kD bands was detected for the Proform and Mature form of ADAM13 (second blot). Total cell extract was separated by SDS-PAGE and probed with an antibody to ADAM13 (6615F), ADAM19 (C3), GAPDH as a loading control, 9E10 (myc-tag), and PACSIN2 as a second loading control. (B) Immunoprecipitation of endogenous ADAM13 from RFP-expressing XTC cells as a control.
Figure 3.5: ADAM19 Binds to the Cysteine-Rich Domain of ADAM13. Transfected 293T cells that overexpress RFP or ADAM19 with full length ADAM13 (A13+A19), a myc tagged disintegrin-cysteine-rich domains of ADAM13 (DC13), a myc tagged disintegrin domain of ADAM13 (D13), or a myc tagged cysteine-rich domain of ADAM13 (CR13) were extracted for coimmunoprecipitation. A 120kD band was detected in the cotransfected (A13+A19) sample, corresponding to the Proform of ADAM13 (second blot) or the Proform of ADAM19 (top blot). The coimmunoprecipitation with an antibody to ADAM19 (C3) (second blot) showed a strong physical interaction to DC13 and to CR13. Total cell extract was separated by SDS-PAGE and probed with an antibody to ADAM13 (6615F), ADAM19 (C3), 9E10 (myc-tag) and GAPDH as a loading control.
Figure 3.6: ADAM19 Localizes to the Endoplasmic Reticulum in XTC Cells. Immunofluorescence of transfected XTC that overexpress ADAM19 (red) was visualized using an antibody to the cytoplasmic domain of ADAM19 (2C5 E1). Localization to the endoplasmic reticulum is shown by using an antibody to the ER-specific protein, Calnexin (green). Strong colocalization of ADAM19 with Calnexin is shown in the Merge (yellow). DAPI (blue) was used to stain the nuclei. Very little to no expression was seen for ADAM19 at the plasma membrane.
**Figure 3.7**: ADAM13 and ADAM19 Colocalize to a Perinuclear Compartment in XTC Cells. Immunofluorescence of transfected XTC that overexpress ADAM19 (red) was visualized using an antibody to the cytoplasmic domain of ADAM19 (2C5 E1). Immunofluorescence of endogenous ADAM13 (green) was visualized using an antibody to the cytoplasmic domain of ADAM13 (6615F). Colocalization of ADAM19 with ADAM13 is shown in the Merge (yellow). DAPI (blue) was used to stain the nuclei. ADAM13 colocalizes with ADAM19 within a perinuclear compartment. Endogenous ADAM13 also localizes to the cell surface in lamellipodia (white arrowheads).
<table>
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<tr>
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<th>14-3-3 Binding Motifs</th>
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<td></td>
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<td>(RR)</td>
<td>(RPxPxxP)</td>
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<td>A33-Cyto</td>
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**Table 3.2: Amino Acid Analysis of Different ADAM Cytoplasmic Domains.** Consensus ER Retention signals are described in (Wieland et al., 1987). Consensus SH3 Binding Motifs are described in (Ren et al., 1993). Consensus 14-3-3 Binding Motifs are described in (Uchida et al., 2006).
Figure 3.8: Models Explaining How ADAM19 Regulates ADAM13 in a Cell. (A) Hypothesis 1: ADAM19 binds and cleaves ADAM13 into a soluble form. Soluble ADAM13 is shed into the extracellular environment as an active protease to remodel a fibronectin matrix. This is important to regulate cell adhesion and migration on fibronectin. (B) Hypothesis 2: ADAM19 binds to ADAM13 and sequesters ADAM13 in a perinuclear compartment. This interaction inhibits the autocatalysis of ADAM13 into a soluble form and prevents remodeling of a fibronectin matrix. This is important to regulate cell adhesion and migration on fibronectin. Alternatively in (C), ADAM19 regulates the processing of ADAM13. More importantly, ADAM19 regulates the level of Mature ADAM13 at the cell surface. This interaction regulates cell-cell adhesion and cell migration. 14-3-3 dimers may be a key adaptor protein involved in this process.
CHAPTER IV

XENOPUS ADAM13 AND ADAM19 ARE IMPORTANT FOR
PROPER CONVERGENCE AND EXTENSION OF THE NOTOCHORD

All of the work presented in this chapter is data completed by myself and is unpublished.

Abstract

This chapter focuses on the role of ADAM13 and ADAM19 in the dorsal mesoderm during gastrulation. Through a loss of function approach using antisense morpholino oligonucleotides to ADAM13 or ADAM19, I show that ADAM19 affects gene expression important for the A-P polarity of the notochord while ADAM13 does not. The changes in gene expression can be partially rescued by the EGF ligand Neuregulin1β, a known substrate for ADAM19 in the mouse. I show that a loss of ADAM13 or ADAM19 affects convergence and extension movements during notochord formation. Specifically, a loss of ADAM13 or ADAM19 causes a delay in mediolateral cell intercalation resulting in a significantly wider notochord compared to control embryos. These defects occur without affecting dishevelled intracellular localization or the activation of the PCP signaling pathway. However, a loss of ADAM13 or ADAM19 reduces dorsal mesoderm cell spreading on a fibronectin substrate through α5β1 integrin.
SECTION I: Introduction

Cellular movements are essential during gastrulation and can be classified into four evolutionary conserved movements; internalization, epiboly, convergence and extension (Solnica-Krezel, 2005). Internalization marks the beginning of gastrulation and in amphibians, creates the dorsal blastopore lip. The involuting mesoderm enters through the blastopore and migrates on the inner surface of the blastocoel roof. This migration event is highly dependent upon the extracellular matrix protein fibronectin, which is expressed along the blastocoel roof (Boucaut and Darribere, 1983; Lee et al., 1984). Epiboly movements spread tissue by increasing the surface area and decreasing thickness. The driving force behind epiboly occurs when cells interdigitate themselves between deep and superficial layers; a process known as radial intercalation (Keller, 1980).

The dorsal mesoderm undergoes dynamic movements that converge the tissue while extending along the anterior-posterior axis; a process called convergence and extension (Keller et al., 1985). Dorsal mesoderm cells undergo this type of movement by displaying mediolateral polarized protrusions to gain traction on neighboring cells and the surrounding extracellular matrix. These cellular behaviors are known as mediolateral cell intercalation. On the ventral side of the gastrulating embryo, both the lateral and ventral mesoderms undergo dynamic movements to converge the tissue without extension; a process called convergence and thickening (Keller and Danilchik, 1988). As a result of these morphogenetic movements during gastrulation, the physical forces generated cause the blastopore to close asymmetrically towards the ventral side of the
embryo (Figure 1.2) (Keller and Shook, 2008). Mediolateral cell intercalation continues after gastrulation in both the paraxial (somites) and axial (notochord) mesoderm. This drives the process of convergence and extension in order to properly lengthen the embryo along the anterior-posterior axis. These cellular movements are essential for the embryo to develop and survive to the tailbud stage.

Members of the ADAM family of metalloproteases play an active role in the process of cell migration. ADAM proteins can function in cell migration either directly through cleavage of cell surface adhesion molecules such as cadherins (Kohutek et al., 2009; McCusker et al., 2009; Schulz et al., 2008) or indirectly through cleavage of cell signaling molecules such as EGF ligands involved in activating cell migration signaling pathways (Bakken et al., 2009; Sun et al., 2010; Zheng et al., 2009). ADAM10 can cleave E-cadherin (Maretzky et al., 2005) and N-cadherin (Reiss et al., 2005) in the extracellular domain affecting both cell-cell adhesion and cell migration in the embryo. Our lab has shown that both ADAM9 and ADAM13 cleave cadherin-11 in the extracellular domain affecting CNC cell migration in Xenopus embryos (McCusker et al., 2009). The ADAM mediated cleavage of cadherin-11 promotes CNC cell migration and shows that the cadherin-11 cleavage product retains biological activity in the embryo. It is hypothesized that the cadherin-11 cleavage product promotes CNC cell migration by acting as a signaling molecule; however an exact mechanism remains to be determined (unpublished work, communication with Catherine McCusker 2009).

Recent work has uncovered a new role for ADAM13 in the closely related frog species Xenopus tropicalis, for neural crest development (Wei et al., 2010). During gastrulation, ADAM13 cleaves ephrinB1 and ephrinB2 ligands in the dorsal mesoderm.
This cleavage event is important to regulate forward ephrin signaling through the Eph receptor. The ADAM13 cleavage of ephrinB1/B2 upregulates canonical Wnt signaling and in turn, activates the expression of the CNC marker snail2. Reduction of the ADAM13 protein reduces CNC induction by the suppression of the Canonical Wnt/snail2 signaling pathway. This mechanism is important for the initial induction of the CNC and provides a functional role for ADAM13 during gastrulation.

Similar to the role in normal development, ADAM proteins are upregulated in disease such as cancer and are involved in the epithelial-to-mesenchymal transition (EMT) during cancer metastasis (Dittmer et al., 2009; Duffy et al., 2009; Fry and Toker, 2010). This further supports their prominent role in cell adhesion and cell migration events. In this chapter I will investigate the contribution of ADAM13 and ADAM19 to convergence and extension movements of the notochord during gastrulation.

**Results**

**SECTION II: Role of ADAM19 in Establishing the A-P Polarity of the Notochord**

Previously I observed alterations in gene expression at the gastrula stage in ADAM19 knock down embryos (Neuner et al., 2009). Gene expression analysis shows a specific loss of Xbrachyury expression concomitant with an upregulation of Chordin expression in the notochord (Figure 2.4). These two genes are expressed in a countergradient along the anterior-posterior axis in the axial mesoderm (Ninomiya et al., 2004). Chordin is a marker of anterior notochord polarity and Xbrachyury is a marker of
posterior notochord polarity. Together, these two genes establish the anterior-posterior (A-P) polarity of the developing notochord. Therefore, I investigated how ADAM19 is disrupting the A-P polarity of the notochord. In addition, I determined if ADAM13 is involved in establishing notochord A-P polarity. Using whole mount in situ hybridization for Xbrachyury (Xbra), I observed a loss of Xbra expression (40%) specifically in the notochord in MO19 injected embryos when compared to control embryos (0%) (Figure 4.1). The defect could be partially rescued by co-injection of full length ADAM19 RNA, but not the catalytically inactive mutant ADAM19 E/A. This suggests that the proteolytic activity of ADAM19 is important for maintaining Xbra expression in the notochord.

In mice, ADAM19 can cleave some members of the EGF family of ligands including Neuregulin1β (NRG1β) (Shirakabe et al., 2001). In Xenopus, NRG1β expression begins during gastrulation and continues through tailbud formation (Yang et al., 1999; Yang et al., 1998a; Yang et al., 1998b). Purified recombinant NRG1β can induce naïve animal cap cells to become mesoderm and express Xbrachyury (Chung and Chung, 1999). Therefore, I investigated whether overexpression of NRG1β could rescue gene expression in MO19 injected embryos. Co-injection of full length NRG1β RNA (NRG-FL) did not rescue Xbra expression in the notochord. However, co-injection of the extracellular region of NRG1β (NRG-EC), a form that mimics a potential cleavage product of ADAM19, did rescue Xbra expression in the notochord. Therefore, our data suggests that ADAM19 mediated shedding of NRG1β is important to maintain Xbra expression in the notochord.
Using whole mount \textit{in situ} hybridization for Chordin, I observed a shift to a more anterior localization of Chordin expression (50\%) in MO19 injected embryos when compared to control embryos (0\%) (Figure 4.2). The defect could be partially rescued by co-injection of full length ADAM19 RNA, but not the catalytically inactive mutant ADAM19 E/A. Furthermore, gene expression could be partially rescued by co-injection of NRG-EC RNA, but not full length NRG-FL RNA. Therefore, our data suggests that ADAM19 mediated shedding of NRG1\(\beta\) is important to maintain Chordin expression in the posterior notochord.

To determine if ADAM13 is important for notochord A-P polarity, I observed Xbra and Chordin expression in MO13 injected embryos. Xbrachyury expression appeared normal compared to control embryos (Figure 4.3). Chordin expression was wider compared to control embryos, but did not shift to a more anterior localization similar to MO19 injected embryos. In addition, ADAM13 RNA could not rescue gene expression in MO19 injected embryos (Figure 4.3). These results suggest that ADAM19 (not ADAM13) mediated shedding of Neuregulin1\(\beta\) is important to establish the A-P polarity of the developing notochord (Figure 4.4). Interestingly, I observed that the expression patterns of Chordin and Xbrachyury appear to be wider in MO13 and MO19 injected notochords compared to control notochords. This suggests that the notochord morphology might be perturbed as a result of a loss of ADAM13 or ADAM19. The morphology of the notochord will be investigated next in Section III of this chapter.
SECTION III: ADAM13 and ADAM19 Delay Convergence and Extension in the Notochord

The notochord is a temporary, rod-shaped structure that provides mechanical support for the developing embryo. It is located directly below the neural tube dictating the primary body axis. In addition, the notochord is important for patterning the surrounding tissues such as the ventral half of the neural tube (floor plate), the paraxial mesoderm (somites), and in controlling L-R asymmetry (Stemple, 2005). To visualize the morphology of the notochord, I performed a double whole mount immunostain using the monoclonal antibody Tor70 to stain the notochord. In addition, the monoclonal antibody 12/101 was used to visualize the surrounding somites (Figure 4.5). Embryos were injected with MO13 or MO19 at the one-cell stage and allowed to develop until the tailbud stage (stage 24). The notochord appears morphologically wider in MO13 and MO19 injected embryos compared to control embryos. In MO19 injected embryos, the notochord was frequently bent or misshapen and is thus likely to contribute to the embryos overall shorter and wider body size. The wide notochords observed in embryos lacking ADAM13 or ADAM19 could be explained by defects in convergence and extension movements of the axial mesoderm. The somites appear morphologically normal in MO13 injected embryos but have gross defects in MO19 injected embryos. The overall segmentation of each somite is not affected in embryos lacking ADAM19; however some of the individual somitic cells are mislocalized and protrude into the intersomitic boundary (Figure 2.8 and 4.4). In addition, the overall level of 12/101
expression is lower in MO19 injected embryos confirming previous results where a reduction of this marker was observed (Figure 2.8, (Neuner et al., 2009)). These results support the role of ADAM19 in muscle differentiation. In final, I observed that the archenteron or internal cavity is significantly larger in MO19 injected embryos. At this point, it is unclear whether this is a direct result of defects in endoderm formation or an indirect result of the embryo being shorter and wider due to defects in the dorsal mesoderm. For the remainder of this chapter I will focus my efforts on exploring the defects observed in the notochord.

To investigate if convergence and extension movements are affected in MO13 or MO19 injected embryos, I analyzed the axial mesoderm ex vivo by fluorescence time lapse microscopy. The microdissection of the dorsal mesoderm from a late gastrula stage embryo is a well established assay called an ‘Open Face Explant’ (Wilson et al., 1989). The explants are dissected from embryos that were microinjected twice. The first injection is at the one-cell stage with either a control Morpholino, MO13 or MO19. The second injection is with RNA encoding for a fluorescent membrane-bound Cherry protein (mbCherry) into one blastomere at the two cell stage (Figure 4.6). Since the first cleavage bisects the embryo into left and right halves, the second injection with mbCherry RNA enabled us to visualize the contribution of the axial mesoderm from one half of the embryo to the non-injected half. More importantly, I could observe axial mesoderm cell movements during notochord formation. The dorsal mesoderm was dissected into an Open Face Explant at the end of gastrulation once the blastopore has closed (stage 12.5/13) and observed by fluorescence time lapse microscopy. In all three explants (CMO, MO13, MO19), the axial mesoderm cells can be seen undergoing
convergence and extension movements to lengthen the embryo along the anterior-posterior axis. Time lapse montages of the recorded movies are shown for CMO in Figure 4.7, MO13 in Figure 4.8 and MO19 in Figure 4.9.

To investigate axial mesoderm cell morphology, the mbCherry localization of four cells was pseudo-colored black to improve visualization. Axial mesoderm cells in the control explant have a bipolar morphology and show directed protrusions along the mediolateral axis (Figure 4.10A). This type of cell morphology is essential for proper mediolateral cell intercalation. The axial mesoderm cells in explants lacking ADAM13 or ADAM19 have a multipolar morphology without directed protrusions along the mediolateral axis. This type of cell morphology suggests that the axial mesoderm will not undergo proper mediolateral cell intercalation. The length-to-width (L:W) ratio of an individual axial mesoderm cell was quantified by measuring the mediolateral length and anterior-posterior width (Figure 4.10B). Axial mesoderm cells lacking ADAM13 or ADAM19 have reduced L:W ratios compared to control cells, suggesting cell morphology is being affected. To determine if mediolateral cell intercalation is being perturbed, I traced three axial mesoderm cells in six explants to follow the cell movements over an eight hour time course in three independent experiments. Control axial mesoderm cells properly extend from one side of the notochord-somite boundary to the opposite side (Figure 4.11A). This allows for each cell to align in a row along the anterior-posterior axis as the notochord forms. However, axial mesoderm cells from explants lacking ADAM13 or ADAM19 do not properly extend from one side of the notochord-somite boundary to the opposite side. This suggests that there is a delay in the process of mediolateral cell intercalation. Moreover, the delay in mediolateral cell
intercalation may explain why the notochords are wider in embryos lacking ADAM13 or ADAM19.

To investigate notochord morphology, the length and width of the notochord was measured at three different time points during notochord formation. These measurements were used to calculate a length-to-width (L:W) ratio from fourteen different cell pairs in three independent experiments. The length of the notochord was measured between two axial mesoderm cells approximately 150µm apart. The width of the notochord was measured between both notochord-somite boundaries. In control notochords, the L:W ratio increased over time, showing that the notochord tissue converged and extended properly (Figure 4.11B). However in explants lacking ADAM13 or ADAM19, the notochord L:W ratios were reduced at all three time points. The notochords from explants lacking ADAM13 or ADAM19 do converge and extend, just not at the same rate as the control explants. Therefore, there is a delay in axial mesoderm convergence and extension movements in embryos lacking ADAM13 or ADAM19 resulting in a significantly wider notochord compared to control embryos.

SECTION IV: The Mislocalization of ECM Proteins in the Notochord

Axial mesoderm cells begin the process of mediolateral cell intercalation after coming in contact with one side of the notochord-somite boundary. This is described as the mechanism of ‘boundary capture’ (Keller and Tibbetts, 1989; Shih and Keller, 1992). This mechanism occurs when the border of the cell in contact becomes quiescent and no longer sends protrusions in its lateral direction. Therefore, the cell forms a stable contact.
or is ‘captured’ by the extracellular matrix proteins that make up the notochord-somite boundary. This event is critical for the proper convergence and extension of the notochord (Keller et al., 2000). To investigate if the notochord-somite boundary is intact, I performed a double immunostain on open face explants. I visualized two extracellular matrix (ECM) proteins fibronectin and laminin for their localization at the notochord-somite boundary by fluorescence microscopy. In control explants, both ECM proteins localized to either side of notochord and at the intersomitic boundaries (Figure 4.12). In explants lacking ADAM13, both ECM proteins are localized on either side of the notochord and at the intersomitic boundaries. However, punctate localization of both ECM proteins was also found mislocalized in the notochord (arrows). In explants lacking ADAM19, strong mislocalization of both ECM proteins is found throughout the notochord. This result was seen previously for fibronectin expression in MO19 injected embryos (Figure 2.8, (Neuner et al., 2009)). Therefore, the notochords of embryos lacking ADAM13 or ADAM19 appear to have normal notochord-somite boundaries but an abnormal presence of ectopic fibronectin and laminin in the notochord. Since the notochord-somite boundary is formed simultaneously as the notochord develops, it is unclear whether the mislocalization of fibronectin and laminin cause the delay in mediolateral cell intercalation or that their mislocalization is a result. Previously, fibronectin localization by confocal microscopy showed that fibronectin fibrils are cleared from the dorsal (floor plate) and ventral (archenteron) sides of the developing notochord during gastrulation (Davidson et al., 2004). As the fibronectin fibrils are remodeled they become enriched along the lateral notochord-somite boundaries. Our lab has previously shown that ADAM13 can cleave and remodel a fibronectin matrix in
cultured cells (Alfandari et al., 2001). Therefore if the dorsal mesoderm cells from embryos lacking ADAM13 or ADAM19 actively secrete fibronectin into the extracellular environments, not having a proper level of ADAM13 at the cell surface to remodel the fibronectin matrix could cause a delay in mediolateral cell intercalation. Future experiments should repeat the double immunostain for fibronectin and laminin in open face explants utilizing a confocal microscope. A Z-stack reconstruction of the entire notochord will determine if the ECM proteins are being mislocalized inside of the notochord or to the dorsal and ventral sides where they are normally absent (Davidson et al., 2004). The mislocalization of fibronectin in the somites of explants lacking ADAM19 is evident of the strong defects occurring in this tissue. Previously, I observed that cell organization within each somite is affected and that two muscle differentiation markers are reduced in embryos lacking ADAM19 (Neuner et al., 2009). Future experiments should investigate whether individual somitic cell organization and the formation of the intersomitic boundary are linked to muscle differentiation.

The major signaling pathways that are critical for establishing convergence and extension movements during gastrulation are the Planar Cell Polarity Pathway (or the Non-canonical Wnt signaling pathway) (Yin et al., 2008) and the interaction between Integrins and the extracellular matrix, specifically fibronectin (Davidson et al., 2006; Rozario et al., 2009). The PCP Signaling Pathway is essential for establishing dorsal mesoderm cell polarity. The interaction between integrin receptors such as α5β1 integrin and fibronectin are critical for dorsal mesoderm cell adhesion. I propose that ADAM13 and ADAM19 function in convergence and extension movements by regulating dorsal
mesoderm cell polarity or cell adhesion to fibronectin. These two hypotheses will be investigated in the next section of this chapter.

**SECTION V: Does ADAM13 and ADAM19 Affect Dorsal Mesoderm Cell Polarity or Cell Adhesion to Fibronectin?**

**Role of ADAM13 and ADAM19 in Dorsal Mesoderm Cell Polarity**

The Planar Cell Polarity pathway is functionally conserved from flies to vertebrates and is essential for the morphogenetic movements during gastrulation. In Drosophila, components of this pathway are specifically localized to cell borders in order to establish cell polarity and coordinate cell motility (Adler, 2002; Zallen and Wieschaus, 2004). Vertebrate counterparts of the PCP pathway are required for convergence and extension movements during gastrulation (Goto and Keller, 2002; Keller, 2002). A loss of any of the pathway components results in the failure of the dorsal mesoderm to undergo convergence and extension (Roszko et al., 2009; Torban et al., 2004). Moreover, it is known that activation of the PCP pathway can be visualized through the translocation of dishevelled (Dsh), an intracellular signaling component, from the cytosol to the plasma membrane in dorsal mesoderm undergoing convergence and extension (Wallingford et al., 2000). This translocation event was not observed in the posterior dorsal mesoderm because this tissue does not undergo convergence and extension. Therefore, there is a direct link between the PCP signaling pathway in establishing cell polarity with the convergence and extension movements of gastrulation.
To investigate if the activation of the PCP signaling pathway is affected, I analyzed the localization of dishevelled in the axial mesoderm of embryos lacking ADAM13 or ADAM19. This technique was performed on embryos that were microinjected twice. The first injection is at the one-cell stage with either a control Morpholino, MO13 or MO19. The second injection is with RNA encoding for dishevelled fused to green fluorescent protein (GFP-dishevelled) into the two dorsal blastomeres at the four cell stage. Overexpression of dishevelled can block convergence and extension movements (Sokol, 1996). However, if the level of RNA is titrated down to a small finite dosage (50pg) of GFP-Dishevelled RNA this does not affect normal development. More importantly, this assay can be used as a tool for visualizing the activation of the PCP signaling pathway *in vivo* (Marsden and DeSimone, 2001; Wallingford et al., 2000). The injected embryos were allowed to develop until the late gastrula stage. Open face explants were dissected and analyzed for dishevelled localization by confocal microscopy. In explants lacking ADAM13 or ADAM19, the localization of dishevelled is found at the plasma membrane similar to control explants (Figure 4.13). To determine if there is a quantitative difference in dishevelled localization, I normalized the level of GFP-dishevelled by co-injecting RNA encoding monomeric red fluorescent protein (RFP) (Figure 4.14A). The same dose of RFP RNA and GFP-dishevelled RNA were coinjected into one dorsal blastomere at the four cell stage. In the open face explants, the fluorescence profile of GFP-dishevelled was measured using Axiovision software along the anterior-posterior axis and the medio-lateral axis of the notochord. The fluorescence profiles of GFP-dishevelled were normalized to the fluorescence profiles of RFP (Figure 4.14B). Compared to control
notochords, there was no significant difference in the GFP-dishevelled fluorescence profiles of notochords lacking ADAM13 or ADAM19. Therefore, these results suggest that ADAM13 and ADAM19 do not affect the activation of the PCP signaling pathway of the dorsal mesoderm during gastrulation.

**Role of ADAM13 and ADAM19 in Dorsal Mesoderm Cell Adhesion to Fibronectin**

During gastrulation, the involuting mesoderm enters through the blastopore and migrates on the inner surface of the blastocoel roof. This migration event is highly dependent upon the extracellular matrix protein fibronectin (FN), which is expressed along the blastocoel roof (Boucaut and Darribere, 1983; Lee et al., 1984; Ramos and DeSimone, 1996). Cell adhesion to FN involves many cell-extracellular matrix interactive sites (Hynes, 2009; Rozario and DeSimone, 2010). The central binding domain for many cell types to FN is found at the tripeptide sequence RGD site (Pierschbacher and Ruoslahti, 1984). Together with an adjacent site called the synergy site, these two domains act cooperatively to support cell adhesion (Aota et al., 1994; Bowditch et al., 1994). Cell attachment to the RGD site, in cooperation with the synergy site, is mediated through the α5β1 integrin receptor that recognizes fibronectin at the cell surface (Pytela et al., 1985). During dorsal mesoderm cell migration on the blastocoel roof, the notochord-somite boundary is formed and cells begin to undergo convergence and extension. To investigate if the dorsal mesoderm cells can properly spread on a fibronectin substrate, I dissociated the dorsal mesoderm cells from MO13 or MO19 injected embryos and plated the cells onto FN. Dorsal mesoderm cells were dissected
from the dorsal blastopore lips of early gastrula (stage 10.5) embryos. The cells were
dissociated in calcium and magnesium-free media, plated onto a FN substrate and
analyzed by time lapse microscopy. After one hour, 85% of the dorsal mesoderm cells
from control embryos spread on FN (Figure 4.15). In MO13 injected embryos, only 60%
of the dorsal mesoderm cells spread on FN. In MO19 injected embryos, only 70% of the
dorsal mesoderm cells spread on FN. If both ADAM13 and ADAM19 were knocked
down by injecting MO13 and MO19, only 60% of the dorsal mesoderm cells spread on
FN. Since an additive affect was not observed, this suggests that the function of
ADAM13 and ADAM19 do not compensate in cell adhesion to fibronectin.

To determine if the defects were due to the involvement of \( \alpha_5\beta_1 \) integrin, I
repeated the cell adhesion assay by plating the cells on a GST fusion protein containing
only the RGD and synergy site called 9.11 (Ramos and DeSimone, 1996). The same
results as seen on FN were observed for dorsal mesoderm cells to spread on 9.11. After
one hour, 85% of the dorsal mesoderm cells from control embryos spread on 9.11 (Figure
4.15). In MO13 injected embryos, only 60% of the dorsal mesoderm cells spread on
9.11. In MO19 injected embryos, only 70% of the dorsal mesoderm cells spread on 9.11.
In MO13 and MO19 injected embryos, only 60% of the dorsal mesoderm cells spread on
9.11 suggesting that the function of ADAM13 and ADAM19 do not compensate in cell
adhesion to FN. Therefore, dorsal mesoderm cells from embryos lacking ADAM13 and
ADAM19 have a reduced ability to spread on a FN substrate. The defects observed may
affect the interaction of \( \alpha_5\beta_1 \) integrins with fibronectin.

To investigate if the expression of \( \beta_1 \) integrin is affected, I determined the protein
level of \( \beta_1 \) integrin in the dorsal mesoderm of MO13 or MO19 injected embryos.
Compared to control embryos, there was no significant change in the overall protein level of β1 integrin in the dorsal mesoderm (Figure 4.18). However, this assay only analyzed the total protein level of β1 integrin. An additional experiment should be completed to determine the cell surface expression of β1 integrin. Immunoprecipitation of β1 integrin from cell surface biotinylated dorsal mesoderm cells of CMO, MO13 or MO19 injected embryos will determine if ADAM13 or ADAM19 affect the protein level of β1 integrin at the plasma membrane.

To rescue the cell adhesion defects on FN and 9.11, I injected RNA encoding for full length ADAM13 or full length ADAM19 into the two dorsal blastomeres at the four cell stage. ADAM13 RNA was able to partially rescue the MO13 effect on 9.11 but did not rescue on FN (Figure 4.16). ADAM19 RNA was able to partially rescue the MO19 effect on both FN and 9.11, as well as, the MO13 effect on 9.11 (Figure 4.16B and 4.17). The ability of ADAM19 to rescue MO13 cell adhesion on 9.11 further supports the intimate relationship between ADAM13 and ADAM19 proteins in the embryo.

Our lab has shown that the cytoplasmic domain of ADAM13 is cleaved off, translocates into the nucleus and promotes cranial neural crest cell migration in vivo (Cousin et al., 2010). Injection of RNA encoding the cytoplasmic domain of ADAM13 fused to GFP (GFP-C13) in the dorsal mesoderm was able to partially rescue the MO19 cell adhesion defects on both FN and 9.11 (Figure 4.17). This suggests that GFP-C13 has an independent biological function in the embryo. GFP-C13 did not rescue the MO13 effect on FN and 9.11 suggesting that the extracellular domain of ADAM13 is important for cell adhesion on FN. An alternative hypothesis could be explained by GFP-C13 acting through a positive feedback mechanism important for upregulating ADAM13.
expression in the embryo (Figure 4.19). In the presence of a MO13 background, the level of ADAM13 could not be rescued because of the effects of the morpholino. However, in the presence of a MO19 background, the protein level of ADAM13 rescues to near control levels. This experiment is preliminary and needs to be repeated.

When GFP-C13 translocates into the nucleus it regulates gene expression by an unknown mechanism. Xenopus Marginal Coil (Xmc) is a cytoplasmic coiled-coil protein that is regulated by GFP-C13 and plays an important role in regulating gastrulation movements (Frazzetto et al., 2002). To determine if Xmc is able to rescue the cell adhesion defects in the dorsal mesoderm, I injected RNA encoding full length Xmc containing an N-terminal myc tag (Xmc-MT) into the two dorsal blastomeres at the four cell stage. However, Xmc-MT RNA could not rescue dorsal mesoderm cell spreading on FN and 9.11 in MO13 or MO19 injected embryos (Figure 4.16 and 4.17). This result suggests that Xmc does not function with ADAM13 or ADAM19 in regulating dorsal mesoderm cell adhesion to FN.

In addition, PI3K signaling and the downstream component AKT are shown to regulate cell adhesion to FN (Nie and Chang, 2007a; Somanath and Byzova, 2009; Somanath et al., 2007). To determine if PI3K is able to rescue the cell adhesion defects in the dorsal mesoderm, I injected RNA encoding the constitutively active p110 subunit of PI3K (Nie and Chang, 2007a). However, p110 RNA could not rescue dorsal mesoderm cell spreading on FN and 9.11 in MO13 or MO19 injected embryos (Figure 4.16 and 4.17). Previously at the gastrula stage, I showed a significant reduction in active phosphorylated AKT in embryos lacking ADAM19 (Figure 2.9). Therefore, I analyzed the level of phosphorylated AKT (AKTp) in the dorsal mesoderm. However in MO13 or
MO19 injected embryos, there was no significant change in the level of AKTp in the dorsal mesoderm (Figure 4.18). The effects on AKT by ADAM19 were seen previously at the late gastrula stage where the expression level of ADAM19 is much higher compared to the early gastrula stage in this assay (Figure 2.1 and 2.9). These results suggest that ADAM13 or ADAM19 regulation of cell spreading to fibronectin is not mediated by PI3K/AKT.

**SECTION VI: Discussion**

In this chapter I have shown that ADAM13 and ADAM19 proteins are important for gastrulation. ADAM19 affects gene expression important for establishing the A-P polarity of the notochord, while ADAM13 does not. Both ADAM13 and ADAM19 are important for convergence and extension movements. Embryos lacking ADAM13 or ADAM19 have a delay in mediolateral cell intercalation of the axial mesoderm, resulting in a significantly wider notochord compared to control embryos. The defects seen in convergence and extension movements do not appear to affect the activation of the PCP signaling pathway. However, dorsal mesoderm cells from embryos lacking ADAM13 or ADAM19 have a reduced ability to spread on fibronectin.

My results show that a loss of ADAM13 or ADAM19 in the embryo does not affect the activation of the PCP signaling pathway by the translocation of dishevelled to the plasma membrane. Dishevelled sub-cellular localization has been studied extensively before to determine the activation state of the PCP signaling pathway in Xenopus embryos (Axelrod et al., 1998; Marsden and DeSimone, 2001; Tada and Smith, 2000;
Wallingford et al., 2000). However, dishevelled protein is also essential for controlling signal transduction of the canonical Wnt signaling pathway via β-catenin (Boutros and Mlodzik, 1999). Dishevelled is an intercellular protein that interacts with the Wnt receptor Frizzled at the plasma membrane and deciphers the Wnt signal to activate the appropriate intracellular signaling cascade (Canonical vs Non-canonical) (Roszko et al., 2009). Therefore, to definitively show that the PCP signaling pathway is affected in embryos lacking ADAM13 or ADAM19 further experiments need to be done. I would propose to investigate the downstream PCP target genes for any changes in gene expression profiles.

My results suggest that ADAM13, and possibly ADAM19, function by regulating dorsal mesoderm cell spreading on fibronectin. A single knock down of ADAM13 reduces cell spreading on FN to 60%. A single knock down of ADAM19 reduces cell spreading on FN to 70%. A double knock down of ADAM13 and ADAM19 reduces cell spreading on FN to 60%, showing there is not an additive effect by the loss of both ADAM proteins. This suggests that the function of ADAM13 and ADAM19 do not compensate in cell spreading on fibronectin. Since the double knock down cell adhesion levels are similar to the knock down of ADAM13 alone, I propose that dorsal mesoderm cell spreading on FN is largely due to ADAM13 with minimal contribution from ADAM19. Moreover, a loss of ADAM19 reduces ADAM13 protein levels at the tailbud stage (Figure 3.2) and at the early gastrula stage (Figure 4.19). Therefore, my results suggest that the dorsal mesoderm cell spreading defects observed in MO19 injected embryos are an indirect result of a reduction in ADAM13 protein levels.
Dorsal mesoderm cell spreading on the fusion protein 9.11 or full length fibronectin were similar. A single knock down of ADAM13 reduces cell spreading on 9.11 to 60%. A single knock down of ADAM19 reduces cell spreading on 9.11 to 70%. A double knock down of ADAM13 and ADAM19 reduces cell spreading on 9.11 to 60%, showing there is not an additive effect by the loss of both ADAM proteins. This strongly suggests that our defect in dorsal mesoderm cell spreading to FN is mediated through the RGD/synergy site via the α5β1 integrin receptor and not through other cell-ECM interactive sites. Previously, the disintegrin and cysteine rich domains of ADAM13 were shown to promote cell adhesion through an interaction with activated β1 integrin (Gaultier et al., 2002). Therefore, ADAM13 (and possibly ADAM19) may function through α5β1 integrin to promote dorsal mesoderm cell adhesion to FN. Since ADAM13 or ADAM19 do not appear to affect the overall expression of β1 integrin (Figure 4.18), it is likely that either the cell surface protein level of α5β1 integrin or the activation of α5β1 integrin could be regulated by ADAM13. Integrins are cell surface heterodimers functioning as receptors in cell adhesion and migration to the extracellular matrix (Hynes, 2009). Using three-dimensional models, it is shown that the integrin α and β tails of α5β1 form a heterodimer and exist in a low affinity state or closed conformation. In this inactive state, α5β1 can bind to the RGD site of fibronectin. In order to change to a high affinity state and to become activated, changes in their three-dimensional conformation occur in relation to the position of each tail and relative to the plasma membrane. Once activated, α5β1 integrin has an open conformation and can bind to both the RGD and Synergy sites on fibronectin. Furthermore, once an integrin heterodimer becomes activated this promotes a physical interaction with the intracellular adaptor protein talin
to the β cytoplasmic tail (Tadokoro et al., 2003). The two main kinases downstream of an activated integrin heterodimer are focal adhesion kinase (FAK) and Src kinase. These kinases regulate other downstream proteins that control the small GTPases Rac1 and RhoA to induce cytoskeletal changes. This is essential for proper cell adhesion and migration on fibronectin.

Two techniques have been developed in zebrafish and humans to analyze activated integrin heterodimers in vivo. Bimolecular Fluorescence Complementation (BiFC) can visualize active integrin heterodimers in vivo (Julich et al., 2009). The α5 integrin was tagged at the C-terminus with the N-terminal fragment of Venus yellow fluorescent protein (α5-nVenus). The β1 integrin was tagged at the C-terminus with the C-terminal fragment of Venus yellow fluorescent protein (β1-cVenus). RNA was transcribed for both constructs and injected into zebrafish embryos. Co-expression of α5-nVenus and β1-cVenus produced strong fluorescence at the intersomitic boundaries compared to weak fluorescence within individual somitic cells. This suggests an active integrin heterodimer forms at the intersomitic boundaries. The second technique utilizes a monoclonal antibody (MAB2079Z) made against human β1 integrin (amino acids 355-425, Millipore) that specifically recognizes its active conformation (Barberis et al., 2004). For our system, injection of human β1 integrin RNA would enable us to visualize activated integrin heterodimers in the dorsal mesoderm by immunohistochemistry. These techniques will allow us to investigate if ADAM13 (or ADAM19) affects α5β1 integrin activation.

Reintroducing ADAM13 RNA (200pg) in the dorsal mesoderm was able to partially rescue cell spreading on 9.11 but not on full length FN (Figure 4.16). ADAM13
is known to bind to the second heparin-binding domain (Hep II) of fibronectin in order to cleave and remodel a fibronectin matrix (Alfandari et al., 2001; Gaultier et al., 2002). It is likely that reintroducing ADAM13 in the dorsal mesoderm cells allows ADAM13 to bind and cleave FN. This would cause a decrease in cell adhesion to full length FN. I observed an even further reduction (42%) in cell adhesion when injecting a high dosage (400pg) of ADAM13 RNA (Figure 4.16A). Furthermore, our lab has shown that a loss of ADAM13, as well as, ADAM13 overexpression in the embryo delays blastopore closure by two hours. Therefore, it is possible that a finite dosage of ADAM13 is needed to obtain a complete rescue on full length FN. Since the 9.11 substrate does not contain the Hep II domain, this might explain why ADAM13 can rescue on the 9.11 substrate but cannot rescue cell adhesion on full length FN. These results suggest that ADAM13 functions through \(\alpha5\beta1\) integrin to regulate cell adhesion on fibronectin, in addition to having a role in binding and cleaving fibronectin.

Work completed by our lab on ADAM13 and another group on ADAM10 has recently shown that the cytoplasmic domains are cleaved off by \(\gamma\)-secretase and translocate into the nucleus to perform an independent function in the embryo (Cousin et al., 2010; Tousseyn et al., 2009). Our lab has shown that overexpression of a GFP fusion to the cytoplasmic domain of ADAM13 (GFP-C13) in cranial neural crest cells affects gene expression in approximately 2000 genes. Co-expression with the extracellular domain of ADAM13 (ADAM13\(\Delta\)cyto) is capable of rescuing CNC cell migration \textit{in vivo}. In addition, the cytoplasmic domain of ADAM19 (GFP-C19) co-expressed with the ADAM13\(\Delta\)cyto is capable of rescuing CNC cell migration \textit{in vivo}. This suggests that the ADAM13 cytoplasmic domain (and possibly ADAM19 cytoplasmic domain) regulates
gene expression when inside the nucleus and that the overlapping functions of ADAM13 and ADAM19 may be explained by the actions of their cytoplasmic domains.

GFP-C13 can rescue dorsal mesoderm cell spreading on FN and 9.11 in MO19 injected embryos but not in MO13 injected embryos (Figure 4.17). In addition, the co-injection at the one cell stage of MO19 and GFP-C13 RNA (1 ng) can upregulate ADAM13 protein level to near control levels (Figure 4.19). This result was not seen by co-injection of MO13 and GFP-C13 RNA (1 ng) because of the effects of the morpholino. Therefore, GFP-C13 may function through a positive feedback mechanism to regulate ADAM13 gene expression. To determine if GFP-C13 can function through a positive feedback mechanism to upregulate ADAM13 transcription, I propose to isolate RNA from embryos overexpressing GFP-C13. I will detect ADAM13 gene expression by real time PCR. The PCR primer sets will have to recognize a specific sequence in the extracellular domain of ADAM13 to avoid amplification of the cytoplasmic domain and cross-reactivity with other ADAM members. Furthermore, our lab has shown the cytoplasmic domain of ADAM19 (GFP-C19) can compensate for GFP-C13 by rescuing CNC cell migration \textit{in vivo} (Cousin et al., 2010). If GFP-C19 can compensate for GFP-C13 in CNC cells, it would be interesting to determine if GFP-C19 could rescue dorsal mesoderm cell spreading on FN and 9.11 substrates. Lastly, it would be interesting to determine if GFP-C19 may function in a similar positive feedback mechanism.

Although ADAM13 and ADAM19 proteins are required for normal morphogenetic movements during gastrulation, my data suggests they function through different mechanisms. I propose that ADAM19 functions by shedding Neuregulin to help establish gene expression for the A-P polarity of the notochord. I propose that
ADAM13 functions to control dorsal mesoderm cell adhesion on fibronectin by regulating the activation of α5β1 integrin. These hypotheses will be explored further in Chapter V of this thesis.

SECTION VII: Materials and Methods used in Chapter IV

Embryo Dissections - Open Face Explants

Embryos were injected in the animal pole region at the one-cell stage with either CMO, a Morpholino to ADAM13 (MO13), or MO19 to knock down protein expression in the entire embryo. The same embryos were injected again into one blastomere at the two-cell stage with mRNA encoding a membrane bound Cherry (mCherry) to visualize individual cells. The resultant embryos were allowed to develop until the end of gastrulation (stage 12.5). At this point, the dorsal mesoderm was dissected from each embryo and the resulting open face explant was prepared as described previously (Wilson, 1989). Time lapse movies were made using an Axiovert inverted microscope (Zeiss) equipped with a Ludl XY-stage control and an Orca camera (Hamamatsu) and controlled by the OpenLab software (Improvision). The explants were allowed to develop until approximately early tailbud (stage 22). Individual cell tracking and notochord measurements were done using the OpenLab software. At the end of each time lapse movie, the explants were fixed in MEMFA and dehydrated in methanol for Whole Mount Immunostain analysis.
Double Whole Mount Immunostain

Embryos were fixed at tailbud stage (st. 24) using MEMFA and stored in methanol. Whole mount immunostaining was performed as previously described (Dent et al., 1989). The Tor70 antibody was a generous gift from Dr. Richard Harland at the University of California, Berkeley. The 12/101 antibody was purchased from the Developmental Studies Hybridoma Bank (Iowa City, Iowa).

Cell Adhesion Assay

Embryos were injected in the animal pole region at the one-cell stage with CMO, MO13, or MO19 to knock down protein expression in the entire embryo. The resultant embryos were allowed to develop until the beginning of gastrulation (stage 10.5), at which point the dorsal mesoderm was dissected from each embryo. Dorsal mesoderm cells were dissociated on an agarose coated plastic dish in calcium and magnesium free media (1X CMF) for 30 minutes. The cells were plated onto a fibronectin (FN) substrate or a GST-fusion protein containing only the RGD/synergy site of fibronectin (GST-9.11) as described previously (Ramos, 1996). Time lapse movies were made using an Axiovert inverted microscope (Zeiss) equipped with a Ludl XY-stage control and an Orca camera (Hamamatsu) and controlled by the OpenLab software (Improvision). Dorsal mesoderm cell spreading was analyzed at the one hour time point for all experiments.

Confocal Microscopy

GFP-dishevelled RNA was injected into the two dorsal blastomeres at the four-cell stage and allowed to develop until late gastrula (stage 12.5/13). Open face explants were
dissected as described above. The explants were cultured in Danilchik media on glass bottom petri dishes. The live explants were allowed to heal for one hour and then imaged at approximately stage 14 using a Zeiss Confocal Scanning Laser Microscope equipped with LSM 510M software. The images were captured under an oil immersion 25x magnification objective lens.
Figure 4.1: A-P Polarity Analysis for Xbrachyury Expression at the Gastrula Stage. Embryos were microinjected with 10 ng of MO19 or a control MO at the one-cell stage and allowed to develop until gastrula stage (stage 12). Embryos were also co-injected at the one-cell stage with RNA encoding full length ADAM19 (250pg), ADAM19E/A (250pg), full length Neuregulin1β (NRG-FL) (100pg) or the extracellular region of Neuregulin1β (NRG-EC) (100pg). Whole mount *in situ* hybridization was performed using the marker Xbrachyury. For all embryos, the dorsal view is presented with the anterior up. The frequencies of the morphotypes observed are indicated in the bottom graph.
Figure 4.2: A-P Polarity Analysis for Chordin Expression at the Gastrula Stage. Embryos were microinjected with 10 ng of MO19 or a control MO at the one-cell stage and allowed to develop until gastrula stage (stage 12). Embryos were also co-injected at the one-cell stage with RNA encoding full length ADAM19 (250pg), ADAM19E/A (250pg), full length Neuregulin1β (NRG-FL) (100pg) or the extracellular region of Neuregulin1β (NRG-EC) (100pg). Whole mount in situ hybridization was performed using the marker Chordin. For all embryos, the dorsal view is presented with the anterior up and the red bar indicates the relative distance from the blastopore. The frequencies of the morphotypes observed are indicated in the bottom graph.
Figure 4.3: ADAM13 affect on the A-P Polarity of the Notochord.
Embryos were microinjected with 10 ng of MO13, MO19 or a control MO at the one-cell stage and allowed to develop until gastrula stage (stage 12). MO19 embryos were also co-injected at the one-cell stage with RNA encoding full length ADAM13 (200pg). Whole mount in situ hybridization was performed using the marker Xbrachyury and Chordin. For all embryos, the dorsal view is presented with the anterior up and the red bar indicates the relative distance from the blastopore. The frequencies of the morphotypes observed are indicated in the bottom graph.
Figure 4.4: Visual Summary showing the A-P Polarity of the Notochord at the Gastrula Stage. Wild type notochords are compared to ADAM13 knock down, ADAM19 knock down, and NRG-EC Rescue notochords showing Xbrahchury (blue) and Chordin (red) expression profiles. Anterior (A), posterior (P). ADAM13 knock down notochords do not affect A-P polarity gene expression. The expression patterns are wider compared to control notochords, suggesting possible notochord morphology defects. ADAM19 knock down notochords do affect A-P polarity gene expression. Specifically, a loss of Xbrahchury concomitant with an anterior shift in Chordin expression. Gene expression can be rescued by co-injecting the extracellular fragment of Neuregulin1β RNA, a form that mimics a potential cleavage product of ADAM19.
Figure 4.5: Double Whole Mount Immunostain on Embryos Lacking ADAM13 or ADAM19. The monoclonal antibody, Tor70, stains the notochord in brown (Kushner, 1984). The monoclonal antibody, 12/101, stains the somites in green (Kintner and Brockes, 1985). The notochord morphologically appears wider in MO13 and MO19 injected embryos. The somites morphologically appear normal in MO13 injected embryos but show gross morphology in MO19 injected embryos. The defects observed in these two dorsal tissues are also evident in the smaller and shorter body size of MO19 injected embryos.
Figure 4.6: Microdissection of an Open Face Explant. (A) Bisect a late gastrula (stage 12.5/13) embryo along the dotted line. (B) Peel away the underlying 'sticky' endoderm layer, exposing the dorsal mesoderm. (C) Cut away the ends to shape the explant into a rectangle. (D) Place the explant mesoderm face down onto a glass bottom Petri dish filled with Danilchik media. Place four small droplets of vacuum grease around the explant and position a glass coverslip on top. Press down gently. (E) All Open Face Explants are positioned with the Anterior at the top and Posterior at the bottom. The notochord is in the center surrounded by the developing somites. This figure was adapted from (Wilson et al., 1989).
Figure 4.7: Time Lapse Microscopy of a Control Open Face Explant. Explants from embryos injected with CMO at the one-cell stage. Membrane bound Cherry RNA was injected into one blastomere at the two-cell stage to visualize axial mesoderm cell movement from one half of the embryo. The montage represents an image every 30 minutes from a ten hour time lapse movie. The movie file is included with this Thesis.
Figure 4.8: Time Lapse Microscopy of MO13 Injected Open Face Explant. Explants from embryos injected with MO13 at the one-cell stage. Membrane bound Cherry RNA was injected into one blastomere at the two-cell stage to visualize axial mesoderm cell movement from one half of the embryo. The montage represents an image every 30 minutes from a ten hour time lapse movie. The movie file is included with this Thesis.
Figure 4.9: Time Lapse Microscopy of MO19 Injected Open Face Explant. Explants from embryos injected with MO19 at the one-cell stage. Membrane bound Cherry RNA was injected into one blastomere at the two-cell stage to visualize axial mesoderm cell movement from one half of the embryo. The montage represents an image every 30 minutes from a ten hour time lapse movie. The movie file is included with this Thesis.
Figure 4.10: Axial Mesoderm Cell Morphology. (A) Open face explants from embryos injected with CMO, MO13 or MO19 at the one-cell stage. Membrane bound Cherry RNA was injected into one blastomere at the two-cell stage. Four cells are outlined in black to better visualize the axial mesoderm cell morphology. (B) Axial mesoderm cell length-to-width ratio. The mediolateral length and anterior-posterior width were measured in axial mesoderm cells of open face explants at the end of gastrulation (st. 13). The graph represents 20 cells from four independent experiments. Control axial mesoderm cells have a bipolar morphology. Axial mesoderm cells from embryos lacking ADAM13 or ADAM19 have a multipolar morphology with reduced axial mesoderm cell L:W ratios.
Figure 4.11: Mediolateral Cell Intercalation of the Axial Mesoderm. Open face explants from embryos injected with CMO, MO13 or MO19 at the one-cell stage. Membrane bound Cherry RNA was injected into one blastomere at the two-cell stage. Three cells were outlined in different colors to better visualize the axial mesoderm cell movement over an eight hour time period. The length was measured between two cells at each time point and divided by the width of the notochord. This generated a length-to-width ratio (L:W). The graph below represents 14 different L:W measurements from three independent experiments. In embryos lacking ADAM13 or ADAM19, the L:W did not change, showing a delay in mediolateral cell intercalation.
**Figure 4.12: Notochord-Somite Boundary Analysis.** Open face explants from embryos injected with CMO, MO13 or MO19 at the one-cell stage. The explants were allowed to develop in Danilchik media until approximately stage 20 and then fixed. Double immunostain was done using antibodies to the extracellular matrix proteins fibronectin (4H2, red) and laminin (anti-rabbit-laminin, Sigma, green). These proteins accumulate at the notochord-somite boundary and at the intersomitic boundaries. In MO13 injected embryos, punctate expression of both ECM proteins was found in the notochord (white arrowheads). In MO19 injected embryos, abnormal expression of both ECM proteins was found throughout the notochord. The images represent maximum projections taken under fluorescence microscopy using an Apotome and Axiovision 4.5 software analysis.
**Figure 4.13: GFP-Dishevelled Localization in the Dorsal Mesoderm.** Open face explants from embryos injected with CMO, MO13 or MO19 at the one-cell stage. 50pg of GFP-dishevelled RNA was injected into the two dorsal blastomeres at the four-cell stage. The explants were allowed to develop in Danilchik media while the images were captured with confocal microscopy. The approximate developmental stage of each image is stage 14. The localization of GFP-dishevelled was found at the plasma membrane under all three conditions.
Figure 4.14: GFP-Dishevelled Localization Normalized to mRFP in the Dorsal Mesoderm. (A) Open face explants from embryos injected with CMO, MO13 or MO19 at the one-cell stage. 50pg of GFP-dishevelled RNA and monomeric-RFP RNA was injected into one dorsal blastomere at the four-cell stage. The explants were allowed to develop in Danilchik media while the images were captured with confocal microscopy. The approximate developmental stage of each image is stage 14. The localization of GFP-dishevelled was found at the plasma membrane under all three conditions with mRFP localized throughout the cytosol. (B) The fluorescence profile of GFP-dishevelled is normalized to the fluorescence profile of mRFP and the average ratios are plotted in the graph. (C) All of the ratio data is plotted in a Box and Whisker Plot. For both data representations, the ratio for GFP-dishevelled does not change under all three conditions.
Figure 4.15: Dorsal Mesoderm Cell Adhesion Assay. (A) Dorsal mesoderm cells were taken from CMO, MO13, MO19, or MO13+MO19 (2MO) embryos, dissociated and plated onto a fibronectin (FN) or 9.11 substrate. Cell attachment and spreading was analyzed after one hour. (B) In control embryos, 85% of the cells spread on FN and 9.11. In MO13 injected embryos, 60% of the cells spread on FN and 9.11. In MO19 injected embryos, 70% of the cells spread on FN and 9.11. In 2MO injected embryos, 60% of the cells spread on FN and 9.11. The images represent cells from one experiment. The graphs shown below represent data from twelve experiments.
Figure 4.16: Summary of Rescue Experiments in Dorsal Mesoderm of MO13 Injected Embryos. Dorsal mesoderm cells were taken from MO13 embryos injected with various rescue RNA constructs into the two dorsal blastomeres at the four-cell stage. The cells were dissociated and plated onto a (A) fibronectin (FN) or (B) 9.11 substrate. Cell attachment and spreading was analyzed after one hour and compared to Control cells. The rescue RNA is full length ADAM13 (A13), full length ADAM19 (A19), the cytoplasmic domain of ADAM13 (C13), Xenopus marginal coil (Xmc-MT), and the constitutively active p110 subunit of PI3K (p110). The dosages are indicated in parentheses.
Figure 4.17: Summary of Rescue Experiments in Dorsal Mesoderm of MO19 Injected Embryos. Dorsal mesoderm cells were taken from MO19 embryos injected with various rescue RNA constructs into the two dorsal blastomeres at the four-cell stage. The cells were dissociated and plated onto a (A) fibronectin (FN) or (B) 9.11 substrate. Cell attachment and spreading was analyzed after one hour and compared to Control cells. The rescue RNA is full length ADAM13 (A13), full length ADAM19 (A19), the cytoplasmic domain of ADAM13 (C13), Xenopus marginal coil (Xmc-MT), and the constitutively active p110 subunit of PI3K (p110). The dosages are indicated in parentheses.
Figure 4.18: Protein Expression in the Dorsal Mesoderm. Embryos were injected with CMO, MO13 or MO19 at the one-cell stage and allowed to develop to early gastrula (stage 10.5). Total protein extract from five dissected dorsal blastopore lips was probed for integrin \(\beta_1\) (8C8 antibody) and phosphorylated AKT (AKTp, anti-rabbit-AKTp, Cell Signaling Technology). GAPDH (Millipore) was probed as a loading control. The below graph shows percent change from three independent experiments normalized to GAPDH expression.
Figure 4.19: C13 Affect on ADAM13 Expression. Embryos were injected with CMO, MO13 or MO19 together with GFP-C13 RNA (1ng) at the one-cell stage and allowed to develop to early gastrula (stage 10.5). Total protein extract from five embryos were immunoprecipitated using our goat ADAM13 antibody and probed with our rabbit ADAM13 antibody (6615F). GAPDH (Millipore) was probed as a loading control. The below graph shows the percent change of ADAM13 Proform and ADAM13 Mature form when normalized to GAPDH.
CHAPTER V

EXPLORING THE FUNCTIONS OF ADAM13 AND ADAM19
IN THE DORSAL MESODERM

All of the work presented in this chapter is data completed by myself and is unpublished.

Abstract

This chapter will explore the roles of ADAM13 and ADAM19 in the dorsal mesoderm. I will focus on determining how each protein contributes to convergence and extension movements during gastrulation. Previous data from Chapter IV of this thesis suggests that ADAM13 and ADAM19 have different functions during gastrulation. ADAM13 appears to function in regulating cell movements while ADAM19 appears to function in regulating cell signaling. In addition, ADAM19 may function to regulate cell movements indirectly through the function of ADAM13 or through the function of Xbrachyury in the notochord. I propose and discuss a few models for ADAM13 and ADAM19 function in gastrulation.

SECTION I: Introduction

Gastrulation is a morphogenetic process that reorganizes the primary germ layers to shape the internal and external features of an early embryo. The main focus on this thesis is to investigate the roles of ADAM13 and ADAM19 during gastrulation. Both ADAM family members are expressed in the dorsal mesoderm of the dorsal blastopore
lip. This tissue is known as the Spemann Organizer and is essential for regulating gastrulation movements and for early tissue patterning in the embryo. Although ADAM13 and ADAM19 are expressed in the same tissue and are required for normal morphogenetic movements during gastrulation, my data suggests they have different functions. ADAM13 appears to function in regulating cell movements while ADAM19 appears to function in regulating cell signaling.

ADAM13 functions to regulate cell movements during gastrulation. This hypothesis is supported by three main observations. First, embryos lacking ADAM13 delay blastopore closure by two hours showing that ADAM13 is required for proper blastopore closure. This is specific to ADAM13 because blastopore closure was not affected in embryos lacking ADAM19 (Figure 2.4). Second, ADAM13 regulates dorsal mesoderm cell adhesion to fibronectin cell autonomously. Dorsal mesoderm cells from embryos lacking ADAM13 reduce cell spreading on FN to 60%. This is compared to dorsal mesoderm cells from control embryos that have 85% of the cells spreading on FN. In a similar assay, dorsal mesoderm cells from embryos lacking ADAM13 reduce cell spreading on the fusion protein 9.11 to 60%. The 9.11 fusion protein only contains the integrin binding domain (RGD and Synergy sites) suggesting that the cell adhesion defects involve α5β1 integrin. Third, ADAM13 is important for convergence and extension movements in the embryo. Embryos lacking ADAM13 have a delay in mediolateral cell intercalation in the axial mesoderm. This defect results in a wider notochord compared to control embryos. The cell adhesion defects on FN likely contribute to the defects observed in convergence and extension as well as blastopore
closure in the embryo. Therefore, I propose that ADAM13 functions to regulate cell movements during gastrulation.

ADAM19 functions to regulate cell signaling during gastrulation. This hypothesis is supported by three main observations. First, ADAM19 regulates gene expression important for the A-P polarity of the notochord. Embryos lacking ADAM19 have a specific loss of Xbrachyury expression concomitant with an anterior shift of Chordin expression in the notochord (Figure 2.5, 4.1 and 4.2). Gene expression is unaffected in embryos lacking ADAM13 at the gastrula stage, suggesting a specific role for ADAM19 (Figure 4.3). Second, embryos lacking ADAM19 have reduced phosphorylated AKT levels (Figure 2.9). AKT is a downstream target of the EGF signaling pathway. This is specific to ADAM19 because phosphorylated AKT is unaffected in embryos lacking ADAM13. Third, ADAM19 may affect the canonical Wnt signaling pathway. Embryos lacking ADAM19 have reduced total β-catenin levels (Figure 2.9). β-catenin is an intracellular target of the canonical Wnt signaling pathway. This is specific to ADAM19 because β-catenin levels are unaffected in embryos lacking ADAM13. Therefore, I propose that ADAM19 functions to regulate cell signaling during gastrulation.

In addition, ADAM19 may have an indirect role in regulating cell movements during gastrulation. This hypothesis is supported by two main observations. First, ADAM19 may function to regulate dorsal mesoderm cell adhesion to FN. Dorsal mesoderm cells from embryos lacking ADAM19 reduce cell spreading on FN and 9.11 to 70%. This is compared to dorsal mesoderm cells from control embryos that have 85% of the cells spreading on FN and 9.11. Second, ADAM19 is important for convergence and
extension movements in the embryo. Embryos lacking ADAM19 have a delay in mediolateral cell intercalation in the axial mesoderm. These defects result in a wider notochord compared to control embryos. However, the ADAM19 observations may be an indirect result by affecting ADAM13 protein. Embryos lacking ADAM19 strongly reduce ADAM13 protein levels at the early gastrula stage (Figure 4.19) and at the tailbud stage (Figure 3.2). Therefore, I propose that ADAM19 may function to regulate cell movements indirectly through the function of ADAM13 or through the function of Xbrachyury in the notochord. Both ADAM13 and ADAM19 functions during gastrulation will be explored further in Section II and Section III of this chapter.

SECTION II: ADAM13 Contributes to Convergence and Extension by Regulating Dorsal Mesoderm Cell Adhesion to Fibronectin

Integrin activation and integrin heterodimer clustering at the cell surface are important for increased adhesion to a fibronectin matrix (Larsen et al., 2006). Disruptions in integrin function with the extracellular matrix have led to defects in cell adhesion and cell migration. Mice null for the α5 integrin subunit die early in gestation due to mesoderm defects such as abnormal posterior somite development and degeneration of the notochord tissue (Goh et al., 1997). A loss of FN in the embryo results in convergence and extension defects in zebrafish (Latimer and Jessen, 2009) and in Xenopus (Rozario and DeSimone, 2010). Moreover, studies using function blocking antibodies to α5β1 integrin randomize cell protrusions that are important for mediolateral cell intercalation, thus causing defects in convergence and extension (Davidson et al.,
In addition to the interaction between $\alpha_5\beta_1$ integrin and FN, the physical state of FN fibril assembly also regulates convergence and extension movements in the embryo (Rozario et al., 2009). Therefore, the interaction between $\alpha_5\beta_1$ and fibronectin plays an essential role in the morphogenetic movements during gastrulation. Dorsal mesoderm cells adhere and migrate on a fibronectin matrix along the blastocoel roof. These cell movements are critical for proper blastopore closure during gastrulation. I propose that ADAM13 (and possibly ADAM19) functions to promote dorsal mesoderm cell adhesion to a fibronectin matrix. ADAM13 promotes cell adhesion by regulating the activation of $\alpha_5\beta_1$ integrin. This regulation may occur through two different cell autonomous mechanisms; an intracellular mechanism or an extracellular mechanism.

**How does ADAM13 Regulate $\alpha_5\beta_1$ Integrin Activation Cell Autonomously?**

ADAM metalloproteases are regulated through a variety of different post-translational modifications (Murphy, 2009). One form of regulation is through intracellular binding partners that interact with the ADAM metalloprotease cytoplasmic domain. The proteins physically interact with SH3 binding motifs and proline rich domains found in the ADAM cytoplasmic domain. A large number of the proteins identified so far are important for regulating ADAM protein subcellular localization and trafficking within a cell. However, some important intracellular kinases have also been identified such as PCKδ, Src, PI3K and ERK (Diaz-Rodriguez et al., 2002; Izumi et al., 1998; Kang et al., 2001; Poghosyan et al., 2002). This suggests that ADAM cytoplasmic domains and the attached kinases may have an intracellular function.
The Src family of tyrosine kinases are a large protein kinase family that function downstream of different cell surface receptors (Bradshaw, 2010). Src kinases together with focal adhesion kinase (FAK) are important for regulating the downstream signaling events upon integrin engagement with the ECM (Pellinen and Ivaska, 2006). Src and FAK kinases regulate guanine nucleotide exchange factors (GEF proteins) which activate the small GTPases RhoA and Rac1. RhoA and Rac1 regulate cytoskeletal changes within a cell and are essential for cell adhesion and cell migration. Src kinases were identified as binding partners to the cytoplasmic domains of many different ADAM proteins such as ADAM9 (Weskamp et al., 1996), ADAM12 (Suzuki et al., 2000), ADAM13 (Cousin et al., 2000), and ADAM15 (Poghosyan et al., 2002).

Src kinases can regulate an ADAM protein through two different mechanisms. First, Src kinase binds and phosphorylates the ADAM cytoplasmic domain to regulate the extracellular function of the ADAM metalloprotease. This is important for regulating the ectodomain shedding of an ADAM substrate from a cell. For example, c-Src kinase binds and phosphorylates the cytoplasmic domain of ADAM15. This increases ectodomain shedding of the ADAM15 substrate, fibroblast growth factor receptor 2 (FGFR2) (Maretzky et al., 2009). FGFR2 was not shed in cells treated with a Src kinase inhibitor or in c-Src(-/-) cells but FGFR2 shedding was rescued by reintroducing Src in c-Src(-/-) cells. This shows that c-Src regulates the ectodomain shedding of FGFR2 by ADAM15. Second, ADAM proteins may enhance Src kinase activity in cultured cells. This occurs when an ADAM protein recruits Src kinase to large protein complexes at the cell surface. For example, ADAM12 and Src kinase co-localize to actin-rich structures along membrane ruffles in cultured cells (Stautz et al., 2010). ADAM12 enhances Src
kinase phosphorylation when cells are plated on the extracellular matrix protein vitronectin. This suggests that upon integrin engagement to vitronectin, ADAM12 can enhance Src kinase activity. Therefore, ADAM proteins may recruit Src kinases to specific locations in a cell as a way to regulate their own metalloprotease activity.

My results suggest that ADAM13 promotes dorsal mesoderm cell adhesion to fibronectin by regulating the activation of $\alpha_5\beta_1$ integrin. This occurs cell autonomously because in my assay the dorsal mesoderm cells were dissociated. Src kinase is capable of binding to the cytoplasmic domain of ADAM13 (Cousin et al., 2000). Therefore, I propose that ADAM13 recruits Src kinase to specific adhesion complexes containing $\alpha_5\beta_1$ integrin at the cell surface. Once $\alpha_5\beta_1$ integrin binds fibronectin and becomes activated, Src kinase phosphorylates FAK to regulate downstream Rho/Rac signaling events and cytoskeletal changes. This intracellular mechanism allows the cell to spread on a fibronectin substrate and to promote proper cell adhesion (Figure 5.1A). Alternatively, ADAM13 may regulate the activation of $\alpha_5\beta_1$ integrin through an extracellular mechanism involving the cleavage of fibronectin (Figure 5.1A). ADAM13 binds to the HepII domain of fibronectin and cleaves fibronectin. This mechanism could promote cell migration on a fibronectin matrix.

In the absence of ADAM13, Src is not properly recruited to the specific adhesion complexes containing $\alpha_5\beta_1$ integrin. As a result, Src activity is reduced, FAK activity is reduced and ADAM13 is not present to cleave fibronectin (Figure 5.1B). Therefore, cells will remain round and not be able to spread on a fibronectin substrate. To test the ADAM13 model, I propose the following experiments. First, to investigate Src kinase activity in MO13 injected embryos, I propose to determine phosphorylated Src protein
levels by western blot. Second, to investigate FAK kinase activity in MO13 injected embryos, I propose to determine phosphorylated FAK protein levels by western blot. The ADAM13 model focuses on explaining how ADAM13 could regulate cell adhesion on fibronectin cell autonomously. The next section will discuss the possibility that ADAM13 may function in a non-cell autonomous mechanism to regulate dorsal mesoderm cell adhesion to fibronectin during convergence and extension movements.

How does ADAM13 Regulate $\alpha 5\beta 1$ Integrin Activation Non-Cell Autonomously?

Ephrin receptors (Eph) and ligands (ephrin) have prominent roles in vascular development (Wang et al., 1998), repulsive guidance of neurons (Cheng et al., 1995), and establishment of tissue boundaries in the embryo (Gale et al., 1996). For example, the differential expression of Eph receptors and ephrin ligands prevents intermixing of cells during CNC migration (Smith et al., 1997). Moreover, it is believed that some ephrins can regulate a cell’s affinity towards different ECM substrates through cross talk with integrin receptors. In forward Eph-ephrin signaling, the EphA2 receptor (de Saint-Vis et al., 2003) or EphA4 receptor (Bourgin et al., 2007) regulate dendritic cell adhesion to FN via $\beta 1$ integrin. In reverse Eph-ephrin signaling, the ephrinB1 ligand (Huynh-Do et al., 2002) and ephrinB2 ligand (Meyer et al., 2007) regulate endothelial cell adhesion to FN via $\alpha 5\beta 1$ integrin. Therefore, the cross talk between ephrins and integrin activation appears to be mediated through an inside-out signaling mechanism. Although an exact molecular mechanism has yet to be determined, it appears to involve intracellular kinases (PI3K, Akt, Src, FAK) and the small GTPases (RhoA and Rac1) to induce cytoskeletal
changes that regulate cell-cell adhesion (Arvanitis and Davy, 2008; Pasquale, 2008; Somanath and Byzova, 2009).

ADAM10 was the first ADAM metalloprotease shown to cleave a member of the ephrin signaling pathway. In neurons, ADAM10 cleaves the ephrinA2 ligand in cis important for axon detachment and termination of signaling (Hattori et al., 2000). Another group showed that the ADAM10 cysteine-rich domain binds to the EphA3 receptor in complex with the ephrinA5 ligand (Janes et al., 2005). This physical interaction positions ADAM10 for cleavage of ephrinA5 in trans, with ADAM10 and its substrate on opposing cells. Moreover, ADAM19 can bind the EphA4 receptor in complex with the ephrinA5 ligand (Yumoto et al., 2008). However, no cleavage event occurs but this interaction is important for blocking the endocytosis of the EphA4-ephrinA5 complex. Therefore, ADAM19 functions to stabilize neuronal synapses in the formation of neuromuscular junctions. ADAM13 can cleave the ephrinB1 and B2 ligands to regulate forward ephrin signaling in the dorsal mesoderm of a closely related species Xenopus tropicalis (Wei et al., 2010). Regardless of proteolytic activity, the Eph-ephrin signaling complex may be a physiologically relevant substrate for ADAM metalloproteases. I propose that ADAM13 cleavage of ephrinB1/B2 in the dorsal mesoderm may affect the activation of α5β1 integrin. Specifically through reverse signaling of ephrinB1/B2, cleavage may regulate the intracellular kinase activity of Src and FAK in complex with α5β1 integrin (Figure 5.2). This cleavage event would be important for regulating cell-cell adhesion on a fibronectin matrix. More importantly, ADAM13 cleavage of ephrinB ligands may contribute to the dorsal mesoderm cell movements during gastrulation.
SECTION III: ADAM19 Mediated Shedding of Neuregulin Contributes to

Convergence and Extension

Gene expression is altered in MO19 injected embryos at the late gastrula stage (Neuner et al., 2009). Specifically, Xbrachyury expression is lost concomitant with an anterior shift of Chordin expression in the notochord (Figure 2.5, 4.1 and 4.2). Gene expression was unaffected in embryos lacking ADAM13 at the gastrula stage, suggesting a specific role for ADAM19 (Figure 4.3). Xbrachyury and Chordin are known to be expressed in a countergradient fashion in the axial mesoderm (Ninomiya et al., 2004). Chordin is a marker of anterior notochord and Xbrachyury is a marker of posterior notochord. Together, these two genes help establish the A-P polarity of the developing notochord. Both genes are required for proper convergence and extension movements in explants and in the embryo. This was the first study in Xenopus linking A-P tissue polarity to mesoderm convergence and extension (Ninomiya et al., 2004). Additionally, I show that Brachyury and Chordin gene expression are dependent on ADAM19 proteolytic activity (Figure 4.1 and 4.2). This is shown by a partial rescue in gene expression by co-injection of ADAM19 RNA but not with a catalytically inactive mutant ADAM19 E/A RNA. It is possible that ADAM19 functions by cleaving an unknown protein important for establishing A-P polarity of the notochord. The unknown protein may be a growth factor that stimulates signal transduction to activate Xbrachyury and Chordin expression in the notochord.

In mice, ADAM19 can cleave some members of the EGF family of ligands including Neuregulin1β (Shirakabe et al., 2001). Neuregulin1β binds to the ErbB3 or
ErbB4 receptors to activate the ErbB signaling pathway (Esper et al., 2006; Eto et al., 2006). In Xenopus, the ErbB2/ErbB3 receptors and NRG1β expression begins during gastrulation and continues through tailbud formation (Nie and Chang, 2007b; Yang et al., 1999; Yang et al., 1998a; Yang et al., 1998b). Purified recombinant NRG1β, as well as, the overexpression of ErbB2 or ErbB3 receptors can induce naïve animal cap cells to become mesoderm and express Xbrachyury (Chung and Chung, 1999; Nie and Chang, 2007b). Moreover, blocking ErbB receptors through injection of a Morpholino or dominant-negative RNA, can affect the convergence and extension movements during gastrulation (Nie and Chang, 2006; Nie and Chang, 2007b). I show that expression of the extracellular domain of NRG1β (NRG-EC) is capable of rescuing Xbrachyury expression in the notochord while full length NRG1β does not rescue (Figure 4.1 and 4.2). Therefore, it is possible that ADAM19 mediated shedding of NRG1β allows soluble NRG-EC to interact with the ErbB receptor to activate signal transduction cell autonomously in the axial mesoderm. The NRG1β-ErbB signaling pathway may induce Xbrachyury gene expression specifically as the notochord develops.

Brachyury has been extensively studied in a number of chordates and non-chordates for its prominent role in the morphogenetic movements during gastrulation (Yamada et al., 2010). For example, Brachyury knockout mice have abnormal primitive streaks due to aberrant mesoderm cell migration (Wilson et al., 1995). In Xenopus, loss of Xbrachyury prevents convergence and extension movements during gastrulation (Conlon et al., 1996; Conlon and Smith, 1999; Kwan and Kirschner, 2003). As a transcription factor, Xbrachyury controls convergence and extension by activating gene expression for Wnt11, a known target gene (Tada and Smith, 2000). Wnt11 is a Wnt
family ligand that binds to the Frizzled receptor and signals through dishevelled to regulate downstream RhoA/Rac1 cytoskeletal changes. Signaling through Wnt11 is essential for convergence and extension movements during gastrulation (Smith et al., 2000; Tada et al., 2002; Tada and Smith, 2000). The Xbrachyury-Wnt11-PCP Signaling pathway is briefly outlined in Figure 5.3. Therefore in embryos lacking ADAM19, the loss of Xbrachyury in the notochord may be indirectly linked to the convergence and extension defects. I propose that ADAM19 functions to cleave NRG1β to maintain expression of Xbrachyury in the notochord (Figure 5.4A). Axial mesoderm cells that express Xbrachyury are bipolar and actively undergo mediolateral cell intercalation through the Wnt11-PCP Signaling Pathway. This is a cell autonomous hypothesis because ADAM19 and Xbrachyury are both expressed in the notochord.

In the absence of ADAM19, NRG1β is not cleaved in an axial mesoderm cell. This reduces ErbB signaling by reducing phosphorylated AKT levels and Xbrachyury gene expression in the notochord (Figure 5.4B). Furthermore, a loss of Xbrachyury in the notochord may reduce the Wnt11-PCP Signaling Pathway important for mediolateral cell intercalation. Therefore, cells will remain round or multipolar and delay mediolateral cell intercalation. To test the ADAM19 model, I will propose the following experiments. First, to investigate if the Wnt11-PCP Signaling Pathway is affected in embryos lacking ADAM19, I propose to analyze Wnt11 gene expression by real time PCR. I predict that a loss of Xbrachyury in the notochord would result in a loss of Wnt11 gene expression in the notochord. Second, to investigate if the loss of Xbra contributes to mediolateral cell intercalation, I propose to inject Xbrachyury RNA in the dorsal mesoderm of MO19 injected embryos. Given the known role of Xbrachyury in regulating convergence and
extension movements, I predict this experiment should rescue defects in MO19 injected embryos. However, inappropriate expression of Xbrachyury can cause ectopic posterior mesoderm formation (Cunliffe and Smith, 1992). Therefore, a targeted injection of a finite dosage of Xbrachyury RNA specifically in the axial mesoderm would need to be completed. Lastly, it would be interesting to determine if expression of NRG1β (NRG-FL vs NRG-EC) in the dorsal mesoderm could rescue convergence and extension defects in embryos lacking ADAM19.

**Concluding Remarks**

ADAM metalloproteases function as ectodomain sheddases through the cleavage of other membrane bound proteins. A significant amount of research in the ADAM field has focused on identifying a number of substrates to different ADAM family members. To a lesser extent, some of the research in the ADAM field has focused on investigating the function of ADAM proteins in cell migration. Most of this work was completed in single cell assays investigating the epithelial to mesenchymal transition *in vitro*. While ADAM proteins appear to be important for individual cell migration, very little is known about the role of ADAM metalloproteases in tissue movements. Tissue movements require coordinated cell migration with respect to surrounding cells. This is the primary focus of research in our laboratory.

My work has focused on understanding the role of ADAM13 and ADAM19 in tissue movements known as convergence and extension. Although both ADAM proteins are required for convergence and extension movements, my data suggests that ADAM13
and ADAM19 have different functions. ADAM13 appears to function in regulating cell movements through cell spreading on fibronectin while ADAM19 appears to function in regulating cell movements through the transcription factor Brachyury. The research presented in this thesis has furthered our understanding of how ADAM metalloproteases can function in coordinated cell movements during early embryogenesis. More importantly, this type of cell migration is recapitulated in abnormal development. The knowledge learned from this research can be applied to better understand disease progression such as cancer metastasis.
Figure 5.1: Model for the Cell Autonomous Function of ADAM13. (A) In control dorsal mesoderm cells, ADAM13 functions to recruit Src kinase to adhesion complexes containing α5β1 integrin. Src kinase together with FAK kinase regulates the small GTPases RhoA/Rac1 to induce cytoskeletal changes. In addition, ADAM13 binds to the HepII domain of fibronectin to cleave fibronectin. Both mechanisms regulate the activation of α5β1 integrin and allow a cell to spread properly on fibronectin. (B) In MO13 dorsal mesoderm cells, Src kinase activity is reduced because it is not recruited properly to adhesion complexes containing α5β1 integrin. This reduces both Src and FAK kinase activity causing α5β1 integrin to not be activated properly. In addition, fibronectin is not cleaved. These defects cause a cell to round up and reduce cell spreading on fibronectin.
Figure 5.2: Model for the Non-cell Autonomous Function of ADAM13. (A) In control dorsal mesoderm cells, upon Eph-ephrin engagement ADAM13 cleaves ephrinB ligands. This promotes dorsal mesoderm cell-cell de-adhesion during the migration on the blastocoel roof. (B) In MO13 dorsal mesoderm cells, Eph-ephrin engagement is not properly regulated because ephrinB ligands are not cleaved. This affects dorsal mesoderm cell-cell de-adhesion during the migration on the blastocoel roof. Eph-ephrinB signaling may increase Src and FAK kinase activity creating an imbalance in RhoA/Rac1 cytoskeletal changes.
Figure 5.3: Xbrachyury Signaling Pathway. As a transcription factor Xbrachyury induces expression of the target gene, Wnt11 (Tada and Smith, 2000). Wnt11 is a Wnt family ligand important for activating the Non-canonical Wnt (or PCP) Signaling Pathway. The PCP Signaling pathway is essential for controlling convergence and extension movements during gastrulation.
Figure 5.4: Model for ADAM19 Function in the Axial Mesoderm. (A) In control axial mesoderm cells, ADAM19 cleaves NRG1β. Soluble NRG1β activates ErbB signaling cell autonomously. Through PI3K/AKT signal transduction, Xbrachyury gene expression is induced. This allows a cell to become bipolar and undergo mediolateral cell intercalation. (B) In MO19 axial mesoderm cells, NRG1β is not cleaved. This reduces ErbB signaling, phosphorylated AKT, and Xbrachyury gene expression. These defects cause a cell to become multipolar and delay mediolateral cell intercalation.
APPENDIX I

GENERATION OF ADAM19 ANTIBODIES

The goal at the onset of the project was to identify another meltrin subfamily member that could compensate for ADAM13 function, specifically in the cranial neural crest. Of the other meltrin subfamily members, ADAM19 had the highest sequence homology at the amino acid level to ADAM13 making it an ideal candidate. At this time, there were no ADAM19 antibodies available that could be used to study the endogenous protein in Xenopus. Thus one of the first major objectives was to generate a number of antibodies that could recognize different structural domains of the ADAM19 protein. Three antibodies were successfully made to the ADAM19 protein and are briefly described:

1. 2C5 E1: Mouse monoclonal antibody directed against the cytoplasmic domain of ADAM19
2. C2 and C3: Rabbit polyclonal antibody directed against both variants (C2 and C3) of the cytoplasmic domain of ADAM19
3. DC19: Goat polyclonal antibody directed against the disintegrin-cysteine rich domains of ADAM19

**Monoclonal Antibody 2C5 E1**

To generate the monoclonal antibody 2C5 E1, I cloned cDNA encoding the first 127 COOH-terminal amino acid residues of the ADAM19 cytoplasmic domain (A19cyto) into the pET-His expression vector (Novagen). Fusion proteins were purified using standard methods. 100 microgram of protein was combined with complete Freund’s adjuvant and injected intraperitoneal into Balb/c mice over a series of immunizations. After each immunization, blood samples were taken to test for the reactivity against A19cyto.

A fusion was performed with Splenocytes from an immunoreactive mouse and the immortal myeloma SP20 cells. The fusion protocol was based on “Antibodies: A Laboratory Manual” by Ed Harlow and David Lane. The resulting hybridoma colonies were first screened by ELISA for reactivity against A19cyto. Out of 47 colonies tested, 33 colonies had an OD value between 0.2 and 0.25, 12 colonies had an OD value between 0.25 and 0.3, and two colonies had an OD value greater than 0.3. The top five ELISA hits were further subcloned and re-tested by ELISA. The top four hits were 1F1, 2C2, 2C5, and 2C8 having an OD value of greater than 3.0. There were subcloned again and the 2C5 E1 colony was discovered for further testing. Media from 2C5 E1 colony was tested by Western Blot and it strongly recognized the A19cyto fusion protein, as well as, a multitude of proteins in stage 45 embryo extract compared to stage 6 embryo extract.
The 2C5 E1 supernatant had minimal cross-reactivity with A9cyto and A13cyto, therefore this monoclonal antibody was further used in our experiments.

Immune IgG were purified on a His-tag fusion protein column containing the same COOH-terminal amino acid residues used for immunization (NHS-sepharose, Pharmacia). The affinity purified 2C5 E1 antibody was tested on ADAM19 transfected XTC cells for detection by western blot (Figure A1.1B). This antibody did not recognize endogenous ADAM19 from total embryo extract and glycoproteins purified on Concanavalin-A beads from 10 embryos (data not shown). Further testing by western blot should be completed to get the antibody’s right working conditions. The 2C5 E1 antibody is used for ADAM19 overexpression studies and works beautifully for immunoprecipitation and immunofluorescence. The resulting Affinity Purified ADAM19 2C5 E1 was used for experiments described in Chapter III of this Dissertation.

**Figure A1.1:** Western Blots using mAb 2C5 E1.  (A) First screen using supernatant from 2C5 E1 hybridoma colony on total embryo extract (stage 6 vs stage 45) and the different ADAM cytoplasmic domains (A9, A13, A19).  (B) Affinity Purified 2C5 E1 Western Blot on transfected XTC cells.

**Polyclonal Antibody C2 and C3**

To generate the rabbit polyclonal antibody C2 and C3, I cloned cDNA encoding the first 146 COOH-terminal amino acid residues of the ADAM19 cytoplasmic domain
(A19cyto) into the pGEX-KG vector (Guan and Dixon, 1991). This construct was called A19 C2. In addition, I cloned cDNA encoding the first 160 COOH-terminal amino acid residues of the ADAM19 cytoplasmic domain (A19cyto) into the pGEX-KG vector (Guan and Dixon, 1991). This construct was called A19 C3. Both cytoplasmic domains were used because I discovered both variants in 3’ RACE PCR from cDNA. The A19 C3 construct is most common to the X. tropicalis sequence and was submitted to GenBank (EU770696). Fusion proteins were purified using standard methods. Prior to immunization, I pre-screened six rabbits to select rabbits that had a low immunoreactivity in Xenopus embryos. Through a multi-channel western blot apparatus, I tested the preimmune rabbit serum against stage 35 embryos (Figure A1.2). I selected rabbit #2, #3 and #5 for further immunization because they had the least immunoreactive background by western blot.

150 microgram of each protein (total = 300 microgram) was combined with complete Freund’s adjuvant and injected into New Zealand white rabbits over a series of immunizations. After each immunization, blood samples were taken to test for

**Figure A1.2: Pre-screen of Rabbit serum by Western Blot.** Non-immune serum was obtained from six New Zealand white rabbits. The serum was tested by western blot at primary antibody concentrations of 1:100 (first lane) and 1:1000 (second lane) for each sample against stage 35 Xenopus embryo extract. Rabbits #2, #3, and #5 were chosen for further immunization because of their low immunoreactivity.
immunoreactivity against A19 C2 and C3 by ELISA and western blot. The ELISA results showed an increasing OD value of >1.0 after each immunization. The immunization schedule was monitored by Aileen Thomas at the Immunization Facility here at UMass. Immune IgG were purified on a His-tag fusion protein (pET vectors, Novagen) column containing the same COOH-terminal amino acid residues used for immunization (NHS-sepharose, Pharmacia). The A19 C2 and C3 antibodies are used for ADAM19 overexpression and endogenous ADAM19 studies. A western blot example of the affinity purified antibody is shown for ADAM19 transfected Cos-7 cells (Figure A1.3). The resulting Affinity Purified ADAM19 C3 was used for experiments described in Chapter II and IV of this Dissertation.

![Figure A1.3: ADAM19 expression in Cos-7 cells.](image)

Affinity purified A19 C2 and C3 were mixed at the primary concentration of 1:2000 to detect ADAM19FL2 (C2) and ADAM19FL3 (C3). The Proform of ADAM19 (120kD) is predominantly expressed in Cos-7 cells.
Polyclonal Antibody DC19

To generate the polyclonal antibody DC19, I cloned cDNA encoding the Disintegrin and Cysteine-Rich amino acid residues of the ADAM19 domains into the pET-His vector (Novagen). Fusion proteins were purified using denaturing standard methods. 1.0 milligram of protein was combined with complete Freund’s adjuvant and injected into two Boer goats over a series of immunizations. Each goat received 0.5 milligram of fusion protein by Intramuscular injection and multiple subcutaneous injections. The goats were numbered 704 and 711 at the Hadley Farm and weighed 70 and 80 pounds, respectively. The immunizations were performed by two undergraduate students, Lauryn Benson and Vincent Patanio. The goats were monitored by Dr. Mark Huyler and Alice Newth.

After each immunization, blood samples were taken to test for immunoreactivity against A19 by ELISA and western blot. The ELISA results showed an increasing OD value of >1.5 after each immunization. Immune IgG were purified on a His-tag fusion protein (pET vectors, Novagen) column containing the same disintegrin-cysteine rich amino acid residues used for immunization (NHS-sepharose, Pharmacia). The DC19 antibody is used for ADAM19 overexpression studies and by immunoprecipitation to detect endogenous ADAM19. A western blot example of the affinity purified antibody is shown for ADAM19 transfected Cos-7 cells (Figure A1.4). The resulting Affinity Purified DC19 was used primarily by immunoprecipitation for experiments described in Chapter II and IV of this Dissertation.

Figure A1.4: ADAM19 expression in Cos-7 cells. Affinity purified DC19 was used at the primary concentration of 1:2000 to detect ADAM19FL2 and ADAM19FL3. The Proform of
ADAM19 (120kD) is predominantly expressed in Cos-7 cells. The high background is due to the secondary anti-goat-HRP antibody.
This Appendix will focus on various data that I have completed in my analysis of ADAM19 in the early embryo. The first piece of data that I would like to discuss supports the role for ADAM19 in heart development. Knockout mice for ADAM19 die perinatally due to defects seen in heart formation (Zhou et al., 2004), confirming the importance of ADAM19 in heart morphogenesis. Conditional knockout of ADAM19 specifically in the cardiac neural crest cells reproduced the heart phenotype suggesting that ADAM19 is critical for cardiac neural crest cell specification and or function but not migration (Komatsu et al., 2007). I have shown in Xenopus that ADAM19 knock down reduces three heart specific genes; NKX2.5 (transcription factor), MLC2 (Myosin Light Chain2, terminal differentiation marker), and MHCα (Myosin Heavy Chain α, terminal differentiation marker) (Figure A2.1). This suggests that ADAM19 is also involved in heart development in amphibians.
Figure A2.1: Heart Gene Expression Analysis. Embryos were injected at the one-cell stage with CMO or MO19 and allowed to develop until stage 28 for Whole Mount in situ Hybridization (N=30). Myosin Light Chain2 (MLC2), Myosin Heavy Chain-α (MHCα) and NKX2.5. Percentages are of the morphotype shown.

*Xenopus laevis* Neural Response Analysis

In addition to ADAM19’s prominent role in cardiac morphogenesis, the ADAM19 knockout mice have also a minor neural phenotype. ADAM19 is expressed in the peripheral nervous system and plays a regulatory role in the formation of neuromuscular junctions (Yumoto et al., 2008). ADAM19 physically interacts with the Ephrin receptor, EphA4, to stabilize the EphA4-ephrinA5 complex (Receptor-ligand) from being internalized. This results in the negative regulation of contact-dependent repulsion of neurons at the neuromuscular junction. I briefly analyzed Xenopus tailbud stage embryos for their ability to respond to a touch stimulus and swim away. This assay was performed in MO9 or MO19 injected embryos at stage 27 (Figure A2.2). MO9 injected embryos showed a slight reduction in their ability to respond to touch and swim away. MO19 injected embryos showed a more robust inability to respond to touch and swim away. These observations could be a result of poorly developed skeletal muscles (reduction in muscle specific genes MLC and 12/101) or poorly developed neuromuscular junctions.

Figure A2.2: Neural Touch Response Assay. Embryos were injected at the one-cell stage with CMO, MO9 or MO19 and allowed to develop until stage 27.
The embryos ability to respond to touch and whether the embryo was capable of swimming away from the stimulus were recorded. The knockdown embryos were compared to CMO and to NI (non-injected) embryos. The percentages are shown in the graph.

**Neuregulin1β**

In mice, ADAM19 can cleave some members of the EGF family of ligands including Neuregulin1β (Shirakabe et al., 2001). In Xenopus, the NRG1β expression pattern overlaps with the expression pattern of ADAM19 (Neuner et al., 2009; Yang et al., 1999; Yang et al., 1998a; Yang et al., 1998b). Our lab has obtained all three Xenopus NRG1 clones; IgNRG1α2, IgNRG1β2, and CRDNRG1β2. Our initial focus is on IgNRG1β2 because it is a known substrate of mouse ADAM19. I cloned the full length gene into the pCS2+ vector and added a Flag epitope after the extracellular Ig-like domain but before the EGF-β2 domain. In addition to the full-length gene, I introduced a stop codon immediately before the transmembrane domain to mimic a possible cleavage product containing only the extracellular domain (IgNRG1β2-EC). The Flag epitope location was selected in hopes that an extracellular cleavage fragment could be detected. This location may not be ideal because detection of IgNRG1β2 by western blot has presented some problems. Transfection into Cos-7 cells and glycoproteins purified by Concanavalin-A beads from the cell extract could not detect NRG1β (data not shown). Microinjection of RNA for NRG1β into Xenopus embryos has shown a faint band of the predicted molecular weight size (Figure A2.3).

![Figure A2.3: Neuregulin1β Western Blot.](image)

Embryos were injected at the one-cell stage with 1ng of RNA encoding NRG-FL. Glycoproteins were purified from 10 embryos by Concanavalin-A beads, separated by SDS-PAGE and detected using an antibody to Flag.
However, injection of 1ng of RNA from glycoproteins purified from ten embryos should yield a much stronger band by western blot. Cloning of NRG1β into pCS2+MT (myc tag) should enable successful detection by western blot. A successfully tagged construct could also be used to determine if any of the Xenopus ADAM members can cleave NRG1β.
APPENDIX III

LIST OF SUPPLEMENTARY MOVIE FILES (ON CD)

Movie 1 – Open Face Explant from a CMO embryo
Explants from embryos injected with CMO at the one-cell stage. Membrane bound Cherry RNA was injected into one blastomere at the two-cell stage to visualize axial mesoderm cell movement from one half of the embryo. The time lapse movie lasts ten hours.

Movie 2 – Open Face Explant from a MO13 injected embryo
Explants from embryos injected with MO13 at the one-cell stage. Membrane bound Cherry RNA was injected into one blastomere at the two-cell stage to visualize axial mesoderm cell movement from one half of the embryo. The time lapse movie lasts ten hours.

Movie 3 – Open Face Explant from a MO19 injected embryo
Explants from embryos injected with MO19 at the one-cell stage. Membrane bound Cherry RNA was injected into one blastomere at the two-cell stage to visualize axial mesoderm cell movement from one half of the embryo. The time lapse movie lasts ten hours.

Movie 4 – Dorsal Mesoderm Cell Spreading and Migration on Fibronectin
Dissociated dorsal mesoderm cells from a CMO embryo were plated on a fibronectin substrate. Time lapse analysis of a three hour movie shows cell spreading and cell migration on FN.

Movie 5 – Dorsal Mesoderm Cell Spreading and Migration on Fibronectin
Dissociated dorsal mesoderm cells from a MO13 injected embryo were plated on a fibronectin substrate. Time lapse analysis of a three hour movie shows cell spreading and cell migration on FN.

Movie 6 – Dorsal Mesoderm Cell Spreading and Migration on Fibronectin
Dissociated dorsal mesoderm cells from a MO19 injected embryo were plated on a fibronectin substrate. Time lapse analysis of a three hour movie shows cell spreading and cell migration on FN.
Movie 7 – Dorsal Mesoderm Cell Spreading and Migration on Fibronectin
Dissociated dorsal mesoderm cells from a 2MO (MO13+MO19) embryo were plated on a fibronectin substrate. Time lapse analysis of a three hour movie shows cell spreading and cell migration on FN.

Movie 8 – Dorsal Mesoderm Cell Spreading and Migration on 9.11
Dissociated dorsal mesoderm cells from a CMO embryo were plated on a 9.11 substrate. Time lapse analysis of a three hour movie shows cell spreading and cell migration on 9.11.

Movie 9 – Dorsal Mesoderm Cell Spreading and Migration on 9.11
Dissociated dorsal mesoderm cells from a MO13 injected embryo were plated on a 9.11 substrate. Time lapse analysis of a three hour movie shows cell spreading and cell migration on 9.11.

Movie 10 – Dorsal Mesoderm Cell Spreading and Migration on 9.11
Dissociated dorsal mesoderm cells from a MO19 injected embryo were plated on a 9.11 substrate. Time lapse analysis of a three hour movie shows cell spreading and cell migration on 9.11.

Movie 11 – Dorsal Mesoderm Cell Spreading and Migration on 9.11
Dissociated dorsal mesoderm cells from a 2MO (MO13+MO19) embryo were plated on a 9.11 substrate. Time lapse analysis of a three hour movie shows cell spreading and cell migration on 9.11.
APPENDIX IV

REPRINT 1

Mechanism of Development (Neuner et al., 2009)
Xenopus ADAM19 is involved in neural, neural crest and muscle development

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ABSTRACT

ADAM19 is a member of the melanin subfamily of ADAM metalloproteases. In Xenopus, ADAM19 is present as a maternal transcript. In vivo expression starts during gastrulation and is apparent in the dorsal blastopore lip. ADAM19 expression through neurulation and tailbud formation becomes enriched in dorsal structures such as the neural tube, the notochord and the somites. Using morpholino knock-down, we show that a reduction of ADAM19 protein in gastrula stage embryos results in a decrease of Brachyury expression in the notochord concomitant with an increase in the dorsal markers, Goosecoid and Chordin. These changes in gene expression are accompanied by a decrease in phosphorylated AKT, a downstream target of the EGF signaling pathway, and occur while the blastopore closes at the same rate as the control embryos. During neurulation and tailbud formation, ADAM19 knock-down induces a reduction of the neural markers N-tubulin and NRP1 but not Sox2. In the somitic mesoderm, the expression of MLC is also decreased while MysD is not. ADAM19 knockdown also reduces neural crest markers prior to cell migration. Neural crest induction is also decreased in embryos treated with an EGF receptor inhibitor suggesting that this pathway is necessary for neural crest cell induction. Using targeted knock-down of ADAM19 we show that the reduction of neural and neural crest markers is cell autonomous and that the migration if the cranial neural crest is perturbed. We further show that ADAM19 protein reduction affects somite organization, reduces 12-101 expression and perturbs fibronectin localization at the intersomitic boundary.

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1. Introduction

Post-translational modification of proteins is a critical step in most biological processes. Among these modifications, cleavage of cell surface proteins control a diverse range of cellular processes such as cell signaling, cell adhesion and cell migration. One family of metalloproteases, ADAM (proteins containing A Disintegrin and A Metalloprotease (Wolffberg et al., 1995), is responsible for modulating various signaling pathways by shedding membrane bound growth factors and/or their receptors (Blobel, 2005; Huovila et al., 2005; Moss and Lambert, 2002). In addition, ADAMs have also been shown to cleave cell adhesion molecules such as cadherins to promote epithelial to mesenchymal transition (EMT) essential for proper embryonic development (Mareetzky et al., 2005; Reiss et al., 2006, 2009).

ADAM19 is a member of the melanin subfamily and has been shown, in mice, to cleave TNF-α, KIt Ligand, TRANCE,
HB-EGF and neuregulin-1 (Chesnau et al., 2003; Horikuchi et al., 2005; Kawaguchi et al., 2007; Shirakabe et al., 2003). The knockout (KO) of ADAM19 has been shown to induce defects in heart development, believed to be due to defective HB-EGF signaling (Kuroha et al., 2004; Zhou et al., 2004). Conditional KO of ADAM19 in the cardiac neural crest cells reproduced the heart phenotype suggesting that ADAM19 is critical for cardiac neural crest cell specification and/or function but not migration (Komatsu et al., 2007). In mice, the main enzyme responsible for the cleavage of both HB-EGF, and neuregulin-1 appears to be ADAM17. In addition, mice lacking ADAM17 show very similar heart defects to ADAM19 KO mice. Furthermore, a double knock out of both ADAM17 and 19 results in a more severe phenotype demonstrating possible functional compensation of these ADAMs during embryonic development (Horikuchi et al., 2005). Fibroblasts derived from ADAM19 KO mice do process both neuregulin-1 and HB-EGF, suggesting that the ADAM19 substrate responsible for its phenotype remains to be identified.

To test whether ADAM19 has a unique role during early embryonic development, we have cloned the Xenopus laevis homologue and analyzed its expression pattern. XADAM19 is present at low levels as a maternal transcript with zygotic expression beginning during gastrulation and localizing to the dorsal blastopore lip. During later stages of embryonic development, ADAM19 is expressed in the dorsal mesoderm, neuroectoderm and neural crest cells. Loss of function experiments using antisense morpholino knock-down reveals that the protein is essential for the specification of the dorsal mesoderm, neuronal derivatives and cranial neural crest cells. The apparent lack of functional compensation for ADAM19 in X. laevis may help resolve the biologically relevant substrate for ADAM19 during early embryonic development.

2. Results

2.1. ADAM19 is expressed both maternally and zygotically

We have cloned the X. laevis homologue of ADAM19 using degenerate oligonucleotides to human, mouse, quail and the genomic sequence of X. tropicalis. The full-length sequence was generated by 5' and 3'RACE-PCR and assembled from multiple PCR amplons. The deduced amino acid sequence is 49% identical to human ADAM19 and 84% identical to X. tropicalis ADAM19. We found several variants in the cytoplasmic domain at the amino acid level, one of which is most common to the X. tropicalis sequence and was submitted to GenBank (EU770969). Amplification using multiple primers from genomic DNA suggests that one intron-less allele is present in the X. laevis genome (data not shown).

We investigated ADAM19 expression throughout development using real-time PCR. Since the genomic DNA appears to contain a copy of the gene lacking introns, we used polyA purified mRNA to eliminate any trace of genomic DNA in our samples. ADAM19 is expressed both maternally and zygotically from gastrulation through early tailbud formation (Fig. 1). The expression level of ADAM19 at gastrulation is low but is confirmed by in situ hybridization where it appears restricted to the dorsal lip of the blastopore (Fig. 2A).

2.2. ADAM19 mRNA is enriched in dorsal structures including somites, neural tube and cranial neural crest cells

Using whole mount in situ hybridization, we investigated the distribution of ADAM19 transcript during gastrulation, neurulation and tailbud formation (Fig. 2). To avoid background staining due to either antibody or substrate precipitation in the blastocoel, we performed in situ hybridization on hemi-gastrula embryos. At stage 12, the mRNA encoding ADAM19 is mostly localized to the dorsal lip of the blastopore where the dorsal mesoderm invaginates (Fig. 2A). During early tailbud stages, ADAM19 is expressed in the dorsal region of the embryo including the neural tube and the dorsal mesoderm. ADAM19 is detected in the neural tube, the notochord and the cement gland (Fig. 2C and F). In the mesoderm, ADAM19 is expressed in both the segmented (somite) and non-segmented (caudal) dorsal mesoderm (Fig. 2G and H). When stained embryos are observed in PBS rather than Murray’s clear to visualize superficial staining, ADAM19 is detected in the segments of the cranial neural crest cells (CNC, Fig. 2I). The expression in the CNC cells was confirmed by RT-PCR on explants dissected from stage 17 embryos (Fig. 2J inset). At stage 26, ADAM19 expression is evident in the cement gland, the brain, the somites, the otic vesicle and the branchial arches (Fig. 2D). At stage 36 ADAM19 is still expressed in the entire head, the branchial arches and the
somites (Fig. 2E). The staining intensity decreases in the somite and by contrast appears stronger in the neural tube. In addition, the expression appears within the pronephros (p).

2.3. Morpholino antisense oligonucleotides to ADAM19 reduces endogenous protein levels

To perform loss of function experiments, we designed antisense morpholino oligonucleotides corresponding to the sequence near the ATG start codon and common to all sequences identified (5’-RACE amplicons). We also generated a polyclonal antibody to the cytoplasmic domain of the two variants cloned for ADAM19. This antibody recognized the cloned ADAM19 in both transfected Cos-7 extract and embryos injected with the ADAM19 mRNA (Fig. 3C and data not shown). The antibody also recognizes a 120 kDa polypeptide expressed in embryos at stage 24 but absent at stage 7 (Fig. 3A) together with additional lower bands present at all stages and most likely not specific. In embryos injected with ADAM19 MO (MO19) the polypeptide at 120 kDa recognized by the antibody is reduced by 60–90%, depending on the injection, when compared to the β1 integrin subunit (Fig. 3A and B). In addition to the 120 kDa band, a higher molecular weight product recognized by the antibody is also decreased by the MO. This band could correspond to either a splicing variant or a protein with different post-translational modifications.

In addition to the endogenous protein, MO19 prevents translation of the ADAM19 mRNA while a morpholino to ADAM13 (MO13) does not (Fig. 3C; MO19 + A19 and MO13 + A19). Mutation of four nucleotides in the ADAM19 mRNA allows translation of the protein in injected embryos (Fig. 3C; MO19 + A19R). While translation of this mRNA is still affected by the MO19 (compare lane 2 to lane 4), it generates amount of ADAM19 protein that are in excess compared to the endog-
enous protein (compare lane 4 to N1 lane 1). We believe that the 4 nucleotides changes (out of 26) may still allow binding of the MO to the mRNA when both are concentrated in a tube before the injection. This construct named ADAM19 Rescue (A19R) was used in all of the rescue experiments. Before performing phenotypic analyses of ADAM19 knock-down, we tested by microinjection the effect of 1, 5, 10, 25 and 50 ng of MO19 per embryos. At the highest dose of 50 ng per embryos, most embryos cleaved and underwent gastrulation, however at the end of neurulation a significant percentage failed to close their neural tube and died (data not shown). While this dose produced significant death, we did not see a decrease of the 120 kDa protein more significant than with 10 ng of MO19. For this reason we selected 10 ng in all subsequent experiments.

2.4. ADAM19 reduction has no effect on blastopore closure but affects Brachyury expression in the notochord

Gastrulation is one of the most sensitive stages of early development and can easily be perturbed by a variety of specific or non-specific treatments to the embryo. To investigate the affect of ADAM19 knock-down on gastrulation, we first measured the size of the blastopore during gastrulation. Using the average diameter of the blastopore, we found that embryos injected with MO19 closed their blastopore at the same rate as embryos injected with a control morpholino (Fig. 4).

Fig. 3 – Morpholino oligonucleotide knock-down of ADAM19. Western blot of protein extract from embryos injected with a control morpholino or a morpholino to either ADAM13 (MO13) or ADAM19 (MO19). The morpholinos (10 ng) were injected at the 1 cell-stage and embryos were extracted at stage 22. (A) Glycoproteins from 4 embryos equivalent were purified on Concanavalin A-agarose, separated by SDS-PAGE and probed with a rabbit polyclonal antibody to the ADAM19 cytoplasmic domain (ADAM19 cyt). A monoclonal antibody to integrin β1 (8C8) was used as a loading control. (B) The densitometric analysis of the blot shown in (A) shows that the ADAM19 protein is reduced by approximately 90% by the MO19 when normalized to the integrin β1 expression level. (C) The MO19 is capable of preventing translation of 1 ng of ADAM19 mRNA when co-injected in embryos (A19) but not a messenger with 4 mutations in the sequence corresponding to the MO19 (A19R). The ADAM13 morpholino does not affect ADAM19 translation.

Fig. 4 – ADAM19 is not required for blastopore closure. Time-lapse imaging of gastrula stage embryos injected with either a control MO (CMO) or the MO19. Embryos were microinjected with 10 ng of MO at the one-cell stage and allowed to develop through the end of gastrulation. The blastopore size in micrometers was measured using the Openlab software at three different time points during gastrulation. The values represent the average of three different experiments using 8 embryos each for the control and the experimental. Error bars correspond to standard deviation from the mean. Representative images are shown below the histogram.
As previously stated, ADAM19 mRNA is found in the dorsal blastopore lip, therefore we tested whether the knock-down of ADAM19 perturbed the expression of early developmental markers (Fig. 5). We first investigated Brachyury expression (Xbra) in morphant embryos at stage 12 by whole mount in situ hybridization (Fig. 5A). Our results show that Xbra is expressed in the ventral and lateral mesoderm but is missing in the notochord of 63% of the embryos with reduced ADAM19 protein (n = 30). To test whether the dorsal mesoderm was entirely missing, we assessed Chordin and Goosecoid expression in the notochord and cephalic mesenchyme respectively. At stage 12, we found that most embryos (85% n = 30) had the same expression of chordin while 35% showed a reduced staining. In contrast, 76% of the embryos injected with MO19 had the same level of Goosecoid staining while the remaining 24% (n = 20) showed a wider expression. Another notochord marker (not-1) was not affected by the knock-down of ADAM19 (data not shown). These results show that the dorsal mesoderm and the notochord are present in embryos with reduced level of ADAM19 but that the patterning of the notochord may be altered.

Using quantitative real-time PCR, we tested the level of expression of these genes in embryos injected with a control MO, MO19 and a combination of MO19 and ADAM19 Rescue mRNA (Fig. 5B). The results confirm a small reduction of Xbra expression (16% decrease) possibly due to the absence of expression in the notochord. In contrast, we found that both Chordin and Goosecoid levels were increased by about two

**Fig. 5** - Phenotypical analysis of ADAM19 KD at the gastrula stage. (A) Embryos were microinjected with 10 ng of either the MO19 or a control MO at the one-cell stage and allowed to develop until gastrula stage (stages 12 and 13). Whole mount in situ hybridization were performed using the markers Brachyury, Sox2 (Xbra Sox2, stage 12) and Chordin and Goosecoid (Chd and Gsc, stage 13). For all embryos, the dorsal view is presented with the anterior up. The red arrowheads point to the notochord. The number of embryos and the frequency of the phenotype depicted in the photographs are indicated in the text. (B) Quantitative real-time PCR was performed on embryos injected with either MO19 alone or together with ADAM19 Rescue mRNA (MO19 + A19R). The relative abundance of Chordin (Chd), Goosecoid (Gsc) and Brachyury (Xbra) gene expression was measured by the 2^(-ΔΔct) method using either embryos injected with the control MO or non-injected and normalized to β-tubulin. There was no effect of the control MO to gene expression. The asterisks represent statistical significance at P < 0.05 using a Student t test. There is a small decrease in Xbra (16%, not significant) and a robust increase in both Chd (187%) and Gsc (230%) which are rescued by the injection of the ADAM19 mRNA.
folds in embryos with reduced ADAM19 protein suggesting that there is in fact an increase in certain dorsal mesoderm markers. These changes in gene expression were all rescued by the injection of ADAM19R mRNA.

While the Goosecoid and Xbra results correlate nicely between the in situ and the real-time PCR, the results for Chordin do not. One simple explanation is that the in situ hybridization is not a quantitative technique and a two-fold increase may not be detectable using this technique. Another possible explanation is that the expression of Chordin is wider but that the level in individual cells either remain the same or decrease slightly. This would appear by in situ hybridization as a decrease while the quantitative PCR would detect the increase.

While the expression of Xbra in the notochord is decreased, we have found using targeted injection at the 8-cell stage and β-galactosidase as a lineage tracer that cells with reduced level of ADAM19 can still be included in the notochord and the somite, suggesting that ADAM19 expression is not essential for the formation of these tissues (data not shown).

2.5. Reduction of ADAM19 protein does not interfere with neural induction but inhibits neural crest induction

To test the effects of the knock-down of ADAM19 on neural induction, we performed in situ hybridization using Sox2 as a general neural marker. The results show that MO19 does not prevent neural induction (Fig. 9). While the overall expression of Sox2 was not affected, careful analysis reveal some subtle changes. For example, while Sox2 expression in control embryos is lighter in the neural plate directly above the notochord (future floor plate, Fig. 5A, red arrowhead), this decrease of Sox2 at the midline is not visible in 30% of the embryos injected with MO19 (n = 30), suggesting that a reduction in ADAM19 protein may affect axial tissue patterning across the mesoderm/neuroectoderm tissue boundary. However, at that point it is not clear whether the effect on the neural plate is direct or the result of a defective signaling from the notochord.

ADAM19 is also expressed in the CNC cells, therefore we performed in situ hybridization and real-time PCR at the open neural plate stage (stage 19) before CNC migration to test if ADAM19 was essential for neural crest induction (Fig. 6). Our results show that both Sox8 and Shh are reduced following injection of MO19 but not a control MO (Fig. 6A). Real-time PCR shows a reduction of about two fold for Sox8 (50% reduction) and Shh (40% reduction; Fig. 6B). In contrast, the neural marker Sox2 increased rather than decreased at this early stage. Since neural crest is induced at the border of the neural plate, it is likely that the cells that are not induced to become neural crest will maintain Sox2 expression.

To test the ability of CNC cells with reduced ADAM19 to migrate, we also performed targeted injection of the MO19 together with GFP RNA at the 16-cell stage in a dorso-lateral animal blastomere. Cell migration was evaluated by observing the distribution of GFP in the different CNC segments (Fig. 6C). Our results show that while GFP is found in CNC that migrate in all segments in control embryos, no migration was observed in 33% of the embryos injected with MO19. Together these results show that ADAM19 is required for neural crest cell induction and migration. It is likely that the defect observed for CNC migration is due to the defect in induction but we cannot rule out that ADAM19 may also have an independent role in cell migration.

At later stages, our results show that there is a significant reduction of xTwist expression in the MO19 injected side (Fig. 7A, arrowhead). To test if the knock-down affects the CNC specification or just xTwist expression we used ADAM11 as a marker of CNC in quantitative PCR experiments. While Twist is a widely used marker for CNC by in situ hybridization, it also labels other cells at the tailbud stage. On the other hand, ADAM11 is more restricted with expression in both the CNC and one line of dorsal neurons (Cai et al., 1998). Confirming the in situ hybridization, we observed a decrease of ADAM11 expression at both stages 17 (58%) and 24 (50%) (Fig. 7B). This expression can be rescued with ADAM19 rescue mRNA. To test if the effect on neural crest induction was direct, or a result from the abnormal mesoderm patterning, we injected the embryos at the 8-cell stage in both animal dorsal blastomeres (Fig. 7C, right). The results show that ADAM11 is decreased while the MLC is not confirming that ADAM19 role in neural crest induction is direct.

2.6. ADAM19 knock-down reduces muscle specification

The expression of ADAM19 increases at neurula and tailbud stages and is mainly concentrated in the dorsal mesoderm and neural tissue. To understand the effects of a reduction in the ADAM19 protein on embryonic development, we performed in situ hybridization using MLC, MyoD, N-tubulin, Sox2 and NRP-1 at the tailbud stage (stage 24). Embryos were injected in one blastomere of the two-cell stage with 10 ng of either control MO (CMO) or MO19 with mRNA encoding β-galactosidase (Fig. 7A). The results show that ADAM19 knockdown dramatically reduces MLC expression in the somites. In contrast, MyoD, which is also expressed in the somites, did not appear to be reduced on the injected side. In addition, MyoD staining shows that the correct somites segmentation is achieved even in the presence of the ADAM19 MO. The reduction of MLC expression was confirmed by quantitative PCR (Fig. 7B). This reduction was stronger at stage 17 (83%) but also evident at stage 24 (58% decrease). In addition, both were rescued by the expression of the ADAM19 mRNA (Fig. 7B). In fact, the same pattern of gene expression (reduction of MLC but no changes in MyoD) was also observed at stage 15 (data not shown). These results suggest that a reduction in the ADAM19 protein level does not affect early induction and segmentation (MyoD) of the somitic mesoderm but may interfere with the differentiation of the muscle (MLC).

2.7. Reduction of ADAM19 protein affects neural patterning

To test the effect of ADAM19 knock-down in the neural tissue, we used Sox2 and NRP-1 as general neural markers and N-tubulin as a marker of neuronal precursors. In situ hybridization at the tailbud stage shows that Sox2 was unaffected by a reduction in ADAM19 protein while both N-tubulin and NRP-1 appear reduced on the injected side (Fig. 7A). The reduction of N-tubulin expression was confirmed by quantitative real-time
Fig. 6 - ADAM19 is critical for neural crest induction. (A) In situ hybridization of neurula (stage 15) embryos using Slug and Sox8 to detect neural crest cells. The injected side is to the left. Embryos injected with MO19 present a decrease expression of both neural crest markers (red arrowhead). (B) Real-time PCR analysis at stage 15. Level of gene expression was measured by the 2^(-ΔΔCt) method and normalized to α-tubulin. Results are presented as the Log2 of the fold change compared to non-injected embryos. Variation of Sox2, Sox8 and Slug are all significant (P < 0.01) in embryos injected with MO19 when compared to the control MO. C) Lateral view of tailbud stage embryos (stage 24) visualizing GFP. Embryos were injected at the 16-cell stage in a dorso-lateral-animal blastomere to target neural crest cells. In the control, GFP is found in all CNC segments (white arrows). This is absent in 33% of the embryos injected with MO19.
Fig. 7 – Phenotypical analysis of ADAM19 KD at tailbud stage. (A) Whole-mount in situ hybridization of tailbud stage embryos (stages 22-24). Embryos were injected in one blastomere of the two-cell stage with 10 ng of MO19 or CMO. mRNA encoding β-galactosidase (β-Gal) was co-injected as a tracer. The embryos were processed by whole mount in situ hybridization with the markers myosin light chain (MLC), N-tubulin, NR1, Sox2 and ADAM11. The injected side, as detected by β-Gal activity (light blue), is on the left side for all embryos. The percentages of embryos displaying these phenotypes are indicated and represent the average of three independent experiments. The black arrowheads point to the area of decreased gene expression. For Twist the embryo heads are presented as a side view. The injected side of each embryo is to the left. Decrease in twist expression is visible (arrowhead) in embryos injected with MO19. (B) Quantitative real-time PCR was performed on embryos injected at the one-cell stage with either the MO19 alone or with the ADAM19 rescue mRNA (MO19 + A19R) and allowed to develop until neurula (stage 17) and tailbud stage (stage 24). Alternatively, the MO19 was injected in 2 dorsal-animal blastomeres at the 8-cell stage (MO19 Ectoderm) to target dorsal ectoderm but not mesoderm derivatives. All genes were compared to non-injected control embryos. The relative abundance of MLC, N-tubulin, Sox2 and ADAM11 gene expression was measured by the 2⁻⁰ΔΔCt method and normalized to α-tubulin. Results are presented as the log2 of the fold change. Error bars correspond to the standard error. Asterisks represent a P < 0.05 using a standard t test assuming unequal variance.
PCR at both stages 17 and 24 (Fig. 7B). While the decrease was small (30%), it was highly reproducible (P < 0.05) and was fully rescued by the injection of ADAM19R mRNA. This suggests that only a fraction of neurons expressing N-tubulin are affected by the ADAM19 knock-down. In contrast to N-tubulin, ADAM19 knock-down had no effect on Sox2 expression at both stage 17 or 24 confirming the in situ hybridization results.

2.8. Changes in neural and neural crest markers are not due to secondary defect of the dorsal mesoderm patterning

To investigate whether the reduction of the neural and neural crest markers were due to the reduction of ADAM19 in these tissues or a secondary effect of the dorsal mesoderm patterning, we injected MO19 in both dorsal animal cells at
the 8-cell stage to target only ectodermal derivatives (Fig. 7B, right graph). Only 1 ng of MO19 was injected in each cell at that stage to account for the reduction in cell size. The results show that a reduction of ADAM19 protein in the ectoderm is sufficient to reduce N-tubulin and ADAM11 in the same proportion as those observed when the MO19 is injected at the one cell stage. In contrast no change was observed in the somitic mesoderm marker MLC, confirming the proper targeting of our injection. These results suggest that ADAM19 is required in the neural ectoderm and neural crest cells to perform its function.

2.9. ADAM19 is required for proper somite organization

To further study the role of ADAM19 in somite formation, we first use the ADAM19 antibody to detect the protein localization on sections of tailbud stage embryos (Fig. 8). We found that ADAM19 localizes to the notochord, the inter somitic boundary and the ventral half of the neural tube (Fig. 8A–C). We also found some staining within the branchial arches (Fig. 8A). The distribution of the protein is consistent with the mRNA expression pattern (Fig. 2) and provides new information about where the protein may be acting. In addition, we performed the same staining on embryos injected on one side with MO19. The most dramatic change is the complete absence of ADAM19 staining at the inter somitic junction (Fig. 8D). As seen for the in situ hybridization with MyoD (Fig. 7A), the segmentation of the somites is apparent in both sides of the embryos. While this metamorphosis is conserved, the organization of the cells within each somite is abnormal in embryos with reduced ADAM19 protein. While the nuclei on the control side are aligned at the center of each cell, the alignment is disrupted on the side with reduced ADAM19 (Fig. 8D). In addition to this disruption, staining with mAb 12-101, a marker of muscle differentiation, is decreased on the MO18 injected side (Fig. 8E). This decrease was observed in the posterior somites but not in the most anterior ones suggesting a delay in 12-101 expression rather than a complete inhibition (data not shown). This decrease of 12-101 expression was confirmed by Western blot on embryos injected at the one cell stage with MO19 (Fig. 8G). Finally, to visualize the inter somitic boundary of embryos injected with the MO19, we performed staining with antibodies to fibronectin (FN). The staining confirms the presence of somite boundaries in the injected side similar to the control side. Interestingly, we also found that some of the boundaries lacked FN (Fig. 8F). This was confirmed by whole mount immunostaining in which there was no embryo that could be visualized at once (Fig. 8H). Using this technique we found that the fibronectin staining was present but abnormal. The defect was seen only in the somites located between the “chevron” and the “straight” shaped somites (approximately 7th somite). Compared to this wild and restricted FN defect, the abnormal distribution of the nuclei affected a larger number of somites. Together, the results presented in Fig. 7 and 8 show that a reduction of the ADAM19 protein affects both the organization and differentiation of somitic cells into muscle but not the initial induction of the paraxial mesoderm or its metamorphosis.

2.10. ADAM19 knock-down affects EGF signaling

The best characterized targets of ADAM19 in mice are members of the epidermal growth factor family (EGF). Upon cleavage ligands bind to the EGF receptor and activate a signaling cascade involving AKT/p38 (protein kinase B). To test if this pathway was affected by the ADAM19 MO, we performed Western blot at stage 12 with embryos injected with the control MO or MO19. Our results show that phosphorylated AKT (pAKT) is decreased significantly in embryos with reduced ADAM19 protein (Fig. 9). In contrast, neither phosphorylated MAPK nor the cytoplasmic adaptor protein PAC-SAP2 was reduced. To test whether the EGF pathway was involved in neural crest cell induction, we used a pharmacological inhibitor of the EGF receptor (AG1478, 100 μM) and treated embryos between stages 10 and 12. In addition we
also used a broad-spectrum inhibitor of metalloprotease (Marimastat 10 μM) that inhibits most ADAM proteases activity. Based on the blastopore size, neither inhibitor affected gastrulation movements (data not shown). Using real-time PCR we found that both Marimastat and AG1478 significantly decreased Slug expression confirming the importance of the EGF signaling pathway and metalloprotease activity in neural crest cell induction. As a control, we performed Western blot with the γtubulin antibody and found a 50% reduction in embryos treated with the EGF receptor inhibitor confirming its activity in vivo. Similar to what was observed with MO19 (Fig. 9A–B), we also found that Marimastat reduced the level of phosphorylated AKT (data not shown), suggesting that one metalloprotease, possibly ADAM19, is essential for EGF signaling during gastrulation.

3. Discussion

This study reports the cloning and molecular analysis of the X. laevis homologue of ADAM19. The ADAM19 mRNA is present during the initial cleavage stages. Zygotic expression is detected during gastrulation and appears to be enriched in the dorsal blastopore lip. During later development, ADAM19 mRNA is localized to dorsal structures including the dorsal mesoderm and neural derivatives. Loss of function experiments using antisense morpholino oligonucleotides show a reduction of Brachyury in the notochord of gastrula stage embryos and an increase in dorsal markers such as Goosecoid and Chordin. During neurulation and tailbud formation a reduction of ADAM19 protein results in the reduction of neuronal, neural crest and somitic markers. The metamerication of somites is not perturbed but the organization of cells within each somite is. We present evidence that the EGF signaling pathway is affected by ADAM19 knock-down.

3.1. ADAM19 gene expression

ADAM19 mRNA is expressed during early mouse embryonic development in a pattern similar to the one described here in Xenopus. It is found in dorsal tissues including the spinal chord, craniofacial and dorsal root ganglia, as well as peripheral neuronal cells. It is also found in mesenchymal condensation specifically in the tailbud and heart (Kurikawa et al., 1998). Mice lacking ADAM19 die peri-natally due to defects seen in heart formation (Zhao et al., 2001), confirming the importance of ADAM19 in heart morphogenesis. Surprisingly, while ADAM19 is expressed early during embryogenesis, no major defects in other organs or tissues where ADAM19 is expressed were described, suggesting functional compensation. The heart defects were shown to be due to a lack of the ADAM19 protein in cardiac neural crest cells rather than in the endothelial cells (Komatsu et al., 2007). The cardiac neural crest cell migration was not affected by the lack of ADAM19, however, the ability of the cells to participate in heart morphogenesis was inhibited. In Xenopus, a reduction of ADAM19 protein has some profound effects on early gene expression in the gastrula, neurula an early tailbud stage. We also find decrease expression of molecular marker during heart formation (data not shown), suggesting that ADAM19 is also involved in heart morphogenesis in amphibians, but focused on the analysis of early phenotypes. It is interesting to note that ADAM2 (meltrin-γ) was not found during early embryogenesis in X. laevis, while ADAM9 and ADAM13 are both expressed in overlapping patterns with ADAM19 (Alfandari et al., 1997; Cai et al., 1998). This suggests that X. laevis may be a good model to study the function of ADAM19 in early embryogenesis with less functional compensation than in the mouse.

3.2. Early phenotype of ADAM19

A reduction of ADAM19 in gastrula stage embryos results in a decrease of Brachury expression in the notochord and an increase of the dorsal markers, Goosecoid and Chordin. This result is consistent with the observation that upregulation of Goosecoid can repress Brachury expression (Boucher et al., 2000). Thus ADAM19, which is expressed in the Spemann’s Organizer, may participate in restricting Goosecoid expression. On the other hand, ADAM19 may be important to maintain Brachury expression in the notochord. At the moment, it is not clear whether Goosecoid overexpression is preventing Brachury expression in the notochord or if these are independently affected by ADAM19. It was previously shown that high doses of TGF-β induce Goosecoid and Chordin and repress Brachury (Lemaitre and Smith, 1999; Lemaître et al., 1997), thus ADAM19 knock-down may increase TGF-β signaling in the embryo. This would mean that ADAM19 function is to reduce TGF-β signaling. In mouse, ADAM12 has been shown to promote TGF-β signaling by associating with the type II receptor and controlling its subcellular localization (Att et al., 2007). It is therefore possible that ADAM19 also interacts with the TGF-β receptor but decreases rather than increases its signaling. Recently, EGF-CFC/FLR1, also known as crypto, was shown to modulate TGF-β signaling by nodal related proteins (Cheng et al., 2003; Dorey and Hilt, 2006; Yabe et al., 2003). Cleavage of FLR-1 by ADAM19 may reduce this signaling at the end of gastrulation when the mRNA for FLR-1 disappears. Thus there are several possible ways that ADAM19 may control TGF-β signaling at gastrulation. Whatever the mechanism of ADAM19 regulation of gene expression, the ratio between Brachury and Chordin expression in the notochord, has been shown to control the antero/posterior identity of notochord cells. Chordin defines the anterior while Brachury defines the posterior (Nimni et al., 2004). This suggests that embryos with reduced ADAM19 have notochords that are anteriorized compared to wild-type embryos. This may in turn alter the patterning of the neural tube in a similar fashion.

To date, the best characterized signaling pathway for ADAM19 is the one involving EGF receptors (Grob et al., 2005). ADAM19 has been shown in mice to cleave some members of the EGF family including neuregulin-1p (NRG1p), which is expressed in Xenopus as a maternal transcript. Zygotic expression begins during gastrulation and continues through tailbud formation (Chung and Chung, 1999; Yang et al., 1999; Yang, 1998). Purified recombinant NRG1 can induce animal cap cells to become mesoderm and express Brachury (Chung and Chung, 1999). It is therefore possible that ADAM19 medi-
ated cleavage of NRG18 in the dorsal blastopore lip is critical for maintaining Brachyury expression in the notochord. NRG18 signals through the EGF receptor ErbB tyrosine kinase, and this signaling pathway has been shown to contribute to convergent and extension movements during gastrulation. However, a knock-down of ErbB3 or ErbB4 did not inhibit Brachyury expression in the notochord, suggesting that ADAM19 function may act through a different pathway (Nie and Chang, 2005, 2007a,b). On the other hand, we found that ADAM19 knock-down decreased pAkt, a known target of the EGF signaling pathway. In addition, the EGF receptor inhibitor AG1478 affects neural crest gene expression in a way similar to that of the ADAM19 knock-down suggesting that ADAM19 is important for EGF activation and that this pathway is critical for neural crest cell induction.

Finally, using various markers for the notochord, paraxial mesoderm and neural ectoderm, we have found that the separation between the axial and paraxial tissues may be delayed or impaired in embryos with reduced ADAM19 protein. Given the growing role of ADAM proteins in cleaving cadherins, it is also possible that ADAM19 is involved in separating the axial from paraxial mesoderm by cleaving cell adhesion molecules such as protocadherins. The role of the protocadherins AXPc and PACPC in notochord and somite formation has been elegantly demonstrated in Xenopus (Chen and Gumbiner, 2006; Kim et al., 2000, 1998; Kuroda et al., 2002; Medina et al., 2004; Untheker et al., 2004; Wang et al., 2008) and it is possible that the activity of these proteins is regulated by proteolytic processing.

3.3. Late phenotype of ADAM19 MO

During neurulation and tailbud formation, embryos with reduced ADAM19 protein show a reduction of N-tubulin and NRP1 in the neural tube and MLC in the somites. Interestingly, for both the dorsal mesoderm and neural tissue, the overall induction is not affected as shown by the expression of Sox2 in the neural tube and MyoD in the dorsal mesoderm, suggesting that ADAM19 may act as a modulator rather than a master controller of cell fate. ADAM19 knock-down reduces CNC cell markers xTwist, Sox8, Slug and ADAM11. In situ hybridization using xTwist reveals that at least some CNC maintain xTwist expression and are capable of migration. Together with the mouse ADAM19 KO, these results suggest that ADAM19 may be required both for neural crest cell induction and function. In addition, we have shown that CNC with reduced level of ADAM19 do not migrate as efficiently as wild-type cells in vivo. This could be due to either a direct affect on migration or the observed defect in neural crest induction. Additional experiments are required to test these two hypotheses. Once again, neuregulin appears as an

Fig. 9 - ADAM19 may interfere with EGF signaling. (A) Western blot using antibody to phosphorylated Akt, phosphorylated MAP kinase, and PACSIN2. Embryos were injected at the one-cell stage with either the control MO (CMO) or MO19. Total protein from 0.5 embryo equivalent were used for each blot. (B) Histogram representing the quantification of the Western blot from three independent injection experiments. All measures were normalized to PACSIN2. Variations in pMAPK were not significant while the decrease in pAkt was significant. (C) Real-time PCR analysis of Slug expression. Embryos were treated with 10 μM of Marimastat (Mar), 100 μM of AG1478 or DMSO (1/1000) as a control, between stages 10 and 12, based on the blastopore size (none of the treatment affected gastrulation timing in these conditions). Marimastat decreased Slug expression by an average of 34% while AG1478 decreases Slug by 60%.
attractive candidate as a substrate for ADAM19 as it has been shown to induce both neural and muscle specific markers in Xenopus animal cap assays (Chung and Chung, 1999). Moreover, the role of NGF in neuronal cell specification has been studied extensively (Britsch et al., 1998; Cameron et al., 2001).

In the somitic mesoderm, a reduction of ADAM19 protein does not prevent induction and metamerization. In addition, the fibronectin rich extracellular matrix is deposited at the intersomatic boundary. While the general organization of somitic mesoderm does not depend on ADAM19, cell organization within each somite appears to be affected by a reduction of ADAM19 protein. In particular, the phenotype suggests that cell rotation may be delayed or abnormal. Together with somite organization, at least two markers of muscle differentiation (MLC and 12–101) are reduced in morphant embryos. It is not clear at the moment whether the effect on cell organization and muscle differentiation are linked. As for the early phenotype (gastrulation) both a signaling role of ADAM19 or a structural role via cleavage of a cell adhesion molecule could account for the defects seen following ADAM19 knock-down. Loss of function and complementation experiments using the putative substrates for ADAM19 will be necessary to determine which of the signaling pathways ADAM19 regulates. The Xenopus system may provide a unique way to dissect in vivo the role of ADAM19 in early embryonic development.

4. Material and methods

4.1. Embryo culture

Xenopus laevis eggs were fertilized and the resultant embryos were dejellied in 2% cysteine and cultured as previously described (Newport and Kirschner, 1982). Staging of embryos was according to (Nieuwkoop and Faber, 1967).

4.2. Cloning of ADAM19

Degenerate oligonucleotide primers were designed from sequences conserved in human, mouse, quail and Xenopus tropicalis. PCR was carried out with cDNA from stage 20, stage 36 and adult liver using S1 (amino acids LNFIR) and AS3 (amino acids CNNNEN) at an annealing temperature of 50 C. The corresponding PCR product was cloned into TOPO-TA (Invitrogen) and sequenced. Specific primers were designed and used in 5′- and 3′-RACE-PCR with the Genrazer kit (Invitrogen) according to the manufacturer’s instructions. Clones from at least 3 independent PCR reactions were sequenced to obtain the full-length ADAM19.

4.3. Quantitative PCR

Total RNA was isolated from 5 embryos according to Chomczynski and Sacchi, (1987) followed by LiCl precipitation and poly A purification using the Oligotex kit (Qiagen) according to the manufacturer instructions. The cDNA was reverse transcribed using the Quantum Script kit (Quanta Bioscience). All PCR reactions were carried out using Taq green (Takara) on a MJG9005P light cycler (Stratagene). All experiments were repeated at least 3 times on separate injection days and the real-time PCR was also performed in triplicate on separate days to maximize variation due to PCR efficiency. The results were analyzed using the 2−ΔΔCt method (Livak and Schmittgen, 2001) and are presented as the Log2 fold change. All data were normalized using a-tubulin. The list of the real-time PCR primers is given in Table 1.

4.4. Plasmid constructs and miRNA microinjection

Full-length Xenopus ADAM19 was amplified by PCR using 2.5U of Pfu DNA polymerase (Stratagene) and cloned into the pCS2+ vector (Turner and Weintraub, 1994). Rescue ADAM19 mRNA was generated using a sense oligonucleotide containing 4 nucleotide silent mutations at the MO binding site (5′-CAATGGAACCCGAGACAGAG-3′, mutation are in bold). Non-rescue ADAM19 mRNA corresponds to the original cDNA sequence. The pCS2-ADAM19 construct was linearized by NotI and transcribed by SP6 RNA polymerase. All mRNA was prepared for microinjection as described in (Cousin et al., 2000). Transcripts (0.3 or 1 ng total) were injected close to the animal pole region of one blastomere at the two-cell stage. The un.injected half of the embryo served as an internal control for each embryo.

4.5. Antisense morpholino experiments

An antisense MO was synthesized (Gene Tools Carvals, OR) just upstream of the ATG start codon for ADAM19 (MO19). The sequence was selected to be complementary

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with all the 5′ cDNA clone obtained by RACE-PCR and also a perfect match to the published sequence for ADAM19 in X. tropicalis. A control Mo (CMO) was used to test for non-specific toxicity in the dose-response experiments. The sequence for Mo19 is 5′ GAGCTCCTGATGCTCCCTCATTTCAGA-3′ and CMO is 5′ CCTCTAAGCTTCAAGTAAATTTA-3′. Serial dilutions of Mo19 (50-1 ng) were injected in 5 nl volume into wild-type embryos to test for toxicity. A dose of 10 ng of Mo19 was selected and was shown to inhibit translation of 1 ng of the wild-type ADAM19 mRNA in embryos while allowing translation of a messenger containing the 4 nt mismatch. When the Mo where injected in a subset of cells to target specific tissues, β-galactosidase or GFP-myc RNA was injected to trace the lineage with reduced ADAM19 protein.

4.6. Whole mount β-galactosidase detection and in situ hybridization

Embryos were fixed in MEMFA (0.1 M MOPS pH 7.4, 2 mM EGTA pH 8, 1 mM MgSO4 and 3% formaldehyde) for 1 h at 20°C. They were washed in PBS and stained at 37°C in PBS solution containing 1 mg/ml X-gal, 20 mM K4Fe(CN)6, 20 mM K3Fe(CN)6, 2 mM MgCl2. The embryos were dehydrated in methanol and stored at –20°C. Whole mount in situ hybridization was performed exactly as described previously using albinoid or wild-type embryos (Harland, 1992).

4.7. Antibody preparation

Two glutathione S-transferase (GST) fusion proteins were prepared by cloning both variants of cDNA encoding the COOH-terminal amino acid residues of the ADAM19 cytoplasmic domains into the pGEX-KG vector (Guan and Dixon, 1991). Fusion proteins were purified using standard methods, combined with complete Freund’s adjuvant and injected into New Zealand white rabbits. Immune IgG were purified on a His-tag fusion protein (pET vectors, Novagen) column containing the same COOH-terminal amino acid residues used for immunization (NHS-sepharose, Pharmacia). Purified IgG (Ab A19) were stored at concentrations of 1 mg/ml in 50% glycerol at –20°C.

4.8. Western blot analysis

Embryo protein extraction and Western blot analysis were performed exactly as described previously (Alfandari et al., 1997). Monoclonal antibody to FACSIN2 (3D8) was used as a loading control as described previously (Conlin et al., 2000). For quantification, blots were detected using chemiluminescence on a G-Box HR16 (Syngene) and analyzed using the Genetool analysis software. Alternatively for weaker signals, the blots were exposed on X-ray films for various time points and the films were scanned and analyzed by densitometry using the same software.

4.9. Immunofluorescence and whole mount immunostaining

Embryos were fixed using either MEMFA or DENTS (20% DMSO in 80% methanol) and stored in methanol before sectioning. Embryos were performed by increasing doses of 15% fish skin gelatin (Sigma) and 15% sucrose in PBS. After a final impregnation overnight at 4°C, embryos were mounted in OCT (Tissue Tek). Cryosections (14–16 μm) were blocked in PBS containing 20% goat serum and 1% BSA (Sigma). Primary antibodies were used at 10 μg/ml and secondary antibodies (Jackson Immunoresearch) were used at 5 μg/ml. Slides were mounted using Vectashield (Vector) and imaged using a Zeiss Axiosvert 200 M inverted microscope equipped with a Ludi XY-stage control and a Hamamatsu Orca camera. Images were taken using the ApoTome structured light illumination system (Zeiss) and the AxiosVision software (Zeiss).

Whole mount immunostaining was performed as previously described (Dent et al., 1989).

4.10. Time-lapse imaging

Embryos at late blastula stage were individually placed in holes made in an agarose coated plastic dish. Time-lapse movies were made using an Axiosvert inverted microscope (Zeiss) equipped with a Ludi XY-stage control and an Orca camera (Hamamatsu) and controlled by the Olysoft software. Measurements were done using the same software.

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